Emerging Concepts of Stem Cell Organization in the Normal Lung and in Lung Cancer

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Abstract

Gaps in our knowledge about the organization and regulation of regenerative cells in the normal and diseased adult lung have been a major impediment to the identification of targets around which improved therapies for intractable lung diseases and lung cancer could be developed. During the last decade, specific lung injury models, the availability of genetically engineered reporter mice, and the development of robust cell separative methods and clonogenic assays for the identification and characterization of adult lung epithelial stem cells have provided the field with powerful tools for the identification and validation of predictive stem cell biomarkers and critical molecular pathways and microenvironmental signals which regulate their behavior. These approaches are key to the development of new therapies to restore healthy lung epithelium following injury or disease.

Introduction

The respiratory epithelium forms a physical barrier to inhaled foreign particles and microbes, protecting the lung from infections. Epithelial maintenance in the steady-state, and epithelial regeneration and repair after damage, is thought to be mediated by the proliferation of continuously renewing endogenous stem cells and their differentiation into the specialized epithelial cells of the conducting airway and alveolar beds.

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However, progress in characterizing epithelial stem and progenitor cells in the adult lung and in identifying the critical microenvironmental signals which regulate their activity has been hampered by the lack of specific markers for their isolation and of robust functional assays to measure their potential. These limitations are being overcome with the increasing availability of instructive, genetically engineered reporter mouse models and the development and refinement of flow cytometric cell sorting strategies and *in vitro* clonogenic assays for their identification and quantitation. These approaches provide powerful tools for the analysis of the organization and regulation of regenerative cells in the healthy lung, and in advancing our knowledge of the dysregulated behavior of lung stem cells following injury, and in understanding the nature and properties of lung cancer initiating cells. In this chapter we discuss what is known about the identity and regulation of epithelial stem and progenitor cells in the developing and adult lung, and how this knowledge informs the characterization of lung cancer initiating cells and the development of novel therapies for lung diseases.

Lung Development

The analysis of fetal lung development has contributed significantly to our understanding of adult lung regeneration and repair after injury. The embryonic lung originates from epithelial progenitors located in the anterior foregut endoderm which form the bronchial airway tree by the process of branching morphogenesis. Briefly, following the outpouching of the foregut, epithelial lung buds grow into the surrounding mesenchyme where fibroblast growth factor-10 (Fgf-10) secreted by smooth muscle progenitors acts to promote epithelial progenitor cell proliferation and inhibit differentiation. As the airway branches, the descendants of these progenitors remain in the airway stalks where they begin to differentiate, while the progenitors continue to proliferate at the growing distal tips of the branching lung epithelium (Rawlins 2008). Cell lineage-tracing has confirmed that self-renewing epithelial progenitor cells give rise to all lung epithelial cell lineages during the pseudoglandular stage of lung development and are characterized by the expression of inhibitor-of-differentiation-2 (Id2) (Rawlins et al. 2009a). Tracheobronchial and bronchiolar airway lineages are subsequently established during the transition from the pseudoglandular to canalicular stage of lung development when proximal airway cells lose the ability to respond to mesenchymally-derived signals capable of inducing distal airway differentiation (Hong et al. 2004a).

Although the process of adult lung regeneration and repair is largely thought to recapitulate ontogeny (Warburton et al. 2001) current evidence suggests that separate epithelial progenitor cell populations are responsible for building the lung during fetal development, and for maintaining and replenishing epithelial cell lineages in the adult lung. This includes the observation that the majority of genes expressed in distal tip progenitor cells in the embryonic lung are not expressed in the adult lung (Rawlins 2008) and that Id2-positive progenitor cells which play a significant role in lung development are not retained in the adult lung (Rawlins et al. 2009a).

Heterogeneity of Adult Mouse Lung Epithelial Stem and Progenitor Cells

The composition of cells that comprise healthy adult lung epithelium varies along the proximal-distal axis of the lung. The tracheobronchial airways are lined by pseudostratified basal, non-ciliated club (previously named Clara cells) and ciliated cells, interspersed with neuroendocrine cells and collecting duct cells which extend into submucosal glands (SMG). The bronchiolar airways are lined by columnar epithelium largely comprising ciliated and club cells, as well as foci of neuroendocrine cells which are referred to as neuroepithelial bodies (NEB). Proximal airways and bronchiolar epithelia also contain interspersed mucus-producing goblet cells which increase markedly in abundance in chronic lung

diseases such as asthma and chronic obstructive pulmonary disease (COPD). On the other hand, alveolar epithelium comprises alveolar type I (squamous) and type II (cuboidal) cells which are the primary sources of surfactant protein-C (SPC, Sftpc).

While it has long been accepted that damaged and senescent epithelial cells in the healthy lung are continually replenished by proliferating and differentiating stem and progenitor cells throughout life, it is only recently that significant inroads have been made into understanding the precise identity and organization of epithelial stem and progenitor cell pools responsible for epithelial homeostasis, regeneration and repair. This has been made possible by exploiting the selective toxicity of drugs to analyze the temporal pattern of epithelial cell regeneration following ablation of specific lung epithelial cell lineages; the development of genetically-engineered reporter mouse models for cell lineage tracing; and the development of robust flow cytometric cell separative strategies for the identification and prospective isolation of candidate lung stem and progenitor cells, and functional *in vitro* clonogenic assays to measure their incidence and proliferative and differentiative potential. The naphthalene-injury model has proven especially useful in describing the organization and properties of airway epithelial stem and progenitor cells. Mature club cells, which comprise the majority of the proximal airway epithelium, are selectively ablated by systemic administration of naphthalene because they express the CYP2F1 and CYP2F2 isoforms of cytochrome P450 enabling them to metabolize naphthalene to generate a cytotoxic derivative, while their precursors are spared (Buckpitt et al. 1992; Reynolds et al. 2000).

Temporal analysis of airway epithelial regeneration in naphthalene and hypoxic injury models has identified distinct progenitor cell populations in the submucosal glands (SMG) and proximal airways. In particular, experiments have identified that murine SMG duct cells that express Krt14 are resistant to severe hypoxic-ischemic injury (Hegab et al. 2011). This population was shown to be self-renewing and was capable of differentiation along ciliated and secretory lineages to SMGs tubules and ducts, as well as the epithelium overlying SMGs, suggesting that there is a multipotent progenitor cell responsible for maintaining the SMGs. In contrast, basal cells in the proximal airways act only as progenitors responsible for repopulation of airway epithelium (Hong et al. 2004b; Rawlins et al. 2009b). Lineage tracing using a cytokeratin-14 (Krt14)-CreER transgene to follow the fate of basal cells in the tracheobronchial airways of mice following naphthalene injury, showed that Krt14-positive basal cells were able to self-renew and labeled basal, ciliated and club cells (Hong et al. 2004a, b). Other studies have demonstrated that Krt5-labeled basal cells are also able to give rise to both ciliated and club cells in both the steady state and following injury (Rock et al. 2009). The relationship between Krt5 and Krt14-positive cells is unclear.

In the bronchiolar airways, epithelial stem/progenitor cells have been identified as naphthalene-resistant cells that also express club cell secretory protein (CCSP, Scgb1a1). These 'variant club cells' are located at the bronchioalveolar duct junction (BADJ) and are associated with calcitonin gene-related peptide (CGRP) expressing neuroepithelial bodies in the branching airways (Giangreco et al. 2002; Reynolds et al. 2000). Variant club cells are transit amplifying cells that give rise to mature club cells and ciliated cells following naphthalene-induced epithelial injury (Hong et al. 2004a, b). Another study has shown that progenitor cells at the BADJ express both airway (CCSP) and alveolar (SPC) epithelial lineage markers. Termed bronchioalveolar stem cells (BASCs), these cells are resistance to naphthalene-treatment and have been shown to proliferate in response to both bronchiolar and alveolar injury (Kim et al. 2005).

In the alveoli, it has long been established that alveolar type II (AT2) cells function as progenitor cells capable of giving rise to alveolar type I (AT1) cells. A recent study by Chapman and colleagues has shown that a subset of murine alveolar epithelial cells that express the integrin-receptor α6β4, but are deficient in pro-SPC, serve as alveolar progenitors during lung regeneration following bleomycin induced lung injury (Chapman

et al. 2011). More recently, studies have also shown that despite being absent from the distal lung in the steady state, p63-expressing cells proliferate and contribute to alveolar regeneration in response to epithelial damage following H1N1 influenza infection (Kumar et al. 2011). The cell surface marker CD74, has also been identified as a positive-selection marker that enriches murine AT2 cells capable of generating alveolar-like colonies when cultured in Matrigel (Lee et al. 2012).

The diversity of epithelial progenitor cells identified in the adult lung in the various injury models suggests that the fate of epithelial stem and progenitor cells is context dependent. One study using an aggregation chimera mouse model showed that lung epithelium was maintained by randomly distributed progenitor cells during homeostasis and repair after moderate injury. In contrast stem cells associated with putative stem cell niches (BADJ and NEB) were necessary to regenerate the denuded lung epithelium after severe injury (Giangreco et al. 2009). Other studies using flow cytometry-based sorting strategies in combination with *in vitro* colony-forming assays (Bertoncello and McQualter 2011) have identified multipotent EpCAM^{pos} Sca-1^{low} α6^{pos} β4^{pos} CD24^{low} epithelial stem/progenitor cell in the adult mouse lung (McQualter et al. 2010). When co-cultured with EpCAM^{neg} Sca-1^{pos} lung mesenchymal feeder cells in a Matrigel-based clonogenic assay, these cells form distinct airway, alveolar or mixed lineage epithelial colonies. Furthermore, when mixed-lineage colonies are dissociated and reseeded they are able to form lineage-restricted airway and alveolar colonies as well as reform mixed lineage colonies, while reseeding of airway and alveolar colonies gives rise to their respective lineages (McQualter et al. 2010). Separate studies have demonstrated that α6^{pos} β4^{pos} progenitor cells give rise to airway and alveolar epithelial cells (Chapman et al. 2011). These findings support the existence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung, in which multipotent epithelial stem/progenitor cells are able to self renew and contribute descendants to airway and alveolar lineages.

Another recent study also reports that three different progenitor populations can be segregated from the heterogeneous EpCAM^{pos} CD24^{low} epithelial stem/progenitor cell population in mice on the basis of the differential expression of an Sftpc-GFP transgene (Chen et al. 2012). The different subsets were distributed along the proximal-distal lung axis, with GFP expression ranging from undetectable in the proximal conducting airways, to low in the terminal bronchioles and high in the alveoli. Furthermore, when placed into culture, GFP^{neg} cells largely produced colonies that resembled pseudostratified epithelium, while GFP^{low} and GFP^{hi} cells formed epithelial colonies that resembled distal airway epithelium. These findings support the concept that local hierarchies of epithelial progenitors are responsible for maintaining different epithelial regions along the proximal-distal lung axis.

Despite the recent advances in the identification of multipotent epithelial stem cells and lineage restricted progenitor cell populations in the adult lung, it remains an open question whether the adult lung epithelium is maintained by an epithelial stem/progenitor cell hierarchy with a single multipotent stem cell subpopulation that gives rise to regional lineage-specific progenitors or by discrete functionally distinct progenitor cells that maintain the anatomically diverse regions of the airways and alveoli.

The Role of the Niche Microenvironment in Regulating Stem and Progenitor Cell Potential

It is also important to remember that the regenerative potential of stem and progenitor cells is not only determined by their intrinsic potential, but also by external regulatory factors provided by their microenvironment. These include cytokines, adhesion molecules, stromal cells, immunomodulatory cells and extracellular matrix proteins. For example, *in vivo* studies have shown that mesenchymal stromal cells (MSC) are involved in re-epithelialization following naphthalene-induced lung injury (Volckaert et al. 2011), and we have also demonstrated that the colony-forming potential of mouse lung epithelial stem/progenitor cells in culture is conditional on the

presence of co-cultured CD45^{neg} CD31^{neg} Sca-1^{pos} lung MSC (McQualter et al. 2010).

In vivo interaction between stem cells and the microenvironment is likely to be even more complex considering that the adult lung comprises at least 40–60 different cell types. Importantly, stem and progenitor cell behavior will also be influenced by the significant changes in the composition of the microenvironment in chronic respiratory diseases, such as smooth muscle hyperplasia, matrix deposition, angiogenesis, and inflammation and cytokine production. Consequently, the physiological and pathophysiological behavior of lung epithelial stem/progenitor cells will be dictated by temporospatial changes in their physical microenvironment and elaboration of regulatory factors in the steady state or following perturbation or injury. Further work is needed to understand if these changes in the airway microenvironment are coincidental or contributory to dysregulation of epithelial stem/progenitor cells and the associated epithelial remodeling.

Human Lung Epithelial Stem and Progenitor Cells

To date, most studies aimed at identifying, isolating and characterizing stem and progenitor cells in the adult lung have concentrated on the analysis of mouse models, and comparatively few have reported progress in isolating and characterizing stem and progenitor cells in the human lung. This may be attributed to the limited availability of adequate amounts of normal human tissue, which is generally restricted to samples from the trachea and proximal airways, and also to the lack of specific cell surface markers to enable their isolation. While some progress has been made despite these impediments, the nature and properties of candidate human epithelial stem and progenitor cells in the normal lung remains unclear at this time.

One of the first reports describing the properties of human lung stem/progenitor cells, showed that unfractionated normal lung cell suspensions were able to form bronchospheric colonies of mixed alveolar, airway and mesenchymal cells (Tesei et al. 2009). More recently Kajstura et al. (2011) described a population of putative human lung stem cells isolated on the basis of their differential expression of c-kit (Kajstura et al. 2011). *In vitro* culture of c-kit^{pos} cells in this study showed that they were clonogenic and multipotent. When injected into an injured mouse lung they appeared to regenerate bronchiolar and alveolar epithelium as well as vasculature, challenging the concept that cells of embryonic endodermal and mesodermal origin remain as distinct lineages in the adult lung. However, significant reservations have been raised about the experimental designs employed in this study (Lung stem cells: Looking beyond the hype. 2011. Nat. Med. 17: 788–789) and the results need to be interpreted with care. More recently, Oeztuerk-Winder et al. (2012), have described an E-cadherin ^{pos}Lrg6^{pos} lung progenitor cell cohort able to regenerate damaged bronchioalveolar epithelium following bleomycin-induced lung injury in mice, as well as regenerate bronchioalveolar tissue when transplanted under the kidney capsule. Significantly, c-kit^{pos} cells isolated in this study were not able to do so.

Perhaps, a recent study of epithelial cells isolated from human trachea provides the most compelling analysis of candidate human lung stem/progenitor cells (Hegab et al. 2012). In this study, basal and SMG duct stem/progenitor cells were isolated from the surface epithelium of human trachea on the basis of their differential expression of nerve growth factor receptor (NGRF), CD166 and CD44. When cultured in a three-dimensional Matrigel-based assay, both basal and SMG duct progenitors demonstrated the capacity to self-renew and differentiate under various conditions in culture.

Lung Stem/Progenitor Cells as Cancer-Initiating Cells

While the precise cellular origin of lung cancer is unknown, comparisons between normal tissue stem cells and cancer cells have revealed many similarities in their behavior, including the capacity for self-renewal and ability to differentiate

into a variety of cells. The concept that cancer is a caricature of normal tissue development was originally proposed by Pierce 40 years ago (Pierce 1974; Pierce and Speers 1988) and has been influential in framing the cancer stem cell paradigm which proposes: (1) that cancers arise from the genetic mutation or epigenetic transformation of rare normal stem or progenitor cells; (2) that the hierarchical organization of cancer stem cells and their progeny within a tumor mimics that of regenerative cells within normal tissue; (3) that cancer stem cells retain the potential to renew, proliferate and generate descendent cell lineages characteristic of their normal counterparts; and (4) that like their normal counterparts, their proliferation and differentiation is dictated by their intrinsic potential and by microenvironmental cues (Castano et al. 2012; Sneddon and Werb 2007).

The regional distribution of specific lung tumor sub-types broadly reflects specific genetic and epigenetic changes in distinct regional stem/progenitor cells distributed along the proximal-distal axis of the airway tree (Asselin-Labat and Filby 2012; Sullivan et al. 2010; Sutherland and Berns 2010), as does the analysis of mice harboring oncogenic mutations. For example, squamous cell carcinomas are generally located in the main bronchus and upper airways, while small cell lung cancers are found in bronchiolar airways, and adenocarcinomas and bronchioalveolar carcinoma are restricted to the alveoli and bronchiolar airways. Evidence that SP-C^{pos} AT2 cells and/or BASC are the cells of origin for lung adenocarcinoma is also consistent with studies reporting an increased incidence of BASC in mice harboring the oncogenic K-ras mutation (Kim et al. 2005) expressed in 15–22% of non-small cell lung cancers (Riely et al. 2009), and the development of lung adenocarcinoma in p53-deficient mice that conditionally express K-ras in SP-C^{pos} cells, but not CCSP^{pos} cells (Xu et al. 2012). On the other hand, another study utilizing cell type-restricted adeno-Cre viral vectors targeting neuroendocrine, club and AT2 cells has shown that neuroendocrine cells are the predominant cell of origin of small cell lung cancer following inactivation of the Trp53 and Rb1 tumor suppressor genes in these lineages (Sutherland et al. 2011).

However, efforts to establish the precise identity of lung cancer initiating cells is confounded by the development of heterogeneity within the tumor during disease progression due to the clonal evolution of tumors resulting from genomic and epigenetic instability (Marusyk and Polyak 2013; Nowell 1976). These temporal genomic and epigenetic changes during tumor progression result in plasticity in the immunophenotypic signature profiles of cancer cells during disease progression. This blurs differences between cells of differing proliferative and differentiative potential and makes it difficult to identify, prospectively isolate and hierarchically order cancer stem cells and their progeny to establish the precise relationship of cancer initiating and propagating cells to their normal counterparts (Sneddon and Werb 2007; Sullivan and Minna 2010).

The analysis of human lung cancer stem cells has been significantly impeded by the intrinsic biological variability and heterogeneity of primary lung cancer biopsied tissue, as well as the lack of defining biomarkers and robust assays for their identification, prospective isolation and functional characterization. Cell-separative strategies devised for the prospective isolation of candidate lung cancer stem cells in human cancer cell lines and primary tumor biopsies have mostly relied on the differential expression of cancer associated biomarkers including aldehyde dehydrogenase (ALDH), CD133, or Hoechst-33342 dye efflux as a measure of multidrug resistance gene activity (Eramo et al. 2010). Candidate lung cancer stem cells isolated on this basis (ALDH^{pos}, or CD133^{pos}, or Hoechst^{dull} side population cells) exhibit proliferative characteristics *in vitro* and in *in vivo* tumor xenografted mouse models that are consistent with the characteristics of stem cells. This includes the ability to recapitulate the histology, heterogeneity and growth characteristics of the parent tumor when xenografted in immune-compromised mice, and the ability to renew and exhibit clonal growth and a high proliferative potential *in vitro*. The ability of these xenografts to mimic the original tumor and to clone *in vitro*

will enable the correlation of biomarker expression profiles with functional assay readouts and patient outcomes, as well as identify key signaling pathways and mutations important in driving the dysregulated behavior of lung cancer propagating cells.

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