·Basic Research·

Expression of transcription factors Slug in the lens epithelial cells undergoing epithelial-mesenchymal transition induced by connective tissue growth factor

Ying-Na Wang[1], Li Qin[2], Jing-Ming Li[2], Li Chen[2], Cheng Pei[2]

[1]Medical School of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
[2]Department of Ophthalmology, the First Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Correspondence to: Cheng Pei. Department of Ophthalmology, the First Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. peich71@163.com
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Abstract

• **AIM: To investigate the expression of transcription factors Slug in human lens epithelial cells (HLECs) undergoing epithelial-mesenchymal transition (EMT) induced by connective tissue growth factor (CTGF).**

• **METHODS: HLECs were treated with CTGF of different concentrations (20, 50 and 100 ng/mL) or without CTGF (control) for 24h. The morphological changes of HLECs were analysed by microscopy. The expression and cellular localization of Slug was evaluated by immumo-fluorescence. Expressions of Slug, E-cadherin and alpha smooth muscle actin (α-SMA) were further determined by Western blot analysis.**

• **RESULTS: HLECs showed spidle fibrolasts-like characteristics and loosely connected each other after CTGF treatment. The immuno-fluorescence staining indicated that Slug was localized in the nuclei and its expression was induced by CTGF. The relative expressions of Slug protein were 1.64±0.11, 1.96±0.03, 3.12±0.10, and 4.08±0.14, respectively, in response to control group and treatment with CTGF of 20, 50 and 100 ng/mL (F=443.86, P<0.01). The increased Slug protein levels were correlated well with up-expression of α-SMA (0.78±0.05, 0.85±0.06, 2.17±0.15, 2.86±0.10; F=449.85, P<0.01) and down-expression of E-cadherin (2.50±0.11, 1.79±0.26, 1.05±0.14, 0.63±0.08; F=101.55, P<0.01).**

• **CONCLUSION: Transcription factor Slug may be involved in EMT of HLECs induced by CTGF *in vitro*.**

• **KEYWORDS:** transcription factors Slug; human lens epithelial cells; connective tissue growth factor; epithelial-mesenchymal transition; alpha smooth muscle actin; adhesion molecules E-cadherin

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INTRODUCTION

Cataract is the most common cause of blindness and is conventionally treated with surgery [1]. In recent years, the cataract patients tend to be younger, who have the greater potential of developing postoperative posterior capsular opacification (PCO) [2]. At present, neodymium: yttrium aluminum garnet (Nd:YAG) laser is routinely performed to disrupt the opacified posterior lens capsule. However, this treatment can cause secondary damage to intraocular lens resulting in iridemia and macular oedema[3,4]. Therefore, it is desirable to search for non-invasive early prevention methods of PCO.

There is evidence that PCO is directly caused by transdifferentiation of lens epithelial cells in the capsule after cataract surgery [5]. We have previously shown that connective tissue growth factor (CTGF) plays a key role in the transdifferentiation of lens epithelial cells [6]. CTGF also known as CCN2 is a matricellular protein of the CCN family[7]. CTGF plays important roles in many biological processes, including cell adhesion, migration, proliferation, angiogenesis, skeletal development, and tissue wound repair, and is critically involved in fibrotic disease and several forms of cancers [8,9]. ven Setten *et al*[10] had extracted CTGF from the aqueous humor of the volunteers. Wunderlich *et al*[11] have discovered that CTGF mRNA was detected in human cataractous plaques of anterior subcapsular cataract (ASC) and human PCO membranes and appeared simultaneously with the expression of type I collagen, alpha smooth muscle actin (α-SMA) and tenascin. This suggests a significant role of CTGF in the pathological course of these ocular disorders.

We have hypothesized that CTGF acts on Slug to dictate epithelial-mesenchymal transition (EMT) process because there is also evidence that Slug is involved in regulation of EMT process in many kinds of cells [12]. Slug is the member of Snail family. It is a highly conserved transcription factor

containing zinc finger structure. In recent years many studies have indicated that Slug involved in the process of EMT of all kinds of cells and was an important regulatory factor[13]. In the development of embryonic mouse heart, Slug participates in atrioventricular canal and the formation of outflow of the endocardial cushions. Upon Slug depletion, mouse embryos of 9.5d suffer endocardial cushions dysplasia [14]. In the fibrosis diseases, Slug involves in the EMT of alveolar epithelial cells and results in pulmonary fibrosis[15]. Slug also participates in the formation of corneal scarring and proliferative vitreoretinopathy (PVR) [16,17]. Slug binds the E-box near the promoter of E-cadherin, preventing the transcription of E-cadherin, in turn reduce the cell adherence[18]. Albeit the existing evidence in other cell-types, Slug's role in PCO formation due to EMT in human lens epithelial cells (HLECs) has not been demonstrated. In this study, we have taken advantages of CTGF that is potent in inducing EMT in HLECs. We have investigated the connection of Slug expression and distribution in HLECs in response to CTGF treatment and EMT induction. Additionally, we have analyzed the expression of E-cadherin and α-SMA, which play important roles in mediating cell-matrix adherence, and myofibroblast, respectively. Both E-cadherin and α-SMA have been implicated in EMT in HLECs[19].

MATERIALS AND METHODS

Cells and Cell Culture Human lens epithelial cell-line SRA01/04 was purchased from ATCC (Manassas, USA). Cells were cultured with modified alpha-Minimum Essential medium (α-MEM; Hyclone, Logan, Utah, USA) supplemented with 1% sodium pyruvate, 1% non-essential amino acid (Gibco, Carlsbad, USA), penicillin (100 U/L), streptomycin (100 U/L) and 10% fetal bovine serum (FBS; Gibco, Carlsbad, USA) in humidified 5% CO_2 at 37℃. Spent medium was discarded when cells were approximately 80% confluence. Cells were then cultured in serum-free medium supplemented with various concentrations of CTGF for 24h. Cell morphological changes were observed under microscope.

Reagents and Antibodies Recombinant human CTGF was purchased from Peprotech (Rocky, Hill, NJ, USA). Rabbit anti-human E-cadherin and anti-human Slug monoclonal antibodies were purchased from Cell Signaling (Beverly, MA, USA) Mouse anti-human α-SMA monoclonal antibody was purchased from Millipore (Billerica, MA, USA). Fluorescent isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were from Biosynthesis Biotechnology (Beijing, China)[20].

Immunocytochemistry HLECs were grown on glass coverslips in 6-well culture plates in the presence or absence of CTGF (50 ng/mL) for 24h. Cells were washed with PBS and fixed in 3.7% paraformaldehyde for 10min, and permeabilized with 0.5% Triton X-100 in phosphate buffer saline (PBS) for 10min at room temperature. Cells were incubated with 10% bovine serum albumin (BSA) in PBS for 1h after thorough wash with PBS and then incubated with a monoclonal anti-Slug antibody (1:50 dilution) or anti-E-cadherin antibody (1:50 dilution) in a humid chamber overnight at 4℃. After washing with PBS, cells were incubated with anti-rabbit IgG antibodies conjugated with FITC for 1h at room temperature. Cells were counterstained with 4,6-diamino-2-phenylindole (DAPI) and mounted on glass slides. Fluorescent images were obtained using a fluorescence microscope (IX71; OLYMPUS, Tokyo, Japan).

Immunoblotting HLECs were seeded in 6-well culture plates and treated with different concentrations (20, 50 and 100 ng/mL) of CTGF or without CTGF (control) for 24h. HLECs were lysed in ice-cold RIPA lysis buffer containing 1% protease inhibitors (Gibco, Carlsbad, USA). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). After blocking with 5% nonfat dry milk in Tris buffered saline solution with 0.1% Tween-20 (TBST) for 1h at room temperature, membranes were incubated overnight with rabbit anti-human E-cadherin, or anti-human Slug, or mouse anti-human α-SMA, anti-human β-actin primary antibodies at 4℃. All antibodies were diluted 1:1000 in TBST containing 1% BSA. Nitrocellulose membranes were washed four times in TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:6700; Cwbio, Beijing, China) or goat anti-rabbit IgG HRP (1:6700; Cwbio, Beijing, China) for 1h at room temperature. After four washes in TBST, specific bands were detected using SuperSignal West Dura (Thermo, USA) for chemiluminescence and exposed to X-ray film (Kodak, Rochester, USA) in dark room.

Image Acquisition and Statistical Analysis The protein expression levels were measured using densitometry with Image J software. Statistical analysis used SPSS18.0 statistical software for data analysis. The experimental data were expressed as mean ±SD. All the experiments were repeated at least 3 times.

Statistical Analysis Statistical analyses were performed using one-way ANOVA test and Turkey HSD when comparing two groups. The correlation analyses of Slug expression with α-SMA and E-cadherin expression induced by CTGF were performed using Pearson correlation analysis. Statistical differences were considered significant at $P<0.05$.

RESULTS

Connective Tissue Growth Factor Treatment Causes Human Lens Epithelial Cells Morphological Change HLECs normally form polygonal monolayer under microscope (Figure 1A). After treatement with 50 ng/mL of CTGF, cells become spindle fibroblasts-like and have gaps between them (Figure 1B). This cell morphological change became more pronounced when cells were treated with 100 ng/mL CTGF. Cells were also became larger and more losely adhered with each other (Figure 1C).

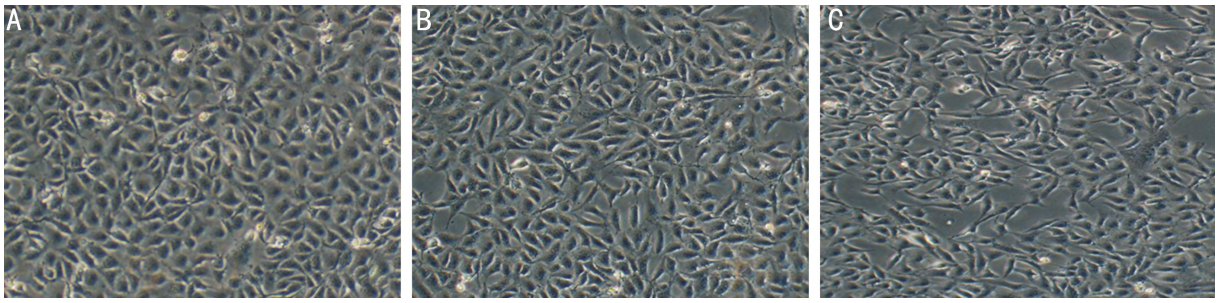

Figure 1 The morphological change of HLECs induced by CTGF A: Untreated HLECs show characteristic polygonal monolayer; B: CTGF (50 ng/mL) treated HLECs show long spindle, losing close patchy distribution; C: CTGF (100 ng/mL) treated HLECs show elongation and become much larger than untreated cells.

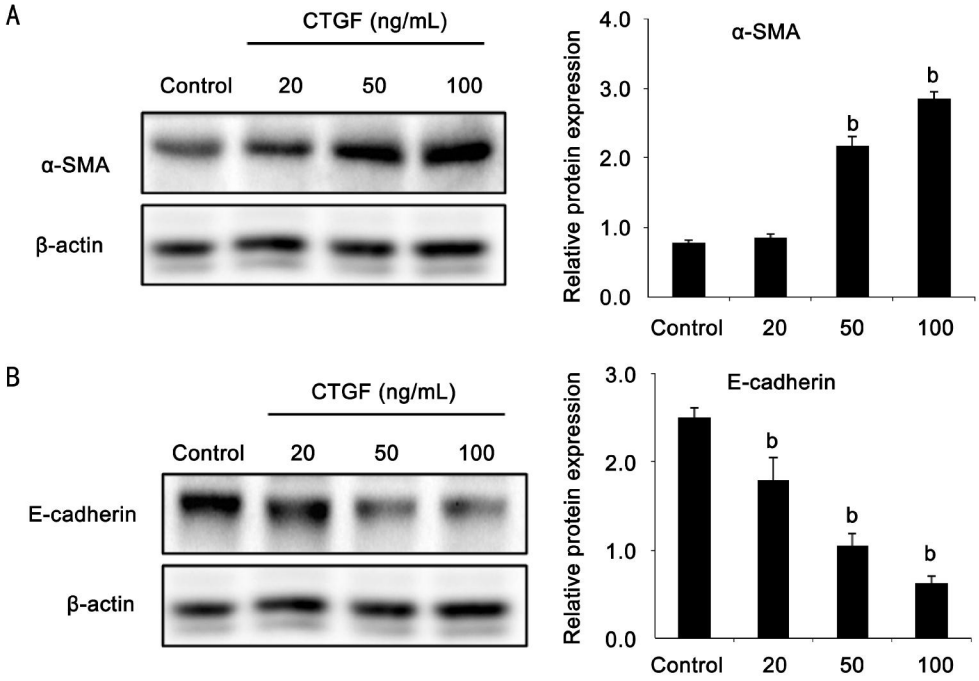

Figure 2 Expression of E-cadherin and α-SMA in HLECs induced by CTGF A: Western blot using anti-α -SMA and anti-β-actin antibodies (left penal) and quantitative analysis using densitometry (right penal). The relative expression of α-SMA in HLECs were 0.78±0.05, 0.85±0.06, 2.17±0.15, and 2.86±0.10, respectively in control and under 20, 50, and 100 ng/mL of CTGF treatment (F=449.85, P<0.01; n=4). B. Western blot using anti-E-cadherin and anti-β-actin antibodies (left penal) and quantitative analysis using densitometry (right penal). The relative expression of E-cadherin in HLECs were 2.50±0.11, 1.79±0.26, 1.05±0.14, and 0.63±0.08, respectively in control and under 20, 50, and 100 ng/mL of CTGF treatment (F=101.55, P<0.01; n=4), ^{b}P<0.01 *vs* control.

Connective Tissue Growth Factor Induced Up- and Down-expression of α-SMA and E-cadherin in Human Lens Epithelial Cells Respectively EMT is a process with characteristic of reduced expression of E-cadherin and increased expression of α-SMA [21]. Therefore, we investigated the expression of these two proteins using Western blot. The results showed that CTGF could induce HLECs to up- and down-express of α-SMA and E-cadherin respectively (Figure 2). Over-expression of α-SMA was observed when cells were treated with CTGF in a dose-dependent manner (Figure 2A). In contrast, down-expression of E-cadherin was observed when cells treated with CTGF, also in a dose-dependent manner (Figure 2B).

Connective Tissue Growth Factor Induced Up-expression of Slug in Human Lens Epithelial Cells Next, we investigated whether CTGF can induce expression of Slug in HLECs induced by CTGF because there is evidence that Slug is involved in regulation of EMT process in many kinds of cells [12]. HLECs were treated with different concentration of CTGF the same way as described above using Western blots and densitometry. Cells showed a statistical significant up-expression of Slug (P<0.01) when treated with as little as 20 ng/mL of CTGF (Figure 3). Pronounced up-expression was observed when cells treated with 50 and 100 ng/mL of CTGF (P <0.001). The expression of Slug was highly positive correlated with α-SMA (Pearson correlation coefficient: r=0.98, P<0.001) and highly negative correlated with E-cadherin (Pearson correlation coefficient: r=-0.94; P<0.001).

Distribution of Slug in Human Lens Epithelial Cells Induced by Connective Tissue Growth Factor We investigated the distribution of Slug in HLECs using immunocytochemistry. We treated HLECs with CTGF (50 ng/mL) and stained cells with anti-Slug antibody as well

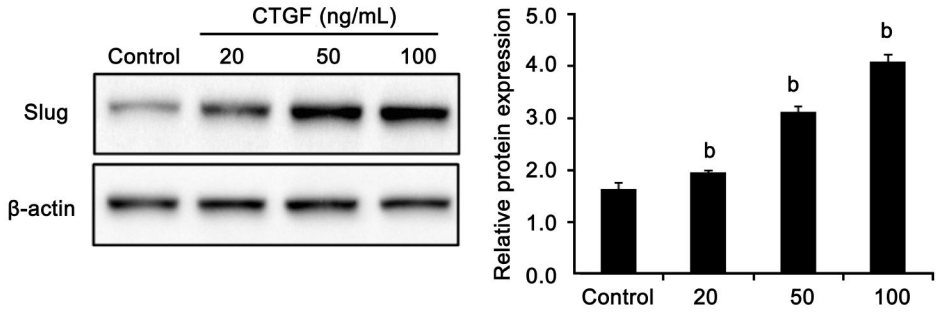

Figure 3 Expression of Slug in HLECs induced by CTGF Western blot was done with anti-Slug antibody (left penal) and quantitative analysis was done using densitometry (right penal). Under 20, 50, 100 (ng/mL) of CTGF, the relative expression of Slug in HLECs were 1.96±0.03, 3.12±0.10, 4.08±0.14 whereas the control group was 1.64±0.11 (F=443.86, P<0.01; n=4), ^bP<0.01 *vs* control.

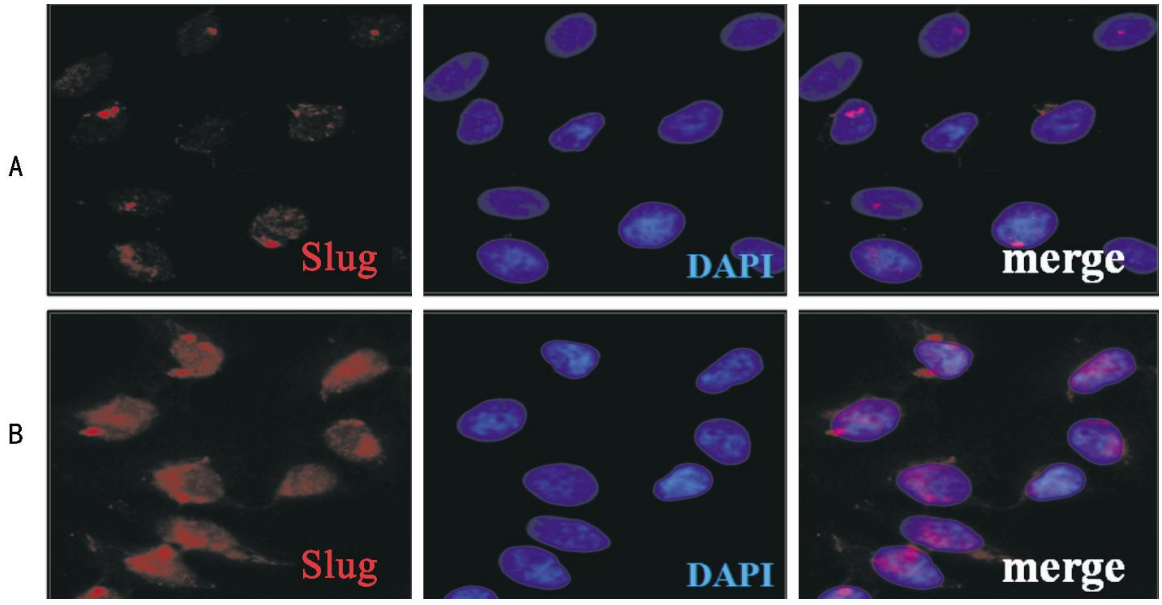

Figure 4 The distribution of Slug in HLECs induced by CTGF Slug was detected with anti-Slug monoclonal anti-body and anti-rabbit antibody conjugated with FITC. Cell nuclei were stained with DAPI. A: In control untreated cells, there was very little Slug being detected; B: Slug protein was exclusively co-localized with DAPI within nuclei in CTGF treated cells.

as DAPI. Under fluorescent microscope, Slug was found exclusively co-localized with DAPI within cell nuclei in CTGF treated cells (Figure 4B). In contrast, in control untreated cells, there was very little Slug being detected (Figure 4A).

DISCUSSION

It is generally believed that the mechanism of PCO formation was wound healing, in response to postoperative residual lens epithelial cells activated by surgical stress [22]. The residual epithelial cells proliferate and migrate from the anterior capsular or equator to the centre of posterior capsule, developing into EMT [23]. The cells gradually lose epithelial properties and obtain the character of fibroblasts. During this process, the expression of E-cadherin, which is responsible for cell adhesion, is reduced or disappeared, whereas α-SMA, the marker of fibroblasts, is increased. At the same time a large amount of extracellular matrix, including glycoprotein, fibrin and various kinds of collagen fibers synthesized and deposited on the surface of the posterior capsule. These deposits resulted in the posterior capsular shrinking and led to the formation of PCO[24].

EMT is the polarity change of epithelial cell. The process transform epithelial cell into fibroblasts. This characteristic change involves in down-expression of the adhesion factor E-cadherin. EMT is initially considered being a characteristic process of embryonic development, directed organ morphological development [25]. In recent years a number of studies have found that EMT is involved in the physiological state of various cellular functions, including cell adhesion function, extracellular matrix formation, and expression of cytoskeletal proteins. Accordingly, various kinds of cell behavior change including migration, proliferation, which is a central part of the wound repair process. The pathological state of EMT also mediated tissue fibrosis, and malignant tumor cell proliferation and migration[26].

Multiple growth factor signaling pathway (TGF-β, EGF, FGF, HGF, Wnt/β-catenin and Notch) and hypoxia can induce the occurrence of EMT. TGF-β can induce lens epithelial cells transdifferentiation and CTGF can strengthen the role of TGF-β through a variety of signaling pathways[27]. Blocking CTGF and related signaling pathway would not appear adverse response that producing by blocking TGF-β.

Our data obtained from this study agree with the previous reports. CTGF treatment causes cell morphological changes from epithelial to fibroblast characteristics (Figure 1). CTGF induces the increased expression of α-SMA and decreased of E-cadherin in HLECs in a dose-dependent manner (Figure 2). In this study we observed that the expressions of Slug and α-SMA were increased whereas the expression of E-cadherin was decreased as a result of the CTGF treatment (Figures 2, 3). Furthermore, by using statistical analysis, up-expression of Slug was highly positive correlated with α-SMA and highly negative correlated with E-cadherin. Taken together, we confidently conclude that Slug participates in EMT of HLECs induced by CTGF *in vitro*.

Formation of PCO is a dynamic process that involves in complex cytokines and signaling pathways. All evidence indicates that CTGF likely induce HLECs through transcription factor Slug. Blocking CTGF expression or inhibit its activity and/or transcription factor Slug may be an alternative and effective way of prevention PCO. Our data suggest that interference of the functions of CTGF or Slug might be developed into a non-invasive procedure for treating PCO. Investigations are under the way to explore CTGF inhibitor and Slug blockers on prevention of PCO in HLECs.

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