# 5-Aminolaevulinic Acid-Based Photodynamic Therapy Restrains Pathological Hyperplasia of Fibroblasts

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Background: This study aimed to explore whether 5-aminolaevulinic acid-based photodynamic therapy (ALA-PDT) restrains pathological hyperplasia of fibroblasts from hyperplastic scar tissues, and to investigate the potential mechanism.

Material/Methods: We used MTT assay, flow cytometry, and terminal-deoxynucleotidyl transferase mediated nick-end labeling (TUNEL) to examine the effects of ALA-PDT on proliferation, cell cycle, and apoptosis of fibroblasts isolated from hyperplastic scar tissues. The growth-promoting effect of fibroblasts on vascular endothelial cells was measured by cell co-culture. Real-time PCR and Western blot analysis were performed to detect the expression levels of transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), Collagen I, Collagen III, vascular endothelial growth factor-A (VEGFA), and basic fibroblast growth factor (bFGF).

Results: ALA-PDT inhibited proliferation delayed cell cycle progress, promoted apoptosis of fibroblasts, and suppressed its growth-promoting effect on vascular endothelial cells, and decreased expression of TGF-β1, α-SMA, Collagen I, Collagen III, VEGFA, and bFGF.

Conclusions: ALA-PDT effectively restrained pathological hyperplasia of fibroblasts from hyperplastic scar tissues, which may provide a research basis for clinical therapy of hyperplastic scars.

MeSH Keywords: Apoptosis • Cell Proliferation • Cicatrix, Hypertrophic • Fibroblasts • Photochemotherapy

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## Background

Fibroblasts are the most abundant cells in connective tissue, playing a crucial role in the wound healing process [1]. Wound healing is a complex process, including 3 phases: inflammatory phase, proliferative phase, and regeneration phase [2]. The inflammatory phase begins with capillary damage, in which a variety of inflammatory cells and fibroblasts are recruited to the lesion. In the proliferative phase, new capillaries grow and transport oxygen and nutrients to enhance cell proliferation. In neonatal granulation tissue, fibroblasts are activated and transform into myofibroblasts to contract the wound. In the third phase, scar tissue forms through remodeling of the granulation tissue and massive apoptosis of vascular cells and myofibroblasts, and surplus myofibroblasts return to fibroblast phenotype [3,4].

In the process of wound healing, myofibroblasts secrete extracellular matrix and collagen to rebuild the damaged tissue. However, excessive proliferation and insufficient apoptosis of myofibroblasts, abnormal secretion of extracellular matrix and collagen would cause pathologic hyperplasia and deposition of fibroblasts, and formed hyperplastic scar [5,6]. Scar is the natural result of wound healing, but hyperplastic scar is a result of cell proliferation and matrix metabolism disorder. Hyperplastic scar tissues are generally thicker and harder than normal skin tissues, appearing red or purple, some of which are accompanied with itching or infiltrating to surrounding tissue, and form chronic ulcer even cancerate. To date, there has been a variety of methods used to prevent or treat the pathological proliferation of fibroblasts during wound healing process to decrease scars formation [7].

5-Aminolaevulinic acid-based photodynamic therapy (ALA-PDT) is a drug-instrument combination technology, utilizing the selective photosensitivity of lesion site to destroy the pathological tissue. Lesion tissue absorbed photosensitizer is exposed with light of appropriate wavelength, and by activating with photosensitizer and oxygen molecule, produces cytotoxic reactive oxygen species (ROS) to damage organelles and then cause the apoptosis and necrosis of target cells [8,9]. The 3 key elements of ALA-PDT are photosensitizer, light irradiation and oxygenation. 5-Aminolaevulinic acid (ALA) is one of second generation photosensitizers, which has no photosensitive activity in itself, but generates endogenous protoporphyrin IX in mitochondria catalyzed by heme synthetase. Light should match with absorption spectrum of the photosensitizer, having enough intensity. As its single wavelength and high energy, laser is an ideal choice. In addition, sufficient oxygen is essential for the satisfactory effect. ALA-PDT has been used to cure squamous carcinoma for several decades, and widely applied for treatment of skin diseases, including flat wart [10], actinic keratosis [11] and acne vulgaris [12]. In addition, ALA-PDT

can induce or promote various cancer cells apoptosis, including breast cancer [13], pituitary adenomas [14] and laryngeal papilloma [15]. However, there are few reports describing whether ALA-PDT affects scar formation. It has been recently proved that ALA-PDT significantly increased apoptotic cell death and reactive oxygen species (ROS) generation in fibroblasts [16], suggesting that ALA-PDT may be able to prevent or remit scars caused by human skin fibroblast excessive proliferation.

In this study, we isolated fibroblasts from skin hyperplastic scar tissues and detected whether ALA-PDT affects proliferation, cell cycle and apoptosis of fibroblasts.

## Material and Methods

### Ethical statement

The human hyperplastic scar tissues were collected from anterior chest wall of 4 patients (2 males and 2 females, 20–42 years old) in the Department of Dermatology, the First People's Hospital of Yunnan Province. These tissues were all caused by wound and presence of more 6 months with swell, hyperplasia, itch and ache. The matched normal skin tissues were collected from abdominal normal skin tissues of the previous 4 patients. Verbal informed consent was obtained from all patients. The tissue collection and experimental procedures were approved by the Ethics Committee of the First People's Hospital of Yunnan Province.

### Cell isolation and culture

Human normal skin tissues (or hyperplastic scar tissues) were washed with phosphate buffer solution (PBS) (Double-helix, Shanghai, China) to remove the intima and connective tissue, and cut into pieces of 1–3 $mm^3$. The tissue pieces were cultivated in culture flasks coated by fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) at 37°C with 5% $CO_2$. 12h later, Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) and 10% FBS were added into the flasks to culture. About 5-6 days later, when the cell confluence reached approximately 70%, the cells were passaged with 0.25% trypsin (Beyotime, Haimen, Jiangsu, China). The fifth generations could be used for subsequent experiments.

### Immunofluorescence

Immunofluorescence was performed to identify the fibroblasts isolated from hyperplastic scar tissues. Cells were seeded onto glass slide beforehand. When the confluence reached 70%, the cells were fixed with 4% paraformaldehyde (Sinopharm, Beijing, China) for 15 min and permeated with 0.1% Triton

**Table 1.** Information of real-time PCR primers used in this study.

| Name | Sequence (5'-3') | Tm | Length of amplicon | Gene ID |
|------|------------------|-----|--------------------|---------|
| TGF-β1 F | 5'-TGGAAACCCACAACGAAATCT-3' | 59.5°C | 319 bp | NM_000660.4 |
| TGF-β1 R | 5'-CTAAGGCGAAAGCCCTCAAT-3' | 59°C | | |
| α-SMA F | 5'-CCCTTGAGAAGAGTTACGAGTT-3' | 58.9°C | 146 bp | NM_001100.3 |
| α-SMA R | 5'-ATGATGCTGTTGTAGGTGGTT-3' | 57.6°C | | |
| Collagen I F | 5'-GCTTGGTCCACTTGCTTGAA-3' | 58.6°C | 212 bp | NM_000088.3 |
| Collagen I R | 5'-CCACTTGGGTGTTTGAGC-3' | 53.7°C | | |
| CollagenIII F | 5'-GCTGTTGAAGGAGGATGTT-3' | 51.8°C | 209 bp | NM_000090.3 |
| CollagenIII R | 5'-GTAGGAGCAGTTGGAGGCTGT-3' | 58.5°C | | |
| β-actin F | 5'-CTTAGTTGCGTTACACCCTTTCTTG-3' | 62°C | 156 bp | NM_001101.3 |
| β-actin R | 5'-CTGTCACCTTCACCGTTCCAGTTT-3' | 64.4°C | | |

TGF-β1 – transforming growth factor-β1; α-SMA – α-smooth muscle actin; F – forward; R – reverse; Tm – melting temperature; bp – base pairs.

X-100 (Amresco, Solon, OH, USA) at room temperature for 30 min. After blocking with goat serum (Solarbio, Beijing, China) for 15 min, the cells were incubated with rabbit anti-Vimentin antibody (1:200, diluted with PBS) (Bioss, Beijing, China) at 4°C overnight. After rinsing with PBS, the cells were incubated with goat anti-rabbit immunoglobulin G (IgG) labeled with Cy3 (1:200, diluted with PBS) (Beyotime) at room temperature in dark for 60 min. After being counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Biosharp, Hefei, Anhui, China), the cells were mounted in the presence of anti-fluorescence quenching agent (Solarbio), observed and photographed with a fluorescence microscope (Olympus, Tokyo, Japan) at 400× magnification.

### ALA-laser irradiation

After being treated with 5-aminolaevulinic acid (ALA) (Sigma, St. Louis, MO, USA), the fibroblasts were irradiated with laser at 633 nm. The maximum density of laser instrument (Yage, Wuhan, Hubei, China) was 100 mW. ALA concentration, treating time, laser intensity and irradiating time were adjusted according to requirement.

### RNA extraction, reverse transcription (RT) and real-time PCR

RNA of cells was extracted using total RNA rapid extraction kit (BioTeke, Beijing, China) according to manufacturer's protocol. After detecting the concentration, 1 μg of RNA sample was reversely transcribed into cDNA with M-MLV reverse transcriptase (BioTeke) in the presence of oligo(dT) and random primers (Sangon, Shanghai, China). The instruments in this section were pro-treated by surface RNase Erase (TIANDZ, Beijing, China) and the reagents were RNase-free.

The cDNA (1 μl for each reaction) was used for real-time PCR to detect the transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), Collagen I, and Collagen III using 2×Power Taq PCR MasterMix (BioTeke) and SYBR Green (Solarbio), with β-actin as the internal control. The PCR procedure was set as follows: 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 30 s, and finally 4°C for 5 min. Calculations were performed using the $2^{-\Delta\Delta Ct}$ method. Information on real-time primers is shown in Table 1.

### Western blot analysis

Protein was extracted using a whole-cell lysis kit (Wanleibio, Shenyang, Liaoning, China) from cells and the concentration of protein was measured by use of a BCA protein quantitative kit (Wanleibio). After being denatured by boiling, the protein sample (40 μg for each lane) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA). After blocking with 5% skim milk (YILI, Hohhot, Inner Mongolia, China) at room temperature for 1 h, the membrane was incubated with the following antibodies at 4°C overnight: rabbit anti-TGF-β1 (1:200) (Santa Cruz, CA), mouse anti-α-SMA (1:400) (Boster, Wuhan, Hubei, China), rabbit anti-Collagen I (1:400) (Boster), mouse anti-Collagen III (1:400) (Boster), rabbit anti-vascular endothelial factor-A (VEGFA) (1:400) (Boster), and rabbit anti-basic fibroblast growth factor (bFGF) (1:200) (Santa Cruz). After rinsing with TBST, the membrane was incubated with goat anti-rabbit IgG labeled with horseradish peroxidase (HRP) (1:5000) (Wanleibio) or goat anti-mouse IgG-HRP (1:5000) (Wanleibio) at 37°C for 45 min, and explored with ECL reagent (Wanleibio). After removing antibodies by stripping buffer (Wanleibio), the membrane was incubated with mouse anti-β-actin (1:1000) (Santa

Cruz) and goat anti-mouse IgG-HRP (1:5000) (Wanleibio) to detect the internal control, β-actin. Optical density values of bands were analyzed by a gel image processing system (Liuyi, Beijing, China).

### MTT assay

Fibroblasts were seeded into 96-well plates with $5 \times 10^3$ per pore to culture. After adhering to the plate, the fibroblasts were treated with drug or laser (different in groups), then 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (Sigma) was added into the medium with 0.2 mg/ml, and 4 h later, the plate was centrifuged at 1000 rpm for 10 min and the supernatant was discarded. The crystal was dissolved with dimethyl sulfoxide (DMSO) (Sigma) (200 µl per pore), and the optical density of the solution was measured at 490 nm.

### Cell co-culture

Human umbilical vein endothelial cell line HUVEC cells were kept in our lab and cultured in DMEM (Gibco) supplemented with 10% FBS (Hyclone), 100 U/ml penicillin, and 100 µg/ml streptomycin (Beyotime) in 5% $CO_2$ at 37°C. Co-culture of fibroblasts with HUVEC cells was performed with transwell chambers (Corning, NYA) using a polycarbonate membrane with a pore size of 8 µM to detect the growth-promoting effect of fibroblasts on HUVE cells. Fibroblasts from human normal skin tissues or hyperplastic scar tissues were cultured on the membrane in a transwell chamber, which sat in the well of a 24-well plate, and the HUVEC cells were cultured in the lower well with M131+MVGS medium. After co-culturing for 5 days, the HUVEC cells were digested, collected, and seeded into 96-well plates. After adhering to the plate, we performed MTT assay of the HUVEC cells to assess cell viability.

### Terminal-deoxynucleotidyl transferase mediated nick-end labeling (TUNEL) assay

TUNEL assay was performed to detect apoptosis of fibroblasts. Cells cultured on glass slides were fixed by 4% paraformaldehyde (Sinopharm) for 15 min, permeated with 0.1% TritonX-100 for 8 min, blocked with 3% $H_2O_2$ (Sinopharm) for 10 min, and incubated with TUNEL reaction mixture (Roche, Basel, Switzerland) at 37°C for 60 min. After rinsing with PBS, cells were incubated with Converter-POD (Roche) at 37°C for 30 min, counterstained with DAB reagent (Solarbio), and counterstained with hematoxylin (Solarbio) for 3 min. Then the cells were treated with 1% hydrochloric acid alcohol, rinsed with water for 20 min, and mounted with glycerine ethanol. Thereafter, the cells were observed and photographed with a microscope (Olympus, Tokyo, Japan) at 400× magnification.

### Flow cytometry

The cells were collected and treated with an Annexin V-FITC/PI apoptosis detection kit (KeyGEN, Nanjing, Jiangsu, China) according to the manufacturer's protocol, and tested by flow cytometer (BD, Franklin Lakers, NJ). Then the apoptosis rates of cells in each group were calculated and analyzed.

Cell cycle detection was performed with a cell cycle analysis kit (Beyotime) and flow cytometer (BD), following the manufacturers' protocol. The cell number in each phase and the proliferation index (PI) of cells in each group were calculated as PI=(S+G2/M)/(G1/G0).

### Enzyme-linked immune sorbent assay (ELISA)

Culture supernatant of fibroblasts was collected and used for ELISA assay to detect VEGFA and bFGF content by use of the VEGFA ELISA kit (Boster) or ELISA kit for FGF2 (USCN, Wuhan, Hubei, China) according to the manufacturers' protocols.

### Statistical analysis

The data in this study are presented as mean ± standard deviation (SD) of 3 individual experiments, and analyzed by one-way ANOVA test, which was considered statistically significant at p<0.05. (* p<0.05, ** p<0.01, *** p<0.001, ns – no significance).

## Results

### Identification of fibroblasts and condition optimization of ALA-PDT

Fibroblasts were isolated from human normal skin tissues or hyperplastic scar tissues and cultured. Microscopy showed that fibroblasts isolated from hyperplastic scar tissues were plumper and their cytoplasm is more abundant than fibroblasts from normal tissues (Figure 1A). Then fibroblasts from hyperplastic scar tissues were identified by immunofluorescence of Vimentin, which is one of features of hyperplastic fibroblasts (Figure 1B).

In order to gain more obvious results, we optimized the experimental conditions of ALA-PDT. After alone treating with ALA of different concentration for 2 h, the fibroblasts from hyperplastic scar tissues were detected cell viability by MTT assay. The results showed that ALA of ≥1.5 mM obviously affected cell growth (Figure 1C). After irradiating alone with lasers of different intensities for 10 min, the fibroblasts were subjected to MTT assay to detect cell viability. The results showed that laser irradiation significantly affected cell growth at the intensity of ≥50 J/cm² (Figure 1D). Next, the orthogonal experiment with ALA at
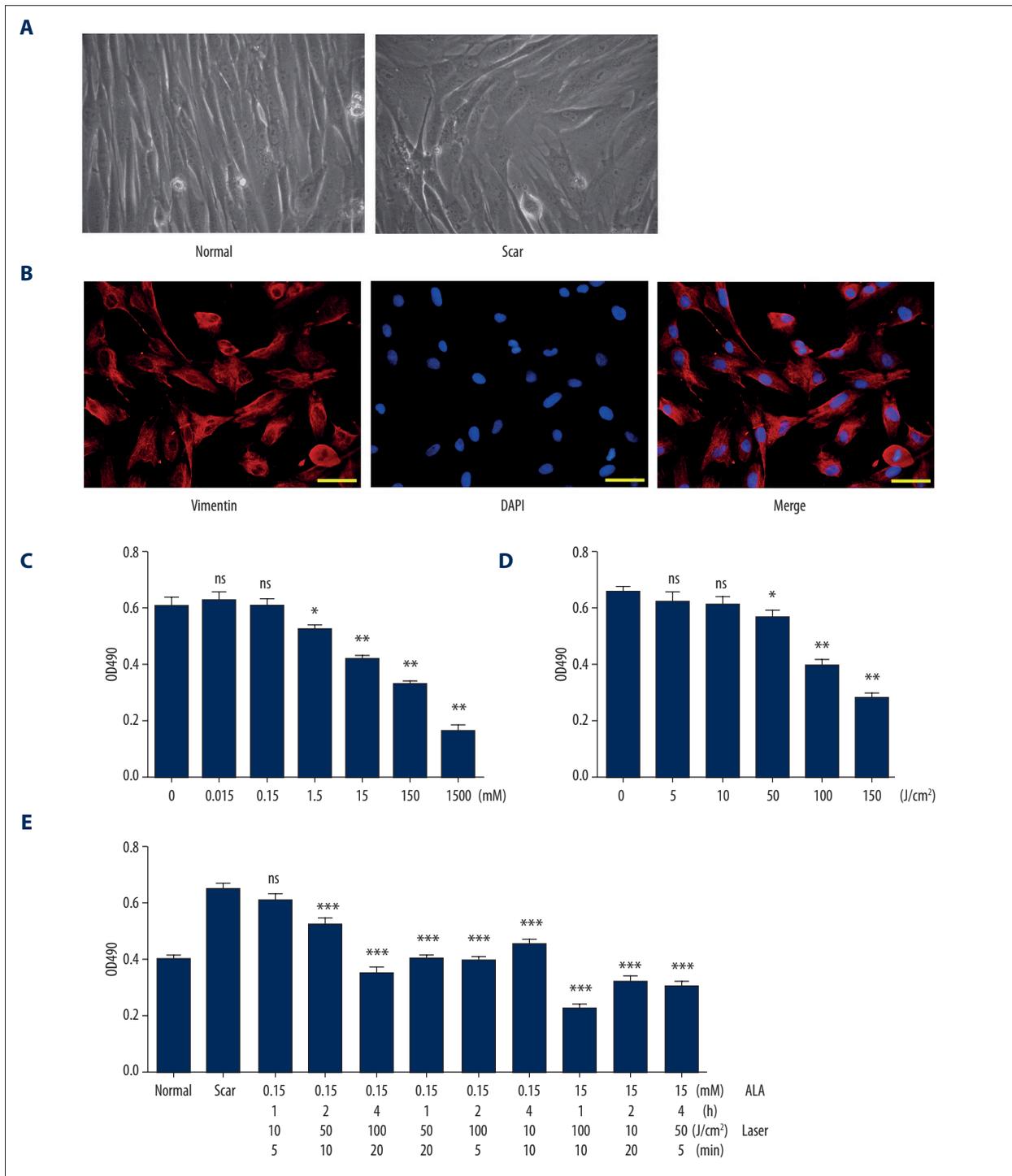
**Figure 1.** Fibroblast identification and 5-aminolaevulinic acid-based photodynamic therapy (ALA-PDT) condition optimization. (**A**)4Fibroblasts isolated from human normal skin tissues (left panel) and hyperplastic scar tissues (right panel) under 400× magnification. (B) Identification of fibroblasts from hyperplastic scar tissues by immunofluorescence of anti-Vimentin (the scale bar in the figure represents 50 µm). (**C**) MTT assay was performed to detect the cell viability of fibroblasts treated with ALA of different concentrations. (**D**) Cell viability of fibroblasts irradiated with lasers of different intensities, detected by MTT assay. (**E**) MTT orthogonal assay of fibroblasts was performed to determine the optimal condition of 5-aminolaevulinic acid (ALA) concentration, time, laser intensity, and irradiating time, with the untreated fibroblasts from scar tissue as the control. The data were analyzed by one-way ANOVA test, *$P<0.05$, ** $P<0.01$, *** $P<0.001$, ns – no significance.

**Table 2.** MTT grouping.

| Groups | ALA concern (mM) | ALA treating time (h) | Laser intensity (J/cm²) | Laser irradiating time (min) |
|--------|------------------|------------------------|--------------------------|-------------------------------|
| 1 | 0.15 | 1 | 10 | 5 |
| 2 | 0.15 | 2 | 50 | 10 |
| 3 | 0.15 | 4 | 100 | 20 |
| 4 | 1.50 | 1 | 50 | 20 |
| 5 | 1.50 | 2 | 100 | 5 |
| 6 | 1.50 | 4 | 10 | 10 |
| 7 | 15 | 1 | 100 | 10 |
| 8 | 15 | 2 | 10 | 20 |
| 9 | 15 | 4 | 50 | 5 |

ALA – 5-aminolaevulinic acid.

3 different concentrations (0.15 mM, 1.5 mM, and 15 mM) and treatment time (1 h, 2 h, and 4 h), lasers of different intensities (10 J/cm², 50 J/cm², and 100 J/cm²) and 3 different irradiating times (5 min, 10 min, and 20 min) was performed. Details of grouping are shown in Table 2, with untreated fibroblasts from hyperplastic scar tissues as the control. As shown in Figure 1E, cell viability was decreased by 65%, most significantly among the 9 groups, when treating with 15 mM ALA for 1 h and irradiating with 100 J/cm² for 10 min. Therefore, this condition was used as the optimal condition in the subsequent experiments.

### ALA-PDT inhibits proliferation and enhances apoptosis in fibroblasts

Flow cytometry was performed to detect cell proliferation. The results showed that fibroblasts from hyperplastic scar tissues proliferated significantly faster than fibroblasts from normal skin tissues, and ALA-PDT significantly delayed transition of cells in G1/G0 phase to S phase (Figure 2A, 2B). Furthermore, ALA-PDT decreased the proliferation index (PI) of fibroblasts by 52%, and treatment with ALA (15 mM for 1 h) or irradiation with laser (100 J/cm² for 10 min) alone also decreased PI of fibroblasts, but not as obviously as ALA-PDT (Figure 2C). Then, apoptosis of fibroblasts was detected by flow cytometry and TUNEL assay. The flow cytometry results showed that ALA-PDT increased apoptosis rate of fibroblasts by 2.02-fold (Figure 2D, 2E), and TUNEL assay results showed similar outcomes (Figure 2F). Moreover, treatment with ALA or irradiation with laser alone enhanced apoptosis of fibroblasts to some degree (Figure 2D–2F).

### ALA-PDT decreases immoderate fibrosis of fibroblasts

In addition to excessive proliferation and inadequate apoptosis, the formation of scar tissue is related to immoderate fibrosis.

Therefore, we detected the expression levels of several fibrosis related factors, TGF-β1, α-SMA, Collagen I, and Collagen III by real-time PCR and Western blot. Real-time PCR results showed that the mRNA levels of TGF-β1, α-SMA, Collagen I, and Collagen III in fibroblasts from hyperplastic scar tissues were obviously higher than in fibroblasts from normal skin tissues, and ALA-PDT decreased the mRNA level of TGF-β1 by 68%, α-SMA by 50%, Collagen I by 54%, and Collagen III by 58% in fibroblasts from hyperplastic scar tissues (Figure 3A–3D). In addition, treatment with ALA of 15 mM for 1 h alone decreased TGF-β1 mRNA level by 45%, α-SMA mRNA level by 34%, Collagen I mRNA level by 30%, and Collagen III mRNA level by 25%. Irradiation with laser of 100 J/cm² for 10 min alone decreased TGF-β1 by 60%, α-SMA by 51%, Collagen I mRNA level by 40%, and Collagen III by 50% compared with untreated fibroblasts from hyperplastic scar tissues (Figure 3A–3D). Western blot results showed similar declines of TGF-β1, α-SMA, Collagen I, and Collagen III in protein level in fibroblasts from hyperplastic scar tissue (Figure 3E–3H).

### ALA-PDT reduces the growth-promoting effect of fibroblasts on vascular endothelial cells

The significance of fibroblasts in wound healing is also due to secretion of growth factors to promote other cells and tissues to grow, primarily vascular tissue and vascular endothelial cells. Therefore, we detected the effect of fibroblasts on the growth of vascular endothelial cell line HUVEC by co-culture and MTT assay. MTT assay showed that fibroblasts from hyperplastic scar tissue promoted the growth of HUVEC cells more obviously than fibroblasts from normal skin, and this promotion was effectively inhibited by ALA-PDT (Figure 4A). Thereafter, we detected the expression of 2 growth factors, VEGFA and bFGF, in culture supernatant and fibroblasts by
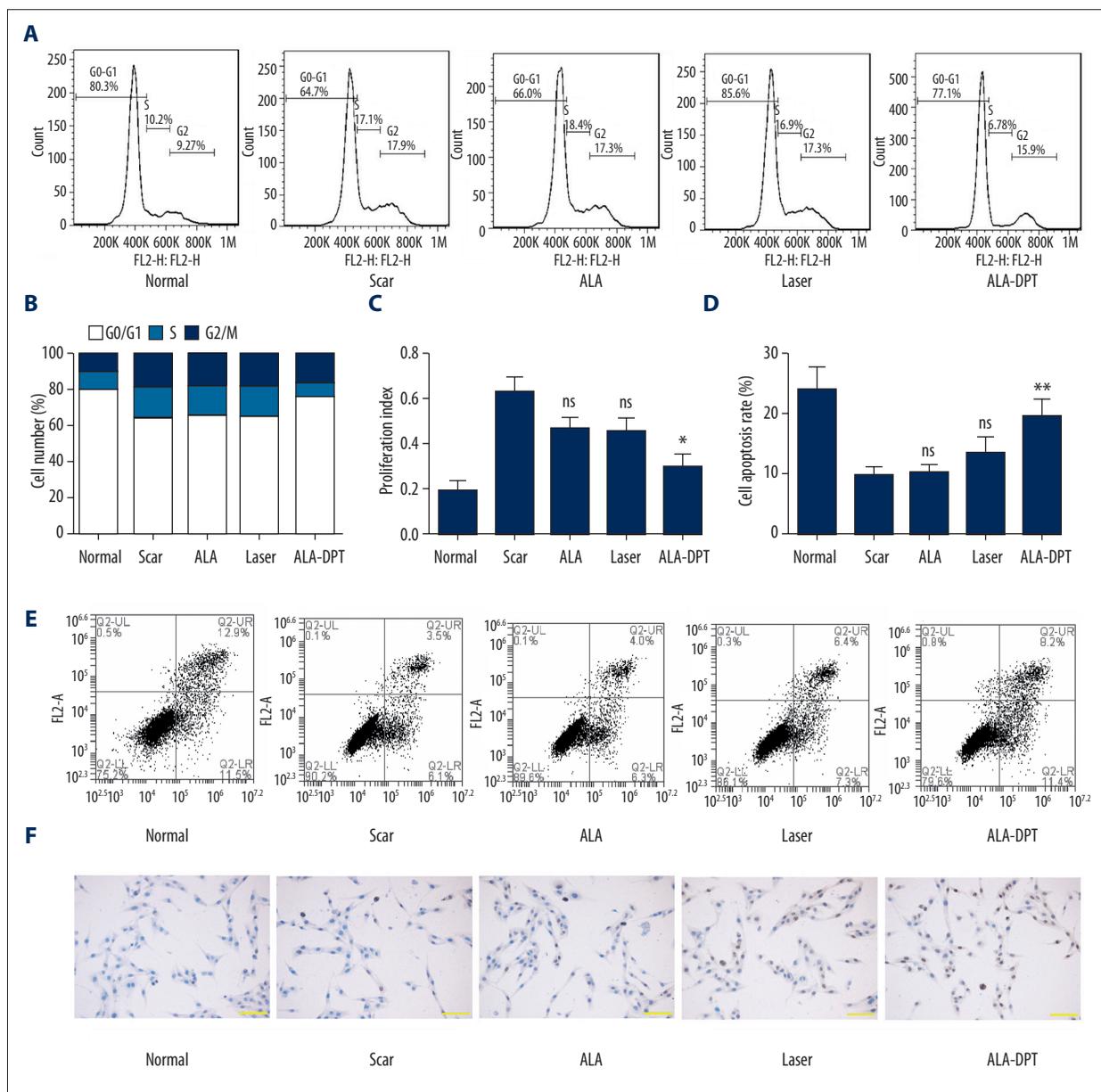
**Figure 2.** ALA-PDT inhibits proliferation and enhances apoptosis of fibroblasts. (**A**) Flow cytometry detection of images of cell cycle of fibroblasts in different groups. (**B**) Histogram of different phases of cell cycle of fibroblasts. (**C**) Proliferation index of fibroblasts. (**D**) Apoptosis rate of fibroblasts in each group. (E) Flow cytometry detection images of apoptosis of fibroblasts in different groups. (**F**) Terminal-deoxynucleotidyl transferase mediated nick-end labeling (TUNEL) assay was performed to test the apoptosis of fibroblasts in each group. The scale bar in the figure represents 50 μm. The data were analyzed by one-way ANOVA test, with the fibroblasts in the scar tissue group as the control. * P<0.05, ** P<0.01, ns – no significance.

ELISA and Western blot, respectively. ELISA results showed that the levels of VEGFA and bFGF in culture supernatant of fibroblasts from hyperplastic scar tissue were higher than culture supernatant of fibroblasts from normal skin, and ALA-PDT decreased the levels of VEGFA and bFGF in culture supernatant of fibroblasts from hyperplastic scar by 45% and 51% (Figure 4B, 4C). Similar to ELISA results, Western blot results showed that the levels of VEGFA and bFGF in fibroblasts from hyperplastic

scar tissue were higher than in fibroblasts from normal skin, and ALA-PDT decreased the levels of VEGFA and bFGF in fibroblasts from hyperplastic scar tissue by 47% and 42%, respectively (Figure 4D, 4E). In addition, treatment with ALA of 15 mM for 1 h or irradiating with laser of 100 J/cm² for 10 min also suppressed promotion of fibroblast vascular endothelial cell growth, and inhibited synthesis and secretion of VEGFA and bFGF in fibroblasts from hyperplastic scar tissue (Figure 4).
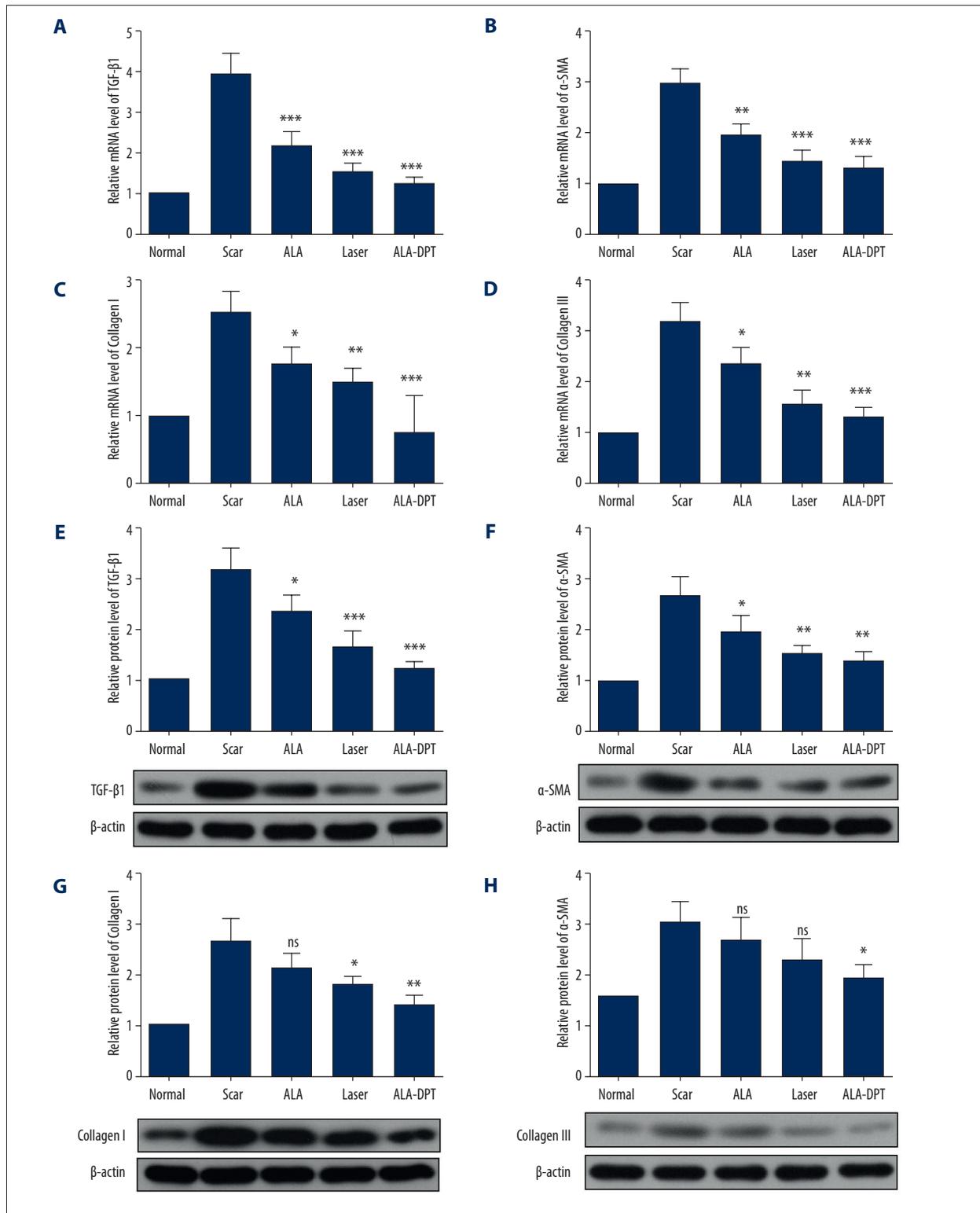
**Figure 3.** ALA-PDT decreases TGF-β1, α-SMA, Collagen I, and Collagen III expression levels in fibroblasts. (**A–D**) The mRNA levels of transforming growth factor-β1 (TGF-β1), α-smooth muscle actin (α-SMA), Collagen I, and Collagen III were detected by real-time PCR. (**E–H**) The protein expression levels of TGF-β1, α-SMA, Collagen I, and Collagen III were examined by Western blot analysis. The data were analyzed by one-way ANOVA test, with the fibroblasts in the scar tissue group as the control. * P<0.05, ** P<0.01, *** P<0.001, ns – no significance.
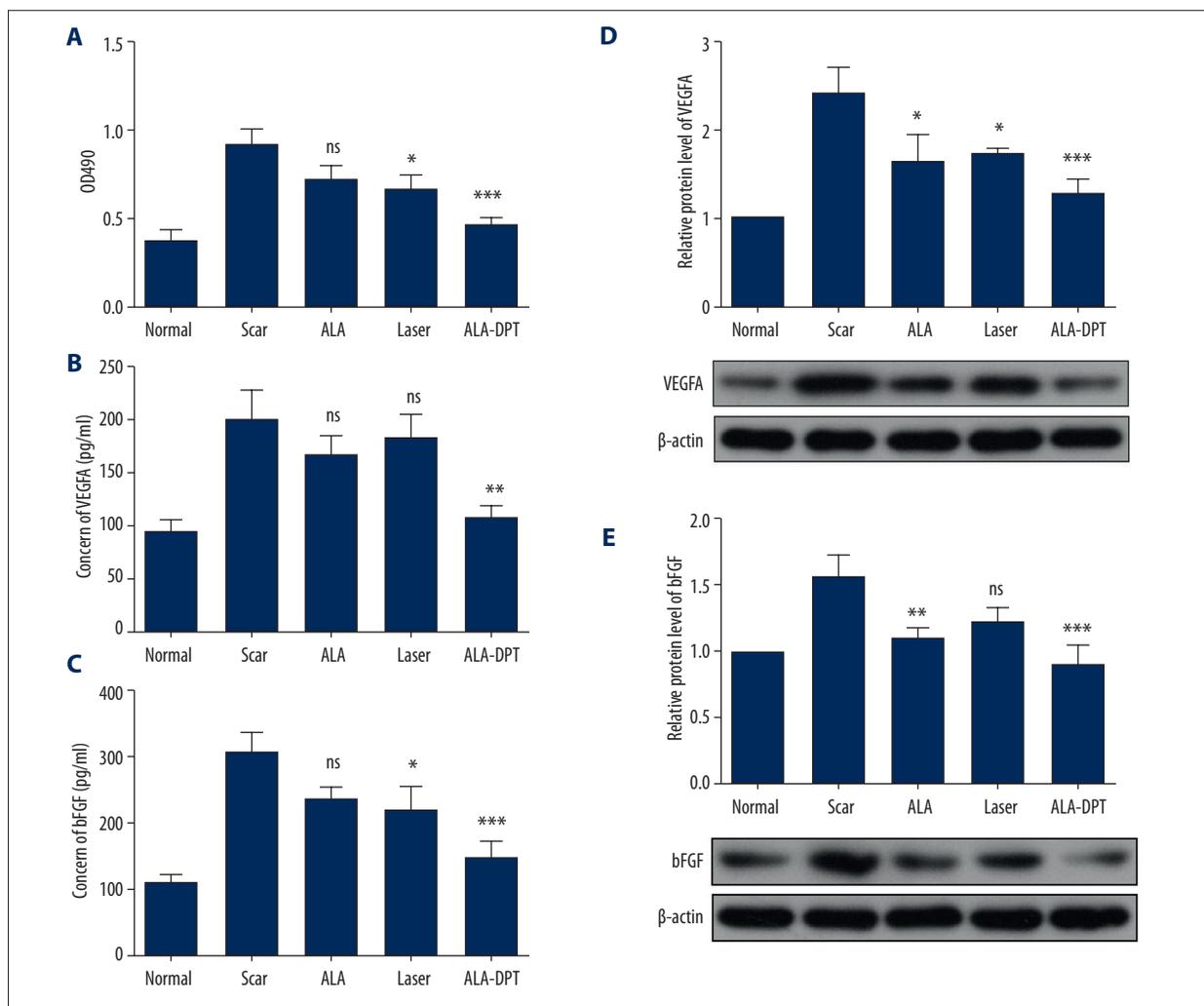
**Figure 4.** ALA-PDT reduces the growth-promoting effect of fibroblasts on HUVEC cells. (**A**) MTT assay was carried out to test the cell viability of HUVEC cells cultured together with fibroblasts. (**B, C**) The expression levels of vascular endothelial growth factor-A (VEGFA) and basic fibroblast growth factor (bFGF) in culture supernatant were tested by ELISA. (**D, E**) The expression levels of VEGFA and bFGF in fibroblasts were detected by Western blot analysis. The data were analyzed by one-way ANOVA test, with the fibroblasts in scar tissue group as the control. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns – no significance.

## Discussion

In this study, we isolated fibroblasts from hyperplastic scar tissues and normal skin tissues and found that fibroblasts from hyperplastic scar tissue proliferated faster and had better survival than fibroblasts from normal skin. Moreover, the expression levels of TGF-β1, α-SMA, Collagen I, Collagen III, VEGFA, and bFGF in fibroblasts from hyperplastic scar tissue were higher than fibroblasts from normal skin, and fibroblasts from hyperplastic scar tissue promoted growth of vascular endothelial cells.

α-SMA is generally expressed at low levels in normal skin tissue, but is expressed at high levels in myofibroblasts [17,18]. Myofibroblasts in scar tissue are differentiated from fibroblasts,

combining characteristics of fibroblasts and smooth muscle cells [19,20]. Compared with fibroblasts, myofibroblasts contain more abundant microtubules and microfilaments, and promote wound shrinkage via utilizing actin and myosin, including α-SMA [21,22]. Myofibroblasts and fibroblasts also synthesize and secrete collagen to form extracellular matrix (ECM).

It has been believed that α-SMA is upregulated by TGF-β. TGF-β belongs to the TGF-β super family, which regulates cells growth and differentiation. *In vivo,* TGF-β stimulates vascular endothelial cells growth and promotes fibroblast proliferation and differentiation. TGF-β also upregulates Collagen I and Collagen III, which are important for formation of hyperplastic scar tissue [23,24].

In addition, angiogenesis is essential for neonatal tissues, including scar tissue, wherein VEGF is crucial for angiogenesis. VEGF strongly promotes vascular endothelial cells mitosis, and induces several enzymes to degrade the basement membrane to promote epithelial cells to infiltrate and migrate. In addition, VEGF inhibits upstream caspase activation and increases anti-apoptosis factors to promote epithelial cells survival [25]. Another growth factor, bFGF, also promotes vascular endothelial cells mitosis and induces epithelial cells migration, as well as stimulating fibroblast proliferation. Through analysis of cell cycle, bFGF was found to induce transition of cells in G0/G1 phase to S phase to realize the rapid proliferation and differentiation of fibroblasts, epithelial cells, and vascular endothelial cells [26]. In addition, the wound healing process is modulated by some other factors. For example, platelet-derived growth factor (PDGF) induces fibroblasts, vascular smooth muscle cells, and keratinocytes in contact inhibition status into switch to proliferation status, and stimulates fibroblasts to synthesize collagen and activates collagen enzyme to regulate the regeneration of ECM [27]. PDGF also induces secretion of insulin-like growth factor-1 (IGF-1), which enhances fibroblasts proliferation by interdicting apoptosis [28]. Furthermore, fibroblast proliferation is enhanced by tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-1 (IL-1), and is suppressed by $\gamma$-interferon ($\gamma$-IFN).

In our study, TGF-$\beta$1, $\alpha$-SMA, Collagen I, Collagen III, VEGFA, and bFGF were selected as markers to measure changes of fibroblasts under different treatments. ALA-PDT was used to cause excessive proliferation of fibroblasts from hyperplastic scar tissues. Under optimal condition, we found that the expression levels of TGF-$\beta$1, $\alpha$-SMA, Collagen I, Collagen III, VEGFA, and bFGF in fibroblasts from hyperplastic scar were significantly downregulated by ALA-PDT. Moreover, ALA-PDT inhibited proliferation and enhanced apoptosis of fibroblasts, and obviously restrained the growth-promoting effect of fibroblasts on vascular endothelial cells.

## Conclusions

We demonstrated that ALA-PDT inhibited fibroblasts from causing excessive proliferation of hyperplastic scar tissues, enhanced apoptosis, decreased immoderate fibrosis, and restrained the growth-promoting effect of fibroblasts on vascular endothelial cells by suppressing expression of TGF-$\beta$1, $\alpha$-SMA, Collagen I, and Collagen III, and reducing the synthesis and secretion of VEGFA and bFGF. These findings may be of great significance for the treatment of pathological scar tissues.

### Conflict of interest

The authors declare that they have no conflict of interest in this work.

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