Epithelial Stem/Progenitor Cells in Lung Postnatal Growth, Maintenance, and Repair

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The adult lung consists of a trachea leading into a system of branched airways ending in millions of alveolar sacs. It contains many different epithelial cell types arranged in precise patterns along the proximodistal axis. Each region of the lung has the capacity to repair through the proliferation of different epithelial cell types. However, the precise identity of the cells mediating repair is not fully resolved. To address this problem, we are using genetic lineage-labeling techniques in the mouse. The tools we have made will also be useful for understanding how progenitor cell behavior is regulated under normal and pathological conditions.

The lung is a complex and sophisticated organ. It is designed to function in gas exchange day in and day out over many decades while protecting itself against infectious agents, allergens, and toxic agents brought in with the air. Multiple mechanisms are used for this protection. Those initiated within the epithelium include the secretion of mucus and surfactant proteins, the mechanical activity of the ciliated cells, and the function of cells of the innate immune system, such as dendritic cells, embedded within the epithelial cells (Holt et al. 2008). In addition, the normal lung epithelium has a robust capacity to replace endogenous cells lost by “wear and tear” or specific injuries. Under normal circumstances, and particularly in experimental animals under laboratory conditions, cell turnover in the respiratory epithelium is very low (Kauffman 1980; Rawlins and Hogan 2008). This is in sharp contrast to the normal high turnover of epithelial cells in organs such as the stomach and intestine that, like the lung, are derived from the definitive endoderm during development (see below). However, if subsets of lung epithelial cells are killed experimentally, then under some circumstances, they can be replaced by the rapid proliferation and differentiation of cells from within the surviving population. Considerable effort has gone into identifying the stem and progenitor cells that affect this lung epithelial repair, and real progress has been made. One advance has been the generation of a series of gene-targeted and transgenic mouse lines that can be used in the living animal to trace the fate of specific populations of epithelial cells and their descendants during lung development and repair (Perl et al. 2002; Hong et al. 2004a, 2004b; Rawlins et al. 2007). Such experiments will allow lineage relationships between the different epithelial cell types to be established. In addition, these lines will be useful for understanding pathological processes. For example, they will likely have a key role in developing new insights into the cell of origin of different kinds of lung cancer. Another potential use for these lines is in studying “mucus metaplasia,” a prominent feature of several lung diseases in which mucus-producing cells accumulate in the air-

ways. One possibility is that these mucus-producing cells arise from differentiated secretory, and possibly ciliated, cells of the adult respiratory epithelium that change their program of gene expression (“transdifferentiate”) without undergoing cell division (Evans et al. 2004; Tyner et al. 2006). This hypothesis could be tested by following the phenotype of lineage-traced secretory or ciliated cells under experimental conditions that promote mucus metaplasia.

MODELS FOR EPITHELIAL STEM/PROGENITOR CELLS OF THE MOUSE LUNG

Studies of the intestine and insulin-producing β cells of the pancreatic islets provide two very different models for how endodermal organs maintain their epithelial population throughout life. In the intestine, a small number of undifferentiated stem cells, residing at or near the base of the crypts, divide infrequently to give rise to transit-amplifying (TA) cells (Barker et al. 2007; Sangiorgi and Capecchi 2008). Classically, TA cells are defined as cells that proliferate rapidly, self-renew over the short term, and undergo lineage diversification, in this case, into goblet, absorptive, and enteroendocrine cells. In the mouse pancreas, in contrast, there is strong evidence that over several months at steady state, or after partial pancreatectomy, the insulin-producing cells of the islets self-duplicate and are not renewed from an undifferentiated, quiescent stem cell population (Dor et al. 2004; Teta et al. 2007). As an organ, the lung fits neither the intestinal nor β -cell model exactly. Rather, it contains different stem and progenitor cells, depending on the region examined (for review, see Borok et al. 2006; Rawlins and Hogan 2006). In the trachea and main bronchi, there has been good (but not watertight) evidence that during steady state, relatively undifferentiated, Trp63 (also known as p63)- and cytokeratin-5-positive basal cells function as classical stem cells (Fig. 1). Basal cells divide infrequently, self-renew over an extended period, and

Figure 1. Schematic representation of the epithelial cells of the mouse lung. In the trachea, the main epithelial cell types are Clara-like secretory cells, mucus-producing goblet cells, ciliated cells, and undifferentiated basal cells that express p63 and cytokeratin Krt5. The upper regions also contain submucosal glands that open into the lumen through ducts (Rawlins and Hogan 2005). There are only a few neuroendocrine clusters in the trachea. In the interlobular airways (bronchi and bronchioles) of the mouse, there are no basal cells and more clusters of neuroendocrine cells. At the junction between the bronchioles and alveoli (known as the bronchioalveolar duct junction or BADJ), a very few cells coexpress proteins normally expressed at high levels by Clara cells (*Scgb1a1*) and alveolar type II cells (surfactant protein C or *Sftpc*). It has been proposed that these dual-positive cells are bronchioalveolar stem cells (BASCs), with the potential to give rise to descendants in both the airways and alveoli (see text). In the alveoli, there are two different epithelial cells types: the type II cells that produce large amounts of surfactant protein and highly attenuated type I cells across which gas exchange takes place. In the human lung, the BADJ is longer and contains cuboidal epithelial cells that have not yet been well defined. In addition, submucosal glands and p63⁺ basal cells extend more distally into the lung than in the mouse, and even the bronchioles contain small numbers of basal cells.

give rise to differentiated descendants, in this case, ciliated and secretory cells (Borthwick et al. 2001; Hong et al. 2004a). However, it is unclear whether *all* basal cells have the same properties or whether there are subsets with higher proliferative capacity or different lineage potential (Hong et al. 2004b; Schoch et al. 2004). For example, the tracheal submucosal glands may be a protected niche environment for a subset of basal stem cells (Borthwick et al. 2001; Rawlins and Hogan 2005). In the intralobar airways (bronchioles) of the mouse lung, there are no basal cells (Fig. 1). Here, the evidence suggests that Clara cells expressing the secretoglobin gene *Scgb1a1* (also known as *CCSP* or *CC10*) self-renew over the long term and give rise to ciliated cells. Some debate exists, however, about whether *all* Clara cells have the same capacity to self-renew and give rise to ciliated cells or whether there are subsets with different properties. According to one theoretical model, all Clara cells have the same potential. In this case, the cells would be analogous to the population of “committed progenitors” that makes up the basal layer of the interfollicular epidermis of the mouse tail (Clayton et al. 2007; Jones and Simons 2008). According to other published models, at least two subpopulations of Clara cells are revealed during the response of the lung to treatment with naphthalene, which specifically kills differentiated Clara cells (Reynolds et al. 2000; Giangreco et al. 2002; Kim et al. 2005). One subpopulation has been called “variant

Clara” or Clara^V cells. These are thought to be relatively undifferentiated (but still to express the Clara cell marker *Scgb1a1*), to be resistant to killing by naphthalene, and to reside in specific niches around the neuroendocrine bodies and at the bronchioalveolar duct junctions (BADJs). They are also thought to be relatively quiescent. However, when the majority of Clara cells are killed by naphthalene, the surviving Clara^V cells rapidly proliferate and give rise to more Clara cells and to ciliated cells. According to this model, Clara^V cells behave more as classical stem cells than committed progenitors because they are relatively undifferentiated (compared to most Clara cells) and are thought to be relatively quiescent at steady state. Another potential subclass of *Scgb1a1*-expressing epithelial cells has been termed bronchioalveolar stem cells or BASCs (Kim et al. 2005). These express both *Scgb1a1* and *Sftpc* (surfactant protein C, also known as *SpC*) and reside in small numbers exclusively in the BADJs. Like Clara^V cells, BASCs are resistant to naphthalene and proliferate rapidly when most of the Clara cells are killed. However, unlike Clara^V cells, it has been argued that BASCs are bipotential and their daughters can give rise not only to new Clara cells, but also to alveolar type II cells. Finally, in the alveoli of the lung (Fig. 1), there is evidence from injury models that type II cells can proliferate and give rise to type I cells, the flattened cells that are critical for gas exchange (Evans et al. 1973, 1975).

CELL LINEAGE ANALYSIS OF THE MOUSE LUNG EPITHELIUM

The only way to definitively test the models described above is to perform lineage-tracing experiments *in vivo*. This technique is based on the irreversible activation of a conditional reporter allele in the genome of specific cell types. This allows the cell, and any of its descendants, to express a reporter protein that can be detected histologically or by live imaging. The goal of these experiments is twofold: (1) to follow the fate of the labeled cells, and their descendants, over time and under different conditions (steady state or repair) and (2) to measure the extent to which the proportion of labeled cells in the population is maintained or diluted over time. From the first parameter, it is possible to define the developmental potential (potency) and fate of specific individual cells, or populations of cells, under the different conditions. From the second parameter, one can judge whether a population is self-maintaining over time or renewed from another, unlabeled, population. This approach is not without its pitfalls, and interpretation of results can be subject to many caveats. However, lineage tracing is generally accepted as the best way to follow the behavior of a cell and its descendants over time *in vivo*. In other words, the method allows us to determine whether cells are functioning as stem cells, TA cells, committed precursors, self-renewing differentiated cells, or terminally differentiated cells. During the past few years, we have generated a number of transgenic and gene-targeted mice for following cell fate and cell lineages in the embryonic and adult lung. These are all based on the CreER^{T2}/loxP system in which Cre recombinase activity in the cell nucleus is activated by the estrogen analog tamoxifen (Tmx) (Joyner and Zervas 2006). We have used a variety of conditional reporter alleles, including *Rosa26R-lacZ*, *Rosa26R-eYFP*, and *Rosa26R-CAG-farnesylatedGFP*. In all experiments, it is important to have controls to test for the potential toxicity of the Cre recombinase in different cell types and for the leakiness of the CreER allele (activity in the absence of exogenous Tmx) (Naiche and Papaioannou 2007).

In the first series of experiments, we used a *FOXJ1-CreER* transgenic mouse line to activate the reporter specifically in ciliated cells of the adult mouse lung (Fig. 2D,G) (Rawlins et al. 2007). We followed the behavior of labeled ciliated cells in response to two very different lung injuries in multiple experiments. We were never able to observe either the division or transdifferentiation of the labeled ciliated cells, strongly suggesting that ciliated cells are terminally differentiated. Subsequently, we observed that the labeled ciliated cell population was diluted by new, unlabeled ciliated cells over an 18-month period at steady state (Rawlins and Hogan 2008). This confirmed that ciliated cells are a terminally differentiated population and provided an estimate for rates of epithelial cell turnover in the steady-state lungs of the laboratory mouse. We have not yet used this mouse strain to test models of mucus cell metaplasia or cancer cell of origin.

More recently, we have generated Clara-cell- and basal-cell-specific CreER mouse strains: *Scgb1a1-CreER*

Figure 2. In vivo lineage-tracing of individual cell types in the mouse trachea and intralobular airways. Paraffin sections of adult mouse trachea (A–D) and bronchioles (E–G). (A,E) Hematoxylin and eosin staining show cell morphology; (B) lineage-labeled basal cells in section of X-gal-stained trachea from *K5-CreER;Rosa26R-LacZ* mouse; (C,F) lineage-labeled Clara and Clara-like cells from *Scgb1a1-CreER;Rosa26R-LacZ* mouse; (D,G) lineage-labeled ciliated cells from *FoxJ1-CreER;Rosa26R-LacZ* mouse. Bar, 200 μ m in all sections.

(Fig. 2C,F) and *K5-CreER* (Fig. 2B). Our unpublished data confirm that in the intralobar airways of the adult mouse at steady state, Clara cells are able to self-renew and give rise to ciliated cells. However, we have not observed Clara cells, or cells coexpressing Clara and type II cell markers (putative BASCs), giving rise to type II cells under any of the conditions that we have tested. These include postnatal growth (embryonic day 18.5 to 1 year), exposure of neonatal and adult mice to hyperoxia (which damages alveoli and induces epithelial proliferation), and treatment with naphthalene. In contrast, in the trachea, basal cells indeed function as a classical stem cell, both self-renewing and generating new Clara cells. It is not yet clear whether a tracheal basal cell can give rise to a ciliated cell directly or whether Clara cells act as a TA intermediate between the basal cells and postmitotic ciliated cells. Certainly, our data suggest that Clara cells in the trachea do not have the capacity to self-renew extensively at steady state. This conclusion is based on the observation that the proportion of lineage-labeled Clara cells is gradually diluted by nonlabeled cells over time. We are currently testing whether all Clara and basal cells in the various regions of the mouse airways have the same properties. We are also studying the fate of Clara cells in the trachea after large numbers of the epithelial cells have been destroyed by exposure to sulfur dioxide.

CONCLUSIONS

In conclusion, our recent studies support the concept that in the mouse lung, the epithelial populations in each anatomical region—the trachea, the bronchioles, and the alveoli—are sustained and repaired by different kinds of stem and progenitor cells (Fig. 3). However, it should be kept in mind that the mouse lung is not exactly anatomically comparable to the human lung. Our own studies, and reports in the literature, show that in the human lung,

Figure 3. Summary of our current ideas about the stem/progenitor cell relationships in the trachea, intralobar airways, and alveoli of the adult mouse. (Solid lines) Evidence based on pulse-chase lineage-labeling experiments in the mouse. (Curly arrows) Self-renewal; (dashed lines) evidence based on experiments or hypotheses not yet validated by in vivo lineage tracing.

basal cells that express Trp63 and cytokeratin 5 extend much more distally than in the mouse (Sheikh et al. 2004). In other words, the distal mouse lung lacks a stem cell population that is present in the human. This means that caution must be exercised when extrapolating to the human the results, for example, of experiments in the mouse designed to identify the cell of origin of lung cancer. Finally, our most recent studies support the concept that the mesothelium of the mammalian lung functions as another stem cell population. In this case, our lineage-tracing studies demonstrate that mesothelial cells give rise to mesenchymal cells internal to the lung, including vascular smooth muscle. This brings the lung into line with other major visceral organs such as the heart and the gut, in which the outer mesothelial layer is a significant source of internal mesenchymal cells (Wilm et al. 2005; Zhou et al. 2008).

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