Supplementary Information: Figures and Tables

Human iPS cell- derived alveolar epithelium repopulates lung extracellular matrix

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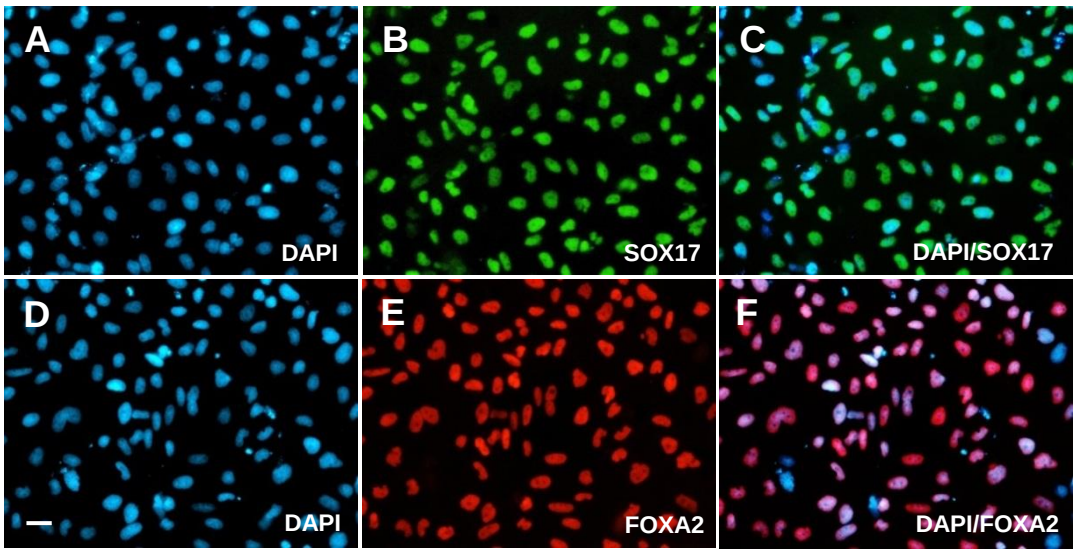
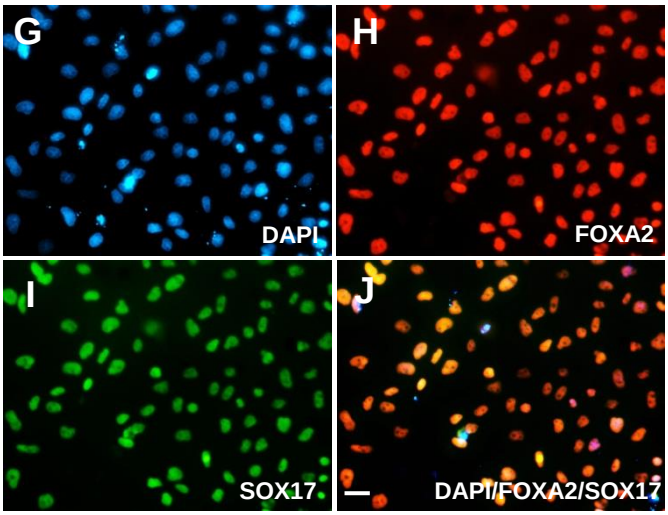
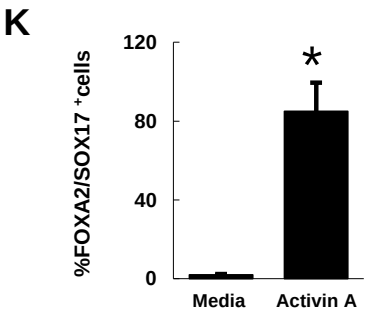
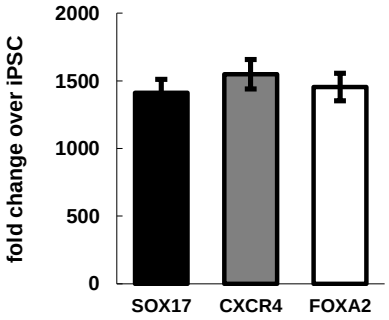
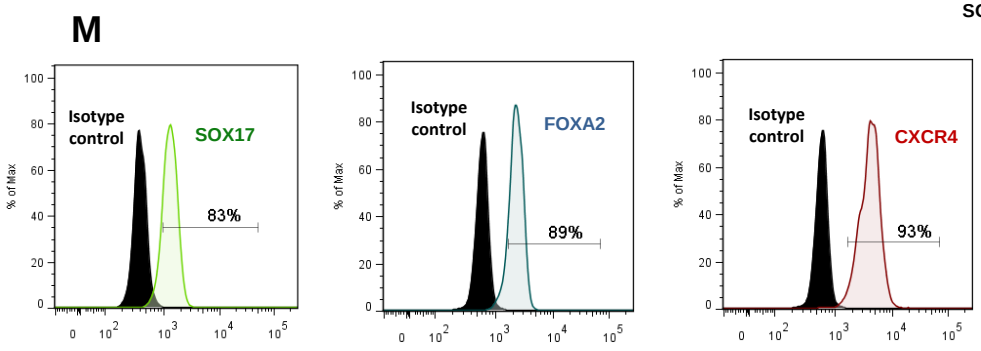


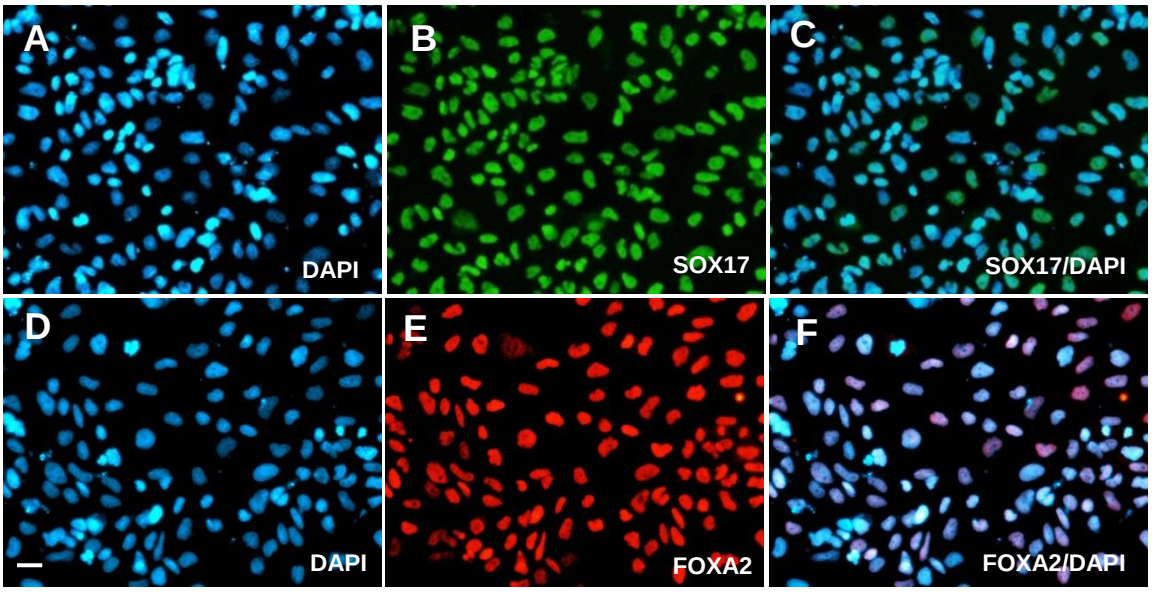


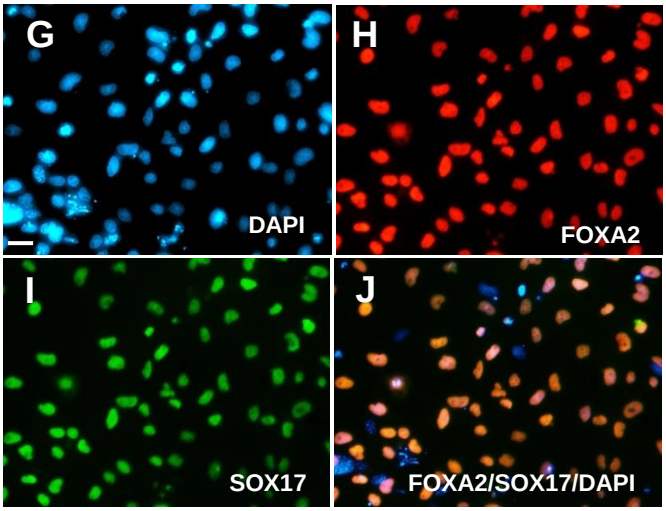


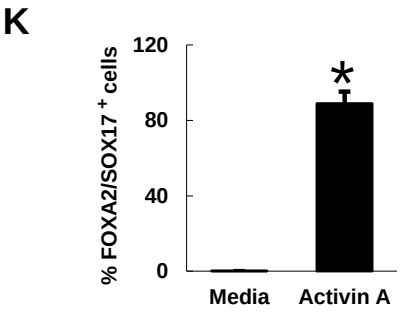


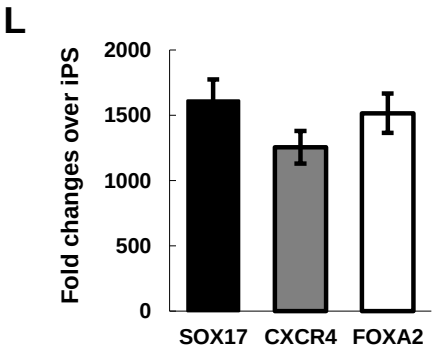


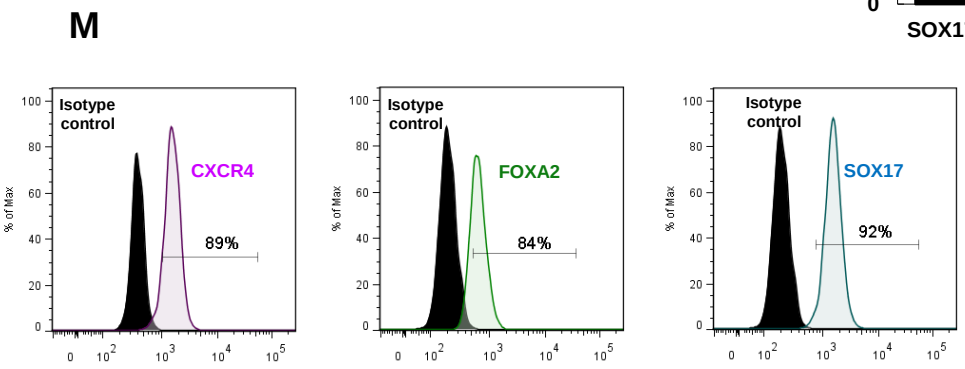
Supplementary fig.1











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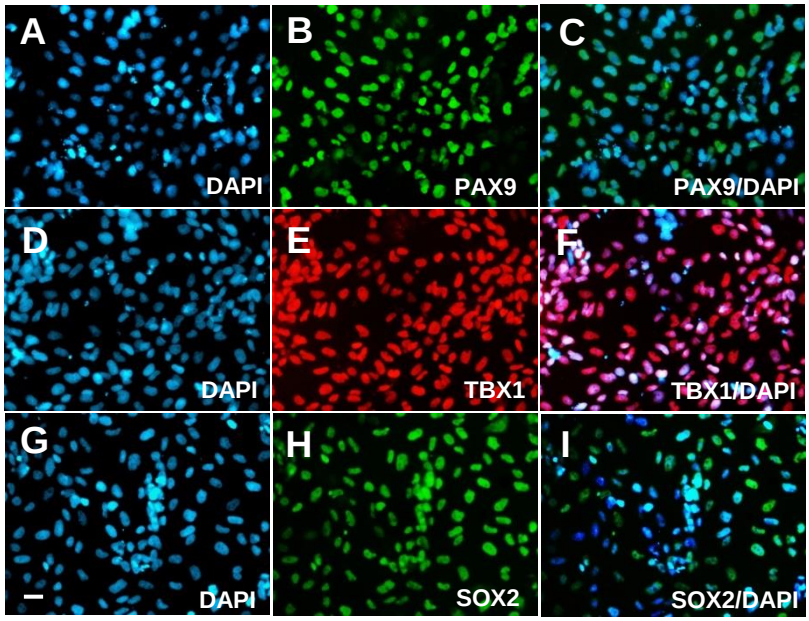

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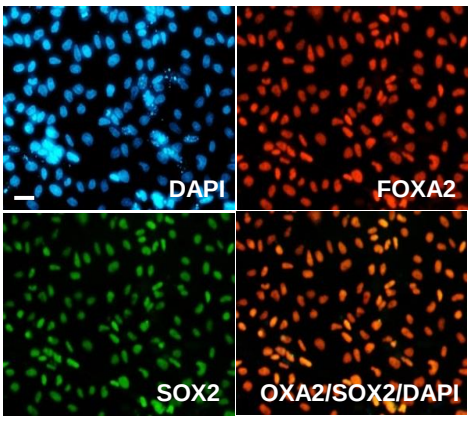

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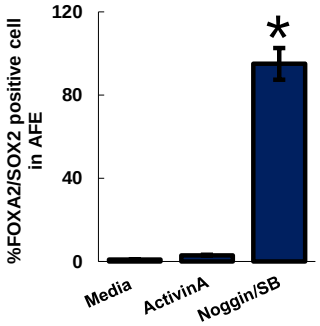

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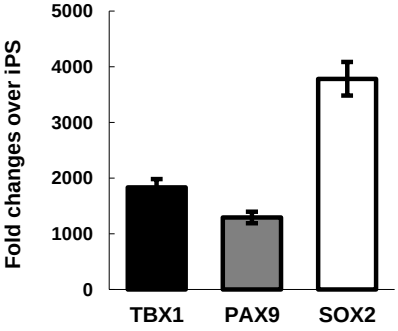

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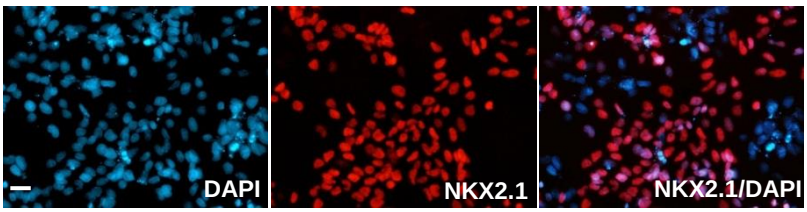

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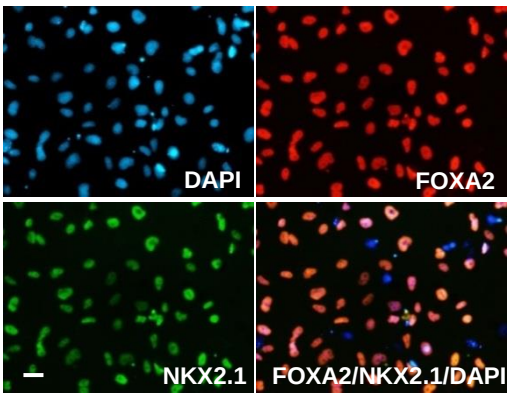

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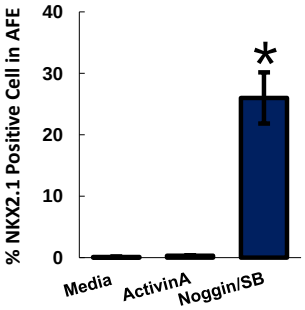
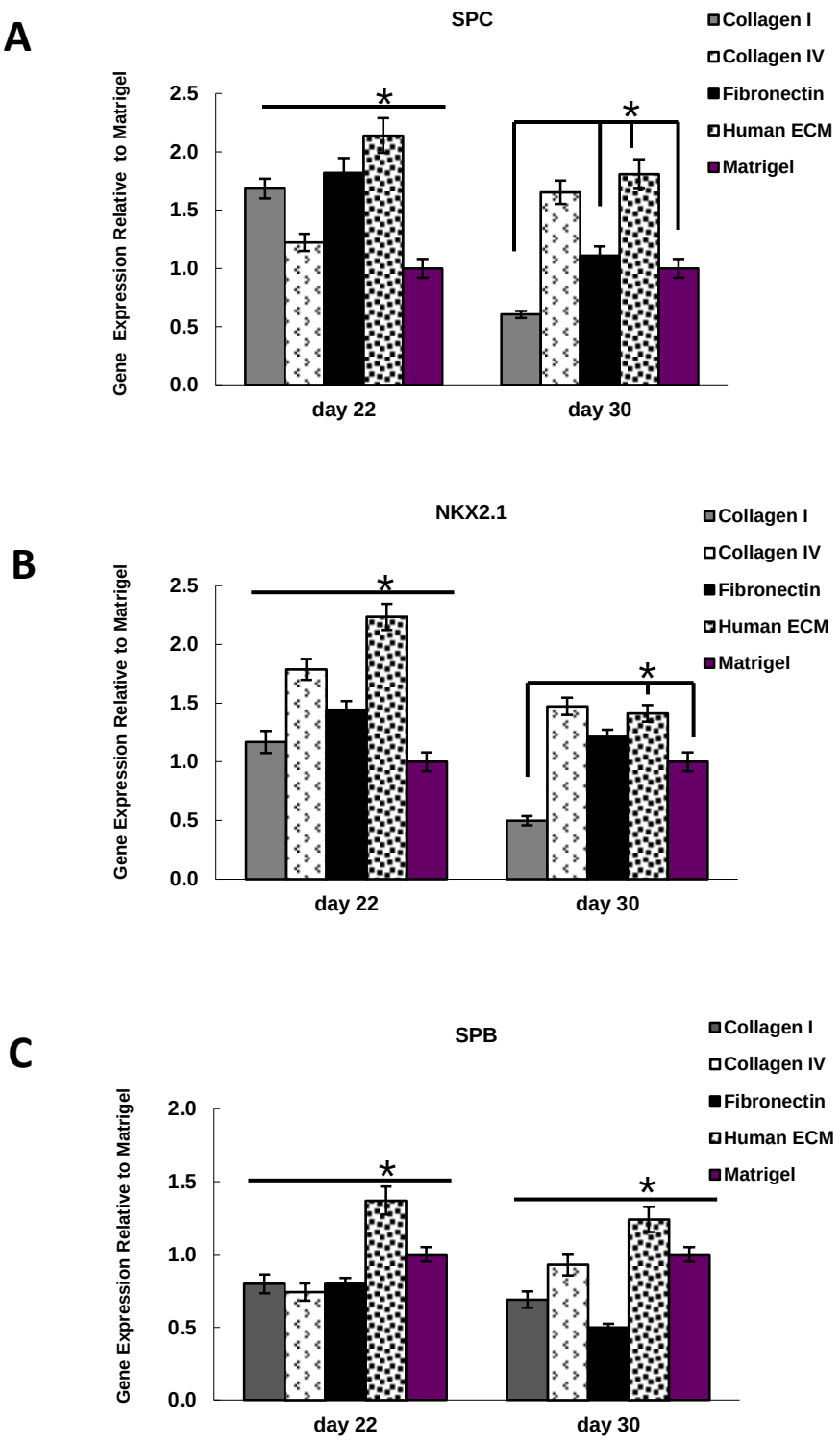


Supplementary fig.3



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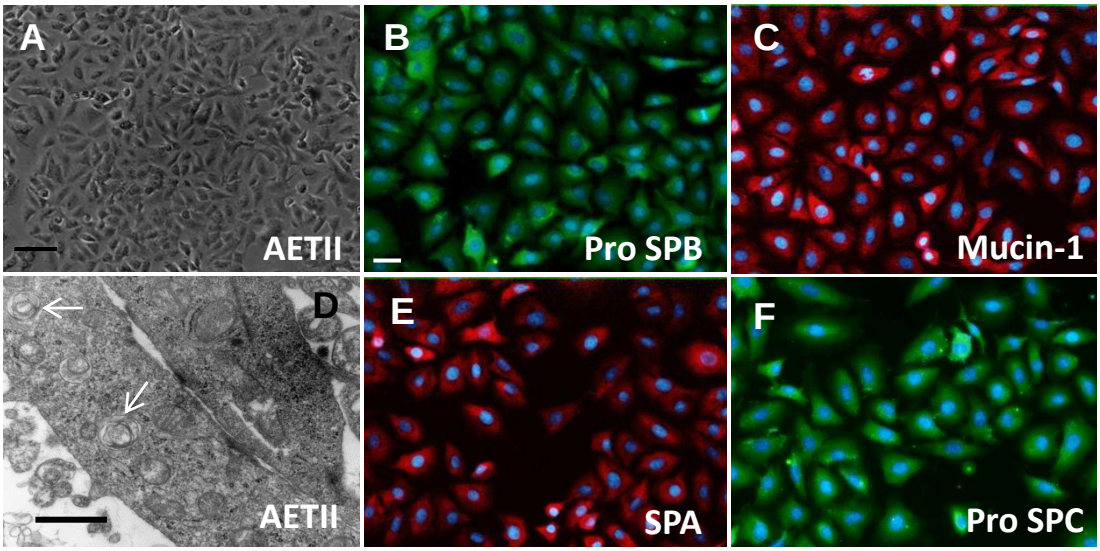

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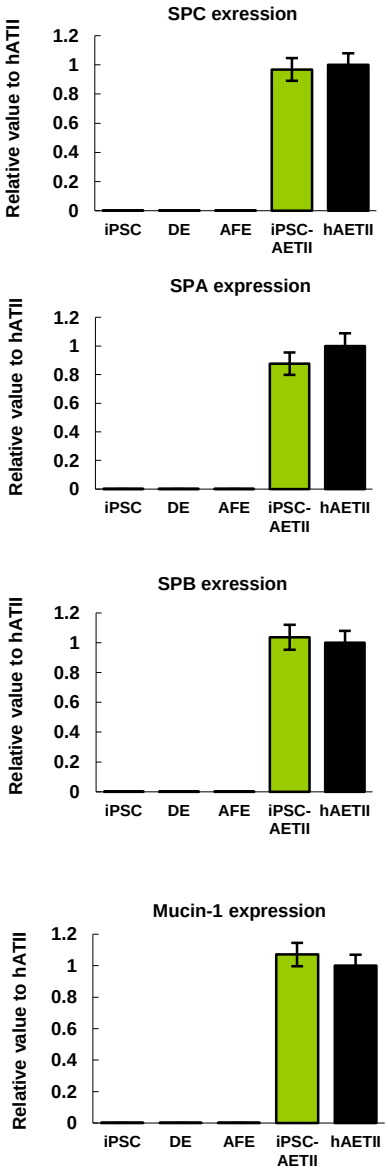

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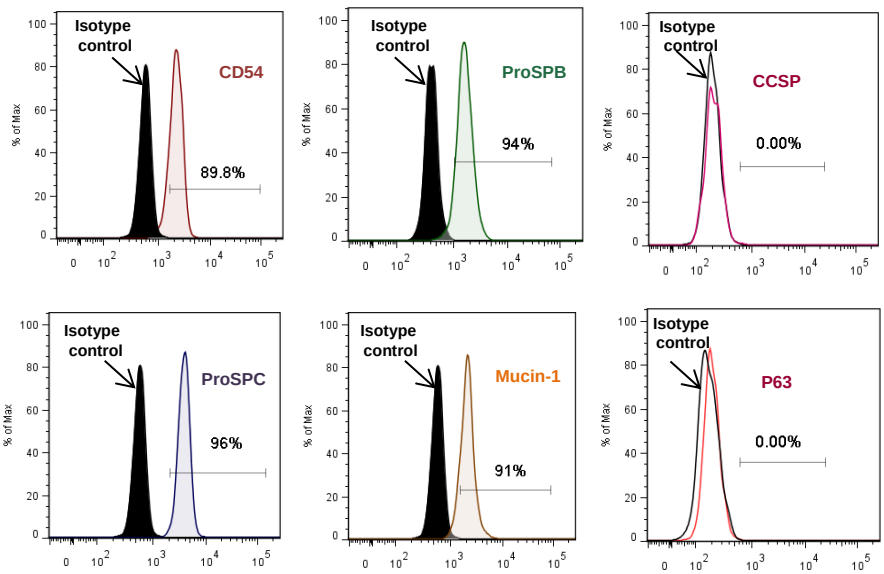

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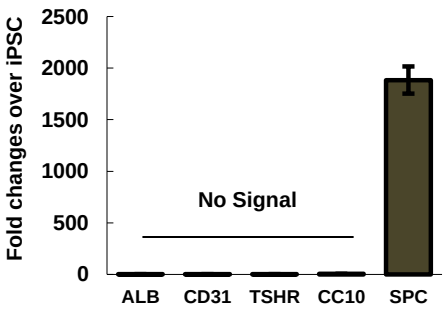

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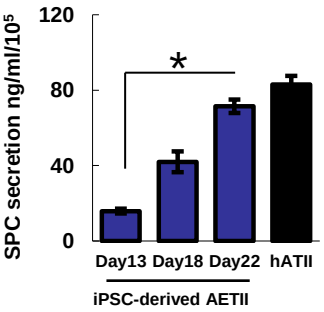
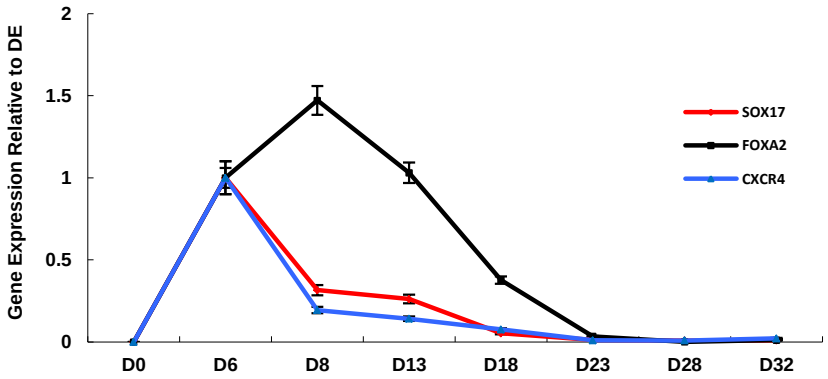


Supplementary fig.5

A



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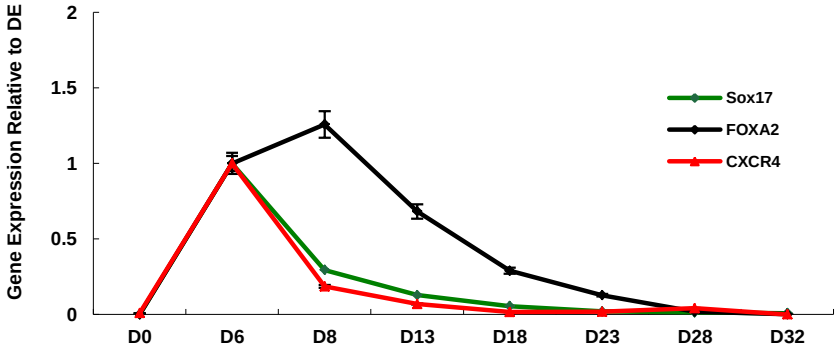

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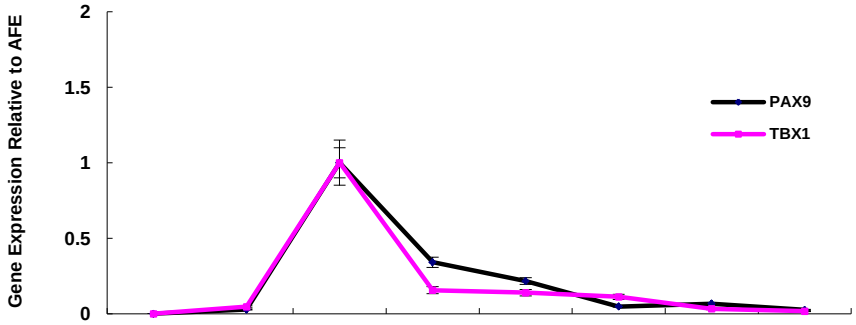

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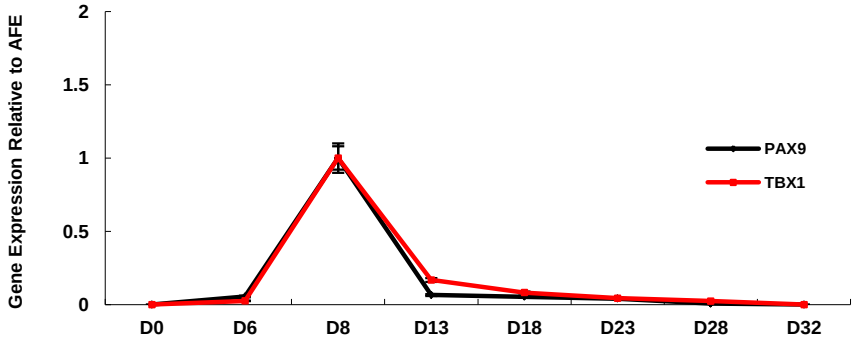

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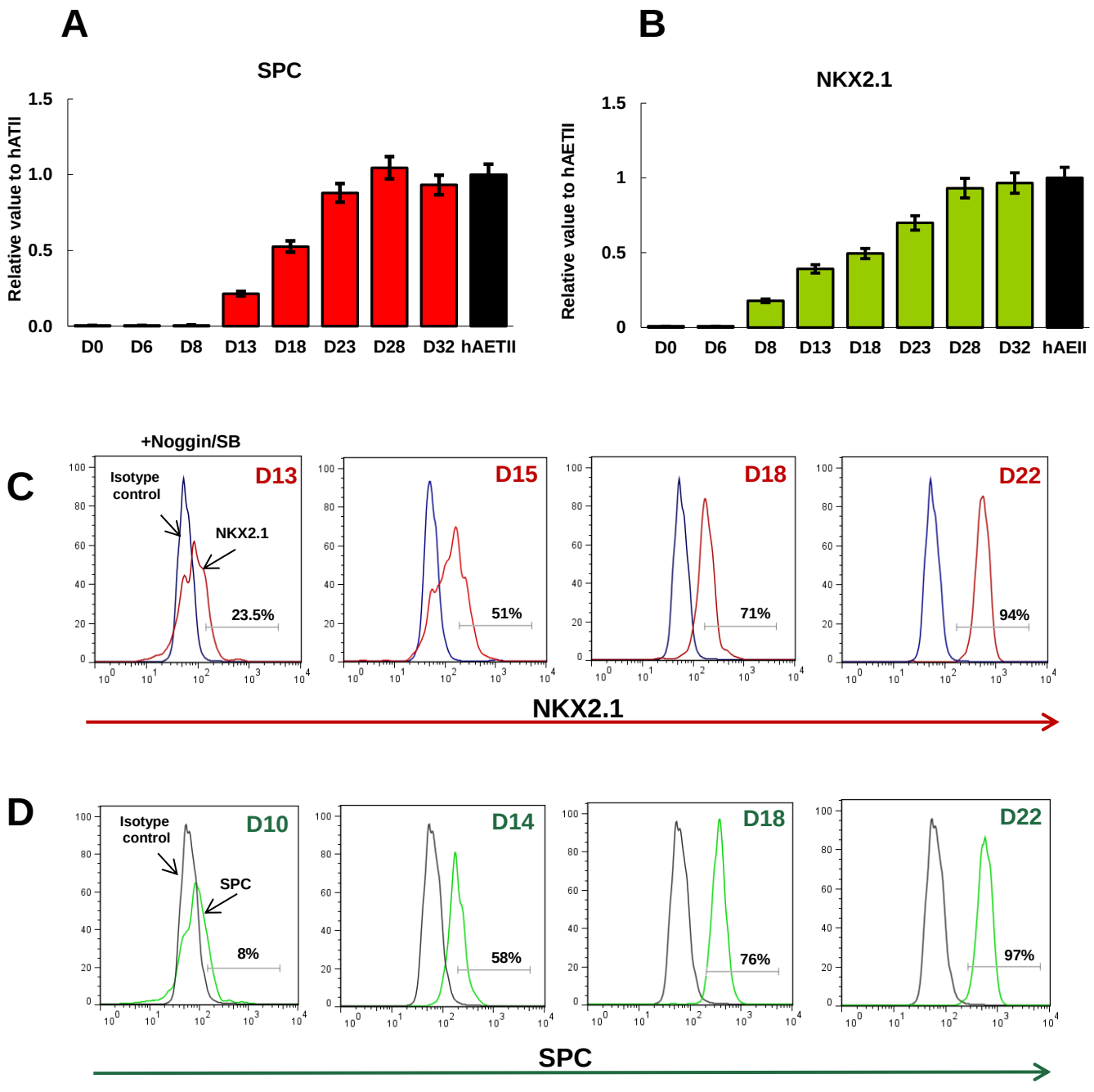
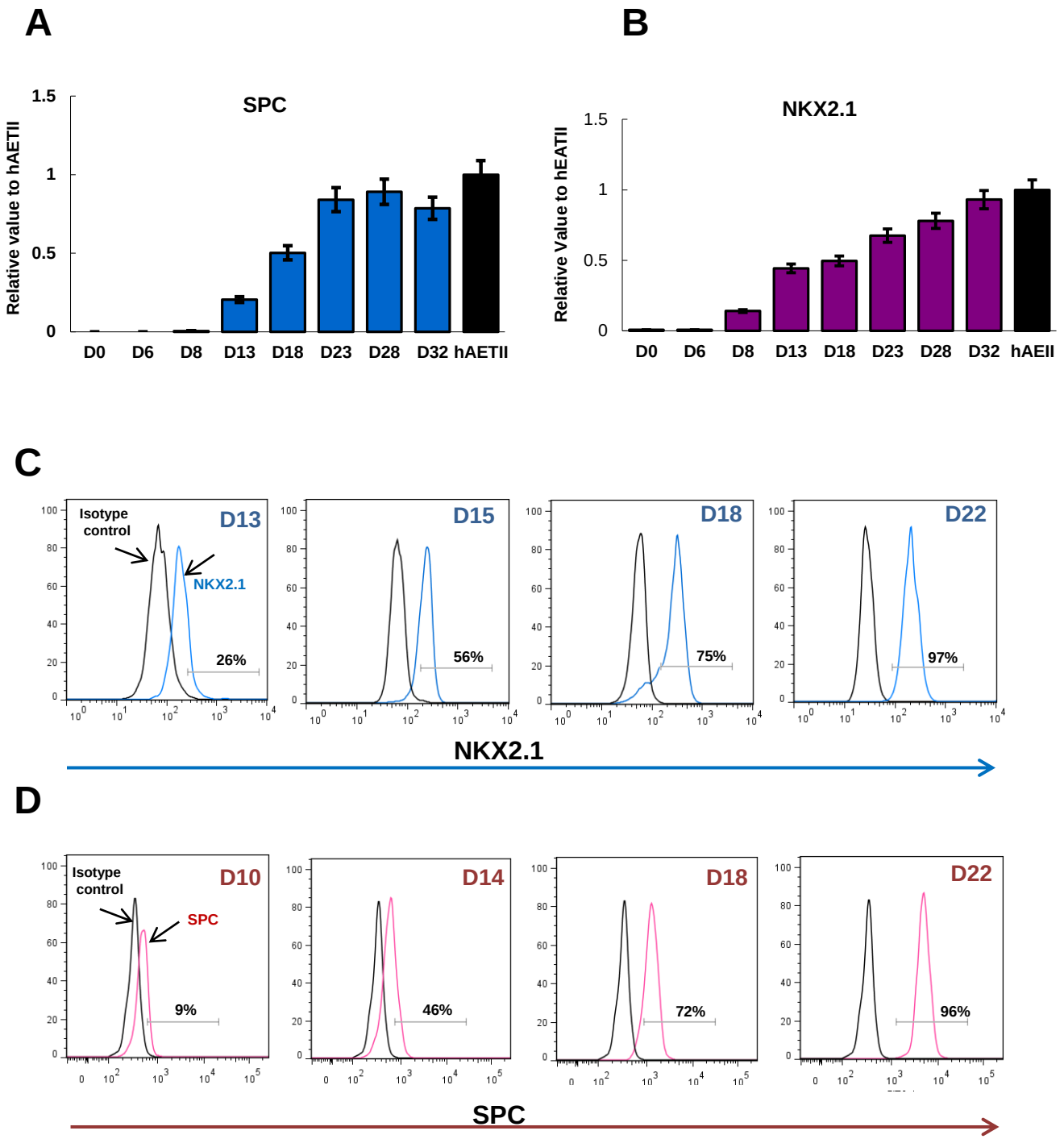


Supplementary Fig. 7



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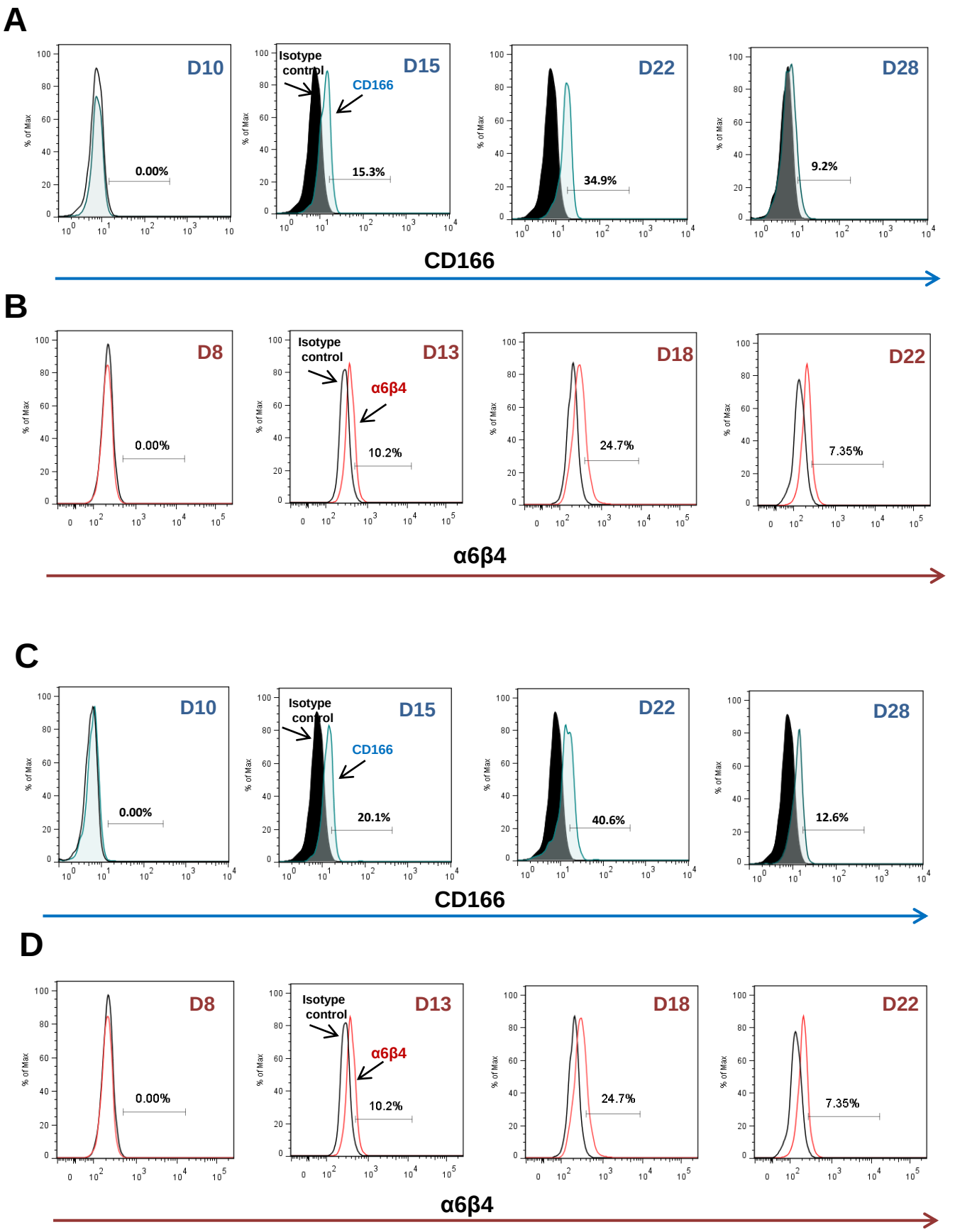

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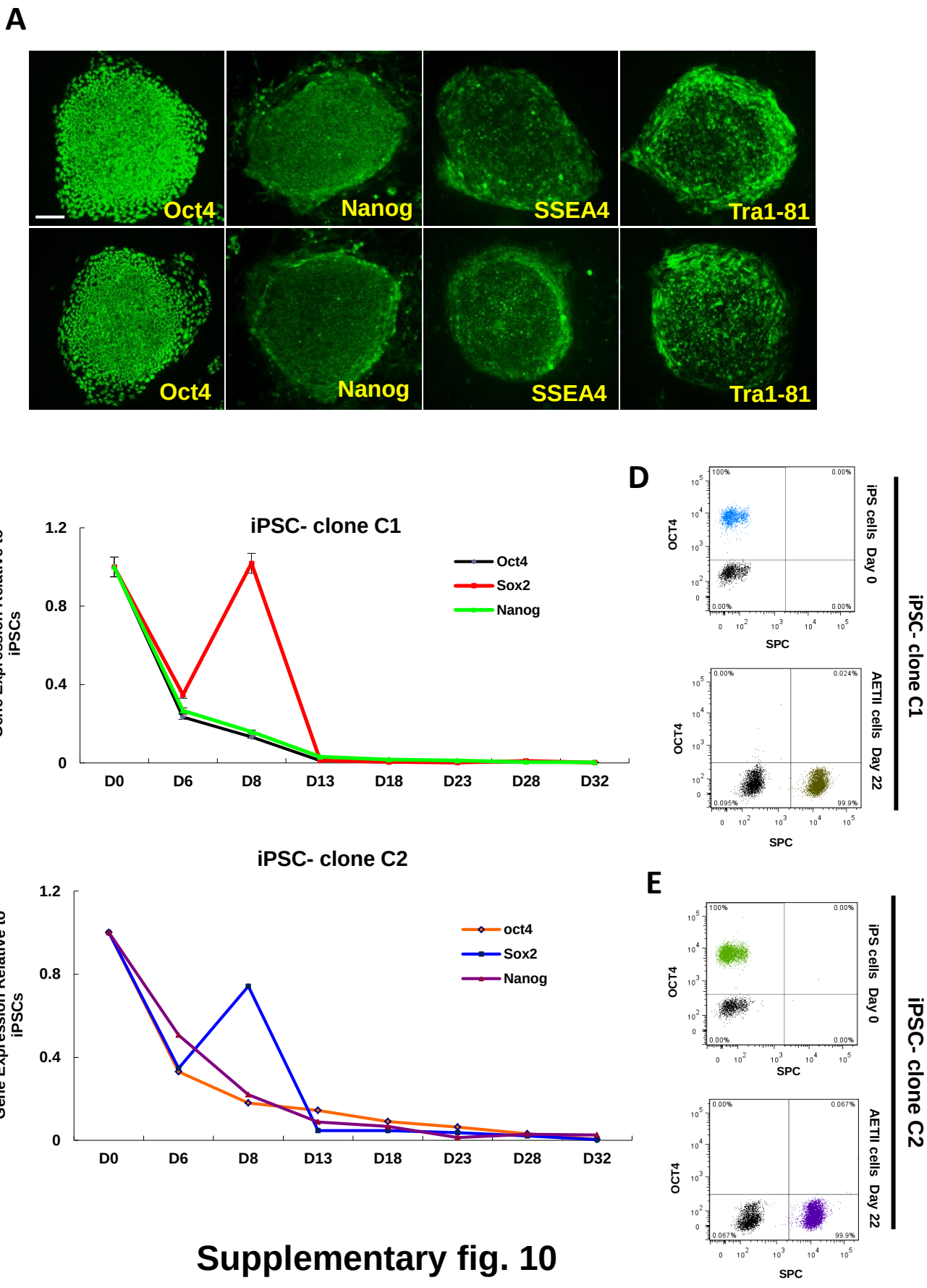
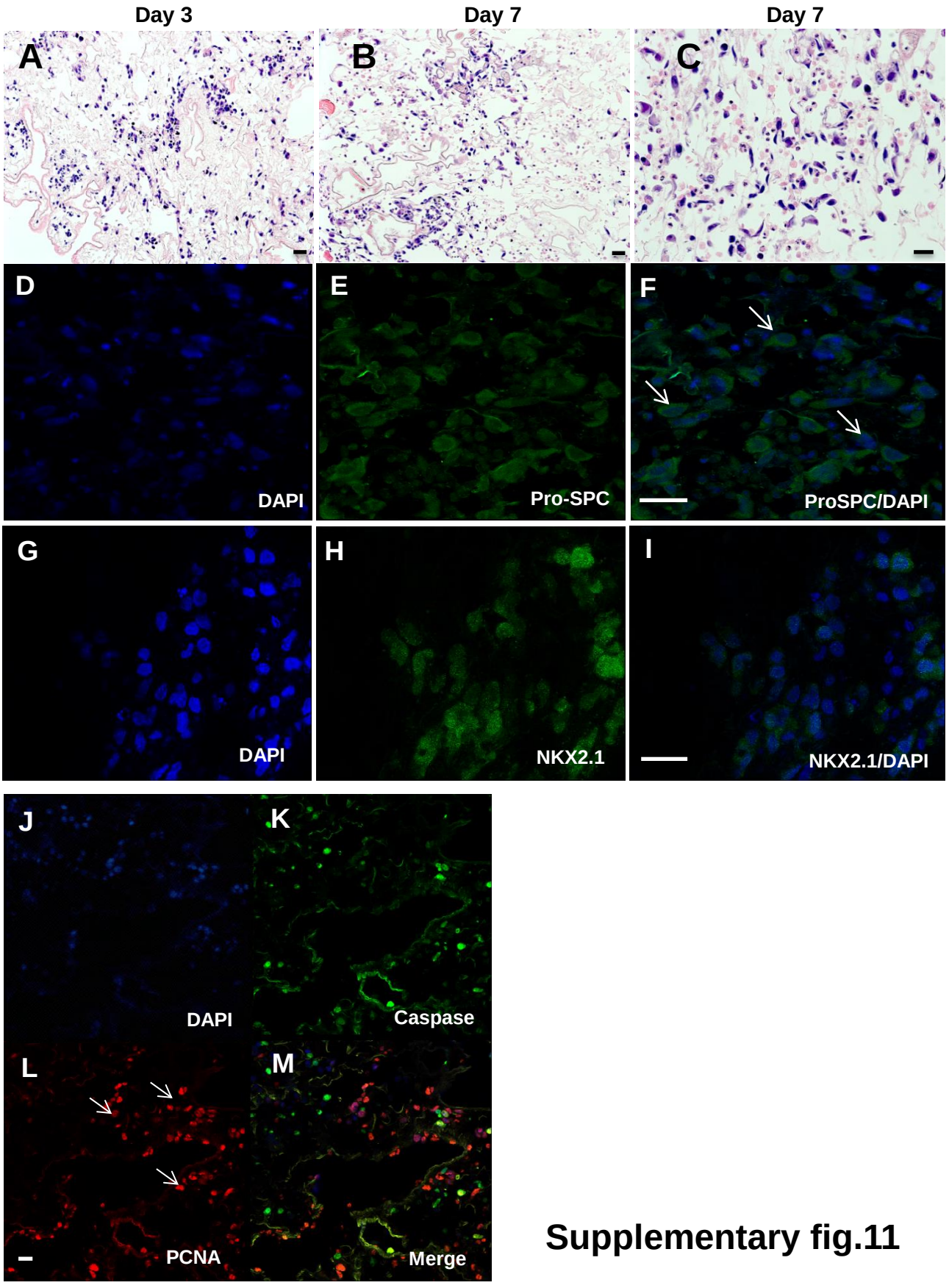


Supplementary fig. 10

iPSC-derived AETII C2 on rat lung scaffold in bioreactor



Supplementary fig.11

Supplementary Figure 1. Functional characteristics of definitive endoderm (DE) cells derived from iPSCs (C1 clone), at day 6: (A-F) Immunofluorescence analysis of DE marker proteins, SOX17 and FOXA2 at day 6. (A, D) Nuclei were stained with DAPI, (B, E) shows SOX17, FOXA2 staining in DE cells, (C, F) Merge. (G-J) immunofluorescence staining showing DE cells are positive for both SOX17 and FOXA2 at day 6, (K) flow cytometric analysis of double positive cells for SOX17/FOXA2 in DE cells exposed to activin A at day 6 compare to iPS cultured in media without activin A, (L) mRNA expression of SOX17, FOXA2 and CXCR4 from three independent experiments by qRT-PCR. (Data expressed as quantification of mRNA normalized to GAPDH and average fold change in gene expression over iPSCs), (M) Flow cytometric analysis of SOX17, FOXA2 and CXCR4 during activin A–mediated induction of definitive endoderm in iPSC cells at day 6 (compare to DE cells stained with corresponding isotype). (Bar indicate ± SEM and n = 3 independent experiments for qRT-PCR and flow cytometry. * on the graph denotes statistically significant difference p-value < 0.005). Scale bar, 31 μm

Supplementary Figure 2: Characteristics of definitive endoderm cells derived from iPSCs C2 clone, at day 6: (A-F) Immunofluorescent staining of definitive endodermal markers, SOX17 and FOXA2 at day 6 of activin A induction (Scale bar, 31 μm), (G-J) Immunofluorescence staining showing DE cells are positive for both SOX17 and FOXA2, (K) flow cytometric analysis of double positive cells for SOX17/FOXA2 in DE cells exposed to activin A at day 6 compare to iPS cultured in media without activin A, (L) Expression of SOX17, CXCR4 and FOXA2 mRNA in C2 iPSCs quantified by qRT-PCR at day 6. (Data expressed as quantification of mRNA normalized to GAPDH and average fold change in gene expression over iPS cells), (M) Representative flow cytometric analysis of SOX17, CXCR4 and FOXA2 in C2 iPSCs derived DE at 6 day. (Bar indicate ± SEM and n = 3 independent experiments for qRT-PCR and flow cytometry. * on the graph denotes statistically significant difference p-value < 0.005). Scale bar, 31 μm

Supplementary Figure 3: Analysis of AFE markers in NOGGIN/SB-431542-treated definitive endoderm in C2 iPS cells. (A-I) Immunofluorescent staining of AFE markers; SOX2, TBX1, PAX9, after 2 day of NOGGIN/SB431542 induction in C2 iPS cells (at day 8). Scale bar,

31 μm, (J) Immunofluorescence staining showing AFE cells are positive for both SOX2 and FOXA2 at day 8, (K) Flow cytometric analysis of double positive cells for SOX2/FOXA2 in AFE cells at day 8. More than 85% of cells were double positive for both SOX2 and FOXA2. (L) Expression of TBX1, SOX2, and PAX9 mRNA quantified by qRT-PCR in C2 iPS cells at day 8 (Data expressed as quantification of mRNA normalized to GAPDH and average fold change in gene expression over iPSCs cells). (M) Immunofluorescence staining of NKX2.1 in AFE derived from clone C2 at day 8. (Scale bar, 31 μm) (N) Immunofluorescence staining showing AFE cells are positive for both NKX2.1 and FOXA2 at day 8; Most NKX2.1 positive cells were stained positive for FOXA2.(Scale bar, 31 μm) (O) Flow cytometric analysis of positive cells for NKX2.1 in AFE cells at day 8. Exposing DE to NOGGIN/SB431542 yield 26% positive cells for NKX2. (Bar indicate ± SEM and n = 3 biological triplicate replicates for qRT-PCR and flow cytometry, * denotes statistically significant difference p-value < 0.05).

Supplementary Figure 4. Differentiation of DE cells (day 6) to AETII (day 22) on different extracellular matrix proteins. (A) SPC, (B) SPB and (C) NKX2.1 expression in AETII differentiated on collagen I, collagen IV, fibronection, and human ECM protein and matrigel, quantified qRT-PCR. The iPSC- derived DE differentiated to AETII on different ECM protein. The gene expression in iPS derived-AETII cells on different ECM proteins were compared to the level seen in iPS derived-hAETII cells on matrigel. Ct values from three independent experiments from the triplicate PCR reactions for a gene of interest (SPB, SPC, NKX2.1) were normalized against average GAPDH Ct values from the same cDNA sample. Fold change of GOI transcript levels between iPS derived-AETII on each ECM protein and iPS derived-AETII cells on matrigel equals $2^{-\Delta\Delta Ct}$, where $\Delta Ct=Ct_{(GOI)} - Ct_{(GAPDH)}$, and $\Delta\Delta Ct=\Delta Ct_{(\text{iPSC-AETII on ECM of interest})} - \Delta Ct_{(\text{iPSC-AETII on Matrigel})}$. Human ECM induced significantly higher levels of SPC, SPB and NKX2.1 expression compared to each ECM proteins individually. (Bar indicate ± SEM and n =3 independent experiments).

Supplementary Figure 5. Functional characterization of differentiated AETII from C2 iPSCs line. (A) Phase-contrast images AETII cells. (B-C,E-F) Immunofluorescent staining of alveolar type II markers; (B) Pro surfactant protein B (ProSPB), (C) Mucin-1, (E) Surfactant

protein A (SPA), (F) Pro surfactant protein C (ProSPC) Scale bar, 63 μm , (D) Transmission electron microscopy, represent AETII contain characteristic cytoplasmic laminar bodies(scale bar, 1μm) (G) qRT-PCR analysis in undifferentiated iPSC, DE, AFE and AETII cells derived from C2 clone compared to hATII cells that were derived from fresh human lung, from three independent experiments values from the triplicate PCR reactions for a gene of interest (SPA, SPB, SPC, Mucin-1) were normalized against average GAPDH Ct values from the same cDNA sample. Fold change of GOI transcript levels between iPS derived-AETII and human type II cells equals $2^{-\Delta\Delta Ct}$, where $\Delta Ct=Ct_{(GOI)} - Ct_{(GAPDH)}$, and $\Delta\Delta Ct=\Delta Ct_{(AETII)} - \Delta Ct_{(ATII)}$ **,** (H) Flow cytometry analysis for the percentage of positive cells for alveolar type II markers at day 22. More than 95% of population were positive for type II cells marker (CD54, SPB, SPC, Mucin-1) when they were negative for CCSP (Clara cell marker), p63 (basal stem cell marker), (I) Expression of albumin, CD31, TSHR, CC10 (CCSP) in iPSC-derived AETII; they were negative for genes indicative of other lineages at day 22. (J) The amount of secreted SPC in the iPSC-derived AETII during the time course of differentiation compared to SPC secretion from isolated AETII from human lung determined by enzyme-linked immunosorbent assay. Bars indicate ± SEM and n = 3 independent experiments for qRT-PCR, ELISA and flow cytometry. * denotes statistically significant difference p-value < 0.05

Supplementary Figure 6. (A-B) Sequential up and downregulation of DE-specific and AFE-specific genes during differentiation to AETII cells quantified by qRT-PCR. (A-B) Ratio of gene expression in DE cells compare to iPSCs during differentiation quantified by qRT-PCR in (A) C1 clone and (B) C2 clone (Data expressed as quantification of mRNA normalized to GAPDH and average fold change in gene expression over DE cells) (C-D) Sequential up and downregulation of AFE-specific proteins during differentiation of iPSCs to AETII quantified by qRT-PCR in (C) C1 clone and (D) C2 clone (Data expressed as quantification of mRNA normalized to GAPDH and average fold change in gene expression over AFE cells). (bar indicate ± SEM and n = 3 independent experiments)

Supplementary Figure 7. Kinetics of NKX2.1 and SPC expression during differentiation of iPSC cells (C1 clone) to lung alveolar epithelium. (A-B) Kinetics of NKX2.1 and SPC mRNA

expression at different days, quantified by real time qRT-PCR. (A) SPC expression during differentiation of iPS cells to AETII. Ct values of SPC is normalized to GAPDH and expressed to levels seen in ATII cells isolated from human lung (hATII) (B) NKX2.1 during differentiation of iPS cells to AETII. Data expressed as quantification of mRNA normalized to GAPDH and expressed to the level of seen in isolated human primary type II. (C-D) Flow cytometry analysis for the percentage of positive cells for (C) NKX2.1 from day 8 to day 22 and (D) SPC from day 10 to day 22. (Bars indicate ± SEM and n = 3 independent experiments for PCR and flow cytometry).

Supplementary Figure 8. **Kinetics of NKX2.1 and SPC expression during differentiation of iPSC cells (C2 clone) to lung alveolar epithelium**. (A-B) NKX2.1 and SPC mRNA expression at different days, quantified by real time RT-PCR in iPSC-derived AETII (C2 clone). (A) SPC expression during differentiation from day 0 to day 32. Ct value for SPC is normalized to GAPDH and expressed to levels seen in AETII cells isolated from human lung (hATII) (B) NKX2.1 expression during differentiation. Data expressed as quantification of mRNA normalized to GAPDH and expressed to the levels seen in isolated human type II (C-D) Flow cytometry analysis for the percentage of positive cells for (C) NKX2.1 from day 8 to day 22 and (D) SPC from day 10 to day 22. (Bars indicate ± SEM and n = 3 independent experiments for PCR and flow cytometry)

Supplementary Figure 9. **Kinetics of α6β4 and CD166 expression during differentiation of iPSC cells to lung alveolar epithelium. (**A,C) Flow cytometry analysis for the percentage of positive cells for CD166 from day 10 to day 28 in AETII derived from (A) C1 clone and (C) C2 clone. **(**B, D) Flow cytometry analysis for the percentage of positive cells for α6β4 from day 8 to day 22 in AETII derived from (B) C1 clone and (D) C2 clone

Supplementary Figure 10. **Pluripotency marker analysis in C2 clone in day 0 and during differentiation to AETII.** (A). Immunofluorescent staining of iPSC markers; OCT4 , Nanog , SSEA4 , Tra1-81 in both iPSC clone C1 and C2. (Scale bar, 100 μm) Both clones were positive for pluripotency genes at day 0. (B-C) Downregulation of iPSC-specific genes OCT4, SOX2, and Nanog during differentiation to AETI. The expression of OCT4, SOX2, and Nanog were

downregulated over the time and by day 32 these markers were undetectable in iPSC-derived AETII derived from both (B)clone C1 and (C)clone C2 (Data expressed as quantification of mRNA normalized to GAPDH and average fold change in gene expression over iPS cells at day 0, bar indicates SEM and n = 3 independent experiments), (D-E) Expression of OCT4 and SPC in differentiated AETII cells on day 0 compare to day 22 analyzed by flow cytometry for (D) C1 clone and (E) C2 clone. At day 22 of differentiation, SPC positive iPSC- derived AETII cells were negative for OCT4.

Supplementary Figure 11. AETII derived from iPSC C2 clone respond to recellularize 3D lung tissue scaffolds in bioreactor (A-C) H&E staining of seeded rat lung scaffold with iPSC-derived AETII cells at (A) day 3 and (B-C) at day 7 in bioreactor. Scale bar, 200 μm. (D-F) Immunostaining for SPC on seeded rat lung scafold with iPSC-derived AETII cells cultured in bioreactor at day 7 (D) Nuclei were stained with DAPI; (E) shows Pro-SPC staining (F) Merge, Scale bar, 50 μm (G-I) Immunostaining for NKX2.1 on seeded rat lung scafold with iPSC-derived AETII cells cultured in bioreactor at day 7 (G) Nuclei were stained with DAPI; (H) shows NKX2.1 staining (I) Merge, Scale bar, 50 μm (J-M) Immunostaining for PCNA and caspase of bioreactor cultured iPSC-derived APTII cells at day 7. Scale bar, 49 μm

Supplementary Table S1. List of antibodies used in staining, flow cytometry and western blot for various experiments

Primary antibodies			
Antigen	Type	Provider (Cat #) (lot #)	Application
Beta-actin	Monoclonal	Abcam (Cat# ab8226, Lot# GR88207-1)	WB
Caspase3	Rabbit polyclonal	Abcam (Cat# ab13847, Lot# GR62173-2)	IHC
CCSP	Rabbit polyclonal	Millipore (Cat# 07-623, Lot# 1972321)	IHC
CCSP	Goat polyclonal	Biovender (Cat# RD81022220, Lot# RD2412)	IHC
CD54-PE	Mouse Monoclonal	BD Pharmingen (Cat# 560971, Lot# 10609)	FC
CXCR4-APC	Monoclonal	BD Pharmingen (Cat# 560936, Lot# 41560)	FC
Cytokeratin-5	Rabbit polyclonal	Abbiotec (Cat# 251431, Lot# 11092101)	IHC
FoxA2	Goat polyclonal	R&D Systems (Cat# AF2400, Lot# ULB0311101)	ICC, FC
Muc-1	Monoclonal	R&D Systems (Cat# MAB6298, Lot# CDYA0111031)	ICC, FC
Nanog	Rabbit monoclonal	Abcam (Cat# ab80892, Lot# GR40243-14)	ICC
Nkx2.1	Rabbit polyclonal	Abcam (Cat# ab76013, Lot# GR76790-2)	ICC, IHC, FC
Oct4	Goat polyclonal	Abcam (Cat# ab27985, Lot# GR56247-1)	ICC, FC
p63	Monoclonal	Santa Cruz (Cat# sc-71825, Lot# H2510)	IHC
Pax9	Rat monoclonal	Abcam (Cat# ab28538, Lot# GR53993-1)	ICC
Pax9	Goat polyclonal	Santa Cruz (Cat# sc-7746, Lot# K121)	ICC
PCNA	Monoclonal	Abcam (Cat# ab29, Lot# GR70504-2)	IHC
proSPB	Rabbit polyclonal	Millipore (Cat# ab3430, Lot# NG1820771)	ICC, FC
proSPC	Rabbit monoclonal	Millipore (Cat# ab3786, Lot# 2117989)	ICC, IHC, FC
proSPC	Rabbit polyclonal	Abcam (Cat# ab40879, Lot# GR86765-1)	WB
Sox2	Rabbit polyclonal	Abcam (Cat# ab97959, Lot# Unknown)	ICC
Sox2-AF647	Monoclonal	BD Pharmingen (Cat# 562139, Lot# 18245)	ICC, FC
Sox17	Goat polyclonal	R&D Systems (Cat# AF1924, Lot# KGA0411031)	ICC, FC
Sox17	Mouse monoclonal	Abcam (Cat#ab84990)	ICC
SPA	Rabbit polyclonal	Millipore (Cat# ab3420, Lot# NG1888873)	ICC
SPA	Rabbit polyclonal	Santa Cruz (Cat# sc-13977, Lot# K0807)	WB
SPC	Rabbit polyclonal	Santa Cruz (Cat# sc-13979, Lot# L1710)	ICC, IHC, FC
SPC	Monoclonal	Life Sciences Advanced Tech.(Cat #E01S0168)	ELISA
SSEA4	Monoclonal	Millipore(Cat# MAB4304, Lot # LV1488380	ICC
T1α	Monoclonal	Abcam (Cat# ab10288, Lot# GR47830-3)	IHC
Tbx1	Rabbit polyclonal	Abcam(Cat # ab18530)	ICC
TRA-1-81	Monoclonal	Millipore(Cat#MAB4381, Lot # LV1512392)	ICC
CD166-PE	Mouse Monoclonal	(Cat# 559263, Lot# 3018832)	FC
CD104-FITC	Mouse Monoclonal	(Cat# 64233, Lot # 3039557	FC

Supplementary Table S1. (continued)

Detection antibodies		
Type	Provider(Cat #) (lot #)	Application
Alexa Fluor® 555 Donkey Anti-Goat IgG (H+L)	Invitrogen (Cat# A21432, Lot# 439379)	ICC, IHC
Alexa Fluor® 568 Donkey Anti-Mouse IgG	Invitrogen (Cat# A10037, Lot# 1110068)	ICC, IHC
Alexa Fluor® 488 Donkey Anti-Rat IgG (H+L)	Invitrogen (Cat# A21208, Lot# 1017330)	ICC
Alexa Fluor® 488 Rabbit Anti-Goat IgG (H+L)	Invitrogen (Cat# A11078, Lot# 1069847)	ICC, IHC
Alexa Fluor® 555 Goat Anti-Rabbit IgG (H+L), highly cross-absorbed	Invitrogen (Cat# A21429, Lot# 1010124)	ICC, IHC
Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L), highly cross-adsorbed	Invitrogen (Cat# A11034, Lot# 1008720)	ICC, IHC
Alexa Fluor® 555 Goat Anti-Mouse IgG (H+L), highly cross-adsorbed	Invitrogen (Cat# A21424, Lot# 1214852)	ICC, IHC
Alexa Fluor® 555 Goat Anti-Rat IgG (H+L)	Invitrogen (Cat# A21434, Lot# 1008806)	ICC
Alexa Fluor® 488 Chicken Anti-Rabbit IgG (H+L)	Invitrogen (Cat# A21441, Lot# 1003212)	ICC, IHC
Alexa Fluor® 488 Chicken Anti-Goat IgG (H+L)	Invitrogen (Cat# A21467, Lot# 474697)	ICC, IHC
Goat anti-Mouse IgG – H&L (FITC)	Abcam (Cat# ab6785, Lot# GR6891-4)	ICC, IHC
Goat anti-Rabbit IgG-HRP	Santa Cruz (Cat# sc-2004, Lot# H2806)	WB
HRP-conjugated … details not provided	Life Sciences Advanced Tech.(Cat #E01S0168	ELISA
APC Mouse IgG2a,κ Isotype Control	BD Pharmingen (Cat# 555576, Lot# 33828)	Isotype control
PE Mouse IgG1, κ Isotype Control	eBiosciences (Cat# 12-4714-82, Lot# E01672-1630)	Isotype control
Alexa Fluor® 647 Mouse IgG1, κ Isotype Control	BD Pharmingen (Cat# 557714, Lot# 34876)	Isotype control
Mouse IgG2a κ Isotype Control FTIC –	eBiosciences (Cat# 11-4724-81 , Lot# E00590-1630)	Isotype control
Isotype FITC Goat Anti mouse Ig	(Cat# 611233 , Lot# 039557)	
PE mouse IgG κ isotype control	(Cat# 555749, Lot# 38193)	

Abbreviations: IHC, immunohistochemistry; ICC, immunocytochemistry; FC, flow cytometry; WB, Western Blot; ELISA, Enzyme-linked immunosorbent assay; PE, Phycoerythrin, APC, Allophycocyanin;

FITC, Fluorescein isothiocyanate; HRP, horseradish peroxidase; CCSP, Clara cell secretory protein; PCNA, Proliferating cell nuclear antigen; SSEA4, Stage-Specific Embryonic Antigen-4; Oct4, Octamer-binding transcription factor 4; Muc-1, Mucin 1; SPA, Surfactant protein A; SPB, Surfactant protein B; SPC, Surfactant protein C; Nkx2.1, NK2 homeobox 1; Sox2, SRY (sex determining region Y)-box 2; Sox17, SRY-box 17; Tbx1, T-box 1; Pax 9, Paired box gene 9; CXCR4, C-X-C chemokine receptor type 4; FoxA2, forkhead box protein A2; AF647, Alexa Fluor® 647.

Supplementary Table S2.Sequences of primers used in qRT- PCR for various experiments.

Gene	Length (bp)	Primer Sequences
hSPA	**180**	Forward: TCCAAGCCACACTCCACGA Reverse: TTCCTCTGGATTCCTTGGG
hSPB	**69**	Forward: TGGGAGCCGATGACCTATG Reverse: GCCTCCTTGGCCATCTTGT
hNKX2.1	**93**	Forward: GGACGTGAGCAAGAACATG Reverse: TCGCTCCAGCTCGTACACC
hSPC	**94**	Forward: CCTTCTTATCGTGGTGGTGGT Reverse: TCTCCGTGTGTTTCTGGCTCAT
hMucin-1	**88**	Forward: AGCTTCTACTCTGGTGCACAA Reverse: GGTGGCTGGGAATTGAGA
hOCT4 endogenous	**164**	Forward: CCTCACTTCACTGCACTGTA Reverse: CAGGTTTTCTTTCCCTAGCT
hSOX2 endogenous	**151**	Forward: CCCAGCAGACTTCACATGT Reverse: CCTCCCATTTCCCTCGTTTT
hNANOG endogenous	**239**	Forward: CCAAATTCTCCTGCCAGTGAC Reverse: CACGTGGTTTCCAAACAAGAAA
hCC10	**105**	Forward: CCCTGGTCACACTGGCTCTC Reverse: TCATAACTGGAGGGTGTGTC
hCXCR4	**79**	Forward: CACCGCATCTGGAGAACCA Reverse: GCCCATTTCCTCGGTGTAGTT
hFOXA2	**89**	Forward: GGGAGCGGTGAAGATGGA Reverse: TCATGTTGCTCACGGAGGAGTA
hSOX17	**61**	Forward: GGCGCAGCAGAATCCAGA Reverse: CCACGACTTGCCCAGCAT
hPAX9	**132**	Forward: GTTATGTTGCTGGACATGGGT Reverse: GAAGCCGTGACAGAATGACTA C
hTBX1	**117**	Forward: GCTCCTACGACTATTGCCC Reverse: CGTATTCCTTGCTTGCCCT
hCD31	**140**	Forward: ATTGCAGTGGTTATCATCGGAGTG Reverse: CTCGTTGTTGGAGTTCAGAAGTGG
hTSHR	**156**	Forward: TTTCTTACCCAAGCCACTGC Reverse: TTCTCTTCATATTCCTGGTGG
hALB	**149**	Forward: AAACGCCAGTAAGTGACAGAG Reverse: ATATCTGCATGGAAGGTGAAT
hGAPDH	**122**	Forward: GACAACAGCCTCAAGATCATCAG Reverse: ATGGCATGGACTGTGGTCATGAG
hCaveolin-1	**122**	Forward: CTACAAGCCCAACAACAAGG Reverse: CATCGTTGAGGTGTTTAGGGT
hAQ5	**79**	Forward: ACTGGGTTTTCTGGGTAGGG Reverse: ATGGTCTTCTTCCGCTCTTC
hT1α	**110**	Forward:TCCAGGAACCAGCGAAGAC Reverse: CGTGGACTGTGCTTTCTGA