• Basic Research •

Sulforaphane modulates TGFβ2-induced conjunctival fibroblasts activation and fibrosis by inhibiting PI3K/Akt signaling

Han–Ruo Liu, Zi–Yao Xia, Ning–Li Wang

Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital of Capital Medical University; Beijing Key Laboratory of Ophthalmology & Visual Sciences, Beijing 100005, China
Correspondence to: Ning-Li Wang. Beijing Institute of Ophthalmology, Beijing Tongren Eye Center, Beijing Tongren Hospital of Capital Medical University; Beijing Key Laboratory of Ophthalmology & Visual Sciences, 17 Hougouhutong Road, Beijing 100005, China. wningli@vip.163.com

Abstract

● **AIM:** To examine the effects of sulforaphane on fibrotic changes of transforming growth factor (TGFβ2) induced human conjunctival fibroblast (HConFs).
● **METHODS:** HConFs were cultured and divided into control, TGFβ2 (1 ng/mL), sulforaphane and TGFβ2+sulforaphane groups. Cell viability and apoptosis were detected using the MTT and ApoTox-Glo Triplex assay. Cell migration was detected using scratch and Transwell assay. Real-time quantitative PCR method was used to evaluate mRNA expression of TGFβ2, matrix metalloproteinase-2 (MMP2), myosin light chain kinase (MYLK), integrin αV, integrin α5, fibronectin 1 and α-smooth muscle actin (α-SMA). The protein expression of α-SMA, p-PI3K, PI3K, p-Akt, and Akt were detected by Western blot.
● **RESULTS:** The proliferation of HConFs was significantly (P<0.05) suppressed by sulforaphane compared to control cells with the increase of the concentration and treatment time. Cell proliferation after 48h incubation was significantly reduced with 100 µmol/L sulforaphane treatment by 17.53% (P<0.05). The Transwell assay showed sulforaphane decreased cell migration by 18.73% compared with TGFβ2-induced HConF (P<0.05). TGFβ2-induced the increasing expression of fibronectin, type I collagen and α-SMA, and the phosphorylation of PI3K and Akt were all significantly suppressed by sulforaphane pretreatment.
● **CONCLUSION:** Sulforaphane inhibits proliferation,

migration, and synthesis of the extracellular matrix in HConFs, and inhibiting the PI3K/Akt signaling pathway. Sulforaphane could be a potential therapeutic drug for prevention of scar formation in filtering bleb after trabeculectomy.

INTRODUCTION

Glaucoma is one of the most common causes of irreversible blindness in the world. Glaucoma filtration surgery is the most effective method for controlling intraocular pressure (IOP), and postoperative scar formation is the most typical reason for failure of the surgery[1]. Nowadays, anti-scarring reagents are used to prevent the formation of subconjunctival scars and improve the results of glaucoma filtration surgery[2-3]. However, some severe complications associated with the use of these agents limit their clinical application, such as filtering bleb leakage, corneal epithelial dysfunction and macular degeneration. The identification of high-security and high-effective methods to reduce the postoperative scaring is of utmost significance[4].

Postoperative scaring after the glaucoma filtration surgery is a multifactorial process, these are the important steps of these process: the transformation and migration of fibroblasts, and the excessive depositing of extracellular matrix (ECM)[5]. And human conjunctival fibroblasts (HConFs), which deposit in subconjunctival connective tissue, also play an essential role in regulating wound healing. Besides, transforming growth factor β (TGFβ) play as a potent stimulus during the promote transformation of fibroblast and the migration of myofibroblasts in scar formation[6]. Intracellular signaling cascades, including the canonical (Smad) signaling pathway

and the other uncanonical pathways such as p38 MAPK signaling and the RAS/ERK MAPK signaling pathways, were activated *via* the binding of TGFβ to its heterodimeric receptor. In addition, the phosphatidylinositide 3-kinase/protein kinase B (PI3K/Akt) signaling pathway was reported to regulate cell survival, migration, proliferation and ECM synthesis, and which could be activated by TGFβ[7].

Sulfaphane [1-isothiocyanato-4-(methylsulfinyl)-butane, sulforaphane (SFN)] is an isothiocyanate which precursor glucoraphanin are abundantly found in broccoli and other cruciferous plants[8]. In recent research, the researchers found that SFN have the protective effects in several pathological models including diabetic cardiomyopathy, carcinogenesis and fibrosis[9-11]. This substance can also reduce lens cell proliferation and viability in both a human lens epithelial cell line and a tissue culture model[12]. In rat cardiac cells, SFN attenuates TGFβ1-induced transformation of fibroblasts and contractile activity, and it can also reduce expression of collagen-binding integrins and inhibits canonical and noncanonical TGFβ signaling pathways[13]. While SFN has been reported to be anti-fibrotic in cardiac fibroblasts models, the effects of SFN on activation of HConFs or their interactions with the ECM is still illiterate for us. In the present study, we aimed to evaluate whether SFN effect HConFs on TGFβ-induced fibroblast events, that is, cell proliferation, ECM remodeling, and expression of genes involved in these processes.

MATERIALS AND METHODS

Cell Culture HConFs were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA), and routinely cultured at 37℃ with 5% CO_2, 95% air in fibroblast medium (Gibco, Paisley, UK) containing fibroblast growth supplement (FGS; ScienCell Research Laboratories), 2% foetal bovine serum (FBS; ScienCell Research Laboratories), 100 U/mL penicillin and 100 μg/mL streptomycin (ScienCell Research Laboratories).

Cell Proliferation and Viability Assay Each group of logarithmic phases HConFs was seeded on a 96-well plate at a density of $1×10^4$/well and incubated for 24h. Each group was set up with 4 replicate wells. After adding 10, 30, 50, 100, 150 and 200 μmol/L SFN for 24h, replaced the medium containing 1% FBS and added 5 mg/mL methyl thiazol tetrazolium (MTT) 20 μL to each well and place in 37℃ incubator for 4h, absorbed the supernatant, added 150 μL DMSO to each well, shaked until the crystals were completely dissolved, and measured the absorbance A value at a wavelength of 570 nm. Cell proliferation rate=(value of each group/value of control group)×100%. The experiment was repeated three times and the average value was taken.

ApoTox-Glo Triplex Assay The ApoTox-Glo Triplex Assay was used to evaluate HConFs apoptosis following manufacturer's instructions. HConFs cells were inoculated into 96-well plates at a density of 5000 cells per well, and after treatment with experimental conditions for 24h, culture medium was replaced with 200 μL. Briefly, apoptosis events were assessed by the addition of a luminogenic caspase-3/7 substrate (Caspase-Glo 3/7, Promega), which could be evaluated *via* production of a luminescent signal in apoptotic cells. Fluorescence signal was measured at luminescence with an FLUO star Omega plate reader (BMG LabTech, Aylesbury, Bucks, UK).

Scratch Wound Assay HConF cells of each group were grown in 6-well plates at $5×10^5$/well and cultured for 12h. Then the medium was aspirated, treatment of each group was applied and cultured for 24h. A 200 μL pipette tip was used to create a single line scratch along the center of the well. The culture solution was aspirated and rinsed with phosphate buffer saline (PBS) three times to remove cell debris. Then added serum-free culture medium to each well and place in a 37℃ incubator. At 24h after scratching, the width of the scratch was visualized under an inverted microscope. Image J software version 1.5 (produced by Java 2HTML) was used to measure the scratch area at each time point and analyze the cell migration ability.

Transwell Migration Assay The chemo migration assay was performed in Transwell plates (pore size, 8 μm; Thermo Scientific, Basingstoke, UK). The paved Transwell chamber was placed in 24-well plates. Cells of each group were made into cell suspension for 150 μL with a density of $5×10^5$/mL into the upper chamber, 600 μL of DMEM containing 20% FBS were added to the lower chamber. Then placed the Transwell chamber in 37℃, 5% CO_2 incubator for 24h. The upper cells of the chamber were erased gently. The membrane of the Transwell chamber was immersed in 4% paraformaldehyde and stained with 0.1% crystal violet for 20min. Each membrane is randomly chosen five fields of view, using an inverted microscope to observe and count the number of cells in each field of view, calculating the average value to evaluate the cell migration ability. The experiment was repeated thrice.

Immunofluorescence Staining Analysis Cells of each group were seeded in a 24-well plate, washed 3 times with PBS after treatment, fixed with 4% paraformaldehyde solution, Triton X permeated the cells, blocked with sheep serum for 60min, and incubated the primary antibodies Smad2 and Smad3 overnight. After incubation for 60min, the nuclei were stained with DAPI and mounted. Take a picture under the microscope and calculate the fluorescence brightness using Image J software. The relative fluorescence ratio=(fluorescence

brightness of each group/fluorescence brightness of the control group)×100%.

RNA Extraction and qRT-PCR Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The concentrations of RNA were measured spectrophotometrically. Then, complementary DNA (cDNA) was synthesized using reverse transcription kit (Takara Bio, Inc.), according to the manufacturer's protocol. Specific cDNAs were subsequently amplified by PCR using the following probes (Life Technologies, Carlsbad, CA, USA): collagen I, F: 5'-TCCTCTTTAGCACCCTTTCG-3' and R: 5'-GGACCAGCAACACCATCTG-3'; fibronectin, F: 5'-CCAGCAGAGGCATAAGGTTC-3' and R: 5'-CACTCATCTCCAACGGCATA-3'; β-actin, F: 5'-TGGAATCCTGTGGCATCCATGAAA-3' and R: 5'-CT AAAACGCAGCTCAGTAACAGTCCG-3'. The real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed using Thunderbird SYBR Green qPCR Mix (Bioline, London, UK). The parameters were set at 95℃ for initial denaturation for 3min, and 95℃ denaturation for 15s, 61℃ annealing for 15s, 72℃ extension for 15s, total for 40 cycles. β-actin was used as the internal reference. The relative expression of each target gene was analyzed using the $2^{-\Delta\Delta Ct}$ method. The experiment was repeated thrice, and the average value was taken.

Western Blot Analysis Cells cultured in 6-well plates were treated in each group for 48h, and 200 μL protein lysate was added to each well to extract the protein on ice. The protein content was determined by bicinchoninic acid assay and equal amounts of protein per sample were loaded onto 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (PVDF) membrane using a Trans-Blot Turbo Transfer System (Danvers, MA, USA). The membrane was blocked with PBS containing 0.5% wt/vol non-fat dry milk for 1h and 0.1% vol/vol Tween-20, hybridized with rabbit anti-α-SMA (1:1000), mouse anti-PI3K p85 (1:500), rat anti-p-PI3K p85α (1:500), mouse anti-Akt l (1:500), mouse anti-p-Akt l (1:500), and mouse anti-β-actin (1:1000) primary antibody at 4℃ overnight. Blots washed with TBST, followed by incubation with horseradish peroxidase-labeled goat anti-mouse and anti-rabbit secondary antibody (1:5000) at room temperature for 1h then wash with PBS. The blots were detected using the ECL reagent plus blotting analysis system. Semi-quantitative analysis of the data was performed by densitometry using Image J software. Actin used as the internal reference protein.

Statistical Analysis Data analysis was performed using SPSS version 20.0 (IBM Corp., USA). The data are expressed as the mean±standard error (number of observations). Student's *t*-test is for differences analysis between two variables and one-way ANOVA test is for differences analysis among multiple data sets. Statistical significance was defined as *P*<0.05. All experiments were repeated 4 times.

RESULTS

Effect of SFN on HConFs Viability and Apoptosis SFN reduced cell viability in a concentration-dependent manner, such that significant differences from control were seen with treatments at 50 μmol/L and above. HConFs viability relative to the untreated control group was not significantly affected by SFN exposure of 30 μmol/L and below following 24h exposure periods. However, at SFN concentrations of 50 μmol/L and above, a significant reduction in cell viability was observed; this effect became more pronounced with increasing concentrations of SFN (Figure 1A). Apoptosis was detected by caspase 3/7 activity and, consistent with the other measurements, a significant increase was identified following 50 to 200 μmol/L SFN exposure (Figure 1B).

Effect of SFN on HConFs Migration In the scratch wound assay, the cell culture dishes of each group showed neat and sharp edges immediately after scratching. Then 24h after scratching, cells were seen to migrate inwards, and the edges of the scratches were rough. SFN treatment of 24h significantly impeded wound closure by TGFβ2 compared with HConFs maintained under control conditions (Figure 2A). Wound healing was increased in the TGFβ2 group compared with the control group (89.58%±2.78%, *P*<0.05) and reduced HConFs migration was observed for the SFN treatment group (52.83%±3.51%; *P*<0.01; Figure 2B). Similar results were observed for the Transwell migration assays. It showed a significant increase migration for the TGFβ2 group compared with the SFN treatment group (137.86±2.47 *vs* 31.55±2.28 cells, *P*<0.01; Figure 3B).

Effect of SFN on ECM Synthesize and Fibroblast Transdifferentiation Exposure of HConFs to 1 ng/mL TGFβ2 for 24h induced the upregulation of collagen I and fibronectin, whereas 100 μmol/L SFN inhibited this upregulation (*P*<0.05; Figure 4). The relative quantification results demonstrated that pretreatment with SFN for 24h before TGFβ2 stimulation significantly suppressed TGFβ2-driven α-SMA expression (Figure 5). Immunocytochemistry revealed that SFN inhibited TGFβ2-induced α-SMA expression (Figure 6).

Effect of SFN on PI3K/Akt Pathway in HConFs To investigate the possible effects of SFN on PI3K/Akt pathway in HConFs, we examined the phosphorylation levels of PI3K (p-PI3K) and Akt (p-Akt) in HConFs. Western blotting revealed that the exposure of HConFs to 1 ng/mL TGFβ2 for 24h induced the phosphorylation of PI3K and Akt which were inhibited by SFN pretreatment significantly (Figure 7).
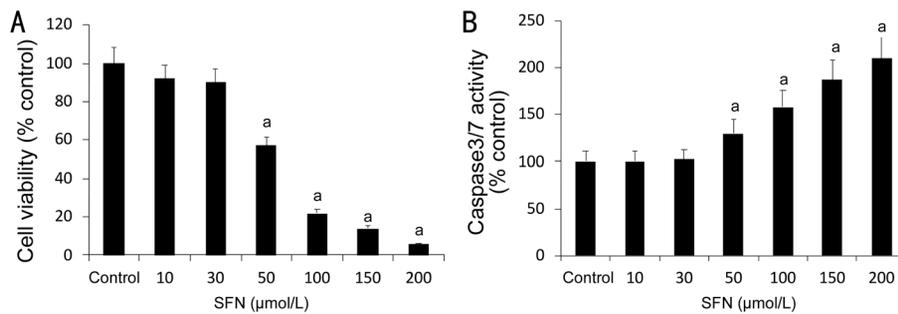
Figure 1 The effect of SFN on cell viability and apoptosis of HConFs Concentration-dependent effects of SFN on HConFs viability (A) and apoptosis (B) following a 24h culture period with SFN. The data are presented as mean±SD (n=4). [a]P<0.05 vs control.
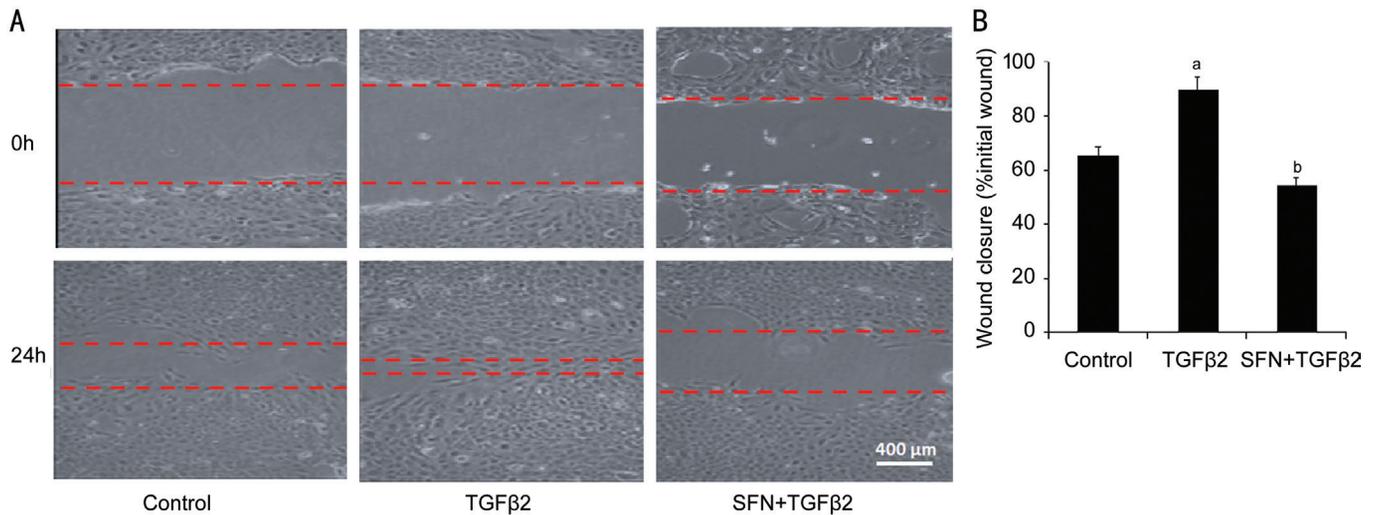

Figure 2 Effect of SFN on TGFβ2-induced HConFs migration assessed by scratch-wound assay Wound closure was assessed at 24h following the initial wound or treated with 100 μmol/L SFN. A: Representative images of scratch-wound assay results. Scale bar, 400 μm. B: Quantitative data from multiple experiments (n=4). Wound closure was quantified as % original wound. [a]P<0.05 vs control; [b]P<0.05 vs TGFβ2 group.
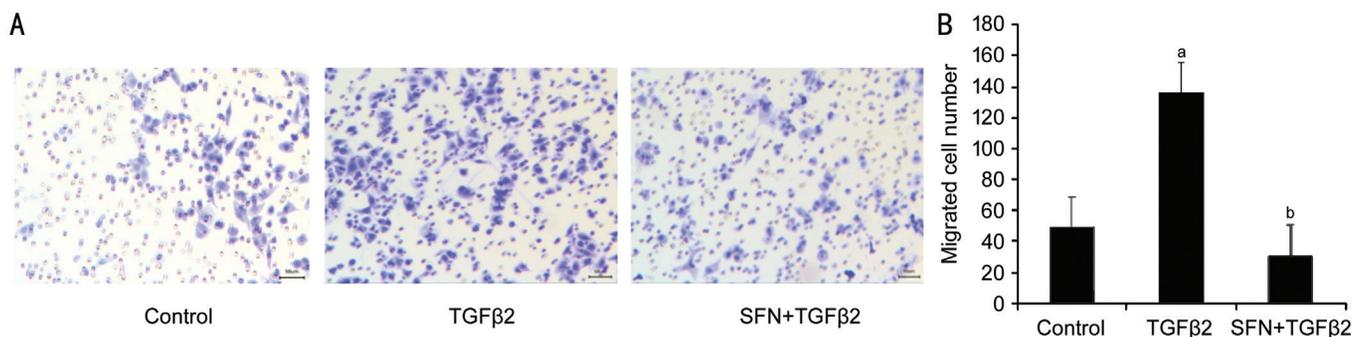

Figure 3 Effect of SFN on TGFβ2-induced HConFs migration assessed by Transwell migration assays A: Representative images of Transwell migration assay results at 24h (200× magnification); B: Quantitative data from multiple experiments (n=4). [a]P<0.05 vs control; [b]P<0.05 vs TGFβ2 group.

DISCUSSION

This research was revealed that SFN inhibited TGFβ2-induced migration, proliferation and expression of collagen I and fibronectin in HConFs. Furthermore, SFN was found to promote HConF cell apoptosis and inhibit the TGFβ2-induced phosphorylation of PI3K and Akt in HConFs. These results suggest another potential strategy for control of wound healing after glaucoma filtration.

The healing process after glaucoma filtration is the main determinant underlying trabeculectomy failure. This process consists of a series of activation, such as transdifferentiation of fibroblasts to myofibroblasts, and fibroblast migration proliferation, deposition, and contraction of dense collagen fibers into the wound site[14]. Wound contractile activity takes place when granulation tissue is populated with myofibroblasts that are characterized by the expression of α-smooth muscle
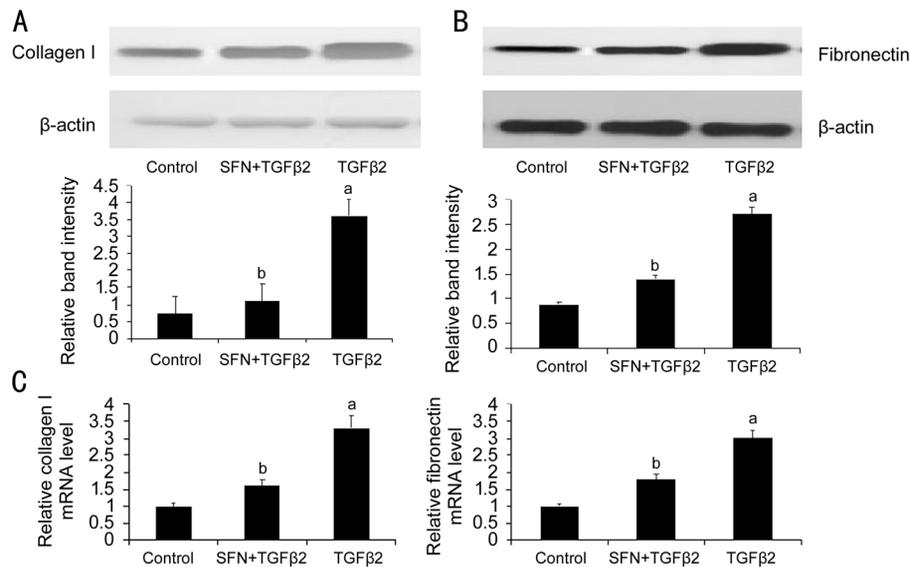
Figure 4 Effect of SFN on TGFβ2-induced collagen I and fibronectin expression in HConFs SFN inhibits collagen I and fibronectin expression in HConFs at the mRNA and protein levels. A: Protein expression of collagen I in HConFs; B: Protein expression of fibronectin in HConFs; C: mRNA expression of collagen I and fibronectin in HConFs. $^aP<0.05$ *vs* control; $^bP<0.05$ *vs* TGFβ2 group.
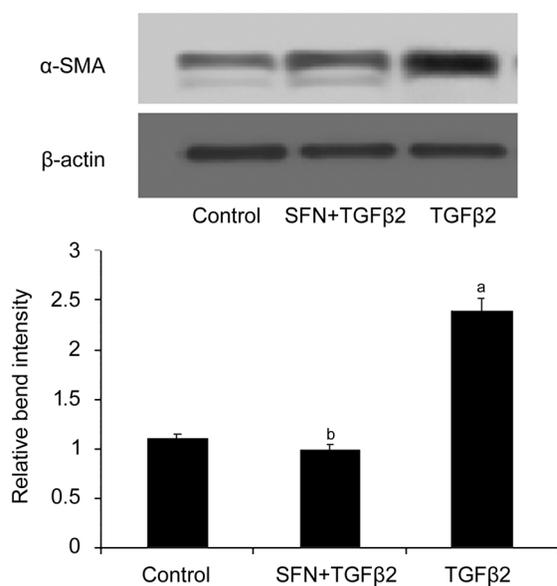

Figure 5 Effect of SFN treatment on α-SMA expression in HConFs by Western blot $^aP<0.05$ *vs* control; $^bP<0.05$ *vs* TGFβ2 group.

actin (α-SMA). Incorporation of α-SMA into actin stress fibers has been proved to promote myofibroblast contraction and wound healing *in vivo* and *in vitro*[15]. Increased synthesis of ECM productions, such as collagen type I and fibronectin, in an activated fibroblast phenotype associate with myofibroblasts[16]. TGFβ has been shown to affect conjunctival fibroblasts in multiple phases of wound healing, including myofibroblasts transdifferentiation and induction of deposition of ECM[17]. In the previous studies, it suggested the expression of TGFβ in normal conjunctiva presence of all the three isoforms. Especially TGFβ2 is predominant as it is elsewhere in the ocular tissue[18-19]. Therefore, TGFβ2 was applied in this study as an inducer of fibrosis to conduct experiments and to induce a series of events similar to wound healing process. As expected, in this study TGFβ2 was found to significantly increase HConF cell migration, and transdifferentiation of fibroblasts into myofibroblasts characterized by α-SMA expression, was further supported by a significant increase in protein expression of the fibronectin and collagen I.

SFN is a phytochemical that mainly exists in cruciferous vegetables, such as broccoli and cabbage, and its safety and lack of toxicity have been demonstrated[20]. The chemo-preventive effect of SFN involves cell cycle arrest and apoptosis, and it was found to have anti-fibrotic activity in liver fibrosis[21], pulmonary fibrosis[22], and keloids[23]. It was demonstrated that SFN inhibited the TGFβ-induced migration and proliferation of HConFs, and inhibited ECM synthesis in HConFs. Collectively, these results suggested that SFN attenuates conjunctival scarring by modulating the function of HConFs and ECM synthesis.

Furthermore, we further explored the potential target of SFN on HConFs. Signaling pathways driven by TGFβ are generally divided into Smad-dependent and Smad-independent pathways, including PI3K/Akt pathways and MAPK pathways. TGFβ2 induced phosphorylation of Smads (Smad2 and Smad3), as well as non-Smad proteins (p38, ERK, and Akt). The PI3K/Akt pathway modulates cell growth, proliferation, migration, differentiation, apoptosis, motility, survival and glucose metabolism[24]. PI3K is an intracellular signaling molecule that is activated to produce inositol 3-phosphate, which then recruit target proteins to the plasma membrane. Akt as a downstream kinase of PI3K is phosphorylated and recruited to the membrane, thus plays a role. PI3K/Akt
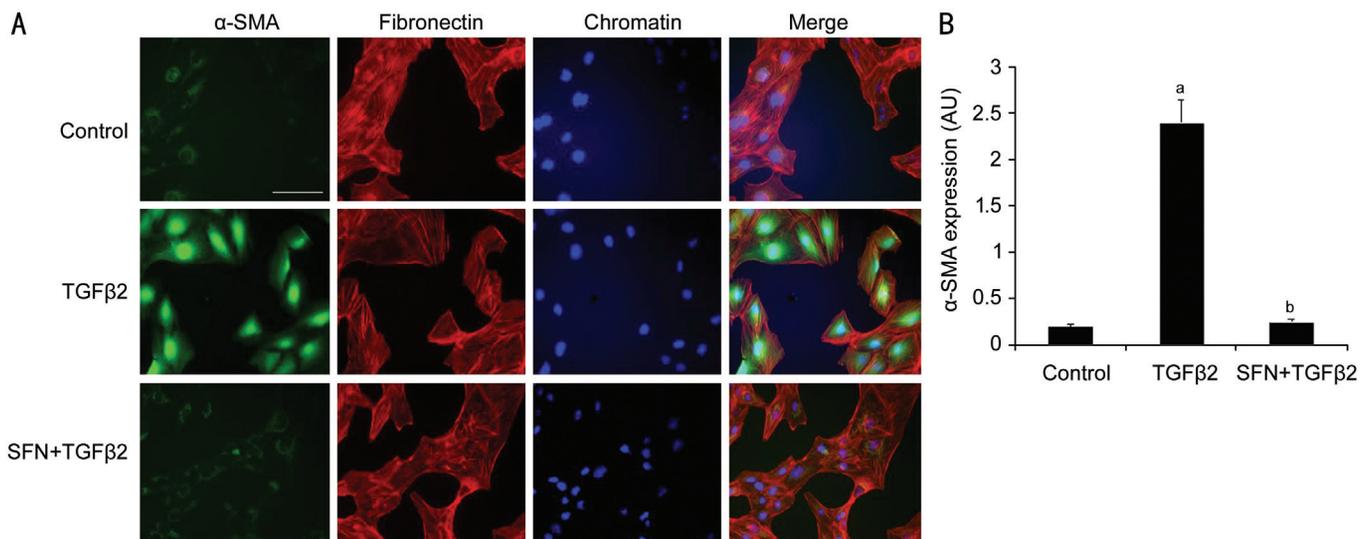
Figure 6 Effect of SFN treatment on α-SMA expression in HConFs A: α-SMA (green) or fibronectin (red) was assessed by immunocytochemistry; B: Quantitative data from multiple experiments. Scale bar, 500 μm. [a]$P<0.05$ vs control; [b]$P<0.05$ vs TGFβ2 group.
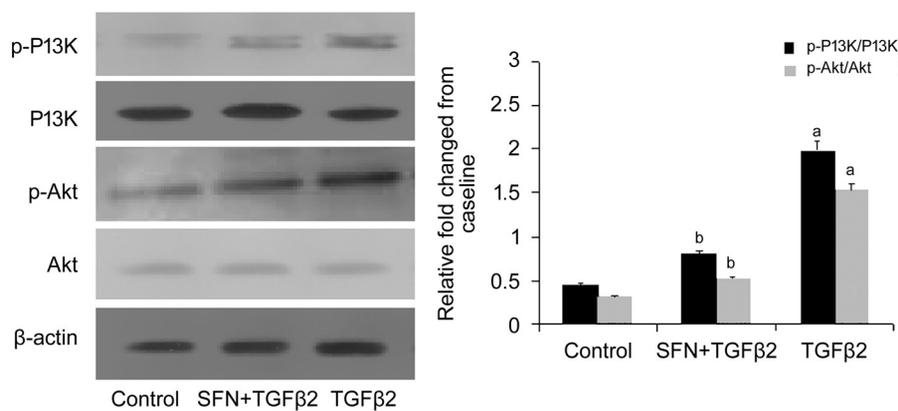

Figure 7 Effect of SFN on TGFβ2-induced p-PI3K and p-Akt expression in HConFs The expression levels of p-PI3K and p-Akt protein expression were examined by Western blot. SFN suppressed activation of the phosphorylation of PI3K and Akt. [a]$P<0.05$ vs control; [b]$P<0.05$ vs TGFβ2 group.

signaling pathway may be involved in ECM secretion in several cell types. In human retinal pigment epithelium (RPE) cells, PI3K/Akt pathway may be involved in TGFβ2-induced collagen synthesis. Mechanical stress can upregulate TGFβ2 expression and induce epithelial mesenchymal transition in RPE cells, besides, this process can activate the PI3K/Akt pathway[25]. In this study, for the first time, we found that SFN could inhibit TGFβ2-induced expression of p-PI3K and p-Akt protein. These results suggested that the inhibitory effects of SFN on HConFs are likely mediated by inhibition of the PI3K/Akt signaling pathway. However, the effects of SFN on the PI3K/Akt signaling and the other signaling pathways associated with conjunctival scarring should be investigated in further studies.

In summary, the results of this study indicate that SFN can reduce cell viability and inhibit TGFβ2-induced proliferation, migration and ECM synthesis in HConFs by modulating PI3K/Akt signaling pathway. It is proposed that SFN may be a promising compound capable of preventing scarring of filtering bleb after glaucoma filtration surgery.

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