

Original Research

## Knockdown of eIF3a attenuates the pro-fibrogenic response of hepatic stellate cells induced by TGF- $\beta$ 1

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**Abstract:** Activation of hepatic stellate cells (HSCs) plays an important role in the development of liver fibrosis. The eukaryotic translation initiation factor (eIF) 3a is the largest subunit of the eIF3 complex and has been involved in pulmonary fibrosis. However, the role of eIF3a in liver fibrosis remains largely unknown. Therefore, in this study, we investigated the role of eIF3a in transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)-induced HSC activation. Our results demonstrated that the expression of eIF3a was up-regulated in human liver fibrotic tissues and activated HSCs. In addition, knockdown of eIF3a suppressed TGF- $\beta$ -induced HSC proliferation and the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen I. Furthermore, knockdown of eIF3a inhibited the expression of p-Smad3 induced by TGF- $\beta$ 1 in HSCs. These results suggest that eIF3a may function as a novel regulator to modulate HSC activation, potentially through inhibiting the TGF- $\beta$ 1/Smad3 signaling pathway.

**Key words:** Eukaryotic translation initiation factor (eIF) 3a, hepatic stellate cells (HSC), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA).

### Introduction

Liver fibrosis, as the final common end-stage of most chronic liver diseases, is triggered by various etiologies, such as viral infection, toxins, metabolic diseases and alcohol consumption (1). It is characterized by the accumulation of extracellular matrix (ECM) (2). Despite significant advances have been made in the development of anti-fibrosis, the appropriate strategies to treat hepatic fibrosis have not been well established.

Hepatic stellate cells (HSCs) are the major mesenchymal cells in liver, and their activation is the main feature in the process of liver fibrosis (3). Recent studies have shown that HSC activation can be induced by many factors, such as transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), endothelin 1 and platelet derived growth factor (PDGF) (4-6). Activated HSCs lose their lipid droplets, migrate to injured sites and are transformed into myofibroblast-like cells that secrete excess production of ECM components leading finally to liver fibrosis (7). Therefore, anti-fibrotic therapeutic strategies include the suppression of HSC proliferation and the down-regulation of collagen production.

The eukaryotic translation initiation factor (eIF) 3a, the largest subunit of the eIF3 complex, is a key functional entity in ribosome establishment and translation initiation (8). There is substantial evidence that eIF3a plays an important role in regulation of cell growth, proliferation, cell cycle, differentiation, cancer progression and the DNA repair pathways (9-11). In addition, several studies suggest a role of eIF3a on fibrosis disease and cellular proliferation and differentiation of fibroblast (12-14). For example, Zhang *et al.* reported that eIF3a was up-regulated in renal fibrotic tissues and knockdown of eIF3a significantly inhibited TGF- $\beta$ 1-induced expression levels of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen I in HK-2 cells (12). However, the role of eIF3a in liver fibrosis remains largely un-

known. Therefore, in this study, we investigated the role of eIF3a in TGF- $\beta$ 1-induced HSC activation.

### Materials and Methods

#### Specimen Collection

Liver biopsies were collected by trans-parietal puncture from 8 healthy individuals and 8 patients with liver fibrosis. Written informed consent was obtained from all patients, and the study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University (China).

#### Cell culture

HSC-T6 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), L-glutamine (4 mmol/L), penicillin (100 IU/mL), and streptomycin (100  $\mu$ g/mL) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

#### Knockdown of siRNA

Scrambled siRNA (si-Con) and small-interfering RNA targeting eIF3a (si-eIF3a) was purchased from GenePharma (Shanghai, China). For transfection, For transfection, 5 $\times$ 10<sup>4</sup> cells/well were seeded in each cell of a 24-well micro-plate, grown for 24 h to reach 30%-50% confluence, and then transfected with si-Con or si-eIF3a using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruc-

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tions. Results were checked by real time PCR (RT-PCR) and western blot at 48 h after transfection.

### Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Trizol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Up to 5  $\mu$ g of the total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Sigma, St. Louis, MO, USA). RT-qPCR was performed with the Applied Biosystems 7900HT Fast Real-Time PCR System using the SYBR Premix Ex Taq. The following primers were used: eIF3a, 5'-TCAAGTCGCCGGACGATA-3' (sense), 5'-CC-TGTCATCAGCACGTCTCCA-3' (antisense);  $\alpha$ -SMA, 5'-CTATTCCTTCGTGACTACT-3' (sense), 5'-ATGC-TGTTATAGGTGGTGGT-3' (antisense); collagen I, 5'-TGACTGGAAGAGCGGAGAGTACT-3' (sense), 5'-GCTGTGGGCATATTGCACAA-3' (antisense); and  $\beta$ -actin 5'-CCGTGAAAAGATGACCCAGATC-3' (sense), 5'-CACAGCCTGGATGGCTACGT-3' (antisense). The steps used for RT-qPCR were as follows: 94°C for 2 min for initial denaturation; 94°C for 30 sec, 59°C for 15 sec, and 72°C for 20 sec; 2 sec for plate reading for 35 cycles; and a melt curve from 65 to 95°C. Relative quantification of gene expression was performed using the  $2^{-\Delta\Delta C_t}$  method and with  $\beta$ -actin mRNA as an internal control.

### Western blot

Total protein was extracted from hepatic fibrosis tissues or HSCs using RIPA Cell Lysis Buffer (Takara Biotechnology, Dalian, China) and protein concentrations were determined by the BCA method. Proteins (40  $\mu$ g protein/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Sigma, St. Louis, MO, USA). Then, nonspecific binding was blocked by incubating with 5 % non-fat milk in TBST buffer at room temperature for 1 h. The blots were incubated with primary antibodies (anti-eIF3a, anti-collagen I, anti- $\alpha$ -SMA, anti-Smad3, anti-p-Smad or GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Subsequently, the membrane was incubated in peroxidase-conjugated secondary antibody goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. The blots were visualized by super ECL and quantified by the Quantity ONE (Bio-Rad, Hercules, CA, USA) software.

### Cell proliferation assay

Cell proliferation was determined using the MTT assay. Briefly, the transfected cells were plated at a density of  $1.0 \times 10^4$  cells/well in a 96-well tissue culture plate and treated with or without TGF- $\beta$ 1. After incubation for 24 h, 20  $\mu$ l of MTT (5 mg/mL; Sigma, St. Louis, MO, USA) solution was added to each well for 4 h. Subsequently, the medium was replaced and the formazan crystals were dissolved in 150  $\mu$ l dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA). Absorbance at 490 nm was measured using a Bio-Rad microplate reader (Bio-Rad, Hercules, CA, USA).

### Statistical analysis

The statistical analysis was performed with SPSS 16.0 software (SPSS, Chicago, IL). Results were pre-

sented as mean  $\pm$  S.D. Statistical significance was analyzed with one-way ANOVA or Student's two-tailed *t* test. Statistical significance was determined at the level of  $P < 0.05$ .

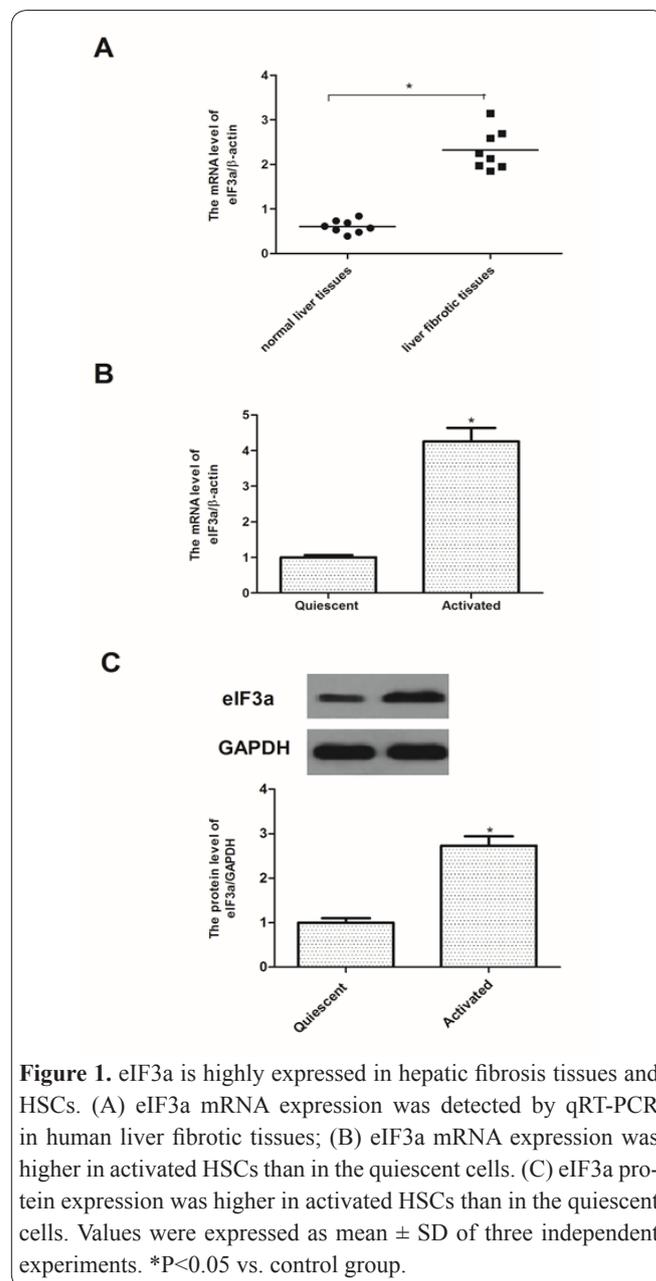
## Results

### eIF3a is highly expressed in hepatic fibrosis tissues and HSCs

To understand the role of eIF3a in the development of liver fibrosis, we examined the expression pattern of eIF3a in human hepatic fibrosis tissues and HSCs. As shown in Figure 1A, eIF3a mRNA expression is highly overexpressed in human hepatic fibrosis tissues compared with normal liver tissues. Similarly, the expression levels of mRNA and protein were also higher in activated HSCs than in the quiescent cells (Figure 1B and 1C).

### Knockdown of eIF3 inhibits the proliferation of activated HSC

To investigate whether high expression of eIF3a in HSCs might regulate the proliferation of HSCs, we generated eIF3a knockdown HSCs by transfection with



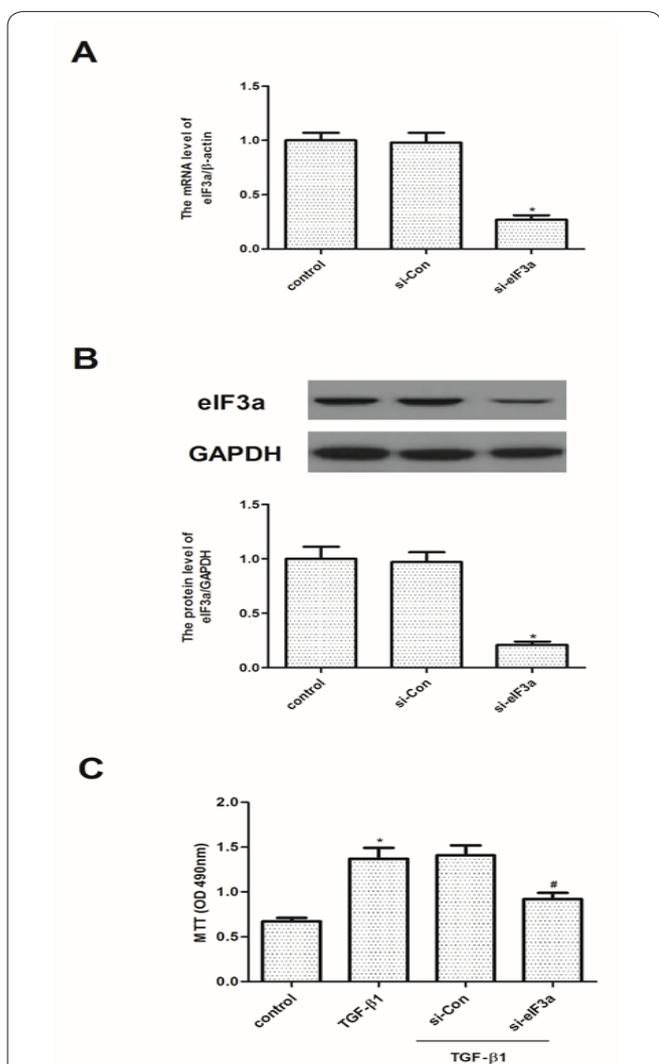
**Figure 1.** eIF3a is highly expressed in hepatic fibrosis tissues and HSCs. (A) eIF3a mRNA expression was detected by qRT-PCR in human liver fibrotic tissues; (B) eIF3a mRNA expression was higher in activated HSCs than in the quiescent cells. (C) eIF3a protein expression was higher in activated HSCs than in the quiescent cells. Values were expressed as mean  $\pm$  SD of three independent experiments. \* $P < 0.05$  vs. control group.

si-eIF3a. As shown in Figure 2A and 2B, the si-eIF3a significantly reduced eIF3a mRNA level to 27.3% of the si-Con and decreased eIF3a protein level to 21.2% of the si-Con.

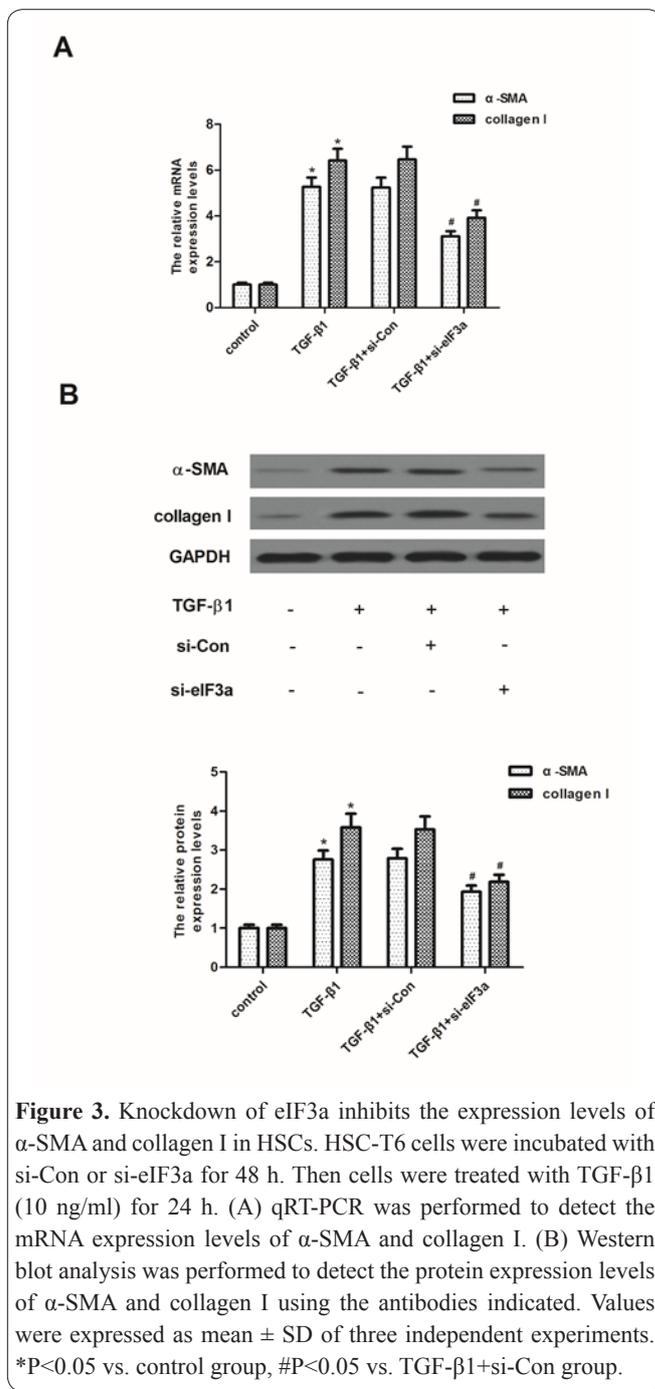
Then, we performed the MTT assay to examine the effect of eIF3a down-regulation on HSC proliferation. As shown in Figure 2C, compared with the control, TGF-β1 significantly increased the proliferation of HSCs. However, compared with the si-Con-transfected TGF-β1-treated HSCs, eIF3a significantly suppressed cell proliferation.

**Knockdown of eIF3a inhibits the expression levels of α-SMA and collagen I in HSCs**

Next, the effects of eIF3a down-regulation on TGF-β1-induced HSC activation and ECM protein production were examined. The results of qRT-PCR analysis showed that knockdown of eIF3a obviously inhibited the mRNA expression levels of α-SMA and collagen I in TGF-β1-stimulated HSCs (Figure 3A). Similarly, wes-



**Figure 2.** Knockdown of eIF3 inhibits the proliferation of activated HSC. HSC-T6 cells were incubated with si-Con or si-eIF3a for 48 h. (A) eIF3a mRNA expression was detected by qRT-PCR; (B) eIF3a protein expression was detected by western blot. HSC-T6 cells were incubated with si-Con or si-eIF3a for 48 h. Then cells were treated with TGF-β1 (10 ng/ml) for 24 h. (C) The cell proliferation was determined with MTT assay. Values were expressed as mean ± SD of three independent experiments. \*P<0.05 vs. control group, #P<0.05 vs. TGF-β1+si-Con group.

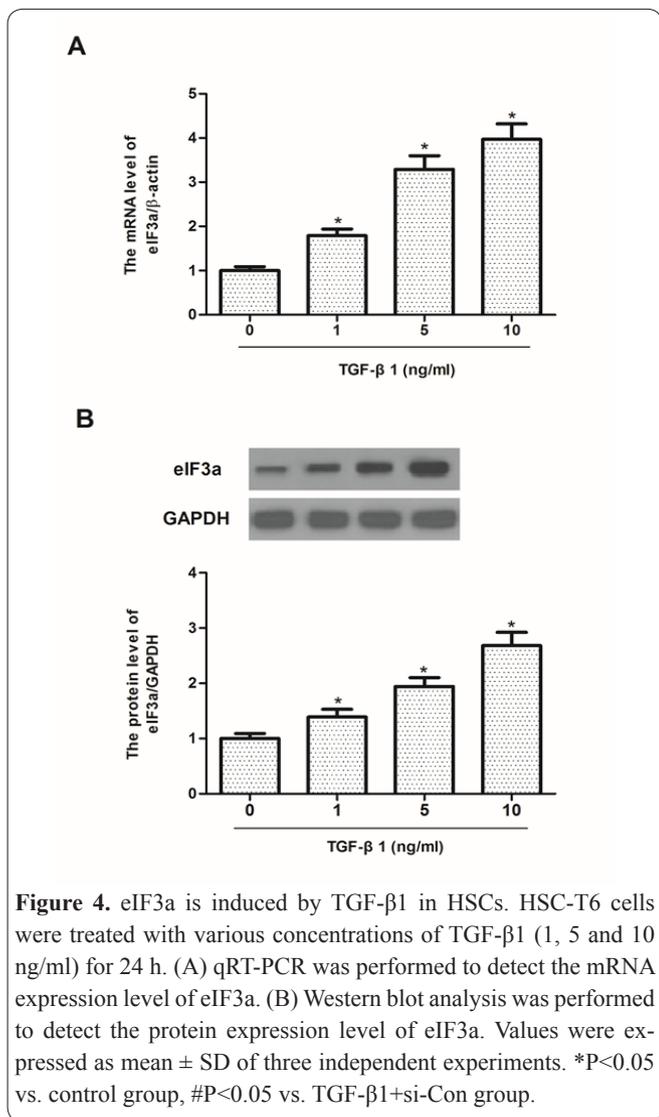


**Figure 3.** Knockdown of eIF3a inhibits the expression levels of α-SMA and collagen I in HSCs. HSC-T6 cells were incubated with si-Con or si-eIF3a for 48 h. Then cells were treated with TGF-β1 (10 ng/ml) for 24 h. (A) qRT-PCR was performed to detect the mRNA expression levels of α-SMA and collagen I. (B) Western blot analysis was performed to detect the protein expression levels of α-SMA and collagen I using the antibodies indicated. Values were expressed as mean ± SD of three independent experiments. \*P<0.05 vs. control group, #P<0.05 vs. TGF-β1+si-Con group.

tern blot analysis also demonstrated that eIF3a down-regulation resulted in a significant decrease in the protein expression levels of α-SMA and collagen I compared with si-Con-transfected TGF-β1-treated HSCs (Figure 3B).

**eIF3a is involved in the regulation of TGF-β1-mediated signaling pathways in HSCs**

To identify potential molecular mechanisms of eIF3a-mediated pro-fibrogenic effect during liver fibrosis, TGF-β1/Smad3 signaling pathway connected with liver fibrosis was investigated. We showed that TGF-β1 induced the elevation of eIF3a mRNA and protein levels both in dose-dependent manner (Figure 4A and 4B). Furthermore, eIF3a down-regulation remarkably decreased the phosphorylation of Smad3 in TGF-β1-induced HSCs. There was no statistical difference regarding to their inhibition abilities on Smad3 expression among different pretreatment groups (Figure 5).



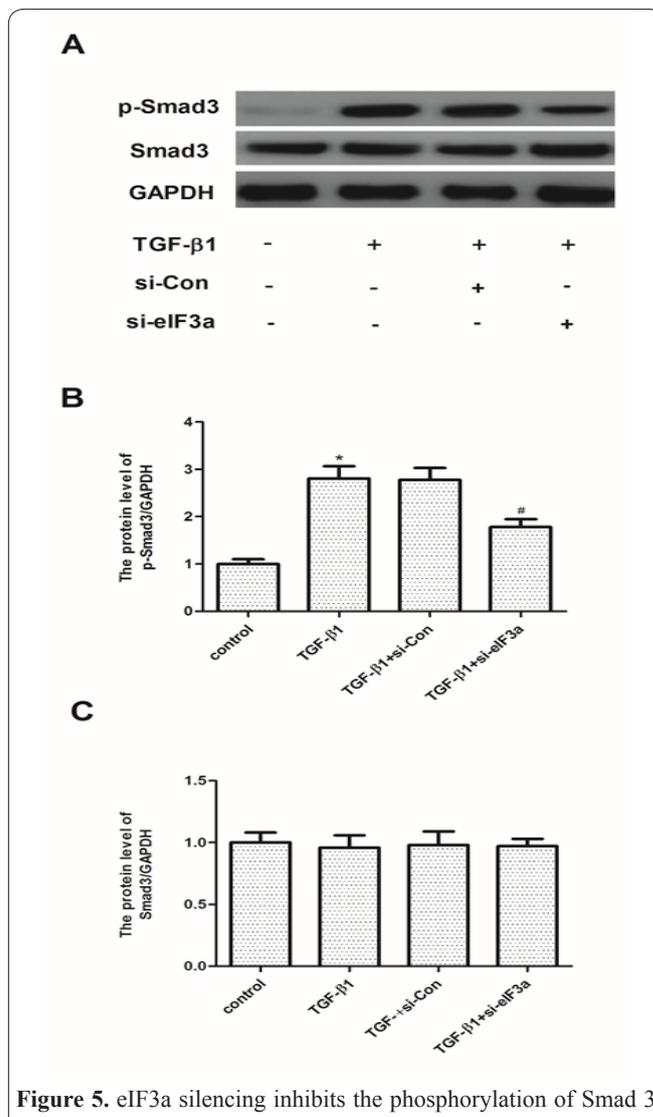
**Figure 4.** eIF3a is induced by TGF- $\beta$ 1 in HSCs. HSC-T6 cells were treated with various concentrations of TGF- $\beta$ 1 (1, 5 and 10 ng/ml) for 24 h. (A) qRT-PCR was performed to detect the mRNA expression level of eIF3a. (B) Western blot analysis was performed to detect the protein expression level of eIF3a. Values were expressed as mean  $\pm$  SD of three independent experiments. \* $P$ <0.05 vs. control group, # $P$ <0.05 vs. TGF- $\beta$ 1+si-Con group.

**Discussion**

In this study, our results showed the expression pattern of eIF3a was significantly increased in human hepatic fibrosis tissues and HSCs. Knockdown of eIF3a significantly suppressed HSC proliferation and the expression levels of  $\alpha$ -SMA and collagen I in TGF- $\beta$ 1-treated HSCs. Furthermore, eIF3a down-regulation remarkably decreased the phosphorylation of Smad3 in TGF- $\beta$ 1-induced HSCs.

eIF3a is a multifunctional protein and plays an important role in regulation of fibrosis. It was reported that the expression of eIF3a was obviously increased in lungs of pulmonary fibrosis rats (15). Li *et al.* confirmed that the expression of eIF3a was significantly up-regulated in right ventricle of right ventricular remodeling rats accompanied by elevation of  $\alpha$ -SMA and collagens (14). In agree with the previous studies, herein, we found that the expression pattern of eIF3a was significantly increased in human hepatic fibrosis tissues and HSCs, which suggested that eIF3a may play an important role in the process of liver fibrosis.

TGF- $\beta$ 1 is a potent stimulator of HSC proliferation and activation *in vitro* (16-18). Studies have shown that siRNA targeting TGF- $\beta$ 1 may inhibit the activation and proliferation of HSCs, and prevent liver fibrosis (19). In the present study, we used TGF- $\beta$ 1 to induce HSC activation and found that eIF3a down-regulation signifi-



**Figure 5.** eIF3a silencing inhibits the phosphorylation of Smad 3 in TGF- $\beta$ 1-stimulated HSC-T6 cells. HSC-T6 cells were incubated with si-Con or si-eIF3a for 48 h. Then cells were treated with TGF- $\beta$ 1 (10 ng/ml) for 30 min. (A) The expression of p-Smad3 and Smad3 were detected by western blot analysis. The relative protein expression levels of p-Smad3 (B) and Smad3 (C) were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Values were expressed as mean  $\pm$  SD of three independent experiments. \* $P$ <0.05 vs. control group, # $P$ <0.05 vs. TGF- $\beta$ 1+si-Con group.

cantly suppressed TGF- $\beta$ 1-induced HSC proliferation. Thus our data strongly suggest that eIF3a silencing attenuated liver fibrosis by inhibition of TGF- $\beta$ 1-induced HSC proliferation.

Numerous studies have reported that liver fibrosis results from the excessive synthesis and deposition of ECM (20-22). In addition, TGF- $\beta$ 1 can induce the activation of HSCs to produce a large amount of ECM (23, 24), and knockdown of TGF- $\beta$ 1 inhibits ECM synthesis in HSCs (19). Similarly, in the current study, we found that TGF- $\beta$ 1 treatment increased the expression levels of  $\alpha$ -SMA and collagen I in HSCs, and eIF3a silencing significantly reduced their expression. Thus our data strongly suggest that eIF3a silencing attenuated liver fibrosis by reducing the amount of ECM in HSCs.

Activation of the TGF- $\beta$  pathway is considered a key event in the development of liver fibrosis and the blunting of TGF- $\beta$  signaling in HSCs can effectively prevent liver fibrosis (25-27). During fibrogenesis, TGF- $\beta$ 1

exerts its biological functions through interaction with TGF- $\beta$ 1 receptors, which are composed of type I (TbetaRI) and type II (TbetaRII) receptors. After TGF- $\beta$  binding to the receptor complex, Smad 2/3 is phosphorylated and binds with Smad4 to form multimers, then activated R-Smads translocate to the nucleus and induce the expression of target genes, including ECM proteins (28). Most recently, one study showed that knockdown of Smad3 significantly reduced TGF- $\beta$ 1-induced collagen I production (29). In the present study, we found that eIF3a down-regulation remarkably decreased the phosphorylation of Smad3 in TGF- $\beta$ 1-induced HSCs. These results suggest that si-eIF3a exerts its anti-fibrosis effects on HSCs activation and collagen synthesis via a significant suppression on the phosphorylation of Smad3 *in vitro*.

Overall, the current study found that eIF3a may function as a novel regulator to modulate HSC activation, potentially through inhibiting the TGF- $\beta$ 1/Smad3 signaling pathway. These results introduce a new aspect of eIF3a biology potentially related to the pathogenesis of liver fibrosis. Thus, eIF3a inhibition appears to be a promising strategy for the prevention of liver fibrosis.

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