

# Developmental Pathways and Specification of Intrapulmonary Stem Cells

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**ABSTRACT:** Tissues have the capacity to maintain a homeostatic balance between wear-and-tear and regeneration. Repair of non-lethal injury also activates cell proliferation to repopulate the injured sites with appropriate cell types and to restore function. Although controversial, the source of the material appears to be at least partly from pools of unique, multipotent stem cells that reside in specialized locations referred to as “niches.” Molecular interactions between the niche and the intracellular factors within stem cells are crucial in maintaining stem cell functions, particularly the balance between self-renewal and differentiation. Many of the mediators of the stem cell-niche interactions are similar or identical to those that control developmental pathways during organogenesis. In this review, we present a systematic discussion and evaluation of the relevant literature with a focused emphasis on three primary signaling pathways, WNT, SHH and BMP with potentially overlapping roles during both development and stem cell maintenance. (*Pediatr Res* 59: 84R–93R, 2006)

Classical observations by early embryologists on tissue regeneration in segmented worms such as polychaetes and oligochaetes suggested that fully differentiated adult tissues may harbor unique cells that retain the capacity for regeneration. Some tissues, such as the intestine, hair follicle and liver, have been more amenable to identification and characterization of these so-called resident stem cells due primarily to faster turnover rate. In other organs, including the lung, these cells have been more elusive and their contribution to regeneration in the adult more difficult to decipher. What appears certain, however, is that adult tissues have the capacity to maintain a homeostatic balance between wear-and-tear and regeneration. Furthermore, the response of tissues to nonlethal injury in most cases activates repair mechanisms that involve cell proliferation and repopulation of injured areas with appropriate cell types to restore function. Although controversial, the source of the repairing tissue is at least

partly from pools of unique cells present in various tissues whose identity can be determined by two simple criteria: 1) the capacity for self-renewal; and 2) the ability to differentiate into more than one cell type. These two criteria are commonly used to define “stem cells” in general, including those embedded within the bone marrow (BM). BM-derived stem cells recently have been a topic of special interest, particularly as far as their therapeutic potential is concerned. This review’s focus will be solely on lung-embedded stem cells. There are other highly informative recent reviews on related topics that complement the specific areas covered in this review (1,2). In addition, the hotly debated topic of BM stem cells is excellently treated by Thebaud *et al.* in the same issue of the journal.

## DEVELOPMENTAL POTENTIAL AND STEM CELLS

Stem cells are thought to be developmentally primitive and rare in number. The ability of cells to differentiate along various lines is referred to as developmental potential. Hence the zygote is developmentally totipotent since it can give rise to all cells within the embryo and the placenta (Fig. 1). Pluripotent stem cells have a more restricted developmental potential and can form only the cells found in the embryo. Further restrictions during embryonic development give rise to multipotent stem cells that form all three germ layers: endoderm, mesoderm and ectoderm. Tissue-committed stem cells, sometimes referred to as “progenitor” cells are also multipotent, but are more restricted in their differentiation potential, being capable of giving rise only to a limited subset of the cell lineages found within a given organ. Recent demonstrations of adult stem cell plasticity however, have challenged this concept (3). Thus, simply based on their developmental potential, stem cells can be divided into two major types: embryonic stem cells are derived from the inner

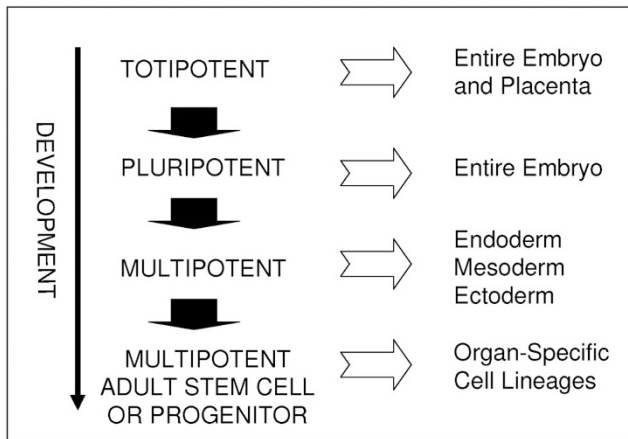
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**Abbreviations:** APC, adenomatous polyposis coli; AT1 (2), alveolar epithelial type 1 (2) cells; BADJ, bronchoalveolar duct junction; BASC, bronchoalveolar stem cell; BM, bone marrow; BMP, bone morphogenetic protein; CCSP, clara cell specific protein; GSC, germline stem cell; K5 (14), keratin 5 (14) promotor; LRC, label-retaining cell; NEB, neuroepithelial body; PNEC, pulmonary neuroendocrine cell; PTC, patched; SHH, sonic hedgehog; SP, side population; SP-C, surfactant protein C; SVZ, subventricular zone; TA, transit amplifying; WNT, wingless; vCE, variant CCSP-expressing



**Figure 1.** Simplified scheme of the relationship between development and stem cell potential.

cell mass of early blastocysts and retain the capacity to contribute to all three germ layers, whereas adult stem cells are restricted in their potential for generating various differentiated cell phenotypes.

Adult stem cells are slow-cycling undifferentiated cells (4,5). These cells can undergo asymmetric division giving rise to daughter cells, one of which can be committed to differentiation and the other retaining the renewal capacity of the original stem cells. Studies performed largely in skin and small intestine have given rise to the concept of a classical stem cell hierarchy in which adult stem cells serve as the source of transit amplifying (TA) cells that cycle more rapidly but have more limited renewal capacity and exhibit more differentiated functions. TA cells contribute much of the increase in cell number that occurs in the steady state, eventually becoming incapable of proliferation and giving rise to cells that enter the terminally differentiated compartment. As a result of their slow-cycling phenotype, stem cells have been identified by their prolonged retention of nucleotide analogs such as bromodeoxyuridine (BrdU), and accordingly are referred to as label-retaining cells (LRCs).

### THE CONCEPT OF THE NICHE

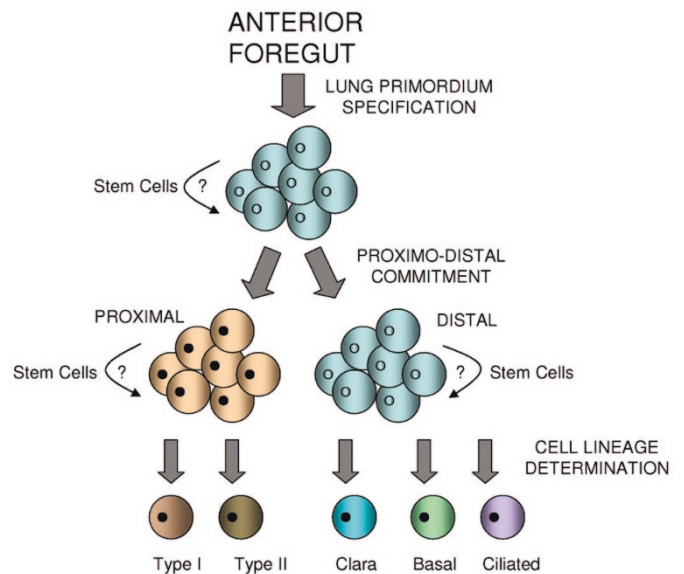
Adult somatic stem cells are thought to exist in a specialized environment called a “niche.” The niche is further thought to provide the necessary environmental signaling that interacts with intrinsic factors within stem cells that act in concert to determine “stemness.” These interactions maintain stem cells by balancing self-renewal against differentiation. The importance of this balance is well illustrated by the recognition that failure in its maintenance leads to cancer on the one hand, and organ failure on the other.

Much of the evidence for existence of a stem cell niche has come from identification of germline stem cells (GSCs) in *Drosophila* and *C. elegans* (6). In mammals, the bulk of the information has emerged from detailed studies of tissues with high rates of proliferation and turnover such as the intestine and the hair follicles. The initial identification of the niche has been in large part due to the ability of stem cells to retain mitotic labeling for prolonged periods (7,8). However, re-

cently, molecular markers such as CD34 and specific cell adhesion molecules such as  $\alpha$ -6-integrins also have been used to identify niches (9). Adhesion molecules in particular are thought to be central to the process of niche-stem cell interactions (10). It is of interest that a number of the molecules implicated in the maintenance of stem cells within the niche have demonstrated roles in lung development.

### LUNG MORPHOGENESIS AND STEM CELL BIOLOGY

The lungs are derived from at least two sources of distinct cellular origins, endoderm and mesoderm. The progenitor of the pulmonary epithelium is the endoderm that is specified within a distinct region of the anterior foregut. The molecular mechanisms for lung specification are currently not well understood. Once specified, the process does not appear to be irreversible since over-expression of a constitutively active beta-Catenin-LEF-1 fusion protein, which is known to activate the WNT canonical pathway (please see below) results in reprogramming of the lung endoderm as evidenced by expression of markers for gut endoderm, the embryonic tissue from which it is originally derived (11). The lung primordium is thought to consist of a cluster of progenitor cells from which numerous differentiated epithelial cell types in the mature lung, including, but not limited to alveolar type I, and type II, Clara, basal and ciliated cells are derived (Fig. 2). In this way, the lung primordium can be viewed as a pool of multipotent cells with the capacity to generate multiple cell lineages found in the mature organ. Whether the pool consists of a group of homogeneous multipotential cells, or cells that are already



**Figure 2.** A simplified schematic representation of lung endodermal morphogenesis. The lung primordium is specified within the anterior foregut. It is not known whether at this point the cells are developmentally equal. Stem cells may emerge at this point, but become developmentally more restricted by localization in proximal *versus* distal niches as lung morphogenesis proceeds. Proximo-distal commitment occurs early during lung development resulting in emergence of distinct cell lineages, some of which are depicted here. The possibility that distinct stem cells emerge during the proximo-distal commitment also exists.

committed along specific, albeit multiple cell lineages, remains unknown. Perl *et al.* (12) found that specification of the proximal *versus* distal lung compartments occurs very early during embryonic lung development suggesting that some differentiation occurs perhaps immediately subsequent to specification of the primordium. In the larval tracheal system of the fruit fly *Drosophila*, the entire individual organ is derived from a primordium that consists of a cluster of eighty presumably homogeneous ectodermal cells (13).

An important player in morphogenesis of the mammalian lung is the mesodermally-derived splanchnic mesenchyme. Growth and differentiation of the lung endoderm is strictly dependent on signaling from the mesenchyme. Classical recombination experiments mixing lung endoderm with distal *versus* proximal lung mesenchyme showed that the mesenchyme provides instructional cues for endodermal morphogenesis and cell lineage determination (14). It is important to note that the mesenchyme is also a source of cells contributing to a number of functional compartments in the lung including the vasculature and the airway smooth muscle. The focus of the current discussion will be on the lung endoderm about which more information is currently available.

Owing largely to analysis of various genetically manipulated mice, we now have some information about the molecules that figure importantly in lung cell proliferation and differentiation. These molecules can be grouped into three broad functional categories: 1) signaling molecules, 2) transcription factors, and 3) extracellular matrix proteins and their receptors (15–18). The signaling molecules category is most relevant to the areas of overlap between morphogenesis and stem cell biology. Of these the three signaling pathways, WNT, SHH and BMP play major roles in both processes.

**The WNT pathway.** WNTs are cysteine-rich secreted glycoproteins that are vertebrate homologues of *Drosophila* wingless. The WNT pathway is complex consisting of at least 18 ligands, and 10 receptors, known after their *Drosophila* counterparts as Frizzled (Fzd) (19). At least three intracellular pathways mediate WNT signaling (20,21). The “canonical pathway” works through stabilization of  $\beta$ -catenin. The “non-canonical,”  $\beta$ -catenin independent pathway is mediated through activation of protein kinase C (PKC) (22,23), or c-jun N-terminal kinase (JNK) (24). Two functional features of the WNT pathway are of special interest to stem cell biology. First, members of the WNT family have distinct expression patterns in the embryo and adult organisms and are critical for regulation of tissue differentiation and organogenesis ([www.stanford.edu/rnusse/Wntwindow.html](http://www.stanford.edu/rnusse/Wntwindow.html)). Second, and more importantly, WNT ligands are critical regulators of cell proliferation and some, including WNT1, WNT3a, WNT7a, and WNT8 exhibit transforming ability in mammalian cells (19). In addition, purified WNT3a protein was shown to induce self-renewal of hematopoietic stem cells (HSCs) (25) and WNT2b/WNT13 functions to maintain undifferentiated progenitor cells in ciliary marginal zone of the vertebrate retina (26). Thus, the canonical pathway appears to control stem cell activation, fate determination and differentiation.

A key factor in the canonical WNT pathway is the molecule known as adenomatous polyposis coli (APC). This

molecule was initially identified as the product of a cancer suppressor gene lost during the development of colonic carcinomas with a putative role in regulating cellular proliferation (27). APC interacts directly with microtubules to establish epithelial cell polarity during mitosis (28). In *Drosophila*, APC is an important factor in regulating and maintaining the homeostatic balance between stem cell division and commitment of daughter cells to differentiation (29). Therefore, a potential underlying cause of neoplastic transformation in epithelial carcinomas, which can arise through an imbalance between self-renewal and differentiation commitment (increased cell proliferation) may be mutations in, or aberration of APC gene expression (30). This model of carcinogenesis has been recently invoked following the identification of a bronchoalveolar stem cell (BASC) population that responds to injury and K-ras-induced oncogenesis (31). Overall, the data from various sources are consistent with a role for WNT signaling in favoring stem cell proliferation in the self-renewal/differentiation balance within the niche.

In the lung, two members of the WNT family have been shown to play key roles during embryonic development. We have shown that WNT5a controls lung branching morphogenesis and maturation by interacting with SHH and FGF10 (32,33). A critical role in regulation of mesenchymal proliferation and vascular development has been described for WNT7b in lung development (34). In addition, WNT signaling through the canonical pathway participates in morphogenesis of submucosal glands in airway epithelium (35). Activation of the canonical pathway leads to nuclear localization of  $\beta$ -catenin and its interactions with transcription factors of the TCF/LEF family, which in turn regulate target gene transcription. LEF-1 is required for development of airway submucosal glands and LEF-1 mRNA is expressed in the glandular buds at early stages of development (35). In addition, the LEF-1 promoter can be induced by Wnt3a and a Wnt-responsive element in the LEF-1 promoter is required for LEF-1 expression in submucosal gland buds (36,37). Given that submucosal gland duct cells have been identified as potential stem cells for regeneration of airway surface epithelium as well as submucosal glands (see below), these findings suggest a potential role for involvement of the WNT/ $\beta$ -catenin/LEF/TCF pathway in stem cell maintenance in the airway epithelium.

**The sonic hedgehog pathway.** Both WNT and Sonic hedgehog (SHH) are lipid-modified proteins and their signaling mechanisms at the cell surface show many similarities (38). SHH is the mammalian ortholog of *Drosophila* Hedgehog (HH) and is highly expressed in the developing pulmonary epithelium (39). SHH signaling is mediated through its receptor Patched (PTC), a twelve transmembrane protein, and is transduced to changes in target gene regulation via the activity of the transcription factors Gli (40,41). The role of SHH signaling in morphogenesis of the lung as well as in stem cell biology has been a target of investigation. During lung development, SHH is primarily made by the epithelium. PTC, on the other hand is expressed abundantly on the mesenchyme and receives and implements SHH signaling to pattern branching morphogenesis (39). Deletion of SHH signaling in the lung results in gross abnormalities with little evidence of

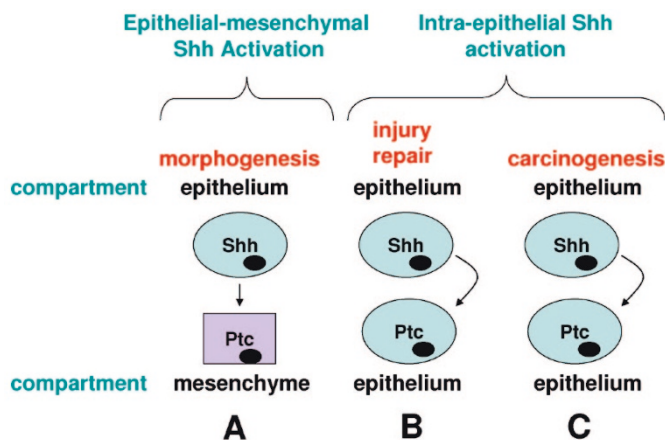
cellular differentiation. In contrast, overexpression of SHH causes increased proliferation and perturbation of cellular differentiation. Based on the latter properties, SHH may be a strong candidate as a potential player in lung stem cell maintenance. In support of this model, transient activation of SHH was documented in the airway epithelium of adult lung during repopulation of injured airways in response to acute proximal injury (42). The latter observation suggests, but does not prove that activation of SHH may drive epithelial cell proliferation during repair of injury. Aberrant activation of SHH pathway, in which subverted stem cells express both SHH and PTC and thus escape the control mechanisms of the niche, has been proposed as a novel mechanism for pulmonary carcinogenesis (43). It is noteworthy that persistent activation of SHH signaling has been found in a subset of small cell lung carcinomas, consisting of neuroendocrine cells (42). The SHH pathway is active in the progenitors of neuroendocrine cells during lung development (42). Thus, it is tempting to speculate that uncontrolled growth of tumor cells may be explained by persistent activation of a signaling pathway such as SHH that would otherwise maintain the balance between proliferation and differentiation of stem cells within a niche (Fig. 3).

In both *Drosophila* and vertebrates, regulation of WNT and Hedgehog are linked. In *Drosophila*, the two signaling pathways interact during gastrulation and germ band extension. Hedgehog stimulates WNT in the adjacent cells and WNT functions to maintain engrailed (*en*), which controls the expression of Hedgehog (41,44,45). In vertebrates, WNT2 expression appears to be dependent on SHH since it is decreased in *Shh(-/-)* lungs (46). We have found that SHH is negatively regulated in the lung by WNT5a, a noncanonical Wnt (47) that antagonizes the activity of canonical WNTs such as WNT1 and WNT3a (48,49). Absence of WNT5a results in increased expression of SHH and PTC (32), and overexpression of WNT5a leads to decreased expression of SHH

and PTC (33). Importantly, interaction between WNT and SHH plays a critical role in regulating progenitor cell behavior in tissues such as neural subventricular zone (SVZ) (50).

**The bone morphogenetic protein pathway.** Bone morphogenetic proteins (BMPs) are multifunctional signaling molecules in the TGF- $\beta$  superfamily that regulate a vast array of cellular processes, including proliferation, differentiation, apoptosis, adhesion, and migration (51). As other members of the TGF- $\beta$  pathway, BMPs act through cell surface receptors to activate a family of transcription factors known as SMADs. BMPs play key roles during embryonic development and remodeling and repair of tissue, wound healing, and inflammatory responses (52). Stem cell behavior within the niche is known to be regulated by BMPs. The most direct evidence for involvement of BMP signaling in regulation of self-renewal and differentiation of stem cells within a niche comes from the studies on *Drosophila* ovaries (53). In this system, the niche provides BMP signaling for maintenance of GSCs. The product of the gene *Bam*, on the other hand promotes differentiation of GSCs. Thus, it is likely that functional and opposing interactions between *Bam* and *Bmp* may constitute the molecular basis for maintenance of homeostasis within the ovarian niche. In support of this hypothesis, BMP signaling was found to directly repress *Bam* transcription in GSCs. These observations demonstrate that niche signaling directly represses differentiation-promoting genes in stem cells to maintain stem cell self-renewal status (53). In mammals, the critical role of BMP signaling has been demonstrated (54). In the adult mouse SVZ, large numbers of new neurons are born continuously. BMPs potentially inhibit neurogenesis both *in vitro* and *in vivo*. The BMP antagonist Noggin is expressed by ependymal cells adjacent to the SVZ. Noggin promotes the creation of a neurogenic environment by blocking endogenous BMP signaling (54). Coincident with the roles of BMP and WNT signaling in stem cell biology, these two pathways are functionally closely related. Not only is WNT signaling required for activation of BMP4 (55,56), WNT and BMP4 can synergistically activate target genes such as *Msx1* (57). Furthermore, mediators of WNT signaling, including beta-catenin and LEF-1/Tcf transcription regulators, physically and functionally interact with Smad4, a downstream component of TGF- $\beta$  superfamily pathway, and synergistically regulate expression of genes during formation of Spemann's organizer in *Xenopus* (58).

In the lung, although no direct evidence for the involvement of BMP signaling in the maintenance of lung stem cells is currently available, its expression in both mesenchymal and epithelial compartments suggests that this signaling pathway may indeed be critical for maintenance and activation of stem cells within pulmonary niches. BMP-5 & BMP7 are expressed ubiquitously in the mesenchyme and epithelium of the lung respectively (59). BMP-4 is confined to the lung epithelium and is a major participant in lung distal morphogenesis. Targeted disruption of BMP-4 results in embryonic lethality before E10, indicating the essential role of this signaling molecule in early embryogenesis (60,61). Lung-specific suppression of BMP-4, through over-expression of noggin, a secreted BMP-4 antagonist, or a dominant negative form of



**Figure 3.** SHH pathway is implicated in stem cell maintenance and carcinogenesis. During morphogenesis, epithelial-mesenchymal interactions occur through paracrine SHH signaling between the two tissues, or compartments (A). In response to injury, intra-epithelial activation of SHH pathway was observed, suggesting that epithelial-epithelial paracrine signaling may underlie the mechanisms of cellular repopulation in injured airways (B). Small cell lung cancer also manifests paracrine SHH signaling through expression of both ligand and receptor (C), wherein there is distinct compartmentalization of signaling and receiving cells, similar to development (A) and repair (B).

the BMP-4 type I receptor, Alk6, does not alter the differentiation of proximal lung cells as evidenced by expression of Clara cell specific markers, but reduces the number of differentiated alveolar type II (AT2) cells (62). Therefore, if BMP-4 figures importantly in lung stem cell maintenance and specification of the environment of the niche, its effect may be confined to distal lung.

### INTRAPULMONARY STEM CELLS

**Proximal versus distal niches.** In the mature lung, there are estimates for the existence of over 40 distinct cell types organized into distinct anatomical regions along the proximo-distal axis of the lung. The epithelium of the conducting airways is composed of ciliated, secretory, basal and other specialized cell types (63,64). Quite distinct from these cells are the epithelial cells lining the alveolar compartments in the distal lung, such as alveolar epithelial type II (AT2) and type I (AT1) cells. As discussed above, it is generally believed that resident tissue-specific stem cells become established during embryogenesis and remain important for repair of self-renewing tissues in the adult. However, the anatomic and histologic complexity of the lung and cellular quiescence in the basal state together with a paucity of specific markers for definitive identification of a stem cell phenotype have considerably impeded characterization of endogenous lung stem cells. Differences in the regional composition of airway cell populations between rodents and humans have further complicated this issue and our understanding of the role of resident lung stem cells in tissue maintenance and repair remains rudimentary.

Using the BrdU labeling approach to identify stem cells, as well as more recently developed surface markers, putative stem cells have been localized to specific sites (niches) throughout the conducting airways and distal lung epithelium (3,65,66). An important question relates to whether there are distinct stem cells embedded within the proximal *versus* distal compartments of the lung. Evidence suggests that multipotent progenitors of the conducting airways (proximal) and gas-

exchange regions (distal) of the lung are derived from distinct populations of cells whose lineage fate is determined relatively early during development (12). Consistent with early specification of either a proximal or distal fate, no single stem cell that can give rise to all lung epithelial cell types has been identified in the postnatal lung. However, a possible exception may be a recently identified population of BASCs discussed below that co-express markers of both proximal and distal lineages although their differentiation potential *in vivo* remains to be determined (31). In the adult lung, there appear to be distinct niches in the proximal and distal conducting airways harboring epithelial progenitor cells with different proliferative potential (Table 1).

**The tracheobronchial niches.** The major cell types constituting the pseudostratified epithelium of the tracheobronchial airways are basal cells, mucous secretory (goblet) cells and ciliated cells. In the proximal conducting airways of the mouse, cells that reside in the ducts of submucosal glands, basal cells and nonciliated secretory cells have been shown to proliferate and to contribute to repopulation of the airway epithelium (67–70). However, there has been lack of consensus on the exact differentiation capacity of each cell type and the degree to which each cell type is capable only of self-renewal (69,71). Several early studies suggested that basal cells function as multipotent progenitors capable of giving rise to all major tracheal epithelial cell types (68,69,72–75). However, following mild NO<sub>2</sub> injury basal cell proliferation was not significantly increased and regeneration of airway epithelium was carried out by secretory cells (70). Clonal analysis of retrovirally-tagged airway surface epithelial cells identified multiple progenitors with either limited or multipotent differentiation capacity (76). The type and degree of airway injury therefore seems to be an important determinant of specific activation of progenitor cell populations. Furthermore, the specific criteria used to determine stemness are key to determining whether cells participating in airway regeneration are truly stem cells capable of giving rise to all cell types, or

**Table 1.** Putative stem cell niches in mouse lung

Location	Cell type	Criteria for 'stemness'
Tracheobronchial	Submucosal gland ducts (proximal trachea)	Label retaining cells <i>in vivo</i> [77]
	Foci at cartilage-intercartilaginous junction (distal trachea)	Label retaining cells <i>in vivo</i> [77]
	Basal cells	Multipotent differentiation of K14 expressing basal cells following Clara cell ablation <i>in vivo</i> [79,84] Clonogenicity of K5 expressing basal cells <i>in vitro</i> [78]
Bronchiolar	Neuroepithelial body-associated variant CCSP expressing cells	Lack of airway regeneration following depletion of CCSP-expressing cells [84]
	Bronchioalveolar duct junction-associated vCE cells Bronchioalveolar stem cells*	Airway regeneration following Clara cell ablation with naphthalene [85] CCSP-SPC +ve cells proliferate <i>in vivo</i> , surface markers (Sca-1, CD34), clonality, multilineage differentiation <i>in vitro</i> [31]
Alveolar	? Subsets of type II cells <i>versus</i> unidentified multipotent progenitor**	Proliferation and differentiation to type I cells <i>in vitro</i> and <i>in vivo</i>
	? BASCs	CCSP-SPC +ve cells proliferate <i>in vivo</i> , surface markers (Sca-1, CD34), clonality, multilineage differentiation <i>in vitro</i> [31]

BASC, Bronchioalveolar stem cells; vCE:variant, CCSP expressing cells.

\* Although identified by different means, BASCs may be the same as vCE at BADJ.

\*\* It is not clear if type II cells are true stem cells or function as a transit amplifying (TA) population.

whether they are progenitors with more limited differentiation potential more closely resembling TA cells.

Polidocanol or SO<sub>2</sub> can be used in an injury model to stimulate cell division in the normally quiescent tracheal epithelium of mice. Using this approach, Borthwick *et al.* (77) identified niches of keratin-rich LRCs in the ducts of submucosal glands in the upper trachea and at foci near the cartilage-intercartilage junctions in association with neuroepithelial bodies (NEBs). The ratio of basal to columnar LRCs in the distal trachea was 2:1. Tracheal xenografts in which surface epithelium was removed by protease digestion could be re-epithelialized from submucosal gland remnants following transplantation into nude mice, supporting the notion that submucosal gland ducts may serve as a stem cell niche. LRCs did not express the neuroendocrine marker calcitonin gene-regulated peptide (CGRP) but small numbers of pulmonary neuroendocrine cells (PNECs) localized to LRC niches suggesting that PNECs, while not themselves precursors for regeneration of airway epithelium, may contribute to the niche microenvironment. These results are consistent with previous studies using retroviral lineage tracing in adult human proximal airway in which a subset of pluripotent cells was identified with the capacity for both glandular and surface airway epithelial differentiation, although the identity of the putative stem cells, tagged by this approach could not be determined (76).

To examine the clonal potential of tracheal basal cells, the keratin 5 (K5) promoter, which is active in niches of LRC in mouse trachea, was used to drive expression of enhanced green fluorescence protein (EGFP) specifically in a subset of basal cells in transgenic mice, corresponding generally to the pattern of LRCs observed in a previous study (78). Examination of sorted populations identified 4.5-fold greater colony forming efficiency in EGFP-positive cells (*i.e.* within the basal cell compartment) than within the EGFP-negative fraction. A small number of cells that were highly enriched for K5 were able to give rise to large colonies. Examination of colonies revealed expression of Clara cell specific protein (CCSP) and  $\beta$ -tubulin 4 (ciliated cell marker) consistent with differentiation to both secretory and ciliated cell types respectively, indicating clonogenicity and multi-lineage differentiation capacity of this subset of basal cells. The subset of K5-expressing cells capable of generating large colonies may represent a population of airway stem cells; however, whether these K5-expressing cells absolutely co-localize with the LRC compartment or represent part of the TA population has not been definitively established.

Lineage tagging using a Cre-loxP reporter approach was used in conjunction with *in vivo* Clara cell depletion to investigate repair of injured mouse tracheal epithelium and address specifically the contribution of basal cells to tracheal regeneration (79). Cytokeratin 14 (K14) is highly expressed within a subpopulation of tracheal basal cells. The K14 promoter was therefore used to drive expression of Cre, which, following recombination, led to expression of LacZ in a subset of tracheal basal cells. Subsets of K14-expressing cells were capable of multipotent or unipotent differentiation *in vivo*, giving rise to basal, ciliated and Clara cells or only basal cells,

respectively. In a separate study using a similar approach, basal cells were identified as being capable of multipotent progenitor repopulation of bronchial epithelium (79). The airway epithelium has a high degree of plasticity which could account for previous observations from this group and others demonstrating that both basal and nonbasal cells could regenerate a complete mucociliary epithelium (70,71). The relative contributions of basal cells *versus* submucosal gland cells under different conditions of injury remains to be absolutely determined. The ability to track the fate of specific progenitor cells using cell-specific promoters to permanently label cells as well as development of better specific surface markers for the stem cell phenotype will be key to determining the differentiation potential of putative regional stem cells.

**The bronchiolar niches.** The terminal bronchioles are lined with simple epithelium composed of ciliated and non-ciliated cells which include Clara cells (64). Clara cells appear to be capable of self-renewal and of differentiation into ciliated cells, functioning as the major TA population within the bronchiolar epithelium after oxidant-mediated damage (70,80,81). Clara cells have the capacity to metabolize lipophilic compounds through cytochrome P450-mediated oxidation, making them susceptible to injury by such compounds (82). Depletion of Clara cells by administration of the Clara cell-specific cytotoxicant, naphthalene, fails to diminish the regenerative potential of terminal bronchioles indicating the presence of pollutant resistant stem cells that are able to participate in airway regeneration after TA cell depletion (83).

In the bronchiolar epithelium, the NEB has been identified as a unique microenvironment harboring progenitor cells capable of restoring airway epithelium after injury of pollutant-sensitive Clara cells (83). Consistent with the existence of a stem cell pool in the NEB microenvironment, Reynolds *et al.* (83) demonstrated that depletion of the TA (Clara cell) pool with naphthalene results in activation of two proliferative populations within the NEB microenvironment, namely PNECs and CCSP-IR Clara cells. Subsets of CCSP-expressing cells located within NEBs appear to have different characteristics from classical Clara cells in that they express lower levels of CCSP, are deficient in the phase I xenobiotic enzyme CYP-2F2, and appear to be relatively resistant to naphthalene suggesting that they may represent a stem cell pool (83). This resistant population of cells has been termed variant CCSP-expressing (vCE) cells. The relative contributions of PNEC and CCSP-expressing cells to epithelial regeneration was further investigated by ablation of CCSP-expressing cells using a model in which the CCSP promoter drives expression of the herpes simplex virus thymidine kinase (84). Ablation of CCSP-expressing cells by administration of gancyclovir led to failure of regeneration of the airway epithelium despite proliferation/hyperplasia of PNECs indicating a requirement of naphthalene resistant vCE cells for regeneration of airway epithelium after TA cell depletion. These studies indicate that although PNECs proliferate in response to naphthalene, they are not sufficient for regeneration of airway epithelium. Similar pollutant-resistant vCE have been identified in terminal bronchioles associated with the bronchoalveolar duct junction (BADJ) (85). Despite the identification within bronchiolar

epithelium of these two niches of naphthalene-resistant vCE cells, the complexity of this issue is highlighted by the recent suggestion that ciliated cells, previously considered to be terminally differentiated, are able to undergo squamous metaplasia and re-differentiate into cuboidal and columnar cell types to restore the bronchiolar epithelium following naphthalene injury (86). Although not definitively proven by lineage tagging of ciliated cells, these results illustrate the difficulty in distinguishing the relative contributions of stem cells *versus* progenitors with more limited differentiation capacity to airway regeneration under different conditions of injury.

A population of cells that co-express CCSP and surfactant protein C (SP-C), markers of Clara cells and AT2 cells, respectively, and originally identified in K-ras-expressing lung tumors was recently identified at the BADJ, a putative stem cell niche in normal lung (31). These cells were resistant to bronchiolar and alveolar damage induced by naphthalene and bleomycin respectively, proliferated during epithelial cell renewal *in vivo*, underwent clonal colony formation, exhibited extensive self-renewal in culture and were multipotent in clonal assays. When grown on Matrigel, colonies gave rise to cells expressing CCSP, SP-C and aquaporin-5 (a marker of AT1 cells). Although difficult to identify morphologically as Clara or AT2 cells, these findings suggested that these double-positive cells may represent a stem cell population for Clara cells and alveolar epithelial cells. Based on these properties, the cells were named BASCs. By FACS analysis, CCSP+ SP-C+ BASCs were identified within the *ScaI*<sup>+</sup> CD45<sup>-</sup> PECAM<sup>-</sup> population. Within the *ScaI*<sup>+</sup> CD45<sup>-</sup> PECAM<sup>-</sup> population, the CD34<sup>+</sup> population was enriched for CCSP+ SP-C+ positive cells. Further immunostaining confirmed *ScaI*<sup>+</sup> CCSP+ CD34<sup>+</sup> cells that were CD45<sup>-</sup> exclusively at the BADJ. The observation that these cells expanded in response to oncogenic K-ras *in vitro* and in precursors of lung tumors *in vivo*, suggested this stem cell population may also be putative precursor cells for adenocarcinomas demonstrating the importance of the appropriate balance between differentiation and proliferation for maintaining the normal composition of airway cell populations. Based on their location and expression of CCSP, these cells likely represent the same population as vCE identified previously at the BADJ (87).

**The alveolar niches.** The alveolar epithelium consists of two morphologically distinct cell types, AT1 and AT2 cells (88,89). AT2 cells are cuboidal and surfactant-producing whereas AT1 cells are larger squamous cells with long cytoplasmic processes that cover the majority of the gas exchange surface of the lung (90,91). AT2 cells are capable of self-renewal and have been thought to serve as progenitors for repair of distal lung epithelium during normal maintenance and repair following injury, whereas AT1 cells *in vivo* have been considered to be terminally differentiated (92,93). Studies of oxygen toxicity in monkeys led to the suggestion that AT2 cells may serve as progenitors for replacement of AT1 cells (94). The concept of the AT2 cells as progenitor was further investigated by serial analyses of thymidine incorporation using light and electron microscopy combined with autoradiographic techniques following exposure to NO<sub>2</sub> (92,95). Tritiated thymidine first appeared in AT2 cells fol-

lowed by appearance of label in AT1 cells. Labeled cells with an intermediate phenotype also were identified suggesting that AT2 cells served as the progenitors for AT1 cells. Similar studies following oxygen exposure in mice (93) also suggested transition from AT2 to AT1 cell following injury. Whether this transition can occur *in vivo* without an intervening cell division has not been determined. The differentiated characteristics of AT2 cells together with rapid proliferation following injury suggests that AT2 cells more closely resemble those of a TA cell than a true stem cell population. Subpopulations of AT2 cells that are more resistant to injury have been identified (96). The possible existence of other as yet unidentified, undifferentiated distal lung cells that can give rise to either or both AT1 and AT2 cells has not been ruled out, while the relative contribution of BASCs to differentiated alveolar epithelium *in vivo* remains to be determined.

Similar to observations *in vivo*, isolated AT2 cells in primary culture lose their phenotypic hallmarks and gradually acquire the morphologic features of AT1 cells (97,98). The cells increasingly express all available AT1 cell phenotypic markers suggesting that AT2 cells *in vitro* transdifferentiate toward an AT1 cell phenotype (AT1-like cells) (99–101). Experimental conditions have been developed that promote retention of the AT2 cell phenotype (*e.g.*, culture with rat serum, with keratinocyte growth factor or on contracted gels) (99,100,102). Importantly, cells that have transdifferentiated toward an AT1 cell-like phenotype can be induced to re-express the phenotypic characteristics of AT2 cells, suggesting far greater plasticity in expression of the differentiated alveolar epithelial cell phenotypes than believed from previous *in vivo* studies. Whether AT1 cells *in vivo* are capable of such reversible transdifferentiation, perhaps enabling them to contribute to the proliferative pool of progenitors following injury has not yet been determined.

**Side populations.** Side population (SP) cells are rare cells defined by their ability to exclude Hoechst 33342 dye via the action of cell-surface-localized ATP binding cassette (ABC) half-transporter breast cancer resistance protein (BRCP1), and are highly enriched for stem cell activity (103,104). BRCP1 (-/-) mice have a normal number of HSCs indicating that while BRCP1 is a marker of SP cells, it is not required for stem cell function (105). BM SP cells express the stem cell markers Sca-1 and c-kit, and lack expression of mature hematopoietic lineage markers (Lin<sup>-</sup>). BM-derived SP reconstitute lethally irradiated mice in smaller numbers than whole BM and may also have the capacity to reconstitute nonhematopoietic tissues (*e.g.* skeletal muscle, cardiac muscle, vascular endothelium and liver (106,107). The property of Hoechst efflux (SP phenotype) has been used in an attempt to identify stem cells from organs other than BM. Using this approach, SP cells have been identified in a variety of nonhematopoietic tissues such as skeletal muscle, liver, kidney and lung and share phenotypic characteristics with BM SP cells (108,109). In contrast to BM SP, tissue SP are characterized by heterogeneity in expression of CD45 which varies between tissues. Asakura and Rudnicki (110) first demonstrated that whole lung tissue gave rise to hematopoietic colonies and determined that the hematopoietic ability resided in the CD45<sup>+</sup> fraction

but only gave rise to granulocytic and monocytic colonies. Summer *et al.* (111) demonstrated that mouse lung SP cells constitute 0.03–0.07% of total lung cells and characterized their surface phenotype as *Sca1*<sup>+</sup>, *BRCPI*<sup>+</sup>, *Lin*<sup>-</sup> and heterogeneous at the *CD45* locus (75% *CD45*-positive). However, SP cells could not be localized by immunostaining for *BRCPI* because of the rarity of the SP cells as well as the lack of correlation of expression with function. Lung SP cells express hepatocyte nuclear factor-3 $\beta$  (*HNF-3 $\beta$* ) but not *NKX2.1*, two critical transcription factors in the lung epithelium. To further define the origin of lung SP cells, transplantation studies were performed in which wild type BM was transplanted into *BRCPI*-deficient mice (which do not have an identifiable SP population) or GFP-expressing BM or SP cells were transplanted into wild-type mice. 6–8 mo. post-transplantation SP were identified in similar numbers as in wild type animals. Both *CD45*<sup>+</sup> and *CD45*<sup>-</sup> cells were present in the SP gate suggesting that lung SP are BM-derived (112). There was heterogeneity in expression of *SMA* and cytokeratin suggestive of subpopulations of cells with differing potential for differentiation. *CD45*<sup>+</sup> lung SP cells express *PU.1* a transcription factor characteristic of myeloid progenitors, suggesting that the *CD45*<sup>+</sup> fraction serves as local progenitors for myeloid cells in the lung. The fate of tissue *CD45*<sup>-</sup> SP cells is less clear although recent gene profiling studies of embryonic *CD45*<sup>-</sup> SP cells identified several genes associated with an endothelial phenotype consistent with a role in vascular development (113). Giangreco and colleagues (87) evaluated SP from elastase digested lungs which were enriched for cells expressing airway-specific markers. In contrast to the findings of Summer *et al.* (112), *Sca-1* reactivity was enriched among *CD45*<sup>-</sup> SP cells and was not unique to SP cells. Furthermore, *CD45*<sup>-</sup> SP cells included cells expressing vimentin and *CCSP*, consistent with the presence of mesenchymal cells and conducting airway cells in this fraction. Presence of *CCSP*, coupled with absence of *CYP450-2F2*, suggests enrichment within this SP for vCE identified by other methods as bronchiolar stem cells as discussed above. The apparent discrepancies among the studies may reflect differences in methods used for enzymatic dissociation of lung tissue which likely sampled different cellular compartments, as well as variability in FACS analysis. Further characterization of these SP populations using standardized approaches for isolation and sorting is needed to understand their exact differentiation potential and the functional contribution of this heterogeneous population to tissue homeostasis and repair following lung injury.

### THERAPEUTIC POTENTIAL OF ADULT STEM CELLS

The progressive nature of a number of pediatric and adult lung diseases (*e.g.* pulmonary fibrosis, emphysema, acute respiratory distress syndrome and bronchopulmonary dysplasia) suggests an inadequate response of normal regenerative pathways following lung injury. It has been suggested that such diseases reflect a stem cell failure possibly due to either absolute loss of stem cells or disruption of the niche or both. Several putative stem cells located in specific niches have

been identified within the conducting airways and distal lung epithelium. However, little is known of their molecular phenotype or the factors that regulate their properties. Characterization of somatic stem cell populations within the lung and the factors that determine stemness will be critical to augmenting their proliferative potential and harnessing their capacity for differentiation along specific lineages to improve tissue regeneration. In particular, identification of key signals involved in maintenance of the stem cell niche as discussed above may allow modulation of the local environment to favor stem cell survival and regeneration. Obviously, any therapeutic approach will have to be balanced by the potential for unlimited proliferation and malignant transformation. In addition to the possibility of manipulating endogenous stem cells to augment local repair, intrapulmonary stem cells may be amenable to manipulation for treatment of genetic diseases (*e.g.* cystic fibrosis). Although these possible applications are intriguing, the true potential of endogenous stem cells as therapeutic targets remains to be determined.

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