The role of TGFβ-HGF-Smad4 axis in regulating the proliferation of mouse airway progenitor cells

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Abstract. The interaction between airway epithelial progenitor cells and their microenvironment is critical for maintaining lung homeostasis. This microenvironment includes fibroblast cells, which support the growth of airway progenitor cells. However, the mechanism of this support is not fully understood. In the present study, the authors observed that inhibition of transforming growth factor (TGF)-β signal with SB431542 promotes the expression of hepatocyte growth factor (HGF) in fibroblast cells. The HGF receptor, c-Met, is expressed on airway progenitor cells; HGF promotes the colony-forming ability of airway progenitor cells. The deletion of Smad4 in airway progenitor cells increases the colony-forming ability, suggesting that Smad4 plays a negative role in the regulating the proliferation of airway progenitor cells. These data demonstrated that the regulation of airway progenitor cells by TGF-β depends on TGF-βR1/2 on stromal cells, rather than on epithelial progenitor cells. These data suggested a role for the TGF-β-TGF-βR1/2-HGF-Smad4 axis in airway epithelial homeostasis and sheds new light on the interaction between airway progenitor cells and their microenvironment.

Introduction

The airway epithelium is pivotal to host defense against foreign microbial pathogens. There is increasing evidence that epithelial alterations are associated with multiple airway diseases, including asthma, chronic obstructive pulmonary diseases (COPD), obliterative bronchiolitis and cystic fibrosis (1-5). Following injury to the epithelium, airway progenitor cells are a crucial part of the repair process (6). These progenitor cells interact with their microenvironment to determine their proliferation, differentiation and capacity for self-renewal. The growth of these progenitor cells is reported to be supported by the production of growth factors by stromal cells (7). Additionally, the research of Lee *et al* (8) suggests that endothelial cells direct the specification and differentiation of airway progenitor cells. Furthermore, parabronchial smooth muscle cells activate airway epithelial progenitor cells to undergo a post-injury epithelial to mesenchymal transition (9). Vimentin-positive lung fibroblasts can create a niche for airway progenitor cells (10). Airway progenitor cells cross talk with their microenvironmental elements through direct contact or autocrine and/or paracrine signals. These include Wnt-β-catenin signaling, BMP signaling, Notch signaling and TGF-β signaling (7,11-13).

The TGF-β signal is overactivated in response to pulmonary fibrosis (14,15). Moreover, TGF-β has been reported to be upregulated in COPD and allergic asthma (16). Inhibition of TGF-β signaling with SB431542 promotes the proliferation of airway epithelial progenitor cells (7). TGF-β signal in fibroblasts can act FGF10 to regulate epithelial stem cell growth (7). It was shown that TGF-β inhibits HGF expression in mesenchymal cells through a TGF-β inhibitory element (17). HGF acts as a ligand with its receptor tyrosine kinase, c-Met to fulfill its function (18). HGF modulates the function of Smad4 through activating the Ras/MAPK pathway (19). However, the role of Smad4 in the regulation of airway progenitor cells has not been addressed.

In the present study, the authors adopted an *in vitro* epithelial-fibroblast co-culture assay developed previously (20). It was observed that TGF-β inhibits the proliferation of airway progenitor cells through the TGF-β receptors on fibroblasts. Inhibition of the TGF-β/TGFR2 pathway alters the secretory properties of fibroblasts, including production of HGF. Deletion of Smad4 resulted in an increase in the colony forming ability of airway progenitor cells.

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Materials and methods

Ethics statement. Experimental mice were maintained under pathogen-free conditions in Tianjin Haihe Hospital's animal facility, and the permit number is SYXK (Jin) 2016-0002. Adult mice between the ages of 2-4 months old were sacrificed for experiments according to protocols approved by the Haihe Hospital Animal Care and Use Committee (Tianjin, China). All surgery was performed under 1% sodium pentobarbital 50 mg/kg intraperitoneal injection anesthesia, and all efforts were made to minimize suffering.

Mice. *β-actin-GFP, Sftpc-Cre*, *TGFβR2$^{f/f}$*, and *Smad4$^{f/f}$* mice were donated by Stripp B.R. (Cedars-Sinai Medical Center, Los Angeles, CA, USA). The authors crossed *Sftpc-Cre* mice with *TGFβR2$^{f/f}$* mice to generate *Sftpc-Cre; TGFβR2$^{f/f}$* mice. Additionally, *Sftpc-Cre* mice and *Smad4$^{f/f}$* mice were crossed to generate *Sftpc-Cre; Smad4$^{f/f}$* mice.

Fractionation of airway epithelial progenitor cells. Lung cell suspensions were prepared using an elastase digestion and stained for fluorescence-activated cell sorting (FACS), as previously described (20). Briefly, cells were resuspended in Hanks' balanced saline solution buffer supplemented with 2% fetal bovine serum, 0.1 mM EDTA, 10 mM HEPES, 100 IU/ml penicillin and 100 μg/ml streptomycin (HBSS^{+}). Cells were then stained with the primary antibodies on ice for 45 min. The following antibodies were employed: EpCAM-PE-Cy7 (25-5791-80, 1:100), CD31-Biotin (13-0311-81, 1:40), CD34-Biotin (13-0341-81, 1:10), CD45-Biotin (13-0451-81, 1:100), Sca-1-APC (17-5981-81, 1:100), and CD24-PE (12-0242-81, 1:20) (all from eBioscience, Inc., San Diego, CA, USA). Cells were subsequently stained with the secondary antibody on ice for 40 min using streptavidin-APC-Cy7 (47-4317-82, 1:100; eBioscience, Inc.). Dead cells were identified using 7-aminoactinomycin D staining (BD Biosciences, Franlkin Lakes, NJ, USA).

Cell cultures and treatment. MLg cells (CCL-206; American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For the experiments, 6-well culture plates (Thermo Fisher Scientific, Inc.) were seeded with MLg cells at 5,000 cells/well and maintained at 37˚C in a humidified atmosphere with 5% CO_2. MLg cells were then exposed to 10 μM SB431542 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 48 h. Cultures were visualized under an OLYMPUS IX73 (Olympus Corporation, Tokyo, Japan) inverted microscope before being harvested for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Matrigel cultures of airway progenitor cells. Mouse airway progenitor cells were co-cultured with MLg cells in Matrigel, as described previously (20). In brief, sorted airway progenitor cells were mixed with MLg cells in growth factor-reduced Matrigel (BD Biosciences) and basic medium (BM) at a 1:1 ratio. The basic medium consisted of DMEM/F12 medium (Cellgro, Manassas, VA, USA), 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), Insulin-Transferrin-Selenium supplement (Sigma-Aldrich; Merck KGaA), 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell mixture was placed in 24-well Transwell filter inserts (BD Biosciences) in a 24-well flat-bottom culture plate containing culture medium (BM + SB431542). Cultures were incubated at 37˚C in a humidified atmosphere with 5% CO_2, and medium was replaced every other day. Colony-forming efficiency (CFE) was determined by counting the number of colonies with a diameter of ≥100 μm in each culture and representing this number as a percentage of seeded progenitor cells.

Total RNA isolation and RT-qPCR. Total RNA was extracted from MLg cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. RNA concentration was assessed and cDNA was synthesized using SuperScript III reagents (Invitrogen; Thermo Fisher Scientific, Inc.) with Oligo-dT and random hexamer priming (Takara Bio, Inc., Otsu, Japan). RT-qPCR was performed using SYBR Green SuperMix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the Light Cycler 96 Real-Time PCR system (Roche Diagnostics, Indianapolis, IN, USA) under the following reaction conditions: Initial heating cycle of 95˚C for 2 min; and 40 cycles of denaturation at 95˚C for 25 sec, primer annealing at 60˚C for 25 sec and extension at 72˚C for 20 sec. Melting curves were used to clarify the identity of amplicons, and the housekeeping gene, β-actin, served as an internal control. The relative mRNA expression levels of targeted genes were calculated using the comparative threshold cycle (CT) method (21) normalized to β-actin mRNA in the same sample. Primers were designed as follows: β-actin forward, 5'-GGCCAACCGTGAAAAGATGA-3' and reverse, 5'-CAGCCTGGATGGCTACGTACA-3'; Hgf forward, 5'-CCTGGTGTTTCACAAGCAATC-3' and reverse, 5'-CATGGGACCTCTGTAGCTTTC-3'. β-actin was used as a housekeeping gene.

Microarray analysis. EpCAM^{+} lung epithelial cells or EpCAM^{+}Sca-1^{+} airway progenitor cells were sorted and pooled for total RNA extraction using RNeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA). RNA (0.1 μg) was used for Affymetrix microarray analysis using mouse genome 430 2.0 arrays (Affymetrix, Inc., Santa Clara, CA, USA). Data were annotated with Affymetrix Expression Console software (Affymetrix, Inc.). Pathway analysis was performed by online Gather KEGG analysis (Kyoto Encyclopedia of Genes and Genomes; http://gather.genome.duke.edu/). A Bayes factor was also included in the consideration of KEGG pathway association.

Statistical analysis. The data were analyzed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean. The significance of the results was assessed using Student's t test between paired groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TGF-β regulates mouse airway progenitor cells through its receptors on MLg cells. To investigate the role of fibroblasts

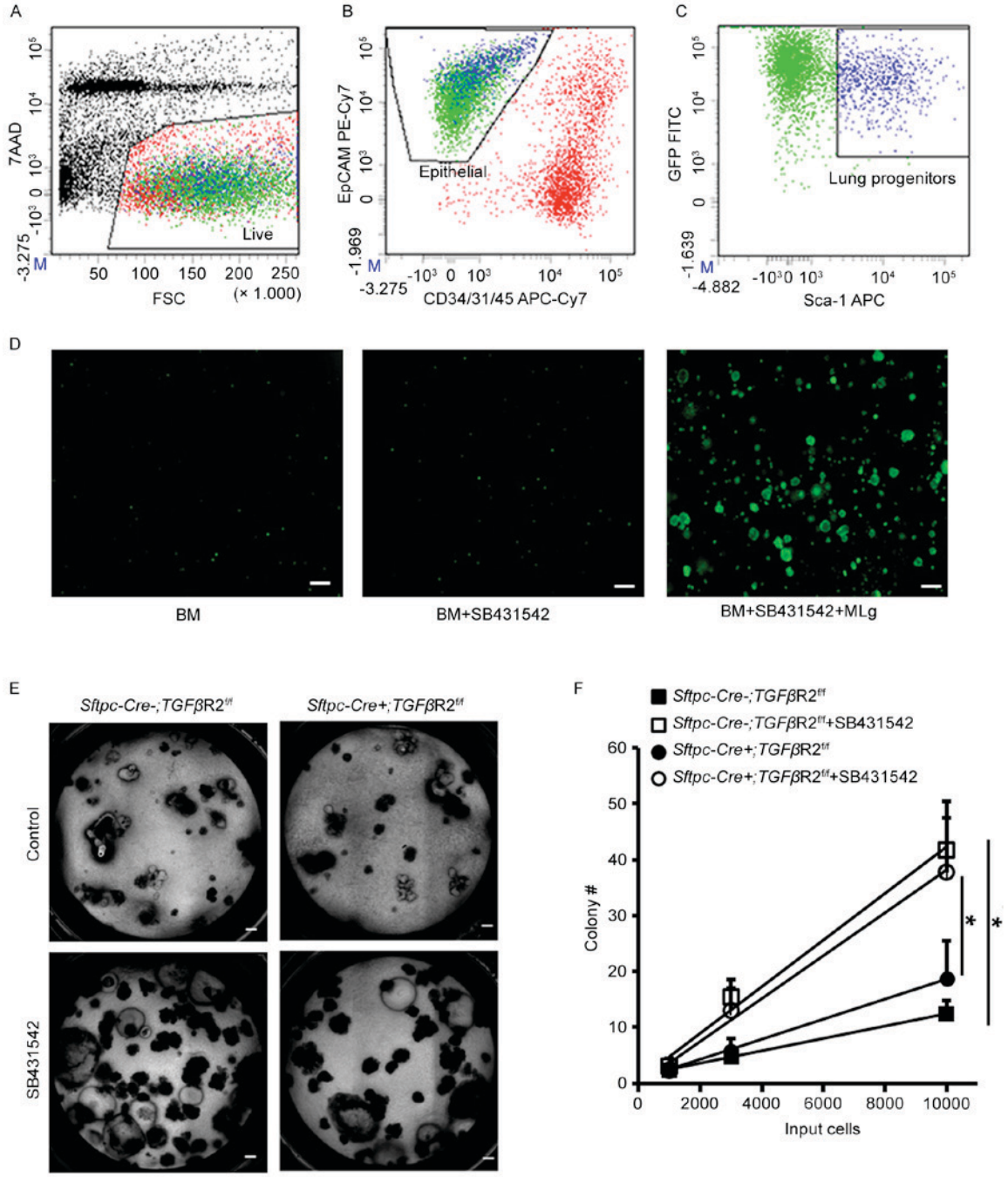

Figure 1. TGF-β regulates mouse airway progenitor cells through its receptors on MLg cells. (A) Exclusion of 7AAD^{pos} cells (y-axis). (B) CD34/31/45(Lin) (x-axis) vs. EpCAM (y-axis) analysis of 7AAD^{neg} population from A. (C) Sca-1 (x-axis) vs. GFP (y-axis) analysis of Lin^{neg}/EpCAM^{pos} population from B represents airway progenitor cells. (D) Sorted progenitor cells were cultured in basic medium, with an addition of 10 μM SB431542, 10 μM SB431542 and MLg cells at day 6. (E) Sorted progenitor cells from *Sftpc-Cre^{-}; TGF-β2R$^{f/f}$* and *Sftpc-Cre^{+}; TGF-β2R$^{f/f}$* mice were cultured under control conditions and with an addition of 10 μM SB431542 for 4 weeks. (F) Quantitative analysis of the colony-forming efficiency in different culture conditions. Data are expressed as the mean ± standard error of the mean. *P<0.05 as indicated. Scale bars, 200 μm. TGF-β, transforming growth factor-β; FSC, forward scatter; GFP, green fluorescent protein; 7-AAD, 7-amino-actinomycin D.

in the regulation of mouse airway progenitor cells by TGF-β, the authors fractionated mouse airway progenitor cells from *β-actin-GFP* mice using a FACS-based strategy. Lung cells from *β-actin-GFP* mice were isolated by elastase digestion. The cells were stained with fluorescent antibodies and a viability dye. Dead cells were detected by 7-AAD staining (Fig. 1A). Endothelial, stromal and hematopoietic cells were excluded by surface staining for CD31, CD34 and CD45 (Fig. 1B). Airway epithelial progenitor cells, also positive for GFP, were further enriched by surface EpCAM and Sca-1 staining (Fig. 1B and C). A significant number of colonies were formed in presence of both SB431542 and MLg cells compared to stromal-free and SB431542 alone cultures (Fig. 1D), which was consistent with the authors' previous findings (20). The authors further examined the TGF-β role in the regulation of airway progenitor cells using *Sftpc-Cre^{+}; TGFβR2$^{f/f}$* mice, in which TGFβR2 is absent in their airway progenitor cells. *In vitro* cultures of airway progenitor cells in presence of MLg

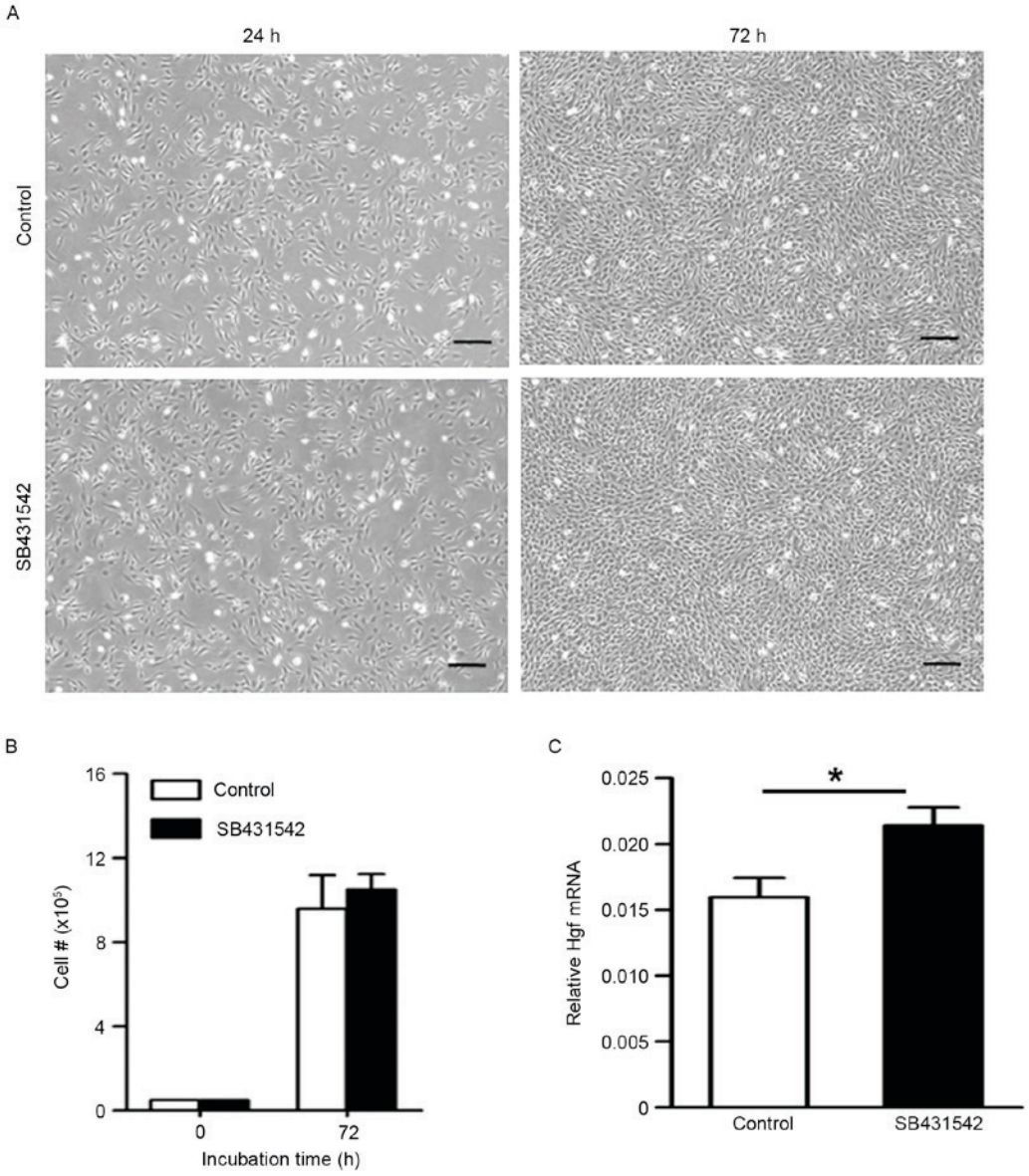

Figure 2. Inhibition of transforming growth factor-β signaling promotes the secretion of HGF in fibroblasts. (A) MLg cells were cultured in control condition or with 10 μM SB431542 for 48 h. (B) Quantitative analysis of the number of MLg cells. (C) Reverse transcription-quantitative polymerase chain reaction analysis was performed to quantify *Hgf* gene expression. Scale bars, 200 μm. Data are expressed as the mean ± standard error of the mean. *P<0.05 as indicated. HGF, hepatocyte growth factor.

cells indicated that the colony-forming ability was comparable between *Sftpc-Cre^{-}; TGFβR2$^{f/f}$* and *Sftpc-Cre^{+}; TGFβR2$^{f/f}$* (Fig. 1E and F). SB431542 enhanced the colony-forming ability of airway progenitor cells in both *Sftpc-Cre^{-}; TGFβR2$^{f/f}$* and *Sftpc-Cre^{+}; TGFβR2$^{f/f}$* (Fig. 1E and F). These data suggested that TGF-β exerts its regulatory role in the colony-forming ability of airway progenitor cells by acting on MLg cells, rather than on airway progenitor cells.

Inhibition of TGF-β signaling promotes the secretion of HGF in fibroblasts. Fibroblasts have been shown to promote airway epithelial progenitor cells by producing growth factors. HGF expression was measured in the absence or presence of SB431542. Within 48 h, the number of MLg cells increased by approximately tenfold in the control group (Fig. 2A). There was no difference in the number of MLg cells between control and SB431542 treatment (Fig. 2B). Additionally, the morphology of MLg cells did not differ between the control and SB431542 treatment (Fig. 2A). Using RT-qPCR, the authors observed that the mRNA expression of *Hgf* in the SB431542 treatment was higher than that in the control (Fig. 2C). These data suggested that activation of the TGF-β signal pathway inhibits the production of growth factor HGF in fibroblasts.

Stromal-derived HGF exerts its function through its specific receptor c-Met. Following this, the c-Met expression was determined in airway progenitor cells, alveolar type 2 (AT2) cells, ciliated cells, and MLg cells using quantitative RT-PCR (Fig. 3A). Mouse AT2 cells and ciliated cells were fractionated as previously (20). c-Met expression was indicated to be more abundant in lung epithelial cells than in stromal cells (Fig. 3A). AT2 cells express more c-Met than airway progenitor cells and ciliated cells in mouse lung (Fig. 3A). *In vitro* 3-D Matrigel culture indicated that HGF promotes the growth of airway progenitor cells in absence of MLg

Table I. Top genes expressed in airway progenitor cells compared to total lung epithelial cells.

Gene ID	Gene name	Gene symbol	Sca-1$^+$/EpCAM
742	Proline/serine-rich coiled-coil 1	Psrc1	12.0
12623	Carboxylesterase 1	Ces1	8.6
232400	cDNA sequence BC048546	BC048546	7.4
17984	Necdin	Ndn	7.4
67005	Polymerase (RNA) III (DNA directed) polypeptide K	Polr3k	4.8
140474	Mucin 4	Muc4	4.7
23886	Growth differentiation factor 15	Gdf15	4.6
22270	Uroplakin 3A	Upk3a	4.3
13078	Cytochrome P450, family 1, subfamily b, polypeptide 1	Cyp1b1	3.7
11670	Aldehyde dehydrogenase family 3, subfamily A1	Aldh3a1	3.6
27280	Pleckstrin homology-like domain, family A, member 3	Phlda3	3.6
70337	Iodotyrosine deiodinase	Iyd	3.6
21808	Transforming growth factor, β	TGF-β	3.5
399638	RIKEN cDNA A030001D16 gene	A030001D16Rik	3.5
215446	Ectonucleoside triphosphate diphosphohydrolase 3	Entpd3	3.4
18104	NAD(P)H dehydrogenase, quinone 1	Nqo1	3.4
217830	RIKEN cDNA 9030617O03 gene	9030617O03Rik	3.4
77914	Keratin associated protein 17-1	Krtap17-1	3.4
70536	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	Qpct	3.4
19363	RAD51-like 1 (*S. cerevisiae*)	Rad51l1	3.3

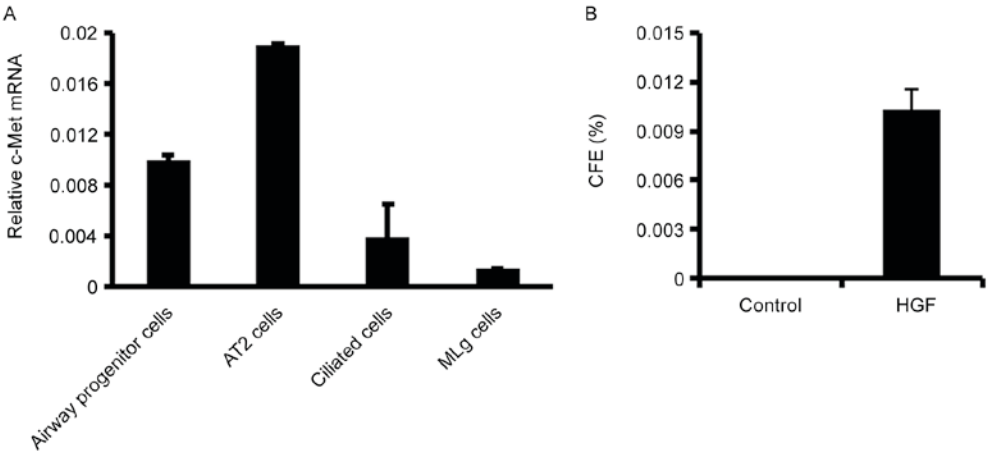

Figure 3. HGF/c-Met regulates airway progenitor cells. (A) The c-Met expression in airway progenitor cells, alveolar type 2 (AT2) cells, ciliated cells, and MLg cells using quantitative reverse transcription-quantitative polymerase chain reaction. (B) CFE of airway progenitor cells is increased in presence of HGF in stromal free 3D Matrigel culture. Data are expressed as the mean ± standard error of the mean. HGF, hepatocyte growth factor; CFE, colony forming efficiency.

cells (Fig. 3B), suggesting that HGF acts directly on airway progenitor cells to promote their growth.

Smad4 regulates the proliferation of airway progenitor cells. Smad elements are implicated in downstream TGF-β signal, and have been shown to be modulated by HGF (22). Smad elements were hypothesized to play a role in the regulation of airway progenitor cells by HGF. Microarray analysis was used to identify enriched transcripts of airway progenitor cells as compared to EpCAM$^+$ lung epithelial cells. The top 20 genes enriched in airway progenitor cells were listed in Table I. Notably, TGF-β transcript is enriched by 3.5 fold over total lung epithelial cells (Table I). In addition, the authors sorted the list of transcripts by their relative expression level, and 1213 transcripts were observed that read 1800 or greater which indicate their potential expression in real samples (Table II). As expected, Scgb1a1, Cyp2f2 and Plunc were presented in the list of highly expressed gene list of airway progenitor cells (Table II). Smad4 was in the list even it did not show up in Table II. Gathered analysis of top genes in airway progenitor cells indicated that Smad4 signaling pathway may participate in the regulation of airway progenitor

Table II. Highly expressed genes in airway progenitor cells.

Entrez gene	Gene name	Gene symbol	Ave.Sca1$^+$	Ave.EpCAM$^+$
287	Secretoglobin, family 1A, member 1 (uteroglobin)	Scgb1a1	33358	33006
13107	Cytochrome P450, family 2, subfamily f, polypeptide 2	Cyp2f2	31168	26482
12409	Carbonyl reductase 2	Cbr2	28700	24926
117158	Secretoglobin, family 3A, member 2	Scgb3a2	28154	25734
15439	Haptoglobin	Hp	25201	19952
67701	WAP four-disulfide core domain 2	Wfdc2	24106	19162
22070	Tumor protein, translationally-controlled 1	Tpt1	23147	22975
13627	Eukaryotic translation elongation factor 1 alpha 1	Eef1a1	21612	21224
68662	Secretoglobin, family 3A, member 1	Scgb3a1	21599	19061
56615	Microsomal glutathione S-transferase 1	Mgst1	21468	18682
19241	Thymosin, β 4, X chromosome	Tmsb4x	21214	22595
20387	Surfactant associated protein A1	Sftpa1	20590	27427
22190	Ubiquitin C	Ubc	20572	18226
18843	Palate, lung, and nasal epithelium associated	Plunc	20304	13823
20341	Selenium binding protein 1	Selenbp1	20207	15170
14319	Ferritin heavy chain 1	Fth1	19942	17755
100042862	Predicted gene 4076	Gm4076	19843	18573
14281	FBJ osteosarcoma oncogene	Fos	19779	18595
65019	Ribosomal protein L23	Rpl23	19029	18042
14319	Ferritin heavy chain 1	Fth1	18279	15370

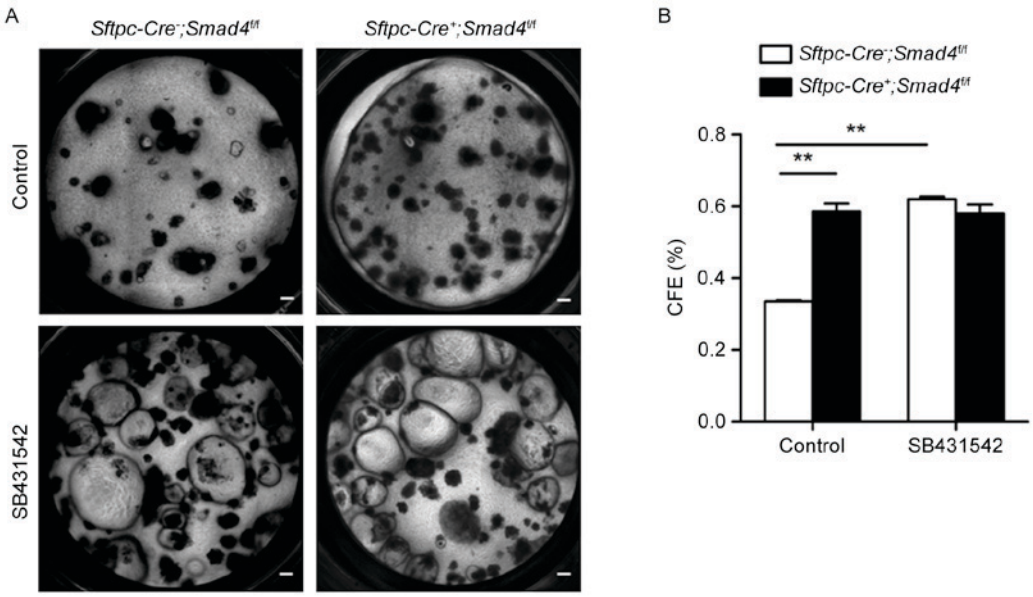

Figure 4. Smad4 regulates the proliferation of airway progenitor cells. (A) Airway progenitor cells from *Sftpc-Cre-; Smad4$^{f/f}$* or *Sftpc-Cre+; Smad4$^{f/f}$* mice were cultured in control condition or with the addition of 10 μM SB431542 at week 4. (B) Quantitative analysis of the colony-forming efficiency in different culture conditions. Scale bars, 200 μm. Data are expressed as the mean ± standard error of the mean. **P<0.01 as indicated. CFE, colony forming efficiency.

cells proliferation (Table III). In addition, *Xenopus* fork head domain factor 3 and direct repeat 4 transcription factors may also serve a role in regulating airway progenitor cells. These data suggested that Smad4 may be a regulator of airway progenitor cells.

To examine this, the authors used a transgenic mouse model, *Sftpc-Cre$^-$; Smad4$^{f/f}$*, in which Smad4 is absent in all lung epithelia. Airway progenitor cells from *Sftpc-Cre$^-$; Smad4$^{f/f}$* or *Sftpc-Cre$^+$; Smad4$^{f/f}$* mice were sorted and cultured in the presence of MLg cells. More epithelial colonies were observed in the *Sftpc-Cre$^+$; Smad4$^{f/f}$* group compared to the *Sftpc-Cre$^-$; Smad4$^{f/f}$* control group in the absence of SB431542 (Fig. 4A and B), suggesting that Smad4 plays a negative role in the proliferation of airway progenitor cells.

Table III. Potential transcription factors active in airway progenitor cells by Gather analysis.

n	Gene name	Total gene (n)	Bayes factor	P-value	Genes
1	SMAD4	88	3.9	0.0004	AW210596, Abcc3, Acas2, Acp5, Adhfe1, Afp, Aox1, Arhgap24, Asgr1, Atp2b2, BC004853, BC010843, BC018371, BC024561, Btbd11, Bucs1, C030018L16Rik, Cbs, Ccng1, Ces1, Chn2, Col4a6, Cyp1b1, Cyp2b10, Cyp4f15, D430038H04Rik, Daf1, Dapk2, Dp1l1, Edg7, Efna5, Entpd3, Epdr2, Ephx1, Fmo2, Fmo3, Foxq1, Gabrp, Gdpd2, Gmnn, Gpr120, Gpx2, Gpx3, Grasp, Gss, Gsta3, Gstk1, Gstm2, Gsto1, Hod, Hpgd, Hspa5bp1, Ifit1, Itih5, Kdr, Lnx1, Ltf, Ly6a, Mscp, Nupr1, Oact1, Palmd, Pgam2, Pgd, Pglyrp1, Phlda3 Polr3k, Ppp2r2b, Prdx6, Qpct, Rab6b, Rarb, Rbp4, Samd4, Sema3a Serpinf1, Slc23a1, Stat4, Stxbp6, Sulf2, Tacstd2, Tgfb2, Tmem40, Tnfrsf21, Tnrc9, Trim3, Trim30 U46068
2	XFD3	7	2.3	0.002	Abca4, Ace2, Ces1, Epdr2, Hpgd, Igf1r, Lmyc1
3	DR4	148	1.6	0.005	1810008K03Rik, 4933428I03Rik, 5430413I02Rik, 9030408N13Rik, A630065K24Rik, AW210596, Abca4, Abcc3, Acas2, Ace2, Acp5, Acpp, Adhfe1, Afp, Aldh3a1, Aqp4, Arhgap24, Armcx2, Asgr1, Atp2b2, Atp6v0d2, Azgp1, BC004853, BC010843, BC018371, BC024561, BC048546, Bax, Btbd11, Bucs1, C030018L16Rik, Cbs, Cckar, Ccng1, Ces1, Chn2, Cldn10, Cldn23, Cnga2, Col23a1, Col4a6 Cyp1b1, Cyp2b10, Cyp4f15, D430038H04Rik, Daf1, Dapk2, Dp1l1, Dtna, Edg7, Efna5, Enah, Entpd3, Epdr2, Ephx1, Fign, Fmo1, Fmo2, Foxq1, Frem1, Gabrp, Gcnt2, Gdf15, Gdpd2, Ggh, Gmnn, Gpr120, Gpx2, Gpx3, Grasp, Gss, Gsta2, Gsta3, Gstk1, Gstm1, Gstm2, Gsto1, Hck, Hmox1, Hod, Hpgd, Hspa5bp1, Igf1r, Il1f9, Il6ra, Itih2, Itih5, Kcnk2, Kdr, Kitl, Ldh2, Lif, Lmyc1, Lnx1, Ltf, Ly6a, MGC25972, Mgat3, Mia1, Mscp, Ndn, Nfe2l2, Notch3, Nqo1, Nrarp, Oact1, Palmd, Pgam2, Pgd, Pglyrp1, Phlda3, Pir, Polr3k, Pparg, Prdx6, Ptger4, Qpct, Rab6b, Rad51l1, Rarb, Rbp4, Rpgrip1, Samd4, Scnn1b, Scnn1g, Sdpr, Sec8l1, Sema3a, Serpinf1, Slc1a1, Slc23a1, Slc38a1, Slc9a9, Sphk1, Stat4, Stxbp6, Sulf2, Tacstd2, Tgfb2, Tmem40, Tmie, Tnfrsf21, Tnrc9, Trim30, Ttpa, U46068, Upk3a, Zfhx1a

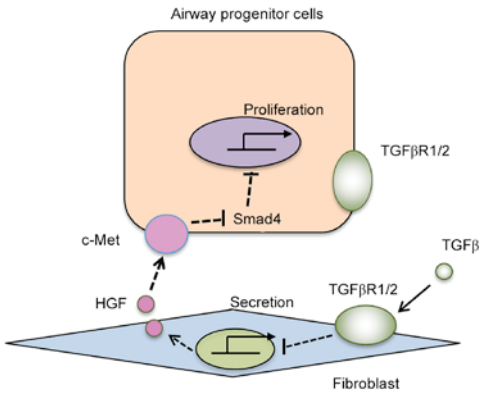

Figure 5. Model for Fibroblasts-airway progenitor cell interaction. The TGF-β signaling inhibits the secretion of HGF in fibroblasts, which binds to its receptor c-Met on airway progenitor cells to promote their growth. Downstream Smad4 may serve as a repressor in the regulating airway progenitor cell function. TGF-β, transforming growth factor-β; HGF, hepatocyte growth factor.

This change was absent, however, between these two groups in the presence of SB431542 (Fig. 4A and B). These data suggest that TGF-β activation is required for the regulation of airway progenitor cells by Smad4.

It was proposed, therefore, that the TGFβ/TGFβR1/2 axis plays an inhibitory role in the expression and/or secretion of growth factors HGF in fibroblasts (Fig. 5). Fibroblast-derived HGF, through its receptor c-Met on airway progenitor cells, supports the proliferation of airway epithelial progenitor cells (Fig. 5). Smad4 may serve as a negative regulator, given its deletion promotes the proliferation of airway progenitor cells (Fig. 5). Epithelial TGFβR1/2 seems not to be involved in the regulation of airway progenitor cell proliferation.

Discussion

In the present study, the authors demonstrated that airway progenitor cell proliferation is not affected by inhibition

of the TGF-β signal or deletion of the TGF-β receptor 2 on progenitor cells in particular. Rather, inhibition of TGF-β signaling specifically in fibroblasts contributes to the proliferation of airway progenitor cells, likely due to the inducible role of TGF-β signaling in the secretion of fibroblast growth factors HGF.

TGF-β signal controls lung progenitor cell fate by altering the microenvironment of the lung progenitor cells niche (23). The TGF-β signal has been implicated in the regeneration of airway epithelium following injury. For instance, the upregulation of TGF-β stimulates extracellular matrix secretion and activates the profibrotic signaling pathway in lung fibrosis (24). Li *et al* (25) demonstrated that deletion of TGF-βR2 on the epithelia prevented mice from bleomycin-induced lung fibrosis. Furthermore, TGF-β signaling promotes differentiation of club cells and alveolar type 2 cells (26-28). These results strongly suggest that TGF-β signaling reduces the proliferation of epithelial progenitor cells and inhibits the regeneration of injured epithelium. In the present study, the authors found that TGF-β indirectly regulates airway progenitor cells. More specifically, TGF-β ligand binds to a cell membrane serine/threonine kinase heteromeric receptor complex, which is composed of type 1 and type 2 receptors. TGFβ1R is known to be inhibited by SB431542 (29). Stimulation of TGFβ1R leads to phosphorylation of Smad2 and Smad3, which combine with Smad4 and translocate into the nucleus (30). Smad plays a critical role in the TGF-β1/Smad signal pathway. Inhibition of TGF-β1/Smad3 signaling induces alveolar repair in the lung of hyperoxic mice (31). It also has been shown that the deletion of Smad4 promotes lung cancer growth and metastasis (32). The authors observed that the proliferation of airway progenitor cells was also promoted in absence of Smad4. Conversely, Smad4 can direct the repair of epithelium under microenvironmental signals.

Growth factors are critical in the regulation of airway epithelial cells by TGF-β. McQualter *et al* (7) found that inhibition of TGF-β can restore stromal cell epithelial-supportive capacity, and upregulate the expression of FGF10 in stromal cells (7). In addition, colonies of airway progenitor cells were apparent in cultures supplemented with FGF10 and HGF in stromal-free conditions by Mungunsukh and Day (33). Similar to FGF10, HGF expression is suppressed by TGF-β signaling post-transcription in a human adult fibroblast culture. In contrast, HGF expression is upregulated by knocking out TGF-β receptor 2 in mammary fibroblasts (34). Consistent with these data, the authors observed that inhibition of TGF-β promotes HGF expression. These findings suggest that blocking TGF-β activates HGF, after which HGF binds to its receptor c-Met on epithelial and endothelial cells to fulfill its functions.

Theoretically, HGF and FGF have been demonstrated to inhibit Smad4 function through activating the Ras/MAPK pathway (19). In the present study, the authors observed that as compared with fibroblasts, airway progenitor cells express c-Met abundantly. c-Met may mediate HGF's role in regulating airway progenitor cells which may be dependent on Smad4. Therefore, the TGFβ-TGFβR1/2-HGF-Smad4 axis may play a role in airway epithelial homeostasis.

Nonetheless, there are still some limitations of the current study. The relationship between HGF and Smad4 was not detected directly, which can be addressed by creating *Sftpc-Cre*$^{+}$*; Met*$^{f/f}$ mice to understand the interactions between c-Met and Smad4 in the regulation of airway epithelial regeneration.

In conclusion, the study suggests that TGF-β represses the proliferation of airway progenitor cells via the TGF-β receptor on fibroblasts, thereby altering their secretory properties including the secretion of HGF. Additionally, Smad4 may play a mechanistic role in the regulation of airway progenitor cells by HGF.

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