SUPPLEMENTAL MATERIALS

Nascent Lung Organoids Reveal Epithelium- and BMP-Mediated Suppression of Fibroblast Activation

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Materials and Methods

Mice and bleomycin-induced lung injury.

All experiments were carried out in accordance with the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). *Col1α1*-GFP transgenic mice were kindly provided by Dr. Derek Radisky and generated as previously described (1). Bleomycin (APP Pharmaceutical, Schaumburg, IL) was delivered to the lungs as previously described (2). 8-week old mice were anaesthetized and 1.2 U/kg bleomycin or PBS was intratracheally delivered using a MicroSprayer (Penn-Century, Philadelphia, PA). The mice were sacrificed on day 7 or 14 after bleomycin delivery.

Cell sorting, cell types and 2D cell culture

Mouse lung fibroblasts were isolated from *Col1α1*-GFP+ mice. Mouse type 2 alveolar epithelial cells were isolated from Sftpc-EGFP+ mice (B6N.Cg-Tg(Sftpc,-EGFP)1Dobb/J), which were purchased from Jackson laboratory (3). Briefly, *Col1α1*-GFP+ mice or Sftpc-EGFP+ mice were anaesthetized and perfused. The lungs were immediately harvested and minced with a razor blade in a 100 mm petri dish in cold DMEM medium (ThermoFisher, Waltham, MA) containing 0.2 mg/ml Liberase DL and 100 U/ml DNase I (Roche, Basel, Switzerland). The mixture was enzymatic digested for 35 minutes and red blood cells were removed with red blood cell lysis buffer (Biolegend, San Diego, CA, USA). The single cell suspension was then incubated with DAPI (1:1000), anti-CD45: PerCp-Cy5.5 (1:200), anti-CD31:PE (1:200), and anti-CD326:APC (1:200) antibodies (Biolegend, San Diego, CA, USA) for 30 minutes on ice. FACS sorting was conducted using a BD FACS Aria II (BD Biosciences, San Jose, CA, USA). DAPI-/CD45-/CD326-/CD31-/GFP+ population were used to isolate *Col1α1*-GFP+ mouse lung fibroblasts. DAPI-/CD45-/CD326+/CD31-/GFP- population were used to isolate lung epithelial cells from Col1α1-GFP+ mouse. DAPI-/CD45-/CD31-/GFP+ were used to isolate *Sftpc*-EGFP+ mouse type 2 alveolar epithelial cells.

C57BL/6 Mouse Primary Tracheal and Bronchial Epithelial Cells (mTEs, Cell Biologics, Chicago, IL) were purchased from Cell Biologics and were cultured in Complete Epithelial Cell Medium (Cell

Biologics, Chicago, IL). Normal Human Lung Fibroblasts (hLFs, Lonza, Basel, Switzerland) were purchased from Lonza and cultured in DMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic. Normal Bronchial epithelial cells (NHBEs, Lonza, Basel, Switzerland) were purchased from Lonza and cultured in Bronchial Epithelial Cell Growth Medium BulletKit (BEGM) (Lonza, Basel, Switzerland). All cells were regularly maintained in at 37 °C in a humidified, 5% CO2 atmosphere. The culture media were changed every other day. Cells were split when reaching 70-90% confluence. Cells from passage 3 to 7 were used in the experiments. For the 2D co-culture experiment, $3x10^5$ mTEs and $3x10^5$ hLFs were seeded in a well of 6 well plate. Epithelial cell medium was used to maintain both cell types. Cells were harvested for RNA extraction at day 3 and day 7.

3D culture, organoid generation and treatment

We adapted a 3D organoid culture model to study interactions between fibroblasts and epithelial cells from prior work (4). Fig 1A represents a schematic of 3D culture used in this study. PneumaCult-ALI Maintennance Medium (Stemcell Technologies, Vancouver, Canada) was combined with Matrigel Matrix (Corning, Corning, NY) in a 3:2 ratio to produce a 40% Matrigel solution. Each well of a 96 well culture plate was coated with 100 µl of the 40% Matrigel solution. The Matrigel layers were then incubated at 37°C for 45 minutes to allow time for polymerization. For the co-culture samples, we did not pre-treat hLFs with mitomycin C, which is used to suppress fibroblast proliferation in other methods(5-7). $1x10^5$ mTEs (or NHBEs) and $1x10^5$ hLFs (mLFs) were suspended in PneumaCult- ALI Maintenance Medium at a 1:1 ratio (unless specified) and seeded on the freshly solidified Matrigel layer. For the mono-culture samples, $1x10^5$ hLF was suspended with same medium and seeded on the top of 40% Matrigel layer. After 24 hours, the cells in the organoids compacted into cell aggregates and were ready for treatment. Cells were then treated with 10 ng/ml BMP4 recombinant human protein (Life Technologies, Carlsbad, CA) 5ng/ml TGF-β1(ThermoFisher, Waltham, MA) or 1µM LDN-193189 (Sigma, St. Louis, MO)alone or in various combinations, all diluted in PneumaCult- ALI Maintenance Medium for 48 hours. The culture media was changed every day during this time frame.

RNA extraction and RT-PCR analysis

Cells in the 3D organoid were removed from Matrigel with Cell Recovery Solution (Corning, Corning, NY). Total RNA was isolated with RNeasy Plus Mini kit (Qiagen, Hilden, Germany) or RNeasy Micro Kit (Qiagen, Hilden, Germany), depending on the number of cells. cDNA was synthesized with SuperScript™ IV Reverse Transcriptase (ThermoFisher, Waltham, MA). Gene expression levels were quantified by qRT-PCR on the Lightcycler 96 Real-Time PCR System (Roche, Basel, Switzerland) according to the manufacturer's instructions. qRT-PCR was performed by incubating the plates at 95 °C for 10 minutes and then cycling 40 times at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Ct values within each experiment were normalized against *GAPDH* or *Actb*. The primers were designed to be mouse or human gene-specific (based on specific sequence differences) and are listed in Table 1. The amplification Ct value from mouse and human fibroblasts 2D monolayer culture were evaluated using human specific *GAPDH* and other primers to demonstrate species specificity, and are presented in table1.

Table E1. Human and Mouse gene primers

Human gene names	**Sequences**	**Ct Value in mouse fibroblasts**	**Ct Value in human fibroblasts**
GAPDH-F	AATGAAGGGGTCATTGATGG	33.74	16.35
GAPDH-R	AAGGTGAAGGTCGGAGTCAA		
ACTA2-F	GTGTTGCCCCTGAAGAGCAT	39.33	21.52
ACTA2-R	GCTGGGACATTGAAAGTCTCA		
COL1A1-F	GAGGGCCAAGACGAAGACATC	43.10	14.96
COL1A1-R	CAGATCACGTCATCGCACAAC		
FN1-F	AGGAAGCCGAGGTTTTAACTG	39.25	13.78
FN1-R	AGGACGCTCATAAGTGTCACC		
CTGF-F	CAGCATGGACGTTCGTCTG	35.82	21.23
CTGF-R	AACCACGGTTTGGTCCTTGG		
Mouse gene names	**Sequences**	**Ct Value in mouse epithelial cells**	**Ct Value in human epithelial cells**
Gapdh-f	AGGTCGGTGTGAACGGATTTG	18.87	39.23
Gapdh-r	TGTAGACCATGTAGTTGAGGTCA		
Epcam-f	GCGGCTCAGAGAGACTGTG	28.21	37.89
Epcam-r	CCAAGCATTTAGACGCCAGTTT		
Cdh1-f	CAGGTCTCCTCATGGCTTTGC	26.78	41.14
Cdh1-r	CTTCCGAAAAGAAGGCTGTCC		
Foxa2-f	TTTTAAACCGCCATGCACTCG	30.56	Not amplifying
Foxa2-r	GTAGTAGCTGCTCCAGTCGG		
Bmp4-f	CCCGGAAGCTAGGTGAGTTC	28.87	Not amplifying
Bmp4-r	AATCCCATCAGGGACGGAGA		

RNA-seq analysis on sorted cell population.

RNA quality was determined using the Fragment Analyzer from AATI (AATI, Ankeny, IA). RNA samples that have RQN values ⩾6 were approved for library prep and sequencing. RNA libraries were prepared using 200 ng of good quality total RNA according to the manufacturer's instructions for the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA), employing poly-A mRNA enrichment using oligo dT magnetic beads. The final adapter-modified cDNA fragments were enriched by 12 cycles of PCR using Illumina TruSeq PCR primers. The concentration and size distribution of the completed libraries were determined using a Fragment Analyzer (AATI, Ankeny, IA) and Qubit fluorometry (Invitrogen, Carlsbad, CA). Libraries were sequenced following Illumina's standard protocol using the Illumina cBot and HiSeq 3000/4000 PE Cluster Kit, yielding 33-40 million fragment reads per sample. The flow cells were sequenced as 100 X 2 paired end reads on an Illumina HiSeq 4000 using HiSeq 3000/4000 sequencing kit and HCS v3.3.52 collection software. Base-calling was performed using Illumina's RTA version 2.7.3. Mayo Clinic's MAPR-Seq software was used to process the raw paired end reads from the RNA sequencing experiments. The raw gene count expression values from MAPR-Seq were then processed by the R package, edgeR, to evaluate differential expression. Genes with an average raw gene count less than 25 in both the 3D Co-culture fibroblasts and 3D fibroblasts groups were excluded from the differential expression analysis. Differentially expressed genes of sorted Col1α1-GFP+ fibroblasts in the presence or absence of epithelial cells were identified using Smyth's moderated t test and Benjamini-Hochberg procedure for adjusted P value (FDR). Genes with a false discovery rate below 0.05, absolute log2 fold change greater than 1, and were annotated by Ensembl as being protein coding were defined as being differentially expressed. The PCA analysis and heatmap were created using a web tool for visualizing clustering of multivariate data (https://biit.cs.ut.ee/clustvis/) and online software Morpheus (https://software.broadinstitute.org/morpheus) based on RPKM (Reads Per Kilobase of transcript, per Million mapped reads) value. Gene set enrichment and pathway analyses were performed using KEGG Pathway Analysis from WEB-based GEne SeT AnaLysis Toolkit (http://www.webgestalt.org/).

Immunofluorescence microscopy and image quantification.

For mice lung tissue, the *Sftpc*-EGFP+ mice were anaesthetized and perfused. The mice lung was inflated with 4% paraformaldehyde (PFA) (Polysciences, Warrington, PA) and placed in 4% paraformaldehyde at 4°C overnight. Tissue was cryo-protected in 15% sucrose at least 24 hours at 4°C and then in 30% sucrose for another 24 hours at 4°C. Tissue was frozen in optimal cutting temperature compound (OCT compound, Fisher HealthCare, Pittsburgh, PA) at -80 °C. For organoids, Each organoid was removed and separated from Matrigel layer with Cell Recovery Solution (Conring), embedded in OCT and frozen at -80°C. 10 μm cryosections were placed on Poly-l-Lysine Coated Slides (Fisher Scientific, Hampton, NH) for immunostaining. Tissues were fixed in 4% paraformaldehyde for 15 minutes and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 15 minutes. The organoids were then treated with 5% goat serum in 1% BSA/PBS (block solution) for 1 hour. After two washes with PBS, the samples were incubated with primary antibody for FITC conjugated α-Smooth Muscle Actin (Sigma-Aldrich, St. Louis, MO) diluted 1:200 or BMP-4 Antibody (Santa Cruz, Dallas, Texas) diluted 1:50 at 4 °C overnight. Specimens were washed twice in PBS and incubated with DAPI (Thermo Fisher Scientific, Waltham, MA) diluted 1:1000 for 1 hour. Samples were mounted with Aqua-Poly/Mount solution (Polysciences, Warrington, PA). Images were acquired using the LSM780 inverted laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) or Olympus CKX53 Inverted Microscope (Olympus, Tokyo, Japan). Digitized images of three representative areas of the organoids or whole organoid were captured and were quantified with ImageJ software. The whole field of each image was selected for acquiring the GFP channel of Integrated Density in the ImageJ software.

Contraction collagen gel assay

The hLFs and mTEs were harvested and suspended in DMEM medium and cold collagen solution. The final collagen (Advanced BioMatrix, San Diego, CA) concentration was 1 mg/mL; the final cell

concentration was hLF= $5x10^5$/mL and mTEs = $5x10^5$/mL. To generate cell suspended hydrogels, 1ml cells and collagen mixture were added into each well of a 12 well plate. The gels were incubated at 37ºC for 40 minutes to promote collagen fibrillogenesis. After collagen fibrillogenesis, 1.0 mL of culture medium (DMEM) was added to each well. The gels were freed from the well edges by gently running the tip of a 200-μL pipet tip along gel edges without shearing or tearing gels. The plates were cultured at 37°C in a humidified 5% CO2 environment. The contraction of gels was recorded using a digital camera to obtain images after 24 hours and measured by ruler.

Western blot assay.

Cells in the 3D organoid were removed from Matrigel with Cell Recovery Solution (Conring) and were harvested into RIPA Lysis Buffer (Thermo Fisher Scientific, Waltham, MA) with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA) Lysates were then quantitated, and equal amounts of protein were subjected to SDS-PAGE on 4-15% gradient gels (Bio-Rad). Due to the close molecular weight of GAPDH and α-SMA, we prepared two gels from the same samples and loading conditions and quantified them separately. PVDF membranes were probed with GAPDH (Cell Signaling Technology, Danvers, MA), α-SMA (Sigma-Aldrich, St. Louis, MO) and Fibronectin (Santa Cruz, Dallas, Texas) antibodies) diluted 1:2000 at 4 °C overnight followed by detection with HRP-conjugated goat anti-rabbit or anti-mouse IgG antibodies diluted 1:2000 in the room temperature for 1 hour. Bands were visualized by using Super Signal West Pico Plus (Thermo Fisher Scientific, Waltham, MA) and ChemiDoc (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's protocol.

Immuno-ECM assay.

Organoids were kept in the original 96 well plate for the immune-ECM assay. The culture media was gently removed from the samples, followed by two washes with PBS. Samples were fixed in 4% paraformaldehyde for 15 minutes. After two additional PBS washes, organoids were treated with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) for 45 minutes. Organoids were incubated with primary polyclonal rabbit antibody for collagen I (Novus NB600-408, Novus Biologicals, Littleton, CO) or

fibronectin (Santa Cruz, Dallas, Texas) diluted 1:200 in Odyssey Blocking Buffer at 4 °C overnight . Organoids were washed twice in PBS and incubated with 100 µL of the corresponding secondary antibodies solution, which were prepared from IRDye® 800CW and IRDye® 680CW secondary antibodies (LI-COR, Lincoln, NE) in Odyssey Blocking Buffer at a 1:750 ratio. The plate was imaged on the Li-Cor Odyssey system (LI-COR, Lincoln, NE). The organoid regions are picked for quantification performed via densitometry and analyzed with Image Studio software. Data are expressed as IR intensity fold changes relative to fibroblasts only control.

BMP luciferase assay.

pGL3 BRE Luciferase was originally generated by Martine Roussel & Peter ten Dijke (Addgene plasmid # 45126)(8). Cells were transfected by Lipofectamine 3000 (Invitrogen, Carlsbad, CA). Luciferase activity was measured via a dual luciferase reporter assay (Promega, Madison, WI) according to manufacturer's protocol, with firefly luciferase activity normalized to renilla activity.

Statistical analysis

All results are expressed as mean ± SEM. Comparisons between groups were made by using an unpaired two-tailed Student's t test. The statistics were analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). P values of < 0.05 were considered statistically significant. All experiments were repeated at least three times, and representative data are shown.

Results

Figure E1. Immunofluorescence imaging of αSMA staining in fibroblasts pre-treated with TGF-β1 followed by organoid culture as hLF alone (A, C, E) or in hLF+mTE co-cultures (B, D, F). Scale bar=50μm.

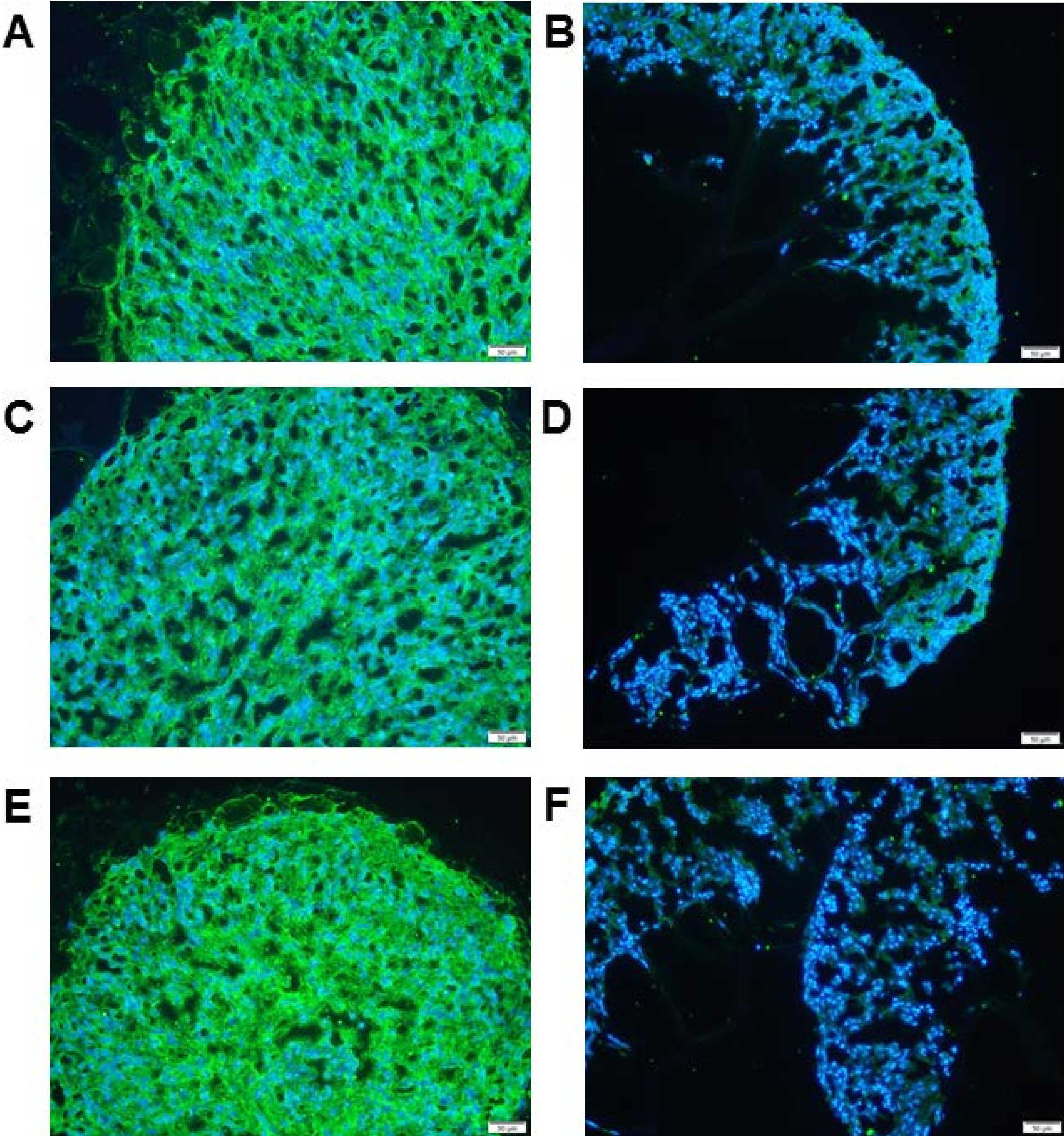
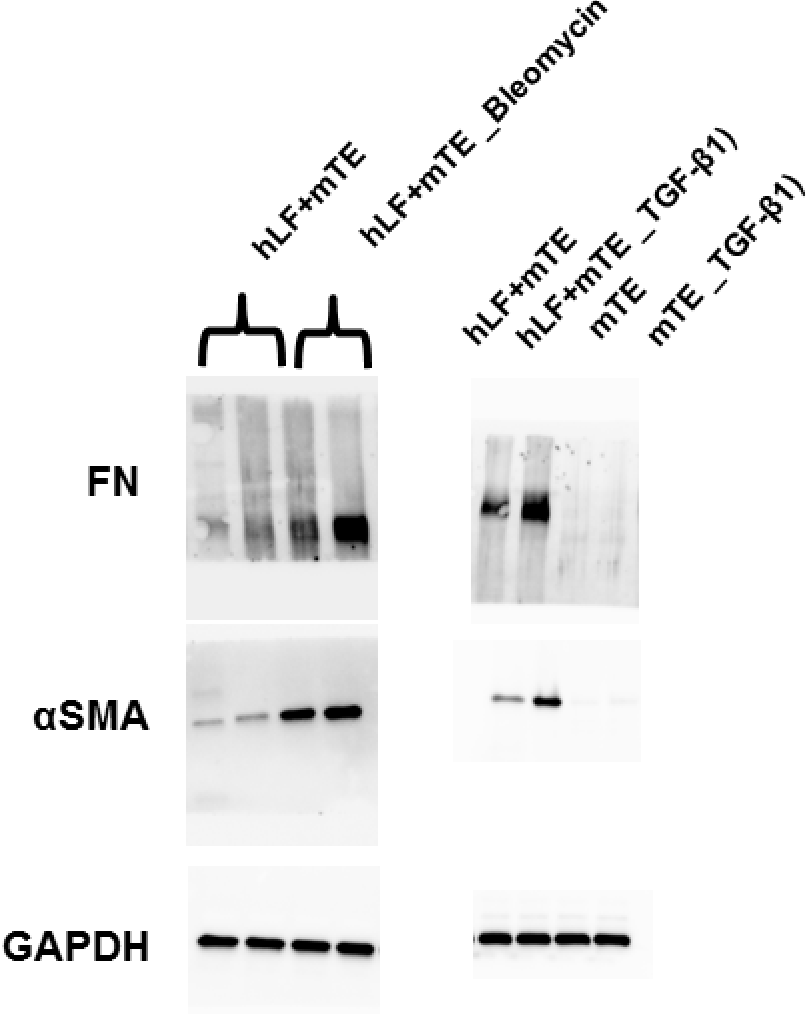


Figure E2. Original blots without any photographic modification from Bio-rad chemidoc imaging system are provided in the online supplement.



References

1. Yata Y, Scanga A, Gillan A, Yang L, Reif S, Breindl M, Brenner DA, Rippe RA. DNase I-hypersensitive sites enhance alpha1(I) collagen gene expression in hepatic stellate cells. *Hepatology* 2003; 37: 267-276.

2. Oh RS, Haak AJ, Smith KMJ, Ligresti G, Choi KM, Xie T, Wang S, Walters PR, Thompson MA, Freeman MR, Manlove LJ, Chu VM, Feghali-Bostwick C, Roden AC, Schymeinsky J, Pabelick CM, Prakash YS, Vassallo R, Tschumperlin DJ. RNAi screening identifies a mechanosensitive ROCK-JAK2-STAT3 network central to myofibroblast activation. *J Cell Sci* 2018; 131.

3. Vanderbilt JN, Gonzalez RF, Allen L, Gillespie A, Leaffer D, Dean WB, Chapin C, Dobbs LG. High-efficiency type II cell-enhanced green fluorescent protein expression facilitates cellular identification, tracking, and isolation. *Am J Respir Cell Mol Biol* 2015; 53: 14-21.

4. Tan Q, Choi KM, Sicard D, Tschumperlin DJ. Human airway organoid engineering as a step toward lung regeneration and disease modeling. *Biomaterials* 2017; 113: 118-132.

5. Hegab AE, Arai D, Gao J, Kuroda A, Yasuda H, Ishii M, Naoki K, Soejima K, Betsuyaku T. Mimicking the niche of lung epithelial stem cells and characterization of several effectors of their in vitro behavior. *Stem Cell Res* 2015; 15: 109-121.

6. Magro-Lopez E, Palmer C, Manso J, Liste I, Zambrano A. Effects of lung and airway epithelial maturation cocktail on the structure of lung bud organoids. *Stem Cell Res Ther* 2018; 9: 186.

7. Ng-Blichfeldt JP, Schrik A, Kortekaas RK, Noordhoek JA, Heijink IH, Hiemstra PS, Stolk J, Konigshoff M, Gosens R. Retinoic acid signaling balances adult distal lung epithelial progenitor cell growth and differentiation. *EBioMedicine* 2018; 36: 461-474.

8. Korchynskyi O, ten Dijke P. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. *J Biol Chem* 2002; 277: 4883-4891.