1521-0103/351/1/87–95$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.114.216044
J Pharmacol Exp Ther 351:87–95, October 2014

Therapeutic Targeting of Src Kinase in Myofibroblast Differentiation and Pulmonary Fibrosis

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Received April 28, 2014; accepted July 18, 2014

ABSTRACT

Myofibroblasts are effector cells in fibrotic disorders that synthesize and remodel the extracellular matrix (ECM). This study investigated the role of the Src kinase pathway in myofibroblast activation in vitro and fibrogenesis in vivo. The profibrotic cytokine, transforming growth factor β1 (TGF-β1), induced rapid activation of Src kinase, which led to myofibroblast differentiation of human lung fibroblasts. The Src kinase inhibitor AZD0530 (saracatinib) blocked TGF-β1–induced Src kinase activation in a dose-dependent manner. Inhibition of Src kinase significantly reduced α-smooth muscle actin (α-SMA) expression, a marker of myofibroblast differentiation, in TGF-β1–treated lung fibroblasts. In addition, the induced expression of collagen and fibronectin and three-dimensional collagen gel contraction were also significantly inhibited in AZD0530-treated fibroblasts. The therapeutic efficiency of Src kinase inhibition in vivo was tested in the bleomycin murine lung fibrosis model. Src kinase activation and collagen accumulation were significantly reduced in the lungs of AZD0530-treated mice when compared with controls. Furthermore, the total fibrotic area and expression of α-SMA and ECM proteins were significantly decreased in lungs of AZD0530-treated mice. These results indicate that Src kinase promotes myofibroblast differentiation and activation of lung fibroblasts. Additionally, these studies provide proof-of-concept for targeting the noncanonical TGF-β signaling pathway involving Src kinase as an effective therapeutic strategy for lung fibrosis.

Introduction

In both normal wound healing and fibrotic lesions, fibroblasts differentiate into "activated" fibroblasts, termed myofibroblasts (Hinz et al., 2007). De novo expression of α-smooth muscle actin (α-SMA), the actin isoform classically expressed in smooth muscle cells, is a hallmark of myofibroblast differentiation (Darby et al., 1990; Hinz et al., 2007). Myofibroblasts possess enhanced contractility and synthetic capacity, producing profibrotic cytokines and extracellular matrix (ECM) proteins (Hinz et al., 2007). These functions of myofibroblasts expedite wound closure during normal wound healing (Gabbiani et al., 1971). Although myofibroblasts facilitate wound healing, they also contribute to the development of fibrotic disorders in multiple organs, such as in the progressive fibrotic lung disease, idiopathic pulmonary fibrosis (IPF) (Hinz et al., 2007; Ding et al., 2011; Kis et al., 2011; Ley et al., 2011). It is important to fully understand the mechanisms involved because therapeutic targeting of key signaling pathways regulating myofibroblast differentiation and activation may reduce the progression of fibrotic disorders, such as IPF.

Expression of α-SMA is a hallmark of myofibroblast differentiation and is important for myofibroblast function. α-SMA enhances contractility and is important to form mature focal adhesions of myofibroblasts (Hinz et al., 2001). The formation of large focal adhesions seems to be a part of myofibroblast maturation. In fully differentiated or mature myofibroblasts, the cytoplasmic filament network is reorganized due to the incorporation of newly synthesized α-SMA, and they connect directly to the focal adhesions (Hinz et al., 2007). The appearance of α-SMA–containing filaments is often used as the second hallmark of myofibroblasts and to confirm the change of phenotype from fibroblasts or other type cells to myofibroblasts (Hinz et al., 2003). Transforming growth factor β1 (TGF-β1) is a potent cytokine that induces myofibroblast differentiation. TGF-β1 is well documented for its profibrotic role and is blamed for fibrotic responses in multiple organs, such as the lung, liver, kidney, and skin (Hales et al., 1994; Tuan and Nichter, 1998; Iwano and Neilson, 2004; Gressner and Weiskirchen, 2006; Romeo et al., 2006; Ding et al., 2011).

This work was supported by the National Institutes of Health National Heart, Lung, and Blood Institute [Grants R01-HL085324 (to Q.D.), R01-HL105473 (to G.L.), and P01-HL114470 (to V.J.T.)]. The authors declare no conflicts of interest.

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dx.doi.org/10.1124/jpet.114.216044.

ABBREVIATIONS: AG1879 (PP2), 4-amino-5-[4-chlorophenyl]-7-[*t*-butyl]pyrazolo-[3,4-D]pyrimidine; α-SMA, α-smooth muscle actin; AZD0530, saracatinib; BIBF-1120, nintedanib; ECM, extracellular matrix; FAK, focal adhesion kinase; IPF, idiopathic pulmonary fibrosis; PARP, poly-ADP-ribose polymerase; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor β1.

Myofibroblast differentiation is a complex process and requires active TGF-β1, the proper ECM, and integrin signaling (Munger et al., 1999; Hagood and Olman, 2007; Hinz et al., 2007; Horowitz et al., 2007; Muro et al., 2008). Integrins are cell surface receptors and are important in myofibroblast differentiation and function (Hinz et al., 2007). The α-SMA–containing filaments connect to focal adhesions, and they likely contract ECM through integrin-mediated signaling. Focal adhesion kinase (FAK) plays a critical role in integrin-mediated signaling (Parsons et al., 2000; Reiske et al., 2000; Ding et al., 2002), and FAK activation is required for TGF-β1–induced α-SMA expression and myofibroblast differentiation (Thannickal et al., 2003; Ding et al., 2008).

Src kinase regulates FAK activation. Src kinase binds to FAK and activates FAK through phosphorylation of the tyrosine 397 (Y397) of FAK (Reiske et al., 2000; Hauck et al., 2002; Siesser et al., 2008). The Src family kinases are a group of nonreceptor tyrosine kinases, and they regulate broad cell functions, including migration, invasion, and growth (Calalb et al., 1995; Mariotti et al., 2001; Ding et al., 2003; Boggon and Eck, 2004; Okutani et al., 2006; Ahluwalia et al., 2010). Src kinase is activated by autophosphorylation of tyrosine residue 418 (Y418) (Calalb et al., 1995; Ahluwalia et al., 2010). The C-terminal domain of Src kinase is often myristoylated or palmitoylated to allow for association with the cell membrane receptors, such as integrins (Ahluwalia et al., 2010; Aleshin and Finn, 2010). This association facilitates the binding of Src kinase to other signaling proteins around focal adhesions or integrins, such as FAK, and activates them (Calalb et al., 1995; Ding et al., 2003; Ahluwalia et al., 2010). Although it is known that integrin-matrix interaction and FAK activation are involved in myofibroblast differentiation, the role of Src kinase in myofibroblast differentiation and lung fibrosis is still underexplored.

Our study investigated the role of the Src kinase pathway in myofibroblast activation in vitro and lung fibrogenesis in vivo. The results show that Src kinase is activated in response to TGF-β1 stimulation. Pharmacologic inhibition of Src kinase blocks TGF-β1–induced myofibroblast differentiation and functions, including contraction and ECM protein expression, in lung fibroblasts. In vivo, bleomycin-induced lung fibrosis is significantly attenuated in mice treated with a Src kinase inhibitor. Suppression of lung fibrosis is associated with significantly decreased Src activation, myofibroblast differentiation, and ECM accumulation, all of which can contribute to the reduced severity of lung fibrosis in mice treated with a Src kinase inhibitor.

Materials and Methods

Reagents. TGF-β1 was obtained from R&D Systems (Minneapolis, MN). The following purified antibodies were purchased: α-SMA (American Research Products, Belmont, MA), Src kinase (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-tyrosine 416 of Src and cleaved poly-ADP-ribose polymerase (PARP) (Cell Signaling Technology, Danvers, MA), FAK (Millipore, Billerica, MA), green fluorescent protein (Santa Cruz Biotechnology), Cy3-conjugated anti–α-SMA antibody (clone 1A4; Sigma-Aldrich, Saint Louis, MO), phosphotyrosine 397 of FAK (pY397-FAK; Biosource, Camarillo, CA), procollagen α1 type 1 (1A1) and fibronectin (Santa Cruz Biotechnology), and anti–glyceraldehyde 3-phosphate dehydrogenase (Research Diagnostics, Flanders, NJ). The Src kinase inhibitor AZD0530 (saracatinib) compound was kindly provided by AstraZeneca UK Limited (London, UK) and was used per the manufacturer's instruction. Chemicals were purchased from Sigma-Aldrich and Fisher Scientific (Waltham, MA).

Cells and Cell Culture. Adult primary normal human lung fibroblasts were purchased from the American Type Culture Collection (Manassas, VA) and Cambrex (Walkersville, MD, now Lonza). Lung fibroblasts from both male and female adults were used. Murine lung fibroblasts were derived from 7- to 10-week-old C57BL/6 mice, as previously described elsewhere (Ding et al., 2008). Procedures and protocols were approved by local institutional animal care and use committee. Fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin as described by us in a previous report (Cai et al., 2010).

Animal Model of Lung Fibrosis. All animal interventions were approved by local institutional animal care and use committee. The bleomycin-induced animal lung fibrosis model was used and described by us in a previous report (Ding et al., 2013). Briefly, the C57Bl6 female mice (8–11 weeks) were anesthetized, and bleomycin (2 U) or saline alone was slowly instilled through the airways into the lungs by using an intratracheal catheter. To study the effect of Src kinase inhibitor on the development of lung fibrosis, bleomycin-challenged mice were treated daily with either AZD0530 (20 mg/kg body weight) or control vehicle (saline) by oral gavage starting from day 7 after bleomycin instillation when lung inflammation slowed and fibrosis started. The lung tissues were harvested at day 21 after bleomycin instillation for lung fibrosis analysis and for histologic and biochemical studies. To collect lung tissue for histochemical studies, the lungs were perfused with cold phosphate-buffered saline, then inflated with 10% formalin, fixed overnight, and embedded in paraffin as described elsewhere (Simmons et al., 1998; Ding et al., 2013).

Analysis of Lung Fibrosis. The severity of lung fibrosis in bleomycin-challenged mice was determined by lung collagen accumulation and morphometric fibrotic area quantification. Lung collagen accumulation or the whole lung collagen level was determined by whole lung hydroxyproline level. Briefly, the harvested lungs were hydrolyzed in 6 M HCl at 110°C for 24 hours, and the amount of hydroxyproline in the lung acid-hydrolysates was determined by colorimetric assays as described in a previous report (Ding et al., 2013). In addition, 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). Lung fibrotic areas were measured on H&E-stained sections by morphometric methodologies (5 μm sections, paraffin-embedded tissues). Manual tracing of lesional and tissue perimeters was performed at a 2$\times$ magnification on a digitized image analysis system (BioQuant NOVA; R&M Biometrics, Nashville, TN). The total fibrotic area was also traced in bleomycin-challenged mice, and fibrotic lesional areas in AZD0530-treated mice were reported as the percentage of fibrotic lesional areas relative to that in vehicle-treated control mice.

Western Blot Analysis. Western blot analysis was performed as described by us in a previous report (Ding et al., 2005). Briefly, cells or lung tissues were detergent-lysed with the following inhibitors: phenylmethanesulfonyl fluoride, aprotinin, leupeptin, and sodium vanadate. The protein concentration of the whole-cell lysate was determined by using a BCA kit (Pierce, Rockford, IL). Equivalent micrograms of whole-cell lysates were electrophoresed on SDS-PAGE, transferred to an Immobilon-P membrane (Millipore), probed with indicated antibodies, and developed with the electrochemiluminescence system (Pharmacia Biotech, Piscataway, NJ). Densitometry analysis of band density was performed as described elsewhere (Cai et al., 2012). The expression of glyceraldehyde 3-phosphate dehydrogenase protein was used as a loading control.

Src Kinase Activity Assay In Vitro and In Vivo. Src kinase activity in cultured lung fibroblasts or lung tissues was measured using a commercially available kinase activity assay kit (ADP-Glo Kinase Assay kit; Promega, Madison, WI). Cultured fibroblasts or tissues were lysed, immunoprecipitated with anti–Src kinase antibody, and the

samples were processed using ADP-Glo and Kinase detection kit reagents followed by the kinase activity assays according to the manufacturer's instructions. Luminescence was measured and quantified by using a plate reader (BioTek Instruments, Winooski, VT). Data in samples were presented as the percentage of relative luminescence unit relative to that of lung fibroblasts cultured in serum-free medium only (as 100% baseline) or to that of lung tissues from only vehicle-treated mice (as 100% baseline).

Immunofluorescence Analysis. Immunofluorescence analysis was performed as described elsewhere (Ding et al., 2002). Briefly, cells cultured on glass-coverslips were fixed in 4% buffered paraformaldehyde, and permeabilized. To study the α-SMA–incorporated cytoplasmic filaments, we reacted cells with Cy3-conjugated anti–α-SMA monoclonal antibody, followed by counterstaining by blue fluorescent DNA dye (Hoechst, nuclei dye). To determine the effect of AZD0530 (Src kinase inhibitor) on myofibroblast differentiation, we quantified the percentage of cells containing α-SMA–incorporated stress fibers over total cells (according to Hoechst staining).

Collagen-Gel Contraction Assay. Collagen-gel contraction assays were performed as described previously elsewhere (Cai et al., 2012). Briefly, collagen gels were cast in six-well plates using type I collagen/Dulbecco's modified Eagle's medium solution composed of bovine skin collagen type I (Invitrogen, Palo Alto, CA). Lung fibroblasts were seeded into the collagen gel (100,000 cells/well) and incubated at 37°C, at a CO_2 level of 5% for the indicated time. Fibroblasts-gel complex contraction was monitored by standardized photography at time 0 hours and at indicated time points. The area of fibroblast-gel complex in digitized photographs was measured, and the ratio of collagen gel area against the culture well area was calculated. The data were pooled and were presented as the percentage of contraction relative to the vehicle-treated group.

Cell Migration Assays. The two-dimensional wound closure monolayer/scratch motility assay was performed as described elsewhere (Cai et al., 2010). Briefly, fibroblasts were plated into 24-well format tissue culture plates and scratched. Digital pictures were taken immediately and again at the end of the assay. The wound area covered by cell migration after scratching was equal to the difference between the two areas above and normalized. Conditions were assayed in replicas of three or four, repeated two to three times, and the data were analyzed and presented as the mean ± S.E.

Statistical Analysis. Data were analyzed using Student's t test analysis for differences between two groups, and expressed as mean ± S.E. All experiments were repeated two to three times with duplicates. $P < 0.05$ was considered statistically significant.

Results

TGF-β1 Induces Src Kinase Activation in Human Lung Fibroblasts; AZD0530 Inhibits TGF-β1–Induced Src Activation and Kinase Activity in a Dose-Dependent Manner. TGF-β1 is a potent profibrotic cytokine that induces myofibroblast differentiation. To understand the role of Src kinase in myofibroblast differentiation, we first examined Src kinase activation in response to TGF-β1 stimulation in serum-starved human lung fibroblasts as well as the time course of Src kinase activation (Fig. 1A). TGF-β1 treatment (10 ng/ml) induced Src activation (determined by phosphorylation of tyrosine 416 of Src, pY416-Src) in lung fibroblasts in a time-dependent manner (Fig. 1A). In response to TGF-β1, Src phosphorylation was increased as early as 30 minutes (Fig. 1A). A biphasic response of Src activation was observed. After TGF-β1 stimulation, Src activation peaked at 1 hour, decreased, then peaked again at 24 hours with a downward trend (Fig. 1A).

It has been well demonstrated that AZD0530 effectively inhibits Src kinases in cancer cells (Chang et al., 2008; Morrow et al., 2010; Laurie et al., 2014). To determine the optimal dose required to inhibit Src kinase activation induced by TGF-β1, the dose effect of AZD0530 on Src activation was examined in human lung fibroblasts (Fig. 1B). Serum-starved lung fibroblasts were treated with AZD0530 or control vehicle at the indicated dose, stimulated with TGF-β1 (10 ng/ml, 1 hour), and then lysed and analyzed via Western blot for Src activation (pY416-Src). AZD0530 inhibited TGF-β1–induced Src activation in a dose-dependent manner, with the optimal inhibition of Src activation at a concentration of 0.1 μM in lung fibroblasts (Fig. 1B). Src kinase activity was also determined to confirm the inhibitory effect of AZD0530 on Src kinase. TGF-β1 stimulation (10 ng/ml) resulted in an approximate 3.3-fold increase in the Src kinase activity in lung fibroblasts (Fig. 1C, bar 2 versus bar 1, 331% ± 10.8% versus 100% ± 4.6%, $P < 0.01$). AZD0530 efficiently inhibited the TGF-β1–stimulated Src kinase activity in lung fibroblasts treated with the optimized dose (0.1 μM) (Fig. 1C, bar 4 versus bar 2, 63.8% ± 4.3% versus 331% ± 10.8%, $P < 0.01$). These data show that TGF-β1 induced Src kinase activation in lung fibroblasts and that AZD0530 treatment can efficiently inhibit TGF-β1–induced Src kinase activation and kinase activity in lung fibroblasts.

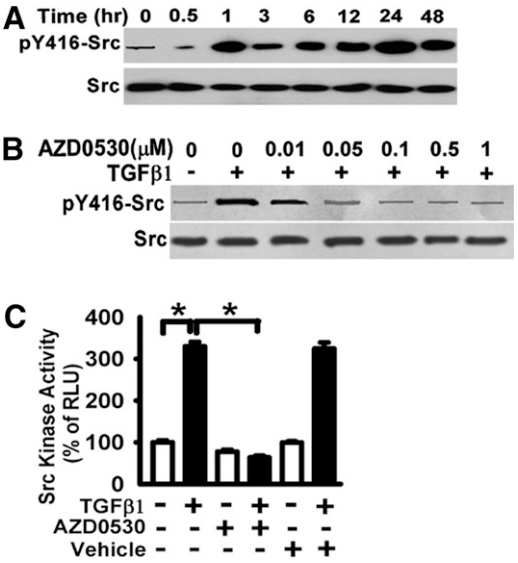

Fig. 1. TGF-β1 induces Src activation, and AZD0530 inhibits TGF-β1–induced Src activation in a dose-dependent manner in human lung fibroblasts. (A) Serum-starved human lung fibroblasts were treated with TGF-β1 (10 ng/ml) in serum-free medium for the indicated time and were detergent lysed; whole-cell lysates were Western blotted with the indicated antibodies. (B) These lung fibroblasts were treated with the AZD0530 inhibitor at the indicated dose or with control vehicle, then treated with TGF-β1 (10 ng/ml) for 1 hour. Cells were detergent-lysed, and the lysates were analyzed by Western blot with the indicated antibodies. Src activation was determined by phosphorylation of tyrosine 416 of Src (pY416-Src). (C) Src kinase activities in these lung fibroblasts were examined by a luminescent kinase assay kit, and the data are presented as the percentage of relative luminescence to those fibroblasts cultured in serum-free medium (as 100%). The optimized AZD0530 dose (0.1 μM) was used in all tests. The data are presented as mean + S.E. *$P < 0.01$. RLU, relative light units.

Src Kinase Activation Is Required for TGF-β1–Induced α-SMA Expression and Formation of α-SMA–Containing Cytoplasmic Filaments. De novo expression of α-SMA expression is one hallmark of myofibroblast differentiation (Hinz et al., 2007). To determine the role of Src kinase in

myofibroblast differentiation, serum-starved human lung fibroblasts were treated with or without AZD0530 (0.1 μm) followed by TGF-β1 (10 ng/ml) for 48 hours. Minimal basal level of α-SMA expression was detected in lung fibroblasts without TGF-β1 treatment (Fig. 2A, lane 1). The α-SMA expression was significantly increased in lung fibroblasts in response to TGF-β1 (Fig. 2A, lane 3 versus lane 1), indicating a phenotype transition from fibroblast to myofibroblast. The TGF-β1–increased α-SMA expression was significantly inhibited by AZD0530 treatment (Fig. 2A, lane 4 versus lane 3). The data demonstrate that inhibition of Src kinase blocked TGF-β1–induced α-SMA expression, supporting a role of Src kinase in myofibroblast differentiation.

To further confirm the role of Src kinase in myofibroblast differentiation, the effect of AZD0530 on the formation of α-SMA–containing filaments induced by TGF-β1 was examined in human lung fibroblasts. The α-SMA–containing filaments are another hallmark of myofibroblast differentiation, particularly in fully differentiated or matured myofibroblasts (Hinz et al., 2007). As expected, TGF-β1 induced newly formed α-SMA–containing filaments (Fig. 2B, top right panel versus top left panel), and the percentage of cells with

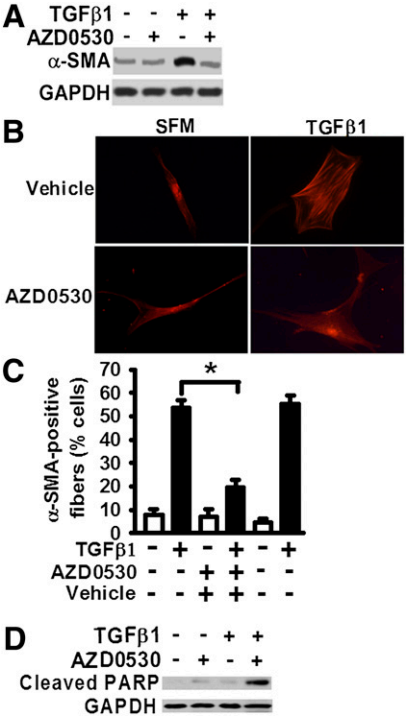

Fig. 2. AZD0530 inhibits TGF-β1–induced α-SMA expression and formation of α-SMA–containing fibers in human lung fibroblasts. (A) Serum-starved human lung fibroblasts were treated with or without AZD0530 (0.1 μM) followed by TGF-β1 (10 ng/ml) for 48 hours. Fibroblasts were lysed and Western blotted with the indicated antibodies. (B) These fibroblasts were fixed and immunofluorescently stained with the Cy-3–labeled monoclonal antibody toward α-SMA. Fluorescent microscopic digital images were taken (original 200×), and representative pictures are shown. (C) Quantification of the percentage of cells with highly organized, thickened α-SMA–containing fibers as described in *Materials and Methods*. The data are presented as mean + S.E. $^*P < 0.01$. (D) These fibroblasts were lysed, and an equivalent amount of whole cell lysates were Western blotted with the indicated antibodies. The antibody specifically recognizes the cleaved PARP and does not cross with the full-length form. The experiments were repeated three times, and representative pictures are shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

α-SMA–containing filaments was significantly increased (Fig. 2C, bar 2 versus bar 1, 53.8% ± 3.2% versus 7.7% ± 3.5%, $P < 0.01$). Inhibition of Src kinase by AZD0530 significantly impaired the formation of α-SMA–containing filaments in TGF-β1–treated fibroblasts (Fig. 2B, bottom right panel versus top right panel), and the percentage of cells with α-SMA–containing filaments was significantly decreased (Fig. 2C, bar 4 versus bar 2, 19.5% ± 3.5% versus 53.8% ± 3.2%, $P < 0.01$). AZD0530 induced significant cleavage of PARP in fibroblasts treated with both TGF-β1 and AZD0530 (Fig. 2D). There is minimal increase of cleaved PARP in fibroblasts treated with only AZD0530 (or only TGF-β1) when compared with vehicle-treated fibroblasts (Fig. 2D). These data support the theory that Src kinase plays an essential role in myofibroblast differentiation and maturation. These data also suggest that Src kinase is involved in prosurvival signaling in myofibroblasts.

Src Kinase Activation Is Required for Myofibroblast Function and Fibroblast Migration. When compared with undifferentiated fibroblasts, one prominent function of myofibroblast is the enhanced ability to contract ECM (Hinz et al., 2007). To examine the potential role of Src kianse in myofibroblast function, TGF-β1–induced collagen-gel contraction was examined in human lung fibroblasts. Lung fibroblasts were treated with or without AZD0530 (0.1 μm) followed by TGF-β1 (10 ng/ml) and subjected to three-dimensional collagen-gel contraction assays. The data show that TGF-β1 significantly induced collagen-gel contraction in lung fibroblasts (Fig. 3A, top right panel versus top left panel). The collagen gels were significantly contracted down to about 29% of the original gel area (equivalent to 71% reduction of gel size) in response to TGF-β1 (Fig. 3B, bar 2 versus bar 1, 58.6% ± 5.4% versus 100% ± 3.1%, $P < 0.01$). In contrast, inhibition of Src kinase activation by AZD0530 significantly impaired the ability of fibroblasts to contract collagen-gels in response to TGF-β1 (Fig. 3A, bottom right panel versus top right panel). These AZD0530-treated fibroblasts only slightly contracted collagen-gels in response to TGF-β1 (down to about 91% of the original gel area, equivalent to only 9% reduction of gel size) (Fig. 3B, bar 4 versus bar 2, 89.4% ± 3.4% versus 58.6% ± 5.4%, $P < 0.01$). These data suggest that activation of Src kinase is required for myofibroblast contractility.

Fibroblast migration is a response to tissue injury, and increased fibroblast motility is considered profibrotic. Fibroblasts derived from human IPF lungs have increased cell migration and invasion (White et al., 2003; Tager et al., 2004; Cai et al., 2010; Li et al., 2011). The role of Src kinases in cell migration is well documented. To determine the effect of AZD0530 on fibroblast migration, we used the two-dimensional monolayer wound closure assay system. Human lung fibroblasts were treated with or without AZD0530 and stimulated with platelet-derived growth factor (PDGF), and subjected to monolayer wound closure assays for 24 hours. The findings demonstrate that inhibition of the Src kinase pathway reduces fibroblast migration induced by the profibrotic factor PDGF-BB (Fig. 3C).

Inhibition of Src Kinase Reduces ECM Protein Production and Decreases FAK Activation in Response to TGF-β1 in Lung Fibroblasts. During TGF-β1–induced myofibroblast differentiation, the production of ECM proteins is greatly increased. To determine whether Src kinase plays a role in ECM protein production, we treated human lung fibroblasts with or without AZD0530 (0.1 μm) followed by

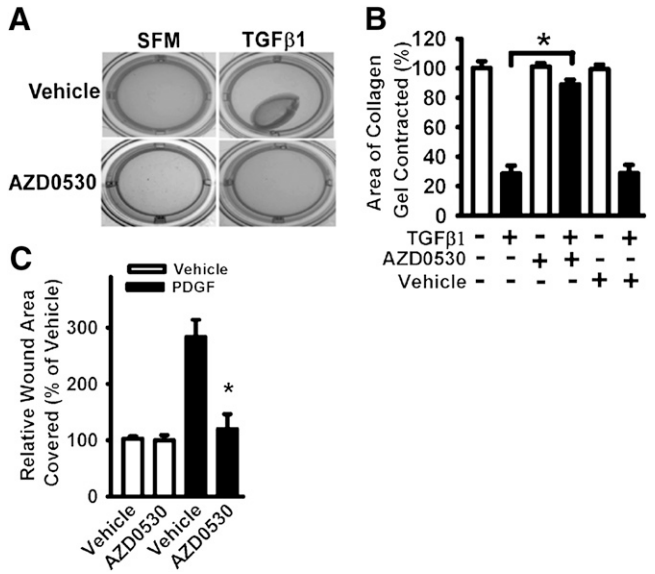

Fig. 3. AZD0530 inhibits TGF-β1–induced three-dimensional collagen gel contraction and PDGF-induced migration in human lung fibroblasts. (A) Serum-starved human lung fibroblasts were treated with or without AZD0530 (0.1 μM) followed by TGF-β1 (10 ng/ml) treatment, and a collagen-gel contraction assay at 37°C, 5% CO_2, for 60 hours. Representative digital images are shown. (B) Data are pooled from three individual experiments (each performed at least in duplicate) and are presented as the percentage of contracted collagen gel area relative to the area of culture wells (mean ± S.E.). The lower percentage represents a stronger gel contraction. (C) Serum-starved human lung fibroblasts were wounded, and treated with or without AZD0530 (0.1 μM) or vehicle followed by PDGF-BB (4 ng/ml) in serum-free medium (SFM) with 1% BSA for 24 hours. Monolayer wound closure assays were performed as described in *Materials and Methods*. Data obtained are pooled (n = 4 per group) and plotted as the percentage of wound area covered over 24 hours relative to the control (fibroblasts in vehicle-treated only). All data are presented as mean + S.E. *$P < 0.01$.

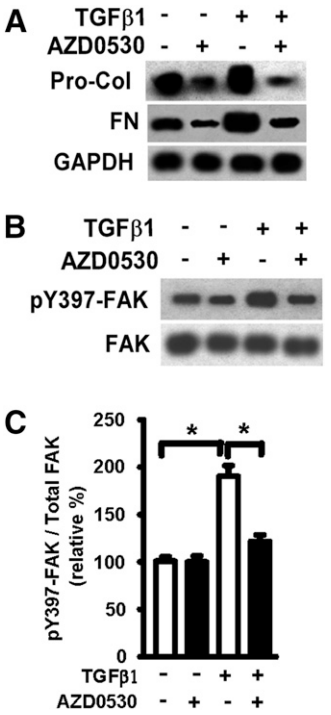

Fig. 4. AZD0530 inhibits TGF-β1–induced procollagen and fibronectin expression, and reduces TGF-β1–induced FAK activation in human lung fibroblasts. Serum-starved human lung fibroblasts were treated with or without AZD0530 (0.1 μM) followed by TGF-β1 (10 ng/ml) for 24 hours. Fibroblasts were lysed and analyzed by Western blot with the indicated antibodies. (A) Expression of collagen and fibronectin (FN). Expression of collagen was examined through expression of procollagen 1A1 (Pro-Col). Representative images are shown. (B) FAK activation was examined through phosphorylation of the tyrosine 397 (pY397) of FAK. Representative images are shown. (C) Densitometry analysis of band intensity for pY397 of FAK. Results are normalized to the total FAK protein level, and basal pY397-FAK was used as 100%. Results are pooled from three individual experiments. *$P <$ 0.01. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

TGF-β1 (10 ng/ml), and then subjected the lung fibroblasts to examination of collagen and fibronectin expression. TGF-β1 treatment greatly enhances collagen (by procollagen 1A1) and fibronectin expression when compared with that seen in vehicle-treated lung fibroblasts (Fig. 4A, lane 3 versus lane 1). The increased expression of collagen and fibronectin induced by TGF-β1 was significantly blocked by AZD0530 treatment (Fig. 4B, lane 4 versus lane 3). The findings indicate that Src kinasae is important for TGF-β1–induced ECM protein expression in lung fibroblasts.

We have previously shown that TGF-β1 induces FAK activation (Thannickal et al., 2003; Ding et al., 2008). FAK activation is required for myofibroblast differentiation as inhibition of FAK blocks the α-SMA expression and formation of α-SMA–containing filaments in fibroblasts (Thannickal et al., 2003; Ding et al., 2008). To understand whether Src kinase regulates the myofibroblast differentiation through a FAK-dependent or FAK-independent pathway, the effect of inhibition of Src kinase (by AZD00530) on TGF-β1–induced FAK activation was examined. TGF-β1 induced significant FAK activation (determined by phosphorylation of the tyrosine 397 of FAK, pY397-FAK) in fibroblasts (193% ± 19%, $P < 0.01$) when compared with basal FAK activation in vehicle-treated fibroblasts (Fig. 4, B and C). Inhibition of Src kinase greatly reduced TGF-β1–induced FAK activation in fibroblasts (Fig. 4, B and C), suggesting that Src kinase likely regulates myofibroblast differentiation through a FAK-dependent pathway.

Inhibition of Src Kinase In Vivo Attenuates the Development of Lung Fibrosis Induced by Bleomycin. The above data support the hypothesis that Src kinase plays an essential role in myofibroblast differentiation and functions. As myofibroblast is one of the major cell types contributing to the accumulation of ECM protein in fibrotic lesions, inhibition of Src kinase by AZD0530 may affect the fibrotic response in vivo. To understand the role of Src kinase in lung fibrosis in vivo, we used the established bleomycin-induced lung fibrosis model. The fibrotic remodeling in lungs is generally more obvious 7 days after bleomycin injection. The bleomycin-injected mice were treated daily with AZD0530 (20 mg/kg body weight) or control vehicle (both by oral gavage) starting at day 7 after bleomycin treatment. The results demonstrate that inhibition of Src kinase significantly attenuates the development of lung fibrosis in mice (Fig. 5). In contrast to the significant lung fibrosis at day 21 after bleomycin injection in vehicle-treated mice, lung fibrosis in AZD0530-treated mice was significantly decreased (Fig. 5). Compared with saline-injected mice, fibrotic lesions (Fig. 5A, top left versus bottom left) and lung collagen accumulation (by hydroxyproline level; Fig. 5D, bar 3 versus bar 1) was significantly increased in bleomycin-injected mice. In contrast, these bleomycin-induced fibrotic responses were significantly reduced in AZD0530-treated mice. The fibrotic lesions (Fig. 5A, bottom

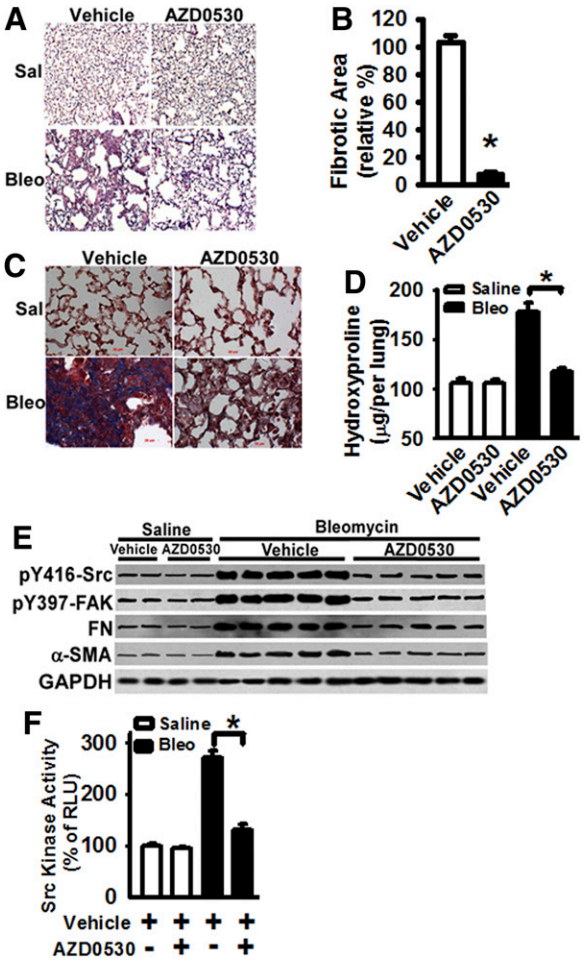

Fig. 5. AZD0530 is protective against lung fibrosis. (A) Mice were intratracheally instilled with bleomycin (Bleo) or saline (Sal) control, and then were treated daily with AZD0530 compound (20 mg/kg body weight) or vehicle by oral gavage starting at day 7 after bleomycin treatment. Lung tissues were harvested at day 21 after bleomycin or saline treatment, sectioned, and H&E-stained. Representative lung sections are shown (magnification, 200×). (B) The lung fibrotic/lesion areas of bleomycin-challenged mice were examined morphometrically and reported by relative fibrotic areas (% of fibrotic area of mice treated with vehicle only). (C) Lung tissue sections were stained by a Masson's trichrome staining kit to demonstrate the areas of collagen deposition (magnification, 400×). (D) Total lung collagen accumulation was determined by hydroxyproline assays. (E) Lungs were harvested at day 14 after bleomycin instillation. Whole-lung lysates were Western blotted for Src activation (pY416-Src), FAK activation (pY397-FAK), and expression of fibronectin (FN) and α-SMA. (F) Lungs were harvested at day 14 after bleomycin instillation, and Src kinase activities were examined (as in Fig. 1). These data are pooled and presented as mean + S.E. (n = 6–8 mice per group). *$P < 0.01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RLU, relative light units.

right versus bottom left, and Fig. 5B) and total lung collagen accumulation (Fig. 5D, bar 4 versus bar 3, 118.6 ± 4.3 μg versus 178.7 ± 8.9 μg per lung, $P < 0.01$) were significantly reduced in AZD0530-treated mice. Masson's trichrome staining also confirmed that collagen-stained areas were significantly reduced in AZD0530-treated mice when compared with vehicle-treated mice in response to bleomycin (Fig. 5C, bottom right versus bottom left).

AZD0530 treatment significantly inhibited Src kinase activation and kinase activity in vivo (Fig. 5, E and F). Bleomycin increased Src kinase activation and kinase activity in vivo when compared with saline-treated control mice (Fig. 5, E and F). AZD0530 effectively blocked bleomycin-induced Src kinase activation (pY416-Src) (Fig. 5E) and kinase activity (Fig. 5F, bar 4 versus bar 3, 132% ± 11.7% versus 273% ± 11.6%, $P < 0.01$). AZD0530-treated mice also had significantly decreased α-SMA expression in lungs when compared with that in vehicle-treated mice in response to bleomycin (Fig. 5E), supporting the idea that inhibition of Src kinase decreased the myofibroblast differentiation in vivo. Bleomycin increased fibronectin expression, and bleomycin-induced fibronectin expression was significantly attenuated in AZD0530-treated mice (Fig. 5E). These results indicate Src kinase inhibition blocks ECM protein expression induced by bleomycin in mice. Furthermore, the downstream FAK signaling was also decreased. Bleomcyin increased FAK activation, and bleomycin-induced FAK activation was inhibited in AZD0530-treated mice (Fig. 5E). Together, these data demonstrate that Src kinase plays an important role in lung fibrosis, and that inhibition of Src kinase has antifibrotic effects in mice.

Discussion

Our results demonstrate that the Src kinase regulates myofibroblast differentiation and activation. Inhibition of Src kinase activity by the pharmacologic inhibitor AZD0530 effectively blocked the expression of α-SMA, reduced the production of collagen and fibronectin, and inhibited the three-dimensional collagen-gel contraction in response to TGF-β1. These in vitro experiments demonstrate that the Src kinase pathway is essential for myofibroblast activation. Furthermore, we show that the Src kinase pathway mediates fibrogenesis in vivo. Blockade of Src kinase by AZD0530 significantly reduced the severity of bleomycin-induced lung fibrosis in mice. Inhibition of Src signaling is associated with significantly reduced collagen production and myofibroblast differentiation in lungs of bleomycin-injured mice. Taken together, these results support that an essential role of Src kinase in myofibroblast differentiation and activation and provides proof-of-concept that targeting Src kinase may provide an effective approach to the treatment of lung fibrosis.

IPF is a fibrotic lung disease (Ley et al., 2011). A central role for myofibroblasts in fibrotic responses in the lungs, liver, kidney, and skin is well documented (Tuan and Nichter, 1998; Iwano and Neilson, 2004; Gressner and Weiskirchen, 2006; Romeo et al., 2006; Kis et al., 2011). Previous studies have implicated Src family kinases in fibrotic reactions. Thy-1–negative fibroblasts are located in fibrotic areas and contribute to lung fibrosis (Hagood et al., 2005). Tumor necrosis factor-α activates Src kinase in Thy-1–negative fibroblasts, and blockade of Src activation abrogates the tumor necrosis factor-α–activated profibrotic gene expression in these Thy-1–negative fibroblasts (Shan et al., 2010). Activation of Hck is associated with spontaneous inflammation and lung fibrosis in mice (Ernst et al., 2002). Excessive deposition of ECM is found in the alveolar septa of transgenic mice expressing the constitutive active form of Hck, a member of the Src family kinases (Ernst et al., 2002). Our findings indicate that the Src kinase pathway is directly involved in myofibroblast differentiation and functions in lung fibroblasts. This is supported by the fact that inhibition of Src kinase effectively blocked α-SMA expression and the

formation of α-SMA–containing fibers, both being hallmarks of myofibroblast differentiation. TGF-β1 greatly increased ECM production and induced collagen-gel contraction in lung fibroblasts. These profibrotic effects of TGF-β1 were inhibited by AZD0530 in human lung fibroblasts. Our results are consistent with previous findings that inhibition of Src kinase prevents collagen synthesis in cardiac and skin fibroblasts (Elkareh et al., 2007; Skhirtladze et al., 2008). α-SMA is critical for myofibroblast contractility (Hinz et al., 2007); therefore, reduced α-SMA expression leads to the reduced contractility in lung fibroblasts treated with AZD030.

Src kinase likely regulates myofibroblast differentiation and function though FAK. FAK is involved in the signaling cascade initiated by integrin receptors and ECM proteins (Parsons et al., 2000; Reiske et al., 2000; Ding et al., 2002). FAK has been identified as a key signaling protein in myofibroblast differentiation, as FAK activation is required for TGF-β1–induced myofibroblast differentiation (Thannickal et al., 2003; Ding et al., 2008; Grove et al., 2014). FAK promotes the formation of α-SMA–containing fibers through neuronal Wiskott-Aldrich syndrome protein (Cai et al., 2012). FAK inhibition also reduced bleomycin-induced lung fibrosis in mice (Lagares et al., 2012; Ding et al., 2013). Cross-talk between Src kinase and FAK is necessary to sustain FAK activity (Calalb et al., 1995; Cary et al., 1996). A biphasic response of TGF-β1–induced Src activation was observed (Fig. 1). We believe the early peak is a receptor-mediated event that closely precedes the activation of FAK, which is also activated in response to TGF-β1 (Ding et al., 2008). We speculate that the delayed activation of Src may be mediated by autocrine secretion of growth factors that have been shown to activate the phosphatidylinositide 3-kinase–Akt pathway in a delayed manner after TGF-β stimulation (Horowitz et al., 2004). Our data demonstrate that TGF-β1 induces FAK activation, and blockade of Src kinase inhibits FAK activation induced by TGF-β1 (Fig. 4). These results support that Src kinase is required for TGF-β1–induced FAK activation. We speculate that Src kinase regulates myofibroblast differentiation at least in part through a FAK-dependent pathway. AZD0530 treatment also induces significant cleavage of PARP in fibroblasts treated with both TGF-β1 and AZD0530 (Fig. 2D). There is only a minimal increase of cleaved PARP in fibroblasts treated with only AZD0530 when compared with vehicle-treated fibroblasts (Fig. 2D). These data suggest a role of Src kinase in prosurvival signaling in myofibroblasts.

The effect of Src inhibition in vivo on lung fibrosis was studied by using the established bleomycin murine lung fibrosis model. Bleomycin treatment induces lung fibrosis in rodents, and the resulting fibrosis shares many key features of human lung fibrosis but does not replicate human IPF (Adamson and Dowden, 1974; Snider et al., 1978; Phan et al., 1980; Moore and Hogaboam, 2008; Hecker et al., 2014). This murine model is dependent on an acute inflammatory response to injury, and it is not a model of progressive fibrosis. Previous studies have shown that neutrophil influx peaks at day 3 after injury (Izbicki et al., 2002), and the acute inflammatory response largely subsides by day 7 (Izbicki et al., 2002; Moore and Hogaboam, 2008; Mouratis and Aidinis, 2011). In this study, we treated animals starting at day 7 to test the efficacy of AZD0530 on lung fibrosis and to limit the potential effects of the drug on inflammation. Future studies will evaluate the role of AZD0530 in persistent or progressive models of lung fibrosis, for example, as recently described by our group in aged mice (Hecker et al., 2014).

Our results support that inhibition of the Src kinase pathway results in decreased myofibroblast differentiation and ECM expression, and attenuated lung fibrosis in vivo. Other mechanisms are likely mediated by the Src kinase pathway in vivo, but they are not specifically explored in our current study. Src kinase is well known for its function in cell migration. Therefore, Src kinase likely promotes lung fibrosis through modulation of cell migration and invasion. Src kinase may be involved in movement of multiple cell types during lung injury and repair. Our studies demonstrate that the Src kinase pathway is required for fibroblast migration induced by PDGF-BB. Fibroblasts derived from human IPF lungs have increased cell migration and invasion (White et al., 2003; Tager et al., 2004; Li et al., 2011), and increased IPF migration is associated with increased FAK activation (Cai et al., 2010). This study shows that Src kinase inhibition leads to decreased FAK activation, and that could contribute to reduced fibroblast migration induced by PDGF-BB (Fig. 3). Src kinase also mediates epithelial-to-mesenchymal transition (Tanjore et al., 2011; Zhong et al., 2011). The epithelial-to-mesenchymal transition is considered to be one potential source of myofibroblast accumulation in fibrotic lungs (Hinz et al., 2007; Tanjore et al., 2011; Zhong et al., 2011). It is likely that blockade of the Src kinase pathway has some beneficial effects on epithelial cells and epithelium integrity, and that may contribute to the reduced lung fibrosis in AZD0530-treated mice.

There are nine identified Src kinase family members so far. Our current study was not designed to specifically address the individual roles of each Src kinase member, as the goal of this study is to evaluate the therapeutic effect of AZD0530 on lung fibrosis (by inhibition of all Src kinase members). Based on our previous studies, Src and Fyn are dominant members of Src family kinase family in lung fibroblasts (Ding et al., 2003). Src kinase members may have overlapping functions, and it is currently not known which specific Src kinase mediates myofibroblast differentiation.

Recent evidence has reignited interest in protein tyrosine kinase inhibitors for the treatment of IPF. The tyrosine kinase inhibitor BIBF-1120 (nintedanib) has been tested in IPF clinical trials (Richeldi et al., 2011). BIBF-1120 targets the PDGF receptor, vascular endothelial growth factor receptor, and fibroblast growth factor receptor. BIBF 1120 treatment is associated with a trend toward a reduction in the decline in lung function with fewer acute exacerbations in a phase II clinical trial (Richeldi et al., 2011). Previous studies have shown that the protein kinase inhibitor AG1879 ([PP2], 4-amino-5-[4-chlorophenyl]-7-[*t*-butyl]pyrazolo-[3,4-D]pyrimidine) reduces in vivo fibrogenesis (Vittal et al., 2005). AG1879 targets the Src and FAK kinases and inhibits the Akt-mediated prosurvival pathway in fibroblasts/myofibroblasts (Vittal et al., 2005). Interestingly, another protein kinase inhibitor imatinib mesylate (Gleevec; Novartis, East Hanover, NJ), which targets c-Abl tyrosine kinase and the PDGF receptor, failed to significantly alter fibrogenic responses (Vittal et al., 2007). One advantage of AZD0530 over AG1879 is that it is has been proven to be safe in humans and is currently being evaluated in several clinical trials (www.clinicaltrials.gov). Based on our studies reported here, repurposing this protein kinase inhibitor for fibrotic diseases, such as IPF, should be considered.

Taken together, our current study demonstrates that the Src kinase pathway plays an essential role in myofibroblast differentiation and activation. Furthermore, delayed administration of AZD0530 in the late reparative phase of lung injury protects against fibrosis in mice, providing proof-of-concept that targeting the noncanonical TGF-β signaling pathway involving Src kinase(s) may serve as an effective therapeutic strategy for lung fibrosis.

Acknowledgments

The authors thank Anni Zheng and Haotian Fang for expert technical assistance.

Authorship Contributions

Participated in research design: Hu, Che, Cai, Han, Ding.

Conducted experiments: Hu, Che, Siegal, Cai.

Contributed new reagents or analytic tools: Siegal, Han, O'Reilly.

Performed data analysis: Hu, Che, Cai, Ding.

Wrote or contributed to the writing of the manuscript: Hu, Che, Han, G. Liu, Antony, Luckhardt, Zhou, R. Liu, Desai, O'Reilly, Siegal, Thannickal, Ding.

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