• Basic Research •

Effects of epidermal growth factor on transforming growth factor-beta1-induced epithelial-mesenchymal transition and potential mechanism in human corneal epithelial cells

Shu-Yang Chen[1,2], Chen Xie[1,3], Hong Zhu[1,3], Ye Shen[1]

[1]Department of Ophthalmology, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
[2]Tongde Hospital of Zhejiang Province, Hangzhou 310012, Zhejiang Province, China
[3]Clinical Research Center, the First Affiliate Hospital, School of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China
Co-first authors: Shu-Yang Chen and Chen Xie
Correspondence to: Ye Shen. Department of Ophthalmology, First Affiliated Hospital, College of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou 310003, Zhejiang Province, China. idrshen@zju.edu.cn
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Abstract

● **AIM:** To evaluate the effects of epidermal growth factor (EGF) on transforming growth factor-beta1 (TGF-β1)-induced epithelial-mesenchymal transition (EMT) in human corneal epithelial cells (HCECs).

● **METHODS:** HCECs were cultured and treated with TGF-β1 for establishing the model of EMT *in vitro*. Biological effect of EGF on TGF-β1-induced EMT was evaluated. Proteins and mRNAs expression changes of E-cadherin, N-cadherin and Fibronectin (EMT-relative markers) after TGF-β1 or TGF-β1 combined EGF treatment were detected by Western blot and RT-PCR, respectively. Viability and migration of HCECs were measured by CCK-8, transwell cell migration assay and cell scratch wound healing assay. Activation of Smad2, ERK, p38, JNK and Akt signaling pathways were evaluated by Western blot. Inhibitors of relevant signaling pathways were added to the HCECs to explore the key signal mechanism.

● **RESULTS:** With treatment of TGF-β1 only, three EMT-relative proteins and mRNA expression showed that EMT up-regulated in a concentration-dependent and time-dependent manner, with significantly decreasing cell viability (TGF-β1≥5 ng/mL, $P<0.05$) and increasing cell migration (TGF-β1≥5 ng/mL, $P<0.01$). The phosphorylation of Smad2 and p38 was a key process of TGF-β1-induced EMT. Meanwhile, EMT-relative proteins and mRNA expression showed that EGF inhibited TGF-β1-indued EMT, with significantly increasing cell viability (EGF≥10 ng/mL, $P<0.01$). It was noteworthy that EGF significantly enhanced cell migration although EMT was inhibited (EGF≥10 ng/mL, $P<0.01$), and the blockage of p38 (by SB202190, a p38 inhibitor) was a potential mechanism of this phenomenon.

● **CONCLUSION:** EGF inhibits TGF-β1-induced EMT *via* suppressive p38, and promotes cells proliferation and migration in a non-EMT process by inhibiting p38 pathway.

● **KEYWORDS:** epidermal growth factor; p38; epithelial-mesenchymal transition; corneal epithelial cell
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INTRODUCTION

The first layer of human corneal structure is primarily composed of human corneal epithelial cells that play a vital role in maintaining the integrity of ocular surface[1-2]. Both traumatic and pathologic factors will destroy the structural integrity, thus leading to ophthalmodynia, tearing, foreign body sensation and decreased visual quality[3-4]. A well-timed corneal epithelium reconstructionis required for symptom relieving and better prognosis. Numerous researches demonstrate that epithelial-mesenchymal transition (EMT) is a potential mechanism in ophthalmic diseases progression as well as damage repair, including corneal epithelium healing[5-6].

EMT, a progress of phenotypic changes from epithelial cells to mesenchyme type, is known as a mechanism related to embryonic development, wound healing, tissue fibrosis and cancer progression[7]. The transition progress is induced by several key transcription factors such as Snail, zinc finger

E-box-binding homeobox1 (ZEB1), ZEB2 and some other basic helix-loop-helix factors[7-10]. Certain signaling pathways have been testified to get involved such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Wnt/β-catenin, insulin-like growth factor (IGF), *etc*.[11]. Among these, the TGF-β family are the most significant and well-demonstrated. TGF-β induces EMT *via* both Smad and non-Smad signaling pathways, the latter include ERK, p38, JUN N-terminal kinase (JNK), Akt, *etc*.[11-12].

After being wounded, the cornea shows a higher expression of TGF-β than normal situation, and up-regulates epithelial cells migration and proliferation as a result. Clinically, the impairment of cornea is better to be cured at the first stage. However, not all of ophthalmic drug will bring advantages for corneal wound healing, because the interaction between TGF-β and other factors remain unclear. According to published studies, EGF has opposite effects in different tissues. One side, for example, EGF can promote TGF-β-induced EMT in lung and pancreatic cancer cells[13]. On the other hand, EGF can suppress and even reverse TGF-β-induced transition in normal hepatic cells[14]. In ocular tissues, sometime EGF and its analogues will be used in clinical therapy. Such as recombinant EGF, which is beneficial to ocular surface defects healing[15-17]. Nevertheless, the effect and mechanism of EGF combining TGF-β remain unclear. The aim of our research is to figure out the exact effect of EGF on TGF-β-induced EMT in human corneal epithelial cells, and the signaling pathways potentially involved.

MATERIALS AND METHODS

Culture of Human Corneal Epithelial Cells In our research, the human corneal epithelial cell (HCEC) line from the Riken cell bank was used. The basal culture medium was Dulbecco's modified Eagle's/Ham's 12 medium (DMEM/F12), containing heat-inactivated (56℃, 0.5h) 10% fetal bovine serum (FBS), 15 mmol/L HEPES buffer, penicillin (100 U/mL) and streptomycin (100 mg/mL) (Gibco Company). The HCECs were cultured in constant temperature incubator at 5%CO_2, 95% air, 37℃ atmosphere, and passaged by trypsin/EDTA every 2-3d (the doubling time of the HCECs with this condition was around 24h). During the experiment, the HCECs were seeded in 6-well plates and Transwell chambers.

Study Design and Study Groups The study design is mainly divided into three parts: 1) Establishing the TGF-β1 induced EMT models with the concentration of 10 ng/mL for various periods of time (1, 3, 6d) or with different concentrations (0, 1, 2, 5, 10, 20 ng/mL) for 2d. Evaluation of EMT-relative markers protein and mRNA expression, cell viability and mobility. 2) Analysing Signaling pathways (Smad2, ERK, p38, JNK and Akt) and exploring the possible signaling pathways involved in TGF-β1 induced EMT of HCECs. 3) Exploring the biological functions of EGF (5, 10, 20 ng/mL) on the TGF-β1 (10 ng/mL) induced EMT, including three EMT-relative markers protein and mRNA expression, cell viability and mobility. Furtherly, exploring the potential mechanism and signaling pathway involved. The study groups were defined as followed: 1) control group, treated with normal culture medium; 2) TGF-β1 treated groups; 3) TGF-β1 (10 ng/mL) combined with signal inhibitors (SB431542, Smad2 inhibitor, 10 μmol/L; PD98059, ERK inhibitor, 20 μmol/L; SB202190, p38 inhibitor, 10 μmol/L; SP600125, JNK inhibitor, 10 μmol/L; Wortmannin, Akt inhibitor, 1 μmol/L) treated groups; 4) EGF (5, 10, 20 ng/mL) treated groups; 5) EGF (5, 10, 20 ng/mL) combined with TGF-β1 (10 ng/mL) treated groups.

Cell Viability Assay Cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) had been used to evaluate the viability of HCECs in different groups. Living cells can turn colorless WST-8 into soluble WST-8 formazan. The latter can be measured at the wavelength of 450 nm and the density is in proportion to the content of metabolically normal cells.

RNA Extraction and Real-time Quantitative PCR Total RNA was isolated from HCECs by TRIzol reagent (Takara, Dalian, China), and then reverse transcribed to cDNA by an RT reagent kit (Takara, Dalian, China). The sequences of the PCR primers were Fibronectin (forward: 5-CGGTGGCTGTCAGTCAAAG-3; reverse: 5-AAACCTCGGCTTCCTCCATAA-3), N-cadhein (forward: 5'-TCAGGCGTCTGTAGAGGCTT-3'; reverse: 5'-ATGCACATCCTTCGATAAGACTG-3'), E-cadherin (forward: 5'-AAAGGCCCATTTCCTAAAAACCT-3'; reverse: 5'-TGCGTTCTCTATCCAGAGGCT-3'), GAPDH (forward: 5'-GGCCTCCAAGGAGTAAGACC-3'; reverse: 5'-AGGGGTCTACATGGAAACTG-3'). The primers above were provided from Sango Biotech (Shanghai, China). The cDNA combining primers and other required ingredients was amplified and evaluated by an ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad, USA).

Western Blot Analysis The total cellular protein was extracted by cell total protein extraction kit (Sangon, Shanghai, China). Same amount of protein lysates (volumes at 20-30 μL) were electrophoresed in 10% or 20% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE; Bio-Rad), and then transferred (at 4℃, 350 mA for 2h) to polyvinylidene difluoride (PVDF) membranes. After blocked with 5% nonfat milk or 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBST), the transferred PVDF membranes were incubated with primary antibodies at 4℃ overnight, and incubated with second antibody at room temperature for 60min. The results were detected by chemiluminescence reagents (ECL, Millipore) and exposed by an imaging system (ChemiDoc MP, Bio-Rad).

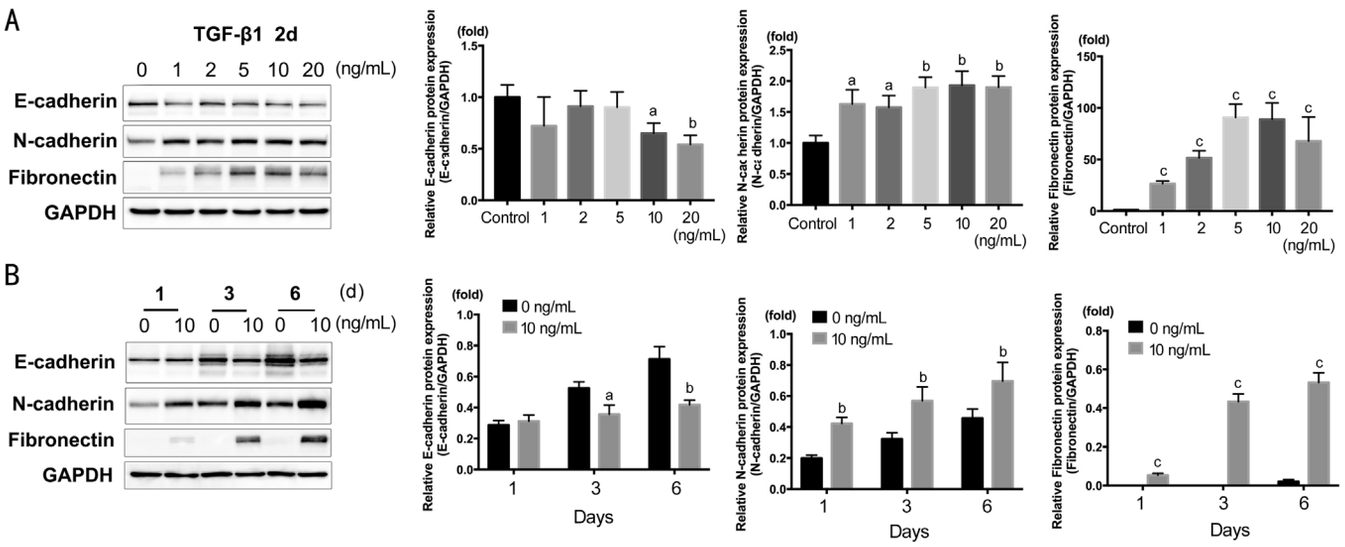

Figure 1 Western blot showing the dose response and time course of E-cadherin, N-cadherin and Fibronectin actived by TGF-β1 in HCECs A: TGF-β1 concentration gradient of 0, 1, 2, 5, 10, 20 ng/mL in 2d; B: Time course of 1, 3, 6d with TGF-β1 at 10 ng/mL. [a]P<0.05, [b]P<0.01, [c]P<0.001 *vs* control group.

Transwell Cell Migration Assay Cell migration was performed in Transwell chambers with polycarbonate membrane, 8.0 μm pore size. HCECs (5×10^4 cells/mL) were combined with 200 μL FBS-free medium with or without TGF-β1 or EGF, and then added to the upper chamber. Medium 600 μL with 10% FBS were added to the lower chamber. The system was incubated for 48h at 37°C. The cells on the upside of the filters were removed by cotton swab. The cells on the underside of the filters were fixed by 75% methyl alcohol and stained by crystal violet. Cells were counted to evaluate the migration across the filters.

Cell Scratch Wound Healing Assay HCECs (1.5×10^5 cells/mL) were seeded into 6-well plates, with DMEM/F12 containing 10% FBS. After 48h, the monolayer was scratched with 1 mL pipette tip. The instant and 24-hour photomicrographs were taken (Nikon ECLIPSE Ti, Japan), and measured by an imaging analyzing software (NIS Elements 4.0, Nikon, Japan).

Statistical Analysis Each result was experimented independently at least three times and was given as mean±standard deviation (SD). Data were analyzed by one-way ANOVA test with Bonferroni correction in comparing data from more than two groups and Student's *t*-test in comparing data between two groups. Data analyzing was carried out by SPSS Statistics (Version 21.0, SPSS Inc., USA). P<0.05 indicated statistical significance.

RESULTS

Effects of TGF-β1 on HCECs Epithelial-mesenchymal Transition, Proliferation and Migration E-cadherin, N-cadherin, and Fibronectin[18] were detected by Western blot (Figure 1). HCECs were treated with TGF-β1 of 1, 2, 5, 10, 20 ng/mL for 2d. As shown in Figure 1A, E-cadherin significantly down-regulated in 10 and 20 ng/mL (P<0.05); N-cadherin and Fibronectin significantly up-regulated in all concentration and peaked around 5-10 ng/mL (N-cadherin, P<0.01; Fibronectin, P<0.001). According to the results, we chose TGF-β1 at 10 ng/mL for the subsequent experiments. The time course of 1, 3, 6d with TGF-β1 at 10 ng/mL was also treated on HCECs. As shown in Figure 1B, N-cadherin, Fibronectin significantly increased at 1d (N-cadherin, P<0.01; Fibronectin, P<0.001), and E-cadherin significantly decreased at 3 and 6d (P<0.05, P<0.01).

We also demonstrated the mRNA expression in Figure 2. The result of N-cadherin and Fibronectin was the same to protein expression, showing increasing concentration-dependence and time-dependence. But the E-cadherin mRNA showed stimulated rather than decreasing as above. The remarkable stimulation of E-cadherin started from 10 ng/mL, and after 3d declined to the level of TGF-β1 free group. This indicate that the regulation of E-cadherin was after transcriptional level in TGF-β1-induced EMT.

The cell viability assay was detected by CCK-8 (Figure 3A), and showed inversely concentration-dependent manner from 5 ng/mL with TGF-β1 treatment (P<0.05). TGF-β1 inhibited the proliferation of HCECs. The transwell assay (Figure 3B) revealed that TGF-β1 promoted cell migration with concentration-dependent manner. All concentration showed significant increasing, and peaked at 10 ng/mL (P<0.001). Cell scratch wound healing assay was the consistent result (Figure 3C) to transwell. After TGF-β1 (10 ng/mL) exposure for 24h, the greater migration distance of HCECs was remarkable (P<0.001).

Signaling Pathways Involved in TGF-β1-induced Epithelial-Mesenchymal Transition The activation of

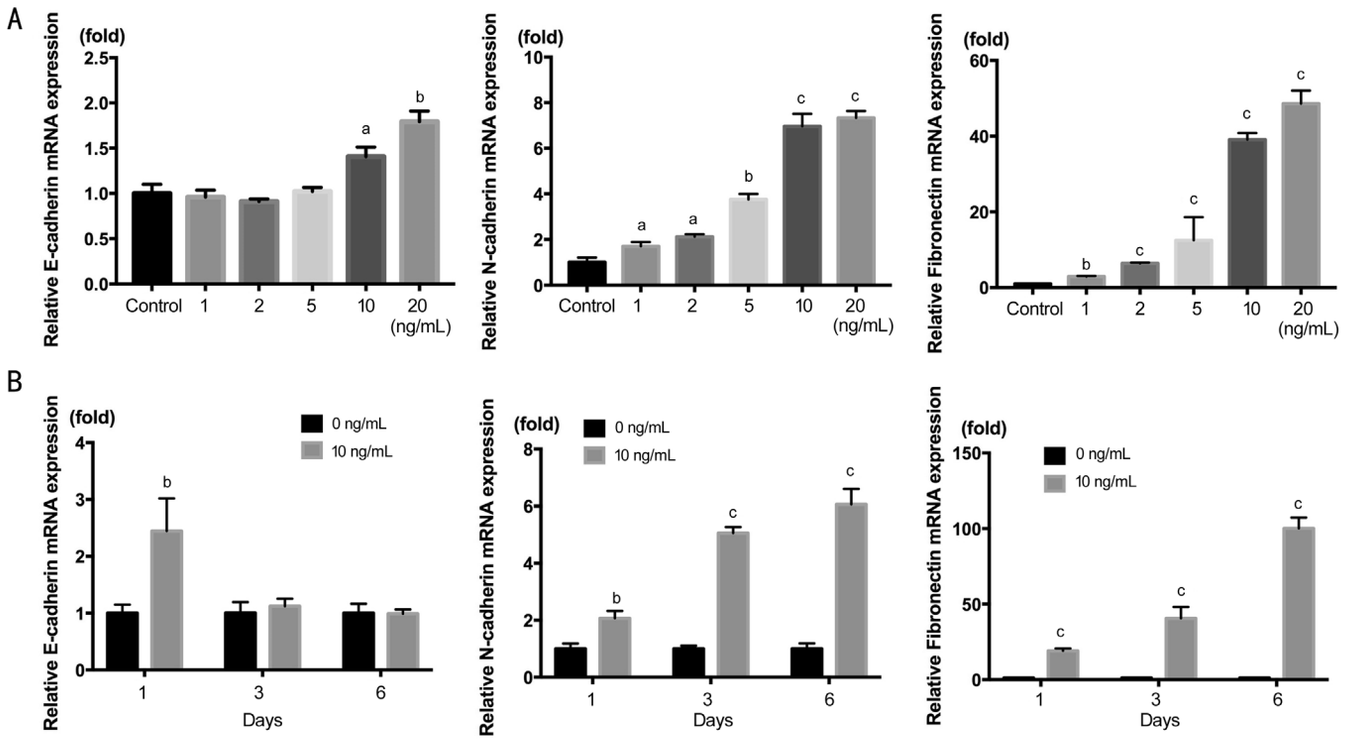

Figure 2 RT-PCR showing the dose response and time course of E-cadherin mRNA, N-cadherin and Fibronectin mRNA actived by TGF-β1 in HCECs A: TGF-β1 concentration gradient of 0, 1, 2, 5, 10, 20 ng/mL in 2d; B: Time course of 1, 3, 6d with TGF-β1 at 10 ng/mL. [a]P<0.05, [b]P<0.01, [c]P<0.001 *vs* control group.

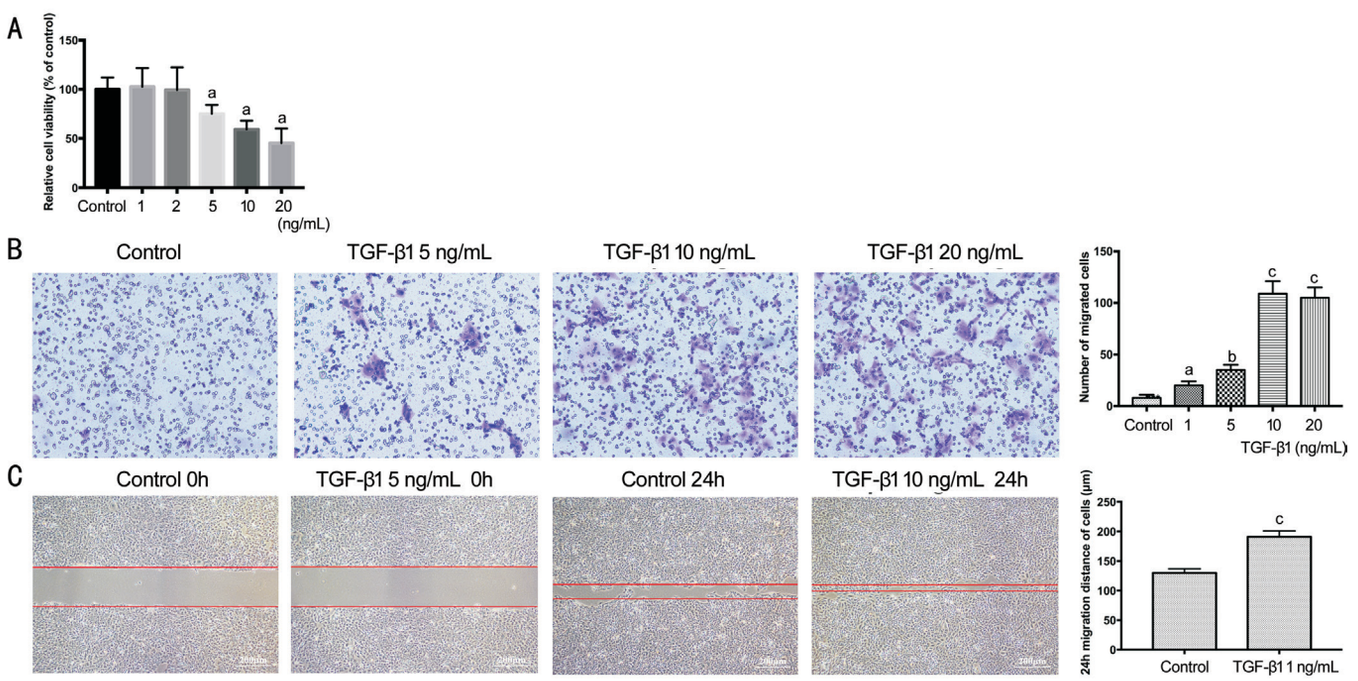

Figure 3 Viability and migration of HCECs treated with TGF-β1 A: The viability of HCECs actived by TGF-β1 concentration gradient of 0, 1, 2, 5, 10, 20 ng/mL in 2d, expressed as a percentage of the control group; B: HCECs migration in transwell assay; C: HCECs migration in cell scratch wound healing assay. [a]P<0.05, [b]P<0.01, [c]P<0.001 *vs* control group.

Signaling pathways were detected by Western blot (Figure 4A). The results showed significant phosphorylated of Smad2 and p38. The maximal expression presented at 30min for p-Smad2 (P<0.001), and at 60min for p-p38 (P<0.001). In contrast, p-ERK1/2, p-JNK, p-Akt remained unchanged. In the meantime, with the treatment of TGF-β1 (10 ng/mL) combining Smad2 inhibitor (SB431542), ERK inhibitor (PD98059), p38 inhibitor (SB202190), JNK inhibitor (SP600125), and Akt inhibitor (Wortmannin) for 2d. According to the result of Fibronectin and N-cadherin, the inhibition of

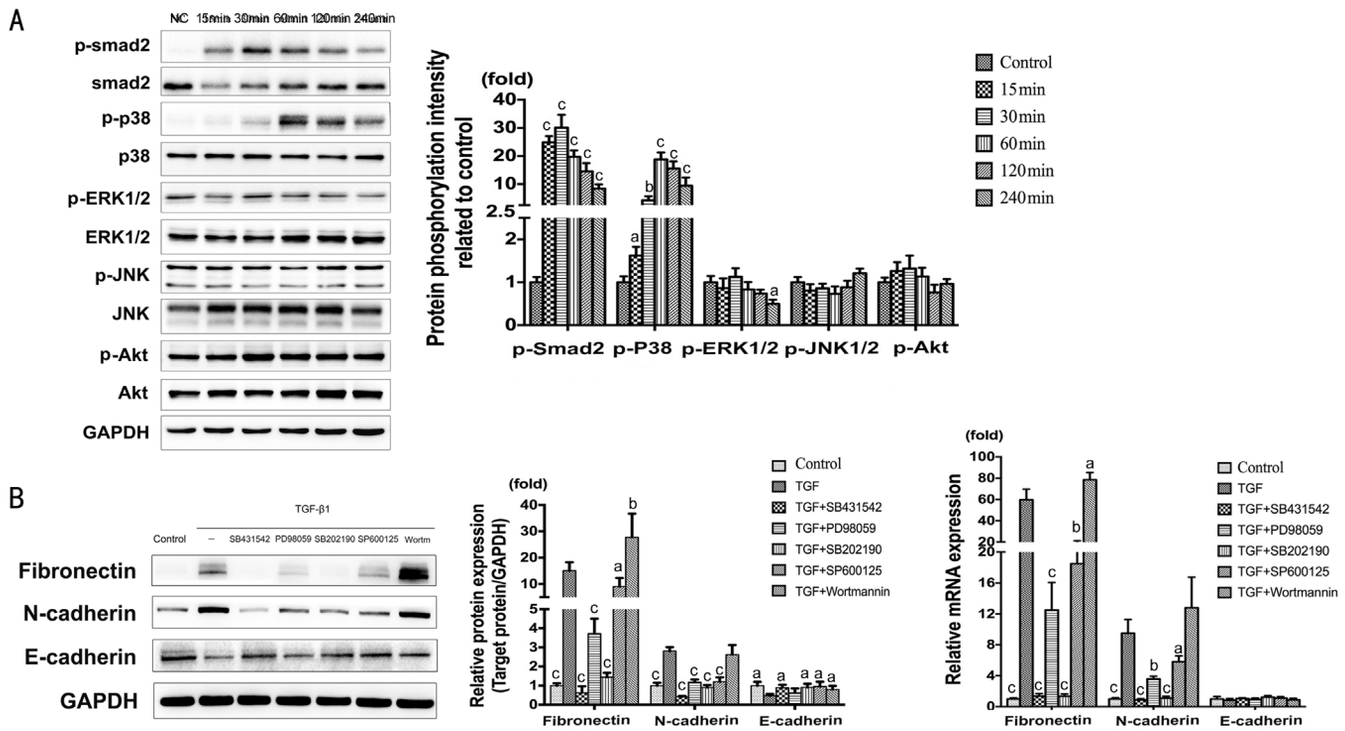

Figure 4 Signaling pathways involved in TGF-β1-induced EMT A: The expression of p-Smad2/Smad2, p-p38/p38, p-ERK/ERK, p-JNK/JNK and p-Akt/Akt at 0, 15, 30, 60, 120, 240min, treated with TGF-β1 (10 ng/mL) in HCECs. [a]P<0.05, [b]P<0.01, [c]P<0.001 *vs* control group. B: The expression of Fibronectin, N-cadherin and E-cadherin treated with TGF-β1 (10 ng/mL) combining Smad2 inhibitor (SB431542, 10 μmol/L), ERK inhibitor (PD98059, 20 μmol/L), p38 inhibitor (SB202190, 10 μmol/L), JNK inhibitor (SP600125, 10 μmol/L), and Akt inhibitor (Wortmannin, 1 μmol/L) for 2d. [a]P<0.05, [b]P<0.01, [c]P<0.001 *vs* TGF-β1 group.

Smad2, ERK, p38 and JNK would distinctly suppress TGF-β1-induced EMT; on the contrary, the inhibition of Akt promote EMT (Figure 4B). The change of E-cadherin was less than Fibronectin and N-cadherin, especially the mRNA expression which did not show any significant difference.

The proliferation and migration of HCECs were taken into consideration (Figure 5). The cell viability assay (CCK-8) showed that the inhibition of ERK and JNK pathways significantly suppress the proliferation of HCECs (P<0.01), but the inhibition of Smad2 and p38 pathways was positive impact compared with TGF-β1 group (P<0.05; Figure 5A-5B). As for the cell migration assay (transwell), the inhibition of Smad2, ERK, JNK and Akt decreased the migration of HCECs, and it deserved to be mentioned that the inhibition of p38 prominently increased cell migration (P<0.01).

Effect of EGF on TGF-β1-induced Epithelial-Mesenchymal Transition, Proliferation and Migration In comparison to TGF-β1 (10 ng/mL) group, Fibronectin and N-cadherin showed evident low expression in the groups with EGF (5, 10, 20 ng/mL, with or without TGF-β1; P<0.01), while E-cadherin showed up-regulation of protein level (P<0.05) and remained unchanged of mRNA expression. Concentration-dependence was not obvious (Figure 6).

The proliferation of HCECs treated with EGF (10 ng/mL) was promoted (P<0.001; Figure 7A). A cluster of cells like huge islands could be seen in EGF group and EGF+TGF group (P<0.001). The cell viability showed that EGF enhanced proliferation of HCECs with concentration-dependence, and could reverse the suppressive effect of TGF-β1 on cells proliferation (Figure 7B). Cells migration showed that EGF (10 ng/mL) remarkably promoted HCECs' migration. Interestingly, the combinative group of TGF-β1 (10 ng/mL) and EGF (10 ng/mL) developed more migration capability than the groups with TGF-β1 or EGF only (P<0.001; Figure 7C, 7D).

Effect of EGF on Signaling Pathways in TGF-β1-induced Epithelial-Mesenchymal Transition The phosphorylation of Akt, ERK, p38 and Smad2 in HCECs was detected after TGF-β1 and EGF treatment (Figure 8). For p38 Signaling pathway, TGF-β1 brought a significant promotion, but EGF remarkably blocked this effect. The blockage of EGF was more obvious at 2h, approaching the control group. The activation of Smad2 signaling pathway induced by TGF-β1 was quite strong (over 30 times of control group), and was also inhibited by EGF, but the inhibition could not be detected until 2h. ERK signaling pathway was activated in groups with EGF, and the group with both TGF-β1 and EGF showed stronger activation, especially at 1h. As for Akt signaling pathway was inhibited in

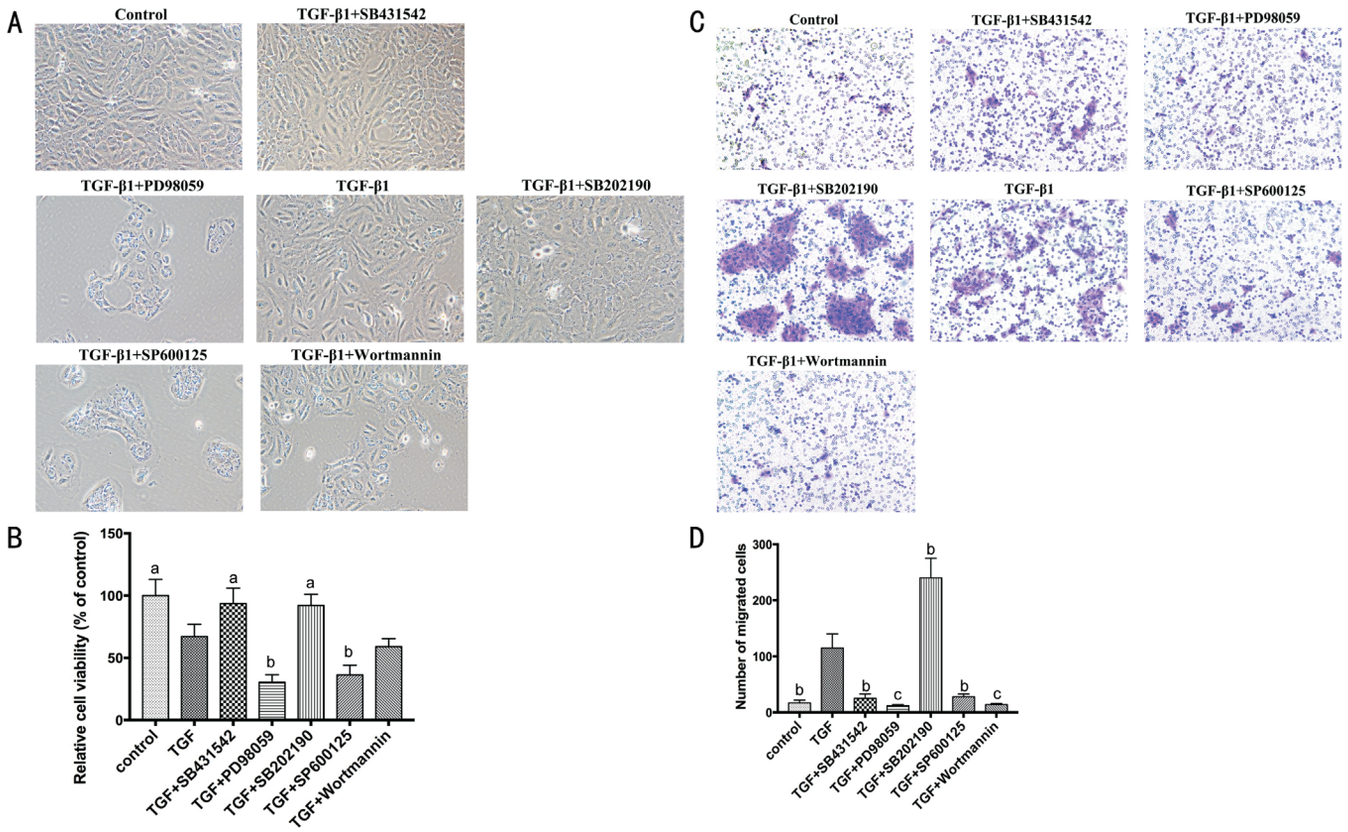

Figure 5 Signaling pathways involved in proliferation and migration of HCECs A: HCECs treated with TGF-β1 (10 ng/mL) combining Smad2 inhibitor (SB431542, 10 μmol/L), ERK inhibitor (PD98059, 20 μmol/L), p38 inhibitor (SB202190, 10 μmol/L), JNK inhibitor (SP600125, 10 μmol/L), and Akt inhibitor (Wortmannin, 1 μmol/L); B: The viability of HCECs; C-D: HCECs migration in transwell assay. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ *vs* TGF-β1 group.

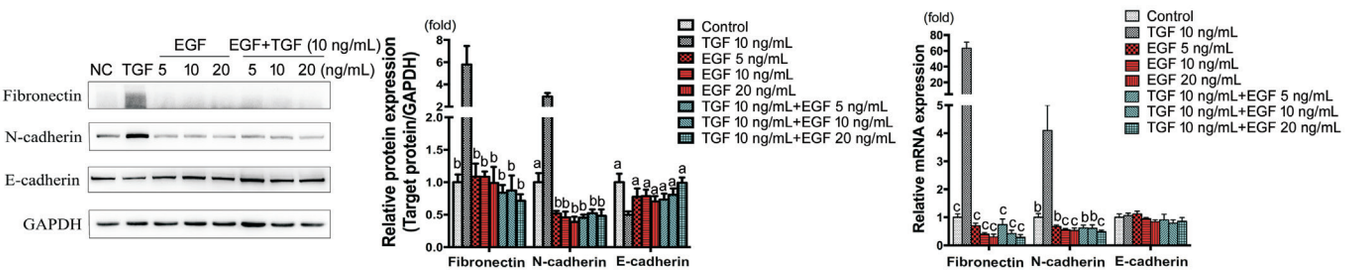

Figure 6 Fibronectin, N-cadherin and E-cadherin expression in HCECs treated with TGF-β1 and EGF HCECs were treated with TGF-β1, EGF, or TGF-β1+EGF and Fibronectin, N-cadherin and E-cadherin protein and mRNA expressions were detected by Western blot and RT-PCR respectively. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ *vs* TGF-β1 group.

groups with EGF, and the inhibition was more significant in 2h group.

DISCUSSION

There are three isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3). TGF-β1 is at a low level in normal cornea, and after wounding it will show a high level along the Bowman's layer. TGF-β2 keeps prominent expression in normal cornea, and up-regulates in epithelial cells migration after wounding. TGF-β3 is observed only in the basal cells, and showed weak relationship to epithelial cells migration[19]. In this study, we chose TGF-β1 in order to figure out the mechanism of TGF-β-induced EMT.

In the present study, we observed that TGF-β1 presented promotion of EMT in HCECs, on both levels of mRNA and protein (Figures 1, 2). And it showed suppressive proliferation and enhanced migration of cells (Figure 3). In lots of studies, TGF-β family proteins are well demonstrated to be the inducer of EMT[20], not only in ocular tissues, but also in many other organs. TGF-β is implicated in numerous pathological processes such as carcinogenesis, immoderate tissue fibrosis, and immunological disorders. TGF-β plays a profitable role in some physiological activities[21]. For example, investigators reveal that in early stages of cancer, TGF-β shows tumor suppressive effects, but in the late stages, TGF-β promotes

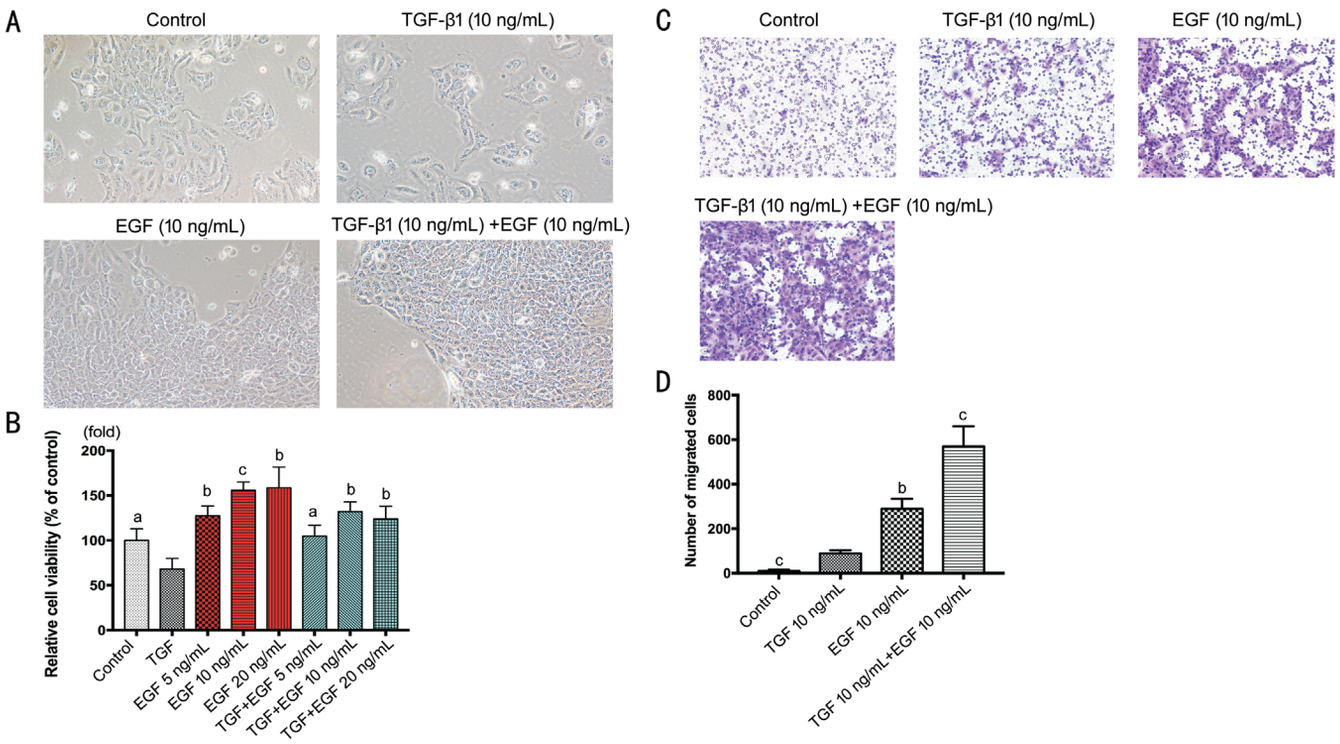

Figure 7 Effect of EGF on TGF-β1-induced proliferation and migration of HCECs A: HCECs treated with TGF-β1, EGF, or TGF-β1+EGF; B: The viability of TGF-β1 treated HCECs with or without EGF; C-D: HCECs migration in transwell treated with TGF-β1 with or without EGF. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ *vs* TGF-β1 group.

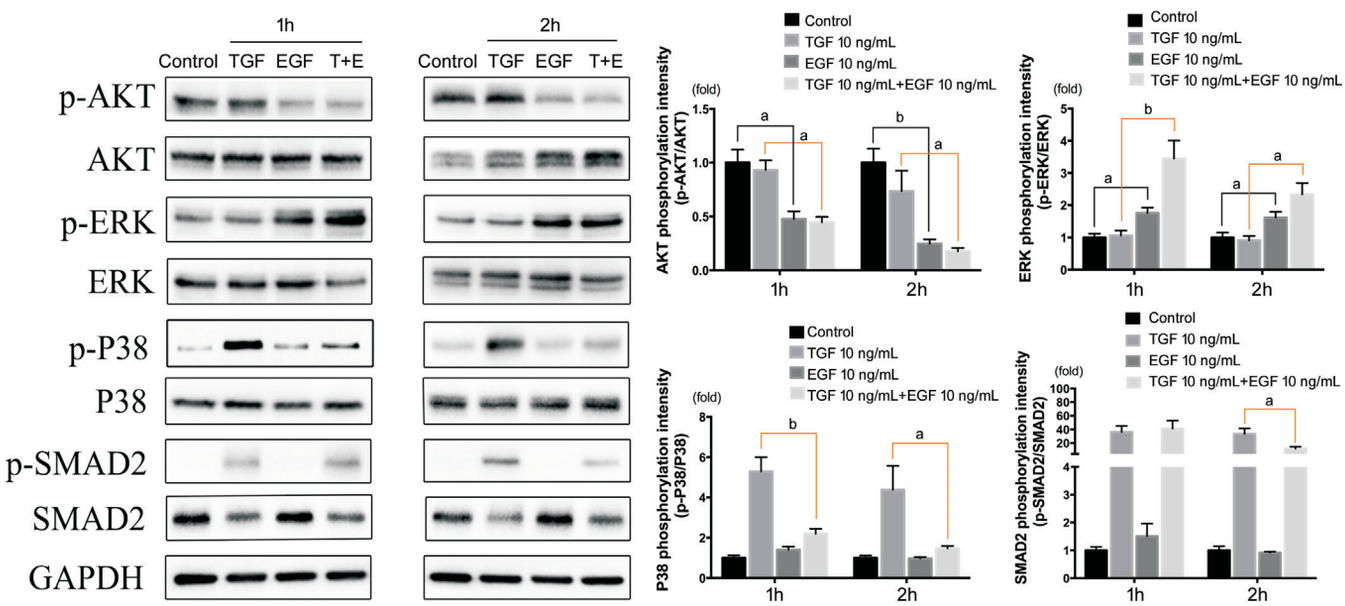

Figure 8 Effect of EGF on signaling pathways in TGF-β1-induced EMT The expression of p-Akt/Akt, p-ERK/ERK, p-p38/p38, p-Smad2/Smad2 in HCECs treated with TGF-β1 and EGF. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

invasiveness and metastasis[22]. For eyes, TGF-β exerts influence on both sides too, participating in inflammation, fibrosis, or acting as a helper for wound healing[23].

Signaling pathways are key processes for TGF-β exerting effect. In the control group and at the very beginning of the treatment by TGF-β1, the expression of p-Smad2 and p38 was at an extraordinary low level. As time course going on, these two pathways showed peak level at 30 and 60min (Figure 4A). It indicated that TGF-β1 acted as the trigger of p-Smad2 and p38 pathways' activation. In numbers of researches, TGF-β is well demonstrated to be an important participator of EMT *via* Smad or non-Smad pathways[24-25]. Smad2/3 are key signaling molecules that are phosphorylated after TGF binding to TGF receptor. In this process, lots of Smads participate in Smad-

depending signaling, such as coactivator Smad4, inhibitory regulator Smad6 and Smad7[11,26]. The non-Smad pathways consist of many Smad-independent signaling, like p38, ERK, JNK and Akt, as we selected in this study. Some researchers mention that there are certain interactions between Smad and non-Smad pathways. For instance, p38 pathway activates phosphorylation of Smad3 thus leading to the enhancement of Smad3/4 complex formation[27].

The treatment of inhibitors revealed the parallel conclusion. When Smad2 and p38 pathways were blocked, EMT was inhibited on mRNA and protein levels (Figure 4B), and cells proliferation increased (Figure 5A, 5B). As for the cells migration (Figure 5C, 5D), Smad2's inhibition showed down-regulation as mentioned, but p38 was a little different. The blockage of p38 brought a high promotion of migration in HCECs like EMT process, however the EMT-relative mRNA and protein expression was decreased. Researchers investigate that inhibition of p38 partially reverses EMT changes in breast cancer cells, with decreasing gene expression of the EMT markers Twist, Snail, Slug and ZEB, as well as N-cadherin protein[28]. And p38 MAPKs have been implicated in phosphorylation of serine 68 which is a major phosphorylation site of Twist1, thus promoting EMT[29]. Moreover, our study revealed that the inhibition of p38 pathway would promote cellular viability and migration of HCECs, and this phenomenon has rarely been mentioned. In cardiomyocytes, some research shows that the blockage of p38 signaling pathway can rescue the reduced cell viability[30]. The suppression of ERK and JNK also showed decreasing EMT-relative mRNA and protein expression. However, the proliferation of HCECs was remarkably inhibited (Figure 5A, 5B), and it was considered to be an indispensable reason of decreasing EMT. In our point of view, ERK and JNK are essential for HCECs' survival, which is the foundation of EMT's initiation and development[31-33].

When the corneal epithelium is injuried, growth factors (EGF, PDGF, TGF, FGF, IGF-I, KGF and HGF) and inflammatory factors (IL-1, IL-6, and IL-10, and TNF-α) are released, which are important regulators that stimulate corneal epithelial cell growth, proliferation, migration, differentiation, adhesion involved in wound healing. They also mediate different cell functions including intracellular and intercellular signaling molecules. TGF-β stimulates corneal epithelial cell migration *via* integrin β1, which mediates p38 MAPK activation, extracellular matrix expression and EMT leading to increased cell mobility. The fundamental purpose of this study was to figure out the effect of EGF on TGF-β1-induced EMT and its potential mechanism within HCECs. We observed that EGF itself would not induce EMT, and it could restrain TGF-β1-induced EMT on both mRNA and protein levels, within the concentration from 5 to 20 ng/mL (Figure 6). The suppressive effect of EGF on TGF-β-induced EMT has been reported in some organs and tissues. EGF will restrain the initiation of TGF-β1-induced EMT, and even reverse the transition[14]. Besides, some researchers prove that EGF can induce phosphorylation and expression of TGF-β/Smad repressor, thus inhibiting TGF-β1-stimulated markers of EMT[34].

Furthermore, EGF brought prominent raising of HCECs' proliferation (Figure 7A, 7B). The micrographs showed different cells morphological characteristic in comparison to control group and TGF-β1 group. HCECs treated with EGF tended to be clusters of cells like huge island, rather than scattered. The exist of EGF relieved the suppressive impact of TGF-β1 on HCECs' proliferation.

In addition, the cells migration with EGF was enhanced (Figure 7C, 7D). We observed that the EGF group showed higher migration than TGF-β1 group, and the combing group with both EGF and TGF-β1 showed the highest. EGF significantly inhibited TGF-β1-induced EMT in HCECs, but the cell motility remarkably enhanced. This is an interesting discovery, because numerous researches point out that EMT is positively correlated to cells motility and migration[6,35]. In some studies, it has been demonstrated that EGF promotes motility and migration of cells including HCECs in a non-EMT way, for instance, through ROS, MEK/ERK and JNK pathways[36-38]. In our research, targeted inhibition of p38 brought decreasing effect on EMT, but the proliferation and migration of HCECs increased. This phenomenon was parallel to the effect of EGF combining TGF-β1. Furthermore, we found that EGF significantly inhibited the phosphorylation of p38 (Figure 8), thus producing biological effects. We suspect that p38 is a key signaling pathway for EGF to inhibit TGF-β1-induced EMT and promote HCECs' proliferation and migration.

We hold the opinion that EGF inhibits TGF-β1-induced EMT *via* suppressive p38 and Smad2, and promotes cells proliferation and migration in a non-EMT process by inhibiting p38-pathway. The combination of EGF and TGF-β1 enhances HCECs' proliferation and migration, bringing advantage for corneal epithelium wound healing. It reveals that EMT is not the only process for corneal epithelium healing. EGF up-regulates the motility and migration of HCECs *via* p38 inhibition, in a non-EMT manner.

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Authors' contributions: Chen SY completed the main part of the experimental research; Xie C analyzed the experimental data, and was a co-author in writing the manuscript; Zhu H completed part of this study, and provided experimental materials; Shen Y designed the methods of this study. All authors read and approved the final manuscript

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