• Original Article •

Transfection of CTGF siRNA inhibits transdifferentiation in human lens epithelium cell line B3 *in vitro*

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Abstract

• AIM: To investigate the expression of connective tissue growth factor (CTGF) and α-smooth muscle actin (α-SMA) in human lens epithelium cell (HLEC) line B3 after transfection by liposome-coated small interfering RNA (siRNA) targeting CTGF.

• METHODS: HLECs were transfected with siRNA targeting CTGF, labeled with 5'-fluorescein isothiocyanate (5'-FITC) and coated with lipofectamine. The transfection ratio was evaluated *via* fluorescence intensity. Cell counting kit-8 (CCK-8) assay was performed to assess cytoviability of both non-transfected and transfected HLECs. Quantitative reverse transcription-polymerase chain reaction (RT-PCR), cell immunochemistry and Western blot analysis were conducted to detect the expression changes of CTGF and α-SMA after transfection.

• RESULTS: A highly effective transfection ratio was observed in siRNA co-transfected with lipofectamine. The transfection ratio reached 95% at 24h. The proliferation of HLECs was inhibited by siRNA after 72h transfection. The expression of CTGF and α-SMA significantly decreased in HLECs after transfected by CTGF siRNA for 24h. This effect was not found in negative control siRNA.

• CONCLUSION: siRNA targeting CTGF decrease CTGF and α-SMA expression in HLECs, which is a potential therapeutic strategy for posterior capsular opacification.

• KEYWORDS: connective tissue growth factor; small interfering RNA; lipofectamine; transdifferentiation
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INTRODUCTION

Posterior capsule opacification (PCO) is a common complication of cataract phacoemulsification surgery. It acutely affects vision. It is caused mainly by proliferation and differentiation of residual lens epithelial cells (LECs) migration and epithelial mesenchymal transition (EMT). EMT, in turn, is characterized by decreased expression of E-cadherin and increased expression of α-smooth muscle actin (α-SMA)[1]. PCO manifests elschnig-pearl, peripheral Soemmering's ring (central PCO in the visual axis), cortex proliferation and cholesterol crystal in some cases. It responds to neodymium-doped yttrium aluminium garnet (YAG) laser capsulotomy quite well and the eyesight can be restored effectively. The operation, however, has vision-related complications, and may damage the intraocular lens (IOLs).

TGF-β, on the other hand, can activate proliferation and promote stromal-derived cells such as fibroblasts, smooth muscle cells. They can also aid cell proliferation, differentiation, adhesion and other important physiological activities[2]. It has been known that neutralizing the expression of connective tissue growth factor (CTGF) can significantly block the TGF-β_2-induced EMT synthesis due to failure to activate Smad signaling pathway in HLECs[3]. TGF-β_2, CTGF and gremlin have been demonstrated to interact in the course of EMT in different tissues and organs, and TGF-β_2 can increase the expression of CTGF and gremlin in different tissue[4]. Previous studies have testified that we could inhibit the EMT of TGF-β_2 on astrocytes and renal tubular epithelial cells by specifically blocking CTGF and gremlin[5-6]. TGF-β_2 is regarded as a prime factor leading to PCO[7-9]. The effect of TGF-β_1 on HLECs, however, stays unknown to us.

CTGF is one of the downstream products of TGF-β_1[10]. It is a chief variable in the induction of EMT, proliferation and the transdifferentiation of residual LECs[11]. It was detected that there were increased expression of CTGF-mRNA accompanied with collagen I and α-SMA in the residual debris of PCO[12].

α-SMA is an important sign of EMT and extracellular matrix (ECM) synthesis in HLECs. E-cadherin and α-SMA help mediate cell-matrix adherence and myofibroblast[13]. Both E-cadherin and α-SMA involve in EMT in HLECs[14]. The complicated relationship among TGF-β_1, CTGF and α-SMA, nevertheless, has not been clarified.

In this study, we were interfering with the growth of HLECs by liposomal transfection of CTGF siRNA *in vitro* cell culture experiment to explore the interaction among TGF-β_1, CTGF and PCO.

MATERIALS AND METHODS

Culture and Treatment of HLECs HLEC line-B3 was purchased from ATCC (Manassas, VA, USA). HLEC-B3 is adherent cell. They were seeded into culture dishes with DMEM containing 5% fetal bovine serum (FBS). The HLECs were synchronized by replacing the nutrient medium with serum-free DMEM and cultured for 24h when the cells were 75% confluence.

siRNA Preparation CTGF specific small interfering RNA (CTGF.siRNA) was purchased from Dharmacon (Lafayette, USA). Control non-targeting pool siRNA (Dharmacon) was used as the transfection control (con.siRNA). The concentration of CTGF.siRNA and con.siRNA were diluted to 100 nmol/L.

Measurement of Transfection The cells in the experimental group were then treated with 3 mL of serum-free medium containing TGF-β_1 and 4 μg CTGF.siRNA marked with 5'-FITC and then mixed with 50 μL Opti-MEMI. The mixture was treated with lipofectamine RNAiMAX for a further 12, and 24h before cells were harvested for further analysis.

Cell Counting Kit-8 Assay The effect of CTGF.siRNA on proliferation of HLECs was detected by cell counting kit-8 (CCK-8) assay (Beyotime Company, ShangHai, China) to evaluate the effect on cell proliferation. HLECs were cultivated in DMEM medium with CTGF.siRNA or negatives control siRNA 8.0 μL, and then mixed with 50 μL Opti-MEMI. The mixture was treated with lipofectamine RNAiMAX and then seeded into 96-well plates at a density of 5000 cells/well. After that, the mixture was cultured for 24, 48, and 72h respectively. At the said time points, 100 μL DMEM and 10 μL CCK-8 were added to each well, and the cells were incubated for additional 2h at 37℃. After the supernatant was removed, the absorbance at 450 nm wavelength was recorded by a microplate reader (Bio-Rad Laboratories, California, USA).

HLECs Grouping Methods Mock group (C group): HLECs were cultivated in DMEM medium with high glucose and mixed with 50 μL Opti-MEMI. The mixture was treated with lipofectamine RNAiMAX (Invitrogen, ShangHai, China) for 24h as per the lipofectamine manufacturer's instructions; TGF-β_1+siRNA group (T+SI group): HLECs were cultivated in DMEM/high glucose medium with TGF-β_1 10 ng/mL and CTGF.siRNA 8.0 μL, and then mixed with 50 μL Opti-MEMI. The mixture was treated with lipofectamine RNAiMAX for 24h; TGF-β_1 group (T group): HLECs were cultivated in DMEM/high glucose medium with TGF-β_1 10 ng/mL, and then mixed with 50 μL Opti-MEMI. The mixture was treated with lipofectamine RNAiMAX for 24h; TGF-β_1+control siRNA group (T+SC group): HLECs were cultivated in DMEM/high glucose medium with TGF-β_1 10 ng/mL and CTGF con.siRNA 8.0 μL, then mixed with 50 μL Opti-MEMI. The mixture was treated with lipofectamine RNAiMAX for 24h.

Quantitative Reverse Transcription-Polymerase Chain Reaction HLECs in culture bottles were washed for four times with DMEM after grouping treatments for 24h. Total HLECs RNAs were extracted by using a Trizol total RNA extraction kit (Invitrogen Company, ShangHai, China) as per the manufacturer's instructions. Reverse transcription was then performed by using cDNA synthesis kit from Fermentas Co., Ltd. (Lithuania). The PCR primers were designed and synthesized by Invitrogen Biotechnology (ShangHai, China) as follows: α-SMA, F 5'-GACAATGGCTCTGGGCTCTGTAA-3' and R 5'-CTGTGCTTCGTCACCCACGTA-3'; CTGF, F 5'-CTTGCGAAGCTGACCTGGAA-3' and R 5'-TCTGTACGCAGGTGATTGGTG-3'. qPCR reaction was performed on Bio-Rad IQ5 thermal cycler (Bio-Rad, California, USA). The results were analyzed with BioQ software to obtain Ct value for each PCR reaction, and $2^{\triangle\triangle Ct}$ method was used to calculate the levels of gene expression.

Cell Immunochemistry The direct visual observation of CTGF and α-SMA protein was performed by immunochemistry after 24-hour grouping treatments. HLECs was plated at a density of 6×10^4 cells/mL. The cells were fixed with 4% paraformaldehyde for 15min. The fixed HLECs cells were permeabilized with 0.1% Triton X-100 in PBS for 10min. The cells were subsequently incubated in 3% H_2O_2 for 10min. The HLECs were blocked in 5% goat serum for 20min and incubated with rabbit anti-human CTGF (1:100 dilution) or mouse anti-human α-SMA (1:100 dilution) overnight. Following three washes with PBS, the slides were incubated with the secondary antibody (polymer helper and then polyperoxidase-anti-mouse/rabbit IgG) for 30min at 37℃. The cells were treated with DAB reagent box (ZSGB-BIO Company, Beijing, China). HLECs were stained with hematoxylin for 20s. The slides were embedded in neutral balsam. The HLECs were seen through a microscope. Representative images were captured with the incorporated digital camera (Olympus image analysis system, Japan). Average optical density (AOD) of the positive were detected and analyzed by image analysis system.

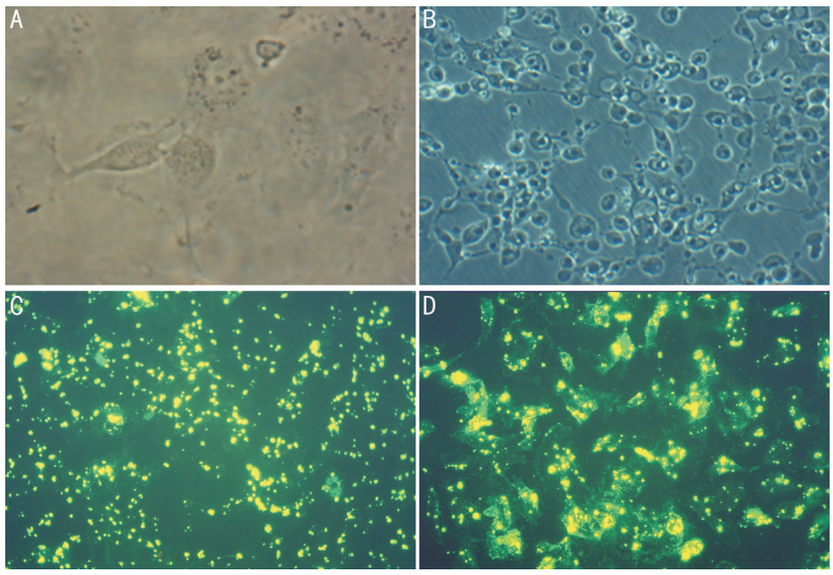

Figure 1 The morphology and fluorescent image of HLECs *in vitro* HLECs were spindled, starred, pyramidal or irregular in shape (A: ×100); The cells cultured under lower osmotic pressure appeared to be swollen and round in shape (B: ×100); The positive rate of siRNA CTGF-5'-FITC transfection with lipofectamine for 12h (C) was 85% and for 24h (D) was 95% (×200).

Western Blot After grouping treatments for 24h, the monolayer cultures were collected with cell scrapers and then lysed with 100 μL of cell lysis buffer on ice for 30min. The cell lysates were centrifuged and supernatants were collected. Total protein was prepared from each group. The protein concentrations in the supernatants were aliquoted and kept using BCA method (Biosynthesis Biotechnology Company, Beijing, China) for further experiments. A total of 50 μg protein per sample was electrophoresed by 10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Millipore Corp., Massachusetts, USA). It was blocked with 5% skimmed milk for 1h at room temperature and incubated overnight at 4°C with primary antibody specific to CTGF (1:2000; Abcam, Shanghai, China), α-SMA (1:1000; Abcam, Shanghai, China), and β-actin (1:1000; Santa Cruz, Shanghai, China). After washed, the membrane was incubated with secondary antibodies (anti-mouse antibody conjugated, Sigma, USA). The membrane was immersed in enhanced chemiluminescence solution, and then exposed to an X-ray film. After the hybridization of secondary antibodies, the resulting images were analyzed with ChemiImager 4000 (Alpha Innotech Corporation, California, USA).

RESULTS

The Morphology Observation of HLECs *in vitro* The HLECs grew in good condition *in vitro* and the positive rate of siRNA CTGF-5'-FITC transfection with lipofectamine was very high (Figure 1).

The Effect of CTGF.siRNA on the HLECs Proliferation The control group and treated groups show no significant difference in 24 or 48h (P>0.05, all datas were analyzed by SPSS software). The proliferation of HLECs after cell culture for 72h had significant difference in both groups (P<0.05). The cell proliferation was significantly inhibited (Figure 2).

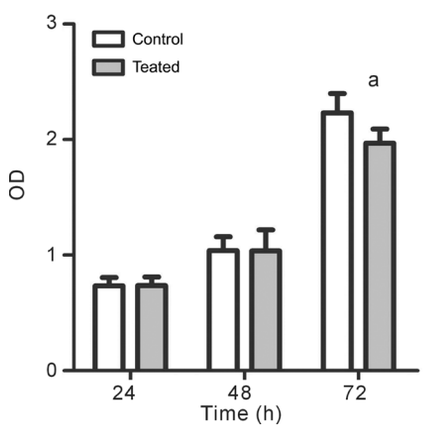

Figure 2 Effect of CTGF.siRNA on the HLECs proliferation [a]P<0.05.

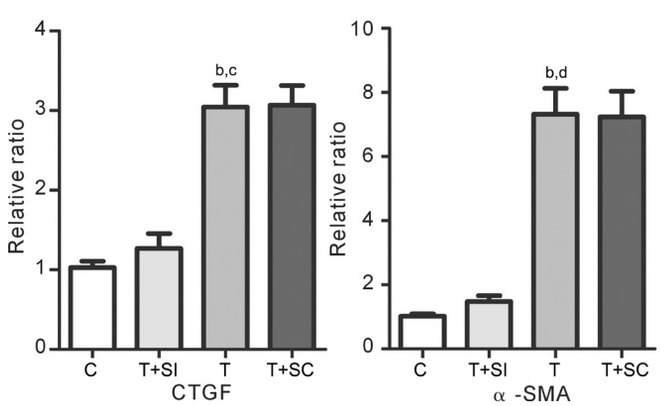

Figure 3 Effect of CTGF.siRNA and con.siRNA on mRNA expression of CTGF and α-SMA analyzed by qPCR [b]P<0.01 *vs* C group; [c]P<0.05, [d]P<0.01 *vs* T+SI group.

The Effect of CTGF.siRNA on mRNA Expression of CTGF and α-SMA After 24-hour treatment of HLECs with TGF-β_1, the mRNA levels of CTGF and α-SMA were significantly up-regulated (Figure 3). In contrast, the increased mRNA levels

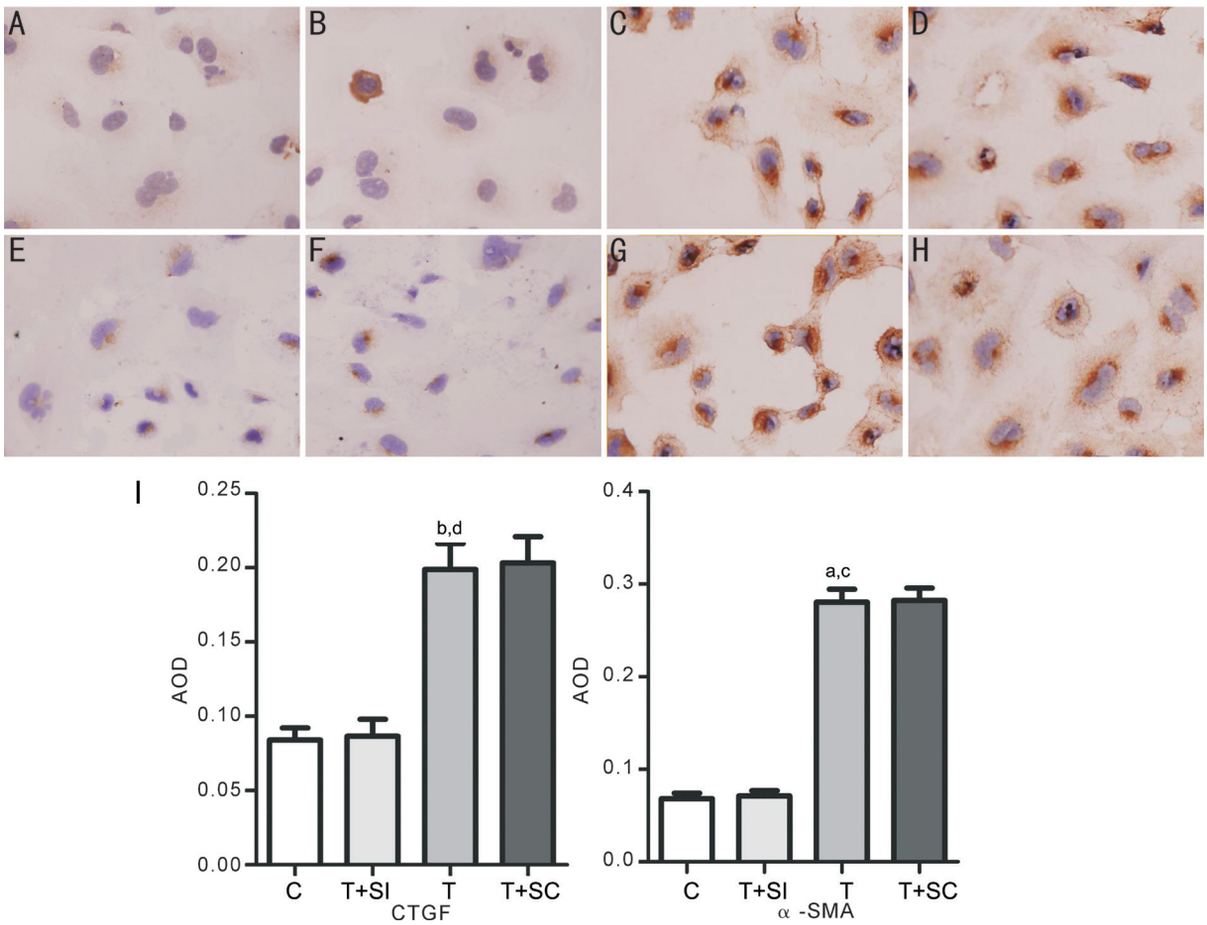

Figure 4 Effect of CTGF.siRNA and con.siRNA on protein expression of CTGF and α-SMA analyzed by cell immunochemistry Brown granules were found in the cytoplasm of HLECs. A, B, C and D respectively indicated CTGF protein expression in C, T+SI, T, and T+SC group, on the contrary, E, F, G, H respectively indicated α-SMA protein expression in C, T+SI, T, and T+SC group. Average optical density of the granules were statistically analyzed (I). [a]*P*<0.05, [b]*P*<0.01 *vs* C group; [c]*P*<0.05, [d]*P*<0.01 *vs* T+SI group.

of CTGF and α-SMA induced by TGF-β_1 was suppressed after 24h treatment with CTGF.siRNA (Figure 3). There was no major difference between the T and T+SC group. It indicated the CTGF con.siRNA had no effect on the mRNA expression of CTGF and α-SMA.

The Effect of CTGF.siRNA on Protein Expression of CTGF and α-SMA Analyzed by Cell Immunochemistry The expression of CTGF and α-SMA were effectively promoted after 24-hour treatment of HLECs with 10 ng/mL TGF-β_1 (Figure 4). The increased levels of CTGF and α-SMA induced by TGF-β_1 were, nonetheless, reduced after 24h of being transfected with CTGF.siRNA (Figure 4). Simultaneously, CTGF con.siRNA had no effect on the expression of CTGF and α-SMA and there was also no significant difference between the C and T+SI group probably due to the insensitivity of cell immunochemistry analysis (Figure 4).

The Effect of CTGF.siRNA on Protein Expression of CTGF and α-SMA Analyzed by Western blot The protein expression of CTGF and α-SMA increased notably after 24h treatment of HLECs with 10 ng/mL TGF-β_1 (Figure 5). However, transfection with CTGF.siRNA effectively suppressed TGF-β_1-induction of CTGF and a-SMA in HLECs (Figure 5). CTGF con.siRNA did not affect the expression of CTGF and α-SMA (Figure 5). And there was no significant difference of the α-SMA between the C and T+SI group. However, CTGF analysis showed the difference between the C and T+SI group (Figure 5).

DISCUSSION

It is widely known that the lens epithelia proliferation and migration from equator of anterior capsular to the center of posterior capsule are the common cytological basis of PCO[15]. The EMT and collagen deposition is also the pathological process in PCO. Antimetabolites such as colchicine, mitomycin (MMC), 5-FU and daunorubicin were used to treat PCO. They have highly inhibited the growth of LECs. However, antimetabolites do have side-effect on exposed cells and create numerous toxic actions to human tissues. YAG laser capsulotomy can quickly and effectively restore vision. The operation, however, might lead to severe complications such as breakup of anterior vitreous membrane, posterior vitreous detachment, retinal detachment and macular edema. Besides, postoperative residual LECs could be activated by surgical

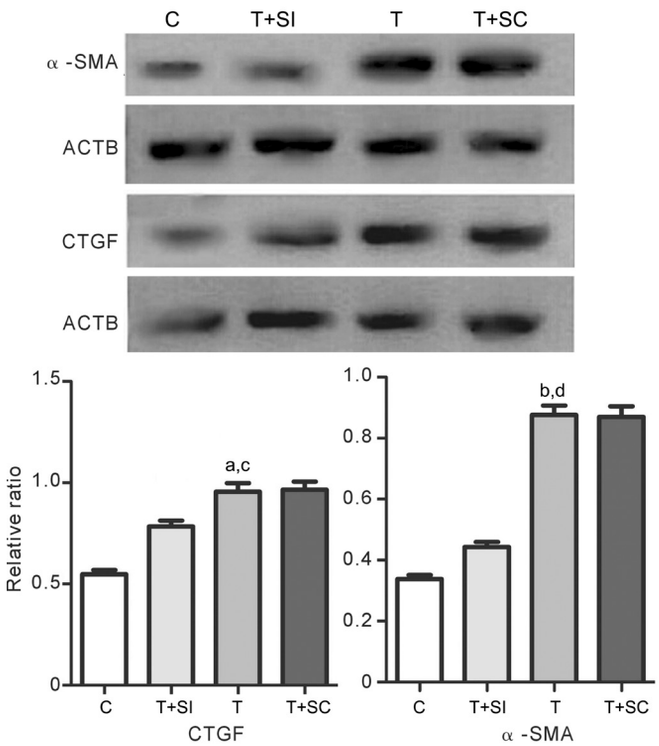

Figure 5 The effect of CTGF.siRNA and con.siRNA on protein expression of CTGF and α-SMA analyzed by Western blot
[a]*P*<0.05, [b]*P*<0.01 *vs* C group; [c]*P*<0.05, [d]*P*<0.01 *vs* T+SI group.

stress. PCO, consequently, will be exacerbated[16]. On the other hand, infant surgery toward PCO might also fail due to non-compliance. New therapy, therefore, need to be developed to treat this problem.

It has been demonstrated that TGF-β, CTGF, and gremlin are connected to Smad signaling[17-18]. As an activating factor, TGF-β unites with TβR-II (one of TGF-βs ligands). Then the conformation changes to compose TβR-II-TGF-β-TβR-I tripolymer. The tripolymer transmits signals to HLECs nucleus by Smad pathway to active the following biological effects. CTGF, as a downstream cytokine of Smad pathway, enhances the effect of proliferation and fibrosis. CTGF stimulates ECM synthesis in HLECs transdifferentiated cells, leading to the formation of plaque-like aggregation and excessive ECM production. The mechanism how Smad proteins interact with CTGF, however, is very complicated. It is associated with different cell types and microenvironments. The mechanism, therefore, should be explored by different biotechnology methods.

There are also other signaling pathways, including ERK1/2, p38 MAPK, JNK, STAT3 and PKC. The pathways are involved in the TGF-β_1-induced up-regulation of CTGF expression in other cell types[19-22]. Many transcription factors and microRNAs are also involved in the various growth factors, including cytokines and hormones that regulate CTGF expression[23]. Among them, TGF-β_1 can profoundly up-regulate CTGF expression in many different cell types[24]. CTGF can promote cell mitosis and proliferation of fibroblasts as well as synthesize collagen, mediate cell adhesion, enhance fibrosis, and regulate ECM synthesis[25-26]. TGF-β and CTGF are catalytic in transdifferentiation of intraocular LECs in the eye[27-28].

In qPCR, cell immunochemistry and Western blot experiment, the expression of CTGF and α-SMA had been significantly increased by transfection with 10 ng/mL TGF-β_1 for 24h compared to control group. It may support the conclusion that the expression of CTGF and α-SMA could be remarkably increased by TGF-β_1. However the induction effect of TGF-β_1 could be inhibited by 30 μg/mL CTGF.siRNA on the siRNA group and it may manifest that the induction to expression of CTGF and α-SMA by TGF-β_1 could be blocked by siRNA at mRNA and protein level. On the SC group, there was no significant difference of the expression of CTGF and α-SMA compared to TGF-β_1 group. It was indicated in our experiment that CTGF.siRNA specially inhibited HLECs transdifferentiation induced by TGF-β_1. We could get the conclusion that cells transdifferentiation is stimulated by CTGF pathway. It is induced by TGF-β_1 Smad signal ways. The synthesis of α-SMA is, furthermore, mediated by CTGF. CTGF is a downstream mediator of TGF-β_1. It regulates some of biological functions. In our knowledge, this is the first study clarifying the relationship among TGF-β_1, CTGF and α-SMA in HLECs. As α-SMA is an important sign of EMT, CTGF. siRNA could stop the EMT process induced by TGF-β_1.

In addition, there was also no significant difference of CTGF and α-SMA expression between the C and T+SI group by cell immunochemistry analysis. Western blot, however, showed the difference of CTGF expression. The discrepancy may be related to the low sensitivity of cell immunochemistry analysis. In summary, cell immunochemistry may be more presentational and easier to perform. Western blot, however, is much more accurate when quantitative eye research is needed.

In the experiments toward HLECs *in vitro*, when it reached 24h, transfection approached to the peak when there were tiny fluorescent particles in cytoplasm of 95% cells. It means that Opti-MEMI and lipofectamine can be effectively applied on the transfection of siRNA in HLECs.

Our experiments, moreover, manifested that the effect of siRNA on HLECs proliferation showed no significant difference compared to negatives con.siRNA until it reached 72h. It suggested siRNA at 72h started to work and specially inhibited the HLECs proliferation.

In conclusion, learning from the data of other researches and our study, we have explored a possible mechanism of TGF-β_1 function on PCO. Cataract surgery may elevate the level of TGF-β in aqueous humor and lens and increase TGF-β expression in anterior chamber[29]. Activated TGF-β_1

subsequently increases the expressions of CTGF in HLECs. It, in turn, activates proliferation and transdifferentiation of LECs into myofibroblast. Our study demonstrated that CTGF.siRNA had notable effect on the EMT induced by TGF-β_1 in HLECs. It, therefore, may be a new strategy to inhibit the expression of CTGF and to prevent and treat anterior subcapsular cataract. CTGF.siRNA, furthermore, may prevent the PCO infants from reoperations to remove the cloudy posterior capsule.

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