·Basic Research·

Effects of transforming growth factor β2 and connective tissue growth factor on induction of epithelial mesenchymal transition and extracellular matrix synthesis in human lens epithelial cells

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Abstract

• AIM: To investigate the effects of transforming growth factor β2 (TGF-β2) and connective tissue growth factor (CTGF) on transdifferentiation of human lens epithelial cells (HLECs) cultured *in vitro* and synthesis of extracellular matrix (ECM).

• METHODS: HLECs were treated with TGF-β2 (0, 0.5, 1.0, 5, 10μg/L) and CTGF (0, 15, 30, 60, 100μg/L) for different times (0, 24, 48, 72h) *in vitro* and the expression of α-smooth muscle actin (α-SMA), the main component of the extracellular matrix type I collagen (Col-1) and fibronectin (Fn) were measured by using real-time polymerase chain reaction (PCR) and western-blot.

• RESULTS: TGF-β2 and CTGF significantly increased expression of α-SMA mRNA and protein (P<0.05, P<0.001), Fn mRNA and protein (P<0.001), Col-1 mRNA and protein (P<0.001). TGF-β2 could induce HLECs expression of CTGF mRNA and protein in dose-dependent manner (P<0.05, P<0.001). TGF-β2 and CTGF could induce HLECs to express α-SMA, Fn and Col-1 in time-dependent manner. Each time of TGF-β2 and CTGF induced HELCs expression of α-SMA, Fn, Col-1 mRNA and protein was significant increase compared with control (P<0.05, P<0.001).

• CONCLUSION: TGF-β2 and CTGF could induce HLECs epithelial mesenchymal transition and ECM synthesis.

• **KEYWORDS:** transforming growth factor β2; connective tissue growth factor; posterior capsular opacification; human lens epithelial cells; extracellular matrix; α-smooth muscle actin; type I collagen; fibronectin

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INTRODUCTION

Posterior capsular opacification (PCO) is the most common postoperative complication of extracapsular cataract or phacoemulsification extraction surgery. Its incidence is about 20% to 50% for adults and almost 100% for children [1]. PCO is mainly caused by the transdifferentiation, proliferation, and migration on the capsular membrane of residual lens epithelial cells, and collagen production after cataract surgery. Studies have found that a variety of cytokines are involved in the development of PCO. Among them, transforming growth factor β2 (TGF-β2) is considered to be an important factor causing PCO occurrence[2-4]. Connective tissue growth factor (CTGF) is an important secreting growth factor found in recent years. It could promote mitosis and proliferation of fibroblasts, collagen synthesis, cell adhesion and migration, and fibrosis. Studies have also confirmed that CTGF as a downstream effector of TGF-β2, plays an important role in the pathological process of PCO[5,6].

In this study, we treated *in vitro* cultured human lens epithelial cells (HLECs) with TGF-β2 and CTGF and examined their effects on HLECs transdifferentiation at mRNA and protein levels, and synthesis of extracellular matrix (ECM), hoping to provide experimental evidences for further exploring the relationship of TGF-β2 and CTGF with PCO and the underlying mechanisms.

MATERIALS AND METHODS

Culture and Treatment of Human Lens Epithelial Cells

HLEC line SRA01/04 was purchased from ATCC (Manassas,

Table 1 Primers used in qPCR

Gene	Forward (5'-3')	Reverse (5'-3')
α-SMA	GACAATGGCTCTGGGCTCTGTAA	CTGTGCTTCGTCACCCACGTA
Fn	CAGGATCACTTACGGAGAAACAG	GCCAGTGACAGCATACACAGTG
Col-1	TCTAGACATGTTCAGCTTTGTGGAC	TCTGTACGCAGGTGATTGGTG
CTGF	CTTGCGAAGCTGACCTGGAA	TCTGTACGCAGGTGATTGGTG
ACTB	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

α-SMA:α-smooth muscle actin; Fn: fibronectin; Col-1:type I collagen; CTGF: connective tissue growth factor; ATCB:β-actin.

VA, USA). Cells within 20 passages were seeded into culture flask at 1×10^6 cells and cultured with DMEM containing 10% fetal bovine serum (FBS). The culture medium was removed when the cells approached 70% confluence, and the cells were then cultured in serum-free DMEM for 24h. The experimental group was then treated with 3ml of serum-free medium containing TGF-β2 and CTGF for 24h respectively, at a final concentration as the following: TGF-β2, 0.5, 1, 5, and 10μg/L and CTGF, 15, 30, 60, and 100μg/L. Control group was treated with an equal volume of medium only.

Extraction of Total Ribonucleic Acid HLEC cells were washed with PBS for 3 times, detached with trypsin digestion, and collected by centrifugation. Total RNAs were extracted using a FASTAgen-RNAfast200 kit (Fastagen, Shanghai, China) according to the manufacturer's manual. The quality of the RNA samples was controlled by measuring A260/280; absorptions between 1.8 and 2.1 indicate a good quality.

Reverse Transcription Reverse transcription was performed using cDNA synthesis kit from TaKaRa Biotechnology (Dalian Co., Ltd., China). Briefly, the 20μL of reaction was set up, containing RNA 2μL, 5X PrimeScript Buffer (for Real-Time) 4μL, PrimeScript RT Enzyme Mix 1μL, Ologo dT Primer (50μmol/L) 1μL, Random heximers (100μmol/L) 1μL, and RNase Free dH_2O 11μL. The reaction was carried out at 37℃ for 45min and at 85℃ for 5s to obtain the cDNA.

Quantitative Real-time Polymerase Chain Reaction The polymerase chain reaction (PCR) primers were designed and synthesized by TaKaRa Biotechnology (DalianCo., Ltd., China) , as shown in Table 1. qPCR was performed using SYBR Primix Ex Taq II PCR kit (TaKaRa Biotechnology, Dalian Co., Ltd., China). Briefly, PCR reaction (20μL) contained SYBR of Primix Ex Taq II (2×) 5.0μL, primer (10μmol/L) 0.8μL, reverse primer (10μmol/L) 0.8μL, DNA template 2.0μL, and dH_2O (sterile distilled water) 6.4μL. PCR reaction was carried out on Bio-Rad IQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) under the following condition: 95℃, 30s; 95℃, 5s, 60℃, 20s, 40 cycles; and 65℃, 15s.

Western Blot After removal of the culture medium, the HLECs were harvested using cell scraper and lysed with 100μL of cell lysis buffer on ice for 30min. The cell lysates were centrifuged at 4℃ at 12 000 ×g for 15min. The supernatant was collected. The protein concentrations were measured using BCA method (Joincare Biosciences, Zhuhai, China). A total of 50μg protein sample was separated by 10% polyacrylamide gel electrophoresis and transferred onto NC membrane, which was blocked with Tris buffered saline Tween (TBST) containing 5% skim milk at room temperature for 3h. The membrane was incubated with CTGF (Abcam,1:1 000), α-SMA (Millipore,1:1 000), Fn (Millipore,1:200), Col-1 (Millipore,1:300) antibodies at 4℃ overnight. After 3 times of washing in TBST for 10min, the membrane was incubated with secondary antibody (Sigma, 1:10 000) at room temperature for 2h and washed with TBST for 15min each time for 3 times. The membrane was then immersed in enhanced chemiluminescence solution for 5min and exposed to X-ray film in the dark for 2min, which was then developed and observed.

Image Acquisition and Statistical Analysis qPCR results were statistically analyzed with Bio-Q software to obtain Ct value for each PCR reaction, and ΔΔCt method was used to calculate the levels of gene expression. Western-blot results were scanned, and the protein expression levels were measured using densitometry with Image J software. SPSS 13.0 statistics software was employed to carry out all the statistical analyses. All tests were repeated 3 times. After treatment of HLECs with different concentrations of TGF-β2 and CTGF, respectively, the overall comparison of protein and mRNA expressions with control group was analyzed using one-way ANOVA. Differences with $P<0.05$ were considered statistically significant.

RESULTS

Dose-dependent Effects of Transforming Growth Factor β2 and Connective Tissue Growth Factor on Expression of CTGF, α-SMA, Fn and Col-1 in Human Lens Epithelial Cells We first explored the dose effects of TGF-β2 and CTGF treatment on the mRNA and protein levels of CTGF, α-SMA, Fn and Col-1 in HLECs using real-time PCR and western blot. The results showed that treatment of HLECs with TGF-β2 at concentrations of 0.5, 1.0, 5 and 10μg/L for 24h dose-dependently enhanced the mRNA and protein levels of CTGF (2.63±0.17, 4.23±0.41, 7.29±0.92, 10.53±0.89), α-SMA (2.64±0.28, 5.57±0.83, 14.69±1.94, 25.16±2.88), Fn (3.39±0.67, 6.77±1.77, 16.13±6.11, 28.60±3.19) and Col-1 (3.41±0.85, 9.42±1.82, 15.88±

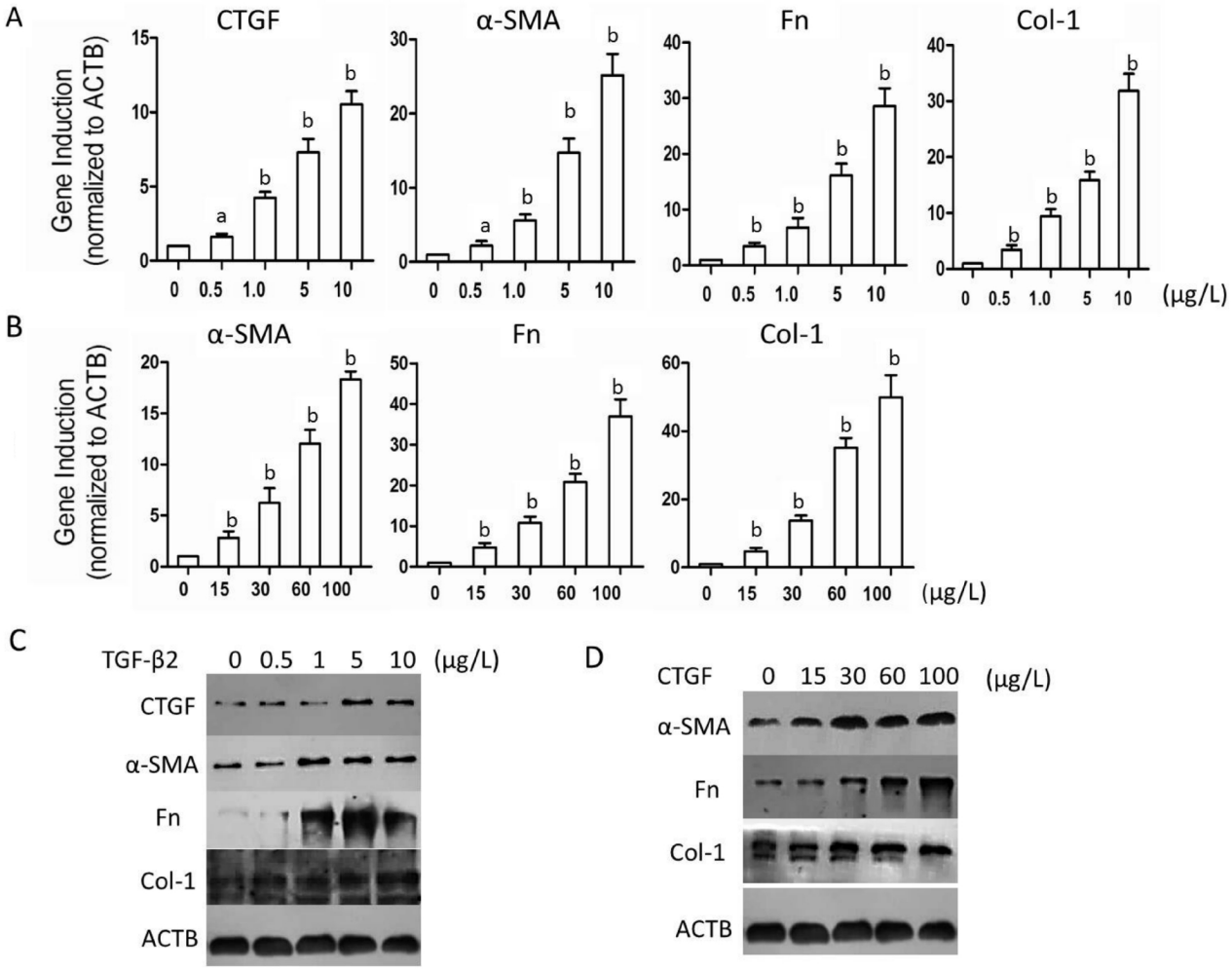

Figure 1 Dose effects of TGF−β2 and CTGF on expression of α−SMA, Fn and COL−1 in HLECs A, C: Dose effects of TGF-β2 (0-10μg/L) on mRNA and protein level of α-SMA, Fn and Col-1 in HLECs ($^aP<0.05$, $^bP<0.001$); B, D: Dose effects of CTGF (0-100μg/L) on mRNA and protein level of α-SMA, Fn and Col-1 in HLECs ($^aP<0.05$, $^bP<0.001$).

1.52, 31.82 ±3.08) (Figure 1A, 1C, $^aP<0.05$, $^bP<0.001$). Similarly, treatment of HLECs by CTGF at 15, 30, 60 and 100μg/L for 24h also dose- dependently enhanced the mRNA and protein levels of α-SMA (3.17±0.56, 6.24±1.44, 12.02± 1.35, 18.31±2.32), Fn (4.73±1.10, 10.77±1.57, 20.80±2.06, 36.93±4.18) and Col-1 (4.75±1.20, 13.78±3.12, 35.15±4.27, 48.53±7.06) (Figure 1B, 1D, $^aP<0.05$, $^bP<0.001$). Overall, the results showed that TGF-β2 could induce HLECs expressing CTGF and both TGF-β2 and CTGF could induce HLECs expressing α-SMA, Fn and Col-1 in a dose-dependent manner.

Time −dependent Effects of Transforming Growth Factor β2 and Connective Tissue Growth Factor on Expression of CTGF, α−SMA, Fn and Col−1 in Human Lens Epithelial Cells We then explored the time-dependent effects of TGF-β2 and CTGF treatment on the mRNA and protein levels of CTGF, α-SMA, Fn and COL-1 in HLECs using real-time PCR and western-blot. The results showed that treatment of HLECs with TGF-β2 at 1.0μg/L for 24, 48 and 72 h significantly enhanced the mRNA and protein levels of CTGF (3.06±0.48, 5.11±0.56, 8.62±0.75), α-SMA (2.35± 0.48, 5.77 ±1.62, 12.39 ±1.23), Fn (1.86 ±0.89, 4.13 ±1.56, 9.90±1.93) and Col-1 (3.73±0.82, 11.44±2.68, 20.87±3.10) (Figure 2A, 2C, $^aP<0.05$, $^bP<0.001$). Similarly, treatment of HLECs by CTGF at 30μg/L for 24, 48 and 72h also significantly enhanced the mRNA and protein levels of α-SMA (3.01±0.74, 7.11±1.16, 12.06±0.85), Fn (2.20±0.33, 4.80±0.60, 11.24±1.15) and Col-1 (3.73±0.52, 11.11±1.44, 23.21±3.62) (Figure 2B, 2D, $^aP<0.05$, $^bP<0.001$). Overall, the results showed that TGF-β2 could induce HLECs expressing CTGF and both TGF-β2 and CTGF could induce HLECs expressing α-SMA, Fn and Col-1 in a time-dependent manner.

DISCUSSION

Transdifferentiation, proliferation and migration of residual lens epithelial cells on the capsular membrane and collagen synthesis after cataract surgery are the main causes for PCO. TGF-β, epidermal growth factor and fibroblast growth factor play important roles in PCO development. TGF-β is a class of multi-functional growth factor, which typically functions as a ligand to induce signal transduction, inhibiting growth, proliferation and activity of both normal and tumor cells.

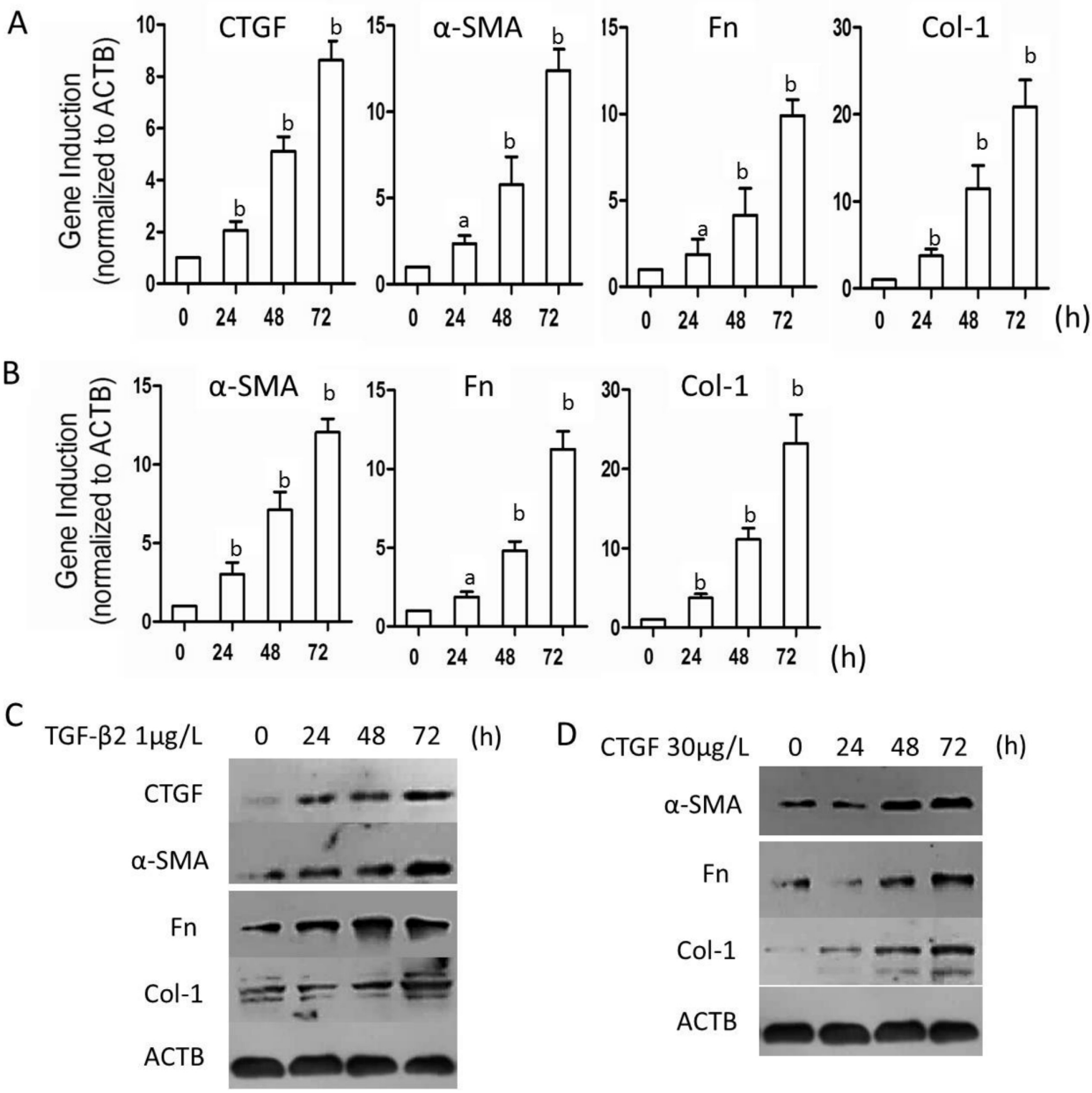

Figure 2 Time effects of TGF-β2 and CTGF on expression of α-SMA, Fn and Col-1 in HLECs A, C: Effects of 1μg/L TGF-β2 treatment for 0-72h on mRNA and protein level of α-SMA, Fn and Col-1 in HLECs ($^aP<0.05$, $^bP<0.001$); B, D: Effects of 30μg/L CTGF treatment for 0-72h on mRNA and protein level of α-SMA, Fn and Col-1 in HLECs ($^aP<0.05$, $^bP<0.001$).

However, it could also promote proliferation and activate stromal-derived cells such as fibroblasts, smooth muscle cells and other cells, and participate cell proliferation, differentiation, adhesion and other important physiological activities [7]. CTGF is widely present in a variety of human tissues and organs, with the highest content in kidney, and has different biological effects in different tissues and locations by binding to different receptors. It can promote cell mitosis and proliferation of fibroblasts as well as collagen synthesis, mediate cell adhesion and migration, enhance fibrosis, regulate extracellular matrix (ECM) synthesis, and so on [8,9]. Both TGF-β2 and CTGF play important roles in proliferative vitreoretinal diseases, glaucoma and other eye diseases [10]. CTGF is present at low concentration in tear, thus it may have potential to promote ocular fibrosis and wound healing [11]. Previous studies found that CTGF and TGF-β were expressed in conjunctival bleb of rabbits after glaucoma filtration surgery. In addition, injecting exogenous CTGF and TGF-β2 to mitomycin C-treated follicles could significantly increase follicle failure rate [12]. Furthermore, mRNA and protein levels of CTGF and TGF-β2 were significantly up-regulated in retinal pigment epithelium on PVR proliferative membrane [13]. Although the biological functions of TGF-β2 and CTGF in lens epithelial cells (LECs) have been reported at home and aboard, studies on the roles of TGF-β2 and CTGF in HLECs and ECM synthesis at mRNA and protein levels were rare.

Research has shown that many factors are involved in CTGF regulation. Among them, TGF-β is the most potent and direct stimulating factor regulating CTGF transcription[14,15]. Studies

have found that CTGF is a downstream effector mediating some biological functions of TGF-β, such as profibrotic effect, stimulating fibroblast proliferation and ECM synthesis, and plays an important role in promoting wound healing and tissue fibrosis [16-18]. CTGF as a downstream molecule of TGF-β1, played an important role in epithelial-mesenchymal transition of renal tubule and in the pathogenesis of diabetic nephropathy [19]. Studies have also found that TGF-β could significantly promote the proliferation of HLECs and CTGF mRNA expression [20]. In addition, CTGF as a cofactor of TGF-β2 could promote TGF-β2-induced fibrosis of retinal pigment epithelial cells [21,22]. In the present study, we used real-time PCR and western-blot methods to explore the effects of TGF-β2 on CTGF expression in HLECs. The results showed that TGF-β2 treatment could enhance CTGF protein and mRNA levels in dose- and time-dependent manners, implying that CTGF may act as a downstream effector of TGF-β2 factor mediating TGF-β2-promoted fibrosis.

Epithelial-mesenchymal transition of lens epithelial cells is the major change of PCO and is characterized by mesenchymal cell phenotypes such as cell transformation, loss of epithelial phenotype, actin reorganization, *etc*[23,24]. Among them, expression of α-SMA is an important process during the epithelial-mesenchymal transition of lens epithelial cells [23,25] and is considered as a key characteristic of fibroblasts differentiating into myofibroblasts in granulation tissue and a key factor involving tissue fibrosis. Actin cytoskeleton rearrangement and α-SMA induction provide a structure basis for migration, invasion and contraction of transdifferentiation cells. α-SMA is similarly expressed in normal lens and lens with cortical, nuclear cataract, but overexpressed in lens with anterior subcapsular and posterior capsular opacification [26]. Fn and Col-1 are the major components of ECM distributed between cells or cell surfaces, forming a network structure to provide a scaffold for cell migration. Studies found that TGF-β2 could induce synthesis of α-SMA, Col-1 and Fn in LECs [27]. In this study, we examined the expression of α-SMA, Fn and Col-1 using real-time PCR and western-blot methods in HLECs treated with TGF-β2 or CTGF at different concentrations for different times. The results showed that TGF-β2 and CTGF could dose- and time-dependently enhance the expression of α-SMA, Fn and Col-1 at both protein and mRNA levels in HLECs, suggesting that TGF-β2 and CTGF could promote epithelial-mesenchymal transition of HLECs and ECM synthesis.

In summary, our results showed that TGF-β2 and CTGF could promote the expression of α-SMA, Fn, Col-1 at both protein and mRNA levels in HLECs in dose- and time-dependent manners, which suggested the following mechanisms for TGF-β2 and CTGF-induced after cataract.

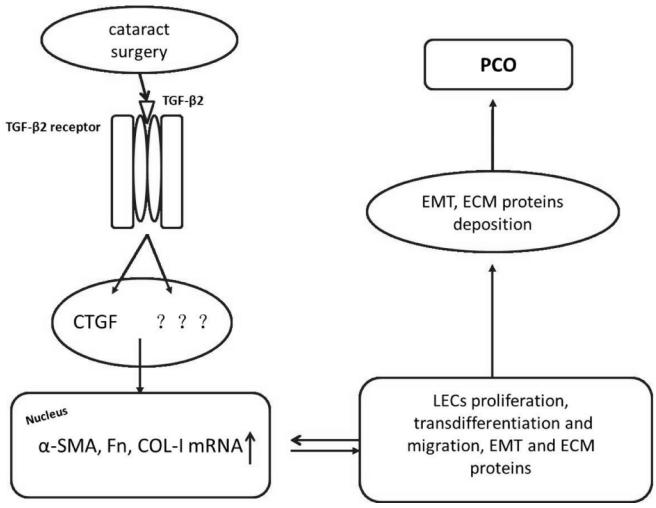

Figure 3 The mechanism of the involvement of TGF-β2 and CTGF in PCO TGF-β2 in aqueous humor and lens is activated after cataract surgery. Activated TGF-β2 in LECs enhances CTGF expression. CTGF further promotes proliferation of the transdifferentiated cells and ECM synthesis, plaque accumulation, as well as synthesis and accumulation of large amounts of excessive ECM, eventually leading to subcapsular lens opacities.

First, TGF-β2 in aqueous humor and lens is activated after cataract surgery and highly expressed in anterior chamber[28,29]; Second, activated TGF-β2 in LECs enhances CTGF expression, thereby inducing transdifferentiation of LECs into spindle myofibroblasts; Third, CTGF further promotes proliferation of the transdifferentiated cells and ECM synthesis, plaque accumulation, as well as synthesis and accumulation of large amounts of excessive ECM, eventually leading to subcapsular lens opacities (Figure 3).

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