

All-trans-retinoic acid modulates TGF- β -induced apoptosis, proliferation, migration and extracellular matrix synthesis of conjunctival fibroblasts by inhibiting PI3K/AKT signaling

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Abstract. Conjunctival fiber generation is implicated in a wide spectrum of ocular diseases. Conjunctival wound healing is characterized by inflammation followed by re-epithelialization, synthesis of new extracellular matrix (ECM), wound contraction and subconjunctival scar formation. The primary cause for the failure of glaucoma filtration surgery results from the excessive scarring of the filtering bleb. All-trans-retinoic acid (ATRA), a derivative of vitamin A, is a potent regulator of ECM synthesis, growth and differentiation. Following a previous study, which revealed that ATRA could inhibit transforming growth factor- β -induced human conjunctival fibroblast (HConF)-mediated collagen gel contraction, the present study aimed to investigate the effects of ATRA on HConF migration, apoptosis, proliferation and ECM synthesis. To achieve this, the present study used Transwell migration, wound healing and Cell Counting Kit-8 assays, flow cytometry and western blot analysis. In addition, the present study aimed to elucidate the mechanism of ATRA in mediating resistance to conjunctival scar formation. ATRA treatment resulted in an increased level of HConF apoptosis, reduced proliferation and migration, decreased collagen I and fibronectin expression, and decreased phosphorylation of PI3K and AKT. Thus, the present study showed a role for ATRA in inhibiting HConF migration, proliferation and ECM synthesis, and in promoting

HConF apoptosis through the inhibition of the PI3K/AKT signaling pathway.

Introduction

Glaucoma is the most common cause of irreversible blindness worldwide, accounting for ~15% of all cases and $\geq 500,000$ new cases annually in both developed and developing countries (1-3). It is characterized by the development of a specific pattern of visual field loss and optic neuropathy. Patients are usually treated using filtration surgery which serves to reduce intraocular pressure in the eye (4). Other treatments include topical medication, laser treatment and surgical modalities. Of the treatments available, surgery is the most effective (5,6). Glaucoma filtration surgery may stimulate conjunctival fibroblast proliferation, migration, differentiation and promote ECM secretion, which are important processes in wound healing and scar formation (7-9). However, glaucoma filtration surgery is not always effective, and excessive scarring of the filtering bleb after surgery is a major problem following surgery.

Scar formation at the filtering bleb after glaucoma filtration surgery is a multifactorial process; human conjunctival fibroblasts (HConFs) play an essential role in subconjunctival wound healing (10). In addition, the cytokine transforming growth factor (TGF)- β is an important factor in regulating wound healing and scar formation (10). The binding of TGF- β to its heterodimeric receptor activates intracellular signaling cascades, including the canonical SMAD pathway and the mitogen-activated protein kinase (MAPK) pathway, and leads to fibrosis (11). The PI3K/AKT signaling pathway, which regulates survival, was also reported to function in regulating cell migration, proliferation and apoptosis (12,13). The PI3K/AKT pathway can be activated by TGF- β and also plays an important role in modulating ECM synthesis (14).

All-trans-retinoic acid (ATRA), a derivative of vitamin A, can inhibit TGF- β and inhibit fibrosis (15). In a previous study, it was reported that ATRA inhibited TGF- β -induced HConF-mediated collagen gel contraction via the SMAD signaling pathway (16). However, the effects of ATRA on

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TGF- β -induced HConF migration, proliferation and ECM synthesis remain unclear. In the present study, HConFs were cultured to investigate the effects of ATRA on the proliferation, migration, apoptosis and ECM synthesis in conjunctival fibroblasts. The aim of the present study was to improve understanding of the mechanism of action of ATRA. The mechanistic insights provided by this study may be applicable to preventing scar formation following glaucoma filtration surgery.

Materials and methods

Materials. ATRA and TGF- β (Sigma-Aldrich; Merck KGaA) were prepared as 0.4 and 50 ng/ml stock solutions in DMSO, respectively, and were stored below -20°C in the dark. ATRA and TGF- β were diluted to 10–200 μM and 1 ng/ml, respectively, in DMEM (Sigma-Aldrich; Merck KGaA) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) on the day of the experiment. The highest concentration of DMSO in the test solutions was 0.1%. To exclude the possibility that proliferation may be inhibited by DMSO, all cells were exposed to a final DMSO concentration of 0.1% in DMEM containing 10% FBS.

Cell culture. Primary HConFs (ScienCell Research Laboratories, Inc.) were cultured according to the manufacturer's instructions; three individual lots of HConFs were obtained. The HConFs were maintained and cultured at 37°C in fibroblast medium (ScienCell Research Laboratories, Inc.) containing 2% FBS, fibroblast growth supplement (ScienCell Research Laboratories, Inc.), 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in a 5% CO_2 humidified incubator. The HConFs were characterized by a spindle-shaped morphology and were positively stained with anti-fibronectin antibodies.

Cell proliferation assay. A HConF proliferation assay was performed as previously described (17). Briefly, HConF suspensions (0.5×10^4 cells/well) were cultured in fibroblast medium in a 96-well plate (200 $\mu\text{l}/\text{well}$) for 24 h at 37°C . ATRA (0.01–10 μM) was added to the fibroblast medium and the cells were cultured for a further 48 h at 37°C in the absence or presence of TGF- β (1 ng/ml), with 0.1% DMSO as the control. The concentrations of ATRA and TGF- β used were determined according to a previous study (15). Subsequently, each well was incubated with 10 μl Cell Counting Kit-8 solution (BestBio; Sigma) for a further 3 h at 37°C . The absorbance was measured at 450 nm using an automated microplate reader (model 3001-1387; Thermo Fisher Scientific, Inc.).

Apoptosis assay. HConF apoptosis was determined using an Annexin V-FITC apoptosis kit (BestBio) as previously described (17). Briefly, the HConFs (5×10^5 cells/well) were plated in 6-well plates and cultured for 48 h. After rinsing twice with PBS, the cells were resuspended in 400 μl 1X binding buffer [10 mM HEPES, 140 mM sodium chloride, 2.5 mM calcium chloride (pH 7.4)], into which 5 μl of Annexin V-FITC was added. After 15 min incubation at $2-8^{\circ}\text{C}$ in the dark, 10 μl of propidium iodide (BestBio) was added to the cells prior to further incubation for 5 min at $2-8^{\circ}\text{C}$ in the dark. Within 15 min after staining, the cells were analyzed using a flow cytometer (Cytomics FV 500) and CytExpert 2.0

software (both Beckman Coulter, Inc.). The apoptotic rate was calculated as the sum of the percentage of early + late apoptotic cells.

Wound healing assay. HConFs were seeded at a density of 5×10^5 cells/well in 6-well plates. The culture medium for the HConFs was replaced after 24 h with serum-free medium, and then cells were incubated for a further 2 h at 37°C . Next, a sterile 20- μl pipette tip was used to scratch a line in the confluent cell monolayer, after which the cells were washed three times with PBS. The scratch wound healed after 24 h of incubation in the following conditions: 1 μM ATRA + 1 ng/ml TGF- β , 1 ng/ml TGF- β and fibroblast medium without ATRA and TGF- β . The effects on HConF migration were evaluated by measuring the area of the wound from the images captured at a magnification of $\times 100$ at 0 and 24 h using a light microscope (Olympus Corporation); analysis was performed using ImageJ version 1.5 (National Institutes of Health).

Transwell migration assay. A chemomigration assay was performed using Transwell plates (pore size, 8 μm) as previously described (18). Briefly, the cells in the upper Transwell chamber (1×10^5) were suspended in 200 μl of DMEM, and DMEM containing 20% FBS was added to the lower chamber. Following a 12-h incubation at 37°C , the medium with non-migrated cells in the upper chamber was removed with a cotton swab. The cells that had migrated to the lower chamber were fixed with 4% paraformaldehyde for 30 min at 37°C and then stained with 0.5% crystal violet for 10 min at 37°C . The cells were counted at $\times 200$ magnification using a light microscope in six different fields of view.

Cell treatment. HConFs were incubated in fibroblast medium in 6-well plates for 24 h. Then, the medium was replaced with the serum-free medium. Serum-starved cells were incubated for 6 h in the presence or absence of ATRA (1 μM), and then for 48 h in the presence or absence of TGF- β (1 ng/ml). Then, cell lysates were analyzed via western blotting.

Western blot analysis. Western blot analysis was performed as previously described (17,19,20). Cells were washed with pre-cooled PBS and lysed with RIPA buffer (Nanjing KeyGen Biotech Co., Ltd.) containing the protease inhibitor phenylmethylsulfonyl fluoride and the phosphatase inhibitor sodium orthovanadate (Nanjing KeyGen Biotech Co., Ltd.) on ice for 30 min. Subsequently, the cells were gently scraped from the plate and centrifuged at $1,500 \times g$ for 12 min at 4°C . Protein concentrations were measured using the bicinchoninic acid method. A total of 20 $\mu\text{g}/\text{sample}$ of protein was separated via 6–15% SDS-PAGE and then transferred onto PVDF membranes. Membranes were blocked with 5% non-fat dry milk for 1 h at 37°C before overnight incubation at 4°C with mouse monoclonal anti-collagen I (1:2,500; cat. no. ab88147; Abcam), rabbit polyclonal anti-fibronectin (1:1,500; cat. no. ab137720; Abcam), mouse antibodies against PI3K p85 α (1:500; cat. no. sc-1637), phosphorylated (p)-PI3K p85 α (1:500; cat. no. sc-12929), AKT (1:500; cat. no. sc-5298) and p-AKT (1:500; cat. no. sc-293125; all from Santa Cruz Biotechnology, Inc.), and mouse monoclonal anti-GAPDH (1:1,000; cat. no. A01020; Abbkine Scientific Co., Ltd.). Each membrane was incubated with

anti-rabbit (1:5,000; cat. no. A0208) or anti-mouse (1:5,000; cat. no. A0216) horseradish peroxidase-conjugated secondary antibodies (both from Beyotime Institute of Biotechnology) for 1 h at room temperature. The blots were visualized using ECL reagent (Beyotime Institute of Biotechnology) and the bands were analyzed using ImageJ software.

Inhibition of TGF- β -induced collagen I and fibronectin expression in HConFs using a PI3K inhibitor. HConFs were incubated in fibroblast medium in 6-well plates for 24 h, then the medium was replaced with serum-free medium. Serum-starved cells were incubated with 10 μ M LY294002 (Calbiochem; Merck KGaA) for 1 h at 37°C. Subsequently, the cells were treated with 1 ng/ml TGF- β for 48 h at 37°C. The effects of the PI3K inhibitor on the TGF- β -induced expression of collagen I and fibronectin in HConF were investigated via western blot analysis.

Statistical analysis. Data analysis was performed using SPSS version 19.0 (IBM Corp.). The data are presented as the mean \pm SD. Each experiment was repeated at least three times. Statistical analysis was performed using Student's t-test or one-way ANOVA; following ANOVA, the least significant difference test was used for pairwise comparisons between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ATRA inhibits TGF- β -induced HConF proliferation. The effect of ATRA on HConF proliferation was examined. The cells were incubated for 48 h with 0.01–10 μ M ATRA in the absence or presence of 1 ng/ml TGF- β with 0.1% DMSO as the control. ATRA was revealed to inhibit HConF proliferation in a concentration-dependent manner ($P < 0.05$; Fig. 1A). HConFs were divided into three treatment groups: The ATRA group (1 μ M ATRA + 1 ng/ml TGF- β), the TGF- β group (1 ng/ml TGF- β) and the control group (0.1% DMSO). ATRA inhibited TGF- β -induced HConF proliferation by 77.50 \pm 1.88% ($P < 0.01$ vs. TGF- β ; Fig. 1B).

ATRA induces HConF apoptosis. The effect of ATRA on HConF apoptosis was examined using flow cytometry. A significantly increased number of apoptotic HConFs were observed in the ATRA group compared with the control group (53.25 \pm 1.2 vs. 22.5 \pm 1.1%, respectively; $P < 0.01$). The TGF- β group showed a significantly decreased apoptotic rate compared with the control group (14.75 \pm 1.4%; $P < 0.01$; Fig. 2).

ATRA inhibits TGF- β -induced cell migration. The migratory ability of the HConFs was evaluated using *in vitro* wound healing and Transwell assays. Similar results were observed for the two assays. The ATRA group exhibited a ~30% reduction in wound healing compared with the control group (48.9 \pm 1.34 vs. 71.30 \pm 1.55%, respectively; $P < 0.05$). Wound healing was increased in the TGF- β group compared with the control group (90.50 \pm 1.22%; $P < 0.05$; Fig. 3A). Reduced HConF migration was observed for the ATRA group (14.85 \pm 1.13 cells; $P < 0.01$), whereas significantly increased migration was noted for the TGF- β group compared with the control group

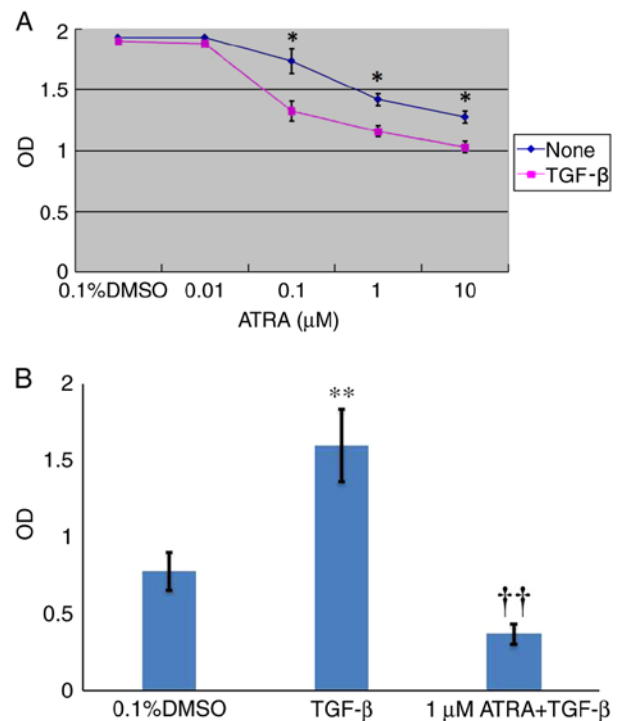


Figure 1. Effect of ATRA on HConF proliferation. Cells were cultured for 48 h in the presence or absence of TGF- β (1 ng/ml) and in the presence of the indicated concentrations of ATRA. The relative cell numbers were detected with a Cell Counting Kit-8 assay. (A) Concentration dependency of ATRA on TGF- β -induced HConFs proliferation * $P < 0.05$ vs. TGF- β . (B) HConFs were divided into 3 groups: ATRA group (treated with 1 μ M ATRA + 1 ng/ml TGF- β), TGF- β group (treated with 1 ng/ml TGF- β), and 0.1% DMSO control group. Data are presented as the mean \pm SD. ** $P < 0.01$ vs. 0.1% DMSO; †† $P < 0.01$ vs. TGF- β . ATRA, all-trans-retinoic acid; HConFs, human conjunctival fibroblasts; TGF- β , transforming growth factor- β .

(135.55 \pm 1.12 cells vs. 28.65 \pm 1.02 cells, respectively; $P < 0.01$; Fig. 3B) in the Transwell migration assays.

ATRA inhibits TGF- β -induced collagen I and fibronectin expression. The effect of ATRA on TGF- β -induced ECM production was investigated. Western blotting analysis and densitometric analysis revealed that exposure of HConFs to 1 ng/ml TGF- β for 48 h induced the production of collagen I and fibronectin, whereas 1 μ M ATRA inhibited TGF- β -induced synthesis of collagen I and fibronectin by HConFs ($P < 0.01$; Fig. 4).

ATRA inhibits the PI3K/AKT pathway. The effect of ATRA on TGF- β -induced PI3K and AKT phosphorylation in HConFs was examined (Fig. 5). Western blotting analysis and densitometric analysis revealed that the exposure of HConFs to 1 ng/ml TGF- β for 48 h induced the phosphorylation/activation of PI3K and AKT. Treatment with 1 μ M ATRA inhibited TGF- β -induced PI3K and AKT phosphorylation, indicating that ATRA inhibited the TGF- β -induced PI3K/AKT signaling pathway ($P < 0.05$; Fig. 5).

Inhibition of TGF- β -induced collagen I and fibronectin expression in HConFs by a PI3K inhibitor. The effect of the PI3K inhibitor LY294002 on the TGF- β -induced expression of collagen I and fibronectin in HConF was investigated. Cells were incubated with 10 μ M LY294002 for 1 h before treatment

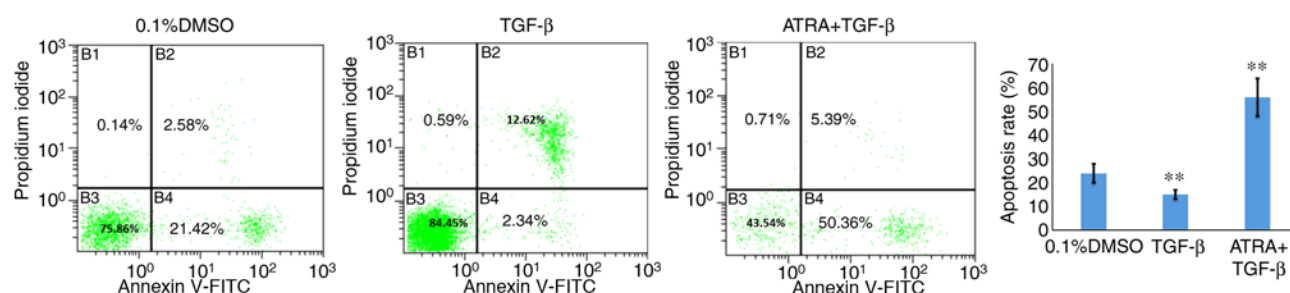


Figure 2. Effect of ATRA on HConF apoptosis. HConFs were cultured with 1 ng/ml TGF- β or 1 μ M ATRA + 1 ng/ml TGF- β for 48 h. Cells were stained with propidium iodide (y-axis) and Annexin V-FITC (x-axis), and analyzed by flow cytometry. For each plot, the upper right quadrant represents late apoptotic cells and the lower right quadrant represents early apoptotic cells. The total apoptosis rate was calculated by the addition of the early apoptosis and the late apoptosis values. Data are presented as the mean \pm SD. ** P <0.01; vs. 0.01% DMSO. ATRA, all-trans-retinoic acid; HConFs, human conjunctival fibroblasts; TGF- β , transforming growth factor- β .

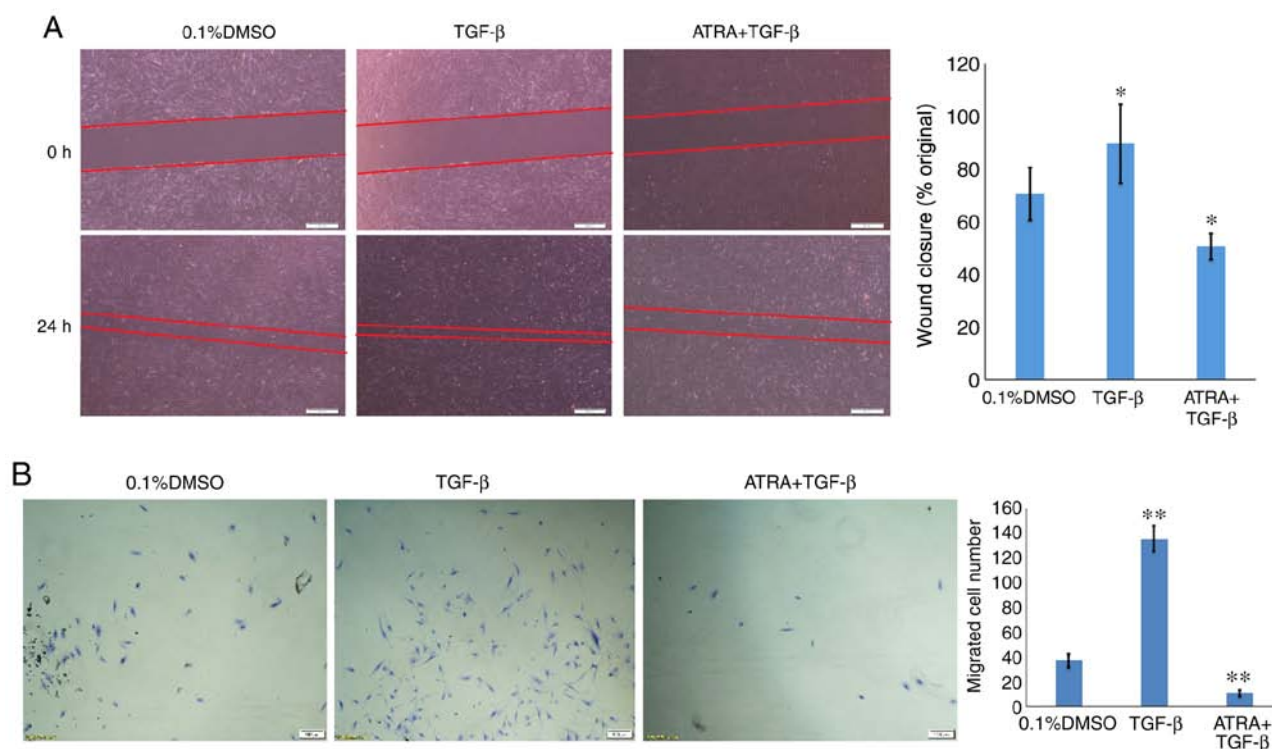


Figure 3. Effect of ATRA on TGF- β -induced HConF migration. The effect of ATRA on HConF migration was assessed using *in vitro* wound healing and Transwell assays. The migratory ability of the HConFs was determined by measuring the width of the wound after 24 h or the number of cells that had migrated to the lower chamber after 12 h. (A) Representative images of the wound healing assay results and quantification of the inhibitory effect of ATRA on HConF migration; magnification: x100. (B) Representative images of the Transwell migration assay and quantification of the inhibitory effect of ATRA on HConF migration; magnification: x200. Data are presented as the mean \pm SD. * P <0.05, ** P <0.01 vs. 0.01% DMSO. ATRA, all-trans-retinoic acid; HConFs, human conjunctival fibroblasts; TGF- β , transforming growth factor- β .

with 1 ng/ml TGF- β for 48 h. Western blotting analysis and densitometric analysis revealed that LY294002 significantly inhibited TGF- β -induced expression of collagen I and fibronectin in HConFs (P <0.05; Fig. 6). The TGF- β -induced expression of collagen I and fibronectin in HConFs was inhibited by 27 and 47% in the presence of LY294002, respectively. The inhibitor had no effect on collagen I and fibronectin expression in the absence of TGF- β (data not shown).

Discussion

In the present study, it was revealed that ATRA inhibited TGF- β -induced migration, proliferation and ECM synthesis in

HConFs. Furthermore, ATRA was found to promote apoptosis and inhibit the TGF- β -induced phosphorylation of PI3K and AKT in HConFs. In addition, the present study showed that the PI3K inhibitor LY294002 attenuated the TGF- β -induced expression of collagen I and fibronectin.

Preventing conjunctival scarring remains a challenge in clinical ophthalmology. TGF- β is a key factor implicated in postoperative scarring as it stimulates the migration, proliferation and differentiation HConFs, and promotes ECM deposition and remodeling (21). ATRA, a derivative of vitamin A, has been shown to regulate ECM expression and serve an important role in fibrotic diseases through the inhibition of TGF- β 1 (22). Moreover, ATRA was found to

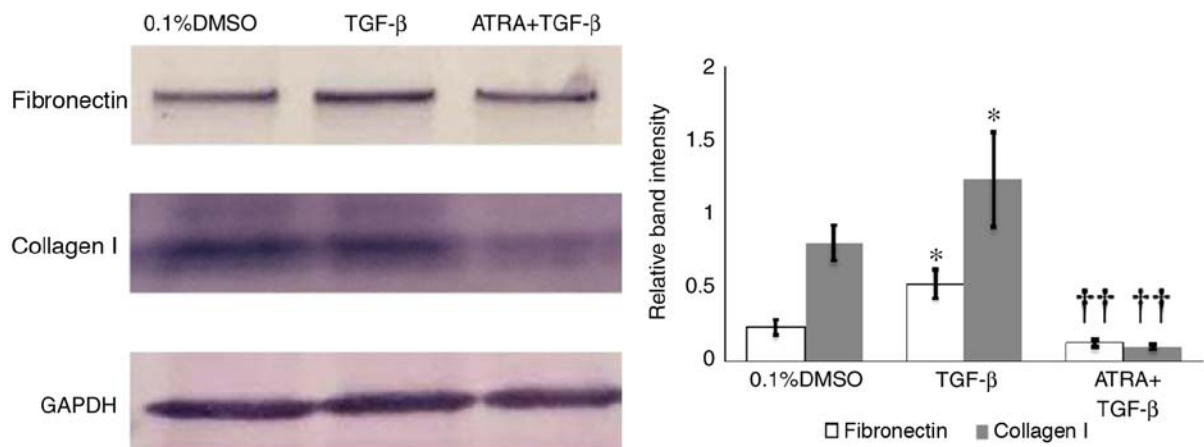


Figure 4. Effect of ATRA on TGF- β -induced collagen I and fibronectin expression in HConFs. Serum-deprived cells were incubated for 6 h in the absence or presence of ATRA (1 μ M) and then for 48 h in the absence or presence of TGF- β (1 ng/ml). Cell lysates were subjected to western blot analysis with collagen I, fibronectin and GAPDH antibodies. The data are representative of three independent experiments. The western blots were subjected to densitometric analysis in order to determine the intensity of the bands corresponding to collagen I and fibronectin. The data are expressed relative to the intensity of the corresponding GAPDH band. Data are presented as the mean \pm SD. * P <0.05 vs. 0.1% DMSO; †† P <0.01 vs. TGF- β . ATRA, all-trans-retinoic acid; HConFs, human conjunctival fibroblasts; TGF- β , transforming growth factor- β .

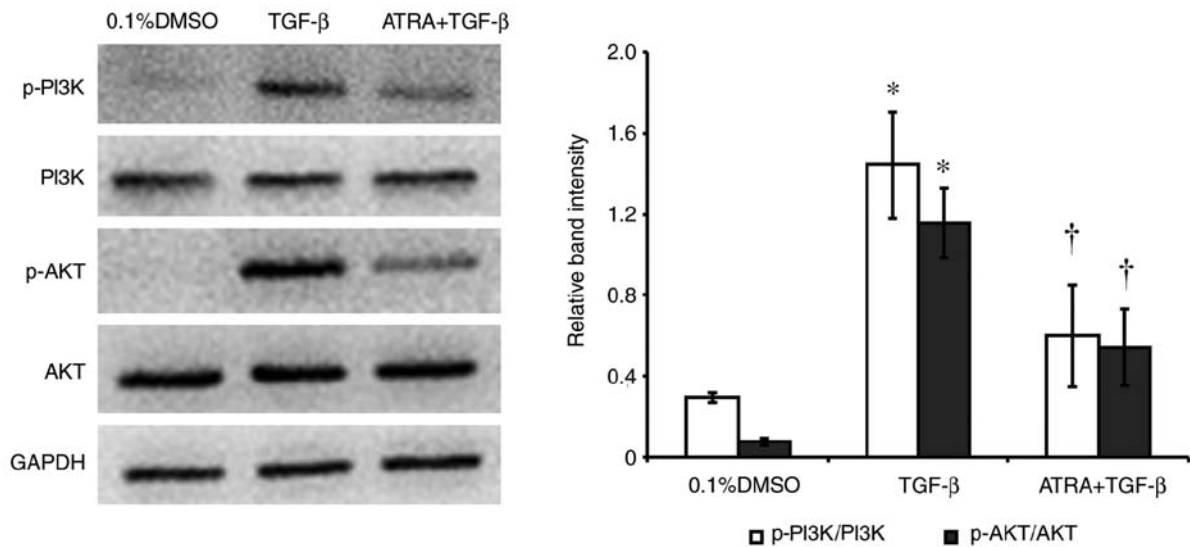


Figure 5. ATRA-mediated inhibition of TGF- β -induced p-PI3K and p-AKT in HConFs. Serum-deprived cells were incubated for 6 h in the presence or absence of ATRA (1 μ M) and then for 48 h in the presence or absence of TGF- β (1 ng/ml). Cell lysates were subjected to western blot analysis with p-PI3K, PI3K, p-AKT, AKT and GAPDH antibodies. The data are representative of three independent experiments. The western blots were subjected to densitometric analysis in order to determine the intensity of the bands corresponding to p-PI3K and p-AKT. The data are expressed relative to the intensity of the corresponding GAPDH band. Data are presented as the mean \pm SD. * P <0.05 vs. 0.1% DMSO; † P <0.05 vs. TGF- β . ATRA, all-trans-retinoic acid; HConFs, human conjunctival fibroblasts; TGF- β , transforming growth factor- β ; p, phosphorylated.

serve a protective role against collagen accumulation, cell injury and proliferation in various types of fibrosis, including liver fibrosis, pulmonary fibrosis and kidney fibrosis (22-25). In a previous study, it was demonstrated that ATRA inhibited TGF- β -induced HConF-mediated collagen gel contraction by attenuating the formation of actin stress fibers and focal adhesions, as well as by inhibiting MAPK, c-Jun and SMAD signaling (16). In the present study, it was demonstrated that ATRA inhibited the TGF- β -induced migration and proliferation of HConFs, inhibited ECM synthesis and increased the apoptosis in HConFs. Collectively, these results suggested that ATRA attenuates conjunctival scarring by modulating the function of HConFs and ECM synthesis.

The PI3K/AKT pathway modulates cell proliferation, differentiation, apoptosis, motility, survival and glucose metabolism (26). PI3K generates 3'-phosphorylated phosphoinositides, including phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-triphosphate, which then recruit target proteins to the plasma membrane (27). AKT is a serine/threonine kinase that acts as an effector of PI3K (27). It has been reported that activation of the PI3K/AKT signaling pathway may induce ECM secretion in several cell types (28,29). LY294002 and AKT small interfering RNA were reported to significantly reduce TGF- β 1-induced α -smooth muscle actin expression, a marker of fibroblasts, in conjunctival fibroblasts, indicating that these changes were mediated by the PI3K/AKT

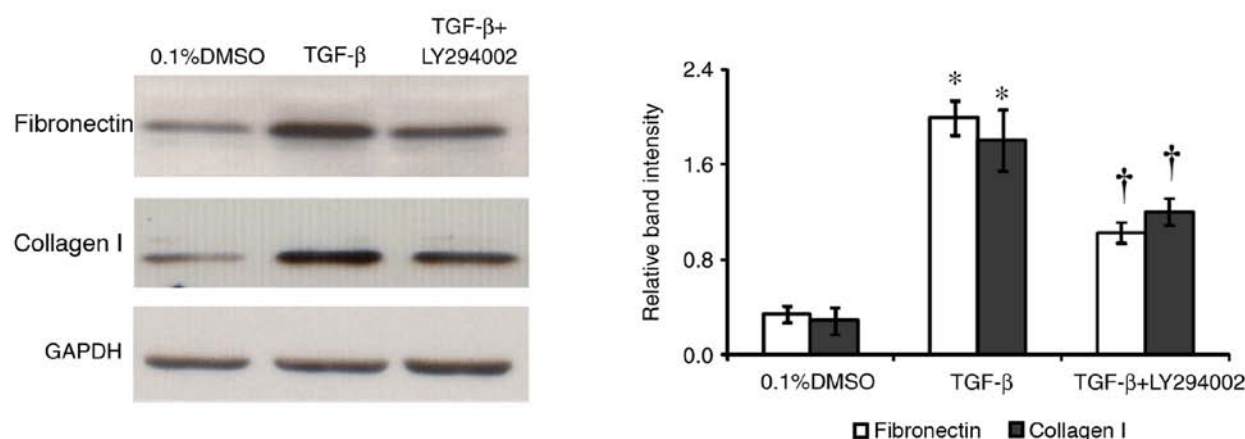


Figure 6. Effect of PI3K inhibition on TGF- β -induced expression of collagen I and fibronectin in HConFs. Serum-deprived cells were incubated for 1 h in the presence or absence of the PI3K inhibitor LY294002 (10 μ M) and then for 48 h in the presence or absence of TGF- β (1 ng/ml). Cell lysates were subjected to western blot analysis with collagen I, fibronectin or and GAPDH antibodies. The data are representative of three independent experiments. The western blots were subjected to densitometric analysis to determine the intensity of the bands corresponding to collagen I and fibronectin. The data are expressed relative to the intensity of the corresponding GAPDH band. Data are presented as the mean \pm SD. *P<0.05 vs. 0.1% DMSO; †P<0.05 vs. TGF- β . ATRA, all-trans-retinoic acid; HConFs, human conjunctival fibroblasts; TGF- β , transforming growth factor- β .

pathway (30). In addition, LY294002 could inhibit the proliferation and migration of conjunctival fibroblasts (31,32). In the present study, it was shown that ATRA promoted apoptosis and inhibited proliferation, migration and ECM synthesis. In addition, it was demonstrated that LY294002 could inhibit TGF- β -induced expression of collagen I and fibronectin, similar to ATRA; this is consistent with the results of a previous study (33). Hence, it was deduced that the inhibitory effects of ATRA on HConFs are likely mediated by inhibition of the PI3K/AKT signaling pathway.

TGF- β -mediated signaling can occur via SMAD-dependent or SMAD-independent pathways; the SMAD-independent pathway includes the MAPK and PI3K/AKT pathways (34). TGF- β activates the SMAD pathway via the phosphorylation of Smad2 and Smad3, which then leads to the formation of a Smad2/3 complex; the Smad2/3 complex and Smad4 are subsequently translocated to the nucleus in order to regulate the expression of genes associated with fibroblast proliferation, migration and ECM deposition (35). In our previous study, a role for SMAD signaling was found in the ATRA-mediated inhibition of TGF- β -induced, HConF-mediated collagen gel contraction (16). Therefore, it was hypothesized that the SMAD signaling pathway is also involved in the inhibitory effects of ATRA on proliferation, migration and ECM synthesis in HConFs.

In conclusion, the findings of the present study revealed the mechanism of action of ATRA. ATRA was found to modulate PI3K/AKT signaling and impact on TGF- β -induced apoptosis, proliferation, migration and ECM synthesis. Given the significant global impact of glaucoma and the inadequacy in its treatment methods, conjunctival scarring at the surgical site after glaucoma filtration surgery (36,37) and the adverse side effects of anti-scarring anti-metabolite medication, such as mitomycin-C and 5-fluorouracil, which can potentially lead to blindness (38-41), there is a requirement for improved therapeutic approaches. From the present study, it is proposed that ATRA may be a promising compound capable of modulating scar formation after glaucoma filtration surgery. However, as the

present study was performed *in vitro*, future research should be directed towards characterizing the *in vivo* effects of ATRA.

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Availability of data and materials

All the data generated and analyzed in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YL and YZ conceived and designed the experiments. LL and XW performed the experiments. LL, XW, and YZ analyzed the data. LL and YL wrote the manuscript. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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