Loureirin B inhibits fibroblast proliferation and extracellular matrix deposition in hypertrophic scar via TGF-β/Smad pathway

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Abstract: The ethanolic extract of Resina Draconis (RDEE) has been reported beneficial to normal wound healing yielding more regularly arranged collagen fibres. Loureirin B, a major component in RDEE, has been supposed to be effective on the prevention and treatment of pathological scars. To investigate the therapeutic effects of loureirin B on hypertrophic scar (HS), fibroblasts from human HS and normal skin (NS) were isolated. Results showed that loureirin B dose-dependently downregulated both mRNA and protein levels of type I collagen (ColI), type III collagen (ColIII) and α-smooth muscle actin (α-SMA) in HS fibroblasts. Loureirin B also suppressed fibroblast proliferative activity and redistributed cell cycle, but did not affect cell apoptosis. In vivo rabbit ear scar model, loureirin B significantly improved the arrangement and deposition of collagen fibres, decreased protein levels of Coll, ColIII and α-SMA and suppressed myofibroblast differentiation and scar proliferative activity. In NS fibroblasts, loureirin B effectively inhibited TGF-β1-induced upregulation of Coll, ColIII and α-SMA levels, myofibroblast differentiation and the activation of Smad2 and Smad3. Loureirin B also affected mRNA levels of major MMPs and TIMPs in TGF-β1-stimulated fibroblasts. Taken together, this study demonstrates that loureirin B could downregulate the expression of fibrosis-related molecules by regulating MMPs and TIMPs levels, inhibit scar fibroblast proliferation and suppress TGF-β1-induced fibrosis, during which TGF-β1/Smad2/3 pathway is likely involved. These findings suggest that loureirin B is a potential therapeutic compound for HS treatment.

Key words: extracellular matrix – fibroblasts – hypertrophic scar – loureirin B – TGF-β/Smad

Accepted for publication 5 February 2015

Introduction

There are numerous patients suffering from pathological scars as a result of surgeries, burns or traumas (1). Individuals with deep dermal injuries are at high risk for hypertrophic scarring, which is characterized as red, raised, rigid and sometimes itchy skins, leading to both physical and psychological disorders. Hypertrophic scar (HS) on exposed skin sites leads to appearance change, while that across the joints often leads to functional impairment. HS displays excessive production of various cytokines, abnormal proliferation of fibroblasts and disorder of extracellular matrix (ECM) (2,3). Although scientists have put lots of efforts into the research of HS, the precise mechanism on its formation, development and treatment is largely unknown, leading to the limitation of effective management and unsatisfactory outcomes.

When the skin is damaged, fibroblasts around the wound edge are activated and migrate into the wound region, where they synthesize and secrete collagens and other extracellular matrix proteins, and participate in the processes of wound healing and tissue repair. During scarless healing, fibroblasts are in a quiescent state with inactive proliferation and synthesis capabilities (1,3,4), redundant collagens fast degrade, myofibroblasts disappear via apoptosis (5), all these effectively prevent HS formation. During the pathogenesis of HS, however, fibroblasts are activated and trans-differentiate into myofibroblasts which express excessive α-SMA, the most widely used marker for myofibroblasts (6–8) and closely related to the texture and contracture of scars (9,10), and produce massive ECM proteins (2,9). Thus, fibroblasts have become the potential therapeutic target for antifibrosis treatment.

Resina Draconis, used as a kind of traditional Chinese medicine, is a kind of red resin extracted from the tree stem of Draea caena cochinchinensis. A recent study has showed that the ethanolic extract of Resina Draconis (RDEE) was beneficial to wound healing that results in more orderly arranged collagen fibres (11), indicating its potential effects on wound healing and inhibition of scar formation. However, RDEE is a complex mixture containing several unknown chemicals, and the mechanism of its pharmacological actions is unclear, leading to limited application and development. Interestingly, loureirin B (CID No. 189670), a known ingredient in RDEE, has been found to take effects on pain relief via modulating sodium currents in trigeminal ganglion neurons (12). Given the potential efficacy of RDEE, we are curious to know whether loureirin B would also work on treating HS or other fibrosis-related diseases. In this study, we have focused on investigating the pharmacological effects of loureirin B on HS formation.

Materials and methods

Ethics statement

All experimental procedures including the use of human and animal samples were conducted under the protocol (No: XIYYLL-2013190) reviewed and approved by Institutional Ethical Committee of the Fourth Military Medical University.
**Cell culture and treatment**

Hypertrophic scar and surrounding NS samples were collected from four patients (two males and two females, age range 18–44 years) between January 2013 and January 2014. Patients received no treatment before HS excision surgery performed in Department of Burns and Cutaneous Surgery, Xijing Hospital, Xi’an, China, and written consents were obtained from all patients or their legal guardians. Samples were first trimmed to remove excessive adipose tissues, rinsed with phosphate buffer solution (PBS) three times, and then minced and incubated in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, New York, USA) containing 0.1% collagenase type I (Sigma, St. Louis, Missouri, USA) at 37°C for 3 h to isolate fibroblasts. Obtained cells were then cultured in DMEM containing 10% foetal calf serum (Gibco), 1% penicillin and 1% streptomycin, at 37°C in a humidified atmosphere of 5% CO₂. Fibroblasts used in all experiments were from 3rd to 5th passage. Before any treatment, fibroblasts reaching 70–80% confluence were incubated in serum-depleted medium for another 12 h. Twenty 60-mm dishes of HS-derived fibroblasts were randomly divided into five groups (n = 4) with each group being treated with lourerine B at 0 μg/ml (control), 5 μg/ml, 10 μg/ml, 25 μg/ml or 50 μg/ml for 24 h. Sixteen 60-mm dishes of NS-derived fibroblasts were randomly divided into four groups (n = 4) with each being treated with DMSO (control), TGF-β1 (5 ng/ml), lourerine B (25 μg/ml) or TGF-β1 (5 ng/ml) + lourerine B (25 μg/ml) for 24 h. Lourerine B was obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China and reconstituted in DMSO at a final stock concentration of 20 mg/ml. Recombinant human TGF-β1 was purchased from PeproTech (London, UK) and dissolved in 10 molar citric acid (pH 3.0) yielding a final stock concentration of 10 ng/ml.

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted from human scar-derived fibroblasts using Total RNA Isolation Kit (Takara, Otsu, Shiga, Japan). The purity of obtained RNA was determined by A260/A280 ratio. 2.0 μg of RNA sample was reversely transcribed with PrimeScript™ RT Reagent Kit (Takara). The resulting cDNA was then amplified using SYBR® Premix Ex Taq™ Kit (Takara) with primer pairs specific to target genes as shown in Table S1, which were finally normalized against the transcriptional level of housekeeping gene GAPDH. The thermal cycle condition was optimized as follows: initial denaturation at 95°C for 30 s, denaturation at 95°C for 15 s, annealing at 64°C for 30 s, elongation at 72°C for 10 s for 40 cycles. At the end of the reaction, a melting curve analysis (65–105°C) was carried out to check for the presence of primer dimers. The relative concentration of target genes was determined by cycle threshold (Ct) which specifies fluorescence became detectable. The Ct value was used for kinetic analysis and was proportional to the initial number of target copies in the sample. The qRT-PCR data were exported and processed using the ΔΔCT method (13).

**Western blot**

Three gram of fresh tissue from rabbit ear was grinded into powder using liquid nitrogen with a cold sterile mortar and pestle. The tissue powder was then transferred into a precooled 5-ml sterile tube containing 3 ml of RIPA (supplemented with PMSF). The tube was then kept on ice for 30 min and centrifuged at 12000 rpm at 4°C for 15 min. The supernatant was collected and transferred into a 5-ml tube and then boiled in 100°C water for 10 min. BCA Kit was used for protein quantification. Forty microgram total protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk, membrane was incubated with specific primary antibody as shown in Table S2 at 4°C overnight. On the following day, membrane was washed three times with TBST (Tris-buffered saline with 0.1% Tween-20) and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at 37°C for 1 h. Immunoreactive traces were detected with Chemiluminescent HRP Substrate ECL kit (Millipore). The density of each protein band on the membrane was scanned by an Alpha Imager scanner (Alpha Innotech, San Jose, California, USA) and analysed by AlphaEase FC image processing software (Alpha Innotech). β-actin was used as equal protein loading control.

**WST-1 assay**

The viability of HS-derived fibroblasts was detected by WST-1 Cell Proliferation Assay Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Fibroblasts were seeded into 96-well plates at a density of 3.0 × 10⁴ cells per well and treated with vehicle control (0.125% DMSO) or 25 μg/ml lourerine B for 24 h, followed by incubation with WST-1 reagent for another 4 h. The amount of newly formed formazan dye was quantitated with a scanning multiwell spectrophotometer (ELISA reader) at 450 nm. The measured absorbance directly correlates with the number of viable cells. This experiment was repeated four times using cells from four different patients.

**Flow cytometry analysis**

Cell apoptosis was detected using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instruction. HS-derived fibroblasts were treated with vehicle control (0.125% DMSO) or 25 μg/ml lourerine B for 48 h. Cells were then digested with 0.25% trypsin, washed twice with cold PBS, resuspended in binding buffer and then incubated with Annexin V-FITC and PI for 15 min at room temperature in dark. Samples were then analysed by FACS Calibur (BD Bioscience). The percentage of stained cells was determined using BD FACS Diva software. This experiment was repeated four times using cells from four different patients.

**Immunocytofluorescence**

Normal skin-derived fibroblasts were seeded into 12-well plates at a density of 1.0 × 10⁴ cells per well for cell climbing. Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, blocked with 1% bovine serum albumin (BSA, Invitrogen, Grand Island, New York, USA) at 37°C for 30 min and then incubated with rabbit anti-α-SMA primary antibody (Table S2) at room temperature overnight. On the next day, cells were incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody (Cwbio, Beijing, China) at 37°C for 1 h. DAPI was used for nuclear staining. Images were analysed by Image-Pro Plus 6.0 system (Media Cybernetics, Silver Spring, MD, USA).

**Rabbit ear scar model**

The rabbit ear scar model was established as described (12,14). For short, 10 adult New Zealand white male rabbits (2.0–2.5 kg b.w./each), provided by the Fourth Military Medical University Animal Center, were acclimated and housed under the standard 12-h light: 12-h dark cycle with free access of water and SPF basal diet. Rabbit
was first anaesthetized with 1% pentobarbital (1.5 mg/kg b.w.), and then, a dermal punch biopsy (10 × 4 mm) was created down to bare cartilage on the ventral surface of each ear to outline a full-thickness wound. Four punch wounds were made on each ear of the eight rabbits. A dissecting microscope was used to ensure the complete removal of epidermis, dermis and perichondrium in each wound. Forty-eight hours after surgery, wounded rabbits were randomly divided into two groups with each being subcutaneously injected with DMSO solution (0.125% in PBS, 0.25 ml/kg b.w.) on the left ear or loureirin B solution (25 μg/ml in PBS, 0.25 ml/kg b.w.) on the right ear once every other day for total six times. Two rabbits were used for pilot experiment, four rabbits were sacrificed 14 days after injury (n = 4), and the rest four were sacrificed 28 days after injury (n = 4). Two of the four scar tissues on the same ear were processed for Western blot, and the other two were used for Masson staining.

**Masson staining**

After ear wounds healed and scars formed, rabbits were sacrificed and scar tissues in situ were excised for Masson staining using Masson Trichrome Staining Kit (Nanjing Biotech, Nanjing, Jiangsu, China). Paraaffin-embedded tissue sections from day-14 and day-28 scars were examined for the expression and arrangement of collagen fibres under a FSX100 microscope (OLYMPUS, Shinjuku-ku, Tokyo, Japan), and images were recorded digitally onto a computer and analysed with Image-Pro Plus 6.0 system. The relative area of collagen fibres below epidermis and above fascia was defined by the ‘irregular AOI’ tool and then measured and recorded automatically by ‘CountsSize’ tool.

**Immunohistofluorescence**

Rabbit ear scar tissues were excised, embedded in paraaffin and cut into 4-μm-thick sections for immunofluorescent staining. Sections were subject to deparaaffinization, dehydration and antigen retrieval as described (15). Sections were then incubated with anti-Ki67 or anti-α-SMA primary antibody (Table S2) at room temperature overnight. On the next day, sections were incubated with Cy3-conjugated goat anti-rabbit IgG secondary antibody at 37°C for 1 h. DAPI was used for nuclear staining. Images were analysed by Image-Pro Plus 6.0 system.

**Statistical analysis**

Results were presented as mean ± SEM. Data were analysed for significance by analysis of variance (ANOVA) using a SPSS 17.0 software (Chicago, IL, USA). P < 0.05 was considered statistically significant.

**Results**

**Loureirin B dose-dependently downregulates the mRNA and protein levels of several fibrosis-related molecules**

It is well known that the expression of fibrosis-related molecules such as ColI, ColIII and α-SMA in HS is upregulated compared to that in NS (3–5,9). To demonstrate the suppressive effect of loureirin B (chemical structure as shown in Fig. 1a) on HS, HS-derived fibroblasts were isolated and treated with loureirin B at different concentrations of 0, 5, 10, 25 and 50 μg/ml to examine whether it would affect the expression of fibrosis-related molecules. qRT-PCR results showed that the mRNA levels of ColI, ColIII and α-SMA dose-dependently decreased by loureirin B treatment (Fig. 1b). Loureirin B remarkably downregulated Coll mRNA level beginning at the concentration of 10 μg/ml and reduced both ColIII and α-SMA mRNA levels from the concentra-

![Figure 1](image)
as earlier described (12,14). Loureirin B or its corresponding vehicle DMSO control was subcutaneously injected into rabbit ear scars 48 h postinjury, and this was repeated on every other day for total six times. The wound closed on days 6–7 postinjury, and the scar developed after that. Rabbit ear scars on days 14 and 28 were excised and subjected to the following analysis. Masson staining results showed that collagen fibres became thinner and more orderly arranged in loureirin B-treated group than those in control (Fig. 3a). The relative area of collagen fibres was further quantified in Fig. 3b, and results showed the reduced relative collagen area in loureirin B-treated group compared to that in TGF-β1 alone (5 ng/ml) + loureirin B co-treatment group compared to that in TGF-β1 alone group.

The immunohistofluorescence staining of α-SMA on loureirin B treated rabbit ear scar was also remarkably attenuated (Fig. 3e).

Loureirin B suppresses the upregulated level of fibrosis-related molecules induced by TGF-β1 in NS-derived fibroblasts

Figure 2. Effects of loureirin B on scar fibroblast proliferation and apoptosis in vitro cell culture and in vivo rabbit ear scar model. (a) Assessment of the proliferative activity of HS fibroblasts following 25 μg/ml loureirin B treatment by WST-1 assay. OD – optical density. (b) Flow cytometry analysis showing the effect of loureirin B on cell cycle distribution. Cell numbers at G1, G2 and S phases were counted and the percentage was calculated. (c) Assessment of the apoptosis rate of HS fibroblasts following loureirin B treatment by flow cytometry. (d) Immunohistofluorescence staining of Ki67 (red) on rabbit ear scar paraffin section on day 14 (D14) postinjury to examine loureirin B’s effect on scar proliferative activity in vivo. DAPI was used for nuclear counterstaining (blue). Scale bar: 100 μm. Results were from four independent experiments using cell/tissues from four different individuals. Each bar was a mean ± SEM of n = 4. *P < 0.05, compared to vehicle control (0.125% DMSO).

Activation of fibroblasts by TGF-β1 is an essential step during HS formation and development (16); thus, it is of great importance to investigate whether loureirin B has any inhibitory effect on TGF-β1-induced fibroblast activation. NS fibroblasts were treated with vehicle control (0.125% DMSO), TGF-β1 alone (5 ng/ml), loureirin B alone (25 μg/ml) or TGF-β1 (5 ng/ml) + loureirin B (25 μg/ml) for 24 h. Results showed that both the elevated mRNA (Fig. 4a) and protein levels (Fig. 4b-d) of Coll, ColIII and α-SMA induced by TGF-β1 were significantly repressed in TGF-β1 + loureirin B co-treatment group compared to that in TGF-β1 alone group. Immunocytofluorescence staining of α-SMA was performed to visualize the effect of loureirin B on TGF-β1-induced transition of fibroblasts into myofibroblasts. As shown in Fig. 4d, TGF-β1 significantly increased the expression of α-SMA in NS fibroblasts, indicating an activation of fibroblasts into myofibroblasts, while loureirin B remarkably suppressed TGF-β1-induced upregulation of α-SMA.

Loureirin B affects the expression of MMPs and TIMPs, and the phosphorylation status of Smad2/3 in TGF-β1-stimulated NS fibroblasts

In addition to collagen deposition in fibrotic wounds or its over-expression in fibroblasts in response to TGF-β1, the degradation of ECM mediated by MMPs and TIMPs might be disturbed. Thus, we further investigated the effect of loureirin B on the expression of selected MMPs and TIMPs in TGF-β1-stimulated fibroblasts. Results showed that loureirin B significantly upregulated mRNA
Previous research has shown that Smad2 and Smad3 are involved in the differentiation and proliferation of fibroblasts and play a key role in the progression of scarring (4,17). Thus, it is important to clarify whether the downregulation of collagens and α-SMA by loureirin B is mediated via TGF-β1/Smad pathway. Western blot analysis showed that loureirin B significantly suppressed the phosphorylation of Smad2 and Smad3, while did not affect their corresponding total protein contents in TGF-β1-stimulated fibroblasts (Fig. 4f–g).

Discussion

Pathological scars are inevitable results from abnormal wound healing after surgeries, burns or traumas. There is currently no single satisfactory therapeutic approach available for HS treatment. Pressure garment therapy (PGT) for both prophylaxis and treatment of HS has been widely used since decades ago while the mechanism still needs further investigation (18,19). A meta-analysis published in 2009 showed that PGT did not alter global scar scores (20). The laser therapy as well as antiscar drugs also leads to high recurrence and unacceptable side effects (21–24). Silicone gel sheet (SGS) is also widely used for HS treatment. SGS is a kind of organic polymer material with flat surface and has no pores, which enables the maintenance of scar hydration status by decreasing the water vapour transmission rate to almost half that of NS and thus suppresses scar formation (23,24). However, a recent review on silicone therapy for HS suggested that there were several defects on experiment design in some published studies on silicone, like the defect in blinding methods and unclear randomization (25,26). Thus, the precise mechanism of silicone therapy for HS is far from fully understood.

Moreover, the combination therapy of surgery, PGT, laser and medicine is also not successful for complete cure of HS.

Fibroblasts, the key cells participating in scar formation, are usually in a quiescent status in NS while become activated after skin injury (9). The aberrant proliferation of fibroblasts and excessive deposition of ECM often result in scarring (23). Compared to the scarless healing, the persistent activation of fibroblasts and increasing deposition of ECM were usually observed in pathological scars (3,27). Collagen is one of the key components in ECM. Coll and ColIII are the two major collagen types in human skin. It is known that deep dermal fibroblasts synthesized more Coll and α-SMA than superficial ones did (3,27). The continuous expression of Coll and ColIII, and the imbalance of Coll/ColIII ratio are important histological features that distinguish HS from NS (23,28,29). In addition, activated fibroblasts overexpress α-SMA, which is a well-known marker for myofibroblasts and promotes scar contraction (30). Therefore, the regulation of fibroblast proliferation/activation and ECM protein synthesis, such as α-SMA and collagens, respectively, is the main focus to study the prevention and treatment of HS.

In the present study, effects of loureirin B on HS-derived fibroblasts were first investigated. Results showed that loureirin B significantly suppressed both the mRNA and protein levels of Coll, CollIII and α-SMA, suggesting its inhibitory effects on the overexpression of ECM in scar tissues (Fig. 1). WST-1 assay showed that loureirin B inhibited the proliferative activity of HS fibroblasts (Fig. 2a). Flow cytometry analysis showed that loureirin B increased the percentage of cells in G1 and G2 phases, while decreased that in S phase (Fig. 2b). These results indicate that loureirin B might inhibit HS formation by reducing ECM expres-
sion and deposition, and suppressing fibroblast proliferation via redistributing cell cycle. In addition, loureirin B did not affect fibroblast apoptosis (Fig. 2c), suggesting that the possible inhibitory effects of loureirin B on HS are unrelated with cell apoptosis.

A rabbit ear scar model was established and used to examine the effects of loureirin B in vivo. Results showed that the expression of collagens and α-SMA in loureirin B-treated scars was lower (Fig. 3c–d) and the arrangement of collagen fibres was more regular (Fig. 3a–b) than those in vehicle control-treated scars. Also, loureirin B inhibited scar proliferative activity (Fig. 2d) and myofibroblast differentiation (Fig. 3e). These in vivo studies demonstrate that loureirin B could suppress scar formation by inhibiting scar proliferation, fibroblast trans-differentiation, and ECM synthesis and deposition.

TGF-β is a superfamily of cytokines that is closely related to wound healing and tissue repair and regulates cell proliferation and ECM synthesis (31). TGF-β1 is found to be a positive regulator that stimulates ECM expression and deposition, and thus closely related to fibrotic diseases including HS (9,16,32,33). During HS formation, TGF-β1 activates fibroblasts directly, promotes the trans-differentiation of fibroblasts into myofibroblasts and induces ECM synthesis and deposition (7,30,34,35). Considering the crucial role of TGF-β1 in scar formation, we further examined the effects of loureirin B in TGF-β1-stimulated fibroblasts. Results showed that TGF-β1-upregulated the expression of Col1 and CollIII and α-SMA in NS fibroblasts, while loureirin B reversely suppressed this induction (Fig. 4a–c). Loureirin B also significantly inhibited TGF-β1-induced myofibroblast differentiation (Fig. 4d).

In addition, the downregulated MMP1 (also called collagenase-1) or the upregulated TIMP1 (the inhibitor of MMP1) mRNA level induced by TGF-β1 was remarkably increased or decreased, respectively, after loureirin B treatment (Fig. 4e). Loureirin B also affected the mRNA levels of several other MMPs, such as MMP2, MMP3, MMP9 and MMP13 (Fig. 4e). Above results suggest that loureirin B inhibits HS formation through ECM degradation mediated by MMPs and TIMPs.

TGF-β1 has been noted to exert biological effects through Smad-dependent and independent signalling pathway in several fibrotic diseases (4). Our results showed that loureirin B decreased the phosphorylation status of Smad2 and Smad3 and thus suppressed their activation, indicating that the inhibitory effects of loureirin B on TGF-β1-induced HS might be through Smad-dependent pathway.

Taken together, our present study demonstrates that loureirin B, a natural pure compound from Resina Draconis, effectively downregulates ECM expression and deposition, inhibits trans-differentiation of fibroblasts into myofibroblasts, as well as improves collagen arrangement. Furthermore, this study suggests that loureirin B suppresses TGF-β1-induced HS via a Smad-dependent pathway. Thus, this preliminary research on loureirin B reveals its potential therapeutic value for HS treatment; however, the precise underlying mechanism needs further elucidation.

Acknowledgements

Bai X and He T designed and performed the research, analysed the data and drafted the paper; Liu J and Wang Y provided HS and NS samples and analysed the data; Fan L, Tao K, Shi J and Tang C contributed essential reagents and tools; Su L and Hu D designed the research study; Su L rewrote and revised the paper and approved the submitted and final versions; Hu D, Liu J and Wang Y provided the funding support.

Funding source

This work was supported by the National Natural Science Foundation of China (Grant Number: 81372069, 81301632 and 81201470).

Conflict of interests

The authors have declared no conflicting interests.

References


Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Figure S1. Evaluation of the effects of vehicle control DMSO at its highest concentration of 0.25% used in this study. (a) The effect of 0.25% DMSO on Coll, CollIII and α-SMA levels in HS fibroblasts by Western blot. (b) Histogram summarizing results in (a). (c) Assessment of the effect of 0.25% DMSO on fibroblast proliferative activity by WST-1 assay. OD’optical density. (d) Assessment of the effect of 0.25% DMSO on the apoptosis rate of HS fibroblasts by flow cytometry. (Blank ctrl = Blank control).

table S1. Primer pairs used for qRT-PCR in this study.

table S2. Antibodies used in this study.