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# Protein Tyrosine Phosphatase α Mediates Profibrotic Signaling in Lung Fibroblasts through TGF-β Responsiveness

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Fibrotic lung diseases represent a diverse group of progressive and often fatal disorders with limited treatment options. Although the pathogenesis of these conditions remains incompletely understood, receptor type protein tyrosine phosphatase α (PTP-α encoded by *PTPRA*) has emerged as a key regulator of fibroblast signaling. We previously reported that PTP-α regulates cellular responses to cytokines and growth factors through integrin-mediated signaling and that PTP-α promotes fibroblast expression of matrix metalloproteinase 3, a matrix-degrading proteinase linked to pulmonary fibrosis. Here, we sought to determine more directly the role of PTP-α in pulmonary fibrosis. Mice genetically deficient in PTP-α (*Ptpra*$^{-/-}$) were protected from pulmonary fibrosis induced by intratracheal bleomycin, with minimal alterations in the early inflammatory response or production of TGF-β. *Ptpra*$^{-/-}$ mice were also protected from pulmonary fibrosis induced by adenoviral-mediated expression of active TGF-β1. In reciprocal bone marrow chimera experiments, the protective phenotype tracked with lung parenchymal cells but not bone marrow—derived cells. Because fibroblasts are key contributors to tissue fibrosis, we compared profibrotic responses in wild-type and *Ptpra*$^{-/-}$ mouse embryonic and lung fibroblasts. *Ptpra*$^{-/-}$ fibroblasts exhibited hyporesponsiveness to TGF-β, manifested by diminished expression of αSMA, EDA-fibronectin, collagen 1A, and CTGF. *Ptpra*$^{-/-}$ fibroblasts exhibited markedly attenuated TGF-β—induced Smad2/3 transcriptional activity. We conclude that PTP-α promotes profibrotic signaling pathways in fibroblasts through control of cellular responsiveness to TGF-β. *(Am J Pathol 2014, 184: 1489—1502; http://dx.doi.org/10.1016/j.ajpath.2014.01.016)*

Fibrosis within the lung may be a consequence of infectious or noninfectious injury and is usually a self-limited process. However, pulmonary fibrosis can also be diffuse and progressive, as occurs in the idiopathic interstitial pneumonias, the most common of which is idiopathic pulmonary fibrosis (IPF).[1—3] The most frequent histopathological pattern of IPF is usual interstitial pneumonia, a process that is characterized by heterogeneous areas of dense fibrosis, fibroblastic foci, and honeycombing, with distortion of the lung architecture.[1,3] IPF is a progressive and frequently fatal disorder, with a median survival of 2.5 to 3.5 years after diagnosis.[1,2,4] Despite intensive investigation, the pathogenesis of IPF remains incompletely understood, and treatment options remain limited.[1,3—8] Both genetic and environmental

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factors have been implicated in the pathogenesis of IPF.[9–15] Notably, the incidence of IPF increases with age,[16] possibly reflecting the cumulative effect of genetic alterations that impair the ability of the lung to repair itself after repeated injury, leading to release of cytokines that induce myofibroblast accumulation, deposition of extracellular matrix (ECM) (including collagen and fibronectin), and progressive diffuse fibrosis.[3,14,17]

Many different cell types appear to participate in the fibrotic process. These include fibroblasts,[18,19] cells of hematopoietic origin (eg, macrophages[20–25] and lymphocytes[26,27]), and lung epithelial cells,[17,28,29] which can promote progressive fibrosis by diverse mechanisms, including production of profibrotic cytokines. Interestingly, although a characteristic feature of pulmonary fibrosis is the presence of increased fibroblasts and myofibroblasts, the origin of these mesenchymal cells remains uncertain and has been the subject of recent controversy.[14,30] Possibilities include proliferation and differentiation of resident fibroblasts, recruitment of the bone marrow–derived progenitor cells termed fibrocytes,[31,32] and transformation of lung epithelial cells and pericytes to mesenchymal cells (epithelial–mesenchymal transition).[28]

TGF-β is a pleiotropic cytokine that regulates diverse cellular responses including proliferation, differentiation, apoptosis, and inflammation.[33–35] TGF-β is fundamental to the pathogenesis of pulmonary fibrosis.[3,14,36–39] In the lung, TGF-β is expressed by various cell types (including epithelial cells, macrophages, and fibroblasts), and levels are elevated both in animal models and in clinical pulmonary fibrosis.[39–43] Pulmonary expression of TGF-β is sufficient to induce progressive fibrosis in animal models.[44] *In vitro*, TGF-β can induce myofibroblast transition of fibroblasts.[29,45] Canonical cellular responses to TGF-β are mediated through type II and type I receptors (TGFβR-II and TGFβR-I), leading to phosphorylation of Smad2 and Smad3, which form a complex with Smad4 that translocates to the nucleus and regulates gene transcription.[33–35,46] TGF-β–triggered Smad2/3 signaling is pivotal in the induction of pulmonary fibrosis in animal models.[39,44,47,48] Smad-independent (noncanonical) mechanisms of TGF-β signaling also exist, with extensive cross talk between the canonical and noncanonical pathways.[35,49–52] Signal attenuation represents an important regulatory aspect of TGF-β responses, and both receptor activation and downstream events are subject to regulation. With respect to the latter, reversible phosphorylation,[53,54] ubiquitination,[55–57] nuclear export of Smads,[58] and the inhibitory Smad7[59] all dampen TGF-β signals.

PTP-α (encoded by *PTPRA*) is a widely expressed transmembrane receptor-type protein tyrosine phosphatase.[60–63] The best-known function of PTP-α is physiological regulation of Src family kinases (SFKs); PTP-α dephosphorylates the inhibitory C-terminal tyrosine residue of SFKs (Y529 of Src), leading to kinase activation.[63–66] PTP-α has both positive and negative roles in cell growth, depending on the cell context. For example, PTP-α overexpression promotes fibroblast growth and tumorigenesis through Src activation.[64] Conversely, PTP-α inhibits proliferation of breast cancer cells[67] and oligodendrocytes through Fyn.[68] Mice genetically deficient in PTP-α (*Ptpra*$^{-/-}$) are viable and exhibit no gross morphological defects, but exhibit abnormalities in learning.[63,66,69]

We and others have identified PTP-α as a component of focal adhesions in fibroblasts, where it regulates cell adhesion, spreading, and motility via activation of SFKs.[63,70–73] We have recently reported that PTP-α promotes fibroblast expression of matrix metalloproteinase 3 (MMP-3), a matrix-degrading proteinase linked to pulmonary fibrosis,[71–74] and that PTP-α promotes fibroblast-mediated degradation of periodontal connective tissue.[75] In addition, PTP-α binds to and is phosphorylated by the IGF-I receptor,[76] a pathway implicated in pulmonary fibrosis.[77,78] Given the importance of PTP-α in these fibrogenic pathways, we investigated its role in animal models of pulmonary fibrosis and the control of profibrotic signaling pathways in the lung. Here, we demonstrate that mice genetically deficient in PTP-α are protected from bleomycin-induced and TGF-β–induced pulmonary fibrosis, with minimal alterations in the acute inflammatory response or production of TGF-β, that the protective phenotype resides in resident lung parenchymal cells, and that *Ptpra*$^{-/-}$ fibroblasts exhibit attenuated profibrotic responses to TGF-β.

## Materials and Methods

### Mouse Models of Pulmonary Fibrosis

All mice were housed in a pathogen-free facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and were treated in compliance with National Jewish Health Institutional Animal Care and Use Committee guidelines under an approved protocol. *Ptpra*$^{-/-}$ mice on a C57BL/6 background were generated as described previously.[63] Littermate matched wild-type (WT) *Ptpra*$^{+/+}$ mice were generated from mating heterozygous (*Ptpra*$^{+/-}$) mice. An intraperitoneal injection of 50 mg/kg ketamine and 5 mg/kg xylazine was used for sedation and anesthesia. After adequate anesthesia was achieved, a superficial incision was made in the cervical area for localization and visualization of the trachea. Subsequently, an oral gavage feeding tube was inserted translaryngeally and 1.5 to 2.5 U/kg of pharmaceutical-grade bleomycin (Bedford Laboratories, Bedford, OH) in 50 μL saline was instilled. To determine the specific dose for each set of experiments, each new lot of bleomycin was assessed for bioactivity in preliminary experiments using WT C57BL/6 mice, balancing the extent of pulmonary fibrosis and mortality (desired: <15%). In each set of experiments, bleomycin from a single lot was used. After instillation, the oral gavage feeding tube was removed, and the skin incision was closed using Vetbond tissue adhesive (3M, St. Paul, MN). Each group consisted of 8 to 12 animals, and animals were euthanized at days 2, 7, 14, or 21.

In a second model of pulmonary fibrosis, mice were treated with intratracheal instillation of $1 \times 10^8$ plaque-forming units (PFU) per mouse of replication-deficient adenovirus (Ad5) encoding active porcine TGF-β1$_{223/225}$ [which contains two point mutations in the LAP domain of the molecule, preventing the LAP from forming a homodimer and associating with the mature active TGF-β1 (Ad5TGF-β)].[44] Empty vector (AdDL70) was used as a control ($1 \times 10^8$ PFU per mouse). Both adenoviruses were obtained from Drs. Jack Gauldie and Martin Kolb (McMaster University, Hamilton, ON, Canada).

### Reciprocal Bone Marrow Transplants

Bone marrow transplantation into lethally irradiated (900 cGy of total body radiation) mice was performed as described previously.[79] The following chimeric mice were generated: WT marrow → WT mice; *Ptpra*$^{-/-}$ marrow → *Ptpra*$^{-/-}$ mice; WT marrow → *Ptpra*$^{-/-}$ mice; and *Ptpra*$^{-/-}$ marrow → WT mice. At 12 weeks after bone marrow transplantation, engraftment was confirmed by flow cytometry using CD45.2 and CD45.1 expression of bone marrow leukocytes as markers. The percentage of engrafted cells was >98% in all experiments. Mice were treated with intratracheal bleomycin as described above.

### Pulmonary Physiology

At baseline and at days 2, 7, 14, and 21, mice were anesthetized with an intraperitoneal injection of 50 mg/kg ketamine and 5 mg/kg xylazine. Mice were subjected to cervical dislocation, to prevent aberrations during pulmonary physiology measurements due to spontaneous respiration. Next, the trachea was exposed and isolated using a 1-cm incision; a rigid 10-mm, 21-gauge blunt cannula was inserted and sutured in place. Mice were then connected to a FlexiVent small-rodent ventilator (Scireq Scientific Respiratory Equipment, Montreal, QC, Canada) and were ventilated at set parameters of respiratory rate of 150, tidal volume of 10 mL/kg, and positive end-expiratory pressure of 0. A single recruitment maneuver of 40 mL/kg was performed to eliminate atelectasis; subsequently, a pressure–volume (PV) curve was generated using a stepwise inflation to 40 mL/kg to generate a quasi-static compliance curve. A Salazar–Knowles equation was used to model the deflation limb of the PV curve, and maximal compliance was then calculated based on the slope of the curve at a pressure of 4 cm $H_2O$.[80,81] This pressure value was chosen to correspond to the maximal slope of the Salazar–Knowles equation in WT untreated animals.

### Bronchoalveolar Lavage and Tissue Analysis

After measurement of pulmonary physiology, four serial lung lavages (0.8 mL) with normal saline containing 1 mmol/L EDTA were performed through the rigid intratracheal catheter and pooled. Cell counts were assessed using a hemocytometer, and cell differentials were evaluated using bronchoalveolar lavage (BAL) cytospin preparations stained with Diff-Quick reagent (Andwin Scientific, Tryon, NC). BAL fluid was then centrifuged to remove cells and debris, and the supernatant was stored at −80°C. Lungs were perfused with 10 mL of normal saline to remove intravascular blood, excised after lavage, and rinsed. The left lungs from three mice of each group were inflated to 20 cm $H_2O$ with 10% buffered formalin and were used for histological analysis. The remaining right lung was flash-frozen and stored at −80°C for collagen analysis. BAL concentrations of IL-1β, keratinocyte chemokine, and tumor necrosis factor α (TNF-α) were measured by a Meso Scale assay according to the manufacturer's instructions (Meso Scale Discovery, Rockville, MD). TGF-β enzyme-linked immunosorbent assay (ELISA) was performed using Elisa Tech (Aurora, CO) ELISA plates according to the manufacturer's instructions.

### Tissue Collagen Analysis

Fixed lungs were embedded, sectioned, and stained with H&E, Picrosirius Red, and trichrome by the National Jewish Health histology core. Collagen content was assessed using a Sircol assay (Biocolor, Carrickfergus, UK) as described previously.[74,82] The assay was performed on the whole right lung and was reported as total collagen (mg) per lung.

### Isolation of Primary Mouse Lung Fibroblasts

After mice were euthanized, the lungs were perfused with 10 mL normal saline, excised, and immediately placed into 5 mL ice cold Hanks' balanced salt solution (HyClone; Thermo Fisher Scientific, Waltham, MA). Lungs were then placed in a prescored 100-mm dish and chopped finely with scissors. The resultant small pieces were pressed onto the surface of the dish and 20 mL of medium (Dulbecco's modified Eagle's medium with GlutaMAX; Life Technologies, Carlsbad, CA), 15% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 U/mL penicillin/streptomycin (HyClone; Thermo Fisher Scientific) was added. Cultures were placed in a 37°C incubator in 5% $CO_2$, 95% air for 24 hours. The next day, the medium was carefully removed from the lung pieces and 15 mL of fresh medium was added to the cultures. The fibroblasts were cultured through eight passages.

### Cell Culture

Experiments were performed on early-passage primary mouse lung fibroblasts isolated as described above, mouse embryonic fibroblasts, or NIH 3T3 cells (ATCC, Manassas, VA). The cells were maintained in a medium consisting of Dulbecco's modified Eagle's medium with GlutaMAX supplemented with 15% or 10% heat-inactivated fetal bovine serum for primary lung fibroblasts or embryonic

fibroblasts and NIH 3T3 cells, respectively, as well as 100 U/mL penicillin/streptomycin. Medium was changed every 3 to 5 days. Cells were incubated at 37°C in 5% $CO_2$, 95% air. Cells were plated on tissue culture plastic coated plates (BD Falcon, Franklin Lakes, NJ) which were precoated with fibronectin (Sigma-Aldrich, St. Louis, MO). Cells were growth-arrested by reducing the concentration of fetal bovine serum to 1% for 24 hours before stimulation with recombinant human TGF-β (R&D Systems, Minneapolis, MN) at a concentration of 2 or 5 ng/mL, depending on the experimental conditions.

## Gene Silencing

Knockdown of PTP-α was achieved by transfection using RNAiMAX (Life Technologies) with 20 nmol/L siRNA. PTP-α–specific siRNA with the sequence 5′-GCAA-CAACGGGUUAGAGGAtt-3′ was obtained from Ambion (Life Technologies). Experiments were performed in six-well culture plates. In each well, the transfection reagents were prepared according to the manufacturer's instructions. In parallel, subconfluent NIH 3T3 cells were harvested and $1 \times 10^5$ cells were seeded into each well. Cells were then incubated at 37°C for 48 to 72 hours before use in experiments as described below.

## Analysis of Gene and Protein Expression

RNA was extracted from cells and reverse-transcribed into cDNA using a QuantiTect kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was analyzed by quantitative real-time PCR (qPCR) using individual primers optimized for each gene. qPCR was performed for 40 cycles on a CFX96 system (Bio-Rad Laboratories, Hercules, CA) using iQ SYBR Green supermix (Bio-Rad Laboratories). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.[83]

## Western Blotting

Cells grown on tissue culture plates were gently washed with 6 mL of PBS and lysed in 0.15 mL of cold radioimmunoprecipitation assay lysis buffer (1% NP-40, 0.1% SDS, 50 mmol/L Tris-HCl at pH 7.4, 150 mmol/L NaCl, 0.5% sodium deoxycholate, and 1 mmol/L EDTA). Protein concentrations were determined using a Pierce bicinchoninic acid protein assay (Thermo Fisher Scientific). Cell lysates were boiled at 100°C for 8 minutes in Laemmli sample buffer to denature the protein. Sample mixtures were loaded and subjected to electrophoresis in an 8% polyacrylamide gel, then transferred to a nitrocellulose membrane. After blocking in 5% nonfat milk in Tris–Tween buffered saline, membranes were treated with primary antibody, washed, and then treated with secondary antibody. Labeled proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Nycomed Amersham Canada, Oakville, ON, Canada). Densitometry was performed using ImageJ software version 1.47 (NIH, Bethesda, MD). Densitometry values of blots were normalized so that one represents the densitometry

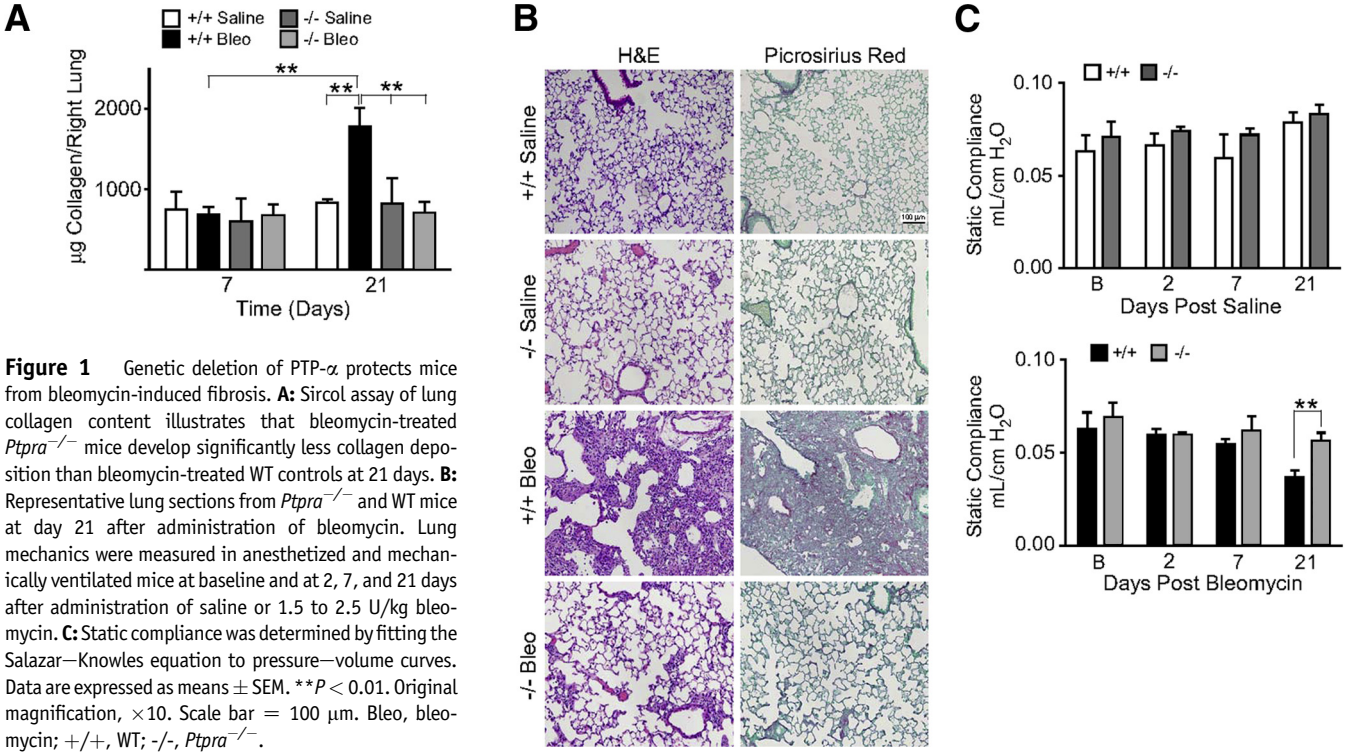



**Figure 1** Genetic deletion of PTP-α protects mice from bleomycin-induced fibrosis. **A:** Sircol assay of lung collagen content illustrates that bleomycin-treated *Ptpra*$^{-/-}$ mice develop significantly less collagen deposition than bleomycin-treated WT controls at 21 days. **B:** Representative lung sections from *Ptpra*$^{-/-}$ and WT mice at day 21 after administration of bleomycin. Lung mechanics were measured in anesthetized and mechanically ventilated mice at baseline and at 2, 7, and 21 days after administration of saline or 1.5 to 2.5 U/kg bleomycin. **C:** Static compliance was determined by fitting the Salazar–Knowles equation to pressure–volume curves. Data are expressed as means ± SEM. $^{**}P < 0.01$. Original magnification, ×10. Scale bar = 100 µm. Bleo, bleomycin; +/+, WT; -/-, *Ptpra*$^{-/-}$.

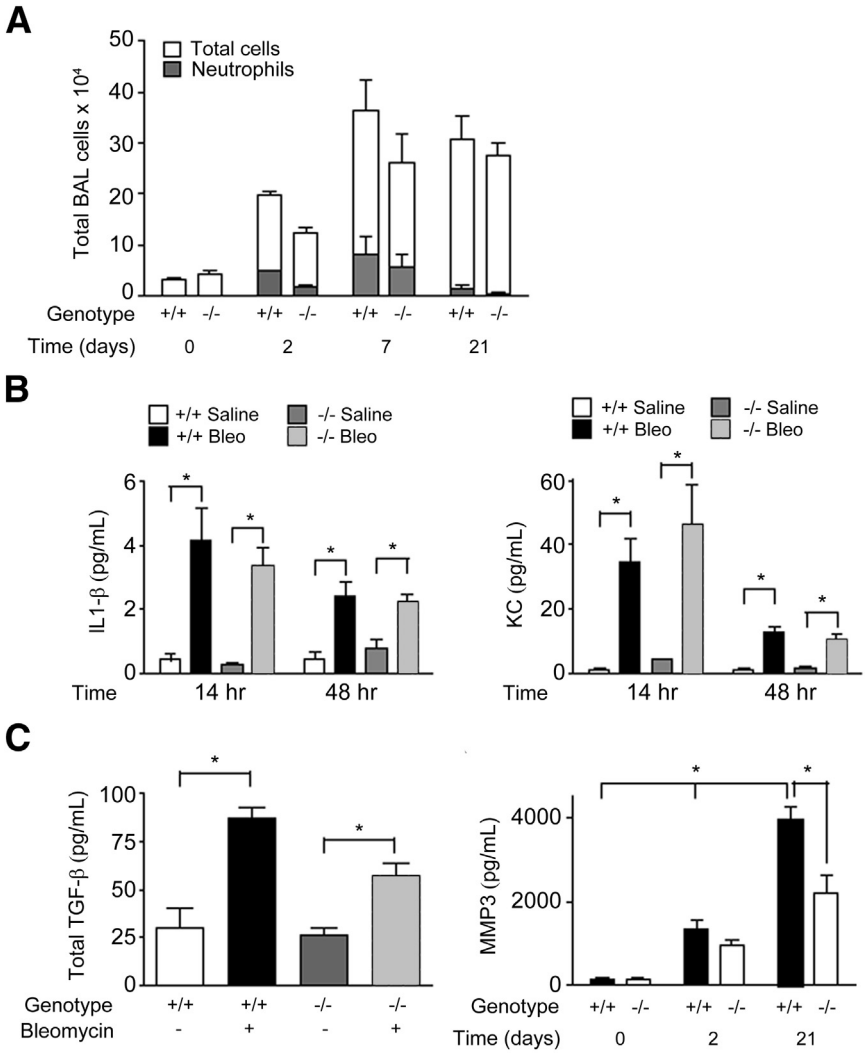



**Figure 2** WT and *Ptpra*$^{-/-}$ mice exhibit similar recruitment of inflammatory cells and production of cytokines in response to bleomycin. **A:** BAL fluid was collected at baseline (day 0) and at 2, 7, and 21 days after bleomycin instillation. Total cell counts and neutrophil counts were determined by differential cell counting. **B:** Meso Scale assay for BAL fluid for proinflammatory cytokines IL-1β and keratinocyte chemokine at 14 and 48 hours after bleomycin administration. **C:** Total TGF-β concentration in BAL fluid determined by ELISA at 7 days after bleomycin instillation. **D:** Levels of BAL matrix metalloproteinase MMP-3 as measured by ELISA at baseline and at 2 and 21 days after bleomycin treatment. Data are expressed as means ± SEM. *$P < 0.05$.

value of control samples. Antibodies to phosphorylated p-AKT (Thr308), p-p38 MAP kinase (Thr180/Tyr182), and p-p44/42 MAP kinase (Thr202/Tyr204) were obtained from Cell Signaling Technology (Danvers, MA).

## Luciferase Reporter Assays

Mouse embryonic fibroblasts were plated at $0.15 \times 10^6$ cells per well in transfection medium (Dulbecco's modified Eagle's medium with GlutaMAX and 10% heat-inactivated fetal bovine serum) in the absence of antibiotics on fibronectin-coated 12-well plates. Twenty-four hours later, cells were transfected with the Smad3 luciferase reporter (SBE4-luc) or Smad2 luciferase reporter (double transfection of ARE-luc and Fast1) plasmids[84–86] in the presence of Lipofectamine 2000 transfection reagent (Life Technologies) according to the manufacturer's instructions. Cotransfection with Renilla luciferase was used for normalization of each transfection. Cells were incubated for 24 hours, washed twice with serum-depleted medium (GlutaMAX and 1% heat-inactivated fetal bovine serum), and then incubated in the absence or presence of 2 ng/mL TGF-β. After 16 hours, cells were washed once with cold PBS and lysed in 1× passive lysis buffer. Lysate supernatants were collected, and reporter activity was assayed on a Synergy luminometer (BioTek Instruments, Winooski, VT), using a Dual-Luciferase Reporter (DLR) assay (Promega, Madison, WI). Firefly luciferase was normalized to Renilla luciferase to obtain normalized units of luciferase activity.

## Collagen Gel Contraction Assay

Collagen gel contraction assays were performed as previously reported.[71,87] In brief, collagen gels were prepared by combining collagen type I (BD Biosciences, San Diego, CA) at a concentration of 3 mg/mL in 0.1% acetic acid with cell suspension. Cultured primary lung fibroblasts were harvested and resuspended in medium at a concentration of $1.5 \times 10^5$ cells/mL. The cells were mixed with collagen solution for a final concentration of $1.0 \times 10^5$ cells/mL, transferred to wells of a 24-well plate, and allowed to

solidify for 20 minutes. Gels were released from the walls of the wells by rimming with a pipette tip. The area of each gel was obtained at various time points by imaging the 24-well plate (Gel Doc imaging system; Bio-Rad Laboratories) and quantified using NIH ImageJ software version 1.47. To measure the area of the gel, the oval measuring tool was used to outline each gel. Each experiment included at least three replicates, and at least three independent experiments were performed with similar results.

### Statistical Analysis

Statistical analysis was performed by Student's paired or unpaired *t*-test, as indicated. Multiple comparisons were performed by one- or two-way analysis of variance with Bonferroni post hoc test for determination of differences between groups. Nonparametric data were analyzed using *U*-test analysis. *P* values of $<0.05$ were considered to be statistically significant. Data are expressed as means $\pm$ SEM. Data were analyzed from $n \geq 4$ independent experiments; *in vitro* experiments were performed in duplicate or triplicate.

## Results

### *Ptpra*$^{-/-}$ Mice Are Protected from Bleomycin-Induced Pulmonary Fibrosis

To investigate the role of PTP-α in the pathogenesis of pulmonary fibrosis, we used mice genetically deficient in PTP-α and the well-established bleomycin model of lung fibrosis.[88,89] WT (*Ptpra*$^{+/+}$) and *Ptpra*$^{-/-}$ mice were treated with 1.5 to 2.5 U/kg of intratracheal bleomycin or saline and were euthanized at days 0, 2, 7, or 21 after treatment. Biochemical analysis of collagen content from the right lung was performed using the Sircol method. Notably, there was a significant increase in lung collagen content at day 21 in WT mice treated with bleomycin, compared with *Ptpra*$^{-/-}$ mice (Figure 1A). Histological findings in lung sections stained with H&E, Picrosirius red (Figure 1B), and trichrome (data not shown) were consistent with the collagen measurements, with increased areas of fibrosis and distortion of the lung architecture in the bleomycin-treated WT mice at day 21. By contrast, the lung architecture of bleomycin-treated *Ptpra*$^{-/-}$ mice was well preserved, and fibrosis was minimal (Figure 1B).

To validate the biochemical and histological analyses by an independent method, we assessed changes in pulmonary physiology in these mice. There was no significant difference in pulmonary compliance at baseline or after saline instillation between WT and *Ptpra*$^{-/-}$ mice (Figure 1C). By contrast, at 21 days after instillation of bleomycin, WT mice had developed a significant decrease in pulmonary compliance, but *Ptpra*$^{-/-}$ mice were protected from development of restrictive physiology (Figure 1C).

### The Inflammatory Response to Bleomycin is Minimally Altered in *Ptpra*$^{-/-}$ Mice

When given intratracheally, bleomycin induces an early (days 1 to 7) acute inflammatory response in the lungs, and fibrosis develops at later time points (days 14 to 21).[89] Given the differences in fibrotic responses to bleomycin in WT and *Ptpra*$^{-/-}$ mice, we sought to determine whether these findings are related to differences in the early inflammatory response. To address this possibility, BAL fluid was collected at days 0, 2, 7, and 21 after instillation of bleomycin, and total and differential cell counts were assessed. We found no difference between WT and *Ptpra*$^{-/-}$ mice for total BAL cell counts or for neutrophil and macrophage counts (Figure 2A).

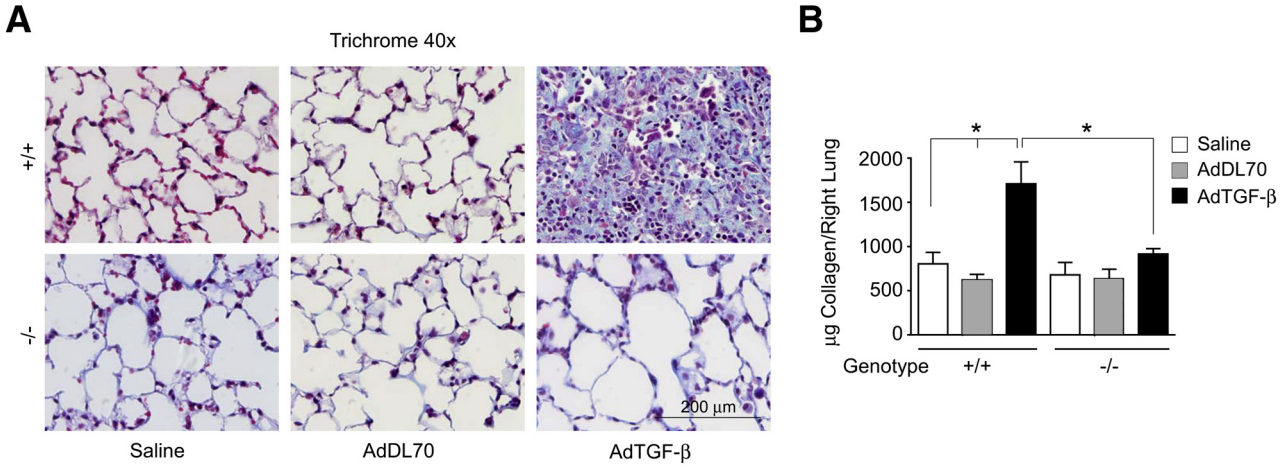
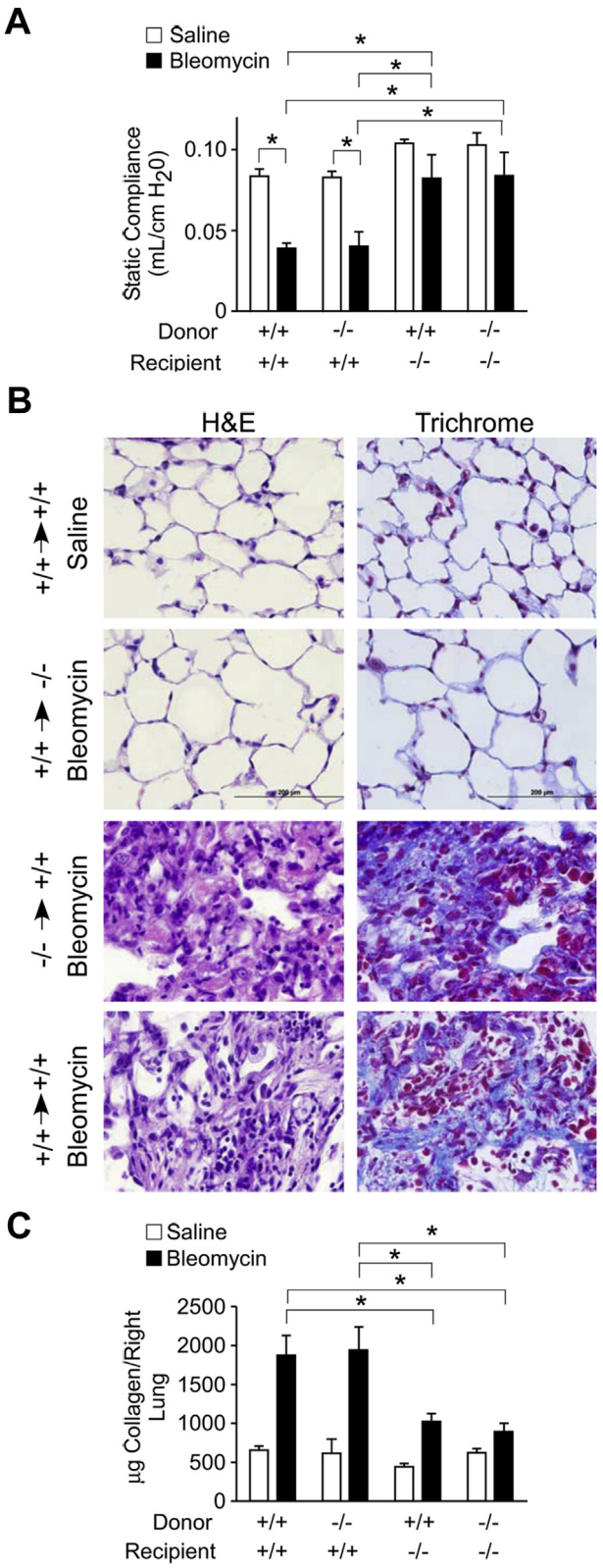



**Figure 3** *Ptpra*$^{-/-}$ mice are protected from pulmonary fibrosis induced by adenoviral-mediated expression of active TGF-β. **A:** Representative lung sections from WT and *Ptpra*$^{-/-}$ mice treated with active vector (AdTGF-β), empty vector (AdDL70), or saline control stained with trichrome. **B:** Collagen content in the lungs of WT or *Ptpra*$^{-/-}$ mice was determined 21 days after treatment using a Sircol assay. Data are expressed as means $\pm$ SEM. $^*P < 0.05$. Original magnification, ×40. Scale bar = 200 μm.

Analysis of cytokine levels in BAL fluid at 14 and 48 hours after bleomycin revealed similar levels of 1L-1β and keratinocyte chemokine in WT and *Ptpra*$^{-/-}$ mice (Figure 2B). Levels of total and active TGF-β were measured at day 7 by ELISA to determine whether the difference in fibrotic response could be attributed to differences in TGF-β production. The 7-day time point was chosen because it marks the peak of TGF-β production in the bleomycin model.[39] No significant difference in total TGF-β levels was noted between bleomycin-treated WT and *Ptpra*$^{-/-}$ mice (Figure 2C). The trend toward a decrease in TGF-β levels in *Ptpra*$^{-/-}$ mice likely reflects attenuated TGF-β signaling, because TGF-β induces its own production.[90] Concentrations of active TGF-β in BAL fluid were very low in WT and *Ptpra*$^{-/-}$ mice and did not differ between genotypes (data not shown). Similarly, TNF-α levels in BAL fluid were very low in WT and *Ptpra*$^{-/-}$ mice and did not differ between genotypes (data not shown). Levels of MMP-3, a matrix-degrading proteinase implicated in pulmonary fibrosis,[74] were significantly higher in bleomycin-treated WT than in with *Ptpra*$^{-/-}$ mice at 21 days (Figure 2D).




## *Ptpra*$^{-/-}$ Mice Are Protected from Pulmonary Fibrosis Induced by Adenoviral-Mediated Expression of Active TGF-β

To verify that the protective effects of PTP-α deficiency reside downstream of TGF-β production, we treated WT and *Ptpra*$^{-/-}$ mice with an adenoviral vector expressing active porcine TGF-β1 by intratracheal instillation (Ad5TGF-β).[44] Empty vector (AdDL70) and saline were used as controls.[74] Mice were euthanized 21 days after treatment. Histologically, the lungs of WT mice treated with vector expressing active TGF-β contained areas of dense fibrosis, whereas *Ptpra*$^{-/-}$ mice were largely protected from fibrosis (Figure 3A). Expression of active TGF-β induced an increase in pulmonary collagen content as measured by the Sircol assay in the lungs of AdTGF-β vector-treated WT mice, compared with either saline or empty vector control (Figure 3B). No increase in lung collagen content was observed in the lungs of *Ptpra*$^{-/-}$ mice (Figure 3B). Levels of TGF-β were similar in the BAL fluid from WT and *Ptpra*$^{-/-}$ mice treated with the AdTGF-β vector (data not shown).

## Resident Lung Parenchymal Cells Mediate the Protective Effect of PTP-α Deficiency

To determine whether resident lung cells or recruited hematopoietic cells are responsible for conferring the protective phenotype associated with genetic deletion of PTP-α,

**Figure 4** Protection from pulmonary fibrosis is mediated by absence of PTP-α in resident lung parenchymal cells. **A:** Compliance measurements obtained via FlexiVent ventilation after reciprocal bone marrow transplantation and treatment with 1.5 to 2.5 U/kg bleomycin for 21 days. **B:** Representative stained lung sections of mice undergoing reciprocal bone marrow transplantation 14 days after administration of bleomycin. **C:** A Sircol assay was performed to determine collagen content after reciprocal bone marrow transplantation and treatment with bleomycin for 21 days. Data are expressed as means ± SEM. *$P < 0.05$. Original magnification, ×40. Scale bar = 200 μm.

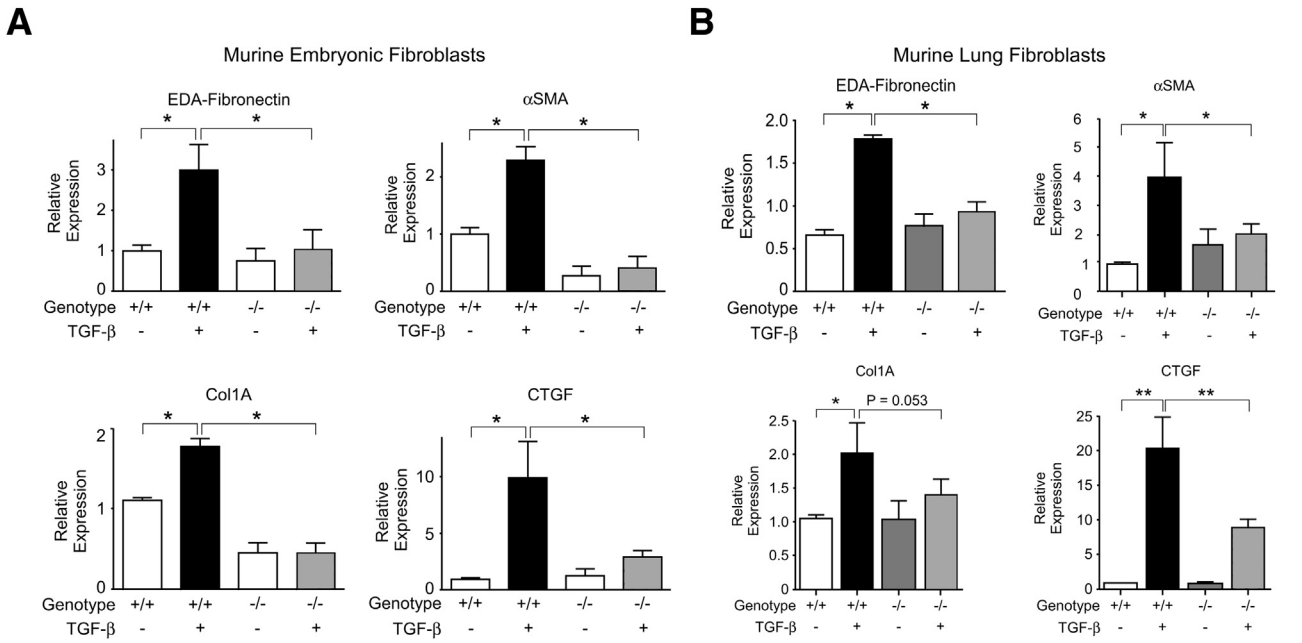



**Figure 5** *Ptpra*$^{-/-}$ fibroblasts exhibit diminished responsiveness to profibrotic TGF-β signals. **A** and **B:** After stimulation with 2 ng/mL TGF-β or buffer control for 6 hours, WT and *Ptpra*$^{-/-}$ mouse embryonic fibroblasts (**A**) and primary mouse lung fibroblasts (**B**) were analyzed by qPCR to assess mRNA expression of EDA-fibronectin, αSMA, collagen 1A, and CTGF. Data are expressed as means ± SEM.*$P < 0.05$; **$P < 0.01$.

we used reciprocal bone marrow transplantation. The following transplants were performed: WT marrow → WT mice; WT marrow → *Ptpra*$^{-/-}$ mice; *Ptpra*$^{-/-}$ marrow → WT mice; and *Ptpra*$^{-/-}$ marrow → *Ptpra*$^{-/-}$ mice. At 12 weeks after transplantation, mice were treated with bleomycin and euthanized at day 21 for measurements of lung compliance, histology, and collagen content. A high degree of chimerism (>98%) was observed in all transplanted mice (data not shown).

WT mice, regardless of the genotype of the transplanted marrow, developed decreased lung compliance by 21 days after intratracheal bleomycin, compared with *Ptpra*$^{-/-}$ mice (Figure 4A). Conversely, lung compliance in *Ptpra*$^{-/-}$ mice did not diminish in response to bleomycin, regardless of the genotype of the transplanted marrow. Histologically, mice with resident WT lung parenchymal cells exhibited an increase in amount and severity of fibrosis after bleomycin, whereas mice with *Ptpra*$^{-/-}$ resident lung parenchymal cells were largely protected from fibrosis and maintained normal lung parenchymal architecture (Figure 4B). These histological findings were not affected by the genotype of the transplanted marrow that was received.

Analogous to the results of compliance and histological data, the lungs of mice with resident WT lung parenchymal cells developed increased collagen content in response to bleomycin, whereas mice with *Ptpra*$^{-/-}$ resident lung parenchymal cells were protected from fibrosis, as demonstrated by biochemical analysis (Figure 4C). These data suggest that PTP-α expression in lung resident cells confers sensitivity to the fibrogenic effects of bleomycin.

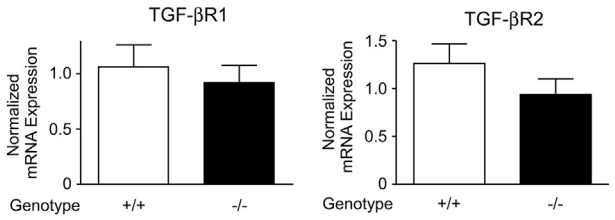



**Figure 6** TGF-β receptor type I and II expression is similar in WT and *Ptpra*$^{-/-}$ fibroblasts. Primary mouse lung fibroblasts were isolated and qPCR analysis performed for TGFβRI and TGFβRII expression. Data are expressed as means ± SEM.

## *Ptpra*$^{-/-}$ Fibroblasts Exhibit Attenuated Profibrotic Responses to TGF-β

Because lung mesenchymal cells are the primary source of excess collagen and other ECM material during fibrotic responses, we next compared the profibrotic responses of isolated WT and *Ptpra*$^{-/-}$ fibroblasts *in vitro* to the profibrotic growth factor TGF-β. For these studies, we used both mouse embryonic fibroblasts and primary mouse lung fibroblasts. Both types of fibroblasts demonstrated similar responses. WT and *Ptpra*$^{-/-}$ embryonic fibroblasts were stimulated with 2 to 5 ng/mL TGF-β and expression of αSMA, collagen 1A (Col1A), EDA-fibronectin (EDA-Fn), and connective tissue growth factor (CTGF) mRNA were assessed by qPCR as markers of profibrotic responsiveness.[91] After stimulation with TGF-β, WT fibroblasts demonstrated a significant increase in the levels of αSMA, EDA-Fn, Col1A, and CTGF mRNA. These responses were markedly attenuated in *Ptpra*$^{-/-}$ embryonic fibroblasts (Figure 5A). A

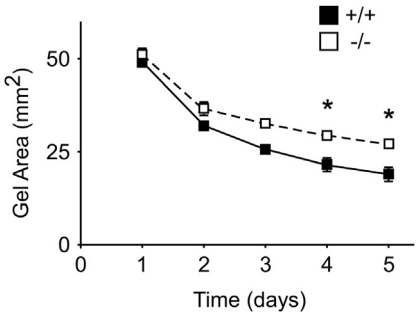



**Figure 7** *Ptpra*$^{-/-}$ fibroblasts exhibit attenuated contractile responses to TGF-β. Mouse lung fibroblasts were cast into collagen gels and allowed to contract over 5 days. Images were captured every 24 hours, and gel area was quantified using ImageJ software. *$P < 0.05$.

similar pattern of attenuated responsiveness to TGF-β was also noted in primary mouse *Ptpra*$^{-/-}$ lung fibroblasts for EDA-Fn, CTGF and αSMA (Figure 5B). A similar trend was observed for Col1A, although it did not achieve statistical significance.

## WT and *Ptpra*$^{-/-}$ Fibroblasts Have Equivalent TGF-β Receptor Expression

To ensure that the differences in fibrogenic responses between WT and *Ptpra*$^{-/-}$ fibroblasts were not due to differences in baseline expression of TGF-β receptors, we compared mRNA expression of both TGFβR-I and TGFβR-II from isolated mouse lung fibroblasts, because these are the major receptors implicated in tissue fibrosis.[42] No significant difference in the expression of either TGFβR-I or TGFβR-II mRNA between isolated primary lung fibroblasts from WT and *Ptpra*$^{-/-}$ mice that would account for differences in TGF-β responses was observed (Figure 6).

## *Ptpra*$^{-/-}$ Fibroblasts Exhibit Attenuated Contractile Responses to TGF-β

To assess the importance of PTP-α in contractile responses of fibroblasts linked to fibrosis,[92,93] we compared the ability of WT and *Ptpra*$^{-/-}$ lung fibroblasts to contract collagen gels.[71,87] The ability of *Ptpra*$^{-/-}$ fibroblasts to contract collagen gels was significantly diminished, relative to that of WT cells (Figure 7). Taken together, these observations demonstrate that *Ptpra*$^{-/-}$ lung fibroblasts exhibit diminished profibrotic responses to TGF-β, relative to WT cells, and are unable to acquire typical myofibroblast features under these conditions.

## PTP-α Promotes TGF-β–Induced Smad-Dependent Transcriptional Activity

Given that genetic deficiency of PTP-α resulted in alterations in expression of Smad-dependent profibrotic genes, we next evaluated the importance of PTP-α in TGF-β–dependent Smad2 and Smad3 transcriptional responses in fibroblasts using Smad luciferase reporter assays. WT and *Ptpra*$^{-/-}$ mouse embryonic fibroblasts were transfected with a Smad2 or Smad3 luciferase reporter and stimulated with TGF-β for 16 hours. *Ptpra*$^{-/-}$ fibroblasts exhibited significantly less activity of both Smad2 and Smad3 reporters (Figure 8, A and B). The basal activity of the reporter was also somewhat less in *Ptpra*$^{-/-}$ fibroblasts; importantly, the TGF-β–induced increase was nearly completely abrogated. To validate these results and to ensure that any differences

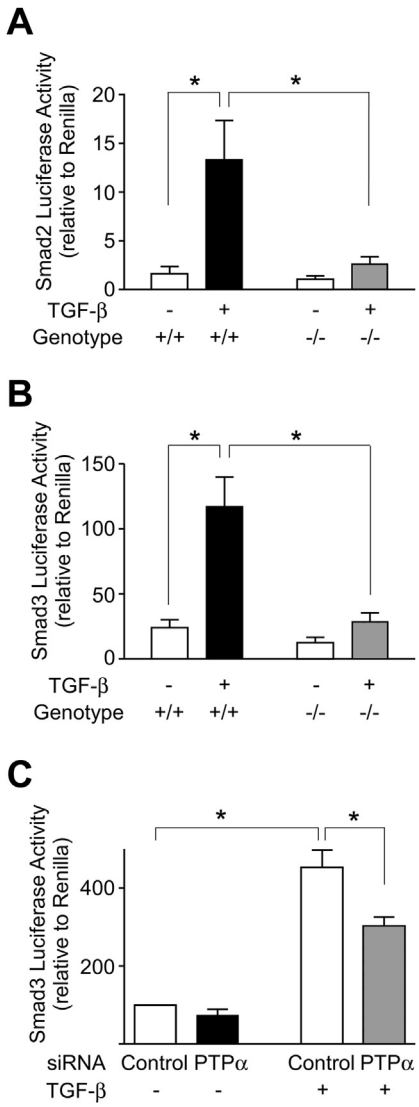



**Figure 8** Smad reporter activity is attenuated in *Ptpra*$^{-/-}$ fibroblasts. **A** and **B:** WT and *Ptpra*$^{-/-}$ mouse embryonic fibroblasts were transfected with Smad2 or Smad3 luciferase reporter constructs for 24 hours and then were incubated in the absence or presence of 2 ng/mL TGF-β for 16 hours. Lysates were assayed for luciferase activity. Both Smad2 (**A**) and Smad3 (**B**) reporter activity were significantly reduced in *Ptpra*$^{-/-}$ fibroblasts, compared with WT. **C:** NIH 3T3 fibroblasts were reverse-transfected with control or PTP-α siRNA, followed by transfection with Smad3 luciferase reporter construct for 24 hours. Cells were then stimulated with 2 ng/mL TGF-β for 16 hours or with buffer medium. Luciferase activity in lysates was normalized to control siRNA treated with buffer. Data are expressed as means ± SEM. *$P < 0.05$.

observed between WT and *Ptpra*$^{-/-}$ fibroblasts were not attributable to genetic compensation, we also used NIH 3T3 fibroblasts in which PTP-α expression was acutely silenced using siRNA, which resulted in 68.3 ± 28.0% knockdown. These cells were then transfected with a Smad3 luciferase reporter and stimulated in an identical manner with TGF-β. As expected, acute gene silencing of PTP-α resulted in significantly attenuated Smad3 reporter activity after TGF-β stimulation (Figure 8C).

To assess the importance of PTP-α in regulation of noncanonical TGF-β signaling pathways involving p38 MAP kinase, p42/44 MAP kinase (ERK), and AKT,[34,35,94] we compared the extent of TGF-β–induced activation of these kinases between WT and *Ptpra*$^{-/-}$ fibroblasts using immunoblot analysis with antibodies that recognize their phosphorylated (activated) state. These studies revealed no apparent differences between WT and *Ptpra*$^{-/-}$ fibroblasts in these pathways (data not shown).

## Discussion

Idiopathic pulmonary fibrosis is a progressive and usually fatal disease that results in destruction of normal lung architecture and ultimately in respiratory failure and death.[3,4] IPF is characterized by remodeling of normal ECM, increased deposition of collagen and other ECM components, and proliferation of fibroblasts within the presence of fibroblastic foci.[1,3] The pathogenesis and underlying mechanisms of this complex disease remain poorly understood, despite intensive study. Concepts of abnormal wound healing and repetitive injury and repair leading to stimulation of myofibroblast differentiation have emerged, as has the importance of profibrotic mediators in driving production of excessive and disorganized fibrous tissue.[14,95,96] Here, we have demonstrated the importance of the transmembrane receptor tyrosine phosphatase PTP-α in the genesis of pulmonary fibrosis in two complementary animal models: intratracheal bleomycin and pulmonary expression of active TGF-β via adenoviral-mediated gene delivery. In addition, our results demonstrate an important role for PTP-α in TGF-β–dependent profibrotic responses in fibroblasts and in profibrotic signaling in the canonical TGF-β–Smad pathway that has been linked to tissue fibrosis.[35,91]

Using the bleomycin and AdTGF-β mouse models of pulmonary fibrosis, we have demonstrated that genetic deficiency of PTP-α protects mice from the development of fibrosis, specifically with respect to preservation of normal lung architecture, prevention of excess collagen deposition, and maintenance of normal pulmonary mechanics after the administration of robust profibrotic stimuli. These observations substantiate an important role for PTP-α in promoting fibrotic responses. Notably, the inflammatory response in these experimental models of pulmonary fibrosis was not significantly altered by the absence of PTP-α, suggesting that PTP-α selectively promotes fibrogenic signaling, rather than acute inflammatory pathways. Results from reciprocal bone marrow transplantation experiments indicate that the protective phenotype arises from cells residing within the lung parenchyma, rather than from circulating bone marrow progenitor cells. Furthermore, the protection from lung fibrosis in the AdTGF-β model that is dependent on overexpression of active TGF-β strongly suggests that PTP-α is situated downstream of TGF-β production and activation in animal models of pulmonary fibrosis. This concept is also supported by the bleomycin model, in which levels of TGF-β in lavage fluid were similar between WT and *Ptpra*$^{-/-}$ mice but the latter were protected from fibrosis.

The importance of fibroblasts in the secretion of excess collagen and other ECM proteins that contribute to the pathogenesis of pulmonary fibrosis prompted us to focus on the role of PTP-α in profibrotic responses in this mesenchymal cell type. TGF-β has been shown to induce myofibroblast differentiation in fibroblasts.[29,45] Myofibroblasts are both contractile and secretory, producing αSMA in stress fibers that are involved in contractile responses, as well as secreting ECM protein components such as fibronectin and collagen.[97] qPCR analysis of mouse embryonic fibroblasts revealed decreased expression of genes involved in ECM production such as collagen 1A and EDA-Fn in *Ptpra*$^{-/-}$ cells in response to TGF-β. Furthermore, *Ptpra*$^{-/-}$ fibroblasts demonstrated impaired ability to contract collagen gels, suggesting an attenuated myofibroblast phenotype.

Expression of TGF-β in the lungs is sufficient for induction of pulmonary fibrosis in animal models via a Smad2/3–dependent profibrotic signaling pathway.[41,44,47,48] TGF-β regulates diverse cellular processes from proliferation and differentiation to apoptosis, and signaling is achieved through type II (TGFβR-II) and type I (TGFβR-I) receptors, which induce phosphorylation of Smad2 and Smad3 after ligand binding and ultimately regulate gene transcription in the nucleus.[33,34,98] Our observation that levels of expression of TGFβRI and TGFβRII do not differ between WT and *Ptpra*$^{-/-}$ fibroblasts indicates that PTP-α does not regulate TGF-β–dependent profibrotic signaling by controlling levels of TGF-β receptor expression but rather that PTP-α likely acts at or downstream of receptor activation.

Previous studies have provided evidence for the importance of TNF-α in up-regulating TGF-β in the pathogenesis of fibroproliferative lung disease induced by bleomycin, silica, and asbestos in mouse models.[25,99] Our present studies expanded on this concept and provide evidence that PTP-α acts at or downstream of TGF-β receptors and therefore also likely downstream of TNF-α in the pathways driving pulmonary fibrosis.

Despite the similar levels of TGF-β receptors, the absence of PTP-α had profound effects on the canonical TGF-β signaling pathway, as evidenced by alterations in receptor-Smad transcriptional responses. Luciferase reporter assays showed that Smad2 and Smad3-dependent transcription in response to TGF-β was significantly attenuated in *Ptpra*$^{-/-}$,

compared with WT mouse embryonic fibroblasts. Similar findings were observed in fibroblast cell lines subjected to acute gene knockdown with siRNA. The ability to recapitulate the findings seen in isolated primary cells by analysis of cells treated with siRNA to PTP-α to achieve knockdown addresses concerns related to genetic compensation that may occur in primary cells derived from mice genetically deficient in PTP-α.

To our knowledge, this is the first report to show a role for a tyrosine phosphatase in the development of pulmonary fibrosis; thus, PTP-α is a novel mediator of the fibrotic process and may provide insights into the pathways that drive progressive fibrosis without affecting the inflammatory responses within the lungs. Signal attenuation represents an important regulatory aspect of TGF-β responses with both receptor activation and downstream events subject to regulation. With respect to the Smad-dependent signaling pathways (which appear to be modified by PTP-α, as evidenced by our *in vitro* and *in vivo* studies), possible mechanisms of regulation or signal attenuation include reversible phosphorylation, degradation, ubiquitination, sumoylation, or alterations in nuclear import and export.[35,46,49] Our working hypothesis is that PTP-α down-regulates one or more of these signaling checkpoints and thus promotes profibrotic TGF-β signaling. Therefore, the absence of PTP-α results in attenuation of profibrotic signals and a subsequent inability of fibroblasts to differentiate to myofibroblasts which accumulate in the interstitium, produce ECM components, and impart contractile force; thus the absence of PTP-α confers a protective phenotype in the setting of a signaling milieu that would otherwise result in the development of fibrosis.

PTP-α control of profibrotic TGF-β signaling could be via effects on SFKs. In this regard, PTP-α dephosphorylates and activates Src, which promotes TGF-β–mediated collagen production in fibroblasts.[100,101] Additionally, inhibition of Src via gene silencing or expression of catalytically inactive (dominant negative) Src resulted in suppression of Smad2- and Smad3-dependent reporter responses.[102] Src can also directly phosphorylate TGFβRII on Y284, promoting activation of p38 MAPK within the noncanonical TGF-β signaling pathway.[94,103] In addition, Src can phosphorylate and activate FAK, which has been shown to promote focal adhesion-dependent signaling,[104–106] which may promote profibrotic signaling cascades.[107] Fyn, another SFK expressed in fibroblasts, mediates myofibroblast differentiation, which may be important in pulmonary fibrosis.[108] In addition to enhancing On signals through TGF-β receptors, PTP-α in association with SFKs could augment TGF-β signaling by inhibiting Off signals. This may occur by inhibition of Smad phosphatases such as PPM1A and PP2A or via an independent mechanism.[53,109] Our studies suggest that PTP-α does not regulate noncanonical TGF-β signaling pathways involving p38 MAP kinase, p42/44 MAP kinase (ERK), or AKT. A more detailed analysis of the molecular mechanisms by which PTP-α enhances profibrotic pathways is currently underway in our laboratory.

Idiopathic pulmonary fibrosis and other fibrosing interstitial pneumonias remain deadly diseases with no current effective therapeutic options. Although some recent clinical trials have shown promising results, including those of pirfenidone and the tyrosine kinase inhibitor BIBF 1120, the overall findings of multiple clinical trials aimed at varied potential target pathways have largely failed to identify ineffective therapies.[5,8,110] Furthermore, patients often present with late-stage disease, and survival time from initial diagnosis is brief.[3,4] In addition to a lack of effective therapies, there is also a need for improved prognostic markers and tools to assess the trajectory of disease progression, which may affect referrals for lung transplantation or palliative care strategies. The novel discovery of the key role of PTP-α and the pathways controlled by it in the pathogenesis of pulmonary fibrosis in animal models could ultimately prove useful as a biomarker to identify patients at risk of developing pulmonary fibrosis or of having a more rapidly progressive disease course. In addition, PTP-α may serve as a target for novel treatment strategies, in particular those that mediate the control of fibrosis without altering the inflammatory response, which is thought to be essential for an effective repair process. Small-molecule inhibitors of PTP-α or targeted antibodies that result in inactivation of the phosphatase activity of PTP-α warrant investigation in animal models, with the ultimate goal of treatment of patients with pulmonary fibrosis. Broader applications to other progressive fibrosing diseases of the liver, kidneys, or heart may also prove relevant as the mechanisms by which PTP-α influences the pathogenesis of pulmonary fibrosis are further defined.

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