Pathological integrin signaling enhances proliferation of primary lung fibroblasts from patients with idiopathic pulmonary fibrosis

Hong Xia, Deanna Diebold, Richard Nho, David Perlman, Jill Kleidon, Judy Kahm, Svetlana Avdulov, Mark Peterson, John Nerva, Peter Bitterman, and Craig Henke

Department of Medicine, University of Minnesota, Minneapolis, MN 55455

The Journal of Experimental Medicine

Idiopathic pulmonary fibrosis (IPF) is a relentlessly progressive lung disease in which fibroblasts accumulate in the alveolar wall within a type I collagen–rich matrix. Although lung fibroblasts derived from patients with IPF display durable pathological alterations in proliferative function, the molecular mechanisms differentiating IPF fibroblasts from their normal counterparts remain unknown. Polymerized type I collagen normally inhibits fibroblast proliferation, providing a physiological mechanism to limit fibroproliferation after tissue injury. We demonstrate that β1 integrin interaction with polymerized collagen inhibits normal fibroblast proliferation by suppression of the phosphoinositide 3-kinase (PI3K)–Akt–S6K1 signal pathway due to maintenance of high phosphatase activity of the tumor suppressor phosphatase and tensin homologue (PTEN). In contrast, IPF fibroblasts eluded this restraint, displaying a pathological pattern of β1 integrin signaling in response to polymerized collagen that leads to aberrant activation of the PI3K–Akt–S6K1 signal pathway caused by inappropriately low PTEN activity. Mice deficient in PTEN showed a prolonged fibroproliferative response after tissue injury, and immunohistochemical analysis of IPF lung tissue demonstrates activation of Akt in cells within fibrotic foci. These results provide direct evidence for defective negative regulation of the proliferative pathway in IPF fibroblasts and support the theory that the pathogenesis of IPF involves an intrinsic fibroblast defect.

CORRESPONDENCE
Craig A. Henke:
henke002@umn.edu

Abbreviations used: EMT, epithelial-mesenchymal transition; FAK, focal adhesion kinase; IPF, idiopathic pulmonary fibrosis; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue; RLUC, *Renilla reniformis* luciferase.

Idiopathic pulmonary fibrosis (IPF) is a chronic, lethal interstitial lung disease of unknown cause. The disease is characterized by scattered fibrotic lesions in various stages of progression (1). The sentinel morphological lesion is the fibroblastic focus, which is composed of fibroblasts in a type I collagen–rich matrix (2). Evidence for the critical role of the fibroblast in the relentless progression of IPF is prima facie: this is the cell that proliferates and deposits type I collagen in the alveolar wall, distorting alveolar architecture and impairing gas exchange (2–5). Although recent studies support the theory that IPF fibroblasts harbor intrinsic alterations of key components of their cellular machinery, large gaps in knowledge remain regarding the mechanisms conferring IPF fibroblasts with the capacity to proliferate and persist in a setting where normal fibroblasts display the tightly regulated proliferation essential for physiological repair (6–12).

The molecular mechanisms regulating fibroblast proliferation on collagen matrices are coming into focus. Seminal studies in the 1980s and 1990s demonstrated that polymerized (fibrillar) collagen, the natural form of type I collagen found in tissues, acts as a negative regulator of fibroblast proliferation, whereas monomeric collagen supports fibroblast proliferation (13–15). On the basis of this work, fibroblast interaction with polymerized collagen is believed to provide an important physiological mechanism to limit fibroproliferation after tissue injury. Integrins mediate cellular interactions with the extracellular matrix and are at the apex of signaling pathways that modulate key components of the cellular proliferative machinery (16–26). Fibroblast interactions with type I collagen are mediated predominantly by the α2β1 integrin (19). In this study, we demonstrate that β1 integrin–polymerized collagen interaction in normal fibroblasts activates the negative growth regulator, tumor suppressor phosphatase and tensin

H. Xia and D. Diebold contributed equally to this paper.

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 205 No. 7 1659–1672 www.jem.org/cgi/doi/10.1084/jem.20080001

homologue (PTEN), whereas in IPF fibroblasts this negative feedback mechanism is defective, enabling these cells to circumvent the negative proliferative effects of polymerized collagen.

RESULTS

IPF fibroblasts display enhanced proliferation on polymerized collagen matrices

Previous studies demonstrate that polymerized collagen inhibits smooth muscle cell and fibroblast proliferation, whereas proliferation is supported on monomeric collagen (13–15). IPF is characterized by the proliferation of fibroblasts in fibrotic foci that contain bundles of polymerized type I collagen (2–5). We therefore examined the integrity of this negative feedback loop in IPF fibroblasts. Compared with monomeric collagen, polymerized collagen markedly inhibited control fibroblast proliferation. As controls, we used lung fibroblasts derived from histologically normal lungs and fibroblasts derived from diseased lung tissue from a patient with chronic obstructive pulmonary disease. In contrast, polymerized collagen was much less effective in inhibiting IPF fibroblast proliferation than that of control fibroblasts, as assessed by cell number (Fig. 1 A) and BrdU incorporation (Fig. 1 B). These data unveil a relaxation of negative growth control in IPF fibroblasts.

Unlike IPF, fibroblast proliferation is self-limited during normal wound repair. To determine whether IPF fibroblast proliferative properties were similar to or distinct from wound fibroblasts, we examined wound fibroblast proliferation on collagen matrices. Because primary human wound fibroblasts were unavailable, we used mouse cutaneous wound fibroblasts. Similar to normal fibroblasts, wound fibroblast proliferation was inhibited by polymerized collagen (Fig. 1 C). These data suggest that the IPF fibroblast phenotype is distinct from the wound fibroblast phenotype.

Exposure of normal fibroblasts to TGF-β for 24–48 h promotes differentiation to a myofibroblast phenotype (27). We cultured normal lung fibroblasts with TGF-β for 3–6 d and examined their proliferation on type I collagen matrices. Treatment of normal fibroblasts with TGF-β inhibited their ability to proliferate on both monomeric and polymerized collagen (Fig. 1 D). These data indicate that exposure of normal lung fibroblasts to TGF-β for 3–6 d does not confer normal fibroblasts with IPF fibroblast proliferative properties.

To determine if this impairment of negative growth regulation in IPF fibroblasts was robust, we examined proliferation on polymerized collagen in defined media containing peptide growth factors. Compared with control fibroblasts, IPF fibroblasts displayed a significantly enhanced proliferative response (Fig. 1 E). These data indicate that IPF fibroblasts robustly resist the negative proliferative effects of polymerized collagen.

Phosphoinositide 3-kinase (PI3K)–Akt–S6K1 signaling in control fibroblasts

To provide a context for our studies of IPF, we examined key signaling events regulating control fibroblast proliferation on type I collagen. Our previous work strongly links fibroblast responses to α2β1 integrin–collagen interaction with PI3K–Akt signal pathway function (20–22). We examined activation of the PI3K–Akt pathway in response to interaction with collagen in both short-term adhesion and long-term proliferation assays. When serum-starved control fibroblasts attach to monomeric collagen, the level of Akt phosphorylation increases in a time- and PI3K-dependent fashion, along with phosphorylation of the downstream kinase S6K1 (Fig. 2 A).

The α2β1 integrin is a major type I collagen receptor (19). To assess the importance of α2β1 in mediating activation of Akt in response to fibroblast attachment to monomeric collagen,

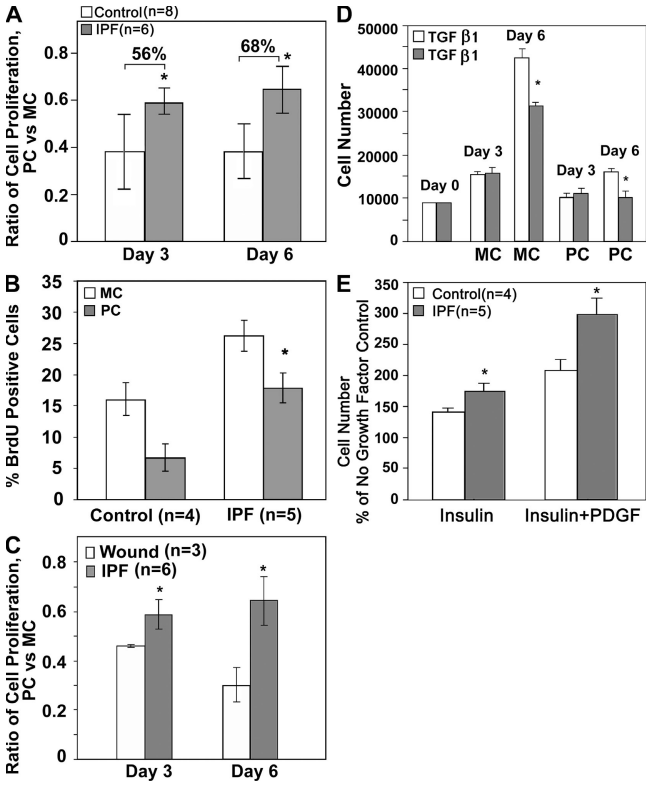

Figure 1. IPF fibroblasts display enhanced proliferation on polymerized collagen matrices. (A) Proliferation assay of control (n = 8) and IPF (n = 6) fibroblasts on monomeric and polymerized collagen. *, P < 0.01 and 0.005 for the ratio of proliferation of IPF fibroblasts on polymerized versus monomeric collagen compared with control fibroblasts at days 3 and 6, respectively. (B) Quantification of DNA synthesis by BrdU incorporation in control (n = 4) and IPF (n = 5) fibroblasts on monomeric and polymerized collagen at day 3. *, P < 0.0001 for the percentage of BrdU-positive IPF and control fibroblasts at the indicated time. (C) Ratio of proliferation of wound (n = 3) and IPF (n = 6) fibroblasts on polymerized versus monomeric collagen at days 3 and 6 (*, P < 0.02 and 0.001, respectively). (D) Normal fibroblasts were exposed to TGF-β for 3–6 d or left untreated, and cell numbers were quantified (*, P < 0.05). (E) Proliferation assay of control (n = 4) and IPF (n = 5) fibroblasts on monomeric and polymerized collagen in defined media. Shown is the percent increase in cell number in response to the indicated growth factor treatment compared with cultures continued in the absence of growth factors at day 3. *, P < 0.05 or 0.001 for the proliferative response of IPF compared with control fibroblasts at the time in defined media containing insulin alone or insulin plus platelet-derived growth factor, respectively. Error bars represent SEM. Data are representative of three independent experiments.

we used integrin blocking antibodies. Preincubation of control fibroblasts with α2 or β1 blocking antibodies attenuated the increase in Akt phosphorylation in response to attachment to monomeric collagen (Fig. 2 B). Reagents blocking the α1, α3, α5, and αV integrin subunits were much less effective in inhibiting Akt activation. Consistent with this, cell spreading on monomeric collagen was significantly impaired by α2 or β1 blocking antibodies (Fig. 2 C). There was no significant synergistic effect on cell spreading using antibodies to α1, α3, α5, and αV in combination with β1 over using the β1 blocking antibody alone (Fig. 2 C). These data demonstrate that ligation of α2β1 by monomeric collagen is primarily responsible for the activation of Akt when fibroblasts attach to collagen.

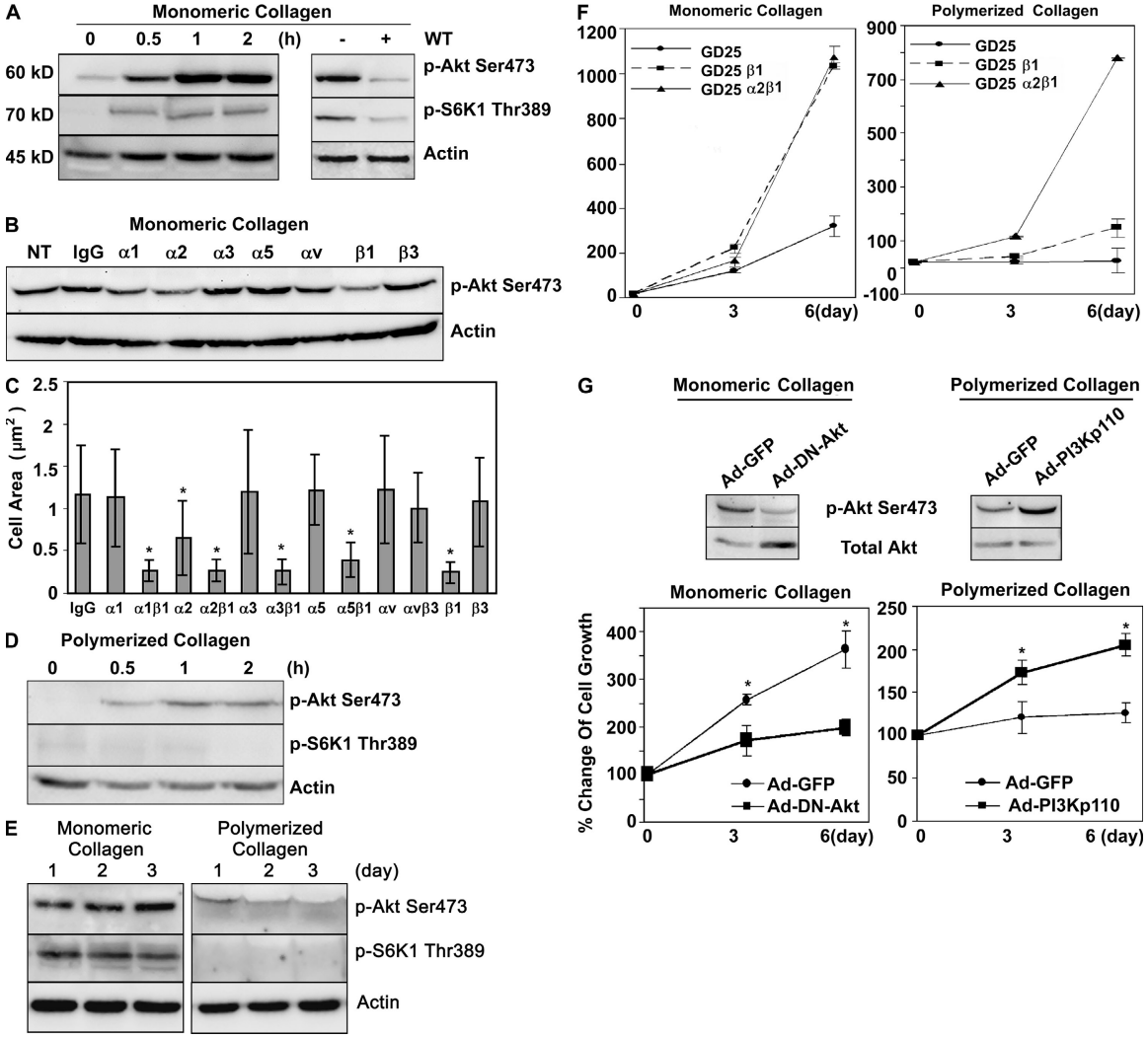

Figure 2. PI3K–Akt–S6K1 signaling in control fibroblasts. (A) Western blot analysis of phospho-Akt and S6K1 in the absence or presence of the PI3K inhibitor wortmannin (WT) in response to control fibroblast attachment to monomeric collagen in the absence of serum. One representative example is shown. (B) Western blot analysis of phospho-Akt in control fibroblasts preincubated with the indicated integrin blocking antibody and plated on monomeric collagen. (C) Fibroblasts were plated on tissue culture plates coated with monomeric collagen and blocked by BSA. Cells were allowed to spread for 30 min. Cell areas of a random 150 cells were quantified by tracing the cell border using ImageJ software (available at http://rsb.info.nih.gov/ij/). (D) Western blot analysis of phospho-Akt and S6K1 in response to control fibroblast attachment to polymerized collagen in the absence of serum. (E) Western blot analysis of phospho-Akt and S6K1 in control fibroblasts cultured on monomeric or polymerized collagen in growth factor–replete media. One representative example is shown. (F) GD25, GD25 β1, and GD25 α2β1 fibroblasts were cultured on monomeric (left) or polymerized (right) collagen in the presence of serum, and cell numbers were quantified. (G) Proliferation assay of control fibroblasts expressing dominant-negative Akt (Ad-DN-Akt; left) or constitutively active p110 subunit of PI3K (Ad-PI3Kp110; right) and cultured on monomeric or polymerized collagen in growth factor–replete media, respectively. (top left) Western blot analyses of phospho- and total Akt. (bottom left) Shown is the percent change in cell growth. *, $P < 0.01$ and 0.001 for the percent change in fibroblast growth on monomeric collagen that was significantly less in control fibroblasts expressing dominant-negative Akt compared with cells expressing control vector at days 3 and 6, respectively. (top right) Western blot analyses of phospho- and total Akt. (bottom right) Shown is the percent change in cell growth. *, $P < 0.01$ and 0.0001 for the percent change in fibroblast growth on polymerized collagen that was significantly greater in control cells expressing constitutively active p110 subunit of PI3K compared with control vector at days 3 and 6, respectively. Error bars represent SEM. Data are representative of three independent experiments.

When we examined the effect of fibroblast attachment to polymerized collagen on the state of the PI3K–Akt–S6K1 pathway, the pattern of signaling differed from that observed on monomeric collagen. The level of phosphorylated Akt increased modestly when fibroblasts attached to polymerized collagen (Fig. 2 D), but S6K1 did not become phosphorylated. These findings indicate that compared with monomeric collagen, the amplitude of the PI3K–Akt–S6K1 signaling pathway on polymerized collagen is attenuated.

After attachment to the matrix in the presence of growth factors, cells enter the cell cycle and proliferate. To examine the relationship between matrix state (polymerized vs. monomeric collagen) and the PI3K–Akt–S6K1 pathway during log-phase growth, control fibroblasts were cultured on monomeric or polymerized collagen in the presence of serum for 3 d. Akt and S6K1 phosphorylation were maintained during fibroblast proliferation on monomeric collagen. In contrast, the levels of phosphorylated Akt and S6K1 were suppressed on polymerized collagen (Fig. 2 E). In the aggregate, these data show a tight relationship between matrix chemistry and activity of the PI3K–Akt–S6K1 pathway in control fibroblasts.

We have found that fibroblasts use $\alpha2\beta1$ to attach to collagen during the adhesion assay (serum-free conditions), and that this is associated with modulation of the PI3K–Akt–S6K1 signal. However, during the proliferation assay the cells are cultured in serum and may interact with other extracellular matrix molecules, such as fibronectin, in addition to collagen. To examine which integrin receptors regulate fibroblast proliferation on collagen matrices, we performed our proliferation assay using GD25 $\alpha2\beta1$ integrin–null fibroblasts (GD25 cells) and GD25 cells reconstituted with $\beta1$ (GD25 $\beta1$A) or $\alpha2\beta1$ integrin (GD25 $\alpha2\beta1$A). GD25-null cells do not express $\beta1$ integrins, but they do express $\alpha v\beta3$, which they use to attach to collagen. GD25 $\beta1$ cells express some $\beta1$ integrins, but not $\alpha2\beta1$. GD25 $\alpha2\beta1$ cells express $\alpha2\beta1$ (28–30). We found that GD25-null cells proliferate poorly on monomeric collagen. Interestingly, GD25 $\beta1$ cells were capable of proliferating on monomeric collagen as well as GD25 cells reconstituted with $\alpha2\beta1$ (Fig. 2 F, left). In contrast, we found that only GD25 $\alpha2\beta1$ cells proliferated on polymerized collagen (Fig. 2 F, right). Both GD25-null and GD25 $\beta1$ cells proliferated poorly on polymerized collagen. Our data suggest a scenario where, in addition to $\alpha2\beta1$, other $\beta1$ integrins may contribute to proliferation signaling on monomeric collagen, whereas $\alpha2\beta1$ predominately regulates proliferation on polymerized collagen.

To examine whether matrix configuration, the PI3K–Akt–S6K1 pathway, and fibroblast proliferation were causally linked, we examined whether inhibiting PI3K using a dominant-negative Akt construct would attenuate fibroblast proliferation on monomeric collagen. Kinase-dead dominant-negative Akt decreased phosphorylated Akt and suppressed fibroblast proliferation on monomeric collagen (48% inhibition of proliferation at day 6 compared with fibroblasts treated with empty vector; Fig. 2 G, left). We also analyzed the effect of enforced activation of PI3K on fibroblast proliferation on polymerized collagen. Enforced activation of PI3K by overexpression of the constitutively active p110 subunit of PI3K increased the level of phosphorylated Akt and enabled fibroblasts to overcome the antiproliferative effect of polymerized collagen (Fig. 2 G, right). These data demonstrate that the activity state of PI3K–Akt is a major component of the mechanism by which polymerized collagen negatively regulates fibroblast proliferation.

The PI3K–Akt–S6K1 pathway is pathologically regulated in IPF fibroblasts

To characterize the PI3K–Akt–S6K1 signaling pathway in IPF fibroblasts, we examined Akt and S6K1 after attachment of IPF fibroblasts to monomeric collagen. Surprisingly, there was a muted increase in Akt and S6K1 phosphorylation compared with control fibroblasts (Fig. 3 A). We have previously shown that ligation of $\beta1$ integrin by the $\beta1$ integrin activating antibody TS2/16 increases Akt phosphorylation in normal fibroblasts (Fig. 3 B, left) (22) and, therefore, tested its effect on Akt in IPF fibroblasts. Ligation of $\beta1$ integrin by TS2/16 in IPF fibroblasts failed to increase Akt phosphorylation (Fig. 3 B, right). These data indicate that IPF fibroblast adhesion to monomeric collagen via $\alpha2\beta1$ results in attenuated activation of the Akt–S6K1 signal, a response opposite of control fibroblasts.

The attenuation of Akt phosphorylation in IPF fibroblasts in response to ligation of $\alpha2\beta1$ by collagen or $\beta1$ integrin activating antibody could result from a reduced level of integrin expression. However, we did not detect significant alterations in $\beta1$ integrin subunit expression in control compared with IPF fibroblasts (unpublished data). Changes in the cytoplasmic domain of integrins can affect the conformation of the extracellular domain. This mechanism is operational in normal cells and is termed inside-out signaling. To investigate the ligand binding/activation state of IPF fibroblasts, we used the 9EG7 monoclonal antibody, which recognizes ligand-induced epitopes on the human $\beta1$ integrin (31, 32). FACS analysis of control and IPF fibroblasts revealed that both fibroblast lines bound comparable levels of 9EG7 antibody, indicating a similar $\beta1$ integrin activation state (Fig. 3 C). Furthermore, full stimulation of $\beta1$ integrin in control and IPF fibroblasts was obtained with the activating TS2/16 antibody. Therefore, the reduced level of Akt and S6K1 activation in response to ligation of $\alpha2\beta1$ by monomeric collagen could not be explained by altered $\beta1$ integrin expression or activation state. To confirm this, we examined focal adhesion kinase (FAK) phosphorylation. FAK is upstream of PI3K–Akt and can be used as a surrogate marker of integrin activation (21). When serum-starved control and IPF fibroblasts were plated on monomeric collagen, the level of FAK tyrosine 397 phosphorylation was similar (Fig. 3 D).

Current models for Akt regulation indicate that Akt phosphorylation is dependent on the activity of the upstream enzyme PI3K (23). We examined PI3K activity in control

and IPF fibroblasts by examining the amounts of phosphatidylinositol 3-phosphate produced in response to ligation of β1 integrin by the TS2/16 activating antibody. PI3K activity was reduced in IPF fibroblasts treated with TS2/16 antibody compared with control (Fig. 3 E). This suggests that the decreased level of Akt and S6K1 phosphorylation seen in

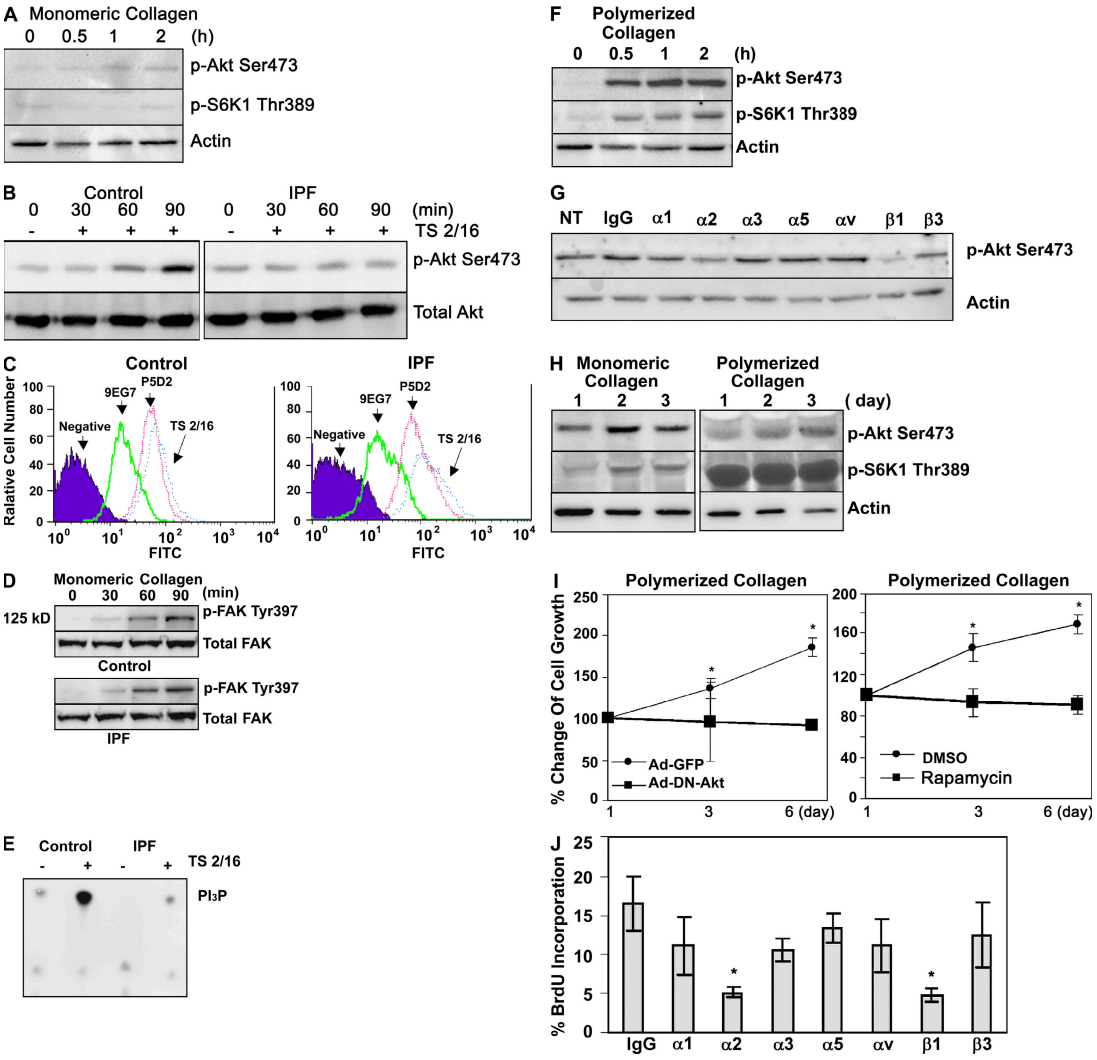

Figure 3. The PI3K–Akt–S6K1 pathway is pathologically regulated in IPF fibroblasts. (A) Western blot analyses of phospho-Akt and S6K1 in response to IPF fibroblast attachment to monomeric collagen in the absence of serum. One representative example is shown. (B) Western blot analysis of phospho- and total Akt in response to treatment of control and IPF fibroblasts with TS2/16 β1 integrin activating antibody. (C) Control ($n = 3$) or IPF ($n = 3$) fibroblasts were stained with anti–β1 integrin antibodies and analyzed by FACS. Anti–β1 integrin antibodies used were against ligand-induced binding sites (9EG7), activating sites (TS2/16), or total β1 integrin regardless of activation state (P5D2). (D) Control and IPF fibroblasts were plated on monomeric collagen. Shown is a Western blot analysis of phosphorylated FAK (tyr397). Data are representative of three independent experiments from one control and one IPF cell line. (E) PI3K activity in control and IPF fibroblasts plated on tissue culture dishes and treated with TS2/16 β1 integrin activating antibody. Data are representative of three independent experiments from one IPF cell line. (F) Western blot analysis of phospho-Akt and S6K1 in response to IPF fibroblast attachment to polymerized collagen. One representative example is shown. (G) Western blot analysis of phospho-Akt in IPF fibroblasts preincubated with the indicated integrin blocking antibody and plated on polymerized collagen. Data are representative of three independent experiments from one representative IPF cell line. (H) Western blot analysis of phospho-Akt and S6K1 in IPF fibroblasts cultured on monomeric or polymerized collagen in growth factor–replete media. Data are representative of three independent experiments from five IFP cell lines. (I) Proliferation assay of IPF fibroblasts expressing dominant-negative Akt (left) or treated with 100 nM rapamycin (right) and cultured on polymerized collagen in growth factor–replete media. Shown is the percent change in cell growth. *, $P < 0.01$ and 0.0001 for the percent change in cell growth on polymerized collagen that was significantly less in IPF fibroblasts expressing dominant-negative Akt at days 3 and 6, respectively; or $P < 0.002$ or 0.001 for the percent change in cell growth on polymerized collagen that was significantly less in IPF fibroblasts treated with rapamycin at days 3 or 6, respectively. Error bars represent SEM. Data are representative of three independent experiments from one IPF cell line. (J) IPF fibroblasts were preincubated with the indicated integrin blocking antibody and plated on polymerized collagen in growth factor–replete media in the presence of antibody. BrdU staining as a measure of DNA synthesis was assessed at 24 h. Error bars represent SEM. Data are representative of three independent experiments from three IPF cell lines.

response to ligation of β1 integrin by monomeric collagen or activating antibody in IPF fibroblasts is caused by a reduced level of PI3K activity.

Having seen aberrant activity of the PI3K–Akt–S6K1 signaling pathway in IPF fibroblasts attaching to monomeric collagen, we examined the pathway after attachment to polymerized collagen. In contrast to control fibroblasts, we found that both Akt and S6K1 became phosphorylated when IPF fibroblasts attached to polymerized collagen (Fig. 3 F). The increase in Akt phosphorylation was largely inhibited by blocking antibodies to the α2 and β1 integrin subunits, indicating that activation of Akt in response to IPF fibroblast adhesion to polymerized collagen is mediated through α2β1 (Fig. 3 G).

We next examined the effect of matrix state on the PI3K–Akt–S6K1 pathway on actively proliferating IPF fibroblasts. In contrast to the pattern observed in control fibroblasts, we found that the levels of phosphorylated Akt and S6K1 were elevated when IPF fibroblasts were cultured on polymerized collagen (Fig. 3 H), indicating that the PI3K pathway was aberrantly activated. These data are consistent with the idea that IPF fibroblasts elude the antiproliferative effects of polymerized collagen by aberrantly activating the PI3K–Akt–S6K1 signaling pathway.

To test this directly, we inhibited the PI3K–Akt pathway by infecting IPF fibroblasts with an adenoviral vector containing a dominant-negative Akt construct and examined proliferation on polymerized collagen. Kinase-dead Akt decreased fibroblast proliferation on polymerized collagen by ∼50% compared with empty vector (Fig. 3 I, left). Consistent with this, inhibition of the downstream Akt effectors mammalian target of rapamycin and S6K1 by rapamycin reversed the aberrant proliferation of IPF fibroblasts on polymerized collagen (Fig. 3 I, right). The ability of IPF fibroblasts to circumvent the negative regulatory effects of polymerized collagen is caused by aberrant activation of the PI3K–Akt–S6K1 pathway.

Similar to control fibroblasts, IPF fibroblasts use α2β1 to attach to polymerized collagen. To address which integrins IPF fibroblasts use for proliferation signaling on polymerized collagen, we cultured IPF fibroblasts pretreated with integrin blocking antibodies on polymerized collagen in the presence of serum. At 24 h, we analyzed BrdU staining as a measure of DNA synthesis. α2 and β1 blocking antibodies attenuated BrdU staining compared with IgG control (Fig. 3 J). Other blocking antibodies had minimal effect on DNA synthesis. Thus, IPF fibroblasts predominantly use α2β1 for proliferation signaling on polymerized collagen.

Regulation of the tumor suppressor PTEN is altered in IPF fibroblasts

PTEN phosphatase negatively regulates integrin growth signaling by inhibiting Akt (33, 34). PTEN activity is determined by its abundance and phosphorylation state (35–38). To determine if changes in PTEN expression were associated with the altered signaling and growth response of IPF fibroblasts on collagen, we examined PTEN protein expression when fibroblasts were cultured on monomeric and polymerized collagen in the presence of serum. As a function of time, control fibroblast PTEN expression decreased on monomeric collagen and increased on polymerized collagen (Fig. 4 A), whereas in IPF fibroblasts PTEN expression was invariant (Fig. 4 B). However, total PTEN protein levels in IPF fibroblasts on polymerized collagen were modestly lower compared with monomeric collagen. Because the abundance of PTEN protein in IPF fibroblasts was only modestly decreased, it remained unclear whether fibroblast interaction with polymerized collagen was associated with a decrease in PTEN activity.

To assess PTEN activity, IPF and control fibroblasts were cultured on collagen, PTEN was immunoprecipitated, and activity was quantified. Fibroblasts in uninjured tissue have a low proliferation rate. To provide a reference value for PTEN activity under proliferation-prohibitive conditions, PTEN activity was measured in quiescent cultures of serum-starved fibroblasts. We then compared fibroblast PTEN activity on monomeric and polymerized collagen in the presence of serum growth factors with PTEN activity under proliferation-prohibitive conditions. In control fibroblasts, PTEN activity under proliferation-prohibitive condition was high. When these fibroblasts were cultured on monomeric collagen in the presence of serum—a condition associated with active proliferation—there was a significant decrease in PTEN activity (Fig. 4 C, left), whereas on polymerized collagen—which represses proliferation—PTEN activity gradually increased over 3 d to the level seen under proliferation-prohibitive conditions. When IPF fibroblasts were cultured under proliferation-prohibitive conditions, PTEN activity was high, similar to control cells (Fig. 4 D, left). However, when we examined PTEN activity on polymerized to monomeric collagen, the response was opposite to that of control fibroblasts. There was a sharp decrease in PTEN activity on polymerized compared with monomeric collagen (Fig. 4 D, left). To confirm these results, we examined PI3K activity in control and IPF fibroblasts by examining the amounts of phosphatidylinositol 3-phosphate produced in response to culture on collagen. When control fibroblasts were cultured on polymerized collagen, the level of PI3K activity decreased 19% compared with monomeric collagen (Fig. 4 C, right). In contrast, the level of PI3K activity increased 45% when IPF fibroblasts were cultured on polymerized collagen (Fig. 4 D, right). These data indicate that in response to polymerized collagen, IPF fibroblasts display inappropriately low PTEN function and concomitant activation of Akt and proliferation.

Recent work suggests that cytosolic PTEN needs to be recruited to the plasma membrane, where it is activated and is then in the right location to inhibit PI3K–Akt (35, 36). Thus, total PTEN levels may not correlate well with activity. To address this issue, we analyzed the levels of membrane-associated PTEN from control and IPF fibroblasts cultured on polymerized collagen. IPF fibroblast membrane–associated PTEN was markedly decreased compared with controls (Fig. 4 E).

This suggests that an inadequate recruitment of cytosolic PTEN to the membrane may account for inappropriately low PTEN activity in IPF fibroblasts on polymerized collagen.

Ectopic expression of PTEN in IPF fibroblasts reverses the abnormal proliferative phenotype

Because inhibition of control fibroblast proliferation on polymerized collagen is associated with suppression of PI3K–Akt–S6K1 and maintenance of high PTEN activity, we examined whether down-regulation of PTEN would enable control fibroblasts to overcome the antiproliferative effects of polymerized collagen. Control fibroblasts were transfected with PTEN or control siRNA and plated on polymerized collagen. PTEN siRNA knocked down PTEN expression (Fig. 5, A and B) and augmented their ability to proliferate on polymerized collagen compared with control (Fig. 5 C).

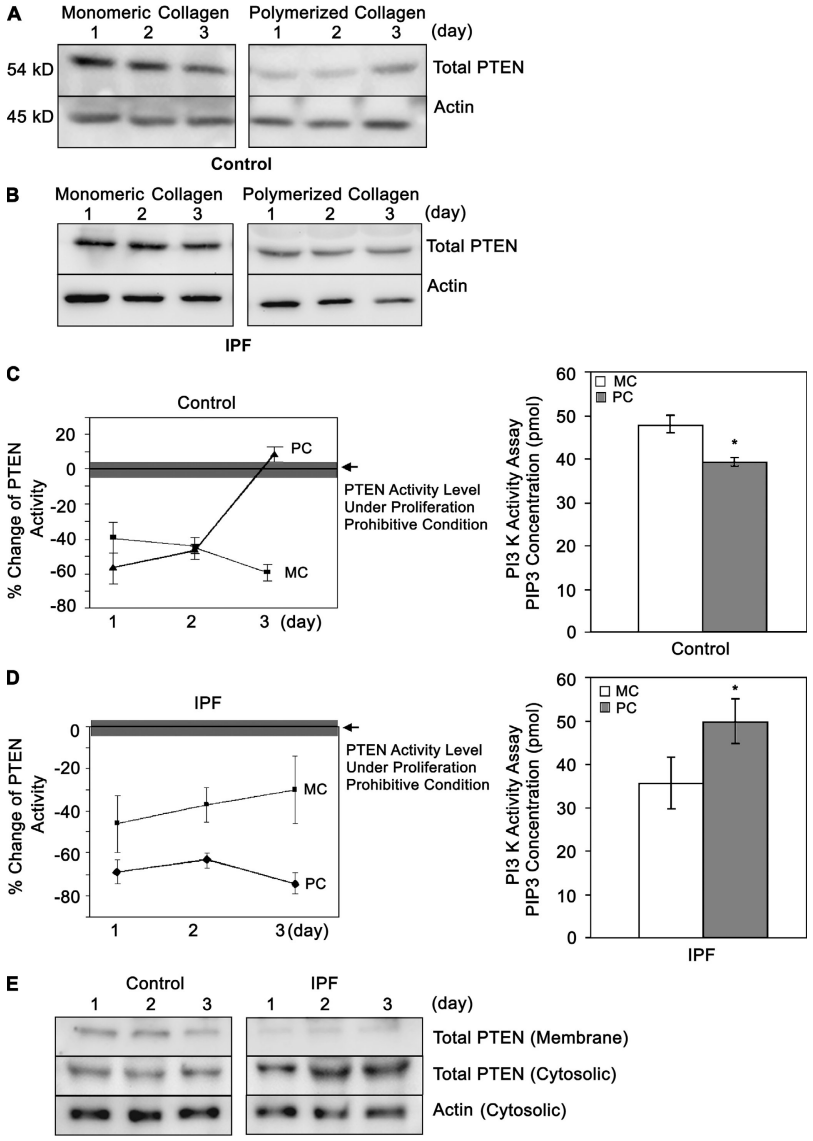

Figure 4. Regulation of PTEN is altered in IPF fibroblasts. (A and B) Western blot analysis of total PTEN in control (A) or IPF (B) fibroblasts cultured on monomeric or polymerized collagen in growth factor–replete media. One representative sample out of five control and five IPF cell lines examined is shown. (C and D) PTEN phosphatase assay showing the percent change in PTEN activity in control ($n = 3$; C, left) or IFP ($n = 3$; D, left) fibroblasts cultured on polymerized or monomeric collagen in growth factor–replete media compared with cells cultured under proliferation-prohibitive conditions. *, $P < 0.001$ for PTEN activity in control fibroblasts on polymerized collagen that was significantly higher at day 3 compared with monomeric collagen; $P < 0.01$ for PTEN activity in IPF fibroblasts on polymerized collagen that was significantly less on day 3 compared with monomeric collagen. Shown is PI3K activity in control (C, right) and IPF (D, right) fibroblasts cultured on monomeric or polymerized collagen in growth factor–replete media for 3 d. *, $P < 0.005$ for PI3K activity in control fibroblasts cultured on polymerized collagen that was significantly less at day 3 compared with monomeric collagen; or $P < 0.05$ for PI3K activity in IPF fibroblasts cultured on polymerized collagen that was significantly higher at day 3 compared with monomeric collagen. Error bars represent SEM. (E) PTEN protein levels in membrane and cytosolic fractions of cell lysates were measured by Western blot analysis. Data are representative of three independent experiments.

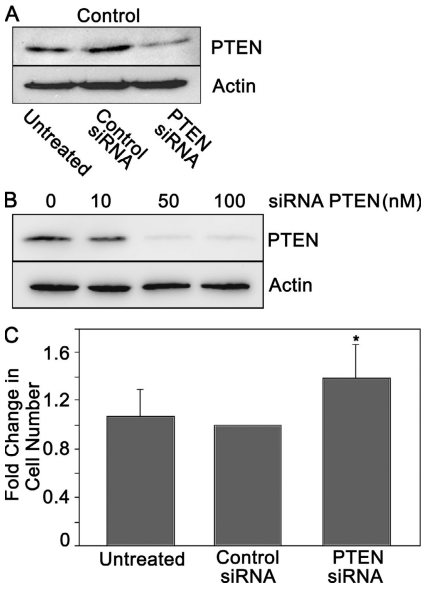

Figure 5. Knockdown of PTEN enables control fibroblasts to elude the antiproliferative effects of polymerized collagen. Control fibroblasts were transfected with PTEN or control siRNA and cultured on polymerized collagen in serum. (A) Western blot analysis of total PTEN levels. (B) Western blot analysis showing PTEN protein expression in response to increasing concentrations of PTEN siRNA. (C) Proliferation assay of control fibroblasts on polymerized collagen in serum-replete media. After 6 d, there was a significant increase in proliferation on polymerized collagen in cells treated with PTEN siRNA compared with control siRNA (*, $P < 0.04$). Error bars represent SEM. Data are representative of three independent experiments.

To determine whether decreased PTEN activity accounted for the enhanced proliferation of IPF fibroblasts on polymerized collagen, we infected IPF fibroblasts with an adenoviral vector containing wild-type PTEN. This elevated PTEN expression and suppressed phospho-Akt (Fig. 6 A). Ectopic PTEN also inhibited two downstream targets of the PI3K–Akt pathway, S6K1 and translation initiation factor eIF4F (Fig. 6, A and B), and inhibited DNA synthesis in IPF fibroblasts cultured on polymerized collagen (Fig. 6 C). Thus, elevation of PTEN activity in IPF fibroblasts restores the normal pattern of proliferation on polymerized collagen, whereas pathologically low PTEN activity can account for the ability of IPF fibroblasts to circumvent the antiproliferative effects of polymerized collagen.

Can PTEN deficiency lead to a more durable fibroproliferative response after tissue injury?

To address this issue, we used PTEN haploinsufficient mice. Using a well-characterized cutaneous wound model, a 2×2–cm skin wound was made on the middorsal surface of PTEN wild-type or haploinsufficient mice (39). We measured the reduction in wound surface area by planimetry as a function of time. There was an ~10-fold greater reduction in wound surface area of PTEN wild-type compared with haploinsufficient mice at day 28 after wounding (Fig. 7 A), indicating that wound repair was delayed in PTEN-deficient mice.

We performed histological analysis of the wounds as a function of time. We focused our analysis at days 18 and 28, as large differences in granulation tissue size were apparent at these times. Histological analysis of day 18 wounds revealed markedly increased cellularity of the granulation tissue of PTEN haploinsufficient (Fig. 7 B, top left) compared with wild-type mice (Fig. 7 B, top right). We sought to determine why the granulation tissue of PTEN haploinsufficient mice displayed increased cellularity. We reasoned that it could be caused by enhanced proliferation, reduced apoptosis, or both. We used Ki67 staining as a marker of proliferation. Abundant Ki67 staining was present both in day 18 wound granulation tissue and in the newly formed epithelium overlying the wound ulcer in PTEN haploinsufficient mice (Fig. 7 B, middle left). There was little Ki67 staining in the granulation tissue of PTEN wild-type mice, whereas the overlying regenerating epithelium stained positive (Fig. 7 B, middle right). Immunohistochemical analysis of PTEN haploinsufficient granulation tissue revealed the presence of abundant α–smooth muscle actin–expressing mesenchymal cells (Fig. 7 B, bottom right).

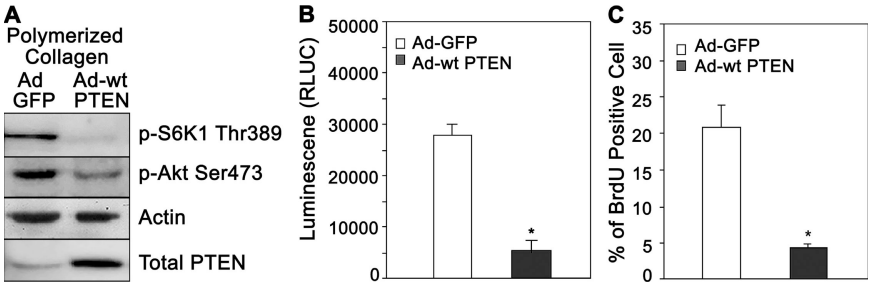

Figure 6. Ectopic expression of PTEN in IPF fibroblasts reverses the abnormal proliferative phenotype. (A) Western blot analysis of total PTEN and phospho-Akt and S6K1 in IPF fibroblasts expressing wild-type PTEN (Ad-wtPTEN) or empty vector (Ad-GFP) and cultured on polymerized collagen in growth factor–replete media. (B) eIF4F activity (RLUC luminescence) in IPF fibroblasts expressing wild-type PTEN or control vector and cultured for 3 d on polymerized collagen. *, $P < 0.0001$ for significantly less eIF4F activity in IPF fibroblasts expressing wild-type PTEN compared with control. (C) Quantification of DNA synthesis by BrdU incorporation in IPF fibroblasts expressing wild-type PTEN or empty vector and cultured on polymerized collagen in growth factor–replete media for 3 d. Shown is the percentage of BrdU-positive IPF fibroblasts. *, $P < 0.0001$ for the percentage of BrdU-positive IPF fibroblasts expressing wild-type PTEN that was significantly less compared with control. Error bars represent SEM. Data are representative of three independent experiments from one IPF cell line.

Double staining for Ki67 and α–smooth muscle actin suggested the presence of proliferating myofibroblasts in the PTEN haploinsufficient granulation tissue (Fig. 7 B, bottom left). By day 28, the wounds of PTEN-deficient mice were incompletely healed, whereas the wounds of the PTEN wild-type mice were nearly completely healed. At this time, there was still a mild persistent proliferative response in PTEN-deficient compared with wild-type mice (unpublished data).

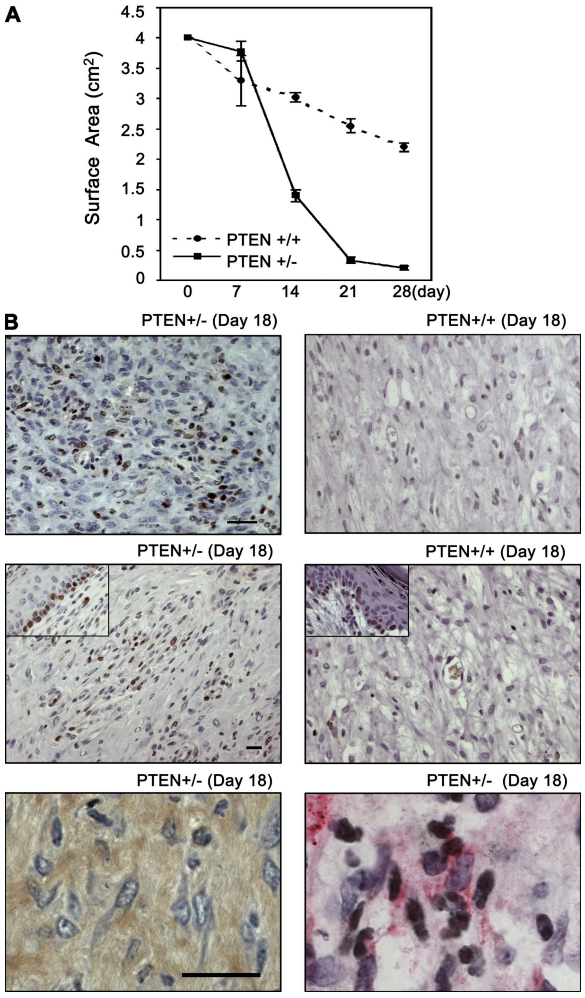

Figure 7. PTEN deficiency leads to a more durable fibroproliferative response after tissue injury. (A) Shown is the surface area of granulation tissue in PTEN wild-type (+/+; $n = 3$) and haploinsufficient (+/−; $n = 3$) mice. There was a significant delay in the resolution of granulation tissue in PTEN +/− compared with +/+ mice ($P < 0.0001$). Error bars represent SEM. (B) Hematoxylin and eosin–stained sections of granulation tissue at day 18 in PTEN +/− (top left) and +/+ (top right) mice. Note the increased cellularity of granulation tissue in PTEN +/− mice. Ki67 staining of day 18 granulation tissue from PTEN +/− (middle left) and +/+ (middle right). Note the abundant Ki67-positive cells (nuclei brown) in the granulation tissue of PTEN +/− mice. Insets show positive Ki67 staining in the newly formed epithelium overlying the wound ulcer. (bottom right) Abundant α–smooth muscle actin staining of the granulation tissue of PTEN +/− mice. (bottom left) Double staining for Ki67 (nuclei brown) and α–smooth muscle actin. Bar, 50 μm.

We also analyzed apoptosis in the granulation tissue of PTEN wild-type and haploinsufficient mice by examining cleaved caspase 3 expression. No differences in cleaved caspase 3 staining were apparent (unpublished data). For controls, no immunoreactivity was seen when IgG isotype control antibodies were substituted for the primary antibody (unpublished data). These data suggest that a more durable fibroproliferative response accounts for the abundance of α–smooth muscle actin–expressing myofibroblasts and the delayed resorption of granulation tissue in PTEN-deficient mice after tissue injury.

PTEN-deficient mice have increased fibrosis after bleomycin-induced lung injury

To examine the effect of PTEN deficiency on the development of pulmonary fibrosis, PTEN haploinsufficient or wild-type mice were given bleomycin intratracheally, and collagen content was analyzed by Sircol assay 21 d later. The collagen content of PTEN-deficient mice was increased 55% compared with wild-type mice (11.12 ± 3.42 μg/g vs. 17.25 ± 2.47 μg/g body weight; $P < 0.005$; Fig. 8 A). Collagen deposition in the mice was evaluated by Trichrome staining. Consistent with the Sircol assay, PTEN-deficient mice displayed augmented collagen deposition after bleomycin compared with wild-type mice (Fig. 8 B, middle and bottom). Collectively, our in vivo studies indicate that PTEN deficiency is associated with a more durable fibroproliferative response leading to exaggerated fibrosis.

Akt is activated in the fibroblastic foci of human IPF tissue

Our in vitro experiments indicate that the Akt–S6K1 pathway is aberrantly activated in IPF fibroblasts cultured on polymerized collagen. To analyze the activation state of Akt in IPF lung tissue in vivo, we performed immunohistochemistry on lung tissue from five IPF patients examining phospho-Akt expression. Consistent with our in vitro studies, phospho-Akt–positive cells were present in the fibroblastic foci of IPF tissue (image is representative of fibrotic foci from the four other patient samples; Fig. 9 C). In contrast, although the epithelium and endothelium of normal lung tissue stained positive for phospho-Akt, no phospho-Akt could be detected in the mesenchymal or smooth muscle cells of normal lung parenchyma (Fig. 9 A). As a control, when fibrotic lung tissue was stained with an irrelevant primary antibody (anti-p53) plus secondary antibody, scant immunoreactivity was present (Fig. 9 E). No immunoreactivity was seen when IPF and normal lung tissue were stained with secondary antibody without primary antibody (unpublished data). When we used an antibody that recognizes total PTEN, we found that PTEN expression was prominent in the epithelium, endothelium, and mesenchymal cells in normal lung tissue (Fig. 9 B). PTEN expression could also be seen in cells in the fibroblastic foci of IPF tissue (Fig. 9 D). This is consistent with our in vitro findings, which demonstrate the presence of PTEN protein expression in IPF fibroblasts cultured on collagen. It is important to note the limitations of the PTEN immunohistochemical studies. PTEN activity is dependent on both its expression and phosphorylation state.

Analyzing total PTEN expression may not accurately reflect PTEN activity. Thus, it remains unclear whether PTEN activity is altered in vivo in IPF. Nevertheless, consistent with our in vitro findings that the Akt pathway is aberrantly activated in IPF fibroblasts, these data verify that Akt is activated in vivo in IPF fibroblastic foci.

DISCUSSION

The pathogenesis of IPF remains incompletely understood. However, prima facie evidence for a key role for the fibroblast in IPF is established by the observation that progressive expansion of the fibroblast focus by proliferating myofibroblasts depositing type I collagen leads to permanently scarred alveoli. Although recent studies indicate that IPF fibroblasts display a distinct pathological phenotype (6–12), the mechanisms differentiating IPF fibroblasts from their normal counterparts remain poorly understood. In this study, we present results providing direct evidence for defective negative regulation of the proliferative pathway in IPF fibroblasts that support the theory that the pathogenesis of IPF involves abnormalities in the fibroblast cellular machinery.

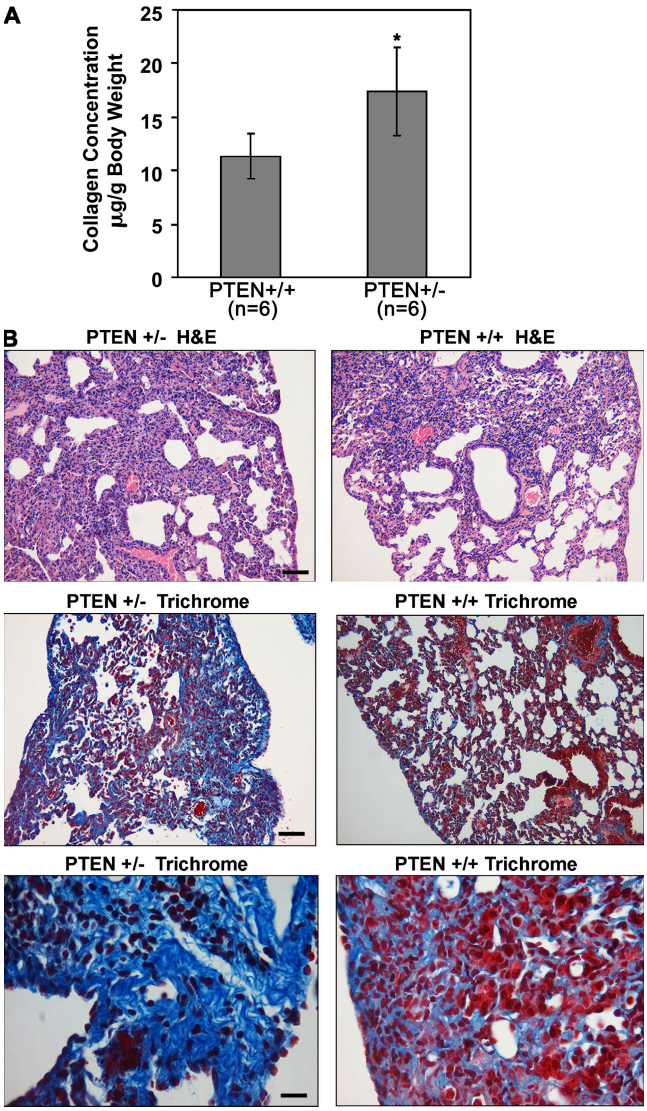

Figure 8. PTEN deficiency exaggerates fibrosis after lung injury. PTEN +/− or +/+ mice were administered bleomycin intratracheally. (A) 21 d later, lungs were harvested and total lung collagen was determined by Sircol assay (n = 6 mice per group). (B) Hematoxylin and eosin (H&E)–stained sections of day 21 lung tissue in PTEN +/− (top left) and +/+ (top right) mice. Trichrome staining of day 21 lung tissue from PTEN +/− (left, middle and bottom) and +/+ (right, middle and bottom) mice. Results are representative of three mice per group. Bars: (top and middle) 200 µm; (bottom) 50 µm.

When cells attach to the extracellular matrix, a matrix recognition signal is generated by integrin–ligand binding. This is termed "outside-in signaling" and activates specific signal transduction pathways that regulate cell function. In the case of normal fibroblasts, ligation of β1 integrin by monomeric collagen generates a matrix recognition signal characterized by an increase in Akt and S6K1 activity in a PI3K-dependent manner. Facilitating this increase in Akt and S6K1 activity is a relaxation in the suppressive activity of PTEN. PTEN is a tumor suppressor phosphatase and a major inhibitor of the PI3K–Akt pathway whose baseline activity is believed to be constitutively high (21). In this study, we demonstrate that a relaxation of PTEN's suppressive activity supports normal fibroblast proliferation on monomeric collagen. In contrast, seminal studies have demonstrated that polymerized collagen functions as a negative regulator of fibroblast proliferation by promoting arrest in the G1 phase of the cell cycle (15). We show that normal fibroblast interaction with polymerized collagen via β1 integrin, which is the physiological form of collagen that fibroblasts interact with, results in high PTEN activity, causing suppression of the PI3K–Akt–S6K1 pathway and proliferation. Thus, the contact of fibroblasts with polymerized collagen provides a physiological mechanism to limit excess fibroproliferation.

It is important to note that for our in vitro studies, the critical comparison was between proliferation signaling in control and IPF fibroblasts on polymerized collagen. In contrast to control fibroblasts where high PTEN activity inhibits PI3K–Akt, we have discovered that in IPF fibroblasts, β1 integrin interaction with polymerized collagen generates an aberrant matrix recognition signal characterized by activated Akt–S6K1 activity because of inappropriately low PTEN activity. Importantly, using PTEN haploinsufficient mice and two in vivo models of tissue injury, a well characterized cutaneous wound healing model and bleomycin-induced lung injury, we confirm that a deficiency in PTEN activity results in a more durable fibroproliferative response after tissue injury and leads to pathological fibrosis.

PTEN activity is a function of abundance and phosphorylation state (35–38, 40). Current models for PTEN activation suggest that cytosolic PTEN is recruited to the plasma membrane, where it is activated by dephosphorylation. Membrane-associated PTEN is then in the right location to inhibit PI3K (35, 36). Our data indicate that PTEN activity is inappropriately low in IPF fibroblasts in response to integrin-polymerized collagen interaction. We have found that membrane-associated PTEN is decreased in IPF fibroblasts compared with controls.

Our data suggest a scenario where in response to IPF fibroblast interaction with polymerized collagen via integrin, there is deficient recruitment of cytosolic PTEN to the membrane. This would result in a failure of PTEN to be activated. Inappropriately low PTEN activity would then facilitate aberrant activation of the PI3K–Akt signal and enable IPF fibroblasts to elude the proliferative-suppressive effects of polymerized collagen.

A previous study presented evidence that PTEN expression is low in IPF tissue compared with normal lung tissue suggesting that a deficiency in PTEN expression is associated with pulmonary fibrosis (41). Our study offers a different explanation for the role of PTEN in IPF. Unlike the findings in this previous study, we have not found that PTEN protein expression is decreased in IPF fibroblasts compared with normal fibroblasts during routine tissue culture. We have found that PTEN activity is inappropriately low in IPF fibroblasts compared with normal fibroblasts when they are cultured on polymerized collagen matrices, and that this is associated with reduced membrane-associated PTEN. Thus, our data do not link IPF with a deficiency of PTEN protein expression. Rather, our studies provide direct mechanistic evidence that the pathological ability of IPF fibroblasts to elude the antiproliferative properties of polymerized collagen is caused by defective regulation of PTEN function in response to their interaction with collagen via integrin.

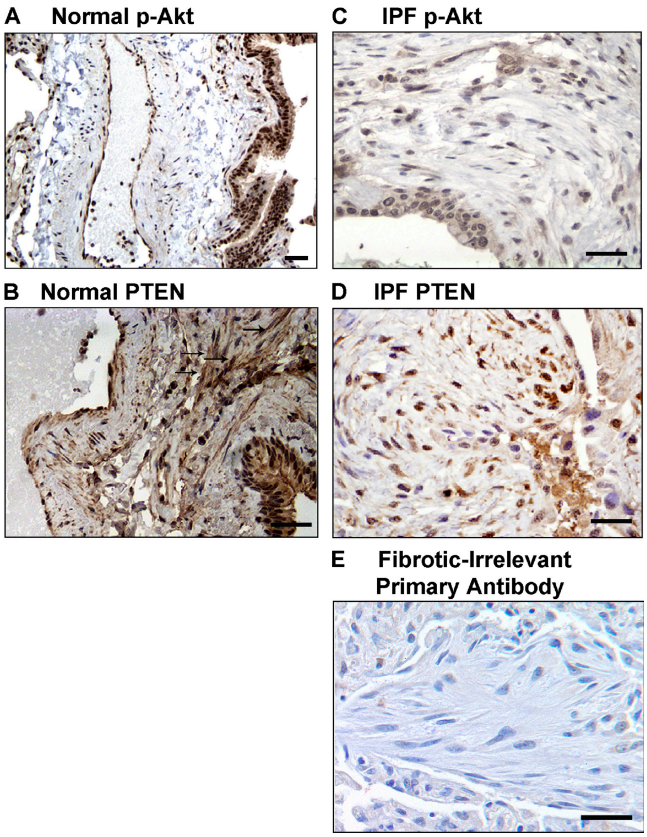

Figure 9. Akt is activated in fibrotic foci in IPF lung tissue. Immunohistochemistry, evaluating phospho-Akt and total PTEN expression, was performed on lung tissue obtained from patients with histologically normal lungs ($n = 5$) and IPF ($n = 5$). (A and B) Normal lung tissue. Note the presence of phospho-Akt in epithelial and endothelial cells (A). No phospho-Akt expression is apparent in mesenchymal cells in the vessel wall or in normal lung parenchyma in A. Arrows denote PTEN expression in spindle-shaped cells within normal lung parenchyma (B). (C and D) IPF lung tissue. Note the phospho-Akt expression in spindle-shaped cells within the fibrotic foci of IPF (C). Cells within fibrotic foci also displayed PTEN expression (D). (E) As a control, shown is fibrotic lung tissue stained with irrelevant primary antibody (anti-p53). Bars, 50 μm.

A central unresolved issue in IPF has revolved around the question of whether IPF fibroblasts are normal fibroblasts that behave abnormally because they reside in a microenvironment consisting of profibrotic cytokines or whether they have acquired a distinct pathological phenotype that is stable and does not depend on continued exposure to profibrotic molecules. Our study suggests that IPF fibroblasts are not normal or wound fibroblasts whose pathological behavior depends on continuous exposure to profibrotic cytokines. Rather, IPF fibroblasts have acquired a distinct stable pathological phenotype that persists despite an absence of profibrotic cytokines. While our study was in progress, a study was published that may provide insight into how these cells have acquired this abnormal phenotype. This study demonstrated that when epithelial cells are chronically exposed to TGF-β (30 d), they undergo epithelial-mesenchymal transition (EMT) (42). These cells display Akt phosphorylation that persists despite withdrawal of TGF-β. In addition, two recent studies suggest an EMT origin for IPF fibroblasts (43, 44). The implication of our work in the context of these studies suggests that IPF fibroblasts may represent cells that have undergone EMT and have acquired a stable pathological phenotype as part of the transdifferentiation process. In conclusion, our data demonstrate that the IPF fibroblast phenotype is characterized by persistent activation of the PI3K–Akt pathway caused by inappropriately low PTEN activity that confers a pathological proliferative response with the capacity to elude the antiproliferative properties of fibrillar collagen.

MATERIALS AND METHODS

Cell culture

Seven 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 typical high resolution chest computed tomography findings of IPF. In all cases, the diagnosis of IPF was confirmed by microscopic analysis of lung tissue and demonstrated the characteristic morphological findings of usual interstitial pneumonia. All patients fulfilled the criteria for the diagnosis of IPF as established by the American Thoracic Society and the European Respiratory Society (45). Seven normal primary control adult human lung fibroblast lines were used. One was purchased from American Type Culture Collection (human lung fibroblast 210), and six were established from histologically normal lung tissue adjacent to carcinoid tumor ($n = 5$) or adjacent to radiation-induced fibrotic lung tissue ($n = 1$). One diseased control primary human fibroblast line was derived from a patient with chronic obstructive pulmonary disease at the time of lung transplantation. Primary lung fibroblast lines were generated by explant culture and cultured in high glucose DMEM containing 10% FCS. Fibroblasts were used between passages five and eight. Cells were characterized as fibroblasts as previously described (46). Use of human tissues was approved by the Institutional Review Board at the University of Minnesota.

Collagen matrices

To prepare two-dimensional monomeric collagen matrices, tissue culture dishes were coated with 100 μg/ml type I collagen solution (PureCol; Allergan). Three-dimensional polymerized collagen matrices (final concentration = 2 mg/ml) were prepared by neutralizing the collagen solution with a one-sixth volume of 6× DMEM medium and diluting to a final volume with 1× DMEM, and incubating the solution at 37°C for 1–2 h.

Proliferation assay

Growth factor–replete media. Serum-starved fibroblasts were plated on collagen in DMEM. After 24 h, the media was replaced with DMEM plus 10% FCS.

Defined media. Fibroblasts were cultured on collagen in serum-free defined medium with no growth factors, with 100 nM insulin alone, or together with 50 pM of platelet-derived growth factor. At the times indicated in the figures, cells were harvested as previously described (15). Cell numbers were quantified by Coulter counter. To assess DNA synthesis, cells were incubated with BrdU for 2 h before harvesting and were stained by anti-BrdU antibody according to the manufacturer's instructions (Roche). DNA synthesis was quantified by assessing the percentage of BrdU-positive cells by microscopic analysis of at least 200 cells per slide.

PTEN activity assay

Cells were lysed and total protein levels were measured from the resulting lysates. PTEN was immunoprecipitated from lysates containing either equal amounts of protein or equal numbers of cells by incubation with anti-PTEN antibody. PTEN activity was assayed using a Malachite green phosphatase kit (Echelon) according to the manufacturer's instructions.

PI3K activity assay

PI3K activity was assessed by the incorporation of [^{32}P]ATP into exogenous phosphoinositide, resulting in the production of PI(3)P, as described by Lei et al. (47). PI3K activity was also quantified using a PI3K ELISA kit (Echelon) according to the manufacturer's instructions.

Adenoviral vectors

Adenoviral vectors containing wild-type PTEN, constitutively active p110 subunit of PI3K (provided by J. Downward, Signal Transduction Laboratory, London, UK), kinase-dead (Lys179, Thr308, and Ser473 mutated to alanine) dominant-negative Akt (provided by J. Downward), and control (Ad-GFP) were constructed and purified according to the manufacturer's instructions (Takara Shuzo Co., Ltd.). Cells were infected at a multiplicity of infection of 1:20.

Cap-dependent translation activity

Fibroblasts were plated on collagen and transfected with a bicistronic dual luciferase reporter plasmid (pMSCV/hygr/rLUC-polIRES-fLUC) in which the translation of *Renilla reniformis* luciferase (RLUC) is cap dependent, whereas translation of firefly luciferase (FLUC) proceeds via an IRES in a cap-independent manner, or infected by Ad-GFP or Ad-wtPTEN-GFP, respectively. 72 h after transduction, the cells were lysed. Extracts were analyzed for RLUC and FLUC activity as previously described (48).

Mice

PTEN haploinsufficient and wild-type mice (C57BL/6 background; both provided by T. Mak, University of Toronto, Toronto, Canada) were used at 8–12 wk of age and weighed between 20–25 g. Animal use was approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

In vivo cutaneous wound model

2 × 2–cm full-thickness cutaneous wounds were made as described by Leslie and Downes (38). Wound surface area was measured by planimetry. Immunohistochemical analysis was performed using the appropriate primary antibody (Ki67 [Abcam]; anti–cleaved caspase 3 [Cell Signaling Technology]; and α–smooth muscle actin [Vector Laboratories]).

Bleomycin model of lung injury

Mice were anesthetized with sodium pentobarbital. 0.05 U bleomycin dissolved in sterile saline was instilled into the trachea. Lungs were harvested on day 21 after bleomycin. Total lung collagen levels were determined in both lungs by Sircol assay according to the manufacturer's instructions (Accurate).

Immunohistochemistry

Immunohistochemical studies were performed on frozen sections prepared from lung specimens obtained from patients undergoing lung transplantation for IPF (n = 5), and from surgical specimens showing normal lung parenchyma distant from tumor nodule (n = 5). The avidin–biotin complex immunoperoxidase technique was used. Primary antibodies included phospho-Akt (ser473; Cell Signaling Technology) and total PTEN (6H2.1; Cascade Bioscience).

Data analysis

Comparisons of data among each experiment were performed with the unipolar unpaired or paired Student's t test. All experiments were replicated a minimum of three times. Data are expressed as mean ± SD. $P < 0.05$ were considered significant.

We thank Dr. Julian Downward for the constitutively active PI3 kinase–p110 subunit and dominant-negative Akt constructs, and Dr. Tak Mak for the PTEN haploinsufficient and wild-type mice.

This work was supported in part by National Heart, Lung, and Blood Institute (NHLBI) grants (HL67794 and HL074882) and an American Heart Association Grant-In-Aid to C.A. Henke; and an NHLBI grant (HL073719) to P.B. Bitterman.

The authors have no conflicting financial interests.

Submitted: 2 January 2008
Accepted: 8 May 2008

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