

Inhibition of α -SMA by the Ectodomain of FGFR2c Attenuates Lung Fibrosis

Wang Ju,^{1*} Yu Zhihong,^{1*} Zhou Zhiyou,¹ Huang Qin,¹ Wang Dingding,² Sun Li,¹ Zhu Baowei,¹ Wei Xing,¹ He Ying,¹ and Hong An¹

¹Guangdong Provincial Key Laboratory of Bioengineering Medicine (National Engineering Research Centre of Genetic Medicine), Guangzhou, Guangdong China; ²Department of Biotechnology, Institute of Life Science and Biological Pharmacy, Guangdong Pharmaceutical University, Guangzhou Higher Education Mega Center, Guangzhou, Guangdong, China

The soluble ectodomain of fibroblast growth factor receptor-IIIc (sFGFR2c) is able to bind to fibroblast growth factor (FGF) ligands and block the activation of the FGF-signaling pathway. In this study, sFGFR2c inhibited lung fibrosis dramatically *in vitro* and *in vivo*. The upregulation of α -smooth muscle actin (α -SMA) in fibroblasts by transforming growth factor- β 1 (TGF- β 1) is an important step in the process of lung fibrosis, in which FGF-2, released by TGF- β 1, is involved. sFGFR2c inhibited α -SMA induction by TGF- β 1 via both the extracellular signal-regulated kinase 1/2 (ERK1/2) and Smad3 pathways in primary mouse lung fibroblasts and the proliferation of mouse lung fibroblasts. In a mouse model of bleomycin (BLM)-induced lung fibrosis, mice were treated with sFGFR2c from d 3 or d 10 to 31 after BLM administration. Then we used hematoxylin and eosin staining, Masson staining and immunohistochemical staining to evaluate the inhibitory effects of sFGFR2c on lung fibrosis. The treatment with sFGFR2c resulted in significant attenuation of the lung fibrosis score and collagen deposition. The expression levels of α -SMA, p-FGFRs, p-ERK1/2 and p-Smad3 in the lungs of sFGFR2c-treated mice were markedly lower. sFGFR2c may have potential for the treatment of lung fibrosis as an FGF-2 antagonist.

Online address: <http://www.molmed.org>

doi: 10.2119/molmed.2011.00425

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive, irreversible and lethal lung disorder of unknown etiology. IPF involves extracellular matrix (ECM) remodeling and deposition, collagen accumulation and fibroblast destruction (1).

Myofibroblasts are generally considered to be key effector cells in the development of fibrosis (2,3), the hallmark of which is the expression of α -smooth muscle actin (α -SMA). Myofibroblasts

are the main source of type I collagen and fibrogenic cytokines in fibrotic lesions and contribute to the altered mechanical properties of affected lungs (4,5). The polypeptide mediators and growth factors believed to be pivotal for fibrosis include transforming growth factor- β (TGF- β), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), platelet-derived growth factor, connective tissue growth factor (CTGF), insulin-like growth factor, epi-

dermal growth factor, interleukin-18 and endothelin (ET) (6). TGF- β plays the most important role in the expression of α -SMA. The SMA promoter harbors Smad binding elements, which specifically bind Smad3 (7). Mitogen-activated protein kinases (MAPKs), including p38 and extracellular signal-regulated kinase 1/2 (ERK1/2), are required for the TGF- β -induced expression of α -SMA (8,9). TGF- β also induces α -SMA expression via a mechanism that involves ET-1 but which is independent of Smad and c-Jun N-terminal kinase (c-JNK) (10,11). In addition to α -SMA, TGF- β induces key markers and effectors of myofibroblast differentiation, including CTGF (CCN2). CTGF is a common target for both TGF- β and ET-1 (12) and is an important mediator of ECM deposition (13).

During the formation of lung fibrosis, FGF signaling is believed to be profibrotic because it promotes the proliferation of fibroblasts and myofibroblasts (14,15). TGF- β stimulates the release of FGF-2 and upregulates the expression of

*WJ and YZ contributed equally to this work.

Address correspondence to Wang Ju, Guangdong Provincial Key Laboratory of Bio-engineering Medicine (National Engineering Research Centre of Genetic Medicine), Guangzhou, Guangdong 510632, China. Fax: + 86 20 85221983; E-mail:

wang_ju1688@yahoo.cn; or Hong An, Guangdong Provincial Key Laboratory of

Bio-engineering Medicine (National Engineering Research Centre of Genetic Medicine), Guangzhou, Guangdong 510632, China. Fax: + 86 20 85221983; E-mail: tha@jnu.edu.cn.

Submitted November 3, 2011; Accepted for publication March 20, 2012; Epub

(www.molmed.org) ahead of print March 21, 2012.

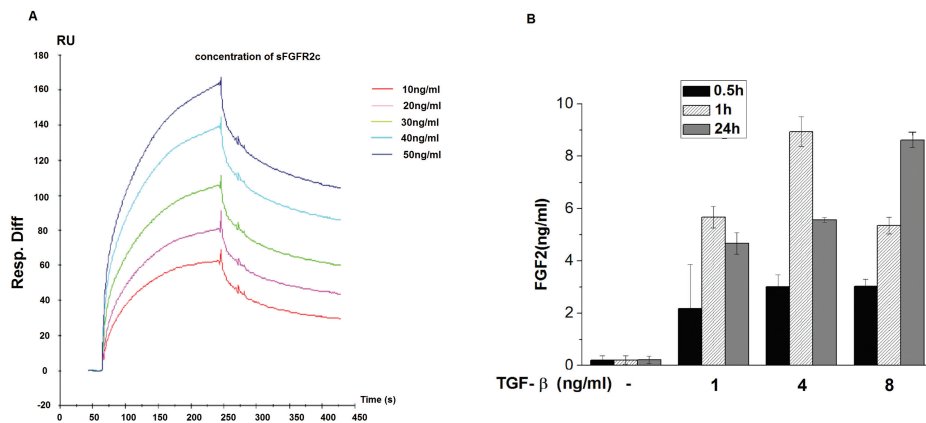


Figure 1. (A) The binding of sFGFR2c to FGF-2 was detected by SPR. (B) The release of FGF-2 from fibroblasts into the medium is promoted by TGF- β 1 (1, 4, 8 ng/mL), as detected by ELISA assay. The experiments were performed three times, and the mean values \pm standard deviation (SD) are presented.

FGFR1 and FGFR2 (16–18). Nevertheless, some studies have led to a different conclusion. For example, FGF-1 was found to display antifibrotic functions by downregulating collagen expression and antagonizing some of the profibrotic effects of TGF- β (7,16,19).

FGFs execute diverse functions by binding and activating members of the FGF receptor (FGFR) family. There are four *FGFR* genes that encode functional receptors, FGFR1–FGFR4. FGFRs consist of three extracellular immunoglobulin (Ig) domains (Ig-I, Ig-II and Ig-III), a single transmembrane domain, and a cytoplasmic tyrosine kinase domain (20). FGFRs have several isoforms because exon skipping can remove the Ig-I domain. Alternative splicing produces the IIIb and IIIc isoforms, which have distinct FGF binding specificities and are predominantly expressed in epithelial and mesenchymal cells, respectively.

The extracellular fragment of FGFRs is called soluble FGFR (sFGFR), and it can competitively bind to FGFs and inhibit the FGFR signaling pathway (21). In our study, we designed sFGFR2c to study the role of FGF signaling in myofibroblast differentiation. sFGFR2c contains two Ig-like domains of the FGFR2IIIc subtype and is the most common receptor of FGF-2 (22,23).

Here, we report that sFGFR2c inhibits the expression of α -SMA in primary mouse lung fibroblasts via both the Smad-dependent and -independent signaling pathways that are activated by TGF- β . *In vivo*, sFGFR2c diminishes the development of bleomycin (BLM)-induced lung fibrosis in mice. The inhibition of sFGFR2c may be related to the FGF-2 signaling pathway.

MATERIALS AND METHODS

Cell Lines, Reagents and Antibodies

Primary lung fibroblasts were purchased from the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences and the School of Basic Medicine of Peking Union Medical College. The following primary antibodies were used. Rabbit anti-FGF-2 was purchased from R&D Systems (Minneapolis, MN, USA). Mouse anti- α -SMA was purchased from Millipore (Billerica, MA, USA). Rabbit anti- β -actin, mouse anti-Smad2 and rabbit anti-Smad3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-p-ERK, rabbit anti-ERK/anti-p38/anti-p-p38/anti-pFGFRs/anti-JNKs/CD34/anti-p-Smad2/3, mouse anti-p-JNKs and horseradish peroxidase-conjugated secondary antibody were purchased from

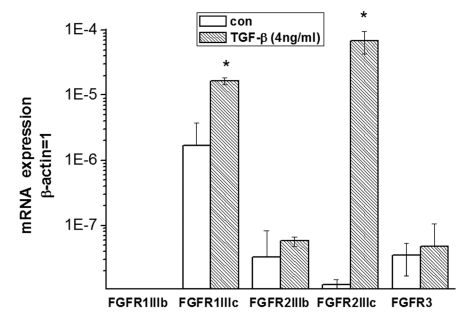


Figure 2. FGFR isoform switching was induced by TGF- β 1 in primary mouse lung fibroblasts, as detected by real-time RT-PCR. The experiments were performed three times, and the mean values \pm SD are presented. The asterisk represents statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 and non-stimulated cells (* p < 0.01). con, Control.

Santa Cruz Biotechnology (Santa Cruz, CA, USA).

The tyrosine kinase selective inhibitors PD98059 and SB203580 were purchased from Tocris Bioscience (Bristol, UK).

sFGFR2c Expression, Renaturation and Purification

The DNA fragment from D2 to D3 of the FGFR2IIIc isoform (amino acids 147–366 of hBEK) was amplified by polymerase chain reaction (PCR), cloned into the pET3c vector and expressed in *Escherichia coli*. After renaturation and purification (referred to in our previous work [23]), sFGFR2c of greater than 95% purity was harvested.

Cell Culture

Primary lung fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) with standard antibiotics in a 95% air, 5% CO₂ humidified atmosphere at 37°C for all experiments.

Animal Treatment

All procedures involving animals were performed in accordance with the institutional animal welfare guidelines of the Experimental Animal Research Center of Sun Yat-Sen University. BLM (3 mg/kg;

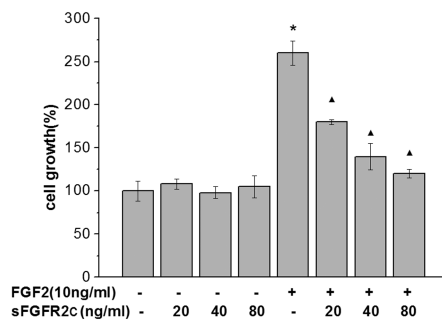


Figure 3. Inhibitory effect of sFGFR2c on the proliferation of fibroblasts as measured by CCK8 assays. The experiments were performed three times, and the mean values \pm SD are presented. The asterisk represents statistically significant differences between cells stimulated with 4 ng/mL FGF-2 and cells stimulated with 4 ng/mL FGF-2 plus sFGFR2c ($*p < 0.01$).

Nippon Kayaku Co., Tokyo, Japan) was intratracheally administered in 25 μ L saline to male C57BL/6 mice (8 wks old, obtained from the Experimental Animal Research Center of Sun Yat-Sen University). The animals were allocated to five groups, as follows: 3 d after the surgery, (a) intratracheal saline, (b) intratracheal saline + intraperitoneal sFGFR2c (50 μ g/daily), (c) intratracheal BLM, (d) intratracheal BLM + intraperitoneal sFGFR2c (50 μ g/daily); 10 d after the surgery, (e) intratracheal BLM + intraperitoneal sFGFR2c (50 μ g/daily). After 10, 17 or 31 d, the animals were killed, the lungs were removed and the lung tissues were incubated in formalin for hematoxylin and eosin (HE) staining, Masson trichrome and immunohistochemical staining. Other lung tissues were directly used for the hydroxyproline assay, bronchoalveolar lavage fluid (BALF) assay and enzyme-linked immunosorbent assay (ELISA).

Surface Plasmon Resonance Analysis of sFGFR2c/FGF-2 Interactions

Kinetic analyses of sFGFR2c/FGF-2 interactions were performed with a BIAcore 3000 instrument GE Healthcare (Chalfont St. Giles, UK) (22). FGF ligands were immobilized on research grade

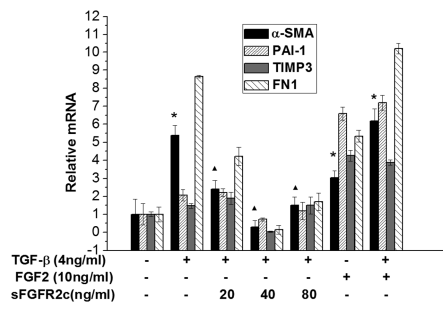


Figure 4. The expression of α -SMA, PAI-1, TIMP3, and FN mRNA in fibroblasts is upregulated by TGF- β 1 and FGF-2, which is a result that is antagonized by sFGFR2c. All of the above genes are fibrosis markers. The experiments were performed three times, and the mean values \pm SD are presented. The asterisks represent statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 and nonstimulated cells ($*p < 0.05$); the triangles represent statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 and cells treated with 4 ng/mL TGF- β 1 plus sFGFR2c (20, 40, 80 ng/mL) ($*p < 0.05$).

CM5 chips. Five different concentrations of sFGFR2c in HBS-EP buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 3 mmol/L EDTA, 0.005% surfactant P-20), from 10 to 50 μ g/mL, were injected into the FGF sensor chips at a flow rate of 50 mL/min. At the end of each sample injection (180 s), HBS-EP buffer was passed over the sensor surface to monitor the dissociation phase. Following 180 s of dissociation, the sensor surface was fully regenerated by the injection of 50 mL of 2 mol/L NaCl in 100 mmol/L sodium acetate buffer (22).

Proliferation Assay

One hundred microliters DMEM supplemented with 10% FBS with 5000 fibroblast cells/mL was added into each well of a 96-well plate for 24 h, and the cells were then serum starved for 24 h in DMEM with 1% FBS. The experiment included a control group, sFGFR2c groups (20, 40, 80 ng/mL) and FGF-2 (10 ng/mL) plus sFGFR2c groups (20, 40, 80 ng/mL). After 48 h of culturing, the cells were incubated with 100 μ L cholecystokinin oc-

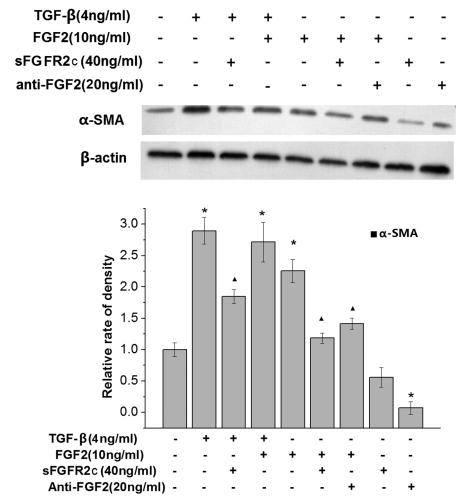


Figure 5. The expression of α -SMA protein was induced by TGF- β 1 as well as by FGF-2 and antagonized by anti-FGF-2 and sFGFR2c as determined by Western blotting. The experiments were performed three times, and the mean values \pm SD are presented. The asterisks represent statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 or 10 ng/mL FGF-2 and nonstimulated cells ($*p < 0.05$); The triangles represent statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 and cells treated with 4 ng/mL TGF- β 1 (or 10 ng/mL FGF-2) plus 40 ng/mL sFGFR2c ($*p < 0.05$).

tapeptide (CCK8) in each well for 1–4 h, and the wavelength absorbance was measured at 450 nm. The mean value of five wells was calculated, and each experiment was repeated three times.

Detecting the Release of FGF-2 after Induction of TGF- β 1 by ELISA

Fibroblasts were seeded in duplicate wells in six-well plates at an initial density of 1×10^5 cells/well and cultured for 1 d. Following starvation for 12 h, the cells were incubated in 2 mL DMEM alone or in DMEM supplemented with recombinant human TGF- β 1 (4 ng/mL). FGF-2 was extracted from the ECM according to the protocol of Nakamura *et al.* (24). After incubation for 0.5, 1 and 24 h, cell culture medium was collected for FGF-2 quantitation. ELISAs were performed in duplicate wells of ELISA kits

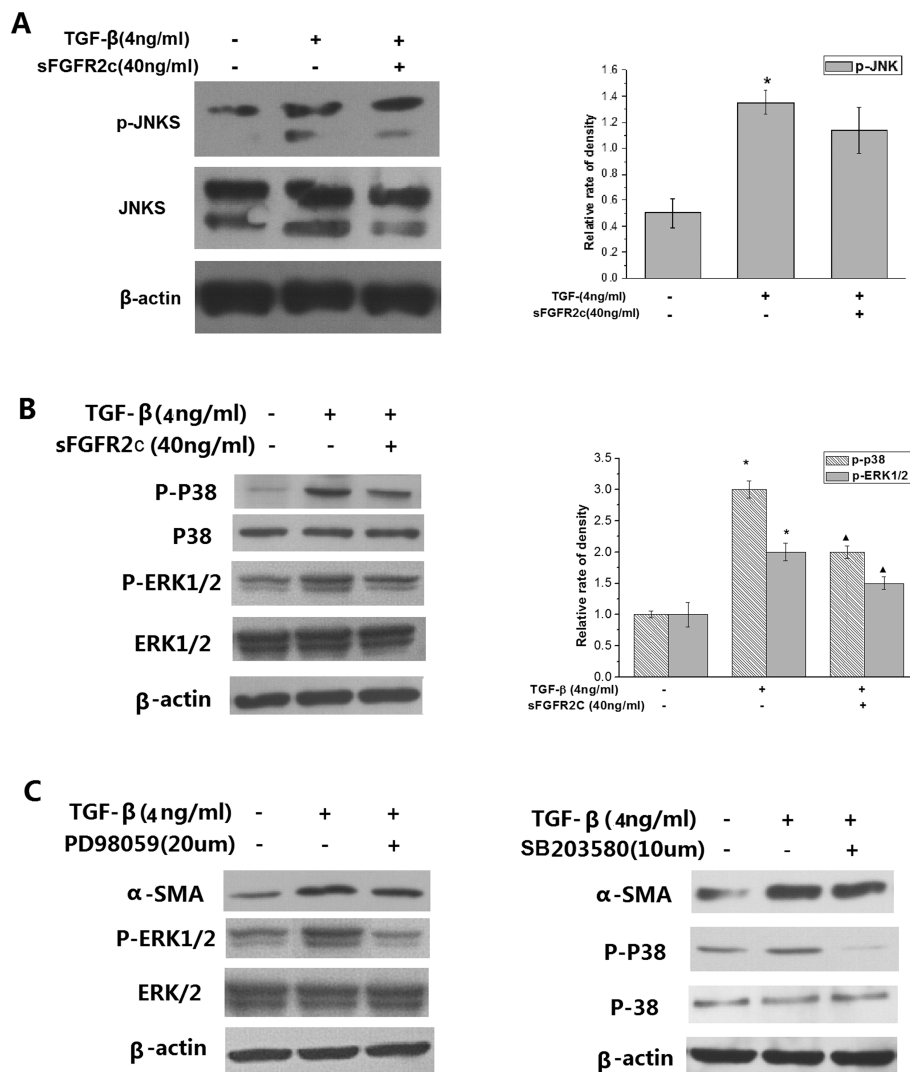


Figure 6. Both TGF- β 1 and sFGFR2c contribute to the activation of the MAPK signaling pathway, which participates in the regulation of α -SMA expression. (A) JNKs are phosphorylated by TGF- β 1 and were not antagonized by sFGFR2c. (B) p38 MAPK and ERK1/2 are phosphorylated by TGF- β 1 and antagonized by sFGFR2c. (C) PD98059 inhibited the phosphorylation of ERK1/2 and the upregulation of α -SMA expression induced by TGF- β 1. SB203580 inhibited the phosphorylation of p38 MAPK but did not inhibit the upregulation of α -SMA expression induced by TGF- β 1. The experiments were performed three times, and the mean values \pm SD are presented. The asterisks represent statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 and nonstimulated cells ($*p < 0.05$); The triangles represent statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 and cells treated with 4 ng/mL TGF- β 1 plus 40 ng/mL sFGFR2c ($*p < 0.05$).

(R&D Systems) according to the manufacturer's instructions.

Real-Time PCR

Total RNA extraction and synthesis of cDNA were carried out using previously published methods (25). Quantitative

real-time reverse transcription (RT)-PCR was performed with the IQ SYBR Green Mix in an iCycler PCR machine (Bio-Rad), using 1 μ L cDNA in triplicate, with 18s rRNA as the internal control. Primers included the following: FGF1, forward 5'-CAGGA GCGAC CAGCA CAT-3'; re-

verse 5'-CGTGT CTGTG AGCCG TAT-3', FGF2, forward 5'-GTGCC AGATT AGCCG ACGC-3', reverse 5'-TCGGC CGCTC TTCTG TCC-3'; 18s rRNA, forward 5'-CGTCT GCCCT ATCAA CTTC-3'; reverse 5'-GATGT GGTAG CCGT TCTCA-3'; α -SMA, forward 5'-GCCAA GCACT GTCAG GAATC C-3', reverse 5'-CACAA TGGAT GGGAA AACAG CC-3'; PAI-1, forward, 5'-GTTCT GCCCA AGTTC TCCCT G-3'; reverse, 5'-GAAAC TGTCT GAACA TGTCG GTCA-3'; TIMP3, forward, 5'-GCAGA TGAAG ATGTA CCGAG GC-3', reverse, 5'-TGGTA CTTGT TGACC TCCAG CTTA-3'; and FN1, forward, 5'-ATGAA GAGGG GCACA TGCTG A-3', reverse, 5'-CTCTG AATCC TGGCA TTGGT CG-3'.

Western Blot

Western blot analysis was performed following a standard procedure. Equal amounts of protein (20 μ g) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, as described previously (18). Immunoreactive bands were visualized by an enhanced chemiluminescence reaction kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was determined by a BCA protein assay kit (Thermo Fisher Scientific).

To inhibit the activation of p38 MAPK and ERK, serum-starved fibroblasts were pretreated with SB203580 (10 μ mol/L) or PD98059 (20 μ mol/L) for 1 h, followed by incubation with TGF- β 1 (4 ng/mL) and sFGFR2c (40 ng/mL). p-FGFRs, FGFR2, ERK1/2, p-ERK1/2, p-p38, JNKs, p-JNKs, Smad2/3, p-Smad2/3 and α -SMA protein levels were analyzed by Western blotting.

RNA Interference

siRNA duplexes against human Smad2, Smad3 and control (scrambled) siRNA were synthesized by Shanghai GenePharma. The sense strands of siRNAs were as follows: negative control: 5'-UUCUC CGAAC GUGUC ACGUT T-3'; Smad3-siRNA: 5'-AGUAG AGACA CCAGU UCUAT T-3'; Smad2-siRNA: 5'-GGUGU UCGAU AGCAU AUUAT T-3'.

Fibroblasts were seeded into six-well plates, grown to 40–60% confluence and then transfected with siRNAs for 4 h using LipofectAMINE 2000 (Invitrogen).

The cells were allocated to three groups: si-negative control group, si-Smad2 group and si-Smad3 group. Each group was treated with phosphate-buffered saline (PBS), TGF- β 1 (4 ng/mL) and sFGFR2c (40 ng/mL) + TGF- β 1 (4 ng/mL). The protein levels were analyzed by Western blotting.

Lung Tissue Sampling and Immunohistochemistry

The lung tissues of mice were fixed in 10% buffered formalin, stained with HE and Masson trichrome, fixed in formalin and embedded in paraffin. Immunohistochemical stainings for α -SMA, p-FGFRs, p-ERK1/2, p-Smad3 and CD34 were performed according to the manufacturer’s protocol.

Hydroxyproline Assay

We measured collagen content in the lungs with a conventional hydroxyproline assay, using a hydroxyproline kit (KeyGEN Biochemical Institute, Nanjing, China) according to the manufacturer’s instructions. The experimental results were quantified by comparison to a standard curve of known hydroxyproline concentrations.

Inflammatory Cell Counts from BALF

Mice were killed by intraperitoneal pentothal injections. The tracheas were exposed, and BALF was obtained by three instillations of 1 mL of ice-cold PBS. BALF was centrifuged, resuspended and cytospun onto slides and Diff-Quik stained according to the manufacturer’s protocol. The number of total cells and the number of macrophages, lymphocytes and neutrophils were counted under a light microscope.

Cytokine Measurement

After the addition of 0.8 mL ice-cold lysis buffer (20 mmol/L HEPES, 100 mmol/L NaCl, 1% NP-40, 10% glycerol, 0.1 mmol/L phenylmethylsulfonyl

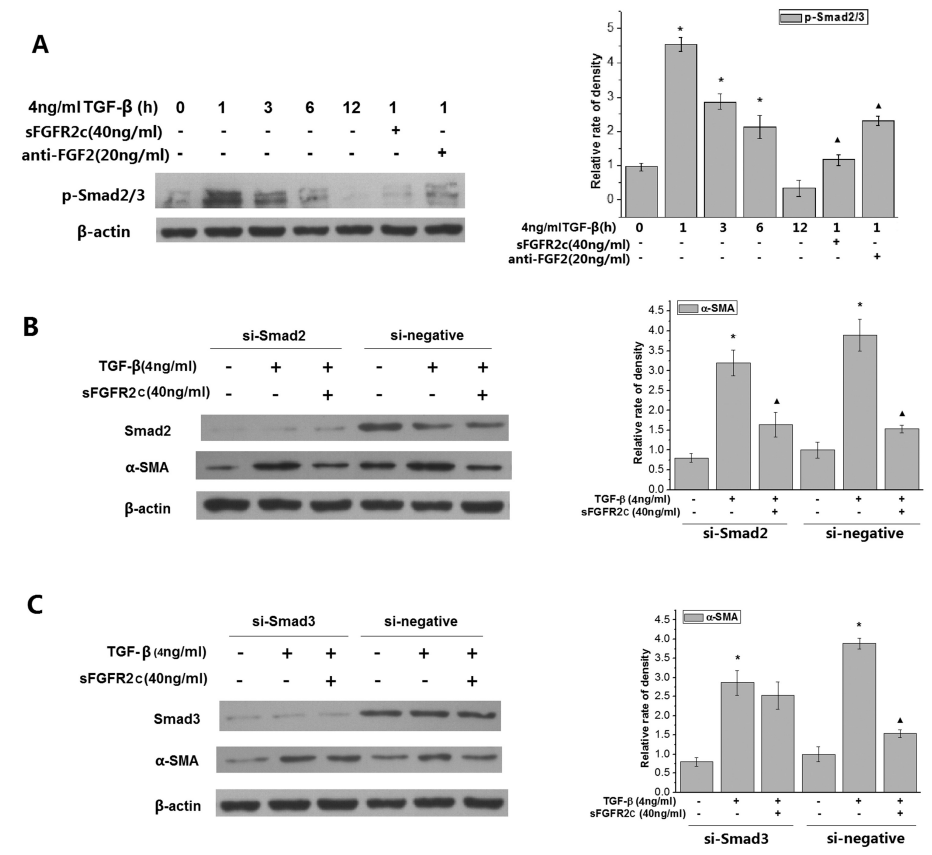


Figure 7. Silencing the expression of Smad 2 and 3 by specific siRNA showed that the Smad3 signaling pathway participated in the regulation of α -SMA expression by TGF- β 1. The inhibitory effect of sFGFR2c on the induction of α -SMA by TGF- β 1 might be linked to the Smad3 signaling pathway. (A) TGF- β 1 phosphorylated Smad2/3, which was antagonized by sFGFR2c and anti-FGF-2. (B) Silencing Smad2 did not affect the inhibitory effect of sFGFR2c on α -SMA expression. (C) Silencing Smad3 abrogated the inhibition of sFGFR2c on α -SMA expression. The data represent the mean values \pm SD of two experiments, each in duplicate. The asterisks represent statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 and nonstimulated cells ($*p < 0.05$); the triangles represent statistically significant differences between cells stimulated with 4 ng/mL TGF- β 1 and cells treated with 4 ng/mL TGF- β 1 plus 40 ng/mL sFGFR2c ($*p < 0.05$).

fluoride, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 0.5 mmol/L sodiumvanadate) to the left or right lung lobes from each mouse, homogenates were prepared with an ULTRATURRAX T8 homogenizer (IKA Labortechnik, Staufen, Germany). After centrifugation, the supernatants were prepared from the lung homogenates and stored in a freezer (-80°C). The ELISA kits for determining CTGF, TGF- β 1 and FGF-2 were all purchased from R&D Systems. All ELISA assays were performed as described above.

Statistical Analysis

The data are expressed as the mean values \pm standard deviation (SD). Statistical significance was determined by a one-way analysis of variance or *t* test. *P* values less than 0.05 were considered significant.

RESULTS

Binding of sFGFR2c to FGF-2 as Detected by SPR

The binding of sFGFR2c to FGF-2 was measured by SPR (Figure 1A). It was

determined that the dissociation constant (Kd) of sFGFR2c to FGF-2 was 5.44×10^{-8} mol/L, consistent with the result of Ibrahim *et al.* (22).

TGF- β 1 Induces the Release of FGF-2

After 0.5, 1 or 24 h of induction with 0, 1, 4 or 8 ng/mL TGF- β 1, ELISA assays determined that FGF-2 was released at up to 9000 pg/mL per 10^5 cells, which was more than 45 times the level of the control (Figure 1B). The highest release occurred when TGF- β 1 was 5 ng/mL and the induction time was 1 h. These results also showed that TGF- β released FGF-2 very quickly, which is consistent with the results of Khalil *et al.* (26). After 24 h induction with 10 ng/mL TGF- β 1, the release of FGF-2 was almost equal to the highest amount, which suggests that new FGF-2 expression was induced by TGF- β .

Expression of FGFRs and Isoform Switching by TGF- β 1 in Primary Mouse Lung Fibroblasts

The expression of FGFRs was detected by real-time RT-PCR in fibroblasts (Figure 2). FGFR2c expression was upregulated dramatically after induction with 4 ng/mL TGF- β , even though the FGFR1c level increased only slightly (27). The function of TGF- β was converted in part by sFGFR2c.

Because the most appropriate ligand of FGFR2c was FGF-2 (28), we speculated that the significance of the intense elevation of FGFR2c by TGF- β was the enhancement of the FGF-2 response in fibroblasts.

sFGFR2c Inhibits Fibroblast Cell Proliferation in the Presence of FGF-2

A CCK8 assay showed that concentrations of 20, 40 and 80 ng/mL sFGFR2c significantly inhibited the stimulatory effect of the proliferation of fibroblasts by FGF-2 (10 ng/mL) (Figure 3). The highest growth rate of FGF-2 was approximately 270%. The lowest growth rate in the presence of FGF-2, at 80 ng/mL sFGFR2c, was 130%. This finding suggests that recombinant sFGFR2c inhibited

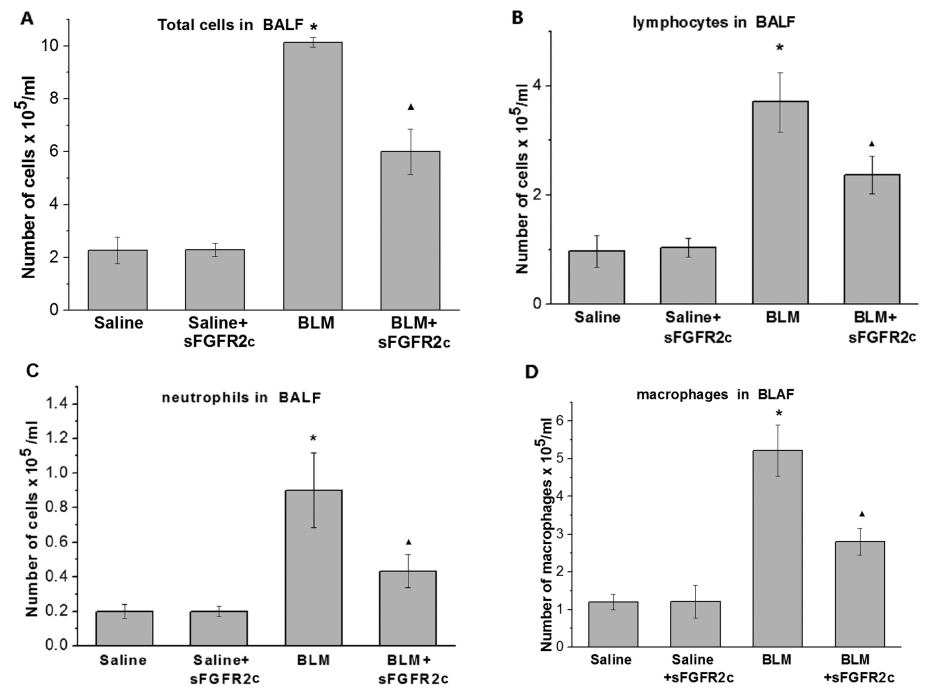


Figure 8. Differential cell counts in BLM + sFGFR2c mice. Bronchoalveolar lavage (BAL) fluid was collected from mice treated with BLM for 10 d to determine (A) the total cell count and the differential cell counts of (B) lymphocytes, (C) neutrophils, and (D) macrophages.

the mitogenic activity of FGF-2 by competing with membrane-bound FGFRs through binding to FGF-2. The inhibitory effect of sFGFR2c was dose dependent. However, in the absence of FGF-2, sFGFR2c did not exhibit any significant inhibitory effect.

sFGFR2c Inhibited the Expression of Genes Related to Fibrosis Induced by TGF- β 1

The genes of α -SMA, PAI-1, TIMP3 and FN1 participate in the deposition of ECM during the process of fibrosis (29–31). In primary mouse lung fibroblasts, the expression of these genes was upregulated strongly by both TGF- β and FGF-2 but was markedly impaired to varying degrees by sFGFR2c (Figure 4). Because our target was α -SMA in this study, we noted that α -SMA expression was affected by TGF- β , FGF-2 and sFGFR2c. The level of α -SMA increased when induced by both FGF-2 and TGF- β . α -SMA reached its highest level when induced by 4 ng/mL TGF- β but did not

increase further when FGF-2 and TGF- β were given at the same time. α -SMA induction by TGF- β was inhibited by concentrations of 20, 40 and 80 ng/mL sFGFR2c, and the lowest amount of α -SMA was found when the concentration of sFGFR2c was 40 ng/mL and the concentration of TGF- β was 4 ng/mL. Therefore, we selected 40 ng/mL as the dose of sFGFR2c used to inhibit the α -SMA expression that was induced by TGF- β in the following work.

sFGFR2c Inhibits the TGF- β 1-Induced Expression of α -SMA

Importantly, in the process of lung fibrosis, TGF- β can induce the differentiation of fibroblasts into myofibroblasts, a transition that is characterized by the presence of α -SMA in cytoplasmic stress fibers (4,32).

Western blotting assays showed that both TGF- β 1 and FGF-2 significantly increased α -SMA levels inside primary mouse lung fibroblast cells 1 d after induction (Figure 5), whereas sFGFR2c and

anti-FGF-2 abrogated the induction of α -SMA expression by TGF- β 1 or FGF-2 (Figure 5). These results suggest that the FGF-2 that was released by TGF- β 1 could promote α -SMA expression and play a role in the differentiation of fibroblasts into myofibroblasts. Meanwhile, sFGFR2c could inhibit this process by inactivating FGF-2, for example, by anti-FGF-2. The Western blotting result also showed that α -SMA expression was not enhanced when FGF-2 and TGF- β were given at the same time.

TGF- β 1 Activates ERK, p38 and JNK MAP Kinase; Activation of ERK and p38, but Not JNK MAP Kinase, Is Diminished by sFGFR2c

It is known that TGF- β 1 activates MAPK signaling pathways in different cell systems, including ERK1/2, JNK and p38 MAPK (26). Immunoblotting revealed that the phosphorylation of ERK1/2, p38 and JNK was detected after 30-min induction by 5 ng/mL TGF- β . Similarly, 40 ng/mL sFGFR2c diminished the activation of ERK1/2 and p38, but not JNK (Figure 6A). Because ET-1 is an important mediator in α -SMA expression, which was induced by TGF- β through the JNK signal pathway (10,11), this result suggests that the inhibition of sFGFR2c on α -SMA expression may not be related to ET-1.

Only the Activation of ERK1/2 Is Involved in the TGF- β 1-Induced Upregulation of α -SMA

To further assess the relationship between the TGF- β 1 induction of α -SMA and the sFGFR2 inhibition of ERK and p38, primary lung fibroblasts were incubated with TGF- β 1 for 24 h in the presence or absence of 10 mmol/L PD98059 (an ERK inhibitor) or SB203580 (a p38 inhibitor). Cellular extracts were prepared, and TGF- β 1-induced α -SMA upregulation was found to be inhibited by PD98059 but not by SB203580 (Figures 6B, C). The results demonstrated that ERK1/2 but not p38 activation played a role in the TGF- β 1 induction of α -SMA, which suggested that the in-

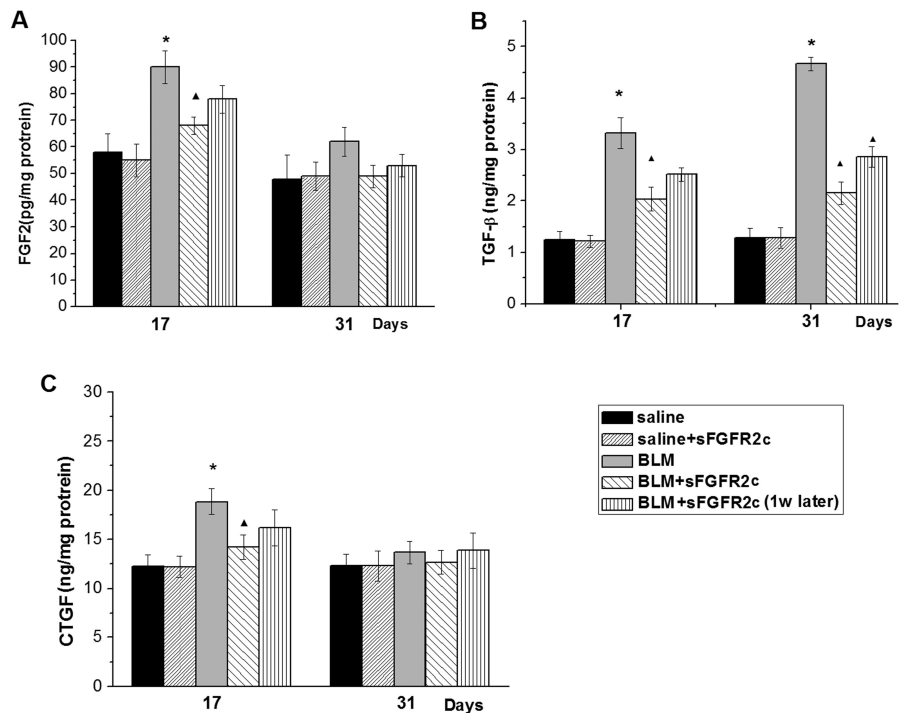


Figure 9. ELISA assay to detect the inhibitory effect of sFGFR2c on the expression of profibrotic marker genes, such as TGF- β , CTGF and FGF-2, in the mouse BLM model at d 17 and 31. The asterisks represent statistically significant differences between the mice treated with BLM and the control group ($*p < 0.05$); the triangles represent statistically significant differences between the mice treated with BLM and treated with BLM + sFGFR2c ($*p < 0.05$).

hibitory activity of sFGFR2c on α -SMA induction by TGF- β 1 is related to the phosphorylation of ERK1/2.

The Smad3 Pathway, but Not the Smad2 Pathway, Is Involved in the Induction of α -SMA by TGF- β 1, and the Activation of α -SMA Is Inhibited by sFGFR2c

In fibroblast cells, TGF- β 1 activated the Smad2/3 signal pathway (Figures 7A, B), which was related to α -SMA induction. Both sFGFR2c and anti-FGF-2 inhibited the phosphorylation of Smad2/3, indicating that there is crosstalk between FGF-2 and the Smad signal pathway. Furthermore, sFGFR2c inhibited the expression of α -SMA in Smad2-silenced cells but not in Smad3-silenced cells, which suggests that the expression of α -SMA is regulated by sFGFR2c through the Smad3 but not the Smad2 pathway (Figures 7B, C).

The BLM-Induced Early Inflammatory Response Is Affected by sFGFR2c Treatment

Inflammation occurs after BLM injury and is thought to contribute to the fibrotic process (33). To analyze the inflammatory response over time, we collected BALF from mice at the 10th d (Figure 8). The total number of cells was determined, and a differential cell count was also performed. Our results indicated that the BLM group as well as the BLM + sFGFR2c group had a large amount of inflammation and cell infiltration in the BALF compared with the saline and saline + sFGFR2c control animals. The total number of cells as well as the percentage of macrophages, lymphocytes and neutrophils in the BLM + sFGFR2c group were distinctly lower than in the BLM group, which suggests that sFGFR2c inhibited BLM-induced fibrosis by suppressing the inflammatory re-

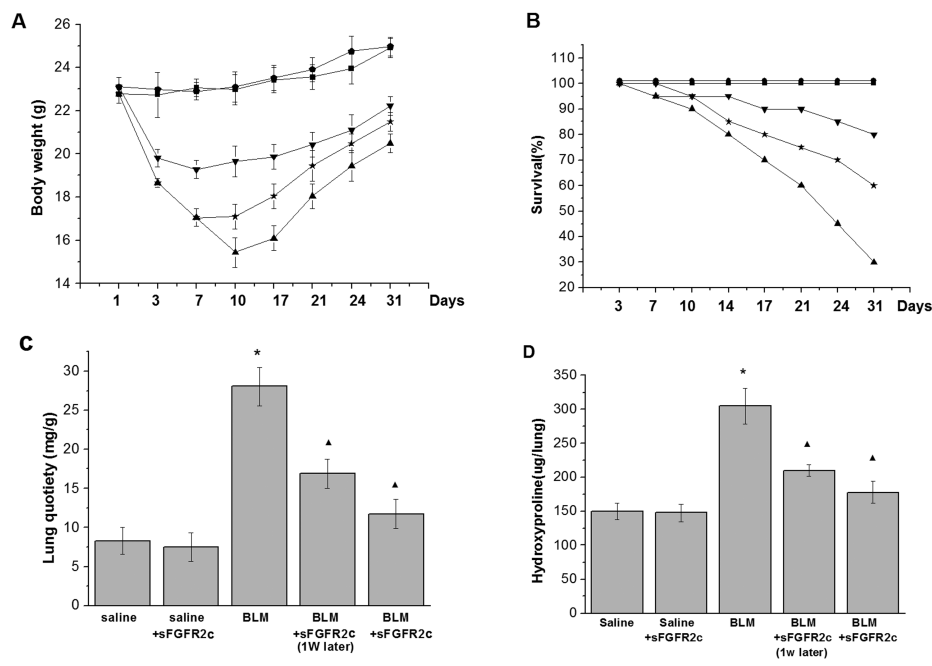


Figure 10. The effects of sFGFR2c on the survival rate and the lung coefficient (lung weight/body weight). (A) The body weights. (B) The survival rates. (C) The lung coefficients. (D) The amount of collagen as measured by hydroxyproline assay. ● represents the saline-treated group; ■ represents the group treated with saline and sFGFR2c; ▼ represents the BLM-treated group; * represents the group treated with BLM and sFGFR2c; ▲ represents the group treated with BLM and sFGFR2c (1 wk later).

response in mice. Although FGF-2 is a known mitogen and angiogenic promoter and is often upregulated after wounding and inflammation, there is no direct evidence to identify its relationship to inflammation. The mechanism by which sFGFR2c inhibits the BLM-induced inflammatory response in the lungs of mice will need to be explored in further studies.

sFGFR2c Inhibits the Expression of Profibrotic Marker Genes in the Mouse BLM Model

After 17 or 31 d, the animals were killed, and the level of fibrosis was determined by the gene expression profiling of TGF- β 1, FGF-2 and CTGF from isolated lung tissue. As shown in Figure 9, the expression of these factors was very low in the saline-treated and saline + sFGFR2c-treated control group and increased after BLM treatment on d 17. In mice exposed to BLM, treatment with 50 μ g/daily sFGFR2c from d 3 to d 31

and d 10 to d 31 resulted in expression levels comparable to those observed in the mice from the two control groups.

sFGFR2c Effectively Inhibited BLM-induced Pulmonary Fibrosis

In vivo, our results demonstrated that the survival rates of the BLM and BLM + sFGFR2c groups were 50% and 80%, respectively, by d 31 (Figure 10B). The lung quotient of the BLM + sFGFR2c group was less than that of the BLM group (Figure 10C). The HE staining assay showed that lung fibrosis increased in severity after mice were given BLM from d 3–31. In BLM-treated mice, 50 μ g daily sFGFR2c for 4 wks significantly prevented BLM-induced lung fibrosis. Using a hydroxyproline assay and Masson staining (Figures 10D, 11A, respectively), we observed that sFGFR2c effectively inhibited the deposition of collagen I during lung fibrosis. We also examined the inhibitory effect of sFGFR2c on BLM-induced fibrosis when it was adminis-

tered intraperitoneally from d 10 to 31. HE staining results showed that sFGFR2c could also reverse the fibrosis successfully, suggesting a therapeutic potential for sFGFR2c in lung fibrosis (Figure 11A). Immunohistochemical staining indicated that sFGFR2c significantly inhibited the phosphorylation of FGFRs, ERK1/2 and Smad3, and the expression of α -SMA (mesenchymal cell markers) (Figure 11B). These results are consistent with the results described above in primary mouse lung fibroblast cells. In addition, the expression of CD34 (a marker of new vessels) was observed. The levels of CD34 were high in the BLM group, the BLM + sFGFR2c group and the BLM + sFGFR2c (1 wk later) group compared with the saline group and the saline + sFGFR2c group (no clear difference between these two groups), which indicated that sFGFR2c had no obvious inhibiting effect on angiogenesis even though it was an FGF-2 antagonist.

DISCUSSION

FGF-2, a well-known mitogen, is believed to play a role in lung fibrosis by promoting the proliferation of fibroblasts and myofibroblasts (14,34). However, scientists have realized that the function of FGF signaling in the fibrosis process is complex. It has been reported that FGF-2 is an important contributor to EMT by stimulating microenvironmental proteases essential for the disaggregation of organ-based epithelial units (14,35). The expression of mesenchymal markers, such as α -SMA and FSP-1, seems to be affected by FGF-2 (36). Furthermore, TGF- β and FGF-2 might cooperate with each other and regulate the EMT of various types of cells in microenvironments during cancer progression (18,37). However, there have been contrary reports, including results that suggest that TGF- β 1-induced EMT is reversed by FGF-1 in IPF (19).

In our study, TGF- β promoted the release of a large amount of FGF-2 quickly, as well as promoting the expression of FGFR2IIIc (the most common receptor of FGF-2 [28]), and formed an integrated

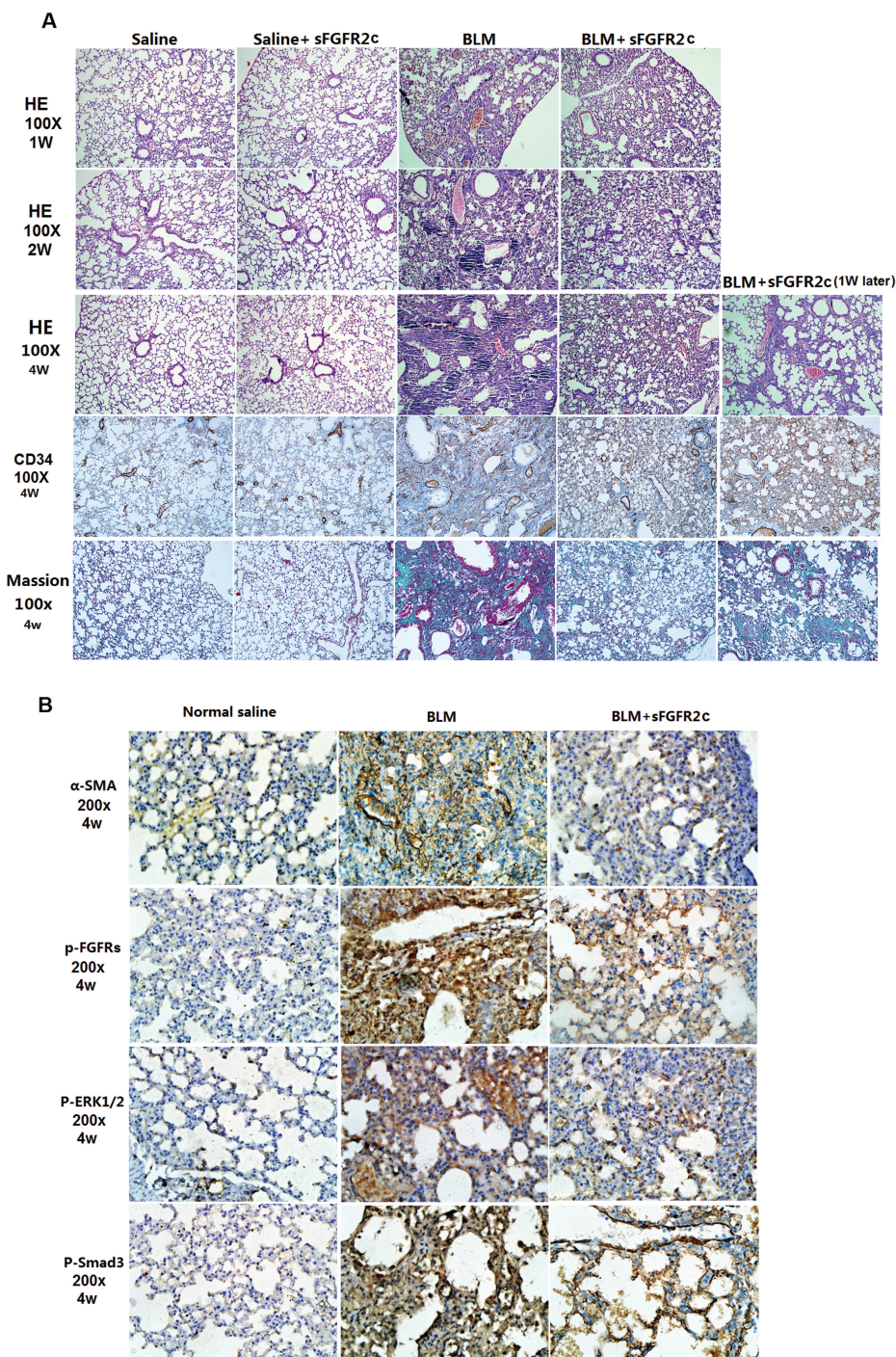


Figure 11. The effects of sFGFR2c on BLM-induced pulmonary fibrosis. Histological analysis of the lungs was performed in the mice treated with saline, saline + sFGFR2c, BLM and BLM + sFGFR2c at d 10, 17 and 31. (A) In the mice treated with BLM, there was more distortion of the alveolar architecture (HE staining) and deposition of collagen (Masson) compared with the mice treated with BLM + sFGFR2c. CD34 (a marker of new vessels) was assayed immunohistochemically, and no clear inhibitory effect of sFGFR2c on angiogenesis was found. (B) The phosphorylation levels of FGFRs, ERK1/2, and Smad3 in the group treated with BLM + sFGFR2c were lower than those in the group treated with BLM.

and enhanced signal pathway for FGF-2. This finding suggests that the relationship between TGF- β and the FGF-2 signaling pathway is very close.

sFGFR2c can bind to the FGF-2 released by TGF- β and inhibit the activation of the FGF signaling pathway by blocking the binding of FGF-2 to membrane FGFRs (21). Previous reports indicated that, in some instances, FGF-2 is profibrotic via the MAPK signal pathway and stimulates the binding of AP-1 to DNA, a nuclear factor required for the activation of multiple genes involved in fibrosis (16), including α -SMA, PAIL, TIMP3 and FN1 (29–31). In our studies, we observed that sFGFR2c inhibited the induction of these genes by TGF- β . Such results suggested that the FGF-2 that is released by TGF- β might play a role in the expression of the fibrotic genes that are induced by AP-1 and that the inhibition of sFGFR2c might function by blocking the FGF-2 signal.

The degree to which sFGFR2c or anti-FGF-2 was found to abrogate α -SMA induction by TGF- β 1 was beyond our expectations. In some reported studies (38,39), δ EF1 was found to be required for the TGF- β -mediated induction of α -SMA. Therefore, we speculated that FGF-2 could activate ctBP1 through the MEK-ERK pathway and bind to δ EF1, thereby playing a role in the α -SMA induction by TGF- β . Because the different signal pathways and their different functions exist in different cells, it is necessary to further explore the relationship between TGF- β and FGF-2 in the induction of α -SMA. Because PD98059, an inhibitor of ERK1/2, could inhibit the induction of α -SMA by TGF- β 1 in our study, the ERK1/2 signaling pathway might be the point at which TGF- β 1 and FGF-2 converge in the induction of α -SMA expression. The inhibitory effect of sFGFR2c on BLM-induced lung fibrosis is also related to this pathway.

In our si-Smad3 experiment, the α -SMA expression that was induced by TGF- β 1 was diminished, indicating that the α -SMA expression was Smad3-dependent, which is consistent with the

work of Masszi *et al.* (40). Meanwhile, the inhibition of α -SMA expression by sFGFR2c suggested that the FGF signaling pathway participated in the Smad3-dependent induction of α -SMA and that the FGF signal might engage in crosstalk with the Smad3 signaling pathway (35,37,41). The cooperation or antagonism between FGF and the TGF- β signaling pathway depends on the different stages of development and/or the different cell contexts.

CONCLUSION

In our study, FGF-2 appeared to be a new target for inhibiting lung fibrosis. As an FGF-2 antagonist, sFGFR2c significantly inhibits lung fibrosis and can be considered a potentially novel antifibrotic drug.

ACKNOWLEDGMENTS

This work was funded by the Ministry of Science and Technology of China (2009ZX09103-632) and by the National Natural Science Foundation of China (91029742). We thank S Fenyong, W Xing and C Xiao-jia for technical help, invaluable discussions and reagents.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES

- American Thoracic Society, European Respiratory Society. (2002) American Thoracic Society / European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. *Am. J. Respir. Crit. Care Med.* 165:277–304.
- Desmouliere A, Darby IA, Gabbiani G (2003) Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab. Invest.* 83:1689–707.
- Phan SH. (2002) The myofibroblast in pulmonary fibrosis. *Chest.* 122(6 Suppl):286-95.
- Hinz B. (2007) Formation and function of the myofibroblast during tissue repair. *J. Invest. Dermatol.* 127:526–37.
- Gabbiani G. (2003) The myofibroblast in wound healing and fibrocontractive diseases. *J. Pathol.* 200;500–3.
- Chaudhary NI, *et al.* (2007) Inhibition of PDGF, VEGF and FGF signalling attenuates fibrosis *Eur. Respir. J.* 29;976–85.
- Hu B, Wu Z, Phan SH. (2003) Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. *Am. J. Respir. Cell Mol. Biol.* 29;397–404.
- Phanish MK, Wahab NA, Colville-Nash P, Hendry BM, Dockrell ME. (2006) The differential role of Smad2 and Smad3 in the regulation of pro-fibrotic TGFbeta1 responses in human proximal-tubule epithelial cells. *Biochem. J.* 393(Pt 2);601–7.
- Munger JS, *et al.* (1999) The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell.* 96;319–28.
- Rodríguez-Pascual F, Redondo-Horcajo M, Lamas S. (2003) Functional cooperation between Smad proteins and activator protein-1 regulates transforming growth factor-b-mediated induction of endothelin-1 expression. *Circ. Res.* 92;1288–95.
- Rodríguez-Pascual F, Reimunde FM, Redondo-Horcajo M, Lamas S. (2004) Transforming growth factor-b induces endothelin-1 expression through activation of the Smad signaling pathway. *J. Cardiovasc. Pharmacol.* 44;S39–42.
- Krieg T, Abraham D, Lafyatis R. (2007) Fibrosis in connective tissue disease: the role of the myofibroblast and fibroblast-epithelial cell interactions. *Arthritis Res. Ther.* 9;(Suppl 2):S4.
- Clozel M, Salloukh H. (2005) Role of endothelin in fibrosis and anti-fibrotic potential of bosentan. *Ann. Med.* 37;2–12.
- Maltseva O, Folger P, Zekaria D, Petridou S, Masur SK. (2001) Fibroblast growth factor reversal of the corneal myofibroblast phenotype. *Invest. Ophthalmol. Vis. Sci.* 42; 2490–5.
- Mulder KM. (2000) Role of Ras and Mapks in TGFbeta signaling. *Cytokine Growth Factor Rev.* 11;23–35.
- Finlay GA, Thannickal VJ, Fanburg BL, Paulson KE. (2000) Transforming growth factor-beta 1-induced activation of the ERK pathway/activator protein-1 in human lung fibroblasts requires the autocrine induction of basic fibroblast growth factor. *J. Biol. Chem.* 275;27650–6.
- Pepper MS, Belin D, Montesano R, Orci L, Vassalli JD. (1990) Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. *J. Cell Biol.* 111;743–55.
- Mukherjee A, Dong SS, Clemens T, Alvarez J, Serra R. (2005) Co-ordination of TGF-beta and FGF signaling pathways in bone organ cultures. *Mech. Dev.* 122;557–571.
- Ramos C, *et al.* (2010) FGF-1 reverts epithelial-mesenchymal transition induced by TGF- β 1 through MAPK/ERK kinase pathway. *Am. J. Physiol. Lung Cell Mol. Physiol.* 299;L222-31.
- Eswarakumar VP, Lax I, Schlessinger J. (2005) Cellular Signaling by FGF receptors *Cytokine Growth Factor Rev.* 16;139–49.
- Gowardhan B, *et al.* (2005) Evaluation of the fibroblast growth factor system as a potential target for therapy in human prostate cancer. *Br. J. Cancer.* 92;320–7.
- Ibrahimi OA, *et al.* (2004) Biochemical analysis of pathogenic ligand-dependent FGFR2 mutations suggests distinct pathophysiological mechanisms for craniofacial and limb abnormalities. *Hum. Mol. Genet.* 13;2313–24.
- Ibrahimi OA, Zhang F, Eliseenkova AV, Linhardt RJ, Mohammadi M. (2004) Proline to arginine mutations in FGF receptors 1 and 3 result in Peifer and Muenke craniosynostosis syndromes through enhancement of FGF binding affinity. *Hum. Mol. Genet.* 13;69–78.
- Nakamura S, Murakami-Mori K, Rao N, Weich HA, Rajeev B. (1997) Vascular endothelial growth factor is a potent angiogenic factor in AIDS-associated Kaposi's sarcoma-derived spindle cells. *J. Immunol.* 158;4992–5001.
- Chaudhary NI, Schnapp A, Park JE (2006) Pharmacologic differentiation of inflammation and fibrosis in the rat bleomycin model. *Am. J. Respir. Crit. Care Med.* 173;769–76.
- Khalil N, Xu YD, O'Connor R, Duronio V. (2005) proliferation of pulmonary interstitial fibroblasts is mediated by transforming growth factor- β 1-induced release of extracellular fibroblast growth factor-2 and phosphorylation of p38 MAPK and JNK. *J. Biol. Chem.* 280;43000–9.
- Powers CJ, McLeskey SW, Wellstein A. (2000) Fibroblast growth factors, their receptors and signaling. *Endocr. Relat. Cancer.* 7;165–97.
- Mohammadi, M. (2005) Structural basis for fibroblast growth factor receptor activation. *Cytokine Growth Factor Rev.* 16;107–37.
- Hu Y, *et al.* (2006) Role of extracellular signal-regulated kinase, p38 kinase, and activator protein-1 in transforming growth factor-beta1-induced alpha smooth muscle actin expression in human fetal lung fibroblasts in vitro. *Lung.* 184;33–42.
- Vayalil PK, *et al.* (2007) Glutathione suppresses TGF-beta-induced PAI-1 expression by inhibiting p38 and JNK MAPK and the binding of AP-1, SP-1, and Smad to the PAI-1 promoter. *Am. J. Physiol. Lung Cell Mol. Physiol.* 293;L1281–92.
- Spence HJ, *et al.* (2006) AP-1 differentially expressed proteins Krp1 and fibronectin cooperatively enhance Rho-ROCK-independent mesenchymal invasion by altering the function, localization, and activity of nondifferentially expressed proteins. *Mol. Cell. Biol.* 26;1480–95.
- Hinz B, *et al.* (2007) The myofibroblast: one function, multiple origins. *Am. J. Pathol.* 170;1807–16.
- Jules-Elysee K, White DA. (1990) Bleomycin-induced pulmonary toxicity. *Clin. Chest Med.* 11;1–20.
- Nugent MA, Iozzo RV. (2000) Fibroblast growth

- factor-2. *Int. J. Biochem. Cell Biol.* 32;115–120.
35. Cushing MC, Mariner PD, Liao JT, Sims EA, Anseth KS. (2008) Fibroblast growth factor represses Smad-mediated myofibroblast activation in aortic valvular interstitial cells. *FASEB J.* 22;1769–77.
36. Rossini M, et al. (2005) Immunolocalization of fibroblast growth factor-1 (FGF-1), its receptor (FGFR-1), and fibroblast-specific protein-1 (FSP-1) in inflammatory renal disease. *Kidney Int.* 68;2621–8.
37. Han L, Gotlieb AI. (2011) Fibroblast growth factor-2 promotes in vitro mitral valve interstitial cell repair through transforming growth factor- β /Smad signaling. *Am. J. Pathol.* 178;119–27.
38. Chinnadurai G. (2009) The transcriptional corepressor CtBP: a foe of multiple tumor suppressors. *Cancer Res.* 69;731–4.
39. Shirakihara T, et al. (2011) TGF-beta regulates isoform switching of FGF receptors and epithelial-mesenchymal transition. *EMBO J.* 30;783–95.
40. Masszi A, et al. (2010) Fate-determining mechanisms in epithelial-myofibroblast transition: major inhibitory role for Smad3. *J. Cell Biol.* 188;383–99.
41. Papetti M, Shujath J, Riley KN, Herman IM. (2003) FGF-2 antagonizes the TGF-beta1-mediated induction of pericyte alpha-smooth muscle actin expression: a role for myf-5 and Smad-mediated signaling pathways. *Invest. Ophthalmol. Vis. Sci.* 44;4994–5005.