The American Journal of Pathology, Vol. 181, No. 1, July 2012

http://dx.doi.org/10.1016/j.ajpath.2012.03.034

Matrix Pathobiology

Low $\alpha_2\beta_1$ Integrin Function Enhances the Proliferation of Fibroblasts from Patients with Idiopathic Pulmonary Fibrosis by Activation of the β-Catenin Pathway

Hong Xia,* Jeremy Seeman,* Jian Hong,† Polla Hergert,* Vidya Bodem,* Jose Jessurun,‡ Karen Smith,* Richard Nho,* Judy Kahm,* Philippe Gaillard,§ and Craig Henke*

From the Departments of Medicine and Laboratory Medicine and Pathology‡ and the Biostatistical Design and Analysis Center-Clinical and Translational Science Institute,§ University of Minnesota, Minneapolis, Minnesota; and the First Affiliated Hospital,† Nanjing Medical University, Nanjing, China*

Idiopathic pulmonary fibrosis (IPF) is a progressive and incurable fibroproliferative disorder characterized by unrelenting proliferation of fibroblasts and their deposition of collagen within alveoli, resulting in permanently scarred, nonfunctional airspaces. Normally, polymerized collagen suppresses fibroblast proliferation and serves as a physiological restraint to limit fibroproliferation after tissue injury. The IPF fibroblast, however, is a pathologically altered cell that has acquired the capacity to elude the proliferation-suppressive effects of polymerized collagen. The mechanism for this phenomenon remains incompletely understood. Here, we demonstrate that expression of $\alpha_2\beta_1$ integrin, a major collagen receptor, is pathologically low in IPF fibroblasts interacting with polymerized collagen. Low integrin expression in IPF fibroblasts is associated with a failure to induce PP2A phosphatase activity, resulting in abnormally high levels of phosphorylated (inactive) GSK-3β and high levels of active β-catenin in the nucleus. Knockdown of β-catenin in IPF fibroblasts inhibits their ability to proliferate on collagen. Interdiction of $\alpha_2\beta_1$ integrin in control fibroblasts reproduces the IPF phenotype and leads to the inability of these cells to activate PP2A, resulting in high levels of phosphorylated GSK-3β and active β-catenin and in enhanced proliferation on collagen. Our findings indicate that the IPF fibroblast phenotype is characterized by low $\alpha_2\beta_1$ integrin expression, resulting in a failure of integrin to activate PP2A phosphatase, which permits inappropriate activation of the β-catenin pathway. *(Am J Pathol 2012, 181:222–233; http://dx.doi.org/10.1016/j.ajpath.2012.03.034)*

Idiopathic pulmonary fibrosis (IPF) is a prevalent and progressive fibroproliferative lung disease that is incurable. Histologically, the fibroblastic focus is the major morphological lesion of IPF. It consists of fibroblasts in a type I collagen-rich matrix.[1] The disease is characterized by unrelenting progression of fibrotic tissue formation, with expansion of fibroblasts within alveolar walls and deposition of type I collagen resulting in the obliteration of airspaces and subsequent impairment of gas exchange that in turn leads to progressive hypoxia.[2–5]

Fibrillar type I collagen, a potent suppressor of normal fibroblast proliferation, functions as a physiological restraint to limit fibroproliferation after injury.[6–8] The β_1 integrin is a cell surface matrix receptor that is critically positioned at the apex of signaling pathways that regulate proliferation.[9–19] We have previously shown that pathological integrin signaling underlies the aberrant proliferative capacity of human IPF lung fibroblasts.[20,21] We have found that β_1 integrin interaction with polymerized collagen suppresses normal fibroblast proliferation by inhibition of the PI3K/Akt/S6K1 signal pathway via maintenance of high PTEN phosphatase activity. IPF fibroblasts circumvent this restraint, displaying a pathological pattern of β_1 integrin signaling in response to polymerized collagen that results in aberrant activation of the PI3K/Akt/S6K1 pathway via inappropriately low PTEN activity. It has remained unclear, however, whether integrin regulation of

Supported by grants from the NIH National Heart, Lung, and Blood Institute (R01-HL074882 and P01-HL91775 to C.A.H.).

Accepted for publication March 28, 2012.

Supplemental material for this article can be found at *http://ajp.amjpathol.org* or at *http://dx.doi.org/10.1016/j.ajpath.2012.03.034*.

Address reprint requests to Craig A. Henke, M.D., Box 276, University of Minnesota, 420 Delaware St. SE, Minneapolis, MN 55455. E-mail: henke002@umn.edu.

IPF fibroblast proliferation operates solely through the PI3K/Akt/S6K1 pathway or whether additional pathways are involved. Although $\alpha_2\beta_1$ integrin is the major type I collagen receptor, neither its function in IPF fibroblasts nor the precise role that $\alpha_2\beta_1$ integrin plays in aberrant proliferation signaling has been previously examined.

Previous studies have found that β-catenin is activated in IPF,[22,23] but the mechanism by which β-catenin is elevated in IPF remains incompletely understood. We have discovered that $\alpha_2\beta_1$ integrin expression is greatly diminished when IPF fibroblasts are cultured on polymerized type I collagen, compared with control fibroblasts. In a seminal study, Heino and colleagues[24] determined that $\alpha_2\beta_1$ integrin is required for activation of the serine/threonine phosphatase PP2A during cell interaction with type I collagen. Here, we demonstrate that low $\alpha_2\beta_1$ integrin expression results in the failure of IPF fibroblasts to appropriately induce PP2A phosphatase activity when interacting with collagen. This is associated with high levels of phosphorylated (inactive) GSK-3β, a PP2A substrate, and very high levels of active β-catenin. We demonstrate that knockdown of β-catenin decreases the ability of IPF fibroblasts to proliferate on polymerized collagen. These findings stand in marked contrast to those for control fibroblasts, which express higher levels of $\alpha_2\beta_1$ integrin and robustly induce PP2A activity, thereby activating GSK-3β and suppressing β-catenin. We demonstrate that inhibition of $\alpha_2\beta_1$ function by blocking antibody or knockdown of β_1 integrin expression in control fibroblasts results in the inability of these cells to appropriately induce PP2A phosphatase activity when these cells interact with polymerized collagen. Interdiction of $\alpha_2\beta_1$ integrin function in control fibroblasts increases the level of phosphorylated (inactive) GSK-3β, increases the level of active β-catenin, and enhances control fibroblast proliferation on polymerized collagen, replicating IPF fibroblast behavior. Our present findings indicate that the pathological IPF fibroblast phenotype is characterized by abnormally low $\alpha_2\beta_1$ integrin expression and function, which is linked to the failure to appropriately induce PP2A phosphatase activity, and thus permitting the aberrant activation of the β-catenin proliferation signal pathway.

Materials and Methods

Primary IPF and Normal Fibroblast Lines

Eight primary fibroblast lines were established from IPF patients. Cells were obtained from lungs removed at the time of transplantation or death. The diagnosis of IPF was supported by history, physical examination, pulmonary function tests, and high-resolution chest computed tomography findings typical of IPF. In all cases, the diagnosis of IPF was confirmed by microscopic analysis of lung tissue, which exhibited the characteristic morphological findings of interstitial pneumonia. All patients fulfilled the criteria for the diagnosis of IPF as established by the American Thoracic Society and the European Respiratory Society.[25] Seven primary control adult human lung fibroblast lines were used. These lines included fibroblasts derived from anatomically normal lung tissue not used at the time of lung transplantation ($n = 4$) and histologically normal lung tissue distant from carcinoid tumor ($n = 2$) or distant from radiation-induced fibrotic lung tissue ($n = 1$). Primary lung fibroblast lines were generated by explant culture and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Fibroblasts were used between passages 3 to 5. Cells were characterized as fibroblasts as described previously.[26] Use of human tissues was approved by the Institutional Review Board at the University of Minnesota.

Antibodies

Anti-α_2 integrin-blocking antibody (MCA743) was obtained from AbD Serotec (Raleigh, NC), anti-β_1 integrin antibody was obtained from BD Biosciences (San Diego, CA), and anti-GSK-3β antibody was obtained from Cell Signaling Technology (Danvers, MA). Anti-$\alpha_2\beta_1$ integrin-blocking antibody (BHA2.1), anti-PP2Ac antibody, and active anti-β-catenin antibody were obtained from Millipore (Billerica, MA). Anti-β_1 integrin, activated antibody (clone HUTS4) was obtained from Millipore.

Collagen Matrices

Three-dimensional polymerized collagen matrices (final concentration, 2 mg/mL) were prepared by neutralizing the collagen solution with 1/6 volume of 6× DMEM and diluting to a final volume with 1× DMEM to which fetal calf serum was added at a final concentration of 1% fetal calf serum. Gels formed after incubation of this solution at 37°C for 1 to 2 hours as described previously.[13–15,20]

Lentiviral Vectors, β_1 Integrin, PP2Ac, and Control shRNA

β_1 Integrin, PP2Ac, β-catenin, and control short hairpin RNA (shRNA) were obtained from the University of Minnesota RNAi Core Facility. The constructs were incorporated into the pGIPZ plasmid vector. The cells were infected with lentiviral vectors at a multiplicity of infection of 1:20. Alternatively, IPF and control fibroblasts were infected with a lentiviral vector containing wild-type β_1 integrin, mutant β_1 integrin (V737N), or control (empty vector) constructs.

Western Blot

Serum-starved fibroblasts were plated on extracellular matrix-coated plates and lysed at the indicated times using cell lysis buffer containing 150 mmol/L NaCl, 1 mmol/L EGTA, 50 mmol/L Tris pH 7.4, 1% Triton X-100, 1% Nonidet P-40, and 1% sodium deoxycholate with protease inhibitors (complete protease inhibitor mixture tablets; Roche Applied Science, Indianapolis, IN). Western blot analysis was performed on the resulting lysates.

Proliferation Assay

Fibroblasts were serum-starved for 2 days and then plated on polymerized collagen (2 mg/mL) matrix in

DMEM with 1% fetal calf serum. After the cells were replated onto polymerized collagen matrix, the medium was replaced with DMEM with 10% fetal calf serum. The cells were incubated with 10 μmol/L bromodeoxyuridine for 5 hours before the cells were harvested at 24 hours. The cells were then stained with anti-bromodeoxyuridine antibody to quantify DNA synthesis and 7-amino actinomycin D as a measure of total DNA. DNA synthesis was quantified by assessing the percentage of bromodeoxyuridine-positive cells by fluorescence-activated cell sorting according to the manufacturer's instructions (BD Biosciences).

PP2A in Vitro *Phosphatase Activity Assay*

PP2A activity was assessed by dephosphorylation of the phosphopeptide K-R-pT-I-R-R. Briefly, PP2Ac was immunoprecipitated from cell lysates with 4 μg of anti-PP2Ac antibody (C subunit, clone 1D6; Millipore) incubated with Protein A agarose slurry, then washed with Tris-buffered saline. Immunoprecipitated PP2A was then incubated with 10 μL Ser/Thr assay buffer (50 mmol/L Tris-HCl, pH 7.0, and 100 μmol/L $CaCl_2$) and 30 μL of the phosphopeptide substrate according to the manufacturer's instructions (Millipore-Chemicon, Temecula, CA). The enzyme reaction was terminated by adding 100 μL of malachite green phosphate detection solution. PP2A activity was measured using a microtiter plate reader at 650 nm.

Immunofluorescent Confocal Microscopy

Immunostaining was performed on paraffin-embedded IPF and control lung tissue using an Axiovert 200M confocal microscope (Carl Zeiss MicroImaging, Göttingen, Germany). Primary antibodies included a polyclonal antibody to α_2 integrin (1:200 dilution; Millipore), a monoclonal antibody to β_1 integrin (1:100 dilution; BD Biosciences), and monoclonal (Sigma-Aldrich, St. Louis, MO) and polyclonal (Abcam, Cambridge, MA) antibodies to α-smooth muscle actin (both at 1:200 dilution).

Immunofluorescence

Immunofluorescence studies were performed on IPF and control fibroblasts cultured on glass coverslips. The cells were then fixed, permeabilized, and incubated (60 minutes, room temperature) with the appropriate primary antibody (β-catenin). The cells were then incubated (60 minutes, room temperature) with Cy2 or Cy3 conjugated secondary antibodies, followed by incubation (10 minutes, room temperature) with DAPI. Primary antibody anti-β-catenin was obtained from Millipore.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin-embedded lung tissue specimens, 5 μm thick, using a polyclonal antibody to human α_2 integrin (Millipore) and a monoclonal antibody to human β_1 integrin (Abcam). The sections were deparaffinized, rehydrated through a series of graded methanol, and heated (95°C, 30 minutes) in citrate buffer (pH 6.0) for antigen retrieval. The sections were quenched with 0.3% H_2O_2 in methanol to inhibit endogenous peroxidases and blocked with 5% normal human serum (30 minutes). Endogenous avidin and biotin binding sites were blocked (15 minutes) sequentially using an avidin/biotin-blocking kit (Vector Laboratories, Burlingame, CA); incubated (16 hours, 4°C) with primary antibodies, and then rinsed and incubated with goat anti-rabbit secondary antibody (30 minutes, room temperature), followed by ready-to-use horseradish peroxidase-streptavidin complex (Vector Laboratories). Controls consisted of secondary antibody only. Specific antibody binding was detected by 3,3′-diaminobenzidine (Vector Laboratories); ready-to-use hematoxylin was used as a counterstain.

Statistical Analysis

Comparisons of data among each experiment were performed with the Satterthwaite *t*-test where applicable. Nonparametric data were assessed using the Wilcoxon rank-sum test. All experiments were replicated a minimum of three times. Data are expressed as means ± SD. $P < 0.05$ was considered significant.

Results

$\alpha_2\beta_1$ Integrin Expression Is Decreased in IPF Fibroblasts in Vitro

Our prior work demonstrated that IPF fibroblasts have escaped the proliferation-suppressive effects of polymerized type I collagen.[20,21] The mechanism involved pathological β_1 integrin signaling, resulting in aberrant activation of the PI3K/Akt/S6K1 proliferation signal pathway. Because $\alpha_2\beta_1$ integrin is the major integrin that mediates interaction with type I collagen, we sought to examine the precise role that $\alpha_2\beta_1$ plays in aberrant proliferation signaling and its function in IPF fibroblasts. We first examined β_1 integrin expression by Western blot analysis in primary lung fibroblasts derived from IPF patients ($n = 7$) and control patients ($n = 7$) and cultured on polymerized type I collagen matrix. On the basis of our prior studies showing aberrant activation of proliferation signaling downstream of integrin,[20,21] we expected that β_1 integrin expression would be high in IPF fibroblasts. Instead, we found that β_1 integrin protein expression was decreased in primary IPF fibroblasts cultured on polymerized collagen, compared with control fibroblasts (Figure 1A). Similar to our results for β_1 integrin, we found that $\alpha 2$ integrin expression also was lower in IPF fibroblasts, compared with control (Figure 1B). We also examined the β_1 integrin activation state of IPF and control fibroblasts cultured on polymerized collagen as a function of time. There was a reduction in the level of activated β_1 integrin in IPF fibroblasts, compared with control (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). To correlate integrin expression with proliferation, we examined the proliferative capacity of IPF and control fibroblasts cul-

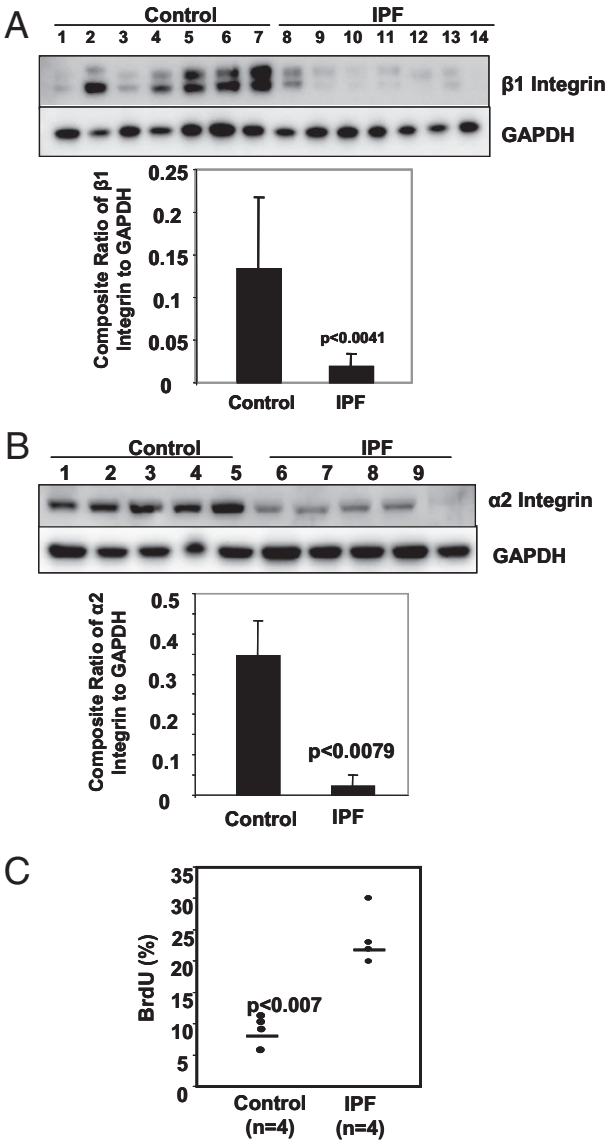

Figure 1. $\alpha_2\beta_1$ integrin expression is decreased in IPF fibroblasts *in vitro*. **A:** Western blot analysis of β_1 integrin expression in primary lung fibroblasts from IPF patients ($n = 7$) and control patients ($n = 7$), cultured on polymerized type I collagen matrix. GAPDH was the loading control. Relative protein expression was determined as the densitometric ratio of β_1 integrin to GAPDH. Shown is the composite ratio of integrin to GAPDH expression comparing IPF with control. **B:** Western blot analysis of α_2 integrin expression in IPF and control fibroblasts from IPF patients ($n = 5$) and control patients ($n = 5$). Relative protein expression was determined as the densitometric ratio of α_2 integrin to GAPDH. Shown is the composite ratio of integrin to GAPDH expression, comparing IPF with control. **C:** Primary lung fibroblasts from IPF patients ($n = 4$) and control patients ($n = 4$) were cultured on 2 mg/mL polymerized collagen matrix for 24 hours, after which cell numbers were quantified. Data are representative of three independent experiments. BrdU, bromodeoxyuridine.

tured on polymerized collagen. Consistent with our prior studies, we found that the ability of IPF fibroblasts to proliferate on polymerized collagen was significantly increased, compared with control fibroblasts (Figure 1C).[20] Thus, during IPF fibroblast interaction with fibrillar type I collagen, the expression of $\alpha_2\beta_1$ integrin is low and the proliferative capacity is increased, compared with control fibroblasts.

$\alpha_2\beta_1$ Integrin Expression Is Decreased in IPF Fibroblastic Foci in Vivo

To determine whether low $\alpha_2\beta_1$ integrin levels in IPF fibroblasts *in vitro* correlated with $\alpha_2\beta_1$ integrin expression *in vivo*, we analyzed $\alpha_2\beta_1$ integrin expression in fibroblasts within type I collagen-rich fibroblastic foci in human IPF lung tissue by immunofluorescence. Using antibodies recognizing α_2 and β_1 integrin subunits, we found that cells within IPF fibroblastic foci displayed diminished immunoreactivity for α_2 or β_1 integrin, compared with epithelial cells overlying the fibroblastic foci, which were strongly immunoreactive for α_2 and β_1 integrin (Figure 2A). Cells within the fibrotic reticulum were strongly immunoreactive for α smooth muscle actin. As a semiquantitative gauge of integrin staining in IPF and control specimens, we compared the intensity of α_2 and β_1 immunoreactivity in epithelial cells lining large airways, using high-power merged immunofluorescent images (Figure 2A; see also Supplemental Figure S2, A and B, at *http://ajp.amjpathol.org*). The integrin expression was similarly intense in both IPF and control large airway epithelium. This is consistent with prior studies indicating strong immunoreactivity to α_2 and β_1 integrin in the bronchial epithelium of normal lung tissue.[27,28] Also consistent with prior reports,[27] diffuse α_2 and β_1 immunoreactivity was apparent in cells comprising normal alveolar septae.

To verify our immunofluorescent results, we performed IHC. Consistent with our immunofluorescent studies, IHC analysis of IPF lung tissue demonstrated that fibroblastic foci displayed a paucity of cells staining for α_2 and β_1 integrin, whereas relatively intense immunoreactivity for both α_2 and β_1 was observed in epithelial cells overlying the fibrotic reticulum (Figure 2B; see also Supplemental Figure S3 at *http://ajp.amjpathol.org*). Diffuse immunoreactivity of α_2 and β_1 integrin was apparent in cells comprising normal alveolar structures in control lung tissue (Figure 2B).

Low $\alpha_2\beta_1$ Integrin Expression Is Associated with a Failure of IPF Fibroblasts to Properly Activate PP2A Phosphatase Activity in Response to Interaction with Polymerized Type 1 Collagen

To reconcile our finding that $\alpha_2\beta_1$ integrin expression is low in IPF fibroblasts with our expectation that proliferation signal pathways downstream from integrin are pathologically activated in IPF, we reasoned that reductions in integrin function might lead to diminished function of one or more key phosphatases that inhibit proliferation signaling. In support of this concept, Heino and colleagues[24] found that $\alpha_2\beta_1$ integrin-mediated cell adhesion to collagen led to the induction of activity of the serine/threonine phosphatase PP2A. This suggested to us that pathologically low $\alpha_2\beta_1$ integrin function in IPF fibroblasts might fail to properly induce PP2A activity when these cells interact with collagen. Consistent with this idea, we found that during IPF fibroblast interaction with polymerized collagen over a 3-day period these cells

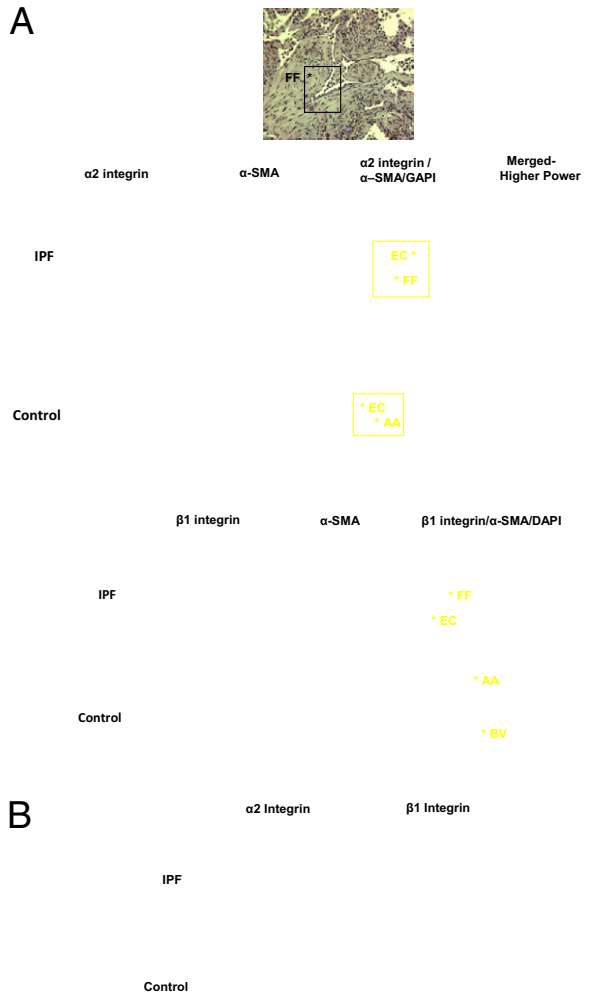

Figure 2. $\alpha_2\beta_1$ integrin expression is decreased in IPF fibroblastic foci *in vivo*. **A:** Immunofluorescent microscopic analysis was performed on IPF and control lung specimens (anatomically normal lung) to analyze for α_2 and β_1 integrin immunoreactivity. In H&E stain of IPF fibroblastic focus (**top**), the **boxed** region corresponds to the area of the immunofluorescent images (**middle**) for α_2 integrin. Immunofluorescent images demonstrate reduced immunoreactivity for α_2 integrin expression in cells comprising the fibrotic reticulum, compared with intense α_2 integrin immunoreactivity in overlying epithelial cells. Strong immunoreactivity for α-smooth muscle actin (α-SMA) was present in the majority of cells comprising the IPF fibroblastic focus. Control lung tissue demonstrates diffuse α_2 integrin staining of anatomically normal alveolar structures. Note that α_2 integrin immunoreactivity is strong in airway epithelial cells. **Bottom:** There is a paucity of β_1 integrin immunoreactive cells within the IPF fibroblastic focus, whereas relatively strong immunoreactivity for β_1 integrin is present in epithelial cells overlying the fibroblastic focus. Cells within the fibroblastic focus are immunoreactive for α-smooth muscle actin. A diffuse pattern of β_1 staining was present in cells comprising normal alveolar septae. The **boxed** region corresponds to the area of the merged image shown at higher power. **B:** IHC microscopic analysis was performed on IPF and anatomically normal control lung specimens to analyze for α_2 and β_1 immunoreactivity. Representative IHC images demonstrate a paucity of α_2 and β_1 immunoreactivity in cells in IPF fibroblastic foci, but with immunoreactivity in epithelial cells overlying the fibroblastic focus. **Asterisks** indicate the features labeled. AA, alveolar area; BV, blood vessel; EC, epithelial cell; FF, fibroblastic focus. Original magnification: ×400 (**A**, H&E stain); ×400 (**A**, unmerged immunofluorescence images); ×630 (**A**, merged images); ×400 (**B**).

failed to properly induce PP2A phosphatase activity (Figure 3A). In marked contrast, control fibroblasts robustly induced PP2A activity. At day 3, PP2A activity was 2.98-fold higher in control fibroblasts, compared with IPF fibroblasts. To determine whether the low PP2A activity in IPF fibroblasts interacting with collagen was due to a lack of PP2A expression or a failure to activate the phosphatase, we examined the expression of the catalytic subunit of PP2A (PP2Ac) in IPF and control fibroblasts cultured on polymerized collagen for 3 days. Both IPF and control fibroblasts expressed PP2Ac. PP2Ac expression was modestly lower in IPF fibroblasts, compared with control fibroblasts (Figure 3B). PP2Ac expression, in both IPF and control fibroblasts, did not appreciably change with time. These results suggested to us that pathologically low $\alpha_2\beta_1$ integrin function fails to appropriately induce PP2A phosphatase activity during IPF fibroblast interaction with polymerized collagen, whereas normal $\alpha_2\beta_1$ integrin function in control fibroblasts facilitates activation of PP2A.

Inhibition of $\alpha_2\beta_1$ Integrin and Knockdown of β_1 Integrin in Control Fibroblasts Results in an Inability of These Cells to Activate PP2A in Response to Interaction with Collagen

To analyze the role of $\alpha_2\beta_1$ integrin in regulating PP2A activity in IPF and control fibroblasts, we used integrin function-blocking antibodies. We used an α_2-blocking antibody that has been shown to specifically interdict $\alpha_2\beta_1$/collagen-induced signals. Blockage of α_2 integrin inhibited the $\alpha_2\beta_1$/collagen-mediated induction of PP2A activity in control fibroblasts (66% decrease in PP2A

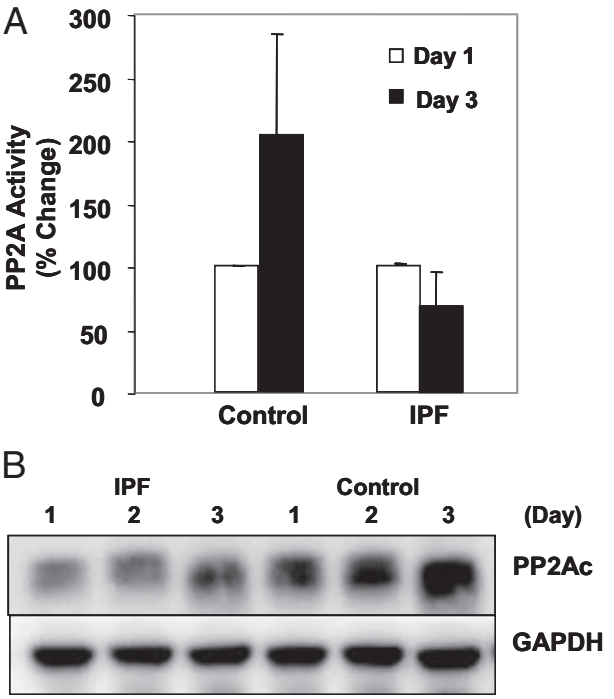

Figure 3. Low $\alpha_2\beta_1$ integrin expression is associated with a failure of IPF fibroblasts to properly activate PP2A phosphatase activity in response to interaction with polymerized type 1 collagen. **A:** Primary lung fibroblasts from IPF patients (n = 7) and control patients (n = 7) were cultured on polymerized collagen and PP2A phosphatase activity was quantified as a function of time. **B:** Western blot analysis for PP2Ac protein expression in IPF and control fibroblasts cultured on polymerized collagen for 3 days.

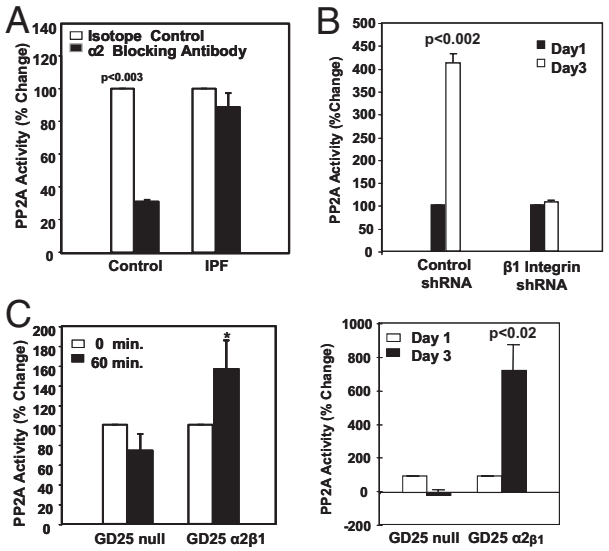

Figure 4. Inhibition of $\alpha_2\beta_1$ integrin and knockdown of β_1 integrin in control fibroblasts results in an inability of these cells to activate PP2A in response to interaction with collagen. **A:** IPF and control fibroblasts were treated with α_2 function-blocking antibody or isotype control antibody and were seeded on collagen matrix. PP2A activity was quantified at 60 minutes. **B:** Control fibroblasts were infected with a lentiviral vector containing shRNA to β_1 integrin or control shRNA. The cells were then cultured on polymerized collagen or tissue culture plates for 3 days, and PP2A phosphatase activity was quantified. **C:** GD25 null and GD25-$\alpha_2\beta_1$ fibroblasts were cultured on polymerized collagen and PP2A activity was quantified. $^*P < 0.01$.

activity in cells treated with blocking antibody, compared with control; Figure 4A). In contrast, PP2A activity was low in IPF fibroblasts and did not significantly change in response to the α_2 or $\alpha_2\beta_1$ integrin-blocking antibody (Figure 4A).

To confirm that the level of $\alpha_2\beta_1$ integrin regulates PP2A activity, we knocked down β_1 integrin expression in control fibroblasts, using shRNA to β_1 integrin. The cells were then plated on polymerized collagen, and PP2A phosphatase activity was examined as a function of time. PP2A activity was 3.84-fold higher in control cells treated with control shRNA, compared with control cells in which β_1 integrin had been knocked down (Figure 4B), indicating that decreasing integrin expression reduces PP2A activation. Of note, we also overexpressed wild-type β_1 integrin in our IPF fibroblasts, as well as the V737N mutant β_1 integrin construct (autoclustered β_1 integrin).[29] Empty vector was used as a control. Overexpression of either of the β_1 integrin constructs was poorly tolerated by IPF fibroblasts, however, and we noted a significant loss of viability (data not shown). Cells treated with empty vector remained viable.

To further verify that the level of $\alpha_2\beta_1$ integrin regulates PP2A activity, we seeded GD25 null fibroblasts, which lack $\alpha_2\beta_1$ integrin, and GD25 fibroblasts reconstituted with $\alpha_2\beta_1$ integrin on polymerized collagen and examined PP2A activity. PP2A activity increased more than sevenfold when GD25-$\alpha_2\beta_1$ cells interacted with polymerized collagen, compared with GD25 null cells. GD25 null cells, which are devoid of $\alpha_2\beta_1$ integrin and adhere poorly to polymerized collagen, failed to activate PP2A (Figure 4C). Taken together, our $\alpha_2\beta_1$ integrin-blocking antibody studies, combined with our integrin knockdown studies using control fibroblasts, support the concept that diminished $\alpha_2\beta_1$ integrin expression in IPF fibroblasts results in a failure of these cells to appropriately activate PP2A in response to interaction with polymerized type I collagen.

Low PP2A Activity in IPF Fibroblasts Is Associated with High Levels of Phosphorylated (Inactive) GSK-3β and Active β-Catenin

PP2A phosphatase has been shown to target multiple substrate serine/threonine kinases for dephosphorylation.[30] Other studies[30] have suggested that PP2A can bind and dephosphorylate Akt. We had previously found that Akt was aberrantly activated in IPF fibroblasts in response to their interaction with collagen, whereas the level of phospho-Akt was low in control fibroblasts.[20] We therefore tested whether PP2A could interact with Akt in control fibroblasts during their interaction with polymerized collagen. Surprisingly, we were unable to detect a binding association of PP2A with Akt in our control fibroblasts (data not shown). We also analyzed the effect of the PP2A inhibitor okadaic acid on the level of Akt phosphorylation in control fibroblasts cultured on polymerized collagen. We did not detect a significant change in the level of phospho-Akt in control fibroblasts treated with the PP2A inhibitor (data not shown). Together, these data indicate that Akt is not a major substrate for PP2A in our primary control fibroblasts during their interaction with polymerized collagen.

The β-catenin pathway has been shown to be activated in IPF.[22,23] GSK-3β has also been shown to be a substrate for PP2A; when dephosphorylated, GSK-3β is active and inhibits β-catenin.[30] Next, therefore, we analyzed the level of phosphorylated (inactive) GSK-3β in IPF and control fibroblasts cultured on polymerized collagen as a function of time. Consistent with a failure of IPF fibroblasts to activate PP2A in response to interaction with collagen, we found that the level of phosphorylated (inactive) GSK was elevated in IPF fibroblasts, compared with control fibroblasts; In control fibroblasts, however, the level of GSK-3β correlated inversely with PP2A activity. As PP2A activity increased during control fibroblast interaction with collagen, the level of inactive GSK-3β decreased (Figure 5A). This suggested that inappropriately low PP2A phosphatase activity in IPF fibroblasts results in elevated levels of phosphorylated (inactive) GSK-3β.

When active, GSK-3β phosphorylates β-catenin, thereby targeting it for proteasomal degradation.[31] Because we have found that the level of inactive GSK-3β is high in IPF fibroblasts cultured on polymerized collagen, we expected that active β-catenin levels would also be high. Consistent with this idea, we found that the level of active β-catenin was elevated in IPF fibroblasts cultured on polymerized collagen, compared with control fibroblasts (Figure 5A). Furthermore, at day 3 of control fibroblast interaction with polymerized collagen, a time when PP2A activity is very high (Figure 3A), the levels of both inactive GSK-3β and active β-catenin were significantly depressed (Figure 5A). These data support the idea that de-

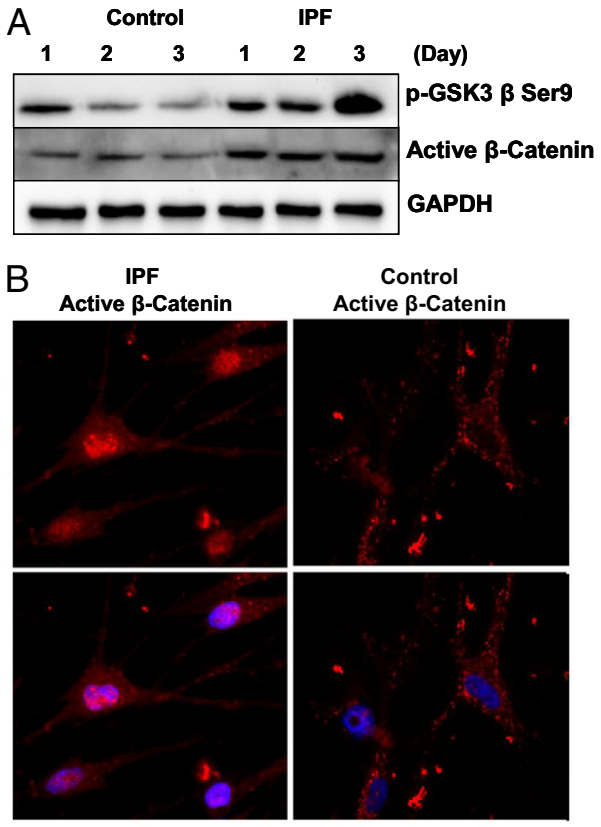

Figure 5. Low PP2A activity in IPF fibroblasts is associated with high levels of phosphorylated (inactive) GSK-3β and active β-catenin. **A:** Western blot analysis of phospho-GSK-3β and active β-catenin in IPF and control fibroblasts cultured on polymerized collagen matrix for up to 3 days. GAPDH was the loading control. **B:** IPF and control fibroblasts were cultured on coverslips coated with polymerized collagen and the location of active β-catenin (red) was examined by immunofluorescent microscopic analysis. DAPI (blue) was used as a nuclear counterstain. Original magnification, ×400.

creased $\alpha_2\beta_1$ integrin levels result in low PP2A activity in IPF fibroblasts, which then leads to inactivation of GSK-3β and consequently elevated levels of active β-catenin.

We also examined the location of active β-catenin in IPF and control fibroblasts cultured on polymerized collagen by immunofluorescent analysis. Consistent with its function as a transcription factor, active β-catenin was present in the nuclei of IPF fibroblasts cultured on collagen. In contrast, little or no nuclear β-catenin could be detected in control fibroblasts (Figure 5B). These data are consistent with reports indicating high levels of active β-catenin in IPF.[22,23]

Inhibition of $\alpha_2\beta_1$ Integrin and Knockdown of β_1 Integrin Increases the Level of Phosphorylated GSK-3β and Active β-Catenin in Control Fibroblasts

Expression of $\alpha_2\beta_1$ integrin and activity levels of PP2A are low in IPF fibroblasts, and the level of inactive or phosphorylated GSK-3β is high. We found the opposite in control fibroblasts, in which $\alpha_2\beta_1$ integrin expression and PP2A activity levels are relatively high but the level of phosphorylated GSK-3β is low. This suggested a link between integrin expression/function and phospho-GSK-3β levels. To analyze the role of $\alpha_2\beta_1$ integrin in regulating GSK-3β and β-catenin activity in IPF and control fibroblasts, we used the α_2 integrin function-blocking antibody. Inhibition of $\alpha_2\beta_1$ function by the blocking antibody augmented the expression of phosphorylated (inactive) GSK-3β expression and increased the level of active β-catenin in control fibroblasts during interaction with polymerized collagen (Figure 6A). Inhibition of $\alpha_2\beta_1$ function by the blocking antibody did not appreciably alter the level of either inactive GSK-3β expression or active β-catenin in IPF fibroblasts, presumably because $\alpha_2\beta_1$ integrin expression is very low in IPF fibroblasts, resulting in a failure of these cells to appropriately activate PP2A. To verify that integrin expression regulates the level of phosphorylated GSK-3β in our primary fibroblasts, we next examined the effect of knockdown of β_1 integrin on the level of phosphorylated GSK-3β and active β-catenin in control fibroblasts. Knockdown decreased the level of total β_1 integrin (Figure 6B). In response to knockdown of β_1 integrin, the level of phospho-GSK-3β was increased, compared with control fibroblasts treated with control shRNA (Figure 6B). Consistent with the result that knockdown of β_1 integrin facilitates inactivation of GSK-3β, knockdown also resulted in augmentation of the level of

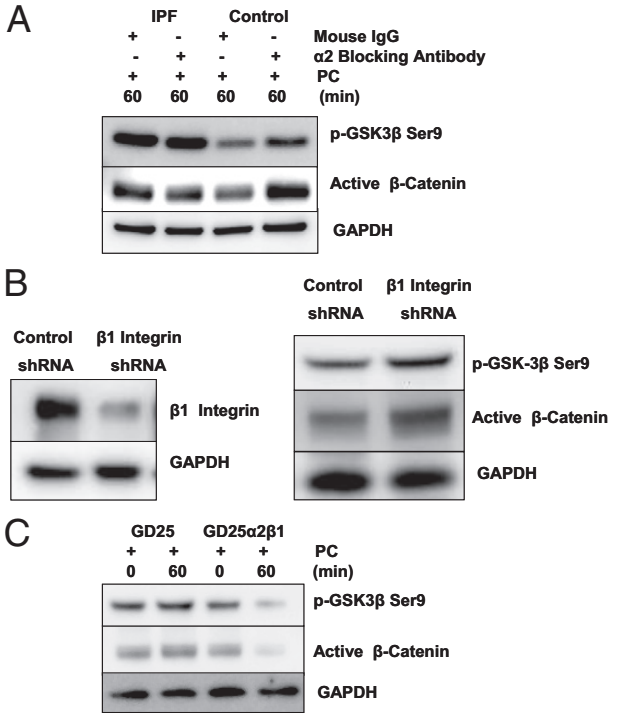

Figure 6. Inhibition of $\alpha_2\beta_1$ integrin and knockdown of β_1 integrin increases the level of phosphorylated GSK-3β and active β-catenin in control fibroblasts. **A:** IPF and control fibroblasts were plated on polymerized collagen (PC) in the presence of α_2 integrin-blocking antibody or isotype control antibody, and the levels of phosphorylated GSK-3β and active β-catenin were examined by Western blot analysis at 60 minutes. **B:** Control fibroblasts were infected with a lentiviral vector containing shRNA to β_1 integrin or control shRNA. The cells were cultured on polymerized collagen, and the levels of total and active β_1 integrin (**left**) and phosphorylated GSK-3β and active β-catenin (**right**) were examined by Western blot analysis at day 3. GAPDH was the loading control. **C:** GD25 null and GD25-$\alpha_2\beta_1$ fibroblasts were cultured on polymerized collagen and the levels of phosphorylated GSK-3β and active β-catenin were examined by Western blot analysis.

active β-catenin (Figure 6B). These data indicate that decreasing β_1 integrin function results in the phosphorylation and inactivation of GSK-3β, thereby augmenting β-catenin activity.

To confirm that the level of $\alpha_2\beta_1$ regulates the levels of phospho-GSK-3β and active β-catenin in response to fibroblast interaction with collagen, we seeded GD25 null cells lacking $\alpha_2\beta_1$ integrin and GD25 cells reconstituted with $\alpha_2\beta_1$ integrin on polymerized collagen and examined phospho-GSK-3β and active β-catenin levels. We found that the levels of phospho-GSK-3β (inactive) and active β-catenin were high in GD25 cells lacking $\alpha_2\beta_1$, compared with GD25 cells reconstituted with $\alpha_2\beta_1$ integrin (Figure 6C). Taken together, these findings strongly support the idea that, when IPF fibroblasts interact with collagen, low $\alpha_2\beta_1$ integrin expression fails to activate PP2A, leading to higher levels of inactive or phosphorylated GSK-3 and high levels of active β-catenin.

Knockdown of PP2Ac in Control Fibroblasts Increases Phospho-GSK-3β Levels

To examine the effect of PP2A activity on GSK-3β phosphorylation, we inhibited PP2A activity in control fibroblasts cultured on collagen using the PP2A inhibitor okadaic acid. Inhibition of PP2A activity by okadaic acid dramatically increased the level of phosphorylated GSK-3β in control fibroblasts and enhanced the level of active β-catenin (Figure 7A). To verify this result, we knocked down the catalytic subunit of PP2A using shRNA to the PP2Acα subunit and examined the effect on the level of phospho-GSK-3β. Knockdown of PP2Ac resulted in increased levels of phosphorylated GSK-3β in control fibroblasts (Figure 7B). Taken together, these results strongly suggest that low $\alpha_2\beta_1$ integrin function reduces PP2A activity, which results in higher levels of phosphorylated (inactive) GSK-3β. We also overexpressed the catalytic subunit of PP2A in our IPF fibroblasts, to examine the effect on GSK-3β. Notably, similar to overexpression of wild-type β_1 integrin or autoclustered β_1 integrin, overexpression of PP2Ac in our IPF fibroblasts resulted in a substantial loss of viability, suggesting that up-regulation of the $\alpha_2\beta_1$ integrin/PP2A pathway is not tolerated in IPF fibroblasts. IPF fibroblasts treated with empty vector behaved similarly to untreated cells.

Knockdown of β_1 Integrin and PP2Ac in Control Fibroblasts, as Well as Inhibition of GSK-3β, Enhances Their Proliferation on Polymerized Collagen

Control fibroblast proliferation is suppressed by polymerized collagen.[6–8] We have shown that knockdown of β_1 integrin in control fibroblasts decreases PP2A activity while increasing the level of inactive GSK-3β and the level of active β-catenin. We therefore analyzed the effect of knockdown of β_1 integrin on control fibroblast proliferation on polymerized collagen. Knockdown of β_1 integrin induced a modest 26% increase in the proliferation of control fibroblasts on polymerized collagen at day 3, compared with cells treated with control shRNA (Figure 8A). This may be due to alterations in cell adhesion due to the reduction in β_1 integrin levels. Because we have found that PP2A activity is robustly induced when control fibroblasts interact with polymerized collagen and that this results in increased levels of active GSK-3β, we next knocked down PP2Ac by shRNA and examined the effect on control fibroblast proliferation. The proliferation of control fibroblasts in which PP2Ac was knocked down was markedly increased, by 51%, compared with cells treated with control shRNA (Figure 8B). These data indicate that a reduction in β_1 integrin or PP2A levels augments control fibroblast proliferation on polymerized collagen, replicating the behavior of IPF fibroblasts on polymerized collagen. Note, however, that because overexpression of our β_1 integrin constructs in IPF fibroblasts was not tolerated, we were unable to examine the effect of overexpression of β_1 integrin on IPF fibroblast proliferation on collagen.

Our data indicate that, when cultured on collagen, control fibroblasts display higher amounts of active GSK-3β and their proliferation is suppressed to a greater extent, compared with IPF fibroblasts, suggesting that

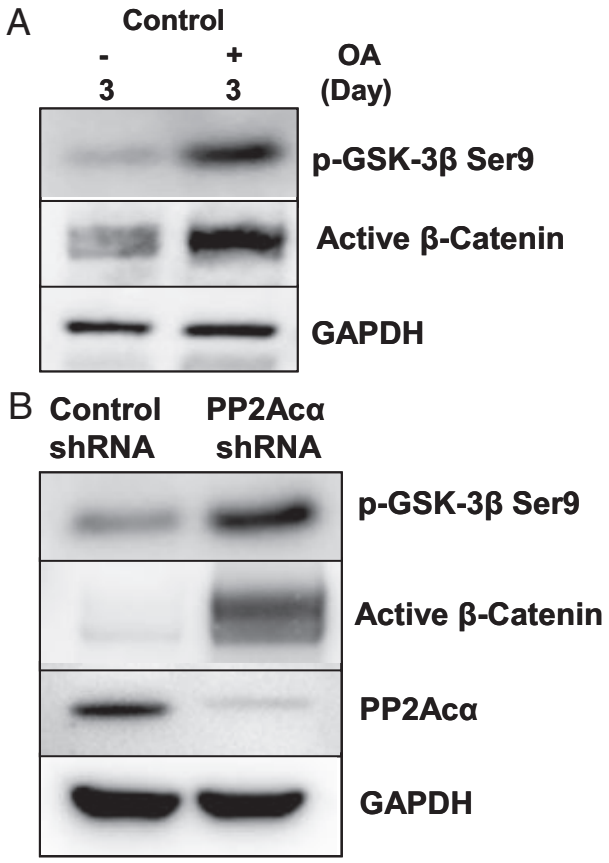

Figure 7. Knockdown of PP2Ac in control fibroblasts increases phospho-GSK-3β levels. **A:** Control fibroblasts were pretreated with 20 μmol okadaic acid (OA) or control vehicle and then were cultured on polymerized collagen for up to 3 days. The levels of phosphorylated GSK-3β and active β-catenin were examined by Western blot analysis. **B:** Control fibroblasts were infected with a lentiviral vector containing shRNA to the catalytic α subunit of PP2A (PP2Acα) or control shRNA. The cells were then cultured on polymerized collagen and the levels of phosphorylated GSK-3β and active β-catenin were examined by Western blot analysis.

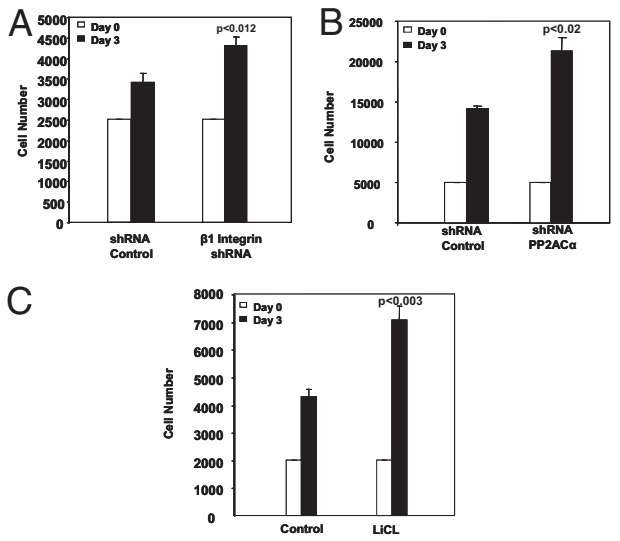

Figure 8. Knockdown of β_1 integrin or PP2Ac and inhibition of GSK-3β in control fibroblasts enhances their proliferation on polymerized collagen. **A:** Control fibroblasts were infected with a lentiviral vector containing shRNA to β_1 integrin or control shRNA. The cells were then cultured on polymerized collagen for 3 days, at which time cell numbers were quantified. **B:** Control fibroblasts were infected with a lentiviral vector containing shRNA to PP2Ac or control shRNA and cultured on polymerized collagen. Cell numbers were quantified at day 3. **C:** Control fibroblasts were cultured on polymerized collagen matrix in DMEM with 10% fetal calf serum containing 20 mmol/L LiCl or vehicle control (sterile water). Cell numbers were quantified at day 3.

GSK-3β inhibition of β-catenin limits control fibroblast proliferation. We therefore examined the effect of inhibition of GSK-3β by LiCl on control fibroblast proliferation on polymerized collagen. Inhibition of GSK-3β by LiCl increased control fibroblast proliferation by approximately threefold, compared with cells treated with vehicle control (Figure 8C). These data, combined with data from our knockdown studies of β_1 integrin and PP2Ac, support the idea that, in control fibroblasts, high levels of $\alpha_2\beta_1$ integrin activate PP2A and increase the level of active GSK-3β. Active GSK-3β reduces active β-catenin, thereby suppressing proliferation.

Knockdown of β-Catenin in IPF Fibroblasts Decreases Their Ability to Proliferate on Polymerized Collagen

Our studies suggest that pathologically low $\alpha_2\beta_1$ integrin results in activation of the β-catenin proliferation signal pathway and that aberrantly high levels of β-catenin facilitate the ability of IPF fibroblasts to elude the proliferation-suppressive properties of polymerized collagen. To investigate this possibility further, we knocked down β-catenin in IPF fibroblasts using β-catenin shRNA and examined their proliferative ability on polymerized collagen. The level of β-catenin in IPF fibroblasts treated with β-catenin shRNA was reduced, compared with cells treated with control shRNA (Figure 9A). Importantly, knockdown of β-catenin reduced the proliferative capacity of IPF fibroblasts cultured on polymerized collagen by 46%, compared with control shRNA (Figure 9B). These data support the hypothesis that activation of β-catenin by deficient $\alpha_2\beta_1$ integrin function enhances IPF fibroblast proliferation on collagen.

Discussion

The pathogenesis of IPF remains incompletely understood. An ongoing question in IPF concerns the unrelenting nature of the fibroproliferative response. A current paradigm for IPF suggests that injury to the epithelium generates a pathological fibroproliferative response.[32–34] During normal tissue repair after injury, fibroproliferation is induced but then rapidly abates. In IPF, however, the fibroproliferative process never appears to abate. Progress in our understanding of this lethal disease and the development of effective medical strategies to combat it depends on an improved understanding of this relentless fibroproliferative process.

In an attempt to understand this progressive fibrotic disease, we have focused our investigations on the fibroproliferative process itself and on its key cellular constituent, the myofibroblast. Several studies indicate that the IPF fibroblast displays a distinct pathological phenotype, one that shares several features in common with the cancer-associated fibroblast phenotype.[35–38] We have found that the IPF fibroblast, similar to cancer-associated fibroblasts, displays low caveolin-1 expression and low PTEN function, resulting in aberrant activation of the PI3K/Akt/S6K1 signal pathway.[20,21]

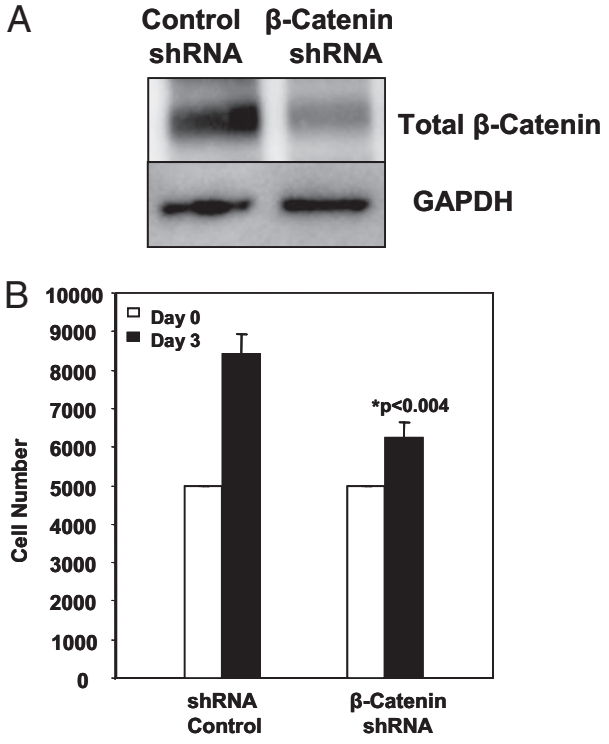

Figure 9. Knockdown of β-catenin in IPF fibroblasts decreases their ability to proliferate on polymerized collagen. IPF fibroblasts were infected with a lentiviral vector containing shRNA to β-catenin or control shRNA and cultured on polymerized collagen matrix in DMEM with 10% fetal calf serum for 3 days. **A:** Western blot analysis of β-catenin levels in IPF fibroblasts treated with β-catenin shRNA or control shRNA. **B:** Cell numbers were quantified at day 3.

Our present findings support the concept that the IPF fibroblast has acquired a distinct phenotype characterized by its ability to proliferate in a type I collagen proliferation-suppressive environment. We have discovered that $\alpha_2\beta_1$ integrin expression is abnormally reduced in IPF fibroblasts and that this altered integrin function confers these cells with their pathological proliferative response. Previous work by Heino and colleagues[24] established that, during cell adhesion to polymerized type I collagen, $\alpha_2\beta_1$ integrin (which is a major collagen receptor) induces activation of the PP2A phosphatase. Here, we have demonstrated that low $\alpha_2\beta_1$ integrin expression is a characteristic of the IPF fibroblast phenotype. Our data support the concept that the pathological decrease in $\alpha_2\beta_1$ integrin expression in IPF fibroblasts results in a failure of the integrin to properly activate PP2A phosphatase. In addition, we show that, during IPF fibroblast interaction with collagen, PP2A activity remains inappropriately low. In contrast, when control fibroblasts interact with collagen, PP2A is induced, with levels rising more than twofold. Importantly, knockdown of β_1 integrin or inhibition of $\alpha_2\beta_1$ by blocking antibody in control fibroblasts impairs the ability of these cells to activate PP2A when interacting with polymerized collagen, mimicking IPF fibroblast behavior. Furthermore, we demonstrate that GD25 null cells, which are devoid of $\alpha_2\beta_1$, are unable to activate PP2A, whereas GD25 cells reconstituted with $\alpha_2\beta_1$ appropriately activate PP2A in response to interaction with polymerized collagen. Note that, similar to IPF fibroblasts, GD25 cells do not activate PP2A, because they are devoid of $\alpha_2\beta_1$ integrin. Unlike IPF fibroblasts, however, GD25 cells display decreased proliferation on polymerized collagen, despite reduced PP2A activity, because their complete lack of $\alpha_2\beta_1$ results in a markedly impaired ability to attach to polymerized collagen. In contrast, despite reduced $\alpha_2\beta_1$, IPF fibroblasts readily attach to polymerized collagen and, because of activation of the β-catenin pathway, they demonstrate the ability to elude the proliferation-suppressive effects of polymerized collagen.

PP2A is a serine/threonine phosphatase that has been shown to target multiple serine/threonine kinases including Akt and GSK-3β.[30] Our data indicate that a major PP2A target in our IPF and control fibroblasts is GSK-3β. Previous studies have identified Akt as a PP2A substrate.[30] We have previously shown that Akt is abnormally activated in IPF, and the present work indicates that IPF fibroblasts display low PP2A activity.[20] Thus, Akt could be elevated because of a failure of the integrin to activate PP2A. However, our present work suggest that this is not the case. We have been unable to detect direct interaction of PP2A with Akt in control fibroblasts, and treatment of control fibroblasts with the PP2A inhibitor okadaic acid did not augment the level of phospho-Akt. Furthermore, our prior studies indicate that inappropriately low PTEN function is responsible for activation of the PI3K/Akt signal pathway in IPF fibroblasts.[20] However the strongest indications that GSK-3β is a major PP2A target come from our present results: i) the consequence of inappropriately low PP2A phosphatase activity in our IPF fibroblasts was an abnormally high level of phosphorylated (inactive) GSK-3β resulting in aberrantly high levels of β-catenin and pathological proliferation (Figure 10); ii) inhibition of PP2A in control fibroblasts increased the levels of phosphorylated GSK-3β and β-catenin; and iii) knockdown of integrin in our control fibroblasts also increased the level of both inactive GSK-3β and active β-catenin.

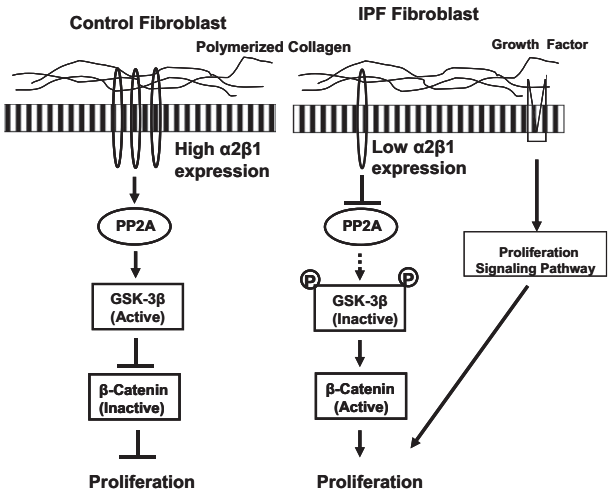

Figure 10. Schematic of a working model for integrin regulation of IPF fibroblast proliferation. **Left:** In response to control fibroblast interaction with fibrillar collagen, high $\alpha_2\beta_1$ integrin expression activates PP2A, promoting dephosphorylation and activation of its target substrate GSK-3β. This causes inactivation of β-catenin and suppression of proliferation. **Right:** During IPF fibroblast interaction with fibrillar collagen, low $\alpha_2\beta_1$ integrin expression fails to appropriately activate PP2A. This causes an accumulation of phosphorylated (inactive) GSK-3β promoting activation of β-catenin and pathological proliferation. Note that data from additional studies[32,33] indicate that certain growth factors, such as TGFβ, also activate proliferation signaling pathways in IPF fibroblasts Thus, available data indicate that a collaboration between matrix and growth factors controls the abnormal proliferation of IPF fibroblasts.

Our present findings indicate that abnormal integrin function leads to a pathological pattern of integrin signaling, resulting in aberrant activation of the β-catenin pathway. Furthermore, we have found that IPF fibroblasts have eluded the proliferation-suppressive effects of polymerized collagen. We therefore sought to examine the consequence of the altered integrin/PP2A axis on proliferation. Because $\alpha_2\beta_1$ expression and PP2A activity are reduced in IPF, compared with control fibroblasts, we chose a knockdown approach, to more closely model this difference in integrin expression and PP2A activity between IPF and control fibroblasts and to assess the role of the $\alpha_2\beta_1$/PP2A axis in regulating proliferation. We avoided complete knockdown of β_1 integrin, so that the cells could still effectively adhere to collagen. We also specifically avoided the use of integrin-blocking antibodies in our attempt to model the effect of reduced integrin expression on proliferation, because blocking antibodies disrupt adhesion and as a consequence disrupt proliferation. We found that reducing β_1 integrin expression or PP2A levels resulted in inactivation of GSK-3β and increased levels of active β-catenin, as well as augmented ability of control fibroblasts to proliferate on polymerized collagen, thereby mimicking IPF fibroblast behavior. Importantly, we show here that the knockdown of β-catenin in

IPF fibroblasts suppressed their ability to proliferate on polymerized collagen. We suggest that this aberrant integrin function will be fundamental to understanding the relentless nature of this prototypical fibroproliferative disease.

A critical remaining issue for future studies will be to delineate the molecular mechanism underlying the pathologically low $\alpha_2\beta_1$ integrin expression and activation in IPF fibroblasts. Another question to be addressed is whether abnormally low $\alpha_2\beta_1$ integrin function results in the compensatory increase of other integrin receptor functions. Whether there is compensatory alteration in other matrix receptors that contribute to pathological proliferation signaling remains unclear at this time. An additional question surrounds the origin of the pathological IPF fibroblast. Experimental evidence suggests that IPF fibroblasts may arise from at least three possible sources: resident fibroblasts, epithelial cells that have undergone epithelial to mesenchymal transition, and/or circulating fibrocytes recruited to the lung from the bone marrow.[39–45] Additional studies are needed to determine the origin of IPF fibroblasts that display this pathological integrin function.

Acknowledgment

We thank Dr. Valerie Weaver (University of California, San Francisco) for supplying the wild-type and autoclustered β_1 integrin constructs.

References

1. Kuhn C 3rd, McDonald JA: The roles of the myofibroblast in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular matrix synthesis. Am J Pathol 1991, 138:1257–1265
2. American Thoracic Society (ATS), European Respiratory Society (ERS): Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. Am J Respir Crit Care Med 2000, 161:646–664
3. Basset F, Ferrans VJ, Soler P, Takemura T, Fukuda Y, Crystal RG: Intraluminal fibrosis in interstitial lung disorders. Am J Pathol 1986, 122:443–461
4. Fukuda Y, Ishizaki M, Masuda Y, Kimura G, Kawanami O, Masugi Y: The role of intraalveolar fibrosis in the process of pulmonary structural remodeling in patients with diffuse alveolar damage. Am J Pathol 1987, 126:171–182
5. Kuhn C 3rd, Boldt J, King TE Jr, Crouch E, Vartio T, McDonald JA: An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. Am Rev Respir Dis 1989, 140:1693–1703
6. Koyama H, Raines EW, Bornfeldt KE, Roberts JM, Ross R: Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors. Cell 1996, 87:1069–1078
7. Schor SL: Cell proliferation and migration on collagen substrata in vitro. J Cell Sci 1980, 41:159–175
8. Rhudy RW, McPherson JM: Influence of the extracellular matrix on the proliferative response of human skin fibroblasts to serum and purified platelet-derived growth factor. J Cell Physiol 1988, 137:185–191
9. Ruoslahti E: Integrin signaling and matrix assembly. Tumour Biol 1996, 17:117–124
10. Khwaja A, Rodriguez-Viciana P, Wennström S, Warne PH, Downward J: Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J 1997, 16:2783–2793
11. Lee JW, Juliano RL: alpha5beta1 integrin protects intestinal epithelial cells from apoptosis through a phosphatidylinositol 3-kinase and protein kinase B-dependent pathway. Mol Biol Cell 2000, 11:1973–1987
12. Jokinen J, Dadu E, Nykvist P, Käpylä J, White DJ, Ivaska J, Vehviläinen P, Reunanen H, Larjava H, Häkkinen L, Heino J: Integrin-mediated cell adhesion to type I collagen fibrils. J Biol Chem 2004, 279:31956–31963
13. Tian B, Lessan K, Kahm J, Kleidon J, Henke CA: beta 1 integrin regulates fibroblast viability during collagen matrix contraction through a phosphatidylinositol 3-kinase Akt/protein kinase B signaling pathway. J Biol Chem 2002, 277:24667–24675
14. Xia H, Nho RS, Kahm J, Kleidon J, Henke CA: Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta 1 integrin viability signaling pathway. J Biol Chem 2004, 279:33024–33034
15. Nho RS, Xia H, Kahm J, Kleidon J, Diebold D, Henke CA: Role of integrin-linked kinase in regulating phosphorylation of Akt and fibroblast survival in type I collagen matrices through a beta1 integrin viability signaling pathway. J Biol Chem 2005, 280:26630–26639
16. Downward J: Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 1998, 10:262–267
17. Chicurel ME, Singer RH, Meyer CJ, Ingber DE: Integrin binding and mechanical tension induce movement of mRNA and ribosomes to focal adhesions. Nature 1998, 392:730–733
18. Chung JC, Bachelder RE, Lipscomb EA, Shaw LM, Mercurio AM: Integrin (alpha 6 beta 4) regulation of eIF-4E activity and VEGF translation: a survival mechanism for carcinoma cells. J Cell Biol 2002, 158:165–174
19. Zhao JJ, Gjoerup OV, Subramanian RR, Cheng Y, Chen W, Roberts TM, Hahn WC: Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell 2003, 3:483–495
20. Xia H, Diebold D, Nho R, Perlman D, Kahm J, Kleidon J, Avdulov A, Peterson M, Bitterman PB, Henke C: Pathological integrin signaling enhances proliferation of primary lung fibroblasts from patients with idiopathic pulmonary fibrosis. J Exp Med 2008, 205:1659–1672
21. Xia H, Khalil W, Kahm J, Jessurun J, Kleidon J, Henke CA: Pathologic caveolin-1 regulation of PTEN in idiopathic pulmonary fibrosis. Am J Pathol 2010, 176:2626–2637
22. Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, Piccoli P, Pedron S, Bertaso M, Scarpa A, Murer B, Cancellieri A, Maestro R, Semenzato G, Doglioni C: Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. Am J Pathol 2003, 162:1495–1502
23. Königshoff M, Balsara N, Pfaff EM, Kramer M, Chrobak I, Seeger W, Eickelberg O: Functional Wnt signaling is increased in idiopathic pulmonary fibrosis. PLoS One 2008, 3:e2142
24. Ivaska J, Nissinen L, Immonen N, Eriksson JE, Kähäri VM, Heino J: Integrin alpha 2 beta 1 promotes activation of protein phosphatase 2A and dephosphorylation of Akt and glycogen synthase kinase 3 beta. Mol Cell Biol 2002, 22:1352–1359
25. American Thoracic Society; European Respiratory Society. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001 [Erratum appeared in Am J Respir Crit Care Med 2002, 166:426]. Am J Respir Crit Care Med 2002, 165:277–304
26. Chen B, Polunovsky V, White J, Blazar B, Nakleh R, Jessurun J, Peterson M, Bitterman P: Mesenchymal cells isolated after acute lung injury manifest an enhanced proliferative phenotype. J Clin Invest 1992, 90:1778–1785
27. Damjanovich L, Albelda SM, Mette SA, Buck CA: Distribution of integrin cell adhesion receptors in normal and malignant lung tissue. Am J Respir Cell Mol Biol 1992, 6:197–206
28. Virtanen I, Laitinen A, Tani T, Pääkkö P, Laitinen LA, Burgeson RE, Lehto VP: Differential expression of laminins and their integrin receptors in developing and adult human lung. Am J Respir Cell Mol Biol 1996, 15:184–196
29. Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, Fong SF, Csiszar K, Giaccia A, Weninger W, Yamauchi M, Gasser DL, Weaver VM: Matrix crosslinking forces tumor progression by enhancing integrin signaling. Cell 2009, 139:891–906
30. Virshup DM: Protein phosphatase 2A: a panoply of enzymes. Curr Opin Cell Biol 2000, 12:180–185

31. Behrens J, Jerchow BA, Würtele M, Grimm J, Asbrand C, Wirtz R, Kühl M, Wedlich D, Birchmeier W: Functional interaction of an axin homolog, conductin, with beta catenin, APC, and GSK3beta. Science 1998, 280:596–599
32. Selman M, King TE, Pardo A; American Thoracic Society; European Respiratory Society; American College of Chest Physicians: Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med 2001, 134:136–151
33. Noble PW, Homer RJ: Idiopathic pulmonary fibrosis: new insights into pathogenesis. Clin Chest Med 2004, 25:749–758, vii
34. Noble PW: Idiopathic pulmonary fibrosis: natural history and prognosis. Clin Chest Med 2006, 27(1 Suppl 1):S11–S16, v
35. Bhowmick NA, Neilson EG, Moses HL: Stromal fibroblasts in cancer initiation and progression. Nature 2004, 432:332–337
36. Sotgia F, Del Galdo F, Casimiro MC, Bonuccelli G, Mercier I, Whitaker-Menezes D, Daumer KM, Zhou J, Wang C, Katiyar S, Xu H, Bosco E, Quong AA, Aronow B, Witkiewicz AK, Minetti C, Frank PG, Jimenez SA, Knudsen ES, Pestell RG, Lisanti MP: Caveolin-1-/- null mammary stromal fibroblasts share characteristics with human breast cancer-associated fibroblasts. Am J Pathol 2009, 174:746–761
37. Mercier I, Casimiro MC, Wang C, Rosenberg AL, Quong J, Minkeu A, Allen KG, Danilo C, Sotgia F, Bonuccelli G, Jasmin JF, Xu H, Bosco E, Aronow B, Witkiewicz A, Pestell RG, Knudsen ES, Lisanti MP: Human breast cancer-associated fibroblasts (CAFs) show caveolin-1 down-regulation and RB tumor suppressor functional inactivation: implications for the response to hormonal therapy. Cancer Biol Ther 2008, 7:1212–1225
38. Trimboli AJ, Cantemir-Stone CZ, Li F, Wallace JA, Merchant A, Creasap N, Thompson JC, Caserta E, Wang H, Chong JL, Naidu S, Wei G, Sharma SM, Stephens JA, Fernandez SA, Gurcan MN, Weinstein MB, Barsky SH, Yee L, Rosol TJ, Stromberg PC, Robinson ML, Pepin F, Hallett M, Park M, Ostrowski MC, Leone G: Pten in stromal fibroblasts suppresses mammary epithelial tumours. Nature 2009, 461:1084–1091
39. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, du Bois RM, Borok Z: Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis. Am J Pathol 2005, 166:1321–1332
40. Willis BC, du Bois RM, Borok Z: Epithelial origin of myofibroblasts during fibrosis in the lung. Proc Am Thorac Soc 2006, 3:337–382
41. Willis BC, Borok Z: TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol 2007, 293:L525–L534
42. Kim KK, Kugler MC, Wolters PJ, Robillard L, Galvez MG, Brumwell AN, Sheppard D, Chapman HA: Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci USA 2006, 103:13180–13185
43. Tanjore H, Xu XC, Polosukhin VV, Degryse AL, Li B, Han L, Sherril TP, Plieth D, Neilson EG, Blackwell TS, Lawson WE: Contribution of epithelial-derived fibroblasts to bleomycin-induced lung fibrosis. Am J Respir Crit Care Med 2009, 180:657–665
44. Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB: CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. Am J Pathol 2005, 166:675–684
45. Moeller A, Gilpin SE, Ask K, Cox G, Cook D, Gauldie J, Margetts PJ, Farkas L, Dobranowski J, Boylan C, O'Byrne PM, Strieter RM, Kolb M: Circulating fibrocytes are an indicator of poor prognosis in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2009, 179:588–594