Sunitinib, a Small-Molecule Kinase Inhibitor, Attenuates Bleomycin-Induced Pulmonary Fibrosis in Mice

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Idiopathic pulmonary fibrosis (IPF) is a chronic and ultimately fatal disease, characterized by excessive accumulation of fibroblasts, extensive deposition of extracellular matrix, and destruction of alveolar architecture. IPF is associated with an epithelial-dependent fibroblast-activated process, termed the epithelial-to-mesenchymal transition (EMT). However, there is still a lack of strategies to target EMT for the treatment of IPF. Sunitinib, a small-molecule multi-targeted tyrosine kinase inhibitor, targets multiple kinases that may play an important role in developing pulmonary fibrosis. Here, we explored the therapeutic potential of sunitinib using a mouse model of pulmonary fibrosis. Mice received intratracheal instillation of bleomycin (BLM). Then, the mice were intragastrically administrated with sunitinib or normal saline until the end of the experiment. Distinguished destruction of pulmonary architecture, conspicuous proliferation of fibroblasts and extensive deposition of collagen fibers were found in BLM mice. Sunitinib attenuated the pulmonary fibrosis and inhibited the accumulation of fibroblasts in the lung of BLM mice. To investigate if the inhibition of fibroblast accumulation in the lung by sunitinib was associated with EMT, we used human bronchial epithelial cells (HBEs) and W138 human lung fibroblasts. Sunitinib suppressed the degree of EMT induced by TGF-β, a profibrotic factor, in HBEs and the proliferation of WI38 fibroblasts. Moreover, sunitinib reduced the degree of phosphorylation of serine residues on Smad2/3 that was induced by TGF-β in HBEs. As EMT and accumulation of fibroblasts are critical for the development of pulmonary fibrosis, targeting multiple pro-fibrosis signaling pathways with sunitinib may be a novel strategy to treat pulmonary fibrosis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic disease characterized by excessive accumulation of fibroblasts, extensive deposition of extracellular matrix (ECM), destruction of alveolar architecture, and a progressive decline in pulmonary function (Camelo et al. 2014; Cottin 2016). Considerable efforts have been made to develop efficient therapeutic strategies for IPF (Spagnolo et al. 2015). Unfortunately, there is barely an essential therapeutic intervention that can reverse an established fibrosis. The long-term survival in IPF patients remains poor (Chakraborty et al. 2014). Recently, the US Food and Drug Administration approved pirfenidone, an inhibitor for the production of transforming growth factor-β (TGF-β) and the TGF-β-stimulated collagen production, and nintedanib, a small tyrosine-kinase inhibitor, for the treatment of IPF (King et al. 2014; Richeldi et al. 2014). However, there are still unavoidable adverse effects, including gastrointestinal disorders, skin-related adverse events and elevation in lev-

els of alanine and aspartate aminotransferase. These drugs merely slow down the progression of pulmonary function failure (Kim et al. 2015). Therefore, further investigations are needed to elucidate critical factors in developing IPF and to identify new potential therapeutic agents.

IPF was associated with an epithelial-dependent fibroblast-activated process, termed the epithelial to mesenchymal transition (EMT) (Kim et al. 2015). Both TGF-β and platelet-derived growth factor (PDGF) signaling play important roles in developing pulmonary fibrosis (Antoniades et al. 1990; Hiwatari et al. 1997). Therefore, therapeutic strategies that disrupt both TGF-β and PDGF signal transduction may have advantages in treating pulmonary fibrosis.

Sunitinib is a small-molecule inhibitor that targets multiple receptor tyrosine kinases, such as platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR). Sunitinib has been used as an inhibitor of angiogenesis for the treatment of several kinds of malignant tumors (Kim et al. 2014). It has been reported that sorafenib, another multikinase inhibitor featured the similar molecular mechanism with sunitinib, can reverse the EMT process through blocking the TGF-β signal transduction in mouse hepatocytes (Chen et al. 2011). Therefore, we have hypothesized that sunitinib may also have a protective effect against IPF via controlling both TGF-β-induced EMT in epithelial cells and PDGF-induced proliferation in fibroblasts.

We therefore evaluated the therapeutic potential of sunitinib using a murine model of pulmonary fibrosis induced by intratracheal instillation of bleomycin (BLM), showing that sunitinib profoundly ameliorated BLM-induced pulmonary fibrosis by reducing the accumulation of fibroblasts. The decrease of the accumulated fibroblasts with sunitinib may be related with an inhibition of EMT or proliferation of fibroblasts. Sunitinib controlled EMT via inhibiting TGF-β signaling. In addition, sunitinib suppressed the proliferation of lung fibroblasts. As both EMT and accumulation of fibroblasts are critical for the development of pulmonary fibrosis, sunitinib may be a potential treatment for IPF.

Materials and Methods

Animals

Female C57BL/6 mice weighing 23-25 g were obtained from the Academy of Military Medical Science (Beijing, China).

Reagents

Recombinant human TGF-β1, PDGF and VEGF were purchased from R&D Systems (Minneapolis, MN, USA). Sunitinib (Sutent) was manufactured by Pfizer Inc (New York, NY, USA). The primary antibodies used in this study include glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich, St Louis, MO, USA), pY-PDGFRβ, pY-VEGFR2, E-cadherin and N-cadherin (Cell Signaling Technology, San Diego, CA, USA), vimentin (Sigma-Aldrich), zonula occludens-1 (ZO-1) (Invitrogen, San Francisco, CA,

USA), fibroblast-specific protein-1 (FSP1) (Dako, Glostrup, Denmark), α-smooth muscle actin (α-SMA) (Sigma-Aldrich), Smad2/3 (BD Biosciences, San Jose, CA), phospho-Smad2 (Ser465/467; Cell Signaling Technology) and phospho-Smad3 (Ser423/425; Invitrogen).

BLM-induced pulmonary fibrosis model

Briefly, C57BL/6 mice were intratracheally injected with 3.5 mg/kg BLM after anaesthesia with an intraperitoneal injection of sodium phenobarbital (20 mg/kg). Sham group received normal saline by the same procedure. Three days after intratracheal injection, the mice were randomized into two groups that were intragastrically administrated with sunitinib (0.5 mg/kg daily) or normal saline until the end of the experiment. Two weeks after sunitinib treatment, the lung tissues from mice were isolated for further examination described below. For the therapeutic treatment of sunitinib, ten days after intratracheal injection, the mice were intragastrically administrated with sunitinib (0.5 mg/kg daily) or normal saline for one week. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Ethics Committee of Nanjing Medical University Experimental Animal Department (Permit Number: 1401023). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Histological analyses

The lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin for histological and immunohistochemical study. Sections (5 μm) were then stained with hematoxylin and eosin (H&E) for routine examination or Masson's trichrome staining for the measurement of collagen content. The lung sections were further immunostained with appropriate dilutions of antibodies against E-cadherin, α-SMA or FSP1. All procedures were in accordance with commercial instruction.

Real-time qPCR

Total RNA from frozen lung tissues was extracted using Trizol (Takara, Shiga, Japan) according to the manufacturer's instructions. Real-time qPCR analysis was performed on Lightcycler480 (Roche, Penzberg, Germany) using SYBR Green PCR Master Mix (Toyobo, Tokyo, Japan). Each measurement was repeated in triplicate and normalized to the levels of GAPDH mRNA. The primers used for real-time PCR assays were listed in Table 1.

Western blotting analysis

To detect the expression of epithelial and mesenchymal markers, HBEs were treated with TGF-β1 (5 ng/ml) or sunitinib as indicated in the figure legends and subjected to western blotting analysis. Approximately 50 μg of total protein was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane and then incubated with the corresponding antibodies. The protein bands were visualized with a chemiluminescence reagent ECL (Millipore Biotechnology Inc., USA).

Hydroxyproline (Hyp) determination

The content of Hyp in 100 mg lung tissues was measured using Hyp test kit (Jiancheng, Nanjing, China) according to a modified method previously described by Jamall et al. (1981). The level of

Table 1. Primer sequences for PCR.

Gene	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')
Mouse TGF-β1	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
Mouse CCN-2	GGGCCTCTTCTGCGATTTC	ATCCAGGCAAGTGCATTGGTA
Mouse COL1	GCTCCTCTTAGGGGGCCACT	CCACGTCTCACCATTGGGG
Mouse COL2	CAGGATGCCCGAAAATTAGGG	ACCACGATCACCTCTGGGT
Mouse COL4	CTGGCACAAAAGGGACGAG	ACGTGGCCGAGAATTTCACC
Mouse TIMP-3	CTTCTGCAACTCCGACATCGT	GGGGCATCTTACTGAAGCCTC
Mouse GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Hyp was expressed as microgram of Hyp per gram of wet weight (mg/g).

Immunofluorescence staining

The lung specimens were fixed in 4% paraformaldehyde and stained with the appropriate dilutions of antibodies against E-cadherin, α-SMA or STAT3. The slides were then incubated with FITC-conjugated anti-mouse IgG or Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA). DAPI was employed for the stain of nuclei. Images of the sections were acquired by a confocal laser scanning microscope (Leica, Wetzlar, Germany).

Cell lines

The human bronchial epithelial cells (HBEs) and a human lung fibroblast cell line WI38 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. HBEs were treated with or without TGF-β1 (5 ng/ml) or plus sunitinib (1, 2 and 5 μM) that were commonly used in *in vitro* experiments (Oechsle et al. 2011; Wen et al. 2015) for 48 hours.

Statistical analysis

All of the experiments were performed in triplicate. The results were presented as the mean values ± standard error (SE). Comparisons were made using Student's t-test or analysis of variance (ANOVA). A two-sided p value < 0.05 was considered statistically significant.

Results

Sunitinib ameliorates BLM-induced pulmonary fibrosis in mice

To evaluate effects of sunitinib *in vivo*, we established an experimental pulmonary fibrosis model by intratracheal administration of BLM. Distinguished destruction of pulmonary architecture, conspicuous proliferation of fibroblasts and extensive deposition of collagen fibers were found in mice treated with BLM (Fig. 1A). However, these pathological fibrotic changes were remarkably attenuated by sunitinib administration (Fig. 1A). The collagen content and distribution in lung tissues were further assessed using Masson's trichrome staining (Fig. 1B). Likewise, BLM treatment significantly increased the collagen deposition,

while sunitinib treatment evidently reduced the deposition of fibrillar collagen, as indicated by the Masson's trichrome-positive areas (Fig. 1B). Moreover, the expression levels of the profibrotic factors, including TGF-β1, connective tissue growth factor (CCN2) were also decreased after sunitinib administration (Fig. 1C). To investigate the therapeutic effect of sunitinib, we administrated mice with sunitinib on day 10 when the mice were in the beginning of fibrotic phase of pulmonary fibrosis. Sunitinib also attenuated the pulmonary fibrosis (Fig. 1D, E). Taken together, sunitinib showed an antifibrotic effect on the BLM-induced pulmonary fibrosis *in vivo*.

Sunitinib reduces the excessive accumulation of ECM in BLM-induced pulmonary fibrosis model.

During the development of IPF, fibroblasts abnormally accumulated, leading to an excessive production of ECM in parenchymal. We therefore assessed the effect of sunitinib on the collagen expression in BLM-induced pulmonary fibrosis model. The mRNA expression of fibrotic matrix component, such as type I collagen, type II collagen and type IV collagen was substantially increased in mice treated with BLM (Fig. 2A). Notably, the increase of the collagen expressions in BLM mice was significantly reduced by sunitinib (Fig. 2A). Moreover, sunitinib attenuated the expression of tissue inhibitor of metalloproteinases-3 (Timp-3), which was increased in pulmonary fibrosis tissue induced by BLM (Fig. 2B). In addition, the content of Hyp, a characteristic amino acid of collagen, was elevated in pulmonary tissue of BLM mice, whereas sunitinib inhibited the accumulation of Hyp induced by BLM. As PDGF or VEGF promoted the proliferation and migration of fibroblasts, we assessed the effect of sunitinib on the phosphorylation of PDGFRβ and VEGFR2. Western blot analysis showed that sunitinib inhibited the phosphorylation of PDGFRβ and VEGFR2 (Fig. 2D).

Sunitinib inhibits the accumulation of fibroblasts in the lung in mice treated with BLM.

A loss of alveolar epithelium with fibroblast proliferation was observed in BLM mice, characterized by immunohistochemistry of epithelial marker E-cadherin and mesenchymal marker FSP1 (Fig. 3A). On the contrary, the level of E-cadherin expression in the alveolar epithelium was
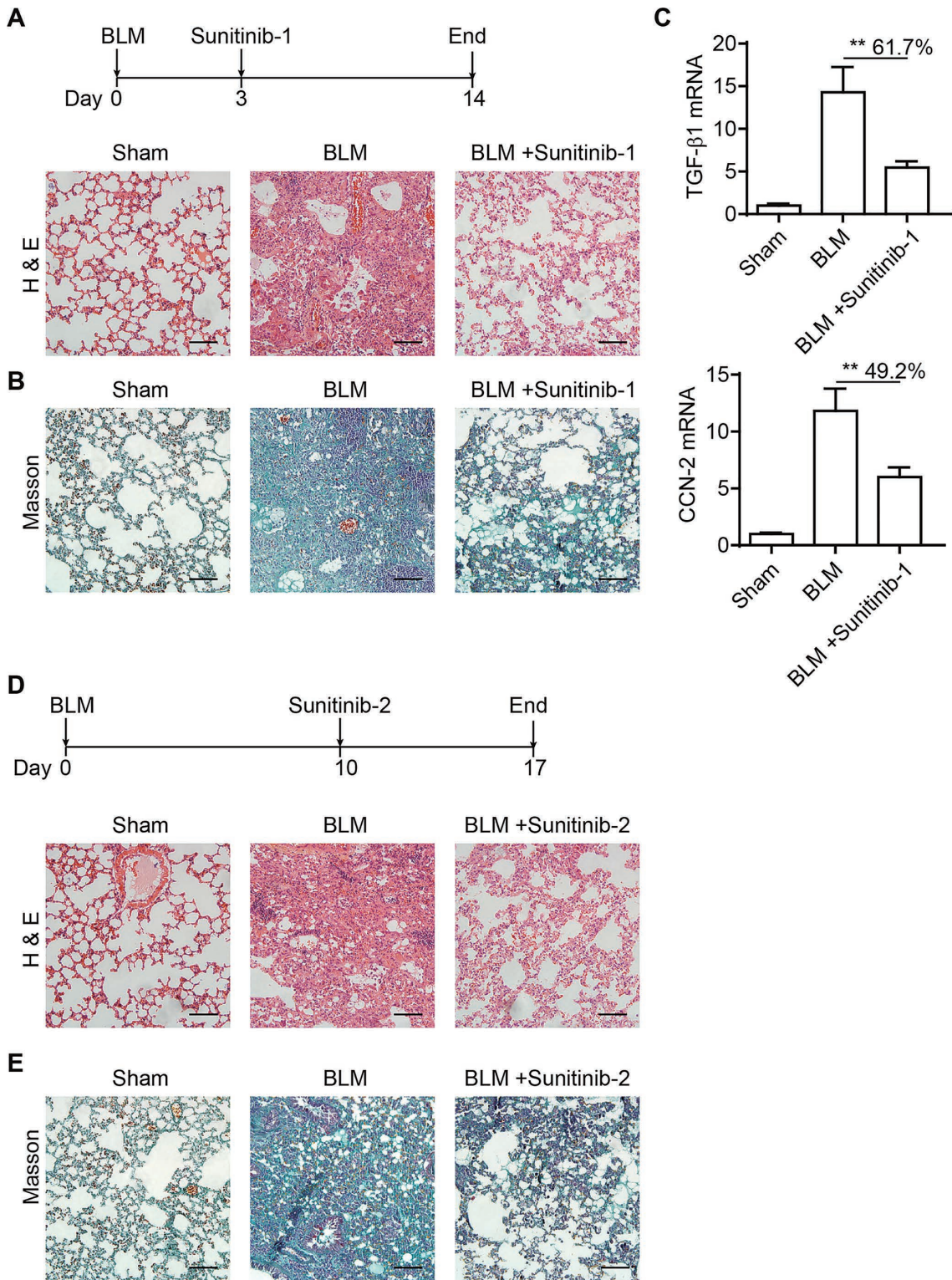
Fig. 1. Sunitinib ameliorates BLM-induced pulmonary fibrosis in mice.
Mice were intratracheally injected with 3.5 mg/kg BLM to establish a murine pulmonary fibrosis model. Three days after intratracheal injection, mice were randomized into two groups that received sunitinib (0.5 mg/kg daily) or normal saline for 12 days. Pulmonary tissue sections were prepared on day 14 and subjected for H&E staining (A) or Masson's trichrome staining (B). (C) Q-PCR analyses of the mRNA expression of TGF-β and CCN-2 in the pulmonary tissues. (C, mean ± SE). **$p < 0.01$. The percentage value indicated the percentage of reduction in BLM + sunitinib-1 group compared with BLM group. BLM mice were randomized into two groups that received sunitinib (0.5 mg/kg daily) or normal saline on day ten for 7 days. Pulmonary tissue sections were prepared on day 17 and subjected for H&E staining (D) or Masson's trichrome staining (E). Sunitinib-1 indicated that sunitinib was received on day 3. Sunitinib-2 indicated that sunitinib was received on day 10.
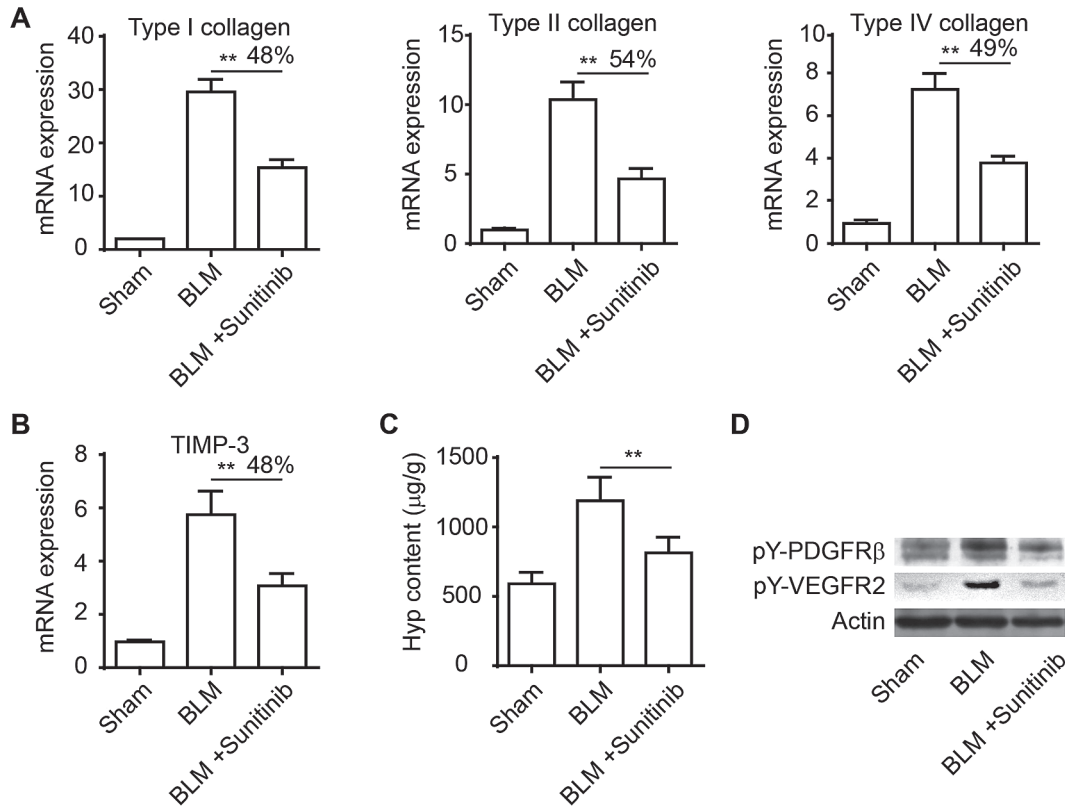
Fig. 2. Sunitinib inhibits pro-fibrosis factors in mice treated with BLM.
Mice were intratracheally injected with 3.5 mg/kg BLM to establish a murine pulmonary fibrosis model. Three days after intratracheal injection, mice were randomized into two groups that received sunitinib (0.5 mg/kg daily) or normal saline for 12 days. Q-PCR analysis of the (A) collagen and (B) Timp-3 mRNA expression levels in pulmonary tissues. The percentage value indicated the percentage of reduction in BLM + sunitinib group compared with BLM group. (C) The content of Hyp (n = 5 for each group) was measured as described in Materials and Methods. (A-C, mean ± SE). **$p < 0.01$. (D) Western blot analyses of pY-PDGFRβ and pY-VEGFR2 expression in pulmonary tissues. These data are representative of three experiments.

largely elevated and the accumulation of FSP1^{+} fibroblasts was dramatically decreased in sunitinib-treated mice (Fig. 3A). Lung sections were then examined for the expression of α-SMA, a reliable marker of activated fibroblasts. As shown in Fig. 3A, α-SMA was only localized in the vessel walls and was not expressed in interstitium in the sham group. After administration of BLM, considerable fibroblasts expressing α-SMA were detected in the interstitium (Fig. 3A). However, fewer α-SMA[+] fibroblasts were detected in the lung sections from mice that received sunitinib treatment. The further immunofluorescence staining for E-cadherin and α-SMA confirmed the results obtained from immunochemistry analysis (Fig. 3B).

Sunitinib suppresses TGF-β-induced mesenchymal transition of HBEs and PDGF/VEGF-induced proliferation of WI38 fibroblasts.

In order to investigate if the inhibition of fibroblast accumulation in the lung by sunitinib was associated with EMT, HBEs and WI38 cells were used. Next we investigated the effect of sunitinib on the mesenchymal transition of HBEs *in vitro*. It has been reported that TGF-β1, a profi-

brotic factor, induces EMT *in vitro*. After exposure to TGF-β, HBEs were differentiated from a polarized epithelial shape to a spindle-like morphology (Fig. 4A, B). Besides, the expression levels of two epithelial markers E-cadherin and ZO-1 were down-regulated, whereas the expression levels of the mesenchymal markers N-cadherin and vimentin were up-regulated (Fig. 4C, D). In consistent with data obtained *in vivo*, sunitinib suppressed TGF-β-induced EMT in HBEs in a dose-dependent manner (Fig. 4). HBEs treated with sunitinib reacquired their epithelial honeycomb-like morphology, accompanied by the elevated expressions of E-cadherin and ZO-1 and decreased expressions of N-cadherin and vimentin. Collectively, these data provided *in vitro* evidence to suggest that sunitinib inhibited the differentiation process of HBEs induced by TGF-β. In addition, PDGF or VEGF-induced proliferation in a human lung fibroblast cell line WI38 cells was also suppressed by sunitinib (Fig. 4E).

Sunitinib antagonizes TGF-β/Smad signaling in HBEs via inhibiting the phosphorylation of Smad2/3.

Due to the prominent role of TGF-β in EMT, we

A

| | Sham | BLM | BLM +Sunitinib |

E-cadherin

FSP1

α-SMA

B

| | Sham | BLM | BLM +Sunitinib |

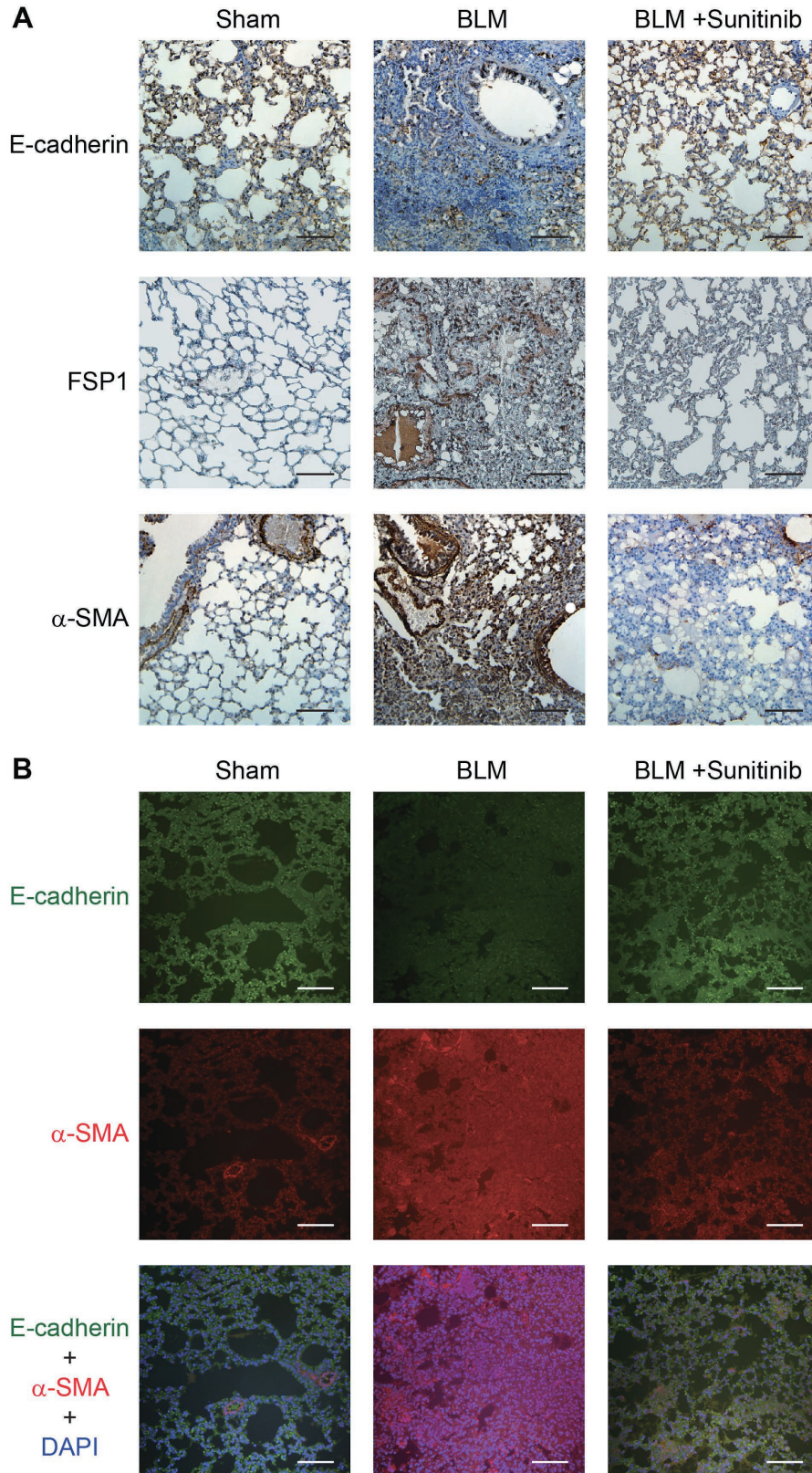E-cadherin

α-SMA

E-cadherin
+
α-SMA
+
DAPI

Fig. 3. Sunitinib reduces the accumulation of fibroblasts in the lung of mice treated with BLM.
     Mice were intratracheally injected with 3.5 mg/kg BLM to establish a murine pulmonary fibrosis model. Three days
     after intratracheal injection, the mice were randomized into two groups that received sunitinib (0.5 mg/kg daily) or nor-
     mal saline for 12 days. (A) Immunohistochemical and (B) immunofluorescence analysis of the pulmonary tissue sec-
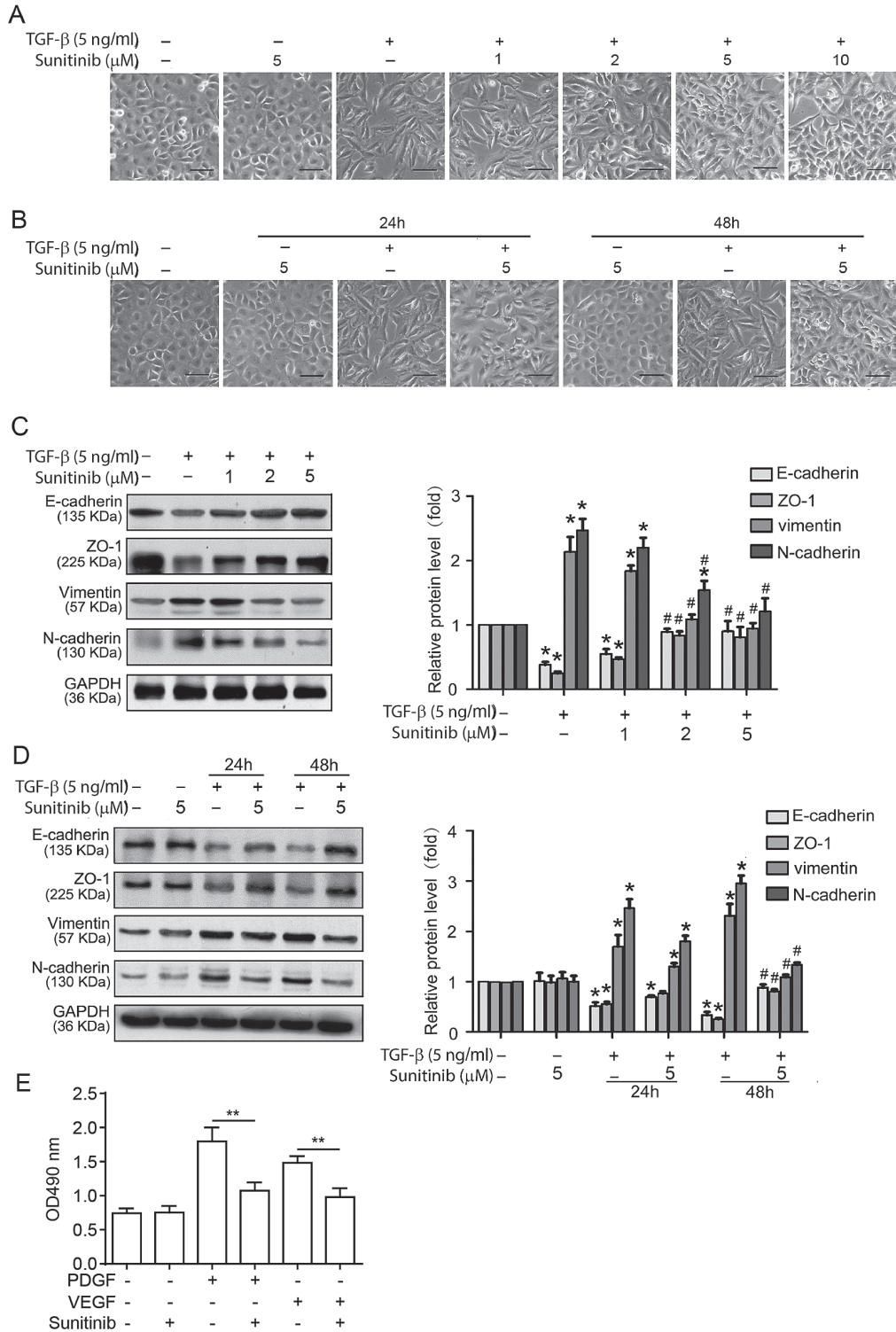   tions.

Fig. 4. Sunitinib inhibits TGF-β-induced mesenchymal transition of HBEs and PDGF- or VEGF-induced proliferation of WI38 fibroblasts.

(A, C) HBEs were treated with TGF-β1 (5 ng/ml) and sunitinib (1, 2, 5 and 10 μM) for 48 hours. (A) The morphological changes of HBEs. (C) Western blot analyses of E-cadherin, ZO-1, N-cadherin, and vimentin expression in HBEs. (B, D) HBEs were treated with TGF-β1 (5 ng/ml) and sunitinib (5 μM) for 24 or 48 hours. (B) The morphological changes of HBEs. (D) Western blot analyses of E-cadherin, ZO-1, N-cadherin, and vimentin expression in HBEs. These data are representative of three experiments. (C-D, mean ± SE, n = 3). *$p < 0.05$ vs. Sham group; #$p < 0.05$ vs. TGF-β1 treated group. (E) Effects of sunitinib on PDGF- or VEGF-induced proliferation in WI38 cells. WI38 cells were treated with or without sunitinib at 500 nM following PDGF (10 ng/ml) or VEGF (10 ng/ml) stimulation for 48 hours. The CytoTox 96 assay was carried out to detect the cell number. **$p < 0.05$ as indicated.
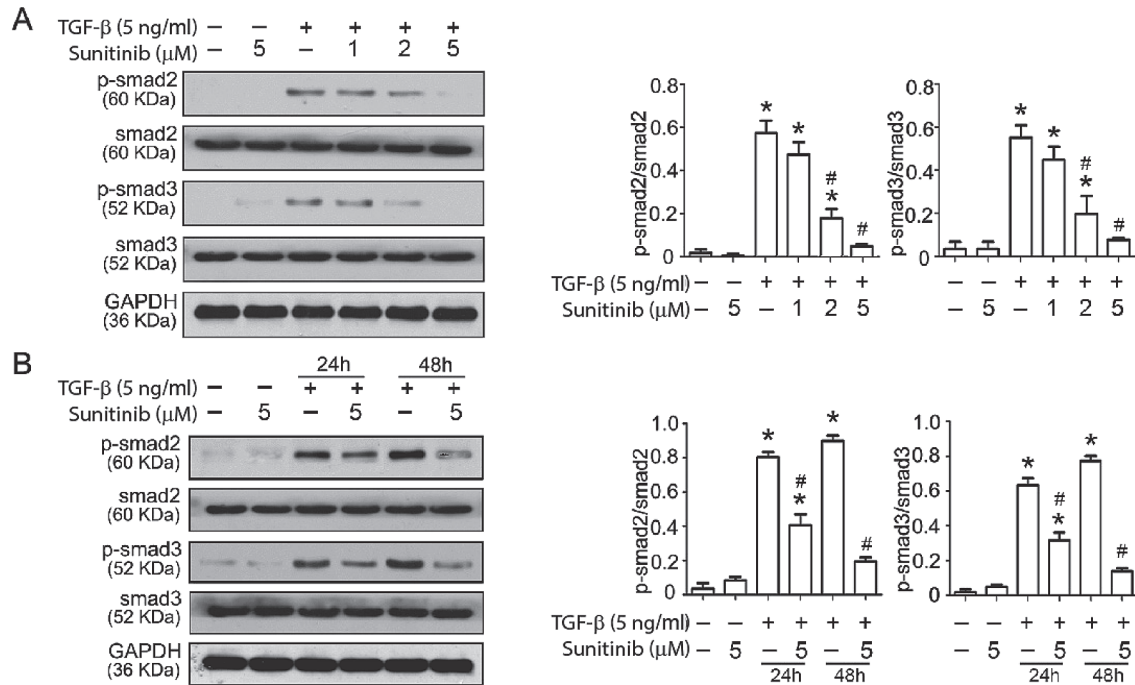
Fig. 5. Sunitinib blocks TGF-β/Smad signaling in HBEs.
(A) HBEs were treated with or without TGF-β1 (5 ng/ml) or plus sunitinib (1, 2 and 5 μM) for 48 hours. (B) HBEs were then treated with or without TGF-β1 (5 ng/ml) or plus sunitinib (5 μM) for 24 or 48 hours. Cell extracts were prepared for Western blot analyses for the determination of smad2 and smad3. These data are representative of three experiments. (A-B, mean ± SE, n = 3). *$p < 0.05$ vs. Sham group; #$p < 0.05$ vs. TGF-β1 treated group.

hypothesized that sunitinib may inhibit the EMT process of HBEs through disrupting TGF-β signaling pathway. To explore the intracellular signal transduction, we examined the effect of sunitinib on the canonical Smad-dependent pathway, which consisted of a family of signal transducers called R-Smads (Smad2 and Smad3). As shown in Fig. 5A and B, sunitinib obviously abrogated TGF-β-mediated phosphorylation of Smad2 and Smad3 at a concentration of 5 μM, indicating that sunitinib acted as an effective inhibitor of TGF-β signaling.

Discussion

As the most common form of interstitial lung disease, IPF is a progressive and generally irreversible lung disease with unknown etiology and few treatment strategies (Staitieh et al. 2015). Here, we find a novel role for sunitinib in controlling TGF-β signaling, which in turn alleviates the pulmonary fibrogenesis in mice. Sunitinib prevented the pulmonary fibrogenesis and attenuated the pulmonary fibrosis (Fig. 1). EMT plays an important role in the development of IPF (Desai et al. 2015). During the progression of IPF, the chronic injuries incite the abnormally activated alveolar epithelial cells (AECs) to secrete TGF-β, which in turn promotes the EMT, by which AECs undergo mesenchymal transformation, lose their cell-cell adhesion, acquire migratory and invasive properties, and ultimately convert into mesenchymal cells, accounting for excessive deposition of ECM in parenchymal (Tian et al.

2015). TGF-β signaling is essential in a number of profibrotic events including EMT, fibroblast activation and eventual ECM deposition (Yang et al. 2014; Eberlein et al. 2015; Okumura et al. 2015; Zerr et al. 2016). Sunitinib profoundly inhibited TGF-β-induced EMT (Fig. 4). Thus, sunitinib could improve the pulmonary fibrosis via inhibiting EMT. Although sunitinib did not promote the death of HBEs or the proliferation of WI38 cells (Fig. 4A, B, E), it might have other effects in addition to EMT. As sunitinib is a multi-targeted kinase inhibitor, it may affect multiple aspects in protecting mice from BLM-induced lung fibrosis. Sunitinib is a strong inhibitor of VEGFR2 and PDGFRβ (Roskoski 2007). PDGF-induced abnormal fibroblast proliferation plays an important role in developing pulmonary fibrosis (Antoniades et al. 1990). Consistently, we found sunitinib at 500 nM inhibited both PDGF and VEGF-induced proliferation in a human lung fibroblast cell line WI38 cells (Fig. 4E). The inhibitory effect of sunitinib on PDGF-induced proliferation may also contribute to the improvement of the BLM-induced fibrosis model by sunitinib. TGF-β protein was proved to trigger and maintain EMT process in the organ fibrogenesis and tumor metastasis primarily via Smad-dependent mechanism. Upon phosphorylation, signal transducers Smad2 and Smad3 form complexes with Smad4, and subsequently translocate into the nucleus to regulate the transcription of target genes responsible for EMT (Kong et al. 2015). Sunitinib as a tyrosine kinase inhibitor reduced the phosphorylation of

serine residues on Smad2/3 induced by TGF-β (Fig. 5). The mechanism of the inhibition of TGF-β-induced Smad2/3 phosphorylation by sunitinib remains unknown. There are several events may be influenced by sunitinib in TGF-β signal transduction, such as TGF-βRI phosphorylation, heterodimerization of TGF-βRI and TGF-βRII, recruitment of Smad2/3 to the receptors, and kinase activity of TGF-βRI. We will investigate these events in the future.

Advances in the understanding of the molecular mechanisms involved in the pathogenesis of IPF have paved the way for identifying the new efficient approaches for the treatment of patients with IPF. Given the important role of TGF-β on EMT process, therapeutic interventions that utilize small chemicals or genetic technology to interfere with TGF-β signaling at various transduction steps have been developed to reverse the established fibrosis (Chakraborty et al. 2014; Fukunaga et al. 2015). For instance, miRNA or chemokine was reported to reverse fibrosis in the rodent animals or cell lines *in vitro* (Fierro-Fernandez et al. 2015; O'Beirne et al. 2015). Apart from the genetic approaches, small chemicals targeting TGF-β signaling cascade have strong therapeutic potential in preclinical settings (Nanthakumar et al. 2015). Notably, sorafenib has been demonstrated to feature the ability to inhibit the profibrogenic activity of TGF-β signaling and ameliorate BLM-mediated pulmonary fibrosis (Chen et al. 2013). Sunitinib malate (Sutent™) is an oral multikinase inhibitor that shows antitumor and antiangiogenic activities. It is reported that the max concnetrations in patients after 28 days of sunitinib administration with doses of 50 mg, 75 mg and 100 mg is about 180 nM, 354 nM and 406/499.4 nM, respectively (Faivre et al. 2006). Prior studies have shown that sunitinib potently inhibited angiogenesis and tumor growth by blocking multiple receptor tyrosine kinases, including VEGFR, PDGFR, c-kit, and other receptors (de Wit et al. 2015). As high concentration may reduce the specificity of sunitinib, sunitinib may affect different signaling pathways in treating different diseases. It has been approved for the clinical treatment of advanced renal cell carcinoma, unresectable gastrointestinal stromal tumors, and well-differentiated metastatic pancreatic neuroendocrine tumors (Imbulgoda et al. 2014). However, besides to its established clinical benefits for patients with a broad range of tumor types, recent studies have demonstrated that sunitinib reversed heart remodeling and significantly improved right ventricular function in experimental models of right ventricular hypertrophy (Kojonazarov et al. 2013). Sunitinib was also reported to block collagen synthesis and mesenchymal transdifferentiation of human hepatic stellate cells, suggesting its potential as a drug candidate in pathological conditions like liver fibrosis (Majumder et al. 2013).

In the present study, we identified a novel antifibrotic property of sunitinib on BLM-mediated pulmonary fibrosis in mice. This agent ameliorated the morphology disorder and decreased the deposition of collagen in murine pulmonary fibrosis induced by BLM. We further demonstrated that sunitinib reversed the process of EMT and fibroblasts activation. However, the detailed molecular mechanism remains elusive. Further *in vitro* studies were conducted to interpret the involved molecular event. When applied to HBEs, TGF-β induced the process of EMT, while sunitinib strikingly suppressed the progression of EMT, suggesting that the antifibrotic effects of sunitinib is at least partly due to its interference with the EMT. We also observed that sunitinib treatment inhibited the phosphorylation of Smad2 and Smad3, the crucial downstream transducers of TGF-β signal pathway. These were consistent with the data generated from sorafenib (Nanthakumar et al. 2015). Based on these encouraging results obtained from animal models of pulmonary fibrosis, sunitinib is expected to be applied for the treatment of IPF. Actually, the agent sunitinib has an exclusive advantage in its efficacy and safety. As an approved oral agent for patients with several types of human malignancies, the safety of sorafenib is strictly guaranteed. Taking into account the therapeutic effects of sunitinib in experimental studies of arthritis and diabetes, we believe this drug may have a much broader indication in clinical application.

Tyrosine kinase signaling transduction plays a pivotal role in a wide variety of cellular processes, including the pathogenesis of pulmonary fibrosis (Wollin et al. 2015). The antifibrotic property of several tyrosine kinase inhibitors has already been investigated in a number of *in vitro* studies and animal models (Chen et al. 2011, 2013). As sunitinib is an inhibitor of multiple receptor tyrosine kinases, it is not surprising to show an inhibitory effect of sunitinib on tyrosine phosphorylation. However we think the inhibition of serine phosphorylation of Smad2/3 by sunitinib is interesting. In spite of improved insights and untiring efforts in this therapeutic avenue, challenges may exist in the course of clinical translation. Further research will be performed to thoroughly elucidate how specific kinases contribute to the pathogenic processes in lung fibrosis, which may ensure its therapeutic potential in the clinical application.

Collectively, we here identified that sunitinib protected mice from BLM-induced pulmonary fibrosis through multiple pro-fibrosis signaling pathways, suggesting a potential agent for the treatment of IPF and other fibrotic disorders.

Acknowledgments

Author Contributions

X.H, W.W., and H.Y. designed, carried out experiments and analyzed data. J.S. and L.L. carried out experiments. X.W., J.H. and Y.G. designed experiments and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

References

Antoniades, H.N., Bravo, M.A., Avila, R.E., Galanopoulos, T., Neville-Golden, J., Maxwell, M. & Selman, M. (1990) Platelet-derived growth factor in idiopathic pulmonary fibrosis. *J. Clin. Invest.*, **86**, 1055-1064.

Camelo, A., Dunmore, R., Sleeman, M.A. & Clarke, D.L. (2014) The epithelium in idiopathic pulmonary fibrosis: breaking the barrier. *Front. Pharmacol.*, **4**, 173.

Chakraborty, S., Chopra, P., Ambi, S.V., Dastidar, S.G. & Ray, A. (2014) Emerging therapeutic interventions for idiopathic pulmonary fibrosis. *Expert Opin. Investig. Drugs*, **23**, 893-910.

Chen, Y.L., Lv, J., Ye, X.L., Sun, M.Y., Xu, Q., Liu, C.H., Min, L.H., Li, H.P., Liu, P. & Ding, X. (2011) Sorafenib inhibits transforming growth factor β1-mediated epithelial-mesenchymal transition and apoptosis in mouse hepatocytes. *Hepatology*, **53**, 1708-1718.

Chen, Y.L., Zhang, X., Bai, J., Gai, L., Ye, X.L., Zhang, L., Xu, Q., Zhang, Y.X., Xu, L., Li, H.P. & Ding, X. (2013) Sorafenib ameliorates bleomycin-induced pulmonary fibrosis: potential roles in the inhibition of epithelial-mesenchymal transition and fibroblast activation. *Cell Death Dis.*, **4**, e665.

Cottin, V. (2016) Idiopathic interstitial pneumonias with connective tissue diseases features: A review. *Respirology*, **21**, 245-258.

de Wit, D., Guchelaar, H.J., den Hartigh, J., Gelderblom, H. & van Erp, N.P. (2015) Individualized dosing of tyrosine kinase inhibitors: are we there yet? *Drug Discov. Today*, **20**, 18-36.

Desai, P., Yang, J., Tian, B., Sun, H., Kalita, M., Ju, H., Paulucci-Holthauzen, A., Zhao, Y., Brasier, A.R. & Sadygov, R.G. (2015) Mixed-effects model of epithelial-mesenchymal transition reveals rewiring of signaling networks. *Cell. Signal.*, **27**, 1413-1425.

Eberlein, C., Rooney, C., Ross, S.J., Farren, M., Weir, H.M. & Barry, S.T. (2015) E-Cadherin and EpCAM expression by NSCLC tumour cells associate with normal fibroblast activation through a pathway initiated by integrin $\alpha v \beta 6$ and maintained through TGFβ signalling. *Oncogene*, **34**, 704-716.

Faivre, S., Delbaldo, C., Vera, K., Robert, C., Lozahic, S., Lassau, N., Bello, C., Deprimo, S., Brega, N., Massimini, G., Armand, J.P., Scigalla, P. & Raymond, E. (2006) Safety, pharmacokinetic, and antitumor activity of SU11248, a novel oral multi-target tyrosine kinase inhibitor, in patients with cancer. *J. Clin. Oncol.*, **24**, 25-35.

Fierro-Fernandez, M., Busnadiego, O., Sandoval, P., Espinosa-Diez, C., Blanco-Ruiz, E., Rodriguez, M., Pian, H., Ramos, R., Lopez-Cabrera, M., Garcia-Bermejo, M.L. & Lamas, S. (2015) miR-9-5p suppresses pro-fibrogenic transformation of fibroblasts and prevents organ fibrosis by targeting NOX4 and TGFBR2. *EMBO Rep.*, **16**, 1358-1377.

Fukunaga, S., Kakehashi, A., Sumida, K., Kushida, M., Asano, H., Gi, M. & Wanibuchi, H. (2015) Integrative analyses of miRNA and proteomics identify potential biological pathways associated with onset of pulmonary fibrosis in the bleomycin rat model. *Toxicol. Appl. Pharmacol.*, **286**, 188-197.

Hiwatari, N., Shimura, S., Yamauchi, K., Nara, M., Hida, W. & Shirato, K. (1997) Significance of elevated procollagen-III-

peptide and transforming growth factor-beta levels of bronchoalveolar lavage fluids from idiopathic pulmonary fibrosis patients. *Tohoku J. Exp. Med.*, **181**, 285-295.

Imbulgoda, A., Heng, D.Y. & Kollmannsberger, C. (2014) Sunitinib in the treatment of advanced solid tumors. *Recent Results Cancer Res.*, **201**, 165-184.

Jamall, I.S., Finelli, V.N. & Que Hee, S.S. (1981) A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. *Anal. Biochem.*, **112**, 70-75.

Kim, H.J., Perlman, D. & Tomic, R. (2015) Natural history of idiopathic pulmonary fibrosis. *Respir. Med.*, **109**, 661-670.

Kim, S., Ding, W., Zhang, L., Tian, W. & Chen, S. (2014) Clinical response to sunitinib as a multitargeted tyrosine-kinase inhibitor (TKI) in solid cancers: a review of clinical trials. *Onco Targets Ther.*, **7**, 719-728.

King, T.E. Jr., Bradford, W.Z., Castro-Bernardini, S., Fagan, E.A., Glaspole, I., Glassberg, M.K., Gorina, E., Hopkins, P.M., Kardatzke, D., Lancaster, L., Lederer, D.J., Nathan, S.D., Pereira, C.A., Sahn, S.A., Sussman, R., et al. (2014) A phase 3 trial of pirfenidone in patients with idiopathic pulmonary fibrosis. *N. Engl. J. Med.*, **370**, 2083-2092.

Kojonazarov, B., Sydykov, A., Pullamsetti, S.S., Luitel, H., Dahal, B.K., Kosanovic, D., Tian, X., Majewski, M., Baumann, C., Evans, S., Phillips, P., Fairman, D., Davie, N., Wayman, C., Kilty, I., et al. (2013) Effects of multikinase inhibitors on pressure overload-induced right ventricular remodeling. *Int. J. Cardiol.*, **167**, 2630-2637.

Kong, D., Zhang, F., Shao, J., Wu, L., Zhang, X., Chen, L., Lu, Y. & Zheng, S. (2015) Curcumin inhibits cobalt chloride-induced epithelial-to-mesenchymal transition associated with interference with TGF-β/Smad signaling in hepatocytes. *Lab. Invest.*, **95**, 1234-1245.

Majumder, S., Piguet, A.C., Dufour, J.F. & Chatterjee, S. (2013) Study of the cellular mechanism of Sunitinib mediated inactivation of activated hepatic stellate cells and its implications in angiogenesis. *Eur. J. Pharmacol.*, **705**, 86-95.

Nanthakumar, C.B., Hatley, R.J., Lemma, S., Gauldie, J., Marshall, R.P. & Macdonald, S.J. (2015) Dissecting fibrosis: therapeutic insights from the small-molecule toolbox. *Nat. Rev. Drug Discov.*, **14**, 693-720.

O'Beirne, S.L., Walsh, S.M., Fabre, A., Reviriego, C., Worrell, J.C., Counihan, I.P., Lumsden, R.V., Cramton-Barnes, J., Belperio, J.A., Donnelly, S.C., Boylan, D., Marchal-Somme, J., Kane, R. & Keane, M.P. (2015) CXCL9 Regulates TGF-β1-Induced Epithelial to Mesenchymal Transition in Human Alveolar Epithelial Cells. *J. Immunol.*, **195**, 2788-2796.

Oechsle, K., Honecker, F., Cheng, T., Mayer, F., Czaykowski, P., Winquist, E., Wood, L., Fenner, M., Glaesener, S., Hartmann, J.T., Chi, K., Bokemeyer, C. & Kollmannsberger, C. (2011) Preclinical and clinical activity of sunitinib in patients with cisplatin-refractory or multiply relapsed germ cell tumors: a Canadian Urologic Oncology Group/German Testicular Cancer Study Group cooperative study. *Ann. Oncol.*, **22**, 2654-2660.

Okumura, N., Minamiyama, R., Ho, L.T., Kay, E.P., Kawasaki, S., Tourtas, T., Schlotzer-Schrehardt, U., Kruse, F.E., Young, R.D., Quantock, A.J., Kinoshita, S. & Koizumi, N. (2015) Involvement of ZEB1 and Snail1 in excessive production of extracellular matrix in Fuchs endothelial corneal dystrophy. *Lab. Invest.*, **95**, 1291-1304.

Richeldi, L., du Bois, R.M., Raghu, G., Azuma, A., Brown, K.K., Costabel, U., Cottin, V., Flaherty, K.R., Hansell, D.M., Inoue, Y., Kim, D.S., Kolb, M., Nicholson, A.G., Noble, P.W., Selman, M., et al. (2014) Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. *N. Engl. J. Med.*, **370**, 2071-2082.

Roskoski, R. Jr. (2007) Sunitinib: a VEGF and PDGF receptor protein kinase and angiogenesis inhibitor. *Biochem. Biophys.*

Res. Commun., **356**, 323-328.

Spagnolo, P., Maher, T.M. & Richeldi, L. (2015) Idiopathic pulmonary fibrosis: Recent advances on pharmacological therapy. *Pharmacol. Ther.*, **152**, 18-27.

Staitieh, B.S., Renzoni, E.A. & Veeraraghavan, S. (2015) Pharmacologic therapies for idiopathic pulmonary fibrosis, past and future. *Ann. Med.*, **47**, 100-105.

Tian, B., Li, X., Kalita, M., Widen, S.G., Yang, J., Bhavnani, S.K., Dang, B., Kudlicki, A., Sinha, M., Kong, F., Wood, T.G., Luxon, B.A. & Brasier, A.R. (2015) Analysis of the TGF*β*-induced program in primary airway epithelial cells shows essential role of NF-*κ*B/RelA signaling network in type II epithelial mesenchymal transition. *BMC Genomics*, **16**, 529.

Wen, J., Li, H.Z., Ji, Z.G. & Jin, J. (2015) Human urothelial carcinoma cell response to Sunitinib malate therapy in vitro.

Cancer Cell Int., **15**, 26.

Wollin, L., Wex, E., Pautsch, A., Schnapp, G., Hostettler, K.E., Stowasser, S. & Kolb, M. (2015) Mode of action of nintedanib in the treatment of idiopathic pulmonary fibrosis. *Eur. Respir. J.*, **45**, 1434-1445.

Yang, J., Velikoff, M., Canalis, E., Horowitz, J.C. & Kim, K.K. (2014) Activated alveolar epithelial cells initiate fibrosis through autocrine and paracrine secretion of connective tissue growth factor. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, **306**, L786-796.

Zerr, P., Palumbo-Zerr, K., Huang, J., Tomcik, M., Sumova, B., Distler, O., Schett, G. & Distler, J.H. (2016) Sirt1 regulates canonical TGF-*β* signalling to control fibroblast activation and tissue fibrosis. *Ann. Rheum. Dis.*, **75**, 226-233.