Functional Analysis of Two Distinct Bronchiolar Progenitors during Lung Injury and Repair

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Air spaces of the mammalian lung are lined by a specialized epithelium that is maintained by endogenous progenitor cells. Within bronchioles, the abundance and distribution of progenitor cells that contribute to epithelial homeostasis change as a function of maintenance versus repair. It is unclear whether functionally distinct progenitor pools or a single progenitor cell type maintain the epithelium and how the behavior is regulated in normal or disease states. To address these questions, we applied fractionation methods for the enrichment of distal airway progenitors. We show that bronchiolar progenitor cells can be subdivided into two functionally distinct populations that differ in their susceptibility to injury and contribution to repair. The proliferative capacity of these progenitors is confirmed in a novel *in vitro* assay. We show that both populations give rise to colonies with a similar dependence on stromal cell interactions and regulation by TGF- β . These findings provide additional insights into mechanisms of epithelial remodeling in the setting of chronic lung disease and offer hope that pharmacologic interventions may be developed to mitigate tissue remodeling.

Keywords: bronchiolar stem cell; Clara; progenitor; fractionation; epithelium

The adult mammalian lung is a quiescent organ composed of many specialized cell types. These cells contribute directly or indirectly to the dual roles of the respiratory system: gas transfer between inspired air and the blood and protection against injurious toxins and microorganisms that gain entry to the body through the air or systemic circulation (1). Epithelial cells lining airways represent the primary barrier to inspired injurious agents. They include progenitor cells, which can proliferate in response to injury, leading to self-renewal or to the generation of other specialized epithelial cell types. The properties of epithelial progenitor cells change as a function of developmental stage and anatomic location within airways (2, 3). During lung development, multipotent progenitor cells located at the growing tips of airways proliferate during the embryonic period to generate progeny that become lineage restricted in the late fetal and adult airway (4, 5). The identity and function of progenitor cells that maintain bronchiolar airways in the adult has been the subject of numerous investigations yet remains somewhat controversial.

Experimental evidence supporting the existence of more than one type of functionally distinct bronchiolar progenitor cell that act in concert to maintain bronchiolar airways comes from

CLINICAL RELEVANCE

This research provides additional insights into mechanisms of epithelial remodeling in the setting of chronic lung disease and offers hope that pharmacologic interventions may be developed to mitigate tissue remodeling.

studies using different chemical injury models to promote epithelial repair. Exposure of rats to inhaled nitrogen dioxide or ozone results in the proliferation of a nonciliated bronchiolar cell, initially referred to as a type A cell (6), that is generated from Clara cells through loss of secretory granules and smooth endoplasmic reticulum (7). Using pulse-chase DNA labeling methods, proliferating type A cells were shown to generate Clara cells and ciliated cells. Thus, Clara cells represent an abundant, broadly distributed pool of progenitor cells that contribute to repair after injury to postmitotic epithelial cell types of airways. The presence of another progenitor cell type that can renew depleted Clara cells is supported by *in vivo* studies involving Clara cell-selective injury models. We and others have used a naphthalene injury model to specifically ablate Clara cells (8). Naphthalene is bioactivated to a toxic derivative by cytochrome P450 isoforms expressed within Clara cells, leading to cell death (9–12). Proliferative naphthalene-resistant progenitor cells localize adjacent to neuroepithelial bodies (NEB) and at bronchoalveolar duct junctions (BADJ) and renew an epithelium containing ciliated and nonciliated cells (13–15). Naphthalene-resistant bronchiolar progenitor cells express the marker Clara cell secretory protein (CCSP) and potentially prosurfactant protein-C (BADJ-associated naphthalene-resistant cells) and expand beyond NEB- and BADJ-microenvironments after endogenous potentiation of canonical Wnt signaling (13, 14, 16, 17). Furthermore, NEB- and BADJ-associated cells proliferating after naphthalene exposure generate progeny with a DNA label-retaining phenotype (13, 14). Together these data suggest that multiple bronchiolar progenitor cells exist within bronchioles that have the potential to play distinct roles in epithelial maintenance and repair.

Recent findings using lineage tracing and chimeric mouse models suggest that broadly distributed abundant progenitor cells rather than rare, specialized progenitor cells maintain the normal airway epithelium (18, 19). Rawlins and colleagues also demonstrate that lineage-tagged CCSP-expressing cells are capable of long-term self-renewal in bronchiolar airways (18). A key question is whether apparently different bronchiolar progenitor cell subsets that are activated in response to ozone or naphthalene-induced airway injury are functionally distinct populations or part of a common progenitor cell pool. Recent efforts have focused on the prospective isolation and development of *in vitro* and transplantation assays to assess the behavior of epithelial progenitor cells (17, 20–24). Kim and colleagues prospectively isolated lung epithelial cells with the

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surface phenotype of CD45^{neg} CD31^{neg} Sca-1^{pos} CD34^{pos} that coexpressed CCSP and pro-SPC. These cells gave rise to clonal populations of cells that expressed alveolar or bronchiolar differentiation markers *in vitro* and were termed “bronchioalveolar stem cells” (17). However, recent lineage-tracing studies investigating the behavior of CCSP-expressing cells *in vivo* have failed to identify cells in terminal bronchioles that give rise to descendants in the alveoli during steady state or after alveolar damage (18). In our previous work, we found that Sca-1^{hi} CD34^{pos} cells do not belong to the bronchiolar lineage and that Sca-1, although expressed on the surface of bronchiolar epithelial cells, does not distinguish between naphthalene-sensitive or -resistant bronchiolar cells (22, 23). We showed that the CD45^{neg} CD31^{neg} CD34^{neg} (Lin^{neg}) Sca-1^{low} fraction contains two specific populations of cells with high or low autofluorescence (AF^{hi} and AF^{low}). Moreover, we show herein and in our previous studies (22) that the AF^{low} population was dramatically increased in the airways of mice in which naphthalene-resistant bronchiolar progenitor cells were expanded through endogenous expression of an activated form of β -catenin. In this article, we further refine methods for the fractionation and enrichment of distinct epithelial progenitor cell subsets that survive and proliferate after various *in vivo* injury models. We show that the AF^{low} population accounts for the bulk of proliferative progenitor cells contributing to repair after naphthalene-induced airway injury, whereas the AF^{hi} population represents a naphthalene-sensitive population that proliferates in response to ozone exposure. These data suggest that two progenitor pools exist within bronchioles that differ in their contribution to epithelial maintenance depending upon the type of airway injury. Furthermore, we show that these progenitor cell populations efficiently proliferate in an *in vitro* assay and that the abundance of colony-forming cells is proportional with the number of CCSP-expressing cells present in each fraction.

MATERIALS AND METHODS

Animal Husbandry

Experimental animals were maintained in pathogen-free conditions in animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care at Duke University. Animal housing and experimentation was performed according to protocols approved by the Institutional Animal Care and Use Committee on mice between 2 and 6 months of age.

Transgenic Mouse Strains and Genotyping

Genomic DNA was isolated from mouse tail according to previously published methods (22). PCR genotyping was performed using previously described conditions and primer pairs that are summarized for each line in Table 1 (16, 25, 26).

Bromo-deoxyuridine Administration

5-Bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO) was resuspended in pyrogen-free 0.9% NaCl solution (B. Brown Medical Inc.,

Irvine, CA) at a concentration of 6 mg/ml and administered intraperitoneally at 50 mg/kg body weight at the indicated intervals.

Ozone Injury

Age- and gender-matched C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were exposed to 1.5 ppm ozone or filtered air for 12 hours. Mice were removed from their housing cages and placed separately into individual stainless steel wire cage units. These units were placed into a 55-liter stainless steel Hinners-style exposure chamber. The chamber was equipped with a high-efficiency filtered air supply, such that chamber air (relative humidity, 50–60%; 20–22°C) was renewed at the rate of approximately 20 changes per hour. Ozone was generated by passing 100% oxygen gas through an ultraviolet light source. The ozone concentration was monitored continuously within the chamber with an ozone ultraviolet light photometer (model 1003AH; Dasibi Environmental Corp., Glendale, CA).

Naphthalene Injury

Naphthalene exposures were performed using 10-week-old male C57BL/6 mice. Naphthalene (Fisher Scientific, Fair Lawn, NJ) was dissolved in corn oil at a concentration of 25 mg/ml and administered by intraperitoneal injection at a dosage of 250 mg/kg body weight.

Flow Cytometry

Suspensions of lung cells were isolated by elastase digestion and stained for flow cytometry as previously described (22). The specificity and source of antibodies used are indicated in Table 2. 7-amino-actinomycin D (0.25 μ g/100 μ l staining buffer; BD Biosciences, San Diego, CA) was used for dead cell discrimination. Intracellular staining for BrdU was performed using the BrdU FITC kit from BD Biosciences according to the manufacturer's instructions. For intracellular staining for CCSP, AF^{hi} and AF^{low} cells were sorted as detailed above and fixed with BD Cytofix/Cytoperm (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Cells were stained with rabbit anti-mouse CCSP antibody (1:100 dilution, in house), followed by Alexa Fluor 594 donkey anti-rabbit IgG (1:100 dilution) (Invitrogen, Carlsbad, CA).

RNA Isolation and Real-Time PCR

Total RNA was isolated from cells using a SV Total RNA (Promega, Madison, WI) isolation kit according to the manufacturer's instructions. Real-time RT-PCR was performed as described previously (27), and samples were read using an Eppendorf realplex Real Time PCR System (Eppendorf, Hauppauge, NY) (4).

Cell Culture

Sorted lung epithelial cells were resuspended in growth factor–reduced Matrigel (BD Biosciences), which was diluted at a ratio of 1:1 with basic culture medium. Basic medium contains DMEM/F12 (Cellgro; Mediatech Inc., Manassas, VA) supplemented with insulin/transferrin/selenium (Invitrogen), 10% FBS (Invitrogen), 0.25 μ g/ml amphotericin B, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 10 μ M SB431542 (Ascent Scientific LLC, Princeton, NJ). Cells suspended in Matrigel were added to the chamber of 24-well Transwell filter inserts (Becton Dickinson, Franklin Lakes, NJ) and placed in 24-well, flat-bottom culture plates containing medium. Where specified, mouse lung fibroblasts (MLg, ATCC) were added to the Matrigel at 2×10^6 cells/ml. Cultures were maintained at 37°C in a humidified in-

TABLE 1. TRANSGENIC ANIMAL MODELS

Genotype	Common Designation	Source or References
CCSP-cre Catnb ^{flloxE3} B6.129(Cg)-Gt(ROSA)26Sor ^{tm4} (ACTB-tdTomato,-EGFP) ^{Luo} /J Germline recombined B6.129(Cg)-Gt(ROSA)26Sor ^{tm4} (ACTB-tdTomato,-EGFP) ^{Luo} /J	ROSA-R/G ROSA-EGFP	Thomas J. Mariani (University of Rochester, Rochester, NY) (16) Makoto M. Taketo (Kyoto University Global COE Program, Japan) (47) Jackson Labs (Bar Harbor, ME)
Scgb1a1-CreER; Foxj1-GFP SP-C-GFP	CCSP/Cre ER Foxj1 GFP SP-C GFP	Brigid L.M. Hogan (Duke University, Durham, NC) (18) Lawrence E. Ostrowski (University of North Carolina, Chapel Hill, NC) (25) Bernice Jo <i>et al.</i> , 2008 (26)

TABLE 2. ANTIBODIES USED FOR FLOW-CYTOMETRY AND TISSUE HISTOLOGY

Antigen	Host	Titer	Source
CCSP	Rabbit polyclonal	1:100 (FC)	In house
CCSP	Rabbit polyclonal	1:10,000 (IF)	In house
proSP-C	Rabbit polyclonal	1:3,000 (IF)	Kind gift from P.Y. Di
BrdU	Mouse	1:100 (FC)	BD Biosciences (San Jose, CA)
GFP	Chicken polyclonal	1:4,000 (IF)	Abcam (Cambridge, MA)
Cytokeratin 18	Mouse monoclonal IgG1, clone # c-04	1:500 (IF)	Abcam
Sca-1	Rat IgG2A, clone D7	1:200 (FC-Biolegend)	Biolegend (San Diego, CA)
EpCAM (PE-Cy7)	Rat IgG2A, clone G8.8	1:200 (FC),	Biolegend
CD45 (biotinylated)	Rat IgG2B, clone 30-F11	1:200 (FC-eBioscience)	eBioscience (San Diego, CA)
CD31 (biotinylated)	Rat IgG2A, clone 390	2.5:100 (FC)	eBioscience
CD34 (biotinylated)	Rat IgG2A, clone RAM34	6.5:100 (FC)	eBioscience

Definition of abbreviations: BrdU = 5-bromo-2'-deoxyuridine; CCSP = Clara cell secretory protein; FC = flow cytometry; GFP = green fluorescent protein; IF = immunofluorescence.

cubator (5% CO₂), and the medium was replaced every other day. Colonies were visualized with an inverted fluorescent microscope (Axiovert40; Carl Zeiss MicroImaging Inc., Thornwood, NY). Colonies were defined as clusters of green fluorescent protein-positive (GFP^{pos}) cells greater than 100 μ m in diameter. Serial passage of GFP^{pos} cells was achieved by dissociation of Matrigel cultures with dispase (1 mg/ml) (Stemcell Technologies, Vancouver, British Columbia, Canada) for 1 hour at 37°C, sorting using a FACS Vantage (BD Biosciences), and reseeding cells into Matrigel cultures in the presence of MLg fibroblasts and SB431542 as described previously.

Histology and Immunostaining

Cultures of lung epithelial cells were fixed by the addition of 4% paraformaldehyde in PBS overnight at 4°C. Fixed colonies in their Matrigel supports were rinsed with PBS, immobilized in 1.5% agarose, embedded in paraffin, and sectioned at 5- μ m thickness. Lung tissue was inflation fixed by instillation of 10% neutral buffered formalin and immersed in 10% neutral buffered formalin for 2 hours at 4°C. Right caudal lobes were embedded in paraffin and sectioned at 5- μ m thickness. Sections were deparaffinized in xylens, transferred to 100% ethanol, and rehydrated in decreasing concentrations of ethanol in PBS. Antigen retrieval was then performed by maintaining sections in 10 mM citrate buffer (pH 6.0) at approximately 95°C for 10 minutes using a microwave. Slides were blocked in 5% BSA (wt/vol) in PBS, followed by incubation with the primary antibody and with a fluorescent secondary antibody (Table 2). Expression of GFP was monitored by immunofluorescence analysis using a chicken anti-GFP antibody followed by Alexa Fluor 488-conjugated goat anti-chicken secondary antibody. Slides were then mounted in DAPI containing Fluoromount-G (2 μ g/ml) (Electron Microscopy Sciences, Hatfield, PA). Fluorescent images were acquired using a Zeiss Axiovert 40 inverted

microscope equipped with AxioCam MRc5 and MR digital cameras (Carl Zeiss MicroImaging Inc.). Images were processed and analyzed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

RESULTS

Phenotypic Characteristics of Fractionated Lung Epithelial Cells

Previous studies by us and others have developed fractionation approaches, allowing enrichment of putative tissue stem cells (17, 20, 22). However, these approaches fail to completely account for the full spectrum of epithelial progenitor cells that may participate in normal tissue maintenance and repair. We have shown that airway epithelial cells are enriched in the CD45^{neg}/CD31^{neg}/CD34^{neg}/Sca-1^{low} (Lin^{neg}/Sca-1^{low}) fraction of total adult mouse lung cells. This population could be further fractionated to enrich for subpopulations of epithelial cells: naphthalene-sensitive Clara cells (high autofluorescence) or naphthalene-resistant cells (low autofluorescence). We have adopted this fractionation strategy and coupled it with surface staining for EpCAM, considered to be an epithelial-specific cell adhesion molecule (28, 29), to further enrich epithelial cells and segregate according to Sca-1 surface expression (Figures 1A and 1B). Consistent with our previous data, the autofluorescence profile of the Sca-1^{low} fraction revealed distinct AF^{hi} and AF^{low} populations (Figure 1C). Furthermore, AF^{hi} cells were depleted by activation of β -catenin signaling within airways (of Lin^{neg}

Figure 1. Cell surface phenotype and autofluorescence characteristics of epithelial cells of the lung. (A–C) Isolated lung cells were stained and analyzed by flow cytometry. (A) Exclusion of Lin^{pos} cells (x axis) and 7AAD^{pos} cells (y axis). (B) Sca-1 (x axis) versus EpCAM (y axis) analysis of 7AAD^{neg}/Lin^{neg} population from A. (C) Autofluorescence analysis of cells in EpCAM^{pos} Sca-1^{low} gate showing the high-autofluorescence (AF^{hi}) and low-autofluorescence (AF^{low}) populations. The representation of AF^{hi} and AF^{low} cells as a function of 7AAD^{neg}/Lin^{neg} cells is indicated as a percent-

age. (D–F) The abundance and distribution of Clara cell secretory protein (CCSP)-expressing cells within AF^{hi} and AF^{low} fractions of the EpCAM^{pos}/Sca-1^{low} population was determined by intracellular staining coupled with flow analysis.

cells, 18.4% for wild-type and 4.8% in *Catnb^{ΔE3}* mice), resulting in the expansion of cell numbers within the AF^{low} fraction (11.4% for wild-type and 20.7% for *Catnb^{ΔE3}* mice) (Figures 1A–1C).

To define the contribution of CCSP-expressing cells to the AF^{hi} and AF^{low} fractions, intracellular staining was performed to quantify CCSP immunoreactivity. Differences were observed between AF^{hi} and AF^{low} fractions in the number of CCSP-positive cells and the level of CCSP expression (Figures 1D–1F). Analysis of cells within the AF^{hi} fraction revealed that greater than 90% showed high levels of intracellular CCSP immunoreactivity (Figure 1E). In contrast, cells within the AF^{low} fraction included weakly CCSP-immunoreactive cells that represented approximately 11% of total cells within the AF^{low} population (Figure 1F).

Cell phenotypes within each fraction were assessed using reporter transgenic mice. We used SPC-GFP (26) or FoxJ1-GFP (25)

transgenic mice for the identification of alveolar type 2 cells (Figure 2A) and ciliated cells, respectively. Immunofluorescence analysis of lung tissue for GFP expression revealed a punctuate distribution of parenchymal alveolar type 2 cells that showed high levels of GFP immunoreactivity and distal conducting airway cells that showed weak GFP immunoreactivity (Figure 2). Similarly, cells dissociated from lungs of SPC-GFP transgenic mice revealed a large population of GFP^{high} cells with minor populations of GFP^{medium-low} cells. Flow cytometric analysis showed that GFP^{hi} cells were Sca1^{neg} and that GFP^{med} and GFP^{low} cells were Sca1^{low} (Figure 2B). Conversely, analysis of cells from lungs of FoxJ1-GFP mice revealed a single population of GFP-expressing cells, consistent with previous analysis of this line that indicated GFP expression within ciliated cells (25). Green fluorescent protein-expressing ciliated cells were largely Sca1^{low} and absent from the large populations of cells showing a Sca1^{neg} staining pattern (Figure 2C).

Figure 2. Molecular validation of cell fractionation strategy. (A) Immunofluorescence detection of green fluorescent protein (GFP) and CCSP within lung tissue of SPC-GFP mice. Representative photomicrographs are shown demonstrating the distribution of CCSP immunoreactivity (red), GFP immunoreactivity (green), and DAPI nuclear counterstaining (blue). GFP immunoreactivity reveals GFP^{hi}, GFP^{low}, and GFP^{neg} epithelial cells on lung sections from SPC-GFP mice. GFP^{hi} cells are CCSP^{neg} and confined to the epithelium of the lung parenchyma (arrowhead). GFP^{low} cells localize to terminal bronchioles and are CCSP^{pos} (closed arrow). CCSP^{pos} cells of proximal intrapulmonary conducting airways are GFP^{neg} (open arrow). (B) GFP^{hi} cells from SPC-GFP transgenic mice are superimposed over an EpCAM/Sca-1 dot plot of the viable Lin^{neg} population. GFP^{hi} type II cells are predominantly EpCAM^{pos}Sca-1^{neg}. (C) GFP^{hi} cells from FoxJ1-GFP transgenic mice are superimposed over an EpCAM/Sca-1 dot plot of the viable Lin^{neg} population. GFP^{hi} ciliated cells are predominantly EpCAM^{pos}Sca-1^{low}. (D) Analysis of mRNA abundance for CCSP, Sftpc, and FoxJ1 in cells fractionated according to their cell surface profile and autofluorescence characteristics. Data are presented for the indicated cell fractions relative to values obtained for total lung RNA.

Quantitative real-time PCR analysis was performed to further validate the cell type-specific composition of fractionated EpCAM^{pos} epithelial cell preparations. The abundance of CCSP mRNA was significantly enriched within total RNA isolated from AF^{hi} and AF^{low} fractions and depleted within total RNA from the Sca-1^{neg} fraction relative to total viable lung cells (Figure 2). High levels of *FoxJ1* mRNA were only observed within the AF^{low} fraction (Figure 2D), a finding that was consistent with the partitioning of GFP^{pos} ciliated cells from FoxJ1-GFP transgenic mice within this fraction (Figure 2C). Expression of *Sftpc* mRNA was greatly enriched within the Sca-1^{neg} fraction, partially enriched within AF^{low}, and absent from AF^{hi} (Figure 2D). Collectively, these data suggest that the AF^{hi} fraction harbors CCSP^{hi} Clara cells and that the AF^{low} fraction harbors a mixed population of ciliated and CCSP^{low} nonciliated cells. Alveolar type 2 cells, the putative progenitor for the alveolar epithelium, are selectively enriched within the Sca-1^{neg} fraction.

Functional Analysis of Epithelial Progenitor Cells *In Vivo*

We next sought to determine how epithelial cell types contained within the Sca-1^{neg}, AF^{hi}, and AF^{low} populations behaved *in vivo* after airway injury. In other studies, Clara cells represent the only proliferative progenitor after ozone exposure and generate progeny that assume Clara or ciliated cell fates. In contrast, two subsets of CCSP-expressing cells can be delineated based upon resistance or sensitivity to naphthalene. Our data demonstrate that CCSP-expressing cells are present in AF^{hi} and AF^{low} fractions and that AF^{low} cells are more numerous after β -catenin-mediated expansion of naphthalene-resistant bronchiolar progenitor cells (Ref. 22 and Figure 1). We used naphthalene and ozone injury models to determine whether the fractionation strategy described above was capable of separating these progenitor cell pools. We initially exposed wild-type C57Bl/6 mice to naphthalene and allowed them to recover for 3 days. Bromodeoxy-uridine (BrdU) was administered by intraperitoneal injection every 12 hours to naphthalene-injured and control mice to label proliferative cell types. Cells were isolated from control and naphthalene-treated lungs, and the distribution of BrdU-labeled cells was determined as a function of cell surface phenotype and autofluorescence characteristics (Figure 3A). To confirm Clara cell injury, we assessed the intracellular complexity (SSC) of the EpCAM^{pos} population in injured and uninjured control mice. Cells with high intracellular complexity (SSC^{hi}, granular Clara cells) are effectively depleted 3 days after naphthalene administration (Figure 3A). Furthermore, analysis of the AF^{hi} population revealed a significant decrease in their abundance after naphthalene exposure (60.5–22.4% in control versus treated mice). Proliferating cells were identified by gating for BrdU-labeled cells using stained cells from mice not treated with BrdU as the negative control. Using this approach, the BrdU-labeling index was determined to be 0.3 and 1.2% of EpCAM^{pos} cells in steady-state and naphthalene-injured airways, respectively. In control and naphthalene-injured populations, greater than 70% of proliferating cells were found in the Sca-1^{low} fraction. Cells labeled with BrdU after naphthalene treatment were preferentially found in the AF^{low} fraction (13.0% in AF^{low} versus 5.5% in AF^{hi}) (Figures 3A, 3C, and 3D). These data demonstrate that most cells with the AF^{hi} phenotype are naphthalene sensitive and that AF^{low} cells are naphthalene resistant and responsible for the bulk of proliferation occurring in the first 3 days after naphthalene-induced airway injury.

To determine the proliferative capacity of AF^{hi} and AF^{low} cell populations after ozone injury, C57Bl/6 mice were exposed to 1.5 ppm ozone for 12 hours. To label proliferating cells,

filtered air (control)–exposed and ozone-exposed mice were treated with BrdU every 12 hours over a 60-hour period. In contrast to naphthalene injury, no changes were observed in the SSC^{hi} population after ozone exposure, demonstrating that ozone does not deplete Clara cells (Figures 3B, 3C, and 3E). A decrease was observed in the size of the AF^{low} fraction, which is consistent with the known selectivity of ozone for ciliated cells and with our earlier data demonstrating enrichment of ciliated cells within this fraction (Figures 2A, 2C, 3B, and 3E). Cells labeled with BrdU were of high and intermediate granularity, consistent with a Clara cell phenotype, and accounted for 0.4 and 6.6% of the EpCAM^{pos} fraction of lung cells in control and ozone-exposed mice, respectively (Figure 3B). In control and ozone-injured populations, greater than 75 and 95%, respectively, of proliferating cells were found in the Sca-1^{low} fraction. Cells labeled with BrdU were preferentially found in the AF^{hi} fraction of EpCAM^{pos}/Sca-1^{low} cells (3.2% in AF^{low} versus 10.6% in AF^{hi}) (Figure 3B–3D). These data suggest that ciliated cells of the AF^{low} fraction are ozone sensitive and that AF^{hi} cells are responsible for the bulk of proliferation occurring after ozone-induced airway injury.

In Vitro Behavior of Isolated Airway Epithelial Progenitor Cells

A clonogenic assay was used to reveal their clonogenic behavior in the absence of differences in microenvironment-specific extrinsic signals. Lin^{neg}/7AAD^{neg}/EpCAM^{pos} cells were sorted from lungs of ROSA26-EGFP mice (a germline recombined variant of ROSA-R/G). Reanalysis of sorted populations indicated that purity of the EpCAM^{pos} population was greater than 99%. Cells were then cultured in growth factor–reduced Matrigel. Under these conditions, when EpCAM^{pos} lung cells were cultured alone, no significant colony-forming ability was observed (Figures 4A and 4H). However, when EpCAM^{pos} lung cells were cocultured with mouse lung stromal (MLg) cells, the growth of spherical colonies was observed (Figures 4B and 4H). Colony-forming efficiency was further enhanced by approximately 4-fold (from 1 to 4%) by addition of the Alk5 inhibitor SB431542 (Figures 4C and 4H). Media additives, including noggin (0.1 μ g/ml), the Rho kinase inhibitor Y-27632 (10 μ g/ml), and Jagged-1 (1 μ M), all of which have previously been shown to facilitate *in vitro* propagation of intestinal epithelial stem/progenitor cells (30), had no effect on colony growth in the absence or presence of MLg cells (data not shown). Dual immunofluorescent staining for GFP and the epithelial cell-specific marker cytokeratin 18 revealed that all colonies derived from GFP^{pos}/EpCAM^{pos} cells were of epithelial origin (Figures 4D–4F). Coculture of equivalent numbers of input EpCAM^{pos} cells from ROSA-EGFP or ROSA-R/G transgenic mice resulted in the formation of either green or red colonies with no evidence of mixing. These data suggest that colonies are derived from single cells (Figure 4G). Next we used lineage tracing to determine the identity of the epithelial cells responsible for colony formation. We have shown previously that CCSP-expressing cells are necessary for airway repair *in vivo* (13, 14). To determine roles for CCSP-expressing cells in the formation of epithelial colonies *in vitro*, we enriched GFP-expressing lineage-tagged cells from tamoxifen-treated CCSP^{cre}ER ROSA-R/G mice and evaluated their colony-forming ability in culture. These cells gave rise to GFP^{pos} colonies similar to those generated using total EpCAM^{pos} cells (compare Figures 4C and 4I). Because the lineage tracing strategy had the potential to include nascent ciliated cells (derived from lineage-tagged CCSP-expressing progenitors) as well as CCSP-expressing cells, we sought to exclude the possibility that ciliated cells give rise to colonies. Thus, GFP^{pos} cells from FoxJ1-GFP transgenic mice were isolated and cultured in the same conditions. These

Figure 3. *In vivo* behavior of Lin^{neg} EpCAM^{pos} Sca-1^{pos} progenitor cells during lung injury and repair. Proliferating cells were labeled with 5-bromo-2'-deoxyuridine (BrdU) after injury by exposure to (A) naphthalene or (B) ozone, using unexposed mice as controls. Isolated cells were permeabilized and stained for detection of cell surface markers or nuclear BrdU. BrdU-positive events (black dots) are superimposed on total events (gray dots) on dot plots of Lin^{neg} cells displayed as a function of side scatter (SSC, left panels), Sca-1 versus EpCAM (center panels), or BrdU labeling versus autofluorescence (right panels). (C) Frequency of BrdU-labeled cells within EpCAM^{pos} cells in control lungs or lungs from mice with ozone injury or naphthalene (Naph.) injury. (D and E) Quantitative data for BrdU labeling within AF^{hi} and AF^{low} fractions of control or injured lungs for naphthalene or ozone exposures.

cells did not form epithelial colonies, demonstrating that CCSP-expressing cells were the only bronchiolar epithelial cells with colony-forming ability (Figure 4J). To exclude a potential ATII origin of colonies, GFP^{hi} cells from SPC-GFP transgenic mice were isolated and cultured in parallel with CCSP-lineage tagged cells or FoxJ1-GFP^{pos} cells. No colony formation was observed under these conditions, demonstrating that the only cells with colony-forming ability within the EpCAM^{pos} fraction of total lung cells were of the CCSP-expressing phenotype.

Using this coculture assay and SB431542 supplementation, we have shown that colony-forming progenitor cells are contained within the AF^{hi} and AF^{low} fractions (Figures 5A and 5B). However, differences were observed in colony-forming efficiency between the AF^{hi} and AF^{low} fractions. The colony-forming efficiency of the AF^{hi} cell fraction was approximately 10.4% (Figures 5A and 5B). In contrast, the AF^{low} cell fraction gave rise

to colonies with a frequency of approximately 1.1% (Figures 5A and 5B). The lower colony-forming efficiency of cells within the AF^{low} fraction is consistent with earlier data, revealing that this population includes ciliated cells and significantly lower numbers of CCSP-immunoreactive cells relative to the AF^{hi} fraction (see Figures 1D–1F and 2). These data suggest that colony-forming efficiency is directly related to the incidence of progenitor cells contained within each fraction.

To better establish the relationship between *in vivo* progenitor cell activity and colony-forming ability *in vitro*, we determined the colony-forming efficiency of AF^{low} cells recovered from lungs of *Catnb*^{ΔE3} mice. Airway epithelial cells of *Catnb*^{ΔE3} mice have an expanded pool of naphthalene-resistant AF^{low} airway progenitor cells due to ectopic activation of canonical Wnt signaling (16). *In vitro* culture of AF^{low} airway progenitor cells from *Catnb*^{ΔE3} mice yielded colonies with the same morphologic features as those generated from AF^{hi} and

Figure 4. *In vitro* culture model for propagation of epithelial progenitor cells. Culture conditions were optimized using a $\text{Lin}^{\text{neg}}/\text{EpCAM}^{\text{pos}}$ cell population of β -actin/GFP transgenic mice. Representative images of Day 6 cultures of cells seeded in growth factor-reduced Matrigel and cultured under (A) basic medium (BM), (B) BM and in the presence of mouse lung fibroblast cells (MLg), or (C) BM supplemented with SB431542 in the presence of MLg cells (1×10^4 cells/condition). (D) Analysis of cytokeratin (CK)-18 expression within GFP^{pos} colonies by dual immunofluorescence analysis. Photomicrographs demonstrate CK-18-immunoreactive cells (D), GFP-immunoreactive cells (E), and a merged image showing coexpression of CK-18 and GFP (F). DAPI is included as a nuclear counterstain (blue). (G) Analysis of clonality of epithelial colonies. $\text{Lin}^{\text{neg}} \text{EpCAM}^{\text{pos}}$ cells were isolated from mice expressing ubiquitous GFP (green signal) or TdTomato (red signal) and cocultured in the presence of MLg cells and SB431542. No mixing of red and green fluorescent cells is observed within individual colonies, indicating their clonal origin. (H) Quantitative analysis of the colony-forming ability of $\text{Lin}^{\text{neg}}/\text{EpCAM}^{\text{pos}}$ cells in BM, BM+MLg, and BM+MLg+SB431542. Cultures of GFP^+ cells (input cell number: 5,000) from lineage tracing mice, (I) CCSPcreER, (J) SPC-GFP, and (K) FoxJ1-GFP were photographed at Day 6. Scale bars: 200 μm for A, B, C, G, I, J, and K; 100 μm for F.

Figure 5. *In vitro* behavior of epithelial progenitor cells from wild-type mice and $\text{Catnb}^{\Delta\text{E3}}$ mice with conditional potentiation of β -catenin signaling within airways. (A) Isolated cells from GFP transgenic mice (WT) or tritransgenic mice β -actin-GFP/CCSP-cre/ $\text{Catnb}^{\text{lox}^{\text{E3}}}$ (ΔE3) were sorted into $\text{Lin}^{\text{neg}} \text{EpCAM}^{\text{pos}} \text{Sca-1}^{\text{pos}} \text{AF}^{\text{hi}}$ (WT AF^{hi}) and $\text{Lin}^{\text{neg}} \text{EpCAM}^{\text{pos}} \text{Sca-1}^{\text{pos}} \text{AF}^{\text{low}}$ (WT AF^{low} or $\Delta\text{E3 AF}^{\text{low}}$) and cocultured with MLg fibroblasts in basic medium supplemented with SB431542. GFP fluorescence images were captured on Day 6 after plating (3×10^4 epithelial cells/ml Matrigel). (B) Clonogenic frequency of cells in each fraction shown in A. (C) Serial passage of epithelial colonies from the indicated cell fractions (* $P < 0.05$).

AF^{low} cells from wild-type mice (Figure 5A). However, the colony-forming efficiency of AF^{low} cells from *Catnb*^{ΔE3} mice was increased by 13-fold over that observed in cultures of wild-type AF^{low} cells (1.1% for wild type versus 14.3% for *Catnb*^{ΔE3}) (Figures 5A and 5B). These data further support the conclusion that our culture system is sufficient for the clonal growth of bronchiolar progenitor cells within fractionated lung cell preparations.

Cultured epithelial cells were passaged to assess the self-renewal potential of AF^{hi} or AF^{low} fractions of wild-type and *Catnb*^{ΔE3} mice. Colony-forming efficiency was assessed in 5-day cultures during the active growth phase of epithelial colonies and before harvesting cells for serial passage. In 5-day cultures AF^{hi}, AF^{low}, and *Catnb*^{ΔE3}AF^{low} cells were found to show 2.4, 0.5, and 9.3% colony-forming efficiency, respectively (Figure 5C). Harvesting and reseeded of sorted GFP^{pos} epithelial cells from AF^{hi} and AF^{low} cultures were associated with increases in colony-forming efficiency to 4.8 and 4.5%, with no significant change observed between passages P1 and P2 (Figure 5C). No further increases in colony-forming efficiency were observed with serial passage of GFP^{pos} cells from *Catnb*^{ΔE3} mice (Figure 5C). These data suggest that the initial *in vitro* expansion of epithelial progenitor cells within wild-type AF^{hi} and AF^{low} fractions results in selection of progenitor cells that exhibit a similar capacity for self-renewal. Furthermore, genetic potentiation of β-catenin signaling within AF^{low} cells from *Catnb*^{ΔE3} results in an increase in clonogenic potential.

DISCUSSION

We have validated a refined fractionation approach that yields three distinct epithelial progenitor cell fractions that encompass bronchiolar and alveolar compartments. When coupled with established mouse lung injury models, progenitor cells contributing to repair after ozone-induced depletion of ciliated cells are predominantly localized to a lung cell fraction characterized by CD45^{neg}CD34^{neg}CD31^{neg}EpCAM^{pos}Sca-1^{low}AF^{hi}. Cells within this fraction are predominantly composed of CCSP-expressing, naphthalene-sensitive Clara cells, a finding that is consistent with data from other laboratories that this cell type represents the proliferative progenitor cell after ozone- or nitrogen dioxide-induced airway injury (7). We show that the pool of progenitor cells that proliferate in response to naphthalene-induced Clara cell ablation is principally CD45^{neg}CD34^{neg}CD31^{neg}EpCAM^{pos}Sca-1^{low}AF^{low}. These cells lack cellular complexity and express low levels of CCSP, characteristics that distinguish them from Clara cells present within the AF^{hi} fraction. Our past and present findings *in vivo* suggest that naphthalene-resistant CCSP^{low} cells (AF^{low}) exhibit distinct functional properties to the broader population of naphthalene-sensitive CCSP^{hi} Clara cells (AF^{hi}).

Roles for Clara cells in epithelial maintenance have recently been reevaluated based upon findings by Giangreco and colleagues and Rawlins and colleagues using somatic chimeras or lineage tracing approaches to follow the behavior of airway progenitors (18, 19). These studies are in agreement that an abundant progenitor rather than a rare undifferentiated progenitor contributes to normal epithelial maintenance in the uninjured state. An important question is whether relatively quiescent tissues such as the lung harbor latent tissue stem cells that can be distinguished from an abundant pool of progenitor cells involved in normal tissue maintenance (8, 13–15, 31, 17–19, 32). We and others have previously used naphthalene exposure to functionally distinguish Clara cells from a pool of naphthalene-resistant progenitor cells that exhibit some characteristics of tissue stem cells (13–15, 17, 19). In the present study, we have functionally

verified that epithelial progenitor cells contributing to airway renewal after naphthalene-induced lung injury are selectively enriched within the CD45^{neg}CD34^{neg}CD31^{neg}EpCAM^{pos}Sca-1^{low}AF^{low} fraction of dissociated lung cells. Naphthalene-resistant progenitor cells have a more restricted distribution within bronchioles than Clara cells, suggesting that regional differences in the airway microenvironment may contribute to their unique behavior (8, 13, 14).

Recent findings in the hematopoietic, hair follicle, and intestinal systems suggest the existence of two separate stem cell pools: A population of stem cells proliferates frequently and is thought to be responsible for normal tissue maintenance, while a “reserve” stem cell population is mainly quiescent and can be mobilized during the repair process after injury (reviewed by Li and Clevers [33]). Similarly, we demonstrate the existence of two distinct progenitor pools in the bronchiolar epithelium with distinct phenotype and functional behavior. Due to the low proliferative index of steady-state epithelium, specific injury models were used to reveal the proliferative potential of these two fractions of AF^{hi} and AF^{low} cells. Thus, AF^{hi} cells replenish the pool of terminally differentiated ciliated cells depleted during ozone injury, whereas the proliferative potential of AF^{low} cells is only revealed in a more severe injury model that specifically targets the more abundant pool of AF^{hi} (Clara) cells. However, whether airway progenitor cells represent functionally distinct stem cell pools, such as those in the gut, skin, and hematopoietic systems, or whether they are more representative of other progenitor cell relationships, such as that between follicular bulge stem cells versus interfollicular keratinocytes, has yet to be determined.

We used an *in vitro* model to determine how subpopulations of bronchiolar progenitor cells that show functionally distinct properties *in vivo* behave when placed in a homogeneous culture environment. We demonstrated that only cells tagged by ligand activation of a CCSP promoter-driven CreER recombinase developed epithelial colonies *in vitro*. Neither ciliated nor alveolar type 2 cells demonstrated colony-forming ability in our *in vitro* assay. These data are consistent with our previous findings that CCSP-expressing cells of conducting airways are necessary for epithelial maintenance and repair (13, 14). Based on the size and growth pattern of individual colonies, we found that progenitor cells within AF^{hi} or AF^{low} fractions of lung epithelial cells displayed very similar capacities for clonal growth and in each case were maintained long after serial passage. Furthermore, both populations of bronchiolar progenitors gave rise to morphologically similar colonies under the conditions of culture used. In related studies, it was recently shown that an EpCAM^{hi} fraction of lung cells includes progenitors that can generate three colony types when cultured in the presence of EpCAM^{neg}Sca-1^{pos} primary lung stromal cells (34). Under these conditions, epithelial colonies displayed molecular characteristics indicative of airway, alveolar, or mixed differentiation. Differences in the outcomes of these studies reinforce the impact that microenvironmental cues, provided by the *in vitro* culture environment or through the natural heterogeneity of the *in vivo* environment, regulate the clonogenic behavior and differentiation potential of epithelial progenitor cells. Dynamic interactions between developing lung endoderm and surrounding mesoderm play critical roles in patterning branching airways and lineage specification (5, 35). Our data suggest that interactions between epithelial and stromal components are critical for clonal expansion of adult bronchiolar epithelial progenitor cells and play important roles in regulating their differentiation. Potentiation of clonal growth by SB431542-mediated inhibition of TGFβRI/ALK5 signaling provides insight into potential regulatory mechanisms that may operate in normal tissue maintenance

in vivo. TGF- β signaling has been shown to inhibit the proliferation of epithelial cells in several organs, including the lung, and has been proposed as a mediator of defective epithelial maintenance in the asthmatic airway (36–39). Other candidate signaling pathways that may regulate bronchiolar progenitor cells include PTEN pathways (40, 41), the mitogen-activated protein kinase pathway (42), Wnt (16, 43, 44), and pathways regulating the activity of K-ras/PI-3-kinase (17, 45, 46). Further *in vitro* analysis of molecular signals regulating the behavior of bronchiolar progenitor cells will provide important insights into intrinsic versus microenvironmental control mechanisms that differentially regulate their behavior in normal tissue maintenance or in the remodeling that accompanies chronic lung disease.

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