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Inhibitory effects of pirfenidone on fibroblast to myofibroblast transition in rheumatoid arthritis-associated interstitial lung disease via the downregulation of activating transcription factor 3 (ATF3)

Chaochen Wu[1], Haobo Lin[1], Xiao Zhang*

Department of Rheumatology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, PR China

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ABSTRACT

Objective: Pirfenidone (PFD) is an oral anti-fibrotic drug used for idiopathic pulmonary fibrosis (IPF) therapy. We determined the role of activating transcription factor 3 (ATF3) and the effect of PFD on fibroblast to myofibroblast transition (FMT) in rheumatoid arthritis-associated interstitial lung disease (RA-ILD).

Methods: RA-ILD lung specimens were obtained by CT-guided percutaneous transthoracic biopsy. A pulmonary fibrosis mouse model was established by the intratracheal injection of bleomycin. Pathological variation and the expression of α-SMA and ATF3 were observed by H&E, Masson and immunofluorescence staining. Primary human lung fibroblasts (pHLFs) were isolated from lung tissues that were pathologically confirmed to be normal by pneumonectomy. Cell viability was detected using an MTT assay. Cell migration and invasion were detected using a Transwell chamber. The protein levels of α-SMA, ATF3, Smad3 and p-Smad3 were measured by Western blot. HLFs were infected with lentiviruses expressing ATF3 or scrambled shRNA.

Results: ATF3 was dramatically upregulated in lung tissues from both bleomycin-induced mice and patients with RA-ILD compared with controls. The upregulation of ATF3 and the accumulation of collagen in the lung tissues of mice with pulmonary fibrosis were reduced by PFD. PFD significantly inhibited increases in the proliferation, invasion and migration of pHLFs stimulated by TGF-β1. Moreover, we observed the inhibitory effect of PFD on FMT via the downregulation of ATF3, which was further confirmed in ATF3 knockdown (KD) pHLFs.

Conclusions: This work shows the inhibitory effect of PFD on FMT in pHLFs, which is mediated by the downregulation of ATF3. Our findings suggest that PFD might have therapeutic potential for the treatment of RA-ILD.

1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by severe inflammatory arthritis as well as extra-articular manifestations, including pulmonary complications. The prevalence of interstitial lung disease (ILD) varies from 1.6% to 41% in RA patients [1]. In a large-scale study, RA-ILD was associated with shortened life expectancy, and 10,725 (6.6%) of 162,032 RA-associated deaths had occurred in patients diagnosed with RA-ILD [2]. Immunosuppressive therapy continues to be used for RA, and no widely accepted treatment alternatives are available for RA-ILD [3].

The presence of myofibroblasts in fibroblast foci, which is one of the histopathological hallmarks of pulmonary fibrosis, can be induced by inflammatory cytokines and chemokines such as TGF-β1, which is a key mediator of pulmonary fibrosis and other fibrotic diseases [4–6]. Myofibroblasts exhibit cytoskeletal synthesis and contraction. This process, known as TGF-β1-induced fibroblast to myofibroblast transition (FMT), results in abnormal hyperplasia within collagen in lung interstitial tissues and leads to reduced lung function [7,8]. Smad3, which is downstream of TGF-β1, is an important mediator of pathological fibrotic conditions [9]. The inhibition of FMT may serve as an effective means to prevent the progression of ILD [10].

Activating transcription factor 3 (ATF3), which is a member of the ATF/cAMP-responsive element-binding (ATF/CREB) family of basic leucine zipper (bZIP) transcription factors, can regulate gene expression under a variety of conditions [11]. The expression of ATF3 is relatively low in resting cells, but it can be rapidly induced by various signals of cellular stress, such as oxidative stress and cell damage, and several cytokines, such as TGF-β1, interleukin-1β, and tumour necrosis factor-α [12–14]. ATF3 was previously reported to be upregulated in the skin in

* Corresponding author at: Department of Rheumatology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, No.58 Zhongshan Road 2, Guangzhou 510080, Guangdong, PR China.
E-mail address: xiaozhangggh@126.com (X. Zhang).
[1] Wu C and Lin H shared first authorship.

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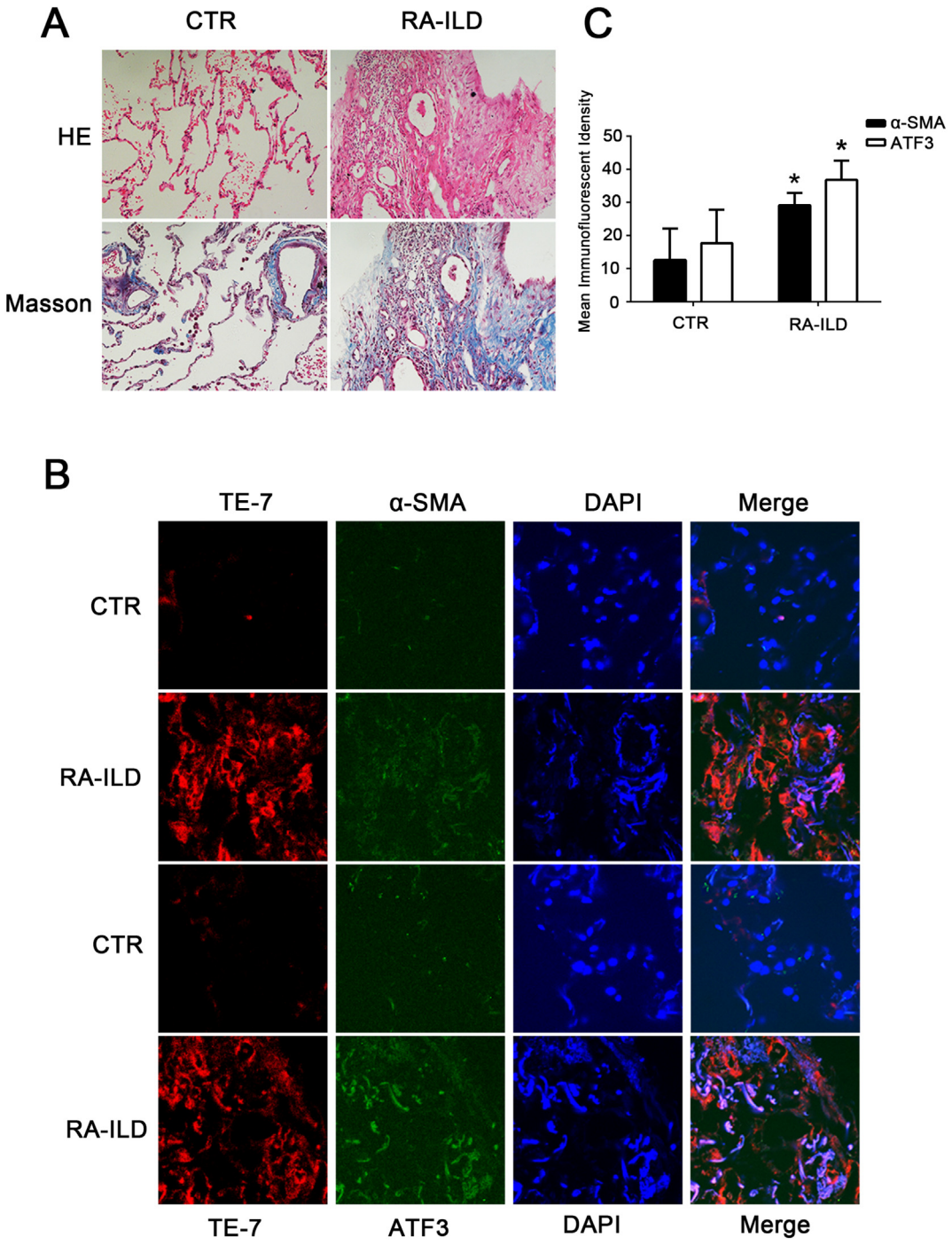

Fig. 1. The upregulation of ATF3 in lung tissues from RA-ILD patients.
(A) Paraffin-embedded lung tissues ($n = 6$) from RA-ILD patients and normal control subjects ($n = 8$) were examined by H&E and Masson trichrome staining (original magnification 200×). Damage of the alveolar architecture and interstitial fibrosis of the alveolar wall occurred in the lungs of RA-ILD patients. (B) Representative confocal microscopy images of lung sections. TE-7 (red), which is specific for fibroblasts, was co-localized with α-SMA (green) and ATF3 (green) in lung sections (original magnification 600×). (C) Mean fluorescence intensities of α-SMA and ATF3 were measured by ImageJ. ATF3 was upregulated, which was accompanied by an increase in α-SMA-positive cells in lung tissues from RA-ILD patients. Values are the means ± S.E.M. $^*P < 0.05$ vs. control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

systemic sclerosis (SSc), and the deletion of ATF3 protected mice from bleomycin-induced and TGF-β receptor I-induced skin fibrosis, which was mediated by canonical TGF-β signalling, suggesting that ATF3 might play a vital role in the pathogenesis of fibrosis [15]. These observations indicate that ATF3 may mediate primary human lung fibroblasts (pHLFs) activation and that the targeting of ATF3 might be promising for the treatment of ILD resulting from RA.

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone, PFD) is an orally active small molecule that has been reported to have therapeutic effects in idiopathic pulmonary fibrosis (IPF) in several large clinical trials [16,17]. This compound exerts certain anti-fibrotic and anti-inflammatory effects in a variety of animal models of pulmonary, renal, hepatic and myocardial fibrosis [18–21]. These effects are believed to be relevant to the modulation of cytokines and growth factors,

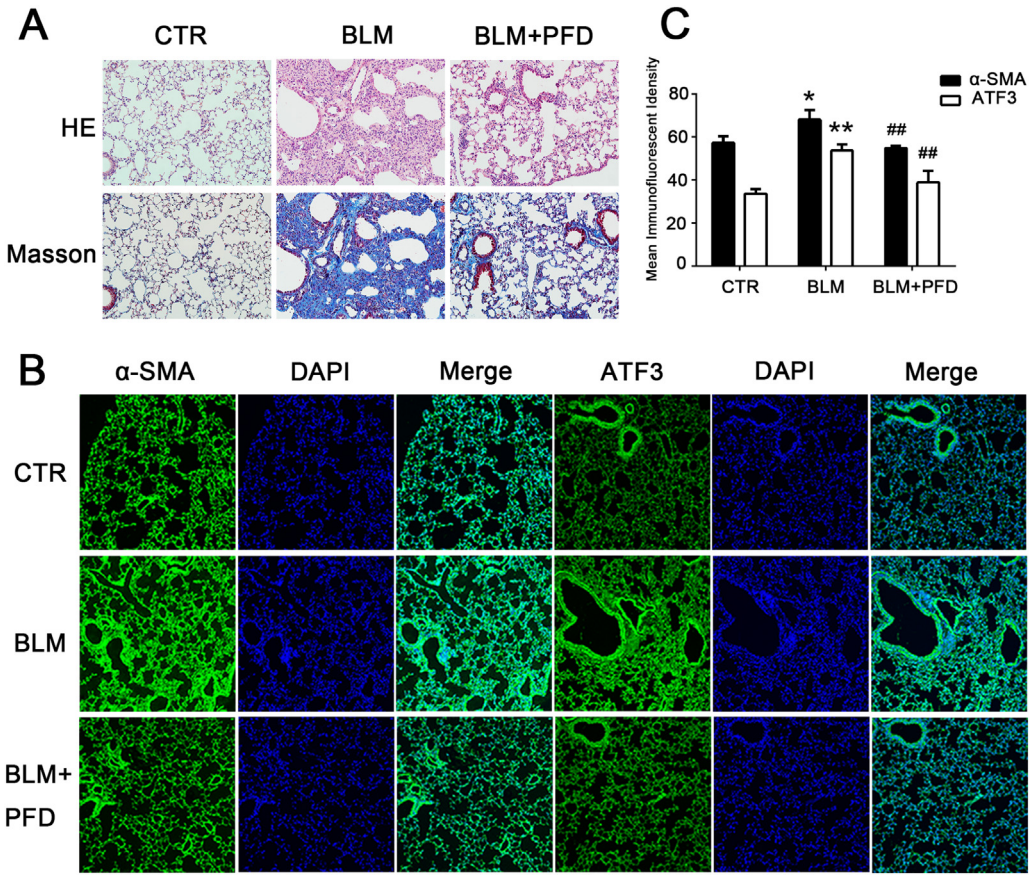

Fig. 2. The effect of PFD treatment on bleomycin-induced pulmonary fibrosis. (A) PFD reduced collagen deposition in lung tissues. Lung tissues from the control group, the BLM group and the BLM + PFD group were observed by H &E and Masson trichrome staining (original magnification 200×). The thickening of the interalveolar septa was significant, and the infiltration of interstitial inflammatory cells was high in the group receiving BLM. (B) Representative confocal microscopy images of lung sections. ATF3 staining was more intense in BLM-induced mice and was markedly decreased in PFD-treated mice (original magnification 200×). (C) Mean fluorescence intensities of α-SMA and ATF3 were measured by ImageJ. Values are the means ± S.E.M. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, $^{\#\#}P < 0.01$ vs. the bleomycin group.

including the most commonly studied cytokine, TGF-β1, which can control cell growth, proliferation and differentiation and promote fibrosis progression [20]. In the present study, we evaluated the effects of PFD on FMT in pHLFs and subsequently explored whether PFD inhibited FMT in pHLFs via the ATF3 pathway.

2. Materials and methods

2.1. Materials

Bleomycin (BLM), pirfenidone (PFD), anti-β-actin antibody, methyl thiazolyl tetrazolium (MTT) and phalloidin-tetramethyl-rhodamine B isothiocyanate were purchased from Sigma (Saint Louis, MO, USA). DAPI was obtained from Molecular Probes (Oregon, USA). Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN, USA). The CellLight™ EdU Apollo®567 In Vitro Imaging Kit was obtained from RiboBio (Guangzhou, China). Anti-ATF3, anti-α-SMA, anti-phospho-Smad3 and anti-Smad3 antibodies were purchased from Abcam (Cambridge, MA, USA). An anti-fibroblast antibody (clone TE-7) was purchased from Merck Millipore (Darmstadt, Germany). Other standard cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Research participants

Paraffin-embedded lung tissues were obtained from the study population, which consisted of 6 patients diagnosed with RA-ILD who underwent CT-guided percutaneous transthoracic biopsies and 8 age- and sex-matched subjects, from whom certain portions of lung tissues were obtained and pathologically confirmed to be normal lung tissue after pneumonectomy. RA was diagnosed according to the revised criteria (1987) of the American College of Rheumatology [22]. ILD was diagnosed according to the international multidisciplinary classification system devised by the official American Thoracic Society/European Respiratory Society [23]. The average age of patients with RA-ILD was 56.2 years (range 44.6–67.8 years), and the median disease duration was 3 years (range 3–13 years). The study was approved by the Research Ethics Committee of Guangdong Provincial People's Hospital and the Guangdong Academy of Medical Sciences, Guangzhou, China. All patients and control subjects signed the informed consent to participate in the study.

2.3. Animal models

Eight- to ten-week-old male C57BL/6 mice (Guangdong Medical Laboratory Animal Center, Guangzhou, China) were used in the following experiments. These mice were randomized into three groups before the initiation of our experimental protocols, which were approved by the Research Ethics Committee of Guangdong Provincial People's Hospital and the Guangdong Academy of Medical Sciences (Guangzhou, China). The animals were assigned to one of 3 groups (n = 10/group): control (CTR), BLM and BLM + PFD. Then, saline with or without BLM was injected into the trachea of each mouse. The following day, the mice who received BLM were randomly assigned to groups that were gavaged with either saline or PFD daily (100 mg/kg) for two weeks. All mice were anaesthetized and sacrificed on day 28, and their lung tissues were excised and fixed with paraformaldehyde.

2.4. Cell culture

Primary human lung fibroblasts (pHLFs) were isolated from lung tissues confirmed to be pathologically normal from lung cancer patients and were cultured in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2. The HLFs were confirmed to be free of *mycoplasma* contamination

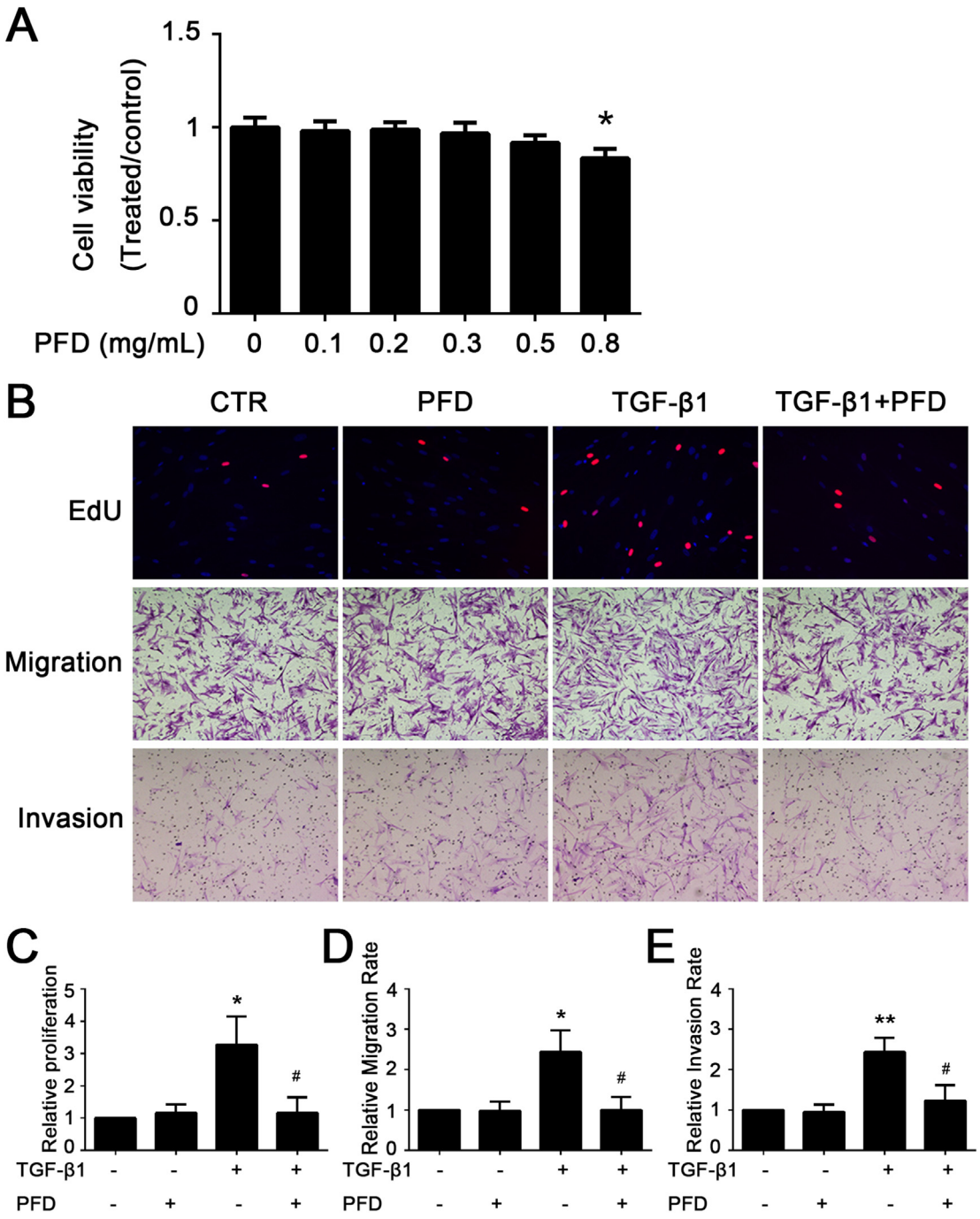

Fig. 3. The effect of PFD treatment on the proliferation, migration and invasion of pHLFs induced by TGF-β1.
(A) HLFs were treated for 72 h with pirfenidone at different concentrations (0, 0.1, 0.2, 0.3, 0.5, 0.8 mg/mL). Cell viability was detected by an MTT assay. The viability of pHLFs was reduced by treatment with PFD only at a concentration of 0.8 mg/mL. (B and C) pHLFs were incubated with PFD (0.2 mg/mL) for 72 h prior to a proliferation assay (original magnification 200×). (B, D and E) A Transwell Boyden chamber method was used to measure cell migration and invasion (original magnification 100×). The cells were incubated for 8 and 12 h for the migration and invasion assays, respectively. PFD treatment reduced the proliferative, migratory and invasive capacities of the pHLFs. The values are the mean ± S.E.M. from at least 3 independent experiments. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. TGF-β1.

by PCR using primers specifically designed to amplify a highly conserved 16S rRNA coding region in the *mycoplasma* genome according to a previously described method [23]. The HLFs were collected from passages 3 to 5 in all experiments and were treated with or without PFD for 72 h and then stimulated with TGF-β1 (5 ng/mL) for 1 or 24 h.

2.5. MTT assay

HLFs were treated with PFD at different concentrations (0, 0.1, 0.2, 0.3, 0.5, 0.8 mg/mL) for 72 h. Then, MTT reagent was added to each well, and the cells were incubated at 37 °C in 5% CO_2 for 4–6 h. The absorbance of each sample was measured with a spectrophotometer at a wavelength of 490 nm.

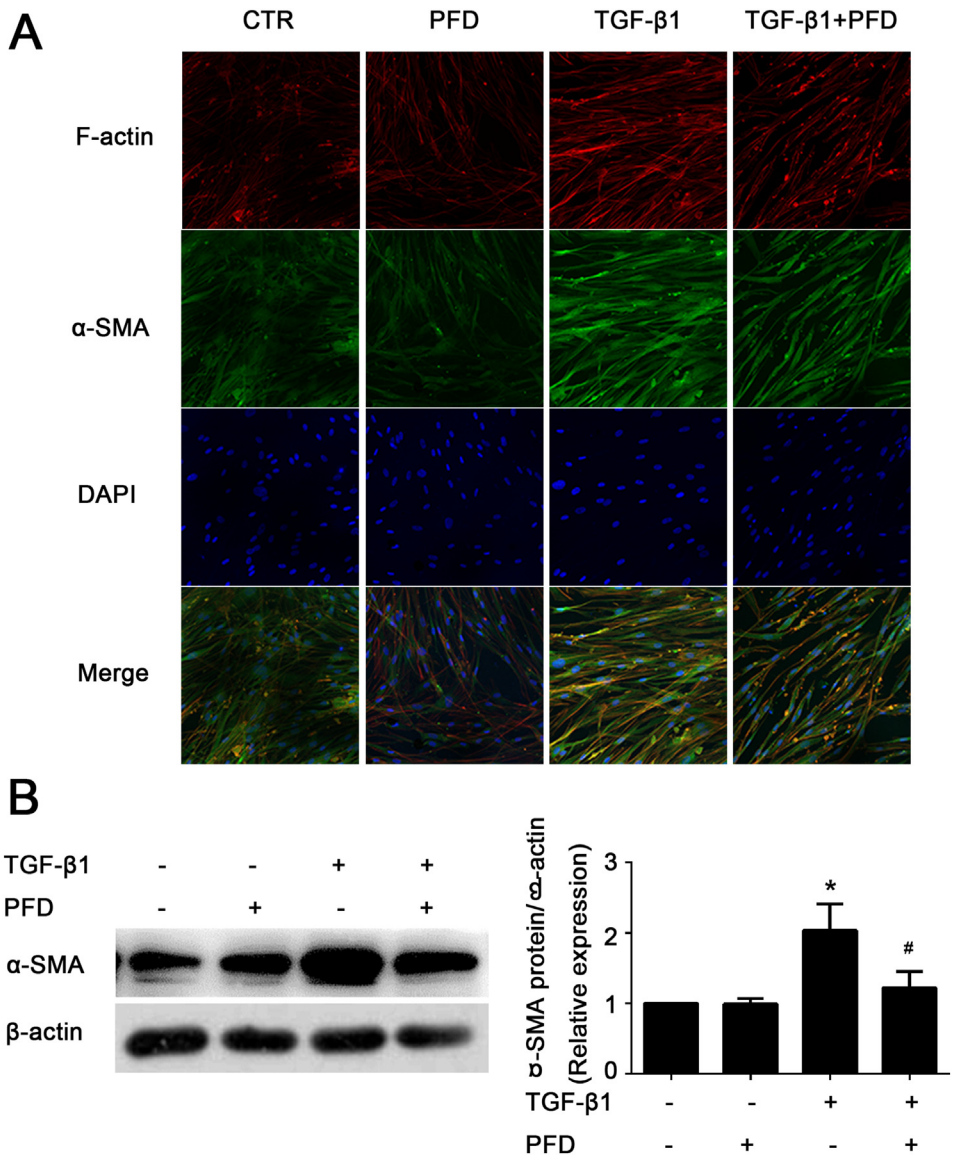

Fig. 4. The effect of PFD treatment on FMT in pHLFs induced by TGF-β1.
(A) PHLFs on glass coverslips were treated with or without PFD (0.2 mg/mL) for 72 h and then stimulated with TGF-β1 (5 ng/mL) for 24 h. The formation and assembly of F-actin stress fibres (detected by fluorescent phalloidin staining) was induced by TGF-β1, but this effect was blocked by treatment with PFD at a concentration of 0.2 mg/mL. The intensity of α-SMA staining was reduced by PFD in pHLFs induced by TGF-β1 (original magnification 200×). (B) Western blot analysis showed that PFD decreased the protein expression of α-SMA in pHLFs. The right panel shows a densitometric analysis of Western blots from 3 independent experiments. All values represent the mean ± S.E.M. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. TGF-β1.

2.6. Cell proliferation assay

HLFs were cultured for 24 h at a density of 1×10^4/well in 96-well plates in serum-free medium. After starvation, the cells were incubated with or without PFD (0.2 mg/mL) for 72 h and were subsequently stimulated with TGF-β1 (5 ng/mL) for 24 h. The cell proliferation assays were then performed using a CellLight™ EdU Apollo®567 In Vitro Imaging Kit according to the manufacturer's instructions.

2.7. Invasion and migration assays

A chemotaxis assay was performed with pHLFs using the Boyden chamber method with a filter with 8.0-mm-sized pores (Transwell, Corning, NY, USA) according to a previously described method [24]. The HLFs were loaded into the upper chamber and incubated for 8 h. Chemotaxis was quantified with ImageJ software by counting the migrating cells that were stained on the underside of the filter using an optical microscope. For the invasion assay, similar experiments were performed for 12 h using inserts coated with Matrigel basement membrane matrix (BD Biosciences, USA).

2.8. Histological examination

Lung specimens were fixed in 10% formalin buffer prior to histological examination. The paraffin sections were 2- to 4-μm thick and stained with haematoxylin and eosin (H&E) and Masson's trichrome stain.

2.9. Confocal laser scanning microscopy

Formalin-fixed, paraffin-embedded lung sections or fibroblasts, which were fixed in 4% paraformaldehyde and permeated with 0.3% Triton X-100 in PBS, were stained with anti-α-SMA, anti-ATF3, and anti-fibroblast antibodies (clone TE-7) and phalloidin-rhodamine. Alexa Fluor-conjugated antibodies were used as secondary antibodies. The nuclei were counterstained using DAPI (4′,6-diamidino-2-phenylindole dihydrochloride). The staining was analysed using a laser scanning confocal microscope (LSM 710, Carl Zeiss, Germany).

2.10. Western blot analysis

Proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE, Pierce, Rockford, IL, USA) and transferred to a poly (vinylidene difluoride) membrane (Amersham,

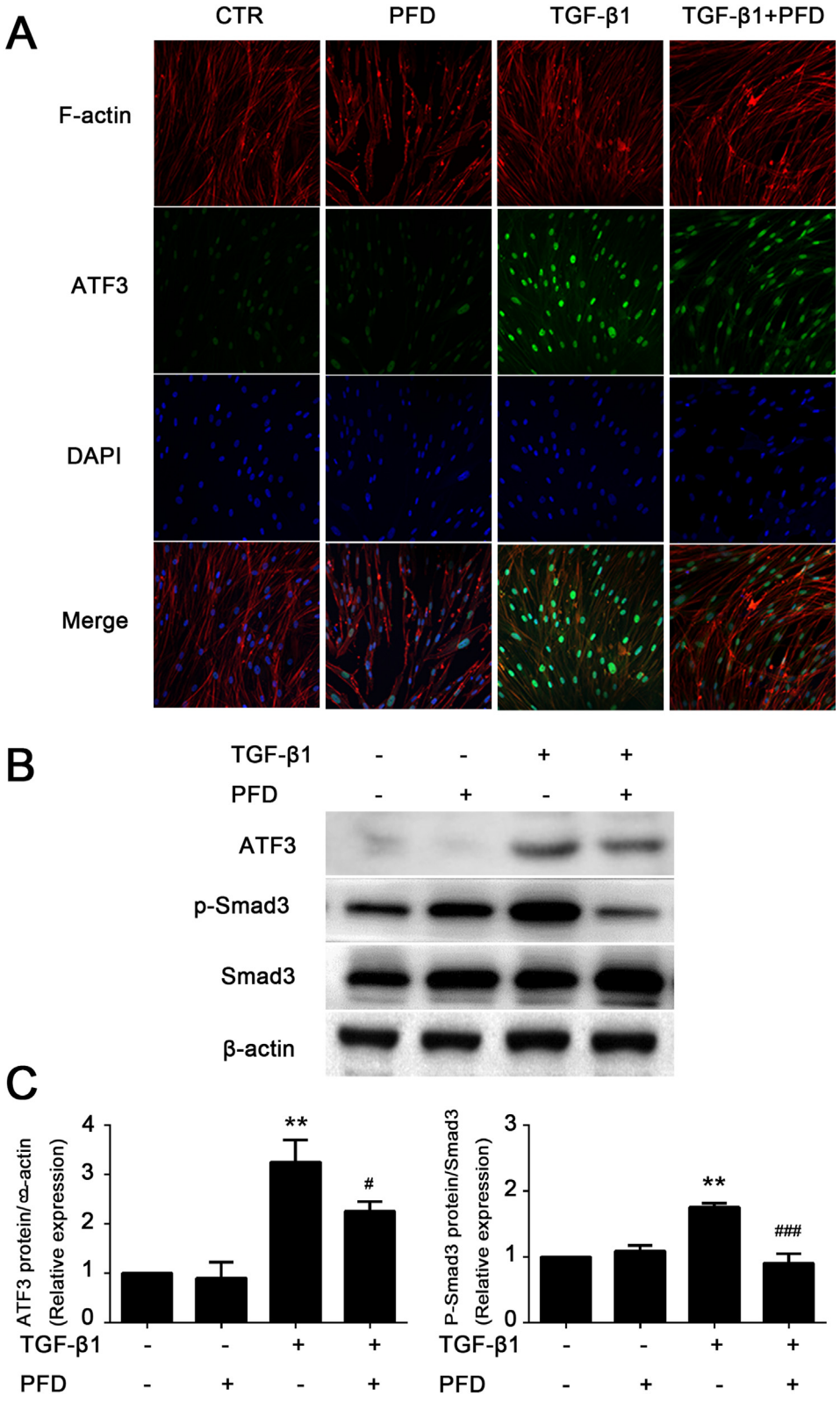

Fig. 5. The effect of PFD treatment on the ATF3/Smad3 signalling pathway in pHLFs.
(A) PHLFs on glass coverslips were treated with or without PFD (0.2 mg/mL) for 72 h and then stimulated with TGF-β1 (5 ng/mL) for 1 h. The intensity of ATF3 staining was suppressed by PFD in pHLFs induced by TGF-β1 (original magnification 200×). (B and C) Western blot analysis was performed to determine the expression of ATF3, p-Smad3 and total Smad3. PFD downregulated the expression of ATF3 and the phosphorylation of Smad3 induced by TGF-β1. The panels show a densitometric analysis of Western blots from 3 independent experiments. All values represent the mean ± S.E.M. $^{**}P < 0.01$ vs. control, $^{\#}P < 0.05$ vs. TGF-β1, $^{\#\#\#}P < 0.001$ vs. TGF-β1.

Arlington Heights, IL). The membrane was incubated with antibodies against ATF3, α-SMA, p-Smad3 and total Smad3 overnight and then incubated with secondary antibodies for 2 h. β-Actin served as the loading control.

2.11. Construction and infection of shRNA-expressing lentiviruses

The sequences of the lentivirus-based shRNA oligonucleotides are as follows:

shATF3 forward primer 5′-AATTGCTGAACTGAAGGCTCAGATTCT CGAGAATCTGAGCCTTCAGTTCAGCTTTTTAT-3′ and reverse primer 5′-AAAAAGCTGAACTGAAGGCTCAGATTCTCGAGAATCTGAGCCTTCA

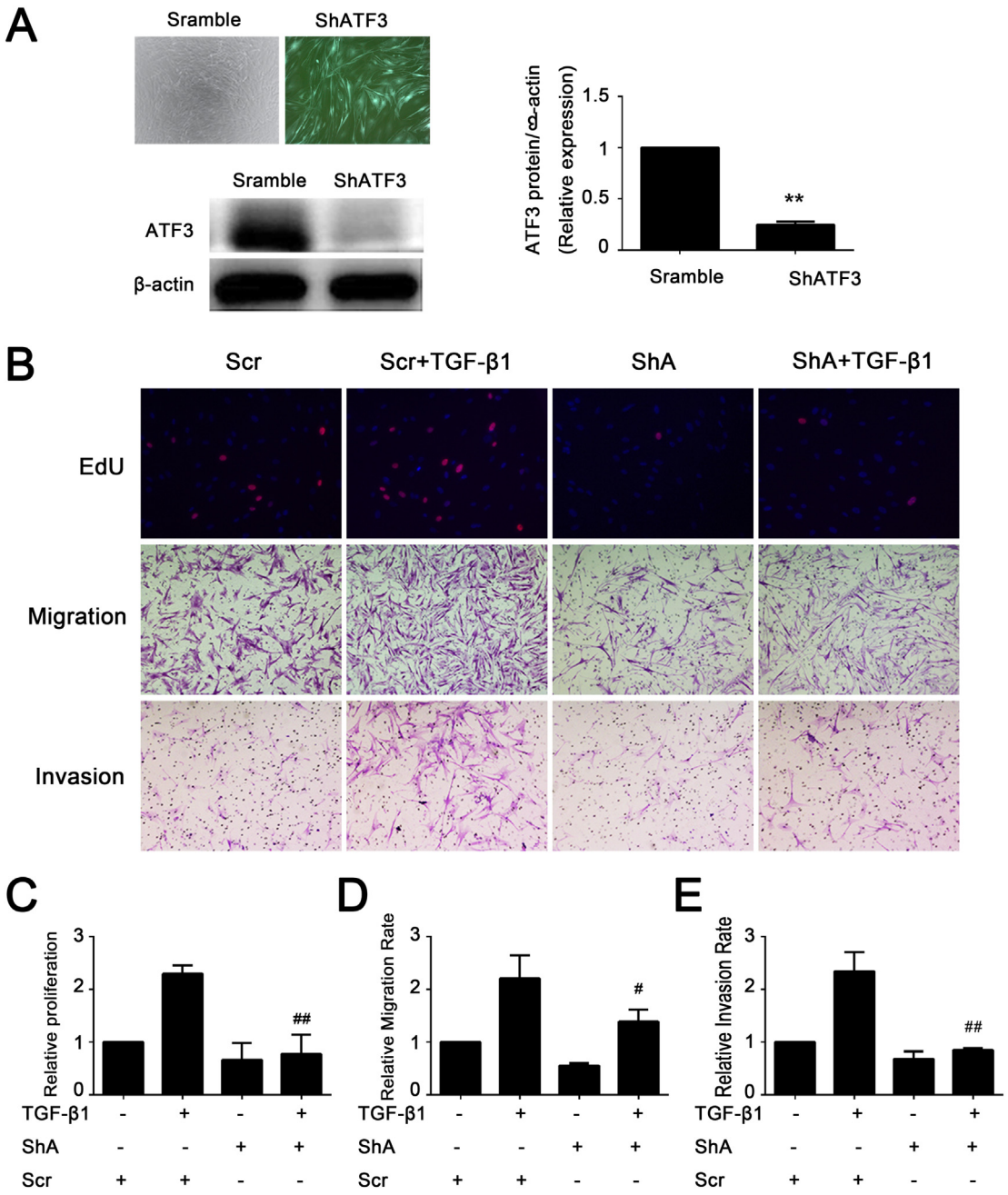

Fig. 6. ATF3 knockdown impedes the cellular functioning of pHLFs.
(A) ATF3 was knocked down in pHLFs, which was confirmed by Western blotting (original magnification 200×). (B and C) pHLFs were incubated with PFD (0.2 mg/mL) for 72 h for the proliferation assay (original magnification 200×). (B, C, D and E) Knockdown (KD) of ATF3 in pHLFs reduced the response to TGF-β1 stimulation versus that observed in fibroblasts treated with scramble shRNA in terms of decreases in the capacities for proliferation (original magnification 200×), migration and invasion (original magnification 100×). The values are the mean ± S.E.M from at least 3 independent experiments. $^{\#}P < 0.05$ vs. Scr + TGF-β1, $^{\#\#}P < 0.01$ vs. Scr + TGF-β1. Scr, scramble; ShA, shRNA ATF3.

GTTCAGC-3′; scramble forward primer 5′-AATTCCTAAGGTTAAGTCG CCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTAT-3′ and reverse primer 5′-AAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGG GCGACTTAACCTTAGG-3′. During infection, pHLFs were treated with shRNA-expressing lentiviral vectors (Sangon Biotech, Shanghai, China) and polybrene (10 mg/mL; Santa Cruz Biotechnology, Santa Cruz, CA) for 12 h at 37 °C. Afterwards, the virus-containing medium was replaced with fresh medium. Infected cells were selected by the addition of puromycin (5 mg/mL) for 48 h post-infection and then propagated for further use. The effect of these shRNAs on ATF3 protein expression was examined using Western blotting. The ATF3 KD vector was a gift from Prof. Xu H in the Department of Rheumatology and Clinical Immunology at the First Affiliated Hospital of Sun Yat-sen University.

2.12. Statistical analysis

All data are presented as the mean ± SEM, and the differences between the groups were analysed by one-way ANOVA or an independent sample *t*-test using SPSS software 21.0. All experiments were independently repeated at least three times. *P* values less than or equal to 0.05 were considered significant.

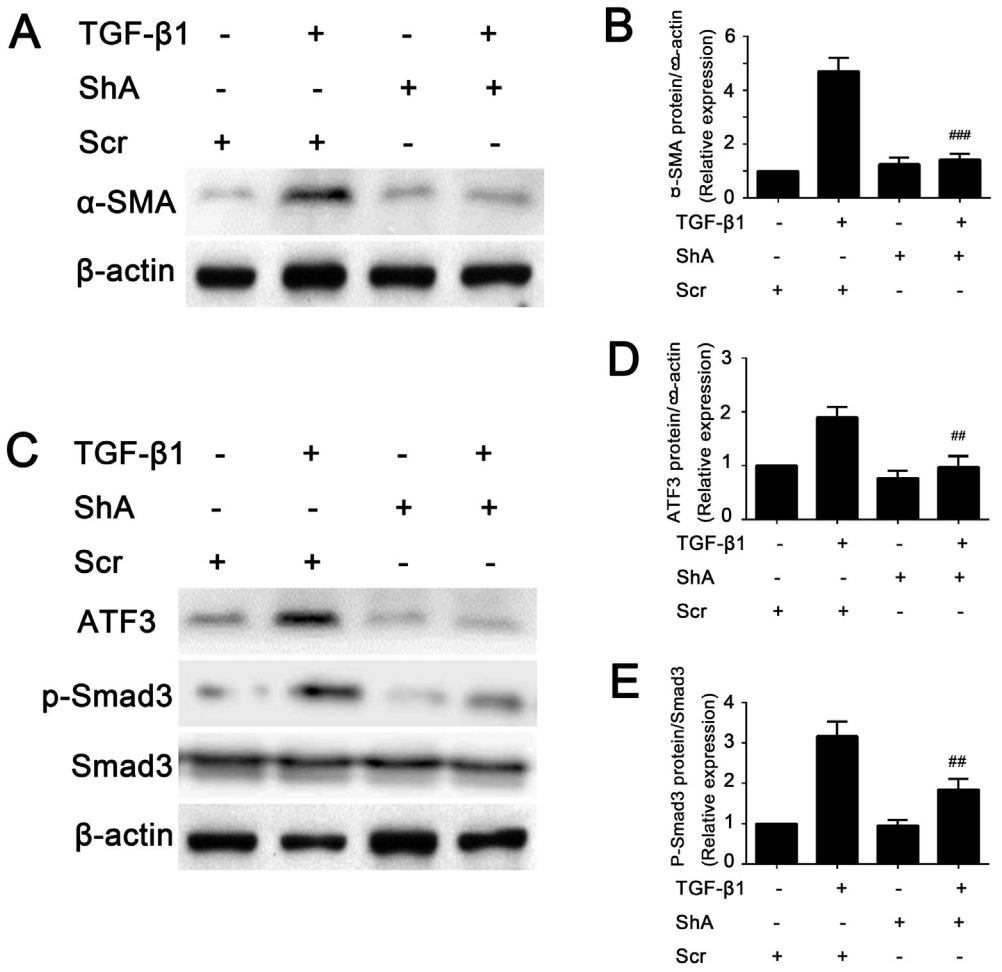

Fig. 7. ATF3 KD downregulates the ATF3/Smad3 signalling pathway in pHLFs.
(A and B) ATF3 was knocked down in pHLFs by short hairpin (sh) RNA. The pHLFs were then stimulated with TGF-β1 (5 ng/mL) for 24 h. Western blot analysis was performed to determine the expression of α-SMA. The right panel shows a densitometric analysis of Western blots from 3 independent experiments. (C, D and E) The pHLFs were stimulated with TGF-β1 (5 ng/mL) for 1 h. Western blot analysis was performed to determine the expression of ATF3, p-Smad3 and total Smad3. All values represent the mean ± S.E.M. $^{\#\#}P < 0.01$ vs. Scr + TGF-β1, $^{\#\#\#}P < 0.001$ vs. Scr + TGF-β1. Scr, scramble; ShA, shRNA ATF3.

3. Results

3.1. The upregulation of ATF3 in the lung tissues of RA-ILD patients

Patients with RA-ILD underwent CT-guided percutaneous transthoracic biopsies to obtain histopathological specimens. The histopathological sections showed that there was greater damage to the alveolar architecture and increased interstitial fibrosis in the alveolar wall in the lungs of RA-ILD patients than in those of normal controls (Fig. 1A). Immunofluorescence assays indicated that ATF3 was upregulated, which was accompanied by an increase in α-SMA-positive cells in lung tissues from RA-ILD patients compared with that in lung tissues from matched individuals. Furthermore, because of the specificity of TE-7 expression in fibroblasts, double-staining with TE-7 antibody and anti-α-SMA showed that fibroblasts were activated in the lungs of RA-ILD patients [25]. Double-staining with TE-7 antibody and anti-ATF3 showed that ATF3 was mainly upregulated in fibroblasts (Fig. 1B and C).

3.2. The effect of PFD treatment on bleomycin-induced pulmonary fibrosis

To investigate whether PFD treatment could ameliorate the fibrotic response induced by BLM in mice, we examined lung sections obtained on day 28 of BLM administration and found that the thickening of the interalveolar septa was significant and the infiltration of interstitial inflammatory cells was high in the group receiving BLM but not in the CTR group. Compared with that in the model group (BLM), structural deformation, inflammation, and collagen deposition in the lungs was obviously reduced in mice treated with PFD (Fig. 2A). The upregulation of α-SMA in the lung tissues of bleomycin-induced mice was inhibited by PFD (Fig. 2B and C).

To determine whether PFD directly inhibited ATF3 in mice with pulmonary fibrosis induced by bleomycin, we tested the expression of ATF3 in lung tissues by immunofluorescent analysis. ATF3 was dramatically upregulated in BLM-induced mice and was markedly decreased in PFD-treated mice (Fig. 2B and C).

3.3. The effect of PFD treatment on the proliferation, migration and invasion of pHLFs induced by TGF-β1

TGF-β1 is the primary cytokine that mediates the differentiation of fibroblasts into myofibroblasts. TGF-β1 at a concentration of 5 ng/mL could effectively enhance the functioning of pHLFs.

Our results showed that the viability of pHLFs was reduced by treatment with PFD at a concentration of 0.8 mg/mL but not at other concentrations (Fig. 3A). In addition, we determined the effect of PFD on pHLF proliferation using an EdU assay. We used PFD at a concentration of 0.2 mg/mL to prevent cytotoxic effects in subsequent experiments. We demonstrated that pHLF proliferation was significantly inhibited by PFD (Fig. 3B and C).

To determine the inhibitory effect of PFD on migration, pHLFs were stimulated with TGF-β1. As shown in Fig. 3B and D, fewer pHLFs were observed on the undersides of the membranes in the PFD-treated group (0.2 mg/mL) compared with the TGF-β1-induced group. Consistent with its effect on the migration of pHLFs, PFD treatment also reduced the invasive capacity of pHLFs (Fig. 3B and E).

3.4. The effect of PFD treatment on FMT in pHLFs induced by TGF-β1

Myofibroblast differentiation is considered to be important in the development of pulmonary fibrosis. The expression and organization of α-SMA is an indicator of myofibroblast differentiation. To determine the role of PFD in regulating actin organization in pHLFs, fluorescent phalloidin staining was used to visualize polymerized actin (F-actin). The formation and assembly of F-actin stress fibres was induced by TGF-β1, which was not observed in unstimulated pHLFs, but this effect was blocked by treatment with PFD at a concentration of 0.2 mg/mL (Fig. 4A). The intensity of α-SMA staining was reduced by PFD in pHLFs induced by TGF-β1. Western blotting analysis further confirmed that PFD decreased the protein expression of α-SMA in pHLFs (Fig. 4B).

3.5. The effect of PFD treatment on signalling pathways in pHLFs

The above results indicated that bleomycin upregulated the expression of ATF3 in pulmonary fibrosis, while PFD attenuated fibrogenesis by inhibiting ATF3. We next investigated whether PFD modulated the ATF3/Smad3 signalling pathway in pHLFs. The stimulation of pHLFs with recombinant TGF-β1 (5 ng/mL) rapidly increased the protein level of ATF3 and increased the intensity of ATF3 staining (Fig. 5). Therefore, we co-treated pHLFs with TGF-β1 and PFD (0.2 mg/mL) and found that PFD reduced the TGF-β1-induced expression of ATF3 and the phosphorylation of Smad3 (Fig. 5). These results showed the inhibitory effect of PFD on the activation of the ATF3/Smad3 signalling pathway.

Primary HLFs were infected with short hairpin RNA-expressing (ShA) or scramble control (Scr)-expressing lentiviral vectors for 12 h at 37 °C and then collected. ShATF3 infection was observed using a fluorescence microscope. ShATF3 inhibited the protein expression of ATF3 effectively at a rate of > 70% (Fig. 6A). It was found that the proportion of EdU-positive cells in pHLFs infected with shATF3 was significantly decreased compared with that in pHLFs infected with the scramble control after 24 h of stimulation by TGF-β1 ($P = 0.003$), suggesting that the downregulation of ATF3 might inhibit the stimulation of proliferation by TGF-β1 (Fig. 6B and C). Similarly, the knockdown (KD) of ATF3 in pHLFs resulted in a reduced response to TGF-β1 stimulation compared with that in pHLFs treated with the scramble control, and this also produced fibroblasts with decreased migration and invasion capacity (Fig. 6B, D and E).

The protein level of α-SMA, which is an indicator of myofibroblast differentiation, was lower in the ShA group than in the Scr group after TGF-β1 stimulation for 24 h ($P < 0.001$, Fig. 7A and B). Similar results were obtained for the expression of ATF3 and p-Smad3 in ATF3 KD pHLFs (Fig. 7C, D and E). Overall, these results demonstrated that the knockdown of ATF3 could decrease the expression of α-SMA and p-Smad3 and attenuate the ability of pHLFs to respond to TGF-β1 stimulation, suggesting that PFD inhibits myofibroblast differentiation by inhibiting the ATF3/Smad3 signalling pathway.

4. Discussion

In the present study, we found that ATF3 was dramatically upregulated in lung tissues from mice with bleomycin-induced pulmonary fibrosis and patients with RA-ILD. In addition, the upregulation of ATF3 and the accumulation of collagen in the lung tissues of bleomycin-induced mice were greatly reduced by PFD. PFD treatment inhibited increases in the proliferation, invasion and migration of pHLFs induced by TGF-β1, regulated F-actin fibre formation and decreased the expression of α-SMA. Furthermore, we observed the inhibitory effect of PFD on the activation of the p-Smad3 and ATF3 pathways, which was further confirmed in ATF3 KD pHLFs. These data suggest that PFD could regulate pHLF functioning and FMT via Smad3-ATF3 signalling.

RA-ILD is one of the most severe extra-articular manifestations of RA and accounts for a considerable proportion of the excess mortality in RA patients [2]. Immunosuppressive therapy would be helpful in stabilizing the pulmonary physiology; controlled clinical studies are urgently needed for RA-ILD, as the side effects of immunosuppressants might be harmful to RA-ILD patients [26]. In addition, PFD was reported to have a protective effect in IPF [16]. The expression of ATF3 was increased in synoviocytes from RA patients [27]. Upon consideration of these results, we speculated that PFD might alleviate pulmonary fibrosis in RA-ILD patients via interference with Smad3-ATF3 signalling. In our study, we found that ATF3 upregulation was accompanied by an increase in α-SMA-positive cells in lung tissues from RA-ILD patients as well as bleomycin-induced mice, and PFD treatment could ameliorate pulmonary fibrosis induced by bleomycin. The upregulation of α-SMA and ATF3 in lung tissues from bleomycin-induced mice was also suppressed by PFD.

The effector cells of ILD are activated fibroblasts and myofibroblasts, which can be recruited to sites of injury and remodel the extracellular matrix. The process of FMT is characterized by an increase in α-SMA and enhanced proliferative, migratory and invasive capabilities. We found that PFD at 0.2 mg/mL suppressed the protein expression of α-SMA and the proliferative ability of pHLFs induced by TGF-β1. We further observed the inhibitory effect of PFD on the migratory and invasive capabilities of TGF-β1-induced pHLFs, which were in line with the results of a previously published study on pHLF functioning that utilized MTT and BrdU assays [28]. Moreover, we demonstrated that PFD treatment impeded F-actin stress fibre formation and assembly that was induced by TGF-β1. These findings indicated that the prevention of FMT may serve as a vital strategy for the therapy of ILD with different underlying causes as long as the pathogenesis of fibrosis is similar.

ATF3 is an adaptive response gene that responds to extra- and/or intracellular changes and regulates the cell cycle and apoptosis, which indicates that it may be involved in susceptibility to cancers [29]. In breast cancer, ATF3 was reported to be associated with cancer metastasis and used as an independent predictor of breast cancer death, suggesting that ATF3 might promote the proliferation, migration and invasion of cancer cells [30]. More research has demonstrated that ATF3 is induced by TGF-β1 and enhances breast cancer cell features [31]. Our study showed that ATF3 was upregulated by TGF-β1, which was accompanied by enhanced proliferative, migratory and invasive capabilities, while the knockdown of ATF3 significantly impeded these cellular functions compared to the TGF-β1 group. In addition, the expression of ATF3 in adult mice leads to rapid ventricle hypertrophy and is increased in fibrotic skin from patients with systemic sclerosis [15,32]. In our study, we observed the suppression of ATF3 by PFD treatment, demonstrating the effect of PFD on ATF3 expression. TGF-β1 plays a crucial role in fibrosis and contributes to the activation of inflammatory cells and FMT [8,33]. Smad3 acts as a mediator of signalling initiated by TGF-β1, and Smad3 null mice show resistance to bleomycin-induced pulmonary fibrosis and carbon tetrachloride-induced hepatic fibrosis [9,34]. In this study, we demonstrated the decreased expression of phosphorylated Smad3 (p-Smad3) in pHLFs after PFD treatment, which is consistent with results in previous reports [28,33]. We also found that p-Smad3 was inhibited in ATF3 KD pHLFs. These results showed that there might be cross-talk between ATF3 and Smad3, both of which mediate mechanisms underlying TGF-β1 function in fibrosis. Dermal fibroblasts isolated from ATF3 knockout (KO) mice had reduced Smad/CAGA reporter activity, while the small interfering (si)RNA-mediated knockdown of Smad3 in dermal fibroblasts prevented the TGFβ-induced upregulation of ATF3 mRNA and protein [15]. Moreover, MCF10CA1a cells ectopically expressing ATF3 had higher levels of phospho-Smad3 than control cells [31]. Here, we further confirmed the positive correlation of ATF3 and p-Smad3. The interruption of Smad3-ATF3 signalling shows promise as a means to reduce the progression of fibrosis.

In summary, our results demonstrate that PFD is capable of modulating FMT and cell functioning in pHLFs via the downregulation of Smad3-ATF3 signalling as well as the alleviation of pulmonary fibrosis.

Further studies are needed to determine the therapeutic potential of PFD for RA-ILD.

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Declaration of Competing Interest

The authors have declared no conflicts of interest.

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