

## Original Article

# c-Jun is increased in hypertrophic scar and inhibits apoptosis in fibroblasts

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**Abstract:** To detect the expression pattern of c-Jun in human hypertrophic scars and investigate its role in regulation of cell proliferation and apoptosis in fibroblasts and its underlying action mechanism. In this study, we examined the expression of c-Jun in hypertrophic scars and fibroblasts using quantitative real-time polymerase chain reaction and western blot analysis and investigated the functional role of c-Jun in fibroblasts through detecting cell proliferation and apoptosis using cell viability assay and flow cytometry. The results showed that the c-Jun was increased in human hypertrophic scar tissues and fibroblasts compared with the respective matched human normal skin tissues or human keratinocyte HaCat cells. Overexpression of c-Jun enhanced proliferation and reduced apoptosis in fibroblasts, however, knockdown of c-Jun resulted in a converse tendency. The c-Jun upregulation could induce PPAR $\beta$ , PDK, pAKT, collagen I production and repress cleaved-caspase 3, caspase 8 and caspase 9 expression in fibroblasts. It is demonstrated that c-Jun contribute to proliferation and repress apoptosis in fibroblasts and the action mechanism is involved in activation of PPAR $\beta$ /pAkt. Our findings provide an insight into hypertrophic scarring and a potential novel target for management of human hypertrophic scar.

**Keywords:** c-Jun, hypertrophic scar, fibroblast, proliferation, apoptosis

## Introduction

Hypertrophic scar (HS), which often develops after thermal or traumatic injury to the deep dermis, is itchy, raised, painful, rigid and disfiguring scar and characterized by proliferation of the dermal tissue, with excessive deposition of fibroblast-derived extracellular matrix (ECM) and collagen proteins [1-3]. The development of hypertrophic scar involves complex pathological process, which is regulated by growth factors, cytokines, ECM molecules and proteolytic enzymes [4-7]. However, the exact mechanisms by which hypertrophic scar is initiated, evolved and regulated remain to be fully elucidated.

The over-proliferation of fibroblasts and excessive deposition of collagen-dominated extracellular matrix are key pathological events in hypertrophic scar formation [8]. The proliferation and function of fibroblast is regulated by various factors such as transforming growth factor (TGF- $\beta$ ) [9], epidermal growth factor (EGF) [10], fibroblast growth factor (FGF) [11], tumor necrosis factor (TNF- $\alpha$ ) [12], peroxisome

proliferator-activated receptors (PPAR $\beta$ ) [13], etc. The c-Jun, as a component of the transcription factor AP-1, is activated by a wide variety of extracellular stimuli. Studies indicate that c-Jun is implicated in many cellular processes and plays an important role in regulation of cell proliferation and apoptosis [14-16]. Ron and his colleagues [17] showed that, in fibroblasts derived from c-Jun null embryos, c-Jun was required for cell cycle progression, and could prevent TNF- $\alpha$ -induced apoptosis. c-Jun, serving as a proto-oncoprotein, is commonly upregulated in tumor with ability to regulate cell proliferation and apoptosis [18]. However, the expression pattern and functional role of c-Jun in hypertrophic scar formation is unclear. We conjecture that c-Jun may contribute to hypertrophic scar formation by regulation of proliferation and function of fibroblasts.

To validate the above supposition, we firstly examined the expression of c-Jun mRNAs and proteins in human HS tissues and fibroblast cell lines, and then investigated the effects of c-Jun on cell viability and apoptosis of HS fibroblasts

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and its underlying molecular mechanisms. In this study, we seek to provide some insight into c-Jun involving in HS development that may allow clinicians to improve treatment and prevention in the future.

### Materials and method

#### *Sample collection*

A total of nine paired hypertrophic scar tissue samples and matched normal skin tissues were obtained from the Xiangya Hospital of Central South University in accordance with the hospital's ethical guidelines and board. The profile of each sample has been delineated in our previous study [19]. All subjects or their caregivers provided written informed consent. All samples were collected and identified by histopathological evaluation. They were stored at 80°C until used.

#### *Cell culture and stimulation*

Human hypertrophic scar fibroblasts (HSF) were purchased from Bioleaf Corporation (Shanghai, China). Human keratinocyte (HaCat) cells were purchased from the cell bank of the Institute of Cell Biology of Chinese Academy of Science (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% (w/v) penicillin/streptomycin in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. The HSF cells were transfected with pcDNA3.1 negative control (pcDNA3.1), pcDNA3.1-c-Jun (c-Jun) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), or infected with lentiviral vector negative control (Lv-NC), lentiviral vectors comprising c-Jun-shRNA (Lv-c-Jun-shRNA).

#### *Quantitative real-time PCR (qPCR)*

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and then the RNA was reverse transcribed using the PrimeScript RT Master Mix Perfect Real Time kit (TaKaRa, Dalian, China) to obtain the cDNA. Using the cDNA as the template, a real-time PCR assay was performed using the following pairs of primers: c-Jun forward, 5'-GGCGATTC-TCTCCAGCTTCC-3' and c-Jun reverse, 5'-TCGACATGGAGTCCCAGGA-3', and  $\beta$ -actin forward, 5'-AGGGGCCGGACTCGTCATACT-3' and  $\beta$ -actin reverse, 5'-GGCGGCACCACCATGTACCCT-3'. The

20  $\mu$ L real-time PCR reaction included 0.5  $\mu$ L of cDNA template, 0.25  $\mu$ L of Primer F, 0.25  $\mu$ L of Primer R, 10  $\mu$ L of RNase-free dH<sub>2</sub>O, and 8  $\mu$ L of 2.5 $\times$  RealMasterMix (SYBR Green I). The reaction conditions included a pre-denaturation step at 95°C for 10 s, and 40 cycles of 95°C for 15 s and 60°C for 60 s. c-Jun expression was normalized by  $\beta$ -actin.

#### *Western blot*

RIPA lysis buffer (Boster, Wuhan, China) was used to extract the total protein from the tissues or cells. BCA Protein Assay Kit (Thermo, Waltham, MA, USA) was used to measure the protein concentration. Then, 60- $\mu$ g total protein was separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked and incubated with a primary antibody anti-c-Jun (1:1000 dilution, Proteintech, UK), anti-PPAR $\beta$ , anti-PDK, anti-Akt, anti-pAkt (1:2000 dilution, Abcam, UK), anti-collagen I, anti-cleaved-caspase 3 (1:2000 dilution, Santa Cruz, USA), anti-caspase 8, anti-caspase 9 (1:2000 dilution, Abcam, UK) and anti- $\beta$ -actin (1:3000 dilution, Boster, USA) overnight at 4°C. The membrane was washed and incubated with a secondary antibody for 90 min at 37°C. The signals on the membrane were detected by enhanced chemiluminescence reagent. Data were analyzed by densitometry using Image-ProPlus software 6.0 (Media Cybernetics, Rockville, MD, USA) and normalized to internal control expression ( $\beta$ -actin).

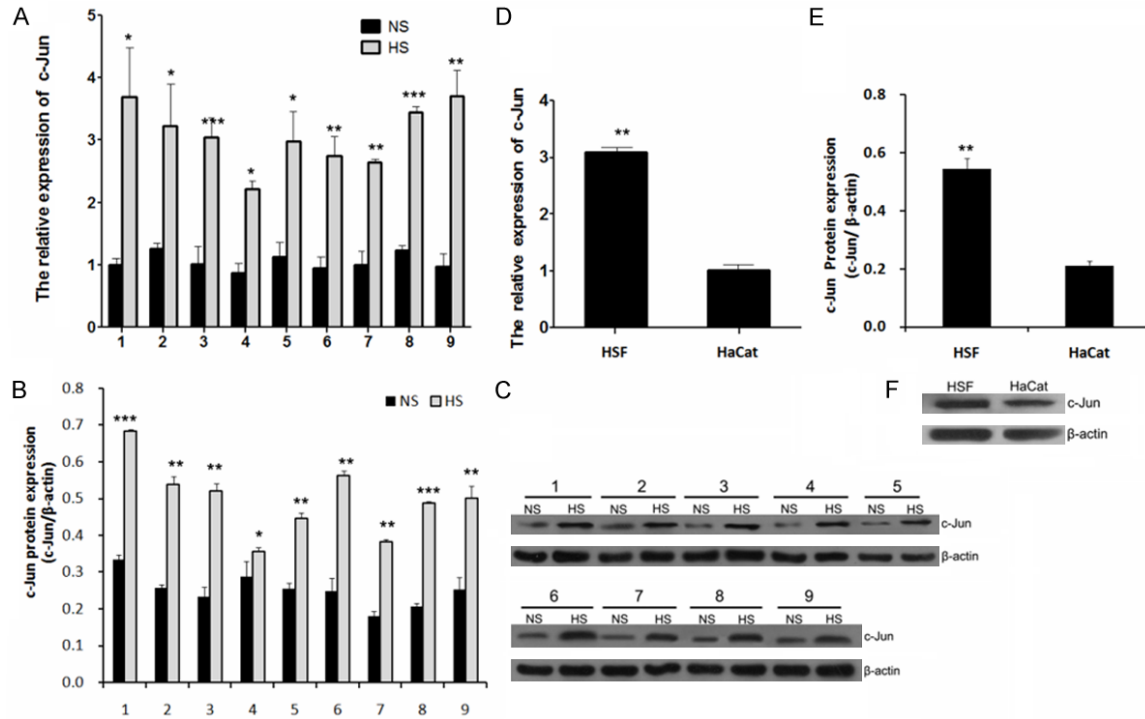
#### *Cell proliferation assay*

Cells treated with the indicated reagents or samples in exponential growth were plated at a final concentration of  $2 \times 10^3$  cells per well in 96-well plates. The viability of cells was evaluated by MTT assay after 24, 48 and 72 of seeding. The optical density at 570 nm (OD570) of each well was measured with an ELISA reader (ELX-800 type, BioTek).

#### *Cell apoptosis assay*

Cells were harvested and treated with FITC (BioVision, Mountain View, CA) according to the manufacturer's instructions. Cell apoptosis was detected by analyzing annexin V-FITC binding by flow cytometry (Ex = 488 nm; Em = 530 nm) using a FITC signal detector and a PI signal detector.

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**Figure 1.** The expression of c-Jun in hypertrophic scar tissues and fibroblasts. A. qPCR detected the relative expression of c-Jun mRNA in hypertrophic scar tissues (HS) and normal skin tissues (NS). B and C. Western blot analyzed and quantified the expression of c-Jun protein in HS and NS. D. qPCR detected the expression of c-Jun mRNA in Human hypertrophic scar fibroblasts (HSF) and human keratinocyte HaCat cells (HaCat). E and F. Western blot analyzed and quantified the expression of c-Jun protein in HSF and HaCat cells. Data are shown as mean  $\pm$  SD, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control (Con).

### Statistical analysis

Data were expressed as mean  $\pm$  SD from at least three separate experiments. Statistical analysis was carried out using SPSS 16.0 software. Student's t-tests or one-way ANOVA was used depending on the experimental conditions. A value of  $P < 0.05$  was considered as statistical significance.

### Results

#### *c-Jun is increased in HS tissues and fibroblast*

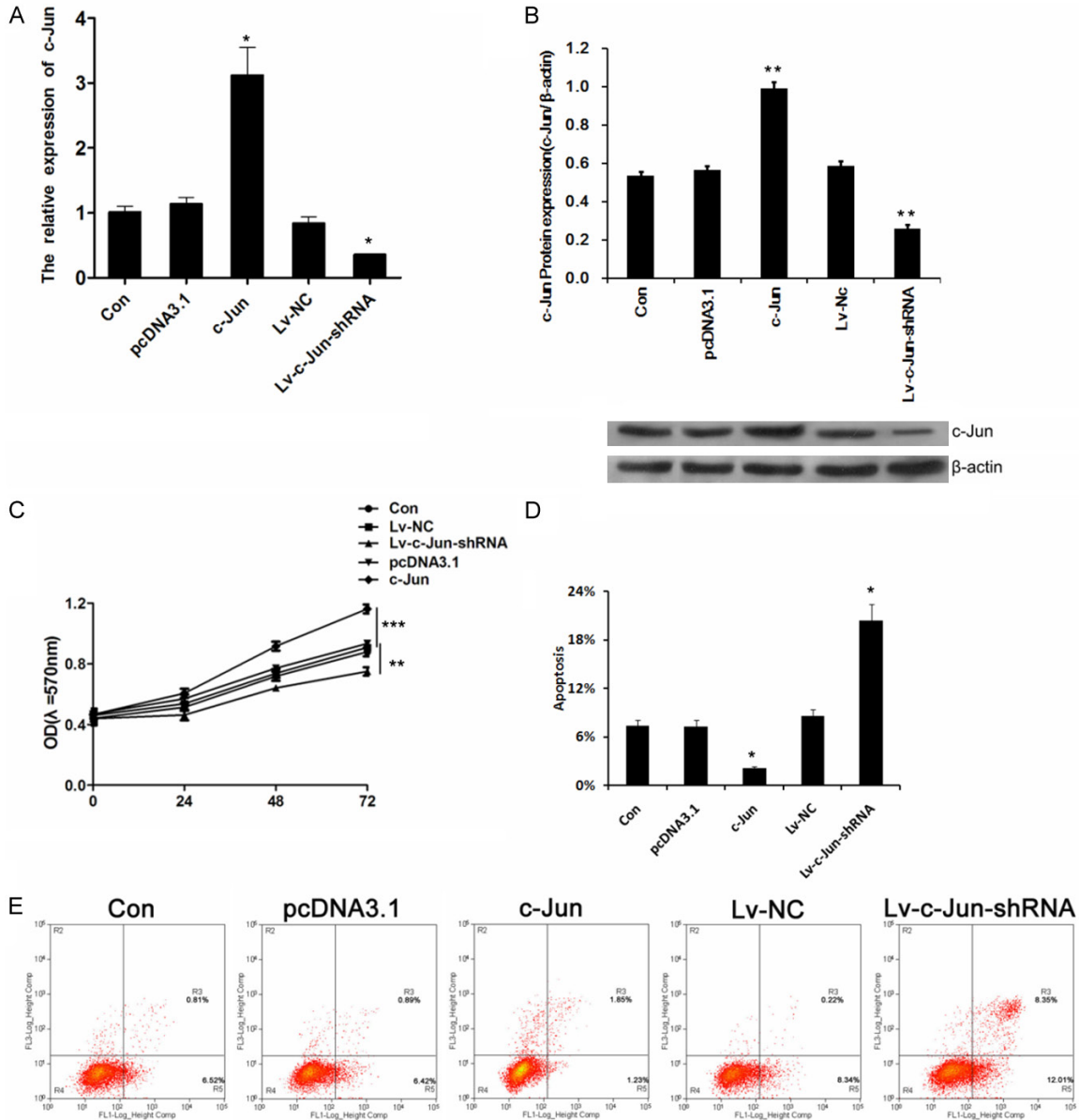
To investigate whether c-Jun is involved in the HS, a total of nine paired human hypertrophic scar tissue samples and matched normal skin tissues (NS) were prepared. The qRT-PCR and western blot analysis showed that the c-Jun mRNA (Figure 1A) and protein (Figure 1B, 1C) levels were increased in hypertrophic scar tissues compared with the respective matched NS. Then, the expression of c-Jun in Human hypertrophic scar fibroblasts (HSF) and human

keratinocyte HaCat cells was detected. The results showed that the expressions of c-Jun mRNA (Figure 1D) and protein (Figure 1E, 1F) in HSF were significantly higher than that in HaCat cells. These results confirm that c-Jun is increased in human hypertrophic scar fibroblasts and tissues.

#### *c-Jun contributes to proliferation of HSF*

To further determine whether c-Jun functions in proliferation of human hypertrophic scar fibroblasts, the c-Jun was overexpressed or knockdowned in fibroblasts transfected with pcDNA3.1-c-Jun (c-Jun) or infected with Lv-c-Jun-shRNA. The qRT-PCR and western blot assays indicated that the c-Jun mRNA (Figure 2A) and protein (Figure 2B) levels were increased in fibroblasts transfected with pcDNA3.1-c-Jun (c-Jun) and decreased in fibroblasts infected with Lv-c-Jun-shRNA compared with the control (Con). Then, the MTT assay showed that overexpression of c-Jun enhanced the cell viability in fibroblasts, however, knockdown of

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**Figure 2.** The effects of c-Jun on proliferation and apoptosis of fibroblasts. A. qPCR detected the relative expression of c-Jun mRNA in fibroblasts transfected with pcDNA3.1-c-Jun (c-Jun) or infected with Lv-c-Jun-shRNA. B. Western blot analyzed and quantified the expression of c-Jun protein in fibroblasts treated as the indicated. C. MTT determined the viability of fibroblasts treated as the indicated at 0, 24, 48 and 72 h. D and E. FCM was used to analyzed the apoptotic rate of fibroblasts treated as the indicated. Data are shown as mean  $\pm$  SD, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 versus control (Con).

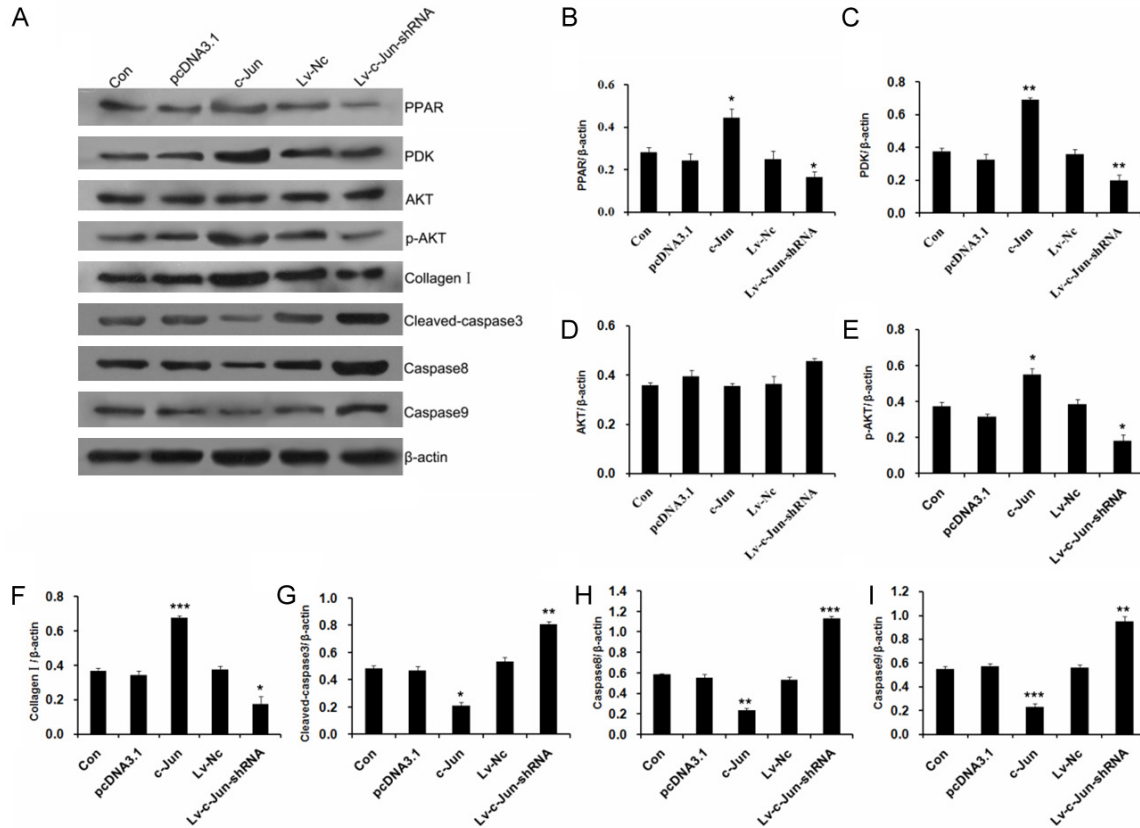
c-Jun reduced it. These demonstrate that c-Jun contributes to proliferation of human hypertrophic scar fibroblasts.

*Over-expression of c-Jun decreases apoptosis in HSF*

It has been reported that c-Jun is involved in regulation of apoptosis. To make clear whether

c-Jun-mediated increased proliferation in human hypertrophic scar fibroblasts was associated with its effects on apoptosis, the FMC assay was used. It was shown that overexpression of c-Jun significantly decreased the apoptosis rate of fibroblasts, however, knock-down of c-Jun remarkably increased the apoptosis rate in fibroblasts. It suggests that c-Jun contributes to proliferation in human hypertro-

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**Figure 3.** The effects of c-Jun on the expressions of PPAR $\beta$ , PDK, pAKT, collagen I, cleaved-caspase 3, caspase 8 and caspase 9 in fibroblasts. (A) Representative images of the expression of the action mechanism-related proteins, detected by western blot. c-Jun induced the increased PPAR $\beta$  (B), PDK (C), AKT (D), pAKT (E), collagen I (F) and the decreased cleaved-caspase 3 (G), caspase 8 (H) and caspase 9 (I). Data are shown as mean  $\pm$  SD, \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 versus control (Con).

phic scar fibroblasts probably by inhibition its apoptosis.

### *The effects of c-Jun on expressions of the action mechanism-related signal molecules*

AS shown in **Figure 3**, overexpression of c-Jun induced the increased PPAR $\beta$ , 3-phosphoinositide-dependent protein kinase (PDK), pAKT, collagen I and decreased cleaved-caspase 3, caspase 8 and caspase 9 in human hypertrophic scar fibroblasts. However, knockdown of c-Jun induced a converse expression pattern of them in human hypertrophic scar fibroblasts, compared with overexpression of it. Neither overexpression nor knockdown of c-Jun had apparent effects on total Akt expression in human hypertrophic scar fibroblasts. These results imply that c-Jun may promote the activation and proliferation of fibroblasts via increasing PPAR $\beta$ , PDK, pAKT and collagen I and suppress the apoptosis of fibroblasts by decreasing caspase 8, caspase 9 and cleaved-caspase 3.

### Discussion

In recent years, with the progress of the research, we have a deeper understanding of wound healing and scar formation mechanism, but the etiology and pathogenesis of hypertrophic scars are not clear. Clinically, there is a great need for effective treatment because hypertrophic scars have a high incidence, are disfiguring and, in some cases, cause functional disability due to contractures [20]. Therefore, there is an urgent need for making clear the molecular mechanisms underlying hypertrophic scarring.

In cancers, c-Jun is identified as a proto-oncoprotein that is commonly overexpressed in many types of cancer and is believed to regulate cell proliferation, the cell cycle, and apoptosis by controlling AP-1 activity [18]. In this study, using qPCR and western blot analysis, we discovered that c-Jun was upregulated in hypertrophic scar tissues and fibroblasts compared with normal



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skin tissues or human keratinocyte cells. Our findings are agreement with the immunohistochemistry study results that c-Jun was existed and increased in hypertrophic scar tissues [21]. The abnormal c-Jun expression in hypertrophic scar tissues suggests that c-Jun may involve in pathologic process of hypertrophic scarring. In addition, that c-Jun was higher expressed in fibroblasts than in keratinocyte cells implies c-Jun regulation of hypertrophic scarring involves its functioning in fibroblasts, since it is well known that the activation and continuous proliferation of fibroblasts play a pivotal role in hypertrophic scar formation [22-24].

Therefore, the functional role of c-Jun in fibroblasts was then investigated. We showed that overexpression of c-Jun significantly suppressed the apoptosis in fibroblasts and enhanced its proliferation. Correspondingly, c-Jun inhibition induced increased apoptosis and decreased proliferation in fibroblasts. It is demonstrated that c-Jun contributes to proliferation and suppresses apoptosis of fibroblasts. We then found that c-Jun could induce PPAR $\beta$ , PDK, pAKT, collagen I production and repress cleaved-caspase 3, caspase 8 and caspase 9 expression in human hypertrophic scar fibroblasts. In the previous study, we have discovered that PPAR $\beta$  was upregulated in hypertrophic scar tissues compared to paired normal skin tissues and confirmed that PPAR $\beta$  could promote proliferative and migratory capacity of hypertrophic scar fibroblast [19]. Studies indicate that AP-1, consists of c-Jun and c-Fos, is an important transcription factor of PPAR $\beta$ . In process of TGF- $\beta$ 1/SMAD3 regulation of wound repair, SMAD3 phosphorylated by TGF- $\beta$ 1 directly binds to c-Jun, which leads to inhibition of AP-1 transcription of PPAR $\beta$  [25, 26]. Therefore, upregulation of c-Jun would promote the transcription of PPAR $\beta$ . The Akt signaling is an important pathway for maintenance of cell survival [27]. PPAR $\beta$  can activate Akt via transcriptional upregulation of PDK1 and ILK [28]. And the phosphorylated Akt can downregulate caspase 9. The reduction of caspase 8 and 9 will decrease the activation of apoptosis effector caspase 3 resulting in reduced apoptosis in fibroblasts. In addition, c-Jun may affect cell cycle progress or arrest, and apoptosis by transcriptional regulation of cyclin D1, p53 and so on [17, 29]. However, whether these molecules also participate in the process of c-Jun regulation of prolif-

eration and apoptosis in fibroblasts needs further study in future.

In summary, we identified that the c-Jun was increased in hypertrophic scar tissues and fibroblasts, and demonstrated that upregulation of c-Jun induced increased proliferation and decreased apoptosis in fibroblasts likely by activation of PPAR $\beta$ /pAkt. Our data provide an insight into hypertrophic scarring and a potential novel target for management of human hypertrophic scar.

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### Disclosure of conflict of interest

None.

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