Multi-directional differentiation of Ascl1-defined progenitors in lung development and injury repair

Yan Li and Ilona Linnoila

ONLINE DATA SUPPLEMENT

Supplementary Method: X-Gal Staining

For X-Gal staining, whole embryos (E10.5-E14.5) or dissected lungs (E12.5-P42) were fixed in 4% paraformaldehyde (PFA) for 1-2 hours at room temperature. After washing in phosphate buffer, the tissues were incubated overnight in X-Gal staining solution (29). Tissues were washed in PBS and post-fixed with 4% PFA overnight at 4°C, dehydrated in 70% ethanol, embedded in paraffin, sectioned at 5µm and stained with Nuclear Fast Red (Vector Laboratories). For cryosectioning, embryos were fixed in 4% PFA for 2 hours at 4°C, rinsed in phosphate buffer, cryoprotected in 30% sucrose, and embedded frozen in OCT (Tissue-Tek) and sectioned at 12µm. Lungs were inflated with 1ml 4% PFA, fixed 1 hour (prenatal), 2 hours (postnatal 1-20 days) and 4 hours (over 3 weeks old) at 4°C before processing for cryosection as above. For paraffin sectioning, lungs were inflated with 1ml 4% PFA, lung lobes were dissected from mice or embryos older than E12.5 and fixed in 4% PFA for 16 hours at 4°C, dehydrated in 70% ethanol and sectioned at 5µm.

Supplementary Table 1.

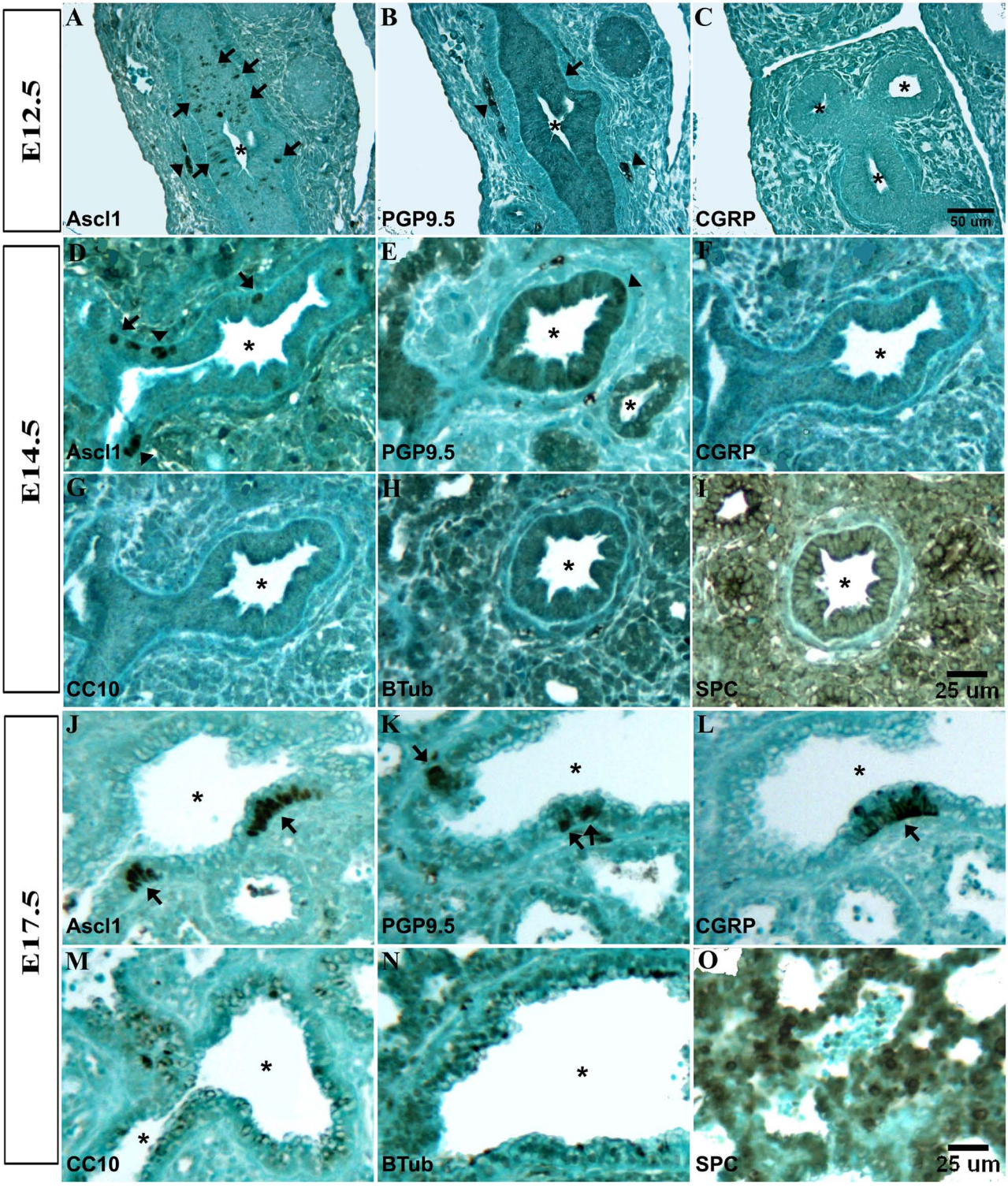
Antibodies	Manufacturer	Dilution	Specificity
Ascl1	BD Pharmingen	1:20	PNEC, neuron, ganglia
PGP9.5	AbD SeroTec	1:6000	PNEC, neuron, ganglia
CGRP	Sigma	1:3000	PNEC, neuron
CC10/UP-1	DAKO	1:1000	Clara cells
FoxJ1/HFH4	abcam	1:400	Ciliated cells
BTub	Gift from Dr. Charles Bevins	1:1000	Ciliated cells
pro-SPC	Chemicon	1:200	Alveolar type 2 cells
α-SMA	abcam	1:400	Smooth muscle cells
TTF-1	Leica microsystem	1:100	Epithelial cells (embryonic)
P75NTR	Promega	1:200	Migrated neuron cells
Aquaporin 5	Millipore	1:1000	Alveolar type 1 cells
GFP	Nacalai tesque	1:500	YFP-expressing cells

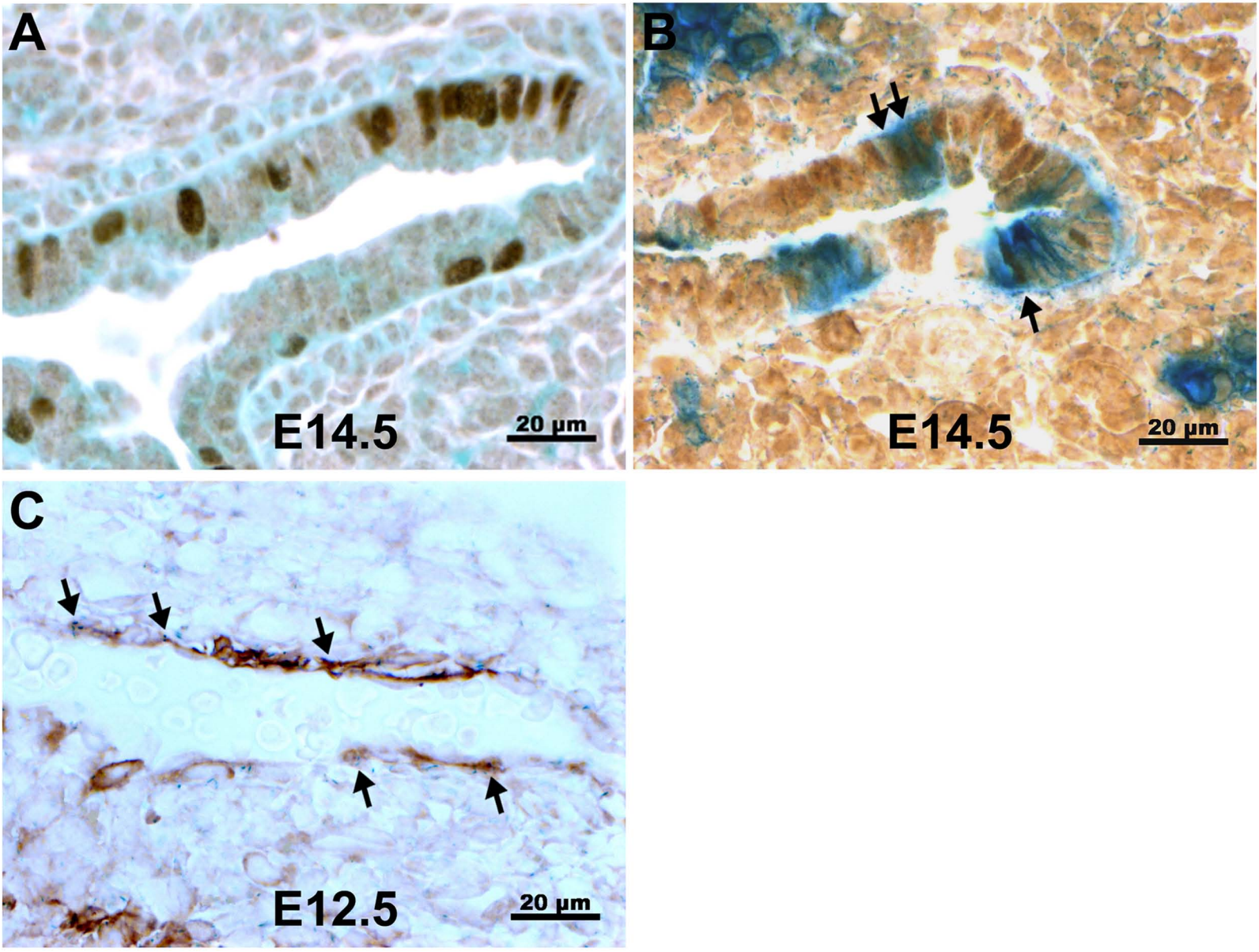
Supplementary Figure 1. Photomicrographs of PNEC and epithelial cell differentiation during development.

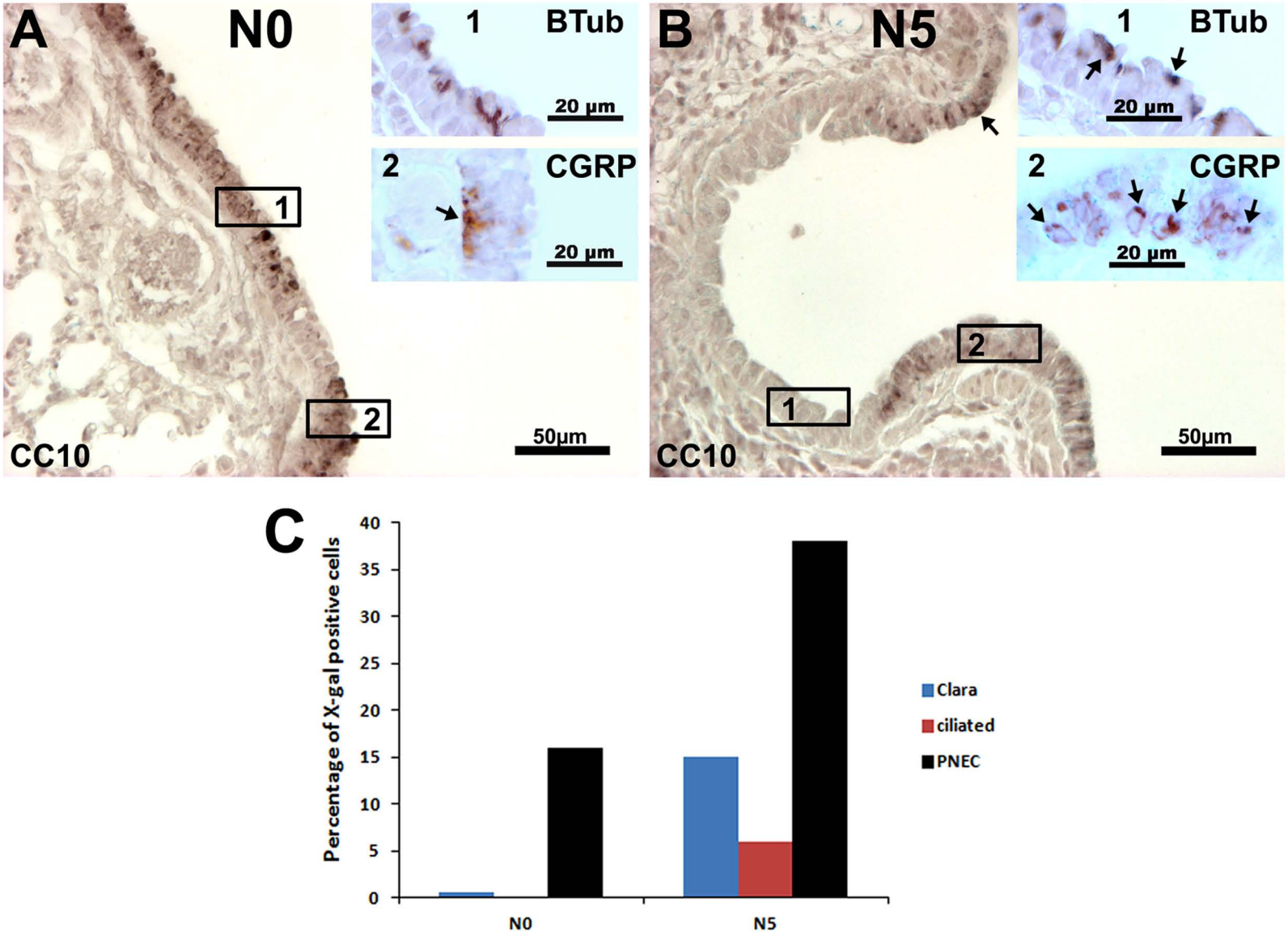
(A-C) PNEC markers in E12.5 embryonic lung. (A) Ascl1 is expressed in solitary PNECs along the proximal airway lining (arrow) and in an adjacent ganglion (arrow head). (B) PGP9.5 is expressed in all the airway epithelial cells (arrow) and ganglia (arrow head). (C) No CGRP is detected. (D-I) Differentiation in E14.5 embryonic lung. (D-E) NEBs start to form in the proximal airways. (D) Ascl1 is expressed in both NEBs (arrow heads) and solitary PNECs (arrows). (E) PGP9.5 is expressed in all airway epithelial cells and more intensely in the NEBs (arrow head). (F-H) No CGRP positive PNECs, Clara, or ciliated cells are present. (I) SPC is expressed in all the epithelial cells. (J-O) Cell differentiation in E17.5 embryonic lung. (J-L) NE markers Ascl1, PGP9.5 and CGRP in NEBs (arrows). (M-O) Epithelial cells are differentiated into Clara cells (M), ciliated cells (N), and alveolar type 2 cells (O) (immunoperoxidase stain; * = airway lumen; bar = 50 μm for A-C; bar = 25 μm for D-O).

Supplementary Figure 2. (A-B) Photomicrographs of emerging ciliated and smooth muscle cells in embryonic lungs. (A) FoxJ1 in proximal airways of E14.5 lung. The immunoreactivity is nuclear (immunoperoxidase stain; bar = 20 μm). (B) Co-localization of ASDCs by X-Gal staining (blue) with brown FoxJ1 immunoreactive signal (arrows; bar = 20 μm). (C) Co-localization of ASDCs by X-Gal staining (blue) with α-SMA immunoreactivity (arrows) in vascular smooth muscle at E12.5 (arrows; bar = 20 μm).

Supplementary Figure 3. Co-localization of lacZ labeling with differentiation markers in naphthalene injury repair. (A) Airway epithelium from control tamoxifen-induced Ascl1-CreERTM;R26-lacZ mouse 5 days after i.p. injection of corn oil (N0). Most cells are positive for CC10 (left panel). Insets illustrate corresponding areas of epithelium in adjacent sections positive for β-tubulin (1) or CGRP (2) (right panels). Note co-localization of blue labeling in cells with brown immunoreactivity (arrows). (B) Airway epithelium from tamoxifen-induced Ascl1-CreERTM;R26-lacZ mouse 5 days after i.p. injection of naphthalene (N5). Groups of CC10 positive cells (left panel). Insets illustrate corresponding areas of epithelium in adjacent sections positive for β-tubulin (1) or CGRP (2) (right panels). Note co-localization of blue labeling in cells with brown immunoreactivity (arrows). bar= 50 μm in B and C; bar= 20 μm in (1) and (2). (C) Bar graph of ASDC quantification. Percentage of Clara (blue bar), ciliated (red bar) cells or PNECs (black bar) that are also X-Gal positive (ASDCs). N0 = oil-treated control; N5= five days post-naphthalene. A total of 1000 CC10 and BTub positive cells, and 30-45 NEBs (200-350 PNECs) were counted per animal (n=3).


E12.5
A
Ascl1
B
PGP9.5
C
CGRP
50 um
E14.5
D
Ascl1
E
PGP9.5
F
CGRP
G
CC10
H
BTub
I
SPC
25 um
E17.5
J
Ascl1
K
PGP9.5
L
CGRP
M
CC10
N
BTub
O
SPC
25 um


A
E14.5
20 µm
B
E14.5
20 µm
C
E12.5
20 µm


A
N0
1
2
CC10
50µm
1
BTub
20 µm
2
CGRP
20 µm
B
N5
1
2
CC10
50µm
1
BTub
20 µm
2
CGRP
20 µm
C
Percentage of X-gal positive cells
40
35
30
25
20
15
10
5
0
N0
N5
Clara
ciliated
PNEC