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TGF-β2 induces transdifferentiation and fibrosis in human lens epithelial cells via regulating gremlin and CTGF

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ABSTRACT

Transforming growth factor (TGF)-β2, gremlin and connective tissue growth factor (CTGF) are known to play important roles in the induction of epithelial mesenchymal transition (EMT) and extracellular matrix (ECM) synthesis. However, the complex functional relationship among gremlin, CTGF and TGF-β2 in the induction of EMT and ECM synthesis in human lens epithelial cells (HLECs) has not been reported. In this study, we found that TGF-β2, CTGF and gremlin can individually induce the expression of α-smooth muscle actin (α-SMA), fibronectin (Fn), collagen type I (COL-I), Smad2 and Smad3 in HLECs. Blockade of CTGF and gremlin effectively inhibited TGF-β2-induced expression of α-SMA, Fn, COL-I, Smad2, and Smad3 in HLECs. Furthermore blockade of Smad2 and Smad3 effectively inhibited CTGF and gremlin induced expression of α-SMA, Fn, COL-I in HLECs. In conclusion, TGF-β2, CTGF and gremlin are all involved in EMT and ECM synthesis via activation of Smad signaling pathway in HLECs. Specifically silencing CTGF and gremlin can effectively block the TGF-β2-induced EMT, ECM synthesis due to failure in activation of Smad signaling pathway in HLECs.

1. Introduction

Posterior capsule opacification (PCO) is the most common postoperative complication after extracapsular cataract extraction or phacoemulsification surgery, it is mainly caused by the transdifferentiation, proliferation, migration, and collagen-production of the residual lens epithelial cells (LECs) in the capsule.

A wide range of cytokines and growth factors are involved in the development of PCO [1]. Among them, TGF-β2 and CTGF have been shown to be critical. Both TGF-β2 and CTGF exert biological functions by binding to their respective receptors and induce cell growth and differentiation, extracellular matrix (ECM) synthesis, and tissue fibrosis [2]. A recent report from Lee and Joo has shown that both TGF-β2 and CTGF enhance the synthesis of epithelial mesenchymal transition (EMT)-specific proteins α-SMA and various ECM proteins including Fn, COL-I, collagen type IV, but reduce the expression of E-cadherin and other intrinsic proteins of LECs [3]. As α-SMA, Fn and COL-I are primary constituents of ECM and crucial for EMT induction, and E-cadherin plays an important role in the maintenance of morphology and structural integrity of normal epithelial cells, the role of TGF-β and CTGF in transdifferentiation and fibrosis of intraocular LECs, trabecular meshwork (TM) cells and retinal pigment epithelial cells (RPE) has been considered to be the main causes for many of the pathological processes in the eye [4,5].

Gremlin, a member of the DAN family protein, is one of the major endogenous antagonists of bone morphogenetic protein (BMP) which plays an important role in many organ development [6]. BMP has a few isoforms such as BMP2, BMP4 and BMP7, they selectively regulate the proliferation and differentiation of many kinds of tissue cells such as osteocytes and tumor cells [7,8]. Gremlin can effectively inhibit BMP activity by binding to BMP extra- and intra-cellularly. Binding of gremlin to intracellular BMP prevents the secretion of mature BMP [9], therefore it in essence regulates the embryonic development, growth, and cell differentiation [10]. Some recent reports have demonstrated that gremlin induces ECM synthesis in TM cells and it further increases the expression of Fn protein when added together with BMP4 and TGF-β, whereas addition of BMP4 and TGF-β2 without gremlin fails to do so [11,12]. The hypothesis is that BMP can inhibit the profibrotic and transdifferentiation effect of TGF-β, while gremlin can bind to BMP and abolish its inhibitory effect on TGF-β, thus enhancing the profibrotic effect of TGF-β [13]. Although the effects of TGF-β2 and CTGF on the EMT and ECM synthesis of LECs have been reported, it remains to be determined whether gremlin plays a role in the EMT and ECM synthesis of LECs, and what are underlying mechanisms of its function.

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The signaling pathway of TGF-β2 is complex and it involves the reciprocal interactions among different signal transduction pathways [14,15] such as the canonical Smad signaling pathway. TGF-β2 binds to a type II receptor, which phosphorylates a type I receptor. The type I receptor then phosphorylates receptor-regulated Smad2 and Smad3 which can bind the coSmad Smad4. CoSmad complexes accumulate in the nucleus where they act as transcription factors and participate in the regulation of target gene expression. Some recent studies have demonstrated that Smad signaling pathway plays an important role in assisting TGF-β2 on EMT and ECM production in HLECs [14], and that gremlin can activate Smad signaling pathway too [11]. However, it is not clear whether CTGF and/or gremlin are regulated by the TGF-β/Smad signaling pathway and blockade of Smad signaling pathway can effectively inhibited gremlin induced expression of α-SMA, Fn, COL-I, in HLECs.

In this study we first induced *in vitro* cultured HLECs with various concentrations of TGF-β2, CTGF, and gremlin, respectively, to examine the expression of TGF-β2, CTGF and gremlin-induced EMT-associated proteins and ECM synthesis, as well as the activation of Smad signaling pathway. Following that we investigated the induction of CTGF and gremlin expressions, in HLECs by TGF-β2 and whether the effect of TGF-β2 on the expression of EMT-associated proteins, ECM synthesis, and activation of Smad in HLECs can be blocked by specifically silencing CTGF and gremlin. Data obtained from this study will provide experimental basis for better understanding the functional relationship among TGF-β2, CTGF and gremlin, and the potential underlying mechanisms for PCO.

2. Materials and methods

2.1. Culture and treatment of HLECs

HLEC line SRA01/04 was purchased from ATCC (Manassas, VA, USA). 1×10^6 cells within 20 passages were seeded into culture flask with DMEM containing 10% fetal bovine serum (FBS). The culture medium was replaced with serum-free DMEM when the cells approached 70% confluence and cells were cultured for 24 h. The cells in the experimental group were then treated with 3 ml of serum-free medium containing TGF-β2, CTGF, or gremlin at various concentrations for a further 24 h before cells were harvested for further analysis. Control group cells were treated with an equal volume of medium only since all reagents are water soluble.

2.2. Quantitative real-time PCR (qPCR)

HLEC cells in culture flasks were washed with PBS for 3 times and treated with trypsin before being collected. Total RNAs were extracted using a FASTAgen-RNAfast200 kit (Fastagen, Shanghai, China) according to the manufacturer's instruction. Reverse transcription was then performed using cDNA synthesis kit from TaKaRa Biotechnology (Dalian Co., Ltd., China). The PCR primers were designed and synthesized by TaKaRa Biotechnology (Dalian Co., Ltd., China) as follows: α-SMA_F, 5′-GACAATGGCTCTGGGCTCTGTAA-3′ and SMA_R, 5′-CTGTGCTTCGTCACCCACGTA-3′; Fn_F, 5′-CAGGATCACTTACGGAGAAACAG-3′ and Fn_R, 5′-GCCAGTGACAGCATACACAGTG-3′; Col-F, 5′-TCTAGACATGTTCAGCTTTGTGGAC-3′ and Col_R, 5′-TCTGTACGCAGGTGATTGGTG-3′; CTGF_F, 5′-CTTGCGAAGCTGACCTGGAA-3′ and CTGF_R, 5′-TCTGTACGCAGGTGATTGGTG-3′; Gremlin_F, 5′-AAGCGAGACTGGTGCAAAAC-3′ and Gremlin_R, 5′-CTTGCAGAAGGAGCAGGACT-3′; CDH1_F, 5′-GAGTGCCAACTGGACCATTCAGTA-3′ and CDH1_R, 5′-AGTCACCCACCTCTAAGGCCATC-3′; ACTB_F, 5′-TGGCACCCAGCACAATGAA-3′ and ACTB_R, 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′.

qPCR reaction was carried out on Bio-Rad IQ5 thermal cycler (Bio-Rad, Hercules, CA, USA). The results were 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.

2.3. Western blot

After appropriate treatments, culture medium was removed, and the HLECs were washed and harvested using cell scraper and lysed with 100 μl of cell lysis buffer on ice for 30 min. The cell lysates were centrifuged and supernatants were collected. The protein concentrations in the supernatants were measured using BCA method (Joincare Biosciences, Zhuhai, China). A total of 50 μg protein per sample was separated by 10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane, which was blocked with TBST buffer containing 5% skim milk at room temperature for 3 h. Following that the membrane was incubated at 4 °C overnight with mouse monoclonal antibodies specific to CTGF (Abcam, UK), gremlin (Abcam), α-SMA (Millipore, USA), Fn (Millipore), Col-1 (Millipore), E-cadherin (Proteintech, USA), phospho-Smad2 (Santa Cruz, USA), phospho-Smad3 (Santa Cruz), Smad2 (Proteintech), Smad3 (Proteintech). After further washing, the membrane was incubated with anti-mouse antibody conjugated HRP (Sigma, USA) at room temperature for 2 h. The membrane was then washed and immersed in enhanced chemiluminescence solution before being exposed to X-ray film. Western-blot results were scanned, and the protein expression levels were measured using densitometry with Image J software.

2.4. Transfection of HLECs with siRNA

The recombinant lentiviruses expressing CTGF specific small-interfering (CTGF.siRNA), Gremlin.siRNA, Smad2.siRNA or Smad3.siRNA respectively were purchased from Neuron Biotech Co., Ltd. (Shanghai, China). Cells were cultured into 6-well plate at 2×10^5 cells/well for 12 h. Then, the medium was removed and the cells were washed with PBS before adding serum-free medium containing 5 μl of lentivirus (4×10^6 virus, MOI = 1:20). Cells were further incubated for 24 h before the medium was replaced with 2 ml of DMEM containing 10% FBS and cultured for a further 24 h. Mock Con.siRNA(a) and Con.siRNA(b) of viral preparation were used as negative controls for CTGF and gremlin respectively.

2.5. Image acquisition and statistical analysis

SPSS13.0 statistics software was employed to carry out all the statistical analyses. After treatment of HLECs with different concentrations of TGF-β2, gremlin, and CTGF, respectively, the overall comparison of protein and mRNA expressions with control group was analyzed using one-way ANOVA, while the difference between groups was compared using Turkey HSD test. Differences with $P < 0.05$ were considered statistically significant. All experiments were repeated 3 times.

3. Results

3.1. The effect of TGF-β2, CTGF and gremlin on the mRNA and protein expressions of α-SMA, Fn, COL-I, and E-cadherin in HLECs

TGF-β2, CTGF, and gremlin are believed to be involved in the process of fibrosis in different cells and tissues, and TGF-β2 can induce the expression of CTGF and gremlin in some cells [2,16]. Our results confirmed these findings in HLECs. After 24 h treatment of HLECs with TGF-β2, the mRNA (Fig. 1A, $^{*}P < 0.05$; $^{**}P < 0.001$)

and protein (Fig. 1D) levels of CTGF, gremlin, α-SMA, Fn, and COL-I were significantly up-regulated in a dose-dependent manner. In contrast, E-cadherin expression was down-regulated with the increase of TGF-β2 concentration (Fig. 1A and D). HLECs responded to CTGF (Fig. 1B and E) and gremlin (Fig. 1C and F) treatment in the similar manner: the expression of α-SMA, Fn, and COL-I were increased in a dose-dependent manner, whereas E-cadherin expression reduced (Fig. 1B, C, E, F, $^{*}P < 0.05$; $^{**}P < 0.001$).

3.2. The effect of CTGF.siRNA and Gremlin.siRNA on expressions of α-SMA and ECM in HLECs via induction of TGF-β2, gremlin and CTGF

TGF-β2, CTGF, and gremlin can individually promote the expression of ECM proteins in HLECs, we next examined whether the endogenous expression of CTGF and gremlin was essential in TGF-β2-induced expression of ECM proteins in HLECs. HLECs were transfected with CTGF.siRNA and Gremlin.siRNA alone, or in combination. The expressions of CTGF, α-SMA, Fn, and COL-I by the cells after treatment with 1 μg/L TGF-β2, 200 μg/L gremlin and 60 μg/L CTGF for 48 h were then determined by qPCR and Western-blot. Our results show that indicated that transfection with either CTGF.siRNA or Gremlin.siRNA effectively suppressed TGF-β2-induction of α-SMA, Fn, and COL-I in HLECs (Fig. 2A and D). Simultaneously silencing of both CTGF and gremlin further reduced the expression of α-SMA, Fn (Fig. 2A, and D, $^{**}P < 0.001$; $^{\#}P < 0.05$). Gremlin.siRNA (Fig. 2B and E) and CTGF.siRNA (Fig. 2C and F, $^{*}P < 0.05$; $^{**}P < 0.001$) also effectively blocked the synthesis of α-SMA, Fn, and COL-I in HLECs with the presence of gremlin or CTGF in the culture.

3.3. The role of CTGF and gremlin in TGF-β2/Smad signaling pathway

As we have shown TGF-β is able to upregulate the expression of CFGF and gremlin in HLECs and Smad signaling pathway is important for TGF-β function, next we investigated the role of CTGF and gremlin in the TGF-β2/Smad signaling pathway. HLECs were treated with different concentrations of TGF-β2 (0–10 μg/L), CTGF (0–100 μg/L), and gremlin (0–400 μg/L), respectively, for 120 min, and the expressions and total and phosphorylated Smad2 and Smad3 were analyzed by Western blotting. Our data indicated that although the total proteins of Smad2 and Smad3 were not significantly changed, the phosphorylated forms were significantly increasedin a dose dependent matter after treatment of TGF-β2, CTGF or gremlin (Fig. 3A–C). Our data also showed that RNAi targeting either CTGF or gremlin significantly reduced the phosphorylation of Smad2 and Smad3 (Fig. 3D) in HLECs treated with TGF-β2, suggesting CTGF and gremlin are important mediators of TGF-β activated Smad signaling pathway.

To further confirm that Smad2 and Smad3 are responsible for the functions of CTGF and gremlin in EMT and ECM synthesis, HLECs were transfected with Smad2.siRNA or Smad3.siRNA. Cells were then treated with 60 μg/L CTGF or 200 μg/L gremlin for

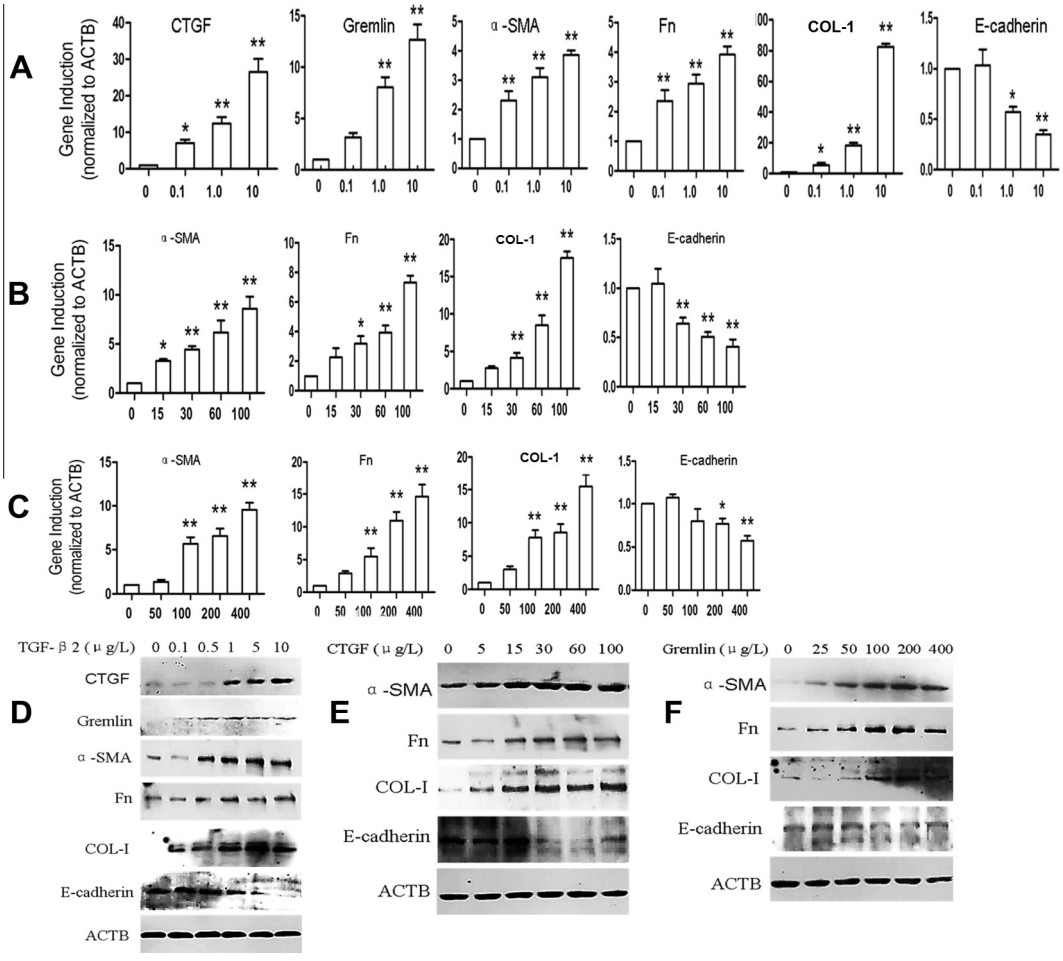

Fig. 1. qPCR and Western blot analysis on expression of CTGF, gremlin, α-SMA, Fn, COL-I, and E-cadherin (Ed) in HLECs. Cells were treated with different concentrations of TGF-β2 (0–10 μg/L) (A and D), CTGF (0–100 μg/L) (B and E), gremlin (0–400 μg/L) (C and F) for 24 h and the expression of mRNA and protein relevant to ACTB were studied by qPCR (A–C) or western blot (D–F). *P* values are given as per respective '0' concentration: $^{*}P < 0.05$, $^{**}P < 0.001$.

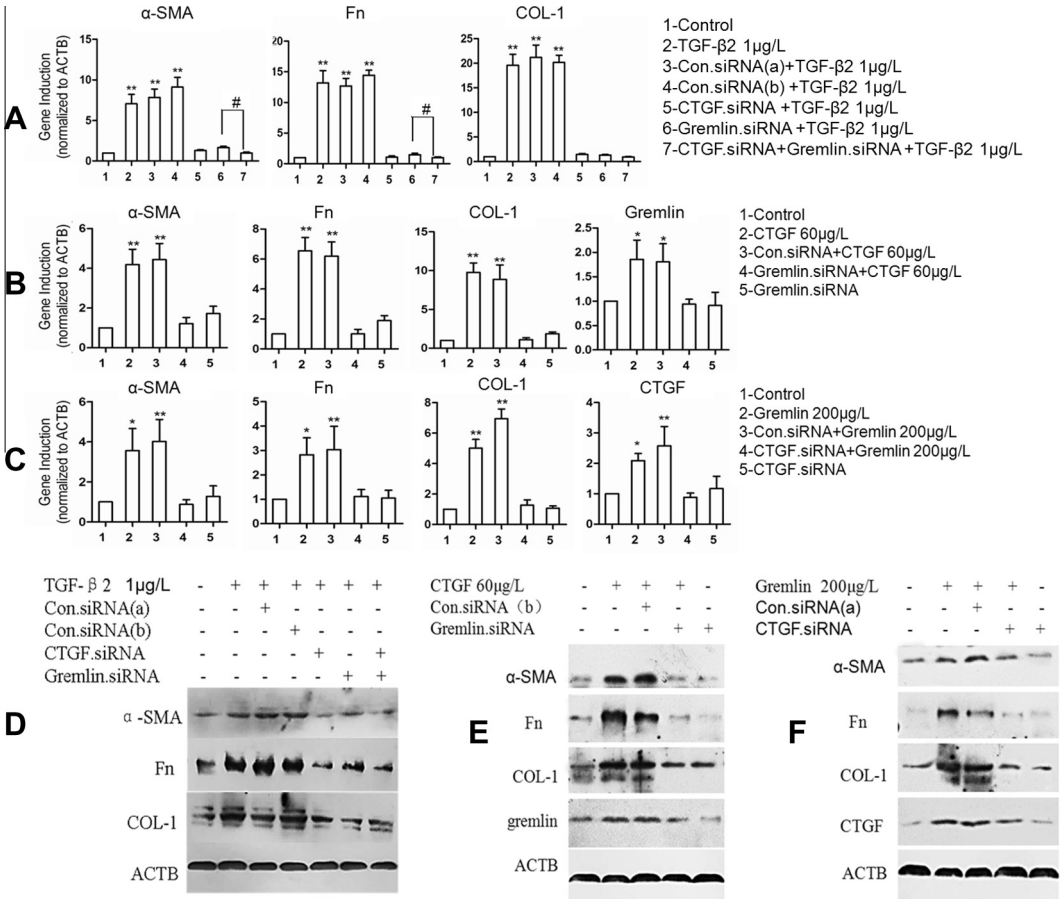

Fig. 2. Effects of CTGF.siRNA and Gremlin.siRNA on expressions of α-SMA and ECM molecules in HLECs after induction with TGF-β2, gremlin and CTGF. TGF-β2 induced expression of α-SMA, Fn and Col-1 mRNA (A) and protein (D) in HLECs; CTGF induced expression of α-SMA, Fn and Col-1 mRNA (B) and protein (D) in HLECs; gremlin induced expression of α-SMA, Fn and Col-1 mRNA (C) and protein (F) in HLECs. $^{*}P < 0.05$; $^{**}P < 0.001$; $^{\#}P < 0.05$.

48 h, and the expression of α-SMA, Fn and COL-I were determined by Western-blot. Our result in Fig. 4 show that the expressions of α-SMA, Fn and COL-I were decrease by Smad2.siRNA (Fig. 4A and C) and Smad3.siRNA (Fig. 4B and D) in the presence of CTGF (Fig. 4A and B) and gremlin (Fig. 4C and D).

4. Discussion

Cell transdifferentiation, proliferation, migration, and collagen synthesis in the capsule after cataract surgery are the main reasons of PCO. A variety of regulatory factors have been implicated in this pathological process. Among them, TGF-β plays a key role in the development of the disease. CTGF is functionally related to TGF-β as the downstream factor of TGF-β and is involved in the pathogenesis of LECs and vascular smooth muscle cells related diseases [17,18], while gremlin enhances the effect of TGF-β in fibrosis and transdifferentiation as an antagonist of BMP [12,19].

It has been found that TGF-β2 can induce the secretion of CTGF and gremlin from TM and RPE cells [11,20]. TGF-β2 and CTGF induce the expressions of α-SMA, COL-I, and Fn in LECs [4,21] and promote the synthesis of ECM in optic nerve astrocytes, in which the expressions of COL-I and Fn are increased over the time; both factors also enhance the expression of matrix metalloproteinases (MMP) [22].

Fn and COL-I are the main components of the ECM and also associated with cell surface, forming a network structure to provide a scaffold for cell migration, and playing critical roles for the migration of transdifferentiated cells [23]. In this study, we have shown that TGF-β2 is able to promote the expressions of CTGF, gremlin and these ECM proteins in HLECs in a dose-dependent manner. And CTGF and gremlin can individually induced the expression of ECM proteins in HLECs. Furthermore, all three molecules were able to induce the expression of α-SMA, a key molecule involved in the differentiation of fibroblasts to myofibroblasts in granulation tissue, tissue fibrosis and during the EMT of LECs. E-cadherin is an adhesion molecule of epithelial cells involved in the formation and maintenance of the connection between normal cells, and thus plays an important role in the maintenance of morphology and structural integrity of normal epithelial cells. Decreased expression of E-cadherin would reduce the adherence between cells, which likely leads to the destruction of the integrity of HLECs [24]. The data from this study suggest that the expression of E-cadherin was significantly decreased in HLECs after treatment with TGF-β2, CTGF or gremlin. These results indicate that TGF-β2, CTGF and gremlin may promote EMT and ECM synthesis through inhibiting E-cadherin.

While TGF-β2 induces the expression of gremlin and CTGF, which then further enhance the expression of α-SMA, Fn and COL-I, it is likely that CTGF and gremlin are potential mediators of TGF-β2-induced expression of EMT-associated proteins and ECM synthesis as the downstream factors of TGF-β2, thus involved in the PCO. By knocking down the endogenous expression of either CTGF or gremlin or both using siRNA, our data revealed that expression of TGF-β2-induced ECM proteins in HLECs were significantly reduced.

Previous studies have demonstrated that specifically blocking CTGF and gremlin can inhibit the profibrotic effect of TGF-β2 on astrocytes in optic nerve and on renal tubular epithelial cells

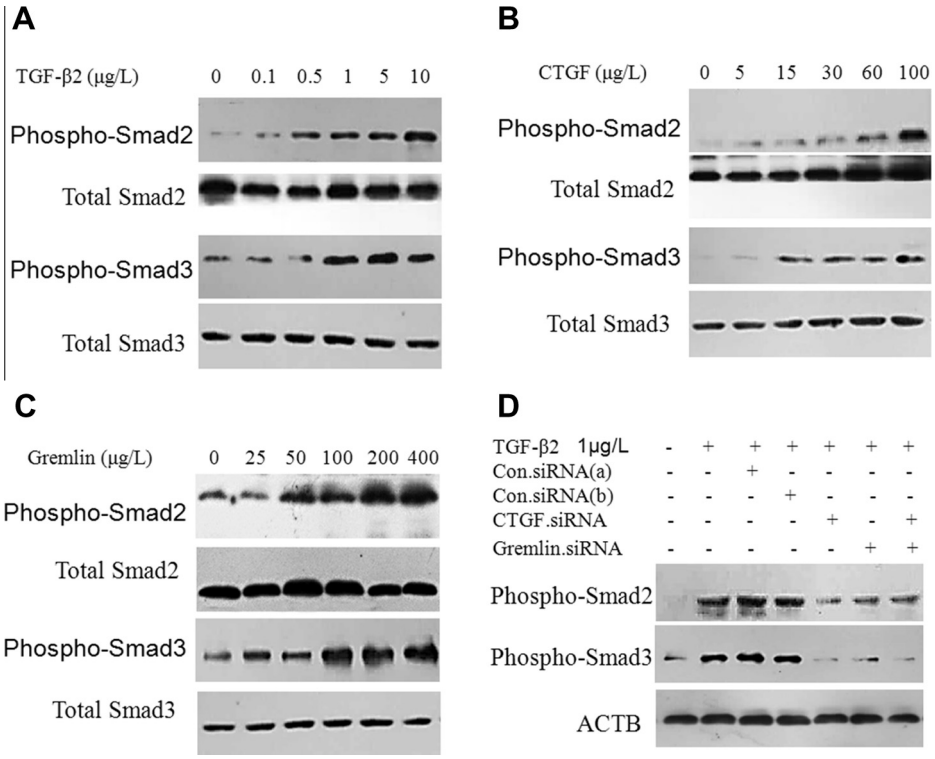

Fig. 3. Detection of Smad2 and Smad3 activation in HLECs by Western-blot. (A–C) The total and phosphorylated Smad2 and Smad3 proteins in HELCs after treatment with TGF-β2 (0–10 μg/L), CTGF (0–100 μg/L), or gremlin (0–400 μg/L) for 120 min; (D) the phosphorylated Smad2 and Smad3 proteins in HLECs transfected with CTGF. siRNA- and/or Gremlin. siRNA lentivirus in the presence of 1.0 μg/L TGF-β2 for 120 min.

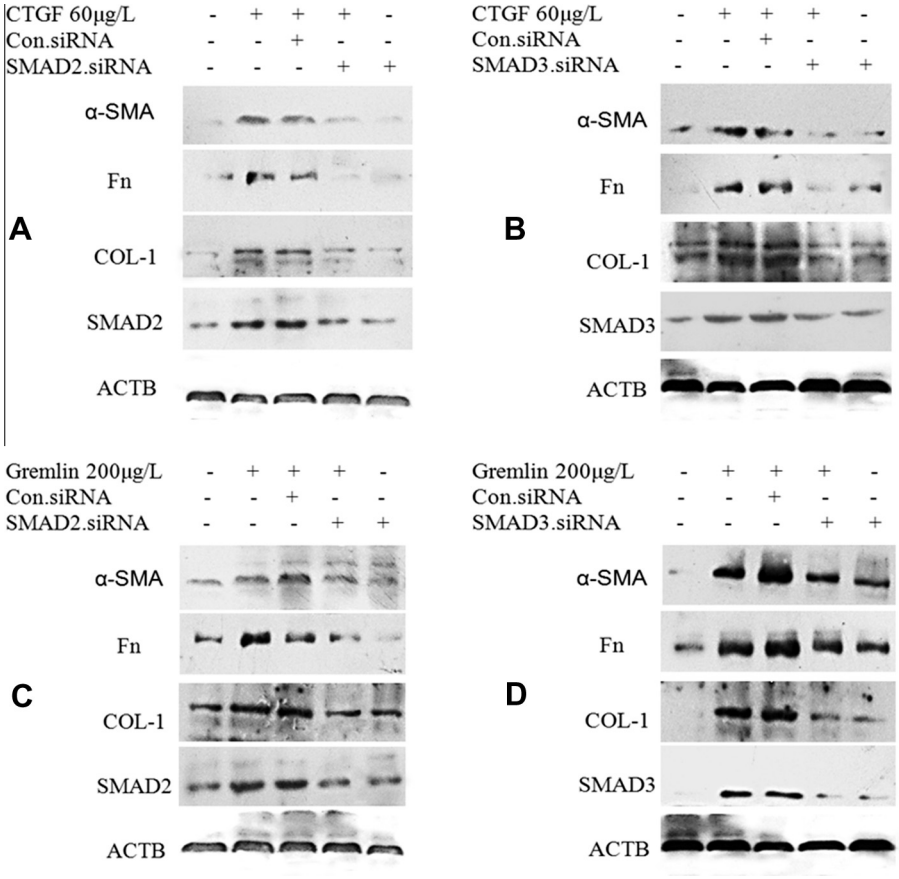

Fig. 4. Effects of smad2.siRNA and smad3.siRNA on CTGF and gremlin induced expressions of α-SMA and ECM proteins. (A, C) CTGF and gremlin induced expression of α-SMA, Fn and Col-1 proteins in HLECs with Smad2.siRNA. (B, D) CTGF and gremlin induced expression of α-SMA, Fn and Col-1 in HLECs with Smad3.siRNA.

[22,25,26]. Sethi et al. reported that TGF-β2 and gremlin are mutually regulated as TGF-β2 increased TGF-β2 expression, and TGF-β2 also increased gremlin expression in TM cells [11]. However, the relationship between gremlin and CTGF in HLECs has not been reported. Our result showed that gremlin induced CTGF expression, and visa versa CTGF also increased gremlin expression in HLECs. It has been speculated that gremlin and CTGF are involved in a "feed-forward" pathogenic pathway [11], which would further exacerbate ECM deposition and lead EMT within HLECs. We suggest that the process of TGF-β2 promoting EMT and ECM synthesis in HLECs is an integrated outcome of actions of multiple factors and signaling pathways, and inhibition of EMT and ECM synthesis may be achieved by blocking or suppressing multiple factors/pathways simultaneously.

It has been demonstrated that TGF-β, CTGF, and gremlin are functionally connected to Smad signaling [11,27]. Our data show that TGF-β2, gremlin and CTGF dose-dependently induced the activation of Smad2 and Smad3 in HLECs, and silencing CTGF and gremlin, individually or in combination, effectively blocked the activation of TGF-β2 induced phosphorylation of Smad2 and Smad3. Regarding to TGF-β2-induced expression of EMT-associated proteins and ECM synthesis in HLECs, similar effect was observed between silencing CTGF or gremlin, or in combination in inhibiting the TGF-β2/Smad signaling. The data suggest that CTGF and gremlin may function sequentially in activating Smad signaling pathway, and interference at either stages that governed by them can effectively inhibit the activation of Smad pathway.

Our results show that TGF-β2 can induce HLECs to express CTGF and gremlin. Furthermore, TGF-β2, gremlin and CTGF can activate Smad signaling pathway in HLECs, and silencing CTGF or gremlin effectively inhibit the TGF-β2/Smad signaling pathway. TGF-β2/Smad signaling pathway involves phosphorylation of Smad2 and -3, which, together or individually, form a complex with co-Smad4 to up-regulate transcription of their target genes including α-SMA, Fn and COL-I [28]. Thus it is likely that TGF-β2 induces the expressions of CTGF and gremlin through Smad mediated signaling pathway. This is confirmed by our experiment with siRNA of Smad2 and Smad3 in HLECs, knocking down the endogenous Smad2 and 3 significantly reduced or not abolished CTGF and gremlin-induced expression of EMT-associated proteins such as α-SMA, Fn and COL-I. To our knowledge, this is the first report demonstrating that the Smad signaling pathway is involved in CTGF- and gremlin-induced expression of EMT-associated proteins and ECM synthesis in HLECs. As siRNA.Smad2 and siRNA.Smad2 did not completely abolish the expression of EMT proteins, other signaling pathways such mitogen-activated protein kinase (MAPK) pathway and PI3K/Akt pathway are also likely to be involved.

In summary, the data from other reports and our own study have suggested a possible mechanism of TGF-β2 function in PCO: cataract surgery activates the originally non-active TGF-β2 in the aqueous humor and lens [29] and increases the level of TGF-β2 expression in the anterior chamber [30]. Activated TGF-β2 subsequently induces the expressions of CTGF and gremlin in LECs, which in turn suppress protective proteins such as BMPs and E-cadherin, and activate the Smad signaling pathway, thereby inducing transdifferentiation of LECs into fusiform myofibroblast. CTGF and gremlin further promote the proliferation and ECM synthesis in transdifferentiated cells, leading to the formation of plaque-like aggregation and excessive ECM production and accumulation, finally resulting in PCO.

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