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Article

The Role of Scgb1a1$^+$ Clara Cells in the Long-Term Maintenance and Repair of Lung Airway, but Not Alveolar, Epithelium

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SUMMARY

To directly test the contribution of Scgb1a1$^+$ Clara cells to postnatal growth, homeostasis, and repair of lung epithelium, we generated a *Scgb1a1-CreER^{TM}* "knockin" mouse for lineage-tracing these cells. Under all conditions tested, the majority of Clara cells in the bronchioles both self-renews and generates ciliated cells. In the trachea, Clara cells give rise to ciliated cells but do not self-renew extensively. Nevertheless, they can contribute to tracheal repair. In the postnatal mouse lung, it has been proposed that bronchioalveolar stem cells (BASCs) which coexpress *Scgb1a1* (*Secretoglobin1a1*) and *SftpC* (*Surfactant Protein C*), contribute descendants to both bronchioles and alveoli. The putative BASCs were lineage labeled in our studies. However, we find no evidence for the function of a special BASC population during postnatal growth, adult homeostasis, or repair. Rather, our results support a model in which the trachea, bronchioles, and alveoli are maintained by distinct populations of epithelial progenitor cells.

INTRODUCTION

The lung consists of three anatomically distinct regions (trachea, bronchioles, and alveoli), each with specialized roles in respiration and host defense. Following postnatal growth, the lung reaches a steady state in which epithelial turnover is low. However, all regions can repair after injury. The identity of the resident stem or progenitor cells responsible for lung postnatal growth, homeostasis, and repair remains controversial (Kim, 2007; Neuringer and Randell, 2006; Rawlins and Hogan, 2006). Here, we exploit the classic technique of lineage tracing to address this critical question.

The composition of the lung epithelium varies along the proximal-distal axis. In the mouse, the tracheal epithelium is pseudostratified and consists mainly of basal cells, secretory nonciliated cells (here called Clara cells for convenience), and ciliated cells. In the interlobar airways (bronchioles), a mixture of Clara and ciliated cells is interspersed with clusters of neuroendocrine (NE) cells. The alveolar epithelium consists of type 2 and type 1 cells. There is compelling indirect evidence that following injury to the mouse bronchioles, the Clara cells can both self-renew and give rise to new ciliated cells (for example, Evans et al., 1978 and Reynolds et al., 2000b). However, it is not clear whether all Clara cells, or only restricted subpopulations, have this capacity. Following naphthalene injury, the only surviving Clara cells are located near NE cells and at the bronchioalveolar duct junction (BADJ); these survivors are both necessary and sufficient to restore the epithelium (Giangreco et al., 2002; Hong et al., 2001; Reynolds et al., 2000a). Another subset of Clara cells has been identified, based on their location at the BADJ and their coexpression of *Scgb1a1* (*Secretoglobin 1a1*, also known as *CC10* or *CCSP*), a marker of Clara cells, and an alveolar type 2 cell marker *Surfactant protein C* (*SftpC*, also known as *SpC*). These cells proliferate following lung injury. Based on their in vitro differentiation potential, it has been proposed that they are bronchioalveolar stem cells (BASCs) that give rise to both bronchiolar and alveolar cells in vivo (Kim et al., 2005). However, their lineage fate has not been followed. Moreover, there is considerable indirect evidence that type 2 cells are a major progenitor for the alveolar epithelium (Evans et al., 1973, 1975). Many questions remain: does only a subset of Clara cells behave as stem cells for airway maintenance and/or repair, or do all, or most, Clara cells fulfill these functions? Are there facultative progenitors that are only activated in response to injury? How do the putative BASCs fit into these models?

In the trachea, lineage-labeling studies have shown that after Clara cell injury, basal cells are an important stem cell population (Hong et al., 2004). However, in response to injury of ciliated cells, only Clara cells, not basal cells, divide (Evans et al., 1986). This raises the possibility that there are two separate populations of stem cells in the trachea: basal and Clara cells. Alternatively, do basal cells function as a long-term, self-renewing stem cell population and Clara cells as a transiently amplifying (TA) population?

To address these questions, we have generated mice in which the gene encoding a Cre Recombinase-modified Estrogen Receptor fusion protein (CreER) is "knocked into" the endogenous

Scgb1a1 locus. *Scgb1a1* is expressed in bronchiolar Clara cells, including putative BASCs, and tracheal Clara cells. Using these mice, we have lineage-labeled Clara cells and followed their descendents during postnatal growth in the steady-state adult and in response to several models of epithelial lung injury. Our results do not support models in which either unlabeled cells or highly spatially-restricted subpopulations of Scgb1a1$^+$ cells (such as BASCs) function as stem cells to maintain the postnatal bronchioles. Nor do Scgb1a1$^+$ cells contribute significantly to the alveoli. Rather, our data suggest that in the bronchioles many Scgb1a1$^+$ cells function as long-term progenitors. By contrast, in the trachea, most Clara cells are a TA population derived from Scgb1a1$^-$ basal cells.

RESULTS

Characterization of *Scgb1a1-CreER^{TM}* Mice

To generate *Scgb1a1-CreER^{TM}* mice, we targeted CreER^{TM} to the 3′ UTR of the endogenous *Scgb1a1* locus (Figure S1A available online). Using this allele, we established conditions for lineage-labeling both putative BASCs and Clara cells at different developmental stages. In CreER^{TM}/LoxP lineage-tracing studies, recombination of the floxed reporter allele is dependent on the dose of tamoxifen (tmx) and occurs stochastically in a fraction of the cells that express Cre (Hayashi and McMahon, 2002). The level of Cre expression also affects recombination efficiency. Adult *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* mice were given one, two, three, or four injections of tmx every other day, and their lungs were harvested 4 days after the final injection (Figure 1A). As expected, recombination occurred in Scgb1a1$^+$ cells throughout the bronchioles and trachea in a dose-dependent manner (Figures 1B–1D). The proportion of labeled cells varied by region and was always trachea < proximal bronchioles < terminal bronchioles (data not shown). This is consistent with published differences in the amount of Scgb1a1 protein per cell between these regions (Dodge et al., 1993).

Lineage-labeled cells were identified by immunohistochemistry, location, and morphology (Table S1). A single dose of tmx labeled Clara cells throughout the bronchioles, but few putative BASCs. Two doses labeled 50% of bronchiolar Clara cells and 60% of BASCs. In addition, a few (1%) type 2 cells were also labeled. Finally, four consecutive doses labeled 80% of bronchiolar Clara cells, more than 90% of BASCs, and 8% of type 2 cells (summarized in Table 1). Many of the lineage-labeled type 2 cells, in this and all subsequent experiments, are in the vicinity of a BADJ. Estimates for cell turnover in the adult alveoli vary, but most are >100 days (Kauffman, 1980). This makes it unlikely that the labeled type 2 cells were derived from the bronchiolar epithelium during the 11 days from the first tmx exposure

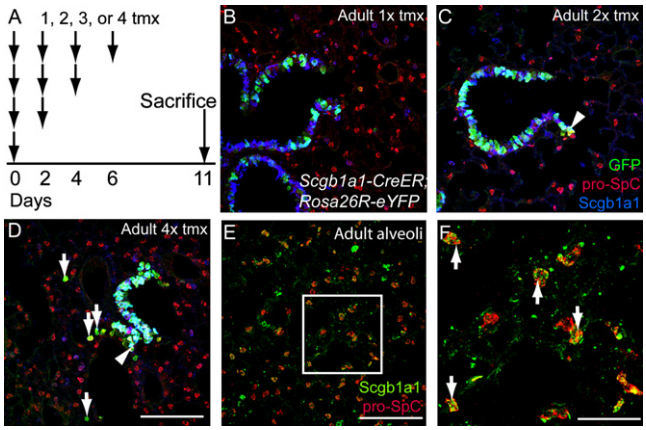

Figure 1. *Scgb1a1-CreER^{TM}* Mice Show Dose-Sensitive Activation of Reporter Gene Expression in Response to Tamoxifen

(A) Time course of tmx injections. Every adult mouse was injected on day 0 so 11 days always elapsed between the first tmx injection and sacrifice.

(B–D) *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* adult lung sections. Green: anti-GFP, lineage label. Red: anti-pro-SpC, type 2 cells. Blue: anti-Scgb1a1, Clara cells. Note the increase in the number of lineage-labeled cells with increasing tmx dose. Arrowheads indicate lineage-labeled putative BASCs (always contiguous with bronchiolar epithelium). Arrows indicate lineage-labeled type 2 cells. (C) One, (D) two, or (E) four tmx injections.

(E and F) Alveoli from a 12-month-old mouse. Green: anti-Scgb1a1. Red: anti-pro-SpC. (F) Higher magnification of box in (E). Note that Scgb1a1 and pro-SftpC are both present in many type 2 cells, in separate vesicles. Arrows indicate (Scgb1a1$^+$, SftpC$^+$) type 2 cells. The level of Scgb1a1 protein in type 2 cells is low and can only be detected by overexposing the bronchiolar Scgb1a1 signal. Scale bars: 100 μm, (B)–(E); 160 μm, (F).

(Figure 1A). More likely, the lineage-labeled type 2 cells themselves express low levels of *Scgb1a1* and hence, CreER. In previous studies, low levels of Scgb1a1 protein were observed in alveolar type 2 cells in the adult rabbit, and *Scgb1a1* mRNA has been detected in adult rat type 2 cells (Patton et al., 1991; Strum et al., 1992). Moreover, rat *Scgb1a1* promoter fragments can drive gene expression in a subset of type 2 cells in transgenic mice (Perl et al., 2005). We confirmed by immunohistochemistry that some SftpC$^+$ type 2 cells in the alveoli of adult mice contain very low levels of Scgb1a1, located in cytoplasmic vesicles (Figures 1E and 1F). These cells could be detected throughout postnatal life, using multiple type 2 cell markers (Figure S2). These data account for the lineage-labeling of some type 2 cells and demonstrate that the putative BASCs are not unique in coexpressing Scgb1a1 and SftpC.

To test for tmx-independent recombination of the reporter, adult *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* mice were treated with vehicle alone. Variable levels (~5%) of recombination

Table 1. Summary of the Extent of Recombination in Scgb1a1$^+$ Cell Populations when *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* Mice Are Exposed to Varying Amounts of Tamoxifen

Time and Dose of tmx	Tracheal Clara Cells	Bronchiolar Clara Cells	Putative BASCs	Alveolar Type 2 Cells
E18.5 1× 0.1 mg/g	++	++	++	+
Adult 2× 0.25 mg/g	++	++	++	+
Adult 4× 0.25 mg/g	+++++	+++++	+++++	++
Adult carrier control	—	+	—	—

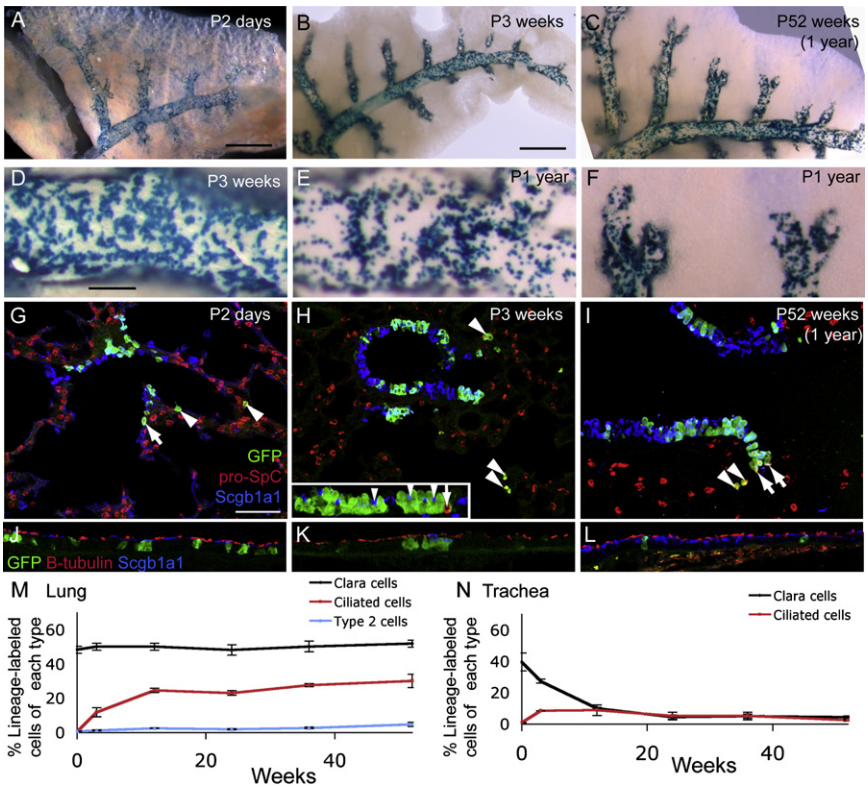

Figure 2. Clara Cells Are a Self-Renewing Progenitor for Postnatal Growth of the Bronchioles, but Not the Trachea

(A–F) *Scgb1a1-CreER^{TM}; Rosa26R-LacZ*. (G–L) *Scgb1a1-CreER^{TM}; Rosa26R-eYFP*. Embryos were exposed to tmx at E18.5.

(A–F) Whole-mount lungs. (A) P2, (B and D) P3 weeks, and (C, E, and F) P52 weeks (1 year).

(G–I) Lung sections. Green: anti-GFP, lineage label. Red: anti-pro-SpC, type 2 cells. Blue: anti-Scgb1a1, Clara cells. Arrowheads indicate lineage-labeled type 2 cells. Arrows indicate lineage-labeled putative BASCs. (G) P2, (H) P3 weeks, and (I) P52 weeks. Inset in (H) shows lineage labeled ciliated cells (arrowheads), but not neuroendocrine cells (arrow). Green: anti-GFP, lineage label. Red: anti-CGRP, neuroendocrine cells. Blue: anti-β-tubulin, ciliated cells.

(J–L) Trachea sections. Green: anti-GFP, lineage label. Red: anti-β-tubulin, ciliated cells. Blue: anti-Scgb1a1, Clara cells. Animals sacrificed at (J) P2, (K) P3 weeks, or (L) P52 weeks (1 year).

(M and N) Graphs to show mean percentage lineage-labeled Clara, ciliated, and type 2 cells following tmx exposure at E18.5. Error bars show SEM. (M) lung, (N) trachea. Scale bars: 2 mm, (A); 1 mm, (B and C); 200 μm, (D–F); and 50 μm (G–L).

were observed at P12 weeks in bronchiolar Clara cells, but never in tracheal Clara cells, putative BASCs, or type 2 cells (Table S2).

To lineage-label Scgb1a1^{+} cells during postnatal growth, we administered a single dose of tmx to pregnant *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* females on embryonic day (E) 18.5. On postnatal day (P) 2, their offspring had recombined the reporter in 50% of bronchiolar Clara cells, 70% of putative BASCs, and a small subset (1%) of type 2 cells (Table S3).

During mouse development, Scgb1a1 protein is first detected at low levels in the bronchioles at E15.5 and at higher levels at E16.5 (Rawlins et al., 2007; Wuenschell et al., 1996). Consistent with this, we were able to activate the reporter by injecting pregnant *Scgb1a1-CreER^{TM}; Rosa26R-LacZ* females with tmx from E14.5 onward, but not at earlier stages (Figures S3A–S3E). When embryos were exposed to tmx at E14.5 and their lungs were assessed at P2, small groups of lineage-labeled cells, including both Clara and ciliated cells, were scattered throughout the bronchioles (Figures S3C and S3D). If tmx was administered at E16.5, the percentage of Clara cells labeling, and the descendents they produced, were indistinguishable from exposure at E18.5 (Figure S3F).

During Postnatal Growth, Scgb1a1^{+} Lineage-Labeled Bronchiolar Cells Self-Renew and Give Rise to Ciliated Cells

During the postnatal period, there is a significant increase in size of the mouse lung. This includes increases in airway diameter and length (Figures 2A–2C, note scale bars), and number of alveoli (Randell et al., 1989). To lineage-trace Scgb1a1^{+} cells during this period, we injected pregnant females once at E18.5 and sacrificed offspring at P2 days; 3 weeks; 3, 6, and 9 months; and 1 year. This period includes rapid postnatal growth followed by slower growth and then steady-state maintenance of the lung (Kauffman, 1980). We confirmed by BrdU labeling that proliferation of epithelial cells throughout the lung continues during the entire experimental period (Figure S4).

To follow the lineage of Scgb1a1^{+} cells, the proportions of labeled basal, Clara, ciliated, NE, putative BASCs, and type 2 cells were scored in sections of each lung after immunohistochemistry and confocal microscopy (Table S1). Because the extent of recombination varied within the organ, we were careful to assess the same range of anatomical regions per lung. For example, 3 proximal airways, 20 terminal bronchioles (including BADJs), and 10 alveolar regions (distal from any visible BADJs) were imaged and scored (see the Experimental Procedures).

In the bronchioles, the percentage of lineage-labeled Clara cells and putative BASCs remained constant for 1 year (Figures 2G–2I and 2M; Table S3). Therefore, throughout this period, the labeled and unlabeled Scgb1a1^{+} Clara cells self-renew at the same rate. By contrast, the percentage of labeled ciliated cells gradually increased from 0 at P2 to 25% between 3 weeks and 3 months and, subsequently, rose slightly (Figure 2H, inset, and 2M). Lineage label was never observed in the NE cells or in the mesenchymal or endothelial compartments (Figure 2H, inset; Table S3). These data show that, overall, the Scgb1a1^{+} cell population in the bronchioles (Clara cells and putative BASCs) both self-renews and gives rise to ciliated cells. In whole-mount assays, the patches of labeled and unlabeled bronchiolar cells are of roughly equal size and appear randomly distributed throughout the airways (Figures 2A–2F). This suggests that the ability to self-renew is widespread within the total bronchiolar Scgb1a1^{+} cell population.

Labeled Bronchiolar Scgb1a1$^+$ Cells Do Not Contribute to the Alveoli during Postnatal Growth

In the alveoli, 1% of type 2 cells were initially labeled by giving tmx at E18.5 (Figures 2G and 2M; Table S3). If alveolar type 2 cells are a self-renewing population in which many cells have equivalent progenitor potential, we would expect the percentage of lineage-labeled type 2 cells to remain steady throughout the experiment. Alternatively, if (Scgb1a1$^+$, SftpC$^+$) cells, either the lineage-labeled type 2 cells or the putative BASCs, are acting as stem cells, the proportion of lineage-labeled type 2 cells would increase over time, as these cells would contribute more descendents to the alveoli than unlabeled type 2 cells. The labeling of type 2 cells varied between 1% and 5% over 1 year (Figures 2G–2I and 2M; Table S3). This suggests that the lineage-labeled (Scgb1a1$^+$, SftpC$^+$) type 2 cells do not self-renew or produce new type 2 cells at a significantly greater rate than the unlabeled type 2 cell population. Moreover, the results are not consistent with the bronchiolar Scgb1a1$^+$ cells (either Clara cells or putative BASCs) contributing to the growth and maintenance of the alveoli. In support of these conclusions, using confocal reconstruction, we did not observe lines, or patches, of lineage-labeled alveolar cells appearing over time in proximity to the BADJs. This might have been expected if cells in the bronchioles are contributing substantial numbers of descendents to the alveoli. This result was confirmed by exposing *Scgb1a1-CreER^{TM}; Rosa26R-LacZ* embryos to tmx at E18.5 and examining lungs by whole-mount X-gal staining at P2 days, 3 weeks, 6 months, and 1 year (n = 3 lungs each time). At all times, these lungs displayed a distinct boundary between the bronchioles, which contained a large number of lineage-labeled cells, and the alveoli which contained only a few LacZ$^+$ cells (Figures 2A–2C and 2F). In the older lungs, P3 weeks and beyond, a small number (not quantitated) of labeled type 1 cells were observed (data not shown). This is consistent with the labeled type 2 cells generating type 1 cells over time (Adamson and Bowden, 1975; Kauffman et al., 1974).

In conclusion, we have found no evidence for any significant contribution of descendents of Scgb1a1$^+$ bronchiolar cells (Clara cells or putative BASCs) to the alveoli during normal postnatal growth and for up to 1 year. This is the case even though many new alveoli are formed in early postnatal life (Randell et al., 1989), and there is proliferation of cells throughout the period as judged by BrdU labeling (Figure S4).

Bronchiolar Scgb1a1$^+$ Cells in the Steady-State Adult Lung

We next investigated the lineage fate of Scgb1a1$^+$ cells in the steady-state adult. Adult *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* mice were given four injections of tmx. This labeled 80% of Clara cells, 90% of putative BASCs, and 8% of type 2 cells (Table S4). Lungs were harvested over a 1 year period and scored as before. Widespread proliferation was detected in adult mice by BrdU incorporation (Figure S4), confirming that epithelial cell turnover continues throughout this period.

In the bronchioles, the percentage of lineage-labeled Clara cells and putative BASCs remained constant during the experiment. By contrast, the percentage of lineage-labeled ciliated cells increased 40-fold (Figures 3A–3C, 3G, and 3J; Table S4). This result is consistent with the Clara cells both self-renewing and giving rise to new ciliated cells. If we assume that all new ciliated cells are derived from Clara cells, the rate of ciliated cell replacement estimated from these data is 0.9% per week. This is consistent with our previous independent estimate of 0.7% ciliated cell turnover per week (Rawlins and Hogan, 2008), supporting the hypothesis that all new ciliated cells in the adult bronchioles are derived from Clara cells.

The percentage of lineage-labeled type 2 cells in the adult alveoli did not increase consistently throughout the experiment. This suggests that (Scgb1a1$^+$, SftpC$^+$) cells, either lineage-labeled type 2 cells or putative BASCs, do not self-renew or generate new type 2 cells at a different rate to the SftpC$^+$ type 2 cell population. It also supports the idea that bronchiolar Scgb1a1$^+$ cells are *not* contributing descendents to the alveoli at steady state. Alveolar epithelial cell turnover has been postulated to be very slow (Kauffman, 1980). However, after the 1 year chase period, we observed lineage-labeled type 1 cells, presumably derived from the initial population of labeled type 2 cells (Figure 3C, marked by an asterisk). These data suggest that turnover of the alveoli did occur.

Finally, we exposed animals to two, rather than four, tmx injections to label a smaller proportion of Clara cells, putative BASCs, and very few type 2 cells (Figures 3E and 3F; Table S5). Similar patterns of cell behavior were also seen in these mice (Table S5).

The Response of Bronchiolar Scgb1a1$^+$ Cells to Injury

We now address the question of how Scgb1a1$^+$ cells contribute to the repair of the lung in three different injury models: exposure of adults to naphthalene and exposure of adults and neonates to hyperoxia. Following naphthalene injury to *Scgb1a1-CreER^{TM}; Rosa26R-LacZ* mice, we observed small numbers of surviving lineage-labeled cells (Figure S5A and S5B). After 3 weeks, large patches of airway epithelium, including both Clara and ciliated cells, were lineage labeled (Figure S5C–S5E). This confirms previous reports that surviving Scgb1a1$^+$ cells can divide and regenerate the airway epithelium following naphthalene injury (Giangreco et al., 2002; Hong et al., 2001; Reynolds et al., 2000a). This result also demonstrates that the *Scgb1a1-CreER^{TM}* allele is active in naphthalene-resistant Clara cells.

Adult hyperoxia was used to test the ability of bronchiolar Scgb1a1$^+$ cells, including putative BASCs, to contribute to the alveoli following injury. We exposed adult *Scgb1a1-CreER^{TM}; Rosa26R* males, which had received different doses of tmx, to >99% oxygen (O_2) for 54 or 60 hr, followed by recovery in 30% O_2 for 12 hr and 2 to 3 weeks in room air. Consistent with previous reports, there were focal regions of severe damage and inflammation throughout the alveoli and terminal bronchioles following O_2 exposure (Figure 4A) (Crapo, 1986; Tryka et al., 1986). Moreover, 48–72 hr following exposure, there was widespread BrdU uptake and Ki67 expression in cells throughout the alveoli and the terminal bronchioles, including cells located at the BADJ (Figures 4B and 4C).

To distinguish between the relative contributions of bronchiolar or alveolar lineage-labeled cells to alveolar repair, two regimens of tmx exposure were employed. In the first two experiments, mice were given 4 or 5 tmx injections to label Clara cells, putative BASCs, and some type 2 cells. In the second

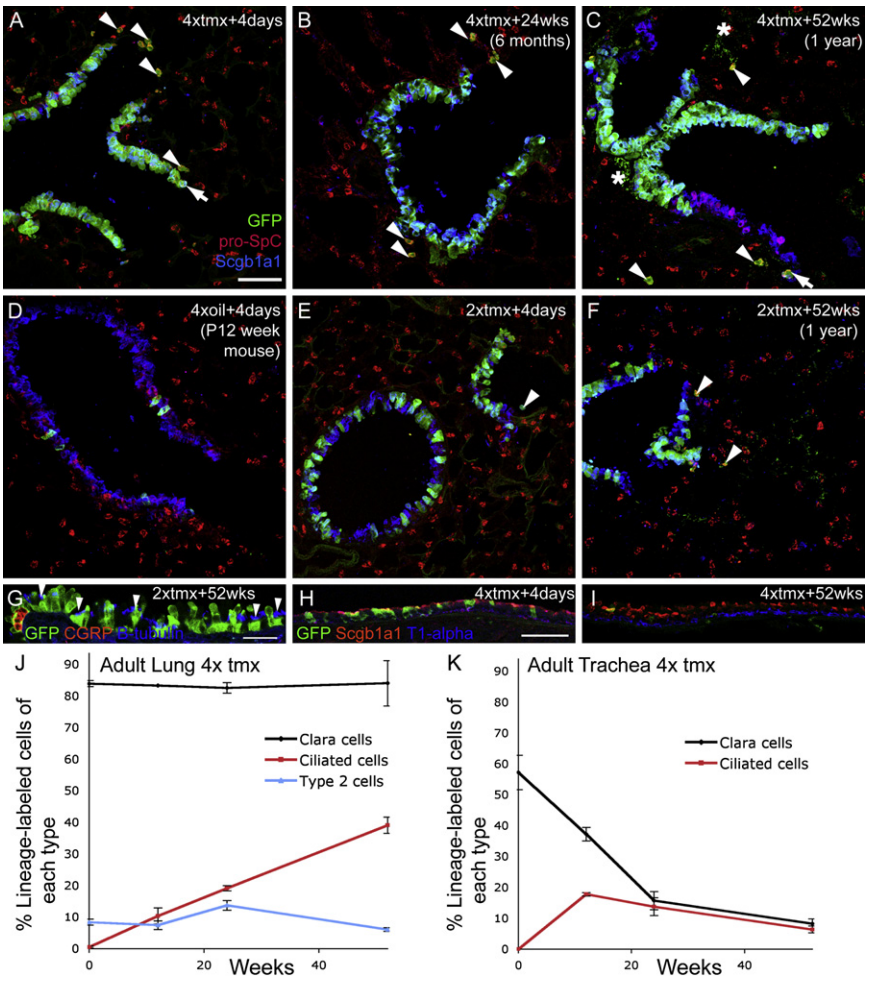

Figure 3. Clara Cells Are a Self-Renewing Progenitor in the Adult Steady-State Bronchioles, but Not the Trachea

(A–I) Adult *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* mice injected twice (E–G) or four times (A–D, H, and I) with tmx or vehicle. (A–F) Lung sections. Green: anti-GFP, lineage label. Red: anti-pro-SpC, type 2 cells. Blue: anti-Scgb1a1, Clara cells. Arrowheads indicate lineage-labeled type 2 cells. Arrows indicate lineage-labeled putative BASCs. (A–C) 4× tmx followed by (A) 4 days, (B) 24 weeks (6 months), or (C) 52 weeks (1 year) chase. Asterisks (*) indicate lineage-labeled type 1 cells. (D) Control plus 4 days chase. (E and F) 2× tmx followed by (E) 4 days or (F) 52 weeks chase. (G) Bronchiolar section, 2× tmx followed by 52 week chase. Green: anti-GFP, lineage label. Red: anti-CGRP, neuroendocrine cells. Blue: anti-β-tubulin, ciliated cells. Arrowheads indicate lineage-labeled ciliated cells. Neuroendocrine cells are not lineage labeled. (H and I) Tracheal sections. Green: anti-GFP, lineage label. Red: anti-Scgb1a1, Clara cells. Blue: anti-T1α, basal cells. 4× tmx followed by (H) 4 days or (I) 52 weeks chase.

(J and K) Graphs to show mean percentage lineage-labeled Clara, ciliated, and type 2 cells following 4× adult tmx injections. Error bars show SEM. (J) lung, (K) trachea. Scale bars are all 50 μm.

two experiments, mice were given 1 or 2 tmx injections to label Clara cells, 50%–60% of putative BASCs, and only a very few (<1%) type 2 cells. Following hyperoxia and recovery, the lungs were assessed in two ways. First, we asked if lineage-labeled bronchiolar cells had contributed *any* descendents to the alveolar compartment. To do this quantitatively, we scored 100–300 alveolar regions from control and recovered groups of mice for the presence/absence of alveolar lineage labeling. We positioned a square with one edge intersecting the terminal bronchiole 5 or 6 cell diameters from the BADJ (diagram in Figure 4D) and scored for the presence/absence of alveolar lineage labeling within the square. The position of the square ensured that the alveolar type 2 cells immediately adjacent to the terminal bronchioles were included. If lineage-labeled cells in the bronchioles were contributing descendents to the alveoli, then following injury and repair, more of the squares would score as "present" for lineage-labeled alveolar cells. This was not observed in any of the four experiments (Figure 4H; Table S6). Rather, in experiments 1 and 2 (where the proportion of lineage-labeled type 2 cells was originally highest), the proportion of BADJs that scored positive actually decreased (statistically significant, $p = 0.008$ and 0.0003). This suggests that when lineage-labeled type 2 cells were killed by O_2 exposure, they were not replaced by lineage-labeled cells from the bronchioles. Second, we asked if any lineage-labeled type 1 cells had been generated by the repair process. Lineage-labeled type 1 cells were observed in animals that had received 4 or 5 tmx injections and, therefore, had labeled type 2 cells. However, none were seen in the animals without significant type 2 cell labeling, even though there were abundant labeled cells in the BADJ (Figures 4D–4G). Therefore, we have been unable to find evidence for Scgb1a1$^+$ bronchiolar cells (Clara cells or putative BASCs) contributing to the alveoli during repair. Rather, our results suggest that following O_2 injury, new type 1 cells are produced from type 2 cells, consistent with previous reports (Evans et al., 1975).

We also tested the response of Scgb1a1$^+$ cells to alveolar injury during neonatal growth, when the cells might be expected to have higher reparative potential. We exposed *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* litters to tmx at E18.5 and then to >95% O_2 from P0 to P7. This was followed by 3 days recovery in 60% O_2 and 4–10 days in room air. Consistent with previous reports (Ahmed et al., 2003), we observed alveolar simplification and an increase in cell proliferation, as measured by Ki67 expression, in treated animals compared with controls (Figure S6). Alveolar regions adjacent to BADJs were scored in sections for the presence or absence of lineage-labeled cells as before. No difference in the percentage of alveolar regions containing lineage-labeled cells was observed between control and O_2-exposed mice (Figure S6; Table S7). Therefore, bronchiolar Scgb1a1$^+$ cells did not contribute to alveolar repair during postnatal development.

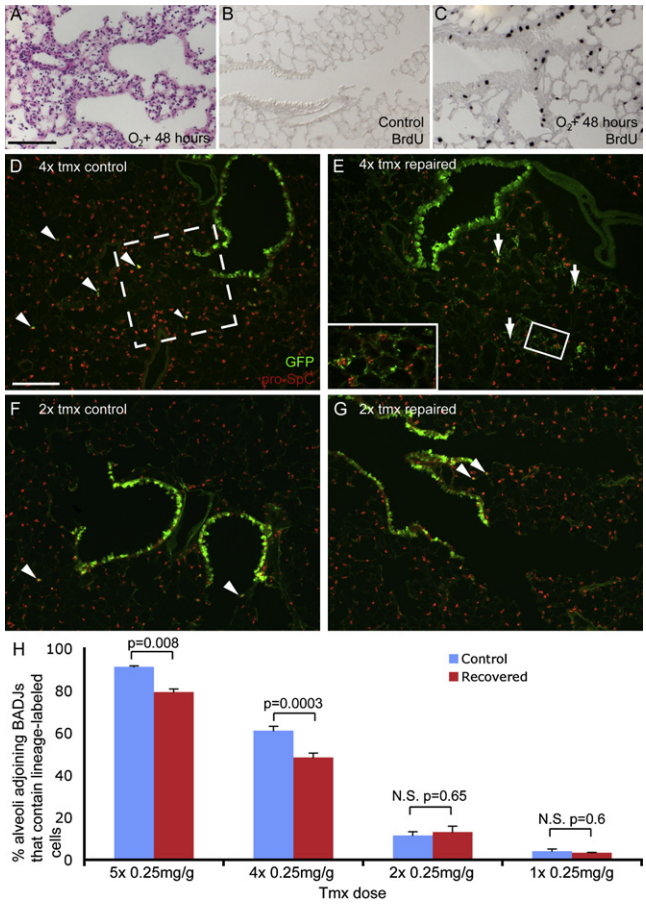

Figure 4. Scgb1a1$^+$ Bronchiolar Cells, Including Putative BASCs, Do Not Contribute to the Alveolar Compartment following O_2-Induced Alveolar Injury

Adult wild-type C57Bl/6 (A–C) or *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* (D–G) lung sections. (A) 48 hr post-O_2, hemotoxylin and eosin. Note extensive inflammatory cells around BADJ and terminal bronchioles, consistent with injury and repair.

(B and C) Black: anti-BrdU, S phase cells. (B) Control, 48 hr post-room air, (C) 48 hr post-O_2. Note extensive proliferation in terminal bronchioles and alveoli.

(D–G) Green: anti-GFP, lineage label. Red: anti-pro-SpC, type 2 cells. Arrowheads indicate lineage-labeled type 2 cells. Arrows indicate lineage-labeled type 1 cells. (D and E) 4× tmx. (D) Control, room air plus 3 weeks recovery. (Dotted box represents the alveolar area around each BADJ scored for presence/absence of lineage-labeled cells in H.) (E) 56 hr O_2 plus 3 weeks recovery. Inset shows lineage-labeled type 1 cells at higher magnification. (F and G) 2× tmx. (F) Control, room air plus 3 weeks recovery. (G) 56 hr O_2 plus 3 weeks recovery. No lineage-labeled type 1 cells were found.

(H) Bar graph showing percentage of alveolar regions adjacent to a BADJ which contain lineage-labeled cells in control (blue bars) and recovered (red bars) animals in four independent O_2 experiments. Error bars show SEM. Means in experiments 1 and 2 are statistically different in a two-tailed t test. Scale bars: 500 μm, (A)–(C); 1 mm, (D)–(G).

Tracheal Scgb1a1$^+$ Cells during Postnatal Growth and Adult Homeostasis

The behavior of lineage-labeled Scgb1a1$^+$ cells in the trachea during postnatal growth was different from that seen in the bronchioles. In the trachea, the percentage of lineage labeling of Scgb1a1$^+$ cells showed a significant *decrease* over time, rather than remaining constant (Figures 2J–2L and 2N; Table S3). Therefore, in the trachea, the majority of Clara cells are not long-term self-renewing progenitors, but are replenished by descendents of an unlabeled cell population. The percentage of lineage-labeled tracheal ciliated cells initially increased, showing that tracheal Clara cells can give rise to ciliated cells. However, the level of lineage labeling was not maintained in the ciliated cell population, a result again consistent with an unlabeled cell population acting as the major progenitor (Figure 2N; Table S3). The lineage label was never observed in the basal cells (Table S3). Similar results were obtained in the adult trachea at steady-state (Figure 3H, 3I, and 3K; Tables S4 and S5). These data are also consistent with an unlabeled cell population acting as the major epithelial progenitor in the steady-state adult trachea.

The Response of Scgb1a1$^+$ Cells to Tracheal Injury

We tested the ability of Scgb1a1$^+$ Clara cells to repair the trachea. Adult *Scgb1a1-CreER^{TM}; Rosa26R* males were given four tmx injections and then exposed to sulfur dioxide (SO_2) to damage the tracheal epithelium. In response to SO_2, many tracheal epithelial cells slough off, leaving cuboidal survivors tightly adherent to the basement membrane (Rawlins et al., 2007). Animals were sacrificed at the peak of epithelial cell proliferation, 24 hr post-SO_2, or after epithelial recovery, 2 weeks post-SO_2. Analysis of whole-mount trachea showed that the number of lineage-labeled cells surviving the injury was variable (Figures 5A–5C). However, in repaired tracheas, clusters of lineage-labeled cells were observed, suggesting that surviving Clara cells can proliferate (Figures 5D and 5E). We confirmed this by exposing injured mice to BrdU to label S phase cells (Figures 5F and 5G). Surviving lineage-labeled cells incorporated BrdU, suggesting that they entered the cell cycle (28 ± 1.8% X-gal$^+$ cells colabeled with BrdU, n = 5 mice, 561 X-gal$^+$ cells). However, the X-gal$^+$ cells were only a small fraction of the total BrdU$^+$ epithelial population (6.3 ± 2.6% BrdU$^+$ cells colabeled with X-gal, n = 5 mice, 2304 BrdU$^+$ cells). Thus, where Clara cells survive, they can proliferate to contribute to tracheal repair. However, Clara cells do not form the bulk of the proliferating population.

Clusters of lineage-labeled cells, consisting of both Clara and ciliated cells, were larger following repair than in control mice (Figures 5H and 5I). This suggests that tracheal Clara cells proliferate at a greater rate following injury than at steady state. Moreover, a greater proportion of ciliated cells were lineage labeled following injury. In control animals, 7.3 ± 3.6% ciliated cells were lineage labeled 3 weeks following the final tmx injection (n = 4 mice, 1392 ciliated cells), compared with 18.1 ± 3.2% lineage-labeled ciliated cells following repair (n = 10 mice, 4364 ciliated cells). We used T1-alpha antibody staining to detect basal cells (Farr et al., 1992). Very rare lineage-labeled basal cells were observed following repair (0.34 ± 0.09%-labeled basal cells, n = 6 mice, 2457 T1α^+ cells), but never in control animals (0 ± 0%-labeled basal cells n = 3 mice, 1102 T1α^+ cells) (Figure 5J, arrow marks lineage-labeled basal cell). Therefore, injury changes the proportion of different cell types that dividing tracheal Clara cells give rise to.

Single Cell Labeling of Scgb1a1$^+$ Cells

Our results suggest that Clara cells are not a long-term self-renewing progenitor in the trachea. However, tracheal Clara cells can give rise to ciliated cells and do self-renew in response to

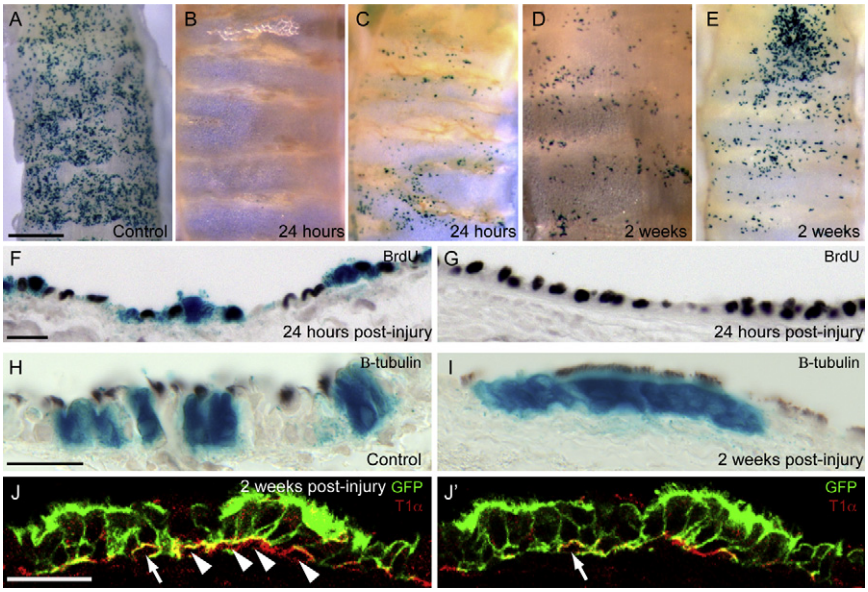

Figure 5. Tracheal Clara Cells Have Greater Proliferative and Differentiative Potential following Injury Than at Steady-State

(A–I) *Scgb1a1-CreER^{TM}; Rosa26R-LacZ* trachea, 4× tmx. Blue: X-gal staining, lineage label. (A–E) Ventral half of bisected whole-mount trachea. (A) No injury control. (B and C) 24 hr post-SO_2 showing variable levels of Clara cell survival. (D and E) 2 weeks post-SO_2. (F–I) Tracheal sections. (F and G) 24 hr post-SO_2. Black: anti-BrdU S-phase cells. (F) Surviving lineage-labeled Clara cells can enter the cell cycle. (G) The majority of proliferating cells are not lineage labeled. (H and I) Brown: anti-β-tubulin, ciliated cells. (H) Control plus 2 weeks. Lineage-labeled cells are in small groups. (I) Two weeks post-SO_2. Large patches of lineage-labeled cells, including both ciliated and Clara cells, are present.

(J and J′) *Scgb1a1-CreER^{TM}; Rosa26R-CAG-fGFP* tracheal sections 2 weeks post-SO_2. Green: anti-GFP, lineage label. Red: anti-T1α, basal cells. Two optical sections from a confocal Z stack are shown. Arrow indicates lineage-labeled basal cell in which colocalization of the membrane-associated lineage label and membrane-associated T1α is visible in every optical section. Arrowheads indicate basal cells which appear to be lineage-labeled in some optical sections only; these were scored as *not* lineage labeled. Scale bars: 0.5 mm, (A); 20 μm, (F–J).

injury. We asked whether tracheal Clara cells can self-renew during postnatal growth or if they only give rise to ciliated cells. If they self-renew, they would be considered to be TA progenitors. To answer this question, we lineage-labeled mostly single tracheal Clara cells by exposing E18.5 *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* embryos to a low dose of tmx. Clone size and cellular composition were assessed in whole-mount preparations at P2 days and 3 weeks (n = 6 trachea each time) (Figures 6A and 6B). At P2 days, 70% of clones consisted of single-labeled cells. By P3 weeks, 82% of clones consisted of more than one cell, and 43% had four or more cells (Figure 6E). It has been demonstrated that airway ciliated cells do not divide (Evans et al., 1978; Rawlins and Hogan, 2008; Rawlins et al., 2007). The observed increase in clone size can only have resulted from the self-renewal of tracheal Clara cells. The overall cellular composition of the clones also changed during the experiment. At P2 days, 93% of clones consisted mostly of Clara cells (expressed as the ratio of Clara: ciliated cells within the clone, Figure 6G). By contrast, at P3 weeks, only 26% of clones were mostly Clara cells. These data are consistent with the idea that during postnatal growth, tracheal Clara cells can self-renew to a limited extent and also give rise to ciliated cells, indicating that they are a TA population.

Individual Clara cells in the bronchioles were also observed to self-renew. At P2 days, 75% of bronchiolar clones were single cells (Figures 6C and 6F). By P3 weeks, 73% of clones consisted of at least two cells and 41% had four or more cells (Figures 6D and 6F). However, clone composition differed between the trachea and bronchioles. At P2 days, 98% of bronchiolar clones consisted mostly of Clara cells. By P3 weeks, 88% of clones still consisted mostly of Clara cells, even though clone size had increased (Figures 6F and 6H). Therefore, during postnatal growth, labeled Clara cells in the bronchioles have a greater tendency to self-renew than to make ciliated cells.

DISCUSSION

Our results support a model in which Scgb1a1^{+} cells in different regions of the lung differ in their role as progenitors in vivo. In the bronchioles, Scgb1a1^{+} cells can both self-renew and generate ciliated cells during postnatal growth and adult homeostasis. Importantly, however, they do not make a significant contribution of descendents to the alveoli under the conditions tested. By contrast, in the trachea, the majority of Scgb1a1^{+} Clara cells constitute a TA population during postnatal growth and steady state, and their capacity for self-renewal and multilineage differentiation increases in response to injury.

In the bronchioles, our data demonstrate that during postnatal growth and homeostasis, the Scgb1a1^{+} Clara cells both self-renew and give rise to ciliated cells for up to 1 year. Thus, the overall behavior of the Clara cell population in the bronchioles justifies their classification as stem cells, even though they are not undifferentiated (Figures 2 and 3). Moreover, patch size in whole-mount preparations (Figures 2 and 6) suggests that these characteristics are widely distributed within the bronchiolar Clara cell population. Interestingly, our data also indicate that, within the bronchioles, there are subpopulations of Clara cells that differ in their ability to contribute to the ciliated cell population. In the adult lineage-labeling experiments, the percentage of labeled ciliated cells rose steadily. By contrast, in our postnatal experiments, lineage labeling of the ciliated cells plateaued in the adult (contrast Figure 3J with Figure 2M). This difference cannot be attributed to the lower percentage of Clara cells, or putative BASCs, labeled in the postnatal experiments. Lineage labeling the same lower percentage in the adult also resulted in a steady increase in ciliated cell labeling (Table S5). Consequently, we hypothesize that the bronchioles contain subpopulations of Clara cells differing in their ability to give rise to ciliated cells. Specifically, our data suggest that a population is labeled

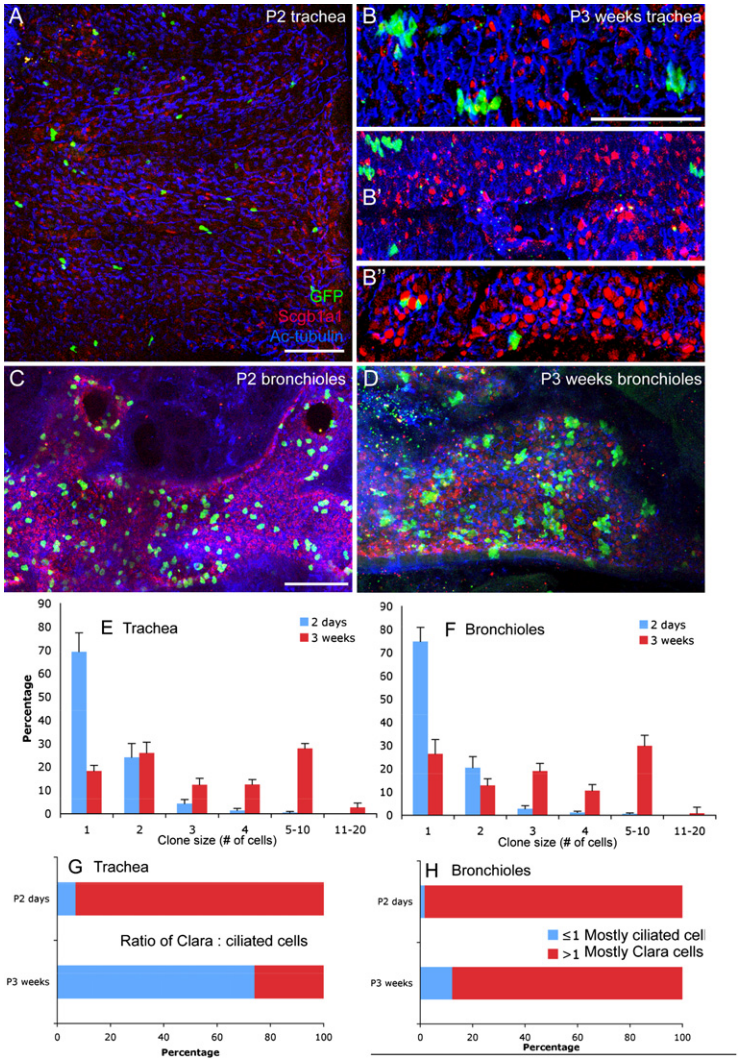

Figure 6. Scgb1a1$^+$ Cells Proliferate during Postnatal Growth of Both the Bronchioles and the Trachea

(A–D) *Scgb1a1-CreER^{TM}; Rosa26R-eYFP* whole-mount lungs exposed to 2.5 μg/gram tmx at E18.5. Green: anti-GFP, lineage label. Red: anti-Scgb1a1, Clara cells. Blue: anti-acetylated tubulin, ciliated cells. (A and B) Trachea (A) P2 or (B) P3 weeks. (C and D) Bronchioles (C) P2 or (D) P3 weeks.

(E and F) Bar graphs showing percentage of different sized clones at P2 (blue bars) and P3 weeks (red bars). (E) trachea; (F), bronchioles.

(G and H) Bar graphs showing cellular composition of the lineage-labeled clones at P2 and P3 weeks. Blue, ratio of Clara: ciliated cells $\leq$ 1, clones consist mostly of ciliated cells. Red, ratio of Clara: ciliated cells > 1, clones consist mostly of Clara cells. (G), trachea; (H), bronchioles. Scale bars all 100 μm.

in the adult, which is more able to give rise to ciliated cells than the Clara cells labeled at E18.5. New experimental tools will be required to investigate how these populations correlate with the already-identified subpopulations of Clara cells, such as the naphthalene-resistant fraction.

By contrast to their behavior in the bronchioles, we have demonstrated that, in the trachea, the majority of Scgb1a1$^+$ Clara cells behave as a TA population and are renewed from an unlabeled progenitor (Figures 2, 3, 5, and 6). In a separate set of experiments, we have lineage-labeled tracheal basal cells and demonstrated that these cells both self-renew over a long period and give rise to Clara and ciliated cells. Therefore, consistent with previously published data, we conclude that tracheal basal cells are a long-term self-renewing stem cell population and tracheal Clara cells are TA progenitors. However, we cannot exclude the possibility that there is a small population of long-term self-renewing Clara cells in the trachea, as the lineage label is maintained in a small percentage of tracheal Clara cells for up to 1 year (Figures 2N and 3K). Following tracheal injury, the Clara cells show increased capacity to both proliferate and self-renew. Moreover, under this condition, they are capable of contributing rare descendents to the basal cell lineage (Figure 5J). This result is consistent with previous observations that mouse tracheal secretory cells can regenerate the entire epithelium, including basal cells, in a xenograft model (Liu et al., 1994). We speculate that in response to severe depletion of basal cells, the TA Clara cells can give rise to basal stem cells. This would be analogous to the behavior of TA cells in the mouse testis and *Drosophila* ovaries in response to stem cell loss (Kai and Spradling, 2004; Nakagawa et al., 2007).

We showed that the putative BASC population located at the BADJ is not unique in coexpressing Scgb1a1 and SftpC. Rather, a proportion of type 2 cells throughout the alveoli express low levels of Scgb1a1, as well as high levels of SftpC in cytoplasmic vesicles (Figures 1E and 1F; Figure S2). These cells can be lineage labeled with high doses of tmx, especially in the vicinity of the BADJ. Lineage analysis shows that these cells can give rise to type 1 cells, but they do not have a higher potential to generate type 2 cells than the overall type 2 cell population (Figures 2, 4, and 5). This strongly suggests, consistent with previously published data, that type 2 cells (regardless of their Scgb1a1 expression status) are the major alveolar stem or progenitor cells. Furthermore, we have been unable to find any evidence that Scgb1a1$^+$ bronchiolar cells (Clara cells or putative BASCs) contribute significant numbers of descendents to the alveoli under any of the physiologically-relevant conditions tested (Figures 2–4). These results are in contrast to previous publications that suggested, based on in vitro data, that the putative BASCs are a stem cell for both the bronchioles and alveoli (Kim, 2007; Kim et al., 2005). It is still possible that the large scale destruction of type 2 cells or some other extreme injury would reveal a latent potential of bronchiolar Scgb1a1$^+$ cells to contribute to the alveoli.

Currently we do not understand the significance of the fact that some Scgb1a1$^+$ bronchiolar Clara cells express SftpC and, conversely, that some alveolar type 2 cells express Scgb1a1. There is accumulating evidence that the (Scgb1a1$^+$, SftpC$^+$) coexpressing cell population increases in number in mouse models of lung cancer (Ventura et al., 2007; Yang et al., 2008). However, it is not clear if this is due to preferential proliferation of pre-existing (Scgb1a1$^+$, SftpC$^+$) cells or to upregulation of *SftpC* or *Scgb1a1* in response to oncogenic activation. It

remains possible that putative BASCs or Scgb1a1$^+$ type 2 cells contribute significantly to some lung pathologies.

Our data demonstrate that the range of epithelial cells that function as stem or long-term self-renewing progenitor cells in the lungs is greater than previously thought. This has implications for studies of human lungs in which BASCs have yet to be identified. Moreover, our data showing that the different cell lineages produced by progenitor cells in the trachea can be altered in response to local conditions is highly relevant to studies on pathogenic epithelial remodeling and regenerative medicine. It will be important in the future to understand the molecular mechanisms that determine progenitor cell self-renewal and lineage allocation in the lung and how these are modulated during repair.

EXPERIMENTAL PROCEDURES

Mouse Strains

9.3 Kb of the 129/SvEv *Scgb1a1* locus was cloned into pBS-KS upstream of a diptheria toxin cassette. An *IRESCreER^{TM}*-floxed-PGK-Neo cassette (kindly provided by Mario Capecchi) was cloned into an Asc1 site in the 3′UTR. The linearized construct was electroporated into 129/SvEv ESCs. Southern blot confirmed correct targeting of multiple ESC lines, and one was used to generate chimeras by blastocyst injection. *Scgb1a1-CreER^{TM}* mice were crossed with FLPeR mice (Farley et al., 2000) to remove the Neo cassette and then backcrossed to C57Bl/6. All experiments were performed at the N2 or N3 backcross generation. Adult mice were 8–12 weeks old. Between two and five animals were used at each time point in every experiment. *Rosa26R-LacZ (Gt(Rosa)26Sor^{tm1Sor})*, *Rosa26R-eYFP (Gt(Rosa)26Sor$^{tm1(eYFP)Cos}$)*, and *Rosa26R-CAG-fGFP* mice were used at the N3 C57Bl/6 backcross generation. The *Rosa26R-CAG-fGFP* allele is described in Figure S1.

Tamoxifen Administration

A 20 mg/ml tmx stock solution was dissolved in Mazola corn oil. Unless otherwise stated, *Scgb1a1-CreER^{TM}; Rosa26R* adults were injected intraperitoneally with 0.25 mg per gram body weight tmx every other day up to five times. Pregnant females were given a single intraperitoneal injection of 0.1 mg or, for low-dose experiments, 2.5 μg tmx per gram body weight. For adult injury experiments, the final tmx injection was administered 1 week (SO_2 and naphthalene) or 2 weeks (O_2) before injury.

Airway Injury

Neonatal Oxygen Exposure

Scgb1a1-CreER$^{TM/+}$ females were mated with homozygous *Rosa26ReYFP* males. Matched pairs of pregnant females were injected with 0.1 mg/gram body weight tmx at E18.5. After birth, the litter was exposed to >95% O_2 or air for 7 days (as described in Ahmed et al., 2003). At P7, litters were switched to 60% O_2 for 3 days, followed by 4–10 days in room air. Two independent experiments were performed.

Adult Oxygen Exposure

Adult male *Scgb1a1-CreER^{TM}; Rosa26R* mice received 1–5 tmx injections. Two weeks later, they were randomly divided into control and hyperoxic groups. The latter were exposed continuously to >99% O_2 (flow rate of 10 l/min, $CO_2 < 0.1\%$) in polystyrene chambers for 54 or 60 hr, then to 30% O_2 for 12 hr, as described previously (Whitehead et al., 2006). Recovery was for up to 3 weeks. To determine the extent of injury, three animals were sacrificed 48 or 72 hr after the start of the recovery period. Four independent experiments were performed.

Naphthalene Exposure

Adult male and female *Scgb1a1-CreER^{TM}; Rosa26R-LacZ* mice were injected i.p. with 250 mg/Kg naphthalene (Sigma) dissolved in Mazola corn oil. Animals were sacrificed 72 hr or 3 weeks following naphthalene exposure.

SO_2 Exposure

Adult males were placed in individual compartments of a polystyrene chamber and exposed to 500 ppm SO_2 in air for 3 hours. Recovery was for 2 weeks (Rawlins et al., 2007). Three independent experiments were performed.

BrdU Exposure

To detect cell proliferation in response to injury, mice were injected i.p. with 10 μl per gram body weight of a solution containing 3 mg/ml BrdU (Amersham Biosciences) 1 hour (SO_2) or 2 hours (O_2) before sacrifice.

Immunohistochemistry and X-Gal Staining

Lungs were inflated with up to 1 ml 4% paraformaldehyde (PFA) and fixed 4 hr (P3 weeks and older) or 1 to 2 hr (P2 to P20) at 4°C in 4% PFA. For immunohistochemistry, lungs were washed in PBS and either sucrose-protected, embedded in OCT (Optimum Cutting Temperature Compound, Tissue-Tek), and sectioned at 12 μm or permeabilized in 0.3% Triton X-100 for whole-mount staining. Primary antibodies were rat anti-GFP (1:500, Nacalai Tesque, 04404-84), chick anti-GFP (1:500, Aveslab, GFP1020), goat anti-Scgb1a1 (1:10000, kindly provided by Barry Stripp), rabbit anti-Scgb1a1 (1:5000, Barry Stripp), rabbit anti-SftpC (1:200, Chemicon, AB3428), mouse anti-β-tubulin (1:1000, BioGenex, MU178-UC), mouse anti-Acetylated-tubulin (1:3000, Sigma, T7451), rabbit anti-CGRP (1:500, Penninsular labs, IHC6006), hamster anti-T1α (1:1000, Developmental Studies Hybridoma Bank, Clone 8.1.1.), and mouse anti-Ki67 (1:100, Vector Labs, VP-K452). Alexa-Fluor-coupled secondary antibodies (Invitrogen) were used at 1:500 dilution. For BrdU staining (mouse anti-BrdU, 1:500, Sigma, BU33, or rat anti-BrdU, 1:500, Accurate Chemical Corporation, OBT0030) lungs were paraffin-sectioned and treated as described (see Rawlins et al., 2007). To detect β-galactosidase activity, whole lungs were stained in X-gal staining solution and then processed for paraffin embedding.

Confocal Microscopy and Cell Counting

All images used for scoring cells consisted of a Z stack of optical sections captured on a Leica Sp2 laser scanning confocal microscope. Multiple optical sections, captured across the entire 12 μm thickness of the tissue section or the entire apical-basal height of the epithelium in whole-mount preparations, were scored to clearly distinguish cell boundaries. Cell identities were assigned using the criteria in Table S1. Images were always captured from the same regions of each lung. In the tracheal sections, cells along the entire proximal-distal axis of a sagittal section cut from the middle of the trachea were scored in all experiments. Bronchiolar ciliated and NE cells were scored from 8 proximal airway images and 2 terminal bronchioles per lung. Bronchiolar Clara cells and alveolar type 2 cells were scored from 20 terminal bronchioles (including BADJs), 3 more proximal airways, and 10 alveolar regions (distal from any visible BADJs) per lung.

Statistical Analysis

All results are reported as mean ± standard error of the mean. All error bars on graphs represent standard error of the mean. Statistical tests are two-tailed t tests.

SUPPLEMENTAL DATA

The Supplemental Data include six figures and seven tables and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00156-8.

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