Negative Regulation of Myofibroblast Differentiation by PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome 10)

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Rationale: **Myofibroblasts are primary effector cells in idiopathic pulmonary fibrosis (IPF). Defining mechanisms of myofibroblast differentiation may be critical to the development of novel therapeutic agents.**

Objective: **To show that myofibroblast differentiation is regulated by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) activity *in vivo*, and to identify a potential mechanism by which this occurs.**

Methods: **We used tissue sections of surgical lung biopsies from patients with IPF to localize expression of PTEN and α–smooth muscle actin (α-SMA). We used cell culture of *pten*$^{-/-}$ and wild-type fibroblasts, as well as adenoviral strategies and pharmacologic inhibitors, to determine the mechanism by which PTEN inhibits α-SMA, fibroblast proliferation, and collagen production.**

Results: **In human lung specimens of IPF, myofibroblasts within fibroblastic foci demonstrated diminished PTEN expression. Furthermore, inhibition of PTEN in mice worsened bleomycin-induced fibrosis. In *pten*$^{-/-}$ fibroblasts, and in normal fibroblasts in which PTEN was inhibited, α-SMA, proliferation, and collagen production was upregulated. Addition of transforming growth factor-β to wild-type cells, but not *pten*$^{-/-}$ cells, resulted in increased α-SMA expression in a time-dependent fashion. In *pten*$^{-/-}$ cells, reconstitution of PTEN decreased α-SMA expression, proliferation, and collagen production, whereas overexpression of PTEN in wild-type cells inhibited transforming growth factor-β–induced myofibroblast differentiation. It was observed that both the protein and lipid phosphatase actions of PTEN were capable of modulating the myofibroblast phenotype.**

Conclusions: **The results indicate that in IPF, myofibroblasts have diminished PTEN expression. Inhibition of PTEN *in vivo* promotes fibrosis, and PTEN inhibits myofibroblast differentiation *in vitro*.**

Keywords: fibrosis; myofibroblast; phosphatase; PTEN; smooth muscle actin

α–Smooth muscle actin (α-SMA) expression in fibroblasts, increased proliferative capacity, and increased generation and secretion of the extracellular matrix (ECM) proteins collagen and fibronectin are key hallmarks of myofibroblast differentiation in fibrotic disorders (1, 2). In idiopathic pulmonary fibrosis (IPF)/usual interstitial pneumonia (UIP), persistence of myofibroblasts is believed to contribute to the development of fibrosis. It has been established that myofibroblast differentiation occurs through combinatorial signals involving transforming growth factor-β_1 (TGF-β) and integrin signaling (3). Conversely, mechanisms that inhibit or reverse myofibroblast differentiation are less well defined. Currently, no effective therapies exist to quell ongoing fibrosis in IPF/UIP. Thus, further investigation into the mechanisms that regulate myofibroblast differentiation may yield better insights into potential therapeutic options for patients with IPF/UIP.

The tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual-specificity protein and lipid phosphatase that uses phosphatidylinositol-3,4,5-trisphosphate as a physiologic substrate (4). Altered PTEN protein expression or activity has increasingly been implicated in the pathogenesis of nonmalignant diseases characterized by tissue destruction and remodeling, such as rheumatoid arthritis (5), asthma (6), and pulmonary fibrosis (7), thereby highlighting the importance of PTEN in physiologic regulation of nontransformed cellular phenotypes. Through its lipid phosphatase activity, PTEN inhibits cell migration (8), promotes cellular apoptosis (9), and inhibits cell growth (10). In addition, PTEN possesses protein phosphatase activity, and can act through the dephosphorylation of focal adhesion kinase (FAK) and Src homology 2–containing protein (11). Interestingly, *pten* expression is markedly decreased by TGF-β (12). We have previously observed diminished PTEN expression in fibroblasts isolated from lungs of patients with pulmonary fibrosis (7), and have hypothesized that this may account for the increased migratory capacity of these cells (7, 13).

Here, we show that in lung biopsy samples of patients with IPF/UIP, only fibroblasts that demonstrate a distinct inhibition or loss of PTEN correlate with the expression of α-SMA. We further show that pharmacologic inhibition of PTEN induces lung fibrosis in mice. Importantly, we show that inhibition of PTEN activity is both necessary and sufficient to induce myofibroblast differentiation. Finally, we demonstrate the novel finding that PTEN overexpression suppresses α-SMA expression, proliferation, and collagen production in myofibroblasts, a process that can occur via either lipid or protein phosphatase activity. Our data suggest that inhibition of PTEN expression in fibroblasts may contribute to the pathogenesis of fibrotic lung disease. Some of the results of this study have been previously reported in the form of an abstract (14).

METHODS

See online supplement for additional details.

Cell Culture and Reagents

C57Bl/6 embryonic mouse fibroblasts and National Institutes of Health 3T3 murine fibroblasts were from the American Type Culture Collection

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(Rockville, MD). Embryonic mouse fibroblasts lacking both *pten* alleles (*pten*$^{-/-}$) were described previously (9). The FAK/Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2), and its inactive control, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3), were from EMD Biosciences (San Diego, CA). The phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 was from Cell Signaling Technologies (Beverly, MA). Porcine TGF-β_1 was from R&D Systems (Minneapolis, MN). Bisperoxo(pyridine-2-carboxyl)oxovanadate (bpV[pic]) was from EMD Biosciences.

Antibodies

Antibodies to phospho-Smad2, total Smad2, and Smad7 were from Cell Signaling Technologies. α-SMA antibody (clone 1A4) was from Dako Corporation (Carpinteria, CA). β-Tubulin antibody was from Upstate (Waltham, MA). Anti-human PTEN (clone 6H2.1) was from Cascade Biosciences (Winchester, MA). Neutralizing antibody against TGF-$\beta_{1,2,3}$ was from R&D Systems.

Intratracheal Bleomycin Administration

Animals were anesthetized with pentobarbital. The trachea was exposed, and sterile bleomycin sulfate (0.05 U/mouse) or sterile phosphate-buffered saline (PBS) alone (50 μl) was delivered via a 26-gauge needle inserted directly into the trachea.

Collagen Measurements

Total lung collagen measurements were determined 21 d after bleomycin instillation. Total lung collagen and secreted collagen from cultured cells were determined using the Sircol collagen dye binding assay (Accurate Chemical Corporation, Westbury, NY) as previously described (15) following the manufacturer's instructions.

Adenovirus Construction

Adenovirus containing cDNA constructs encoding wild-type PTEN (Ad-PTEN) or the empty virus control (Ad-EV) were prepared as previously described (16).

Immunofluorescent Staining

Cells affixed to glass slides were permeabilized, blocked, and incubated with anti–α-SMA antibodies (1:1,000) for 1 h. Cells were washed and then incubated with fluorescein isothiocyanate (FITC)–conjugated secondary antibody for 1 h in the dark. Cells were again washed, mounted with 4′,6-diamidino-2-phenylindole–containing mounting media (Vector Labs, Burlingame, CA), and viewed under an epifluorescent microscope (Nikon Eclipse 50i; Nikon Inc., Melville, NY).

Immunohistochemistry

Immunohistochemistry was performed on 3- to 5-μm sections of formalin-fixed, paraffin-embedded surgical lung biopsy specimens obtained from patients undergoing surgical lung biopsy for diagnosis of idiopathic interstitial pneumonia. Prior written informed consent was obtained from all subjects in accordance with the University of Michigan Institutional Review Board. Rehydrated tissue sections were incubated with primary antibody, followed by biotinylated secondary antibody, and stained using the Vectastain ABC Universal kit (Vector Labs). Color development was achieved using 3,3′-diaminobenzidine, and sections were counterstained with hematoxylin. For immunofluorescent staining, the method of Mason and colleagues (17) was followed.

Fibroblast Proliferation Assay

Fibroblasts were plated in 96-well plates and serum-starved for 24 h. Media were changed, including indicated reagents, and cells cultured for 24 h. ^{3}H-thymidine was added for the last 16 h of culture, and radioactive counts per minute were assessed using a scintillation counter.

Semiquantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) used primers and probes for α-SMA as previously described (7, 18), and glyceraldehyde phosphate dehydrogenase was used to normalize results. Relative gene expression was calculated using the comparative cycle–threshold method (19).

Western Blot Analysis

Western blot analysis under reducing conditions was performed on equal concentrations of protein from whole-cell lysates and immunoprecipitated proteins, as previously described (7).

Statistical Analyses

Statistical analyses were performed using GraphPad InStat 3.05 (San Diego, CA). Differences between groups were evaluated using Student's *t* test. For multiple comparisons, one-way analysis of variance with Bonferroni's post-test analysis was used. Data were considered significant at a p value less than 0.05. Results were plotted using GraphPad Prism 3.02. Densitometry of visualized bands on Western blot was performed using Image J software (version 1.31; National Institutes of Health, Bethesda, MD).

RESULTS

Decreased PTEN Expression in Fibroblasts of Fibrotic Lesions Correlates with Increased α-SMA Expression

We have previously shown that PTEN expression is decreased in lung fibroblasts from patients with fibroproliferative disease compared with normal lung fibroblasts (7). To determine whether decreased PTEN expression was a general feature of lung fibroblasts or was localized to α-SMA–expressing myofibroblasts, we performed immunohistochemical analysis of surgical lung biopsy specimens from 10 patients with UIP, the histologic pattern associated with IPF (20). Consecutive sections were stained for α-SMA and PTEN and compared. A representative sample of fibroblastic foci, shown in Figure 1A, demonstrates that α-SMA expression is observed in spindle-shaped fibroblasts where PTEN staining is diminished or lost, thus indicating that α-SMA and PTEN expression may be inversely related (Figure 1A). Similar findings were observed in all other cases evaluated (data not shown). To confirm that PTEN was not being expressed in the same cell where α-SMA was being expressed, we employed triple immunofluorescent staining (17) on specimens from 10 patients with pulmonary fibrosis. Figure 1B shows a separate section from the same patient in Figure 1A stained for α-SMA (Cy3, red) and PTEN (FITC, green). We observed that Cy3-positive myofibroblasts do not demonstrate significant FITC staining. 4′,6-Diamidino-2-phenylindole (blue) staining identifies nuclei. Similar results were observed in all other samples (data not shown).

Inhibition of PTEN Activity in Fibroblasts Results in Myofibroblast Differentiation

To determine whether a cause-and-effect relationship exists between PTEN inhibition and myofibroblast differentiation, we initiated our studies by examining fibroblasts isolated from the embryos of *pten*-deficient transgenic mice (*pten*$^{-/-}$) compared with wild-type murine embryonic fibroblasts. These cells were defined as fibroblasts based on morphology under a light microscope, as well as by the expression of fibroblast-specific protein-1 and vimentin, and have been previously characterized (9). To determine α-SMA expression, cultured *pten*$^{-/-}$ and wild-type fibroblasts were assessed by immunoblotting. We observed that α-SMA was expressed in both *pten*$^{-/-}$ and wild-type fibroblasts under serum-free, quiescent conditions, although to a significantly greater degree in *pten*$^{-/-}$ fibroblasts ($p < 0.0001$). To ensure equal protein loading, membranes were stripped and reprobed for β-tubulin (Figure 2A). Immunofluorescent staining for α-SMA in serum-deprived wild-type and *pten*$^{-/-}$ cells confirmed this increase in α-SMA, and demonstrated that all *pten*$^{-/-}$ cells expressed α-SMA (Figure 2B). To investigate whether

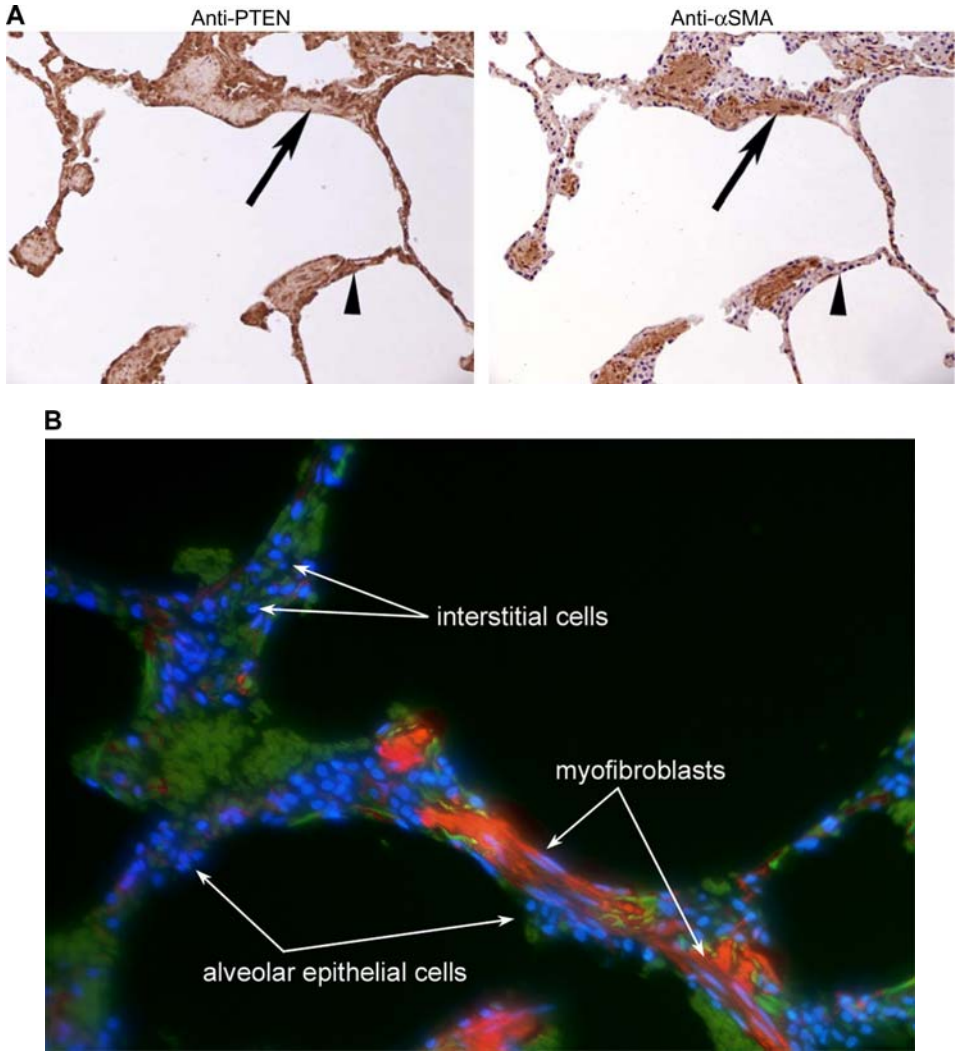

Figure 1. (*A*) Immunohistochemical analysis of phosphatase and tensin homolog deleted on chromosome 10 (PTEN; *left panel*) and α–smooth muscle actin (α-SMA; *right panel*) in a biopsy specimen from a patient with pulmonary fibrosis. *Arrows* identify clusters of spindle-shaped myofibroblasts (fibroblastic foci), which are characterized by α-SMA expression and a relative decrease in PTEN expression. *Arrowheads* delineate cuboidal epithelial cells (which express PTEN but not α-SMA) lining distorted airspaces. Photomicrographs are representative of fibroblastic foci observed throughout the lungs of all 10 patients examined (200× original magnification). (*B*) Triple immunofluorescent staining of the same biopsy specimen from the patient in (*A*) for PTEN (fluorescein isothiocyanate, *green*), α-SMA (Cy3, *red*), and nuclei (4′,6-diamidino-2-phenylindole [DAPI], *blue*). Whereas PTEN is located in epithelial and interstitial cells, it is not observed in α-SMA–expressing myofibroblasts (400× original magnification).

inhibition of PTEN activity is sufficient to promote α-SMA gene and protein expression, PTEN activity was inhibited in wild-type fibroblasts by the PTEN inhibitor dipotassium bpV(pic) (21). Treatment of cells with bpV(pic) does not alter protein levels of PTEN (21), but inhibits PTEN activity, as demonstrated by increasing levels of phosphorylated Akt at serine-473 (21). When we inhibited PTEN activity by treating wild-type cells with 200 nM bpV(pic) for 24 h, we observed a roughly fourfold increase in α-SMA expression. This increase in α-SMA was accompanied by a threefold increase in levels of phosphorylated Akt at serine-473 (Figure 2C).

We next investigated the possibility that PTEN affected other myofibroblast behaviors, namely proliferation and collagen secretion. To achieve this, *pten*$^{-/-}$ and wild-type cells were assessed for basal proliferation rates and collagen secretion. Using a ^{3}H-thymidine incorporation assay, we determined that *pten*$^{-/-}$ fibroblasts proliferated roughly 60 times faster than wild-type cells (Figure 3A). Similarly, cultures of *pten*$^{-/-}$ and wild-type cells were assessed for collagen production. Whole-cell lysates (to evaluate cell-associated collagen) and culture supernatants (to evaluate secreted collagen) were assessed for collagens I–V by Sircol assay. Although we did not observe significant amounts of soluble collagens in supernatants (not shown), we did observe significant elevations in cell- and matrix-associated collagens (Figure 3B, *left panel*).

Overexpression of PTEN Inhibits Myofibroblast Differentiation

To show that PTEN could inhibit myofibroblast differentiation, we next infected *pten*$^{-/-}$ cells with an adenoviral construct encoding either full-length, functional Ad-PTEN, or Ad-EV, and assessed for α-SMA expression, proliferation, and collagen production. We observed a resultant down-regulation of proliferation (Figure 3A, *right panel*), collagen production (Figure 3B, *right panel*), and α-SMA expression (Figure 3C) in cells infected with Ad-PTEN as compared with Ad-EV. Collectively, these data indicate that suppression of PTEN is sufficient to promote

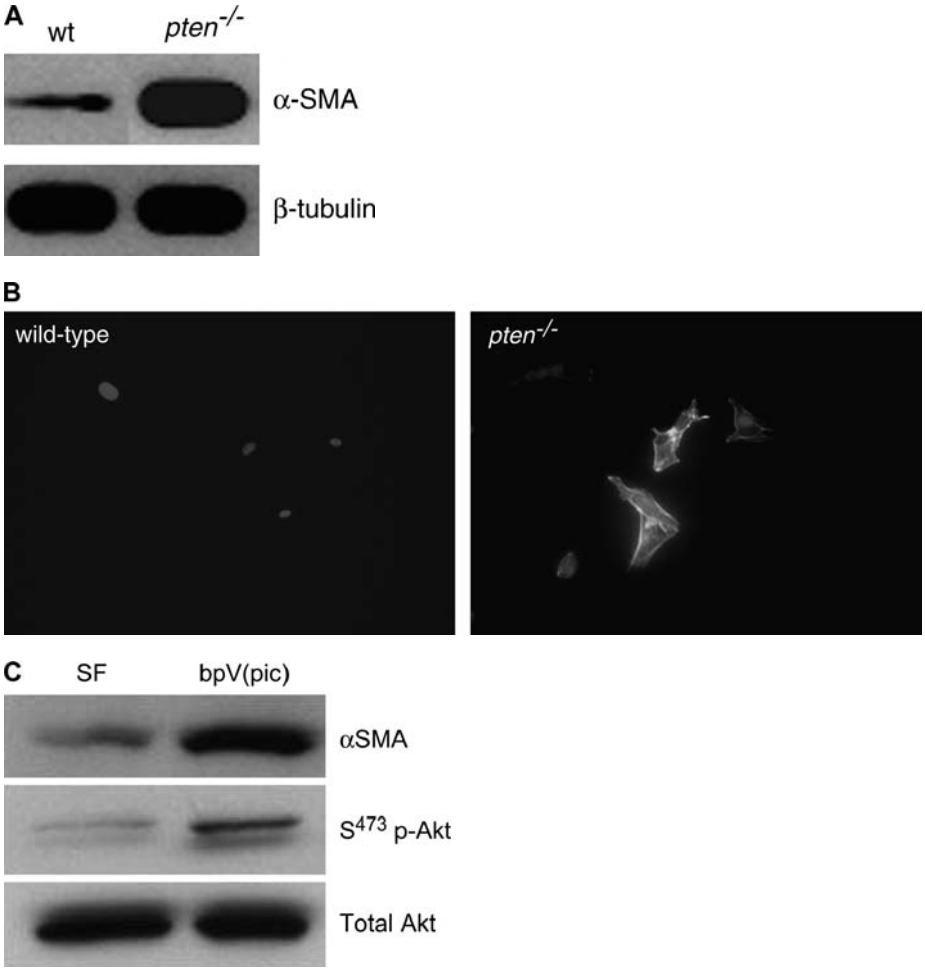

Figure 2. Loss of *pten* corresponds with increased α-SMA expression in fibroblasts. (*A*) Western blot of whole-cell lysates derived from serum-starved wild-type murine embryonic fibroblasts (wt) or embryonic fibroblasts from $pten^{-/-}$ mice ($pten^{-/-}$) probed for α-SMA. Blots were stripped and reprobed for β-tubulin as a loading control. The results are representative of three independent experiments using three separate cultures of cells. (*B*) Indirect immunofluorescent analysis of serum-starved wt fibroblasts (*left panel*) or $pten^{-/-}$ fibroblasts (*right panel*) labeled with anti–α-SMA antibody. Results are representative of two independent experiments. (*C*) The wt fibroblasts were growth-arrested and then treated with either serum-free media (SF) or 200 nM bisperoxo(pyridine-2-carboxyl)-oxovanadate (bpV[pic]) for 24 h, lysed, and immunoblotted for α-SMA, phosphorylated Akt at serine-473 (S^{473} pAkt), and total Akt. Results are representative of three separate experiments.

myofibroblast differentiation, and that reconstitution of PTEN into $pten^{-/-}$ cells will reverse the phenotype.

Increased α-SMA Expression in $pten^{-/-}$ Fibroblasts Is Not Dependent on TGF-β Signaling

TGF-β is a multifunctional cytokine that is a potent inducer of myofibroblast differentiation (22) and repressor of *pten* transcription (12). It has been well documented that autocrine release of TGF-β accounts, at least in part, for ongoing ECM secretion and myofibroblast differentiation in numerous model systems (23–26). Given that $pten^{-/-}$ cells possess a predominant myofibroblast phenotype, it was important to determine whether this was mediated by autocrine signaling by endogenous TGF-β. To address this, wild-type and $pten^{-/-}$ cells were cultured until subconfluent and extensively washed. Cells were then serum-starved and treated for 24 h with either control antibody or an antibody to TGF-β that is capable of neutralizing the activity of the three isoforms of TGF-β. Cells were then lysed and assayed for α-SMA expression by Western blot. As shown in Figure 4A, $pten^{-/-}$ cells expressed higher amounts of α-SMA than wild-types after 24 h of serum starvation (compare *lanes 1* and *4*, Figure 4A), confirming our initial observation. After the addition of neutralizing antibody to TGF-β or control antibody for 24 h, there was no decrease in α-SMA expression in wild-type or $pten^{-/-}$ cells (Figure 4A), suggesting that autocrine TGF-β is not a significant contributor to the constitutive myofibroblast phenotype seen in $pten^{-/-}$ cells.

TGF-β signaling predominantly occurs through the Smad pathway, where receptor–ligand interaction results in rapid phosphorylation and nuclear translocation of Smad2 and Smad3 (27, 28). Our data indicate that autocrine TGF-β signaling did not play a significant role in α-SMA expression in $pten^{-/-}$ fibroblasts. However, it was necessary to exclude the possibility that constitutive Smad2 or Smad3 activity accounted for the differences in α-SMA expression between $pten^{-/-}$ and wild-type fibroblasts. To do this, $pten^{-/-}$ and wild-type fibroblasts were plated until subconfluent, serum-starved overnight, and treated with exogenous TGF-β (2 ng/ml) for 1 h. Cells were then lysed and assayed for Smad2 and Smad3 phosphorylation by Western blot. After serum deprivation, no phosphorylation of Smad2 could be observed in either wild-type or $pten^{-/-}$ cells (Figure 4B). However, treatment of both cells with TGF-β for 1 h resulted in robust Smad2 phosphorylation (Figure 4B, *top panel*). Blots were stripped and probed for total Smad2 to ensure equal loading. Similar results were obtained for Smad3 (data not shown). This finding implies that basal Smad2 and Smad3 phosphorylation in $pten^{-/-}$ fibroblasts does not account for the increased constitutive α-SMA expression observed, but that both cells are equally capable of responding to an exogenous TGF-β challenge. Furthermore, these data suggest that surface TGF-β receptor expression and activity is equivalent between the two cell lines.

Regulation of Smad2/3 nuclear translocation occurs in part via the activity of Smad7. To determine whether dysregulation of Smad signaling could account for the constitutive myofibroblast phenotype observed in $pten^{-/-}$ cells, we assessed the levels of Smad7 in $pten^{-/-}$ and wild-type cells by Western blot. Unexpectedly, we observed that Smad7 levels were *increased* in $pten^{-/-}$

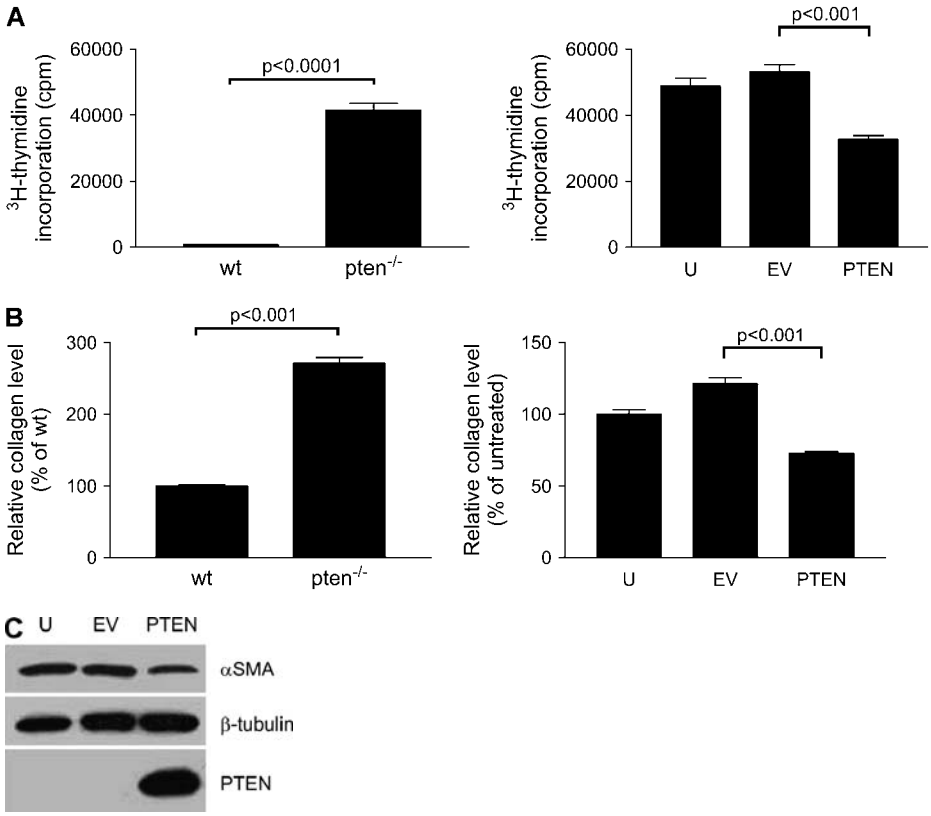

Figure 3. Reconstitution of PTEN into *pten*$^{-/-}$ cells inhibits the myofibroblast phenotype. (*A, left panel*) The wt and *pten*$^{-/-}$ cells were seeded in 96-well plates and allowed to proliferate in the presence of ^{3}H-thymidine for 24 h. ^{3}H-thymidine incorporation was measured by scintillation counting. *Right panel*: After infection with adenovirus encoding full-length, active PTEN (PTEN), but not empty virus (EV), proliferation was significantly attenuated. (*B, left panel*) Whole-cell lysates from wt and *pten*$^{-/-}$ cells were serum-starved for 24 h before evaluation for collagen production by Sircol assay. *Right panel*: The *pten*$^{-/-}$ cells were untreated (U) or infected with EV or active PTEN (Ad-PTEN, PTEN) for 24 h before lysis and evaluation of collagen production by Sircol assay. (C) The *pten*$^{-/-}$ cells were untreated (U), or treated with EV or with Ad-PTEN (PTEN) for 24 h. Lysates were assessed for α-SMA expression by Western blot. Blots were stripped and probed for β-tubulin as a loading control. Subsequently, the blot was stripped and reprobed to confirm PTEN expression.

cells compared with wild-type cells (Figure 4B, *bottom panel*). This interesting observation suggests that another possible action of PTEN is to inhibit Smad7 expression. Because Smad7 is a regulatory Smad, our data would appear to support the theory that PTEN inhibition or loss in nonmalignant cells disrupts normal cellular homeostatic mechanisms, promoting aberrant behaviors.

TGF-β Markedly Induces α-SMA Expression in Wild-Type Fibroblasts, but Only Minimal Further Increases in *pten*$^{-/-}$ Fibroblasts

Our data indicate that *pten*$^{-/-}$ fibroblasts express α-SMA independent of TGF-β stimulation, but that these cells are capable of responding to exogenous TGF-β through the Smad signaling pathway. Smad proteins have been shown to mediate TGF-β–induced α-SMA expression (29); we therefore asked whether the addition of TGF-β to *pten*$^{-/-}$ fibroblasts would further increase α-SMA expression. To test this, wild-type and *pten*$^{-/-}$ fibroblasts were growth-arrested for 24 h in serum-free media, washed with PBS, and then cultured in serum-free media in the absence or presence of TGF-β (2 ng/ml) for 24 h. Cell lysates from two separately grown cultures of each cell type were harvested and assessed by Western blot for α-SMA expression. Figure 5A shows that wild-type fibroblasts significantly increased α-SMA expression 24 h after TGF-β exposure ($p < 0.02$), whereas α-SMA expression in *pten*$^{-/-}$ fibroblasts was not substantially altered. This finding appears to be in agreement with our data demonstrating increased levels of Smad7 in *pten*$^{-/-}$ cells.

Because α-SMA is elevated constitutively in *pten*$^{-/-}$ fibroblasts as compared with wild-type fibroblasts, we next assessed whether this was due to increased gene expression. After isolating RNA from quiescent, serum-deprived *pten*$^{-/-}$ and wild-type fibroblasts, we used semiquantitative RT-PCR for α-SMA. We found that α-SMA gene expression was 1.8-fold greater in *pten*$^{-/-}$ cells compared with wild-type cells ($p < 0.0001$; Figure 5B), which correlated with increased α-SMA promoter activity (data not shown). In aggregate, these data establish that loss of PTEN expression resulted in increased α-SMA gene transcription and protein expression. Moreover, these findings imply that α-SMA expression induced by inhibition of PTEN occurs independent of Smad signaling.

Inhibition of PTEN Activity Is Necessary for TGF-β–induced α-SMA Expression

Because our data demonstrate that inhibition of PTEN activity was sufficient to induce α-SMA expression, we questioned whether it was also necessary to promote α-SMA expression in fibroblasts after TGF-β stimulation. To test this, we induced myofibroblast differentiation in normal fibroblasts by treating with TGF-β (2 ng/ml) for 24 h while concomitantly infecting with Ad-PTEN or Ad-EV. Cell lysates were collected and evaluated by Western blot for α-SMA. As shown in Figure 6, TGF-β–induced α-SMA expression in fibroblasts was attenuated in the presence of Ad-PTEN, but not in the presence of Ad-EV. Together, these data indicate that suppression of PTEN expression is both necessary and sufficient for α-SMA expression.

α-SMA Inhibition by PTEN Can Be Accomplished through Both Lipid- and Protein-Phosphatase Activity

Because PTEN is a dual-specificity phosphatase, we next sought to determine whether the lipid- or protein-phosphatase activity was responsible for α-SMA regulation. Thus, serum-starved wild-type fibroblasts were induced to express α-SMA, either through TGF-β treatment (2 ng/ml) or bpV(pic) treatment (200 nM), in the presence or absence of the FAK/Src-specific inhibitor PP2 (or its negative control, PP3; 10 μM), or the PI3K-specific inhibitors LY294002 (50 μM) or wortmannin (50 nM), for 24 h.

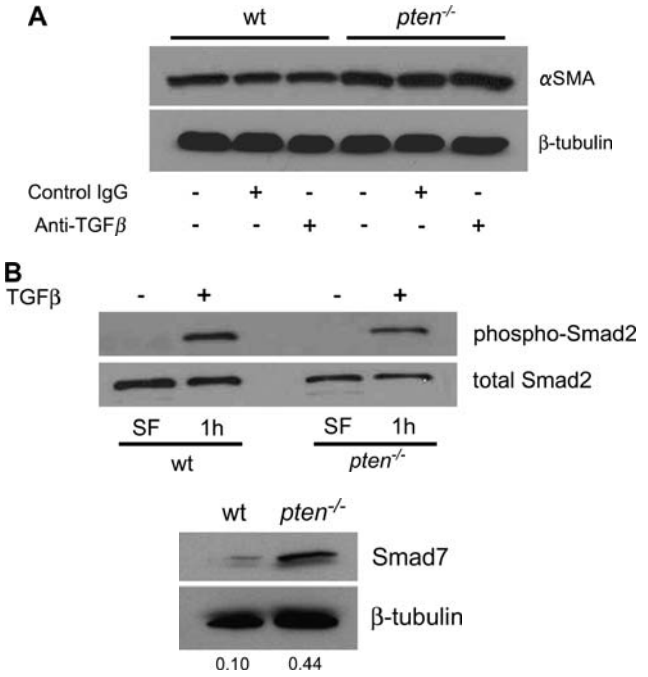

Figure 4. α-SMA expression in *pten*$^{-/-}$ cells is not dependent on autocrine TGF-β signaling or Smad activation. (*A*) The wt or *pten*$^{-/-}$ cells were cultured in serum-free media in the presence or absence of neutralizing antibody to TGF-β or control antibody for 24 h, and cell lysates were harvested for Western blot analysis of α-SMA. The blot was stripped and reprobed for β-tubulin as a loading control. Results are representative of two separate experiments. (*B, top panel*) The wt or *pten*$^{-/-}$ cells were cultured in serum-free media (SF) in the presence or absence of TGF-β (2 ng/ml) for 1 h, lysed, and assessed for phospho-Smad2 by Western blot. The membrane was stripped and reprobed with an antibody against total Smad2 to confirm equal loading. Results are representative of three separate experiments. *Bottom panel*: Lysates from serum-starved wt and *pten*$^{-/-}$ cells were collected and immunoblotted for total Smad7. The blot was stripped and reprobed for β-tubulin. Numbers under each *lane* represent densitometric ratios of Smad7 to β-tubulin.

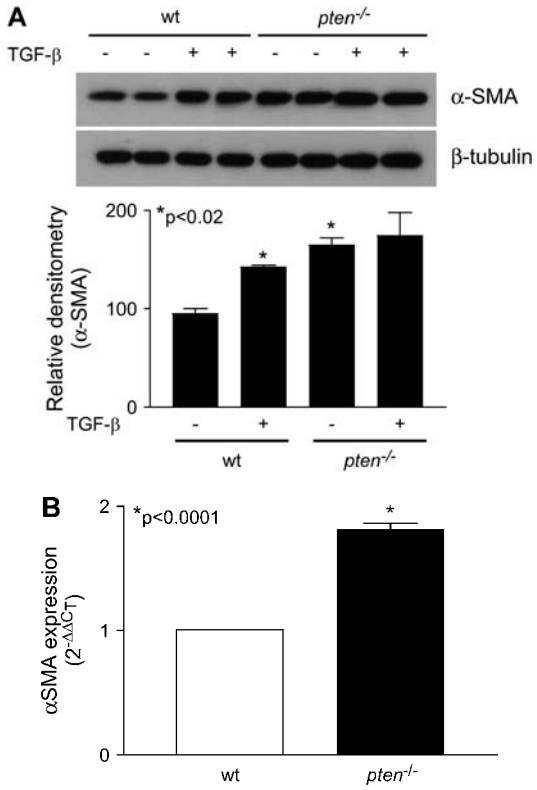

Figure 5. Increased basal expression of α-SMA in *pten*$^{-/-}$ cells is due to increased gene expression and transcription. (*A*) Two separate aliquots of wt and *pten*$^{-/-}$ cells were cultured in serum-free media in the presence or absence of TGF-β (2 ng/ml) for 24 h. Whole-cell lysates were evaluated by Western blot for α-SMA expression. To verify equal protein loading, the membrane was stripped and reprobed for β-tubulin. (*B*) The wt and *pten*$^{-/-}$ cells were cultured in serum-free media for 24 h and RNA was isolated. α-SMA gene expression was evaluated by semiquantitative real-time PCR using glyceraldehyde phosphate dehydrogenase as an internal control. Results are pooled data from two separate experiments performed in triplicate.

Untreated serum-free cells were assessed as a negative control. Whole-cell lysates were prepared and proteins separated by electrophoresis. Immunoblotting for α-SMA revealed the expected increase in α-SMA expression after treatment with TGF-β. Consistent with our previous findings, bpV(pic) also induced α-SMA expression, although to a lesser extent than TGF-β (Figure 7). Importantly, we observed that, in the presence of PP2, but not PP3, TGF-β–induced and bpV(pic)-induced α-SMA expression was attenuated, indicating that FAK and/or Src phosphorylation is necessary for α-SMA expression and that PTEN is capable of dephosphorylating one or both of these proteins (Figure 7), consistent with previous data (30). Similarly, we observed that, in the presence of LY294002 or wortmannin, TGF-β–induced and bpV(pic)-induced α-SMA expression was completely inhibited, providing evidence that unopposed PI3K activity after PTEN inhibition also contributes to α-SMA expression (Figure 7). Together, these data signify that PTEN is capable, through multiple signaling pathways, of inhibiting α-SMA expression during myofibroblast differentiation.

PTEN Inhibition Worsens Experimental Pulmonary Fibrosis and Is Associated with Greater Numbers of α-SMA–expressing Cells

Our aggregate data indicate that inhibition of PTEN is both necessary and sufficient to induce myofibroblast differentiation *in vitro*, a process that may also occur *in vivo* in UIP/IPF. To determine whether inhibition of PTEN would have similar effects in experimental pulmonary fibrosis, we used a murine bleomycin model. On Day 14 after intratracheal injection of bleomycin, a time point corresponding to resolution of the inflammatory phase and progression of the fibrotic phase, animals were treated with daily intraperitoneal injections of bpV(pic) (5 mM diluted in PBS) (31), or PBS as a control. Mice were killed 21 d after bleomycin and lungs were assessed for total collagen and presence of α-SMA–expressing cells. Bleomycin-treated animals demonstrated a significant increase in total lung collagen compared with control animals (Figure 8A). As predicted, bleomycin-exposed animals treated with bpV(pic) had substantially more lung collagen when compared with both control and bleomycin-exposed animals (Figure 8A). Histologically, we found that both collagen deposition (as evidenced by trichrome staining) and α-SMA expression (as evidenced by immunostaining) paralleled the total lung collagen findings (Figure 8B), indicating that PTEN inhibition *in vivo* results in increased α-SMA–expressing cells and worse fibrosis.

DISCUSSION

In this article, we describe the novel finding that PTEN negatively regulates myofibroblast differentiation in fibroblasts, and

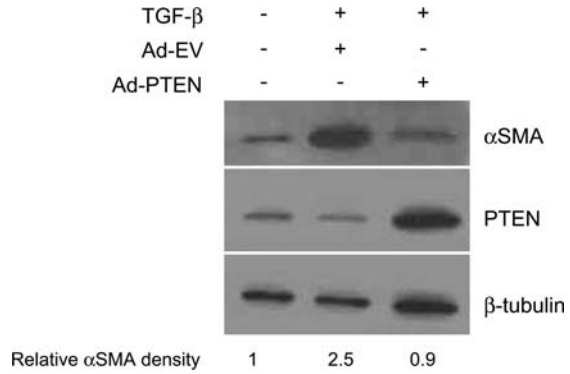

Figure 6. Inhibition of PTEN is necessary for TGF-β–induced α-SMA expression. Wt, serum-starved fibroblasts were induced to express α-SMA with TGF-β (2 ng/ml) in the presence or absence of an adenovirus encoding full-length Ad-PTEN or empty virus alone (Ad-EV). Whole-cell lysates were assessed for α-SMA expression by Western blot. The same blot was sequentially stripped and reprobed for PTEN and β-tubulin. The results are representative of two independent experiments.

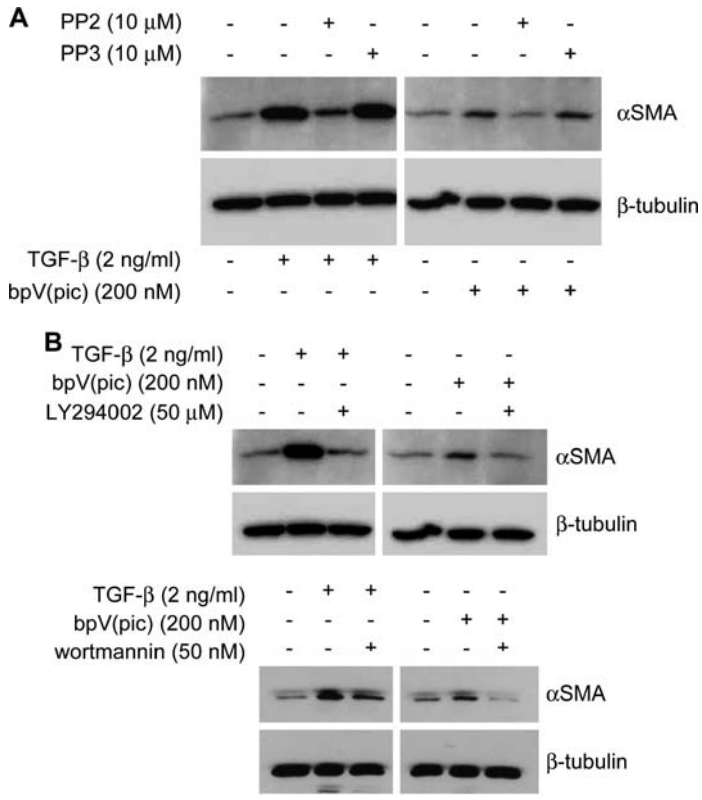

Figure 7. PTEN suppression of α-SMA expression involves both lipid- and protein-phosphatase activity. (*A*) Wt, serum-starved fibroblasts were induced to express α-SMA by treating with TGF-β (2 ng/ml) or bpV(pic) (200 nM) in the presence or absence of PP2 (10 μM) or PP3 (10 μM) for 24 h. Whole-cell lysates were prepared and evaluated by Western blot for α-SMA. The blot was stripped and reprobed for β-tubulin to confirm equal protein loading. The blot is representative of two separate experiments. (*B*) Wt, serum-starved fibroblasts were induced to express α-SMA by treating with TGF-β (2 ng/ml) or bpV(pic) (200 nM) in the presence or absence of the PI3K inhibitors LY294002 (50 μM) or wortmannin (50 nM) for 24 h. Whole-cell lysates were prepared and evaluated by Western blot for α-SMA. The blot was stripped and reprobed for β-tubulin to confirm equal protein loading. The blot is representative of two separate experiments.

that inhibition of PTEN allows for increased myofibroblast differentiation both *in vitro* and *in vivo*. We base this conclusion on our results showing that both *pten*$^{-/-}$ fibroblasts and fibroblasts in which PTEN is inhibited demonstrate increased expression of α-SMA, increased proliferation, and increased production of collagen. In addition, we show that PTEN suppression is necessary for TGF-β–mediated myofibroblast differentiation, and is sufficient to induce myofibroblast differentiation in wild-type cells. Importantly, we confirm the *in vivo* relevance of the association between PTEN and myofibroblast differentiation both in human IPF/UIP and in bleomycin-induced murine lung fibrosis, thereby identifying PTEN as a physiologic regulator of the myofibroblast phenotype. Mechanistically, our data suggest that suppression of PTEN promotes α-SMA expression via unopposed signaling through the PI3K and/or FAK/Src pathways, resulting in increased α-SMA gene expression and transcription.

Smad2/3 activation and nuclear localization following TGF-β exposure results in myofibroblast differentiation and α-SMA transcription (22, 28). Inhibition of Smad7, which binds and prevents nuclear translocation of the Smad2/3 complex, might also allow for increased α-SMA transcription after TGF-β stimulation. Somewhat unexpectedly, we observed that Smad7 levels were increased in *pten*$^{-/-}$ cells compared with wild-type cells. Although this may appear antithetical to our hypothesis, it is possible that elevated Smad7 levels in *pten*$^{-/-}$ cells may account for our observation of the relative inability of TGF-β to promote α-SMA expression further in *pten*$^{-/-}$ cells. This could be interpreted as a compensatory mechanism to prevent further α-SMA induction by external stimuli. Alternatively, Smad7 increases in *pten*$^{-/-}$ cells may indicate that Smad7 is also negatively regulated by PTEN. Regardless, our data describe a novel, non–Smad-dependent pathway of α-SMA expression that is tightly regulated by PTEN. As such, the data presented here support a new paradigm in the concept of fibroblast activation. Whereas previous investigation has focused on direct stimulus-induced activation, our study reveals that loss of negative regulatory elements, independent of Smad activation, can also result in myofibroblast differentiation. Thus, our data provide one possible explanation for the development of fibrotic disease in the absence of an identified inciting injury, and extend our current understanding of myofibroblast differentiation.

Fibroproliferative disorders are characterized by unrelenting, progressive ECM secretion and remodeling by α-SMA–expressing myofibroblasts, which ultimately causes organ dysfunction (32). Heterogeneity in subpopulations of fibroblasts derived from patients with fibroproliferative diseases suggests selective clonal expansion (33) that, although not malignant *per se*, is reminiscent of malignant cells. Features of myofibroblasts in fibroproliferative disorders include a relative resistance to apoptosis (34), increased migration/invasion of tissue (7), and increased proliferation (35)—all characteristics of malignant cells. It has been well established that PTEN negatively regulates cell survival (9) and fibroblast proliferation (36). We have previously shown that PTEN is suppressed in lung fibroblasts isolated from patients with IPF, which may account for increased migratory/invasive behavior (7). We therefore propose that phenotypic behavior of myofibroblasts in fibroproliferative disorders is similar to that of malignant cells in cancers in which PTEN is mutated, deleted, or otherwise inactivated. Thus, it is likely that other features of myofibroblasts not evaluated here, such as contractility or secretion of other ECM proteins, may be

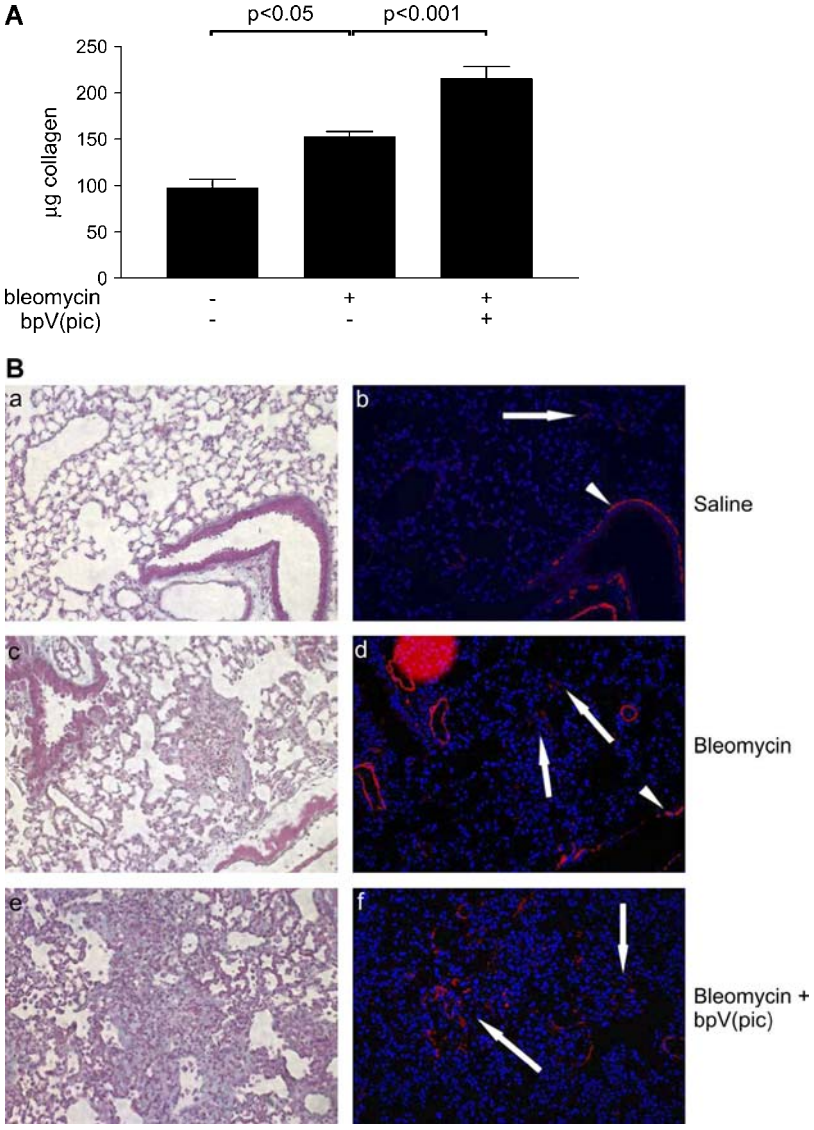

Figure 8. Inhibition of PTEN worsens experimental fibrosis. (*A*) C57Bl/6 mice administered intratracheal bleomycin were treated in the presence or absence of the PTEN inhibitor bpV(pic). Animals were killed 21 d after bleomycin treatment, and lungs were assessed for total collagen. Mice treated with intratracheal saline were used as controls (n = 8 for each experimental group). The figure is representative of two separately performed experiments. (*B*) *a* and *b*: Lung sections from a mouse receiving intratracheal saline. *c* and *d*: Lung sections from a mouse receiving intratracheal bleomycin. *e* and *f*: Lung sections from a mouse receiving intratracheal bleomycin and intraperitoneal bpV(pic). *Left panels* (*a*, *c*, *e*): Trichrome stain. *Right panels* (*b*, *d*, *f*): Immunofluorescent stain for α-SMA (*red*) and nuclear DAPI stain (*blue*). *Arrows* are pointing to individual cells or clusters of myofibroblasts staining positively for α-SMA. *Arrowheads* identify α-SMA–expressing smooth muscle cells lining large airways. α-SMA–expressing myofibroblasts colocalize to fibrotic regions of the lung (200× original magnification).

orchestrated through the activity of PTEN. Further investigation into this area will likely shed more light on the spectrum of phenotypic behaviors modulated by PTEN.

To assess whether PTEN could be involved in the regulation of myofibroblast differentiation, we employed complementary techniques to inhibit or overexpress PTEN. Using *pten*$^{-/-}$ cells, we found a marked increase in α-SMA expression, proliferation, and collagen secretion compared with wild-type cells. Interestingly, a clear difference was observed in the magnitude of effect among α-SMA expression, proliferation, and collagen production in *pten*$^{-/-}$ cells. Whereas α-SMA expression was 3-times greater, proliferation was 60-times greater in *pten*$^{-/-}$ cells compared with wild-type cells. The reason for this is not immediately apparent, but likely reflects the preferential impact of PTEN on intracellular signaling pathways that regulate proliferation compared with those that regulate protein synthesis.

Confirmatory experiments with PTEN inhibition using bpV(pic) in wild-type cells resulted in similar phenotypic findings. At high doses, bisperoxyvanadium compounds are potent, nonspecific phosphatase inhibitors (37). However, we chose to use bpV(pic) at a dose of 200 nM, a dose that inhibits PTEN but not PTP-1B or PTP-β (21); thus, our findings of increased myofibroblast differentiation are likely due to specific PTEN inhibition rather than nonspecific phosphatase inhibition. Hence, our data are consistent with our hypothesis that PTEN negatively regulates the myofibroblast phenotype. It is important to note that our studies demonstrate a correlation between decreased PTEN activity and myofibroblast differentiation. Whether this is due to increased numbers of myofibroblasts (an effect of proliferation) or increased amounts of α-SMA and collagen per cell (an effect of protein synthesis) is not yet clear. Data presented in Figures 2B and 8B would lend support to the former possibility, but the contribution of increased α-SMA and collagen produced per cell cannot be discounted.

To verify that PTEN plays an active role in suppressing myofibroblast differentiation, we used adenoviral infection strategies. These experiments revealed that in *pten*$^{-/-}$ cells, reconstitution of PTEN resulted in inhibition of the myofibroblast phenotype. Furthermore, in wild-type cells stimulated with TGF-β, overexpression of PTEN prevented the expected increase in α-SMA, whereas empty virus had no such effect. Importantly, under both experimental conditions, α-SMA expression was not completely abrogated. This is not unexpected. Indeed, we observed a slight increase in α-SMA transcription and protein

in *pten*$^{-/-}$ cells treated with TGF-β (Figure 5), substantiating our hypothesis that a separate, PTEN-independent pathway also promotes α-SMA expression. Given our findings that loss of PTEN induces α-SMA in a Smad-independent fashion, it is possible that the further increase in α-SMA in *pten*$^{-/-}$ cells after TGF-β reflects the contribution of the Smad pathway. Alternatively, small increases in α-SMA expression seen after TGF-β in *pten*$^{-/-}$ cells may simply reflect a near-maximal basal expression and/or transcription of α-SMA beyond which further increases are unattainable.

Phosphoinositol-3-phosphates generated by the activity of PI3Ks are the primary physiologic target of PTEN, although FAK and Src homology 2–containing protein have been shown to be dephosphorylated as well. One of the best-studied actions of the PI3K product phosphatidylinositol-3,4,5-trisphosphate is to recruit Akt to the plasma membrane through binding to its SH_3 domain, thereby facilitating the phosphorylation and activation of Akt. As such, it is conceivable that α-SMA expression might be induced as a result of PI3K-mediated Akt activity, and our data bolster this hypothesis. It is intriguing to note that induction of α-SMA expression after PTEN inhibition was also abrogated using PP2, a tyrosine kinase inhibitor specific for FAK and the Src-family kinases. Previous data have shown that FAK activity is necessary for α-SMA expression in fibroblasts (38), which is consistent with our findings. However, we cannot exclude the possibility that inhibition of Src kinases also plays a role. Indeed, Src kinases have been shown to phosphorylate PTEN, although the effects of tyrosine phosphorylation on PTEN activity are contradictory (39, 40). The ability of PTEN to inhibit α-SMA expression via antagonizing either the PI3K or FAK/Src pathways (or both) is not surprising, and demonstrates the multiple means by which cellular phenotypes are regulated.

Our previous studies have shown a stable suppression of PTEN at both the gene and the protein level in primary lung fibroblasts isolated from patients with pulmonary fibrosis, at least through 5 to 7 passages *ex vivo* (7). Given the heterogeneity of fibroblast cultures derived from primary tissues, it was unclear whether cultured fibroblasts expressing low levels of PTEN were actually representative of myofibroblasts within fibroblastic foci. Immunohistochemical analysis now conclusively demonstrates that suppression of PTEN in fibroblasts is a marker of a myofibroblast phenotype, as demonstrated by colocalization of α-SMA *in vivo*. To address whether similar findings were observed in a murine model of pulmonary fibrosis, we used bpV(pic) to inhibit PTEN activity. As predicted by our hypothesis, this had the effect of worsening fibrosis and increasing numbers of α-SMA–expressing cells.

It is unclear how stable suppression of *pten* in fibrotic-lung fibroblasts occurs. TGF-β is a potent inhibitor of *pten* (12), and is found in abundance in fibrotic tissue (41); yet we observed no difference in α-SMA expression in our studies using neutralizing antibody to TGF-β, suggesting that this is an unlikely mechanism for prolonged *pten* suppression in our model system. Other investigations have revealed that length of the 5′-untranslated region may regulate the constitutive expression of *pten* (42), as can the tumor suppressor p53 (43), and the transcription factors nuclear factor-κB and early growth response–1 (44, 45). Furthermore, recent studies have shown that epigenetic modifications of *pten* may result in inactivation of the gene in a number of cancers, including endometrial (46) and breast (47). Currently, it is unclear how *pten* may be stably inhibited in mesenchymal cells; nonetheless, studies designed to address these questions will clearly be of importance.

In conclusion, we have established that the tumor suppressor PTEN is a central negative regulator of myofibroblast differentiation. Furthermore, we have shown that this *in vitro* mechanism has *in vivo* relevance to human fibroproliferative disease and experimental pulmonary fibrosis. Finally, we have demonstrated that strategies designed to enhance PTEN expression or activity within cells can inhibit further fibroblast proliferation and collagen secretion, and may be a viable approach to the treatment of these diseases. Indeed, we have recently demonstrated that prostaglandin E_2 inhibits fibroblast migration via augmenting PTEN activity (48), thus highlighting the feasibility of this concept. Future work will be required to more precisely define the regulation of PTEN in fibroblasts.

Conflict of Interest Statement: E.S.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.G.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.H.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.W.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.R.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.D.K. has a patent for PTEN gene delivery that is pending. G.B.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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