

## Protein Tyrosine Phosphatase-N13 Promotes Myofibroblast Resistance to Apoptosis in Idiopathic Pulmonary Fibrosis

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### Abstract

**Rationale:** Idiopathic pulmonary fibrosis (IPF) is a progressive, fibrotic interstitial lung disease characterized by (myo)fibroblast accumulation and collagen deposition. Resistance to Fas-induced apoptosis is thought to facilitate (myo)fibroblast persistence in fibrotic lung tissues by poorly understood mechanisms.

**Objectives:** To test the hypothesis that PTPN13 (protein tyrosine phosphatase-N13) is expressed by IPF lung (myo)fibroblasts, promotes their resistance to Fas-induced apoptosis, and contributes to the development of pulmonary fibrosis.

**Methods:** PTPN13 was localized in lung tissues from patients with IPF and control subjects by immunohistochemical staining. Inhibition of PTPN13 function in primary IPF and normal lung (myo)fibroblasts was accomplished by: 1) downregulation with TNF- $\alpha$  (tumor necrosis factor- $\alpha$ )/IFN- $\gamma$ , 2) siRNA knockdown, or 3) a cell-permeable Fas/PTPN13 interaction inhibitory peptide. The role of PTPN13 in the development of pulmonary fibrosis was assessed in mice with genetic deficiency of PTP-BL, the murine ortholog of PTPN13.

**Measurements and Main Results:** PTPN13 was constitutively expressed by (myo)fibroblasts in the fibroblastic foci of patients with IPF. Human lung (myo)fibroblasts, which are resistant to Fas-induced apoptosis, basally expressed PTPN13 *in vitro*. TNF- $\alpha$ /IFN- $\gamma$  or siRNA-mediated PTPN13 downregulation and peptide-mediated inhibition of the Fas/PTPN13 interaction in human lung (myo)fibroblasts promoted Fas-induced apoptosis. Bleomycin-challenged PTP-BL<sup>-/-</sup> mice, while developing inflammatory lung injury, exhibited reduced pulmonary fibrosis compared with wild-type mice.

**Conclusions:** These findings suggest that PTPN13 mediates the resistance of human lung (myo)fibroblasts to Fas-induced apoptosis and promotes pulmonary fibrosis in mice. Our results suggest that strategies aimed at interfering with PTPN13 expression or function may represent a novel strategy to reduce fibrosis in IPF.

**Keywords:** pulmonary fibrosis; (myo)fibroblasts; apoptosis resistance; PTPN13

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## At a Glance Commentary

### Scientific Knowledge of the

**Subject:** Idiopathic pulmonary fibrosis is an intractable lung disease in which apoptosis-resistant (myo)fibroblasts accumulate and persist in fibroblastic foci leading to ongoing collagen matrix deposition. The mechanisms that promote (myo)fibroblast resistance to apoptosis remain incompletely understood. Improved knowledge of these mechanisms could lead to new therapeutic approaches to control (myo)fibroblast accumulation.

### What This Study Adds to the

**Field:** Through the combined use of human lung tissues, isolated lung (myo)fibroblasts, and a mouse model of pulmonary fibrosis, our study has identified previously unknown roles for the protein tyrosine phosphatase, PTPN13, in the persistence of fibrotic lung (myo)fibroblasts and the development of pulmonary fibrosis. These findings suggest that interfering with PTPN13 expression or function may represent a novel strategy to prevent the development and progression of fibrotic lung diseases.

Idiopathic pulmonary fibrosis (IPF) is a parenchymal lung disease that results in progressive scarring of the alveolar-capillary units and eventual respiratory failure (1). With a median survival of approximately 3 years after diagnosis and limited therapies, the prognosis for patients with IPF is dismal (2, 3). Precise details regarding the initiating events remain poorly understood, although repetitive injury to the alveolar epithelium is thought to drive an aberrant repair process in which an unrestrained accumulation of fibroblasts and  $\alpha$ -SMA ( $\alpha$ -smooth muscle actin)-expressing myofibroblasts (which we collectively refer to as [myo]fibroblasts) within fibroblastic foci results in excessive paraseptal collagen deposition (1, 4). Recent studies have suggested that multiple cell types contribute to (myo)fibroblast accumulation in the granulation tissue that forms during alveolar injury (5–7). However, unlike normal granulation tissue resolution, where (myo)fibroblasts undergo

apoptosis and are removed during repair (8), (myo)fibroblasts persist in the fibroblastic foci of patients with IPF because of a failure to undergo apoptosis (9–11). Thus, improved understanding of the mechanisms contributing to (myo)fibroblast apoptotic failure might reveal new therapeutic targets to reduce lung (myo)fibroblast accumulation and parenchymal fibrosis in patients with IPF.

The death receptor Fas is abundantly expressed on normal lung fibroblasts, and once a predefined threshold has been exceeded, its ligation induces apoptosis (12, 13). Lung (myo)fibroblasts from patients with IPF, however, are largely resistant to Fas-induced apoptosis (12, 14–16). TGF- $\beta$  (transforming growth factor- $\beta$ ), in addition to its profibrotic activities, creates an antiinflammatory microenvironment through its ability to inhibit the production of proinflammatory cytokines, including TNF- $\alpha$  (tumor necrosis factor TNF- $\alpha$ ) and IFN- $\gamma$ . Our previous studies have shown that exposure of (myo)fibroblasts to the combination of TNF- $\alpha$  and IFN- $\gamma$  (TNF- $\alpha$ /IFN- $\gamma$ ) overcomes their intrinsic resistance to Fas-induced apoptosis (12, 13). Furthermore, based on our earlier studies showing a paucity of TNF- $\alpha$  in the lungs of patients with IPF (17), we recently observed that intratracheal delivery of TNF- $\alpha$  to mice with established pulmonary fibrosis accelerates fibrotic resolution (18), whereas genetic TNF- $\alpha$  deficiency impedes fibrosis resolution (18). Together, these findings support the concept that a limited TNF- $\alpha$ -driven proinflammatory signal to the lungs beneficially promotes the resolution of pulmonary fibrosis, in part by sensitizing lung (myo)fibroblasts to Fas-induced apoptosis.

Seeking to understand the mechanisms by which TNF- $\alpha$ /IFN- $\gamma$  promotes

sensitivity of lung fibroblasts to Fas-induced apoptosis, we analyzed the transcriptomes of TNF- $\alpha$ /IFN- $\gamma$ -exposed human primary lung (myo)fibroblasts. We found that among 603 differentially expressed transcripts, the mRNA encoding PTPN13 (protein tyrosine phosphatase-N13), an apoptosis inhibitory Fas-interacting tyrosine phosphatase (also known as FAP-1 in humans, and PTP-BL in mice) was reduced (13). Based on these findings, we hypothesized that PTPN13 mediates the resistance of lung (myo)fibroblasts to Fas-induced apoptosis and promotes pulmonary fibrosis. Herein, we address this hypothesis in fibrotic lung tissues and primary lung (myo)fibroblasts from patients with IPF, and in PTP-BL<sup>-/-</sup> mice.

## Methods

### Human Lung Tissues and Primary Lung (Myo)Fibroblasts

Lungs from nondiseased individuals were obtained from Tissue Transformation Technologies, as described (12). Lung samples from patients with interstitial lung diseases were obtained during diagnostic surgical lung biopsy or lung transplantation. Immunohistochemical staining for PTPN13 and  $\alpha$ -SMA was conducted, as described (9). PTPN13 staining intensity was semiquantified by a board-certified pathologist using a three-tiered scaling system (absent = 0, intermediate = 1, strong = 2).

Primary cultures of normal lung (myo)fibroblasts from nondiseased, male and female human donors (Table 1) were isolated and cultured on plastic, as described (12). IPF (myo)fibroblasts were isolated from peripheral, lower lobe lung

**Table 1.** Patient Demographics

Characteristics	Healthy Volunteers (n = 6)	Patients	
		IPF (n = 8)	Organizing Pneumonia (n = 6)
Male sex, n (%)	2 (33)	5 (63)	5 (83)
Age, yr, mean $\pm$ SD	55 $\pm$ 28	65 $\pm$ 12	63 $\pm$ 16
Age distribution, n (%)			
15 to <25	2 (33)	0 (0)	0 (0)
25 to <50	0 (0)	0 (0)	1 (17)
50 to <80	4 (67)	8 (100)	5 (83)

*Definition of abbreviation:* IPF = idiopathic pulmonary fibrosis.

Two of the patients with organizing pneumonia presented with a mixed usual interstitial pneumonia/organizing pneumonia pathology.

biopsies from patients with IPF. When cultured on rigid plastic substrata, fibroblasts increase  $\alpha$ -SMA expression suggesting they exhibit features of (myo) fibroblasts, hence our definition. (Myo) fibroblast apoptosis and Fas DISC (death-inducing signaling complex) assembly were assessed, as described (12, 13) and in the online supplement. All human studies used deidentified samples and were approved by the National Jewish Health and University of Colorado Denver institutional review boards.

#### Animal Care and Procedures

PTP-BL<sup>+/-</sup> embryos, generously provided by Dr. Michael Grusby, Harvard University TH Chan School of Public Health, were implanted into pseudopregnant females. PTP-BL<sup>-/-</sup> male mice and control C57BL/6J male mice (Jackson Laboratory) aged 8–10 weeks

were studied. Mice were intratracheally instilled with 2.5 U/kg bleomycin (APP Pharmaceuticals), as described (18). Fibrotic parameters were assessed, as described (18, 19) and in the online supplement. All mouse procedures were approved by the National Jewish Health Institutional Animal Care and Use Committee.

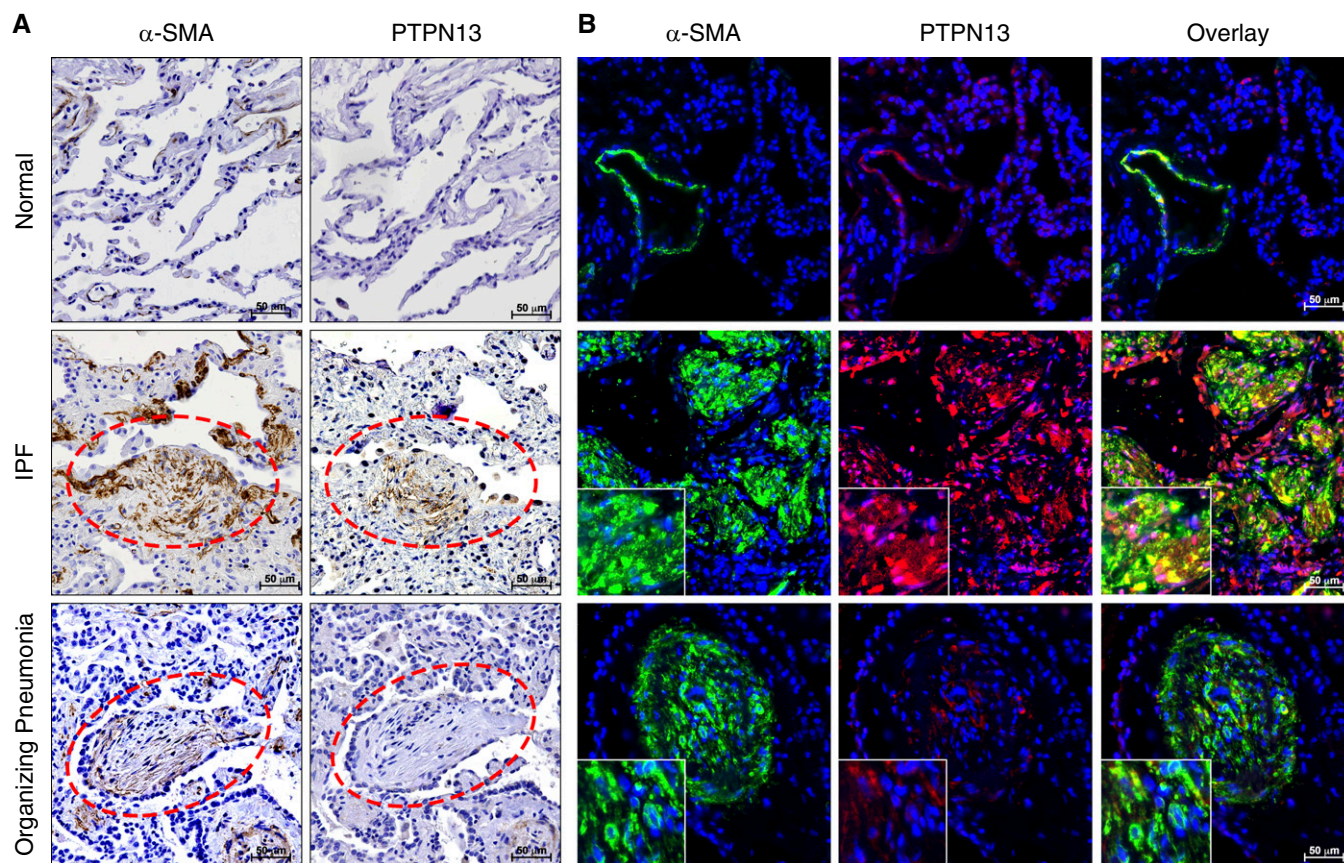
#### Statistics

Data are presented as mean  $\pm$  SEM. Statistical analyses were performed using GraphPad Software or R (R: a Language and Environment for Statistical Computing. <http://www.R-project.org>). Statistical comparisons of means were performed using Student's *t* test or by ANOVA. We used the false discovery rate procedure to control for multiple testing (18). A *P* value less than 0.05 was regarded as statistically significant.

## Results

### PTPN13 Is Localized to Fibroblastic Foci in the Lungs of Patients with IPF

PTPN13 was first identified through its ability to inhibit Fas-induced apoptosis (20). Because primary lung fibroblasts from patients with IPF are impaired in their ability to undergo Fas-induced apoptosis (12, 16), we first determined if PTPN13 might be relevant to (myo)fibroblast resistance to Fas-induced apoptosis by investigating its expression in the fibroblastic foci of patients with IPF. Using both immunohistochemical staining of PTPN13 and  $\alpha$ -SMA on serial sections and dual-label immunofluorescent staining on single sections, PTPN13 was found to be localized to both  $\alpha$ -SMA-positive myofibroblasts and  $\alpha$ -SMA-negative cells of fibroblastic morphology in the fibroblastic foci of patients with IPF (Figures 1A and



**Figure 1.** PTPN13 (protein tyrosine phosphatase-N13) is associated with fibrotic foci in lung tissues from patients with idiopathic pulmonary fibrosis (IPF). (A) PTPN13 and  $\alpha$ -SMA ( $\alpha$ -smooth muscle actin) colocalization of immunohistochemical staining in serial sections of lung tissues from nondiseased human subjects ( $n = 6$ ), patients with IPF ( $n = 8$ ), and patients with organizing pneumonia ( $n = 6$ ). (B) Coimmunofluorescent staining of  $\alpha$ -SMA (green) and PTPN13 (red). Insets are twice the magnification of the other panels. Control nonimmune IgG staining is shown in Figure E1. \*\*\* $P < 0.001$ , mean  $\pm$  SEM. Red dashed ellipses indicate fibroblastic foci or fibroblastic buds.

1B, middle row). Of the 13 IPF lung biopsies studied, 12 (92%) demonstrated PTPN13 staining in fibroblastic foci and PTPN13 expression was significantly increased ( $P < 0.001$ ) in IPF lung tissues compared with normal subjects (see Figure E2 in the online supplement). As a disease control, we evaluated PTPN13 localization in lung biopsies from patients with organizing pneumonia, a subacute, nonprogressive, and largely temporary reparative disorder in which (myo)fibroblasts organize in the alveoli of affected patients. Unlike IPF, organizing pneumonia frequently resolves because of (myo)fibroblast apoptosis (9, 10). We observed minimal expression of PTPN13 in the luminal  $\alpha$ -SMA-positive myofibroblasts in organizing pneumonia compared with IPF (Figures 1A and 1B, lower row). We also observed minimal expression of PTPN13 in normal lung parenchyma, although positive staining was detected in alveolar macrophages and some airway epithelial cells (see Figure E3). These findings support the notion that PTPN13 expression by lung (myo)fibroblasts in IPF contributes to their accumulation via resistance to Fas-induced apoptosis.

#### **TNF- $\alpha$ /IFN- $\gamma$ Downregulates PTPN13 Expression in Human Lung (Myo)Fibroblasts**

Given that PTPN13 is expressed in the fibroblastic foci in patients with IPF and exposure of primary human lung (myo)fibroblasts to TNF- $\alpha$ /IFN- $\gamma$  overcomes their resistance to Fas-induced apoptosis (12, 14, 16, 21), we hypothesized that PTPN13 is basally expressed by cultured primary human lung (myo) fibroblasts, and exposure to TNF- $\alpha$ /IFN- $\gamma$  downregulates PTPN13. Primary normal human and IPF lung (myo)fibroblasts along with human lung (myo)fibroblastic MRC5 cells were grown on rigid plastic tissue culture plates and stimulated with TNF- $\alpha$ , IFN- $\gamma$ , or both cytokines. PTPN13 mRNA and protein levels were quantified by qPCR and Western blotting, respectively. PTPN13 mRNA and protein were found to be basally expressed at similar levels in primary normal human and IPF lung (myo)fibroblasts and MRC5 cells (Figures 2A and 2C). Likewise, basal PTPN13 mRNA expression as quantified by RNA-seq (see Figure E4) and protein levels were similar in multiple normal and IPF (myo)fibroblast lines (see Figure

E5). Incubation with IFN- $\gamma$  had little effect on PTPN13 expression (Figure 2A). However, exposure to TNF- $\alpha$  reduced PTPN13 expression by approximately 50% (Figure 2A), whereas incubation with both cytokines reduced PTPN13 expression by approximately 75% (Figure 2A).

All three types of lung (myo)fibroblasts behaved similarly (Figure 2A; see Figure E5). Because we have previously shown that TNF- $\alpha$ /IFN- $\gamma$  sensitization to Fas-induced apoptosis takes approximately 24–36 hours (12), we compared the kinetics of cytokine-induced downregulation of PTPN13 with acquisition of sensitivity to Fas-induced apoptosis. Exposure to TNF- $\alpha$ /IFN- $\gamma$  led to maximal down-regulation of PTPN13 mRNA and protein by 18–24 hours (Figures 2B and 2C) concurrent with acquisition of sensitivity to Fas-induced apoptosis as reflected by caspase-8 activation (Figure 2D) and mitochondrial depolarization (see Figure E6), which was used as an alternative apoptosis marker.

#### **Reduced PTPN13 Expression or Impaired Interaction with Fas Overcomes Resistance of Lung (Myo)Fibroblasts to Fas-induced Apoptosis**

To determine if reduced expression of PTPN13 is sufficient to overcome the resistance of human (myo)fibroblasts to Fas-induced apoptosis, we knocked-down PTPN13 with two different siRNAs (Figure 3A). Normal, IPF and MRC5 lung (myo)fibroblasts were transfected with PTPN13 or nontargeting siRNAs before assessing their ability to undergo Fas-induced apoptosis. Both PTPN13 siRNAs reduced PTPN13 protein expression by greater than 80% and to a level similar to TNF- $\alpha$ /IFN- $\gamma$  treatment (Figure 3B). Figures 3C–3E show that PTPN13 knockdown sensitized normal, IPF, and MRC5 (myo)fibroblasts to Fas-induced proapoptotic caspase-3 activation, whereas the nontargeting siRNA had no effect on either PTPN13 protein expression or susceptibility to Fas-induced apoptosis (Figures 3B–3E). Similar data were obtained for caspase-8 activation (see Figure E7).

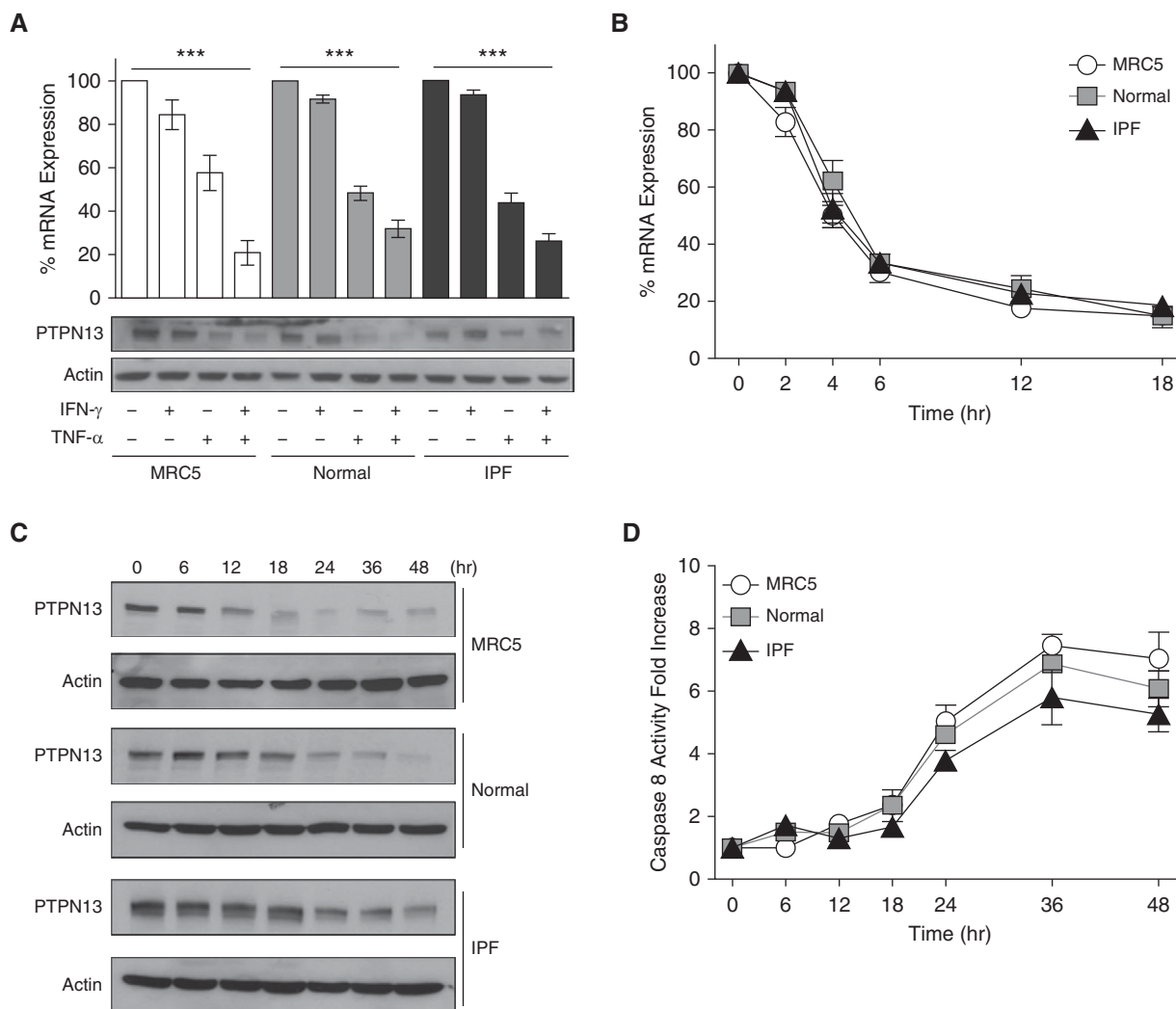
Fas-induced apoptosis is initiated when the adapter protein FADD and procaspase-8 are recruited to the cytoplasmic domain of Fas to form the DISC (22). We tested the hypothesis that PTPN13 knockdown overcomes the resistance of human lung (myo)fibroblasts to Fas-induced apoptosis

by enabling DISC assembly. PTPN13-specific siRNAs were used to knockdown PTPN13 in MRC5 lung (myo)fibroblasts before stimulation with agonistic anti-Fas antibody and assessment of DISC assembly by coimmunoprecipitation of caspase-8 with Fas. Figure 3F shows that PTPN13 knockdown was sufficient to increase Fas-induced recruitment of caspase-8, whereas the nontargeting siRNA was without effect. Together, these data indicate that PTPN13 expression is necessary for the resistance of normal, IPF, and MRC5 human lung (myo)fibroblasts to Fas-induced signaling and apoptosis.

Previous studies have shown that Fas contains a C-terminal tripeptide sequence (SLV) that interacts with PTPN13 via PDZ domain 2 (Figure 3A) (23). To confirm that PTPN13 is necessary for the resistance to Fas-induced fibroblast apoptosis, we interfered with the interaction between Fas and PTPN13 with a previously validated cell-permeable acyl-SLV peptide (23). Pretreatment of MRC5 (myo)fibroblasts with acyl-SLV resulted in a similar level of Fas-induced caspase-8 and caspase-3 activation to that accomplished with TNF- $\alpha$ /IFN- $\gamma$  stimulation (Figures 4A and 4B), whereas pretreatment with the control acyl-SVL peptide was without effect (Figures 4A and 4B). Similarly, the acyl-SLV peptide, but not the acyl-SVL peptide, enabled DISC assembly (Figure 4C). We confirmed the specificity and effectiveness of the SLV peptide in blocking the Fas-PTPN13 interaction using an AlphaScreen assay and found that the SLV peptide, but not the SVL peptide, inhibited the interaction between the PDZ1/2 domain of PTPN13 and the C-terminal SLV sequence of Fas (see Figure E8). Together, these findings indicate that downregulation of PTPN13 expression by TNF- $\alpha$ /IFN- $\gamma$  exposure, PTPN13 siRNA, or interference of the Fas-PTPN13 interaction restores Fas-induced DISC assembly and apoptotic signaling in human lung (myo)fibroblasts.

#### **PTPN13 Tyrosine Phosphatase Activity Protects (Myo)Fibroblasts from Fas-induced Apoptosis by Dephosphorylating Fas**

Fas contains two tyrosine residues (Y232 and Y291) that upon phosphorylation (24, 25), promote Fas oligomerization and apoptosis (26, 27). Because PTPN13 is a protein tyrosine phosphatase, we tested the hypothesis that TNF- $\alpha$ /IFN- $\gamma$ -induced



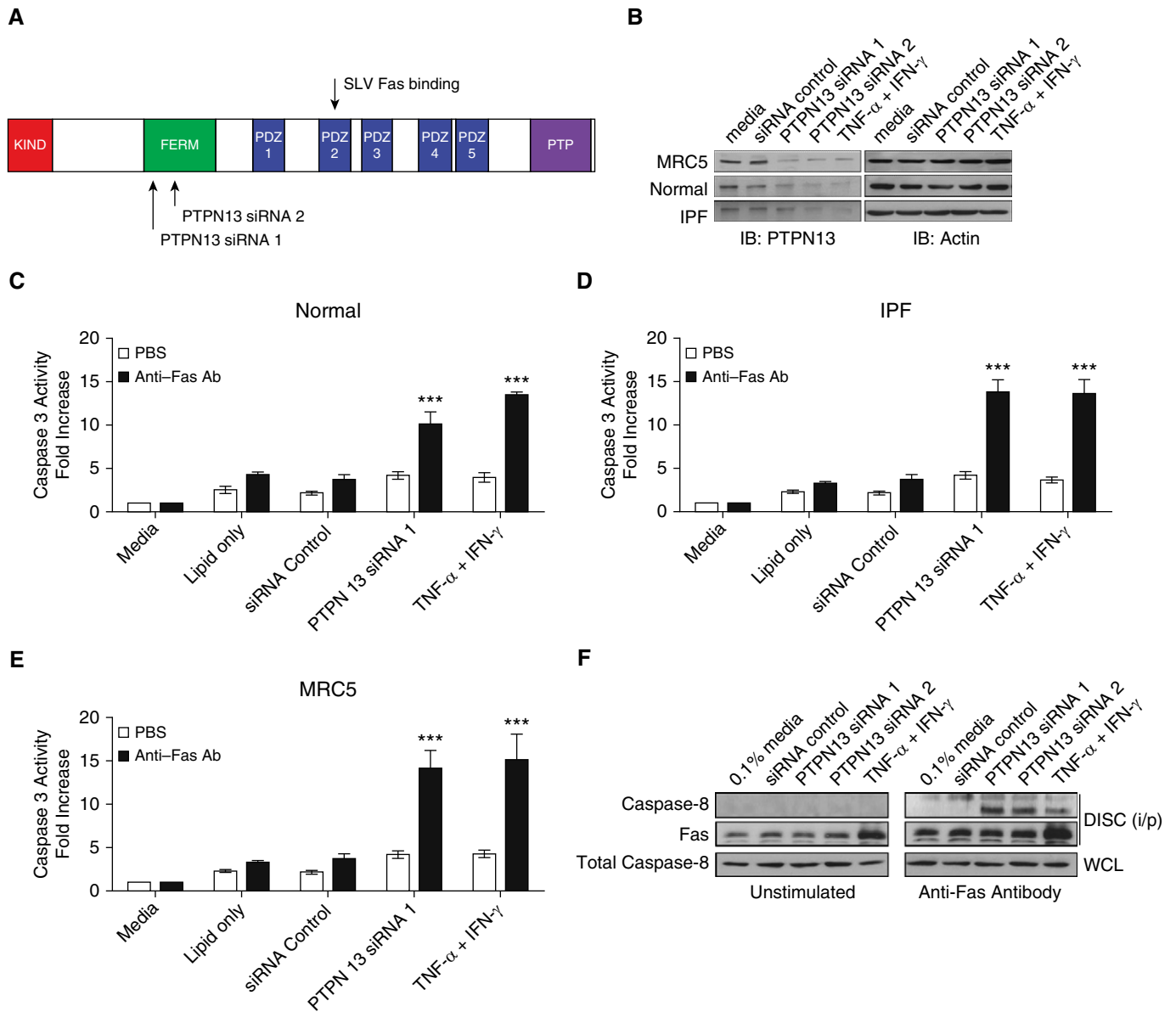
**Figure 2.** TNF- $\alpha$  (tumor necrosis factor- $\alpha$ )/IFN- $\gamma$  decrease PTPN13 (protein tyrosine phosphatase-N13) expression in human lung myfibroblasts. (A, top panel) qPCR analysis of PTPN13 in normal human lung fibroblasts (normal), fibrotic human lung myfibroblasts (IPF), and MRC5 cells. (A, bottom panel) Western blot analysis of PTPN13 protein. (B) qPCR-based assessment of the time course of PTPN13 mRNA expression after TNF- $\alpha$ /IFN- $\gamma$  exposure. (C) Western blot analysis of PTPN13 expression over 48 hours in MRC5, normal, and IPF fibroblasts exposed to TNF- $\alpha$ /IFN- $\gamma$ . (D) Fas-induced caspase-8 activation after sensitization of myfibroblasts to TNF- $\alpha$ /IFN- $\gamma$  over 48 hours before stimulation with agonistic anti-Fas-activating antibody for 4 hours. TNF- $\alpha$  was used at 10 ng/ml and IFN- $\gamma$  at 50 U/ml. \*\*\* $P < 0.001$ , mean  $\pm$  SEM.  $n = 3$  per cell line. IPF = idiopathic pulmonary fibrosis.

PTPN13 downregulation or siRNA silencing would increase Fas tyrosine phosphorylation and sensitivity to Fas-induced apoptosis. First, we incubated MRC5 (myo)fibroblasts with TNF- $\alpha$ /IFN- $\gamma$  and assessed phosphorylation of Fas residues Y232 and Y291 by Western blotting. Exposure to TNF- $\alpha$ /IFN- $\gamma$  increased phosphorylation of Fas Y291 (Figure 5A) coincident with both the downregulation of PTPN13 expression and the acquisition of sensitivity to Fas-induced apoptosis (Figures 2C and 2D). The appearance of slower migrating phospho-Fas bands and SDS-stable higher order receptor complexes (Figure 5A) is consistent with

previous studies showing that Fas undergoes post-translational palmitoylation and phosphorylation in cells destined to undergo apoptosis (28). We confirmed that Fas undergoes tyrosine phosphorylation of both monomeric and higher order complexes by immunoprecipitation with antiphosphotyrosine antibody and immunoblotting with anti-Fas (see Figure E9). Phosphorylation of Fas Y232 was not detected (data not shown). Next, to determine if Fas phosphorylation was mediated by PTPN13, we silenced PTPN13 expression or interfered with its interaction with Fas with the acyl-SLV peptide. Figures 5B and 5C show that both approaches led to

similar increases in Fas Y291 phosphorylation in lung (myo)fibroblasts.

To determine if reduced protein tyrosine phosphatase activity accounted for the increase in Fas Y291 phosphorylation, we incubated MRC5 myofibroblasts with pervanadate, a broad-spectrum PTP inhibitor, and evaluated its effect on Fas Y291 phosphorylation. Figures 5D and 5E show that pervanadate increased Fas Y291 phosphorylation and overcame the resistance of the cells to Fas-induced apoptosis. Thus, the resistance of human lung (myo)fibroblasts to Fas-induced apoptosis is caused by PTPN13-mediated dephosphorylation of Fas Y291.



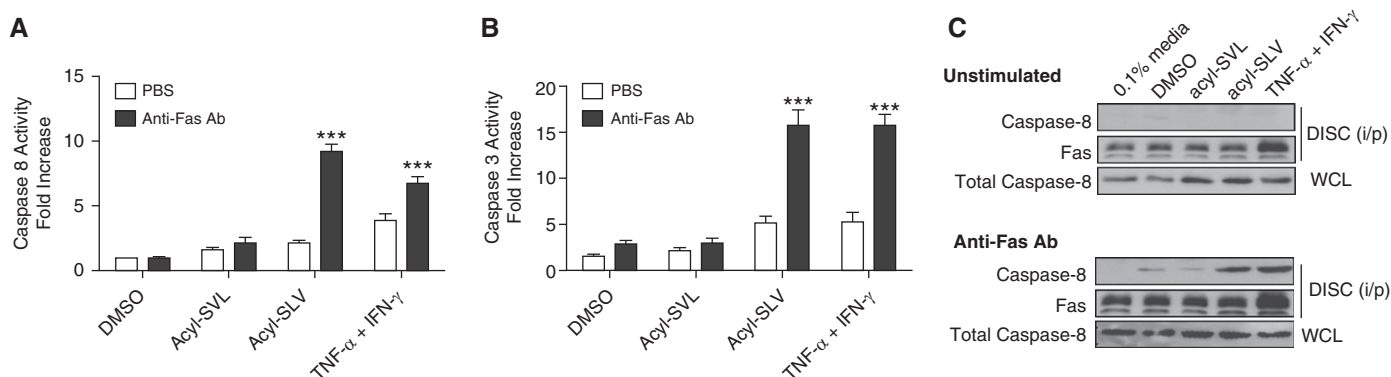
**Figure 3.** Decreased PTPN13 (protein tyrosine phosphatase-N13) expression sensitizes human lung myfibroblasts to Fas-induced apoptosis. (A) Illustration of protein domains within PTPN13. Two PTPN13-specific siRNAs are directed toward the FERM domain. (B) Western blot analysis of siRNA-mediated knockdown of PTPN13. (C–E) Fas-induced caspase-3 activation in PTPN13 siRNA-treated normal, idiopathic pulmonary fibrosis, and MRC5 lung myfibroblasts, respectively. (F) Decreased expression of PTPN13 by siRNA or TNF- $\alpha$  (tumor necrosis factor- $\alpha$ )/IFN- $\gamma$  promotes the DISC (death-inducing signaling complex) assembly after Fas stimulation. Coprecipitated caspase-8 was recruited to the DISC after Fas ligation in lung fibroblasts pretreated with PTPN13 siRNA or with TNF- $\alpha$ /IFN- $\gamma$ . The quantitative increase in immunoprecipitated Fas after stimulation with TNF- $\alpha$ /IFN- $\gamma$  is caused by increased Fas expression as previously reported (13). \*\*\* $P < 0.001$ , mean  $\pm$  SEM.  $n = 3$  per cell line. Ab = antibody; IPF = idiopathic pulmonary fibrosis; PBS = phosphate-buffered saline; WCL = whole-cell lysate.

### PTP-BL Deficiency Reduces Bleomycin-induced Pulmonary Fibrosis in Mice

To assess the *in vivo* role of PTPN13 in pulmonary fibrosis, we studied the consequences of intratracheal instillation of bleomycin (2.5 U/kg) in mice lacking PTP-BL, the highly conserved ortholog of

PTPN13 (29). Whereas wild-type (Wt) mice developed significant mortality 8–10 days after bleomycin, mortality in PTP-BL<sup>-/-</sup> mice was reduced (Figure 6A). The amount of lung collagen, as visualized by trichrome staining (Figure 6D) and lung hydroxyproline measurements, was significantly ( $P = 0.031$ ) reduced in

bleomycin-challenged PTP-BL<sup>-/-</sup> mice at 14 days (Figure 6B). However, histologic examination (Figure 6C) and stereologic quantification of lung inflammation (Figure 6F) in the lungs of bleomycin-instilled mice revealed extensive lung injury with infiltration of macrophages, thickened alveolar septae, protein exudation, and



**Figure 4.** Reduced PTPN13 (protein tyrosine phosphatase-N13) expression or decreased association with Fas enables assembly of the Fas-induced DISC (death-inducing signaling complex) in MRC5 myfibroblasts. (A and B) Inhibition of Fas/PTPN13 interactions with acylated-SLV peptide augments Fas-induced caspase-8 (A) and caspase-3 (B) activation. (C) Inhibition of Fas/PTPN13 interactions with acylated-SLV peptide promotes the formation of the DISC after Fas ligation. Both peptides were used at a concentration of 10  $\mu$ M. \*\*\* $P$  < 0.001, mean  $\pm$  SEM.  $n$  = 3 per cell line. Ab = antibody; PBS = phosphate-buffered saline; TNF = tumor necrosis factor; WCL = whole-cell lysate.

alveolar collapse in both mouse strains ( $P$  = 0.642). Analysis of BAL leukocyte numbers also revealed similar increases in macrophages and neutrophils in bleomycin-instilled Wt and PTP-BL<sup>-/-</sup> mice, although lymphocyte numbers were significantly reduced in PTP-BL<sup>-/-</sup> mice (Figure 6E). Furthermore, BAL fluid levels of albumin (Figure 6F;  $P$  = 0.288) and TGF- $\beta$  (Figure 6G;  $P$  = 0.641) were not significantly different between PTP-BL<sup>-/-</sup> mice and Wt mice. Thus, PTP-BL deficiency had little effect on the development of inflammation and acute lung injury after bleomycin exposure, but significantly inhibited the ensuing collagen accumulation (i.e., fibrosis).

Although PTPN13/PTP-BL was originally described as a mediator of resistance to Fas-induced apoptosis, it has also been shown to participate in additional signaling pathways that may be of relevance to the reduced fibrosis seen in PTP-BL<sup>-/-</sup> mice. To further investigate its *in vivo* function, we assessed PTP-BL expression and localization in saline- and bleomycin-instilled Wt mice (Figure 7C). Figure 7F shows that total lung PTP-BL mRNA levels were significantly increased ( $P$  < 0.001) in response to bleomycin instillation in comparison with saline-instilled control mice. Similar to that seen in human lung tissues, PTP-BL was expressed by lung macrophages and a few epithelial cells. However, in contrast to IPF lung tissues wherein PTPN13 was robustly expressed in (myo)fibroblasts in fibroblastic foci, PTP-BL was less robustly expressed in the (myo)fibroblasts of the fibrotic lesions in

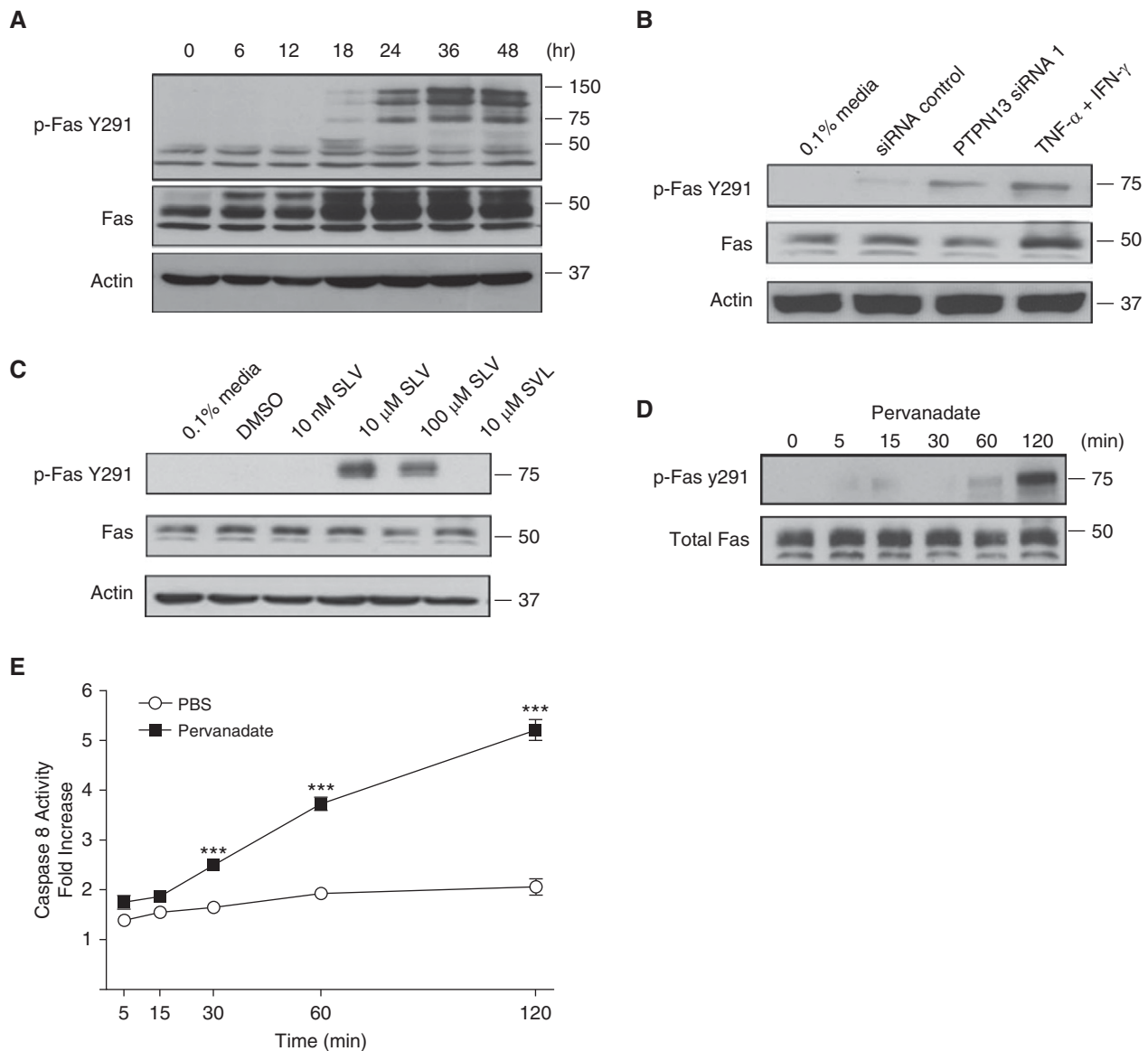
bleomycin-instilled mice (Figures 7A and 7B). In addition, quantification of lung myfibroblast density by immunohistochemistry for  $\alpha$ -SMA unexpectedly indicated that lung (myo)fibroblast density was not significantly ( $P$  > 0.05) affected by whole-body PTP-BL deficiency (Figures 7D and 7E).

PTPN13 (humans) and PTP-BL (mice) are expressed in both hematopoietic and nonhematopoietic (i.e., stromal) cells. Thus, it was possible that the reduced fibrosis seen in PTP-BL<sup>-/-</sup> mice may be caused by loss of PTP-BL in hematopoietic cells, a possibility strengthened by the findings that PTPN13/PTP-BL is expressed in macrophages (Figures 7A and 7B; see Figure E3); and that PTP-BL<sup>-/-</sup> mice recruited fewer lymphocytes into their lungs in response to bleomycin instillation compared with Wt mice (Figure 6E). To address this possibility, we created bone marrow chimeric mice in which PTP-BL<sup>-/-</sup> bone marrow cells were transferred into lethally irradiated Wt recipients (PTP-BL<sup>-/-</sup> to Wt) and *vice versa* (Wt to PTP-BL<sup>-/-</sup>). All groups of mice exhibited >95% chimerism at 8 weeks as reflected by CD45.1 and CD45.2 expression levels on peripheral blood leukocytes (data not shown). The mice were then instilled with bleomycin and assessed for inflammatory, injury-associated, and fibrotic parameters at 2 weeks. Figures 8A–8C show that the lungs of chimeric Wt to PTP-BL<sup>-/-</sup> mice phenocopied whole-body PTP-BL<sup>-/-</sup> mice (Figure 6) and exhibited a significant reduction in fibrosis. In contrast, chimeric PTP-BL<sup>-/-</sup> to Wt mice phenocopied the

typical fibrotic response seen in Wt mice (Figures 6 and 8A–8C). Both Wt to Wt and PTP-BL<sup>-/-</sup> to PTP-BL<sup>-/-</sup> control mice developed the expected fibrotic and nonfibrotic responses, respectively (data not shown). Collectively, our findings support the conclusion that loss of PTP-BL in nonhematopoietic stromal cells is responsible for the reduced fibrosis seen in the lungs of PTP-BL<sup>-/-</sup> mice.

## Discussion

Resistance to apoptosis is thought to contribute to (myo)fibroblast persistence in the lung parenchyma of patients with IPF (9–11). Here we show that the protein tyrosine phosphatase PTPN13 is expressed by (myo)fibroblasts in the fibroblastic foci of patients with IPF and contributes to their resistance to Fas-induced apoptosis. We also show that genetic deficiency of PTP-BL, the mouse ortholog of human PTPN13, reduced bleomycin-induced pulmonary fibrosis in mice, although the mechanism may differ. Additionally, the proinflammatory cytokines TNF- $\alpha$ /IFN- $\gamma$ , which sensitize lung fibroblasts to Fas-induced apoptosis, downregulate PTPN13 expression and augment proapoptotic Fas tyrosine phosphorylation and signaling. Taken together, these findings suggest that a therapeutic approach aimed at reducing PTPN13 expression, activity, or interaction with Fas might serve as novel strategies to facilitate lung (myo)fibroblast apoptosis in the lungs of patients with IPF and reduce pulmonary fibrosis.



**Figure 5.** PTPN13 (protein tyrosine phosphatase-N13) decreases Fas tyrosine phosphorylation and Fas-induced apoptosis. (A) Western blot depicting the time-dependent increase in phosphorylation of Fas at Y291 after tumor necrosis factor- $\alpha$ /IFN- $\gamma$  sensitization. (B) Western blot analysis showing increased tyrosine phosphorylation of Fas at Y291 after siRNA1-mediated PTPN13 knockdown. (C) Western blot showing increased phosphorylation of Fas at Y291 after treatment with acylated-SLV peptide. (D) Western blot analysis illustrating increased phosphorylation of Fas at Y291 after pervanadate treatment. (E) Caspase-8 activation in MRC5 myofibroblasts is significantly increased after pretreatment with pervanadate for 2 hours before stimulation with agonistic anti-Fas antibody.  $***P < 0.001$ , mean  $\pm$  SEM.  $n = 3$ . PBS = phosphate-buffered saline; TNF = tumor necrosis factor.

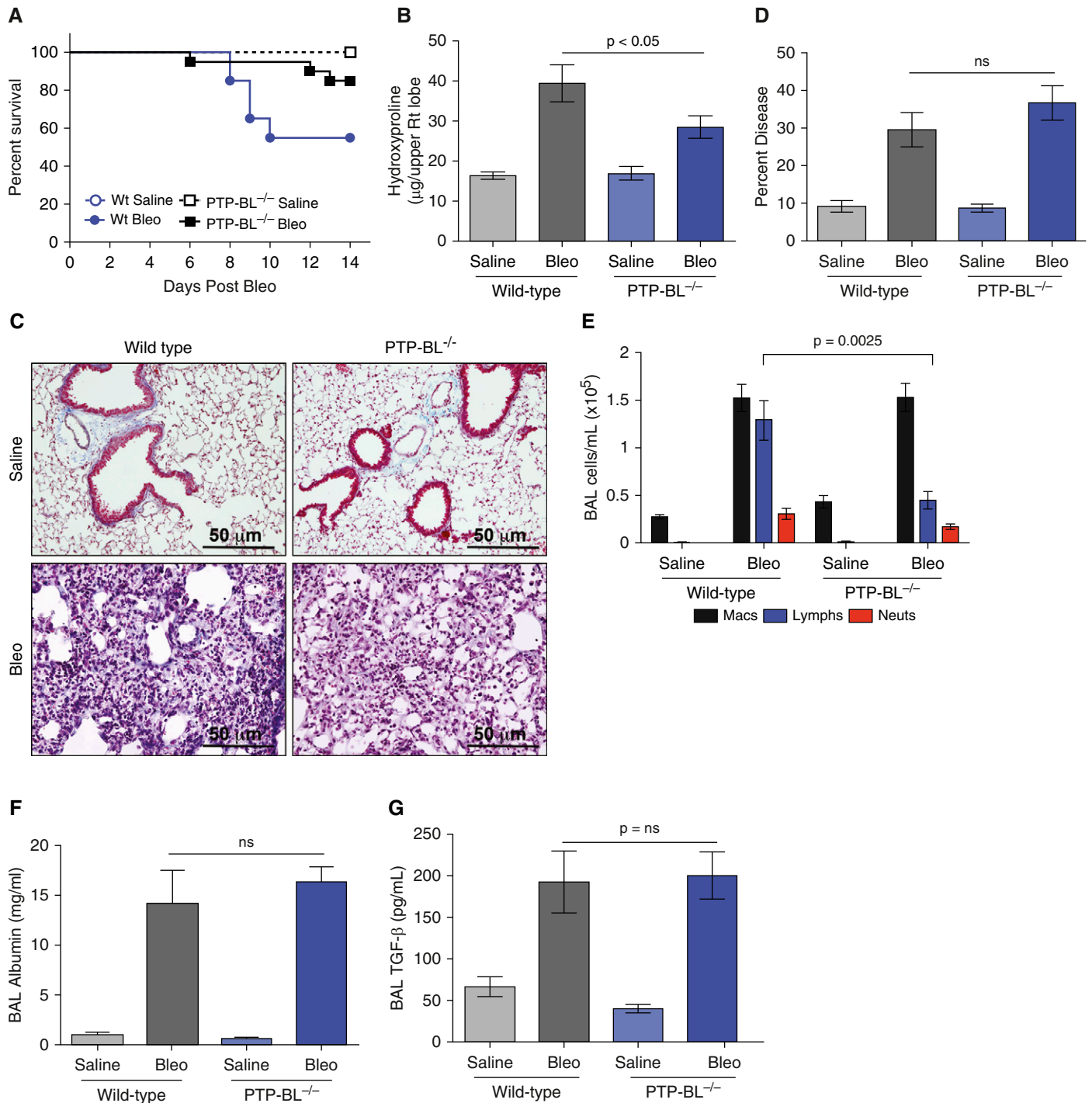
PTPN13 was abundantly detected in  $\alpha$ -SMA-negative fibroblasts and  $\alpha$ -SMA-positive (myo)fibroblasts in the fibroblastic foci of IPF patients but was absent from these cells in the fibrotic buds in organizing pneumonia, a reversible inflammatory/fibrotic lung disease (30). These findings may be significant because unlike the apoptosis-resistant (myo)fibroblasts in the fibroblastic foci of patients with IPF (9, 10, 31), some of the

(myo)fibroblasts in the fibrotic buds in organizing pneumonia are apoptotic, presumably as the fibrotic buds undergo resolution (10). These findings therefore provided a compelling impetus to test the hypothesis that PTPN13 contributes to the resistance of human lung fibroblasts to apoptosis and pulmonary fibrosis.

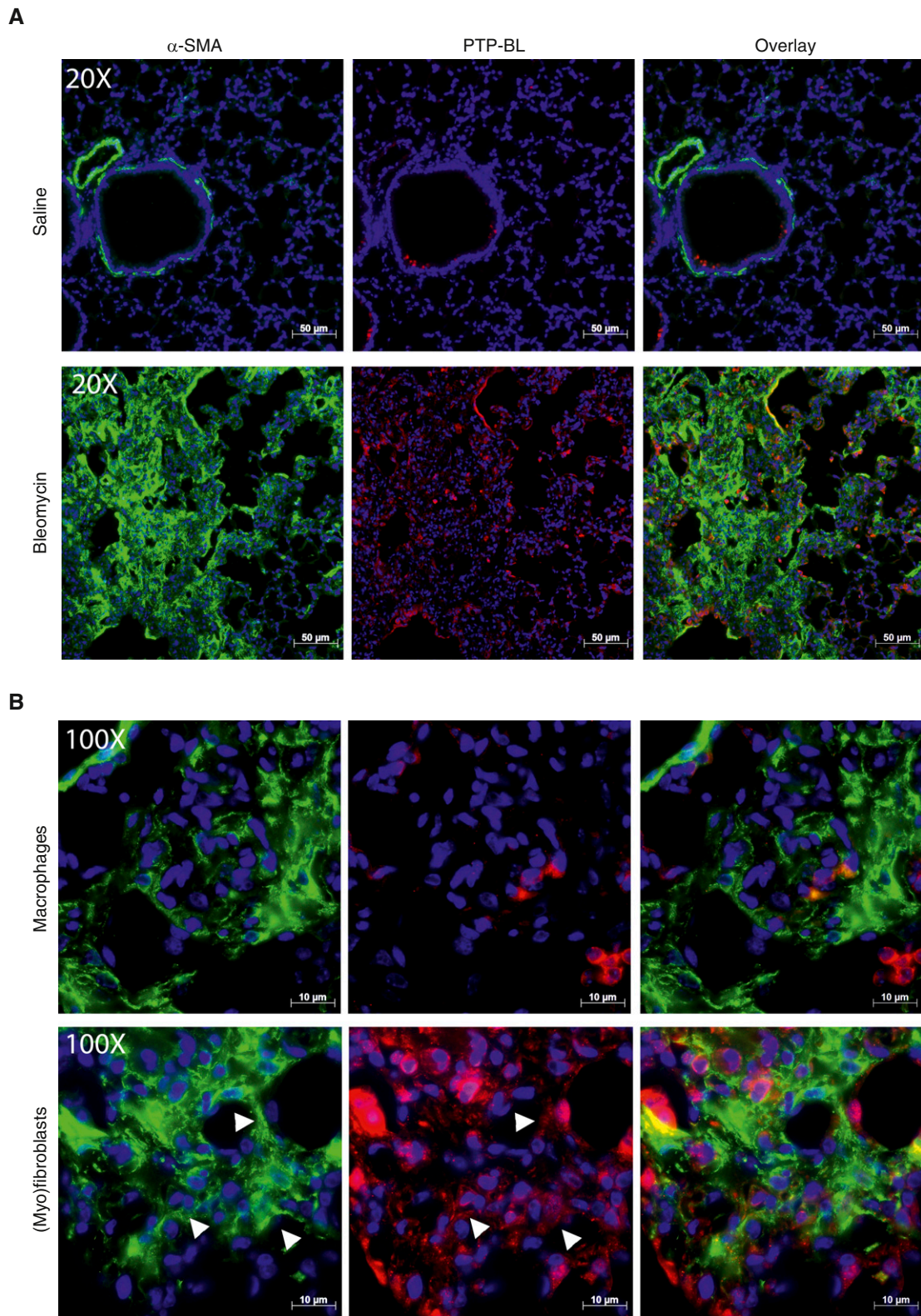
PTPN13 mRNA and protein were found to be basally expressed at similar levels by primary normal and IPF lung

(myo)fibroblasts and MRC5 cells cultured *in vitro* on ultrarigid plastic, a finding that we confirmed by querying publicly available transcriptomic datasets (GSE44723, GSE47460, GSE2052) containing normal and IPF (myo)fibroblasts, and normal and fibrotic whole-lung tissue. Although conceptually one might have expected cultured IPF lung (myo)fibroblasts to express higher levels of PTPN13 than cultured normal lung fibroblasts, these

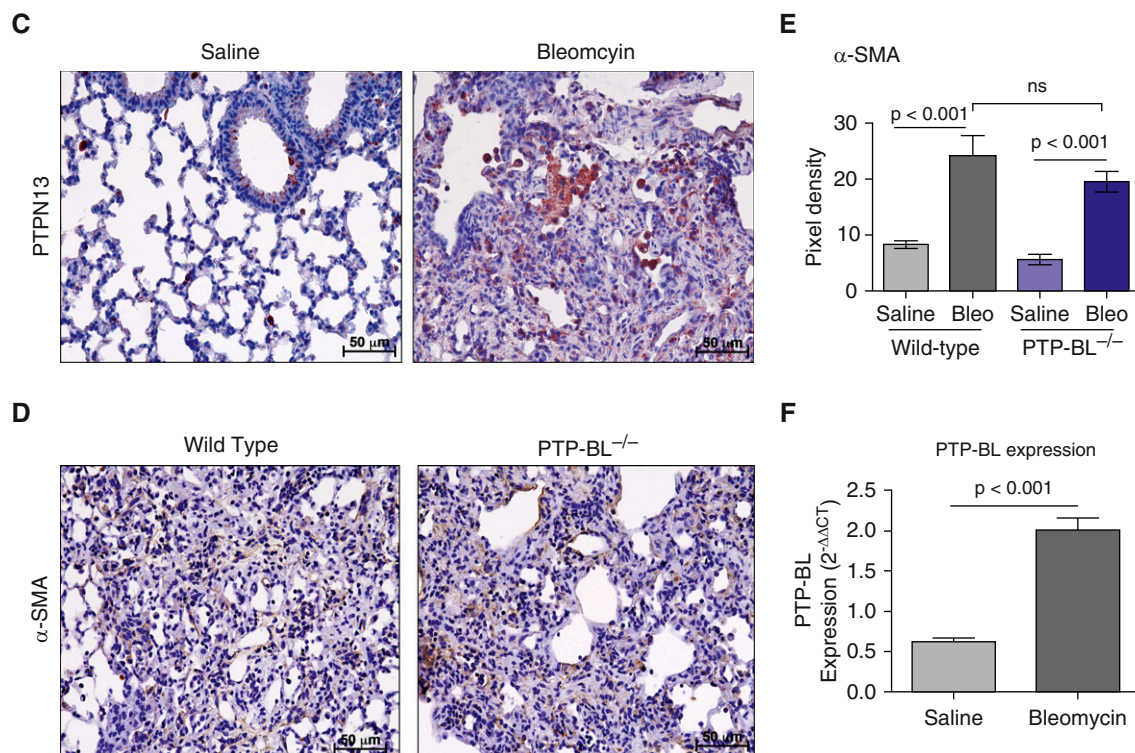




**Figure 6.** PTP-BL deficiency reduces bleomycin-induced pulmonary fibrosis in mice. (A) PTP-BL<sup>-/-</sup> mice have decreased mortality after bleomycin instillation compared with wild-type control animals. (B and C) Bleomycin-instilled PTP-BL<sup>-/-</sup> mice had a significantly reduced percentage of lung collagen compared with bleomycin-instilled wild-type mice as determined by assessment of collagen deposition identified by hydroxyproline and Masson trichrome staining (blue). Hydroxyproline levels were significantly reduced in bleomycin-instilled PTP-BL<sup>-/-</sup> mice compared with wild-type mice. (D) The percentage of diseased lung assessed by quantitative stereology was similar in bleomycin-instilled wild-type and PTP-BL<sup>-/-</sup> mice. (E) PTP-BL<sup>-/-</sup> mice have decreased lymphocyte infiltration into the airspaces after bleomycin instillation. (F) BAL albumin and (G) transforming growth factor- $\beta$  levels were similar in bleomycin-instilled PTP-BL<sup>-/-</sup> and wild-type control animals. Values are mean  $\pm$  SEM difference.  $n = 10$  mice per group. All endpoints were measured at Day 14. Bleo = bleomycin; ns = not significant; TGF = transforming growth factor; Wt = wild type.



**Figure 7.** PTP-BL is modestly expressed in  $\alpha$ -SMA ( $\alpha$ -smooth muscle actin)-positive fibroblasts in lung tissues from fibrotic wild-type mice. (A) Coimmunofluorescent staining of  $\alpha$ -SMA (green) and PTP-BL (red) shows some dual staining  $\alpha$ -SMA<sup>+</sup>/PTPN13<sup>+</sup> myofibroblasts. (B) Higher magnification shows positive PTP-BL staining in macrophages (top) and fibroblasts (bottom). White arrowheads highlight cells in which  $\alpha$ -SMA and PTP-BL are colocalized. (C) Immunohistochemical localization of PTP-BL in saline-instilled (left) and bleomycin-instilled (right) wild-type mice. (D) Immunohistochemical



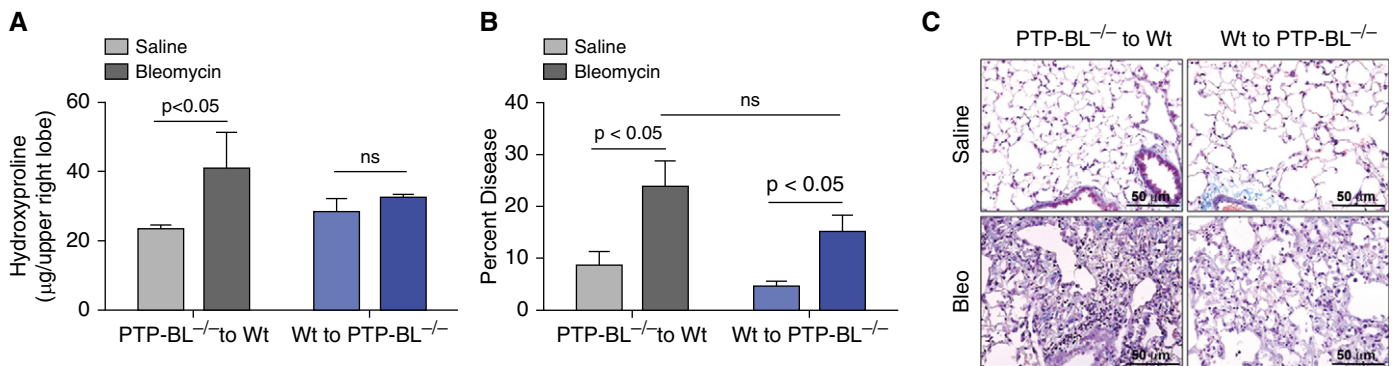
**Figure 7.** (Continued). staining of  $\alpha$ -SMA in bleomycin-instilled wild-type (left) and PTP-BL<sup>-/-</sup> mice (right). (E) Semiquantitative assessment of  $\alpha$ -SMA immunostaining to identify (myo)fibroblasts was not significantly different in bleomycin-treated wild-type and PTP-BL-deficient mice. (F) PTP-BL mRNA expression in whole-lung tissue from saline- and bleomycin-instilled wild-type mice. (A–C and F)  $n = 5$  mice per group. (D and E)  $n = 10$  mice per group. Bleo = bleomycin; ns = not significant; PTPN13 = protein tyrosine phosphatase-N13.

findings are consistent with current knowledge about the consequences of culture of fibroblasts on rigid plastic surfaces, which rapidly result in the development of myofibroblast features, including increased expression of  $\alpha$ -SMA and stretch fiber formation. Furthermore, in unpublished studies (E.F. Redente and D.W.H. Riches) using hydrogels of increasing Young's modulus (i.e., stiffness), we observed that PTPN13 can be regulated by substratum stiffness. Thus, by virtue of their ability to basally express PTPN13, thereby mimicking (myo)fibroblasts in the fibroblastic foci of patients with IPF, these collective findings reinforce the suitability of cultured human lung (myo)fibroblasts as a model to study the mechanisms underlying the resistance of fibrotic lung fibroblasts to Fas-induced apoptosis.

Exposure of normal and IPF lung (myo)fibroblasts and MRC5 cells to TNF- $\alpha$ /IFN- $\gamma$  downregulated PTPN13 expression coincident with the acquisition

of sensitivity to Fas-induced apoptosis. PTPN13 expression has been shown to be regulated at multiple transcriptional and post-transcriptional levels (32–36). Additionally, we have shown that PTPN13 downregulation in lymphoid cell lines involves p62-dependent targeting to autophagosomes in cells that exhibit high autophagic flux (37). Interestingly, and consistent with the notion that impaired autophagy may result in the maintenance of PTPN13 expression, recent studies have suggested that the fibroblastic foci of patients with IPF are impaired in their ability to undergo autophagy via a mechanism involving the profibrotic cytokine, TGF- $\beta$  (38, 39). Clearly many questions remain about how PTPN13 expression is controlled *in vivo* and future studies using freshly isolated fibroblasts and (myo)fibroblasts from normal and IPF lung tissue, which would not be impacted by *in vitro* culture, may help address this fundamental knowledge gap.

Seeking to understand how downregulation of PTPN13 function promoted Fas-induced (myo)fibroblast apoptosis, we investigated the role of its tyrosine phosphatase activity in Fas-signaling. PTPN13 downregulation or interference with its interaction with Fas concordantly increased phosphorylation of Fas tyrosine residue 291 (Y291) and sensitized (myo)fibroblasts to Fas-induced apoptosis. Pervanadate, a pan-tyrosine phosphatase inhibitor, mimicked both events, suggesting that PTPN13 suppresses apoptosis by dephosphorylating Fas phospho-Y291. Y291 (which, using a different numbering system, is also referred to as Y275 [40]) has been shown to be involved in Fas trafficking to the cell surface and in promoting susceptibility to Fas-induced apoptosis (26, 27, 40). In addition, Y291 is a key residue in a consensus internalization motif. Thus, it is plausible that phosphorylation of Y291 may mask this internalization motif, blocking



**Figure 8.** PTP-BL deficiency in stromal, but not hematopoietic, cells phenocopies the reduced bleomycin-induced pulmonary fibrosis seen in whole-body PTP-BL-deficient mice. (A) Lung hydroxyproline levels and (B) stereology-quantified lung injury in saline- and bleomycin-instilled PTP-BL<sup>-/-</sup> to wild-type (Wt) and Wt to PTP-BL<sup>-/-</sup> mice. (C) Trichrome-stained sections from saline- and bleomycin-instilled PTP-BL<sup>-/-</sup> to Wt and Wt to PTP-BL<sup>-/-</sup> mice. Values are mean  $\pm$  SEM.  $n = 5$  mice per group. Bleo = bleomycin; ns = no significant difference.

internalization and increasing Fas cell surface expression and susceptibility to apoptosis (41, 42).

To explore the role of PTPN13 *in vivo*, we investigated fibrosis in mice lacking PTP-BL, the murine ortholog of PTPN13. Loss of PTP-BL had no effect on bleomycin-induced acute lung injury, inflammation, or TGF- $\beta$  production. However, the ensuing fibrosis, as reflected by lung hydroxyproline levels and collagen staining, was reduced in PTP-BL<sup>-/-</sup> mice, through an effect on stromal cells. PTP-BL mRNA was detected in the lungs of bleomycin-instilled mice, although PTP-BL protein was only modestly detected in (myo)fibroblasts in fibrotic areas of Wt mice compared with the robust expression seen in the fibroblastic foci of patients with IPF. Although future studies in PTP-BL<sup>-/-</sup> mice should help resolve these seemingly discordant findings between humans and mice, we speculate that they may be related to differences in the mechanical properties of lung tissues in the transient, resolving bleomycin model of pulmonary fibrosis and the persistent, progressive fibrosis seen in IPF. In contrast to the persistently fibrotic lung tissues of patients with IPF, which have been reported to have a mean stiffness (Young's modulus) of 16.5 kPa (43), the reversibly fibrotic lung tissues of bleomycin-instilled mice are considerably more compliant, with a median stiffness of approximately 3 kPa (44). Thus, it is conceivable that the reduced intensity of PTP-BL protein expression in lung tissues from bleomycin-instilled mice compared with the robust PTPN13 expression lung tissues from patients with IPF may be

caused by difference in matrix stiffness. Consistent with this notion, we found that although murine (myo)fibroblasts expressed only modest PTP-BL staining in fibrotic lung tissues, primary murine lung fibroblasts were capable of expressing high levels of PTP-BL when cultured on rigid plastic dishes (see Figure E10).

PTPN13 binds to human Fas through a C-terminal SLV sequence that interacts with the PDZ2 domain of PTPN13, and via a second interaction with Fas Y291 (23, 40). Although human Fas contains both motifs, mouse Fas lacks the SLV sequence. Thus, mouse Fas and PTP-BL do not interact in the same way as their human orthologs, and PTP-BL does not inhibit Fas-induced apoptosis in mice (45). Consistent with these findings, (myo)fibroblast density in bleomycin-induced fibrotic lung tissues from Wt mice was not significantly different from that of PTP-BL<sup>-/-</sup> mice. Thus, the reduction in the fibrosis seen in PTP-BL<sup>-/-</sup> mice may represent an additional and previously unknown role for PTP-BL and potentially its human ortholog in collagen synthesis or turnover. PTPN13 and PTP-BL have both been shown to control additional signaling pathways including nuclear factor- $\kappa$ B, mitogen-activated protein kinase, signal transducer and activator of transcription, epidermal growth factor receptor signaling (29, 46–48), and Src-family kinases (49, 50). Because many of these pathways have also been implicated in the development of pulmonary fibrosis, PTP-BL deficiency might attenuate the fibrotic response by also affecting these pathways. Furthermore, Schickel and colleagues (36) reported that

the micro-RNA, miR-200c, basally represses PTPN13 in normal epithelial cells. However, during their transition to mesenchymal-like cells, miR-200c expression is repressed leading to increased PTPN13 expression, acquired resistance to Fas-induced apoptosis, and development of an invasive fibroblast phenotype. Thus, it is plausible that PTP-BL deficiency may also reduce fibroblast invasiveness and contribute to the reduced fibrotic lung phenotype. Consistent with this view, Yang and colleagues (51) showed that miR-200c expression is reduced in the lungs of patients with IPF and that pulmonary delivery of miR-200c in mice reduced the development of bleomycin-induced pulmonary fibrosis (51). Thus, miR-200c-induced downregulation of PTP-BL/PTPN13 could plausibly contribute to the reduction of fibrosis in these mice.

Why (myo)fibroblasts undergo apoptosis and clearance during normal repair processes yet persist in the lungs of patients with IPF remains poorly understood. Inflammation, together with elevated levels of TNF- $\alpha$  and IFN- $\gamma$ , accompany acute lung injury (52) before normal repair. Analogous to the epigenetically controlled development of fibroblast anatomic memory (53), it seems plausible that recruited fibroblasts could develop memory of TNF- $\alpha$ /IFN- $\gamma$  exposure during acute lung injury, perhaps via downregulation of PTPN13 expression, thereby becoming poised to undergo apoptosis once the repair process is complete. In contrast to acute lung injury, persistent and progressive fibrosis in patients with IPF develops in the absence of

a robust acute inflammatory response (1), related in part to the TGF- $\beta$ -rich, antiinflammatory environment of the fibrotic lung, which suppresses TNF- $\alpha$  and IFN- $\gamma$  expression (17, 54). Indeed, we have proposed (12, 13, 18) that fibroblast persistence *in vivo* may be caused by limited availability of TNF- $\alpha$ , IFN- $\gamma$ , and other factors (e.g., prostaglandin E<sub>2</sub>) (55, 56), that are required to sensitize fibrotic lung (myo)fibroblasts to apoptosis. Consistent with this idea, we recently showed that lung delivery of recombinant TNF- $\alpha$  accelerates the resolution of established fibrosis in the bleomycin model (18). Intratracheal administration of IFN- $\gamma$  has similarly been shown to reduce fibrosis in this model (57). The notion that

proinflammatory cytokines may play a role in fibrosis resolution is further supported by the finding of persistent fibrosis that we and others have seen in the absence of TNF- $\alpha$  and IFN- $\gamma$ , as demonstrated in bleomycin-challenged TNF- $\alpha$ <sup>-/-</sup> mice and CXCR3<sup>-/-</sup> mice, the latter which fail to recruit IFN- $\gamma$  producing natural killer cells (18, 58).

In summary, our findings reveal novel mechanisms by which PTPN13 promotes the resistance of lung (myo)fibroblasts to Fas-induced apoptosis and contributes to the development of pulmonary fibrosis. Although further studies are required to dissect the complexity of PTPN13 involvement, the current data obtained from the fibrotic lung tissues of patients with IPF,

primary human lung (myo)fibroblasts, and PTP-BL<sup>-/-</sup> mice suggest that PTPN13 may be a novel and relevant therapeutic target to reduce the progression of fibrosis in patients with IPF. Furthermore, because PTPN13 interacts with Fas and not other death receptors, therapeutically targeting the Fas-PTPN13 interaction interface may be a more specific approach to interfere with the function of PTPN13 in the resistance of (myo)fibroblasts to Fas-induced apoptosis, without interfering with its other functions (e.g., its role as a tumor-suppressor in gut epithelia) (35). ■

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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