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Therapeutic Targeting of Src Kinase in Myofibroblast Differentiation and Pulmonary Fibrosis

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d) Abbreviations: ECM, extracellular matrix; TGF-β1, transforming growth factor beta-1; α-SMA, alpha-SMA; IPF, idiopathic pulmonary fibrosis; FAK, Focal adhesion kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, green fluorescent protein; HE, Hematoxylin and Eosin; PDGF platelet-derived growth factor; EMT, epithelial-to-mesenchymal transition; N-WASP, neuronal Wiskott-Aldrich syndrome protein.

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Abstract

Myofibroblasts are effector cells in fibrotic disorders by synthesizing and remodeling the extracellular matrix (ECM). This study investigated the role of the Src kinase pathway in myofibroblast activation *in vitro* and fibrogenesis *in vivo*. The pro-fibrotic cytokine, transforming growth factor beta-1 (TGF-β1), induced rapid activation of Src kinase that leads 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 alpha-smooth muscle actin (α-SMA) expression, a marker of myofibroblast differentiation, in TGF-β1-treated lung fibroblasts. In addition, induced expression of collagen and fibronectin, and 3D-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 to controls. Furthermore, 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 non-canonical 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 pro-fibrotic cytokines and ECM protiens (Hinz et al., 2007). These functions of myofibroblasts expedite wound closure during normal wound healing (Gabbiani et al., 1971). While 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; Ley et al., 2011; Kis et al., 2011; Ding et al., 2011). It is important to fully understand the mechanisms involved, as 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

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from fibroblasts or other type cells to myofibroblasts (Hinz et al., 2003). Transforming growth factor beta-1 (TGF-β1) is a potent cytokine that induces myofibroblast differentiation. TGF-β1 is well documented for its pro-fibrotic role and is blamed for fibrotic responses in multiple organs, such as lung, liver, kidney, and skin (Ding et al., 2011; Gressner and Weiskirchen, 2006; Iwano and Neilson, 2004; Romeo et al., 2006; Tuan and Nichter, 1998; Hales et al., 1994).

Myofibroblast differentiation is a complex process and requires active TGF-β1, the proper ECM, and integrin signaling (Munger et al., 1999; Hinz et al., 2007; Muro et al., 2008; Horowitz et al., 2007; Hagood and Olman, 2007; Munger et al., 1999). Integrins are cell surface receptors and important in myofibroblast differentiation and functions (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 (Reiske et al., 2000; Parsons et al., 2000; Ding et al., 2002), and FAK activation is required for TGF-β1-induced α-SMA expression and myofibroblast differentiation (Ding et al., 2008; Thannickal et al., 2003).

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; Siesser et al., 2008; Hauck et al., 2002). The Src family kinases are a group of non-receptor tyrosine kinases, and regulate broad cell functions, including migration, invasion, and growth (Ahluwalia et al., 2010; Boggon and Eck, 2004; Okutani et al., 2006; Calalb et al., 1995; Mariotti et al., 2001; Ding et al., 2003). Src kinase is activated by autophosphorylation of tyrosine residue 418 (Y418) (Ahluwalia, de Groot et al., 2010; Calalb et al., 1995). 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, de Groot 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 activate them (Calalb et al., 1995; Ding et al., 2003; Ahluwalia, de Groot 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. Pharmacological 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. Transforming growth factor-β1 (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, Danvers, MA), FAK (Milipore, Billerica, MA), green fluorescent protein (GFP) (Santa Cruz, CA). Cy3-conjugated anti-α-SMA antibody (clone 1A4, Sigma-Aldrich, Saint Louis, MO), phospho-tyrosine 397 of FAK (pY397-FAK) (Biosource, Camarillo, CA), procollagen alpha 1 type 1 (1A1) and fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Research Diagnostics, Flanders, NJ). Src kinase inhibitor AZD0530 (saracatinib) compound was kindly provided by AstraZeneca UK Limited and used per manufacturer's instruction. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Waltham, MA).

Cells and Cell Culture. Adult primary normal human lung fibroblasts were purchased from American Type Culture Collection (Manassas, VA) and the Cambrex (Walkersville, Maryland, now Lonza). Lung fibroblasts from both male and female adults were used. Murine lung fibroblasts were derived from 7-10 week-old C57BL/6 mice as described previously (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 (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin as described by us previously (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 previously (Ding et al., 2013). Briefly, the C57Bl6 female mice (8-11 weeks) were anesthetized, and bleomycin (2U) 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 (Saracatinib) (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 histological 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 previously described (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 above lung acid-hydrolysates was determined by colorimetric assays as previously performed (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 Hematoxylin and Eosin (HE) stained sections by morphometric methodologies (5 μm sections, paraffin embedded tissues). Manual tracing of lesional and tissue perimeters were performed at a 2X magnification on a digitized image analysis system (BioQuant NOVA, R&M Biometrics, Inc.). 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 Blotting was performed as described by us previously (Ding et al., 2005). Briefly, cells or lung tissues were detergent lysed containing the following inhibitors (PMSF, Aprotinin, Leupeptin, and Sodium Vanadate). Protein concentration of 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 a Immobilon-P membrane (Millipore Corp., Bedford, MA), probed with indicated antibodies, and developed with the ECL system (Pharmacia Biotech, Piscataway, NJ). Densitometry analysis of band density was described previously (Cai et al., 2012). The expression of GAPDH 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). Data in samples were presented as the percentage of relative luminescence unit (RLU) 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 was carried out as described previously (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, cells were reacted with Cy3-conjugated anti-α-SMA monoclonal antibody, followed by counter staining by blue fluorescent DNA dye (Hoechst, nuclei dye). To determine the effect of AZD0530 (Src kinase inhibitor) on myofibroblast differentiation, the percentage of cells containing α-SMA-incorporated stress fibers over total cells (according to Hoechst staining) was quantified.

Collagen Gel Contraction Assay. Collagen-gel contraction assays were performed as described previously (Cai et al., 2012). Briefly, collagen gels were cast in 6-well plates using type I collagen/DMEM 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 vehicle-treated group.

Cell migration assays. The 2-D wound closure monolayer/scratch motility assay was performed as described previously (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 analyzed and presented as the mean ± SE.

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Statistical Analysis. Data were analyzed using the Student's t-test analysis for differences between two groups, and expressed as mean ± SE. All experiments were repeated 2-3 times with duplicates. A p value < 0.05 was considered to be 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 pro-fibrotic cytokine and 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 was peaked at 1 hour, decreased and then peaked again at 24 hrs. with a downward trend (Fig. 1A).

It has been well demonstrated that AZD0530 (saracatinib) effectively inhibits Src kinases in cancer cells (Laurie et al., 2014; Morrow et al., 2010; Chang et al., 2008). 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 Western blotted 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 $\pm$ 10.8% versus 100 $\pm$ 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 $\pm$ 4.3% versus 331 $\pm$ 10.8%, $p < 0.01$). These data show that TGF-β1 induced Src kinase activation in lung fibroblasts, and that AZD0530 treatment can efficiently inhibited TGF-β1-induced Src kinase activation and kinase activity in lung fibroblasts.

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,

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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, right-top panel versus left-top panel), and the percentage of cells with α-SMA-containing filaments was significantly increased (Fig. 2C, bar 2 versus bar 1, 53.8 $\pm$ 3.2% versus 7.7 $\pm$ 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, right-bottom panel versus right-top panel), and the percentage of cells with α-SMA-containing filaments was significantly decreased (Fig. 2C, bar 4 versus bar 2, 19.5 $\pm$ 3.5% versus 53.8 $\pm$ 3.2%, $p < 0.01$). AZD0530 induced significant cleavage of poly-ADP-ribose polymerase (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 to 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 pro-survival signaling in myofibroblasts.

Src Kinase Activation Is Required for Myofibroblast Function and Fibroblast Migration. When compared to un-differentiated 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 3D-collagen-gel contraction assays. The data show that TGF-β1 significantly induced collagen-gel contraction in lung fibroblasts (Fig. 3A, right-top panel versus left-top 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 $\pm$ 5.4% versus 100 $\pm$ 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, right-bottom panel versus right-top 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 $\pm$ 3.4% versus 58.6 $\pm$ 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 pro-fibrotic. Fibroblasts derived from human IPF lungs have increased cell migration and invasion (White et al., 2003; Tager et al., 2004; Li et al., 2011; Cai et al., 2010). The role of Src kinases in cell migration is well documented. To determine the effect of AZD0530 on fibroblast migration, we used the 2D 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 pro-fibrotic 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, human lung fibroblasts were treated with or without AZD0530 (0.1 μm) followed by TGF-β1 (10 ng/ml), and then subjected to examination of collagen and fibronectin expression. TGF-β1 treatment greatly enhances collagen (by pro-collagen 1A1) and fibronectin expression when compared to 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 focal adhesion kinase (FAK) activation (Ding et al., 2008; Thannickal et al., 2003). 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 (Ding et al., 2008; Thannickal et al., 2003). 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

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FAK, pY397-FAK) in fibroblasts (193 ± 19%, $p < 0.01$) when compared to basal FAK activation in vehicle-treated fibroblasts (Figs. 4B & 4C). Inhibition of Src kinase greatly reduced TGF-β1-induced FAK activation in fibroblasts (Figs. 4B & 4C), 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 major cell type 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 to saline-injected mice, fibrotic lesions (Fig. 5A, top-left versus bottom-left panel) 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-right versus bottom-left panel, and

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Fig. 5B) and total lung collagen accumulation (Fig. 5D, bar 4 versus bar 3, 118.6 $\pm$ 4.3 µg versus 178.7 $\pm$ 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 to vehicle-treated mice, in response to bleomycin (Fig. 5C, bottom right panel versus bottom left panel).

AZD0530 treatment significantly inhibited Src kinase activation and kinase activity *in vivo* (Figs. 5E – 5F). Bleomycin increased Src kinase activation and kinase activity *in vivo* when compared to saline-treated control mice (Figs. 5E – 5F). AZD0530 effectively blocked bleomycin-induced Src kinase activation (pY416-Src) (Fig. 5E) and kinase activity (Fig. 5F, bar 4 versus bar 3, 132 $\pm$ 11.7% versus 273 $\pm$ 11.6%, $p < 0.01$). AZD0530-treated mice also had significantly decreased α-SMA expression in lungs when compared to that in vehicle-treated mice in response to bleomycin (Fig. 5E), supporting that inhibition of Src kinase decreased the myofibroblast differentiation *in vivo*. Bleomycin increased fibronectin expression, and the 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 inhibition of Src kinase has anti-fibrotic effects in mice.

Discussion

Our results demonstrate that the Src kinase regulates myofibroblast differentiation and activation. Inhibition of Src kinase activity by the pharmacological inhibitor, AZD0530 (saracatinib), effectively blocked the expression of α-SMA, reduced the production of collagen and fibronectin, and inhibited the 3D-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 the 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.

Idiopathic pulmonary fibrosis (IPF) is a fibrotic lung disease (Ley et al., 2011). A central role for myofibroblast in fibrotic response in lung, liver, kidney, and skin is well documented (Kis et al., , 2011; Gressner and Weiskirchen, 2006; Iwano and Neilson, 2004; Romeo et al., 2006; Tuan and Nichter, 1998). Previous studies have implicated Src family kinases in fibrotic reactions. Thy-1-negative fibroblasts are located in fibrotic areas and contributes to lung fibrosis (Hagood et al., 2005). Tumor necrosis factor-alpha (TNF-α) activates Src kinase in Thy-1-negative fibroblasts, and blockade of Src activation abrogates the TNF-α-activated pro-fibrotic 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 Src family kinase (Ernst et al., Dunn, 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 pro-fibrotic effects of TGF-β1 were inhibited by AZD0530 in human lung fibroblasts. Our results are consistent with previous findings that inhibition of the Src kinase prevents collagen synthesis in cardiac and skin fibroblasts (Elkareh et al., 2007; Skhirtladze et al., 2008). The α-SMA is critical for myofibroblast contractility (Hinz et al., 2007); therefore, the reduced α-SMA expression leads to the reduced contractility in lung fibroblasts treated with AZD030.

Src kinase likely regulates myofibroblast differentiation and function though focal adhesion kinase (FAK). FAK is involved in signaling cascade initiated by integrin receptors and ECM proteins (Reiske et al., 2000; Parsons 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 (Ding et al., 2008; Thannickal et al., 2003; Grove et al., 2014). FAK promotes the formation of α-SMA-containing fibers through neuronal Wiskott-aldrich Syndrome protein (N-WASP) (Cai et al., 2012). FAK inhibition also reduced bleomycin-induced lung fibrosis in mice (Ding et al., 2013; Lagares et al., 2012). A 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 PI3K-Akt pathway in a delayed manner following TGF-β stimulation (Horowitz et al., JBC, 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 myofiboblast differentiation at least in part through a FAK-dependent pathway. AZD0530 treatment also induces significant cleavage of poly-ADP-ribose polymerase (PARP) in fibroblasts treated with both TGF-β1 and AZD0530 (Fig. 2D). There is only minimal increase of cleaved PARP in fibroblasts treated with only AZD0530 when compared to vehicle-treated fibroblasts (Fig. 2D). These data suggest a role of Src kinase in pro-survival 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 (Phan et al., 1980; Snider et al., 1978; Adamson et al., 1974; 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

post-injury (Ibicki et al., 2002), and the acute inflammatory response largely subsides by day-7 (Ibicki 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 kinse 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 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 (EMT) transition (Tanjore et al., 2011; Zhong et al., 2011). EMT is considered as one potential sources of myofibroblast accumulation in fibrotic lungs (Tanjore et al., 2011; Zhong et al., 2011; Hinz et al., 2007). 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., JBC, 2003). Src kinase members may have overlapping functions, and it is currently not known which specific Src kinase mediates myofibroblast differentiation.

Recent evidence reignites the 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 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 show that the protein kinase inhibitor (AG1879, PP2) reduces *in vivo* fibrogenesis (Vittal et al., 2005). AG1879 targets the Src and FAK kinases and inhibits Akt-mediated prosurvival pathway in fibroblasts/myofibroblasts (Vittal et al., 2005). Interestingly, another protein kinase inhibitor, imatinib mesylate (Gleevec) which targets c-Abl tyrosine kinase and 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.

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Taken together, the 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 protected against fibrosis in mice, providing proof-of-concept that targeting the non-canonical TGF-β signaling pathway involving Src kinase(s) may serve as an effective therapeutic strategy for lung fibrosis.

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Authorship Contributions

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

Conducted experiments: Hu, Che, Siegal, and Cai

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

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

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

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Footnotes

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b) Some of the work has been presented as poster at the American Thoracic Society meeting.

c) The authors declare no conflict of interest.

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Figure Legends

Figure 1. TGF-β1 induces Src activation, and AZD0530 inhibits TGF-β1-induced Src activation in a dose-dependent manner in human lung fibroblasts.

Panel A: Serum starved human lung fibroblasts were treated with TGF-β1 (10ng/ml) in serum-free medium (SFM) for the indicated time, detergent lysed, and whole cell lysates were Western blotted with the indicated antibodies. **Panel B:** The above lung fibroblasts were treated with the AZD0530 inhibitor at the indicated dose or with control vehicle, then treated with TGF-β1 (10ng/ml) for 1 hour. Cell were detergent lysed, and lysates were Western blotted with the indicated antibodies. Src activation was determined by phosphorylation of tyrosine 416 of Src (pY416-Src). **Panel C:** Src kinase activities in the above lung fibroblasts were examined by a luminescent kinase assay kit and data are presented as the percentage of relative luminescence (RLU) to those fibroblasts cultured in SFM (as 100%). The optimized AZD0530 dose (0.1 μM) was used in all tests. The data are presented as the mean + SE. * represents $p < 0.01$.

Figure 2. AZD0530 Inhibits TGF-β1-induced α-SMA expression and formation of α-SMA-containing fibers in human lung fibroblasts. Panel 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. **Panel B:** The above fibroblasts were fixed, immunofluorescently stained with the Cy-3-labelled monoclonal antibody toward α-SMA, and fluorescent

microscopic digital images were taken (original 200X). Representative pictures are shown. **Panel C**: Quantification of the percentage of cells with highly organized, thickened α-SMA-containing fibers as described in Experimental Procedures. The data are presented as the mean + SE. * represents $p < 0.01$. **Panel D**: The above fibroblasts were lysed and equivalent amount of whole cell lysates were Western blotted with indicated antibodies. The antibody specifically recognizes the cleaved PARP and do not cross with the full length form. The experiments were repeated 3 times and Representative pictures are shown.

Figure 3. AZD0530 inhibits TGF-β1-induced 3D collagen gel contraction and PDGF-induced migration in human lung fibroblasts. **Panel 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. **Panel B**: Data are pooled from three individual experiments (each performed with at least in duplicate) and 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. **Panel C**: Serum-starved human lung fibroblasts were wounded, treated with or without AZD0530 (AZD, 0.1 μM) or vehicle followed by PDGF-BB (4 ng/ml) in serum-free medium with 1% BSA for 24 hours. Monolayer wound closure assays were performed as described in the Materials and Methods. Data obtained were pooled (n = 4 per group) and plotted as the percentage of wound area covered over 24 hours relative to control (fibroblasts in vehicle-treated only). All data were presented as mean + SE. * represents $p < 0.01$.

Figure 4. AZD0530 inhibits TGF-β1-induced pro-collagen 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 Western blotted with the indicated antibodies. **Panel A**: Expression of collagen and fibronectin (FN). Expression of collagen was examined through expression of pro-collagen 1A1 (Pro-Col). Representative images are shown. **Panel B**: FAK activation was examined through phosphorylation of the tyrosine 397 (pY397) of FAK. Representative images are shown. **Panel C**: Densitometry analysis of band intensity for pY397 of FAK. Results were normalized to total FAK protein level and basal pY397-FAK was used as 100%. Results were pooled from three individual experiments. * represents $p < 0.01$.

Figure 5. AZD0530 is protective against lung fibrosis.

Panel 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 hematoxylin and eosin (HE) stained. Representative lung sections are shown (200x). **Panel 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). **Panel C:** Lung tissue sections were stained by a Masson's

trichrome staining kit to demonstrate the areas of collagen deposition (400x). **Panel D:** Total lung collagen accumulation was determined by hydroxyproline assays. **Panel 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 fibronection (FN) and α-SMA. **Panel F:** Lungs were harvested at day 14 after bleomycin instillation and Src kinase activities were examined as in Fig. 1. Above data are pooled and presented as mean + SE (n = 6-8 mice per group). * represents $p < 0.01$.

Figure 1.

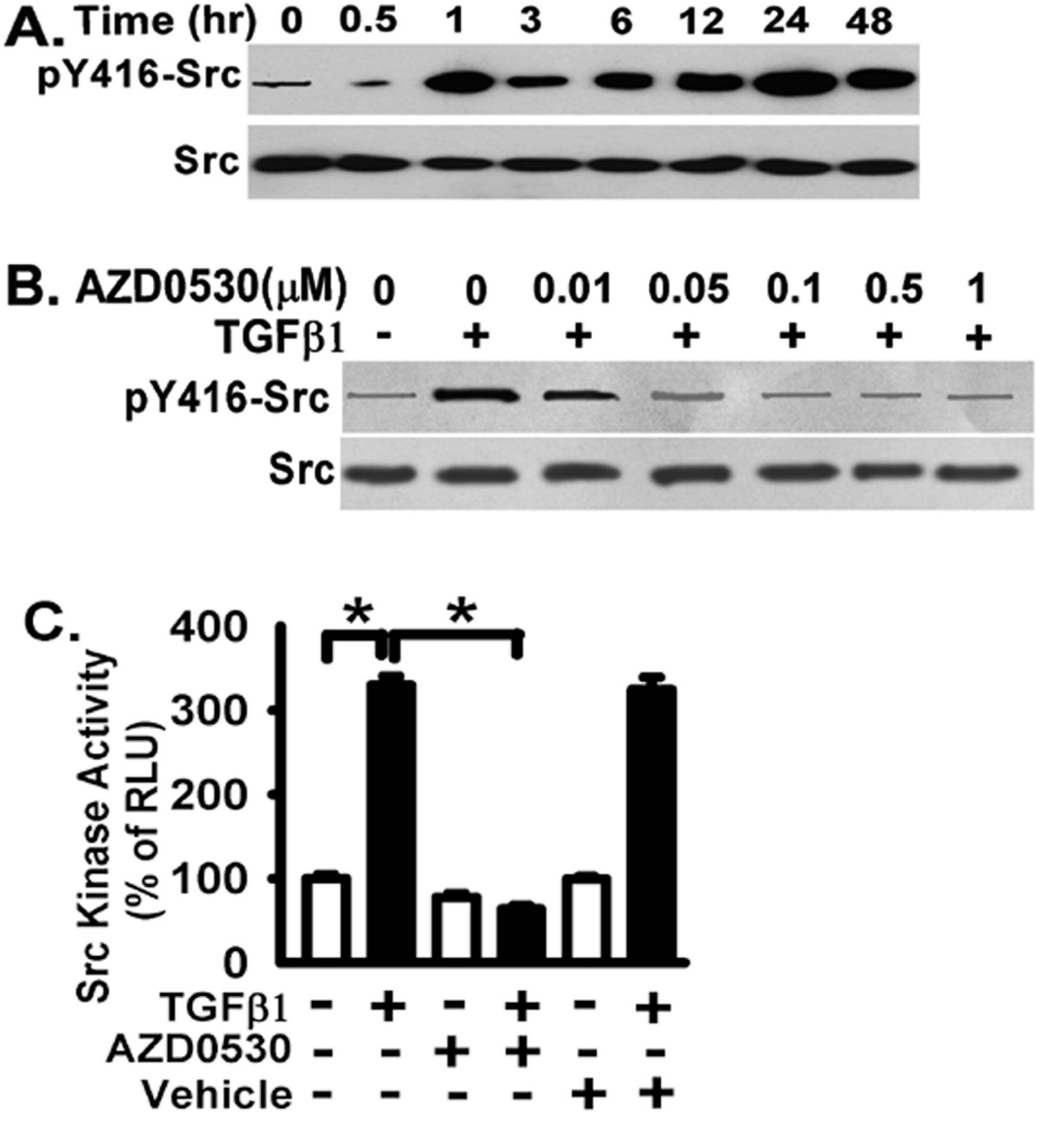
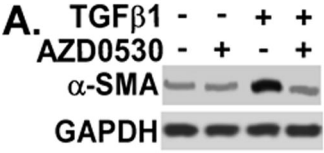
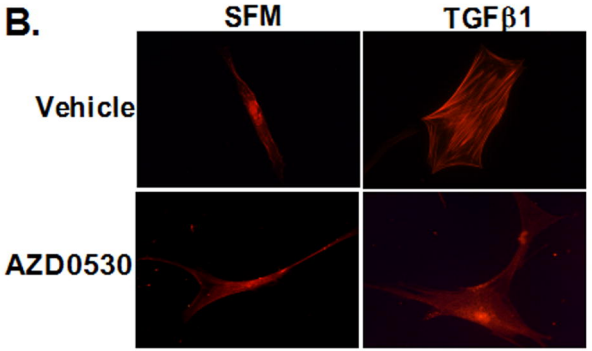
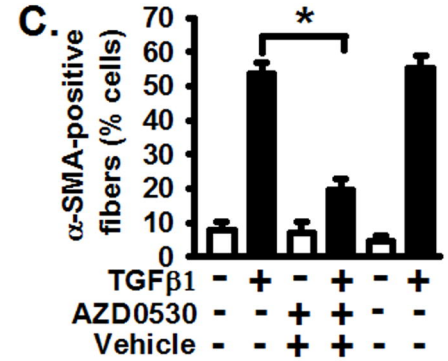

A.
Time (hr) 0 0.5 1 3 6 12 24 48
pY416-Src
Src
B.
AZD0530(μM) 0 0 0.01 0.05 0.1 0.5 1
TGFβ1 - + + + + + +
pY416-Src
Src
C.
Src Kinase Activity (% of RLU)
400
300
200
100
0
*
*
TGFβ1 - + - + - +
AZD0530 - - + + - -
Vehicle - - - - + +

Figure 2.


A.
TGFβ1 - - + +
AZD0530 - + - +
α-SMA
GAPDH


B.
SFM
TGFβ1
Vehicle
AZD0530


C.
α-SMA-positive fibers (% cells)
70
60
50
40
30
20
10
0
*
TGFβ1 - + - + - +
AZD0530 - - + + - -
Vehicle - - + + - -

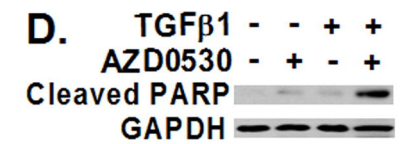
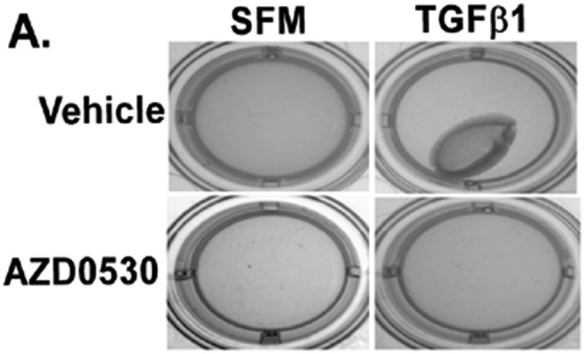
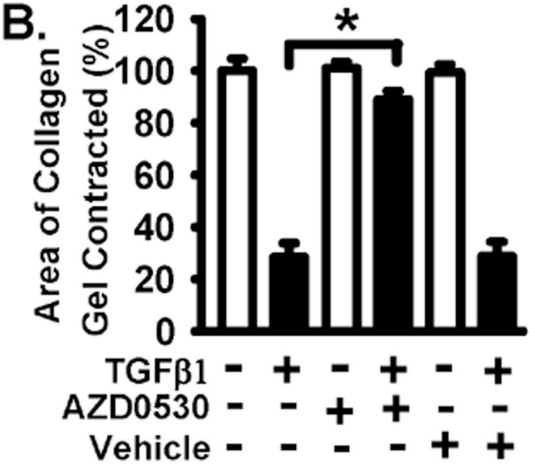

D.
TGFβ1 - - + +
AZD0530 - + - +
Cleaved PARP
GAPDH

Figure 3.


A.
SFM
TGFβ1
Vehicle
AZD0530


B.
Area of Collagen Gel Contracted (%)
120
100
80
60
40
20
0
*
TGFβ1 - + - + - +
AZD0530 - - + + - -
Vehicle - - - - + +

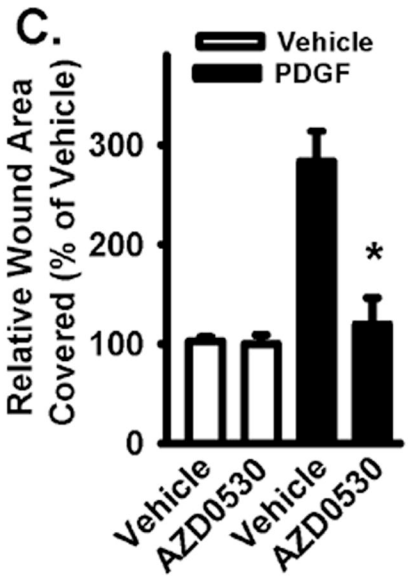

C.
Vehicle
PDGF
Relative Wound Area Covered (% of Vehicle)
300
200
100
0
*
Vehicle
AZD0530
Vehicle
AZD0530

Figure 4.

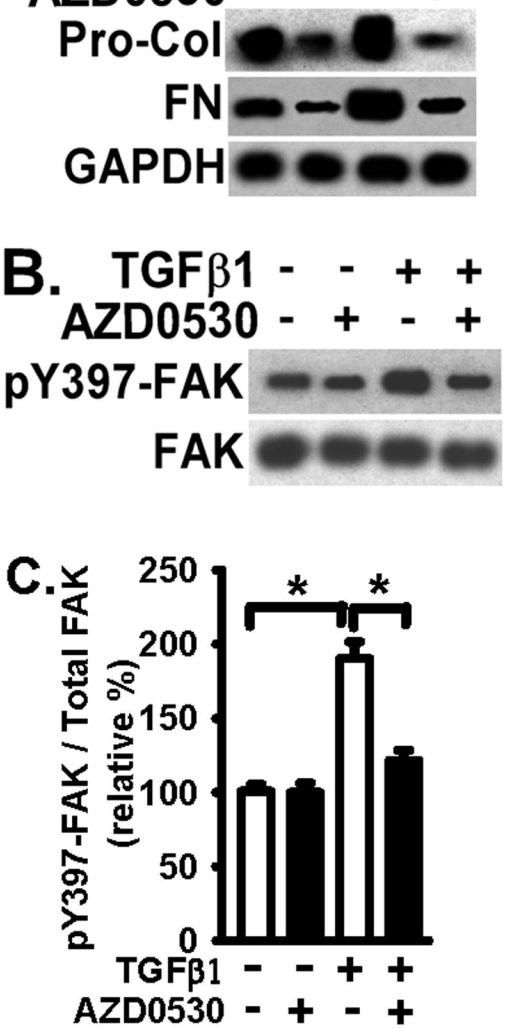
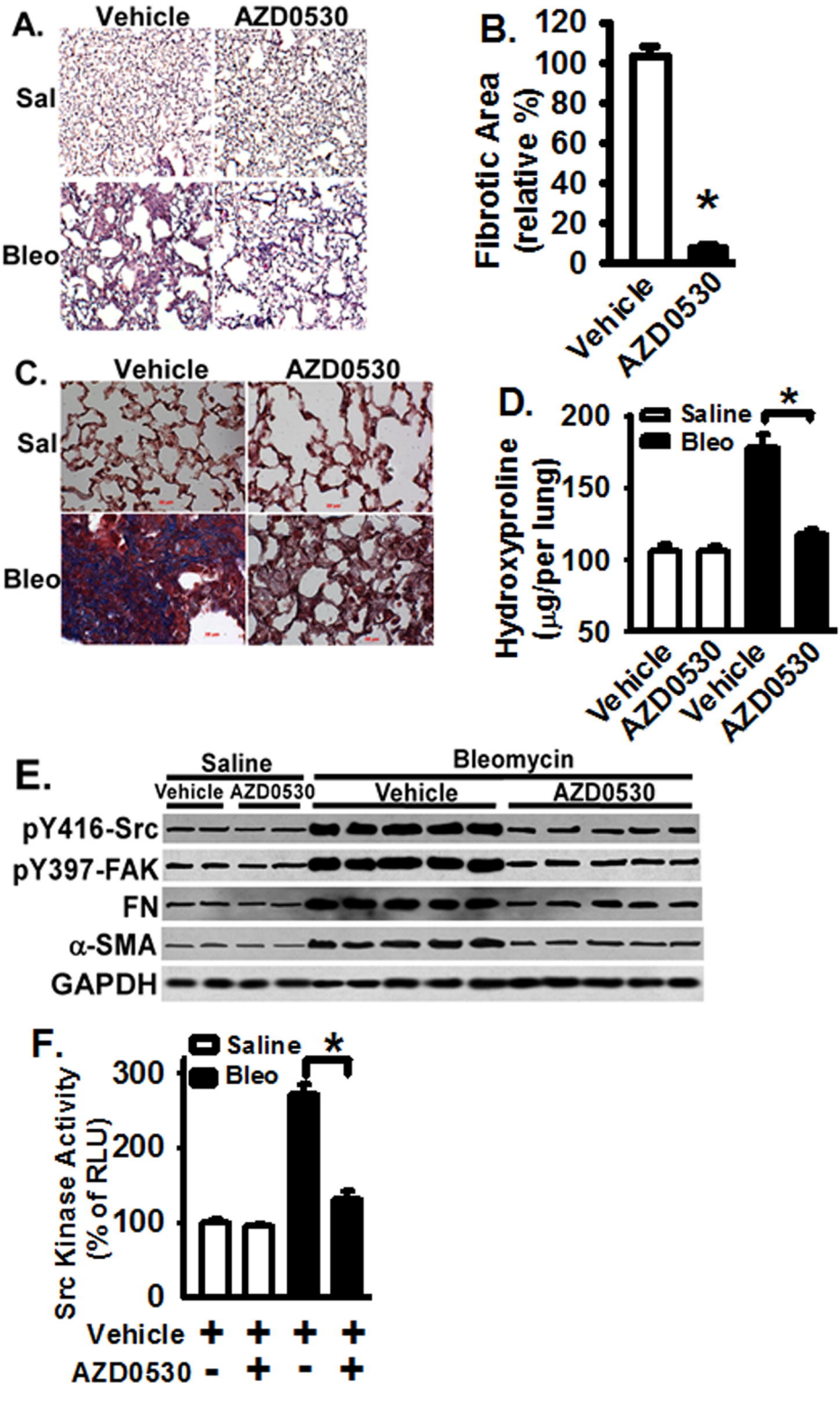

A.
TGFβ1 - - + +
AZD0530 - + - +
Pro-Col
FN
GAPDH
B.
TGFβ1 - - + +
AZD0530 - + - +
pY397-FAK
FAK
C.
pY397-FAK / Total FAK (relative %)
250
200
150
100
50
0
*
*
TGFβ1 - - + +
AZD0530 - + - +

Figure 5.


A.
Vehicle
AZD0530
Sal
Bleo
B.
Fibrotic Area (relative %)
120
100
80
60
40
20
0
*
Vehicle
AZD0530
C.
Vehicle
AZD0530
Sal
Bleo
D.
Hydroxyproline (μg/per lung)
200
150
100
50
Saline
Bleo
*
Vehicle
AZD0530
Vehicle
AZD0530
E.
Saline
Bleomycin
Vehicle
AZD0530
Vehicle
AZD0530
pY416-Src
pY397-FAK
FN
α-SMA
GAPDH
F.
Src Kinase Activity (% of RLU)
300
200
100
0
Saline
Bleo
*
Vehicle + + + +
AZD0530 - + - +