Bronchiolar Progenitor Cells

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A comprehensive appreciation of mechanisms regulating epithelial maintenance and repair in pulmonary airways is fundamental to our understanding of tissue remodeling and dysfunction in chronic lung disease. This review provides an update on current concepts that have emerged from recent work in the field of airway epithelial repair and progenitor cell biology. New models to investigate the behavior of lung epithelial progenitor cells have provided fresh insights into their regulation and organization, and help to clarify their roles in normal maintenance and repair. Emerging technologies for the fractionation and culture of lung epithelial cells also provide opportunities to investigate the behavior and regulation of progenitor cell subsets in controlled systems. These advances hold promise for development of new strategies to modulate epithelial cell behavior and to effect tissue repair in the setting of lung disease.

Keywords: progenitor; bronchiolar epithelium; stem cell; transit-amplifying cell

Chronic obstructive lung disease (COPD) is a global health problem, the leading cause of which is long-term exposure to cigarette smoke. The observation among cigarette smokers with COPD that clinical measures of severity do not always correlate with the extent of exposure suggests that initiation and/or progression of pathologic changes to the lung may be exacerbated when coupled with other intrinsic and/or environmental factors (1–3). From an evolutionary and population-based perspective there is a sufficiently broad spectrum of initial responses to lung injury resulting from cigarette smoking that a subpopulation whose genetic composition is poorly suited to appropriately repair damaged tissue progresses to clinical disease. However, despite advances in clinical diagnosis and interventions aimed at moderating disease progression, there remains relatively little information that sheds light on early events associated with initiation of the disease process. A confounding hurdle is the cellular and functional complexity of the lung coupled with the likelihood that distinct initiating events culminate in related pathologic outcomes. Recent advances in understanding normal mechanisms of lung tissue regeneration provide some insight into repair responses that may be involved in the preclinical lung disease accompanying cigarette smoking and may provide insight into sensitive processes whose deregulation leads to disease progression.

In general, the capacity for tissues to maintain, renew, or regenerate themselves in adulthood requires cell replacement that may occur either through the proliferation of endogenous cell types or recruitment of cells from the systemic circulation. The contribution made by either of these mechanisms varies considerably between tissue types and for distinct cellular

compartments within a tissue. When considering epithelial organs and the lung in particular, both mechanisms of cell replacement have been proposed to operate in epithelial maintenance (4). In the lung, even though circulating progenitor cells have been shown to contribute to fibroblast and endothelial cell replacement, the notion that circulating cells contribute to epithelial maintenance or renewal remains controversial (5, 6). This review will focus on recent data that provide insight into mechanisms regulating epithelial maintenance and replacement with a particular focus on roles for endogenous progenitor cells, signaling pathways that have the potential to modulate the behavior of these cells, and efforts that are being made toward their isolation, fractionation, and *in vitro* propagation.

TERMINOLOGY USED TO CATEGORIZE PROLIFERATIVE CELL TYPES INVOLVED IN TISSUE MAINTENANCE

As discussed in previous reviews, the inability to uniformly apply terminology developed to describe proliferative cell types in rapidly renewing tissues to those of all adult somatic tissues has led to significant confusion in the literature (7). Proliferative cell types of the lung and other slowly renewing tissues, in particular, diverge from the classical terminology that was initially proposed based upon cell replacement in the gut and epidermis (8, 9), two tissues in which continuous cell replacement is necessary due to rapid loss of post-mitotic cells within the tissue. However, familiarity with the classical terminology and how proliferative lung cell types diverge is important in considering their functional importance in epithelial maintenance under normal and injured conditions (7, 10).

A term that is commonly (although not exclusively) used to describe any cell that is in the process of proliferating or capable of entering the cell cycle (i.e., not post-mitotic), is the progenitor cell. In classical stem cell hierarchies, progenitor cells can be subdivided into stem- (S) and transit-amplifying (TA) cells. Neither S nor TA cells of classical stem cell hierarchies fulfill specialized functions other than proliferation. Surprisingly, other than the relative scarcity of S compared with their more abundant TA progeny and their apparent requirement for a supportive niche, there are no other uniformly applicable distinctions between these progenitor cell types, even when considering tissues that harbor classical stem cell hierarchies. The capacity of S cells for unlimited self-renewal has been used to distinguish them from TA cells in the gut and hematopoietic systems (11, 12). However, even though this property was considered to be a distinguishing characteristic of epidermal S and TA cells, this concept was refuted by Jones and Simons (13) and by Clayton and colleagues (14), who demonstrated that basal cells of the interfollicular epidermis, generally considered to be a TA population, were actually capable of long-term self-renewal. Infrequent proliferation has also been used to functionally distinguish S and TA cells in the gut and epidermis (15, 16), a property that is no longer considered to be a functional distinction between intestinal S and TA cells due to the demonstration that long-term repopulating S cells proliferate with a frequency similar to that of TA cells (17). Another property of S cells of the hematopoietic system and epidermis

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that distinguish them from TA cells is their greater differentiation potential, a property that is not shared by intestinal S relative to their TA progeny. As such, even though the capacity for unlimited self-renewal, infrequent proliferation, and capacity for multipotent differentiation are considered to be characteristics of stem cells, they cannot be used as definitive measures of "stemness" in classical stem cell hierarchies.

Despite the differences in functional properties of S and TA cells within rapidly renewing tissues, a common denominator is the role that S cells play in replenishment of depleted TA cells. In these tissues S cells are either constitutively active or undergo natural phases of activation and quiescence to effect replacement of TA cells and ultimately specialized cell types of the tissue. This obligatory role for S cells in tissue maintenance has been demonstrated through use of chimera or genetic lineage tracing strategies (18, 19). Progenitor cells of slowly renewing tissues exhibit similar properties but show patterns of proliferation that are generally less frequent in the normal state and are only elevated to levels seen in rapidly renewing tissues after injury or stress (7).

PROGENITOR CELLS OF THE LUNG EPITHELIUM

Lung progenitor cells with characteristics similar to those discussed above for tissues harboring classical stem cell hierarchies have been revealed *in vivo* using animal models. The identity, functional regulation, and localization of these progenitor cells have been reviewed in detail elsewhere (5, 10, 20–22). Current areas of controversy that will be addressed in the following discussion are how lung tissue S and TA cells are defined and, accordingly, whether endogenous S cells exist within the lung, the potency (differentiation potential) of putative epithelial stem cells, and interpretation of *in vitro* studies investigating the behavior of lung progenitor cells.

In Vivo Models

The demonstration in conducting airways of populations of abundant and broadly distributed progenitor cells suggests that their organization in the lung is more akin to the epidermis than the gut, lacking defined compartments that maintain highly proliferative cells independently of more specialized post-mitotic cells. Evidence for the existence of airway stem cells came from a series of studies using injury models that either specifically or nonspecifically depleted the abundant populations of airway progenitor cells. Selective depletion of bronchiolar Clara cells through parenteral delivery of naphthalene was repaired through activation of naphthalene-resistant cells that localized specifically to airway branchpoints in association with neuroepithelial bodies (23, 24) or bronchoalveolar duct junctions (25). Either intratracheal instillation of polydocanol, a detergent, or sulfur dioxide (SO₂), a toxic gas, have been used to strip the surface epithelium from the trachea (26). Repair of the tracheal epithelium in response to these severe forms of injury was accomplished through migration of proliferative cells from the submucosal gland duct followed by restoration of a normal pseudostratified epithelium. For both bronchiolar and tracheal injury models, the question arises as to whether repairing cells are truly a distinct population of S cells or whether they simply represent the remnant of abundant progenitor cells that survive injury. This is unlikely to be the case following naphthalene-induced lung injury due to the highly stereotypic and focally localized repair process despite systemic delivery of the toxicant (27). In contrast, progenitor cells contributing to repair of the tracheal epithelium after either polydocanol or SO₂ exposure may be preserved due to the inability of these agents to physically penetrate and injure epithelial cells lining the sub-

mucosal gland (26). The finding both within bronchioles and tracheal epithelium of progenitor cells that proliferate infrequently in the steady-state relative to the abundant progenitor cell pool has been proposed as further evidence for the existence of rare progenitor cells that more closely resemble S cells of the epidermis (23, 25, 26).

Early studies demonstrating the existence of abundant progenitor cells, and the more recent analysis of chemically resistant S-like cells that repair airways after depletion of these abundant progenitor pools, suffer from the fact that these acute repair models fail to provide insight into mechanisms of long-term epithelial maintenance. Two model systems have recently been developed to address this issue in the lung. Rawlins and colleagues developed a lineage tracing model in which the tamoxifen-regulated CreER fusion protein was expressed under the regulatory control of the Clara cell secretory protein (CCSP) gene, allowing the introduction of lineage tags in CCSP-expressing cells of the developing or adult epithelium (28). They were able to show that lineage tags introduced into CCSP-expressing cells were stably maintained in the CCSP-expressing population and that the abundance of bronchiolar ciliated cells carrying the lineage tag increased over time. These data argued that bronchiolar CCSP-expressing cells, represented largely by mature Clara cells, are a self-renewing progenitor pool. The observation that lineage tags are chased into ciliated cells over time is also consistent with the early findings of Evans and colleagues (29) that Clara cells are a progenitor for renewal of ciliated cells. They went on to demonstrate that lineage tags introduced into CCSP-expressing cells of tracheobronchial airways were depleted over time within the CCSP-expressing population. Collectively, these data suggest that CCSP-expressing cells of proximal airways behave like classical TA cells such as those of the intestinal epithelium, whereas CCSP-expressing cells of bronchiolar airways behave more like the self-renewing progenitor cell pool present within the interfollicular epidermis. A different approach was used by Giangreco and colleagues to investigate the long-term behavior of airway progenitor cells in normal and injured airways (30). In this study chimeric mice were generated through fusion of morula-stage embryos from wild-type (WT) mouse line and one in which GFP was ubiquitously expressed in all somatic cells. Chimeras were evaluated for the extent of epithelial mixing between somatic cells of each genotype within various tissues, including airways of the lung. Analysis of epithelial cells lining the gut demonstrated the presence of large clonal patches of either WT (GFP[−]) or GFP⁺ cells indicative of stem cell-mediated maintenance. In contrast, epithelial cells lining bronchiolar airways of the adult lung were of completely mixed genotypes, closely resembling genotypic heterogeneity observed within the epithelium of pancreatic islets, suggesting that an abundant progenitor cell maintained the normal airway epithelium rather than rare tissue S cells. These findings were concordant with those of Rawlins and colleagues and suggest that broadly distributed abundant progenitor cells, Clara cells in the bronchiolar epithelium, maintain the normal airway epithelium. In chimeric mice described by Giangreco and coworkers, only Clara cell depletion resulting from naphthalene exposure was able to change the cellular composition of airways from a heterogeneous mix of GFP⁺ and GFP[−] cells to one of large clonal patches of GFP⁺ or GFP[−] cells (30). In this case, bronchiolar airways more closely resembled the renewing epidermis after wounding, where S cells are recruited from the hair follicle bulge to replace the depleted basal cell pool of the interfollicular epidermis. Clonal patches of GFP⁺ cells were centered on airway branch points or were located within terminal bronchioles, a finding that reinforced earlier observations that S-like cells originate from these microenvironments.

Molecular Regulation of Airway Progenitor Cells

The application of contemporary approaches allowing genetic manipulation of the airway epithelium has provided novel insights into molecular pathways that regulate the behavior of airway progenitor cells. The first such study suggestive of genetic regulation of the bronchiolar stem cell pool involved potentiation of K-Ras signaling within airway epithelium as a model of lung cancer (31). Use of the Cre-LoxP system coupled with adenovirus-mediated delivery of Cre recombinase to airway epithelial cells was used to activate a latent K-RasG12D allele. Early pre-neoplastic lesions resulting from K-Ras activation included a population of bronchiolar cells that expressed pro-surfactant protein C (Pro-SPC), a protein product thought to be expressed exclusively by alveolar type 2 cells. Co-expression of CCSP and Pro-SPC was later demonstrated to be a property of naphthalene-resistant cells localizing to the bronchoalveolar duct junctions of wild-type mice (32). The term bronchoalveolar stem cell (BASC) was coined based upon the ability of cultured CCSP/Pro-SPC dual positive cells to express markers of Clara cell (CCSP), alveolar type 2 cell (Pro-SPC), and alveolar type 1 cell (Aquaporin 5) differentiation. Since then a number of signaling pathways have been described that impinge upon lung development and/or epithelial progenitor cell fate in adulthood to expand the pool of cells that resemble bronchiolar stem cells defined either by their resistance to naphthalene and/or capacity to repair injured airways and/or co-expression of CCSP/Pro-SPC. Signaling molecules implicated as regulators of airway progenitor cell behavior in these studies include β -catenin, GATA-6, Pten, PI3-K, Bmi-1, and MAPK (33–39). However, it remains unclear how closely stem-like cells that have been expanded through manipulation of these signaling molecules/pathways relate to endogenous stem/progenitor cells of the normal lung, and whether pathways that include these gene products as critical transducers represent physiologic regulators of the endogenous bronchiolar stem cell pool.

Fractionation and *In Vitro* Culture of Lung Epithelial Progenitor Cells

Basic deficiencies in our understanding of airway stem and progenitor cells include the lack of a rigorous molecular phenotype that allows for their unambiguous identification and the lack of validated *in vitro* methods to study their behavior in an isolated system. The critical importance of a well-defined molecular phenotype coupled with rigorous assays to investigate the behavior of isolated cells has been clearly demonstrated for progenitor cells that maintain the intestinal epithelium. In the small intestine, only recently has the true identity and location of stem cells been defined despite decades of research investigating their functional properties and regulation *in vivo* (17, 40). A number of strategies have been developed to enrich subpopulations of lung cells and fractionate to further select for cell types of interest. Instillation of low-melt agarose is a commonly used approach to limit the activity of proteolytic enzymes used for dissociation of lung tissue to a defined compartment. In so doing, enriched populations of airway and alveolar epithelial cells can be prepared with significantly reduced cross-contamination. Further selection and enrichment of viable lung progenitor cell subsets has been achieved through staining and sorting approaches. These include either differential staining with vital dyes or using monoclonal antibodies to cell surface markers that can be coupled with magnetic bead or fluorescence-activated cell sorting (FACS) approaches for cell enrichment/depletion.

Among the first studies aimed specifically at applying cell fractionation strategies to the isolation of lung epithelial stem cells were those relying upon efflux of the DNA binding dye Hoechst 33342 (41, 42). Previous work by Goodell and colleagues determined that Hoechst efflux, and the resulting generation of a distinct “side population (SP)” of weakly stained cells by flow cytometry, allowed for selective enrichment of hematopoietic stem cells (43). Giangreco and colleagues found that selection of mouse lung cell suspensions prepared by elastase digestion for the Hoechst 33342 effluxing “side population (SP)” cells resulted in significant enrichment for epithelial cells with surface stem cell antigen-1 (Sca-1) immunoreactivity (42). However, the coupling of this approach with *in vitro* culture methods to determine colony-forming ability suggested that clone-forming cells within the SP fraction were of more mesenchymal than epithelial character (44). Kim and colleagues were able to develop a fractionation scheme based upon enrichment for distal mouse airway/alveolar epithelial cells and immunophenotypic fractionation (32). Negative selection for the hematopoietic marker CD45 and the endothelial cell marker CD31, coupled with positive selection for Sca-1 and CD34, resulted in the generation of a cell fraction that was highly enriched in CCSP/Pro-SPC dual immunoreactive cells that retained epithelial character after serial passage *in vitro*. However, more recent studies suggest that Sca-1-positive populations can be further subdivided into high and low-expressing subpopulations; Sca-1 low including Clara and bronchiolar stem cells (45), with Sca-1 high representing a lung stromal population (46). These fractionation strategies have been validated through use of mouse models, allowing expansion of bronchiolar stem cell pools and lineage tracing, yet suffer from the lack of *in vitro* assays allowing unambiguous classification of progenitor cell types based upon functional properties of isolated cells. Parallel approaches applied to tracheal epithelial cell populations have revealed subpopulations of nerve growth factor receptor (NGFR)-expressing basal epithelial cells that are highly enriched for clone-forming cells *in vitro* based upon use of a spheroid-forming assay (47).

Collectively, these reports demonstrate significant progress in the field but leave some large unanswered questions. A key question relates to interpretation of *in vitro* assays of progenitor cell behavior and how this equates to the behavior of counterpart cells *in vivo*. Results from numerous *in vivo* studies have led to the emergence of the concept that “stemness” is a cellular property requiring a combination of extrinsic and intrinsic regulatory cues. Roles for extrinsic signals in the maintenance of tissue stem cells comes from the finding that in most tissues a stem cell “niche” is required for their maintenance (48). The finding that bronchiolar stem cells, defined as the pool of naphthalene-resistant epithelial progenitors, are localized within discrete bronchiolar microenvironments suggests that the lung also harbors niches that contribute to stem cell maintenance (23, 25, 32, 49). In contrast, Clara cells represent a progenitor cell pool that do not require a specialized niche for long-term maintenance (28–30). So how does this relate to *in vitro* observations of clone-forming epithelial cells? Isolated cells are clearly devoid of cell–cell or local paracrine interactions that recapitulate those of the niche. It might reasonably be thought that supplying these factors would be critical for maintenance of the stem cell phenotype *in vitro*. By extrapolation, it is likely that stem cells may assume characteristics of more specialized cells *in vitro*, such as Clara cells, if appropriate cues are not provided for stable stem cell maintenance. Based upon this logic, it is not clear that *in vitro* models of clonogenic potential are a reflection of “stemness” that has been revealed

through *in vivo* studies. The rhetorical nature of these questions result in part from the absence of well-defined molecular markers that unambiguously relate cells propagated *in vitro* with their *in vivo* counterparts. It is likely that future studies aimed at expanding the repertoire of molecular markers allowing definitive molecular definition of lung epithelial cell types, including progenitor cell subsets, will provide critical tools allowing continued advances to be made in the field of lung stem/progenitor cell biology.

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