Beneficial impact of bFGF antisense therapy in a rat model of pulmonary fibrosis

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Abstract. *Objectives:* This study aims to explore basic fibroblast growth factor (bFGF)'s role in the development of pulmonary fibrosis through applying bFGF antisense oligonucleotide therapy in a rat model of pulmonary fibrosis. *Methods:* Thirty rats were randomly divided into five groups: bFGF sense-transfected, bFGF antisense-transfected, null vector-transfected, pulmonary fibrosis (PF), and control groups. Sense, antisense, null, and PF groups were administered bleomycin to induce pulmonary fibrosis. Sense, antisense, and null vectors were intratracheally injected into the lungs of their respective groups followed by sacrifice after 28 days post-injection. Lung coefficients, H&E and Masson trichome staining, and serum and bronchoalveolar lavage fluid bFGF expression were comparatively assessed in addition to lung homogenate mRNA expressions of several select proteins and hydroxyproline content. *Results:* The antisense and sense groups had significantly decreased and increased lung coefficients and pulmonary fibrosis than the PF and null groups, respectively, with the pulmonary fibrosis stage positively correlated with treatment. Antisense, PF, and null groups showed significantly reduced collagen fiber levels compared to the sense group. The antisense group displayed significantly lower serum and lavage fluid bFGF expression in addition to significantly lower bFGF, α-smooth muscle actin, Smad3, transforming growth factor-β1, connective tissue growth factor, collagen I (and significantly higher Smad7) mRNA expression relative to the PF and null groups. The antisense and sense groups showed significantly higher hydroxyproline content relative to the PF and null groups. *Conclusions:* bFGF appears to promote collagen I synthesis and upregulates TGF-β1/Smad signaling to promote lung fibroblast proliferation and differentiation in pulmonary fibrosis. bFGF antisense oligonucleotide therapy shows promise in preventing the development of pulmonary fibrosis, likely though a TGF-β1/Smad-based signaling mechanism. *(Sarcoidosis Vasc Diffuse Lung Dis 2015; 32: 22-31)*

Key words: basic fibroblast growth factor, bFGF; pulmonary fibrosis

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive, fatal lung disease with a median survival period of three years (1). Currently, the pathogenesis of IPF remains unclear, and no effective drugs are available to prevent its progress (2). IPF is characterized by fibroblast proliferation and deposition in extracellular matrix (3). As lung fibroblasts play a critical role in interstitial collagen and extracellular matrix formation, an improved understanding of the biological behavior of fibroblasts can contribute to revealing the mechanism(s) underlying pulmonary fibrosis (3).

To this end, inhibition of fibroblast proliferation has become a novel therapeutic strategy for

treatment-resistant forms of pulmonary fibrosis (3). Excessive activation of the transforming growth factor (TGF)-β1 signaling pathway has been associated with fibroblast proliferation and differentiation to myofibroblasts (4) — a special type of fibroblast that is relatively rare in normal lung tissue and displays the following differences from the normal fibroblast phenotype: (i) larger and more morphologically irregular; (ii) oval or irregular nucleus; (iii) rich rough endoplasmic reticulum and Golgi complex; (iv) microtubules intensively arranged in parallel under the plasma membrane; and (v) α-SMA-coated microtubular actin that enables contractility similar to smooth muscle cells (5).

Mechanistically, the major signaling pathways of the TGF-β1-induced fibroblast-to-myofibroblast differentiation are the TGF-β1/PI3K/Akt and the TGF-β1/Smad signaling pathways (6,7). The TGF-β1/Smad signaling pathway has been closely associated with fibrotic processes across a number of internal organs, including the heart, liver, lungs, and kidneys (8), and Smad proteins play a critical role in mediating TGF-β1 signal transduction from the cell surface to the nucleus (9). Smad signal transduction can be divided into three categories: (i) receptor-activated Smad (R-Smad, including Smadl, Smad2, Smad3, Smad5, and Smad8) — Smad2 and Smad 3 show substrate specificity for TGF-β type I receptor (TβRI), a mediator of TGF-β1 and hormone signaling, that phosphorylates Smad2/3, which activates nuclear Smad4 that regulates connective tissue growth factor (CTGF) and procollagen-α1 gene expression; (ii) co-dielectric-type Smad (Co-Smad, Smad4) — phosphorylated Smad4 combines with R-Smad to form a complex polymer that localizes to the nucleus to affect target gene expression; and (iii) inhibitory Smad (I-Smad, including Smad6 and Smad7) — Smad7 is considered one of the most important negative regulators of TGF-β1/Smad signaling via its binding to TβRI, which further affects R-Smad phosphorylation and activation (9).

Aside from the TGF-β1/Smad activation of CTGF expression, basic fibroblast growth factor (bFGF, FGF-2) has also been identified as a mitogen for fibroblasts and myofibroblasts (10). Both TGF-β and bFGF have been demonstrated to play crucial roles in inducing fibroblast proliferation and differentiation in different stages of a bleomycin-induced pulmonary fibrosis model (11), and bFGF

overexpression has been detected in the lung tissue and bronchoalveolar lavage fluid of patients with pulmonary fibrosis (12). bFGF is produced by a variety of cell types, including alveolar macrophages, fibroblasts, T-lymphocytes, endothelial cells, and mast cells (13), and higher levels of bFGF protein and mRNA expression have been found in the lung mast cells of patients with chronic pulmonary fibrosis with the degree of pulmonary fibrosis associated with the number of mast cells that overexpress bFGF accompanied by larger numbers of mast cells localizing in the extracellular matrix proximate to smooth muscle cell/myofibroblast proliferation (14-16).

These previous findings suggest that bFGF is directly related to the fibroblast proliferation and differentiation underlying pulmonary fibrosis. Therefore, this investigation aims to explore bFGF's role in the development of pulmonary fibrosis through a well-established bleomycin-induced rodent model of pulmonary fibrosis (17). We applied bFGF antisense oligonucleotide therapy in the setting of bleomycin-induced pulmonary fibrosis to (i) determine whether bFGF antisense oligonucleotides negatively regulate lung fibroblast proliferation, differentiation into myofibroblasts, and collagen synthesis via the TGF-β1/Smad signaling pathway through (ii) assessing the influence of bFGF antisense oligonucleotides on the mRNA expression of α-smooth muscle actin (α-SMA), Smad3, Smad 7, TGF-β1, CTGF, and collagen I in lung tissue.

Materials and Methods

Subjects and Materials

All animal experiments were performed according to the guidelines established by the Chinese Council on Animal Care and approved by the Ethics Committee of Wuhan University (license number: 047). Wistar rats (2-3 months old, 180-250 g) were purchased from the Experimental Animal Center at Wuhan University. We purchased ketamine hydrochloride (0.1 g/unit, Fujian Gutian Pharmaceutical Co., Ltd., China), bleomycin (8.0 mg/unit, A5, lot number 051003, Tianjin Taihe Pharmaceutical Co., Ltd., China), a bFGF enzyme-linked immunosorbent assay (ELISA) kit (Hebei Bohai

Biotech Co., China), rat hydroxyproline pre-coated ELISA kits (SouthernBiotech, Birmingham, AL, U.S.), and a One-Step RT-PCR Kit (TaKaRa Co., China).

Oligonucleotide Synthesis and Vector Construction

Oligonucleotide and complementary antisense oligonucleotides of the rat bFGF gene (consisting of 15 nucleotides each) were synthesized as follows: 5'-CGGCAGCCATGGCCC-3'. The following null thiosense oligonucleotide sequence was also synthesized: 5'-GGGCCATGGCTGCCG-3'.

The pSNAV plasmid was purchased from Vector Gene Technology Co. Ltd. (Beijing, China), which contains AAV inverted terminal repeats (ITR) and the cytomegalovirus immediate early (CMV IE) promoter, followed by a multiple clone site (MCS). The rat bFGF sense, antisense, and null oligonucleotides were inserted into pSNAV vectors and transfected into baby hamster kidney-21 (BHK-21) cells (ATCC, Manassas, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Cells stably expressing the oligonucleotides were selected by 800 µg/ml G418 (neomycin) (Invitrogen, Carlsbad, CA, USA) after 24 hours of transfection. These cells were plated in a 15-cm-diameter dish and incubated at 37°C in 5% CO_2 until reaching 90% confluency (about 8×10^8 cells per dish). Then, the cells were infected by the HSV-1 helper virus, which contained the Rep and Cap genes necessary for rAAV virus replication and packaging, at a multiplicity of infection (MOI) of 0.1. After 48 hours, the cells were harvested and submitted to single-step gravity-flow column purification. The titer and purity of the viruses were measured by Southern blot analyses using a dig-labeled probe and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), respectively.

Experimental Groups

We selected 25 female Wistar rats and randomly divided them into the following five groups (five rats per group): bFGF sense-transfected group (sense group), bFGF antisense-transfected group (antisense group), null vector-transfected group (null group), pulmonary fibrosis group (PF group), and healthy control group (control group).

Four groups, including sense group, antisense group, null group, and PF group, were ether anesthesized and induced into pulmonary fibrosis by intratracheal injection of 0.4% bleomycin in saline (5 mg/kg). To control for the effects of the operation, the control group was intratracheally injected with the equivalent amount of saline (1 ml/kg). Immediately after bleomycin instillation, sense, antisense, and null groups were intratracheally injected with equal amounts (3 ml) of the sense, antisense, and null vectors, respectively. All rats were sacrificed at day 28 post-transfection.

To comparatively assess the role of antisense bFGF gene transfection and hydroxyproline content changes rate in lung tissue, the PF group was administered bleomycin at the same time as the transfected groups, and the control group underwent sample collection at the same time points as the other groups.

Specimen Collection

At day 28 post-transfection, all rats were anesthetized, weighed, and laparotomized by saline gauze blunt dissection to expose the abdominal aorta for blood collection. After centrifugation, serum samples were cryopreserved at -70 for later bFGF detection. All rats were then sacrificed and thoracotomized. The tracheas were severed at the neck, stripped down to remove whole lungs, and weighed in order to calculate the lung coefficient= lung weight (mg)/body weight (g) × 100% (18).

Next, we ligatured the hilar of the left lung, and performed a left pneumonectomy for lung homogenate. We also inserted a 12# blunt needle via the trachea into the right lung, ligatured the connection in place, douched with sterile saline (5 ml per time, total of three times), and collected the lavage fluid. After centrifugation, supernatant liquid was cryopreserved in sterile tubes at -70 for later bFGF detection. We then intratracheally injected 10% neutral formalin (4-6 ml) into right lung to expand the pleura, ligatured the trachea, and fixed the right lung in 10% formalin paraffin for histological examination.

Lung Morphology and Histopathology via Light Microscopy

According to a previously described method (19), the right lungs were immersed in 10% forma-

lin, then the lower lobes were removed for paraffin sectioning, H&E, and Masson trichrome staining. Pulmonary fibrosis was graded on a scale from 0-3 for 'no fibrosis,' 'mild fibrosis,' 'moderate fibrosis,' and 'severe fibrosis,' respectively.

ELISA and RT-PCR

We followed the ELISA kit manual instructions to assay the amount of bFGF expression in serum and bronchoalveolar lavage fluid. We used RT-PCR to assay the mRNA expression of bFGF, α-SMA, collagen I, TGF-β1, connective tissue growth factor (CTGF), Smad3, and Smad7 in the lung homogenate samples from the left lung. The housekeeping gene β-actin was used as internal control. Hydroxyproline content in lung homogenate samples from the left lung were assayed according to the ELISA kit manual instructions.

Statistical Methods

Experimental data were expressed as means ± standard deviations using of SPSS 10.1. Differences between groups were assessed using analysis of variance (ANOVA) and q-testing.

RESULTS

Lung Coefficients

The sense group showed a significantly higher lung coefficient (i.e., lung weight (mg)/body weight (g) × 100%) than the control (P=0.000) and antisense groups (P=0.001) (Table 1).

Histopathological Lung Changes

To induce interstitial pulmonary fibrosis, bleomycin was administered after sense, antisense, and null vector transfection in the sense, antisense, and null groups, respectively; the PF group was administered bleomycin with no vector. Pulmonary fibrosis was significantly reduced in the antisense group relative to the PF and null groups (P<0.05; Table 2). The stage of pulmonary fibrosis was positively correlated with treatment (Kendall's tau-b=0.4879, P=0.0039). Masson trichrome staining re-

Table 1. Lung coefficients by experimental group

Group	n	Lung coefficient (mean±SD)*
Control	5	6.98±0.78
PF	5	10.98±1.40
Null	5	11.02±1.32
Antisense	5	8.81±1.48
Sense†	5	3.22±1.44

*Lung coefficient is defined as the lung weight (mg)/body weight (g) × 100%.
†The post hoc test found that the sense group showed a significantly higher lung coefficient than the control (P=0.000) and antisense groups (P=0.001).

Table 2. Pulmonary fibrosis stage by experimental group

Group	Pulmonary fibrosis stage*			
	0	1	2	3
Control	5	0	0	0
PF	0	0	4	1
Null	0	1	2	2
Antisense	1	3	1	0
Sense	0	0	0	5

* Fisher exact Chi-square test, df=12, $chi2$=42.9167, P=0.000. Kendall's tau-b=0.4879 and P=0.0039 revealed that the stage of pulmonary fibrosis was positively correlated with treatment
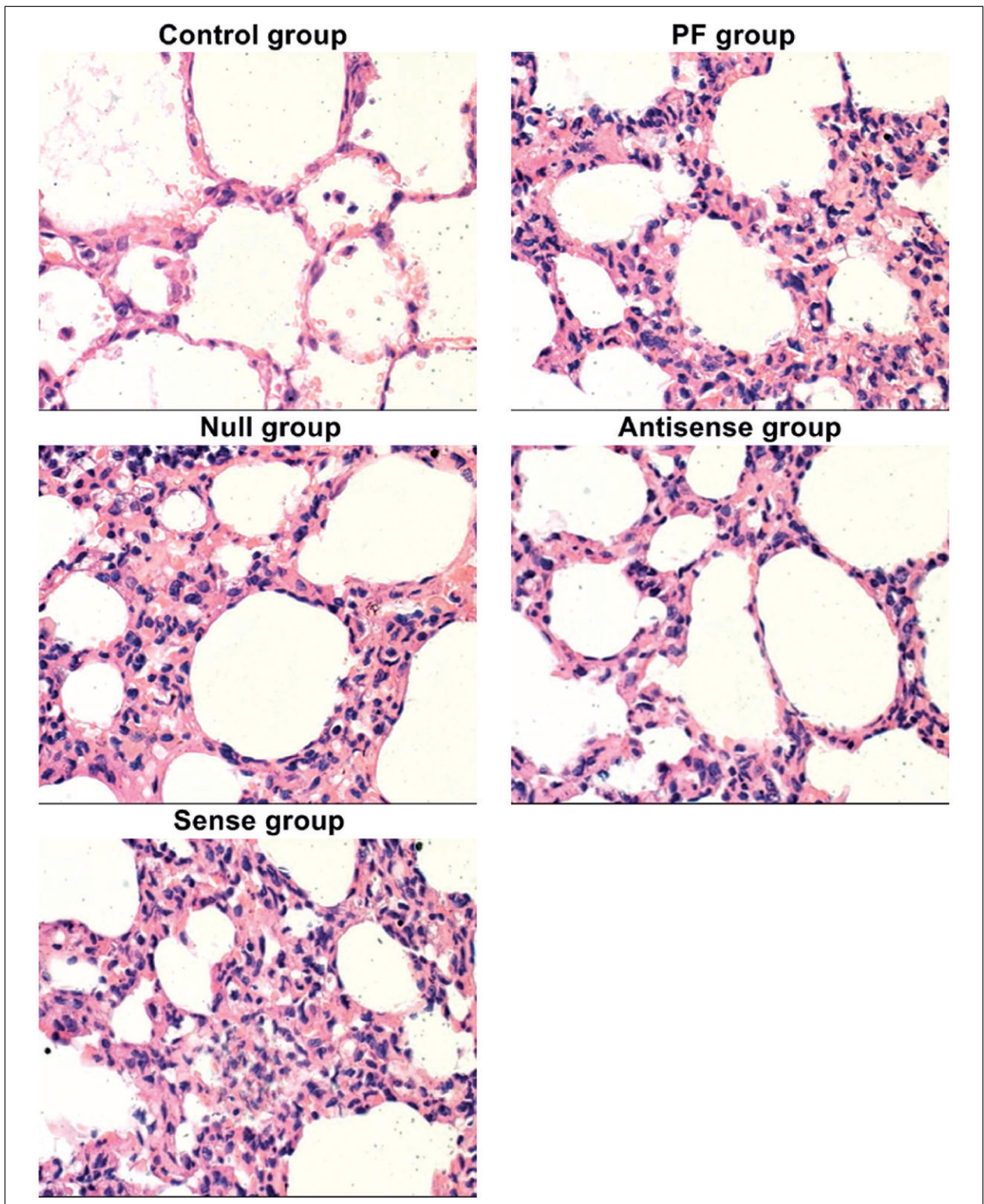
vealed that the PF, null, and antisense groups showed significantly reduced levels of collagen fibers compared to the sense group (Fig. 1). Across all time points, the null group showed no significant differences with the PF group (Fig. 2).

Serum and Bronchoalveolar Lavage Fluid bFGF Expression
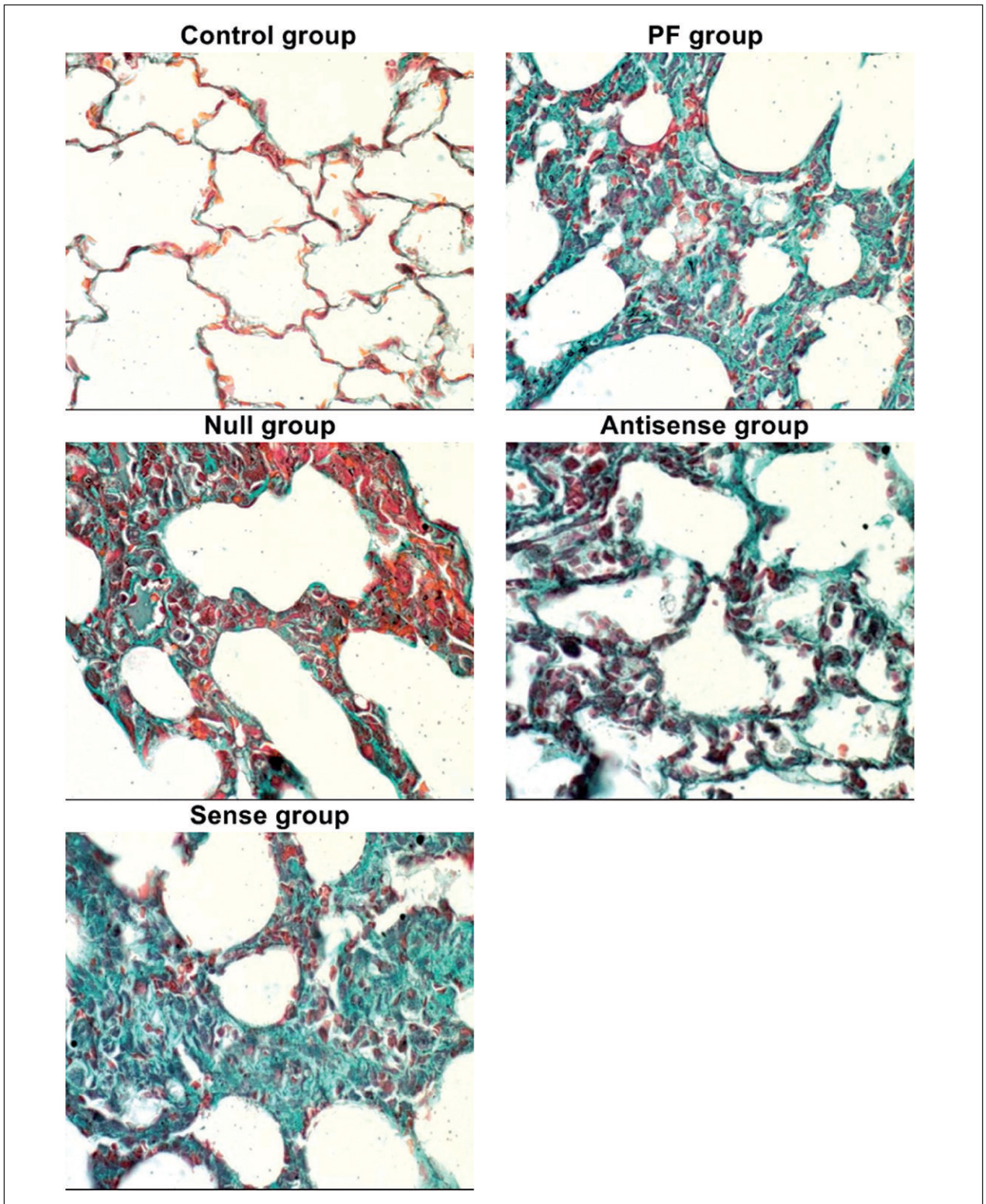
With respect to bFGF expression in serum, the antisense group displayed significantly lower bFGF expression relative to the sense group and higher bFGF expression relative to the control group (P<0.05; Figure 3). As to bFGF expression in lavage fluid, the antisense group displayed significantly lower bFGF expression relative to the sense group and significantly higher bFGF expression relative to the control group (P<0.01). The sense group presented significantly higher lavage fluid bFGF expression relative to all other groups.

mRNA Expression in Lung Homogenate

The antisense group showed significantly lower bFGF, α-SMA, Smad3, TGF-β1, CTGF, and col-

Fig. 1. H&E Staining Comparison of the Five Groups (×100). Pulmonary fibrosis was significantly reduced in the antisense group relative to the PF and null groups.

Fig. 2. Masson Trichrome Staining Comparison of the Five Groups (×100). The PF, null, and antisense groups showed significantly reduced levels of collagen fibers compared to the sense group.
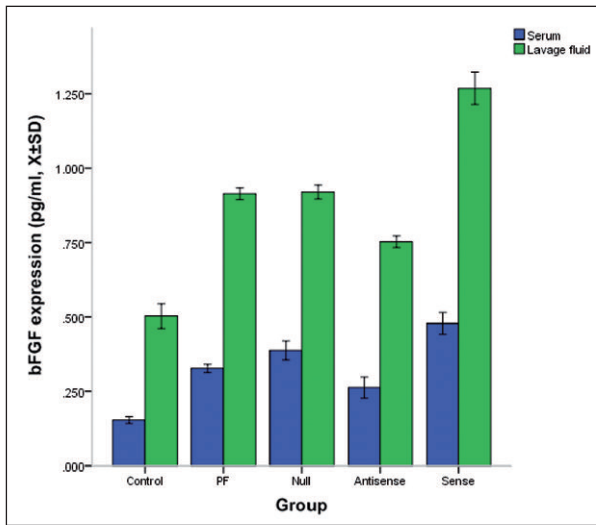
Fig. 3. bFGF Expression in Serum and Lavage Fluid (pg/ml, X±SD). The post hoc test revealed that the antisense group presented significant difference with control both in serum (*P*=0.033) and lavage fluid (*P*=0.003). Post hoc testing revealed that the sense group presented significant differences with both control and antisense groups in serum (*P*=0.000). The sense group displayed significant differences with all groups in lavage fluid (*P*=0.000).

lagen I mRNA expression relative to the PF, null, and sense groups (*P*<0.01; Figure 4) and significantly higher bFGF, α-SMA, Smad3, TGF-β1, CTGF, and collagen I mRNA expression relative to the the control group (*P*<0.01). The antisense group showed significantly higher Smad7 mRNA expression relative to the PF and null groups (*P*<0.01). The sense group showed significantly higher Smad3, TGF-β1, CTGF, and collagen I (in addition to significantly lower Smad 7) mRNA expression relative to all other groups (*P*<0.05). The sense group showed significantly higher bFGF and α-SMA mRNA expression relative to only the control and antisense groups. Across all time points, the null group showed no significant differences with the PF group (*P*>0.05).

Hydroxyproline Content

By ELISA, the antisense group presented with significantly lower lung hydroxyproline content relative to the PF and null groups, while the sense group presented with significantly higher lung hydroxyproline content relative to the PF and null groups (*P*=0.000; Figure 5).
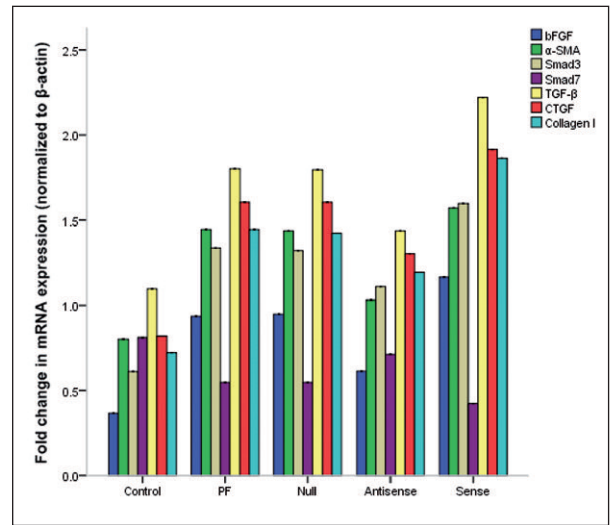
Fig. 4. Relative mRNA Expression in Lung Homogenate (X±SD). Relative mRNA expression levels were calculated as the fold-change relative to β-actin expression (housekeeping gene). Post hoc testing revealed that the antisense group displayed significant differences with all other groups in bFGF, α-SMA, Smad3, TGF-β1, CTGF, and collagen I mRNA expression (*P*=0.000). However, the antisense group only showed significant differences with the PF and null groups in Smad7 mRNA (*P*=0.000). Post hoc testing revealed that the sense group showed significant differences with all other groups in Smad3, Smad7, TGF-β1, CTGF, and collagen I mRNA expression (*P*=0.000). However, the sense group only displayed significant differences with control and antisense groups in bFGF and α-SMA mRNA expression (*P*=0.000).
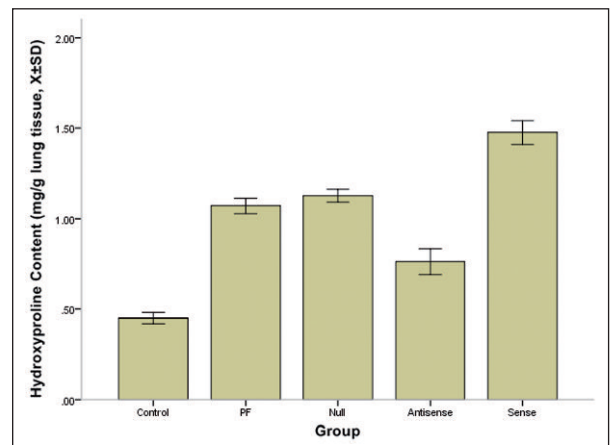
Fig. 5. Hydroxyproline Content (mg/g lung tissue, X±SD). Post hoc testing revealed that the antisense group presented significant differences in hydroxyproline content with all groups (*P*=0.000). Post hoc testing revealed that the sense group showed significant differences in hydroxyproline content with all groups (*P*=0.000).

Discussion

Pulmonary fibrosis is common outcome of a variety of diffuse pulmonary inflammatory diseases; however, its pathogenesis remains unclear (2). Nevertheless, the pathological changes associated with pulmonary fibrosis have been consistently characterized by alveolar epithelial injury, enhanced extracellular matrix deposition, and fibroblast proliferation primarily consisting of fibroblasts and myofibroblasts (20). These findings suggest that myofibroblasts are derived from fibroblast differentiation in the lung interstitium during the course of pulmonary fibrosis. These fibroblasts and myofibroblasts secrete collagen I and , the major components of the extracellular matrix (21). One previous study has shown that pulmonary fibrosis displays increased expression of collagen-producing proteins including TGF-β1, gelatinase B (matrix metalloproteinase (MMP)-9), and the matrix metalloproteinase inhibitors TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (22); however, the same study found no difference in the expression of collagen-degrading proteins, such as collagenase 1 (MMP-1) and gelatinase A (MMP-2), suggesting that the collagen synthesis outpaces collagen degradation in pulmonary fibrosis, resulting in increased collagen deposition.

In the current study, pathological changes consistent with pulmonary fibrosis were observed in lung tissue 28 days after bleomycin administration. At all time points, the bFGF antisense group displayed significantly reduced fibrosis, collagen I mRNA expression, and hydroxyproline content compared to the PF and null groups ($P<0.05$), while the bFGF sense group showed significantly increased fibrosis, collagen I mRNA expression, and hydroxyproline content compared with the PF and null groups ($P<0.05$). Meanwhile, the bFGF antisense group displayed significantly lower bFGF levels in the serum and bronchoalveolar lavage fluid in addition to significantly lower lung bFGF mRNA expression relative to the PF and null groups ($P<0.01$), while the bFGF sense group showed significantly higher levels of all these parameters relative to the PF and null groups ($P<0.05$). These findings indicate that bFGF promotes fibroblast proliferation and collagen fiber synthesis, suggesting that bFGF may be a key factor in pulmonary fibrosis development.

In the present study, to observe bFGF's effect on differentiating lung fibroblasts into myofibroblasts (which characteristically express α-SMA) (5), RT-PCR detection of ±-SMA in lung homogenate showed that ±-SMA expression of the bFGF antisense group was significantly lower than the PF and null groups ($P<0.01$), while the bFGF sense group displayed significantly higher α-SMA expression than the PF and null groups ($P<0.05$), suggesting that bFGF release during pulmonary fibrosis promotes lung fibroblast differentiation into myofibroblasts.

TGF-β1 is a key cytokine that induces pulmonary fibrotic processes, and TGF-β1 transgenic mice have been shown to suffer from severe pulmonary fibrosis (23), suggesting that TGF-²1-induced pulmonary fibrosis not only involves fibroblast proliferation and differentiation to myofibroblasts, but also induction of CTGF expression, increases in extracellular matrix (ECM) synthesis, and inhibition of collagen-degrading enzymes to prevent interstitial collagen dissolution (24,25). In the current study, RT-PCR detection of TGF-β1 and CTGF in lung homogenate found that the bFGF antisense group displayed significantly lower TGF-β1 and CTGF expression than the PF and null groups ($P<0.01$), while the bFGF sense group showed significantly higher expression of these two factors than the PF and null groups ($P<0.05$), suggesting that bFGF promotes TGF-β1 and CTGF secretion by fibroblasts and myofibroblasts during pulmonary fibrosis.

TGF-β1-induced fibroblast-to-myofibroblast differentiation is driven by the TGF-β1/Smad signaling pathway that has been closely associated with pulmonary fibrotic processes (9). In the present study, the bFGF antisense group showed significantly lower Smad3 and higher Smad 7 mRNA expression relative to the PF and null groups ($P <0.01$), while the bFGF sense group showed significantly higher Smad3 and lower Smad7 relative to the PF and null groups ($P<0.05$). As Smad3 is excitatory and Smad7 is inhibitory (9), these findings suggest that bFGF activates the TGF-β1/Smad signaling pathway, thereby promoting downstream CTGF expression.

There are several limitations to this study. First, although bFGF antisense appears to inhibit fibroblast differentiation here, the mRNA results are not sufficient to prove this effect (if any). Thus, further

immunohistochemical and *in vitro* studies are required to detect bFGF effects on myofibroblast differentiation. Second, in addition to mRNA findings, TGF-β1 and CTGF protein data are also needed to clarify the effects of bFGF antisense. Third, this study failed to measure mast cell distribution or the effect of bFGF sense or antisense on mast cell activity. Fourth, in the bleomycin-induced rat model of pulmonary fibrosis, the shift from inflammation to fibrosis typically occurs around day nine post-bleomycin administration (26), so potential anti-fibrotic therapies should be evaluated in the latter phase of established fibrosis (nine days post-bleomycin) as opposed to the earlier period of bleomycin-induced inflammation and early fibrosis. However, in this study, all vectors were administered immediately post-bleomycin administration; therefore, our future studies in this rat model will administer vectors at least nine days post-bleomycin to assess the effect of the vectors on the later fibrotic phase. Fifth, the body/lung weight variation post-bleomycin administration is not totally attributable to fibrotic change, as this phenomenon is partially due to inflammation-related edema between days seven and fourteen post-bleomycin administration (27). Therefore, our future studies in this rat model, we will seek to establish a correlation between the lung coefficient and the fibrosis score and collagen accumulation in the different groups on day 28 post-bleomycin administration and compare the lung coefficients on days seven and fourteen post-bleomycin administration.

In conclusion, bFGF appears to promote collagen I synthesis and upregulates TGF-β1/Smad signaling to promote lung fibroblast proliferation and differentiation in pulmonary fibrosis. bFGF antisense oligonucleotide therapy shows promise in preventing the development of pulmonary fibrosis, likely though the TGF-β1/Smad signaling pathway.

AUTHOR CONTRIBUTIONS

Zhenshun Cheng designed the study; Jiong Yang performed the experiment and conducted the data collection; Weijun Tan analyzed the data; Li Zhang drafted the original manuscript; and Qiuyue Tan and Zhenshun Cheng supervised the data analysis and edited the manuscript for intellectual content and style.

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