Figure S1

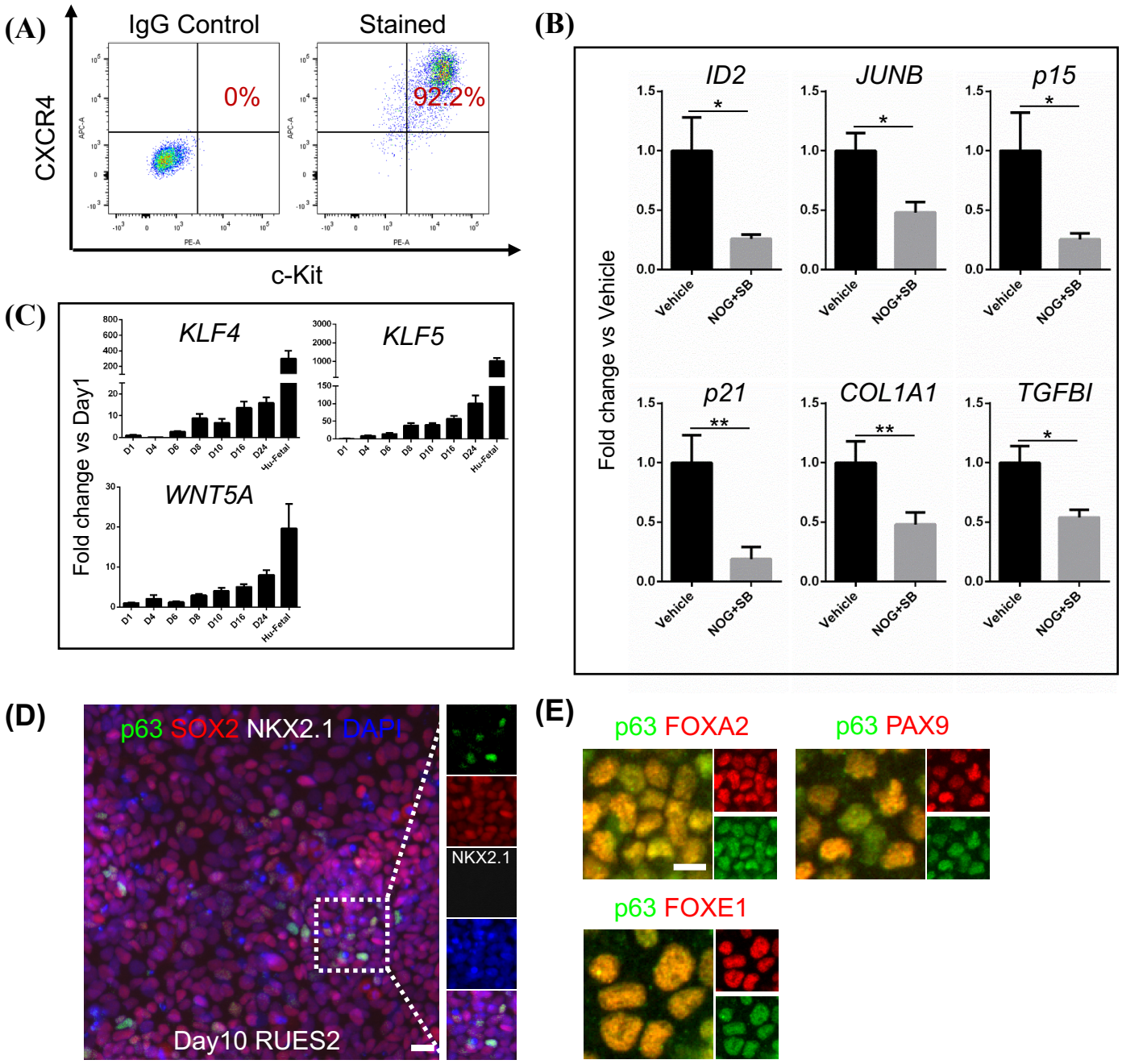
(A)
IgG Control
Stained
0%
92.2%
CXCR4
c-Kit
(B)
ID2
JUNB
p15
p21
COL1A1
TGFBI
Fold change vs Vehicle
Vehicle
NOG+SB
(C)
KLF4
KLF5
WNT5A
Fold change vs Day1
D1
D4
D6
D8
D10
D16
D24
Hu-Fetal
(D)
p63 SOX2 NKX2.1 DAPI
NKX2.1
Day10 RUES2
(E)
p63 FOXA2
p63 PAX9
p63 FOXE1

Figure S1 (Related to Figures 1-2). Combined treatment with signaling pathway regulators promotes hESC specification towards esophageal progenitor cells (EPCs). **(A)** Extensive endodermal commitment ($CXCR4^+$ $c\text{-}KIT^+$) of RUES2 cells after 3-day differentiation as indicated by flowcytometric analysis. **(B)** Treatment with NOGGIN and SB431542 (Day6-16) significantly reduced the expression of the BMP downstream targets ID2 and JUNB and the TGFß downstream targets p15, p21, COL1A1 and TGFBI at day16 of cell culture. The transcript levels were represented by the fold change compared to Vehicle controls. Data represent mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$ by unpaired, two-tailed Student's t test. **(C)** Expression of the esophageal markers *KLF4, KLF5 and WNT5A* during EPC differentiation. Human fetal esophagus (Hu-Fetal) was included as control. The transcript levels were represented by the fold change compared to day 1 (D1) hESC. Data represent mean ± SEM (n = 3). **(D)** A small population of $p63^+$ $SOX2^+$ $NKX2.1^-$ EPCs are present at day10 of differentiation. **(E)** EPCs at day10 differentiation express EPC markers FOXA2, FOXE1 and PAX9. Scale bars: (D) 20 μm, (E) 10 μm.

Figure S2

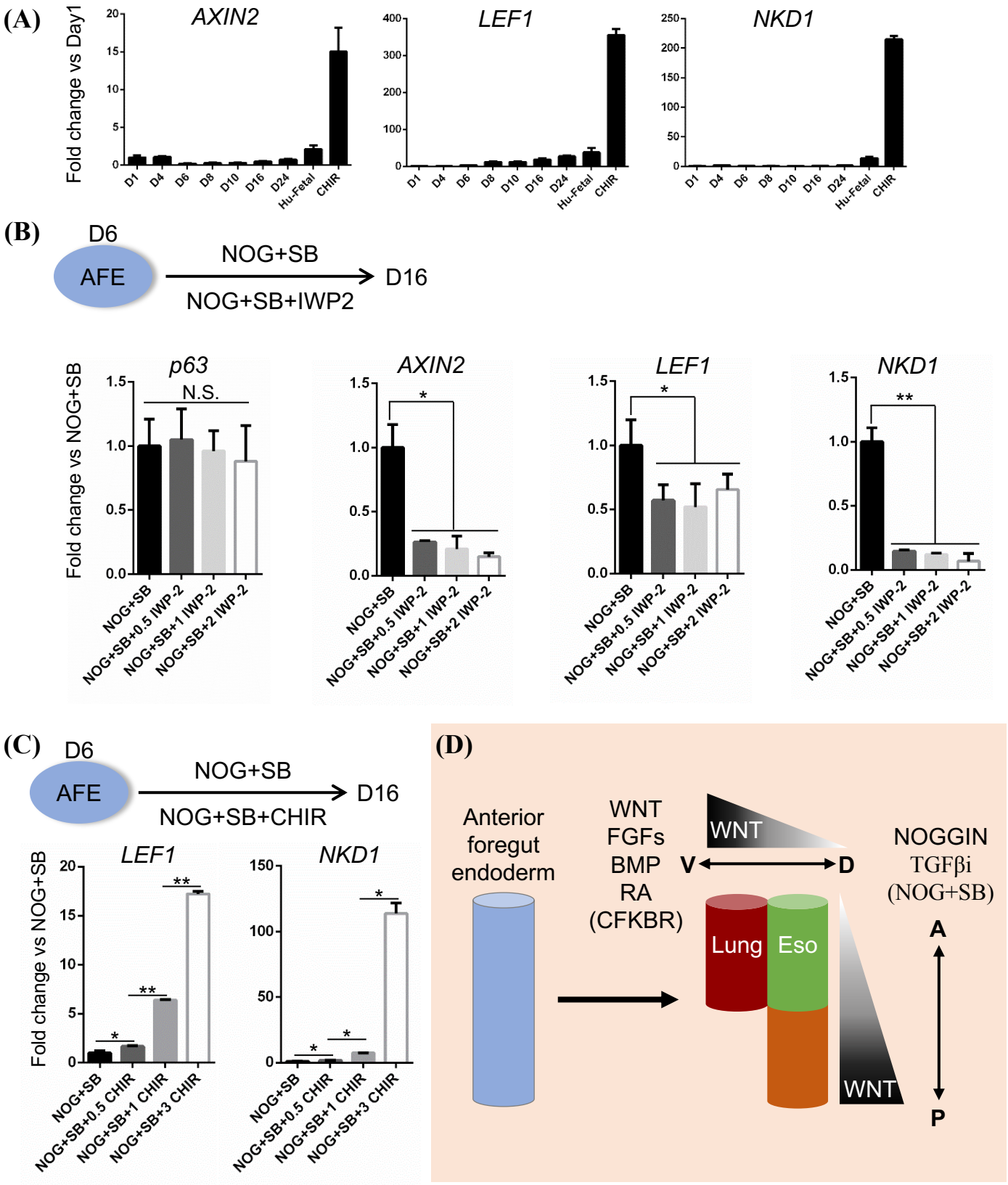
(A)
AXIN2
LEF1
NKD1
Fold change vs Day1
D1
D4
D6
D8
D10
D16
D24
Hu-Fetal
CHIR
(B)
D6
AFE
NOG+SB
NOG+SB+IWP2
D16
p63
N.S.
AXIN2
LEF1
NKD1
Fold change vs NOG+SB
NOG+SB
NOG+SB+0.5 IWP-2
NOG+SB+1 IWP-2
NOG+SB+2 IWP-2
(C)
D6
AFE
NOG+SB
NOG+SB+CHIR
D16
LEF1
NKD1
Fold change vs NOG+SB
NOG+SB
NOG+SB+0.5 CHIR
NOG+SB+1 CHIR
NOG+SB+3 CHIR
(D)
Anterior foregut endoderm
WNT
FGFs
BMP
RA
(CFKBR)
WNT
V
D
Lung
Eso
WNT
NOGGIN
TGFβi
(NOG+SB)
A
P

Figure S2 (Related to Figures 1-2). Combined in- and activation of multiple signaling pathways promotes sequential differentiation of PSCs towards esophageal progenitor cells. (A) Expression of the WNT signaling downstream targets *AXIN2, LEF1* and *NKD1* during EPC differentiation. Human fetal esophagus (Hu-Fetal) was included as control. hESC RUES2-derived AFE cells treated with 3 μM CHIR (Day6-16) was included as a positive control. The transcript levels were represented by the fold change compared to day 1 (D1) hESC. Data represent mean ± SEM (n = 3). **(B)** WNT inhibition by IWP2 does not affect esophageal specification. RUES2-derived AFE cells were treated with Noggin (NOG) plus SB431542 (SB) in combination with 0.5, 1 or 2 μM IWP2 from day6 to day16. Gene expression was determined at day16. The transcript levels of were represented by the fold change compared to NOG+SB. Data represent mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$ by unpaired, two-tailed Student's t test. N.S., not significant. **(C)** WNT activation by 0.5, 1 or 3 μM CHIR increased the transcript levels of the WNT targets LEF1 and NKD1. The transcript levels of were represented by the fold change compared to NOG+SB. Data represent mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$ by unpaired, two-tailed Student's t test. **(D)** Schematics of generating esophageal progenitor cells from the hPSC-derived AFE cells. While WNT, FGF, BMP and retinoic acid signaling activation by the CFKBR cocktail promotes respiratory cell fate, BMP and TGFß dual inhibition by Noggin and SB431542 promotes esophageal cell fate. Additionally, WNT activation combined with BMP and TGFß dual inhibition posteriorizes the foregut. NOG, Noggin; SB, SB431542.

Figure S3

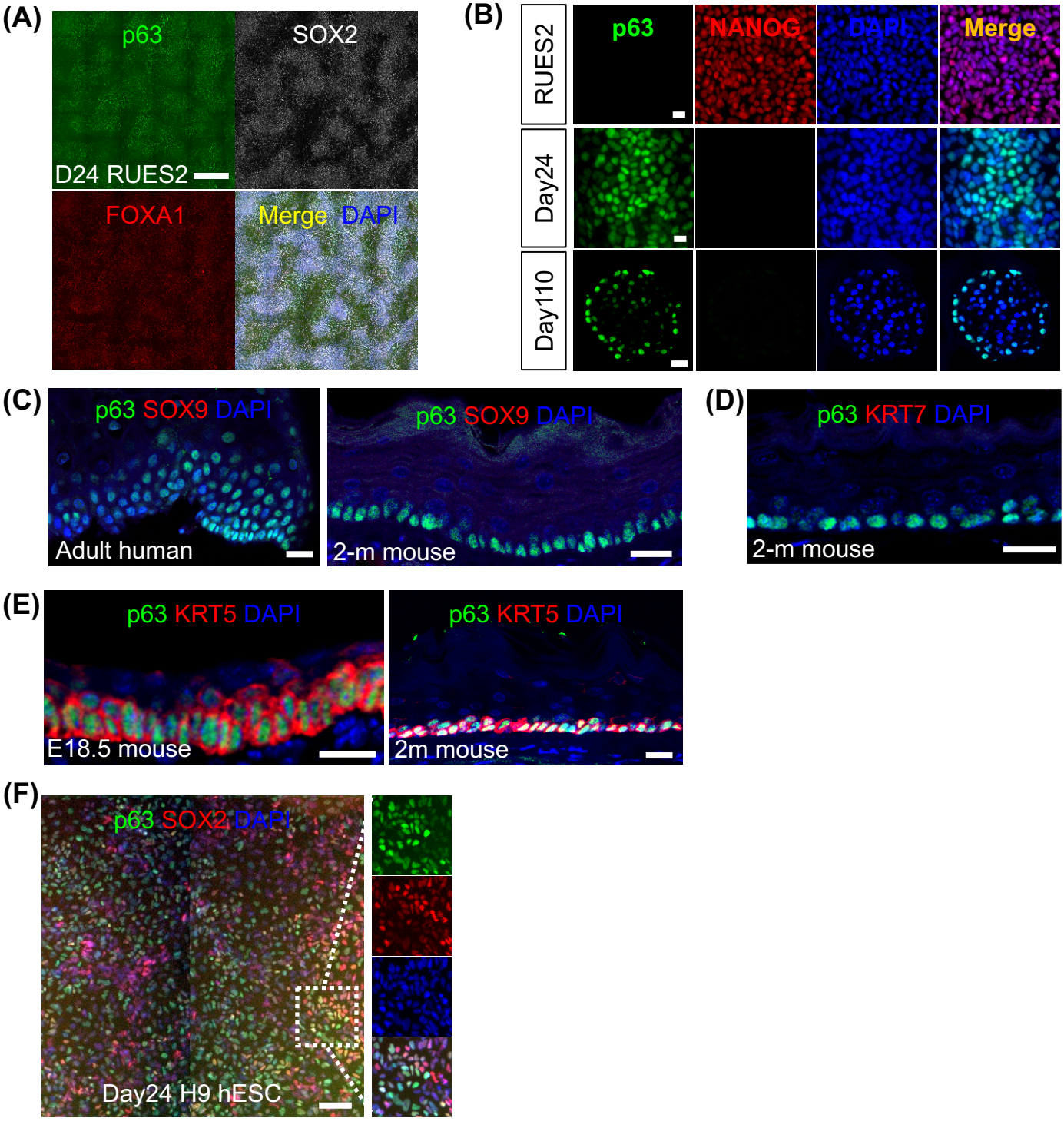
(A)
p63
SOX2
D24 RUES2
FOXA1
Merge DAPI
(B)
RUES2
Day24
Day110
p63
NANOG
DAPI
Merge
(C)
p63 SOX9 DAPI
Adult human
p63 SOX9 DAPI
2-m mouse
(D)
p63 KRT7 DAPI
2-m mouse
(E)
p63 KRT5 DAPI
E18.5 mouse
p63 KRT5 DAPI
2m mouse
(F)
p63 SOX2 DAPI
Day24 H9 hESC

Figure S3 (Related to Figures 1-3). Expression of epithelial markers in the mouse and human esophagus (A) Representative 20x tile scans of RUES2-derived esophageal progenitors cells of day24 culture (n = 5). **(B)** NANOG is expressed in hESC RUES2 but not in $p63^+$ EPCs in 2D differentiation culture at day24 and in 3D organoid culture at day110. **(C)** SOX9 is not expressed in the adult human and mouse esophagus. **(D)** KRT7 expression is lost in the adult mouse esophagus. **(E)** Expression of p63 and KRT5 in the basal progenitor cells of the stratified squamous epithelium in E18.5 and adult mouse esophagus. **(F)** Representative tile scan images of EPCs ($p63^+$ $SOX2^+$) derived from the hESC H9 cell line. Note EPCs coexpress p63 and SOX2. Abbreviation: m, month. Scale bars: (A) 1 mm, (B-F) 20 μm, (H) 100 μm.

Figure S4

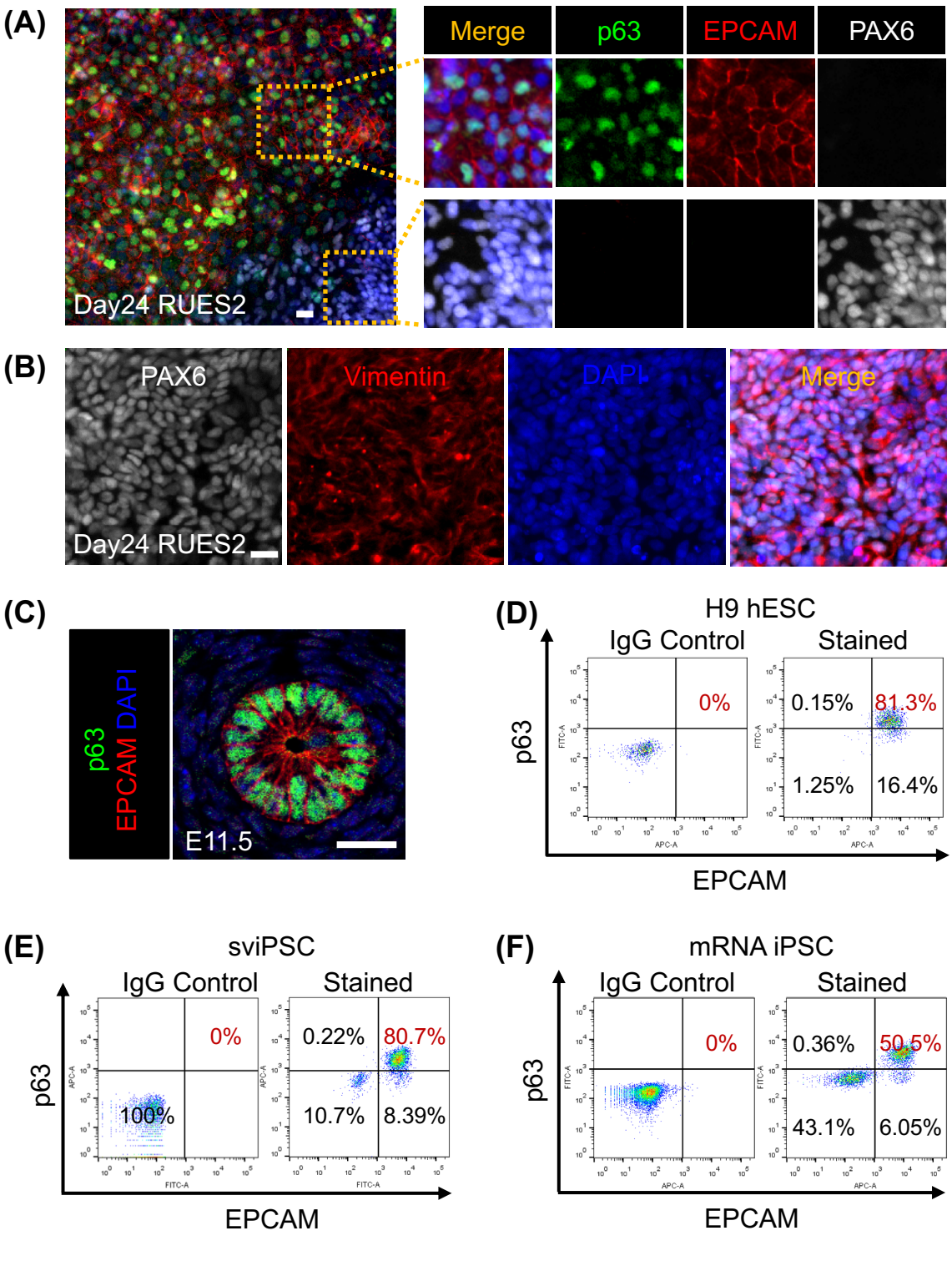
(A)
Merge
p63
EPCAM
PAX6
Day24 RUES2
(B)
PAX6
Vimentin
DAPI
Merge
Day24 RUES2
(C)
p63
EPCAM DAPI
E11.5
(D)
H9 hESC
IgG Control
Stained
p63
EPCAM
0%
0.15%
81.3%
1.25%
16.4%
(E)
sviPSC
IgG Control
Stained
p63
EPCAM
0%
100%
0.22%
80.7%
10.7%
8.39%
(F)
mRNA iPSC
IgG Control
Stained
p63
EPCAM
0%
0.36%
50.5%
43.1%
6.05%

Figure S4 (Related to Figures 3-4). Purification of EPCs from differentiating PSCs. (A-B) The majority of non-epithelial cells ($EPCAM^-$) in day24 RUES2 culture are neuroectodermal cells ($PAX6^+$ $EPCAM^-$ $p63^-$). Note $p63^+$ $EPCAM^+$ cells are $PAX6^-$ (A), and co-expression of PAX6 and Vimentin in RUES2-derived cells (B). **(C)** p63 and EPCAM are co-expressed in the E11.5 mouse esophagus. **(D)** After 24day differentiation, EPC yield ($p63^+$ $EPCAM^+$) for the hESC H9 cell line is 80.6±4.2% (n = 3). **(E-F)** After 24day differentiation, yield for sviPSC- and mRNA iPSC-derived EPCs is 80.2±6.5% (n=3) and 50.1±3.5% (n=3 for each), respectively. Scale bars: 20 μm.

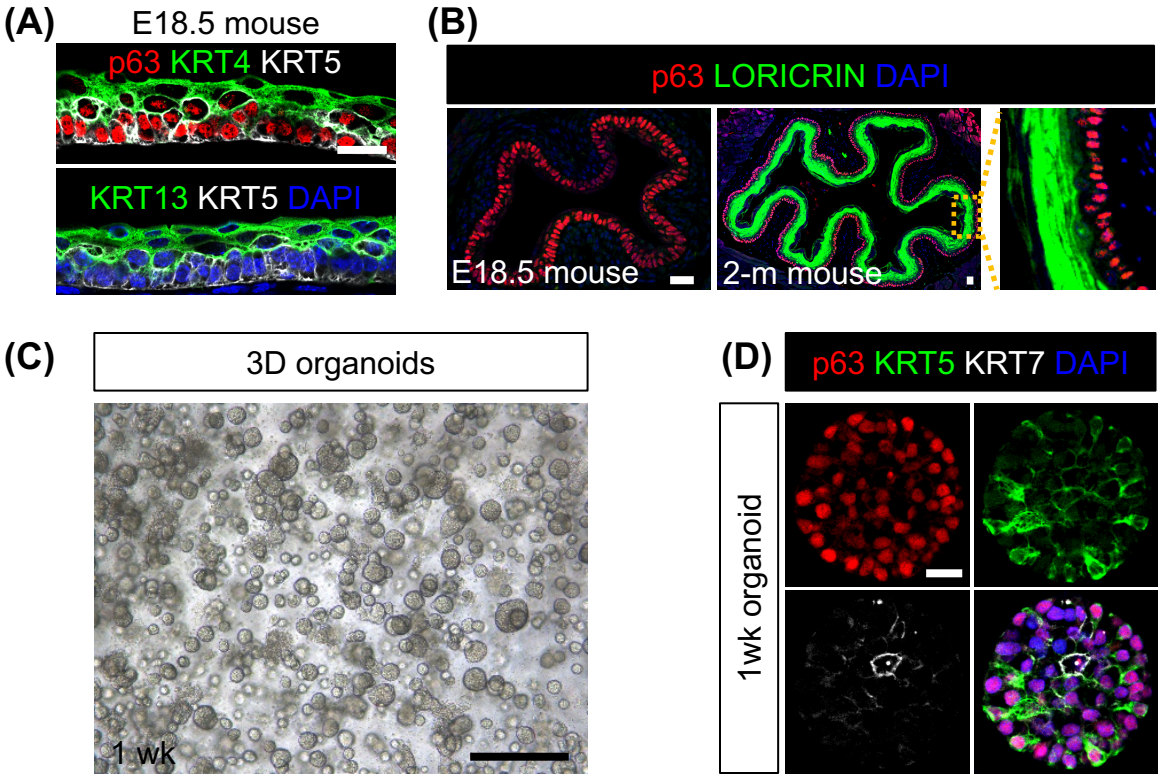
(A)
E18.5 mouse
p63 KRT4 KRT5
KRT13 KRT5 DAPI
(B)
p63 LORICRIN DAPI
E18.5 mouse
2-m mouse
(C)
3D organoids
1 wk
(D)
p63 KRT5 KRT7 DAPI
1wk organoid

Figure S5 (Related to Figures 4-5). hPSC-derived EPCs reconstitute the stratified squamous epithelium. (A) Expression of p63, KRT4, KRT5 and KRT13 in E18.5 mouse esophagus. Note the expression of KRT5 is limited to $p63^{+}$ basal cells and KRT4 and KRT13 are expressed in the suprabasal cells. **(B)** LORICRIN is expressed in 2-month but not E18.5 mouse esophagus. **(C)** Bright images of 1-week 3D organoids established using RUES2-derived EPCs (n = 3). **(D)** The expression of KRT7 is diminished while the expression of the basal cell marker KRT5 is maintained in 1-week 3D esophageal organoids. Scale bars: (A, B, D) 20 μm, (C) 1 mm.

Figure S6

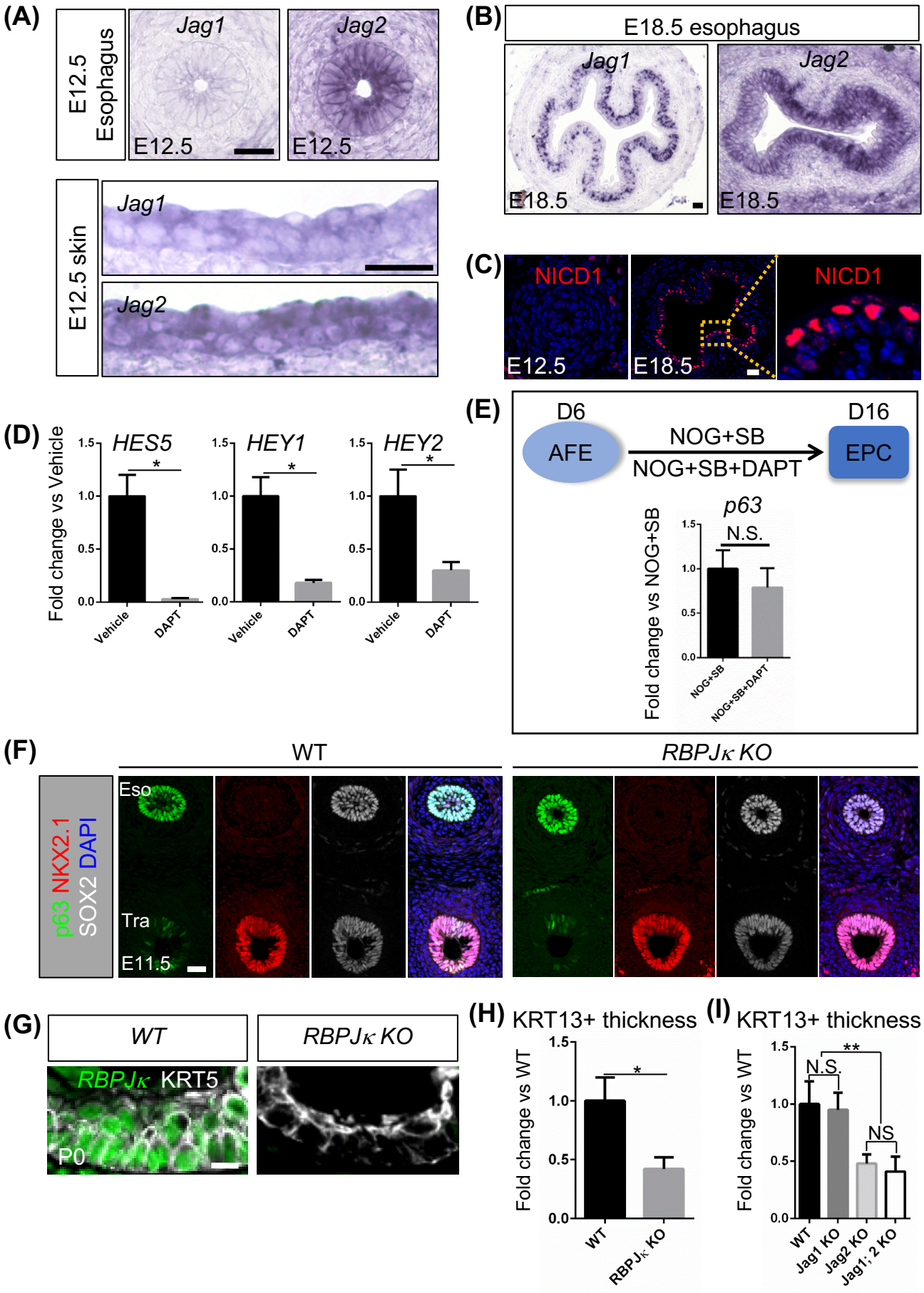
(A)
E12.5 Esophagus
Jag1
Jag2
E12.5
E12.5
E12.5 skin
Jag1
Jag2
(B)
E18.5 esophagus
Jag1
Jag2
E18.5
E18.5
(C)
NICD1
NICD1
E12.5
E18.5
(D)
Fold change vs Vehicle
HES5
HEY1
HEY2
Vehicle
DAPT
(E)
D6
D16
AFE
NOG+SB
NOG+SB+DAPT
EPC
p63
N.S.
Fold change vs NOG+SB
NOG+SB
NOG+SB+DAPT
(F)
WT
RBPJκ KO
p63 NKX2.1
SOX2 DAPI
Eso
Tra
E11.5
(G)
WT
RBPJκ KO
RBPJκ KRT5
P0
(H)
KRT13+ thickness
Fold change vs WT
WT
RBPJκ KO
(I)
KRT13+ thickness
Fold change vs WT
N.S.
NS
WT
Jag1 KO
Jag2 KO
Jag1; 2 KO

Figure S6 (Related to Figures 6-7). NOTCH signaling is required for the formation of the stratified squamous epithelium. (A-B) RNA in situ hybridization of *Jag1* and *Jag2* in E12.5 mouse esophagus and skin (A) and E18.5 mouse esophagus (B). **(C)** Expression of Notch1 Intracellular Domain (NICD1) is correlated with the squamous differentiation of epithelial progenitors. Note at E12.5 the levels of NICD1 are too low to be detected whereas at E18.5 NICD1 is expressed in the differentiated (suprabasal) cell. **(D)** The transcript levels of *HES5*, *HEY1* and *HEY2* are decreased in EPCs following treatment of 10 μM DAPT (γ-secretase inhibitor) for 6 days. The transcript levels were represented by the fold change compared to Vehicle controls. Data represent mean ± SEM (n = 3). *p < 0.05 by unpaired, two-tailed Student's t test. EPCs were derived from RUES2 by culturing for 24days. **(E)** Inhibition of NOTCH signaling by DAPT did not affect EPC specification from the hESC-derived AFE. The transcript levels were represented by the fold change compared to NOG+SB. **(F)** Expression of p63, NKX2.1 and SOX2 in the epithelium of *Shh-Cre; RBPJκ$^{loxp/loxp}$* (*RBPJκ KO*) mutants at E11.5 (n = 4). Note p63 is expressed in *RBPJκ KO* mutants. **(G)** Expression of *RBPJκ* is lost in the epithelium of *Shh-Cre; RBPJκ$^{loxp/loxp}$* (*RBPJκ KO*) mutants at P0. **(H)** Thickness of KRT13^{+} suprabasal layers is reduced in P0 *RBPJκ KO* mutants. Data represent mean ± SEM (n = 4). *p < 0.05 by unpaired, two-tailed Student's t test. **(I)** Thickness of KRT13^{+} suprabasal layers is reduced in the esophagus of *Shh-Cre; Jag2$^{loxp/loxp}$* (*Jag2 KO*) and *Shh-Cre; Jag1$^{loxp/loxp}$; Jag2$^{loxp/loxp}$* (*Jag1; 2 KO*) mutants. Data represent mean ± SEM (n = 3). **p < 0.01 by unpaired, two-tailed Student's t test. N.S., not significant. Abbreviation: Tra, trachea; Eso, esophagus. Scale bars: 20 μm.

Table S1

Genes	Forward primers	Reverse primers
AXIN2	TGTCCAGCAAAACTCTGAGG	GTGCAAAGACATAGCCAGAAC
p21	TGTCACTGTCTTGTACCCTTG	GGCGTTTGGAGTGGTAGAA
COL1A1	CCCCTGGAAAGAATGGAGATG	TCCAAACCACTGAAACCTCTG
FOXA1	AGGGCTGGATGGTTGTATTG	TGAGTTCATGTTGCTGACCG
FOXA2	CTGGTCGTTTGTTGTGGC	TTCATGTTGCTCACGGAGG
FOXE1	GAGCCTGCTACAACCCTG	TGTGTCTATGAGTTTTCGTCCC
HES5	CTACCTGAAGCACAGCAAAG	AGCTTCATCTGCGTGTCG
HEY1	TGGTACCCAGTGCTTTTGAG	CTCCGATAGTCCATAGCAAGG
HEY2	ATTATAGAGAAAAGGCGTCGGG	GCATCTTCAAATGATCCACTGTC
HNF6A	GAGGATGTGGAAGTGGCTG	ACATCTGTGAAGACCAACCTG
ID2	CATCCCACTATTGTCAGCCTG	AGAAGGGAATTCAGAAGCCTG
INVOLUCRIN	CTGCCTCAGCCTTACTGTG	GCTCCTGATGGGTATTGACTG
JAG1	GGACTATGAGGGCAAGAACTG	AAATATACCGCACCCCTTCAG
JAG2	CAGGAAGTGATCGGGTTCG	CAGACAAGGCTTCCATCCG
JUNB	GGACACGCCTTCTGAACG	CGGAGTCCAGTGTGGTTTG
KLF4	ACCTACACAAAGAGTTCCCATC	TGTGTTTACGGTAGTGCCTG
KLF5	GAAGGAGTAACCCCGATTTGG	CTTCCCAGGTACACTTGTATGG
KRT13	AAGACCATTGAAGAGCTCCG	TGGCATTGTCAATCTCCAGG
KRT14	GAAGTGAAGATCCGTGACTGG	GCAGAAGGACATTGGCATTG
KRT4	AGCTAGATACCTTGGGCAATG	CACAAAGTCATTCTCGGCTG
KRT5	AGAGCTGAGAAACATGCAGG	AGCTCCACCTTGTTCATGTAG
KRT7	CAGGATATGGCACGGCAG	CACAGAGATATTCACGGCTCC
LEF1	AGACAAGCACAAACCTCTCAG	TCATTATGTACCCGGAATAACTCG
NANOG	GAAATACCTCAGCCTCCAGC	GCGTCACACCATTGCTATTC
NKD1	CTCGCCGGGATAGAAAACTAC	GGTGTGGGATGTGGATGG
NKX2.1	CAGGACACCATGAGGAACAG	TCATGTTCATGCCGCTCG
p15	GTTAAGTTTACGGCCAACGG	ACCTTCTCCACTAGTCCCC
p63	TTCGGACAGTACAAAGAACGG	GCATTTCATAAGTCTCACGGC
PAX9	GGTGAACGGGTTGGAGAAG	CTGTAGGTCATGTAAGGCGAC
PROX1	TTTTATACCCGTTATCCCAGCTC	TGCGTACTTCTCCATCTGAATG
SOX2	CACACTGCCCCTCTCAC	TCCATGCTGTTTCTTACTCTCC
SOX9	ACTTGCACAACGCCGAG	CTGGTACTTGTAATCCGGGTG
TGFBI	GTCTACACAGTCTTTGCTCCC	TCCGCTAACCAGGATTTCATC
WNT5A	TCGCCCAGGTTGTAATTGAAG	TGAGAAAGTCCTGCCAGTTG

Table S1 (Related to the STAR Methods Section, Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Primer sequences for qRT-PCR.