TGF- β 1-Induced Pulmonary Fibroblasts Proliferation and Differentiation Are Inhibited by Antisense Oligodeoxynucleotide of Basic Fibroblast Growth Factor

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Received December 16, 2014; accepted June 14, 2015

Summary The objective of this study is to investigate the role of antisense oligodeoxynucleotide (ASODN) of basic fibroblast growth factor (bFGF) in the proliferation, differentiation and collagen synthesis of pulmonary fibroblasts (PFs) and the possible mechanism. PFs were isolated from male Wistar Rat and transfected with antisense oligodeoxynucleotide or sense oligodeoxynucleotide (SODN) of bFGF for four experimental conditions: (1) control group: PFs without TGF- β 1; (2) TGF- β 1 group: PFs with TGF- β 1; (3) TGF- β 1+ASODN group: ASODN transfected PFs with TGF- β 1; and (4) TGF- β 1+SODN group: SODN transfected PFs with TGF- β 1. Proliferation of PFs was monitored by growth curves and MTT assays, expression of α -smooth muscle actin (α -SMA) was evaluated by immunocytochemistry, concentrations of bFGF, CTGF, and Type I collagen in culture supernatants were determined by ELISA, and mRNA expression of Smad3, Smad7 and Type I collagen in pulmonary fibroblasts was detected by RT-PCR. We found that SODN of bFGF increased cell proliferation of PFs, enhanced the expression level of bFGF, CTGF, α -SMA, type I collagen and Smad3. However, ASODN of bFGF had the opposite effects. However, Smad7 was increased in TGF- β 1+SODN group but decreased in TGF- β 1+ASODN group. The proliferation, differentiation, and collagen synthesis of PFs could be promoted by bFGF and inhibited by ASODN of bFGF which may be related to the regulation of TGF- β 1-Smad signaling pathway.

Key words Basic fibroblast growth factor, Antisense oligodeoxynucleotide, Pulmonary fibroblast, Proliferation, Differentiation.

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive disease with a rotten prognosis for which, until recently, there were no effective treatments. Pathologically, IPF is characterized by general interstitial pneumonia with excessive fibroblast proliferation and the deposition of large amounts of extracellular matrix (ECM) in the interstitium and matrix remodeling, which leads to distortion of the alveolar architecture, progressive declines in lung function, and ultimately, loss of lung function (American Thoracic Society and European Respiratory Society 2002, Noble *et al.* 2012).

Pulmonary fibroblasts play an important role in the synthesis of collagens and ECM in the pulmonary interstitium. In idiopathic pulmonary fibrosis, there are numerous fibroblast foci in pulmonary connective tissues. These foci are primarily comprised of pulmonary fibroblasts and myofibroblasts, which differentiate from fibroblasts in the pulmonary interstitium. Both fibroblasts and myofibroblasts predominantly secrete Types I and III collagen, the major components of the ECM (Wolters *et al.* 2014). TGF- β 1-Smad signaling pathway is considered to be the most important regulation pathway for proliferation, differentiation and collagen synthesis of pulmonary fibroblasts

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DOI: 10.1508/cytologia.80.331

(Lee *et al.* 2014).

Basic fibroblast growth factor (bFGF) is a potent chemotactic and mitogenic factor for smooth muscle cells, fibroblasts and myofibroblasts (Davis *et al.* 1997, Berndt *et al.* 1995). Previous studies found that quantities of bFGF have been detected in bronchoalveolar lavage fluid and in lung tissue of patients with acute lung injury and pulmonary fibrosis (Henke *et al.* 1991, Inoue *et al.* 1996). Mast cells, a major source of bFGF, have been found to aggregate in areas of pulmonary fibroblast hyperplasia in the lungs of patients with chronic pulmonary fibrosis (Qu *et al.* 1995, Reed *et al.* 1995). Our previous study also found that mast cells which express high levels of bFGF induced rat pulmonary fibroblast proliferation and differentiation (Cheng *et al.* 2012). These findings suggested that bFGF was closely related to the proliferation and differentiation of pulmonary fibroblasts. But the effects of bFGF on the proliferation, differentiation and collagen synthesis of pulmonary fibroblasts and the possible mechanism is still unknown. Based on these findings, the present study is designed to investigate the effects of bFGF on the proliferation, differentiation and collagen synthesis of pulmonary fibroblasts by transfecting rat pulmonary fibroblasts with antisense oligodeoxynucleotide (ASODN) and sense oligodeoxynucleotide (SODN) of bFGF *in vitro* and the relationship with the Smad signaling pathway.

Materials and methods

Ethics statement

This study was approved by The Medical Animal Care & Welfare Committee of Zhongnan Hospital, Wuhan University. All animal work in this study was conducted according to relevant national and international guidelines.

Animals

Ten adult male Wistar rats (2.5±0.22 months old; 211.1±4.46 g) were purchased from the Laboratory Animal Center of Wuhan University.

Reagents and antibody

RMPI-1640 medium, fetal bovine serum (FBS), penicillin–streptomycin, 2-mercaptoethanol, phosphate buffered saline (PBS), lipofectamine 2000 and 0.05% trypsin/0.02% EDTA solution were obtained from Invitrogen-GIBCO (Grand Island, NY, USA). The recombinant TGF- β 1 was obtained from R&D Systems (Minneapolis, MN, USA). The bFGF, Type I collagen ELISA kits and the MTT kits were purchased from R&D Systems (Minneapolis, MN, USA). The CTGF ELISA kit was purchased from CUSABIO. The anti-mouse α -SMA mAb were purchased from Abcam (Cambridge, UK).

Oligodeoxynucleotide

The antisense oligodeoxynucleotide (ASODN) which was complementary with the gene of bFGF mRNA in rat was synthesized by Augct (Beijing, China). The gene sequence of ASODN was 5'-CGG CAG CCA TGG CCC-3' and the oxygen was replaced by sulfur. In addition, the gene sequence of the sense oligodeoxynucleotide (SODN) which was 5'-GGG CCA TGG CTG CCG-3' was synthesized by Augct.

Isolation and culture of primary pulmonary fibroblasts

Rats were put to death through anesthetizing by intraperitoneal injection of urethane (1 g kg⁻¹) and then immersed in disinfectant. Thoracotomy and laparotomy were performed in an aseptic environment and rats were perfused transcardially with Ca²⁺ and Mg²⁺ free PBS through the right ventricle. The abdominal aorta was opened and artificial ventilation was provided *via* endotracheal

intubation. When the lungs became grey, they were lavaged with Ca^{2+} and Mg^{2+} free PBS (50 mL) through the endotracheal tube. The lungs were harvested aseptically and cut into pieces. These pieces were washed with distilled water containing penicillin (1 : 1000) and streptomycin (1 : 400) and then three times with RPMI-1640 medium. The lung pieces were digested in RPMI-1640 containing 3 mg mL^{-1} trypsin and 2.5 mg mL^{-1} DNase I three times at 37°C in a 5% CO_2 atmosphere (fresh solution was prepared for each digestion), 30 min once. After each round of digestion, the supernatant was collected and mixed with an equal volume of RPMI-1640 containing 15% newborn calf serum (NBCS). This mixture was filtered through a mesh and the solution was centrifuged at 1000 rpm at 25°C for 8 min. Cells were re-suspended in RPMI-1640 containing 10% NBCS and then seeded into flasks followed by incubation at 37°C in a 5% CO_2 atmosphere for 24 h. Adherent cells were pulmonary fibroblasts. Pulmonary fibroblasts were cultured in RPMI 1640 medium supplemented with 10% FBS, $100 \mu\text{g mL}^{-1}$ L-glutamine, 100 U mL^{-1} penicillin, $100 \mu\text{g mL}^{-1}$ streptomycin, and $50 \mu\text{M}$ 2-mercaptoethanol in an atmosphere of 5% CO_2 and 95% air at 37°C .

Identification of primary pulmonary fibroblasts

Inverted phase contrast microscope was used to observe the morphology of isolated cells. The cover slips that were prepared for the immunohistochemistry were placed into flasks for the adhering of the lung fibroblasts. Cell-coated cover slips were fixed in acetone for 10 min at 4°C . Immunohistochemistry using a streptavidin–peroxidase method was performed according to the manufacturer's instructions. The cover slips were washed in PBS and then treated with 3% H_2O_2 in methanol to inactivate endogenous peroxidase. Subsequently, cover slips were blocked with normal goat serum and then treated with primary antibody (anti-vimentin monoclonal antibody) and secondary antibody (biotin-conjugated goat anti-mouse IgG). Subsequently, cover slips were incubated with HRP conjugated streptavidin (S-A/HRP) followed by visualization at room temperature with DAB. Counterstaining was with hematoxylin followed by dehydration and mounting with neutral gum. Cells were observed under a light microscope.

Cell transfection

The lung fibroblasts from passage 2 which were digested to $1 \times 10^6 \text{ cells mL}^{-1}$ were seeded in six-well plates with serum-free RPMI 1640 medium. The $100 \mu\text{L}$ RPMI-1640 medium with $100 \mu\text{g}$ ASODN (or SODN) of bFGF and the $100 \mu\text{L}$ RPMI-1640 medium with $4 \mu\text{L}$ Lipofectamine 2000 were mixed uniformly and placed in room temperature for 20 min. The mixture was added in six-well plates which were cultured in an atmosphere of 5% CO_2 and 95% air at 37°C . Four hours later, the 10% PBS was added in six-well plates. The cells of transfection were obtained 24 h later. The expression of bFGF was detected by ELISA to verify the success of transfection.

Experimental cell culture groups

The cells of transfection with ASODN (or SODN) of bFGF and the normal cells from passage 3–4 at a density of $1 \times 10^4 \text{ mL}^{-1}$ were inoculated to 24-well culture plates and fused to 70% confluence in RPMI-1640 medium with 10% PBS. Four groups were included in this study. (1) Blank control group: PFs were cultured in the RPMI-1640 medium absence of TGF- β 1. (2) The TGF- β 1 group: PFs were cultured in the RPMI-1640 medium with 5 ng mL^{-1} TGF- β 1. (3) The TGF- β 1+ASODN group: PFs transfected with ASODN were cultured in the RPMI-1640 medium with 5 ng mL^{-1} TGF- β 1. (4) The TGF- β 1+SODN group: PFs transfected with SODN were cultured in the RPMI-1640 medium with 5 ng mL^{-1} TGF- β 1.

Morphology and growth of pulmonary fibroblasts

The cells from each group were maintained in 24-well plates for 24 h. The pulmonary fibroblast morphology was observed under an inverted phase contrast microscope. At the same time,

cells from three wells were collected for the counting of pulmonary fibroblasts number. Counting was performed three times with averaging each day for five consecutive days to generate pulmonary fibroblast growth curves.

MTT assay pulmonary fibroblast proliferation

Cells were maintained in 24-well plates for 72 h. Then, cells were collected from three wells for each experimental group by digestion with 0.25% trypsin at room temperature for 5 min. Cells were washed three times with HBSS to remove trypsin, re-suspended in 1 mL of culture medium, and 200 μ L of this suspension was transferred to a 96-well plate.

Cells were maintained at 37°C in a 5% CO₂ atmosphere for 24 h to allow cells adhere to the microtest plate wall. The supernatant was carefully removed and 80 μ L of freshly prepared RPMI-1640 was added. Cells in each well were incubated with 20 μ L of MTT solution (5 mg mL⁻¹) for 4 h. Then, the supernatant was removed and 100 μ L of DMSO was added to each well followed by shaking for 10 min to dissolve the crystals. Absorbance was measured at 490 nm with a microplate reader. Results were reported as arbitrary optical density (OD) units.

Detection of α -SMA by immunocytochemistry

Cells in logarithmic growth phase (1×10^5 mL⁻¹) were seeded on sterile cover slips in a 24-well plate followed by incubation for 24 h. When cells confluence reached 90%, the medium was removed and fresh RPMI-1640 medium containing 15% FBS was added. After incubation for 48 h, α -SMA expression was determined by immunocytochemistry using an anti- α -SMA mAb according to the supplier's recommendations.

Measurement of bFGF, CTGF, and Type I collagen levels by ELISA

The PFs, PFs transfected with ASODN, and PFs transfected with SODN were maintained in 24-well plates for 72 h. Then, supernatants were collected and the amounts of bFGF were determined with ELISA kits according to the manufacturer's instructions. Otherwise, the cells from each experiment group were maintained in 24-well plates for 72 h and supernatants were collected and the amounts of CTGF and Type I collagen were determined with ELISA kits according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) detection for the detection of Smad3, Samd7 and Type I collagen mRNA

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). A one-step RT-PCR was performed using the Super-Script One-Step RT-PCR kit (TaKaRa). According to kit instructions, RNA was reverse transcribed into complementary DNA, followed by PCR. PCR primer was synthesized by Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The following primers were used for amplification of target transcripts: Smad3 forward 5'-TGATCCCTCCAAATTCAGAGC-3'; Smad3 reverse 5'-TAAAGACCTCTATGCCAACCA-3'; Smad7 forward 5'-TGTGTCCAAAGAGCCCTCCCT-3'; Smad7 reverse 5'-CACGCCATCCACTTCCTT-3'; Type I collagen forward 5'-TGACTGGAAAGAGCGGAGAGT-3'; Type I collagen reverse 5'-GAATCCATCGGTATGCTCT-3'; β -actin forward 5'-CACGATGGAGGGGCCGGAATC-3'; and β -actin reverse 5'-GAATCCATCGGTATGCTCT-3'. PCR products were electrophoresed through a 1.5% agarose gel and then visualized under gel imaging system. The ultraviolet absorbance at 260 nm was measured, and absorbance ratio of target gene to β -actin was taken as the relative value of target gene mRNA level.

Statistical analysis

All data were statistically processed using the SPSS 17.0 software (SPSS Inc., Chicago, IL,

USA) and were processed as mean $\pm$ SD. The *t* test was used for two groups' comparison and one-way ANOVA test was used for multiple groups' comparison. $p<0.05$ was considered as statistically significant.

Results

The identification of lung fibroblasts

As shown in Fig. 1A, most of the cells were spindle cells, uniformly distributed with good condition. Immunohistochemical staining of vimentin showed that brown granules uniformly distributed in the cytoplasm, which demonstrated the harvest cells were lung fibroblasts (Fig. 1B).

The success of transfection

As shown in Fig. 2, the expression of bFGF in PFs transfected with ASODN was significantly decreased compared to the normal cells ($p<0.01$), and the expression of bFGF in cells transfected with SODN was markedly increased compared to the normal cells ($p<0.01$). This indicated that the cell transfection was successful.

The effects of bFGF on pulmonary fibroblasts proliferation

Pulmonary fibroblasts were cultured for 5d in each group (Fig. 3A). In all groups, the numbers of pulmonary fibroblasts were gradually increased. PFs increased the most rapidly in TGF- β 1+SODN group, followed successively by that of in the TGF- β 1 group, TGF- β 1+ASODN group and control group. For all groups, the numbers of pulmonary fi-

Fig. 1. The identification of lung fibroblasts. A. Cell morphology under the microscope ($\times 100$). B. Cell morphology of immunohistochemical staining ($\times 100$).

Fig. 2. The expression of bFGF was detected by ELISA. Results were means $\pm$ SEM ($n=3$ /group). * $p<0.01$ (vs. normal), $\Delta p<0.01$ (vs. normal).

Fig. 3. Pulmonary fibroblast proliferation. The proliferation of pulmonary fibroblasts in control, TGF- β 1, TGF- β 1+ASODN, TGF- β 1+SODN groups were determined by (A) changes in cell numbers of fibroblast for 5 d, and (B) MTT assays of pulmonary fibroblast proliferation. Results were means $\pm$ SEM ($n=3$ /group). In (A), the change in cell numbers of fibroblast by day was significantly different among the four groups by repeated ANOVA measurements ($p<0.05$). In (B), $\Delta p<0.05$ (vs. control, TGF- β 1, TGF- β 1+ASODN), $*p<0.05$ (vs. control, TGF- β 1, TGF- β 1+SODN).

broblasts peaked at 5 d of culture: TGF- β 1+SODN group = $(4.867 \pm 0.3512) \times 10^6$ cells/well, TGF- β 1+ASODN group = $(2.3 \pm 0.3) \times 10^6$ cells/well, TGF- β 1 group = $(3.5 \pm 0.2646) \times 10^6$ cells/well, control group = $(1.267 \pm 0.3055) \times 10^6$ cells/well. At 5 d, the numbers of pulmonary fibroblasts in the TGF- β 1+SODN group were more than those in the control and TGF- β 1 groups ($p<0.05$), but the numbers in the TGF- β 1+ASODN group were less than those in the TGF- β 1 group ($p<0.05$).

After 5 d of culture, MTT assays were also used to assess fibroblast proliferation (Fig. 3B). The measured absorbance (OD units) for the TGF- β 1+SODN group was significantly higher than the control and TGF- β 1 groups ($p<0.05$), and the TGF- β 1+ASODN group was significantly lower than the TGF- β 1 group ($p<0.05$). This suggested that the proliferation of the pulmonary fibroblasts was more active in the TGF- β 1+SODN group than in the control and TGF- β 1 groups, but less in the TGF- β 1+ASODN group than in the TGF- β 1 group.

Detection of α -SMA in pulmonary fibroblasts

The expression of α -SMA was detected by immunocytochemistry (Fig. 4). Representative photomicrographs of α -SMA expression are shown in Fig. 4 for the (A) control group, (B) TGF- β 1 group, (C) TGF- β 1+ASODN group, and (D) TGF- β 1+SODN group (darker color indicates more intense staining). In the control group, only a few cells were positive for α -SMA. In the TGF- β 1 and TGF- β 1+SODN groups, large numbers of cells exhibited positive staining for α -SMA in the cytoplasm, but compared to the TGF- β 1 and TGF- β 1+SODN groups, the cells exhibited positive staining for α -SMA in TGF- β 1+ASODN group were decreased. Figure 4E showed that the integrated optical density (IOD) for α -SMA expression in the TGF- β 1+SODN group was dramatically higher than the TGF- β 1 and control groups ($p<0.05$), and the IOD of the TGF- β 1+ASODN group was higher than the control group but lower than the TGF- β 1 group ($p<0.05$). It suggested that the differentiation from pulmonary fibroblast into myofibroblast was increased in the TGF- β 1+SODN group and decreased in the TGF- β 1+ASODN group compared to the TGF- β 1 group.

Cell culture production of CTGF and Type I collagen

As shown in Fig. 5, the concentrations of CTGF (Fig. 5A) and Type I collagen (Fig. 5B) were

Fig. 4. The expression of α -SMA was detected by immunocytochemistry. Representative photomicrographs showing α -SMA staining results ($\times 100$) for (A) control group, (B) TGF- β 1 group, (C) TGF- β 1+ASODN group, and (D) TGF- β 1+SODN group. (E) Integrated optical density (IOD) readings for α -SMA staining. Results were means $\pm$ SEM ($n=3$ /group). α -SMA expression levels were: control group = 0.153 ± 0.0252 , TGF- β 1 group = 0.347 ± 0.0153 , TGF- β 1+ASODN group = 0.25 ± 0.0265 , TGF- β 1+SODN group = 0.47 ± 0.04 , $\Delta p < 0.05$ (vs. control, TGF- β 1, TGF- β 1+ASODN), $*p < 0.05$ (vs. control, TGF- β 1, TGF- β 1+SODN).

Fig. 5. Cell culture production of CTGF and Type I collagen. ELISA was used to determine the supernatant concentrations of (A) CTGF and (B) Type I collagen. Results were means $\pm$ SEM ($n=3$ /group). $\Delta p < 0.05$ (vs. control, TGF- β 1, TGF- β 1+ASODN), $*p < 0.05$ (vs. control, TGF- β 1, TGF- β 1+SODN).

Fig. 6. The mRNA expression of Smad3, Smad7 and Type I collagen. RT-PCR was used to detect mRNA expression of (A) Smad3, (B) Smad7 and (C) Type I collagen. The ultraviolet absorbance at 260nm was measured, and absorbance ratio of target gene to β -actin was taken as the relative value of target gene mRNA level. Results were means $\pm$ SEM ($n=3$ /group). $\Delta p<0.05$ (vs. control, TGF- β 1, TGF- β 1+ASODN), * $p<0.05$ (vs. control, TGF- β 1, TGF- β 1+SODN).

significantly increased in the TGF- β 1+SODN group than the TGF- β 1 and control groups ($p<0.05$). However, the concentrations of them in the TGF- β 1+ASODN group were higher than the control ($p<0.05$) but lower than the TGF- β 1 group ($p<0.05$). These results indicated that the secretion of CTGF and Type I collagen increased in the TGF- β 1+SODN group and decreased in the TGF- β 1+ASODN group.

The mRNA expression of Smad3, Smad7 and Type I collagen in pulmonary fibroblasts

As shown in Fig. 6, the mRNA expressions of Smad3 (Fig. 6A) and Type I collagen (Fig. 6C) were significantly increased in the TGF- β 1+SODN group than the TGF- β 1 and control groups ($p<0.05$). However, the mRNA expressions of them in TGF- β 1+ASODN group were higher than the control ($p<0.05$) but lower than the TGF- β 1 group ($p<0.05$). Meanwhile, the mRNA expression of Smad7 was just the opposite. These results indicated that SODN of bFGF increased the expression of Smad3 and decreased the expression of Smad7; however, ASODN of bFGF had the opposite effect. This effect was same for the expression of Type I collagen.

Discussion

Pulmonary fibrosis is a complex, chronic pathological change characterized by excessive fibroblast proliferation and aberrant deposition of extracellular matrix (ECM) proteins which result in irreversible distortion of the lung architecture and permanent loss of function (Noble *et al.* 2012). The activated lung fibroblasts and myofibroblasts which are the main components of fibroblast foci are recognized as key effector cells in producing ECM (Fernandez and Eickelberg 2012,

Sivakumar *et al.* 2012, Lekkerkerker *et al.* 2012). The numbers of fibroblasts and myofibroblasts are correlated with worsening lung function, progression of disease, and poor prognosis (Flaherty *et al.* 2003). These suggest that the abnormal biological behavior of fibroblasts plays an important role in pulmonary fibrosis formation and development. Therefore, it can provide important information for treatment of pulmonary fibrosis to understand the pathogenetic mechanism of pulmonary fibroblast proliferation and differentiation.

Fibroblast growth factors (FGFs) are heparin-binding protein mitogens encoded by the FGF gene family which can stimulate the division of most cell types, such as endothelial cells, fibroblasts and keratinocyte. During embryonic development, FGFs play key roles in regulating cell proliferation, migration and differentiation. In adult tissues, FGFs are homeostatic factors and have important functions in mitogenesis, cellular migration, differentiation, angiogenesis, and wound healing (Yun *et al.* 2010, Moya *et al.* 2010, Shi *et al.* 2013). In the family of FGFs, basic fibroblast growth factor (bFGF) is the most studied factor. In lung disease, bFGF plays an extremely important role in lung cancer cells growth and tumor angiogenesis. Having the synergy with vascular endothelial growth factor (VEGF), bFGF promotes lung cancer cell proliferation, stimulates the formation of tumor blood vessels, and is associated with tumor metastasis (Grozio *et al.* 2007). Studies have shown that bFGF promotes the reconstruction of the vascular structure of COPD and reconstruction of asthma airway by stimulating the proliferation of fibroblasts and smooth muscle cells and being involved in angiogenesis and tissue regeneration process (Ward *et al.* 2004). In the research of pulmonary fibrosis, bFGF and TGF- β have each been found to play a critical role in the different phases of bleomycin-induced pulmonary fibrosis by inducing fibroblast proliferation and transformation, respectively (Osaki *et al.* 2001). Excessive expression of VEGF-A, VEGF-C, bFGF, and PDGF has been detected in cystic fibrosis airway epithelial cells (Verhaeghe *et al.* 2007). These evidences show that bFGF is involved in the process of pulmonary fibrosis.

Results from our research showed that bFGF which was increased by transfecting SODN of bFGF promoted the proliferation of pulmonary fibroblasts, while the proliferation of pulmonary fibroblasts was receded by ASODN of bFGF which could decrease the expression of bFGF. In addition, bFGF promoted the differentiation from fibroblast into myofibroblasts (increased α -SMA expression) which was attenuated by inhibiting the secretion of bFGF. What's more, the synthesis of Type I collagen was markedly increased when the expression of bFGF was promoted by transfecting SODN of bFGF, however, ASODN of bFGF receded the synthesis of Type I collagen. Thus, our results suggest that bFGF facilitates pulmonary fibroblasts proliferation, differentiation and collagen synthesis which can be suppressed by inhibiting expression of bFGF.

Plenty of evidence has shown that fibroblast proliferation and phenotypic transformation is primarily related to excessive activation of TGF- β signal pathway (Gharaee-Kermani *et al.* 2010, Yanagita 2012, Verrecchia and Mauviel 2007). TGF- β , which is produced predominantly by macrophages, plays a central role in the processes inducing EMT, driving differentiation of fibrocytes, and differentiation towards myofibroblasts (Verrecchia and Mauviel 2007, Lamouille *et al.* 2014, Harris *et al.* 2013). TGF- β 1 has been thought to be a critical molecule in pulmonary fibrosis, and play an important role in the process of pulmonary fibrosis. Increased expression of TGF- β 1 has been detected in lung tissues which are obtained from patients of idiopathic pulmonary fibrosis (Khalil *et al.* 1991). The fibrogenic role of TGF- β 1 is affirmed in animal models of pulmonary fibrosis, and the regulation of biological activities of TGF- β 1 has significantly decreased the degree of fibrosis in these models (Das *et al.* 2014, Song *et al.* 2013).

Following extracellular activation of TGF- β , smads protein mediates intracellular signal transmission (Mehra and Wrana 2002). Smads are a kind of highly conserved protein family with a molecular weight of 42–60kDa (Matsuzaki 2013). They can be divided into three categories based on structural and functional characteristics: receptor regulated smads (it was smad2/3 that was related to TGF- β 1 signal transduction), common mediator (primarily smad4), and inhibitory

smads (consisting of smad6 and smad7) (Leask and Abraham 2004, Verrecchia and Mauviel 2002). Smad3 can promote fibroblast proliferation and phenotypic transformation as well as synthesis and secretion of extracellular matrix, playing a key role in the TGF- β 1 signal transduction (Isono *et al.* 2002). Smad7 is the main inhibitory regulatory protein of TGF- β signal pathway, which can competitively bind the TGF- β 1 receptor (Saika *et al.* 2007).

In our research, rat pulmonary fibroblast was stimulated by TGF- β 1. Results showed that the mRNA expression of Smad3 was significantly increased and the mRNA expression of Smad7 was markedly decreased when the synthesis of bFGF was increased by transfecting SODN of bFGF. However, inhibiting the synthesis of bFGF by transfecting ASODN of bFGF could reduce TGF- β 1-induced Smad3 mRNA expression and promote TGF- β 1-induced Smad7 mRNA expression. In addition, our research found that connective tissue growth factor (CTGF) was high expressed in the condition of transfecting SODN of bFGF, while the expression CTGF was decreased following the synthesis of bFGF suppressed by transfecting ASODN of bFGF. CTGF, which is stimulated by TGF- β 1, is an important cell factor in pulmonary fibrosis (Fu *et al.* 2011, Lai *et al.* 2009). These indicate that bFGF can up-regulate the expression of Smad3 and down-regulate the expression of Smad7 in TGF- β 1 stimulated lung fibroblasts, and this effect can be inhibited by ASODN of bFGF. Moreover, bFGF can promote the expression of CTGF and the ASODN of bFGF inhibits TGF- β 1-induced synthesis of CTGF.

In conclusion, this study demonstrates that the increased expression of bFGF promotes TGF- β 1-induced pulmonary fibroblast proliferation, differentiation and collagen synthesis, and the possible mechanism is associated with the regulation of TGF- β 1-Smad signaling pathway. The ASODN of bFGF can inhibit this effect by down-regulating the expression of CTGF and Smad3 and up-regulating the expression of Smad7. Our research provides a new perspective about the treatment of pulmonary fibrosis.

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