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Original Article

Role of thrombospondin-4 in fibroblasts from normal skin and hypertrophic scars

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Abstract: Object: This study aims to investigate the functions of thrombospondin-4 (TSP4) in fibroblasts from normal skin and hypertrophic scars. Methods: Human normal skin-derived and hypertrophic scar-derived fibroblasts were cultured, and TSP4 gene expression was upregulated and downregulated in fibroblasts through viral transfection. TGF-β1, α-SMA, and BCL-2 gene and protein expression levels were measured by Western blot and quantitative real-time PCR assays. The resulting data were statistically analyzed. Results: TGF-β1, α-SMA, and BCL-2 expression levels increased in both normal skin-derived and hypertrophic scar-derived fibroblasts after TSP4 upregulation ($P < 0.05$). In contrast, these levels decreased in hypertrophic scar-derived fibroblasts after TSP4 downregulation ($P < 0.05$), and the difference was statistically significant. Conclusions: These results indicate that TSP4 participates in the physiological processes of hypertrophic scars. Specifically, TSP4 can promote proliferation and inhibit apoptosis in fibroblasts. In addition, TSP4 can also facilitate transformation of fibroblasts to myofibroblasts and improve the contractibility of scar tissues.

Keywords: Hypertrophic scar, normal skin, fibroblast, extra-cellular matrix, thrombospondin-4

Introduction

Hypertrophic scars are a common pathologic wound healing state after repair, which usually manifests as dark red or raised tough tissue blocks near lesions. Superficial hypertrophic scars on the body may present symptoms including pain and itching, and these may also affect appearance. Furthermore, hypertrophic scars that form in organs (e.g. lungs, liver and kidneys) and joints may seriously affect the physiological functions of these organs and the quality of life of patients. Numerous researchers are dedicated to the study of wound healing, and have made great achievements. However, the exact pathogenesis of hypertrophic scars remains unclear, making its prevention and treatment a challenging problem that remains to be solved [1].

Hypertrophic scars are a result of pathological wound healing, and present histopathological changes such as inflammatory infiltration, excessive fibroblast proliferation, and abnormal extracellular matrix (ECM) accumulation [2]. Differences between fibroblasts from normal skin and hypertrophic scars, such as differences in gene and protein expression [3], have been studied. However, the exact mechanism that leads to the formation of scars remains to be further investigated.

Thrombospondins (TSPs), which was originally discovered in the ECM by Baenziger et al., are secreted by platelet α-particles and are involved in regulation of platelet adhesion and aggregation [4, 5]. The TSP family consists of five ECM proteins, which are divided into two subgroups: subgroup A and subgroup B. Subgroup A is a trimer that consists of TSP1 and TSP2, whereas subgroup B is a pentamer that consists of TSP3, TSP4, and TSP5 [6]. TSPs have been shown to participate in cell-cell and cell-ECM interactions, as well as in the formation and degradation of the ECM (e.g. collagen and laminin). Furthermore, TSPs also regulate inflammatory responses after vascular injury, and are related to wound healing, angiogenesis, connective tissue arrangement, and neuronal synapse formation [7, 8].

Table 1. Specimen source information

No.	Type of skin	Gender	Age	Specimen site
S1	Hypertrophic scar	F	28	Abdomen
S2	Hypertrophic scar	M	17	Neck
S3	Hypertrophic scar	F	36	Abdomen
N1	Normal skin	F	47	Eye pouch
N2	Normal skin	F	25	Upper eyelid
N3	Normal skin	M	28	Upper eyelid

In recent years, high-throughput sequencing has been used for comparative analyses of hypertrophic scar tissues and normal skin tissues [3]. Specifically, Paddock et al. found that TSP4 expression is upregulated in hypertrophic scar tissues [9]. TSP4, an ECM glycoprotein, has a C terminus that can interact with collagen types I, II, III, and V, laminin-1, and fibronectin and this interaction takes place during the synthesis of the ECM. TSP4 has primarily been studied in neuromuscular and cardiovascular systems. For example, in 1998, Urry et al. [10] first discovered that TSP4 is a component of the sarcomere and muscle in African clawed frog embryos. Arber et al. [11] found that TSP4 expression is highly induced in neuromuscular junctions after injury, promoting neurite outgrowth in the retinal ganglion. Moreover, Frolova et al. [12] reported that cardiac volume and fibrotic mass are significantly increased in TSP4 knockdown mice with artificial heart failure, mainly due to the considerable deposition of ECM proteins, indicating that TSP4 may regulate the function of cardiac fibroblasts to participate in the regulation of cardiac fibrosis and the remodeling process in the setting of cardiac failure.

Our earlier study showed that hypertrophic scar-derived fibroblasts exhibit significantly higher TSP4 gene and protein expression than normal skin-derived fibroblasts. In order to further investigate the role of TSP4 in hypertrophic scar-derived fibroblasts and further explore the effect of TSP4 on the function of hypertrophic scar-derived fibroblasts in the present study, fibroblasts with upregulated and downregulated TSP4 were obtained by viral transfection.

Materials and methods

*Specimen source (**Table 1**)*

Specimens were obtained from six surgical patients in our department. Specifically, both the hypertrophic scar and normal skin groups consisted of discarded surgical tissues. The hypertrophic scar group comprised three patients with hypertrophic scarring. Among these patients, one patient was male and two patients were female and the age of these patients ranged from 17-36 years, with a mean age of 27 years. Scar tissue was taken from the neck and abdomen, and the causes of injury included burns and surgical incisions. The normal skin group included three patients with normal skin. Among these patients, one patient was male and two patients were female and patient ages ranged from 25-47 years, with a mean age of 33.3 years. Normal tissue was obtained from the eyelids and eye bags under the eyes. All patients provided a signed informed consent prior to providing the specimens.

Culture and passage of human skin fibroblasts

After the fat and epidermis were removed, fresh skin tissue specimens were washed three times with phosphate-buffered saline (PBS; Hyclone, Logan, UT, USA) containing 10% double antibiotics (penicillin-streptomycin mixture, Solarbio, Beijing, China). Subsequently, the specimens were cut into 0.25 × 0.25 cm tissue blocks using a sterile razor blade. The cut surface of the tissue block was attached to the bottom of a sterile 100-mm Petri dish, and incubated in an incubator for 1.5-2 hours. After the tissue blocks were fixed in Petri dishes, complete medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, Hyclone) was added and the tissue blocks were cultured in an incubator (5% CO_2 at 37°C). The culture was observed daily. Four to five days later, the tissue blocks and the old medium were discarded when a large number of fusiform fibroblasts grew around the tissue blocks. Then, fresh medium was added at 10 ml per Petri dish. Cell growth was dynamically observed, and the medium was changed every 3-4 days. When cells grew to 90% confluence in the Petri dishes, the old medium was aspirated and cells were washed once with 10 ml of PBS. Subsequently, 2 ml of trypsin was added for digestion, and cells were cultured in an incubator for 1-2 minutes. The digestion was terminated by adding 4-5 ml of medium containing serum, and adherent cells were mixed by gentle and repeated pipetting. Then, the cell suspension was centrifuged for five minutes (1,000 rpm). After the supernatant was decanted, 1 ml of culture medium was added and the cells

Table 2. TSP4, α-SMA, TGF-β1, and GAPDH primer sequences

	Sequence (5' to 3')
TSP4	Length 148 bp
	Forward Primer: 5'-TTAAATAACGACCAGAAAGACACC-3'
	Reverse Primer: 5'-CCACACCATCATCCTTG-3'
α-SMA	Length 151 bp
	Forward Primer: 5'-AGCGTGGCTATTCCTTCGTT-3'
	Reverse Primer: 5'-CCATCAGGCAACTCGTAACTC-3'
TGF-β1	Length 251 bp
	Forward Primer: 5'-GAAACCCACAACGAAATCTATGAC-3'
	Reverse Primer: 5'-AAAGATAACCACTCTGGCGA-3'
GADPH	Length 191 bp
	Forward Primer: 5'-GAAGGTGAAGGTCGGAGT-3'
	Reverse Primer: 5'-CCATCAGGCAACTCGTAACTC-3'

were completely separated by pipetting. Equal amounts of cells were vertically dropped into four Petri dishes, and complete medium was added before culturing the cells in an incubator at 37°C with 5% CO_2. The cultures were dynamically observed, and the medium was changed, as described above.

Recombinant adenovirus infection of fibroblasts

TSP4 expression vector, interference vector, and the corresponding control recombinant adenovirus were constructed by Neuron Biotech Co. Ltd. (Shanghai, China). The following primers were used: TSP4, 5'-GATGGCTATATCTGTGGAAAGG-3'; GFP-R, TCAGCTTGCCGTAGGTGGCAT; interference vector, 5'-CCTATTTCCCATGATTCCTTCATA-3'; empty vector control, 5'-GTAATACGGTTATCCACGCG-3'; and negative control (NC), 5'-TTCTCCGAACGTGTCACGT-3'. After counting the cells, fibroblasts (1×10^6 cells per Petri dish) were added in serum-free DMEM medium containing 5 μg/ml polybrene and 5×10^7 virus particles (MOI = 50). After four hours of incubation in an incubator, the original medium was removed and 5 ml of DMEM supplemented with 10% fetal bovine serum was added to each Petri dish. Thereafter, cells were observed under an inverted fluorescence microscope every 48 hours. Proteins and nucleic acids were extracted for further analysis.

Protein extraction and western blotting

TSP4 protein expression in normal skin-derived and hypertrophic scar-derived fibroblasts was analyzed using Western blot assay. Briefly, cells were trypsinized, suspended and centrifuged at 1,000 rpm for five minutes. After the supernatant was discarded, 0.5 ml of lysis buffer was added and the cells were mixed, resuspended by vortexing, and allowed to stand at 4°C for two minutes. Subsequently, 1 ml of extraction buffer was added. Then, the cells were mixed by vortexing and allowed to stand at 4°C for 10 minutes. The solution was separated into two phases through an intermediate protein membrane *via* centrifugation at 10,000 g for 10 minutes at 4°C. The upper and lower phases were aspirated, and the intermediate protein floc was retained. The precipitate was washed with 1 ml of anhydrous ethanol and centrifuged at 10,000 g for three minutes at 4°C, allowing the protein to precipitate at the bottom of the tube. After the liquid was removed from the tube, the protein precipitate was air dried at room temperature, dissolved in 80 μl of 2% sodium dodecyl sulfate, and stored at -20°C. The protein concentration was measured using a BCA Protein Assay Kit (Applygen, Beijing, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted using an 8% separation gel and a 4% stacking gel. Then, the proteins were transferred to membranes and incubated with primary antibodies against TSP4 (1:400, Abcam), α-SMA (1:200, Abcam) and BCL-2 (1:2,000, Abcam) to detect TSP4 (molecular weight, approximately 135 kDa), α-SMA (molecular weight, approximately 42 kDa) and BCL-2 (molecular weight, ~25 kDa). The membranes were also incubated with a murine monoclonal antibody against GAPDH (1:5,000; CWBIO, Beijing, China) to detect GAPDH (molecular weight, approximately 43 kDa), which served as an internal reference. The reactions were incubated on a shaker at 4°C overnight. After being washed, TSP4, α-SMA, BCL-2, and GAPDH were detected by incubating the membranes with horseradish peroxidase-labeled goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies (1:5,000; ZSBIO, Beijing, China), respectively, on a shaker at room temperature for two hours. After membrane washing, EZ-ECL solution A and solution B were mixed in equal volumes, and spread onto the membranes. The membranes were placed in a darkroom for two minutes, and images of the protein bands were

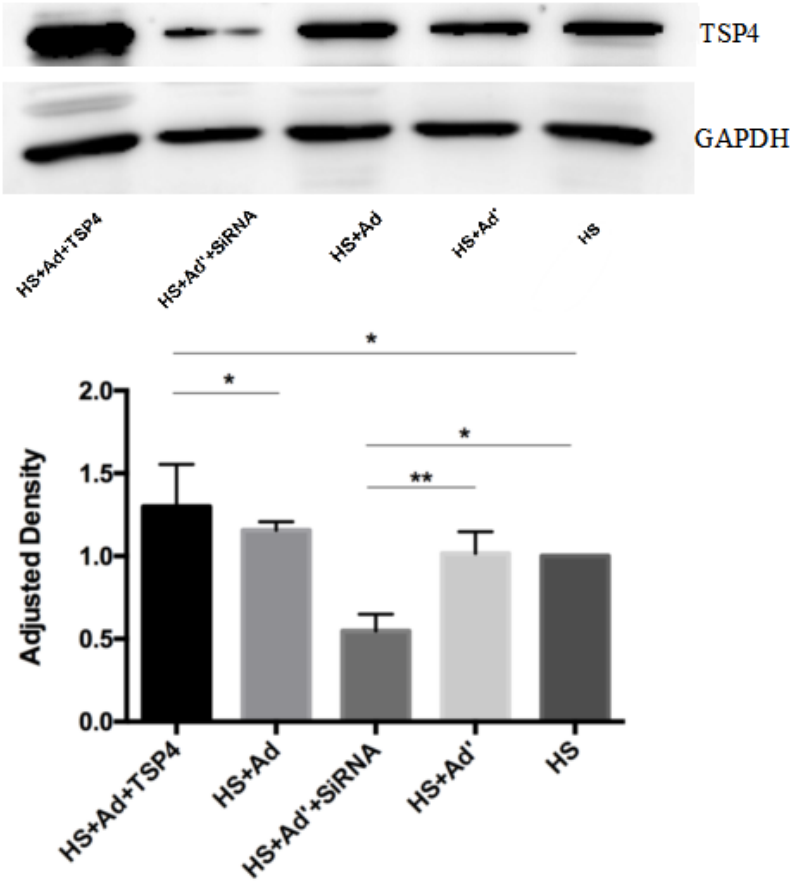

Figure 1. TSP4 protein expression levels in fibroblasts from hypertrophic scars after TSP4 gene up- and downregulation. The following groups were examined: HS + Ad + TSP4 (hypertrophic scar-derived fibroblasts + TSP4), HS + Ad (hypertrophic scar-derived fibroblasts + adenovirus-void vector 1), HS + Ad' + siRNA (hypertrophic scar-derived fibroblasts + siRNA), HS + Ad' (hypertrophic scar-derived fibroblasts + adenovirus-void vector 2) and HS (untreated hypertrophic scar-derived fibroblasts), *P < 0.05 between the two groups, **P < 0.01 between the two groups.

acquired using ImageQuant LAS 4010 (GE Healthcare, Piscataway, NJ, USA).

Quantitative real-time PCR

Total RNA was extracted from fibroblasts using TRIzol (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions, and the RNA concentration and purity were estimated using a UV spectrophotometer. cDNA was synthesized by reverse transcription using a reverse transcriptase system (Promega, Fitchburg, WI, USA). The PCR was then prepared using Fast SYBR Green Master Mix (ABI, Foster City, CA, USA) according to manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was conducted on an ABI 7500 Real Time quantitative PCR System to measure TSP4, α-SMA and TGF-β1 gene amplification in normal skin-derived and hypertrophic scar-derived fibroblasts using GAPDH as an internal reference gene. The forward and reverse primer sequences are listed in **Table 2**. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

A gray-scale analysis of Western blot bands was conducted using Image J (National Institutes of Health, Bethesda, MD, USA). Data are presented as mean ± standard error of the mean, and a statistical analysis was performed using SPSS17.0 software (SPSS Inc., Chicago, IL, USA). The differences between groups were compared using Student-Newman-Keuls (SNK) test or one-way analysis of variance (ANOVA). A *P*-value < 0.05 was considered statistically significant.

Results

TSP4 protein expression was significantly higher in TSP4-overexpressing fibroblasts and significantly lower in TSP-knockdown fibroblasts from hypertrophic scars

Western blotting revealed that TSP4 protein levels were significantly higher in fibroblasts from hypertrophic scars treated with replication-defective adenovirus expression vector containing the human TSP4 gene (TSP4 upregulation). After transfection with siRNA targeting TSP4, Western blotting revealed that the protein levels of TSP4 were markedly downregulated (**Figure 1**), suggesting the successful knockdown of TSP4 in these fibroblasts. Moreover, TSP4 protein expression did not significantly differ between these two negative control groups and the untreated hypertrophic scar fibroblasts.

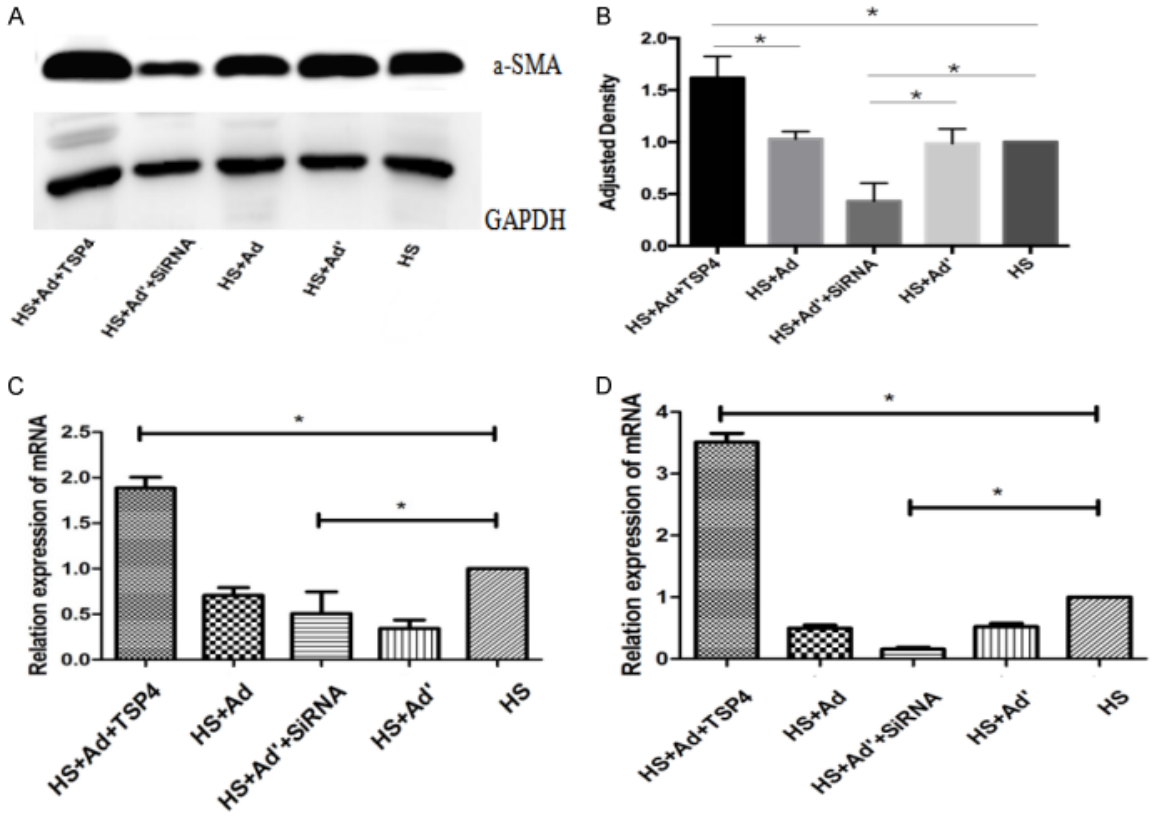

Figure 2. α-SMA and TGF-β1 expression in hypertrophic scar-derived fibroblasts after TSP4 gene overexpression and knockdown. Total RNA and protein were isolated from hypertrophic scar-derived fibroblasts. qRT-PCR was performed to compare the α-SMA and TGF-β1 mRNA expression levels between treated groups, and Western blotting was performed to compare the α-SMA protein expression levels between treated groups. A and B. α-SMA protein expression as demonstrated by Western blotting. C. α-SMA mRNA expression as demonstrated by qRT-PCR. D. TGF-β1 mRNA expression as demonstrated by qRT-PCR. The following groups were examined: HS + Ad + TSP4 (hypertrophic scar-derived fibroblasts + TSP4), HS + Ad (hypertrophic scar-derived fibroblasts + adenovirus-void vector 1), HS + Ad' + siRNA (hypertrophic scar-derived fibroblasts + siRNA), HS + Ad' (hypertrophic scar-derived fibroblasts + adenovirus-void vector 2) and HS (untreated hypertrophic scar-derived fibroblasts), *$P < 0.05$ between the two groups.

Effect of TSP4 overexpression and knockdown on fibroblast function

The effects TSP4 overexpression, siRNA knockdown, and transfection with an empty control virus on hypertrophic scar-derived or normal dermal fibroblasts were examined. Hypertrophic scar-derived dermal fibroblasts constitutively express α-SMA and TGF-β1 (**Figure 2**). Furthermore, qRT-PCR revealed that upregulation of TSP4 increased the expression levels of α-SMA and TGF-β1 mRNA, whereas the downregulation of TSP4 decreased the expression levels of α-SMA and TGF-β1 mRNA (**Figure 2C** and **2D**). Moreover, Western blotting revealed similar results for α-SMA (**Figure 2A** and **2B**).

In contrast, normal skin-derived fibroblasts did not express α-SMA or TGF-β1. After transduction with a TSP4 expression vector, α-SMA and TGF-β1 mRNA levels all increased (**Figure 3A** and **3B**).

TSP4 overexpression inhibited fibroblast apoptosis

The expression levels of BCL-2 protein were also measured by Western blotting (**Figure 4**), which revealed that BCL-2 was higher in the TSP4 upregulation group than in the TSP4 downregulation group. This suggests that upregulating TSP4 gene expression activates the proliferation of fibroblasts and inhibits fibroblast apoptosis.

Discussion

A recent study has shown that the TSP family mainly modulates cell-cell and cell-ECM interactions, regulates the structural support role of

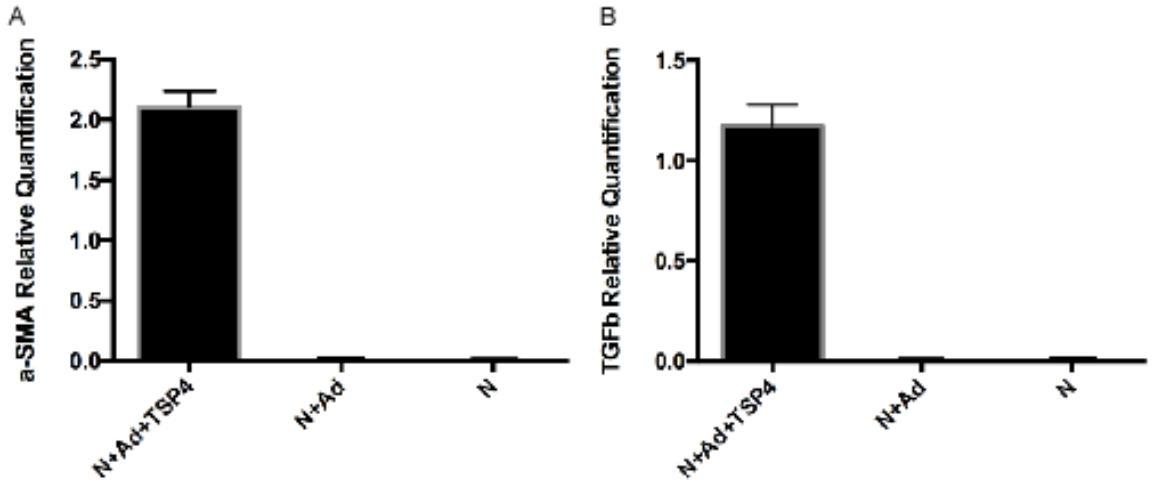

Figure 3. α-SMA and TGF-β1 expression by normal skin-derived fibroblasts after TSP4 gene overexpression. Total RNA was isolated from hypertrophic scar-derived fibroblasts, and qRT-PCR was performed to compare the α-SMA (A) and TGF-β1 (B) mRNA expression levels between treated groups. The following groups were examined: N + Ad + TSP4 (normal skin-derived fibroblasts + TSP4), N + Ad (normal skin-derived fibroblasts + adenovirus-void vector) and N (untreated normal skin-derived fibroblasts).

ECM proteins such as collagen and laminin, and participates in a series of biological activities including wound healing, angiogenesis, connective tissue arrangement, and neuronal synapse formation [7].

Existing research on TSP family members has shown that TSP1 is highly expressed in the skin of patients with dermal fibrotic disorders such as keloids and scleroderma [13, 14]. Additionally, wound healing is markedly slowed in TSP1 knockdown mice, and TSP1 can affect the role of TGF-β1 [15]. Moreover, expression of TSP2, which is mainly produced by fibroblasts and smooth muscle cells, also affects wound healing. In addition, scientists have speculated that TSP2 can regulate metalloproteinase activity in the ECM, thereby affecting ECM remodeling [15]. Lastly, TSP family members exhibit 53-82% homology [16], suggesting that TSP4 may also play a role in wound healing and scar hyperplasia.

Earlier high-throughput sequencing results revealed that the TSP4 gene is upregulated in hypertrophic scar tissues [3]. Our previous experiments demonstrated that TSP4 protein is ubiquitous in the ECM of hypertrophic scars, and that fibroblasts from hypertrophic scars express significantly more TSP4 at the gene and protein levels than fibroblasts from normal skin. These findings strongly suggest that TSP4 plays a role in the formation of hypertrophic scars.

Furthermore, our experimental data reveal that overexpression of TSP4 can increase α-SMA expression in fibroblasts from normal skin and hypertrophic scars (**Figures 2A**, **2B** and **3A**). Rajkumar et al. [17] demonstrated that normal skin-derived fibroblasts do not express α-SMA protein. Since α-SMA is a characteristic cell marker of myofibroblasts [18], the above result suggests that TSP4 induces the transformation of fibroblasts into myofibroblasts.

Moreover, our results reveal that TGF-β1 mRNA levels increased in fibroblasts from normal skin and hypertrophic scars after overexpression of the TSP4 gene, compared with the control group (**Figures 2C** and **3B**). Conversely, expression of TGF-β1 decreased after TSP4 downregulation, suggesting that TSP4 can promote TGF-β production in human fibroblasts. TGF-β1 is considered an important paracrine/autocrine growth factor that leads to hypertrophic scars. Additionally, the TGF-β1 signaling pathway has been shown to initiate and stimulate a differentiation cascade of fibroblasts toward myofibroblasts to subsequently facilitate the production of α-SMA and ECM (collagen type I), thereby increasing tissue contractility [1]. Therefore, we assume that TSP4 induces transformation of fibroblasts to myofibroblasts by affecting the TGF-β1 signaling pathway in human hypertrophic scars.

TSP1 has been shown to activate TGF-β1. However, TSP4 lacks domains analogous to the

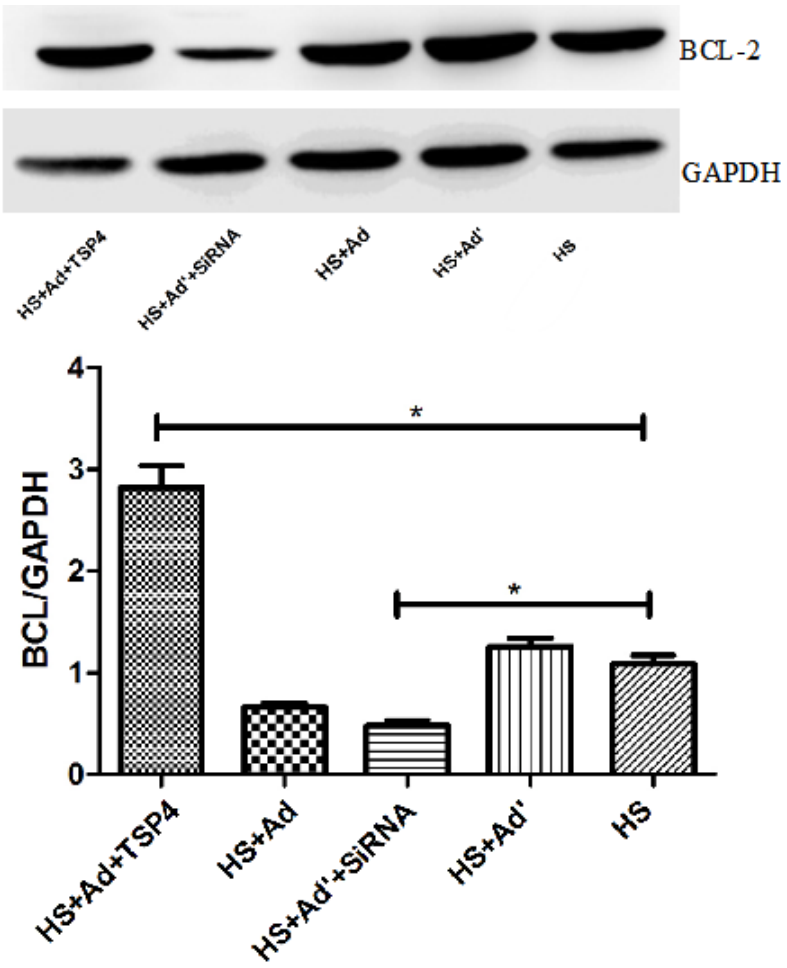

Figure 4. BCL-2 protein expression in fibroblasts from hypertrophic scars after TSP4 gene overexpression and knockdown. The following groups were examined: HS + Ad + TSP4 (hypertrophic scar-derived fibroblasts + TSP4), HS + Ad (hypertrophic scar-derived fibroblasts + adenovirus-void vector 1), HS + Ad' + SiRNA (hypertrophic scar-derived fibroblasts + siRNA), HS + Ad' (hypertrophic scar-derived fibroblasts + adenovirus void vector 2) and HS (untreated hypertrophic scar-derived fibroblasts), *P < 0.05 between the two groups.

TSP1 and TSP2 domains that regulate TGF-β activation [15]. This suggests that TSP4 may activate TGF-β *via* a different pathway than TSP1, and that its interactions with cell surface receptors and binding partners in the ECM may also differ from those of TSP1.

BCL-2 is a proto-oncogene that encodes the BCL-2 protein, which can significantly inhibit apoptosis. Compared to the control group, BCL-2 protein expression was significantly increased in response to the upregulation of the TSP4 gene, whereas it decreased after downregulation of the TSP4 gene. Our study suggests that upregulating the expression of the TSP4 gene activates the proliferation of fibroblasts and inhibits fibroblast apoptosis.

Nevertheless, our experiments are subject to limitations. In principle, normal skin tissue specimens and hypertrophic scar tissue specimens should ideally be collected from the same site and the same patient to eliminate inter-individual and inter-positional differences in gene expression. However, patients often expect minimal trauma while removing scars, making the removal of additional normal skin surrounding the scar difficult. Adequate preoperative communication and surgical planning, as well as increasing the sample size, may help minimize such errors in future studies.

Our study was the first to reveal the role of TSP4 in fibroblasts from hypertrophic scars. Specifically, TSP4 can increase expression levels of TGF-β1, α-SMA, and BCL-2, promote cell proliferation, and inhibit apoptosis in fibroblasts. Moreover, TSP4 can facilitate transformation of fibroblasts to myofibroblasts, and improve contractility of scar tissue. In addition, our experimental data revealed that TSP4 participates in the pathological processes of hypertrophic scars. Further studies of the function of TSP4 in human fibroblasts would help elucidate the mechanism of hypertrophic scar formation and provide new therapeutic strategies to prevent hypertrophic scar formation in clinic.

Disclosure of conflict of interest

None.

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