Pirfenidone and nintedanib attenuate pulmonary fibrosis in mice by inhibiting the expression of JAK2

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Background: Pirfenidone and nintedanib were approved by the Food and Drug Administration (FDA) for the treatment of idiopathic pulmonary fibrosis (IPF). These two drugs can slow the progression of the disease, but the specific mechanisms are not fully understood. In the current study, bleomycin (BLM) induced pulmonary fibrosis in mice was accompanied by high p-JAK2 expression in lung tissue, mainly in the nucleus. The expression of p-JAK2 significantly decreased after intragastric administration of pirfenidone and nintedanib. p-JAK2 is reportedly expressed mainly in the cytoplasm and exerts its effect by activating downstream p-STAT3 in the nucleus.

Methods: *In vivo* experiments, pulmonary fibrosis was induced in mice with BLM and then treated with pirfenidone and nintedanib. The levels of transforming growth factor-β (TGF-β1), SP-A, SP-D and KL-6 in serum were measured by enzyme-linked immunosorbent assay (ELISA). Pathological staining was performed to assess lung fibrosis in mice, Western blot was performed to detect the expression levels of relevant proteins, and immunofluorescence was performed to observe the fluorescence expression of p-JAK2. In cellular experiments, MLE12 was stimulated with TGF-β1 and intervened with TGF-β1 receptor inhibitor and si-JAK2, pirfenidone and nintedanib, respectively, and the related protein expression levels were detected by Western blot.

Results: In both *in vivo* and *in vitro* experiments, pirfenidone and nintedanib were found to attenuate the expression of lung fibrosis markers by inhibiting the expression of JAK2, which may reduce the entry of p-JAK2 into the nucleus by downregulating JAK2 phosphorylation through inhibition of the TGF-β receptor. In contrast, inhibition of JAK2 expression greatly reduced the expression of TGF-β receptor and α-smooth muscles actin (a myofibroblast activation marker).

Conclusions: In both *in vivo* and *in vitro* experiments, the present study demonstrated that TGF-β1 promotes JAK2 phosphorylation through a non-classical pathway, and conversely, inhibition of JAK2 expression affects the TGF-β1 signalling pathway. Therefore, we speculate that TGF-β1 and JAK2 signaling pathways interact with each other and participate in fibrosis.

Keywords: Pirfenidone; nintedanib; pulmonary fibrosis; JAK2; transforming growth factor-β (TGF-β1)

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Introduction

Idiopathic pulmonary fibrosis (IPF) is the most common type of interstitial lung disease (ILD). IPF is most common among men and among people over the age of 50 years. The disease course of IPF is variable and unpredictable, with a median survival of only 2–4 years after diagnosis (1). The intrapulmonary manifestation of IPF is that activated cells in the alveoli release numerous cytokines and growth factors, which promote the recruitment, proliferation and differentiation of lung fibroblasts into myofibroblasts, resulting in excessive collagen deposition, progressive scarring of the lung parenchyma, and irreversible loss of lung function (2). IPF affects three million people worldwide. The currently available antifibrotic drugs include pirfenidone and nintedanib. Although these two drugs can slow disease progression, they cannot improve or stabilize lung function and may cause intolerance issues (3).

Pirfenidone is an oral pyridine that exhibits anti-inflammatory, antioxidant and antifibrotic effects in both cell and animal experiments, including modulation of transforming growth factor-β (TGF-β) expression and inhibition of fibroblast and collagen synthesis (4-7). Nintedanib is an inhibitor of multiple tyrosine kinase receptors involved in the pathogenesis of pulmonary fibrosis, for example, platelet-derived growth factor (PDGF) receptors α and β; vascular endothelial growth factor (VEGF) receptors 1, 2, and 3; and fibroblast growth factor (FGF) receptors 1, 2, and 3. Nintedanib has been shown to prevent the development of pulmonary fibrosis in the bleomycin (BLM) mouse model (8-11). Although pirfenidone and nintedanib

are Food and Drug Administration (FDA)-approved for the treatment of IPF, their mechanisms of action are not fully understood (12).

TGF-β is a multifunctional cytokine with three subtypes: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 plays a major role in pulmonary fibrosis. TGF-β1 is a potent profibrotic mediator that promotes epithelial-mesenchymal transition (EMT), epithelial cell apoptosis, epithelial cell migration, and the production of other profibrotic mediators (13). In fibrosis, TGF-β1 promotes the activation of Smad2/3 by binding and activating its receptor complexes (TβRI and TβRII). Activated Smad2/3 forms a complex with common partner Smads, Smad4; this complex enters the nucleus and binds certain Smad-binding sequences in the promoter regions of target genes, including fibronectin, collagen and plasminogen activator inhibitor-1 (PAI-1), promoting their transcription. Furthermore, TGF-β1 signaling induces fibrosis not only through Smad-dependent canonical pathways but also through Smad-independent noncanonical pathways, such as the JAK/STAT, Ras-MAPK, TAK1-p38/JNK, PI3K-Akt, and Par6-Smurf1 pathways (14,15).

The JAK/STAT signaling pathway is crucial for cell homeostasis and plays important roles in tumors, inflammation and autoimmune diseases. It is also a pathway involved in ILD (16,17). The JAK2/STAT3 pathway is activated in a variety of fibrotic diseases, such as bone marrow, skin, liver, myocardial and kidney fibrosis (18). In addition to the Smad pathway, TGF-β1 can also induce the phosphorylation of JAK2 through noncanonical pathways. JAK2 interacts with STAT3 and phosphorylates the latter to induce fibrosis (16,18). In addition, JAK2 can be activated by other profibrotic mediators, including PDGF, VEGF, interleukin (IL)-6, IL-13, angiotensin II (ANGII), serotonin (5-HT), and endothelin-1 (ET-1) (18,19). Previous study has reported the presence of STAT3 phosphorylation in fibrotic lung tissue from IPF patients and indicated that STAT3 phosphorylation is involved in the transition from fibroblasts to myofibroblasts and causing damage to lung epithelial cells (19). We hypothesized that pirfenidone and nintedanib can inhibit the expression of JAK2 through the TGF-β1 signaling pathway, thereby attenuating pulmonary fibrosis. The results from cell experiments indicated that there may be mutual influence between the TGF-β1 signaling pathway and the JAK2 signaling pathway. We present this article in accordance with the ARRIVE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1057/rc).

Highlight box

Key findings
- In this study, two pathways, JAK2/STAT3 and transforming growth factor-β, were found to interact with each other through anti-fibrotic drugs and to be jointly involved in the development of pulmonary fibrosis.

What is known and what is new?
- Pirfenidone and nintedanib have antifibrotic effects.
- We identified the mechanism of action of these two antifibrotic drugs in bleomycin-induced lung fibres in mice.

What is the implication, and what should change now?
- We can look at different pathway mechanisms to find the most appropriate way to reduce the extent of pulmonary fibrosis.

Methods

Animals and cells

Animals

Forty female C57BL/6 mice (body weight: 18–20 g; age: 8 weeks) were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (Chengdu, China) and raised at a temperature of 20–26 ℃. The animals had free access to food and water. Experiments were started after one week of adaptive feeding with standard chow. BLM (Takasaki Plant, Nippon Kayaku Co., Ltd., Japan), pirfenidone (AdooQ Bioscience, Nanjing, China), and nintedanib (AdooQ Bioscience) were used for subsequent *in vivo* experiments.

Cells

Mouse lung epithelial cells (MLE12; Procell Life Science & Technology Co., Ltd., Wuhan, China) were cultured in F12 medium (Shanghai Fuheng Biotechnology Co., Ltd., Shanghai, China) containing 10% fetal bovine serum (FBS) (Biochannel Company, Nanjing, China) under 37 ℃ and 5% CO_2, and cells at passages 3–5 were used for the experiments. TGF-β1 (PeproTech, USA), LY2109761 (a TGF-β1 receptor inhibitor; AdooQ Bioscience), and si-JAK2 (Guangzhou RiboBio Co., Ltd., Guangzhou, China) were used for subsequent *in vitro* experiments. Experiments were performed under a project license (No. Dossy20210119001) granted by the Ethics Committee of Chengdu Dashuo Biotechnology Co. (Chengdu, China), in compliance with institutional guidelines for the care and use of animals.

Animal modeling and intervention

After 40 8-week-old SPF female C57BL/6 mice were adaptively reared for one week, 10 mice were randomly selected as the control group, and the remaining 30 mice were intratracheally injected with BLM (5 mg/kg) on the first day to generate a pulmonary fibrosis model. On the third day, the 30 mice were randomly divided into three groups, with 10 mice in each group: BLM group (0.1 mL CMC-Na), pirfenidone group (20 mg/kg), and nintedanib group (60 mg/kg) (20,21). After the mice in each group were intragastrically administered the corresponding drug every other day until the 28th day, they were anesthetized and sacrificed for the collection of lung tissue and peripheral plasma.

Enzyme-linked immunosorbent assay (ELISA)

The samples taken for this experiment were serum from mice. ELISAs were used to detect TGF-β1, SP-A, SP-D and KL-6, strictly following the instructions of the ELISAs kit. ELISAs kits were purchased from Jingmei Biotechnology (Jiangsu, China) to complete the experiments. All experimental steps were carried out according to the instructions.

Histological and immunohistochemical analyses

Lung tissue was fixed with 4% formalin for 12–16 h, dehydrated, and embedded in paraffin. The specimen blocks were cut into 5-µm-thick sections. The sections were stained with hematoxylin-eosin (H&E) to observe inflammatory infiltration and the structural integrity of the alveoli. Masson staining was used to assess the extent of collagen deposition.

For immunohistochemical staining of α-smooth muscle actin (α-SMA) and collagen I, tissue slides were deparaffinised with xylene, polarised with decreasing concentrations of alcohol and rinsed with deionised water. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide solution for 30 min at room temperature after antigen repair. The slides were closed with 10% normal goat serum for 1 hour. The primary antibody was incubated overnight at 4 ℃ and then the secondary antibody was incubated for 60 min at room temperature. The slides were visualised by diaminobenzidine (DAB) staining and restained with hematoxylin. Integral optical density (IOD) and positive area (area) were quantified by Image J. The mean optical density (MOD = IOD/area) was calculated to evaluate the expression of α-SMA and collagen I.

Western blotting

Total tissue protein was extracted from cell lysates (Beyotime, Shanghai, China), and the protein concentration was determined using a bicinchoninic acid (BCA) protein quantification kit (Beyotime). Protein samples were electrophoresed on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk powder at room temperature for 2 h, incubated with appropriately diluted primary antibody at

4 ℃ overnight, rinsed 3 times, and incubated with horseradish peroxidase (HRP)-labeled secondary antibody at room temperature for 2 h. The bands were visualized with a fluorescence chemiluminescence imaging system (Clinx Science Instruments, Shanghai, China). Anti-TGF-β1, anti-α-SMA, anti-Fn, anti-GAPDH, anti-JAK2, anti-p-JAK2, anti-STAT3, and anti-p-STAT3 monoclonal antibodies were purchased from Affinity (Jiangsu, China). Anti-TGF-β-R2 antibodies were purchased from Proteintech. Antibody dilutions were purchased from Beyotime. HRP-labeled goat anti-rabbit and goat anti-mouse IgG antibodies were purchased from EarthOx (USA).

Immunofluorescence

Mouse lung tissue sections and treated cells were collected and blocked with serum. Anti-p-JAK2 antibody (1:50) was added to the sections and cells, which were then incubated at 4 ℃ overnight in the dark. Then, fluorescent secondary antibodies [1:100, fluorescein isothiocyanate (FITC)-labeled, green] were added, and the sections and cells were placed in a wet box in an incubator at 37 ℃ for 1 h. Afterwards, 4',6-diamidino-2-phenylindole (DAPI) was added dropwise, and the sections and cells were incubated in the dark for 5 min. The specimens were mounted with anti-fluorescence quenching agent and observed and photographed under a fluorescence microscope.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using GraphPad Prism 9.0 software. The data were presented as the mean ± standard deviation or mean ± standard error. ANOVA was used to analyze differences among multiple groups, and the independent t test was used to analyze differences between two groups. $P<0.05$ indicated that a difference was statistically significant.

Results

Effects of pirfenidone and nintedanib on plasma biomarkers in mice

We evaluated the therapeutic effect of pirfenidone and nintedanib in mouse pulmonary fibrosis by detecting the levels of TGF-β1, SP-A, SP-D and KL-6 in mouse plasma. *Figure 1A* shows that the levels of TGF-β1, KL-6, SP-A and SP-D in the plasma of the pulmonary fibrosis in the mouse model were significantly increased in the BLM group. In the pirfenidone and nintedanib groups, the elevations in TGF-β1, KL-6, SP-A and SP-D levels in the plasma were reversed by pirfenidone and nintedanib.

Pirfenidone and nintedanib attenuated BLM-induced pulmonary fibrosis in mice by inhibiting the expression of JAK2

Figure 1B shows H&E and Masson staining of lung tissue from mice sacrificed on day 28. Regarding the H&E results, the mice in the control group had relatively intact lung tissue structures, thin alveolar septa, and a few inflammatory cells in the alveolar septa; the mice in the BLM group had severely damaged lung tissue structures, markedly thickened alveolar septa, considerable inflammatory cell infiltration, and evident inflammatory responses; and the mice in the pirfenidone and nintedanib groups showed less alveolar structure damage and inflammatory cell infiltration than those in the BLM group. Regarding the Masson staining results, there was no obvious blue staining of lung tissue in the control group. The lung tissue in the BLM group was severely damaged, with a large area of blue staining, indicating the deposition of a large number of collagen fibers. Inflammation and fibrosis in the lung tissue of mice in the pirfenidone and nintedanib groups were attenuated compared with those in the lung tissue of mice in the BLM group.

α-SMA is a recognised biomarker of myofibroblast activation and collagen I is an important factor for ECM deposition in the lung interstitium. *Figure 1C* shows the expression of α-SMA and collagen I in the alveoli and interstitium of the lung observed by immunohistochemistry. The results indicated that α-SMA and collagen I were highly expressed in the interstitium in the BLM group, and the expression of α-SMA and collagen I was significantly reduced in the drug-treated group compared with the BLM group.

To determine whether nintedanib and pirfenidone can reduce pulmonary fibrosis in mice by inhibiting JAK2 expression, Western blotting was performed (*Figure 2A*). The results indicated that the expression of JAK2, p-JAK2 and p-STAT3 was significantly increased in the BLM group and that intervention with pirfenidone and nintedanib significantly reversed the BLM-induced elevations in these proteins. The expression of TGF-β-R2, SMA (a myofibroblast activation marker) and extracellular matrix (ECM) fibronectin in the BLM group increased, and
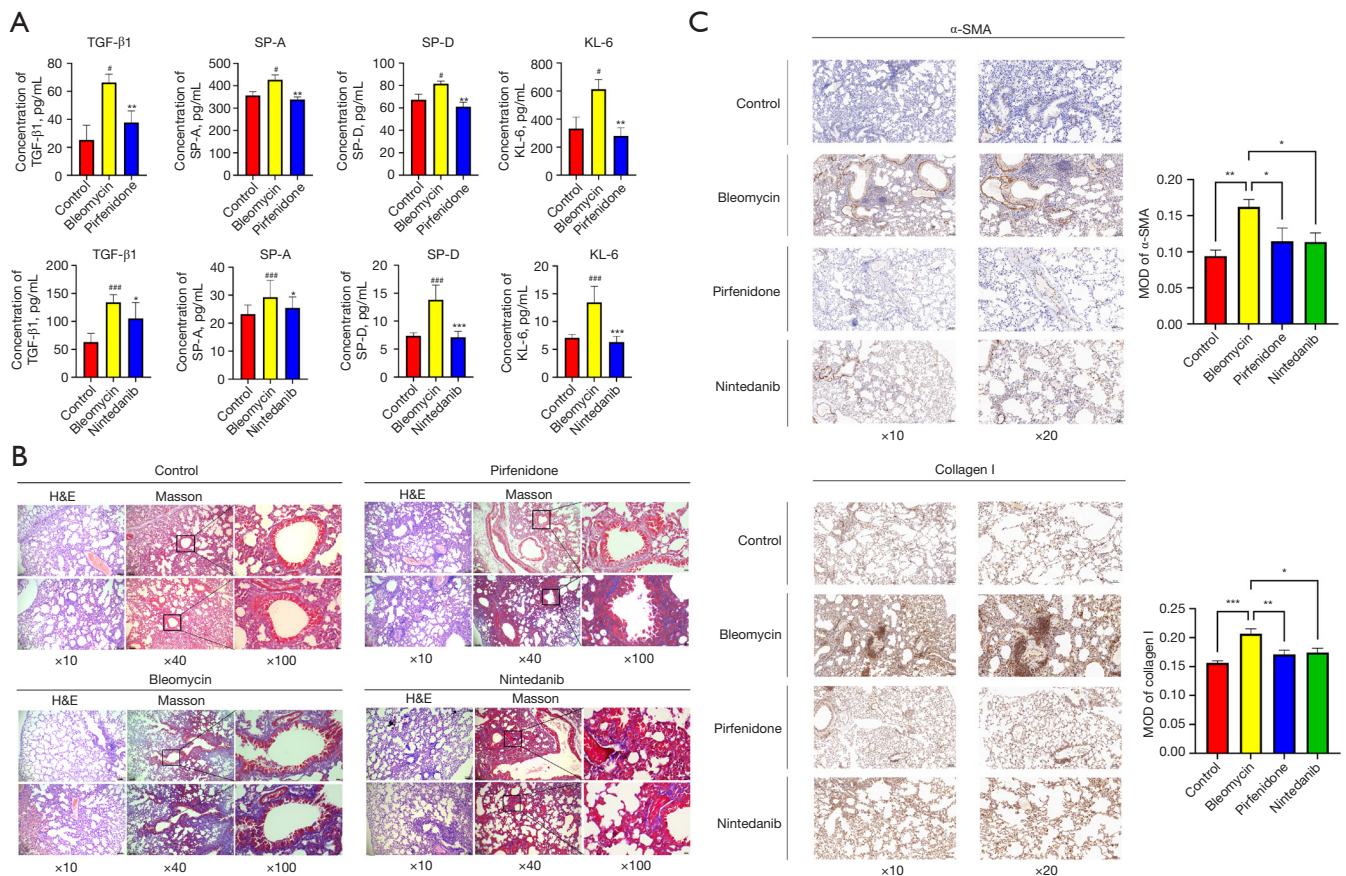
Figure 1 In a mouse lung fibrosis model constructed by bleomycin, fibrosis was significantly attenuated with the intervention of pirfenidone and nintedanib. (A) Levels of TGF-β1, SP-A, SP-D and KL-6 in the plasma of mice. The plasma levels of TGF-β1, KL-6, SP-A and SP-D were significantly increased in mice and were inhibited by pirfenidone and nintedanib (n=3, #, P<0.05; ###, P<0.001, compared with Control. *, P<0.05; **, P<0.01; ***, P<0.001, compared with BLM group). (B) Representative photographs of pirfenidone and nintedanib on bleomycin-induced pulmonary pathological changes in mice (H&E staining and Masson staining of mouse lung tissue). On day 28, interstitial fibrosis was evident in the lungs of mice in the Bleomycin group compared with the Control group. However, lung histopathological changes were significantly reduced in the Pirfenidone group, Nintedanib group. (C) Immunohistochemistry of α-SMA and collagen I (brown areas) in bleomycin-induced pulmonary fibrosis in mice on day 14. Representative images of lung sections from each group are shown. TGF, transforming growth factor; H&E, hematoxylin-eosin; SMA, smooth muscle actin; MOD, mean optical density; BLM, bleomycin.

intervention with pirfenidone and nintedanib reversed the BLM-induced elevations in these proteins. Hence, pirfenidone and nintedanib may inhibit the expression of JAK2 by downregulating the TGF-β1 signaling pathway, thereby attenuating pulmonary fibrosis.

Expression and location of p-JAK2 in mouse lung tissue

As shown in *Figure 2B*, p-JAK2 was located in the nucleus within the fibrotic areas in BLM-induced mouse lung tissue, and the fluorescence signal of p-JAK2 in the fibrotic areas

of BLM-induced mouse lung tissue significantly decreased after pirfenidone and nintedanib treatment. Hence, JAK2 can not only translocate to the nucleus and activate the transcription of target genes through downstream dimerisation of STAT3, but also has the potential to enter the nucleus through phosphorylation and play a role independent of STAT3.

TGF-β1 stimulated JAK2 expression in MLE12 cells

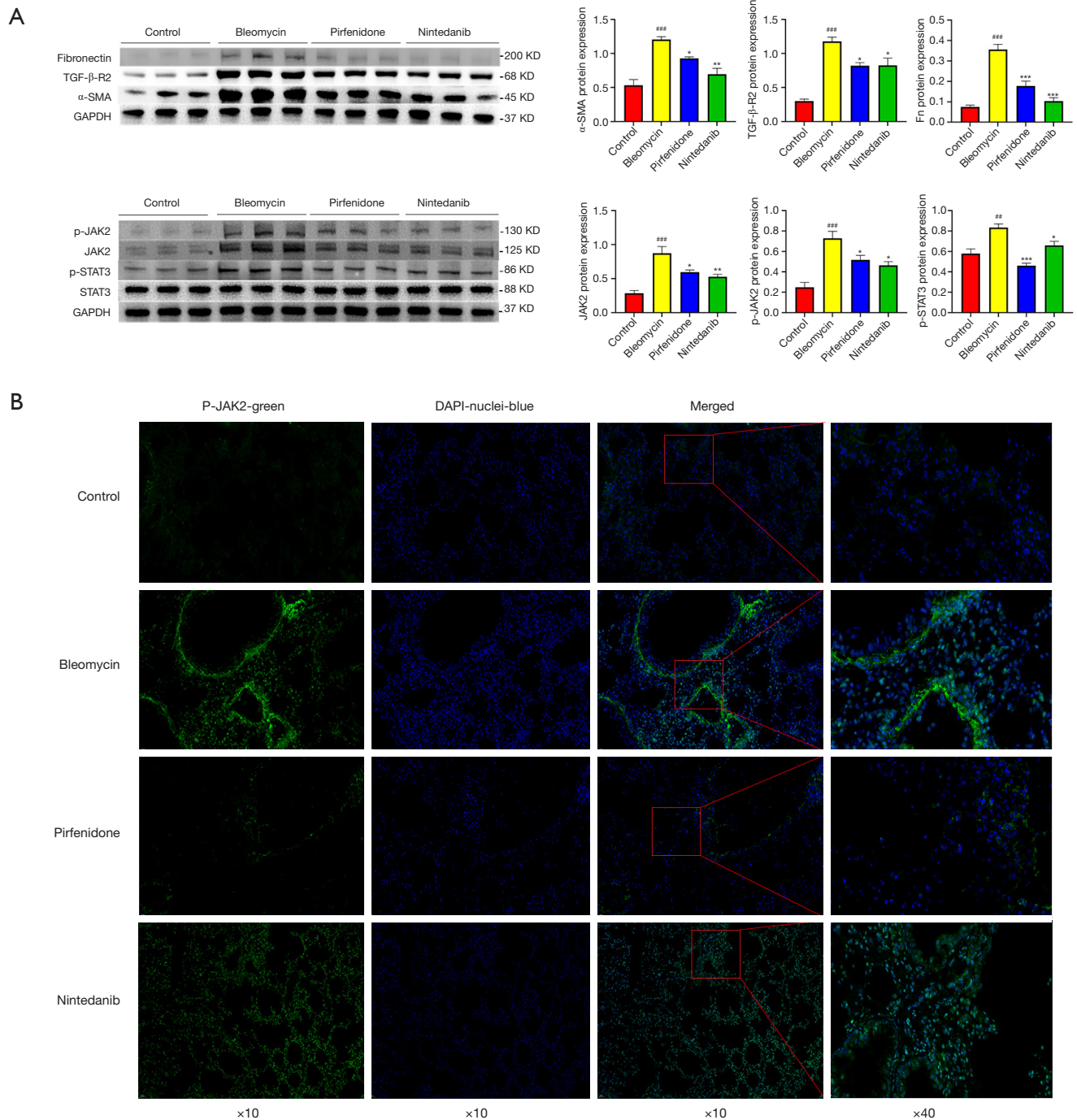Repeated injury of the alveolar epithelium is the main

Figure 2 JAK2 is involved in a model of pulmonary fibrosis in mice constructed with bleomycin. (A) Expression of proteins associated with pulmonary fibrosis in mice by pirfenidone and nintedanib intervention. The protein expression levels of Fn, TGF-β-R2, α-SMA, p-JAK2, JAK2, p-STAT3, and STAT3 in mouse lung was measured by Western blotting. GAPDH was used as the internal control (n=6, ##, P<0.01; ###, P<0.001, compared with Control. *, P<0.05; **, P<0.01; ***, P<0.001, compared with BLM group). (B) Expression of p-JAK2 in pathological sections of mouse lungs was detected by immunofluorescence staining. TGF, transforming growth factor; SMA, smooth muscle actin; DAPI, 4',6-diamidino-2-phenylindole; BLM, bleomycin.

mechanism for the occurrence of IPF. The injured epithelial cells produce numerous cytokines and promote fibrosis. The development of fibrosis involves complex signaling interactions between epithelial and mesenchymal cells. The active TGF-β1 signaling pathway in tissue remodeling has been shown to play an important role in fibrosis. Therefore, we stimulated MLE12 cells with TGF-β1 to construct an epithelial cell injury model.

TGF-β1 induces the phosphorylation of JAK2, which interacts with STAT3 and phosphorylates the latter, promoting fibrosis. In addition, JAK2 can be activated by other profibrotic factors, including PDGF, VEGF, IL-6, IL-13, ANGII, 5-HT, and ET-1. *Figure 3A* shows the expression of JAK2, p-JAK2, p-STAT3, TGF-βR2, SMA, and fibronectin after stimulation of MLE12 cells with different concentrations of TGF-β1 (0, 5, 10, 20, 50, and 100 ng/mL). The expression of these proteins was highest with 50 ng/mL TGF-β1, whereas 100 ng/mL TGF-β1 inhibited the expression of these proteins. To further verify whether TGF-β1 increases JAK2 expression, we treated MLE12 cells with different concentrations of the TGF-β1 receptor inhibitor LY2109761 (0.01, 0.05 and 0.5 µM) and observed the concentration-dependent effect of LY2109761 on JAK2. As shown in *Figure 3B*, with increasing LY2109761 concentrations, the protein expression of JAK2, p-JAK2 and p-STAT3 gradually decreased. Thus, TGF-β1 can stimulate JAK2 expression in MLE12 cells.

Downregulation of JAK2 expression affected the TGF-β1 signaling pathway

To further explore the role of JAK2 in EMT, we observed changes in α-SMA by downregulating the expression of JAK2 (through siRNA or inhibitor treatment). As shown in *Figure 3C*, after downregulating the expression of JAK2, TGF-βR2 and SMA levels significantly decreased. Hence, JAK2 can affect the TGF-β1 signaling pathway, suggesting that there may be mutual influence between the JAK2 and TGF-β1 pathways. We treated MLE12 cells with pirfenidone (1 mM) and nintedanib (1 µM), as shown in *Figure 4A*. JAK2, p-JAK2, p-STAT3 and SMA expression in all treated cells significantly decreased. Therefore, in the *in vitro* experiments, we demonstrated that pirfenidone and nintedanib may attenuate fibrosis by inhibiting JAK2.

JAK2 may function before STAT3 enters the nucleus

In *in vivo* experiments, we observed p-JAK2 in the nuclei of cells in fibrotic areas in mice with pulmonary fibrosis and speculated that p-JAK2 might also be located in the nucleus in cellular experiments. We treated MLE12 cells with TGF-β1 (50 ng/mL) at different time points (0, 1, 2, 6, 12, 24 h) and found that the expression of p-JAK2 increased after treatment at 1 h, decreased after treatment at all time points between 2 and 12 h, and increased after treatment at 24 h (*Figure 4B*). The expression of p-STAT3 was increased after treatment at 2 h, then gradually decreased before increasing again at 24 h. Therefore, we speculate that JAK2 may play a role before STAT3 enters the nucleus.

Discussion

In recent years, increasing evidence has shown that IPF is an epithelial injury-driven disease and that abnormally activated alveolar epithelium can lead to the migration, proliferation, and differentiation of fibroblasts into myofibroblasts. These myofibroblasts secrete large amounts of ECM, leading to lung structure remodeling. At present, despite its limitations, the BLM-induced pulmonary fibrosis model is still a popular model for exploring the pathogenesis of IPF and the effectiveness of new antifibrotic drugs (22,23). In this study, we established a BLM-induced lung fibrosis model in mice and investigated the possible mechanisms of action of two antifibrotic drugs, pirfenidone and nintedanib. MLE12 cells, a widely used mouse capillary alveolar epithelial cell line, were also selected for this experiment, and there is a large body of literature using MLE12 cells to study the biological properties of alveolar epithelial cells and the mechanisms of related diseases (24-26).

Among the animal models of pulmonary fibrosis, in addition to BLM-induced pulmonary fibrosis, they include fluorescein isothiocyanate, silica, and radiation (27) as well as the injury model of butylated hydroxytoluene (BHT) proposed (28). The BHT model is a model with distal alveolar involvement, and the pathology is much closer to that of human pulmonary fibrosis. In contrast, the histology of the BLM animal model shows a proximal airway-centred pattern similar to human airway-centred interstitial pneumonia. However, there is no literature available to suggest whether the molecular mechanisms of signalling that they ultimately lead to pulmonary fibrosis are different. The BLM model is the most widely used and best characterised mouse model. The BLM-induced pulmonary fibrosis model can be readily induced in a short period of time with high reproducibility (29). Therefore, the BLM model was used in this experiment. Although the model
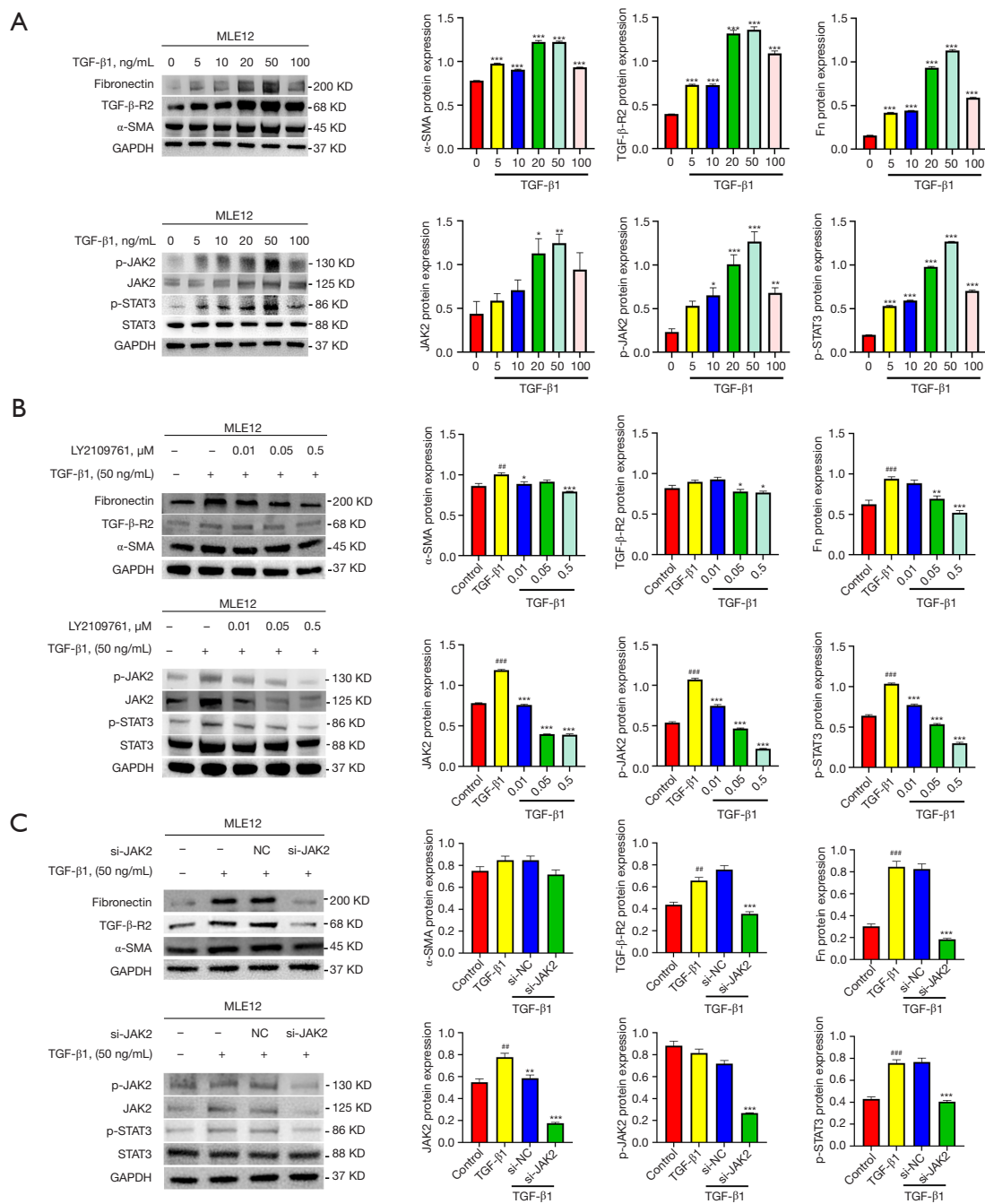
Figure 3 In an *in vitro* model, JAK2 is involved in the signaling pathway of fibrosis. (A) The expression levels of Fn, TGF-β-R2, α-SMA, p-JAK2, JAK2, p-STAT3, and STAT3 proteins were detected by Western blotting after stimulation of MLE12 cell with different concentrations of TGF-β1 (0, 5, 10, 20, 50 and 100 ng/mL) for 48 h; GAPDH was used as the internal control (n=3, *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, compared with 0 ng/mL). (B) After treatment with TGF-β1 (50 ng/mL) for 6 h, the inhibitor of TGF-β1 receptor LY2109761 (0.01, 0.05 and 0.5 μM) was then applied to MLE12 at different concentrations, and Western blotting was performed to detect Fn, TGF-β-R2, α-SMA, p-JAK2, JAK2, p-STAT3, and STAT3 protein expression levels. GAPDH was used as the internal control (n=3, ##, $P<0.01$; ###, $P<0.001$, compared with Control. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, compared with TGF-β1 group). (C) MLE12 was treated with TGF-β1 (50 ng/mL) for 6 h and then treated with NC and si-JAK2, and the expression levels of Fn, TGF-β-R2, α-SMA, p-JAK2, JAK2, p-STAT3, and STAT3 proteins were detected by Western blotting (n=3, ##, $P<0.01$; ###, $P<0.001$, compared with Control. **, $P<0.01$; ***, $P<0.001$, compared with TGF-β1 group). Fn, fibronectin; TGF, transforming growth factor; SMA, smooth muscle actin; NC, negative control.
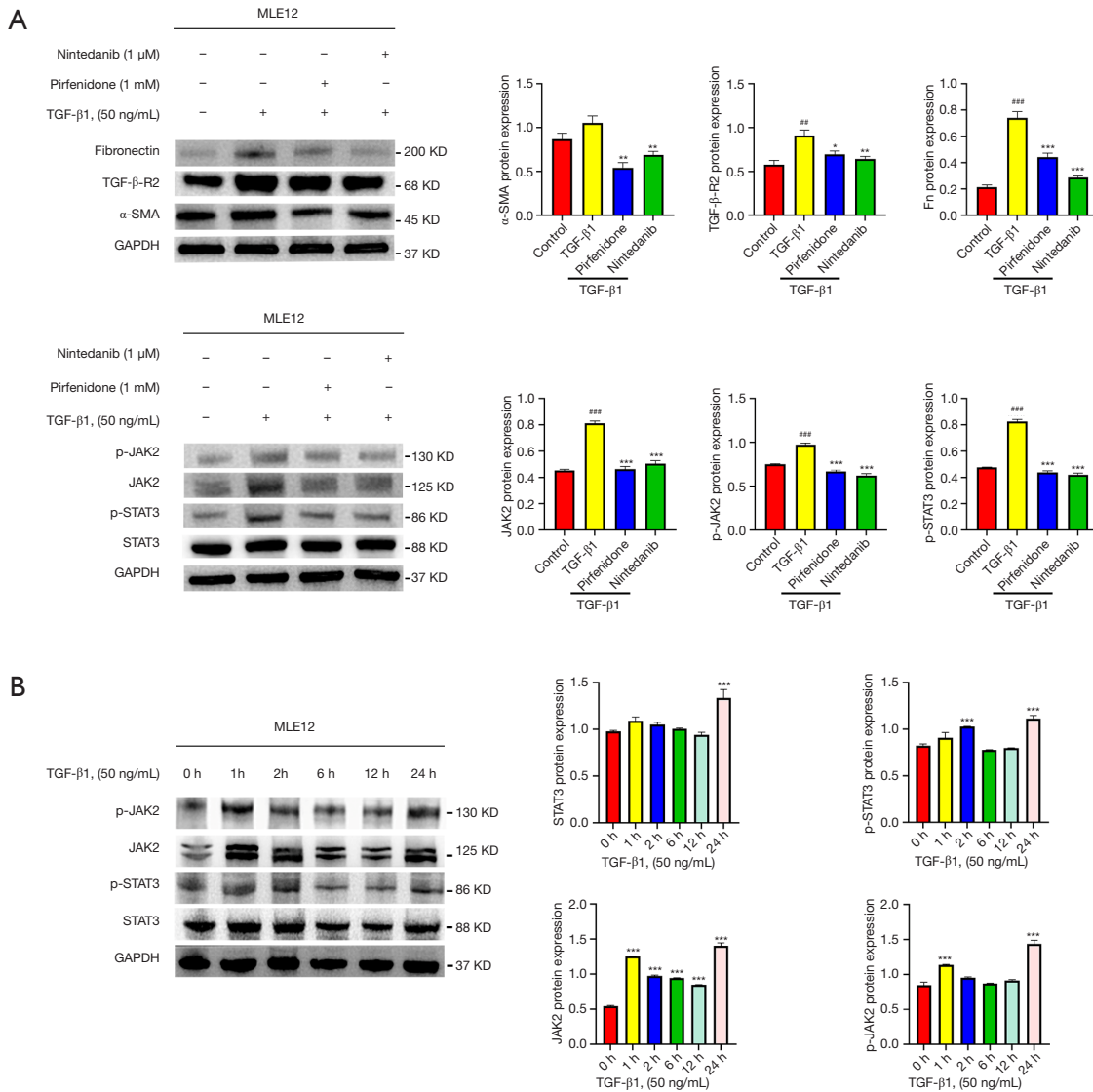
Figure 4 In an *in vitro* model, pirfenidone and nintedanib also significantly attenuated indicators of pulmonary fibrosis. (A) MLE12 was treated with TGF-β1 (50 ng/mL) for 6 h and then intervened with pirfenidone (1 mM) and nintedanib (1 μM), and Western blotting was performed to detect Fn, TGF-β-R2, α-SMA, p-JAK2, JAK2, p-STAT3, and STAT3 protein expression levels. GAPDH was used as the internal control (n=3, ##, P<0.01; ###, P<0.001, compared with Control. *, P<0.05; **, P<0.01; ***, P<0.001, compared with TGF-β1 group). (B) TGF-β1 (50 ng/mL) was treated with MLE12 cell at different time points (0, 1, 2, 6, 12, 24 h), and the expression levels of Fn, TGF-β-R2, α-SMA, p-JAK2, JAK2, p-STAT3, and STAT3 proteins were detected by Western blotting. GAPDH was used as the internal control (n=3, ***, P<0.001, compared with 0 h). Fn, fibronectin; TGF, transforming growth factor; SMA, smooth muscle actin.

still suffers from resorption of pulmonary fibrosis in mice after 30 days of evolution, the model system provides an opportunity to study the regression of fibrosis at these later time points (29).

Due to the variable and unpredictable course of IPF and the lack of easily reproducible surrogate endpoints for patient-related outcomes, molecular biomarkers are urgently needed to improve the prognosis of this chronic lung disease. Therefore, it is of great value to identify easily detectable specific serum biomarkers. Recent studies have reported that peripheral blood can be used to indicate the presence, stage, and prognosis of IPF (30,31). When alveolar epithelial cells are damaged, KL-6, SP-A and SP-D are released from these cells and can be detected in blood. Previous study has suggested that TGF-β1 is involved in fibrotic diseases. This molecule is a fibroblast chemokine that can recruit fibroblasts in surrounding tissues to aggregate, proliferate and differentiate into damaged areas, produce a large amount of collagen, and stimulate the synthesis and secretion of various cytokines (32). KL-6 is a mucin-like glycoprotein expressed on the extracellular surfaces of alveolar type II cells and bronchiolar epithelial cells. As a chemokine, KL-6 promotes the migration, proliferation and survival of lung fibroblasts. SP-A and SP-D are also produced by type II alveolar epithelial cells, and together, the two participate in the formation of alveolar surfactants and in the formation and metabolism of alveolar hyaline membranes (33-36). We detected biomarkers (KL-6, SP-A, and SP-D) in mouse plasma by ELISAs and found that the expression of these markers after pirfenidone and nintedanib treatment was significantly lower than that in the BLM group, suggesting that these biomarkers can potentially be used as indicators of therapeutic efficacy in IPF patients.

Ruan *et al.* reported that oral administration of fedratinib (a JAK2 inhibitor) attenuated the degree of BLM-induced pulmonary fibrosis in mice by reducing myofibroblast activation and reduced the levels of Smad3, Akt, Erk, JNK and P38 phosphorylation, suggested that fedratinib may inhibit the JAK2/STAT3 and TGF-β1/Smad signaling pathways (37). Conte *et al.* demonstrated that pirfenidone attenuated the TGF-β1-mediated differentiation of fibroblasts into myofibroblasts either through a canonical Smad-dependent pathway or a Smad-independent noncanonical pathway (6). We speculated that pirfenidone may inhibit Smad-independent noncanonical pathways to alleviate fibrosis. The results of a previous study suggested that nintedanib can block several signaling pathways simultaneously, for example, growth factor (TGF-β, PDGF, FGF, etc.) signaling pathways (10). Ruan *et al.* reported that some tyrosine kinase inhibitors, such as nintedanib, thalidomide and sunitinib, can strongly inhibit the TGF-β1/Smad3 signaling pathway, suggesting that tyrosine kinase inhibitors also have a relationship with the TGF-β1 signaling pathway (15). Chaudhary *et al.* also noted that many cytokines, such as TGF-β, IL-1 and TNF-α, exhibited PDGF-dependent profibrotic activity and speculated that nintedanib can interfere with JAK2 expression via targeted inhibition of TGF-β1/Smad3 in the PDGF pathway (38). Therefore, we speculated that pirfenidone and nintedanib may attenuate pulmonary fibrosis in mice via the atypical JAK2/STAT3 pathway of TGF-β1.

In this study, to evaluate the antifibrotic effects of pirfenidone and nintedanib, the lung tissues of mice in each group were HE- and Masson-stained. The results indicated that pirfenidone and nintedanib significantly reversed BLM-induced pulmonary fibrosis, a finding that is consistent with the results of previous studies (4,6). Western blots showed that after pirfenidone and nintedanib treatment, the expression of JAK2, p-JAK2, and p-STAT3 significantly decreased, and the expression of TGF-βR2, SMA (a myofibroblast activation marker), and ECM fibronectin decreased. Fluorescence microscopy showed that the expression of p-JAK2 in the lung tissues of mice in the BLM group increased and that the fluorescence was mainly localized in the nucleus. After pirfenidone and nintedanib treatment, the fluorescence signal of p-JAK2 decreased. Previously, the role of JAK in signal transduction was thought to be limited to an activator of STATs in the cytoplasm, but in recent years, p-JAK2 has also been detected in the nucleus (39,40). Montero *et al.* found that p-JAK2 was present at increased levels in BLM-treated rat lung tissue and was located in the nucleus of cells in fibrotic areas (16). The discovery of p-JAK2 in the nucleus means that p-JAK2 can function independently of STAT3. This experiment also revealed that p-JAK2 in mouse lung tissue was mainly localized in the nucleus. Therefore, pirfenidone and nintedanib may inhibit the expression of JAK2 by affecting the TGF-β1/TGF-βR2 signaling pathway, thus attenuating pulmonary fibrosis.

Previous study has indicated that activation of the TGF-β1 receptor and subsequent Smad signaling is not sufficient to drive EMT (38). Therefore, many other signaling pathways can also affect EMT. For example, TGF-β1 can activate some non-Smad signaling pathways (such as the MAPK, AKT, PAR6 and Shc signaling

pathways) and the JAK2 signaling pathway. Dees *et al.* proposed that JAK2 is activated in a TGF-β-dependent manner in systemic sclerosis and that the expression of p-JAK2 and p-STAT3 is elevated under TGF-β stimulation (41). Similarly, You *et al.* noted that the TGF-β1 signaling pathway can induce JAK2 phosphorylation and activate the JAK2/STAT3 signaling pathway (42). Furthermore, Ruan *et al.* reported that fedratinib (a targeted selective JAK2 tyrosine kinase inhibitor) inhibits the inflammation and fibrosis caused by TGF-β1 and IL-6 by targeting the JAK2 receptor (37). In conclusion, there is an interaction between the TGF-β1/SMAD3 and JAK2/STAT3 signaling pathways.

We further explored the relationship between TGF-β1 and JAK2 in cell experiments and found that 50 ng/mL TGF-β1 strongly stimulated the expression of JAK2, p-JAK2, p-STAT3, and SMA in MLE12 cells. JAK2 expression was inhibited by the TGF-β1 receptor inhibitor LY2109761 in a dose-dependent manner; that is, JAK2 expression decreased greatly with increasing concentrations of LY2109761. When pirfenidone and nintedanib were used to inhibit TGF-β1 and TGF-β1 receptors, the expression of JAK2 and p-JAK2 significantly decreased, and the corresponding expression of SMA and ECM also decreased. Conversely, when JAK2 gene expression was downregulated, the expression of TGF-βR2 and SMA decreased, suggesting that downregulating the expression of JAK2 can significantly affect the activation of the TGF-β1 signaling pathway.

Conclusions

In summary, TGF-β1 promotes JAK2 phosphorylation, and by blocking the TGF-β1/TGF-βR signaling pathway inhibits JAK2 phosphorylation. This may represent one of the antifibrotic mechanisms of pirfenidone and nintedanib. Interestingly, after JAK2 expression was downregulated, TGF-βR2 and SMA expression significantly decreased. These results indicate that inhibiting the expression of JAK2 can affect the TGF-β1 signaling pathway and further inhibit the occurrence of EMT and fibrosis. It is speculated that there may be mutual influence between the JAK2 and TGF-β1 signaling pathways. These results provide new insights for the treatment of pulmonary fibrosis.

Acknowledgments

Footnote

and use of animals. Since we do not have an animal breeding facility, all of our animal experiments are animal experiments done at Chengdu Dashuo Biotechnology Co.

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