Lysophosphatidic Acid Receptor–2 Deficiency Confers Protection against Bleomycin-Induced Lung Injury and Fibrosis in Mice

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Idiopathic pulmonary fibrosis is a devastating disease characterized by alveolar epithelial cell injury, the accumulation of fibroblasts/myofibroblasts, and the deposition of extracellular matrix proteins. Lysophosphatidic acid (LPA) signaling through its G protein–coupled receptors is critical for its various biological functions. Recently, LPA and LPA receptor 1 were implicated in lung fibrogenesis. However, the role of other LPA receptors in fibrosis remains unclear. Here, we use a bleomycin-induced pulmonary fibrosis model to investigate the roles of LPA_2 in pulmonary fibrogenesis. In the present study, we found that LPA_2 knockout (*Lpar2*$^{-/-}$) mice were protected against bleomycin-induced lung injury, fibrosis, and mortality, compared with wild-type control mice. Furthermore, LPA_2 deficiency attenuated the bleomycin-induced expression of fibronectin (FN), α–smooth muscle actin (α-SMA), and collagen in lung tissue, as well as levels of IL-6, transforming growth factor–β (TGF-β), and total protein in bronchoalveolar lavage fluid. In human lung fibroblasts, the knockdown of LPA_2 attenuated the LPA-induced expression of TGF-β1 and the differentiation of lung fibroblasts to myofibroblasts, resulting in the decreased expression of FN, α-SMA, and collagen, as well as decreased activation of extracellular regulated kinase 1/2, Akt, Smad3, and p38 mitogen-activated protein kinase. Moreover, the knockdown of LPA_2 with small interfering RNA also mitigated the TGF-β1–induced differentiation of lung fibroblasts. In addition, LPA_2 deficiency significantly attenuated the bleomycin-induced apoptosis of alveolar and bronchial epithelial cells in the mouse lung. Together, our data indicate that the knockdown of LPA_2 attenuated bleomycin-induced lung injury and pulmonary fibrosis, and this may be related to an inhibition of the LPA-induced expression of TGF-β and the activation and differentiation of fibroblasts.

Keywords: lysophosphatidic acid; LPA_2; idiopathic pulmonary fibrosis; transforming growth factor–β

More than five million people are afflicted with idiopathic pulmonary fibrosis (IPF), a progressive and highly devastating interstitial lung disease (1), with an average survival time of 2–5 years from initial diagnosis (2). The pathology of IPF is characterized by alveolar epithelial cell injury, areas of Type II cell hyperplasia, the accumulation of fibroblasts and myofibroblasts, and the deposition of α–smooth muscle actin (α-SMA) and extracellular matrix (ECM) proteins such as fibronectin (FN) and collagen (3–6). Recent investigations have indicated that alveolar injury, followed by abnormal wound-healing, is involved in the pathogenesis of IPF (7, 8). Subsequently, the accumulation of fibroblasts results in excessive scarring and changes in lung architecture, leading to a loss of lung function and death (9). However, the details underlying the pathogenesis of IPF remain elusive, and effective therapies are not yet available for patients with IPF.

Lysophosphatidic acid (LPA) is a bioactive lysophospholipid that regulates numerous cellular functions, and also promotes wound healing and tissue fibrosis (10). LPA signals through its G-protein–coupled receptors, and so far, in humans, six LPA receptors ($LPA_{1–6}$) have been cloned and characterized (11–14). Recent studies have demonstrated a positive role for LPA and LPA_1 in the pathogenesis of fibrosis (15, 16). *In vitro*, LPA is known to induce the proliferation and differentiation of lung fibroblasts (17, 18), and to augment the fibroblast-mediated contraction of released collagen gels (19). Lipid profiling indicates that LPA levels were dramatically higher in bronchoalveolar lavage (BAL) fluid collected from patients with IPF, compared with their normal counterparts (16). In the bleomycin-induced pulmonary fibrosis model, LPA_1 deficiency conferred significant protection against bleomycin-induced fibrosis and mortality (16, 20). Furthermore, LPA-induced fibroblast recruitment, vascular leakage, and epithelial cell apoptosis were also markedly reduced in *Lpar1*$^{-/-}$ mice (16, 20). *In vivo*, the oral administration of an LPA_1 antagonist dramatically prevented bleomycin- induced pulmonary fibrosis in mice (16, 21), and the intraperitoneal injection of an $LPA_{1/3}$ antagonist ameliorated irradiation-induced lung fibrosis (22). In a renal fibrosis model, LPA_1 deficiency or the administration of an LPA_1 antagonist suppressed renal interstitial fibrosis (12, 23). Moreover, LPA induced fibroblast chemotaxis through an LPA_1-dependent mechanism (16). Together, these results point to LPA_1 receptor antagonism as a potentially useful treatment for IPF. However, the role of other LPA receptors in fibrosis remains unknown.

Transforming growth factor–β (TGF-β) is known to play critical roles in the pathogenesis of lung injury and fibrosis (24, 25), and recent reports indicate that LPA_2 induced αvβ6 integrin–mediated TGF-β activation in epithelial cells (26). We therefore hypothesized that LPA_2 is likely to play a functional role in bleomycin-induced lung fibrosis, a well-validated model of chronic pulmonary fibrosis. In this report, we show that *Lpar2*$^{-/-}$ mice are significantly protected against bleomycin-induced lung injury, fibrosis, and mortality, and that the bleomycin-induced expression of FN, α-SMA, and collagen is attenuated in the lungs of *Lpar2*$^{-/-}$ mice. In addition, the increased levels of IL-6, TGF-β1, and total protein found in the BAL fluid of wild-type (WT) control mice challenged with bleomycin are not observed in bleomycin-treated, LPA_2-deficient mice. Mice deficient in LPA_2

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are also protected from bleomycin-induced alveolar and bronchial epithelial cell apoptosis. *In vitro*, the LPA-induced differentiation of lung fibroblasts is characterized by an increased expression of FN, α-SMA, and collagen. The knockdown of LPA_2 attenuated the LPA-induced differentiation of lung fibroblasts and the resultant increase in these ECM proteins through the modulation of Akt, Smad3, and p38 mitogen-activated protein kinase (p38 MAPK). In addition, the LPA-induced expression of TGF-β1 and the differentiation of human lung fibroblasts were suppressed by knocking down LPA_2 expression. Our experiments demonstrate that LPA_2, through the mediation of TGF-β expression and signaling, plays a role in bleomycin-induced pulmonary fibrosis.

MATERIALS AND METHODS

Experimental Pulmonary Fibrosis Model

Sex-matched and age-matched offspring of WT and *Lpar2*$^{-/-}$ mice (male, aged 8–10 wk) used for bleomycin-induced fibrosis experiments were housed in the animal facility at the University of Illinois at Chicago. WT mice (B6129SF2/J, stock number 101045) were purchased from Jackson laboratory (Bar Harbor, Me). WT and *Lpar2*$^{-/-}$ (B6129SF2/J background) mice were anesthetized (with a 3 ml/kg mixture of 25 mg/kg of ketamine in 2.5 ml of xylazine) (27), followed by treatment with either saline or bleomycin sulfate (1.25–2 U/kg of body weight, ∼ 0.025–0.04 U/animal) in saline by an intratracheal injection in a total volume of 50 μl. Animals were killed for analysis on Days 0, 3, 7, 14, 21, or 28 after bleomycin challenge, and BAL fluid was collected by an intratracheal injection of phosphate-buffered saline (PBS) solution (0.5 ml twice) followed by gentle aspiration, with a final volume of 0.7–0.8 ml. BAL fluid was centrifuged, and the supernatants were processed for protein and cytokine measurement. Lungs were removed from the mice and their lobes were sectioned, embedded in paraffin, and cut into 5-μm sections. Hematoxylin and eosin staining, trichrome staining, and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assays were performed by the Pathology Core Facility at the University of Illinois at Chicago. Other related assays were described previously (28, 29), and detailed methods are described in the online supplement. The experiments reported here were approved by the Animal Care Use Committee of the University of Illinois at Chicago, and conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Immunofluorescence Microscopy

Immunofluorescence microscopy to determine the expression of ECM proteins was performed as described elsewhere (30). Briefly, human lung fibroblasts were grown in slide chambers before transfection with scrambled RNA or small interfering RNA (siRNA). After stimulation with TGF-β1 (5 ng/ml) or 18:1 LPA (10 μM) for 48 hours, cells were fixed with 3.7% paraformaldehyde in PBS for 10 minutes, followed by permeabilization for 4 minutes in Tris-buffered saline (TBS) containing 0.25% Triton X-100. Cells were then washed with PBS three times, and blocked with TBS Tween 20 (TBST) blocking buffer (containing 2% BSA) for 3 minutes at room temperature. Cells were then incubated with primary antibodies (1:200 dilutions in blocking buffer) for 1 hour, followed by three rinses (15 min each) in TBST. Cells were then stained with Alexa Fluor secondary antibodies (1:200 dilutions in blocking buffer; Life Technologies, Grand Island, NY) for 1 hour, followed by a TBST wash for 15 minutes. Slides were prepared with mounting media, examined under a Nikon Eclipse TE 2000-S fluorescence microscope (Nikon, Tokyo, Japan), and the images were recorded with a Hamamatsu digital camera (Tokyo, Japan), using a ×60 oil immersion objective lens.

Statistical Analysis

Data are expressed as means ± SEMs. All results were subjected to statistical analysis using one-way ANOVA or a two-tailed Student *t* test. Values of $P < 0.05$ were considered significant. At least three independent sets of experiments were performed (31, 32).

Details of other materials and methods used in this study are outlined in the online supplement.

RESULTS

LPA_2 Deficiency Attenuates Bleomycin-Induced Lung Injury, Inflammation, and Mortality in Mice

Recent studies revealed a key role for LPA in the pathogenesis of lung and kidney fibrosis and the signals transduced through its receptors. LPA_1 deficiency was shown to protect mice from bleomycin-induced lung fibrosis and ensuing mortality by attenuating bleomycin-induced fibroblast recruitment, vascular leakage, and lung epithelial cell apoptosis (15, 16). LPA_2, another LPA receptor, plays a critical role in αvβ6 integrin–mediated TGF-β activation in epithelial cells (26). Given that TGF-β acts as a potent driver of pulmonary fibrosis, we hypothesized that LPA_2 may also play a role in bleomycin-induced lung fibrosis. To investigate whether LPA_2 plays a role in the pathogenesis of lung fibrosis, WT and *Lpar2*$^{-/-}$ mice were treated with bleomycin (2 U/kg of body weight) intratracheally, and the mortality of bleomycin-challenged mice was recorded. As shown in Figure 1A, WT mice challenged with bleomycin began dying at 5 days after bleomycin treatment, and reached a mortality rate of approximately 80% on Day 14, whereas *Lpar2*$^{-/-}$ bleomycin-challenged mice began to die on Day 8, with a final mortality rate of approximately 50% on Day 14. Thus, LPA_2 deficiency clearly delayed the onset of disease severity, and protected against bleomycin-induced mouse mortality ($P = 0.023$).

An analysis of BAL fluid and lung tissue from control and LPA_2-deficient mice with and without bleomycin challenge did not show significant differences in lung morphology between WT and *Lpar2*$^{-/-}$ mice (Figure 1B). However, bleomycin injury responses in terms of the influx of inflammatory cells into the alveolar spaces were attenuated in *Lpar2*$^{-/-}$ mice, compared with WT mice (Figure 1C). On Day 3 after bleomycin challenge, the total cell increase in *Lpar2*$^{-/-}$ mice was significantly reduced compared with that of WT control mice. However, this decrease was not significant on Days 7 and 14 after bleomycin challenge (Figure 1D). Differential cell counts showed that the numbers of infiltrating neutrophils were dramatically reduced in *Lpar2*$^{-/-}$ mice compared with WT control mice, but not the number of macrophages and lymphocytes (Figures E1A–E1C in the online supplement).

To determine whether LPA_2 plays a role in maintaining lung vascular integrity, WT and *Lpar2*$^{-/-}$ mice were treated with bleomycin, BAL fluid protein levels were measured, and the extravasation of Evans blue dye in lung tissue was assessed. As shown in Figure 1E, the administration of bleomycin to WT mice caused a dramatic increase in total BAL protein on Days 3, 7, and 14 after challenge. However, the level of total BAL protein recovered from *Lpar2*$^{-/-}$ mice was significantly lower than what was recovered from WT bleomycin-challenged mice (Figure 1E). In agreement with the decreased total BAL protein levels, the extravasation of Evans blue dye from the vasculature of *Lpar2*$^{-/-}$ mice into their lung tissue 7 days after bleomycin challenge was also significantly less than that in WT control mice (Figure 1F). These results show that LPA_2 deficiency provides protection from bleomycin-induced vascular leakage in mice.

Inflammation was assessed by measuring IL-6 levels in the BAL of WT and *Lpar2*$^{-/-}$ bleomycin-treated mice (Figure 1G). In WT mice, bleomycin caused an elevation of IL-6 levels in BAL fluid. IL-6 levels in the BAL fluid of bleomycin-treated *Lpar2*$^{-/-}$ mice were significantly lower than those of WT bleomycin-treated mice. Furthermore, bleomycin-induced fibrosis was evaluated in WT and LPA_2-deficient mice on Days 21 and 28 after bleomycin challenge, using a lower dose of bleomycin (1.25 U/kg body weight). As shown in Figures E2A and E2B, lung injury and mortality rates

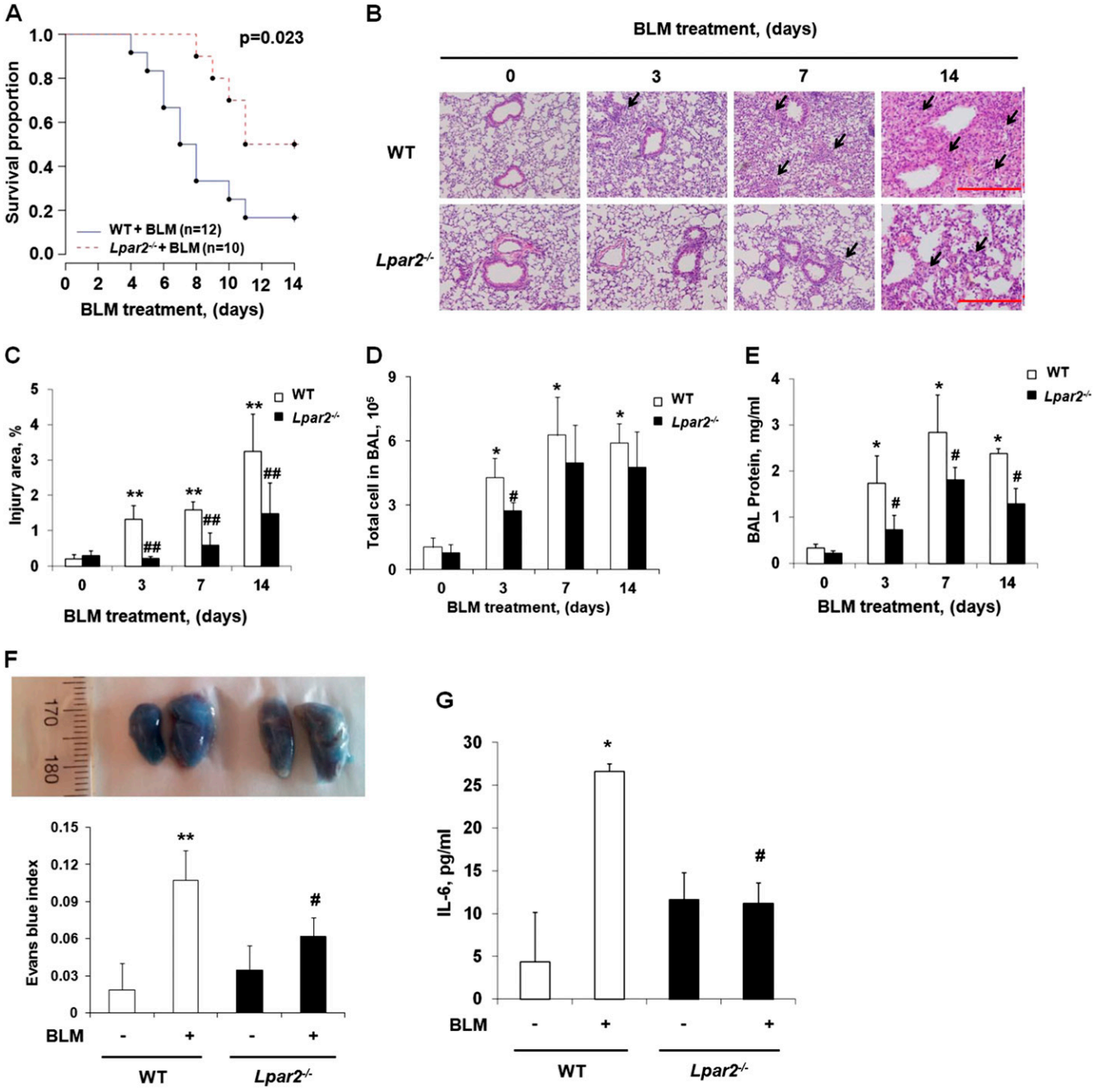

Figure 1. Lysophosphatidic acid receptor–2 (LPA_2) deficiency protects against bleomycin (BLM)–induced lung injury, inflammation, and mortality in mice. Wild-type (WT) and LPA receptor–2 knockout (*Lpar2*$^{-/-}$) mice (aged 8–10 wk, male) were anesthetized with 3 ml/kg of a mixture of ketamine (25 mg/kg) and 2.5 ml of xylazine, followed by an intratracheal injection of saline or bleomycin sulfate (2 U/kg, $\sim$ 0.04 U/animal) in saline in a total volume of 50 μl. Animals were killed for analysis on Days 0, 3, 7, or 14 (after bleomycin administration). Bronchoalveolar lavage (BAL) fluid was collected and analyzed, as described in Materials and Methods. Lungs were removed from the mice and their lobes were sectioned, embedded in paraffin, and cut into 5-μm sections for hematoxylin-and-eosin staining. (*A*) Survival of WT and *Lpar2*$^{-/-}$ mice challenged with bleomycin. (*B*) Representative hematoxylin-and-eosin staining of lung tissue obtained from bleomycin-challenged *Lpar2*$^{-/-}$ and WT mice (*arrows* show injured areas). Original magnification, ×10. *Scale bar*, 200 μm. (*C*) Quantitative analysis of injury area, expressed as the percentage of the total cross-sectional area. (*D*) Total infiltrated cell number. (*E*) Total protein levels in BAL fluid, expressed as means ± SEMs. (*F*) Evans blue dye extravasation assay for pulmonary vascular leakage. Briefly, 7 days after bleomycin challenge (2 U/kg body weight), mice were injected with Evans blue dye (20 mg/kg) intravenously for 3 hours. After perfusion, lungs were excised and imaged by a Canon (Tokyo, Japan) digital camera (*top*), followed by homogenization and analysis, as described in Materials and Methods. The Evans blue dye in lung-tissue supernatants and plasma was quantified and normalized to the plasma level (*bottom*). (*G*) IL-6 level in BAL fluid from WT and *Lpar2*$^{-/-}$ mice on Day 7 after bleomycin challenge. Data are expressed as means ± SEMs. *$P < 0.05$ and **$P < 0.01$, versus WT mice without BLM treatment. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, versus WT mice with BLM challenge at the same time point ($n = 4$–6 per group).

were significantly higher in WT mice compared with LPA_2-deficient mice at 21 and 28 days after bleomycin challenge. Taken together, these results show that LPA_2 deficiency confers protection against bleomycin-induced lung injury, inflammation, and mortality in mice at higher and lower doses of bleomycin.

Bleomycin-Induced Lung Fibrosis Is Suppressed in LPA_2-Deficient Mice

To investigate the effects of LPA_2 deficiency in the pathogenesis of bleomycin-induced lung fibrosis, lung sections from WT and *Lpar2*$^{-/-}$ mice with or without bleomycin challenge (1.25–2.00 U/kg body weight) were stained for trichrome and assayed for collagen. A trichrome and Sircol collagen assay revealed that whole-lung collagen levels were similar in WT and *Lpar2*$^{-/-}$ mice without bleomycin challenge. However, collagen deposition was attenuated in *Lpar2*$^{-/-}$ mice compared with WT mice on Days 14, 21, and 28 after bleomycin challenge (Figures 2A, 2B, E2C, and E2D). Ashcroft scores also indicated that bleomycin-induced fibrogenesis was significantly attenuated in *Lpar2*$^{-/-}$ mice (Figures 2C and E2E). Further, BAL fluid from bleomycin-challenged WT mice contained significantly higher TGF-β1 protein levels relative to vehicle-treated WT and *Lpar2*$^{-/-}$ mice. In sharp contrast to bleomycin-treated WT mice, a lack of increase in TGB-β1 protein levels was evident in the BAL fluid and lung tissue of similarly treated *Lpar2*$^{-/-}$ mice (Figures 2D, E2F, and E2I). In addition, as shown in Figures 2E and E2F–E2H, bleomycin-induced increases of FN and α-SMA levels in the lung tissue of WT mice on Days 14 and 28 after bleomycin challenge were significantly attenuated in *Lpar2*$^{-/-}$ mice.

LPA-Induced Expression of TGF-β, α-SMA, and ECM Proteins in Human Lung Fibroblasts Is Attenuated by Anti–TGF-β Antibody

Recent reports show that LPA induces TGF-β expression in corneal fibroblasts (33). However, a role for LPA receptors in TGF-β expression and fibroblast activation has not been previously demonstrated. To show that LPA is important for the activation of lung fibroblasts, human lung fibroblasts were challenged with LPA, and the expressions of TGF-β1, α-SMA, and ECM proteins

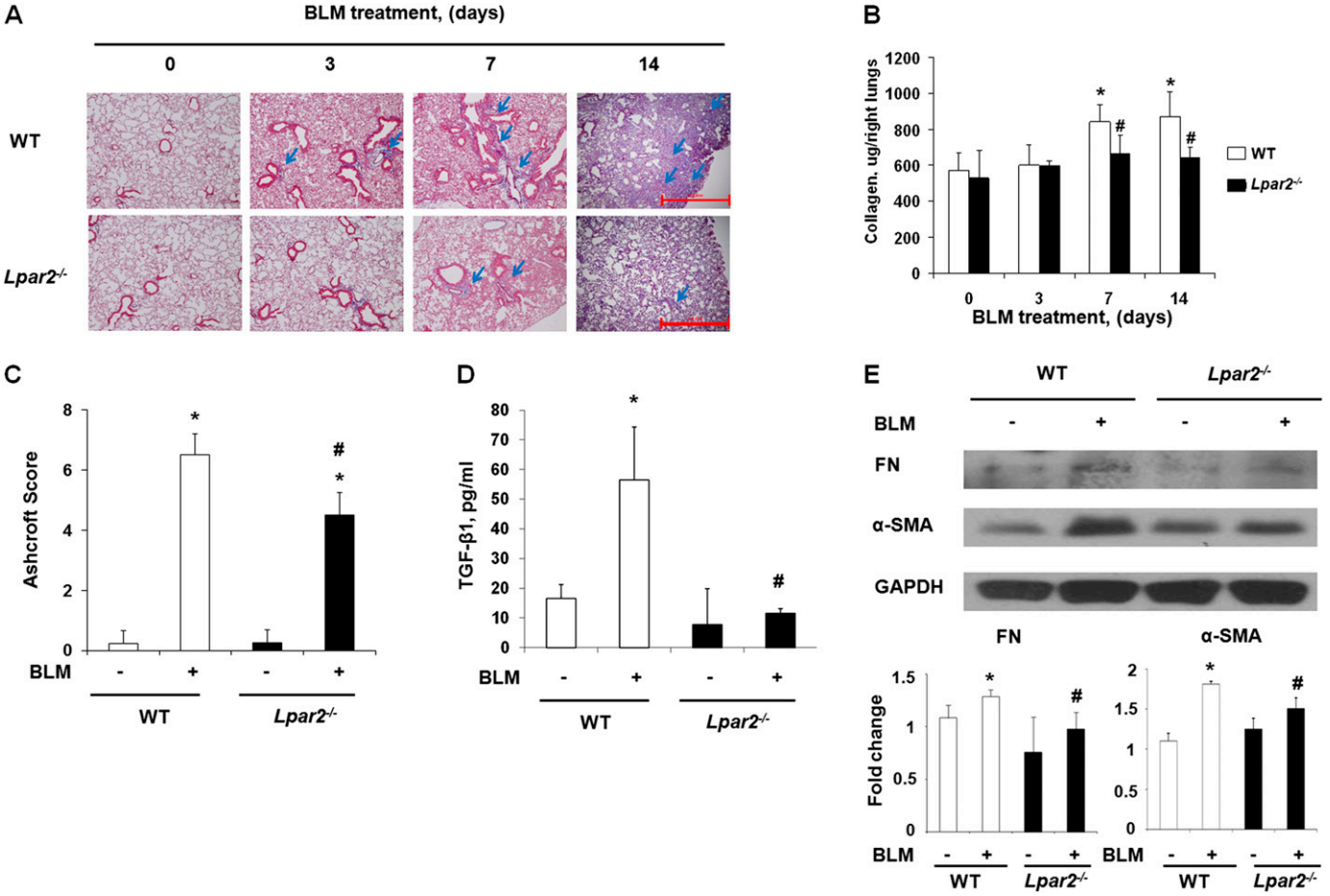

Figure 2. LPA_2 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. *Lpar2*$^{-/-}$ or WT mice (male, aged 8–10 wk) received bleomycin (2 U/kg in 50 μl PBS, ~ 0.04 U/animal) or PBS intratracheally, and were killed on Days 0, 3, 7, and 14 after bleomycin challenge. Lungs were removed, embedded in paraffin, and cut into 5-μm sections for staining. (*A*) Representative images of trichrome-stained lung sections were obtained from *Lpar2*$^{-/-}$ or WT mice, with or without bleomycin challenge (*blue arrows* show collagen deposition areas in *blue*). Original magnification, ×4. *Scale bar*, 1 mm. (*B*) Acid-soluble collagen in lung tissue. (*C*) Ashcroft score of lung sections from lung tissue obtained from *Lpar2*$^{-/-}$or WT mice challenged with bleomycin on Days 0 and 14. (*D*) Transforming growth factor–β1 levels in BAL fluid obtained from *Lpar2*$^{-/-}$ or WT mice after bleomycin challenge on Day 14. (*E*) Protein levels of α–smooth muscle actin (α-SMA) and fibronectin (FN) in lung tissue from *Lpar2*$^{-/-}$ or WT mice after bleomycin challenge on Day 14. Data are expressed as means ± SEMs. *$P < 0.05$ versus wild-type mice without bleomycin treatment. #$P < 0.05$, versus WT mice after bleomycin treatment at the same time point ($n = 4$–6 per group). BLM, bleomycin; GAPDH, glyceraldehyde 3–phosphate dehydrogenase.

(such as FN and collagen 1A2), as well as TGF-β1 secretion, were measured. As shown in Figures 3A and 3B, LPA treatment significantly increased the expression of TGF-β1, α-SMA, and ECM proteins, as well as TGF-β1 secretion. The pretreatment of cells with an anti–TGF-β1 antibody partly blocked the LPA-induced expression of TGF-β1, α-SMA, and ECM proteins and TGF-β1 secretion in human lung fibroblasts (Figures 3C–3I). These results suggest a role for LPA in TGF-β secretion and signaling in human lung fibroblasts.

LPA-Induced Expression of TGF-β1 and Differentiation of Human Lung Fibroblasts Are Mediated through LPA_2

To determine whether LPA-induced TGF-β1 expression and fibroblast differentiation are mediated specifically through LPA_2, human lung fibroblasts were treated with LPA_2 siRNA (si-LPA_2) or a scrambled siRNA. Treatment with si-LPA_2 dramatically down-regulated the LPA_2 mRNA (~ 80%) and protein (~ 45%) levels in human lung fibroblasts (Figures 4A–4C), and exerted no effect on the expression of other LPA receptors (data not shown). The knockdown of LPA_2 significantly reduced the LPA-induced expression of TGF-β1 in human lung fibroblasts, and blocked the LPA-induced differentiation of human lung fibroblasts, as indicated by the reduced levels of ECM proteins (Figures 4B–4G). Immunofluorescence staining also demonstrated that si-LPA_2 diminished the LPA-induced expression of α-SMA and FN in lung fibroblasts (Figure 4H). However, the knockdown of the expression of LPA_1 by si-LPA_1 did not significantly attenuate the LPA-induced expression of TGF-β, α-SMA, and ECM proteins in human lung fibroblasts (Figures E3A–E3E). Similar to the findings in human lung fibroblasts, LPA-induced expressions of TGF-β, FN, α-SMA, and collagen were significantly reduced in lung fibroblasts isolated from *Lpar2*$^{-/-}$ mice, compared with those isolated from WT mice (Figures E4A–E4E). Together, these results show that LPA-induced lung fibroblast differentiation and TGF-β1 expression are specifically mediated by LPA_2.

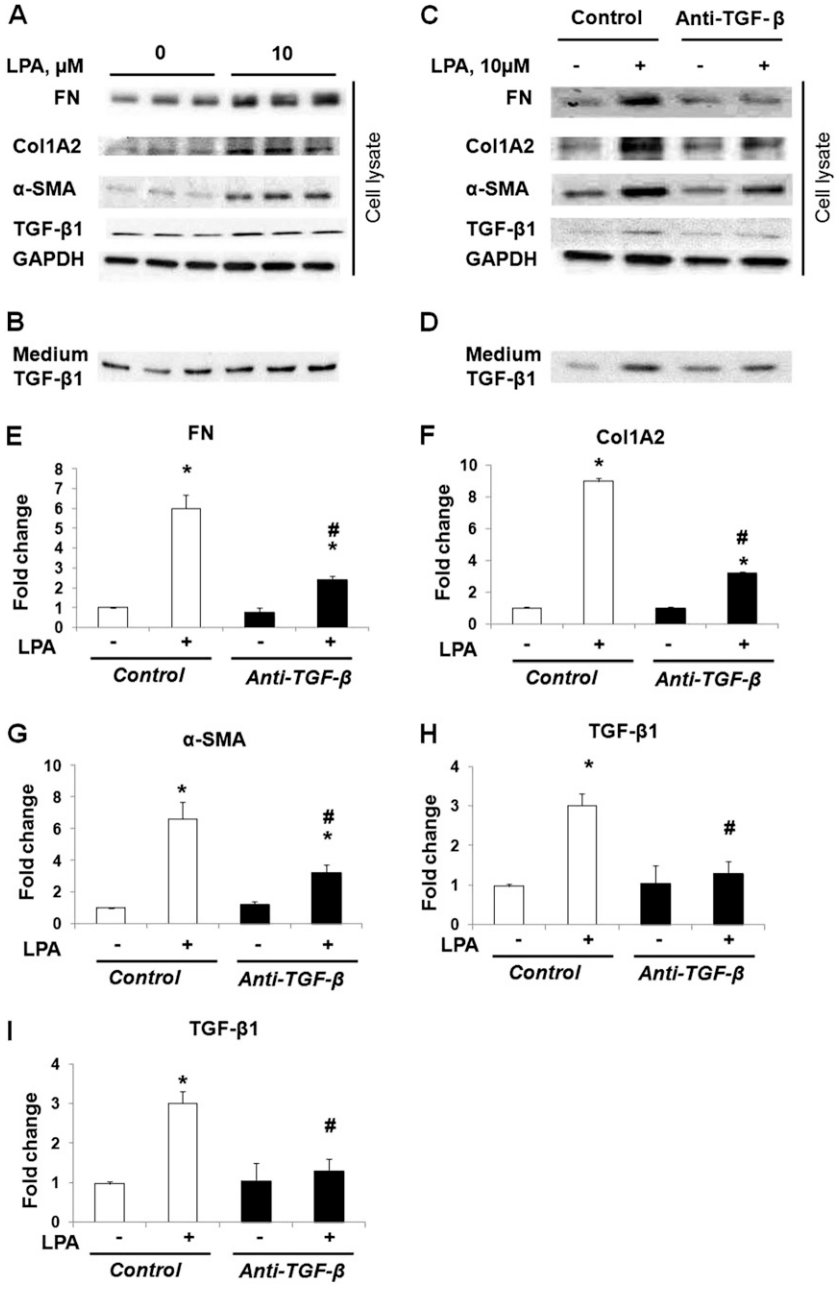

Figure 3. Lysophosphatidic acid (LPA) induces the activation and differentiation of lung fibroblasts via Transforming growth factor (TGF)-β1. Serum-starved (for 20 h) human lung fibroblasts (~ 90% confluence) were pretreated with anti–TGF-β1 antibody or control IgG antibody (5 μg/ml, 1 h), and challenged with 18:1 LPA (10 μM) or vehicle for 48 hours, and cell lysates (20 μg protein) were subjected to SDS-PAGE and immunoblotting. The cell culture medium was concentrated, and TGF-β1 levels were analyzed by SDS-PAGE and immunoblotting. The intensity of the bands with anti-FN or anti–α-SMA, collagen 1A2 (Col1A2), and TGF-β1 antibody were quantified and normalized to GAPDH. LPA (10 μM) at 18:1 induced the expression of FN, α-SMA, Col1A2, and TGF-β1 in human lung fibroblasts (*A*) and the secretion of TGF-β1 into the culture medium (*B*). Effects of anti–TGF-β1 antibody on 18:1 LPA (10 μM)–induced expression of FN, α-SMA, Col1A2, and TGF-β1 in human lung fibroblasts (*C*) and TGF-β levels in culture medium (*D*). (*E–I*) Quantification of the expression of FN (*E*), Col1A2 (*F*), α-SMA (*G*), and TGF-β1 (*H*) induced by LPA in human lung fibroblasts, and the secreted TGF-β1 in culture medium (*I*). Data are expressed as means ± SEMs of three independent experiments. $^{*}P < 0.05$, versus cells with control antibody but without LPA treatment. $^{\#}P < 0.05$, versus LPA-challenged cells with pretreatment of control antibody.

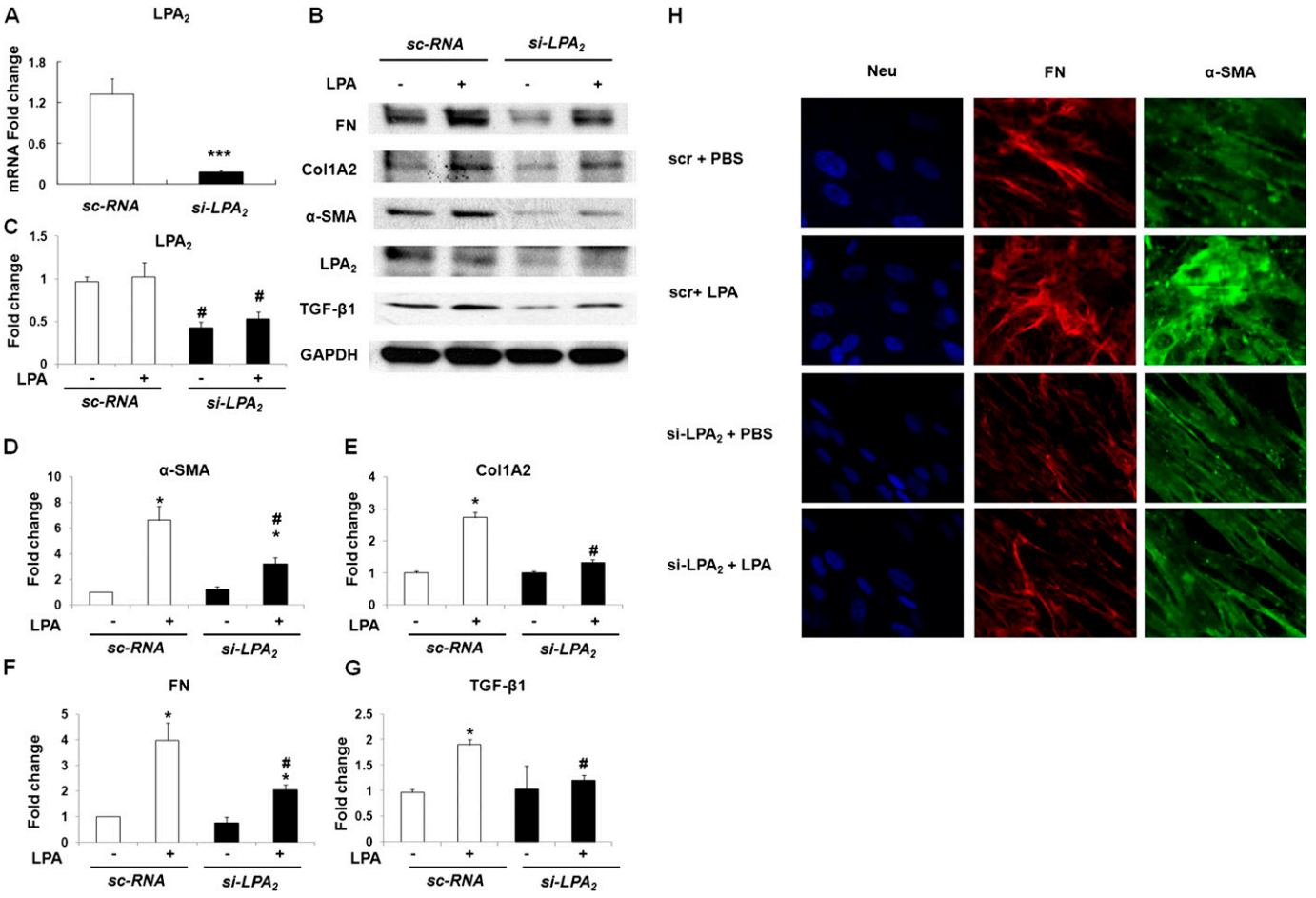

Figure 4. LPA_2 small interfering RNA (siRNA) blocks the LPA-induced expression of transforming growth factor (TGF)-β, as well as the activation and differentiation of lung fibroblasts. Human lung fibroblasts (~ 60% confluence) were treated with control scrambled siRNA (sc-RNA) or LPA_2 siRNA (si-LPA_2) (200 nM) for 24 hours, followed by serum starvation and treatment with 18:1 LPA (0 and 10 μM) for another 48 hours. Cell lysates (20 μg of protein) were subjected to SDS-PAGE and immunoblotting, as described in MATERIALS AND METHODS. The intensity of the bands with anti-FN or anti–α-SMA, Col1A2, and TGF-β1 antibody were quantified and normalized to GAPDH. Data are expressed as the means ± SEMs of three independent experiments. (*A*) mRNA levels of LPA_2 from human lung fibroblasts transfected with scrambled siRNA or LPA_2 siRNA. ***$P < 0.001$, versus cells with sc-RNA treatment. (*B*) Immunoblotting to determine the expression of LPA_2, FN, α-SMA, Col1A2, and TGF-β1. A representative immunoblot from three independent experiments is shown. (*C–G*) Quantification of the expression of LPA_2 (*C*), α-SMA (*D*), Col1A2 (*E*), FN (*F*), and TGF-β1 (*G*) induced by LPA after LPA_2 siRNA treatment. *$P < 0.05$ versus cells with sc-RNA but without LPA treatment. #$P < 0.05$ versus LPA-challenged cells with pretreatment of sc-RNA. (*H*) Human lung fibroblasts grown on eight-well slide chambers were transfected with scrambled or LPA_2 siRNA for 24 hours, exposed to PBS or 18:1 LPA (10 μM) for 48 hours, washed, fixed, permeabilized, probed with anti-FN or anti–α-SMA antibodies, and examined by immunofluorescence microscopy, using a ×60 oil objective. The FN (*red*) and α-SMA (*green*) images showed matched cell fields for each condition. A representative image from three independent experiments is shown.

The LPA-Induced Phosphorylation of Extracellular Regulated Kinase 1/2, Smad3, Akt, and p38 MAPK in Human Lung Fibroblasts Is Mediated through LPA_2

Having established a role for LPA and LPA_2 in lung fibroblast differentiation, we evaluated the effects of LPA_2 deficiency on signaling pathways linked with fibrosis. Similar to TGF-β1, LPA has been shown to induce lung fibroblast differentiation via the increased phosphorylation of Smad2/3, Akt, extracellular regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK (33, 34). In primary human lung fibroblasts, both LPA and TGF-β stimulated Smad3 phosphorylation in a time-dependent fashion (Figures E5A and E5B), and the knockdown of LPA_2 with si-LPA_2 significantly suppressed the LPA-induced phosphorylation of ERK1/2, Smad3, Akt, and p38 MAPK, but exerted no effect on the phosphorylation of Smad2 and JNK (Figures 5A–5G). To assess the role of Akt, ERK1/2, and p38 MAPK in LPA-induced fibroblast differentiation, human lung fibroblasts were pretreated with pharmacological inhibitors of Akt, ERK1/2, and p38 MAPK phosphorylation. As shown in Figures E6–E8, the LPA-induced expressions of TGF-β and ECM proteins were attenuated by inhibitors of Akt, ERK1/2, and p38 MAPK phosphorylation in human lung fibroblasts. Moreover, blocking ERK1/2 phosphorylation with PD98059 also attenuated LPA-mediated Smad3 phosphorylation in human lung fibroblasts (Figure E7A). These results demonstrated that in human lung fibroblasts, LPA/LPA_2 signaling plays a critical role in fibrosis-associated signaling pathways, and particularly those involving ERK1/2, Smad3, Akt, and p38 MAPK. However, Smad2 and JNK are likely activated by an alternative LPA receptor.

TGF-β1–Induced Human Lung Fibroblast Differentiation Requires LPA_2

Having established a role for TGF-β in LPA-mediated fibroblast differentiation via LPA_2, we investigated the role of LPA_2 in the TGF-β–induced expression of α-SMA, FN, and collagen in human lung fibroblasts. The TGF-β1–induced expressions of

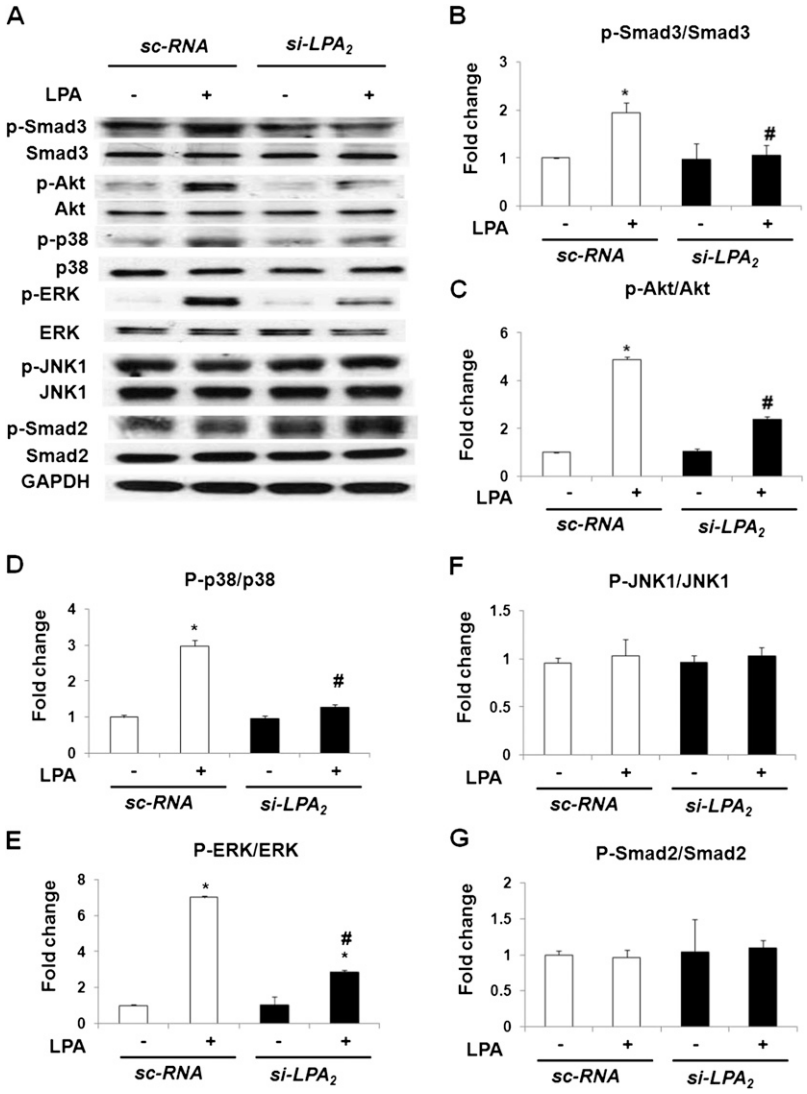

Figure 5. LPA_2 siRNA attenuates the LPA-induced phosphorylation of Smad3, Akt, p38 mitogen-activated protein kinase (p38 MAPK), and extracellular regulated kinase (ERK) in human lung fibroblasts. Human lung fibroblasts ($\sim$ 60% confluence) were treated with scrambled or LPA_2 siRNA (200 nM) for 48 hours, followed by serum starvation and treatment with 18:1 LPA (10 μM) for another 15 minutes. (*A*) Cell lysates (20 μg of protein) were subjected to SDS-PAGE and immunoblotted with anti–p-Smad3, Smad3, p-Akt, Akt, p–p38 MAPK, p38 MAPK, p–c-Jun N-terminal kinase (JNK)–1, JNK1, p-Smad2, Smad2, p-ERK, ERK, and GAPDH antibodies. A representative immunoblot from three independent experiments is shown. (*B–G*) The intensity of each band was quantified using densitometry, and normalized to GAPDH. Data are expressed as the means $\pm$ SEMs of three independent experiments. $^{*}P < 0.05$, versus cells without LPA treatment. $^{\#}P < 0.05$, versus scrambled siRNA–treated cells also treated with LPA.

α-SMA, FN, and collagen 1A2 were significantly reduced in si-LPA_2–transfected human lung fibroblast cells, in which LPA_2 was knocked down. However, scrambled siRNA exerted no effect on the expression of these proteins (Figures 6A–6E). Immunofluorescence staining also showed that si-LPA_2 treatment resulted in the diminished TGF-β–induced expression of α-SMA and FN in human lung fibroblasts (Figure 6F). Similar to human lung fibroblasts, the TGF-β1–induced expression of FN, α-SMA, and collagen was significantly suppressed in lung fibroblasts isolated from *Lpar2*$^{-/-}$ mice, compared with those from WT mice (Figures E9A–E9D). Together, these results suggest that LPA_2 is essential for TGF-β–mediated signal transduction and the differentiation of lung fibroblasts.

Bleomycin-Induced Lung Epithelium Apoptosis Is Decreased in *Lpar2*$^{-/-}$ Mice

An intratracheal administration of bleomycin induces the rapid apoptosis of bronchial and alveolar epithelial cells (20). To determine whether LPA_2 deficiency also suppressed epithelial cell apoptosis during pulmonary fibrogenesis, we compared the amounts of bleomycin-induced alveolar and bronchial epithelial cell apoptosis in WT and *Lpar2*$^{-/-}$ mouse lung parenchyma. Apoptosis, as determined by the number of TUNEL^{+} cells, was increased in the alveolar epithelium of WT mice after bleomycin challenge. However, the numbers of TUNEL^{+} cells in the alveolar epithelium of similarly treated *Lpar2*$^{-/-}$ mice were approximately 80% lower than in those of WT mice (Figures E10A and E10B). Similarly, the bleomycin-induced apoptosis of bronchial epithelial cells was also significantly attenuated in *Lpar2*$^{-/-}$ mice (Figures E10C and E10D). These results show that LPA_2 deficiency offers substantial protection against bleomycin-induced alveolar and bronchial epithelial cell apoptosis in mice.

DISCUSSION

The pathogenesis of IPF has been extensively studied in animal models and human patients, and numerous factors have been implicated in the pathogenesis of IPF (35). However, the specific mechanisms leading to lung fibrosis remain elusive. Previous studies based on animal models and various cell types revealed the involvement of inflammatory cells, epithelial cells, and fibroblasts in the pathology of lung fibrosis (3–6). Recent clinical studies have demonstrated significant elevations in LPA levels in BAL samples collected from patients with IPF compared with normal subjects, suggesting a role for LPA in pulmonary fibrogenesis. In addition, LPA_1 was found to be the most highly expressed LPA receptor in fibroblasts obtained from patients with IPF, and is known to be involved in LPA-

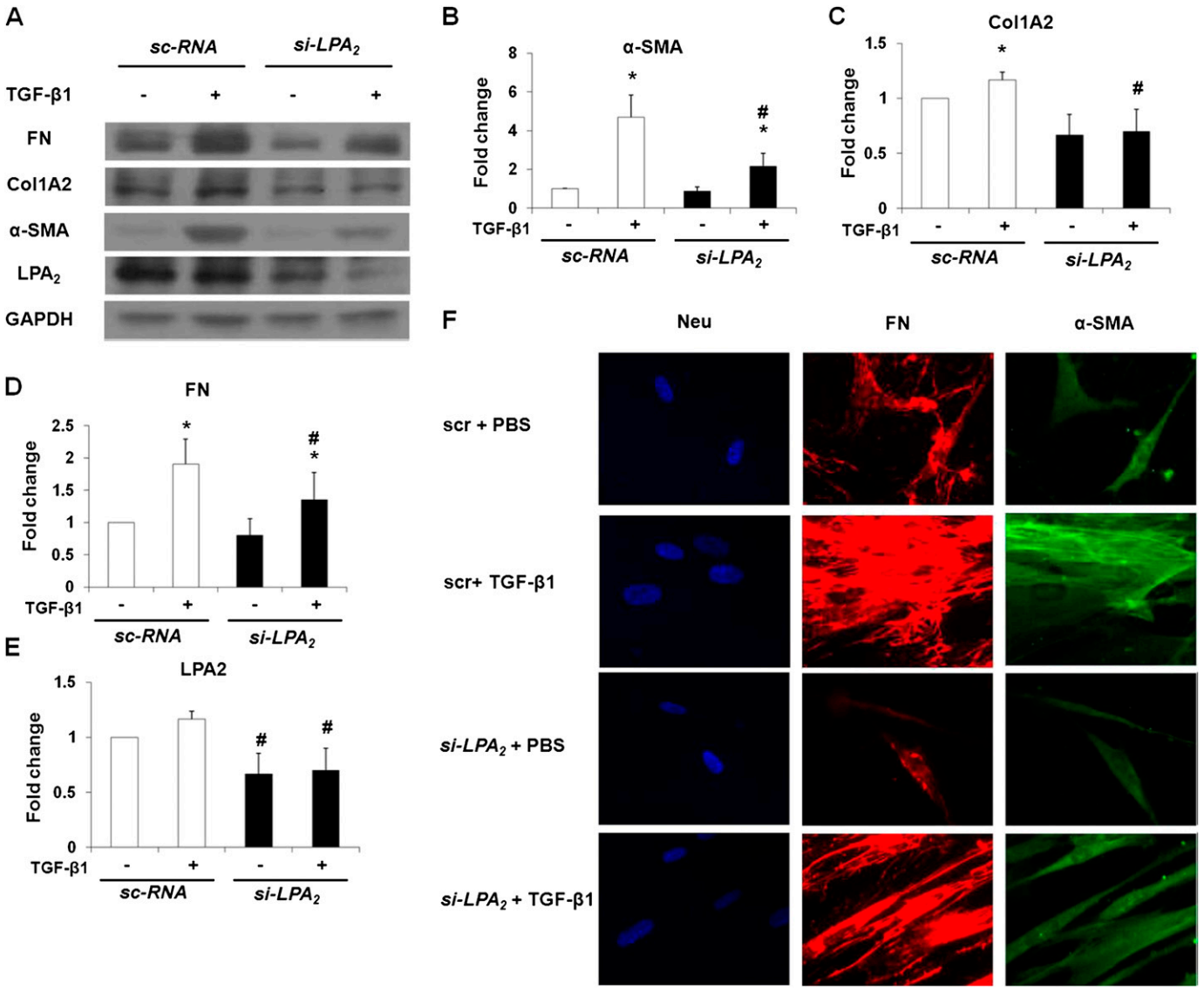

Figure 6. LPA_2 siRNA attenuates the Transforming growth factor (TGF)-β1–induced activation and differentiation of lung fibroblasts. Human lung fibroblasts (~ 60% confluence) were treated with scrambled or LPA_2 siRNA (200 nM) for 24 hours, followed by serum starvation and treatment with TGF-β1 (5 ng/ml) for another 48 hours. Cell lysates (20 μg protein) were subjected to SDS-PAGE and immunoblotting. The intensity of bands for anti-FN or anti–α-SMA and Col1A2 antibodies were quantified and normalized to GAPDH. Data are expressed as the means ± SEMs of three independent experiments. (*A*) Immunoblotting to determine relative expression levels of FN, α-SMA, Col1A2, and TGF-β1. A representative immunoblot from three independent experiments is shown. (*B–E*) Quantification of the expression of α-SMA (*B*), Col1A2 (C), FN (*D*), and LPA_2 (*E*) induced by TGF-β1 after LPA_2 siRNA treatment. $^*P < 0.05$, versus cells without LPA treatment. $^{\#}P < 0.05$, versus scrambled siRNA-treated cells with LPA treatment. (*F*) Human lung fibroblasts grown on eight-well slide chambers were transfected with scrambled or LPA_2 siRNA (for 24 h), exposed to PBS or TGF-β1 (for 48 h), washed, fixed, permeabilized, probed with anti-FN or anti–α-SMA antibodies, and examined according to immunofluorescence microscopy, using a ×60 oil objective. The FN (*red*) and α-SMA (*green*) images showed matched cell fields for each condition. A representative image from three independent experiments is shown.

mediated fibroblast recruitment (16). In the mouse model of fibrosis, LPA_1 deficiency conferred significant protection against fibrosis in lungs (16), kidneys (23), and skin (36). Oral administration of the LPA_1 specific antagonist, (4′-{4-[(R)-1-(2-chloro-phenyl)-ethoxycarbonylamino]-3-methyl-isoxazol-5-yl}-biphenyl-4-yl)–acetic acid (AM966), dramatically prevented bleomycin-induced pulmonary fibrosis (21). Further, the expression of LPA_2 was also up-regulated in lungs from bleomycin-challenged mice, especially in alveolar cells associated with areas of lung injury and fibrosis (26). However, the role of LPA_2 in the development of pulmonary fibrosis in animal models remains unclear. Here, we show that LPA_2 deficiency also offers significant protection against bleomycin-induced lung injury and fibrosis in mice. We show for the first time that LPA_2 is also involved in lung inflammation and injury mediated by bleomycin, which is characterized by vascular leakage and lung epithelial cell apoptosis. In contrast to our current findings, a previous study indicated that LPA_1, but not LPA_2, drove lung fibroblast recruitment in bleomycin-challenged mice (16). Differences in genetic background are known to influence susceptibility to bleomycin-induced fibrosis, and may account for the contrasting results. In addition, we note that the protection we observed was not absolute, and thus the relative degree of fibrosis observed in different genotypes could complicate interpretations. We observe that TGF-β signaling is compromised in *Lpar2*$^{-/-}$ mice,

and that LPA_2 not only transduces LPA signaling but also regulates TGF-β secretion and the TGF-β–induced activation and differentiation of lung fibroblasts, supporting the functionality of LPA_2 receptor loss, albeit producing a relatively smaller protective contribution compared with LPA_1 deficiency.

TGF-β is a key cytokine that plays an essential role in fibrogenesis and regulates the activation, differentiation, and proliferation of fibroblasts (37). Here we investigated the role of LPA_2 and TGF-β in the differentiation and activation of lung fibrosis. Our present study shows that the bleomycin-induced expression of α-SMA and ECM proteins (FN and collagen 1A2) is attenuated in *Lpar2*$^{-/-}$ mice. *In vitro*, the knockdown of LPA_2 expression using siRNA dramatically blocked the LPA-induced phosphorylation of ERK1/2, p38 MAPK, Akt, and Smad3, as well as TGF-β secretion in human lung fibroblasts (Figures 4 and 5). Further, a pharmacological inhibition of the LPA-induced phosphorylation of ERK1/2, p38 MAPK, and Akt attenuated LPA-induced TGF-β secretion. These results suggest a potential link between the LPA/LPA_2 signaling axis (involving the ERK1/2, p38 MAPK, and Akt pathways) and TGF-β secretion in human lung fibroblasts. In fact, earlier studies have shown the involvement of phosphatidylinositol 3–kinase and MAPK pathways in the production of TGF-β by macrophages (38) and the hepatitis C virus–mediated activation of TGF-β1 gene by Src and MAPKs in a human hepatoma cell line (39). LPA levels are elevated in the BAL fluid of patients with IPF and in animal models of pulmonary fibrosis (16), which may provide an important mediator in initiating TGF-β secretion from fibroblasts and other cell types in the lung. A major pathway of increased LPA production in BAL fluid occurs via the autotaxin-catalyzed or lysophospholipase D–catalyzed hydrolysis of lysophosphatidylcholine (40). Recent studies show that limiting LPA synthesis by blocking autotaxin is beneficial in animal models of pulmonary fibrosis (41), suggesting autotaxin as a possible therapeutic target in IPF.

Another intriguing finding of the present study involves the requirement of the LPA_2 for the TGF-β–mediated expressions of FN, collagen 1, and α-SMA (Figure 6). In this study, we have demonstrated that the knockdown of LPA_2 attenuated the LPA-induced and TGF-β–induced expression of fibrogenic markers and the differentiation of lung fibroblasts (Figures 4 and 6). In addition, we also showed that bleomycin-induced TGF-β secretion and accumulation in BAL fluid were dramatically reduced in *Lpar2*$^{-/-}$ mice compared with their WT counterparts. The regulation by LPA_2 of the LPA-induced expression of TGF-β in lung fibroblasts may constitute the underlying reason why *Lpar2*$^{-/-}$ mice are protected from bleomycin-induced fibrosis. The potential mechanisms involved in the cross-talk between LPA/LPA_2 and TGF-β/TGF-β receptors remain unknown, even though cross-talk between LPA receptors and receptor tyrosine kinases such as platelet-derived growth factor receptor (PDGF-R), epidermal growth factor receptor (EGF-R), and hepatocyte growth factor receptor (MET) have been well documented in various cell types, including epithelial cells (27). The elegant work of Xu and colleagues showed that the mRNA expression levels of both LPA_2 and αvβ6 integrin are up-regulated in the lungs of bleomycin-challenged mice, and both LPA_2 and αvβ6 integrin are increased and coassociated in lung epithelium and mesenchymal cells within the regions of fibrosis (26). In this study, the mRNA expression of LPA_2 in fibroblasts was not reported. However, reports have demonstrated the expression of LPA_2 in fibroblasts from humans and mice. Human lung fibroblasts express $LPA_{1–3}$ receptors, with the highest expression for LPA_1, followed by LPA_2 and LPA_3, and in these cells, LPA receptors may be involved in the TGF-β–induced and LPA-induced activation of lung fibroblasts and fibrogenesis *in vivo* (17). Further, the mRNA expression of LPA_2 was not significantly changed in fibroblasts from patients with IPF compared with control subjects (42). Thus, the mRNA for LPA_2 is expressed both in epithelial cells and in fibroblasts, although in none of these studies was LPA_2 protein expression demonstrated.

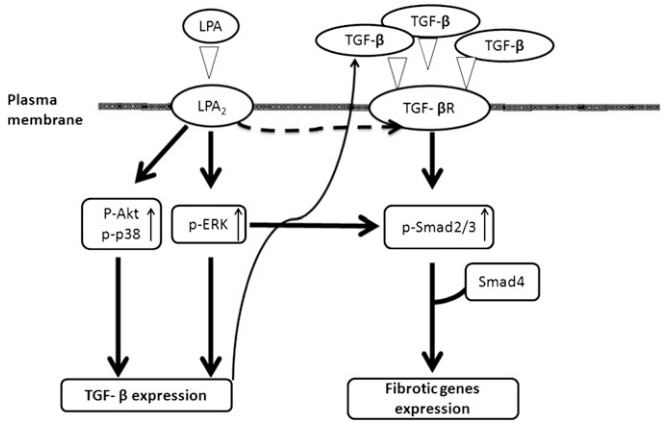

Figure 7. Schematic diagram illustrates the effects of LPA and Transforming growth factor (TGF)-β on fibrotic gene expression, differentiation, and activation in human lung fibroblasts. LPA binding to LPA_2 on the cell surface induces the activation of intracellular signaling pathways and triggers the expression of fibrotic genes (α-SMA, FN, Col1A2, and TGF-β). LPA induces TGF-β expression through the activation of Akt, p38, and ERK, and secreted TGF-β then signals through the TGF-β receptor (TGF-βR) and augments fibrogenesis. LPA_2 is also required for the TGF-β signaling of Smad3 phosphorylation and expression of genes associated with fibrogenesis. However, the potential mechanisms involved in the cross-talk between LPA_2 and the TGF-βR remain unclear.

In addition to LPA, another bioactive lipid, sphingosine-1–phosphate (S1P), may also play a role in pulmonary fibrosis. S1P levels are elevated in the BAL fluid of patients with IPF and bleomycin-challenged mice (43). S1P levels are regulated by a synthetic pathway mediated by two isoforms of sphingosine kinases (SphKs), SphK1/2, that phosphorylate sphingosine and generate S1P, and by the catabolic pathways that break down S1P because of the actions of S1P lyase and other lipid phosphatases (28). Our recent study revealed that the expression of SphK1 was increased in lung-tissue samples from patients with IPF and bleomycin- challenged mice (28). SphK1 is a known effector of the TGF-β–mediated signal transduction and expression of profibrogenic molecules in bleomycin-challenged lung tissue and in human lung fibroblasts (44). *In vivo*, SphK1 deficiency or the inhibition of SphK activity protects mice from bleomycin-induced pulmonary fibrosis, and in these mice, S1P levels in lung tissue were reduced and TGF-β signaling was inhibited (28). Previous studies also indicated that the SphK1/$S1P_3$ signaling axis plays a role in the TGF-β–induced transdifferentiation of myoblasts to myofibroblasts. Prolonged exposure of mice to FTY720, a prodrug for the $S1P_1$ agonist FTY720-phosphate, exacerbated bleomycin-induced vascular leakage, fibrosis, and lung injury in mice (45). Although both S1P and LPA signal through their respective cognate G-protein–coupled receptors, these lipid mediators are known to play opposing roles in regulating pulmonary epithelial integrity or leakiness. S1P is a potent endothelial barrier–protecting agent and prevents vascular leakage by tightening the vascular barrier (46–48), whereas LPA induces endothelial barrier dysfunction and vascular leakage (16, 49, 50). At present, how these two bioactive lipids with opposing actions in the endothelium induce pulmonary fibrosis in bleomycin-challenged mice remains unclear. Plausibly, the potential mechanisms for the induction of fibrosis by S1P and LPA are different. Further studies are necessary to

delineate the similarities and differences in S1P and LPA signaling that contribute to fibrogenesis in the lung.

In conclusion, we have identified a novel role for LPA_2 in pulmonary fibrosis, and demonstrated that LPA_2 deficiency protects against bleomycin-induced lung injury, fibrosis, and mortality in mice. LPA_2 deficiency also attenuated the bleomycin-induced expression of FN, α-SMA, and collagen in lung tissue, whereas the knockdown of LPA_2 affected the LPA-induced expression of TGF-β1 and the differentiation of lung fibroblasts via the decreased activation of Akt, Smad3, ERK1/2, and p38 MAPK (Figure 7). Another major and novel finding involves the requirement of LPA_2 for TGF-β signaling in human lung fibroblasts. However, this finding requires further study (Figure 7). Taken together, the experiments presented here indicate that LPA_2 plays a novel and critical role in bleomycin-induced lung injury and fibrosis in mice.

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