Int J Clin Exp Med 2015;8(6):8551-8560
www.ijcem.com /ISSN:1940-5901/IJCEM0008892

Original Article

Uighur medicine abnormal savda munzip (ASMq) suppresses expression of collagen and TGF-β_1 with concomitant induce Smad7 in human hypertrophic scar fibroblasts

Nan Li*, Menglong Kong*, Tao Ma, Weicheng Gao, Shaolin Ma

Department of Plastic Surgery, 1st Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, China.
**Equal contributors.*

Received April 8, 2015; Accepted June 7, 2015; Epub June 15, 2015; Published June 30, 2015

Abstract: Background: Hypertrophic scar (HS) is a common dermal disease, for which numerous treatments are currently available but they do not always yield excellent therapeutic results. Hence, alternative strategy are needed. Recent basic and clinic research has shown that Uighur medicine abnormal savda munzip (ASMq) has anti-hypertrophic scar properties but its molecular mechanism is unknown. The aim of this study was to explore the effect of ASMq on TGF-β/Smads signaling in fibroblasts derived from hypertrophic scar. Purpose: To investigate the effect of ASMq on the TGF-β/Smads signaling pathway in hypertrophic scar fibroblasts (HSFs). Methods: Hypertrophic scar fibroblasts (HSFs) were isolated from human of hypertrophic scar and passaged to the 3~4 generation, which were treated with the different concentrations of ASMq. Cells treated with 5-Fu served as the positive control group. After treatment for 48 hours, expressions of Smad7, TGF-β_1, type I and III collagen, were examined by immunocytochemistry, reverse transcription PCR and Western blotting, respectively. Results: ASMq markedly enhanced the expression of inhibitory Smad7, with suppression of type I and III collagen and TGF-β_1. We observed that treatment of ASMq induced Smad7 to enter the cytoplasm from the nucleus of hypertrophic fibroblasts. Conclusions: ASMq inhibits scarring probably by enhancing the expression of inhibitory Smad7, and inhibiting TGF-β_1, collagen expression, and is a potential treatment for scarring.

Keywords: Uighur medicine abnormal savda munzip, TGF-β_1, hypertrophic scar fibroblasts, smad7

Introduction

It is well known that the development of hypertrophic scar are associated with an abnormal proliferation of fibroblasts and overproduction of collagen in extracellular matrix (ECM) [1]. Excessive expression of transforming growth factor-β_1 (TGF-β_1) has been demonstrated as a key factor in promoting scar formation [2]. Not only does TGF-β_1 regulate cellular growth, differentiation, adhesion, and apoptosis, but it also induces excessive deposition of collagen by scar fibroblasts. The consequence of TGF-β_1 stimulation is reported to be mediated by an unique signaling pathway of the Smad family [3]. Ten types of Smad protein are included in Smad family and they play a fundamental role in a variety of cellular function regulated by TGF-β_1. It has been reported that continuing activation of Smad pathway may contributes greatly to the formation of hypertrophic scar [4, 5]. Smad7 as the unique negative feedback regulator of this signal pathway prevents Smad2/3-receptor interactions and subsequent Smad phosphorylation. Therefore, an attempt to up-regulate Smad7 may be a promising way to reduce TGF-β_1 excretion and inhibit fibrosis.

Uighur medicine abnormal savda munzip (ASMq), consisted of ten kinds of herbal compound medicine, is the prescription urighur medicine by which complex diseases such as tumor, diabates, coronary heart diseases, that caused by abnormal savda based on the traditional Ugihur Medical theory, have been treated. Traditional Uighur medicine holds that there are four kinds of humors (body fluids) in the

Table 1. Sequences of polymerase chain reaction and amplification

Gene	Sequence	Annealing	Cycles	Products (bps)
Smad7	CAAGAGGCTGTGTTGCTGTGAATC GTTGGTTTGAGAAAATCCATCGG	55°C	30	144
TGF-β_1	CGAAATCTAGACAAGTTCAAGCAG GAGGTATCGCCAGGAATTGTTG	55°C	35	193
Collage I	TGTGCGATGACGTGATCTGTGA CTTGGTCGGTGGGTGACTCTG	55°C	35	111
Collage III	CTCTGCTTCATCCCACTATTATTT TGCGAGTCCTCCTACTGCTAC	55°C	35	470
GAPDH	CGTCTTCACCACCATGGAGA CGGCCATCGCCACAGTTT	55°C	35	300

human body, namely Savda, Belghem, Sapra and Kan, which are associated with the fundamental elements of earth, fire, water and air, respectively. However, excess or deficiency of one of the four humors, called "abnormal humor", When abnormal changes occur in the body fluids, the normal body fluids in turn have certain abnormal characteristics and are termed "abnormal body fluids", forming the basis of various diseases. Previous studies showed that these treatment effect of ASMq involved in eliminating free radicals, repairing DNA and mitochondrial oxidative damage caused by hydroxyl free radical, and thus improves antioxidant ability of patients, suffered abnormal body fluids mature as determined by traditional Uighur Medical theory. Diseases such as cancer, hypertension, coronary heart disease are preventable and treatable by means of these important mechanisms [6-8].

Modern medicine thinks the HS fibrosis for skin disease, is a kind of systemic fibrosis diseases. We assume that the HS is abnormal body fluids in trauma caused by abnormal deposition on the surface of the skin. Our preliminary studies have shown that administration of ASMq by either lavage, external or combination of them reduced formation of hyperplastic scar in scar model in rabbit ear, suggesting that ASMq possesses a potent protective effect against hypertrophic scar formation [9, 10]. However, it is still unknown how ASMq exerts its effects on suppression of hyperplastic scar formation. This study is to examine whether TGF-β_1-mediated signaling pathway involved the effect of ASMq on suppression of collagen deposition by hypertrophic scar fibroblasts.

Materials and methods

Samples of hypertrophic scar tissue were harvested from burn patients who underwent plastic surgery at the department of Burns and Orthopedics of the first affiliated hospital of Xinjiang Medical University. The patients were five males and five females, aging from 19 to 45 years. Hypertrophic scar was identified by clinic observation and confirmed by histological examination. Informed consent was obtained by each participant and the study was approved by the medical and Ethics Committees of the first affiliated hospital of Xinjiang Medical University. Hypertrophic scar fibroblast culture primary fibroblast cultures were established as described previously. Briefly, tissue specimens were repeatedly washed in sterile Dul-becco's Modified Eagle's Medium (DMEM, LOW glicose, HyClone, USA) supplemented with an antibiotic/antimycotic preparation and then were cut into 0.5-1 mm^3 pieces. The epidermis was scraped off, and the dermis pieces were then placed in 100 mm cell culture flasks (Corning, USA) which were added 8ml culture medium containing DMEM with 10% fetal bovine serum (FBS, HyClone, USA), 100 U/ml penicillin, and 0.1 g/ml streptomycin at 37°C in air containing 5% CO_2. When reached 90% confluence, fibroblasts were subcultured with digestion by 0.25% trypsin. Cells passage 4-5 were used for further investigation. In the study, we stochastically chose three or four patient-derived fibroblasts for each experiment, and each experiment was repeated three times.

ASMq stimulation

Cells were seeded at a density of 2 × 10^4 cells ml-1 into 24-well plates for immunocytochemistry staining, into-mm plates for RNA and protein harvested. All experiment was divided into fine groups: Group A: cells were grown in DMEM/10% FBS without the addition of ASMq, Group B-D: ASMq (Xinjiang uygur pharmaceutical co, LTD, China)cells treated with ASMq at concentration of 0.2, 0.4, 0.6 mg/ml, respectively. Group E: cells are grown in DMEM/10% FBS with 5-FU (Jin Yao amino acid co, LTD, China). After treat-

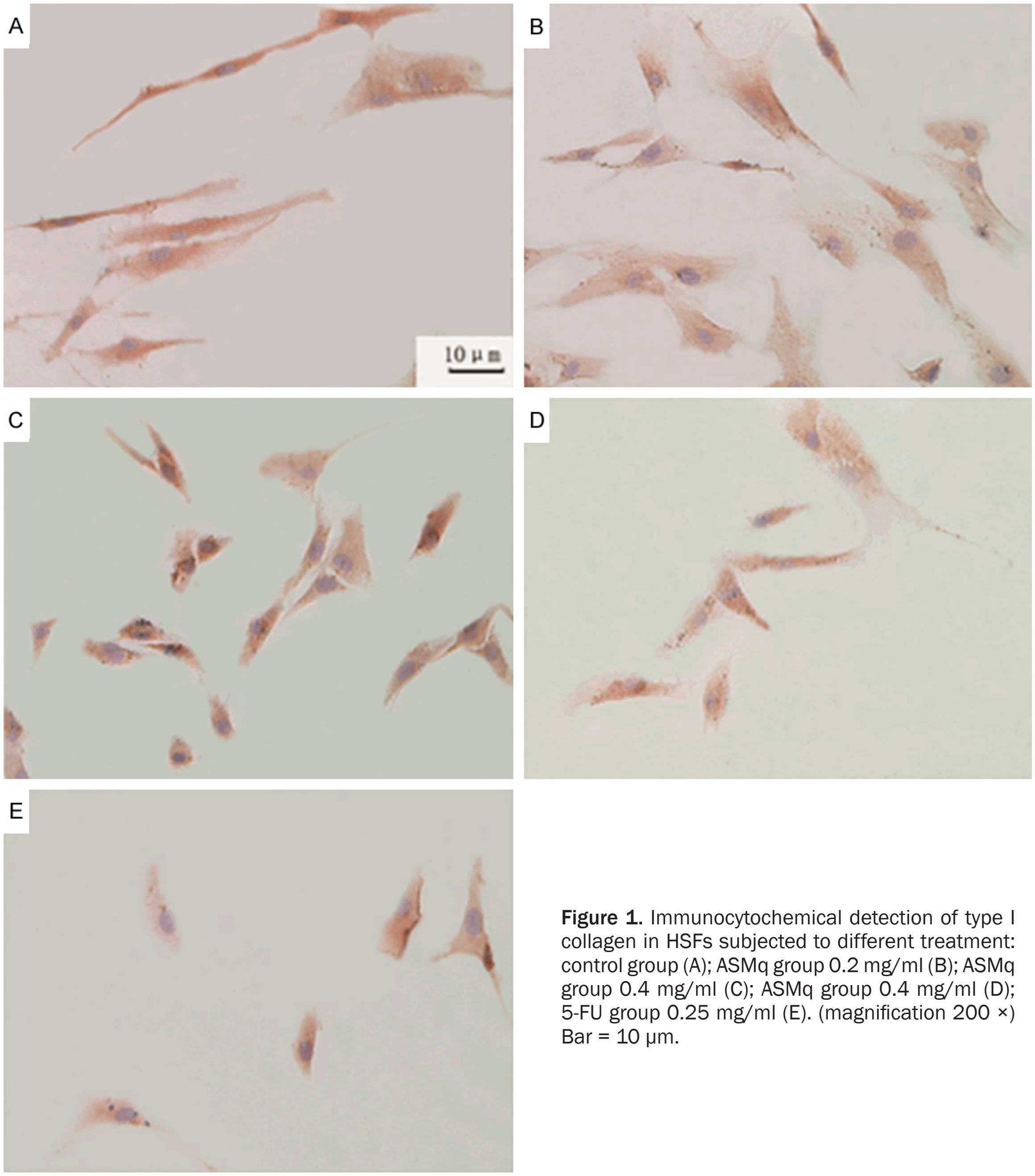

Figure 1. Immunocytochemical detection of type I collagen in HSFs subjected to different treatment: control group (A); ASMq group 0.2 mg/ml (B); ASMq group 0.4 mg/ml (C); ASMq group 0.4 mg/ml (D); 5-FU group 0.25 mg/ml (E). (magnification 200 ×) Bar = 10 μm.

ment for 48 h, the fibroblasts in each group harvested for further analysis.

Immunocytochemistry

Immunohistochemistry was carried out using a commercial avidin-biotin peroxidase complex (ABC) kit (Boster, China) according to the manufacture's recommendations. Briefly, cells from each group was washed three times with PBS, and then fixed 4% cold paraformaldehyde for 15 min. After inactivating endogenous peroxidase with deionization- 3% H_2O_2 for 20 min and blocking with normal goat serum for 15 min at room temperature, cultures were incubated with primary rabbit anti-human antibodies against collagen I and III (1:150, respectively; Boster, China), Smad7 (1:200; CLOUDCLONE CORP, USA), and TGF-β_1 (1:200; CLOUD-CLONE CORP, USA) overnight at 4°C, and then incubated with goat anti-rabbit secondary antibody (Boster, China) for 30 min at 37°C. After wash-

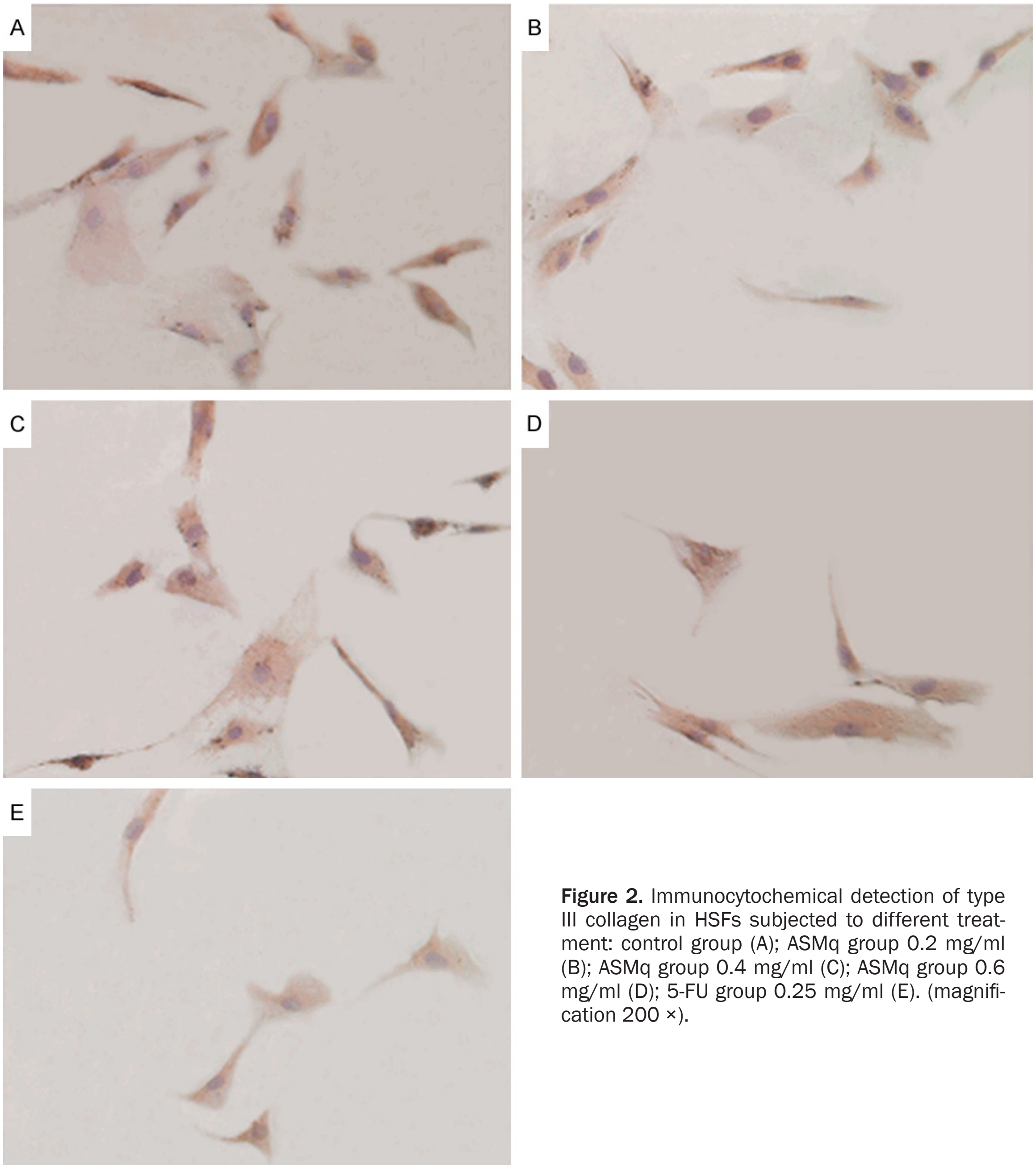

Figure 2. Immunocytochemical detection of type III collagen in HSFs subjected to different treatment: control group (A); ASMq group 0.2 mg/ml (B); ASMq group 0.4 mg/ml (C); ASMq group 0.6 mg/ml (D); 5-FU group 0.25 mg/ml (E). (magnification 200 ×).

ing three times with PBS, the color was developed with DAB (Boster, China) for 5 min. As a negative control, the primary antibody was replaced with phosphate-buffered saline (PBS). Images were acquired using a confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

Reverse transcription polymerase chain reaction (RT-PCR)

After 48 h of culture, total RNA in each group was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). For RT-PCR, 1 ug total RNA was reverse transcribed with Superscript TM (Invitrogen, Carlsbad, CA, USA) and oligo (dt) as primers. cDNAs were amplified using specific sets of primers for each gene. The sequence of each pair of primers, product sizes and amplification conditions were briefly listed in **Table 1**. After PCR amplification, 5 ul of total PCR reaction from each group were analyzed by a 2% agarose gel electrophoresis. The gels were scanned using a densitometer (Furi Science & Technology Ltd, Shanghai, China) and the den-

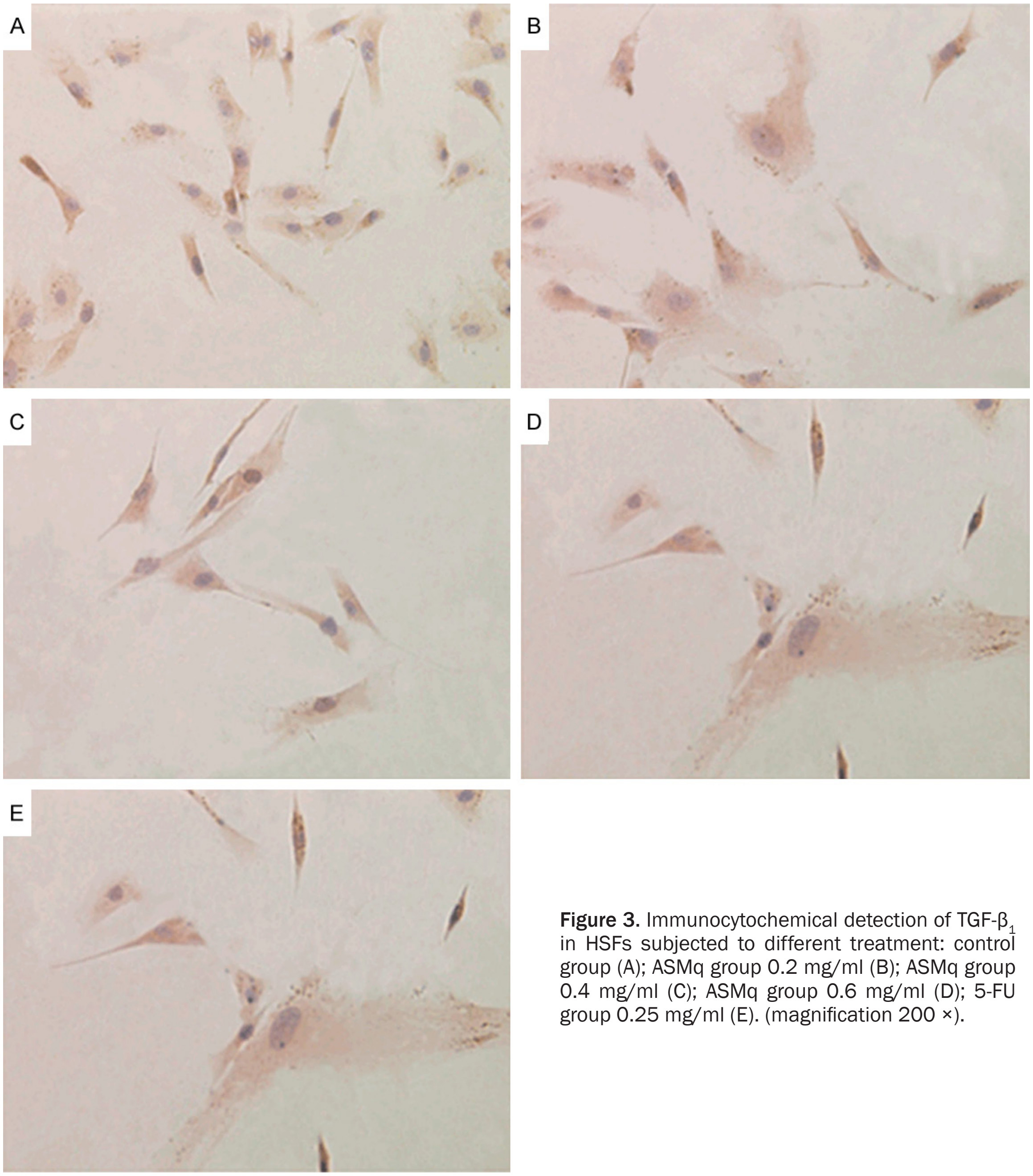

Figure 3. Immunocytochemical detection of TGF-β_1 in HSFs subjected to different treatment: control group (A); ASMq group 0.2 mg/ml (B); ASMq group 0.4 mg/ml (C); ASMq group 0.6 mg/ml (D); 5-FU group 0.25 mg/ml (E). (magnification 200 ×).

sities of the bands were compared with those of the housekeeping gene, GAPDH.

Western blot analysis

HSFs in each group were washed three times with ice cold PBS and lysed with RIPA (mainly containing 1% Noidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, Aprotine, and 1% PMSF was added immediately after the adding of lysis buffer). Cell lysates (30 ug of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA) using a semi-dry transfer cell. Cultures were incubated with primary rabbit anti-human antibodies against collagen I and III (1:4000, CLOUDCLONE CORP, USA), Smad7 (1:300; CLOUDCLONE CORP, USA), and TGF-β_1 (1:400; CLOUD-CLONE CORP, USA) overnight at 4°C. After vigorous washing, the mem-

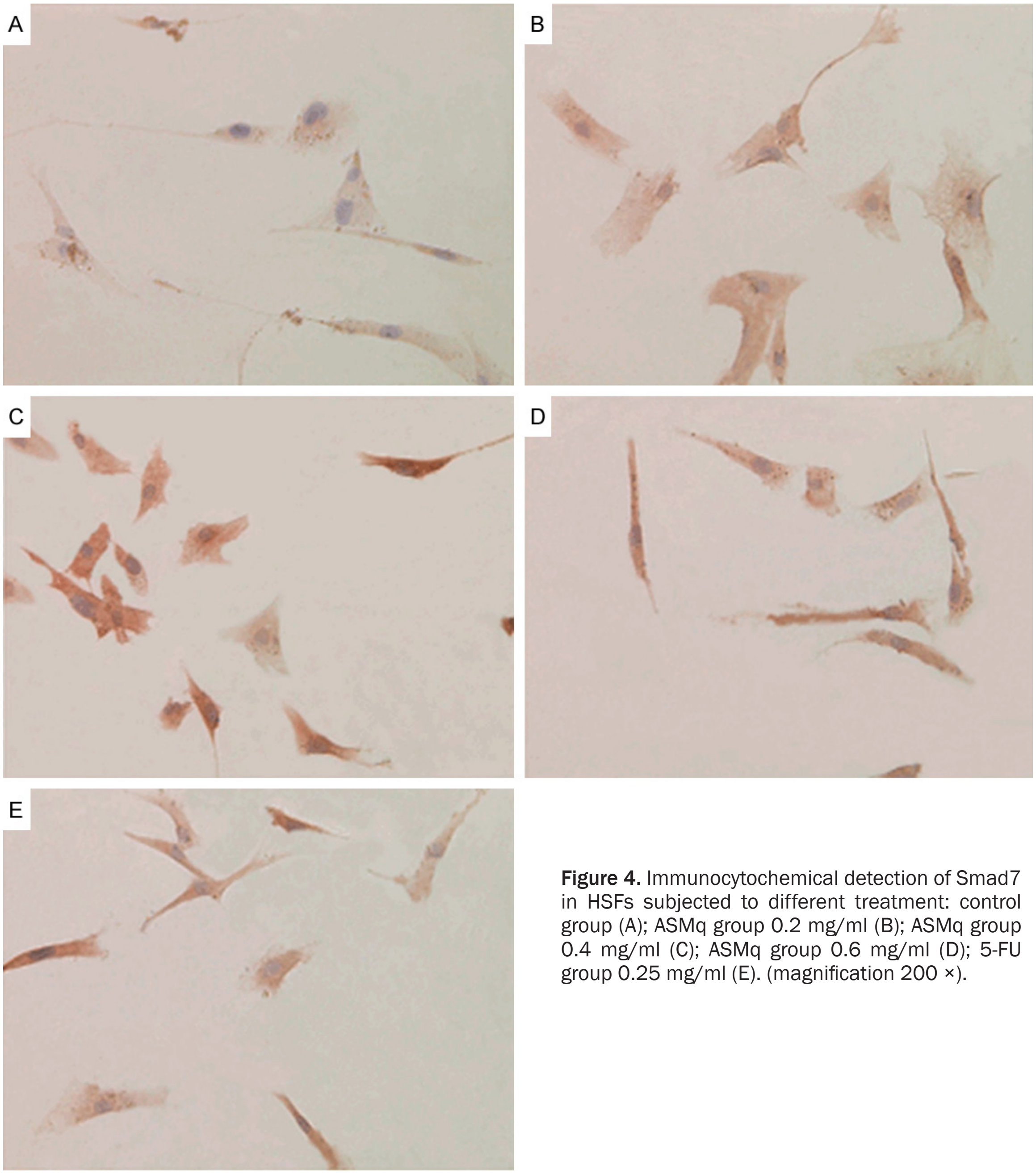

Figure 4. Immunocytochemical detection of Smad7 in HSFs subjected to different treatment: control group (A); ASMq group 0.2 mg/ml (B); ASMq group 0.4 mg/ml (C); ASMq group 0.6 mg/ml (D); 5-FU group 0.25 mg/ml (E). (magnification 200 ×).

brane was then incubated with HRP-conjugated goat anti-rabbit secondary antibody (diluted 1:4000) at room temperature for 2 h. The membrane blot was developed with Super Signal West Pico Chemiluminescent Substrate and the densities of the bands were compared with those of GAPDH.

Data analysis

The experimental data are shown as means and standard deviation, and the between-group comparisons were analyzed by t test. Result of statistics significance of differences was determined with SPSS 11.5 software ($P < 0.05$).

Results

Effect of ASMq on TGF-β_1, Smad7, collagen type I and III expression in HSFs

By immunohistochemical staining, with administration of ASMq, we found that staining of collagen Type I and III were suppressed, respec-

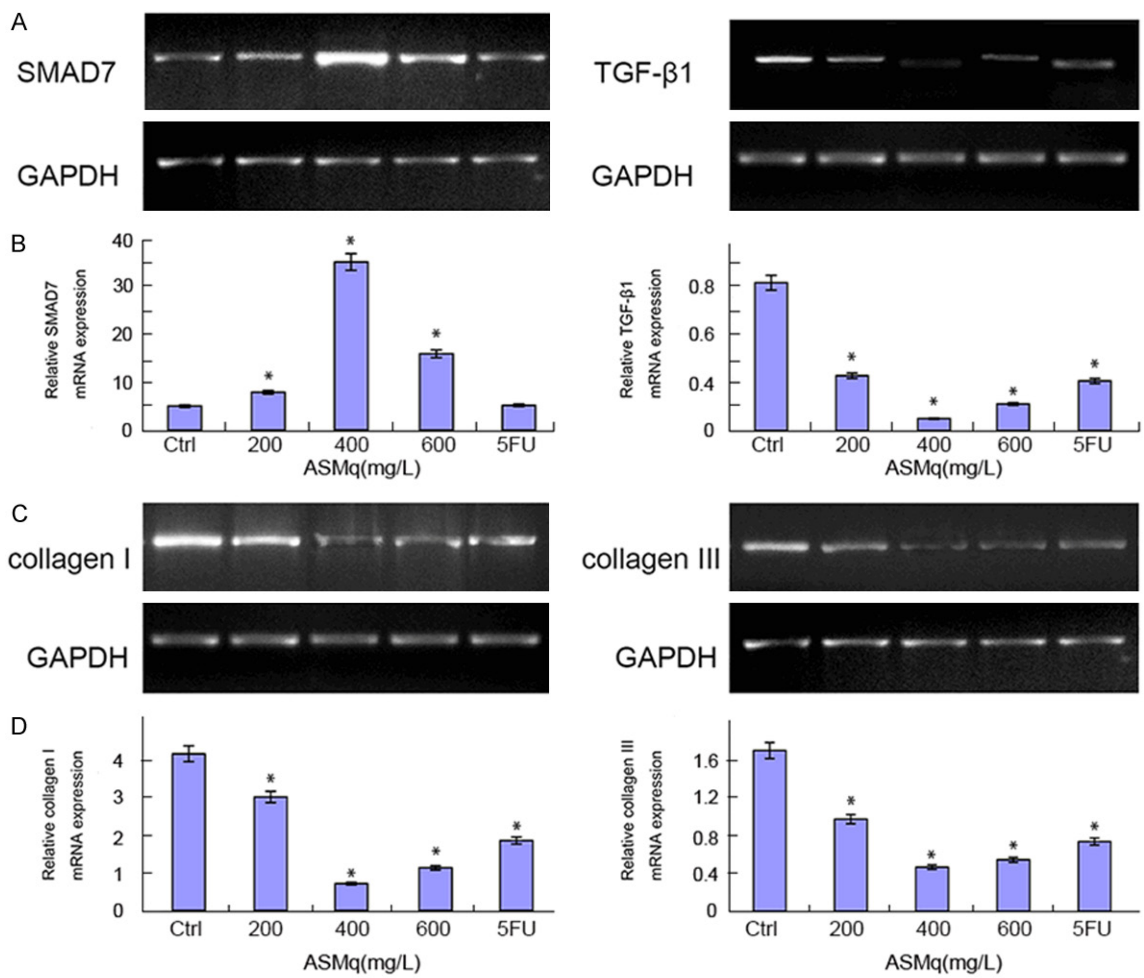

Figure 5. ASMq reduces the expression of TGF-β_1, Smad7, Type I and III collagen mRNA in Hypertrophic scar fibroblasts. Hypertrophic scar fibroblasts were cultured and treated without or with different concentrations (200, 400, and 600 mg/L) of ASMq in DMEM medium containing 10% fetal bovine serum for 48 h. Total mRNA was prepared and subjected to RT-PCR for TGF-β_1, Smad7, Type I and III collagen and GAPDH. GAPDH was used as a loading control. Experiments were repeated thrice with similar results. The graph is the mean densitometric data showing the level of TGF-β_1, Smad7, Type I and III collagen mRNA normalized to that of GAPDH mRNA. $^*P < 0.05$ compared with the values of control (no ASMq). Experiments were repeated thrice with similar results.

tively, with decrease in number of fibroblasts, which was comparable to that in 5-Fu treated control (type I collagen, **Figure 1**; type III collagen, **Figure 2**). And positive staining for TGF-β_1 gradually became weak in fibroblasts treated with ASMq in different Doses (**Figure 3**). Meanwhile, to determine whether the inhibitory Smad involved in the suppressive effect of ASMq, expression of Smad7 was detected in ASMq treated fibroblasts. It was found that staining intensity, which was predominantly detected within cytoplasm, was stronger in ASMq groups than that in normal control group (**Figure 4**).

Effect of ASMq on TGF-β_1, Smad7, collagen Type I and III mRNA expression in cultured HSFs

By RT-PCR detection (**Figure 5**), it was found that mRNA expression of collagen type I and III was inhibited up to 79.3%, 68.1% with treatment of ASMq in a dose of 0.4 respectively. The reduced level of collagen type I and III expression was significantly lower than that in fibroblasts without treatment ($P < 0.05$), but was comparable to that in 5-Fu treated group. This dose dependent effect of ASMq on collagen expression was further confirmed by western-blot analysis.TGF-β_1 mRNA expression in scar

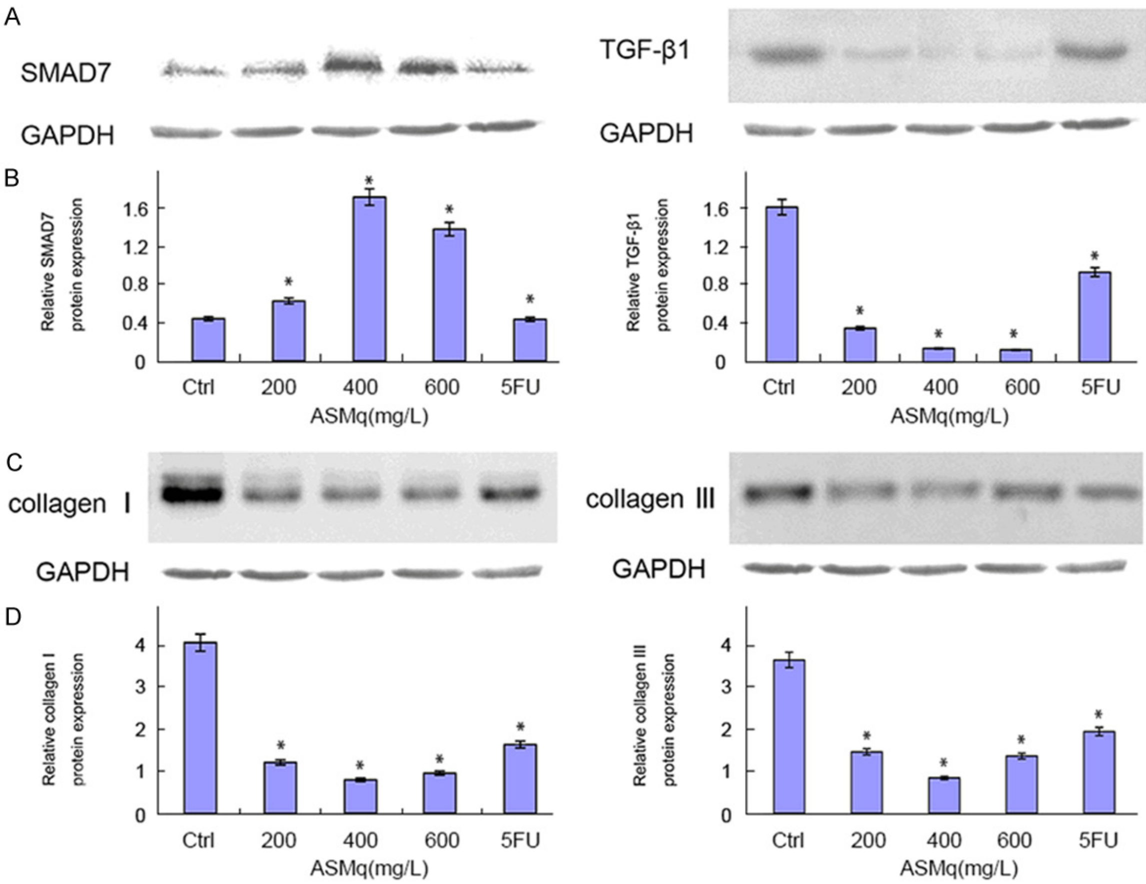

Figure 6. ASMq reduces the expression of TGF--β_1, Smad7, Type I and III collagen protein in Hypertrophic scar fibroblasts. Hypertrophic scar fibroblasts were cultured and treated without or with different concentrations (200, 400, and 600 mg/L) of ASMq in DMEM medium containing 10% fetal bovine serum for 48 h. Cell lysates was prepared and subjected to Western blot for TGF-β_1, Smad7, Type I and III collagen and GAPDH. GAPDH was used as a loading control. Experiments were repeated thrice with similar results. The graph is the mean densitometric data showing the level of TGF--β_1, Smad7, Type I and III collagenprotein normalized to that of GAPDH protein. $^*P < 0.05$ compared with the values of control (no ASMq) and 5FU. Experiments were repeated thrice with similar results.

fibroblasts was suppressed up to 1.9, 4.6 and 2.7 times when treated with 0.2, 0.4 and 0.6 mg/ml ASMq, respectively ($P < 0.05$). The suppressive effect of ASMq on TGF-β_1 expression showed no significant difference when compared with that treated with 5Fu. While the upregulated Smad7 mRNA expression in the experimental group was 1.14, 5.58, 2.01 times to that of the normal control ($P < 0.05$).

Effect of ASMq on TGF-β_1, Smad7, collagen Type I and III protein expression in HSFs

As shown in **Figure 6**, the protein expression of collagen type I and III expression and TGF-β_1 was highly reduced by ASMq in a dose dependent manner as determined by western-blot analysis. While the Smad7 protein expression in the ASMq group was upregulated compared to that of the control group.

Discussion

Hypertrophic scar occur as a result of a pathological wound-healing process, characterized by excess collagen deposition and hyperproliferation of fibroblasts [11]. In the present study, our results indicate that ASMq markedly enhanced suppressed expression of type I and III collagen in htpertrophic scar fibroblasts, with decreased expression of TGF-β_1, and enhance inhibitory Smad7 expression. TGF-β_1 plays a critical role in a wide variety of biological processes, including tissue repair and ECM accumulation [12]. Recently, many studies have demonstrated that TGF-β_1 promotes hypertrophic scar formation by triggering pathological accumulation of extracellular matrix [13, 14]. Therefore, it is reasoned that inhibition of TGF-β_1 activity would have potential benefits in suppressing hypertrophic scar formation. Furthermore, it has been observed that by binding to its receptor, TGF-β_1 induces activation of downstream Smad proteins HSFs [15]. Smad family is the first identified substrate of the TGF-β type I receptor (TβRI) kinases. They play a crucial role in the transduction of receptor signals to specific target genes in nucleus [16]. Smads, mainly including receptor-activated Smads (R-Smads, Smad2/Smad3), the common Smads (co-Smads, Smad4) and the inhibitory Smads (I-Smads, Smad7), participate the complicated biological network of TGF-β_1 in HSFs. R-Smads are phosphorylated by ligand-activated TβRI, leading to activation of a series of downstream events. Phosphorylated R-Smads form a heterodimeric complex with Smad4 and move into the nucleus where the complexes interact with Smads binding elements (SBE) located in the promoter regions of target genes, such as the gene of collagen, Smad7 and TGF-β_1, to enhance gene transcription [17, 18]. Smad7 are the unique negative feedback regulator of TGF-β/Smads signaling which forms complexes with Smurf1 or Smurf2 to mediate the termination of signaling by promoting the poly-ubiquitination and degradation of activated receptor [19-21]. The balance among Smads is the key issue in maintaining normal TGF-β_1 functions. However, alteration in balance of Smads mediated signaling results in dysfunction of fibrolasts that leads to formation of hypertrophic scar [22]. Most importantly, it has been reported that Smad7, the unique negative feedback regulator of TGF-β/Smads signaling, is diminished in HSFs [23]. To verify that cellular function and reproductive activity of HSFs were altered by ASMq via TGF-β/Smads signaling, we tested changed of type I and III collagen expressions. Type I and III collagen expressions were suppressed in ASMq group when compared to the control. Due to the close relationship between TGF-β_1 signaling and the production of collagen, blocking TGF-β/Smads signaling has the potential of repressing fibroblast proliferation and collagen synthesis, thereby preventing the formation of hypertrophic scar [24]. In our study, we observed that ASMq inhibited the expression of TGF-β_1 and increased the expressions of Smad7. This results demonstrated that with attenuation of TGF-β/Smads signaling via increased expression of Smad7, the stimulation effect of TGF-β_1 on ECM deposition and the expression of TGF-β_1 itself was down regulated in HSFs by ASMq administration. Smad7 as the unique negative feedback regulator of TGF-β/Smads signaling forms complexes with Smurf1 or Smurf2 to mediate the termination of signaling by promoting the poly-ubiquitination and degradation of activated receptors. It strongly demonstrated that after blocking TGF-β/Smads signaling via increasing Smad7 by tetrandrine, TGF-β_1 expression decreased. This results is in agreement with the reports which observed that upregulation of Smad7 or blockage of TGF-β_1 activity resulted in inhibiting of HSFs [25]. In conclusion, the results from the present study provide evidences supporting that ASMq can negatively regulate the expression of both type I and III collagen with inhibition of TGF-β_1 and upregulate the expression of Smad7. It has confirmed that ASMq inhabits HSFs at least partially through induction of Smad7 resulting in inhibition of TGF-β_1 transcription and its intracellular signaling. Furthermore, collagen type I and III production can be reduced and reproductive activity can be suppressed by blocking the TGF-β_1 signaling pathway. Thus, ASMq appears to have the potential to prevent hypertrophic scar formation and excessive scaring. Further investigations are required to elucidate the other potential mechanisms involved the mechanism of ASMq in inhibiting scar formation.

Acknowledgements

This research work was supported by National Natural Science Foundation of China (no. 81260291) and Graduate student research innovation project in Xinjiang (Grant No. XJGRI2014086).

Disclosure of conflict of interest

None.

Address correspondence to: Shaolin Ma, Department of Plastic Surgery, 1st Affiliated Hospital of Xinjiang Medical University, 393 Xinyi Road, Urumqi 830011, Xinjiang, China. Tel: 86-13179805849; E-mail: mashaolin9@sina.com

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