Overexpression of Sulf2 in idiopathic pulmonary fibrosis

Xinping Yue1,2, Jingning Lu2, Linda Auduong2, Mark D Sides3, and Joseph A Lasky3

1To whom correspondence should be addressed: Tel: +1-504-568-2024; Fax: +1-504-568-6158; e-mail: xyue@lsuhsc.edu

2Department of Physiology, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA and 3Section of Pulmonary Diseases, Critical Care and Environmental Medicine, Department of Medicine, Tulane University Health Sciences Center, New Orleans, LA 70112, USA

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Previously, we have shown that heparan sulfate (HS) 6-O-endosulfatase 1 (Sulf1) is a transforming growth factor-β1 (TGF-β1)-responsive gene in normal human lung fibroblasts and functions as a negative feedback regulator of TGF-β1 and that TGF-β1 induces the expression of Sulf1 as well as that of the closely related Sulf2 in a murine model of pulmonary fibrosis. In this study, we focused on the role of Sulf2 in modulating TGF-β1 function and the development of pulmonary fibrosis. We found that Sulf2 mRNA was overexpressed in lung samples from human patients with idiopathic pulmonary fibrosis (IPF), and Sulf2 protein was specifically localized to the hyperplastic type II alveolar epithelial cells (AECs). In vitro, TGF-β1 induced the expression of Sulf2 with accompanied HS 6-O-desulfation in A549 cells, adenocarcinoma cells derived from the type II alveolar epithelium. Using small interference RNA to block Sulf2 expression, we observed a biphasic TGF-β1 response with early enhanced Smad activation, but eventually reduced TGF-β1 target gene expression in Sulf2 knockout A549 cells compared with the control cells. To study the role of Sulf2 in normal type II AECs, we isolated primary type II cells from wild-type and Sulf2 knockout mice. We observed enhanced Smad activation as well as enhanced TGF-β1 target gene expression in Sulf2 knockout type II AECs compared with wild-type type II AECs. In conclusion, Sulf2 is overexpressed in IPF and may play a role in regulating TGF-β1 signaling in type II AECs.

Keywords: fibrosis / heparan sulfate / IPF / Sulf2 / TGF-β1

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease of unknown etiology and currently there are no therapies proved to affect its high mortality (Kim and Meyer 2008; Klingsberg et al. 2010). Pathologically, IPF is characterized by scarring at the periphery of secondary lobules, architectural distortion including honeycombing and temporal and spatial heterogeneity (Katzenstein et al. 2008). Fibroblastic foci and type II alveolar epithelial cell (AEC) hyperplasia are two important features of IPF. Fibroblastic foci represent areas of active fibrosis and are composed of activated myofibroblasts, synthesizing and depositing a collagen-rich matrix. Type II AEC hyperplasia represents the proliferative response of type II cells to injury. In the lung, type II AECs are believed to serve as progenitors for repair of the alveolar epithelium following injury, being capable of both self-renewal and giving rise to type I AECs (Adamson and Bowden 1974).

Whether the hyperplastic type II cells in IPF lungs are anti-fibrotic (as a population of AEC renewal) or pro-fibrotic (as producers of pro-fibrotic signals) is not clear.

Studies in both humans and animal models of pulmonary fibrosis have shown that transforming growth factor-β1 (TGF-β1) plays a pivotal role in the development and progression of the disease (Ask et al. 2006). TGF-β1 initiates and sustains fibroblast activation and differentiation into myofibroblasts, exemplified by the de novo expression of α-smooth muscle actin (SMA). Importantly, myofibroblasts are the primary cellular source of the extracellular matrix (ECM) production in the active fibrotic sites (Phan 2002). In type II AECs, TGF-β1 induces growth arrest and apoptosis, thus preventing epithelial repair necessary for the resolution of fibrosis (Selman and Pardo 2006). In addition, TGF-β1 induces the transdifferentiation of type II AECs to fibroblasts/myofibroblasts (Willis et al. 2006), a process known as epithelial-mesenchymal transition (EMT). Although the contribution of type II AEC EMT to the expansion of the fibroblast/myofibroblast population in pulmonary fibrosis is still a matter of debate (Kim et al. 2006; Rock et al. 2011), it is evident that EMT in type II cells would adversely affect epithelial integrity and regeneration following injury, contributing to the progression of fibrosis.

Heparan sulfate (HS) is the glycosaminoglycan side chains of HS proteoglycans (HSPGs). Through their HS chains, HSPGs interact and modulate the functions of a myriad of growth factors and morphogens, performing diverse functions including cell–cell, cell–matrix interactions, matrix assembly and tissue homeostasis (Bernfield et al. 1999; Perrimon and Bernfield 2001). TGF-β1 interacts strongly with heparin and highly sulfated HS (McCaffrey et al. 1992; Lyon et al. 1997). In cultured rat kidney fibroblasts, heparin and highly sulfated HS potentiate TGF-β1 function, and importantly, selective...
loss of N-, 2-O- or 6-O-sulfates leads to reduced TGF-β activity (Lyon et al. 1997).

Sulf1 and Sulf2 are HS specific, extracellular sulfatases that specifically remove 6-O-sulfates from HS intra-chain sites at the cell surface and the ECM, and have been shown to regulate the function of Wnt, fibroblast growth factors, stromal cell-derived factor-1 and bone morphogenetic proteins, among others (Lamanna et al. 2007). In a previous study (Yue et al. 2008), we have shown that Sulf1 is a TGF-β1-responsive gene in normal human lung fibroblasts and functions as a negative feedback regulator of TGF-β1 and that TGF-β1 induces the expression of Sulf1 as well as that of Sulf2 in a murine model of pulmonary fibrosis. In this study, we report the overexpression of Sulf2 in the hyperplastic type II AECs in human IPF lungs, and we provide in vitro evidence that Sulf2 regulates TGF-β1 signaling in type II AECs.

**Results**

**Sulf2 is overexpressed in the hyperplastic type II AECs in IPF**

Control and IPF lung samples were obtained from the Lung Tissue Research Consortium (LTRC). The IPF group (13 patients) consisted of 1 female (62 years of age) and 12 males (53–69 years of age). Lung samples from mild chronic obstructive pulmonary disease (COPD) patients with normal lung function (forced expiratory volume in one second, >80% predicted) were used as the control group because the LTRC has very few specimens that are truly normal, and samples from this group had a relatively normal histology and no features of IPF (based on examination of H&E stained sections, data not shown). The control group (8 patients) consisted of 2 females (78 and 87 years of age) and 6 males (57–78 years of age).

Examination of Sulf2 mRNA expression by quantitative real-time PCR (qRT-PCR) revealed a 2.2-fold increase of Sulf2 mRNA in lung homogenates from the IPF samples compared with the controls (Figure 1A). To examine which cell types were expressing Sulf2, we performed immunostaining for Sulf2 on control and IPF samples, using a rabbit polyclonal antibody against Sulf2 (H2.3) (MORIMOTO-TOMITA et al. 2005). Representative images from three control and five IPF lungs are shown in Figure 1B. Our results showed that Sulf2 was specifically expressed by the hyperplastic type II AECs in the IPF lungs. These included type II cells in areas where alveolar septal thickening was observed (Figure 1Be) as well as in areas where normal alveolar structures were entirely obliterated (Figure 1Bh). Type II AECs in control samples (Figure 1Bb) as well as normal areas in the IPF samples (not shown) had little or no detectable expression of Sulf2. A summary of the lung samples and Sulf2 staining pattern has been provided in Supplementary data, Table S1. The identity of the type II AECs was confirmed by immunostaining for the type II cell marker, pro-surfactant protein C (pro-SPC), on consecutive sections (Figure 1Ba, d and g). No positive staining was detected with nonimmune rabbit IgG at the same (Figure 1Bc, 1 µg/mL) or five times the concentration of the Sulf2 antibody (Figure 1Bf and i, 5 µg/mL).

**TGF-β1 induces Sulf2 expression in A549 cells**

A549 cells are adenocarcinoma cells derived from the type II alveolar epithelium and are commonly used as a type II AEC cell line in vitro (Ramos et al. 2010; Buckley et al. 2011). In contrast to normal human lung fibroblasts, which express both Sulf1 and Sulf2 (Yue et al. 2008), A549 cells mainly express Sulf2 (data not shown). Treatment with TGF-β1 augmented both Sulf2 mRNA (Figure 2A) and protein (Figure 2B) expression. TGF-β1 induced an ~5-fold increase in Sulf2 mRNA expression at 24 h at concentrations of both 1 and 5 ng/mL, and this level of expression was maintained at 5 ng/mL of TGF-β1 at 48 h (Figure 2A). At protein level (Figure 2B), 5 ng/mL of TGF-β1 also induced a greater level of Sulf2 protein expression. Both the unprocessed pro-protein (~125 kDa) and the amino-terminal subunit of Sulf2 (~75 kDa) were present in the total cell extracts, consistent with the previous report (Tang and Rosen 2009). We also tested whether TGF-β1 could induce Sulf2 expression in a murine type II AEC cell line, murine lung epithelial (MLE)-12, and observed an ~2-fold induction (data not shown). These results indicate that the induction of Sulf2 by TGF-β1 could be a common feature of type II AECs. Similar to A549 cells, MLE-12 cells do not express detectable levels of Sulf1 (data not shown).

**TGF-β1 induces HS 6-O-desulfation in A549 cells**

Sulfated disaccharide composition was analyzed by metabolic labeling of HS with [35S]Na2SO4 followed by high performance liquid chromatography (HPLC) analysis. As shown in Figure 3, treatment of TGF-β1 (5 ng/mL for 48 h) resulted in significant 6-O-desulfation in all the 6-O-sulfated disaccharides. These included single-sulfated unsaturated uronic acid (UA)-N-acetylglucosamine (GlcNAc)-6S (4.85 ± 0.26 vs. 8.76 ± 0.16%, P < 0.001), di-sulfated UA-GlcNS-6S (2.04 ± 0.21 vs. 5.44 ± 0.44%, P < 0.001) and tri-sulfated UA2S-GlcNS-6S (13.91 ± 0.60 vs. 26.91 ± 0.68%, P < 0.001). Concurrent increases were observed in UA-GlcNS (37.58 ± 0.41 vs. 32.13 ± 0.47%, P < 0.001) and UA2S-GlcNS (41.62 ± 0.34 vs. 26.77 ± 0.26%, P < 0.001). As Sulf2 was induced by TGF-β1 in these cells (Figure 2) and Sulf2 is a well-characterized HS 6-O-endsulfatase (Lamanna et al. 2006), the 6-O-desulfation observed was likely mediated by TGF-β1-driven Sulf2 expression.

**Sulf2 knockdown alters TGF-β1 response in A549 cells**

To investigate the role of Sulf2 in TGF-β1-induced fibrotic response in A549 cells, we used small interference RNA (siRNA) to block Sulf2 expression. Initially, we tested three siRNA sequences against Sulf2 (siRNA ID 121056, 121057 and 121058), and two sequences (siRNA ID 121056 and 121058) that gave similar knockdown were then combined in the subsequent experiments. Forty-eight hours after siRNA transfection, cells were stimulated with TGF-β1. As shown in Figure 4, TGF-β1 induced Sulf2 mRNA and protein expression in the negative control (NC, sequence that does not target any known mRNA) siRNA-transfected cells similar to what was observed in the parental cells (Figure 2). In Sulf2 siRNA-transfected cells (up to 72 h following TGF-β1 stimulation), Sulf2 siRNA reduced both the baseline and TGF-β1-induced Sulf2 expression. In Sulf2 siRNA transfected
cells without TGF-β1 treatment, Sulf2 mRNA levels were only ~10% of the levels in NC siRNA-transfected cells; with TGF-β1 treatment, Sulf2 mRNA level was ~20% of the levels in NC siRNA-transfected cells without TGF-β1 treatment (Figure 4A). Consistent with the mRNA levels, Sulf2 siRNA reduced baseline as well as TGF-β1-induced Sulf2 protein expression (Figure 4B). Disaccharide analysis confirmed the increase in 6-O-sulfated disaccharides in Sulf2 siRNA-transfected cells (Supplementary data, Figure S1).

To study the effect of Sulf2 knockdown on TGF-β1 signaling, we first examined Smad2 and Smad3 activation following TGF-β1 treatment (Figure 5A). Similar to lung fibroblasts in which siRNA-mediated Sulf1 knockdown results in enhanced Smad activation by TGF-β1 (Yue et al. 2008), phosphorylation (activation) levels of both Smad2 and Smad3 were significantly elevated in Sulf2 knockdown A549 cells following TGF-β1 treatment. In addition, total Smad2 level was also elevated in Sulf2 knockdown A549 cells. Similar Smad2 upregulation was observed previously in lung fibroblasts with Sulf1 siRNA transfection (Yue et al. 2008), suggesting a common regulatory mechanism.

We then examined the expression of TGF-β1 downstream target genes at 72 h. Plasminogen activator inhibitor (PAI)-1 is a TGF-β1-responsive gene involved in the control of ECM synthesis and degradation, whereas matrix metalloproteinase (MMP)-2 and -9 are two enzymes involved in the degradation of matrix molecules during the process of EMT (Keski-Oja et al. 1988; Kasai et al. 2005). Opposite to what would be expected from enhanced Smad2/3 activation, the expression of all the three TGF-β1 target genes was significantly reduced in Sulf2 knockdown A549 cells. The protein level of PAI-1 in the conditioned media was examined by western blotting,
which revealed a 70% reduction in Sulf2 knockdown cells compared with NC cells treated with TGF-β1 (Figure 5B). Gelatin zymography (Figure 5C) showed that both TGF-β1-induced MMP-2 and MMP-9 were significantly lower in Sulf2 knockdown cells (60 and 45% of the levels in NC cells treated with TGF-β1, respectively).

The apparent contradicting Smad activation (enhanced) and downstream TGF-β1 target gene expression (reduced) prompted us to perform a detailed time-course study of PAI-1, MMP-2 and MMP-9 mRNA expression following TGF-β1 treatment. Our results revealed a biphasic TGF-β1 response in Sulf2 knockdown A549 cells (Figure 6A–C). At early time points (4 and 8 h), TGF-β1 induced higher PAI-1, MMP-2 and MMP-9 mRNA expression in Sulf2 knockdown cells, consistent with enhanced Smad2/3 activation. However, this enhanced response was un-sustained in Sulf2 knockdown cells. At 24 h, NC siRNA-transfected cells had higher expressions of PAI-1 and MMP-9 compared with Sulf2 knockdown cells treated with TGF-β1, and at 48 h, the expression of all the three genes was higher in NC cells compared with Sulf2 knockdown cells.

TGF-β1 signaling is tightly regulated by negative feedback mechanisms, including the induction of inhibitory Smad7 as well as downregulation of Smad3 following TGF-β1 stimulation (Yanagisawa et al. 1998; Massague 2000). In order to examine whether these negative feedback mechanisms were at play in Sulf2 knockdown A549 cells, we performed qRT-PCR to examine the changes in Smad3 and Smad7 mRNA in NC and Sulf2 siRNA-transfected cells (Figure 6E and F). As expected, Smad3 mRNA expression was downregulated in both NC and Sulf2 siRNA-transfected cells following TGF-β1 stimulation (Figure 6E). Interestingly, the basal level of Smad3 mRNA was significantly lower in Sulf2 knockdown cells, and stimulation by TGF-β1 further reduced it to 20% of the level in NC cells and 50% of the level in NC cells treated...
A549 cells may not represent a normal type II AEC response. As A549 cells are cancer cells, the response we observed in primary type II AECs from Sulf2 knockout mice exhibit (Supplementary data, Figure S2).

TGF-β1 knockdown cells could account for the un-sustained TGF-β1 response seen in A549 cells treated with or without TGF-β1 stimulation (5 ng/mL). Black open circle, NC siRNA-transfected cells; black solid circle, NC siRNA-transfected cells treated with TGF-β1; red open circle, Sulf2 siRNA-transfected cells; red solid circle, Sulf2 siRNA-transfected cells treated with TGF-β1. *P < 0.05 and ****P < 0.001 between NC siRNA-transfected A549 cells treated with or without TGF-β1. (B) Sulf2 protein expression in NC or Sulf2 siRNA-transfected A549 cells with or without TGF-β1 stimulation (5 ng/mL, at 48 h). The data shown are representative of three independent experiments.

with TGF-β1 (at 48 h). The downregulation of Smad3 in Sulf2 knockout cells could account for the un-sustained TGF-β1 target gene expression. The expression of the inhibitory Smad7 was induced by TGF-β1 in both NC and Sulf2 siRNA-transfected cells, and the induction was higher in NC cells (Figure 6F). Thus, the expression of Smad7 could not explain the un-sustained TGFβ1 response seen in Sulf2 knockout cells. A 20–40% increase (statistically significant) in Smad2 mRNA level was observed in Sulf2 knockout cells compared with control cells without TGF-β1 stimulation (Figure 6D), consistent with increased Smad2 protein expression in Sulf2 knockout cells (Figure 5A).

The dependence of TGF-β1-induced PAI-1, MMP-2 and MMP-9 expression on Smad3 was induced by TGF-β1-stimulated Sulf2 knockdown cells compared with control cells without TGF-β1 stimulation (Figure 5A). In contrast to A549 cells, TGF-β1 did not induce significant MMP-2 expression in primary type II cells (Figure 7D). The above data indicate that, in the absence of Sulf2, primary murine type II AECs gained an enhanced response to TGF-β1.

In contrast to A549 cells and the murine type II AEC cell line MLE-12, TGF-β1 did not significantly induce Sulf2 expression in primary murine type II AECs under our culture condition (data not shown). Because of this negative finding, we went on to test whether epithelial injury could induce Sulf2 expression. Epithelial injury has been proposed to be the initiating event in the development of IPF (Kottmann et al. 2009). We used bleomycin as the cytotoxic agent. Bleomycin is a chemotherapy drug that causes pulmonary fibrosis in certain cancer patients receiving this treatment. In mouse models, bleomycin is the most commonly used drug to induce pulmonary fibrosis (Degryse et al. 2010). In primary murine type II cells from wild-type mice, bleomycin consistently induced Sulf2 mRNA and protein expression (Figure 8). Bleomycin also induced Sulf2 expression in A549 cells (data not shown). Thus, in human as well as in murine type II AECs, epithelial injury caused by bleomycin induces Sulf2 expression, which subsequently could alter their response to TGF-β1.

**Discussion**

HS plays critical roles in a variety of developmental, physiological and pathological processes due to its ability to interact in a structure-dependent manner with numerous growth factors, morphogens and matrix molecules. It is now well established that HS-protein interactions critically depend on the amount and the positions of the O-sulfate groups, in particular, the 6-O-sulfates that form binding sites for proteins (Esko and Lindahl 2001; Nakato and Kimata 2002). The 6-O-sulfation state of HS can be regulated in two ways: 6-O-sulfotransferases and 6-O-desulfation at the cell surface.

**Primary type II AECs from Sulf2 knockout mice exhibit enhanced TGF-β1 response**

As A549 cells are cancer cells, the response we observed in A549 cells may not represent a normal type II AEC response.
and the ECM by the Sulfs. Work in our laboratory has focused on the induction of Sulf1 and Sulf2 by TGF-β1 and the functional significance of these sulfatases in TGF-β1 signaling and the development of pulmonary fibrosis.

Unlike interactions with members of the FGF family, HS does not appear to be directly required for TGF-β1 binding and/or activation of its specific signal transducing receptors (Lyon and Gallagher 1998). However, as TGF-β1-heparin/HS interaction requires 6-O-sulfation (Lyon et al. 1997), the Sulfs could play important roles in modulating the local concentration or availability of TGF-β1 at the cell surface. The induction of Sulf1 in lung fibroblasts (Yue et al. 2008) and Sulf2 in type II AECs (current study) by TGF-β1 could reflect a negative feedback mechanism to reduce TGF-β1 binding to cell surface HS, thus reducing TGF-β1 signaling through its membrane receptors.

In both our previous work on lung fibroblasts and our current study of A549 cells, we used siRNA knockdown approach to study the role of Sulf1 and Sulf2 in TGF-β1 function. In lung fibroblasts, siRNA-mediated knockdown of Sulf1 resulted in elevated total-Smad2 levels and enhanced Smad activation (both Smad2 and Smad3) upon TGF-β1 stimulation, leading to enhanced TGF-β1 target gene expression, including α-SMA, collagen I and fibronectin. In A549 cells, however, we observed a biphasic TGF-β1 response with siRNA-mediated Sulf2 knockdown. At early time points, blocking Sulf2 expression enhanced TGF-β1-induced Smad activation (both Smad2 and Smad3) as well as mRNA expression of PAI-1, MMP-2 and MMP-9. However, this enhanced TGF-β1 target gene expression was not sustained in Sulf2 knockdown cells. Careful examination of Smad expression and activation revealed that although both total and phosphorylated Smad2 were upregulated in Sulf2 knockdown cells, Sulf3 expression was downregulated, which eventually resulted in un-sustained TGF-β1 target gene expression. As A549 cells are cancer cells, the response we observed may not represent a normal type II AEC response. Indeed, in primary type II AECs isolated from Sulf2 knockout mice, both TGF-β1-induced Smad activation and downstream TGF-β1 target gene expression were elevated compared with type II AECs isolated from wild-type mice. In addition, different from A549 cells with siRNA-mediated Sulf2 knockdown,
total-Smad2 and -Smad3 levels were not significantly altered in Sulf2 null primary type II AECs. This could reflect differences in “acute” downregulation of Sulf2 using siRNA in A549 cells and “chronic” downregulation of Sulf2 in the Sulf2 null cells.

In this study, we observed a 2.2-fold increase in Sulf2 mRNA expression in lung samples from IPF patients compared with the controls. This finding is consistent with the results of a gene expression profiling study in which a 2-fold upregulation of Sulf2 was observed in fibrotic lungs compared with normal lung tissues (Yang et al. 2007). The similar fold induction in Sulf2 also indicates that our control group, lung samples from mild COPD patients, had similar baseline Sulf2 expression compared with normal lungs. Immunohistochemistry study further revealed that Sulf2 was specifically localized to the hyperplastic type II AECs in the IPF lungs. The overexpression of Sulf2 was likely induced by TGF-β1a as shown to be overexpressed in the IPF lungs (Khalil et al. 1991). In primary murine type II AECs, however, we did not observe induction of Sulf2 by TGF-β1. It is not clear whether this is due to species difference or our in vitro culture conditions. We went on to test whether epithelial injury could induce Sulf2 expression using bleomycin, a chemotherapy agent that induces epithelial injury and pulmonary fibrosis in both mice and humans. Our results show that bleomycin indeed induces Sulf2 expression in both primary murine type II AECs (Figure 8) and A549 cells (data not shown). It is important to investigate whether other types of epithelial injury such as oxidative stress, which is more relevant in the development of IPF (Kliment and Oury 2010), could also induce Sulf2 expression in type II AECs.

In addition to regulating TGF-β1 signaling as suggested by the current study, Sulf2 could play important roles in other aspects of alveolar epithelial injury and repair. Sulf2 has been shown to regulate Wnt signaling (Dhoot et al. 2001; Ai et al. 2003; Nawroth et al. 2007), and components of Wnt signaling pathways have been shown to be altered in IPF (Chilosi et al. 2003; Konigshoff and Eickelberg 2010). Recently, Sulf2 was identified as a direct transcriptional target of p53 and was shown to be required for p53-mediated cellular senescence (Chau et al. 2009). Understanding the role of Sulf2 in the complex signaling networks of pulmonary fibrosis (TGF-β, Wnt and p53) is important in understanding the role of Sulf2 in the pathogenesis of IPF.

IPF is frequently associated with lung cancer (Konigshoff 2011), and Sulf2 has been shown to be overexpressed in lung cancer cells and promote cancer cell growth and transformation (Lemjabbar-Alaoui et al. 2010). The overexpression of Sulf2 in the hyperplastic type II cells in IPF may contribute to the increased incidence of lung cancer in IPF patients.

**Materials and methods**

**Human IPF samples**

Human IPF and control samples were obtained from the LTRC (Concept Sheet 07-99-0005), a program sponsored by the National Heart, Lung and Blood Institute. The clinical data and specimens have been de-identified by the LTRC.
Fig. 7. Primary type II AECs from Sulf2 knockout mice exhibit enhanced TGF-β1 response. (A) The purity of the type II AEC isolation was assessed by immunostaining for pro-SPC. (B) Phospho- and total-Smad2 and -Smad3 levels were analyzed by western blotting at 30 min following TGF-β1 (1 and 5 ng/mL) stimulation. Results from treatment with 1 and 5 ng/mL of TGF-β1 were similar and combined in the quantification of P-Smad2/T-Smad2 and P-Smad3/T-Smad3 ratios. White bars, WT type II AECs treated with TGF-β1; black bars, Sulf2 knockout type II AECs treated with TGF-β1. *P < 0.05. (C) Protein levels of PAI-1 in the conditioned media were analyzed by western blotting. (D) Protein levels of MMP-2 and MMP-9 in the conditioned media were analyzed by gelatin zymography. White bars, control; black bars, 1 ng/mL of TGF-β1 and horizontal bars, 5 ng/mL of TGF-β1. The data shown are representative of three independent experiments.

Fig. 8. Bleomycin induces Sulf2 expression in primary murine type II AECs. Primary murine type II AECs from wild-type mice were treated without (white bars) or with bleomycin (10 mU/mL). (A) Sulf2 mRNA expression at 24 h was analyzed by qRT-PCR. Fold changes were normalized to the housekeeping gene 36B4. **P < 0.05. (B) Expression of Sulf2 protein at 48 h following bleomycin treatment was analyzed by western blotting using a mouse monoclonal antibody against Sulf2 (2B4). The unprocessed pro-protein (>100 kDa) was the predominant form detected. Expression of γ-tubulin was used as the loading control. The data shown are representative of three independent experiments.
Flash-frozen lung tissues were used for RNA extraction and formalin-fixed and paraffin-embedded lung tissue sections were used for immunohistochemistry.

**Immunohistochemistry**

Formalin-fixed and paraffin-embedded human lung tissue sections were deparaffinized and rehydrated. Antigen retrieval was achieved by microwave on high for 5 min in 300 mM NaCl, 30 mM sodium citrate, pH 6.0, followed by cooling at room temperature for 30 min. Histostain-Plus Kits with DAB as the substrate (Invitrogen, Grand Island, NY) were used for detection of Sulf2 (H2.3, kindly provided by Dr Steven Rosen at University of California, San Francisco) and pro-SPC (Millipore, Billerica, MA) following the manufacturer’s instructions.

**Cell culture**

A549 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen). Cells within 10 passages were used in all experiments. Before treatment with TGF-β1 (R&D Systems, Minneapolis, MN) in DMEM containing 0.5% FBS and penicillin/streptomycin for 48 h at 37°C, Glycosaminoglycan purification, HS digestion and disaccharide analyses were performed as described (Yue et al. 2008).

**siRNA transfection**

Small interference RNA (siRNA) against human Sulf2 (siRNA ID 121056, 121057, 121058) and NC siRNA (Cat #: 4611) were obtained from Ambion. A549 cells (80,000 cells/mL) were reverse transfected in 12-well plates (1 mL/well) with a mixture of 30 nM Sulf2 siRNA 121056 and 30 nM of Sulf2 siRNA 121058, or 60 nM NC siRNA in full serum media without antibiotics using the Lipofectamine™ RNAiMAX reagent (Invitrogen). Transfection media were replaced with DMEM containing 0.5% FBS and penicillin/streptomycin the next day. After another 24 h (which was 48 h after siRNA transfection), cells were treated with TGF-β1. Knockdown of Sulf2 was evaluated by qRT-PCR at different time points following TGF-β1 treatment. Sulf2 protein levels were analyzed by western blotting at 48 h after TGF-β1 stimulation.

**Gelatin zymography**

Conditioned media were analyzed using precast Novex gelatin zymogram gels (Invitrogen) as described previously (Shan et al. 2005). The intensity of MMP-2/-9 gelatinolytic zones on the gels was quantified using the NIH image J software.

**Primary type II AEC isolation and culture**

Sulf2 knockout mice on a C57BL/6 background were kindly provided by Dr Xingbin Ai at Boston University (Ai et al. 2007). Primary type II AECs were isolated from 2–3-month-old Sulf2 knockout mice and their wild-type littermates (control) essentially as described (Corti et al. 1996; Kim et al. 2006). Purity of the isolated type II AECs was assessed by immunofluorescence staining for pro-SPC on cytospin cells. Typically 1–2 × 10⁶ cells containing 90% type II AECs were isolated from a single mouse. Cells were cultured in 24-well tissue culture plates and maintained in SAGM (without hydrocortisone, Lonza, Allendale, NJ) containing 5% FBS and 10 ng/mL KGF (Peprotech) in a 37°C, 5% CO₂ incubator. The media were replaced after 48 h. Treatment with TGF-β1 or bleomycin (10 μM/mL, Zhejiang Hisun Pharmaceutical Co., Zhejiang, China) was carried out in SAGM (without hydrocortisone).
Statistical analysis
All experiments were performed at least three times with similar results. Data were expressed as mean ± standard error of the mean. Statistical analyses were performed using unpaired Student’s t-test for two groups and ANOVA followed by Bonferroni’s multiple comparison tests when more than two groups were compared. Differences were considered statistically significant when P < 0.05.

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest
None declared.

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Abbreviations
Ac, acetyl; AECs, alveolar epithelial cells; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; GlcNAc, N-acetylglucosamine; HPLC, high performance liquid chromatography; HS, heparan sulfate; HSPGs, HS proteoglycans; IPF, idiopathic pulmonary fibrosis; MMP, matrix metalloproteinase; NC, negative control; PAI-1, plasminogen activator inhibitor-1; pro-SPC, pro-surfactant protein C; qRT-PCR, quantitative real-time PCR; siRNA, small interference RNA; SIS3, specific inhibitor of Smad3; alpha-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; UA, unsaturated uronic acid.

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