Online Data Supplement

A Single Cell Functions as a Tissue-Specific Stem Cell and the *In Vitro* Niche-Forming Cell

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Methods

Isolation and culture of tracheal epithelial cells: For air-liquid interphase studies, filler cells were prepared and cultured as previously reported (1). These cells were derived from transgenic mice that express green fluorescent protein under regulation of the ubiquitin promoter (2). These cells are referred to as "filler cells". Cells were counted and viability determined by trypan blue exclusion. ALI cultures were incubated in modified mouse tracheal epithelial culture plus (mMTEC Plus) medium. Supplements were as follows: Epidermal growth factor (EGF) (BD Biosciences): 25 ng/ml; Hydrocortisone (MP Biomedicals) : 0.1 mg/ml; Cholera toxin (Sigma): 0.1 μg/ml; Insulin-Transferrin-Selenite (ITS) (Gibco): 10 μg/ml insulin, 5 μg/ml transferrin, 5 μg/ml sodium selenite; Bovine pituitary extract (Gibco): 0.03 mg/ml; Retinoic acid (Sigma-Aldrich): 50 nM; Fetal bovine serum (FBS) (Hy Clone): 5%. Differentiation was induced by transition to 2% Nuserum plus 50 nM retinoic acid in MTEC-basic (NS medium) (1).

For co-culture analysis, the ratio of rimmed clone cells to tracheal cells was 1:100. For analysis of paracrine signaling, tracheal cells were cultured in the basal compartment of the Transwell culture system at 1 X 10^5 cells/cm^2. Rimmed clone cells were cultured in the apical chamber at 1 X 10^5 cells/cm^2. Both the Transwell membrane and the plastic surface of the basal compartment were coated with a rat tail collagen film. When the rimmed clone epithelium was polarized, the medium was changed to NS medium and the air-liquid-interface established. Cultures were fed on alternate days and fixed as indicated below on differentiation day 10.

Clonal analysis: As an initial test of clonality, tracheal cells isolated from mice expressing green fluorescent protein (GFP) under control of the ubiquitin promoter (2) and from mice expressing red fluorescent protein (RFP) under regulation of the chicken β-actin promoter/CMV enhancer (3) were mixed at a ratio of 1:1, 3:1 and 1:3 and cultured on 3T3 feeders at a density of 3x10^4 cells/cm^2. Cells were cultured in irradiated NIH3T3 fibroblasts as indicated in the main methods section. Cultures were analyzed on culture day 14. The number of colonies expressing only endogenous GFP or DsRed fluorescence was expressed as a function of the total number of colonies. These results were confirmed by independent quantification of mixed color colonies and expression of their number as a function of total colony numbers.

Limiting dilution analysis method: Rimmed clones were generated from

sorted CD49f^{bright}/Sca1^{+}/ALDH^{+} cells and allowed to grow for 14 days. Individual clones were isolated from the rest of the culture using a cloning cylinder and cells recovered by trypsinization. The cells were counted and plated in 96-well plates using 8 dilution levels: 200, 100, 50, 25, 12, 6 and 3 and 1 cell/well. These passage (p) 1 cultures were incubated for 14 days. Wells were scored as positive or negative for clone-growth using an inverted microscope equipped with phase objectives. The positive / negative data were used to calculate the clone forming cell efficiency for each of 5 p1 clones. These data were also used to select a dilution level that resulted in 99% probability that the clone was generated by a single cell. Clones selected from this dilution level were trypsinized and plated in 96-well plates using the 8 dilution levels indicated above. The passage process was repeated until the isolated clone cells failed to form clones.

Source information:

Table 1. Reagents

Name	**Source**
Media	Mediatech
Trypsin	Mediatech
Dispase	Gibco
Collagenase	Sigma
RBC lysis buffer	BD Biosciences
Rat tail collagen I	BD Pharmingen
Bovine serum albumin	Fischer Scientific
DNAse	Sigma-Aldrich
DAPI	Sigma-Aldrich
Assay on demand	Applied Bio system
RNA isolation kit	Stratagene
cDNA synthesis kit	Invitrogen

Table 2: Immunofluorescence Antibodies

Name	**Source**	**Host**	**Dilution**
Keratin 5	Covance Innovative Antibodies	Rabbit	1:1000
Keratin 14	Thermo Scientific	Mouse	1:500
E-cadherin	Santa Cruz Biotechnology	MAb	1:250
Clara cell secretory protein	In house (4)	Rat MAb	1:2500
(CCSP)		Goat	
Acetylated Tubulin (ACT)			
Anti-BrdU	Sigma-Aldrich		1:5000
Ki-67		Mouse	
Aldh1a1	Accurate Chemical and Scientific	MAb	1:250
p63	Corp.		1:100
	Dako Cytomation	Rat	1:500
	BD Pharmingen	Rat MAb	1:250
	BD Pharmingen	Mouse	
		MAb	
		Mouse	
		MAb	

Table 3: Flow Cytometry Antibodies.

Name	**Source**	**Dilution (for 10^5cells)**
CD31	eBiosciences	5 µl
CD45	BD Biosciences	2 µl
TER119	BD Biosciences	2 µl
CD34	BD Biosciences	4 µl
CD49f	BD Biosciences	6 µl
Sca1	BD Biosciences	0.5 µl
CD90	BD Biosciences	3.0 µl
Compensation beads	BD Biosciences	

Results

Detailed explanation of results in Figure1: Rimmed clones formed by unfractionated tracheal cells contain label-retaining and label-diluting cells.

Methods: Unfractionated mouse tracheal epithelial cells were cultured on irradiated 3T3-feeders for up to two weeks. The proliferation characteristics of tracheal colonies was evaluated by BrdU pulse analysis. Control cultures were pulsed with BrdU on culture days 0-3, 3-5, 5-7, or 12-14 and evaluated at the end of each labeling period. As an initial test of clonality, tracheal cells isolated from mice expressing Green fluorescent protein (GFP) under control of the ubiquitin promoter (2) and from mice expressing red fluorescent protein (RFP) under regulation of the chicken β-actin promoter/CMV enhancer (3) were mixed at a ratio of 1:1, 3:1 and 1:3 and cultured on irradiated NIH3T3 feeders at a density of $3x10^4$ cells/cm^2.

Results: Unfractionated tracheal cells generated two kinds of colonies; non-rimmed and rimmed. Non-rimmed colonies had irregular edges and exhibited cobblestone morphology (Fig 1A). Rare colonies had two domains, consisting of a cobblestone central region and a stratified rim (Fig 1B). These rimmed colonies were round, could be viewed with the naked eye on culture days 9-14, and grew at a rate of

0.5 ± 0.1 mm/day. The frequency of rimmed clone forming cells in unfractionated cell preparations was 1 in $4x10^4$ cells.

Numerous $BrdU^+$ cells were observed in rimmed clone cultures pulsed on culture days 0-3, 3-5, 5-7 and 12-14. These data demonstrate that proliferation occurred throughout the 14 day culture period. In contrast, non-rimmed clones did not label with BrdU on or after culture day 3. These data indicated that rimmed clone forming cells had extensive mitotic potential.

Label retention was used as the *initial* indicator of a cell that proliferated infrequently relative to its progeny. Cultures were pulsed with BrdU as indicated above and chased for two weeks (Fig 1F-L). Rare label-retaining cells were present in rimmed clones pulsed between days 0-3 and chased to day 14 (Fig 1F, J). These cells were located primarily in the rim domain. BrdU label-retaining cells were also detected in cultures that were pulsed on days 3-5 and 5-7 and chased to day 14 (Fig 1G, H). However, cells labeled at these time points were infrequent compared with the number that proliferated during culture days 0-3. These data indicated that most rimmed clone cells depleted their label by day 14 and that rimmed clone cells segregated into mitotically quiescent (label-retaining) and active (label-diluting) subsets early in the culture period.

On culture day 14, cultures initiated by a mix of tracheal cells derived from GFP and DsRED expressing mice generated non-rimmed and rimmed colonies. Those composed exclusively of GFP^+ cells or RFP^+ cells were detected with a frequency of 97.22 ± 1.73% (Fig 1C, D and E). This rate of false clonal assignment is within previously reported limits (5, 6). These data indicated that non-rimmed and rimmed colonies were derived from a single cell and allow their designation as clones.

Dual immunofluorescence analysis was used to determine the cellular phenotype of rimmed clone cells. Cells in both the central and rim domains were positive for K5, a basal cell marker (Fig 1K). In contrast, $K14^+$ cells were present only in the rim domain (Fig 1L). BrdU label-retaining cells co-expressed K5 or K14 (Fig 1K and L). Rimmed clones did not express differentiation markers associated with secretory cell types, Clara cell secretory protein (CCSP) and Muc5AC, or a marker associated with ciliated cells, acetylated tubulin (ACT) (data not shown). These data indicated that rimmed clones contain basal cells and are consistent with in vivo analysis demonstrating that highly mitotic tracheal basal cells co-express K5 and K14 (7).

Optimization of tracheal cell recovery and cell surface marker expression:
Methods: Enzymatic digestion was required to recover tracheal cells for FLOW cytometric analysis. Therefore enzyme-sensitivity of the cell surface markers used in separation of tracheal epithelial cell subsets was evaluated using three different cell isolation strategies. To evaluate time-dependent susceptibility to the proteases, tracheas were digested for 30, 60 or 120 minutes. Tracheas were incubated at 37 °C with 0.15% pronase (1), 4 unit/ml elastase (8), or a mixture of dispase (0.3%), collagenase VIII (0.2%) and trypsin (0.05%) (DCT(9)). Cells were stained

for Sca1 and CD34 as indicated in Material and Methods. Fluorescence intensity was evaluated by flow cytometry as indicated in Materials and Methods.

Results: A one-hour incubation of the trachea with pronase significantly decreased the mean fluorescence intensity of Sca1 and CD34 (Supplemental Fig. 1A-C). Digestion with elastase retained these antigens (Supplemental Fig 1D-F) but resulted in poor recovery of basal cells (data not shown). In contrast, digestion of tracheas with DCT preserved Sca1, CD34 (Supplemental Fig 1G-I), and CD49f (data not shown).

Figure Legend

Figure 1
Enzyme susceptibility of Stem cell antigen 1 (Sca1) and CD34: Flow cytometric analysis of Sca1 and CD34 fluorescence intensity on tracheal cells recovered using various digestion protocols. Whole trachea was digested for various times (indicated in each panel) with three enzymes that are typically used to recover tracheal epithelial cells. Cells were then stained for Sca1 and CD34. The four quadrants were set according to isotype controls. Note that the position of the quadrant markers are invariant. A-C. Cells were recovered with 0.15% pronase. D-F. Cells were recovered with 4 Unit/ml elastase. G-I. Cells were recovered with dyspase/collagenase/trypsin (DCT).

Controls for tracheal epithelial cell fractionation:
Procedure: The optimal dilution of the antibodies used in this study was determined by titration on tracheal cells recovered by a 1 hour digestion with DCT. An isotype control appropriate to each antibody was used as a control. Mean fluorescence intensity was determined with a Mo-Flow XDP cell sorter. Post-sort analysis was done on a portion of the sorted cells using cytometer settings identical to those used for the initial collection.

Results: DCT digested tracheal cells were stained with various single color antibodies at different dilutions to determine the optimum antibody concentration (Supplemental Fig 2). To demonstrate that staining pattern was specific for each antibody, samples were stained with similar amount of the respective isotype control. Shifting of mean fluorescence intensity with antibody to CD49f and isotype (Supplemental Fig 2A), Sca1-isotype (Supplemental Fig 2B), CD45-isotype (Supplemental Fig 2C) and CD31-isotype (Supplemental Fig 2D) demonstrated that staining pattern was specific for individual antibody. Optimal titers are noted the Materials and Methods section.

To determine the purity of populations sorted according to $CD49f^{bright}/Sca1^{+}$ and ALDH activity, a portion of the $ALDH^{+}$ and $ALDH^{-}$ sorted populations were re-analyzed. Purity of the $ALDH^{+}$ population was 93% (F) and of the $ALDH^{-}$ population was 97% (G).

Figure 2
Mean fluorescence intensity of tracheal cells isolated with Dispase/Collagenase/Trypsin (DCT) digestion and stained with individual antibody and respective isotype control. A. CD49f-PE-Cy5; B. Sca1-PE; C. CD45-PE-Cy7 and D. CD31-PE-Cy7. E. Representative

bivariate plots for CD49f and ALDEFLUOR activity (ALDH). The CD49f^{bright}/Sca1^{+} population was separated into the ALDH^{+} and ALDH^{-} subpopulations. F. ALDH^{+} subset, post-sort analysis; G. ALDH^{-} subset, post-sort. Purity of the individual subpopulations is indicated in the panel.

Phenotypic analysis of tracheal epithelial cell subpopulations:
Method: Sorted cells were deposited on glass slides using a cytospin and fixed with 3.4% paraformaldehyde / 3% Sucrose / 1x PBS for 20 minutes. Cells were evaluated for expression of various differentiation markers using standard methods (7).

Results: Quantitative data are presented in Tables 1 and 2 in the main text.

Figure 3
A-B. Molecular phenotype of CD49f^{bright} cells. (A) Cells were cytospun and stained for the basal cell marker K5 (green), the secretory cell marker CCSP, and the nuclear stain DAPI (blue). (B) Similar preparations were stained for the ciliated cell marker ACT (red), K5 (green) and DAPI, (blue).
C. Phenotype of CD49f^{bright}/Sca1^{+}/CD34^{-} cells. Cells were cytospun and stained K5 (green), CCSP (red), and DAPI (blue).

Analysis of label retention in rimmed clones using the K5-tTA/TRE-H2B:GFP model:
Method: Rimmed clones were generated from Mice that were bitransgenic for the K5-rTA transgene (10) and the TRE-H2B:GFP transgene (11) were fed chow containing 625 mg/g doxycycline for 6 days prior to tissue recovery. Clones were generated as previously described and photographed on culture day 14.

Results: GFP^{+} cells were detected in the rim domain of rimmed clones. GFP-bright, GFP-dim, and GFP-negative cells were detected. These data supported the existence of label retaining and label-diluting cells and countered the argument that BrdU^{+} label retaining cells were mitotically senescent. GFP^{+} cells were detected in passage 2 rimmed clones (data not shown).

Figure 4
Analysis of H2B:GFP label-retaining cells in rimmed clones.
Arrows serve as fiduciary points and indicate the location of GFP+ cells.
A. Phase contrast image of a rimmed clone;
B. Direct fluorescence analysis of GFP+ cells in the rimmed domain of a rimmed clone.
C. Merged phase contrast and direct fluorescence image.

Analysis of GFP expression in ALI cultures:
Method: ALI cultures were generated using standard methods from mice expressing GFP under regulation of the ubiquitin promoter (UBI-GFP). On culture day 14, differentiation day 9, a set of 3 cultures were fixed with 3% paraformaldelyde/30% sucrose/ 1X PBS and counterstained with DAPI. The epithelial layer was imaged enface using the extended focus function of Axiovision (Zeiss) on an Axioimager Z.1 fluorescence microscope. A

second group of 3 cultures were treated with trypsin/EDTA and cells deposited on glass slides using a cytospin. Endogenous GFP fluorescence was imaged as above.

Results: Endogenous GFP fluorescence was detected in all cells present in the fixed cultures (SFig 3A and B). The GFP signal intensity varied among cells and both cytosolic and nuclear localization of GFP fluorescence was observed (SFig 3D and E). Cytospin analysis demonstrated that each epithelial cell retained GFP fluorescence in ALI culture. Evidence of nuclear GFP fluorescence as also detected in these cell preparations.

Figure 5

Differentiation of GFP-filler cells in ALI culture: (A-C) Analysis of endogenous GFP fluorescence in ALI cultures derived from UBI-GFP mice. A. Dual color image of endogenous GFP fluorescence (green) in fixed cultures counterstained with DAPI (blue), B. the same field as A, showing GFP fluorescence only, C. the same field as A, showing DAPI fluorescence only. The arrows indicate the cluster of cells that are imaged at higher magnification in panels D-F. Original magnifications are mentioned in the lower right-hand corner. (D-F) GFP fluorescence that co-localized with DAPI. D. Dual color image of endogenous GFP fluorescence (green) in fixed cultures counterstained with DAPI (blue), E. the same field as D, showing GFP fluorescence only. F. the same field as D, showing DAPI fluorescence. Arrows indicate cells in which GFP and DAPI fluorescence are coincident. (G-I) Cytospin preparations of trypsinized cells from UBI-GFP ALI cell cultures. G. GFP and DAPI, H. GFP only, I. DAPI only. The arrows indicate nuclear GFP. Original magnifications are mentioned in the lower right-hand corner.

Proliferative potential of $CD49f^{bright}/Sca1^{+}/ALDH^{+}$ cells in the steady state and after NA injury.

Methods; The experimental procedure is detailed in Figure 7C. $CD49f^{bright}/Sca1^{+}/ALDH^{+}$ cells were isolated at each time point by flow sorting and cytospin preparations were immunostained for BrdU.

Figure 6

Cytospin preparations were stained and imaged for DAPI (blue) and BrdU (red). Arrows: BrdU+ cells, red; BrdU- cells, blue. Original magnifications are indicated in the lower right corner of each panel. A BrdU+ cell from each group is shown in the inset at 1000X magnification.

A. Experimental Group I
B. Experimental Group II.
C. Experimental Group III
D. Experimental Group IV.

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