Chapter 3
Stem Cell Niches in the Lung

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Abbreviations

AEC I/II	Alveolar epithelial cell I/II
AF	Autofluorescence
ALDH1	Aldehyde dehydrogenase 1 family
AQP3	Aquaporin 3
BADJ	Bronchioalveolar duct junction
BASC	Bronchioalveolar stem cell
CCSP	Club cell secretory protein
CD16	Fc receptor, IgG, low affinity III
CD24	CD24 molecule
CD31	Platelet/Endothelial cell adhesion molecule 1 (aka PECAM1)
CD32	Fc receptor, IgG, low affinity IIb
CD34	CD34 antigen
CD45	Protein tyrosine phosphatase, receptor type, C (aka Ptprc)
CD73	5′ Nucleotidase, Ecto (aka Nt5e)

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CD151	CD151 antigen
CDH1	Cadherin 1, type 1, E-cadherin (aka E-Cad)
CFTR	Cystic fibrosis transmembrane conductance regulator
CGRP	Calcitonin gene-related peptide
CK	Cytokeratin
CSC	Cancer stem cell
EpCAM	Epithelial cell adhesion molecule
F3	Tissue factor (aka TF)
FGF	Fibroblast growth factor
GSI-A_3B	*Griffonia simplicifolia* isolectin A_3B
H33342	Hoechst 33342
ITGA6	Integrin alpha 6 (aka CD49f)
ITGB4	Integrin beta 4 (aka CD104)
LGR6	Leucine-rich repeat containing g protein-coupled receptor 6
LRC	Label retaining cell
Ly6a	Lymphocyte antigen 6 complex, locus A (aka Sca-1)
Ly76	Lymphocyte antigen 76 (aka TER119)
lrMSC	Lung-resident mesenchymal stromal cell
MSC	Mesenchymal stromal cell
NEB	Neuroendocrine body
NGFR	Nerve growth factor receptor
NSCLC	Non-small cell lung cancer
PDGFRα	Platelet-derived growth factor receptor alpha
PNEC	Pulmonary neuroendocrine cell
SAE	Surface airway epithelium
SCLC	Small cell lung cancer
SMG	Submucosal gland
SPC	Surfactant protein C (aka Sftpc)
TGFβ	Transforming growth factor beta
TROP2	Tumor-associated calcium signal transducer 2 (aka Tacstd2)
TTF1	Thyroid transcription factor 1 (aka NKx2.1)

3.1 Introduction

The epithelium of the conducting and respiratory airways in the adult lung is composed of numerous phenotypically distinct epithelial cell types tailored to perform region-specific functions. Because the lung is exposed to the external environment and to inhaled pathogens, its airways must have a rapid capacity to regenerate if injured; this is essential to preserving an epithelial barrier and normal lung functions. Both during injury repair and in the context of homeostatic turnover, cell regeneration depends on various types of stem/progenitor cells that are positioned throughout the pulmonary tree (Borthwick et al. 2001; Hong et al. 2001; Kim et al. 2005; Liu et al. 2006; Liu and Engelhardt 2008; Rawlins et al. 2009b; Reynolds and Malkinson 2010; Rock and Hogan 2011).

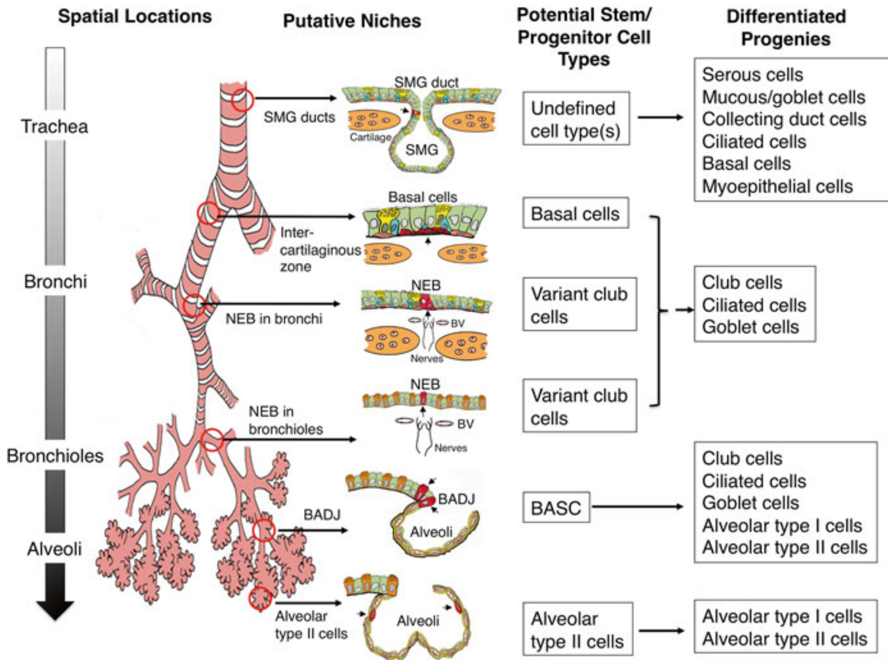

Fig. 3.1 Illustration of potential stem/progenitor cell niches in the lung of the adult mouse. The lung can be divided into three major levels of conducting airways (the trachea, bronchi, and bronchioles) plus the gas-exchanging alveoli. Distinct region-specific stem/progenitor cell niches are thought to exist along the proximal-distal axis of the airway. These include: SMG ducts in the proximal trachea, basal cells within intercartilaginous zones of the trachea and primary bronchi, NEBs in the intralobar bronchi and bronchioles, and the BADJ and alveolar spaces within the alveoli. Progenitor/stem cells (marked in *red* and *listed*) reside in their respective local niches and these environments enable them to maintain their stem/progenitor properties and control their ability to differentiate into various progeny cell types. *SMG* submucosal gland, *NEB* neuroepithelial body, *BADJ* bronchioalveolar duct junction, *BV* blood vessel

As in other adult tissues and organs, the stem/progenitor cells of the adult lung are undifferentiated cells and have the capacity to remain multipotent, self-renew, and produce differentiated progeny present in the physiological domain in which they reside. Throughout the airway tree, several distinct cell types carry out local repair in response to injury (Fig. 3.1). In mice, such cells include a subset of basal cells (within the proximal airway) (Rock et al. 2009, 2010; Cole et al. 2010; Hajj et al. 2007; Hong et al. 2004a), basal-like cells within the ducts of SMGs (Borthwick et al. 2001; Engelhardt 2001; Engelhardt et al. 1995; Hegab et al. 2011, 2012b; Xie et al. 2011; Lynch and Engelhardt 2014), a subset of naphthalene-resistant variant club cells (within the NEBs of the bronchi and bronchioles) (Guha et al. 2012; Hong et al. 2001; Reynolds et al. 2000a; Xing et al. 2012; Reynolds and Malkinson 2010), a subset of SPC expressing club cells at the BADJ (Giangreco et al. 2002; Kim et al. 2005; Rawlins et al. 2009b; Zheng et al. 2013), and a subset of alveolar type II cells (Barkauskas et al. 2013; Fujino et al. 2011).

Studies using murine models have revealed several region-specific stem cell niches along the proximal-distal axis of the airway that maintain distinct subpopulations of progenitors. Stem/progenitor cells are mobilized from these epithelial niches to maintain tissue homeostasis during injury repair and normal cellular turnover. The coordination of molecular and cellular events in the microenvironment of stem cell niches plays a pivotal role in maintaining the balance of stem/progenitors and differentiated cells that are needed for regeneration in the lung (Fig. 3.1). In this chapter, we review the diversity of cell types, including potential stem/progenitor cells, that have been identified in the adult lung, and discuss advances in our understanding of stem/progenitor cell niches and their roles in injury repair and lung cancer.

3.2 Cellular Diversity in the Adult Lung

Based on its anatomical and functional features, the lung epithelium can be divided into three domains: the proximal cartilaginous airways (trachea and bronchi), the bronchioles (bronchioles, terminal bronchioles, and respiratory bronchioles), and the alveoli. The epithelial cell types in each of these domains are distinguished by their morphology, cellular phenotype (i.e., proteins they express), and function. The proximal airway of the mouse is lined with a pseudostratified columnar epithelium composed mainly of basal, club, goblet, and ciliated cells; the secretory SMGs reside beneath this surface airway epithelium (SAE) and are limited to the proximal trachea in mice (Hansell and Moretti 1969; Pack et al. 1980; Widdicombe et al. 2001; Jeffery 1983; Liu et al. 2006). The major cell types in the human proximal airway differ slightly from those in mice and include basal, intermediate, goblet, non-ciliated columnar, and ciliated cells (Jeffery 1983; Liu et al. 2006; Mercer et al. 1994). Furthermore, in humans the SMGs are present throughout the cartilaginous airways, including the trachea and bronchi. These glands are composed of an interconnecting network of serous acini and mucus tubules, which secrete antibacterial factors, mucous, and fluid into the airway lumen (Wine and Joo 2004). In the distal mouse and human airways (i.e., bronchioles), club, ciliated, neuroendocrine, and goblet cells are the major cell types, and neuroendocrine cells are found both individually and in clusters within NEBs (Mercer et al. 1994; Van Lommel et al. 1999; Plopper et al. 1980; Liu et al. 2006). However, the bronchioles of human lungs have also been shown to contain basal cells, albeit at lower abundance than in the proximal regions (Tamai 1983; Rock et al. 2010). The alveolar epithelium is lined by surfactant-producing cuboidal alveolar type II epithelial cells (AECII) and squamous gas-exchanging alveolar type I epithelial cells (AECI) (Liu et al. 2006). The major epithelial cell types that are present at various locations throughout the airway are listed in Table 3.1.

Table 3.1 Major epithelial cell types in the lungs of adult mice and humans

Cell types	Cell-type markers	Stem/ Progenitor cells	Lineage cell type(s)	Candidate niches	Reference(s)
Basal cells	Cytokeratin 5, Cytokeratin 14, P63, NGFR, ITGA6	Yes	Basal, club, goblet, and ciliated cells	Intercartilaginous zones and SMGs	Hong et al. (2004b), Hajj et al. (2007), Hong et al. (2004a), Rock et al. (2009), Schoch et al. (2004), Cole et al. (2010), Engelhardt et al. (1995), Xie et al. (2011), Borthwick et al. (2001), Hegab et al. (2011, 2012b)
Intermediate cells	Cytokeratin 5, Cytokeratin 14, P63	Yes	Goblet and ciliated cells	Undefined (transient amplifying progenitor)	Engelhardt et al. (1995), Evans et al. (1986)
Club cells (conducting airways)	CCSP (Scgb1a1)	Yes	Club, basal[a], goblet, and ciliated cells	NEB	Hong et al. (2001), Boers et al. (1999), Rawlins et al. (2009b), Reynolds et al. (2000b), Reynolds et al. (2000a), Tata et al. (2013)
Club cells (BADJ)	CCSP (Scgb1a1), SPC	Yes	Club, AECII, and AEC1 cells	BADJ	Zacharek et al. (2011), Kim et al. (2005), Regala et al. (2009), Tropea et al. (2012), Zheng et al. (2013), Rock et al. (2011)
Ciliated cells	FoxJ1, Tubulin IV	No	Not applicable	Not applicable	Pardo-Saganta et al. (2013), Rawlins and Hogan (2008)
Goblet cells	Mucin 5AC, Mucin 5B, Spdef	No	Not applicable	Not applicable	Engelhardt et al. (1995), Shimizu et al. (1996)
Non-ciliated columnar cells (non-goblet or non-club)	Undefined	Yes	Undefined	Undefined	Evans et al. (1986)
AECI cells	Aquaporin 5, Podoplanin RAGE	No	Not applicable	Not applicable	Barkauskas et al. (2013), Kinnard et al. (1994)

(continued)

Table 3.1 (continued)

Cell types	Cell-type markers	Stem/ Progenitor cells	Lineage cell type(s)	Candidate niches	Reference(s)
AECII cells	SPB, SPC, Lamp3, Abca3, and integrin α6β4	Yes	ATI and ATII cells	Alveoli space	Reddy et al. (2004), Barkauskas et al. (2013), Fujino et al. (2011), Chapman et al. (2011)
PNEC	CGRP, Ascl1, Pgp9.5	Yes	PNEC, club[b], and ciliated cells[b]	NEB	Song et al. (2012), Hong et al. (2001), Peake et al. (2000), Boers et al. (1996), Guha et al. (2012)
MSC	CD73/90/105, Vimentin, Prolys-4-hydroxylase	Yes	Adipocyte, chondrocyte, osteocyte, myofibroblasts, and fibroblasts	Bone marrow, circulation system, and pulmonary interstitium	Lama et al. (2007), Sabatini et al. (2005), Sinclair et al. (2013), Martin et al. (2008)

NEB neuroepithelial body, *BADJ* bronchioalveolar duct junction, *PNEC* pulmonary neuroendocrine cell, *MSC* mesenchymal stem cell, *AECI* alveolar type I epithelial cells, *AECII* alveolar type II epithelial cells

[a]Occurs only in the setting of basal cell ablation

[b]Findings from studies using different transgenic models conflict with respect to club and ciliated lineages derived cells from PNECs

3.3 Potential Stem Cells in the Adult Lung

Stem/progenitor cells are crucial for development, tissue homeostasis, and injury repair in the lung. Studies using epithelial reconstitution assays, murine injury models, and lineage tracing approaches have identified several region-specific stem/progenitor cell populations in the adult lung of mice and humans. Basal cells in the proximal airways, variant club cells in bronchioles, bronchoalveolar stem cells (BASCs) in BADJs, and a subset of AECII in alveolar spaces have all been identified as stem/progenitor cells (Table 3.1).

In the trachea and main-stem bronchi, basal cells are the principal stem cells involved in homeostasis and injury repair and have the capacity to generate all the major cell types found in the proximal airway, including basal, ciliated, goblet, and granular secretory cells (including club cells) (Hong et al. 2004a, b; Hajj et al. 2007; Rock et al. 2009; Schoch et al. 2004; Cole et al. 2010; Engelhardt et al. 1995). The intermediate cells in the human proximal airway are so named because they are generally thought to represent an intermediate state of differentiation from basal cells and to serve as a transient amplifying cell population with the capacity to differentiate into ciliated and goblet cells (Engelhardt et al. 1995; Mercer et al. 1994). Intermediate cells do not exist in the mouse proximal airway, potentially because of the less pseudostratified nature of their smaller diameter airways. Of note, studies of murine lung injury involving BrdU labeling demonstrated that label-retaining cells (LRCs) reside predominantly in the ducts of SMGs, suggesting that these glands serve as a stem cell niche in the proximal airway (Xie et al. 2011; Borthwick et al. 2001; Engelhardt et al. 1995; Engelhardt 2001; Rock et al. 2009). Importantly, the SMG-localized LRCs have the capacity to undergo sequential rounds of cell division despite their slowly cycling phenotype (Xie et al. 2011; Lynch and Engelhardt 2014). Nevertheless, because lineage tracing of glandular LRCs has not yet been possible, the ability of these stem cells to produce specific airway cell types remains unclear. Several cellular markers have been utilized to identify and isolate basal cells. These include cytokeratin 5 (CK5), cytokeratin 14 (CK14), and aquaporin 3 (Rock et al. 2009, 2010; Schoch et al. 2004). Using a CK5-CreER^{T2} transgenic mouse line, Rock et al. further demonstrated that basal cells are capable of differentiating into club and ciliated cells, both at steady state and during injury repair (Rock et al. 2009). In addition, they identified nerve growth factor receptor (NGFR) and integrin α6 (ITGA6, also called CD49f) as markers on the surfaces of isolated human basal stem cells (Rock et al. 2009). Similarly, Ghosh et al. identified a CD49f^{bright}/Sca-1^{+}/ALDH1^{+} (Aldehyde dehydrogenase 1) subset of tracheal basal cells as region-specific stem cells, and demonstrated that these cells could generate niches in vitro and contribute to tracheal epithelial maintenance and injury repair (Ghosh et al. 2011). These studies suggested that basal cells play key roles in both homeostasis and injury repair of the proximal airway.

In the intralobar bronchiolar airways, a subset of the variant club cells that express club cell secretory protein (CCSP, also called Scgb1a1) but not CyP450-2F2 (CCSP^{+}, CyP450-2F2^{-}) can self-renew and produce both club cells and ciliated

cells (Hong et al. 2001; Rawlins et al. 2009b; Reynolds and Malkinson 2010; Xing et al. 2012; Guha et al. 2012). This CCSP^{+}/CyP450-2F2^{-} subset was also found at the BADJ of distal bronchioles, where it contributed to airway epithelial regeneration following naphthalene-mediated depletion of CyP450-2F2^{+} club cells (Giangreco et al. 2002). Kim et al. subsequently identified BASCs as a subpopulation of cells that express CCSP and pro-surfactant protein C (SPC) and serve as region-specific stem cell at the BADJ (Kim et al. 2005). Using naphthalene- and bleomycin-induced murine models of lung injury repair, Kim et al. further demonstrated that these cells possessed the capacity to self-renew and to produce differentiated epithelial cells in vivo, and that BASCs could differentiate into club cells and alveolar epithelial cells in an ex vivo clonogenic assay (Kim et al. 2005). Conversely, an in vivo lineage tracing experiment using a CCSP(Scgb1a1)-CreER™ knock-in mouse line revealed that club cells generated daughter club cells and ciliated cells but not alveolar cells following hypoxia-induced lung injury (Rawlins et al. 2009b). However, subsequent lineage tracing studies following alveolar injury by influenza infection and bleomycin exposure support the finding that CCSP-expressing stem/progenitors can give rise to AECI and AECII cells (Zheng et al. 2013; Rock et al. 2011). These injury-dependent influences on BASC-derived lineages suggest that either specific injury signals may invoke different responses and/or that multiple subsets of BASCs exist with different capacities for differentiation. The later hypothesis is consistent with findings suggesting that BASCs in the distal airways might include a heterogeneous population of progenitor cells (Teisanu et al. 2009, 2011; Chen et al. 2012). A study by Teisanu et al. classified club cells with the surface antigen profile CD45^{-}/CD31^{-}/CD34^{-}/EpCAM^{+}/Sca-1^{low} into two subgroups based on their autofluorescence (AF) profiles and suggested that club cells in the AF^{low} population are naphthalene resistant, whereas their AF^{high} counterparts were not (Teisanu et al. 2011). Indeed, mice that were exposed to naphthalene showed significantly greater proliferation in AF^{low} club cells compared to AF^{high} club cells, and conversely, mice exposed to ozone showed significantly greater proliferation in the AF^{high} club cell fraction compared to AF^{low} club cells (Teisanu et al. 2011). McQualter et al. demonstrated that an EpCAM^{+}/Sca-1^{low}/Integrin α6β4^{+}/CD24^{low} fraction of epithelial stem/progenitor cells was capable of self-renewing and differentiating into a variety of airway epithelial lineages, including alveolar epithelial cells (McQualter and Bertoncello 2012). These studies provide evidence that BASCs play key roles in the repair of injury to both bronchiolar and alveolar cells, as well as in homeostasis.

In the pulmonary alveolus, surfactant-producing AECII cells have long been recognized as stem/progenitor cells for the squamous AECI cells in the adult lung (Adamson and Bowden 1974; Evans et al. 1975). In vitro assays of cell proliferation and clonogenicity, as well as in vivo analyses following epithelial injury and lineage tracing, have produced mounting evidence that a subset of AECIIs have the capacity to proliferate and restore the alveolar epithelium by producing either new AECII cells or their squamous AECI counterparts (Reddy et al. 2004; Barkauskas et al. 2013; Fujino et al. 2011). Equally noteworthy were findings suggesting that integrin α6β4 is a biomarker for a subset of stem/progenitor cells in alveolar epithelia;

SPC^{-}/integrin α6β4^{+} cells were found resident in the alveolar epithelia, where they were able to regenerate SPC^{+} AECII cells (Chapman et al. 2011). The notion that AECII cells are region-specific stem/progenitors was recently confirmed by work from the Hogan laboratory, which employed a genetic SPC-labeled lineage tracing assay and an in vitro 3D culture model. This study produced convincing evidence that AECII cells were able to maintain the homeostasis of alveolar epithelia during both steady-state turnover and injury repair (Barkauskas et al. 2013). Several strategies that rely on biomarkers to identify and isolate adult lung stem/progenitor cells are listed in Table 3.2.

3.4 Stem Cell Niches in the Adult Lung

As discussed above, a vast body of evidence has demonstrated that distinct stem/progenitor cell populations reside in specific anatomical niches (Fig. 3.1), where diverse cell types and signals coordinate the behavior of stem cells during homeostasis and following injury. Stem cell niches are discrete microenvironmental units within a tissue that can provide one or more of the following features important for stem cell control: a unique extracellular matrix; supporting cell types; unique innervation and nearby vasculature; and diffusible factors that allow stem cells to maintain a capacity to self-renewal and control their proliferation and differentiation in the setting of injury (Fuchs et al. 2004). The anatomical sites of airway stem cell niches are typically epithelial structures associated with these unique features described above (e.g., innervation, support cells). Although much remains to be learned about how components of airway niches coordinate stem/progenitor cell behavior and phenotype, data from organ systems that have been studied more extensively suggest that they are likely important in the lung as well.

In the following discussion of stem cell niches in the airway, we focus on the unique anatomic and biologic properties of each niche within a particular region of the lung, and on how these features may contribute to repair following injury. In particular, we concentrate on studies of slowly cycling stem/progenitor cells in the mouse lung, since nucleotide label retention has been one of the most commonly used methods for tracking the anatomic locations of stem cell niches in the lung.

3.4.1 The Tracheal Surface Airway Epithelium

Within the tracheal SAE, subsets of basal cells are thought to be the major stem/progenitor cells. Following injury, LRCs tend to cluster within intercartilaginous zones of the distal trachea and larger bronchi along the basal lamina of the surface epithelium (Borthwick et al. 2001). These intercartilaginous zones tend to be sites of high blood vessel concentration and nerve penetration to the epithelium (Baker et al. 1986; McDonald 1988). These features are likely important biologic

Table 3.2 Experimental strategies used to isolate and characterize progenitor cells based on phenotypic characteristics

Input	Phenotypic markers[a,b]	Assay	Differentiation/Proliferation potential	References
Human nasal polyps	$CD151^+/F3^+$	In vitro ALI, ex vivo xenograft assays	Fully differentiated and functional airway epithelium including basal, columnar, goblet, and ciliated cells	Hajj et al. (2007)
Human fetal airway xenografts	$AQP3^-$	Ex vivo xenograft assay	Mature epithelia including ciliated, secretory, and basal cells within 4–6 weeks (compared to 6–20 weeks in $AQP3^{hi}$ cells) and by 20 weeks both $AQP3^-$ and $AQP3^{hi}$ cells generated mature epithelia including glands containing mucous and secretory cells	Avril-Delplanque et al. (2005)
Human trachea	$NGFR^+/ITGA6^+$	In vitro 3D colony-forming assay	Colonies containing basal and ciliated cells	Rock et al. (2009)
Human trachea and bronchi	$CD45^-/H33342^{Side\ Pop}$	In vitro limiting dilution assay, ALI culture	Cobblestone colonies of basal cells on plastic and basal, columnar, and goblet cells in ALI cultures	Hackett et al. (2008)
Human lung	$CD45^-/CD31^-/CD73^-/CD34^-/CDH1^+/LGR6^+$	In vitro limiting dilution assay, in vivo kidney organoid assay	Colonies containing club, AECII, and AECI cells and single cells from both freshly isolated and clonally expanded cells generated clones in mouse kidney capsule grafts	Oeztuerk-Winder et al. (2012)
Human lung	$CD45^-/CD31^-/EpCAM^+/HTII\text{-}280^+$	In vitro 3D colony-forming assay	Colonies containing AECII cells	Barkauskas et al. (2013)
Mouse trachea	$GSI\text{-}A_3B^+/NGFR^+$	In vitro 3D colony-forming assay	Colonies containing basal and ciliated cells	Rock et al. (2009)
Mouse trachea	$CD45^-/CD31^-/Ly76^-/Ly6a^+/Itga6^{hi}/Aldefluor^+$	In vitro colony forming assay(s) in vivo airway injury model	Rimmed clones in a co-culture system with irradiated feeder cells; basal, ciliated, and club cells in a mixed ALI culture system; proliferated in vivo following naphthalene injury	Ghosh et al. (2011)
Mouse tracheal surface epithelium	$Trop2^+/Itga6^+/Aldefluor^+$	In vitro 3D colony-forming assay	Luminal colonies containing basal, ciliated, columnar, serous, and mucous cells in 3D cultures	Hegab et al. (2011, 2014)
Mouse tracheal glandular epithelium	$Trop2^+/Itga6^+/Aldefluor^+$	In vitro 3D colony-forming assay, in vivo fat pad assay	Dense colonies containing basal, columnar, serous, and mucous cells in 3D cultures, and basal, serous, mucous, and myoepithelial cells in an in vivo fat pad assay	Hegab et al. (2011, 2014)

Mouse lung	$CD45^-/CD31^-$/ $EpCAM^+/Itgb4^+$/ $LysoTracker^+$	In vitro 3D colony-forming assay	Colonies containing AECII cells	Van der Velden et al. (2013)
Mouse lung	$CD45^-/CD16^-$/ $CD32^-/EpCAM^{hi}$/ $Itgb4^+$	In vitro 3D colony-forming assay, in vivo kidney organoid assay	Colonies containing AECII, club, and cell expressing both SPC and CCSP in a 3D colony-forming assay and within a mixed kidney capsule organiod assay	Chapman et al. (2011)
Mouse lung	$CD45^-/CD31^-$/ $Ly6a^+/CD34^+$	In vitro limiting dilution assay	Mesenchymal colonies with osteogenic and chondrogenic potential (data not shown)	McQualter et al. (2009)
Mouse lung	$CD45^-/CD31^-$/ $EpCAM^{hi}/Itga6^+$/ $Itgb4^+/CD24^{lo}$	in vitro 3D colony-forming assay	Cystic colonies containing ciliated, club, goblet, basal, and secretory cells, saccular colonies containing club, AECII cells, and mixed colonies containing mucous, ciliated, and AECII cells	McQualter et al. (2010)
Transgenic mouse trachea	$CK5\text{-}GFP^+$/ $GSI\text{-}A_3B^+$	In vitro 3D colony-forming assay, in vivo lineage tracing	Colonies containing ciliated and basal cells; lineage-labeled CK5-expressing cells generated basal, club, and ciliated cells both at steady state and following SO_2 injury	Rock et al. (2009)
Transgenic mouse lung	$CD45^-/CD31^-$/ $CD34^-/EpCAM^+$/ $SFTPC\text{-}GFP^{-/lo/hi}$	In vitro 3D colony-forming assay	GFP^{neg} cells generated spheroid colonies containing AECII, club, serous, ciliated, and basal cells, GFP^{lo} cells generated irregularly shaped colonies containing AECII, club, and ciliated cells, and GFP^{hi} cells generated dense colonies containing AECII cells	Chen et al. (2012)
Transgenic mouse lung	$CD45^-/CD31^-$/ $EpCAM^+$/Sftpc-CreER; $Rosa\text{-}Tm^+$	In vitro 3D colony-forming assay, in vivo lineage tracing	Colonies containing AECII and AECI cells; lineage-labeled Sftpc-expressing cells proliferate at steady state and following injury	Barkauskas et al. (2013)

[a]Official gene symbols are substituted for CD49f (Itga6), Sca-1 (Ly6a), TER119 (Ly76), E-Cad (CDH1), and Tissue Factor (F3)

[b]GSI-A_3B *Griffonia simplicifolia* isolectin A_3B has been shown to bind to airway basal cells, Aldefluor substrate reports enzymatic aldehyde dehydrogenase activity, LysoTracker (LysoTracker Green DND-26 (Invitrogen)) is a dye that stains acidic intracellular compartments

components and may function to localize stem/progenitor cells within this niche at homeostasis; they could also mediate injury responses that direct changes in stem/progenitor cell behavior. It has been suggested that these intercartilaginous zones enable a subset of surface basal cells to maintain multipotency within the mouse proximal airway (Borthwick et al. 2001; Engelhardt 2001; Liu and Engelhardt 2008; Rock et al. 2009, 2010; Hong et al. 2004b). Subpopulations of basal cells with the capacity for self-renewal and differentiation have also been described by others, based on clonogenic assays and lineage tracing studies (Cole et al. 2010; Hajj et al. 2007; Hong et al. 2004a; Schoch et al. 2004; Rock et al. 2009). For example, Rock et al. recently identified a subset of basal cells that were marked with $p63^+$/$NGFR^+$/$CK5^+$ that were able to self-renew and to generate luminal daughter cells within an in vitro 3D tracheosphere assay (Rock et al. 2009). Lineage tracing studies in mice expressing a CK5-promoter driven CreER transgene further demonstrated that CK5-expressing basal cells could give rise to ciliated and club cells in the tracheobronchial airways, both at steady state and following injury (Rock and Hogan 2011; Rock et al. 2010).

3.4.2 The Tracheal Submucosal Glands

A link between SMGs and stem/progenitor cells in the SAE was first discovered through retroviral lineage tracing experiments using human airway epithelial cells and a rat trachea xenograft model (Engelhardt et al. 1995). In these studies, retrovirally tagged human tracheobronchial epithelial cells were expanded in a denuded rat trachea that had been subcutaneously implanted into nu/nu mice. These cultures contained diverse populations of airway cells that were capable of clonal expansion within the xenografted airway. Phenotypic analysis of clones established a working model for progenitor/progeny relationships in the adult human proximal airway. Although seven clonal classes were discovered, the most abundant clone phenotype was multipotent and contained basal, intermediate, ciliated, and goblet cells. These multipotent clones were also the largest in size, supporting the hypothesis that they were derived from stem/progenitors with the largest capacity for expansion. Notably, SMGs also formed within these xenografts, and lineage tracing revealed that they were always associated with multipotent clones on the SAE. Expansion of basal cell progenitors in vitro prior to seeding into xenografts reduced the complexity of possible outcomes in clone phenotypes observed, giving rise to multipotent clones almost exclusively. These findings suggested that a small subset of basal cells are multipotent for SAE cell types and also have the capacity to form SMGs (Engelhardt et al. 1995). Additionally, these studies demonstrated with early passage primary human airway epithelial cells that a diverse range of progenitors exist in the human proximal airway with unipotent and bipotent capacities for differentiation. Later, clonal analysis in mice expressing a CK14-CreER transgene confirmed these findings and demonstrated that at least two subsets of basal cells exist with either unipotent or multipotent capacity for differentiation (Hong et al. 2004b). Consistent with

the finding that a subset of adult airway stem cells have the capacity to generate SMGs, LRCs localized within glands or glandular ducts following tracheal epithelial regeneration following injury (in response to both SO_2 and detergent treatment), suggesting that a subset of slowly cycling glandular epithelial cells are tissue-specific stem/progenitor cells and are capable of regenerating the airway epithelium after injury (Borthwick et al. 2001; Engelhardt 2001). Based on dual nucleotide sequential labeling experiments, these glandular LRCs retain the capacity to divide following repeated injury but remain slowly cycling (Xie et al. 2011; Lynch and Engelhardt 2014). Glandular LRCs make up a small fraction of total glandular cells (0.39 % ± 0.03 %) at 90 days after injury and only about 10 % of glandular LRCs reenter the cell cycle following a second injury and remain slowly cycling (Xie et al. 2011). Thus, if slowly cycling glandular LRCs are a stem cell, they represent approximately 0.04 % of total glandular cells.

Similar observations on glandular-derived stem cells were made in a murine model of hypoxic-ischemic injury (Hegab et al. 2011, 2012b). In these studies, Hegab et al. found that the SMG duct cell population included stem/progenitor cells that shared phenotypic features with surface airway basal cells and were resistant to epithelial injury in the context of tracheal hypoxic-ischemic injury. In vitro and ex vivo assays carried out with epithelial stem/progenitor cells isolated from the SMG duct have demonstrated that these cells are capable of self-renew and can generate several cell types found in the SAE and SMGs (Hegab et al. 2011, 2012a, 2014). Furthermore, in vitro colony forming assays using epithelia isolated from the gland-rich proximal region of the mouse trachea have revealed that these cells have a higher potential for proliferation than their counterparts from the gland-free distal trachea (Xie et al. 2011).

Cumulatively, these studies provide convincing evidence that the SMGs serve as a stem/progenitor cell niche for the proximal airway. The positioning of stem/progenitor cell niches within SMGs likely has biologic significance beyond simply the maintenance of glandular cell types. For example, SMGs are less exposed to the external environment and pathogens that threaten the lung, thus glandular stem cell niches are more protected. Additionally, SMGs are highly innervated (Nadel 1983; Wine 2007) and their secretions are regulated (i.e., enhanced) in response to injury of the SAE (Xie et al. 2011). Given that SMGs play an important role in airway innate immunity by producing secretions that regulate the composition of fluid, electrolytes, mucus, and antibacterial factors at the airway surface (Wine and Joo 2004; Wang et al. 2001; Dajani et al. 2005), it is not surprising that the regulation of glandular secretions following airway insults might be coordinated with the mobilization of glandular stem/progenitor cells that regenerate the airway surface. Interestingly, studies of cystic fibrosis suggest that defects in glandular secretions caused by the lack of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel alter the SMG stem/progenitor cell niche by dysregulating the calcitonin gene-related peptide (CGRP) neuropeptide (Xie et al. 2011). Such studies have demonstrated that, in mice deficient for CFTR, slowly cycling LRCs relocate from SMGs to the SAE following naphthalene injury and that this is accompanied by a redistribution of highly proliferative stem/progenitor cells from proximal

gland-rich regions of the trachea to regions of the SAE that lack glands. CGRP is induced in SMGs following airway injury and leads to the induction of gland secretions by activating CFTR, however, CGRP is constitutively upregulated in SMGs of cystic fibrosis humans, ferrets, pigs, and mice and this altered neuroendocrine signaling is thought to be the basis of stem cell niche dysfunction (Xie et al. 2011). Such findings emphasize the plastic nature of airway stem/progenitor cell niches.

3.4.3 The Neuroepithelial Bodies in the Intralobar Airways

Pulmonary NEBs are found within the epithelia of intrapulmonary airways (bronchi and bronchioles) and contain specialized CGRP-expressing pulmonary neuroendocrine cells (PNECs) (Cutz et al. 2013). The NEBs are extensively innervated and intrapulmonary bronchial capillaries are fenestrated at these sites (Lauweryns et al. 1972, 1974; Cutz et al. 2013). Both innervation and NEBs have been shown to be highest at sites of bifurcation in the airway (Elftman 1943; Cutz et al. 2013). Given these unique anatomic characteristics of NEBs, it is not surprising they appear to be stem/progenitor cell niches for the intralobar airways during both normal cell turnover and injury repair (Hong et al. 2001; Reynolds et al. 2000a).

At least two distinct cell types exist within NEBs—CCSP^{+}/CyP450^{-} variant club cells and the above-mentioned CGRP^{+} PNECs (Reynolds et al. 2000a, b). Lineage tracing studies using murine models have demonstrated that both the PNECs, and variant club cells associated with NEBs, have the capacity to self-renew and to differentiate into club and/or ciliated cells following naphthalene injury (Song et al. 2012; Hong et al. 2001; Xing et al. 2012; Guha et al. 2012). A subpopulation of naphthalene-resistant CCSP^{+}/CyP450^{-} variant club cells was identified as stem/progenitor cells in distal airways (Reynolds et al. 2000a, b; Hong et al. 2001; Rawlins et al. 2009b). Guha et al. also recently identified a distinct subset of CCSP^{low}/CyP450^{-}/Scgb3a2^{+}-expressing club cells resident in NEBs for which Notch signals and the transcription factor TTF1 (Nkx2.1) played a crucial role in determining the secretory cell fate in developing murine airways, supporting the idea that the NEB microenvironment is a stem cell niche for variant club-like stem cell precursors (Guha et al. 2012). Notch signaling in club cells was also found in the adult lung, in which Notch1 was required for repopulating lost club cells following airway epithelial injury (Xing et al. 2012). Previously, Hong et al. ablated CCSP-expressing cells—including club and variant club cells—by treating transgenic mice that expressed thymidine kinase from a CCSP promoter with ganciclovir, and then studied the lineage potential of the CGRP-expressing PNEC progenitors (Hong et al. 2001). The group found that, although PNECs replicated following club cell ablation, they were unable to regenerate CCSP-expressing club cells or ciliated cells, suggesting that PNECs are not competent to regenerate the mouse bronchiolar epithelium (Hong et al. 2001). However, Song et al. obtained different results using another approach to tag the PNEC lineage (Song et al. 2012). Specifically, these investigators introduced a CreER transgene into the CGRP locus and used this

transgene to lineage trace or ablate CGRP-expressing PNECs. They found that fate mapped CGRP-expressing PNECs could generate both club and ciliated cells following naphthalene injury, but that when PNECs were ablated using Cre-activated diphtheria toxin (DTA), ciliate cells were not regenerated (Song et al. 2012). The apparent discrepancies between the outcomes in Hong et al. and Song et al., which suggest that PNECs are either unipotent or multipotent, respectively, are likely related to the methods of airway injury used in these studies and may reflect a high level of plasticity in distal airway progenitors.

3.4.4 The Bronchioalveolar Duct Junctions

Mounting evidence suggests that within the terminal bronchioles the BADJ is a niche for BASCs that are capable of regenerating both bronchiolar and alveolar epithelial cell lineages following injury (Zacharek et al. 2011; Kim et al. 2005; Regala et al. 2009; Tropea et al. 2012; Zheng et al. 2013; Rock et al. 2011). In vitro studies suggested that BASCs have the capacity to differentiate into club, AECII, and AECI cells. However, lineage tracing studies using CCSP-CreER knock-in mice did not substantiate these findings in vivo, at least in the cases of naphthalene- and hyperoxia-induced acute injury to the lung; the CCSP-expressing progenitors did not give rise to the alveolar epithelium in these contexts (Rawlins et al. 2009a). Nevertheless, when other models of alveolar injury (influenza infection or bleomycin exposure) were tested, lineage traced CCSP-expressing progenitors gave rise to labeled AECI and AECII cells (Zheng et al. 2013; Rock et al. 2011; Tropea et al. 2012). Thus, the contribution of BASCs to alveolar injury repair may depend on injury-specific regulatory factors within the BADJ microenvironment.

3.4.5 The Alveoli

The terminal end of the respiratory tree is composed of alveolar sacs, whose cellular composition includes AECI and AECII cells, capillaries, and lung-resident mesenchymal stromal cells (lrMSCs). AECII cells, which produce surfactant protein C (SPC), have been suggested to serve as a stem/progenitor cells from which AECI and AECII cells are regenerated after alveolar injury (Adamson and Bowden 1974; Barkauskas et al. 2013). However, a recent study that used an SPC-CreER mouse model to map the fates of AEC cells following bleomycin injury found that the majority of AECII cells in fibrotic areas did not arise from preexisting SPC-expressing AECII cells (Chapman et al. 2011), but rather from a subset of previously unrecognized AECs. These cells expressed the laminin receptor integrin α6β4 but not CCSP or SPC and expanded to form a differentiated alveolar-like epithelium containing CCSP-expressing cells and SPC-expressing AECII cells in an ex vivo kidney capsule model (Chapman et al. 2011). By contrast, in a more recent

study by Barkauskas et al., SPC-expressing AECII cells were found to self-renew and differentiate into AECI cells, both at steady state and following alveolar injury (Barkauskas et al. 2013). These investigators went on to show, using an in vitro differentiation 3D culture model, that individual AECII cells produced self-renewing "alveolospheres" that comprised both AECI and AECII cells. Of note, co-culturing AECII cells with a PDGFRα-expressing subpopulation of lung mesenchymal cells significantly increased the efficiency of formation of self-renewing alveolospheres. Thus, these PDGFRα-expressing lung stromal cells, which include alveolar fibroblasts and lipofibroblasts, appear to be components of the AECII stem/progenitor cell niche within the alveolus (Barkauskas et al. 2013). Taken together with studies on the BADJ, these studies suggest that multiple stem/progenitor cell niches in the distal lung may contribute to repair of the alveolus following injury, and that the active niche in the context of homeostasis resides in the alveolus.

3.5 MSCs and Stem/Progenitor Cell Niches in the Adult Lung

Increasing evidence indicates that MSCs are important components of epithelial stem/progenitor niches in the adult lung, and that they play an essential role in orchestrating epithelial regeneration during both homeostasis and injury repair (McQualter et al. 2010, 2013; Volckaert et al. 2011, 2013; Gong et al. 2014). lrMSCs can be isolated from bronchioalveolar lavage (BAL) fluid (Lama et al. 2007) and lung tissue (Ricciardi et al. 2012) using the techniques of differential plastic adherence and enzymatic dissociation, respectively. Studies evaluating in vitro co-culture models have demonstrated that lrMSCs are not only key for the proliferation and differentiation of epithelial stem cells (McQualter et al. 2010) but also are able to differentiate into AECII cells when co-cultured with AECII cells in a transwell model (Gong et al. 2014). In this context, lrMSCs can contribute to lung repair by secreting FGF-10 and TGF-β, and thereby promoting re-epithelialization (McQualter et al. 2010, 2013; Volckaert et al. 2011, 2013). In the developing lung, FGF-10 is central to regulating BMP, Wnt, and Shh signaling pathways, which are responsible for coordinating differentiation in this context (Morrisey and Hogan 2010). In the adult lung, TGF-β signaling by mesenchymal cells regulates the secretion of FGF-10 and provides a cue that is necessary for epithelial regeneration (McQualter et al. 2010, 2013). Two subpopulations of lrMSCs were found—CD166^{-} lrMSCs, which have the capacity to differentiate into lipofibroblast and myofibroblast cell types and to support epithelial stem cell proliferation and differentiation in vitro, and CD166^{+} lrMSCs, which are limited to producing cells of the myofibroblast lineage and fail to support epithelial stem cell proliferation and differentiation in vitro (McQualter et al. 2013). Studies by Volckaert et al., which used a naphthalene-based model of lung injury, have identified lrMSCs as important components of the bronchiolar stem/progenitor cell niche. These studies implicate parabronchial smooth muscle cells (PSMCs) in the regulation of

naphthalene-resistant club cells at the BADJ and adjacent to NEBs by activating Wnt/FGF-10 signaling (Volckaert et al. 2011). In addition, the Wnt target gene c-Myc was found to be critical for both activating the PSMC niche and inducing FGF-10 expression (Volckaert et al. 2013). Mechanistically, FGF-10 secreted by PMSCs activated Notch signaling and Snail expression in naphthalene-resistant club cells, and subsequently initiated the repair process by promoting club cell proliferation and differentiation (Volckaert et al. 2011, 2013).

3.6 Stem/Progenitor Cell Niches and Cancer-Initiating Stem Cells in the Lung

Lung cancer is a heterogeneous disease in terms of its phenotypic diversity and anatomical sites of origin in the airways. Lung cancers can be subdivided into two major groups—small cell lung cancers (SCLCs) and non-small cell lung cancers (NSCLCs). SCLC is characterized by neuroendocrine cell morphology and accounts for ~15 % of lung malignancies; NSCLC accounts for the remaining cases (~85 %) and can be further subdivided into three distinct histological subtypes: squamous cell carcinoma (SCC), adenocarcinoma, and large cell carcinoma (Travis et al. 2013). The morphologies and molecular properties (e.g., activation of the Wnt, Hedgehog (Hh), and Notch signaling pathways) of each subtype have led to the hypothesis that lung cancers are derived from stem cells in the lung (Alamgeer et al. 2013; Lundin and Driscoll 2013). Currently, it is possible to isolate lung cancer stem cells (CSCs) based on the expression of several tumor markers, including aldehyde dehydrogenase (ALDH), CD133, CD44, and the ability to efflux certain dyes such as Hoechst (Alamgeer et al. 2013).

Although lung CSCs have not been as well characterized as other tumors, the current understanding of the phenotypes of region-specific airway epithelial stem/progenitor cells has led to the hypothesis that cancers initiate at specifically those anatomic locations in which stem cell niches reside. This hypothesis is supported, in part, by findings from animal models of lung cancer; the most common sites of origin for different lung cancer types correlate with distinct, region-specific airway stem/progenitor cell niches (Kitamura et al. 2009; Succony and Janes 2014; Leeman et al. 2014). Notably, mouse adenocarcinomas are characterized by the expression of the transcription factor Nkx2.1 (TTF1), CCSP, and SPC and arise from BADJs, suggesting that cancer-initiating progenitor cells arise from within club or AECII stem/progenitor-cell populations (Kim et al. 2005; Imielinski et al. 2012; Travis et al. 2013; Xu et al. 2012). Lung SCCs are characterized by differentiation into squamous cells with a basal cell phenotype, and can be subdivided based on mRNA expression levels, into classes of cells that resemble basal cell progenitors in the SAE or SMGs (Wilkerson et al. 2010), two sites at which stem cell niches exist. Similarly, SCLCs are found predominantly in the intermediate airways and are characterized by the expression of a range of neuroendocrine cell markers, including CGRP (Song et al. 2012; Kelley et al. 1994; Carraresi et al. 2006). Thus, SCLCs may originate

from CGRP-expressing progenitors within the NEB stem/progenitor cell niche. This hypothesis is further supported by experiments using transgenic mice deficient for Rb1 and p53 in specifically the club cells, AECII cells, or PNECs; in these animals, SCLCs arise most frequently from NEB-resident PNECs (Sutherland et al. 2011). These findings suggest that mutations that dysregulate airway stem/progenitor cell niches play important roles in selecting lung CSCs that outcompete other progenitors and promote cancer initiation, metastasis, and chemoresistance (Takebe and Ivy 2010; Chen et al. 2014).

3.7 Perspective Summary

The results from in vitro and in vivo clonogenic assays and lineage tracing analyses in various experimental models have suggested that region-specific stem/progenitor cells reside within distinct niches in the lung. At least five unique epithelial stem/progenitor cell niches have been proposed in the lung, and the signals that induce the expansion of progenitors and specification of daughter cells from each of these niches appear to be diverse. Moreover, in the mouse models that have been studied, this often depends on the type of injury. The available data also suggest that some progenitors impart a high level of lineage plasticity to the lung, with committed differentiated cell types capable of adopting stem cell properties and reestablishing stem cell niches in the setting of severe airway injury. Given that abnormalities in lung stem/progenitor cell niches can occur in the context of genetic disease, viral infection, and lung cancer, it will be important to define the cues that are intrinsic to lung cells, as well as those that are extrinsic (i.e., present in the unique regional niches of the lung). Such knowledge is expected to provide effective new avenues for the treatment of lung diseases.

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