·Basic Research·

Skullcapflavone II suppresses TGF-β-induced corneal epithelial mesenchymal transition *in vitro*

Meng-Xi Li[1], Zhen Zhang[1,2], Yue Zhang[1,2], Fan-Ru Zhao[1], Yu-Fan Li[1], Yu-Fei Dang[1], Yang-Yang Yue[3], Li Li[1]

[1]Department of Ophthalmology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
[2]Xi'an People's Hospital (Xi'an Fourth Hospital), Shaanxi Eye Hospital, Affiliated Xi'an Fourth Hospital, Northwestern Polytechnical University, Xi'an 710000, Shaanxi Province, China
[3]Department of Vascular Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China
Co-first authors: Meng-Xi Li and Zhen Zhang
Correspondence to: Yang-Yang Yue and Li Li. The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China. 18792890313@163.com; eyelili@126.com
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Abstract
● **AIM:** To investigate the effect of skullcapflavone II (SCF-II) on the epithelial-mesenchymal transition (EMT) induced by transforming growth factor beta (TGF-β) in human corneal epithelial cells (HCECs), as well as to identify the signaling pathways that may be involved.

● **METHODS:** HCECs were cultured *in vitro*. At a SCF-II (5, 10 μmol/L) dose, cell viability was analysed with a cell counting kit-8 (CCK-8) assay, and cell migration was monitored with wound healing and Transwell migration assays. There were 4 groups: SCF-II, TGF-β, SCF-II+TGF-β and Control. Western blotting and immunofluorescence were performed to show the expression of EMT markers and the translocation of nuclear factor kappa-B (NF-κB) into the nucleus in the 4 groups.

● **RESULTS:** Treatment with SCF-II decreased HCEC viability in a dose-dependent manner. A concentration below 10 μmol/L did not present obvious cell toxicity, and survival rates were more than 70% at 48h. Treatment with SCF-II (5 and 10 μmol/L) significantly impeded migration in wound healing and Transwell migration assays ($P<0.05$), and EMT markers and NF-κB translocation into the nucleus were inhibited. After both TGF-β and SCF-II treatment, the migration of TGF-β-treated HCECs were suppressed by SCF-II ($P<0.05$). The expression levels of the mesenchymal markers N-cadherin ($P<0.05$), α-smooth muscle actin (α-SMA; $P<0.05$) and NF-κB ($P<0.05$) in both TGF-β- and SCF-II-treated HCECs were lower than those in the HCECs treated with TGF-β alone and higher than those in HCECs treated with SCF-II alone. Immunofluorescence showed that the entry of NF-κB into the nucleus in both TGF-β- and SCF-II-treated HCECs was less than that in the TGF-β-treated HCECs.

● **CONCLUSION:** SCF-II inhibit TGF-β-induced EMT in HCECs by potentially regulating the NF-κB signalling pathway. Thus, SCF-II represents a candidate putative therapeutic agent in corneal fibrotic diseases.

● **KEYWORDS:** skullcapflavone II; epithelial-mesenchymal transition; transforming growth factor; nuclear factor kappa-B; human corneal epithelial cells
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INTRODUCTION
Blindness resulting from corneal disease is the third most prevalent cause of blindness worldwide[1]. Corneal transplantation remains the primary method for addressing corneal blindness[2]. However, this procedure is significantly limited by issues such as rejection, infection, failure, and a shortage of donors[3]. Identifying drugs that can reverse excessive corneal fibrosis and preserve vision—potentially even partly replacing keratoplasty, is a huge challenge for ophthalmologists. It has been confirmed that epithelial-mesenchymal transition (EMT) is crucial for corneal fibrosis[4]. This condition encompasses several processes, including delayed corneal epithelial healing and the release of various cytokines, such as interleukin-1α, interleukin-1β, and transforming growth factor beta (TGF-β), which facilitate the transformation of keratocytes into mature myofibroblasts[5-6], Corneal opacification results from abnormal deposits of the

extracellular matrix (ECM) deposits[7]. If these processes are not halted, excessive corneal opacity may become irreversible, leading to blurred vision, decreased vision, and even corneal blindness. In summary, while EMT in the early stage of wound healing, excessive EMT is a direct cause of corneal opacity and corneal blindness[6]. Therefore, it is crucial to maintain a balanced EMT and the balance between repair and fibrosis is essential. The development of drugs that prevent excessive corneal fibrosis by suppressing EMT could significantly reduced.

TGF-β plays a crucial role in inducing EMT[8], and the activation of the TGF-β/nuclear factor kappa-B (NF-κB)/ matrix metalloprotein-9 (MMP-9) pathway promotes epithelial repair following corneal injury[9]. Inhibition of NF-κB has been shown to reduce EMT[10]. In the context of the cornea, one study demonstrated that hypercapnic acidosis impaired TGF-β1-induced migration of corneal fibroblasts and the expression of alpha smooth muscle actin (α-SMA), in which the NF-κB and Smad signaling pathways may be involved[11]. These findings indicate that the upregulation of TGF-β primes EMT, and that activation of either the TGF-β or NF-κB pathway may contribute to corneal EMT, which is characterized by promotion of the downregulation of epithelial markers such as E-cadherin, alongside the upregulation of mesenchymal markers including N-cadherin, vimentin, and α-SMA[12-14]. Therefore, our objective was to investigate certain drugs that, *via* the TGF-β or NF-κB pathway, may attenuate EMT and achieve the aim of preserving corneal transparency.

Skullcapflavone II (SCF-II), a traditional Chinese medicine, has been proven to suppress the EMT of tumor cells and thus prevent their and migration[15]. Suppressing the TGF-β-induced EMT is the core signalling pathway. However, in corneal tissue, whether SCF-II, as a drug, can reverse excessive fibrosis and maintain vision is unclear. Therefore, SCF-II was investigated in this study for its effect on EMT induced by TGF-β in human corneal epithelial cells and the potential signalling pathways that might be involved.

MATERIALS AND METHODS

Cell culture Immortalized human corneal epithelial cells (HCECs) were cultured in DMEM-F12 (Gibco; Thermo Fisher Scientific, USA), including 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, USA), epidermal growth factor (EGF); 10 ng/mL; AF-100-15; PeproTech, USA), insulin (5 μg/mL; Sigma-Aldrich, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL). A 37°C incubator with 5% CO_2 was used to culture the cells.

Antibodies and Reagents Human recombinant TGF-β and Cell Counting Kit-8 (CCK-8) were obtained from Selleckchem (Houston, TX, USA). The primary antibodies included: anti-N-cadherin (Rabbit, Abcam, Cambridge, UK, 1:1000 dilution, No.ab245117) and anti-E-cadherin (Rabbit, Abcam, Cambridge, UK, 1:1000 dilution, No.ab11512), anti-α-SMA (Rabbit, Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution, No.19245) and anti-NF-κB (Rabbit, Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution, No.8242), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Mouse, Proteintech, Manchester, UK, 1:1000 dilution, No.60004). All secondary antibodies were purchased from Thermo Fisher Scientific. SCF-II was acquired from Sigma-Aldrich, Germany (No.SMB00545). Other chemicals were purchased from Proteintech, Manchester, UK.

Cell Viability Assay HCECs were seeded at a density of 2000 cells/well in 96-well plates. Then, different concentrations of SCF-II (0-10 μmol/L) were added for 24 or 48h. Next, mixed CCK-8 reagent (10 μL) and serum-free medium (100 μL) were added to each well. Finally, the absorbance was measured with a microplate reader (Bio-Tek Instruments, Winooski, USA) at 450 nm after 4h of incubation.

Wound-Healing Assay HCECs were seeded in 6-well plates, and serum-free medium was used for 6h. When the cell density reached 100%, a 200-μL pipette tip was used to draw cross-line wounds. After washing three times with phosphate buffered saline (PBS), DMEM-F12 with 1% fetal bovine serum (FBS) and SCF-II (5, 10 μmol/L) were added to the cells. Wound healing was shown through a phase-contrast microscope after 24h at the same position, and the rate of wound healing was measured using ImageJ (NIMH, National Institutes of Health, USA).

Transwell Migration Assay Trypsinized and resuspended HCECs were cultured in serum-free medium containing SCF-II (5, 10 μmol/L). One millilitre of medium with 10% FBS was added to each well (the bottom well) in 24-well plates. The cell suspension was seeded in polycarbonate membrane inserts with 8-μm pores (the top well). After incubation for 72h, some of the cells had migrated to the underside side of the polycarbonate membrane. The cells were fixed with 4% paraformaldehyde and stained with hematoxylin. Migratory cells were counted by using a phase-contrast microscope (Nikon Eclipse Ti-S, Tokyo, Japan), and three different locations were counted in each insert.

Western Blotting The HCECs were lysed using radioimmunoprecipitation assay (RIPA) buffer for 10min on ice and centrifuged at 4°C (15000×g, 15min). The proteins were separated by electrophoresis using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, which were loaded with equal amounts of each protein sample. After transferring resolved proteins to nitrocellulose membranes, they were blocked with 5% nonfat milk for 1h. Then the membranes were treated with the primary antibodies overnight. After washing with TBST (a mix of Tris-buffered

saline and Tween 20) 3 times, the membranes were treated with the secondary antibodies. Finally, Trident femto Western HRP Substrate Solutions A and B (GeneTex) were mixed at a 1:1 ratio and added to the PVDF membrane. The protein bands were visualized by the Bio-Rad ChemiDoc XRS+ chemiluminescence imaging system and analysed with ImageJ (NIMH, National Institutes of Health, USA).

Immunofluorescence In 24-well plates, cells were seeded overnight on glass sides. After washing three times with PBS, 4% paraformaldehyde was used to fix the cells for 30min at room temperature. Then, the cells were treated with permeabilization buffer (1% Triton X-100 in PBS) for 30min and blocked for one hour with sealing agent after washing with PBS. Next, they were treated with anti-NF-κB antibody (Rabbit, Cell Signaling Technology, Danvers, MA, USA, 1:200 dilution, No.8242) at 4℃ overnight and then stained with secondary antibody [Goat anti-Rat IgG(H+L) Cross-Adsorbed Secondary Antibody, Cyanine3, Thermo Fisher Scientific, Hanover Park, IL, 1:200 dilution, No.A10522] for 1h in a darkroom after washing. Subsequently, DAPI (1 μg/mL) was used to stain the nuclei for 5min. Finally, a ZEISS Instruments confocal microscope was used to observe and photograph the cells.

Statistical Analysis Each experiment was evaluated statistically with GraphPad Prism software package (GraphPad Prism version 8.0; La Jolla, CA, USA). All data were reported as the mean± standard deviation (SD) of triplicate experiments. The differences between every two groups were compared with an independent *t* test. When the value of *P* was less than 0.05, statistical significance was defined.

RESULTS

Structure and Cell Toxicity of SCF-II for HCECs Since there is limited evidence to confirm the safety of SCF-II on normal corneal cells, we first evaluated the cell toxicity of SCF-II on HCECs using the CCK-8 assay. SCF-II dose-dependently decreased the viability of HCECs (Figure 1B). According to the data analysis, 5 and 10 μmol/L SCF-II were used in the subsequent experiments, which did not induce obvious cell toxicity, and the survival rates were more than 70% at 48h.

SCF-II Influenced Corneal Cell Migration To assess the effects of SCF-II on HCECs migration, we performed in wound-healing and Transwell migration assays. After 24h of treatment with SCF-II, the wound-healing assay showed that SCF-II significantly inhibited the wound healing effect in a concentration-dependent manner compared with the control group (Figure 2A). Additionally, the Transwell assay indicated that compared with the control group, the number of migrated cells was significantly lower in the SCF-II-treated samples (Figure 2A). Overall, SCF-II could influence cell migration.

SCF-II Decreased EMT Through Inhibition of EMT-Related Marker Expression EMT has been confirmed to be crucial in corneal fibrosis. Reasonable EMT is the key to promoting corneal wound repair and maintaining its transparency, but excessive EMT is the direct cause of corneal opacity and corneal blindness. Therefore, maintaining a reasonable EMT and the balance between repair and fibrosis is important. The effect of SCF-II on the expression of EMT markers, including NF-κB, E-cadherin, N-cadherin and α-SMA was investigated in this study. The expression of NF-κB was decreased significantly after treatment with SCF-II for 48h in a dose-dependent manner (Figure 3A). Meanwhile, the epithelial marker E-cadherin was upregulated, and mesenchymal markers, including N-cadherin and α-SMA, were downregulated. These results strongly suggest that SCF-II suppresses EMT progression. Additionally, immunofluorescence showed that the expression of NF-κB in the nucleus was decreased in SCF-II-treated HCECs (Figure 3B). Culturing with SCF-II for 48h caused a significant decrease in NF-κB translocation into the nucleus compared with untreated cells.

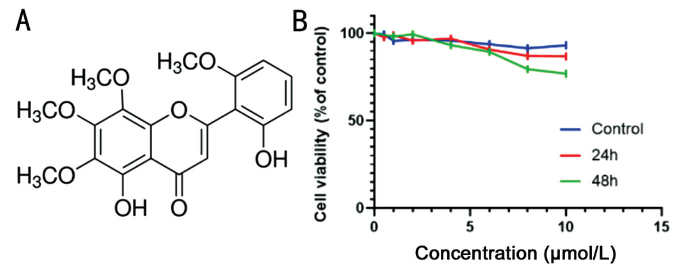

Figure 1 Structure and cell toxicity of SCF-II for HCECs A: Chemical structure of SCF-II; B: Cell viability was assessed using the CCK-8 assay. Compared with the control group, the viability of HCECs decreased after treatment with SCF-II for 24, 48h, respectively, but not lower than 70%. *P*<0.05. SCF-II: Skullcapflavone II; HCECs: Human corneal epithelial cells; CCK-8: Cell counting kit-8.

SCF-II Attenuated Cell Migration of TGF-β-Induced EMT TGF-β is vital for inducing EMT, which could contribute to the development of corneal fibrosis. To determine the inhibitory effect of SCF-II on TGF-β-induced EMT, we analysed morphological changes in HCECs. Treatment with TGF-β induced the mesenchymal phenotype as elongated and spindle-like shapes, but 10 μmol/L SCF-II significantly suppressed this kind of phenotype (Figure 4A). Thus, we speculate that TGF-β-induced EMT is inhibited by SCF-II.

As shown in Figure 4B, after 24h of treatment, the remaining wound area was lower in the group treated with TGF-β than in the control group, which confirmed that upregulation of TGF-β is the priming factor of EMT. Additionally, the remaining wound area was significantly larger in the presence of SCF-II than in the control wells. Moreover, the remaining wound area in the presence of both SCF-II and TGF-β was significantly

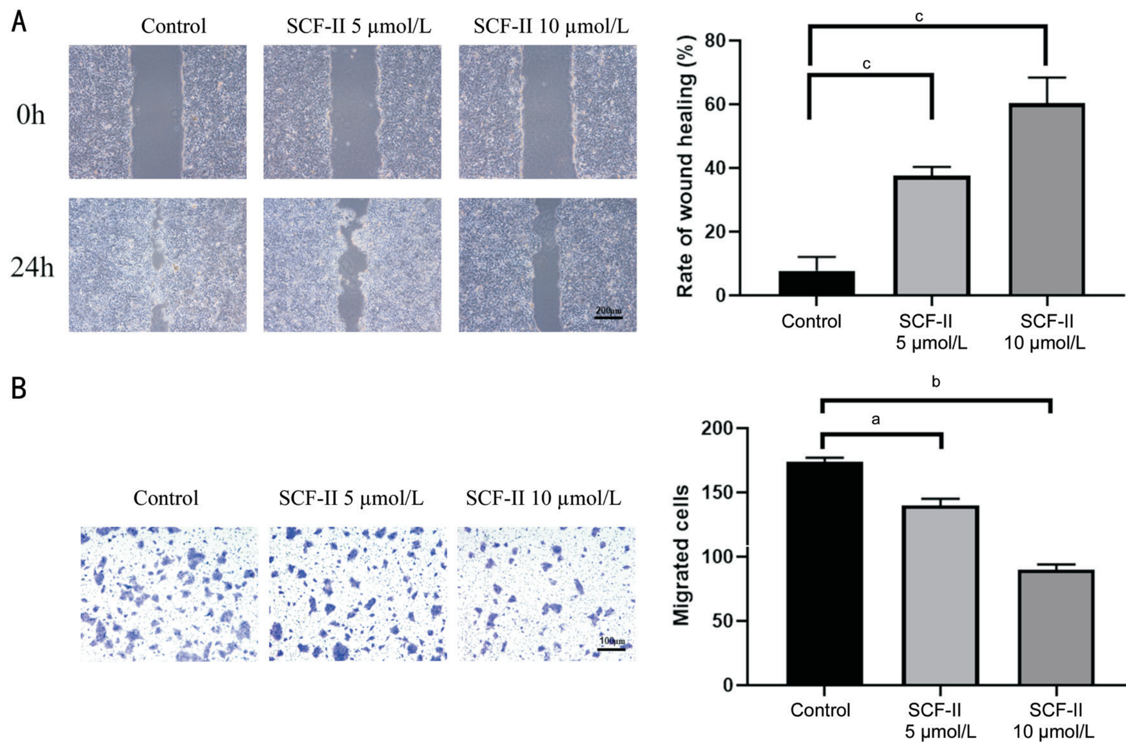

Figure 2 SCF-II attenuated migration of HCECs A: Wound-healing assay showed SCF-II significantly inhibited wound healing in HCECs in a concentration-dependent manner compared with control; B: Transwell migration assays revealed that SCF-II decreased the migration of HCECs in a concentration-dependent manner compared with control. *n*=3, scale bars, 200 μm (A) and 100 μm (B). $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. SCF-II: Skullcapflavone II; HCECs: Human corneal epithelial cells.

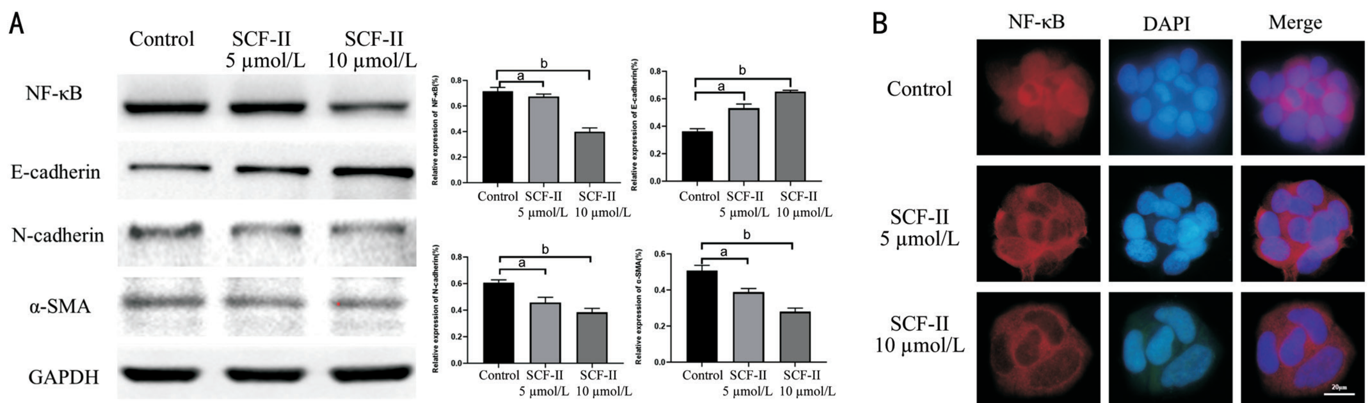

Figure 3 SCF-II attenuated EMT by decreasing expression of markers related to EMT and NF-κB translocation into the nucleus A: Western blotting showed treatment with SCF-II resulted in significant downregulation of mesenchymal factors (NF-κB, N-cadherin, α-SMA) and upregulation of E-cadherin (*n*=3, $^{a}P<0.05$, $^{b}P<0.01$); B: Immunofluorescence analysis confirmed the Western blot analysis results. SCF-II decreased NF-κB translocation into the nucleus. Red represents NF-κB, and blue represents nuclei. Scale bars=20 μm. SCF-II: Skullcapflavone II; EMT: Epithelial-mesenchymal transition; NF-κB: Nuclear factor kappa-B; α-SMA: Alpha smooth muscle actin.

larger than that in wells treated with only TGF-β but lower than that in the wells treated with only SCF-II. It could be concluded that SCF-II could inhibit TGF-β-induced cell migration.

To further verify whether SCF-II could inhibit TGF-β-induced EMT migration progression, we performed a Transwell migration assay, the results of which matched those obtained from the wound-healing assay. The numbers of migrated cells were lower in both the SCF-II- and TGF-β-treated samples than in the TGF-β-treated samples (Figure 4C).

SCF-II Attenuated TGF-β-Induced EMT by Decreasing the Expression of EMT-Related Markers and NF-κB Translocation into the Nucleus Western blotting showed that the expression of E-cadherin decreased significantly, whereas the expression levels of N-cadherin, α-SMA and NF-κB increased in TGF-β-treated HCECs (Figure 5A). In contrast, SCF-II obviously reversed the TGF-β-induced decrease in E-cadherin expression. In addition, the increases in N-cadherin, α-SMA and NF-κB expression induced by TGF-β were clearly inhibited by SCF-II.

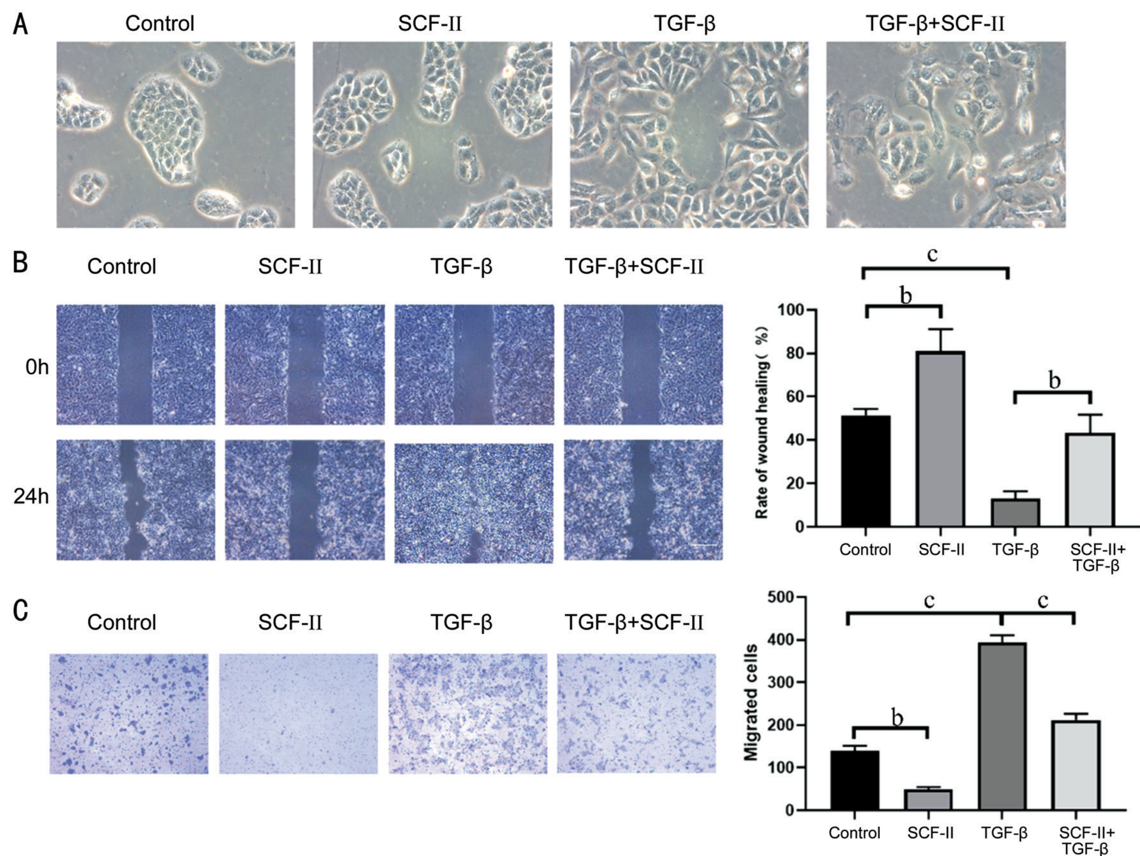

Figure 4 SCF-II attenuated the migration of TGF-β-induced EMT A: HCECs treated with TGF-β or TGF-β+SCF-II; B: Wound-healing assay showed SCF-II significantly inhibited the migration of HCECs in the presence of TGF-β (10 ng/mL); C: Transwell migration assays revealed that SCF-II decreased the migration of HCECs in the presence of TGF-β (10 ng/mL). *n*=3, scale bars, 50 μm (A), 200 μm (B), and 100 μm (C). [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001. SCF-II: Skullcapflavone II; TGF-β: Transforming growth factor β; EMT: Epithelial-mesenchymal transition; HCECs: Human corneal epithelial cells.

Immunofluorescence analysis showed that the expression of NF-κB in the nucleus was lower in both SCF-II- and TGF-β-treated HCECs than in TGF-β-treated cells, which was in accordance with the Western blotting results (Figure 5B). These results strongly suggested that SCF-II suppressed the effect of TGF-β-induced EMT in HCECs and that the NF-κB signalling pathway was involved.

DISCUSSION

Clinically, there is currently no exact and effective method to restore corneal opacity except corneal transplantation. Therefore, finding a drug to inhibit EMT at the optimal time point when the tissue has repaired completely without negative effects and that results in less scarring is important. To that end, our team has evaluated new antifibrotic agents from natural sources, such as SCF-II. As a kind of Chinese herbal medicine, it is extracted from the root of *Scutellaria baicalensis*[16]. Many studies have confirmed its antifibrotic, antioxidant, anti-inflammatory and anticancer effects[15,17-19]. Our research demonstrated the effect of SCF-II on EMT in corneal tissues.

EMT is the fundamental mechanism of fibrosis in many organs[6,20-22]. TGF-β is secreted during EMT, and the persistence of TGF-β promotes EMT[23]. It is reasonable to suppress TGF-β-induced EMT to reduce corneal opacity. In recent years, studies have shown that some drugs can suppress corneal EMT, such as bone morphogenetic protein 4 (BMP4)[24] and hypercapnic acidosis[11]. The key in our study is that the results showed that SCF-II also suppressed the migration of HCECs by wound-healing and Transwell migration assays (Figure 2). Meanwhile, the upregulation of the mesenchymal markers N-cadherin and α-SMA, the downregulation of the epithelial marker E-cadherin, and the entry of NF-κB into the nucleus were shown in Figure 3. These results suggested that EMT was inhibited in SCF-II-treated HCECs.

Moreover, the NF-κB pathway is activated in numerous ocular surface diseases as a key transcription factor pathway for processes such as inflammation, angiogenesis, stress response and corneal wound healing[25-28]. For corneal fibroblasts, stress factor NF-κB-induced inflammation is essential for corneal scarring[27,29]. A study found that olopatadine could significantly reduce corneal opacity in alkali-induced corneal injury in rats by suppressing NF-κB expression[30] and simultaneously resulted in pulmonary fibrosis by reversing the process of EMT through the NF-κB/p65 pathway[31]. SCF-II can reduce

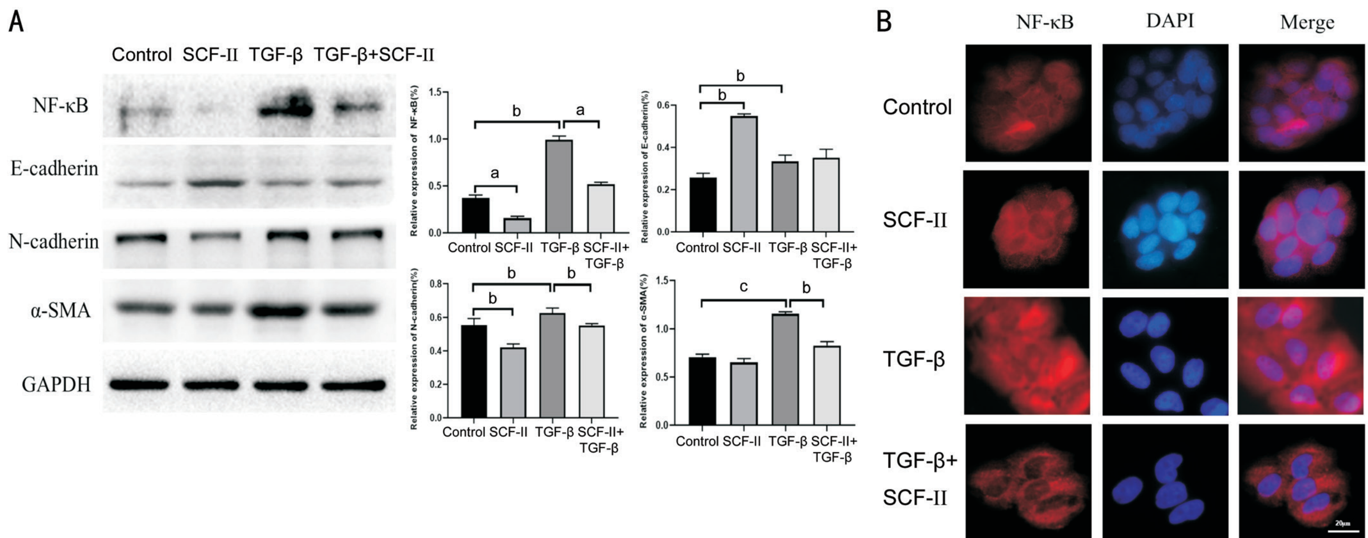

Figure 5 SCF-II attenuated TGF-β-induced EMT by decreasing expression of EMT-related markers and NF-κB translocation into the nucleus A: Western blotting showed treatment with SCF-II (10 μmol/L) decreased mesenchymal factors (NF-κB, N-cadherin, α-SMA) and increased epithelial markers (E-cadherin) in the TGF-β (10 ng/mL)-treated HCECs (n=3, $^aP<0.05$, $^bP<0.01$, $^cP<0.001$); B: Immunofluorescence analysis confirmed the Western blot analysis results. SCF II (10 μmol/L) decreased NF-κB translocation into the nucleus in the TGF-β (10 ng/mL)-treated HCECs. Red represents NF-κB, and blue represents nuclei. Scale bars =20 μm. SCF-II: Skullcapflavone II; TGF-β: Transforming growth factor β; EMT: Epithelial-mesenchymal transition; NF-κB: Nuclear factor kappa-B; α-SMA: Alpha smooth muscle actin.

the main pathophysiological characteristics of allergic asthma by regulating the TGF-β1/Smad signalling pathways[18]. It could also inhibit the activation of NF-κB and the subsequent MMP-1 expression induced by tumor necrosis factor (TNF)-α in foreskin fibroblasts[15]. Therefore, SCF-II has the potential to inhibit TGF-β-induced EMT *via* the NF-κB pathway. Thus, the downregulation of TGF-β and NF-κB is a potential treatment point to inhibit EMT. In our results, after treatment with both TGF-β and SCF-II, morphological changes showed that SCF-II inhibited the TGF-β-induced mesenchymal phenotype, and the migration of TGF-β-treated HCECs were suppressed by SCF-II (Figure 4). Both TGF-β- and SCF-II-cultured HCECs had lower expression levels of N-cadherin, α-SMA and NF-κB than HCECs treated with only TGF-β and higher expression in HCECs treated with only SCF-II (Figure 5). The results indicated that the effect of SCF-II on corneal EMT was related to TGF-β. Moreover, immunofluorescence analysis showed that TGF-β promoted the entry of NF-κB into the nucleus, while SCF-II attenuated NF-κB (Figure 4). Hence, this study confirmed that SCF-II could inhibit TGF-β-induced EMT in HCECs by regulating the NF-κB signalling pathway.

In the present study, we confirmed that SCF-II has few cytotoxic effects when the concentration of SCF-II is 5 and 10 μmol/L and that SCF-II suppresses the NF-κB signalling pathway involved in TGF-β-induced EMT in cultivated HCECs. To the best of our knowledge, this is the first study to evaluate the antifibrotic effect of SCF-II in TGF-β-induced corneal EMT. This study has some limitations. We found that SCF-II could inhibit TGF-β-induced EMT in HCECs by potentially regulating the NF-κB signalling pathway, but the precise mechanism is unclear. In the next step, we will attempt to determine the relationship between TGF-β and NF-κB and the signalling pathways in which they are involved in EMT. To solve the clinical problem of reduced corneal opacity after wound healing, further studies are needed to clarify whether SCF-II suppresses corneal fibrosis and has no negative effect on corneal tissue healing in animal models. SCF-II suppresses TGF-β-induced EMT in cultivated HCECs by potentially regulating the NF-κB signalling pathway. This finding provides a new way to reduce corneal opacity.

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Authors' contributions: Zhang Y, Li MX, Zhang Z acquired the data; Zhang Z and Li MX wrote the manuscript; Zhao FR, Li YF and Dang YF analyzed the data; Yue YY and Li L conceived the research; Li L supervised the research and revised the manuscript.

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Conflicts of Interest: Li MX, None; **Zhang Z,** None; **Zhang Y,** None; **Zhao FR,** None; **Li YF,** None; **Dang YF,** None; **Yue YY,** None; **Li L,** None.

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