Stem Cell Reports

Supplemental Information

Acellular Lung Scaffolds Direct Differentiation of Endoderm to Functional Airway Epithelial Cells: Requirement of Matrix-Bound HS Proteoglycans

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Figure S1

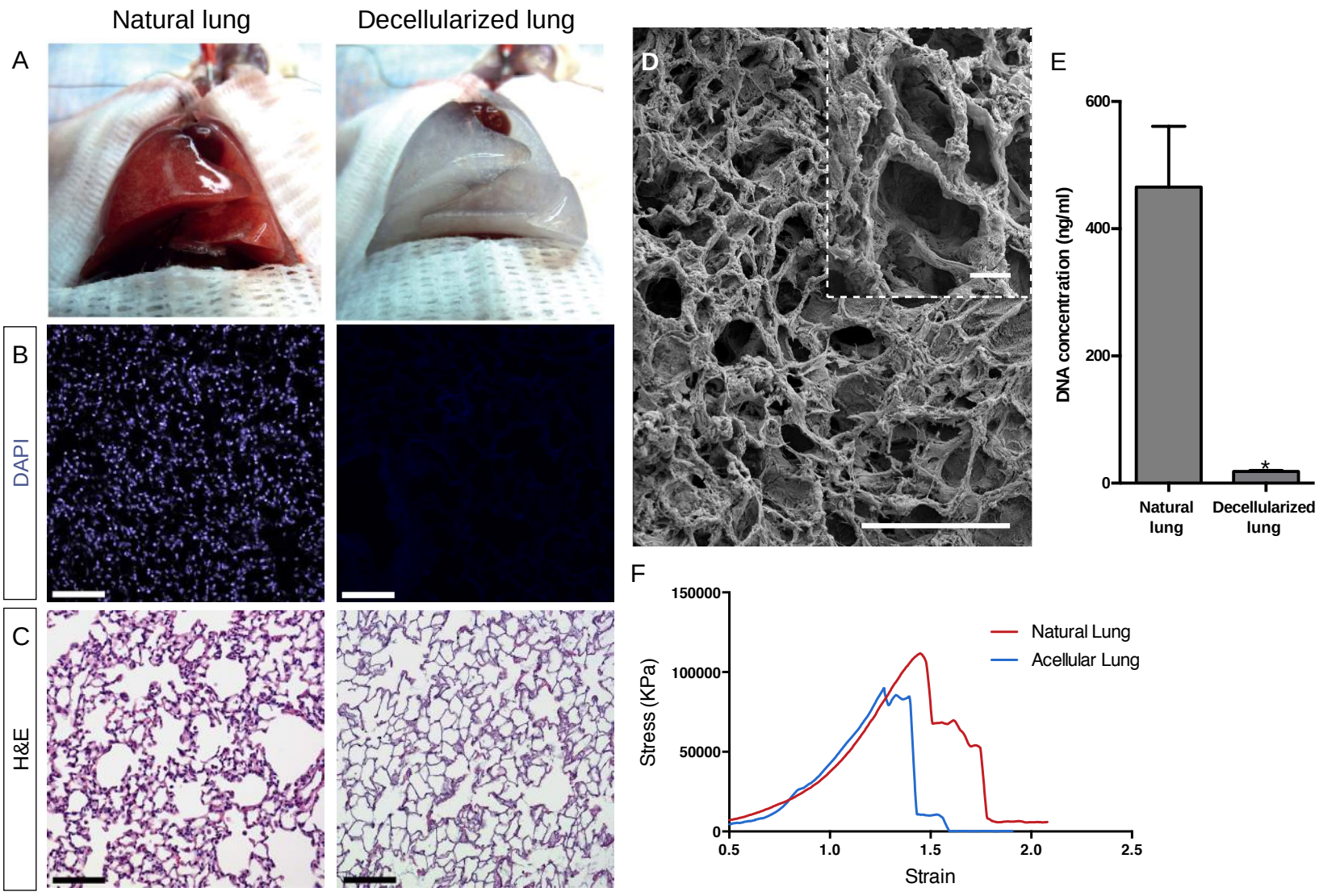
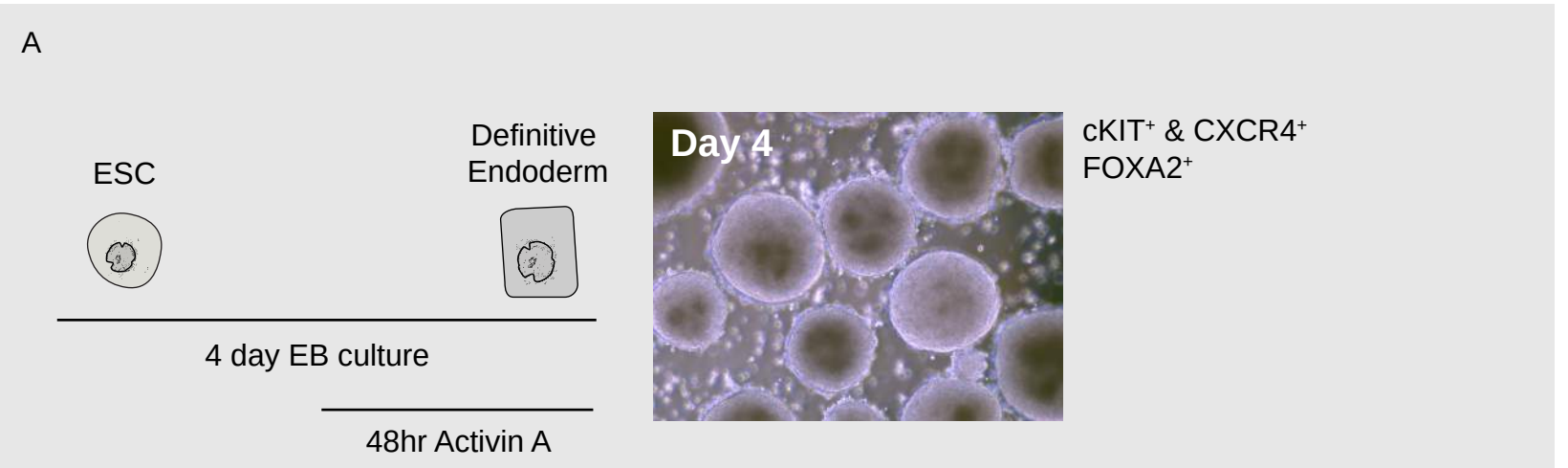
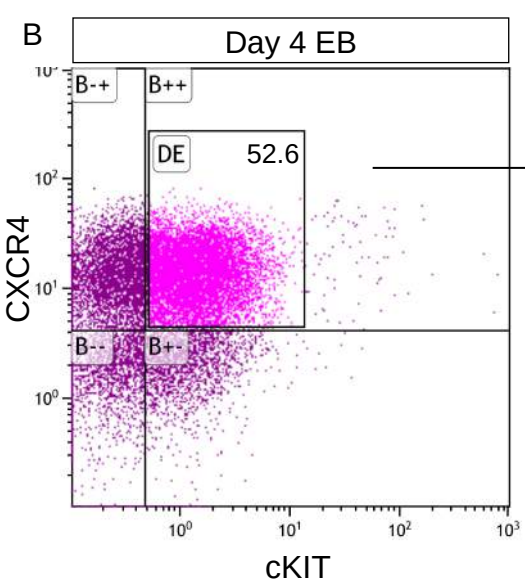
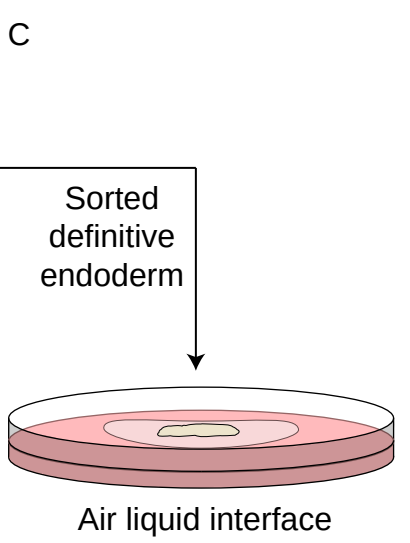
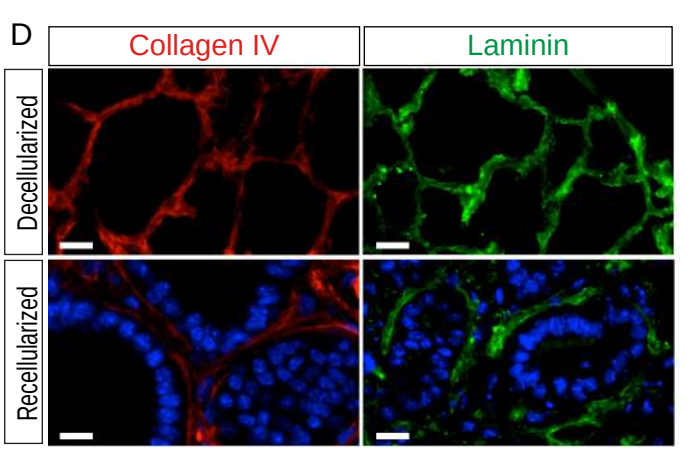

Natural lung
Decellularized lung
A
B
DAPI
C
H&E
D
E
DNA concentration (ng/ml)
600
400
200
0
*
Natural lung
Decellularized lung
F
Stress (KPa)
150000
100000
50000
0
0.5
1.0
1.5
2.0
2.5
Strain
Natural Lung
Acellular Lung
G
Col-I DAPI
Col-IV DAPI
Lmn DAPI
HSPG DAPI
Fn DAPI
Natural lung
Acellular lung
H
Elastin
I
Rinses post-decellularization
5 x
10 x
20 x
Natural lung
Laminin
268
238
Ponceau S
J

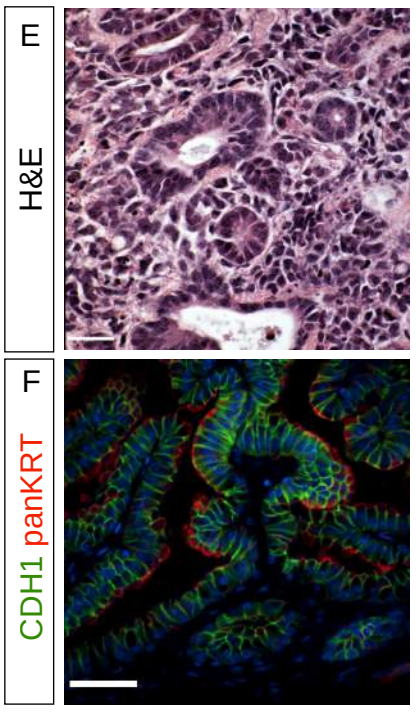
Figure S2


A
ESC
Definitive Endoderm
Day 4
cKIT+ & CXCR4+
FOXA2+
4 day EB culture
48hr Activin A


B
Day 4 EB
B-+
B++
DE
52.6
B--
B+-
CXCR4
cKIT


C
Sorted definitive endoderm
Air liquid interface


D
Collagen IV
Laminin
Decellularized
Recellularized


E
H&E
F
CDH1 panKRT

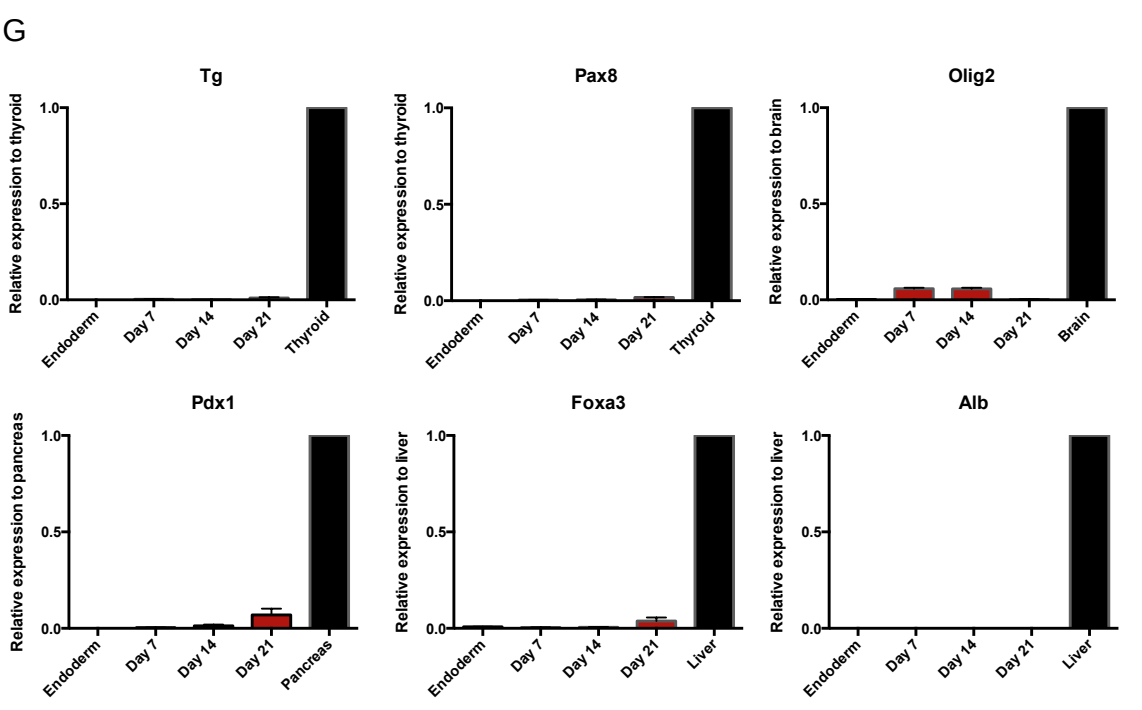

G
Tg
Pax8
Olig2
Pdx1
Foxa3
Alb
Relative expression to thyroid
Relative expression to brain
Relative expression to pancreas
Relative expression to liver
Endoderm
Day 7
Day 14
Day 21
Thyroid
Brain
Pancreas
Liver

Figure S3

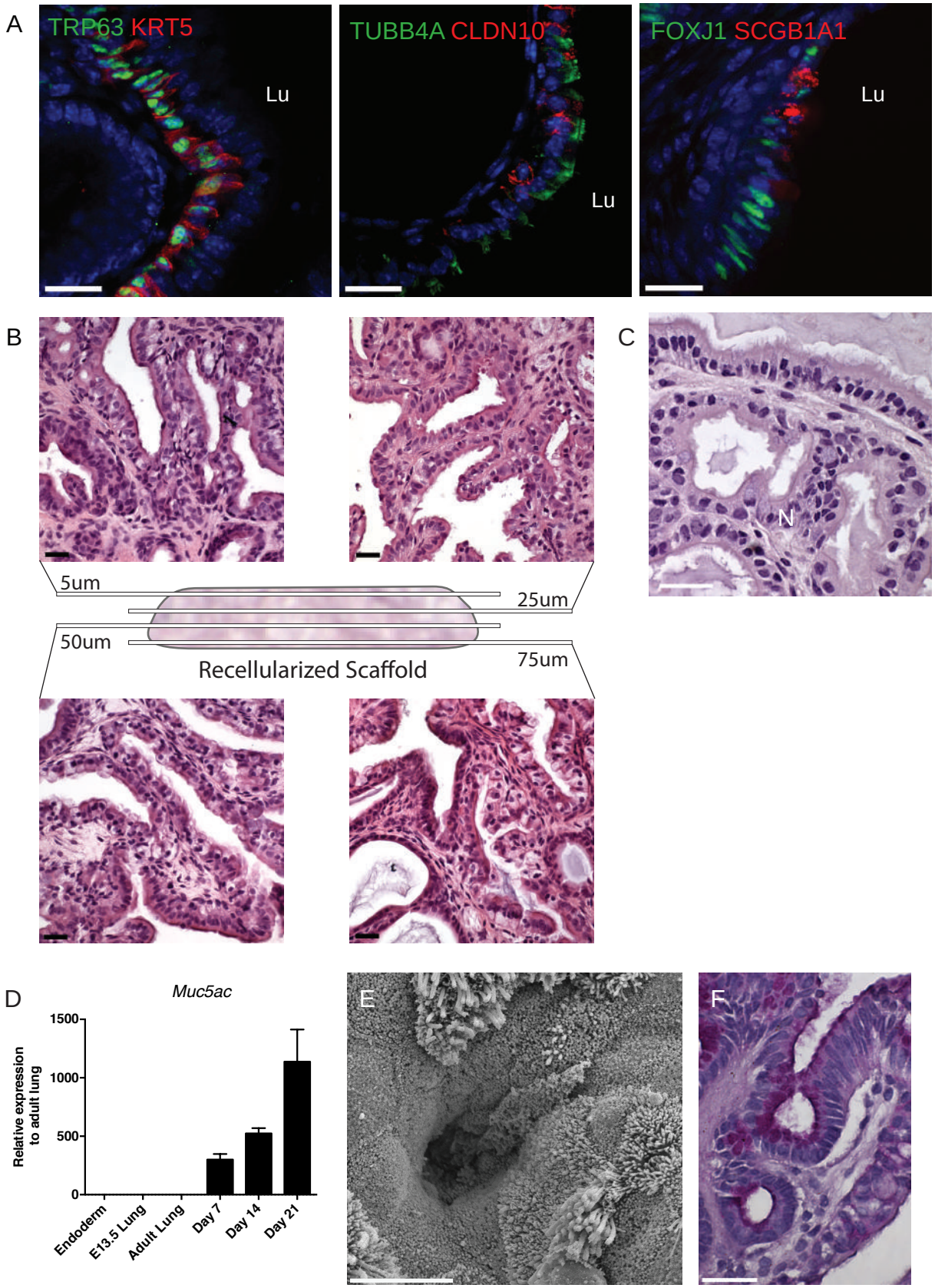

A
TRP63 KRT5
Lu
TUBB4A CLDN10
Lu
FOXJ1 SCGB1A1
Lu
B
5um
25um
50um
75um
Recellularized Scaffold
C
N
D
Muc5ac
Relative expression to adult lung
1500
1000
500
0
Endoderm
E13.5 Lung
Adult Lung
Day 7
Day 14
Day 21
E
F

Figure S4

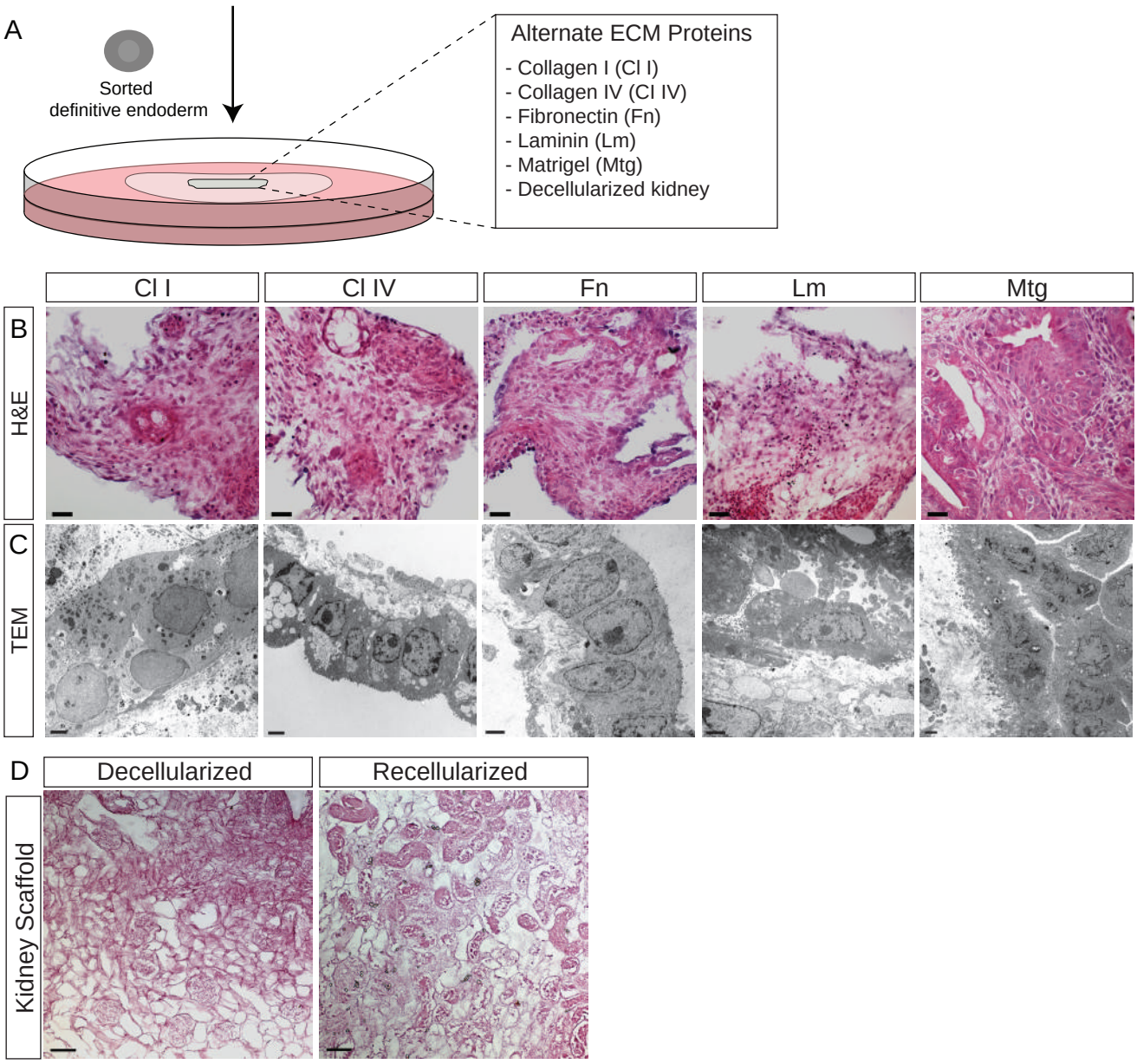

A
Sorted
definitive endoderm
Alternate ECM Proteins
- Collagen I (Cl I)
- Collagen IV (Cl IV)
- Fibronectin (Fn)
- Laminin (Lm)
- Matrigel (Mtg)
- Decellularized kidney
Cl I
Cl IV
Fn
Lm
Mtg
B
H&E
C
TEM
D
Decellularized
Recellularized
Kidney Scaffold

Figure S5

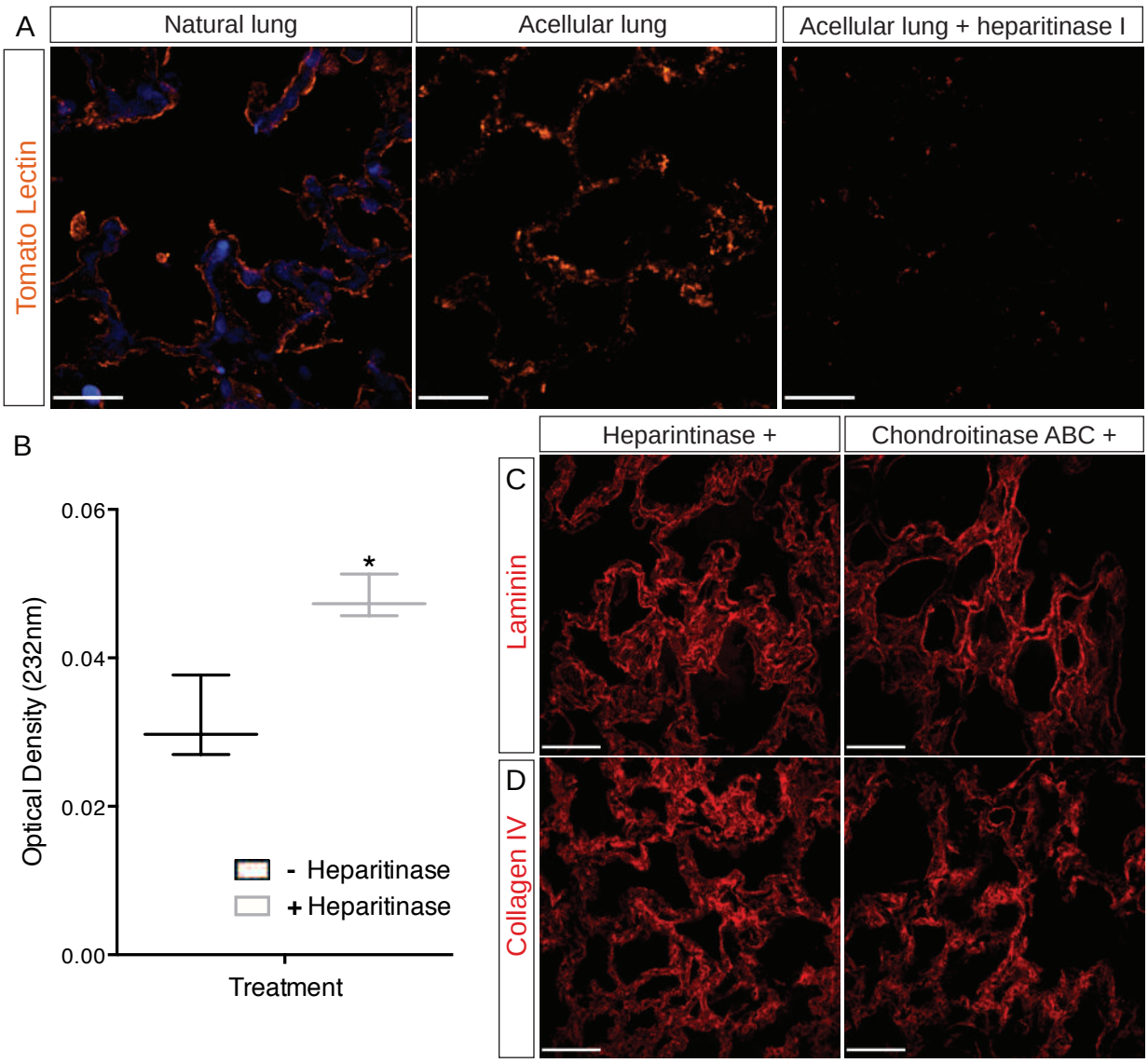

A
Natural lung
Acellular lung
Acellular lung + heparitinase I
Tomato Lectin
B
Optical Density (232nm)
0.06
0.04
0.02
0.00
*
- Heparitinase
+ Heparitinase
Treatment
Heparintinase +
Chondroitinase ABC +
C
Laminin
D
Collagen IV

Figure S1, Related to Figure 1. Decellularization of Adult Rat Lungs Removes Cellular Components While Maintaining Extracellular Matrix Structure and Protein Composition.

(A) Photographic representation of a natural lung (left panel) and an acellular lung (right panel) following decellularization with CHAPS-based solution. **(B-C)** Corresponding hematoxylin & eosin (H&E) and DAPI staining of natural and acellular lung tissue sections show the absence of cellular material including nuclei following decellularization. Scale bars=50µm. **(D)** Scanning electron microscope (EM) analysis following decellularization show the surface of acellular lung scaffolds with intact matrix architecture. Scale bar=100µm, scale bar of inset=10µm. **(E)** Removal of DNA following Benzonase treatment of decellularized scaffolds was confirmed with DNA assay. Mean ± SEM, n=3, *p<0.01. **(F)** Tensile testing represented by stress versus strain plots of natural and decellularized scaffolds characterize mechanical properties of scaffolds. Acellular scaffolds have a similar tensile strength curve profile, although with a lower overall peak strength, to natural lungs. **(G)** Natural (top panel) and acellular lungs (lower panel) were examined for ECM protein composition. Immunostaining analysis shows intact collagen I (Col-I), collagen IV (Col-IV), laminin (Lmn), heparan sulfate proteoglycans (HSPG), and fibronectin (Fn), while DAPI staining is absent from decellularized tissue. Scale bars =25µm. **(H)** Hart's elastin stain shows intact elastin fibers in scaffolds following decellularization. Scale bar=25µm. **(I)** Immunoblot analysis for basement membrane-protein laminin following 5, 10, and 20 washes after decellularization shows that scaffolds maintain laminin protein composition for up to 10 rinses. Ponceau stain was used as loading control, presented band is at 50kDa. **(J)** Transmission EM analysis shows decellularized scaffolds with positive immunogold staining for laminin (black arrowheads). Scale bar=500nm.

Figure S2, Related to Figure 1 and Figure 2. Decellularized Lung Scaffolds are Repopulated with ES-derived Definitive Endoderm Cells and Maintained at Air Liquid Interface.

(A) Definitive endoderm induction ($FOXA2^+$, $cKIT^+$ & $CXCR4^+$) is achieved with 48hr treatment of ES-derived embryoid bodies with 50ng/ml of Activin A. **(B)** Endodermal

cells are sorted for cKIT and CXCR4 using fluorescence-activated cell sorting to obtain an enriched population for recellularization. **(C)** Schematic representation of recellularization setup. Sorted definitive endoderm cells are seeded on thick sections (300-400µm) of acellular lung scaffold and cultured on a floating hydrophobic polycarbonate membrane to achieve an air-liquid interface, in serum- and growth factor-free supportive media. **(D)** Seeded endodermal cells repopulate scaffolds and organize into luminal structures. Immunostaining for collagen IV and laminin shows that these scaffold proteins line the developing cellular (DAPI positive) structures. **(E)** H&E staining of scaffold cultures reveals organization of seeded endodermal cells into luminal structures (day 7). Scale bar=50µm. **(F)** Positive immunostaining for panKRT and CDH1 reveals presence of epithelial cells. Scale bar=50µm. **(G)** Real time-PCR analysis shows that relative to expression of adult tissue (mean ± SEM, n=4 experiments), thyroid lineage makers *Tg* and *Pax8*, and neuroectoderm marker *Olig2* are hardly detected in seeded scaffolds after 7, 14, and 21 days of culture. Expression of additional endoderm lineage markers including *Pdx1* (pancreas), *Foxa3* (posterior endoderm), and *Albumin* (liver) are also marginally detected.

Figure S3, Related to Figure 2 and Figure 3. Prolonged Culture of Mouse- or Rat-derived Endoderm on Lung Scaffolds Promotes Differentiation to Airway Epithelial Cells

(A) Immunostaining of day 21 cultures for airway lineage markers shows the presence of basal cells ($TRP63^+$, $KRT5^+$) (left image), ciliated cells and club cells [$TUBB4A^+$, $CLDN10^+$ (center image), and $FOXJ1^+$, $SCGB1A1^+$ (right image)]. Scale bars=25µm. **(B)** Hematoxylin and eosin staining of day 21 tissue sections generated from various depths shows repopulation of entire decellularized scaffolds. **(C)** Decellularized rat scaffolds support repopulation and differentiation of Rat ESC (DAc8 cell line)-derived definitive endoderm to airway epithelial cells, similar to that achieved with mouse ESC lines. Scale bar=50µm. **(D)** RT-PCR analysis reveals upregulation of secretory cell marker *Muc5ac* in seeded cells with extended culture. Gene expression is presented relative to adult lung; mean ± SEM, n=3 experiments. **(E)** Scanning EM analysis reveals the presence of pit structures with ciliated and secretory cells lining the orifice. Scale

bar=5µm. **(F)** Pit structures present on epithelial surfaces show a strong PAS positive stain suggestive of mucin-producing cells lining these structures. Scale bar=50µm.

Figure S4, Related to Figure 2. Endoderm Culture Without Lung Scaffold Does Not Promote Differentiation to a Lung Phenotype

(A) Schematic representation of alternate scaffold recellularization. Sorted endodermal cells are seeded on specific matrix proteins, Matrigel or decellularized kidney scaffolds and cultured at air-liquid interface in base supportive media. **(B-C)** H&E staining and transmission EM of seeded endodermal cells reveal the inability of isolated matrix proteins and Matrigel to promote differentiation to lung epithelial cells. H&E scale bar=50µm, EM scale bar=2µm. **(D)** Decellularized kidney scaffolds seeded with definitive endoderm and cultured at air liquid interface cannot support the adherence, proliferation, and differentiation of sorted endodermal cells. Scale bar=50µm

Figure S5, Related to Figure 4. Enzymatic Treatment of Acellular Scaffolds with Heparitinase I Cleaves Heparan Sulfate Proteoglycans.

(A) Natural, acellular and heparitinase I-treated acellular scaffolds were stained for heparan sulfate using biotinylated tomato lectin. Immunostaining reveals the effective cleavage of heparan sulfate proteoglycans following heparitinase I treatment. Scale bar=25µm. **(B)** Cleaved heparan sulfate from scaffolds is detected in the remaining supernatant following heparitinase I treatment using UV spectral analysis for optical density at 232nm. This confirms the efficacy of the enzymatic treatment and removal of heparan sulfate proteoglycans from scaffolds. N=3 experiments; mean ± SEM, *$p<0.01$. **(C-D)** Immunostaining for laminin and collagen IV reveals that the basement membrane remains intact following enzymatic treatment with heparitinase I and chondroitinase ABC. Scale bar=50µm.

Movie S1 – Beating Ciliated Cells in Mature Cultures, Related to Figure 3.

Antibody Information

Target	Host, Conjugation	Company	Catalogue Number	Application, Dilution
Primary Antibodies				
CDH1	Mouse, non-conjugated	BD Biosciences	610181	IF, 1:100
CFTR	Rabbit, non-conjugated	Abcam	Ab181782	IF, 1:200
c-KIT	Rat, PE-Cy7	BD Biosciences	558163	FACS, 1:100
CLDN10	Rabbit, non-conjugated	Santa Cruz	sc-25710	IF, 1:350
Collagen I	Rabbit, non-conjugated	Abcam	ab34710	IF, 1:200
Collagen IV	Rabbit, non-conjugated	Abcam	ab6586	IF, 1:200
CXCR4	Rat, APC	BD Biosciences	558644	FACS, 1:100
Fibronectin	Rabbit, non-conjugated	Abcam	ab23750	IF, 1:200
FOXA2	Mouse, non-conjugated	Abcam	ab60721	1:200
FOXJ1	Goat, non-conjugated	Santa Cruz	sc-54371	IF, 1:200
HSPG	Rat, non-conjugated	Abcam	ab2501	IF, 1:100
Ki67	Rat, non-conjugated	DAKO	M7249	IHC, 1:100
pan-KRT	Rabbit, non-conjugated	DAKO	ZO622	IF, 1:800
KRT5	Rabbit, non-conjugated	Abcam	ab24647	IF, 1:1000
Laminin	Rabbit, non-conjugated	Novus Biologicals	NB300-144	IF, 1:200 WB, 1:1000
NKX2-1	Rabbit, non-conjugated	Abcam	ab76013	1:200
POU5F1	Rabbit, non-conjugated	Cell Signaling	2788-s	1:200
SCGB1A1	Goat, non-conjugated	Santa Cruz	sc-9772	IF, 1:1000 WB, 1:1000
SOX2	Goat, non-conjugated	R&D Systems	AF2018	IF, 1:400
SOX9	Goat, non-conjugated	R&D Systems	AF3075	IF, 1:400
TJN1	Rabbit, non-conjugated	Invitrogen	40-2200	IF, 1:200
TRP63	Mouse, non-conjugated	Santa Cruz	sc-8431	IF, 1:200
TUBB4A	Mouse, non-conjugated	BioGenex	MU178-UC	IF, 1:500
Secondary Antibodies				
Goat IgG	Donkey, Alexa Fluor 647	Invitrogen	A-31573	1:200
Goat IgG	Donkey, Alexa Fluor 488	Invitrogen	A-21202	1:200
Mouse IgG	Donkey, Alexa Fluor 488	Invitrogen	A-11055	1:200
Rabbit IgG	Donkey, Alexa Fluor 546	Invitrogen	A-11056	1:200
Rat IgG	Goat, Alexa Fluor 488	Invitrogen	A-11006	1:200

FACS: Fluorescence-activated cell sorting; IF: Immunofluorescent staining; WB: Western blot

RT-PCR Primers

Gene	Primer Source, Catalogue Number / sequence	
Alb	CCTAGGAAGAGTGGGCACCAAGTGT	AGCAGAGAAGCATGGCCGCCTTTC
Aqp5	TATCCATTGGCTTGTCGGTCAC	TCAGCGAGGAGGGGAAAAGCAAGTA
Cftr	Qiagen, QT00114604	
Foxa2	AAAGTATGCTGGGAGCCGTGAA	CGCGGACATGCTCATGTATGTGTT
Foxa3	Qiagen, QT01657705	
Foxj1	Qiagen, QT00111097	
Muc5ac	Qiagen, QT01196006	
Nkx2-1	TATGCTTCATGGCCCTGAACT	TTTCCTATCTCCAGCGTCTGTCCT
Olig2	AATGCGCGATGCGAAGCTCTTT	AAGCCCACGTTGTAATGCAGGT
Pax8	TCGACTCACAGAGCAGCAGCAGT	AGGTTGCGTCCCAGAGGTGTATT
Pdx1	Qiagen, QT00102235	
Scgb1a1	TCCGCTTCTGCAGAGATCTG	TGAAGAGAGCAACAGCTTTG
Sftpb	Qiagen, QT01537529	
Sftpc	Qiagen, QT00109424	
Sox2	Qiagen, QT01539055	
Sox9	Qiagen, QT00163765	
Tg	Qiagen, QT00116592	
Trp63	Qiagen, QT00197904	

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

ESC Culture and Endoderm Induction

Mouse ESC lines (R1 (*Nkx2-1*^{mCherry})(Bilodeau et al., 2014), G4 (*dsRed-MST*), and129/Ola (*Bry-GFP*/*Foxa2-hCD4*)) and rat ESC line (DAc8) were maintained below passage 40, in the pluripotent state under feeder-free, serum-free culture using 2i conditions as outlined in previously published reports (Ying et al., 2008). The 2i media base consists of DMEM/F12 and Neurobasal Medium (GIBCO) in a 1:1 ratio. This is supplemented with B-27 (with RA) and N-2 supplements (GIBCO), fraction V bovine serum albumin (GIBCO), penicillin/streptomycin (GIBCO), Glutamax (GIBCO), 4μM monothioglycerol (sigma), 1000U/mL LIF (Millipore), 1μM PD0325901 (Stemgent), and 3μM CHIR99021 (Stemgent). Endoderm induction was achieved using a four day embryoid body (EB) culture method with activin A treatment previously outlined in published reports (Gouon-Evans et al., 2006) using serum-free differentiation medium (SFDM). Single cells were seeded at a 20,000 cells/ml density in low adherent plates (Nunc, Roskilde, Denmark) to allow for EB formation for 48 hours. Day 2 EBs were collected and reseeded at 1:2 density in SFDM supplemented with 50ng/ml activin A for an additional 48 hours. Day 4 EBs were harvested and sorted by fluorescence activated cell sorting for cKIT^{+}/CXCR4^{+} cells representing definitive endoderm.

Anterior endoderm was generated for use in RT-PCR as a control. Anteriorization was achieved by seeding sorted definitive endoderm cells as a monolayer on gelatin coated 6-well plates in SFDM supplemented with 10μM SB431542 (Sigma, 431542) and 100ng/ml Noggin (R&D Systems, 1967-NV/CF) for 24hours. Anteriorization was confirmed using RT-PCR analysis showing a reduction in *Foxa3* expression (posterior endoderm).

Decellularization of Lungs

All animal experiments were approved and carried out in accordance with the animal care committee guidelines of the Hospital for Sick Children Research Institute. Decellularization of mouse and rat lung scaffolds was achieved by sequential tracheal lavages and retrograde pulmonary arterial perfusion using a CHAPS-based decellularization solution, without requiring a bioreactor. The heart and lungs were

accessed by a median sternotomy and the trachea was cannulated with a plastic catheter near the thyroid cartilage and secured in place with a suture. Lungs were inflated with phosphate buffered saline (PBS)(no Ca^{2+} and Mg^{2+}) via the tracheal catheter to total lung capacity to assist perfusion (<12 cmH_2O in mice, <20 cmH_2O in rats). A 25-gauge syringe inserted through the right ventricle was used to access the pulmonary artery and allow perfusion with 10U/mL heparinized HBSS- (Sigma, H0777) to remove blood cells. The inferior vena cava was ligated and the left atrium slit to indirectly drain the pulmonary veins. The lungs were decellularized with sequential tracheal lavages (10 lavages) with decellularization solution (8mM CHAPS, 25mM EDTA, 1M NaCl in PBS), followed by extensive rinsing with PBS (10-15 lavages). Lungs were then removed from the animal and exposed to 90U/ml Benzonase endonuclease (Novagen, 70664-3) for 12 hours while on a rotator at room temperature to remove cellular components. Scaffolds were then treated with antimicrobial agents, 200U/mL pen/strep (Gibco, 15140) and 25µg/mL amphotericin B (Gibco, 15290) for 6 hours at room temperature to prepare for recellularization. Thick vibratome sections (Leica) of acellular lung were generated at approximately 350µm and conditioned with SFDM media prior to recellularization.

Recellularization of Thick Scaffold Sections

Thick sections of decellularized lung were transferred to Nucleopore hydrophobic floating membranes (8µm pore size, Whatman, 110614) and seeded with 100,000cells/section using sorted $cKIT^+/CXCR4^+$ endodermal cells. Cultures were maintained in 6-well plates in SFDM, without any additional inductive factors. Media was changed every 48 hours for up to 21 days.

DNA Assay

For DNA assay, DNA was extracted from decellularized lung scaffolds using a chloroform/phenol extraction method. DNA was measured by UV absorbance in microplates using a SpectraMax Absorbance Microplate Reader equipped with SoftMax Pro Data Acquisition & Analysis software.

Tensile Testing

Natural and decellularized sections of lung underwent tensile testing using Mach-1 Motion setup and software (Biosyntech). Sample sections (1-2cm^2 cross-sectional area and 500µm thick) were stretched until break point to assess the ultimate tensile strength, represented by stress-strain curves.

Immunostaining

Cell-scaffold cultures were fixed in 4% paraformaldehyde (PFA) at 4°C for 8 hours. Samples were paraffin embedded and sectioned at 5µm. Sample sections were rehydrated and heat-induced epitope retrieval with citrate buffer was performed. Slides were then blocked for one hour with 5% normal donkey serum and 1% BSA in PBS for 1 hour at room temperature. Samples were then incubated with primary antibodies at 4°C overnight in humidified chamber, and detected using Alexa Fluor conjugated secondary antibodies (Invitrogen) for one hour at room temperature. Nuclei were counterstained using DAPI (Invitrogen). Details of antibodies are provided in the Antibody Table. Images were captured with Leica CTRMIC 6000 confocal microscope and Hamamatsu C910013 spinning disc camera (Leica Microsystems Inc.), and analyzed with Volocity software (PerkinElmer).

For immunohistochemical stain of Ki67, sections were rehydrated and heat-induced epitope retrieval with citrate buffer was performed. Sections were immunostained for Ki67 expression using a standard procedures with a Ki-67 primary antibody diluted in blocking buffer and incubated overnight at 4°C in humidified chamber. Slides were washed thoroughly with PBS and primary antibody was detected by using biotinylated rabbit anti-rat secondary antibody (Jackson ImmunoResearch) diluted 1:200, followed by ABC kit (from Vector). The color was developed by addition of diaminobenzidine (DAB) (Sigma) and counterstained with Mayer's hematoxylin (Sigma, H9627).

For transferase (TdT)-mediated dUTP nick end labeling (TUNEL) of apoptotic cells, tissue slides were rehydrated and antigen retrieval was completed as stated above. Assay was completed as per manufacturer's protocol using TUNEL enzyme (Roche, 11767305001) and TUNEL Label Mix (Roche, 11767291910).

Flow cytometry and cell sorting

For definitive endoderm, day 4 EBs were dissociated using TrypLE express (Gibco), for 3 minutes at 37°C. Single cell suspensions were labeled with cKIT and CXCR4 antibodies in Sort Buffer (Hank's balanced salt solution (HBSS-) supplemented with 2% (v/v) fetal bovine serum and 10mM HEPES buffer) for 20 minutes on ice. Cells were sorted using AriaII-GC (BD Biosciences) and data analysis was carried out using Diva (BD Biosciences) and FlowJo (TreeStar) software.

For identification of mcherry positive cells differentiated on scaffolds, day 7, 14, and 21 cultures were dissociated to isolate seeded cells for flow cytometric quantification. Cell-scaffold tissue cultures were incubated in an elastase solution (1mg/mL elastase in SFDM with 2% fetal bovine serum (FBS)), at 37°C for 30 minutes. Tissue was spun to remove elastase, and placed in FBS with 2.5% DNAse and minced for one minute at 4°C, followed by vigorous shaking for 2 minutes to aid in releasing of cells. SFDM was added to mixture, and minced tissue was filtered using a pre-wetted 20µm nylon mesh filter. Cellular recovery using this method were as follows, day 7 cultures: $3.63x10^5 \pm 1.1x10^4$ (n=3), day 14 cultures: $4.55x10^5 \pm 5.3x10^4$ (n=3), day 21 cultures: $9.43x10^5 \pm 1.9x10^4$ (n=3). Cells were collected into Sort Buffer and analyzed for mcherry expression using a MoFlo (Beckman Coulter) apparatus and Diva (BD Biosciences) software.

Real Time Quantitative PCR

RNA was extracted from cell-scaffold cultures using PicoPure RNA Isolation Kit (Life Technologies), and cDNA synthesis was carried out with 1ng of RNA using SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. Ten micrograms of template cDNA was used for real-time PCR (40 cycles of amplification) using SYBR GreenER qPCR SuperMix with murine specific primer sets, listed in RT-PCR Primer Table. Analysis was performed using StepOnePlus qPCR (Applied Biosystems). Gene expression was normalized to RNA Polymerase II and expressed relative to selected positive (adult tissue, E13 lung) or negative controls (definitive endoderm).

Immunoblot Analysis

For laminin detection, 50µg of solubilized extracellular matrix supernatant (in RIPA buffer), and 1µg of purified laminin protein as positive control, were separated on a 5% SDS-PAGE gel. For SCGB1A1 detection, 50µg of day 21 cell-scaffold culture media, and rat lung lysate as positive control, were separated on a 3%–12% gradient SDS-PAGE gel (Invitrogen). Proteins were transferred to nitrocellulose paper (Millipore) and blocked with 3% milk in PBS plus 0.1% Tween-20. The membranes were then incubated overnight at 4°C with anti-laminin or anti-SCGB1A1 antibodies at 1:1000 dilution in PBS-milk 1% containing 0.2% Tween-20. Blots were then incubated for 1 hour with respective secondary antibodies conjugated with peroxidase, and were developed using chemiluminescent substrates (PerkinElmer) according to the manufacturer's instructions and imaged on X-ray film.

Histological Analysis

Cell-scaffold cultures were fixed in 4% paraformaldehyde (PFA) at 4°C for 8 hours. Samples were paraffin embedded and sectioned at 5µm. Slides were rehydrated and standard hematoxylin and eosin staining, Hart's elastin stain, and periodic acid-Schiff (PAS) stain was performed.

Electron Microscope Analysis

Samples were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer pH7.4. Transmission EM specimens were post-fixed in osmium tetroxide, dehydrated in an ascending series of acetone, infiltrated and embedded in Epon Araldite prior to polymerization at 60° C overnight. Ultrathin sections were then cut and mounted on grids, and stained with uranyl acetate and lead citrate prior to microscopy (JEOL, JEM1011) and image acquisition was completed using a CCD camera (AMT corp.). Scanning EM samples were post-fixed in osmium tetroxide, dehydrated in an ascending series of ethanols and critical point dried. Samples were then mounted on aluminum stubs using double-sided carbon tape. Samples were rendered conductive with a thin coat of gold palladium using a sputter coater and examined and photographed in a field emission scanning EM (JEOL, JSM 6700F).

Efflux Assay

Iodide efflux from differentiated cultures following cAMP agonist stimulation was measured periodically to assess CFTR channel activity, as CFTR is permeable to iodide. Day 21 scaffold cultures were loaded with sodium iodide solution (3.0mM KNO_3, 2.0mM $Ca(NO_3)_2$, 11mM glucose, 20mM HEPES, 136mM NaI) in a 6-well plate for 1h at 37°C, for iodide uptake (three cell-scaffold cultures were pooled for each assay) measurements. Samples were then washed 10 times with wash solution (3.0mM KNO_3, 2.0mM $Ca(NO_3)_2$, 11mM glucose, 20mM HEPES, 136mM $NaNO_3$) and epithelium sodium channel (ENaC)-specific inhibitor amiloride (100µM). The last wash was collected as a blank reading, followed by eight sequential readings of cAMP-stimulated halide flux using 300µl of wash solution containing FIG (10µM forskolin, 100µM 3-isobutyl-1-methylxanthine, and 50µM genistein). Each reading represented 1 minute of exposure to FIG wash solution. Vehicle dimethyl sulfoxide (DMSO) was used as a negative control reading. Solutions from each one-minute time point were collected into a 96 well plate and absolute iodide electrode potential value (mV) was measured using a halide-selective microelectrode (Lazar Research Laboratories). Readings were recorded using Digidata 1320A Data Acquisition System and Clampex 8.1 software. Using a calibration curve, recorded mV values were converted to iodide concentration in µM.

Enzymatic Treatment of Scaffolds

Decellularized scaffolds were treated with Heparitinase I solution (0.1M sodium acetate, 10mM calcium acetate, 10mU heparitinase I (Amsbio, 100704)) for 4 hours at 37°C. Enzymatic activity was confirmed by UV spectral analysis of treated scaffolds at 232nm for HS disaccharides in the wash supernatant, as well as Tomato-lectin staining (Vector Laboratories, B-1175 1:500 dilution; detection with PE-conjugated Streptavidin, Biolegend, 405203 1:200 dilution) of the untreated and treated scaffolds. Alternatively, scaffolds were treated with chondroitinase ABC solution (0.4M Tris-HCl buffer pH8.0, 0.4M sodium acetate, 0.1% BSA, 5mU chondroitinase ABC (Amsbio, 100330-1A) for 1 hour at 37°C. Enzymatic activity was confirmed by immunoblot analysis for removal of CS proteoglycans from treated scaffolds. Following enzymatic treatment, scaffolds were

rinsed first with PBS (containing penicillin-streptomycin and amphotericin B) for one hour, followed by SFDM media. Sorted endodermal cells were seeded on scaffolds and cultured at air liquid interface as previously described.

Protein Profiler Array

A protein antibody array (R&D Systems, ARY015) was used to identify candidate analytes remaining on decellularized scaffolds, as per manufacturer's instructions. Briefly, following decellularization, scaffolds treated with or without heparitinase I were homogenized in PBS with protease inhibitors. After homogenization, 1% triton X-100 was added to samples. Samples underwent one freeze-thaw cycle and were then centrifuged at 10,000 x g for 5 minutes to remove cellular debris. Protein concentration was determined using Bradford protein assay (Bio-rad). Sample lysates were diluted (300µg of protein per sample), mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with the protein array membrane. Streptavidin-HRP and chemiluminescent detection reagents were used for detection. A signal was generated at each capture spot on membrane, corresponding to the amount of protein bound. Pixel density for each spot was detected and quantified using ImageJ open access software.

Kidney Scaffold Decellularization

Adult mouse kidneys were sectioned to 1000µm using a vibratome (Leica). Sections were soaked in 0.1% SDS using a peristaltic pump supplying fresh SDS at a flow rate range of 0.4-0.8 rpm for 72 hours. Decellularized kidney scaffolds were soaked in 100x Pen/Strep for 1 hour to remove any contaminates. Scaffolds were then washed with PBS and SFDM prior to seeding with sorted $cKIT^{+}/CXCR4^{+}$ endodermal cells.

Endoderm Culture on Isolated Matrix Proteins

Sorted endodermal cells were seeded onto isolate matrix proteins and cultured under the same conditions as with seeded lung scaffolds in SFDM, at air liquid interface using the hydrophobic floating membranes (Whatman, 110614). Matrix proteins used include mouse laminin (BD, 354232), human fibronectin (BD, 356008), rat-tail collagen I (BD, 356236), and mouse collagen IV (BD, 354233). Matrix proteins were diluted to 10µg/mL

and applied to the floating membranes prior to seeding with endoderm. For Matrigel (BD, 356230), a thick coat (200µl) was placed on floating membranes and allowed to gel prior to seeding with endoderm.

Statistical Analysis

Statistical comparisons were performed using unpaired t-tests, unless specified otherwise. For multiple comparisons of more than two groups, one-way ANOVA was used with Dunnett's test for significance.

Supplemental References

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