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ORIGINAL ARTICLE

Effect of shRNA mediated Smad4 gene silencing on the fibrosis of C2C12 myoblasts

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Summary
Our present study aimed to investigate the effect of lentiviral-mediated RNAi using short hairpin RNA (shRNA) targeting Smad4 on TGF-β1 induced fibrosis. shRNAs targeting Smad4 were designed and the most efficient shRNA was screened. This shRNA was introduced into a lentiviral vector which was used to infect C2C12 myoblasts, and then the Smad4 expression was detected. Cells were divided into: C2C12 cells group, TGF-β1 induction group, transfection group, and transfection after TGF-β1 induction group. C2C12 myoblasts were transfected with lentivirus carrying Smad4-shRNA and treated with TGF-β1 to induce the differentiation into myofibroblasts. Fluorescence Real-time-PCR and the western blot assay were employed to detect the expressions of collagen I and α-SMA. The results showed that the protein and mRNA expression of Smad4 in the C2C12 cells transfected with Smad4-shRNA1 was significantly reduced when compared with C2C12 before transfection. In the TGF-β1 induction group, the mRNA expressions of α-SMA and collagen I were significantly increased as compared to the C2C12 cells group. In the transfection after TGF-β1 induction group, the mRNA expressions of α-SMA and collagen I were significantly increased compared to the transfection group, and the protein expressions significantly increased, respectively. In the transfection after TGF-β1 induction group, the mRNA expressions of α-SMA and collagen I were significantly decreased compared to the TGF-β1 induction group, and the protein expressions significantly reduced, respectively. The results indicate that suppression of Smad4 expression can efficiently inhibit the TGF-β1 induced fibrosis in myoblasts. The findings suggest Smad4 may become a novel target for the treatment of skeletal muscle fibrosis.

Key words: skeletal muscle injury; fibrosis; transforming growth factor-β1; C2C12 myoblast

INTRODUCTION

Skeletal muscle injury is one of the most common sports injuries. However, the natural recovery of skeletal muscle injury requires a relatively long time, the quality of skeletal muscle after recovery is unreliable, and local scars frequently form after recovery. Scars have biomechanical defects which may cause a second injury of the skeletal muscle (Orchard 2001). There is evidence showing that the local expression of the transforming growth factor-β1 (TGF-β1) is significantly increased following skeletal muscle injury (Jackson et al. 2011). TGF-β1 can stimulate the synthesis of extracellular matrix (Cheng and Grande 2002), and has been found to be a key cytokine causing the development of fibrosis. Studies also demonstrate that the inhibition of the TGF-β1

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signalling pathway may also counteract with the fibrosis of the heart, lung, liver and kidney (Tse et al. 2004, Matsuzaki 2009, Bran et al. 2010). Smad2 and Smad3 are the downstream substrates of the TGF-β receptor. Following activation, TGF-β1 ineluctably binds to the Smad4, forming a complex which then enters the nucleus directly or indirectly inducing cell differentiation (Li et al. 2010, 2011). We hypothesize that lentiviral-mediated interference with Smad4-shRNA can significantly inhibit Smad4 expression in myoblasts and thus the Smad2 and Smad3 can not enter the nucleus of muscle cells (Kretschmer et al. 2003, Zhu et al. 2004), which then inhibit the TGF-β1 induced skeletal muscle fibrosis.

MATERIALS AND METHODS

Materials

C2C12 myoblasts were purchased from the Wuhan Cell Bank. The following reagents were used in the present study: TRIzol (Invitrogen, Carlsbad, USA), Real-time PCR kit (TaKaRa Biotechnology Co., Ltd., Dalian, China), ECL kit (Pierce Biotechnology, Inc., Rockford, USA), goat antimouse Smad4 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, USA), GAPDH and anti-mouse IgG (Cell Signalling Technology, Inc., Danvers, USA), Lentivirus Package plasmid mix (System Biosciences, Mountain View, USA), recombinant lenti-siRNA1 plasmid expression vector (our lab), 293TN cells (System Biosciences), 0.25% trypsin (GIBCO®), fetal bovine serum (FBS, GIBCO®), DMEM high glucose (Invitrogen, Carlsbad, USA), Opti-MEM (GIBCO®), Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) and PVDF membrane (Millipore, Billerica, USA).

Cell culture

C2C12 myoblasts were maintained in complete medium (DMEM high glucose with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2.

Preparation of myofibroblasts

The TGF-β1 was used to induce the differentiation of C2C12 myoblasts into myofibroblasts according to the method previous described by Ono et al. (2007). The C2C12 myoblasts were grown in the complete medium containing 5 ng/ml TGF-β1 at 37 °C in a humidified atmosphere with 5% CO_2 for 72 h. The expressions of α-SMA and collagen I were measured by immunofluorescent staining daily. Light microscopy was performed to observe whether these cells were long-spindle shaped or polygonal myofibroblasts, which help determine the differentiation of myoblasts. Trypan blue staining was performed to exclude the unviable cells.

Construction of lentivirus carrying Smad4-shRNA and cell transfection

The siRNA targeting mouse Smad4 sequence (GenBank accession No. NM_008540) and scramble siRNA were designed according to the method described in http://www.invitrogen.com/rnai. The single-stranded DNA corresponding to siRNA was used to synthesize double-stranded oligonucleotide. The shRNA1, shRNA2, shRNA3 and scramble shRNA are shown in Table 1.

The NIH3T3 cells were seeded into 6-well plates (3×10^5 cells/well) followed by incubation for 24 h and subsequent transfection. The siRNA expression vectors were used to transfect NIH3T3 cells using Lipofectamine TM 2000 according to the manufacturer's instructions. Two days after transfection, the total RNA was extracted using TRIzol according to the manufacturer's instruction and then reverse transcribed into cDNA. Then, the cDNA was used for amplification using the Real-time PCR kit to measure the mRNA expression of Smad4, and β-actin served as an internal reference. The primers were as follows:

Smad4: 5'-CGGCCGTGGCAGGGAACA-3' (forward);
5'-CTGCAGAGCTCGGTGAAGGTGAAT-3' (reverse);
β-actin: 5'-CCTCTATGCCAACACAGTGC-3' (forward);
5'-GTACTCCTGCTTGCTGATCC-3' (reverse).

PCR was carried out in 20 μl of reaction mixture and the conditions for PCR were as follows: 40 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 20 s and extension at 72 °C for 20 s. The products were then subjected to 1% agarose gel electrophoresis.

Two days after transfection, cells were lysed in the buffer containing 20 nM Tris-HCl (pH 7.4), 150 mM NaCl and 1% PMSF. The extracted protein of equal content was loaded onto 8% Tris glycine SDS buffer followed by SDS-PAGE. Then, the proteins were transferred onto PVDF membranes which were blocked in 5% slim milk in TBST for 1 h. Subsequently, the membranes were treated with Smad4 antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, USA) or GAPDH (1:200, Cell Signalling Technology, Inc., Danvers, USA) in TBST at 4 °C overnight and then with HRP conjugated anti-mouse IgG (1:6000, Cell Signalling Technology) at room temperature for 2 h. Visualization was carried

Table 1. **shRNA sequence for interfering.**

shRNA	Oligonucleotide sequences
shRNA1	Forward: 5'-GATCCGGATGAGTACGTTCACGACCTTCCTGTCAGAGTCGTGAACGTACTCATCCTTTTTG
	Reverse: 5'-AATTCAAAAAGGATGAGTACGTTCACGACTCTGACAGGAAGGTCGTGAACGTACTCATCCG
shRNA2	Forward: 5'-GATCCGTAATCGCGCATCAACGGACTTCCTGTCAGATCCGTTGATGCGCGATTACTTTTTG
:	Reverse: 5'-AATTCAAAAAGTAATCGCGCATCAACGGATCTGACAGGAAGTCCGTTGATGCGCGATTACG
shRNA3	Forward: 5'-GATCCGTAGGACTGCACCATACACCTTCCTGTCAGAGTGTATGGTGCAGTCCTACTTTTTG
	Reverse: 5'-AATTCAAAAAGTAGGACTGCACCATACACTCTGACAGGAAGGTGTATGGTGCAGTCCTACG
Scr	Forward: 5'-GATCCCGTTTAACTCTCCCAACCACTTCCTGTCAGATGGTTGGGAGAGTTAAACGTTTTTG
	Reverse: 5'-AATTCAAAAACGTTTAACTCTCCCAACCATCTGACAGGAAGTGGTTGGGAGAGTTAAACGG

Notes: a total of 3 shRNAs and a scramble shRNA were designed (Scr: scramble shRNA).

out with ECL kit to detect the protein expression of Smad4. The most efficient shRNA1 was used to construct lentivirus for the transfection of 293TN cells according to the description in Lentivirus Package plasmid mix (System Biosciences, Mountain View, USA).

Construction of cell line with stable Smad4-shRNA expression

C2C12 cells were seeded into 96-well plates followed by incubation for 24 h. Then, lentivirus was added to these cells at the MOI of 6 followed by incubation at 37 °C in an atmosphere with 5% CO_2. Three days later, fluorescence microscopy was carried out to detect the expression of green fluorescent protein. According to the fluorescence intensity, the colonies with stable expression were selected for passaging.

Effect of lentiviral-mediated RNA interference against Smad4 on TGF-β1 induced fibrosis of myofibroblasts

Cells were divided into groups A, B, C and D. In group A, the C2C12 cells were untreated. The C2C12 cells in the group received TGF-β1 induction. In group C, the C2C12 cells were transfected with lentivirus. In group D, the C2C12 cells underwent TGF-β1 induction and were then transfected with lentivirus.

Three days after culture, the GFP expression in group C was observed under a fluorescence microscope. Real-time PCR and Western blot were employed to detect the mRNA and protein expressions of Smad4 in the cells of groups A and C which had high expression of GFP. The method and procedures were similar to those described in the "Construction of lentivirus carrying Smad4-shRNA and cell transfection". In addition, Real-time PCR, Western blot and immuno-fluorescence staining were carried out to detect the expressions of α-SMA and collagen I in the four groups.

1) Real-time PCR:

The primers were as follows: α-SMA: 5'-CTGAAGAGCATCCCACCCT-3' (forward), 5'-TCTCCAGAGTCCAGCACGAT-3' (reverse). Collagen I: 5'-GAGCGGAGAGTACTGGATCGA-3' (forward), 5'-CTGACCTGTCTCCATGTTGCA-3' (reverse).

2) Western blot assay:
   The primary antibodies were α-SMA (1:1,500, Thermo) and collagen I (1:200, Merck). The secondary antibody was anti-mouse IgG (1:1000, Cell Signaling).
3) Immunofluorescence staining: cells were seeded into 6-well plates and washed in PBS. Then, these cells were fixed in 4% paraformaldehyde at room temperature for 15 min followed by perforation with TritonX-100 for 30 min. These cells were subsequently treated with 5–7% goat serum at room temperature for 30 min followed by incubation with α-SMA antibody (1:1,500, Thermo) or collagen I (1:200, Merck) overnight. After washing in PBS, cells were treated with anti-mouse IgG (1:1000, Cell Signalling) and Topro-3 in 0.01% Triton X-100 in PBS (pH 8.0) at room temperature for 2 h. After washing in PBS, these cells were observed under confocal microscope.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was carried out with SPSS version 11.0 (IBM, Armonk, USA). Comparisons between two groups were carried out with t test at the significance level of $2\alpha=0.05$.

RESULTS

Effect of different shRNAs on the Smad4 expression

The Real-time PCR and Western blot assay showed that three shRNAs could significantly inhibit the protein and mRNA expressions of Smad4 which however remained unchanged in the scramble shRNA group (Fig. 1). As shown in Fig. 1B, shRNA1 inhibited the Smad4 expression by 60% (statistically significant). Considering that the transfection efficacy was about 75%, the actual silencing efficacy of shRNA could reach higher than 80%. Thus, shRNA1 was used for the construction of lentivirus.

Lentiviral-mediated Smad4-shRNA1 transfection and suppressed protein expression of Smad4

Immunofluorescence staining showed that about 98% of C2C12 cells were transfected with lentivirus carrying Smad4-shRNA at 2 days after transfection at a MOI of 6. Furthermore, the GFP expression was not significantly reduced in these transfected cells of passage 3. Real-time PCR and Western blot assay showed the cells with high GFP expression had low Smad4 expression when compared with cells in the control group. Furthermore, the mRNA expression was decreased by 85% and the protein expression reduced by 86.93% in the lentiviral mediated Smad4-shRNA1 transfection group (statistically significant) (Fig. 2).

Effect of Smad4-shRNA1 on the TGF-β1 induced expressions of α-SMA and collagen I

In the present study, TGF-β1 was used to induce the differentiation of myoblasts into myofibroblasts (Ono et al. 2007). Immunohistochemistry was employed to detect the expressions of α-SMA and collagen I. In this experiment, cells were divided into groups A, B, C and D which were untreated C2C12 cells, C2C12 cells receiving TGF-β1 induction, C2C12 cells which were transfected with lentivirus carrying Smad4-shRNA and the C2C12 which were transfected with lentivirus carrying Smad4-shRNA and underwent TGF-β1 induction, respectively. The results showed that the mRNA expressions of α-SMA and collagen I in group B were increased by 34.41% and 29.04% (statistically significant), respectively, when compared with group A, and the protein expression increased by 72.14% and 79.33% (statistically significant), respectively. In the group D, the mRNA expressions of α-SMA and collagen I were increased by 6.36% and 7.22% (statistically significant), respectively, compared to group C and the protein expressions elevated by 5.12% and 7.31% (statistically significant), respectively. When compared with group B, the mRNA expressions of α-SMA and collagen I in group D were decreased by 32.51% and 35.17% (statistically significant), respectively, and the protein expressions of α-SMA and collagen I reduced by 65.06% and 72.02% (statistically significant), respectively (Fig. 3).

DISCUSSION

Skeletal muscle injury is a common disease in sports medicine. According to recent statistics, skeletal muscle injury accounts for 31% of all sports injuries (Ekstrand et al. 2011). Studies show that the satellite cells play an important role in the repair following skeletal muscle injury. Normally, the satellite cells are in a quiescent state and have no expression of muscle proteins. Following skeletal muscle injury, these satellite cells are activated, express a lot of muscle regulatory factors in the presence of integrate basal lamina, and synthesize the functional proteins in the muscles leading to the formation of muscle fibers (Charge and Rudnicki 2004, Wagers and Conboy 2005).

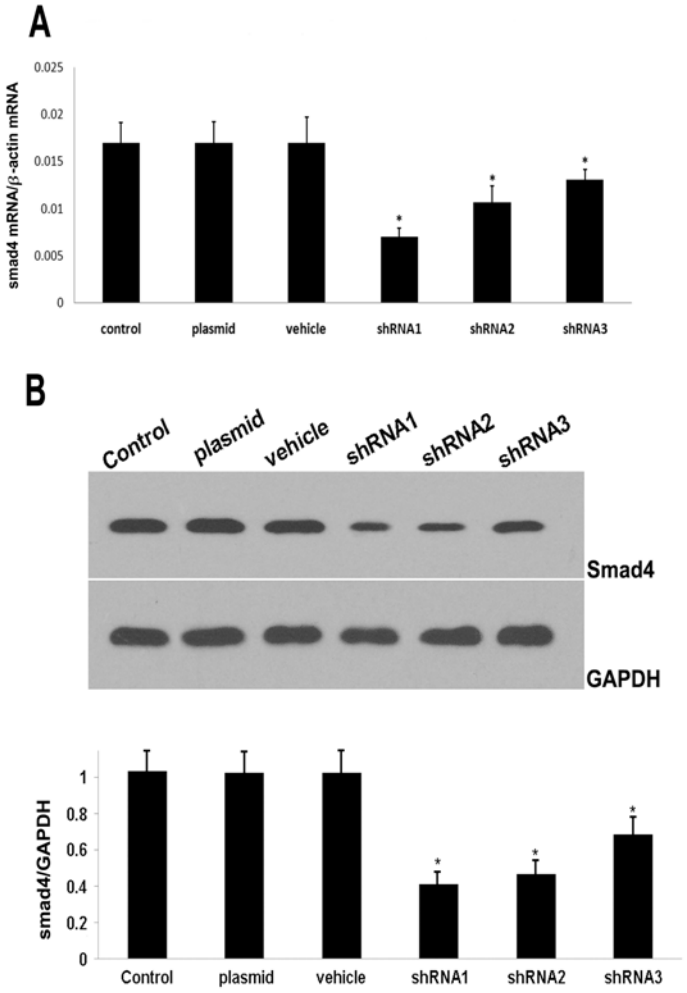

Fig. 1. **Effect of Smad4-shRNA on the expression of Smad4.** shRNA was used to transfect C2C12 cells using Lipofectamine 2000. Two days later, RT-PCR (A) and Western blot assay (B) were employed to detect the silencing efficacy. The mRNA and protein expressions of Smad4 were significantly reduced. *Statistically significant as compared with normal C2C12 cells.

Repair following skeletal muscle injury is a hot topic in sports medicine. It has been found that some cytokines can promote the regeneration of skeletal muscle or inhibit skeletal muscle fibrosis following injury, and thus the gene therapy of skeletal muscle injury has attracted increasing attention from researchers. However, in studies on skeletal muscle injury, the therapeutic efficacy of various measures to promote the recovery of skeletal muscle injury is still unsatisfactory and treatment with these measures is often accompanied by fibrosis of the injured skeletal muscles and the formation of scars (Musaro 2005, Urish et al. 2005). Li and Huard (2002) were the first to report the differentiation of myoblasts into the precursors of myofibroblasts following skeletal muscle injury in a mouse model. Therefore, the activation of myoblasts is usually accompanied by the development of abnormal fibrosis. Thus, to block the differentiation of myoblasts into myofibroblasts may be an effective way to prevent skeletal fibrosis following skeletal muscle injury.

Over deposition of extracellular matrix including type I and III collagen may cause tissue fibrosis. TGF-β1 is a critical cytokine that can promote the synthesis and deposition of extracellular matrix. TGF-β1 can induce the differentiation of different

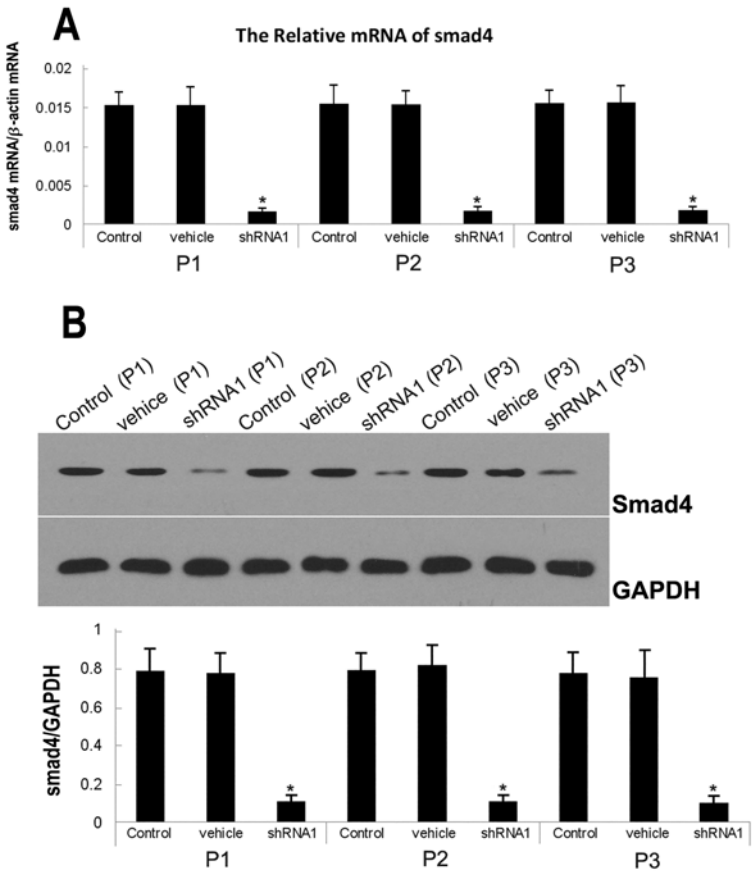

Fig. 2. **Effect of lentiviral mediated Smad4-shRNA1 transfection on the protein expression of Smad4.** Three days after transfection, the Real-time PCR(A) and Western blot analysis(B) showed a lower expression of Smad4 that was mantained in three generations in the lentiviral mediated Smad4-shRNA1 transfected group. *Statistically significant as compared with the control (non-tranfected) and vehicle groups.

mesenchymal cells (fibroblasts, stromal cells, satellite cells and myoblasts) into myofibroblasts (Powell et al. 1999, Leask and Abraham 2004, Li et al. 2004). In the present study, TGF-β1 was used to induce the differentiation of C2C12 cells. In group B, C2C12 myoblasts received differentiation induction with TGF-β1 for 72 h, and results showed the expressions of α-SMA and collagen I, two markers of differentiation into myofibroblasts, at both mRNA and protein levels (statistically significant). This demonstrates that TGF-β1 can inhibit the myogenic differentiation of C2C12 cells and induce the differentiation of C2C12 myoblasts into myofibroblasts (Wicik et al. 2010).

The signal transduction of TGF-β1 depends on the serine kinase phosphorylation of its receptor as well as the activation of transcriptional factors including Smad, MAPK and PI3K/Akt. Ghosh et al. (2001) reported that TGF-β1 could regulate the human a2(I) collagen promoter via the Smad signalling pathway which resulted in the abnormal synthesis and deposition of collagens and a fibrotic response. By using RNA interference, the expressions of down-stream proteins of TGF-β1 signal (Smad2 and Smad3) are inhibited, exhibiting an anti-fibrotic effect, which has been confirmed to be effective in some studies. Gressner et al. (2009) reported that to decrease the Smad2 expression could effectively inhibit the hepatic fibrosis, and Wang et al. (2007) also reported that down-regulation of Smad3 could suppress the synthesis of collagens in skin scars. Following the activation of Smad2 and Smad3

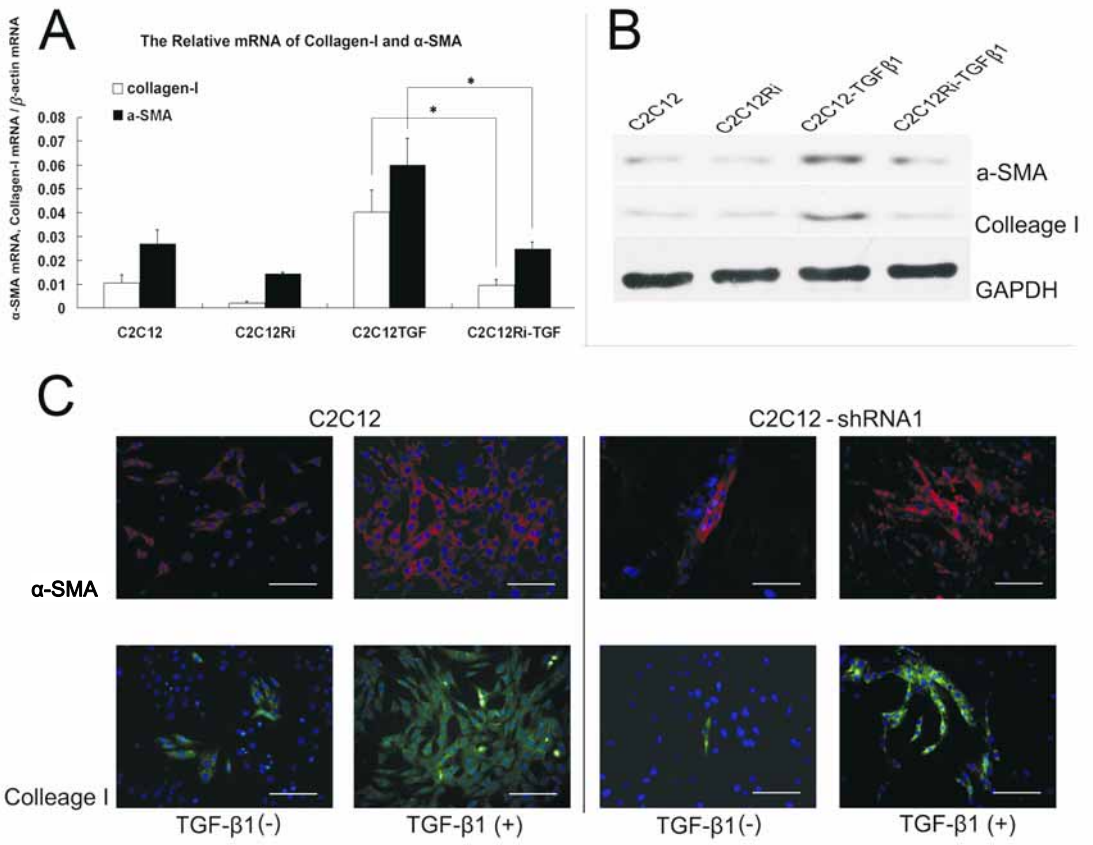

Fig. 3. **Expressions of α-SMA and collagen I following transfection of lentivirus carrying Smad4-shRNA1.** Smad4 could down-regulate the TGF-β1 induced differentiation of C2C12 myoblasts into myofibroblasts. C2C12 cells transfected with lentivirus carrying shRNA and those transfected with blank lentivirus were maintained in the presence of TGF-β1 for 72 h. Fluorescent quantitative PCR (A), Western blot (B) and immunofluorescence (C) were employed to detect the expressions of α-SMA and collagen I. The expressions of α-SMA and collagen I of Smad4-lentivirus mediated C2C12 cells significantly decreased. *Statistically significant as compared with bland letivirus mediated C2C12 cells under TGF-β1 induction, scale: 200 μm.

signals, the initiation of gene transcription depends on the translocation of these proteins into the nucleus. Smad4 is a type of coordination Smad protein, and can bind to the activated Smad 1, 2, 3, 5 or 8 following phosphorylation forming heterodimers, which then translocate into the nucleus and subsequently initiate the gene transcription resulting in cell differentiation. Thus, to inhibit the Smad4 expression may be beneficial for the treatment of fibrosis.

Short hairpin RNA can induce the degradation of mRNAs and silence the gene expression (Nishikawa and Sugiyama 2010). Our results also demonstrate the mRNA and protein expressions of Smad4 in the C2C12 cells transfected with lentivirus carrying Smad4-shRNA were reduced by 85% and 86.93%, respectively, which suggests the Smad4 is significantly down-regulated. In addition, the α-SMA and collagen I expressions in the C2C12 cells transfected with lentivirus carrying Smad4-shRNA in the presence of induction with TGF-β1 are reduced by 65.06% and 72.02% when compared with those in normal C2C12 cells following induction with TGF-β1, which indicates that Smad4 plays a crucial role in TGF-β1 induced fibrosis. Together with the aforementioned, we speculate that Smad4 is also a critical factor in TGF-β1 induced fibrosis. However, the specific mechanism and the role of other signalling pathways are required to be elucidated.

Taken together, our results demonstrate that inhibition of Smad4 expression by using shRNA can effectively inhibit the TGF-β1 induced fibrosis of C2C12 myoblasts. Thus, Smad4 may become a novel target for the treatment of the fibrosis of skeletal muscle.

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