·Basic Research·

Transforming growth factor-β2 induces morphological alteration of human corneal endothelial cells *in vitro*

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Received: 2013-12-19 Accepted: 2014-06-13

Abstract

• AIM: To investigate the morphological altering effect of transforming growth factor -β2 (TGF -β2) on untransfected human corneal endothelial cells (HCECs) *in vitro*.

• METHODS: After untransfected HCECs were treated with TGF-β2 at different concentrations, the morphology, cytoskeleton distribution, and type IV collagen expression of the cells were examined with inverted contrast light microscopy, fluorescence microscopy, immunofluorescence or Western Blot.

• RESULTS: TGF -β2 at the concentration of 3 -15 μg/L had obviously alterative effects on HCECs morphology in dose and time -dependent manner, and 9 μg/L was the peak concentration. TGF -β2 (9 μg/L) altered HCE cell morphology after treatment for 36h, increased the mean optical density (P <0.01) and the length of F -actin, reduced the mean optical density (P <0.01) of the collagen type IV in extracellular matrix (ECM) and induced the rearrangement of F -actin, microtubule in cytoplasm and collagen type IV in ECM after treatment for 72h.

• CONCLUTION: TGF -β2 has obviously alterative effect on the morphology of HCECs from polygonal phenotype to enlarged spindle -shaped phenotype, in dose and time -dependence manner by inducing more, elongation and alignment of F -actin, rearrangement of microtubule and larger spread area of collagen type IV.

• KEYWORDS: human corneal endothelial cell; transforming growth factor-β2; F-actin; microtubule; collagen type IV
DOI:10.3980/j.issn.2222-3959.2014.05.03

Wang J, Fan TJ, Yang XX, Chang SM. Transforming growth factor-β2 induces morphological alteration of human corneal endothelial cells *in vitro*. *Int J Ophthalmol* 2014;7(5):759-763

INTRODUCTION

Corneal endothelium, on the inner surface toward the aqueous humor and essential for maintaining corneal transparency, is composed of a monolayer of hexagonal cells. As adult corneal endothelial cell has limited regenerative capacity, the response of the mature endothelium to cell loss because of illness and/or wounds, normally involves spreading, enlargement and/or migration of neighboring cells to cover the wound area, the result of which is an increase in overall cell size and an morphological alteration from a hexagonal to a polymorphic shape[1-5].

Given that the mean concentration of transforming growth factor-β2 (TGF-β2), which is present mainly in latent form in aqueous humor and capable of mediating epithelial-mesenchymal transition (EMT), repairing after injury and inhibiting proliferation of human corneal endothelial cells (HCECs), elevates higher in injured and unhealthy eyes, it is reasonable to hypothesize that the morphological alteration observed in HCECs is, at least in part, the result of increased concentration of TGF-β2, which is not reported before[6-12]. So in the study, cell shape, arrangement of cytoskeleton and spread of collagen type IV in extracellular matrix (ECM) under HCECs were examined to investigate the effect mechanism of TGF-β2 on the morphology of HCECs and infer the regulatory function of TGF-β2 on the repair after injury of human corneal endothelium by morphological alteration and migration.

MATERIALS AND METHODS

Materials Untransfected HCECs at passage 100 from the HCE cell line which was established previously in the Key Laboratory for Corneal Tissue Engineering, Ocean University of China, was cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 (1:1, v:v) (DMEM/F12) (Invitrogen, Carlsbad, CA, USA) (pH 7.2) medium containing 10% fetal bovine serum (FBS) (HyClone, Logan, Utah, USA) at 37℃ in a 5% CO_2 incubator [13]. TGF-β2 was

purchased from PeproTech (London, UK). Phalloidin conjugated to tetramethylrhodamine B isothiocyanate (TRITC) was from Sigma-Aldrich (St. Louis, USA). Mouse monoclonal anti-α-tubulin antibodies, rabbit polyclonal anti-collagen type IV-α2 antibodies were from Santa Cruz Biotechnology (CA, USA). Fluorescent isothiocyanate (FITC)-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were from Biosynthesis Biotechnology (Beijing, China).

Methods

Cell culture and treatment The HCECs were collected using 0.25% (m:v) trypsin (Sigma-Aldrich, St. Louis, USA), counted and then seeded in 24-well cell culture cluster (Corning, NY, USA) at a density of 2.5×10^4 cells/mL. After cultured in DMEM/F12 medium containing 10% FBS (pH 7.2) at 37℃ in a 5% CO_2 incubator overnight and starved in DMEM/F12 without FBS for 24h, HCECs were treated with TGF-β2 (1-15 μg/L) for 72h until 80%-90% cell confluence was reached as described previously [9]. Specimens were observed under Nikon Ti-S inverted contrast microscope with charge coupled device (CCD) camera to find out a peak concentration of TGF-β2 for altering HCE cell morphology. HCECs were treated at the peak concentration for 72h until 80%-90% cell confluence was reached, and the images were captured every 12h.

Fluorescent and immunofluorescent staining HCECs at a density of 2.5×10^4 cells/cm^2 were treated with the peak concentration of TGF-β2 for 24h or 72h as described above. Cells were fixed in 3.7% (m:v) paraformaldehyde for 10min at 4℃ followed by phosphate buffer saline (PBS) washes, and permeabilized with 0.25% Triton X-100 (F-actin and α-tubulin) for 10min at room temperature (RT). After non-specific binding blocked with 1% FBS (Invitrogen, CA, USA), cells were incubated with mouse monoclonal anti-α-tubulin antibodies (1:50 dilution, v:v) or rabbit polyclonal anti-collagen type IV-α2 antibodies (1:50 dilution, v:v) at 4℃ in a humid chamber overnight and followed by FITC-conjugated goat anti-mouse IgG (α-tubulin and collagen type IV) antibody and Phalloidin-TRITC (F-actin) for 1h at RT, After washed, specimens were observed under Nikon TE2000-U inverted fluorescent microscope with CCD camera and images were exported into the Image-Pro Plus to determine mean optical density (determining staining intensity).

SDS-PAGE and Western Blot After treated as mentioned above, HCECs were washed with ice-cold PBS and lysed on ice in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) (Beyotime, Beijing, China) with 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) (Beyotime, Beijing, China). Protein concentration in the clarified cell extracts was estimated using bicinchoninic acid (BCA) protein assay kits (Beyotime, China). Equal amounts of protein were subjected to 10% polyacrylamide gels for SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham, Braunschweig, Germany). Nonspecific binding was blocked by incubation for 1h at 4℃ in 5 g/L skim milk powder in Tris buffered saline solution with 0.1% (v:v) Tween-20 (TBST). Membranes were incubated with rabbit anti-collagen type IV-α2 polyclonal antibodies (1:100 dilution, v:v) for 2h, washed, reblocked, and then exposed to HRP-conjugated goat anti-rabbit IgG (1:100 dilution, v:v) for 1h in TBST with 0.25% (m:v) bovine serum albumin (Sigma, St. Louis, USA). The same blots were probed with mouse monoclonal anti-α-tubulin antibodies (1:100 dilution, v:v) and HRP-conjugated goat anti-mouse IgG (1:100 dilution, v:v) to control protein load. After a thorough wash, immune complexes were detected using diaminobenzidine (DAB).

Statistical Analysis Data were presented as the mean±SD of at least three independent and analyzed by SPSS 20.0 and t-test. $P<0.05$ was designated as significant, $P<0.01$ was designated as extremely significant.

RESULTS

Morphological Changes After cells treated for 72h, makeable morphological changes of HCECs were induced by TGF-β2 at the concentration of 3-15 μg/L (Figure 1), which was dose-dependent. The addition of TGF-β2 to cultures produced enlarged and ragged cells, which was spindle-shaped phenotype and failed to form a typical polygonal (or cobblestone) monolayer (compare Figure 1A and B-G). The peak concentration 9 μg/L for morphological changes and altered HCE cell morphology after treatment for 36h (Figure 2), and the effect was time-dependent (Figure 2).

Effects of TGF-β2 on Human Corneal Epithelial Cells Cytoskeleton The difference of mean optical density of F-actin and α-tubulin between control and TGF-β2-treated group was a little (Figure 3) after treatment for 24h, while after treatment for 72h the morphology and the configuration (parallel to the long axis of cells) of F-actin and α-tubulin in cytoplasm (Figure 3) between two groups differed widely, besides, the mean optical density ($P<0.01$) and length of

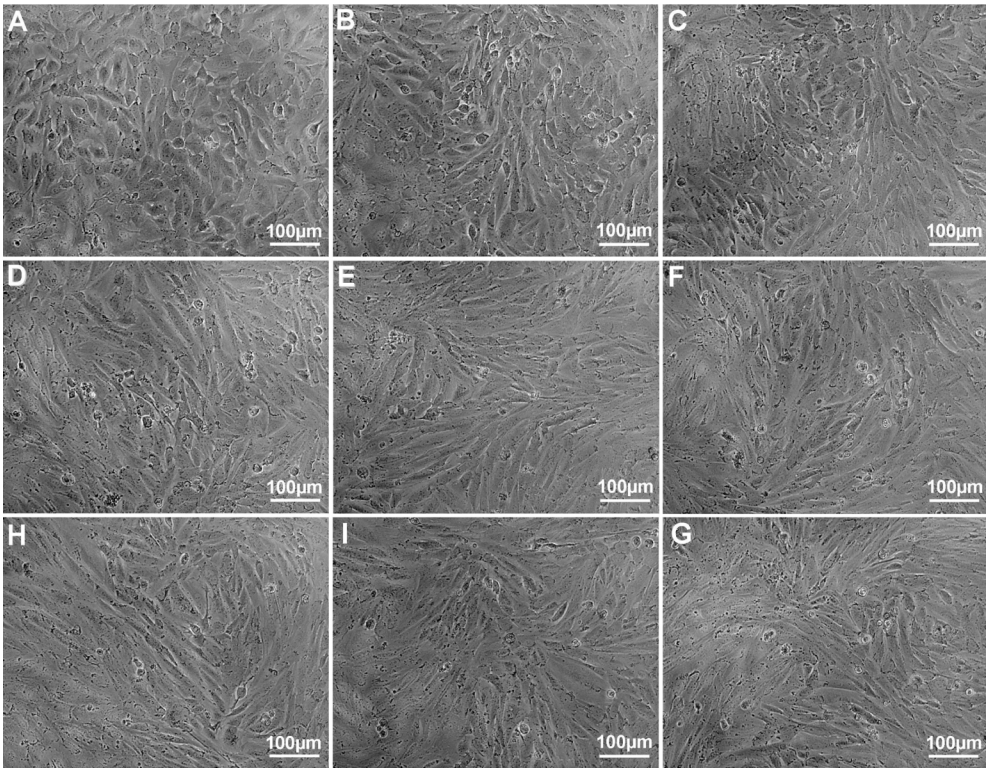

Figure 1 Morphologic changes of HCECs induced by TGF-β2 at different concentration for 72h A-G: 0; 1; 3; 5; 7; 9; 11; 13; 15 μg/L.

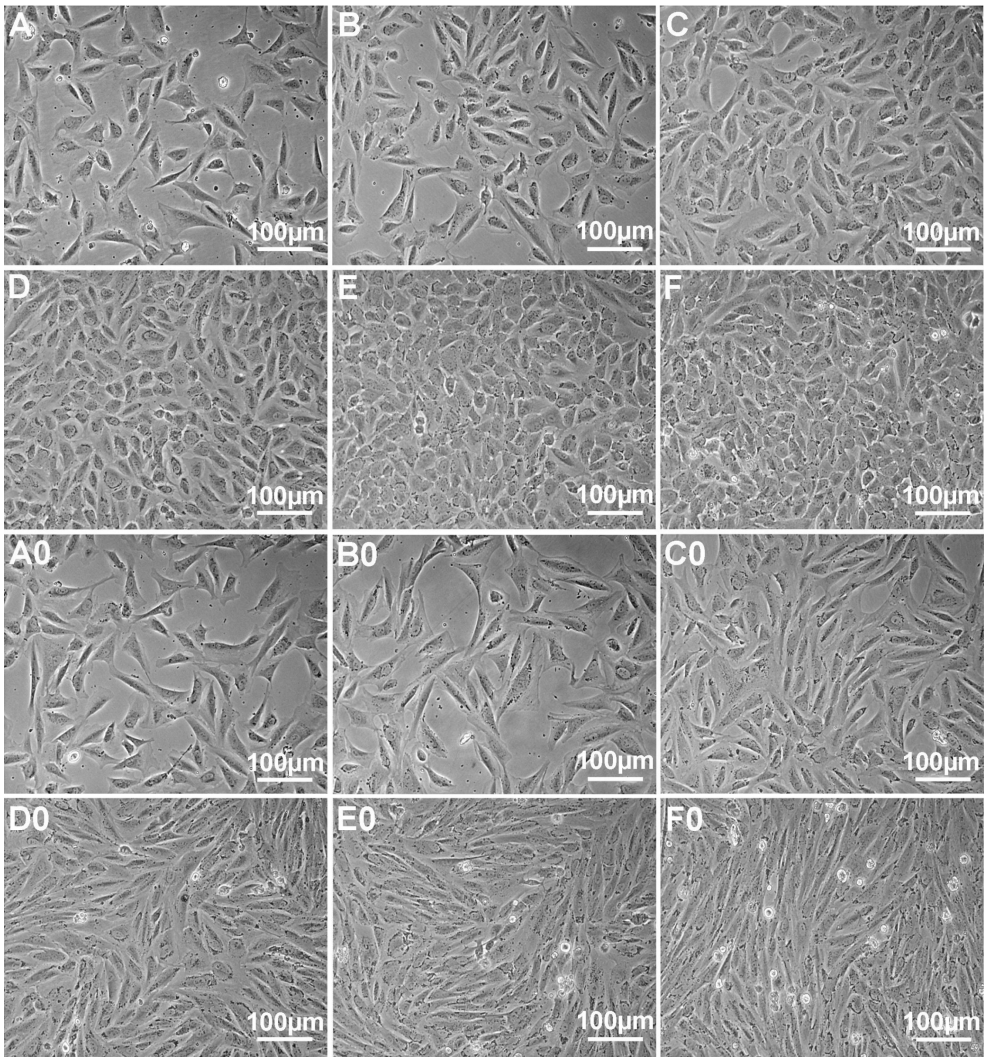

Figure 2 Morphologic changes of HCECs induced by TGF-β2 for different treated time A-F: Control group 12, 24, 36, 48, 60 and 72h; A0-F0: Treated group by TGF-β2 12, 24, 36, 48, 60 and 72h.

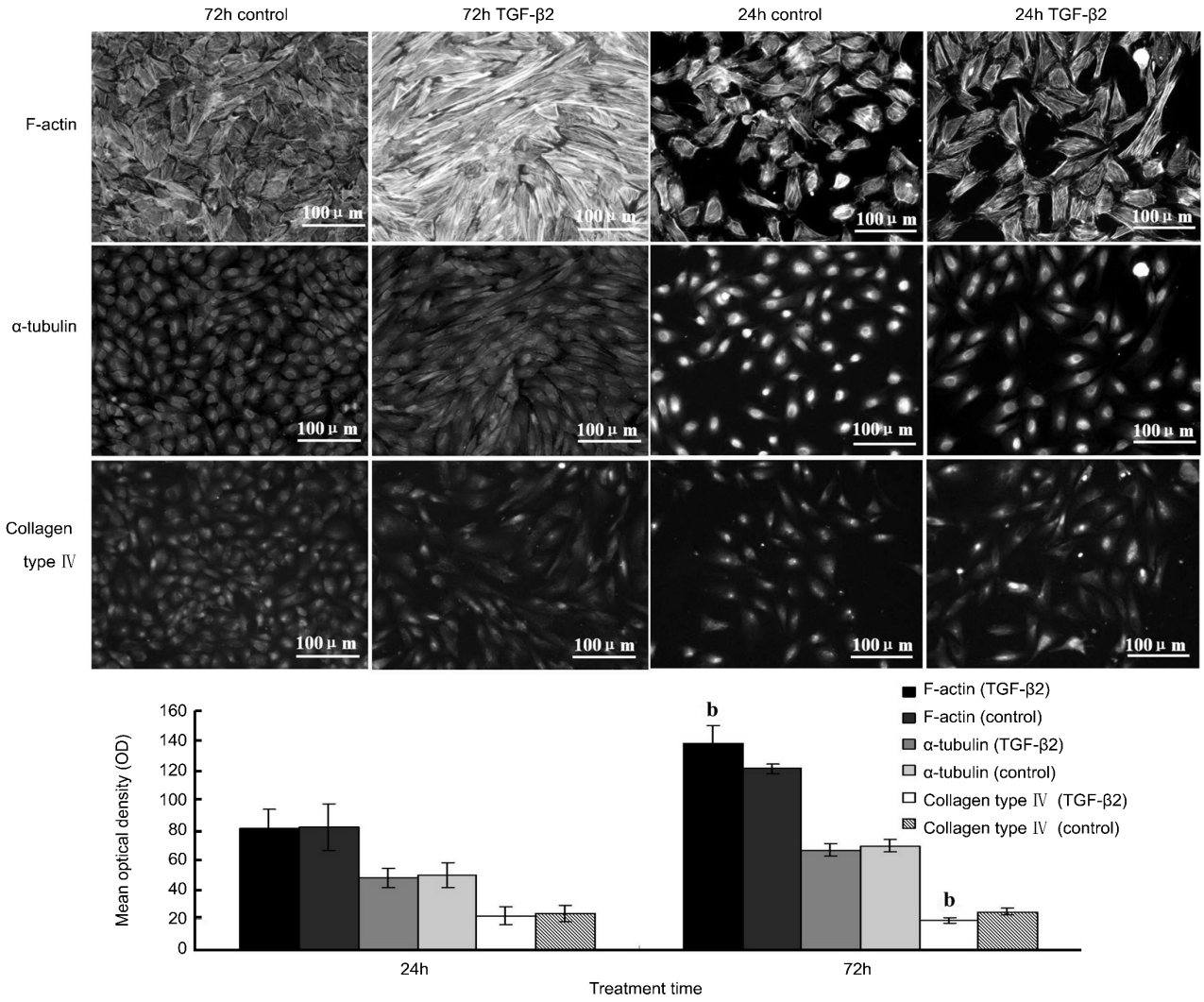

Figure 3 Fluorescence staining for F-actin and immunofluorescence staining for α-tubulin and collagen type IV in ECM $^{b}P<0.01$.

F-actin were increased (Figure 3).

Effects of TGF-β2 on Human Corneal Epithelial Cells Collagen type IV The mean optical density of collagen type IV under single HCEC had no significant change (Figure 3) after treatment for 24h, but was remarkable reduced 72h later ($P<0.01$) (Figure 3). The result of Western Blot showed that TGF-β2 didn't influence the relative content of collagen type IV in the ECM (Figure 4).

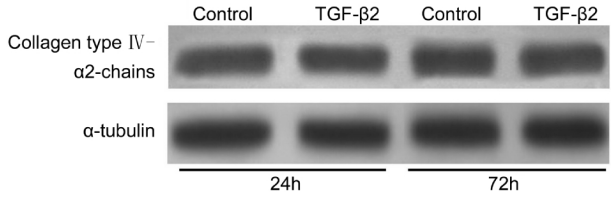

Figure 4 Western Blot for total content of collagen type IV in ECM of HCECs.

DISCUSSION

TGF-β2 may mediate repair after injury and promote wound healing by inducing morphological changes and regulating synthesis and secretion of ECM [14,15]. So the present study was designed to determine whether and how TGF-β2 altered the morphology of untransfected HCECs *in vitro*.

In the present study, the results showed that TGF-β2 at the concentration of 3-15 g/L had obviously alterative effects on HCECs morphology, from cobblestone monolayer to enlarged spindle-shaped monolayer in dose-dependent manner, which was similar with the effect of TGF-β on the shape of the bovine retinal endothelial cells, and 9 μg/L was the peak concentration. TGF-β2 at the peak concentration could alter HCE cell morphology after treatment for 36h, and the effect was time-dependent[14].

Since F-actin, also named as stress fiber, and collagen type IV which is one of main components in Descemet's membrane, play an important role in maintaining morphology of HCECs, in the study arrangement of F-actin and microtubule in cytoplasm, together with the total content and arrangement of collagen type IV under a single HCEC, were investigated to explore the morphological altering effect of TGF-β2 on HCECs [16,17]. After treatment for 24h, TGF-β2 didn't alter the shape of HCECs, the number of F-actin, the

arrangement of F-actin and microtubule or the content of collagen type IV in ECM, while after treatment for 72h, there was obviously different between treated and control group, such as more and longer F-actin, rearrangement of F-actin and microtubule parallel to the long axis of HCECs, which was similar with the findings from other researchers [14,18,19]. However the result of immunofluorescence and Western Blot revealed that after treated for 72h, the spreading area of collagen type IV under single HCEC was increased, and the content of collagen type IV in ECM remained unchanged, which was similar with some results but different from others which treated cells, types of investigated collagens or experimental methods, such as enzyme linked immunosorbent assay (ELISA) for detecting the concentration of collagen in the culture medium and RT-PCR for the total expression of the collagen gene, were different[20-22]. In summary, our data indicated that TGF-β2 altered the morphology of HCECs in dose and time-dependence manner by inducing more, elongation and alignment of F-actin, rearrangement of microtubule and larger spread area of collagen type IV under single HCEC, which help us realize the effect mechanism of TGF-β2 at higher concentration in aqueous humor on repair after injury of human corneal endothelium by morphological changes.

ACKNOWLEDGEMENTS

Foundations: Supported by National High Technology Research and Development Program ("863" Program) of China (No.2006AA02A132); Key Developing Discipline of Hebei Province (No.201221)

Conflicts of Interest: Wang J, None; **Fan TJ,** None; **Yang XX,** None; **Chang SM,** None.

REFERENCES

1 Joyce NC. Proliferative capacity of corneal endothelial cells. *Exp Eye Res* 2012;95(1):16-23

2 Rao GN, Shaw EL, Arthur E, Aquavella JV. Morphologic appearance of the healing corneal endothelium. *Arch Ophthalmol* 1978;96 (11): 2027-2030

3 Honda H, Ogita Y, Higuchi S, Kani K. Cell movements in a living mammalian tissue: long-term observation of individual cells in wounded corneal endothelial of cats. *J Morphol* 1982;174(1):25-39

4 Laing RA, Sanstrom MM, Berrospi AR, Leibowitz HM. Changes in the corneal endothelium as a function of age. *Exp Eye Res* 1976;22(6):587-594

5 Matsuda M, Sawa M, Edelhauser HF, Bartels SP, Neufeld AH, Kenyon KR. Cellular migration and morphology in corneal endothelial wound repair. *Invest Ophthalmol Vis Sci* 1985;26(4):443-449

6 Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-β2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol* 2001;239(2):109-113

7 Heldin CH, Landström M, Moustakas A. Mechanism of TGF-beta signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. *Curr Opin Cell Biol* 2009;21(2):166-176

8 Shah M, Foreman DM, Ferguson MW. Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J Cell Sci* 1995;108(Pt 3):985-1002

9 Fan TJ, Wang J, Zhao J, Yang HS, Zhao WZ, Yang XX. Inhibitory effect of transforming growth factor-β2 on the proliferation of human corneal endothelial cells *in vitro*. *Guoji Yanke Zazhi* 2010;10(5):841-843

10 Li X, Xu JT, Cui H, Hu Q. Expression of TGF-β2 in rat aqueous humor following corneal alkali burns, acid burns and thermal burns. *Guoji Yanke Zazhi* 2008;8(9):1819-1822

11 Picht G, Welge-Luessen U, Grehn F, Lütjen-Drecoll E. Transforming growth factor β2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch Clin Exp Ophthalmol* 2001;239(3):199-207

12 Ochiai Y, Ochiai H. Higher concentration of transforming growth factor-β in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn J Ophthalmol* 2002;6(3):249-253

13 Fan T, Zhao J, Ma X, Xu X, Zhao W, Xu B. Establishment of a continuous untransfected human corneal endothelial cell line and its biocompatibility to denuded amniotic membrane. *Mol Vis* 2011;17:469-480

14 Sutton AB, Canfield AE, Schor SL, Grant ME, Schor AM. The response of endothelial cells to TGF beta-1 is dependent upon cell shape, proliferative state and the nature of the substratum. *J Cell Sci* 1991;99(Pt 4): 777-787

15 Quaglino D Jr, Nanney LB, Ditesheim JA, Davidson JM. Transforming growth factor-beta stimulates wound healing and modulates extracellular matrix gene expression in pig skin: incisional wound model. *J Invest Dermatol* 1991;97(1):34-42

16 Deng LH, Lin WL. Research advances of cornea collagen protein. *Guoji Yanke Zazhi* 2008;8(3):557-560

17 Barry PA, Petroll WM, Andrews PM, Cavanagh HD, Jester JV. The spatial organization of corneal endothelial cytoskeletal proteins and their relationship to the apical junctional complex. *Invest Ophthalmol Vis Sci* 1995;36(6):1115-1124

18 Edlund S, Landström M, Heldin CH, Aspenström P. Transforming Growth Factor-β - induced Mobilization of Actin Cytoskeleton Requires Signaling by Small GTPases Cdc42 and RhoA. *Mol Biol Cell* 2002;13(3): 902-914

19 Moustakas A, Stournaras C. Regulation of actin organisation by TGF-β in H-ras-transformed fibroblasts. *J Cell Sci* 1999;112(Pt 8):1169-1179

20 Batra V, Musani AI, Hastie AT, Khurana S, Carpenter KA, Zangrilli JG, Peters SP. Bronchoalveolar lavage fluid concentrations of transforming growth factor (TGF)-beta1, TGF-beta2, interleukin (IL)-4 and IL-13 after segmental allergen challenge and their effects on alpha-smooth muscle actin and collagen III synthesis by primary human lung fibroblasts. *Clin Exp Allergy* 2004;34(3):437-444

21 Kimoto K, Nakatsuka K, Matsuo N, Yoshioka H. p38 MAPK mediates the expression of type I collagen induced by TGF-beta 2 in human retinal pigment epithelial cells ARPE-19. *Invest Ophthalmol Vis Sci* 2004;45(7): 2431-2437

22 Kottler UB, Jünemann AG, Aigner T, Zenkel M, Rummelt C, Schl tzer-Schrehardt U. Comparative effects of TGF-beta 1 and TGF-beta 2 on extracellular matrix production, proliferation, migration, and collagen contraction of human Tenon's capsule fibroblasts in pseudoexfoliation and primary open-angle glaucoma. *Exp Eye Res* 2005;80(1):121-134