

# Semaphorin 7A plays a critical role in TGF- $\beta_1$ -induced pulmonary fibrosis

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**Semaphorin (SEMA) 7A regulates neuronal and immune function. In these studies, we tested the hypothesis that SEMA 7A is also a critical regulator of tissue remodeling. These studies demonstrate that SEMA 7A and its receptors, plexin C1 and  $\beta_1$  integrins, are stimulated by transforming growth factor (TGF)- $\beta_1$  in the murine lung. They also demonstrate that SEMA 7A plays a critical role in TGF- $\beta_1$ -induced fibrosis, myofibroblast hyperplasia, alveolar remodeling, and apoptosis. TGF- $\beta_1$  stimulated SEMA 7A via a largely Smad 3-independent mechanism and stimulated SEMA 7A receptors, matrix proteins, CCN proteins, fibroblast growth factor 2, interleukin 13 receptor components, proteases, anti-protease, and apoptosis regulators via Smad 2/3-independent and SEMA 7A-dependent mechanisms. SEMA 7A also played an important role in the pathogenesis of bleomycin-induced pulmonary fibrosis. TGF- $\beta_1$  and bleomycin also activated phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB)/AKT via SEMA 7A-dependent mechanisms, and PKB/AKT inhibition diminished TGF- $\beta_1$ -induced fibrosis. These observations demonstrate that SEMA 7A and its receptors are induced by TGF- $\beta_1$  and that SEMA 7A plays a central role in a PI3K/PKB/AKT-dependent pathway that contributes to TGF- $\beta_1$ -induced fibrosis and remodeling. They also demonstrate that the effects of SEMA 7A are not specific for transgenic TGF- $\beta_1$ , highlighting the importance of these findings for other fibrotic stimuli.**

## CORRESPONDENCE

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Abbreviations used: BAL, bronchoalveolar lavage; Dox, doxycycline; ECM, extracellular matrix; Egr, early growth response protein; FGF, fibroblast growth factor; ICAD, inhibitor of caspase-activated DNase; IHC, immunohistochemistry; IPF, idiopathic pulmonary fibrosis; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; SEMA, semaphorin; Tg, transgene; TIMP, tissue inhibitor of metalloproteinase; TUNEL, TdT-mediated dUTP nick-end labeling.

Fibrosis is an important cause of morbidity and mortality in the lung and other organs. This can be seen in the interstitial lung diseases, including idiopathic pulmonary fibrosis (IPF), scleroderma, radiation-induced pulmonary fibrosis, and bleomycin lung, where fibroproliferative matrix molecule deposition, enhanced collagen accumulation, apoptosis, and alveolar septal rupture with honeycombing are often juxtaposed and can lead to fatal consequences (1–4). Airway fibrosis is also an important contributor to the pathogenesis of airway disorders, such as bronchiolitis obliterans syndrome and asthma (5–9). Surprisingly, the mechanisms of tissue fibrosis in these important lung disorders are poorly understood.

The TGF- $\beta$  family proteins are multifunctional cytokines that play pivotal roles in diverse biologic processes, including the regulation of cell growth and survival, cell and tissue differentiation, development, inflammation, immunity, hematopoiesis, and tissue remodeling (10).

TGF- $\beta_1$  is essential for wound healing, stimulates matrix molecule deposition, and has been implicated in the pathogenesis of a variety of fibrotic disorders, including IPF, scleroderma, radiation-induced pulmonary fibrosis, and asthma (10–17). The important roles that TGF- $\beta_1$  might play in IPF can be seen in human studies that demonstrated that bioactive TGF- $\beta_1$  is expressed in an exaggerated fashion in lungs from patients with IPF (14, 15, 18, 19). They can also be seen in animal studies that demonstrated that TGF- $\beta_1$  is a critical mediator of bleomycin-induced pulmonary fibrosis (18, 20, 21) and that high dose adenoviral transfer of TGF- $\beta_1$  causes a progressive fibrotic response in the lung in vivo and an IPF-like disease with fibroblastic foci in an explant culture system (19). Surprisingly, the mechanisms that TGF- $\beta_1$  uses to mediate these tissue responses have not been fully defined.

The semaphorins (SEMAs) are a large family of phylogenetically conserved, secreted, and membrane-bound proteins that are divided into eight classes based on sequence similarities and distinct structural features (22, 23).

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A variety of studies have documented the ability of members of this family to act as axon guidance molecules, and SEMA abnormalities have been implicated in the pathogenesis of neurologic disorders, such as Alzheimer's disease and motor neuron degeneration (22, 23). SEMAs are also expressed on myeloid and lymphoid cells, including B cells, T cells, NK cells, and macrophages, and have been implicated in immune responses and the regulation of organogenesis, angiogenesis, apoptosis, and neoplasia (22–26). SEMA 7A, also called CDw108, was originally discovered based on sequence similarities with the vaccinia virus SEMA homologue A39R and is now known to be the homologue of several viral SEMAs (25, 27–29). It is unique amongst SEMAs in that it is stabilized via a glycosylphosphatidylinositol membrane linkage (22–24, 29). In addition, unlike many SEMAs, which act as repulsive axonal guidance clues, SEMA 7A enhances central and peripheral axonal growth and is required for proper axon track formation during embryonic development (22, 23). SEMA 7A may also play prominent roles in inflammation, immunity, and dental and osseous tissue responses based on its ability to stimulate macrophage chemotaxis and cytokine production and inhibit T cell function and its expression on odontoblasts, stimulation of osteoblast migration, and regulation of osteoclast fusion (24–26, 30). The effects of SEMA 7A are believed to be mediated via at least two receptors, plexin C<sub>1</sub> and the  $\beta$ 1 integrin subunit (8, 23, 25). However, the mechanisms by which SEMA 7A mediates its tissue effects are poorly understood. In particular, a relationship between SEMA 7A and TGF- $\beta$  family proteins has not been defined.

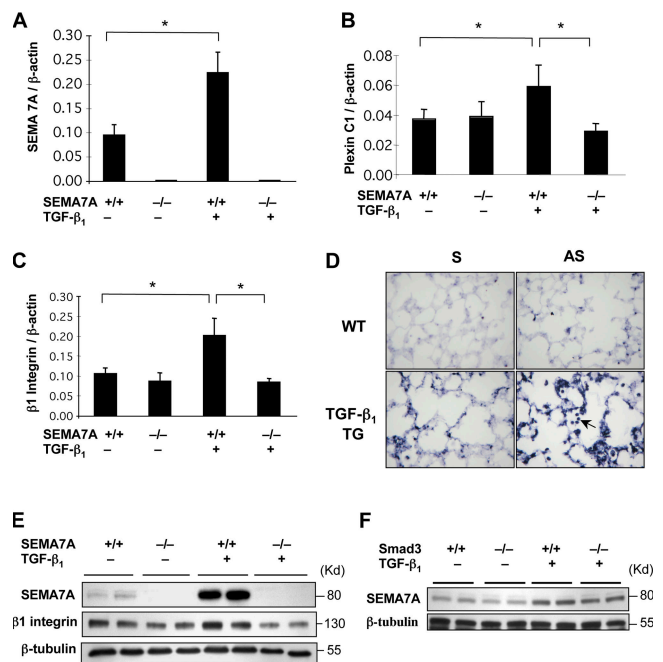
We hypothesized that SEMA 7A is regulated by TGF- $\beta$ <sub>1</sub> and plays a critical role in TGF- $\beta$ <sub>1</sub>-induced tissue fibrotic and remodeling responses. To test this hypothesis, we characterized the effects of transgenic TGF- $\beta$ <sub>1</sub> on the expression of SEMA 7A and its receptors in the murine lung. We also characterized the effector responses induced by transgenic TGF- $\beta$ <sub>1</sub> in mice with WT and null SEMA 7A loci. These studies demonstrate that TGF- $\beta$ <sub>1</sub> is a potent stimulator of SEMA 7A and plexin C<sub>1</sub> and the  $\beta$ 1 integrin subunit. They also demonstrate that SEMA 7A plays a key role in the pathogenesis of TGF- $\beta$ <sub>1</sub>-induced fibrosis and alveolar remodeling, and that TGF- $\beta$ <sub>1</sub> regulates the expression of SEMA 7A receptors, extracellular matrix (ECM) proteins, proteases, antiproteases, transcription factors, fibrogenetic cytokines, apoptosis regulators, and IL-13 receptors via Smad 2/3-independent, SEMA 7A-dependent activation pathways.

## RESULTS

### TGF- $\beta$ <sub>1</sub> regulation of the SEMA 7A system

To address the possibility that SEMA 7A contributes to TGF- $\beta$ <sub>1</sub>-induced responses in the murine lung, studies were first undertaken to determine if the expression of SEMA 7A or its putative receptors were regulated by transgenic TGF- $\beta$ <sub>1</sub>. This was done by comparing the levels of SEMA 7A and plexin C<sub>1</sub> and  $\beta$ 1 integrins in the lungs from transgene (Tg) negative (–) and positive (+) mice at various times after Tg

activation. These studies demonstrate that TGF- $\beta$ <sub>1</sub> is a potent stimulator of SEMA 7A and plexin C<sub>1</sub> and  $\beta$ 1 integrin mRNA accumulation in the lungs from doxycycline (Dox)-treated Tg mice (Fig. 1, A–C). In situ hybridization also highlighted the presence of SEMA 7A mRNA in a variety of cells, including macrophages, epithelial cells, and cells in fibrotic foci (Fig. 1 D and not depicted). The induction of SEMA 7A and its receptors was seen after as little as 2 d of Dox administration and persisted throughout the 28-d study interval (Fig. 1 and not depicted). It was associated with comparable levels of induction of SEMA 7A and  $\beta$ 1 integrin protein accumulation in lung lysates but not in bronchoalveolar lavage (BAL) fluids (Fig. 1 E and not depicted). Interestingly, the induction of plexin C<sub>1</sub> and  $\beta$ 1 integrins was SEMA 7A dependent because TGF- $\beta$ <sub>1</sub> did not induce either SEMA 7A receptor in SEMA 7A null mice (Fig. 1, B and C). This induction of SEMA 7A was also largely independent of Smad 3 signaling because similar levels of SEMA 7A induction were seen in TGF- $\beta$ <sub>1</sub> Tg<sup>+</sup> mice with WT and null Smad 3 loci (Fig. 1 F). These studies demonstrate that TGF- $\beta$ <sub>1</sub> is a potent stimulator of SEMA 7A and its receptors in the murine lung. They also demonstrate that SEMA 7A is induced



**Figure 1. Regulation of the SEMA 7A and its receptors by TGF- $\beta$ <sub>1</sub>.** Tg<sup>–</sup> and Tg<sup>+</sup> mice with WT<sup>+/+</sup> and null<sup>–/–</sup> SEMA 7A loci and (+/+) and (–/–) Smad 3 loci were generated and evaluated after 2 wk of Tg activation. The levels of mRNA encoding SEMA 7A (A), plexin-C<sub>1</sub> (B), and  $\beta$ 1 integrins (C) were evaluated using real-time RT-PCR. In situ hybridization with sense (S) and antisense (AS) probes was used to localize SEMA 7A mRNA (D). The arrow highlights a positively stained macrophage after 2 wk of Tg activation. Western evaluations of lung lysates were used to evaluate the level of SEMA 7A and  $\beta$ 1 integrin proteins (E and F). The values in A–C represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. \**P* < 0.05. D–F are representative of a minimum of four similar evaluations.

by TGF- $\beta_1$  via a largely Smad 3-independent mechanism and plays a critical role in the induction of its own receptors in this setting.

### Role of SEMA 7A in TGF- $\beta_1$ -induced fibrosis

To determine if the fibrotic effects of TGF- $\beta_1$  were altered in the absence of SEMA 7A, we used biochemical (Sircol) and histologic approaches to quantitate the collagen in the lungs from Tg mice with WT and null SEMA 7A loci. In accord with previous studies from our laboratory (31), transgenic TGF- $\beta_1$  caused parenchymal and alveolar fibrosis as well as a significant increase in lung collagen content in mice that expressed SEMA 7A normally ( $P < 0.001$ ; Fig. 2, A and B). Interestingly, the airway and parenchymal fibrotic responses were diminished in the absence of SEMA 7A. After 14 d of Dox water administration, TGF- $\beta_1$ -induced collagen accumulation was decreased by  $80.1 \pm 8.5\%$  ( $P < 0.001$ ) in SEMA 7A null versus WT animals (Fig. 2 B). These studies demonstrate that SEMA 7A plays an important role in the pathogenesis of TGF- $\beta_1$ -induced pulmonary fibrosis in the murine lung.

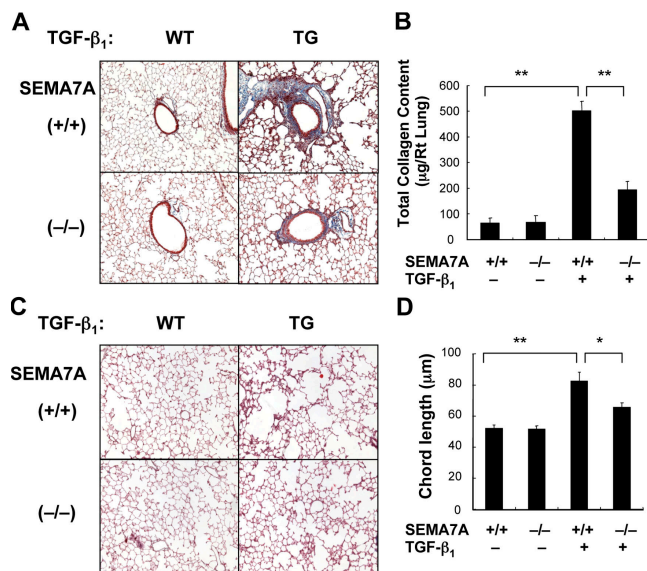
### Role of SEMA 7A in alveolar remodeling

In addition to inducing tissue fibrosis, TGF- $\beta_1$  induces alveolar remodeling with septal destruction and an increase in alveolar chord length (31). To define the role(s) of SEMA 7A

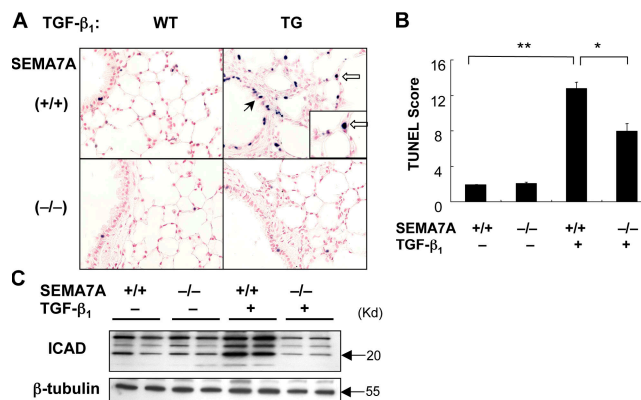
in these responses, we compared the alveoli from Tg mice with WT and null SEMA 7A loci. In accord with our prior report (31), an increase in lung destruction was readily apparent in Tg mice that produced SEMA 7A normally (Fig. 2, C and D). Interestingly, null mutations of SEMA 7A caused a significant decrease in alveolar remodeling that was apparent in histologic and morphometric evaluations (Fig. 2, C and D). Overall, a null mutation of SEMA 7A caused a  $72.3 \pm 8.6\%$  decrease in the TGF- $\beta_1$ -induced increase in alveolar chord length in mice on Dox water for 2 wk ( $P < 0.05$ ). Thus, SEMA 7A plays an important role in the pathogenesis of TGF- $\beta_1$ -induced alveolar remodeling in the murine lung.

### Role of SEMA 7A in TGF- $\beta_1$ -induced DNA injury and cell death

Previous studies from our laboratory demonstrated that TGF- $\beta_1$  induces epithelial apoptosis and that this apoptotic response is a critical precursor of TGF- $\beta_1$ -induced fibrosis (31). Thus, studies were undertaken to test the hypothesis that SEMA 7A plays an important role in TGF- $\beta_1$ -induced apoptosis. This was done using TdT-mediated dUTP nick-end labeling (TUNEL) stains to compare the TGF- $\beta_1$ -induced DNA injury and cell death in transgenic mice with WT and null SEMA 7A loci. Transgenic TGF- $\beta_1$  caused an impressive increase in TUNEL staining in mice with WT SEMA 7A loci (Fig. 3, A and B). These TUNEL<sup>+</sup> cells were largely epithelial cells as indicated by their histologic location and morphology (not depicted). This response was readily appreciated after 2 d of Dox administration and decreased with longer periods of Tg activation (Fig. 3, A and B, and not depicted). At these time points, SEMA 7A appeared to play an important role in this response because the TUNEL staining



**Figure 2. Roles of SEMA 7A in TGF- $\beta_1$ -induced fibrosis and alveolar remodeling.** Tg<sup>-</sup> and Tg<sup>+</sup> mice with WT<sup>+/+</sup> and null<sup>-/-</sup> SEMA 7A loci were generated and evaluated after 2 wk of Tg activation. The ability of TGF- $\beta_1$  to induce tissue fibrosis was assessed with trichrome histology evaluations (A) and Sircol collagen assays (B). The ability of TGF- $\beta_1$  to induce alveolar remodeling was assessed with hematoxylin and eosin histology evaluations (C) and morphometric chord length assessments (D). The panels in A and C are representative of at least five similar experiments. The values in B and D represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ .

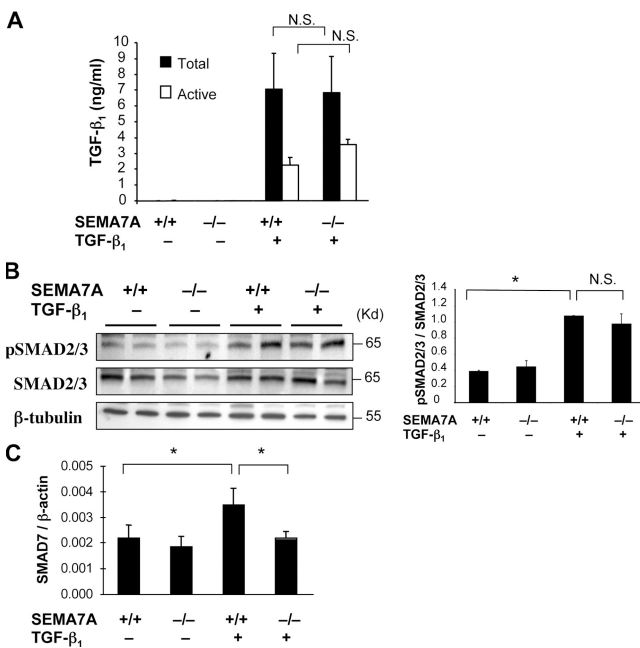


**Figure 3. Roles of SEMA 7A in TGF- $\beta_1$ -induced DNA injury and cell death.** Tg<sup>-</sup> and Tg<sup>+</sup> mice with WT<sup>+/+</sup> and null<sup>-/-</sup> SEMA 7A loci were generated and evaluated after 48 h of Tg activation. The ability of transgenic TGF- $\beta_1$  to induce DNA injury and cell death was assessed with TUNEL evaluations (A and B). In A, the solid and open arrows highlight representative TUNEL staining alveolar epithelial and alveolar type II cells, respectively. Caspase-mediated ICAD cleavage is illustrated in C. A and C are representative of at least five similar experiments. The values in B represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ .

was decreased in lungs from  $Tg^+$  mice with null mutations of SEMA 7A (Fig. 3, A and B). This was readily appreciated after 48 h of Dox administration, where TGF- $\beta_1$ -induced TUNEL staining was decreased by  $43.3 \pm 3.5\%$  compared with Tg mice with WT SEMA 7A loci ( $P < 0.05$ ; Fig. 3 B). This inhibition was associated with a significant decrease in caspase-mediated inhibitor of caspase-activated DNase (ICAD) cleavage (Fig. 3 C). Thus, these studies demonstrate that SEMA 7A is an important contributor to TGF- $\beta_1$ -induced DNA injury and cell death in the murine lung.

**Roles of SEMA 7A in TGF- $\beta_1$ -induced inflammation**

To determine if SEMA 7A contributed to TGF- $\beta_1$ -induced tissue inflammation, we compared the cellularity of BAL fluids and tissues from  $Tg^+$  mice with WT and null SEMA 7A loci. Similar numbers and types of cells were recovered in BAL fluids and tissues from  $Tg^-$  mice regardless of their SEMA 7A genotype (not depicted). In accord with prior studies from our laboratory (31), transgenic TGF- $\beta_1$  augmented BAL and tissue cellularity by increasing macrophage and, to a lesser extent, lymphocyte and eosinophil accumulation (not depicted). Importantly, the absence of SEMA 7A did not alter the magnitude or differential of these responses (not depicted). Thus, these studies demonstrate that SEMA

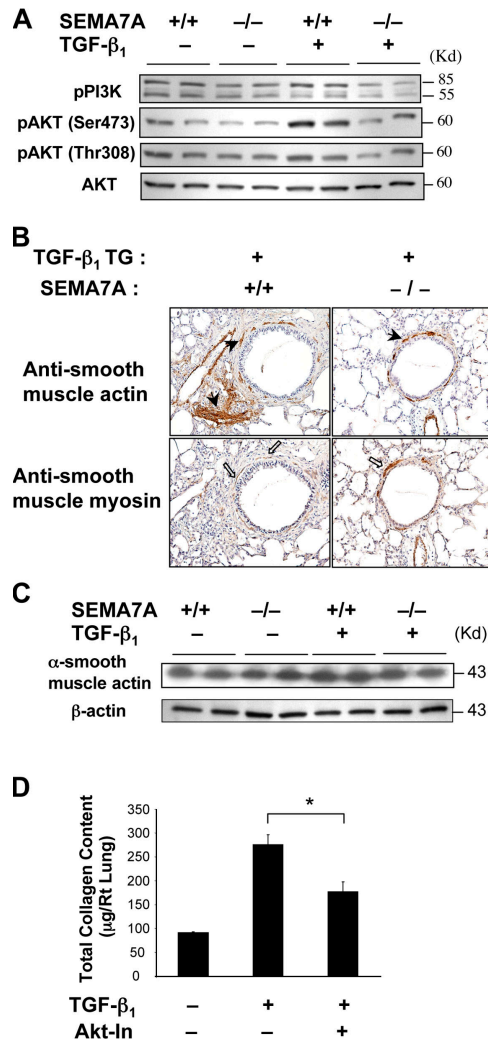


**Figure 4. Roles of SEMA 7A in the regulation of TGF- $\beta_1$  and Smads.**  $Tg^-$  and  $Tg^+$  mice with WT<sup>+/+</sup> and null<sup>-/-</sup> SEMA 7A loci were generated and evaluated after 2 wk of Tg activation. The levels of total (■) and activated (□) TGF- $\beta_1$  in BAL fluids from these mice were evaluated by ELISA (A), and the levels of total and phosphorylated Smads 2 and 3 were evaluated by Western analysis and evaluated with densitometry (B). Real-time RT-PCR was also used to quantitate the levels of mRNA encoding Smad 7 (C). The values in A and C represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. B is representative of four similar evaluations. N.S., not significant; \*,  $P < 0.01$ .

7A is not a major regulator of TGF- $\beta_1$ -induced inflammation in the murine lung.

**SEMA 7A regulation of transgenic TGF- $\beta_1$  and Smads**

The decreased ability of TGF- $\beta_1$  to induce tissue responses in the absence of SEMA 7A could be due to a decrease in the production of transgenic TGF- $\beta_1$  or a decrease in its ability to activate its effector pathways. To differentiate amongst these options, we compared the levels of total and active TGF- $\beta_1$  in the lungs from  $Tg^-$  and  $Tg^+$  mice with WT and null SEMA 7A loci.

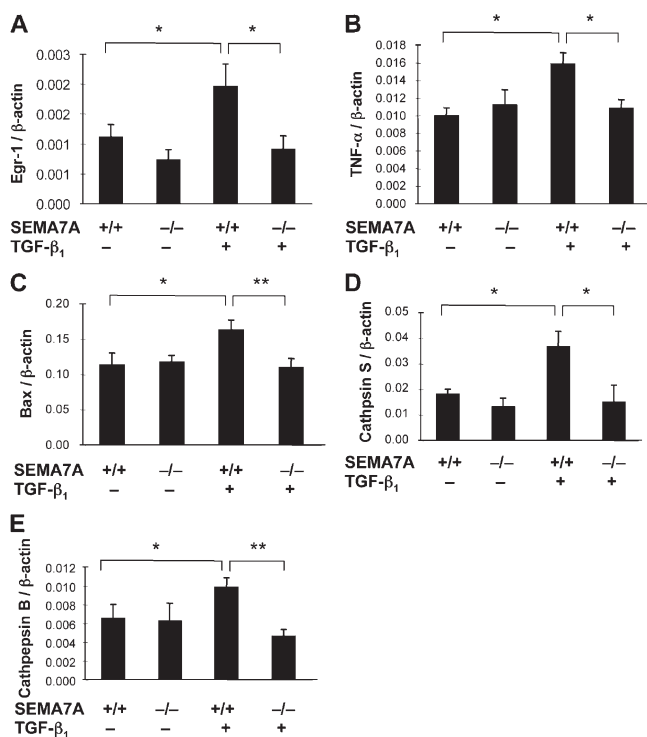


**Figure 5. SEMA 7A, PI3K, PKB/AKT, and myofibroblast accumulation.**  $Tg^-$  and  $Tg^+$  mice with WT and null SEMA 7A loci were generated and evaluated after 2 wk of Tg activation. The levels of phosphorylation of PI3K and PKB/AKT were evaluated using Western blots with antibodies against the two forms of pAKT and PI3K (A). Myofibroblast accumulation was assessed using IHC (B) and Western evaluations (C) with antibodies against  $\alpha$ -smooth muscle actin (solid arrows) and myosin (open arrows). Sircol assays were used to evaluate the effects of PKB/AKT inhibition (Akt-In) on collagen accumulation after 10 d of Dox incubation (D). A–C are representative of five similar evaluations. The values in D represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. \*,  $P < 0.01$ .



As can be seen in Fig. 4 A, these effects appeared to be due, in great extent, to an alteration(s) in TGF- $\beta_1$  effector pathway activation because the levels of total and bioactive TGF- $\beta_1$  in BAL from Tg<sup>+</sup> mice with null SEMA 7A loci were comparable or greater than those in fluids from Tg<sup>+</sup> mice that make SEMA 7A normally.

To address the mechanism by which SEMA 7A contributed to TGF- $\beta_1$  effector responses, we started by determining if a deficiency of SEMA 7A altered the ability of TGF- $\beta_1$  to induce or activate Smads. These were done by comparing the Smads in Tg<sup>+</sup> mice with WT and null SEMA 7A loci. Because Smads 2 and 3 mediate and Smad 7 inhibits TGF- $\beta$  effector pathway activation (10, 20), all three were evaluated. The levels and phosphorylation status of Smads 2 and 3 were similar in the lungs from Tg<sup>-</sup> mice with WT and null SEMA 7A loci (Fig. 4 B). In accord with reports in the literature (10), Smad 2/3 phosphorylation was increased in TGF- $\beta_1$  Tg<sup>+</sup> mice on Dox water (Fig. 4 B). Interestingly, the levels of phosphorylation of Smads 2 and 3 were similar in mice with WT and null SEMA 7A loci (Fig. 4 B). Transgenic TGF- $\beta_1$  also increased the levels of mRNA encoding of Smad 7 in mice with WT SEMA 7A loci (Fig. 4 C). This induction, however, was significantly decreased in Tg<sup>+</sup> mice with null SEMA 7A loci (Fig. 4 C). When viewed in combination,

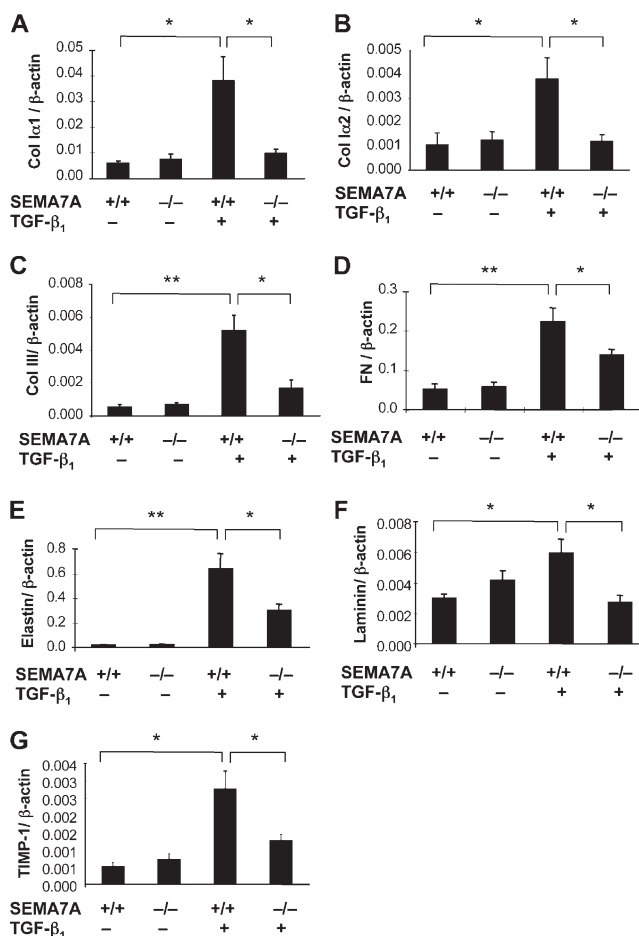


**Figure 6. Roles of SEMA 7A in the regulation of apoptosis regulators.** Tg<sup>-</sup> and Tg<sup>+</sup> mice with WT<sup>+/+</sup> and null<sup>-/-</sup> SEMA 7A loci were generated and evaluated after 2 d of Tg activation. Real-time RT-PCR was used to quantitate the levels of mRNA encoding Egr-1 (A), TNF- $\alpha$  (B), Bax (C), cathepsin S (D), and cathepsin B (E). The values represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. \*, P < 0.05; \*\*, P < 0.01.

these studies demonstrate that the defect in TGF- $\beta_1$  effector activation noted in SEMA 7A null mice was not associated with a decrease in Smad 2/3 phosphorylation or an increase in the expression of Smad 7.

### SEMA 7A, phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB)/AKT, and myofibroblasts

An evolving body of data has implicated a pathway that involves integrin activation of PI3K and its downstream target PKB/AKT in the pathogenesis of myofibroblast hyperplasia and tissue fibrosis (32–35). To gain additional insight into the contributions of SEMA 7A, we compared the activation of PI3K and PKB/AKT and the accumulation of myofibroblasts in Tg<sup>+</sup> mice with WT and null SEMA 7A loci. These studies demonstrated that TGF- $\beta_1$  is a potent stimulator of PI3K phosphorylation, PKB/AKT phosphorylation at amino acids 473 and 308, and  $\alpha$ -smooth muscle actin containing



**Figure 7. Roles of SEMA 7A in the regulation of ECM proteins and antiprotease.** Tg<sup>-</sup> and Tg<sup>+</sup> mice with WT<sup>+/+</sup> and null<sup>-/-</sup> SEMA 7A loci were generated and evaluated after 2 wk of Tg activation. Real-time RT-PCR was used to quantitate the levels of mRNA encoding  $\alpha$ 1(I) collagen (A),  $\alpha$ 2(I) collagen (B), type III collagen (C), fibronectin (FN) (D), elastin (E), laminin (F), and TIMP-1 (G). The values represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. \*, P < 0.05; \*\*, P < 0.01.

myofibroblast accumulation (Fig. 5). In all cases, these events were SEMA 7A dependent, with PI3K and PKB/AKT activation and myofibroblast accumulation being significantly decreased in SEMA 7A-deficient mice (Fig. 5, A–C).  $\alpha$ -smooth muscle myosin containing myocytes were not similarly altered (Fig. 5 B). Importantly, TGF- $\beta_1$ -induced tissue fibrosis was also significantly decreased in mice treated with the PKB/AKT inhibitor (Fig. 5 D) (34, 36). These studies highlight a TGF- $\beta_1$ -activated, SEMA 7A-dependent pathway that involves PI3K and AKT and regulates myofibroblast hyperplasia and tissue fibrosis.

### SEMA 7A and TGF- $\beta_1$ -induced apoptosis

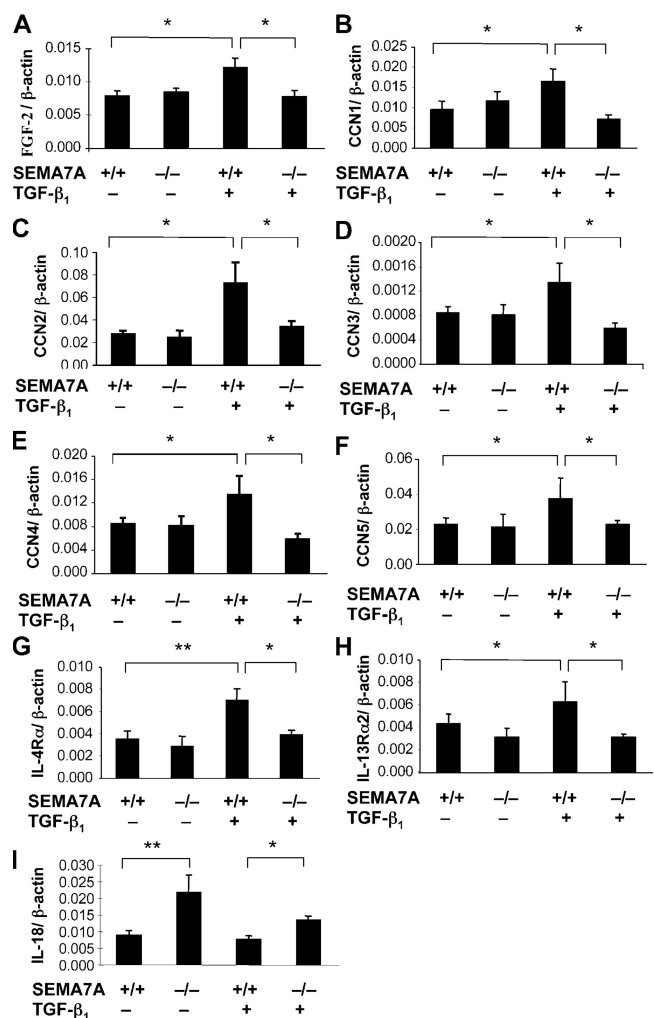
Previous studies from our laboratory demonstrated that TGF- $\beta_1$  induces lung epithelial cell apoptosis via an early growth response protein (Egr)-1-dependent mechanism that involves multiple cell death pathways (31). Thus, to address the mechanisms by which SEMA 7A might contribute to TGF- $\beta_1$ -induced apoptosis, the ability of TGF- $\beta_1$  to regulate Egr-1, the mitochondrial cell death pathway activator Bax, and death receptor cell death pathway activator TNF were evaluated. TGF- $\beta_1$  was a potent stimulator of the levels of mRNA encoding all three moieties (Fig. 6, A–C). Cathepsins S and B, which have also been implicated in lung epithelial cell apoptosis (37), were similarly stimulated (Fig. 6, D and E). These responses were mediated, at least in part, by SEMA 7A because null mutations of SEMA 7A decreased the ability of TGF- $\beta_1$  to stimulate Egr-1, Bax, TNF, and the cathepsins (Fig. 6). When viewed in combination, these studies demonstrate that SEMA 7A plays an important role in TGF- $\beta_1$  stimulation of Egr-1 and the activation of the death receptor, mitochondrial, and cathepsin-mediated cell death pathways.

### SEMA 7A and the mechanisms of pulmonary fibrosis and alveolar remodeling

Studies were next undertaken to compare the regulation of collagens and selected proteases and antiproteases in the lungs from TGF- $\beta_1$  Tg mice with WT and null SEMA 7A loci. These studies demonstrated that TGF- $\beta_1$  is a potent stimulator of the accumulation of mRNA encoding  $\alpha 1(I)$ ,  $\alpha 2(I)$ , and type III collagens, fibronectin, elastin, and laminin (Fig. 7, A–F). Transgenic TGF- $\beta_1$  also stimulated the expression of mRNA encoding tissue inhibitor of metalloproteinase (TIMP)-1 (Fig. 7 G). In all cases, SEMA 7A played an essential role in these inductive events because the ability of TGF- $\beta_1$  to regulate the accumulation of mRNA encoding these moieties was significantly decreased in mice with null mutations of SEMA 7A (Fig. 7).

Studies were also undertaken to determine if the ability of TGF- $\beta_1$  to regulate other known fibroregulatory cytokines and their receptors was altered in the absence of SEMA 7A. Transgenic TGF- $\beta_1$  caused significant increases in the levels of mRNA encoding fibroblast growth factor (FGF)-2 (Fig. 8 A); the CCN growth factor family proteins CCN1 (Cyr-61), CCN2 (connective tissue growth factor; CTGF), CCN3 (NOV), CCN4 (WISP-1), and CCN5 (WISP-2) (Fig. 8, B–F);

and the IL-4 and IL-13 receptor components IL-4R $\alpha$  and IL-13R $\alpha 2$  (Fig. 8, G and H). In accord with our findings with the ECM proteins, these stimulatory effects were also SEMA 7A-dependent because they were all ameliorated in Tg<sup>+</sup> mice with null SEMA 7A loci (Fig. 8, A–H). In contrast, IL-18, which inhibits tissue fibrosis (38), was inhibited by SEMA 7A in Tg<sup>-</sup> and Tg<sup>+</sup> mice (Fig. 8 I). In all cases, comparable alterations in protein accumulation were noted (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20061273/DC1>). These studies demonstrate that SEMA 7A plays a critical role in the ability of TGF- $\beta_1$  to augment the accumulation of mRNA encoding collagens and other ECM proteins, anti-proteases, fibrostimulatory cytokines, and IL-13 receptors while inhibiting the expression of IL-18.



**Figure 8. Roles of SEMA 7A in the regulation of fibroregulatory cytokines and IL-13 receptor components.** Tg<sup>-</sup> and Tg<sup>+</sup> mice with WT<sup>+/+</sup> and null<sup>-/-</sup> SEMA 7A loci were generated and evaluated after 2 wk of Tg activation. Real-time RT-PCR was used to quantitate the levels of mRNA encoding FGF-2 (A), CCN1 (B), CCN2 (C), CCN3 (D), CCN4 (E), CCN5 (F), IL-4R $\alpha$  (G), IL-13R $\alpha 2$  (H), and IL-18 (I). The values represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ .

### SEMA 7A in bleomycin-induced pulmonary fibrosis

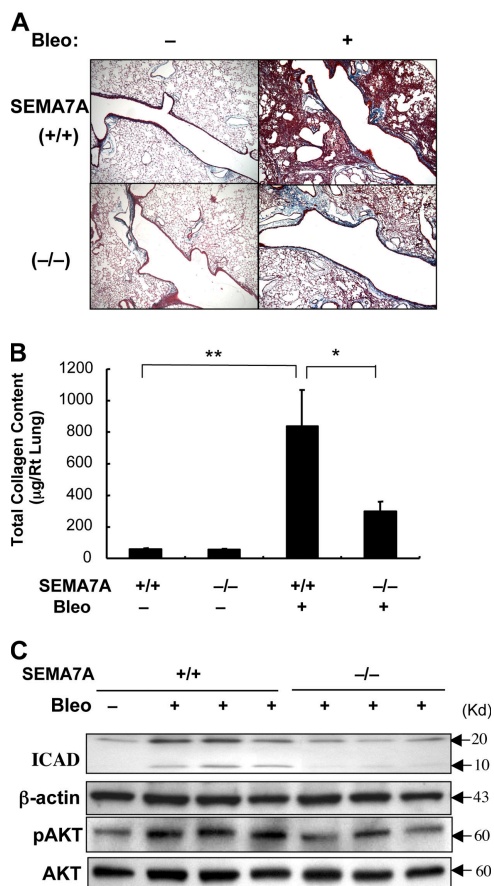
Studies were also undertaken to determine if the biology of SEMA 7A that was defined using our Tg modeling system was also relevant to fibrotic responses that were induced by other stimuli. Bleomycin was chosen because TGF- $\beta_1$  is known to play an important role in the pathogenesis of the fibrosis it induces (18, 20, 21). As seen in Fig. 9, bleomycin caused a significant increase in pulmonary fibrosis and collagen accumulation that was most impressive 3 wk after intratracheal administration. The response was associated with significant levels of inflammation, epithelial apoptosis, ICAD cleavage (Fig. 9 C and not depicted), and PKB/AKT phosphorylation (Fig. 9 C). In keeping with the results noted in our TGF- $\beta_1$  Tg mice, bleomycin-induced epithelial apoptosis, ICAD cleavage, PKB/AKT activation, and tissue fibrosis were significantly diminished and inflammation was not altered in SEMA 7A null animals (Fig. 9, A–C, and not depicted). At the 3-wk time point, SEMA 7A deficiency

decreased the bleomycin-induced increase in pulmonary collagen accumulation by  $90.3 \pm 7\%$  ( $P < 0.001$ ). Thus, SEMA 7A plays an important role in the pathogenesis of the apoptosis, signaling, and fibrotic responses induced by bleomycin as well as transgenic TGF- $\beta_1$ .

### DISCUSSION

To gain insight into the pathogenesis of wound healing, tissue fibrosis, and tissue remodeling, the cellular and molecular events that TGF- $\beta_1$  uses to induce these responses need to be understood. In these studies, we tested the hypothesis that SEMA 7A is induced by and plays a critical role in the pathogenesis of TGF- $\beta_1$ -induced fibrotic and remodeling responses in the lung. These studies highlight previously unrecognized relationships between SEMA 7A and TGF- $\beta_1$ . Specifically, they demonstrate that SEMA 7A and its receptors, plexin C1 and  $\beta 1$  integrins, are stimulated by TGF- $\beta_1$ . They also demonstrate that SEMA 7A plays a critical role in TGF- $\beta_1$ -induced fibrosis, myofibroblast hyperplasia, alveolar remodeling, and apoptosis, but does not contribute to TGF- $\beta_1$ -induced inflammation. Insights were also obtained from studies that demonstrate that TGF- $\beta_1$  stimulates SEMA 7A via a Smad 3-independent mechanism and enhances the expression of ECM proteins, CCN proteins, FGF-2, IL-13 receptors, proteases, antiproteases, transcription factors, and apoptosis using a Smad 2/3-independent and SEMA 7A-dependent mechanism(s). TGF- $\beta_1$  also activated PI3K and PKB/AKT via a SEMA 7A-dependent mechanism, and PKB/AKT inhibition decreased TGF- $\beta_1$ -induced fibrosis. When viewed in combination, these observations demonstrate that SEMA 7A participates in a Smad 2/3-independent pathway that involves PI3K and PKB/AKT and plays a central role in the regulation of the fibrogenic and remodeling effector responses induced by TGF- $\beta_1$ . Because the fibrogenic effects of bleomycin were also ameliorated in SEMA 7A null mice, they also demonstrate that the effects of SEMA 7A are not specific for transgenic TGF- $\beta_1$ , thereby highlighting the importance of our findings to other fibrotic stimuli.

SEMA 7A plays a central role in axonal guidance, and emerging evidence points to diverse functions of a variety of SEMAs (including SEMA 7A) in the immune system. These observations led to the present contention that the nervous and immune systems share SEMA-containing regulatory pathways (22–24). Our studies demonstrate, for the first time, that SEMA 7A is a critical regulator of TGF- $\beta_1$ - and bleomycin-induced tissue fibrosis and remodeling. This contention, that SEMA 7A is an important regulator of tissue remodeling, is in accord with genetic studies highlighting the association of SEMA 7A polymorphisms and abnormal bone mineral density and vertebral fractures in Korean women (39), as well as studies highlighting the expression of SEMA on osteoblasts and the ability of SEMA 7A to regulate osteoblast migration and osteoclast fusion (25). Thus, these studies demonstrate that SEMA 7A-containing pathways, although contributing to neural and immune homeostasis, also contribute to the regulation of tissue fibrosis and remodeling.



**Figure 9. Role of SEMA 7A in bleomycin-induced fibrosis.** Mice with WT<sup>+/+</sup> and null<sup>-/-</sup> SEMA 7A loci were generated, given intratracheal bleomycin or its vehicle control, and evaluated at intervals thereafter. At the 3-wk time point, fibrosis was assessed with tissue trichrome stains (A) and Sircol collagen assays (B). ICAD cleavage and PKB/AKT phosphorylation were evaluated 1 wk after bleomycin administration (C). A and C are representative of five similar experiments. The values in B represent the mean  $\pm$  SEM of evaluations in a minimum of five mice. \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ .

The TGF- $\beta$  isoforms (TGF- $\beta_1$ , TGF- $\beta_2$ , and TGF- $\beta_3$ ) are synthesized as latent precursors that are complexed with latent TGF- $\beta$ -binding proteins. They are activated by a variety of mechanisms that includes proteolytic cleavage and integrin binding (10, 40). Once activated, they bind to heterodimeric receptors containing type I and type II receptor components, both of which contain tyrosine kinase activity (10). The presentation of TGF- $\beta_1$  to the type II receptor may also be assisted by accessory proteins, including  $\beta$  glycan and endoglin (10). In most cases, TGF- $\beta_1$  signals via Smads, with Smads 2 and 3 being phosphorylated by TGF- $\beta$ R1 kinase, binding to Smad 4, and translocating to the nucleus (10). Smad 7, on the other hand, inhibits Smad 2/3 phosphorylation and Smad 2/3/4 heterocomplex nuclear translocation (10, 20). Our studies demonstrate that mice lacking SEMA 7A have a striking defect in their ability to generate TGF- $\beta_1$ -induced lung fibrosis and alveolar remodeling. They also demonstrate that this deficiency is associated with and potentially mediated by the inability of TGF- $\beta_1$  to maximally stimulate collagen and other ECM proteins, fibrostimulatory CCN and other cytokines, antiproteases, transcription factors, and receptor components in the absence of SEMA 7A. Previous studies using in vitro culture systems demonstrated that Smad 3 is required for TGF- $\beta_1$  stimulation of CCN2 or type II collagen (41, 42). In contrast, our studies demonstrate that SEMA 7A contributes to the tissue effects of TGF- $\beta_1$  in a Smad 2/3-independent fashion. On superficial analysis, these observations would appear to conflict with one another. However, there is increasing evidence for Smad 2/3-independent pathways that mediate the effects of TGF- $\beta_1$ . One of the most intriguing pathways involves the activation of PI3K and subsequent activation of PKB/AKT (43). This pathway has been strongly associated with the regulation of cell survival and is felt to play a key role in the differentiation and survival of myofibroblasts at the site of pulmonary fibrosis (34). The PI3K pathway is also known to regulate other fibrosis-relevant remodeling responses, including cell proliferation, angiogenesis, and cell differentiation (44). Our studies demonstrate that TGF- $\beta_1$  induces myofibroblast hyperplasia and activates PI3K and PKB/AKT via a SEMA 7A-dependent mechanism(s). They also demonstrate that PKB/AKT inhibition diminishes TGF- $\beta_1$ -induced fibrosis.  $\beta_1$  integrins are known to participate in PI3K activation (34). This allows for the exciting hypothesis that SEMA 7A- $\beta_1$  integrin binding leads to PI3K activation and subsequent PKB/AKT phosphorylation, myofibroblast accumulation, and matrix molecule production. Additional investigation, however, will be required to more fully understand the nature and importance of this pathway.

Our studies demonstrate that the ability of TGF- $\beta_1$  to stimulate tissue fibrosis is markedly diminished in SEMA 7A null mice. They also highlight several events that can contribute to this response. It is easy to understand how the decreased levels of mRNA encoding ECM proteins and TIMP-1 that are seen in the absence of SEMA 7A could contribute to the inability of TGF- $\beta_1$  to induce fibrosis in

SEMA 7A null animals. Our studies also demonstrate that TGF- $\beta_1$  stimulates CCN1-5, FGF-2, and Egr-1 via SEMA 7A-dependent mechanisms. These are important observations because CCN2 (also called CTGF) and related moieties CCN1 (Cyr-61), CCN3 (NOV3), CCN4 (WISP-1), and CCN5 (WISP-2) are induced during healing and at sites of fibrosis where they play an essential role in the deposition of ECM proteins and are proposed to mediate the tissue effects of TGF- $\beta_1$  (45, 46). FGF-2 is also induced by TGF- $\beta_1$  and has been shown to interact with TGF- $\beta_1$  in the generation of tissue scarring (47-49). Similarly, Egr-1 is a zinc finger transcription factor that stimulates the production of platelet-derived growth factor, and FGF-2 and plays a critical role in TGF- $\beta_1$ -induced epithelial apoptosis (31, 50-52) (see below). Lastly, our studies demonstrate that SEMA 7A contributes to the inhibition of IL-18 production. These are interesting findings in light of the recent observation that IL-18 inhibits tissue fibrosis (38). When viewed in combination, these observations suggest that SEMA 7A is a central mediator of a variety of pathways involved in TGF- $\beta_1$  stimulation of tissue fibrosis, including the stimulation of ECM proteins, stimulation of antiproteases, expression of CCNs and FGF-2, induction of Egr-1, and inhibition of IL-18.

The "type 2 cytokine hypothesis of fibrosis" suggests that tissue scarring occurs in chronic inflammatory disorders when cytokine balance is shifted in a type 2 direction (53, 54). In keeping with this hypothesis, IL-13 is expressed in an exaggerated fashion in a variety of fibrotic diseases, including IPF, scleroderma, and asthma (5, 55-57). Studies from our laboratory and others have also demonstrated that IL-13 mediates its fibrogenic effects, in part, via its ability to induce and activate TGF- $\beta_1$  (58, 59). IL-13 signals via a multimeric receptor complex involving IL-13R $\alpha$ I and IL-4R $\alpha$ . IL-13 also binds to IL-13R $\alpha$ 2, which has, until recently, been believed to be a decoy receptor (60, 61). However, it has recently been proposed to participate in the induction of TGF- $\beta_1$  and the generation of tissue fibrosis in bleomycin-induced lung injury (58). Our studies demonstrate, for the first time, that the expression of IL-13R $\alpha$ 2 and IL-4R $\alpha$  is decreased in the lungs from TGF- $\beta_1$  Tg mice that lack SEMA 7A. These observations highlight another mechanism by which SEMA 7A can contribute to the regulation of tissue fibrotic responses. This mechanism might be particularly relevant to diseases such as asthma, scleroderma, and cirrhosis of the liver, in which IL-13 and TGF- $\beta_1$  are believed to play important roles.

Alveolar remodeling is a final common pathway that contributes to the pathogenesis of a variety of pulmonary diseases and disorders, including the honeycombing that is seen in advanced interstitial lung diseases and the alveolar destruction that is seen in pulmonary emphysema. As noted above, TGF- $\beta_1$  has been implicated in the pathogenesis of IPF and other interstitial lung diseases. Interestingly, TGF- $\beta_1$  has also been implicated in the pathogenesis of cigarette-induced emphysema and the emphysema in patients with Marfan's Syndrome (62-65). Previous studies from our laboratory demonstrated that TGF- $\beta_1$  induces airway remodeling via a mechanism



that involves epithelial apoptosis (31). These studies also demonstrated that Egr-1 plays a central role in this apoptosis response and that interventions that block this epithelial cell death response diminish TGF- $\beta_1$ -induced collagen accumulation (31). The present studies add to our knowledge of this pathway by demonstrating that TGF- $\beta_1$  induces Egr-1 via a SEMA 7A-dependent mechanism. They also demonstrate that TGF- $\beta_1$  uses a similar SEMA 7A-dependent pathway(s) to stimulate TIMP-1 and cathepsins. When viewed in combination, these observations suggest that SEMA 7A is a central regulator of TGF- $\beta_1$ -induced alveolar remodeling and that this regulation occurs at the level of epithelial apoptosis and local protease balance. They also suggest that the ability of SEMA 7A-based alterations to ameliorate TGF- $\beta_1$ -induced fibrosis can be attributed, at least in part, to the effects of these interventions on the TGF- $\beta_1$ -induced epithelial apoptosis response.

In summary, these studies demonstrate that TGF- $\beta_1$  is a potent stimulator of SEMA 7A and its putative receptors. They also highlight the critical roles that SEMA 7A and downstream PI3K and PKB/AKT play in TGF- $\beta_1$  induction of tissue fibrosis, remodeling, myofibroblast hyperplasia, and apoptosis, as well as the important roles that SEMA 7A-dependent pathways play in TGF- $\beta_1$  stimulation of SEMA 7A receptors, ECM proteins, proteases, antiproteases, transcription factors, fibrogenic and fibroregulatory cytokines, and key cytokine receptors in the murine lung. Lastly, they demonstrate that SEMA 7A mediates similar responses in bleomycin-induced fibrosis, thereby highlighting the potential importance of this pathway in other settings. TGF- $\beta_1$ -induced tissue fibrosis and remodeling contribute to the pathogenesis of a wide variety of pulmonary and extra-pulmonary diseases and disorders. These findings suggest that interventions that regulate SEMA 7A, its receptors, or its signaling pathways may be therapeutically useful in disorders characterized by TGF- $\beta_1$ -mediated fibrosis and remodeling. Additional investigations of the roles of SEMA 7A and the SEMA 7A pathway in the pathogenesis of fibrotic and remodeling disorders and the utility of SEMA 7A-based therapeutics in their treatment are warranted.

## MATERIALS AND METHODS

**Overexpression Tg and null mutant mice.** CC10-rTA-TGF- $\beta_1$  Tg mice were generated in our laboratory, bred onto a C57BL/6 background for >10 generations, and used in these studies. These mice use the Clara cell 10-kD protein (CC10) promoter to specifically target bioactive TGF- $\beta_1$  to the lung. The methods that were used to generate and characterize these mice were described previously (31).

Mice with null mutations of SEMA 7A that had been bred onto a C57BL/6 background for >10 generations were provided by A.L. Kolodkin (Johns Hopkins University, Baltimore, MD) (22, 23). Mice with null mutations of Smad 3 were obtained from the late A.B. Roberts (National Cancer Institute, Bethesda, MD). Before use, these mice were bred onto a C57BL/6 background. These mice were bred with the TGF- $\beta_1$  mice to obtain Tg mice with WT and null SEMA 7A or Smad 3 loci. Genotyping of TGF- $\beta_1$  Tg, SEMA 7A null, and Smad 3 null mice was accomplished according to the protocols established in our laboratory, the Kolodkin's laboratory, and the literature (22, 23, 31, 66). All animal experiments were approved by the

Yale School of Medicine Institutional Animal Care and Use Committee in accordance with federal guidelines.

**Dox water administration.** 6-wk-old Tg<sup>+</sup> mice and Tg<sup>-</sup> littermate controls were randomized to normal water or water containing 0.5 mg/ml Dox as described previously (31). Phenotypic alterations were evaluated at intervals thereafter.

**Quantification of lung collagen.** Animals were anesthetized, a median sternotomy was performed, and right heart perfusion was accomplished with calcium and magnesium-free PBS. The heart and lungs were then removed en bloc. The right lung was frozen in liquid nitrogen and stored at -80°C until used. Collagen content was determined by quantifying total soluble collagen using the Sircol Collagen Assay kit (Biocolor) according to the manufacturer's instructions. The data is expressed as the collagen content of the entire right lung.

**Histologic analysis.** The lungs were removed en bloc as described above, inflated at 25 cm pressure with PBS containing 0.5% low melting point agarose gel, fixed, embedded in paraffin, sectioned, and stained. Hematoxylin and eosin, and Mallory's trichrome stains were performed in the Research Histology Laboratory of the Department of Pathology at the Yale University School of Medicine.

**Morphometric analysis.** Alveolar remodeling was estimated from the mean chord length of the airspace as described previously by our laboratory (31, 67).

**BAL and lung inflammation.** Lung inflammation was assessed by BAL as described previously (31). The BAL samples from each animal were pooled and centrifuged. The number and type of cells in the cell pellet were determined with light microscopy.

**TUNEL evaluations.** End labeling of exposed 3'-OH ends of DNA fragments was undertaken with the TUNEL in situ cell death detection kit AP (Roche Diagnostics) as described by the manufacturer. After staining, 20 fields of alveoli were randomly chosen for examination. The labeled cells were expressed as a percentage of total nuclei.

**mRNA analysis.** mRNA levels were assessed using real-time RT-PCR assays as described by our laboratories (31, 68). In these assays, total cellular RNA from the lungs were obtained using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The primer sequences that were used that have not been previously reported by our laboratory can be found in Table S1, available at <http://www.jem.org/cgi/content/full/jem.20061273/DC1>.

**Quantification of TGF- $\beta_1$ .** The levels of BAL TGF- $\beta_1$  were determined by ELISA (R&D Systems) as per the manufacturer's instructions. These evaluations were performed before and after acid activation to assess the levels of activated and total TGF- $\beta_1$ , respectively.

**Immunoblot analysis.** Lung lysates were prepared and Western analysis was undertaken with antibodies that reacted selectively with ICAD (Chemicon International); SEMA 7A (AF1835; R&D Systems); Smads 2/3, phosphorylated Smad 2/3, Egr-1, PI3K, AKT, and phosphorylated AKT (Ser 473 and Thr 308; all from Cell Signaling Technology); and  $\beta_1$  integrins (N-20), cathepsin S (C-19), CCN2 (CTGF, L-20), FGF-2 (H-131), and  $\beta$ -actin (H-196; all from Santa Cruz Biotechnology, Inc.) as described previously (31, 67).

**In situ hybridization.** In situ hybridization was undertaken as described previously by our laboratory (59). The mouse SEMA 7A probe (DNA fragment corresponding to the sequence of mouse EST AA260340) was placed in a pBluescript II KS phagemid (Stratagene) between T7 and T3 promoter sites, and sense and antisense RNA probes were generated, labeled, and used.

**Immunohistochemistry (IHC).** IHC was undertaken to localize  $\alpha$ -smooth muscle actin (DakoCytomation), and  $\alpha$ -smooth muscle myosin heavy chain

(Biomedical Technology). These assays were undertaken as described previously by our laboratories (31, 67).

**Akt inhibition.** To define the role of PKB/AKT in TGF- $\beta_1$ -induced fibrosis, Akt inhibitor VII (15  $\mu\text{g}/\text{mouse}$ ; TAT-*Akt-In*; EMD Bioscience) (34, 36) and its vehicle control were used. They were given daily during the 10-d study interval via an i.p. route starting 1 d before Dox activation.

**Bleomycin administration.** Bleomycin (0.075 U/mouse) or vehicle control was administered to C57BL/6 female mice as described by Jiang et al. (69).

**Statistics.** Normally distributed data are expressed as means  $\pm$  SEM and assessed for significance by Student's *t* test or ANOVA as appropriate. Data that were not normally distributed were assessed for significance using the Wilcoxon rank sum test.

**Online supplemental material.** Fig. S1 is a representative Western blot illustrating accumulation of Egr-1, cathepsin S (Cat-S), CTGF, and FGF-2 in lysates from Tg<sup>-</sup> and Tg<sup>+</sup> mice with WT and null SEMA 7A loci. Table S1 lists the primer sequences used in the real-time RT-PCR reactions. The online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20061273/DC1>.

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