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TECHNICAL REPORT

A novel tree shrew model of pulmonary fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease without effective therapy. Animal models effectively reproducing IPF disease features are needed to study the underlying molecular mechanisms. Tree shrews are genetically, anatomically, and metabolically closer to humans than rodents or dogs; therefore, the tree shrew model presents a unique opportunity for translational research in lung fibrosis. Here we demonstrate that tree shrews have in vivo and in vitro fibrotic responses induced by bleomycin and pro-fibrotic mediators. Bleomycin exposure induced lung fibrosis evidenced by histological and biochemical fibrotic changes. In primary tree shrew lung fibroblasts, transforming growth factor beta-1 (TGF-β1) induced myofibroblast differentiation, increased extracellular matrix (ECM) protein production, and focal adhesion kinase (FAK) activation. Tree shrew lung fibroblasts showed enhanced migration and increased matrix invasion in response to platelet derived growth factor BB (PDGF-BB). Inhibition of FAK significantly attenuated pro-fibrotic responses in lung fibroblasts. The data demonstrate that tree shrews have in vivo and in vitro fibrotic responses similar to that observed in IPF. The data, for the first time, support that the tree shrew model of lung fibrosis is a new and promising experimental animal model for studying the pathophysiology and therapeutics of lung fibrosis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive interstitial lung disease of unknown cause, characterized by fibrosis of the lung parenchyma [1]. Despite considerable research efforts, there is no effective therapy for IPF. Prognosis is poor as the median survival following a diagnosis of IPF remains ~3–5 years [2, 3]. The exact pathogenesis of IPF remains an enigma. Current evidence suggests that IPF is likely the result of loss of control of diverse cellular and molecular pathways. The aberrant intersection of alveolar epithelium injury and repair, fibroblast activation and invasion, the rise of myofibroblast, and dysfunction of other cell types including macrophages, stem cells, and fibrocytes, contribute to the pathogenesis of IPF [2]. Myofibroblasts, compared to other involved cell types, are generally accepted as the cell type predominantly responsible for increased production of extracellular matrix (ECM) proteins, which subsequently leads to destruction of lung structure and fibrosis in IPF [4, 5].

Fibrotic tissues in the lung, liver, and kidney show prominent myofibroblasts [4–6], supporting that myofibroblast differentiation is one critical step during the development of fibrotic disorders. Recent evidence indicates that the sources of myofibroblasts are variable among different organs [4].

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Fibroblast migration into the wounded area and differentiation into myofibroblasts exist in normal and pathologic wound healing, such as fibrotic tissue remodeling. Focal adhesion kinase (FAK) plays an important role in fibroblast migration and myofibroblast differentiation in primary lung fibroblasts derived from IPF patients [7]. Transforming growth factor beta-1 (TGF-β1) is a major pro-fibrotic mediator that induces myofibroblast differentiation and promotes fibrotic responses in lung, liver, and kidney [8]. Enhanced expression of α-smooth muscle actin (α-SMA) is the hallmark of myofibroblast differentiation [4, 5, 9]. Formation of α-SMA-containing cytoplasmic filaments is another hallmark of myofibroblasts; these α-SMA-containing cytoplasmic filaments enhance the contractile phenotype of myofibroblasts, which is required for wound healing [4, 9–11]. Animal models of lung fibrosis, including mouse and rat models, have provided critical insight into the pathogenesis of IPF and were able to recapitulate some but not all pathologic features of human IPF [12].

Tree shrew, a native species to Southeast Asia and Southwest China, belongs to the order of Scandentia. They are small diurnal mammals (150–220 g) closely related to the human and primate [13–18]. Whole genome phylogenetic analysis has confirmed that tree shrews are genetically much closer to humans and nonhuman primates than dogs or rodents [13–15]. Many metabolic and anatomic structures of tree shrew are more similar to humans than dogs or rodent [14–16]. Tree shrew striatum resembles that of humans and primates. Similar to the human eye, a collagenous lamina cribrosa exists in the tree shrew eye, but not in mice or rats [17–21]. Tree shrews have been used in studies of lung cancer [22], Zika virus infection [23], and H1N1 influenza [24]. Tree shrews closely mimic the influenza viral infection seen in humans [24]. Notably, tree shrews infected by Zika virus developed the common rash seen in patients infected by Zika virus [23]. Thus, the tree shrew has unique advantages as animal model of human diseases and presents a unique opportunity to advance translational research.

In this study, we present data for an experimental tree shrew model of lung fibrosis. Bleomycin-induced injury resulted in lung fibrosis and pro-fibrotic responses in tree shrews, with evidences of increased hydroxyproline levels, increased expression of collagen, fibronectin, and α-SMA, and increased activation of FAK in lung tissues. In vitro, in response to TGF-β1, tree shrew primary lung fibroblasts have increased α-SMA expression, the hallmark of myofibroblast differentiation, increased ECM protein production, and increased FAK activation. PDGF-BB treated tree shrew lung fibroblasts have enhanced cell migration and invasion when compared to vehicle controls. Inhibition of FAK signaling significantly blocked TGF-β1 induced myofibroblast differentiation and ECM production, and blocked platelet derived growth factor BB (PDGF-BB) induced cell migration and invasion. These findings support that the tree shrew lung fibrosis model shares common fibrotic responses and pro-fibrotic signaling pathways that are seen in human IPF.

Materials and methods

Reagents

Transforming growth factor-β1 (TGF-β1) was obtained from R&D Systems (Minneapolis, MN). The following antibodies were purchased: phospho-FAK [pY397] (Biosource, Camarillo, CA), procollagen alpha 1 type 1 (1A1) and fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), FAK (Cell Signaling, MA), α-SMA (American Research Products, Belmont, MA), and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Research Diagnostics, Flanders, NJ). All antibodies purchased are against human antigens. Antibodies designed against human antigens detect tree shrew antigens successfully [25–29]. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Waltham, MA).

Tree shrew model of lung fibrosis

All animal interventions were approved by Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. Both male and female tree shrews (3–5 month old) were challenged with bleomycin (1.75 U/kg body weight) using an intratracheal catheter similarly as described previously for mouse model of lung fibrosis [7]. The Ashcroft scoring system was used to assess the pulmonary fibrotic changes in lung tissue sections. Fibrotic lesional areas were measured on hematoxylin and eosin (H&E) stained lung tissue sections by morphometric methodology.

Lung collagen determination and Masson's trichrome staining

The whole lung collagen level was determined by whole lung hydroxyproline level [30]. The harvested lungs were hydrolyzed in 6 M HCl at 110 °C for 24 h, and the amount of hydroxyproline in the above lung acid hydrolysates was performed by colorimetric assay as described [31]. Collagen deposition in lung tissue sections (5–10 μm, paraffin embedded tissues) was localized by Masson's trichrome staining using a commercially available staining kit according to the manufacturer's instructions (Poly Scientific, Bay Shore, NY). Positive area was measured based on pooled results from series sections and represented as the percentage of positive area over the total lung area.

Cell and cell culture

Primary human lung fibroblasts were described previously [7, 32]. Isolation and propagation of primary tree shrew lung fibroblasts were performed similarly as described previously [32]. Briefly, fibroblasts were maintained and propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Experiments were performed on early passages (passage 2–4) of primary lung fibroblasts.

Whole lung and cell protein extracts

Whole lung or cell homogenates were prepared in detergent lysis buffer containing 1% NP-40 with the following inhibitors, 100 μM phenylmethanesulfonyl fluoride (PMSF), 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, and 100 μM sodium vanadate, and 20 μg/ml TLCK [9]. The resultant supernatants after centrifugation (14,000 × *g* for 20 min at 4 °C) were analyzed by immunoblotting immediately or stored at −80 °C until used.

Western blotting

Immunoblotting was performed using 1% NP-40 whole lung tissue lysates or whole cell lysates as described previously [33]. Briefly, equivalent micrograms of lysates were electrophoresed on a disulfide-reduced 8–12% SDS-PAGE, transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) for probing, and developed with the enhanced chemiluminescence system (Fisher Scientific).

Adenoviral vectors

Adenoviral vectors expression FAK-related non-kinase (FRNK) and green fluorescent protein (GFP) is described previously [32].

Cell migration and invasion assays

The wound closure monolayer scratch motility assay and transwell invasion assay were performed similarly as described previously [32]. Briefly, fibroblasts were plated in serum-free DMEM with 1% BSA for 24 h. The monolayer was scratched, and the wound area covered by cell migration over indicated time on digital photomicrograph images was calculated. Mitomycin C was added to inhibit cell proliferation after PDGF-BB treatment. The invasion assay was performed in two-well Boyden-type transwells. Briefly, lung fibroblasts (4×10^4 cells) in serum-free DMEM media with 1% BSA were plated onto 8 μm filters coated with Matrigel on the top surface of the insert well and PDGF-BB containing media at the bottom well, and allowed to invade at 37 °C with 5% CO_2 for 36 h. Remaining cells on the top surface were removed and the invaded cells were fixed, stained, and counted. Conditions were assayed in replicas of three or four, repeated two to four times, and the data analyzed and presented as the mean ± SE.

Lactate assay

Lactate levels were determined by using colorimetric and fluorometric method. Briefly, samples were prepared from tree shrew lung tissue on day 21 after bleomycin treatment, or cell culture lysate or culture medium from primary tree shrew lung fibroblasts treated with or without 5 ng/ml TGF-β1. Lactate levels were determined by lactate assay kit according to the manufacturer's instructions (BioVision, Milpitas, CA).

Statistical analysis

Data were analyzed using the Student's *t* test analysis (Sigma Plot, SPSS Inc.) for differences between two groups, and expressed as mean ± SE. All experiments were repeated at least three times.

Results

Bleomycin induces lung fibrosis in tree shrews

To establish the tree shrew model of lung fibrosis, bleomycin was intratracheally instilled into tree shrews (1.75 U/kg body weight). Lungs were harvested on day 21 after bleomycin exposure and pro-fibrotic responses were determined. Using several measures, the fibrotic responses to bleomycin was significantly increased when compared to that in control (Fig. 1). H&E staining and morphometric evaluation demonstrate that a significant portion of lung is fibrotic in bleomycin-treated tree shrews (Fig. 1a, b). Masson's trichrome staining shows that the fibrotic collagen area is significantly increased in bleomycin-treated tree shrews (Fig. 1a, c). Bleomycin-treated tree shrews have about 1.4-fold greater hydroxyproline levels compared to saline treated tree shrews (Fig. 1d), supporting a significant increase of collagen content in bleomycin-treated tree shrews compared to control.

Bleomycin induces myofibroblast differentiation, increases production of extracellular matrix (ECM) proteins, and activates FAK signaling in tree shrews

To examine the effect of bleomycin on pro-fibrotic responses, expression of α-SMA, a hallmark of myofibroblast differentiation, was examined in bleomycin-treated tree shrews. Whole lung α-SMA expression level is increased about 2.7-fold in bleomycin-treated tree shrews

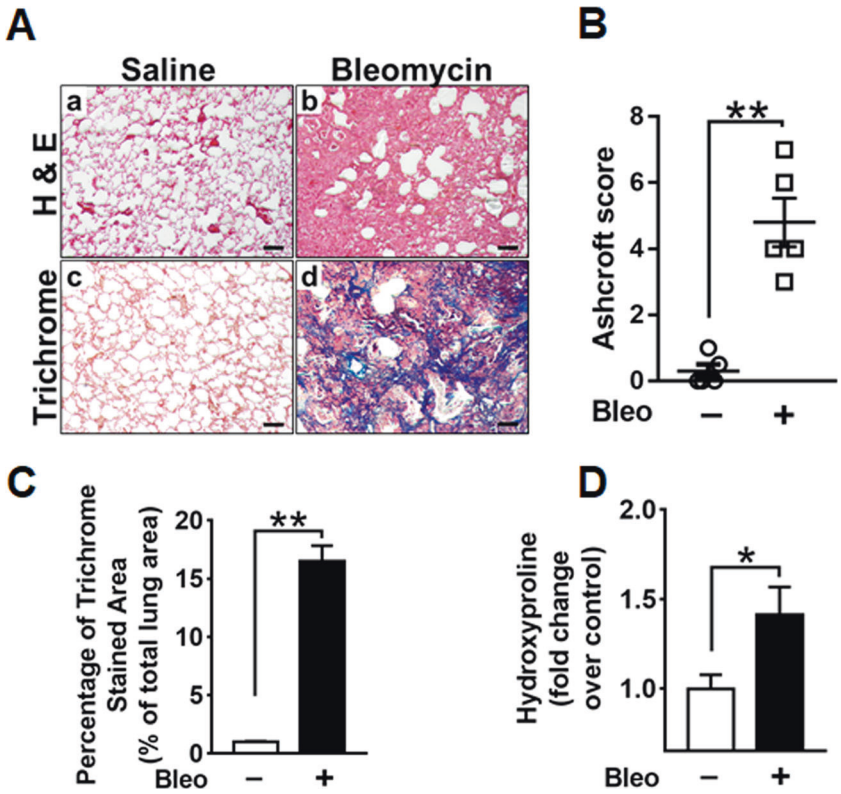

Fig. 1 Bleomycin induces lung fibrosis in tree shrews. a Tree shrews were intratracheally instilled with bleomycin (1.75 U/kg) or saline. Lungs were harvested at day 21 and stained with hematoxylin and eosin (H&E) or Masson's Trichrome (original magnification ×40, scale bar = 100 μm). **b** The severity of lung fibrosis was analyzed by Ashcroft score. **c** Masson's Trichrome positive areas were morphometrically quantified and represented as the percentage of total lung areas in serial lung tissue sections. **d** Lung hydroxyproline levels (normalized to that in saline treated tree shrews). Data were shown as mean ± S.E. $n = 5$ for bleomycin-treated tree shrews. $n = 6$ for saline treated tree shrews. $*p < 0.01$; $**p < 0.001$.

when compared to saline controls (Fig. 2a, b). Expression of procollagen and fibronectin was examined in whole lung lysates by western blot (Fig. 2a). Procollagen level was increased about 2.1-fold in bleomycin-treated tree shrews compared to saline controls (Fig. 2a, c). Fibronectin level was increased about 3.2-fold in bleomycin-treated tree shrews when compared to saline treated controls (Fig. 2a, d). Cell migration toward wounded area is a part of wound healing process, and cell–matrix interaction is necessary. FAK plays an important role in integrin engagement with ECM proteins [34–36]. FAK activation is required for cell migration and invasion, and promotes myofibroblast differentiation and resistance to apoptosis [36–38], which support a role for FAK in the pro-fibrotic responses and lung fibrosis. Therefore, we examined FAK activation in bleomycin-treated tree shrew lung tissues. FAK activation, by phosphorylation of Tyr397 (Y397), was increased nearly 2.5-fold in bleomycin-treated tree shrews compared to control (Fig. 2a, e). These data clearly indicate that tree shrews have increased pro-fibrotic responses and fibrotic remodeling in the lungs of bleomycin exposed tree shrews.

Primary tree shrew lung fibroblasts become pro-fibrotic by differentiation to myofibroblasts through a FAK-mediated pathway in response to TGF-β1

To determine the pro-fibrotic responses of tree shrews, primary tree shrew lung fibroblasts and primary normal human lung fibroblasts were treated with TGF-β1 to compare the pro-fibrotic responses (Fig. 3). In response to TGF-β1, α-SMA expression was increased in tree shrew and human lung fibroblasts (Fig. 3a, b), suggesting myofibroblast differentiation induced by TGF-β1 in the fibroblasts. Next, we examined the ECM production in primary tree shrew fibroblasts in response to TGF-β1, a central pro-fibrotic process. Expression of procollagen and fibronectin was significantly increased in TGF-β1 treated fibroblasts when compared to controls (Fig. 3a). In response to TGF-β1, collagen expression was increased by 2.2- and 3.1-fold in tree shrew and human lung fibroblasts, respectively, and fibronectin expression was increased by 3.3- and 2.8-fold in tree shrew and human lung fibroblasts (Fig. 3c, d). FAK and Smad3 mediate TGF-β1 signal transduction and myofibroblast differentiation [39, 7]. TGF-β1 induced activation of FAK and Smad3 in tree shrew and human lung fibroblasts (Fig. 3a, e, f).

To determine whether FAK plays a similar role in tree shrew fibroblasts as seen in human fibroblasts in response to TGF-β1 [7, 32], FRNK was overexpressed to inhibit FAK in tree shrew fibroblasts. We have previously shown that FRNK overexpression inhibits FAK in normal and IPF human lung fibroblasts [32]. Overexpression of GFP was used as a control. FRNK expression inhibited TGF-β1 induced FAK activation in tree shrew lung fibroblasts (Fig. 3g, k). Similarly, FRNK overexpression reduced TGF-β1 induced increased expression of α-SMA,

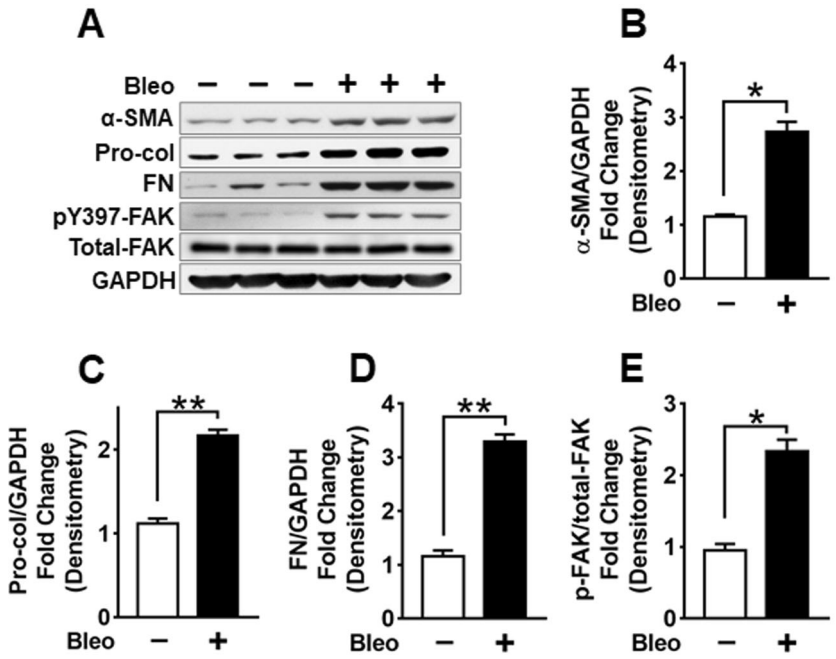

Fig. 2 Bleomycin induces pro-fibrotic responses in tree shrews. a Tree shrews were intratracheally instilled with bleomycin (1.75 U/kg) or saline. Lungs were harvested at day 21. Whole lung lysate was analyzed by western blot for the levels of α-SMA, collagen (through procollagen, pro-col), and fibronectin (FN). GAPDH served as the loading control ($n =$ three individual tree shrews in each group). Representative images are shown. **b** Densitometry analysis of α-SMA expression as shown in **a**. **c** Densitometry analysis of procollagen expression as shown in **a**. **d** Densitometry analysis of FN expression as shown in **a**. **e** Densitometry analysis of FAK Y397 phosphorylation as shown in **a**. Data were shown as mean ± S.E. *$p < 0.01$; **$p < 0.001$.

collagen, and FN in tree shrew lung fibroblasts (Fig. 3g–j). Control GFP expression had no noticeable effect on TGF-β1 induced responses (Fig. 3g–k). These data suggest that FAK signaling is critical for TGF-β1 induced pro-fibrotic responses in tree shrew derived lung fibroblasts.

Platelet derived growth factor BB (PDGF-BB) induces migration and invasion in primary tree shrew lung fibroblasts; FAK is required for migration and invasion induced by PDGF-BB

We next studied whether tree shrew fibroblasts respond to PDGF-BB with enhanced migration and invasion compare to that in human fibroblasts. PDGF-BB significantly promoted cell motility in tree shrew fibroblasts based on wound healing assays, and that is similar to PDGF-BB induced cell motility in human fibroblasts (Fig. 4a). FRNK expression abrogated PDGF-BB induced cell motility in tree shrew and human fibroblasts (Fig. 4a). Whereas GFP expression had no noticeable effect (Fig. 4a). Furthermore, PDGF-BB induced invasion through Matrigel in tree shrew and human fibroblasts (Fig. 4b). Blocking FAK signaling by FRNK overexpression significantly reduced PDGF-BB induced invasion through matrix gel in tree shrew and human fibroblasts (Fig. 4b).

Lactate, the product of glycolysis, is increased in primary tree shrew lung fibroblasts in response to TGF-β1

Dysregulated glycolysis is recently reported as one important mechanism contributing to the pathogenesis of IPF [40, 41]. Dysregulated glycolysis results in increased lactate, an end-product of glycolysis, in fibroblasts from subjects with IPF [40, 41]. Lactate is increased in tree shrews with bleomycin treatment when compared to that in saline controls (Fig. 5a). In line with data in lung tissues, intracellular and extracellular lactate content are increased in response to TGF-β1 in cultured primary tree shrew fibroblasts (Fig. 5b, c). The data show that tree shrews and tree shrew fibroblasts exhibit a phenotype of increased glycolysis in response to bleomycin and TGF-β1 (Fig. 5). Our data support that the molecular mechanisms underlying IPF pathogenesis are shared in the tree shrews, and the tree shrew model of lung fibrosis is a unique and promising experimental animal model for studying the pathophysiology and therapeutics of lung fibrosis.

Discussion

The pathogenesis of IPF still remains an enigma. The prognosis of IPF is poor with median survival being about

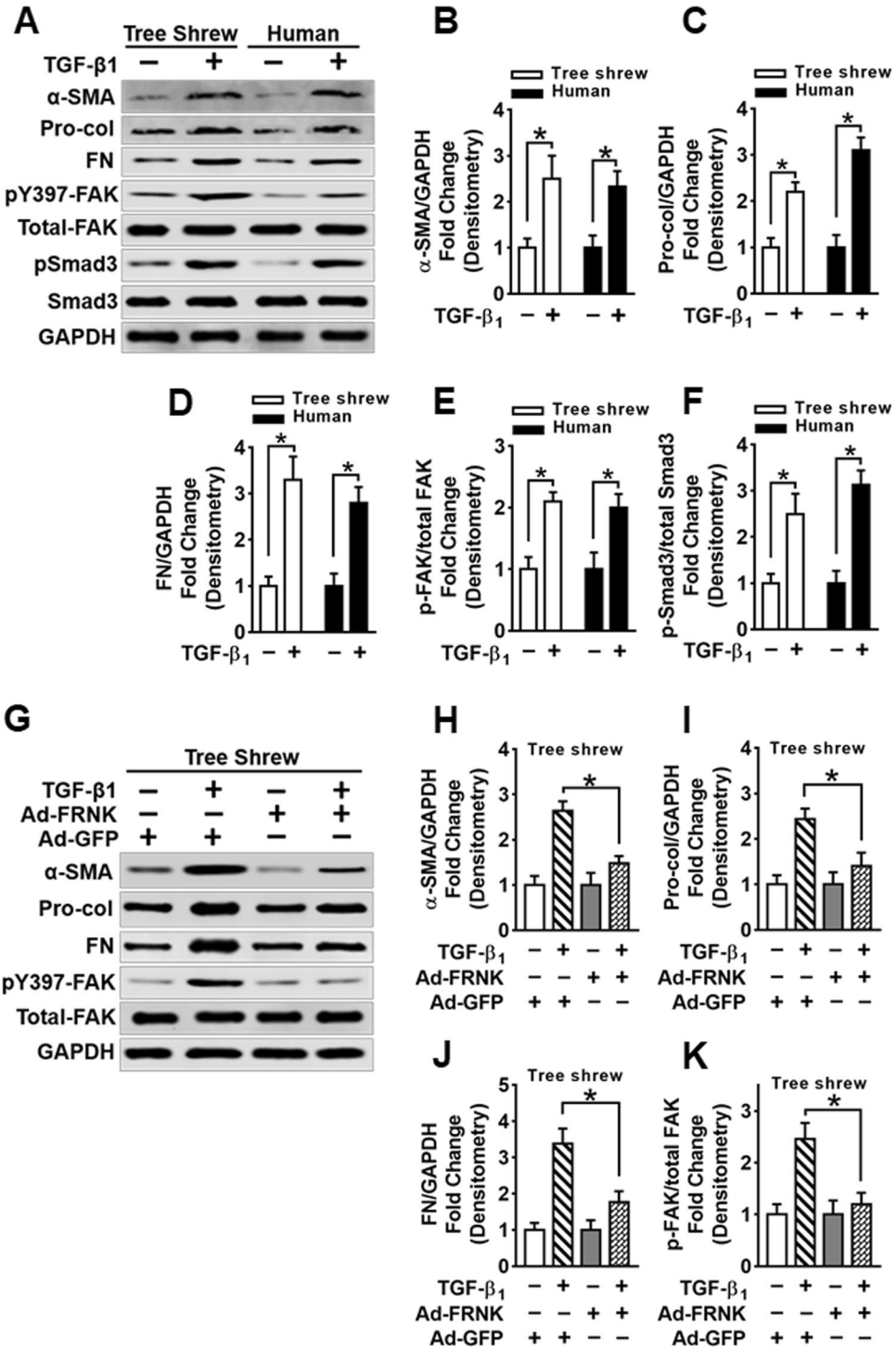

Fig. 3 TGF-β1 induces pro-fibrotic responses in tree shrew lung fibroblasts. a Tree shrew and normal human lung fibroblasts were serum starved and then treated with TGF-β1 (5 ng/ml) for 24 h. Equivalent amount of cell lysate was western blotted with the indicated antibodies. **b–f** Densitometry analysis of expression of α-SMA, pro-col, and FN, also activation of FAK and Smad3, as shown in **a**. Blank bars for tree shrew fibroblasts. Black bars for human fibroblasts. **g** Tree shrew lung fibroblasts were treated as in **a**. We have previously shown that FRNK overexpression inhibits FAK and myofibroblast differentiation in human lung fibroblasts. Here, FRNK and control GFP overexpression were mediated by adenoviral vectors in tree shrew lung fibroblasts. **h–k** Densitometry analysis of expression of α-SMA, pro-col, FN, and activation of FAK, as shown in **g**. Data were shown as mean ± S. E. $*p < 0.01$; $**p < 0.001$.

3–5 years after diagnosis, making IPF the deadliest interstitial lung disease [2, 3, 42]. Animal models of lung fibrosis are critical to understand the pathogenesis of IPF. Preclinical studies with animal models have contributed to clinical trials that led to the FDA approved drugs pirfenidone and nintedanib [43, 44]; however, the efficacy of pirfenidone and nintedanib in IPF is limited. Current animal models of lung fibrosis recapitulate some but not all pathologic features of human IPF [7, 12]. Because of this limitation, new animal models of lung fibrosis that recapitulate pathologic features of human IPF are critical to discover novel, druggable targets to conquer this deadly disease.

Here, we provide data to establish a new and novel experimental lung fibrosis model in tree shrews, a diurnal mammal that shares more similarity in metabolic and

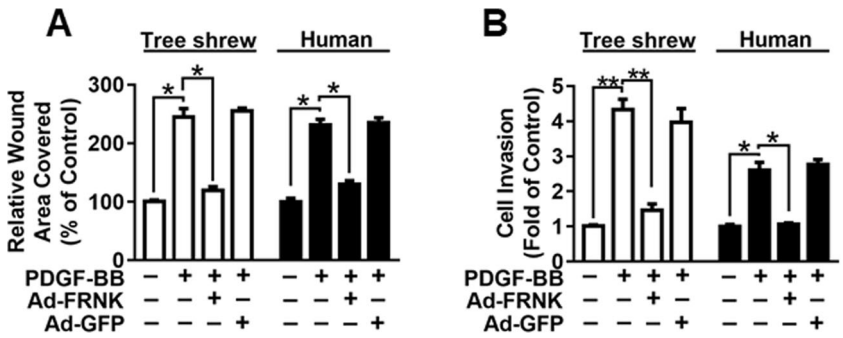

Fig. 4 PDGF-BB promotes cell motility and invasion through FAK-mediated signaling pathway in tree shrew lung fibroblasts. **a** Tree shrew and normal human lung fibroblasts were serum starved and then treated with PDGF-BB (4 ng/ml) and followed by wound closure monolayer scratch motility assays. FRNK and control GFP overexpression were mediated by adenoviral vectors in lung fibroblasts. The percentage of wound area covered by cell migration was normalized to controls (without PDGF-BB) within each type of fibroblasts. **b** Tree shrew and normal human lung fibroblasts were serum starved and subjected to transwell invasion assays. The top surface of transwells were pre-coated with Matrigel. The bottom medium contained PDGF-BB (4 ng/ml). The data were pooled and presented as the fold invasion over the controls (without PDGF-BB) within each type of fibroblasts. Data were shown as mean ± S.E. $*p < 0.01$; $**p < 0.001$.

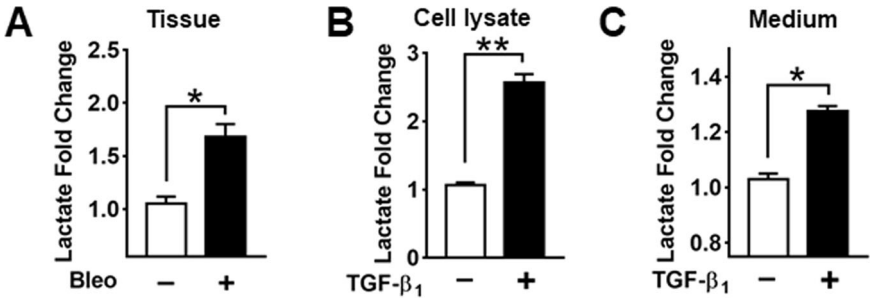

Fig. 5 Tree shrew lung fibroblasts show increased glycolysis reprogramming in response to TGF-β1. **a** lung extract from bleomycin-instilled tree shrew ($n = 5$) have increased lactate compared to saline instilled tree shrews ($n = 6$). **b** Primary tree shrew lung fibroblasts have increased lactate from cell lysate in response to TGF-β1. **c** Primary tree shrew lung fibroblasts have increased lactate in cell culture medium in response to TGF-β1, suggesting increased release of lactate. Data were shown as mean ± S.E. $*p < 0.01$; $**p < 0.001$.

anatomic structure with humans than rodents or dogs [14–16]. Bleomycin instillation through intratracheal route induces pro-fibrotic responses and lung fibrosis in tree shrews that recapitulate some pathologic features in IPF and are comparable to that seen in human lung fibrosis [7, 12]. The in vivo findings were evaluated by histological and biochemical methods in tree shrews challenged with or without bleomycin (Figs. 1, 2). Furthermore, the fibrotic responses were evaluated in vitro in primary lung fibroblasts derived from tree shrews. Tree shrew primary lung fibroblasts respond to the pro-fibrotic effects of TGF-β1 and show increased myofibroblast differentiation, matrix protein synthesis, and activation of Smad3 and FAK (Fig. 3). In response to PDGF-BB, tree shrew lung fibroblasts show increased motility and invasion through Matrigel (Fig. 4). Importantly, these pro-fibrotic responses in tree shrew primary lung fibroblasts are comparable to that in primary human lung fibroblasts.

FAK plays an important role in cell migration and invasion [34]. In cancer, FAK signaling is required for cancer cell migration and metastasis; therefore, FAK inhibitors have been evaluated for cancer treatment [34]. We and others have demonstrated that FAK signaling regulates fibroblast cell motility, increases myofibroblast differentiation, and promotes myofibroblast survival [32, 33, 36–38]. FAK activation is increased in the fibrotic lungs in tree shrews treated with bleomycin (Fig. 1). To gain insights into the FAK signaling involved in pro-fibrotic responses in tree shrews, FAK signaling was inhibited by FRNK overexpression (Fig. 3). Inhibition of FAK activation reduced TGF-β1 induced myofibroblast differentiation and ECM protein expression (Fig. 3), and blocked PDGB-BB induced cell motility and invasion through Matrigel in primary tree shrew and primary human lung fibroblasts (Fig. 4). These findings suggest that FAK is an important player in mediating pro-fibrotic responses and FAK-mediated signaling is shared between tree shrew and human lung fibroblast.

Rodent models have been widely accepted and used for the study of IPF. These experimental animal models

have generated important insights and provided valuable knowledge for the understanding of IPF pathogenesis. However, the biological nature of these rodent animal models set limitations for the study of IPF human diseases [12]. Indeed, all current animal models of lung fibrosis have limitations to fully recapitulate the pathological features seen in human IPF [12]. Thus, new animal models of lung fibrosis are still in demand to study human IPF. Although nonhuman primates may represent the most attractive model to study the complicated human disease like IPF, the high cost in addition to other barriers in nonhuman primate models of human diseases is likely one major weakness for its wide application for IPF research. The current study provide data to support an experimental model of lung fibrosis in tree shrews. The current study cannot fully assess if the tree shrew model of lung fibrosis can recapitulate the pathological features of human IPF better than other animal models, due to that the scope of current work is limited by the number of tree shrews and time points tested. Additional studies are required to fully evaluate the tree shrew as human-like model of IPF; many questions remains to be answered, including but not limited to whether the tree shrew model resembles human IPF pathological features, its versatility, the availability of genetically modified animal lines, the availability to test therapeutically and response to the clinically approved drugs such as nintedanib and pirfenidone, whether the immunological and inflammatory responses resembles human IPF, and whether the fibrosis self-resolve over time or progressive. Bleomycin model itself has limitations [45, 46]. Therefore, it will be ideal if the tree shrew model can be tested by using other fibrotic inducing methods such as FITC, silica, or viral vector-based delivery of active TGF-β1, to induce lung fibrosis in tree shrews, and compare the fibrotic signaling and lung fibrosis results with that obtained from the bleomycin-induced model. Another potential limitation is that tree shrews cost much less than nonhuman primates, but higher than mice. We expect that tree shrews provide many advantages over commonly used animal models since they are genetically, biologically, and structurally closer to humans and nonhuman primates than rodents or dogs [13–18]. Tree shrews share some biological structures with humans and that do not exist in mice or rats [17–21]. Notably, tree shrews share similar physical response with human in response to disease. Tree shrews infected by Zika virus have developed physical signs similar to that of humans, the common rash in infected patients, but that has not been observed in rodents [23]. The current study demonstrates, for the first time, a preclinical tree shrew model of lung fibrosis, which shares common pro-fibrotic responses and signaling pathways observed in human IPF and in human lung fibroblasts. Taken together, the results strongly suggest that the tree shrew model of lung fibrosis is a new and promising experimental animal model for studying the pathophysiology and therapeutics of lung fibrosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. American Thoracic Society, European Respiratory Society IPF: diagnosis and treatment. International consensus statement. Am J Respir Crit Care Med. 2000;161:646–64.
2. Blackwell TS, Tager AM, Borok Z, Moore BB, Schwartz DA, Anstrom KJ, et al. Future directions in idiopathic pulmonary fibrosis research. An NHLBI workshop report. Am J Respir Crit Care Med. 2014;189:214–22.
3. Ley B, Collard HR, King TE Jr. Clinical course and prediction of survival in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2011;183:431–40.
4. Phan SH. The myofibroblast in pulmonary fibrosis. Chest. 2002;122 Suppl 6:286S–9S.
5. Desmouliere A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofiobroblast. Wound Repair Regen. 2005;13:7–12.
6. White ES, Baralle FE, Muro AF. New insights into form and function of fibronectin splice variants. J Pathol. 2008;216:1–14.
7. Ding Q, Cai GQ, Hu M, Yang Y, Zheng A, Tang Q, et al. FAK-related nonkinase is a multifunctional negative regulator of pulmonary fibrosis. Am J Pathol. 2013;182:1572–84.
8. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in grandulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biochem. 1993;122:103–11.
9. Cai GQ, Chou CF, Hu M, Zheng A, Reichardt LF, Guan JL, et al. Neuronal Wiskott-Aldrich syndrome protein (N-WASP) is critical for formation of alpha-smooth muscle actin filaments during myofibroblast differentiation. Am J Physiol Lung Cell Mol Physiol. 2012;303:L692–702.
10. Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. Mol Biol Cell. 2001;12:2730–41.
11. Hinz B, Dugina V, Ballestrem C, Wehrle-Haller B, Chaponnier C. Alpha-smooth muscle actin is crucial for focal adhesion maturation in myofibroblasts. Mol Biol Cell. 2003;14:2508–19.
12. Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol. 2008;294:L152–60.
13. Janecka JE, Miller W, Pringle TH, Wiens F, Zitzmann A, Helgen KM, et al. Molecular and genomic data identify the closest living relative of primates. Science. 2007;318:792–4.
14. Fan Y, Huang ZY, Cao CC, Chen CS, Chen YX, Fan DD, et al. Genome of the Chinese tree shrew. Nat Commun. 2013;4:1426.
15. Zhang XH, Dai ZX, Zhang GH, Han JB, Zheng YT. Molecular characterization, balancing selection, and genomic organization of

the tree shrew (Tupaia belangeri) MHC class I gene. Gene. 2013; 522:147–55.
16. Petruzziello F, Fouillen L, Wadensten H, Kretz R, Andren PE, Rainer G, et al. Extensive characterization of Tupaia belangeri neuropeptidome using an integrated mass spectrometric approach. J Proteome Res. 2012;11:886–96.
17. Samuels BC, Siegwart JT, Zhan W, Hethcox L, Chimento M, Whitley R, et al. A novel tree shrew (Tupaia belangeri) model of glaucoma. Investig Ophthalmol Vis Sci. 2018;59:3136–43.
18. Ni RJ, Huang ZH, Shu YM, Wang Y, Li T, Zhou JN. Atlas of the striatum and globus pallidus in the tree shrew: comparison with rat and mouse. Neurosci Bull. 2018;34:405–18.
19. Rice MW, Roberts RC, Melendez-Ferro M, Perez-Costas E. Neurochemical characterization of the tree shrew dorsal striatum. Front Neuroanat. 2011;5:53.
20. Mooser F, Bosking WH, Fitzpatrick D. A morphological basis for orientation tuning in primary visual cortex. Nat Neurosci. 2004; 7:872–9.
21. Paik SB, Ringach DL. Link between orientation and retinotopic maps in primary visual cortex. Proc Natl Acad Sci USA. 2012; 109:7091–6.
22. Ye L, He M, Huang Y, Zhao G, Lei Y, Zhou Y, et al. Tree shrew as a new animal model for the study of lung cancer. Oncol Lett. 2016;11:2091–5.
23. Zhang L, Shen ZL, Feng Y, Li DQ, Zhang NN, Deng YQ, et al. Infectivity of Zika virus on primary cells support tree shrew as animal model. Emerg Microbes Infect. 2019;8:232–41.
24. Yang ZF, Zhao J, Zhu YT, Wang YT, Liu R, Zhao SS, et al. The tree shrew provides a useful alternative model for the study of influenza H1N1 virus. Virol J. 2013;10:111.
25. Xiao J, Liu R, Chen CS. Tree shrew (Tupaia belangeri) as a novel laboratory disease animal model. Zool Res. 2017;38:127–37.
26. Tu Q, Yang D, Zhang X, Jia X, An S, Yan L, et al. A novel pancreatic cancer model originated from transformation of acinar cells in adult tree shrew, a primate-like animal. Dis Model Mech. 2019;12:1–9.
27. Chen M, Ou C, Yang C, Yang W, Qin Q, Jiang W, et al. A novel animal model of induced breast precancerous lesion in tree shrew. Biol Pharm Bull. 2019;42:580–5.
28. Han Y, Li B, Yin TT, Xu C, Ombati R, Luo L, et al. Molecular mechanism of the tree shrew's insensitivity to spiciness. PLoS Biol. 2018;16:e2004921.
29. Fan Y, Luo R, Su LY, Xiang Q, Yu D, Xu L, et al. Does the genetic feature of the Chinese tree shrew (Tupaia belangeri chinensis) support its potential as a viable model for Alzheimer's disease research? J Alzheimers Dis. 2018;61:1015–28.
30. Hu M, Che P, Han X, Cai GQ, Liu G, Antony V, et al. Therapeutic targeting of SRC kinase in myofibroblast differentiation and pulmonary fibrosis. J Pharmacol Exp Ther. 2014;351:87–95.
31. Berg RA. Determination of 3- and 4-hydroxyproline. Methods Enzymol. 1982;82:372–98.
32. Cai GQ, Zheng A, Tang Q, White ES, Chou CF, Gladson CL, et al. Downregulation of FAK-related non-kinase mediates the migratory phenotype of human fibrotic lung fibroblasts. Exp Cell Res. 2010;316:1600–9.
33. Che P, Yang Y, Han X, Hu M, Sellers JC, Londono-Joshi AI, et al. S100A4 promotes pancreatic cancer progression through a dual signaling pathway mediated by Src and focal adhesion kinase. Sci Rep. 2015;5:8453.
34. Hauck CR, Hsia DA, Schlaepfer DD. The focal adhesion kinase-a regulator of cell migration and invasion. IUBMB Life. 2002;53: 115–9.
35. Siesser PM, Hanks SK. The signaling and biological implications of FAK overexpression in cancer. Clin Cancer Res. 2006;12:3233–7.
36. Parsons JT. Focal adhesion kinase: the first ten years. J Cell Sci. 2003;116:1409–16.
37. Ding Q, Gladson CL, Wu H, Hayasaka H, Olman MA. FAK-related non-kinase inhibits myofibroblast differentiation through differential MAPK activation in a FAK-dependent manner. J Biol Chem. 2008;283:26839–49.
38. Horowitz JC, Ajayi IO, Kulasekaran P, Rogers DS, White JB, Townsend SK, et al. Survivin expression induced by endothelin-1 promotes myofibroblast resistance to apoptosis. Int J Biochem Cell Biol. 2012;44:158–69.
39. Bonniaud P, Kolb M, Galt T, Robertson J, Robbins C, Stampfli M, et al. Smad3 null mice develop airspace enlargement and are resistant to TGF-beta-mediated pulmonary fibrosis. J Immunol. 2004;173:2099–108.
40. Cho SJ, Moon JS, Lee CM, Choi AM, Stout-Delgado HW. Glucose transporter 1-dependent glycolysis is increased during aging-related lung fibrosis, and phloretin inhibits lung fibrosis. Am J Respir Cell Mol Biol. 2017;56:521–31.
41. Kottmann RM, Kulkarni AA, Smolnycki KA, Lyda E, Dahanayake T, Salibi R, et al. Lactic acid is elevated in idiopathic pulmonary fibrosis and induces myofibroblast differentiation via pH-dependent activation of transforming growth factor-beta. Am J Respir Crit Care Med. 2012;186:740–51.
42. Raghu G, Chen SY, Yeh WS, Maroni B, Li Q, Lee YC, et al. Idiopathic pulmonary fibrosis in US medicare beneficiaries aged 65 years and older: incidence, prevalence, and survival, 2001–11. Lancet Respir Med. 2014;2:566–72.
43. King TE Jr., Bradford WZ, Castro-Bernardini S, Fagan EA, Glaspole I, Glassberg MK, et al. A phase 3 trial of pirfenidone in patients with idiopathic pulmonary fibrosis. N Engl J Med. 2014; 370:2083–92.
44. Richeldi L, du Bois RM, Raghu G, Azuma A, Brown KK, Costabel U, et al. Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. N Engl J Med. 2014;370:2071–82.
45. O'Dwyer DN, Moore BB. Animal models of pulmonary fibrosis. Methods Mol Biol. 2018;1809:363–78.
46. Liu T, Los Santos FG, Phan SH. The bleomycin model of pulmonary fibrosis. Methods Mol Biol. 2017;1627:27–42.