Paving the road for lung stem cell biology: bronchioalveolar stem cells and other putative distal lung stem cells

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DEFINING STEM CELLS

The field of stem cell biology continues to grow as numerous types of stem cells are identified in model organisms and in human tissues. Clearly, the identification of these previously elusive cells has led to models redefining the development of tissues and the lineage relationships that exist between adult cells (e.g., Refs. 15, 37, 59). The development of fluorescence-activated cell sorting technology (FACS) has been crucial to the isolation of rare stem cell populations from adult tissues (e.g., Refs. 4, 59, 63, 67). Methods to disaggregate complex tissues and grow these cells in culture and the ability to perform molecular analyses on small numbers of cells have also allowed the stem cell field to expand.

The identification of stem cells relies on their definition. Tissue stem cells are unspecialized cells that are capable of self-renewal and give rise to specialized, or differentiated, cells. In some tissues, cells exhibiting stem cell characteristics have been identified during a particular developmental stage or in the adult tissue after injury. Importantly, for most tissues, a direct lineage relationship between putative embryonic and adult stem cells has not yet been established. Many stem cells maintain proliferative capacity for long durations of the life of an organism, yet are quiescent in normal tissue, and only a fraction of the stem cell population may enter the cell cycle after injury; however, it should be noted that these criteria are not met by all stem cell populations. Progenitor cells (also known as transient amplifying or TA cells) also produce differentiated cells and are usually more mitotically active than stem cells, but they do not self-renew. Thus both stem cells and progenitor cells are capable of proliferation, and self-renewal is a distinguishing feature of stem cells. Differentiated cells are the typically postmitotic cells that carry out the daily functions of a tissue. Finally, stem cells exhibit a range of potency: many stem cells, such as hematopoietic stem cells (HSCs), are multipotent, giving rise to two or more lineages of differentiated cells, whereas germ line stem cells are unipotent (4, 7, 10, 28, 34, 37, 38, 51, 71).

An important functional definition of stem cells relies on the ability to demonstrate that the putative stem cells can give rise to specialized cells and retain self-renewal capacity in vivo. This component of a stem cell definition is often exceedingly difficult to achieve without knowledge of the precise conditions needed for such assays. Bone marrow reconstitution with HSCs and mammary gland reconstitution are excellent examples of in vivo functional assays for stem cells (9, 23, 58, 59, 62, 69), but in vivo transplantation assays for stem cells remains a challenge in other tissue systems including the lung. In contrast, there is no cell culture-based assay for HSC self-renewal as there is for neural crest and skin stem cells among others (4, 39). In vitro and in vivo assays tailored for tissue-specific stem cell function are thus still in demand. At minimum, self-renewal and differentiation capacity should be demonstrated in one of these contexts to define a new stem cell population.

EVIDENCE FOR LUNG STEM CELLS

The pulmonary system contains a variety of epithelial cell populations (2). In humans, basal cells, secretory goblet cells, submucosal glands, and ciliated cells line the trachea and upper
The same regions in the mouse are populated by ciliated and nonciliated columnar cells, and a few submucosal glands are found in the proximal airway. The murine submucosal glands include mucous-producing cells, ciliated cells, and basal cells. Neuroendocrine cells are found mostly within the large proximal airways and reside in clusters referred to as neuroendocrine bodies. The nonciliated, columnar Clara cells that line the bronchioles and terminal bronchioles secrete surfactants to aid in oxygen exchange and provide a protective epithelial barrier in the airways. The alveolar epithelium is composed of alveolar type II (AT2) cells, the cuboidal epithelial cells that produce surfactants and the resulting surface tension required for gas exchange, as well as the alveolar type I (AT1) cells, the flat epithelial cells that deliver oxygen to the blood. Numerous stromal cells are present, and the lung has been described as containing at least 40 different cell types, likely to be an underestimation given the promise of future studies to identify distinct stem, progenitor, and differentiated cells.

Rodent models of lung injury suggest that the lung harbors distinct stem or progenitor cell types distributed in discreet locations along the respiratory track (for further review, see Refs. 40, 41, 49). Studies of the response to damaging agents such as polidocanol, naphthalene, or sulfur dioxide have described at least three different populations in the trachea and/or upper airway that may be stem or progenitor cells: 1) basal cells, 2) keratin-rich cells in the submucosal gland ducts or intercartilaginous regions, and 3) secretory cells (resembling the nonciliated, columnar Clara cells that line the bronchioles; Refs. 5, 57; reviewed in Refs. 20–22, 49). Intraperitoneal injection of the pollutant naphthalene selectively ablates Clara cells. A fraction of the Clara cells (“variant Clara cells”) are resistant to naphthalene and may function as lung stem or progenitor cells (Refs. 16, 20, and references above). Clara cells have also been proposed to give rise to ciliated cells after nitrogen dioxide and ozone treatment (3, 12, 13). Whereas one report suggests that ciliated cells give rise to Clara cells after injury (42), a recently reported in vivo lineage tracing technique indicates that there is no evidence that ciliated cells are stem or progenitor cells with Clara cell potential (48). Finally, AT2 cells were reported to proliferate after bleomycin-mediated AT1 cell ablation and gave rise to cells with AT1 characteristics in culture, lending to the frequent nomenclature for AT2 cells as lung stem or progenitor cells (Refs. 16, 25, and reviewed in Ref. 49). Populations of Hoechst dye-effluxing cells have been isolated from the lung, but their role as stem cells is undefined (17, 64).

The seminal studies highlighted here have provided a solid background to fuel further work in lung stem cell biology. Many of these initial studies provided key molecular markers with which to track putative lung stem or progenitor cells, which made follow-up studies feasible. The development of rodent lung injury models is also invaluable, and they will continue to be used in all-important demonstration of functional read-out for stem or progenitor function in vivo. Most of the conclusions about cell lineage relationships listed above were based on electron microscopy or bromodeoxyuridine (BrdU) incorporation at given time points after lung injury. Thus the observed ability of damage-resistant lung cells to proliferate after injury may have been misconstrued as evidence for self-renewal, and it is important to consider that those reports may have identified progenitor cells rather than stem cells. Although these important prior studies suggest that lung stem cells may exist, reports of methods to isolate these cells and demonstration of the key stem cell properties are limited.

The identities of these and other populations of putative stem or progenitor cells in the rodent lung have been reviewed extensively (41, 49). Therefore, the remainder of this review will focus only on distal lung stem cells. It should, however, be noted that the types of technological advances and approaches discussed for enhancing understanding of distal lung cell biology also apply to other putative stem or progenitor cells niches in the lung.

**IDENTIFICATION OF BASCs**

Expanding on prior studies indicating the presence of a putative stem cell population in the distal lung (Ref. 16 and references therein), we identified and isolated bronchioalveolar stem cells (BASCs), which we hypothesize maintain homeostasis of bronchiolar and alveolar epithelial cells (Ref. 31; Fig. 1). Cells expressing both the AT2 cell marker, pro-surfactant protein C (SP-C), and the Clara cell marker, CCSP (also known as CCA, CC10, urotoglobin, and Scgb1a1), were first identified in murine lung tumors initiated by oncogenic K-ras (27). Given this finding and an earlier report that embryonic lung epithelial cells that may give rise to alveolar and bronchiolar lineages may also coexpress similar markers (70), we reasoned that cells of this nature might exist in the normal lung and, if so, may play a role in lung tumorigenesis. Indeed, we found CCSP+ SP-C+ cells exclusively at the bronchioalveolar ductal junction (BADJ), the portion of the respiratory epithelium where the terminal bronchioles abut the alveolar epithelium and the same location where Giangreco et al. (16)
localized the distal lung naphthalene-resistant cells (31). The CCSP+ SP-C+ cells are quiescent in normal lung and proliferate in response to bronchiolar and alveolar epithelial injury. We are currently investigating the response of these cells to diverse forms of lung injury to determine whether they indeed have a region-specific response to lung injury.

We next reasoned that the expression of two lineage markers in these cells might indicate their ability to function as a stem or progenitor cell population for both lineages. Since the cytoplasmic markers were not useful for their isolation and further live cell analyses, we developed a protocol for purification of CCSP+ SP-C+ cells using positive selection for the cell surface markers Sca-1 and CD34 and the absence of the hematopoietic and endothelial cell markers CD45 and CD31, respectively. We had initially tested these and other surface markers based on their previous utility in isolating skin epithelial and hematopoietic stem cells (4, 26, 38, 47, 67). It should be noted that positive expression of these markers alone does not constitute “stemness,” which should be tested functionally; in some tissues, stem cells are excluded with the same markers (60).

We developed methods to demonstrate that the CCSP+ SP-C+ Sca-1+ CD34+ cells are able to self-renew and differentiate in culture, suggesting that they are a stem cell population, and therefore we termed them BASCs (31). FACs-isolated BASCs formed colonies when grown on irradiated mouse embryonic fibroblasts (feeders), and the cells remained CCSP+ SP-C+, suggesting that growth on feeders maintained their undifferentiated state. BASCs cultured on Matrigel contained CCSP+ SP-C+ cells (Clara-like cells), SP-C+ CCSP+ cells (AT2-like cells), and SP-C+ aquaporin 5-positive cells (AT1-like cells) as determined by immunostaining, showing that they had multilineage differentiation capacity. Importantly, single BASCs gave rise to epithelial colonies, and neither the frequency of clonal growth nor the ability of the cells to differentiate into bronchiolar and alveolar cells diminished with each passage when we performed limiting dilution analysis by plating varied numbers of BASCs per well, demonstrating the self-renewal capacity of BASCs (for an additional example of limiting dilution for stem cell analysis, see Ref. 66). In contrast to BASCs, our AT2 cell cultures did not produce secondary colonies or differentiate. We hypothesize that this difference in AT2 cell differentiation capacity compared with previous reports (for example, Ref. 25), is either reflective of culture condition variations or the AT2 cell isolation procedures used by other groups may have yielded BASCs along with AT2 cells. Thus, in our studies, AT2 cells did not self-renew or differentiate, suggesting that they are either progenitors or differentiated cells rather than stem cells.

**BASC RELATIONSHIP TO OTHER DISTAL LUNG CELLS**

Genetic evidence that new Clara, AT1, or AT2 cells can arise from BASCs, variant Clara cells, or AT2 cells in vivo is lacking, leaving open the possibility that any or all of these cell types, or other as yet unidentified cells, are the stem cells that maintain distal lung homeostasis. Both BASCs and variant Clara cells were identified in the BADJ, express CCSP, and are resistant to naphthalene. The shared characteristics suggest that BASCs and variant Clara cells are the same cell population. Alternatively, a subset of variant Clara cells may be BASCs, or BASCs may give rise to variant Clara cells that, in turn, function as progenitors for more specialized Clara cells (31). Data from CCSP herpes simplex virus thymidine kinase (HSVtk) mice (53) suggest that the stem cells required for repair of Clara cell injury express CCSP or that CCSP-expressing cells are required to support the stem cells that give rise to new Clara cells. Both AT2 cells and a population of bronchiolar cells “strikingly similar to fetal epithelium” exhibited growth after alveolar injury (1). This latter mysterious population of cells may be BASCs, and furthermore, this study supports the possibility that multiple cell types play a role during repair of alveolar epithelium.

Alternative, a subset of variant Clara cells may be BASCs, or BASCs and variant Clara cells are the same cell population.

**THE BRONCHOALVEOLAR DUCT JUNCTION NICHE**

In addition to a need to characterize the relationship of the diverse distal lung cells, it remains to be determined if and how the pulmonary region where these cells reside regulates their activity. Specific cell types and/or specific signaling molecules may be present in the BADJ that exert influence on neighboring epithelial cells. As a specific example, it is also unclear whether the conditions for BASC cultures on feeders or Matrigel more closely resemble the environment in the BADJ.

The BADJ was first described as a putative lung stem cell niche that housed variant Clara cells and represents the divide between the conducting airway and the alveolar space (16). Notably, transition areas or regions between two different types of epithelial cells in other tissues have also been proposed to be stem cell niches (esophagus, cornea, etc.; Ref. 48 and references therein; Refs. 46, 55). Whether these regions in diverse tissues share unique signaling environments is unknown, but discovery of such common mechanisms could provide insight to stem cell regulatory factors.

The BADJ has also been implicated in lung pathologies. We observed that oncogenic K-ras-induced lung tumors appeared to grow in the BADJ at the earliest stages of tumor development (31). This finding supported the hypothesis that BASCs are the cells of origin of murine lung adenocarcinoma. Both human and murine lung tumor cells transplanted into the lung via intratracheal injection produce secondary tumors that may grow selectively in the BADJ (Ref. 36 and Kim C, unpublished results). Furthermore, the postnatal cells with colony-forming ability isolated by Ling et al. (35), described above, may reside in the BADJ and may be the cells targeted by severe acute respiratory syndrome coronavirus infection. Clearly, characterization of the BADJ will enhance comprehension of lung disease.

Aside from the BADJ endogenous epithelial and stromal cells, exogenous cells that traffic to the lung have been implicated in lung homeostasis. These include circulating epithelial cells, fibrocytes, and bone marrow cells (Refs. 18, 19, 33, 45,
PATHWAYS IMPLICATED IN PUTATIVE DISTAL LUNG STEM CELL REGULATION

As methods to isolate and characterize distal lung stem cell populations are emerging, it is possible to begin to map out their regulatory pathways. Two new studies point to a requirement for MAPK14 (also known as p38α) in regulation of lung stem or progenitor cell proliferation and differentiation in lung development and adult lung (Refs. 24, 68; see Ref. 30 for further discussion; Fig. 2). In separate studies, deletion of the INK4 family cyclin-dependent kinase inhibitor p18INK4c led to an expansion of the incidence of BASCs as detected by dual SP-C and CCSP staining (43). It remains to be determined whether p18 deletion specifically affects BASCs or also other putative lung stem, progenitor, or differentiated cells, as other cell types were not quantified in the reported studies. In contrast to deletion of Mapk14, p18 deficiency at early ages expanded BASCs but did not seem to affect BAC location or lung architecture. Interestingly, the studies indicating the possible role of these pathways in BAC or other distal lung stem cell function also link the same molecules to lung tumorigenesis as did our prior studies regarding activation of oncogenic K-ras. This point further highlights the delicate balance between homeostasis, regeneration, and tumorigenesis that is likely maintained by stem cell regulation. Clearly, these studies are just the beginning of lung stem cell pathway delineation.

![Diagram of pathways in lung stem cell regulation](image)

**Fig. 2.** A model for the potential regulators of distal lung stem cell function is shown. **Top:** in normal adult lung, Clara cells, BASCs (or other putative distal lung stem cells), and AT1 and AT2 cells are quiescent. As recently shown, p38α and p18 may inhibit the proliferation of BASCs. **Middle:** in the context of lung development or injured lung, p38α function may be required to activate a pathway that drives differentiation of BASCs to produce alveolar cells including AT1 and AT2 cells. **Bottom:** several pathways have been shown to result in increased numbers of lung cells with a BAC phenotype as well as lung tumorigenesis, suggesting that pathways involving these molecules regulate distal lung cell homeostasis. p18INK4c, INK4 family cyclin-dependent kinase inhibitor; C/EBP, CCAAT/enhancer binding protein; HNF3β, hepatocyte nuclear factor.

NEXT STEPS IN UNDERSTANDING LUNG STEM CELL BIOLOGY

Further characterization of BASCs and other putative distal lung stem cells is warranted to gain insight in lung stem cell biology. Although we have demonstrated the multipotency of BASCs in culture, it remains possible that BASCs function as progenitor or differentiated cells in vivo; it will be important to define their potential in vivo (31). The lineage from long-term HSCs to specialized blood cells has been well-defined (reviewed in Ref. 51), but it remains to be determined whether a classical hierarchy of stem, progenitor, and differentiated cells exists for pulmonary epithelia. Precise lineage tracing in adult lung has only been performed for limited cell populations such as keratin-expressing tracheal cells and ciliated cells (21, 22, 49, 50). Knowing the connections between adult lung cell lineages and how they are regulated at the molecular level will be a crucial foundation for studying the mechanisms of lung disease and tumorigenesis. Finally, it will be important to determine whether there is a BAC counterpart or other stem cell populations in human lung.

Just as isolation of BASCs and other tissue-specific stem cells required technological developments, further characterization of distal lung stem cells will require several key tools. First, a transplantation assay is needed to test the function of isolated putative lung stem cells in vivo. One assay has been established for reconstitution of epithelial cells in denuded rat trachea (8), but this system likely provides a test for putative tracheal or upper airway stem cells rather than for stem cells that give rise to distal Clara cells or alveolar cells; the involved denuded trachea and subcutaneous implantation of the tracheal graft do not mimic the BADJ that is likely relevant for testing distal lung stem cell function. Second, appropriate mouse strains and genetic tools for lineage tracing in precise cell populations in adult lung are required. Lineage tracing experiments will be crucial for testing the function of stem cells without removing them from their natural surroundings and without potentially confounding factors that may limit the potential of isolated cells. Third, additional molecular markers to define BASCs and other lung cells are needed to allow these cells to be isolated with greater purity and for other cellular and genetic manipulations.

Based on data from other tissues, it is conceivable that establishment of a transplantation assay for distal lung stem cells in vivo will require 1) depletion of endogenous differentiated epithelial cells, such as Clara cells or AT2 cells, and 2) depletion of endogenous stem cells. For example, the HSC bone marrow reconstitution assay is facilitated by the fact that irradiation destroys endogenous differentiated hematopoietic cells as well as HSCs (65). The number of HSCs within the bone marrow is tightly regulated because HSCs occupy discrete locations in their microenvironment (6, 72). To allow injected HSCs to engraft, one must first create space in the microenvironment or create an “empty niche” for donor cells (11). In other systems such as the mammary gland, the endogenous epithelial cells are depleted before injection of putative stem cells to demonstrate engraftment and differentiation potential (9, 23, 59, 69). Even the tracheal grafting assay relies on freezing to kill endogenous epithelial cells to create a substrate for test cells to engraft and give rise to new cells (8). An additional requirement for a lung stem cell transplantation...
assay is that 3) the system allows for enough empty stem cell niches to allow injected stem cell engraftment while simultaneously maintaining enough lung function for survival. Genetic techniques to deplete a fraction of stem and differentiated cells may make an in vivo lung cell transplantation assay feasible (furthermore, see Ref. 29).

Lineage tracing has been performed in vivo in other tissues using several strategies and is important for future distal lung stem cell biology. Transgenic or knock-in constructs relying on stem cell-specific expression of a particular marker could make it possible to track cell fate in vivo. Limited knowledge of BASC- or other lung stem cell-specific markers precludes this approach. Many of the available lung-specific transgenic strains exhibit sporadic expression in target cells, transgene expression that significantly differs from the endogenous gene expression, limited expression or activity in adult lung cells, and/or aberration of lung function solely from transgene expression (14, 44, 54, 61). These findings underscore the importance of using an approach that relies on endogenous transcriptional regulation (e.g., knock-in alleles) rather than using transgenic strains for lineage tracing experiments. Such an approach has recently been used to label and track the fate of ciliated cells in the lung (50). Gene-specific manipulation using a combination of the currently known lung cells markers (Fig. 1) will likely provide the most plausible, immediate approach to distal lung cell lineage tracing (furthermore, see Ref. 29).

Gene expression analysis or other means of identifying new markers for lung cells will have implications for murine and human lung biology. Using BASCs as an example, currently known cell surface markers are quite limiting for identifying a counterpart in human lung; Sca-1 does not have a known functional human homolog. CD34 could be used with exclusion of CD31 and CD45 to attempt to isolate human BASCs. However, several other cell populations have been identified that share the same marker phenotype, including circulating fibrocytes (45, 56). Therefore, additional markers will likely be needed to identify BASCs in human lung.

Although previous studies identified culture conditions that support putative lung stem cell self-renewal and differentiation, future studies to more carefully define lung stem cell culture components will be important. In our studies, BASCs were cultured in DMEM with fetal bovine serum; the conditions for BSC growth could be better defined in future experiments. To enable further cell biological and biochemical analyses, enhanced expansion of BASCs (e.g., larger colony size or improved clonal colony-forming ability) would be ideal. The frequency of colony-forming cells in the BSC population did not significantly increase with passage, suggesting that the feeder culture does not completely prevent BSC differentiation, some BASCs die after passage, BASCs asymmetrically divide in this context, or some combination of these factors. Matrigel is a basement membrane preparation of extracellular matrix proteins and growth factors harvested from mouse sarcoma cells (32). It will be important to determine which components of Matrigel direct BSC differentiation. Identification of media supplements that specify BSC differentiation toward only the bronchiolar or alveolar lineages would also provide needed insight for lung cell biology.

THE IMPACT OF DISTAL LUNG STEM CELL BIOLOGY

IN THE CLINIC

We have shown that BASCs are likely to be the cells of origin in a mouse model that closely recapitulates the features of human lung adenocarcinoma (31), drawing important connections between normal lung stem cell biology and lung cancer. Further studies implicating BASCs in tumor initiation may elucidate more effective molecular markers and chemoprevention strategies for lung cancer by targeting BASCs in the early stage of malignancy. Tumor-associated BASCs may be the cells that are required for tumor progression, maintenance, and/or metastasis and thus the crucial cells to eliminate for anticancer therapy.

Distal lung stem cell biology may also provide novel approaches to therapy for devastating pulmonary diseases such as emphysema, bronchiolitis obliterans, and chronic obstructive pulmonary disease that have essentially no cure aside from lung transplant (40). Chronic lung diseases could be alleviated by directed differentiation of BASCs or other putative lung stem cells to restore defective lung epithelia. For example, this could involve using stem cells to directly replace damaged lung cells in patients or using stem cells to generate functional alveolar structures or enhanced surfactant (40). Stem cells might also be useful for delivering functional genes to other lung epithelia, such as ex vivo gene therapy of cystic fibrosis. Finally, it may be possible to elucidate ways to design drugs that stimulate a patient’s own endogenous lung stem cells to repair defective tissue.

Our limited knowledge of the functions of lung stem cells serves as a roadblock to more detailed understanding of the cellular mechanisms of lung pathologies. The new tools described above will make it possible to better characterize preclinical models of lung injury and lung disease and to determine whether epithelial stem cell function is altered in lung disease. Future work with mouse models will elucidate the connections between lung homeostasis, lung disease, and lung cancer.

CONCLUSION

Already, the identification of BASCs and other putative distal lung stem or progenitor cells has provided new avenues for research aimed to understand development, homeostasis, and disease in the distal lung. It is likely that other endogenous lung stem or progenitor cells that maintain distinct pulmonary regions will be isolated in the future. Carefully designed genetic and cell biology approaches to characterize distal lung stem cells, progenitor cells, specialized lung cells, and their respective environment(s) will provide the strong foundation needed to build the road to the future of lung stem cell biology.

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