Chapter 4
Bronchioalveolar Stem Cells in Cancer

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Abbreviations

AAH	Atypical adenomatous hyperplasia's
AEC1/1	Alveolar epithelial cell 1/2
BASC	Bronchioalveolar stem cell
CC10	Clara cell 10 kDa
CCSP	Clara cell secretory protein
COPD	Chronic obstructive pulmonary disease
CSC	Cancer stem cell
EGFR	Epithelial growth factor receptor
Hh	Hedgehog
IPF	Idiopathic pulmonary hypertension
Krt	Keratin
NE	Neuroendocrine cell
NSCLC	None small cell lung cancer
OB	Obliterative bronchitis
SCGB1A1	Secretoglobin family 1A member 1 protein
SCLC	Small cell lung cancer
SFTPC	Surfactant protein C
TTF-1	Thyroid transcription factor 1 (aka NKx2.1)
Trp63	Tumor repressor protein 63
WNT	Wingless-related integration site

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4.1 Introduction: Stem Cells and Cancer

Stem cells are defined by their expression of pluripotent and/or multipotent markers and are considered the undifferentiated source for the generation and regeneration of all tissues. The stem cell ability to self-renew via symmetrical division ensures that an undifferentiated pool of cells is maintained within each tissue and throughout the lifespan of the organism (Reya et al. 2001; He et al. 2009). The stem cell ability to differentiate via asymmetrical division drives the generation of specialized tissues during development and during regeneration and/or repair of injury in the adult organism (Morrison and Kimble 2006). The choice to divide symmetrically or asymmetrically appears to depend on a variety of factors, including the potency of the cell in question, the position of the stem cell within its niche, and the requirements of the tissue supported by the stem cell for regeneration or repair (Snippert et al. 2010; Scadden 2014). One underlying mechanism for asymmetric versus symmetric division is controlled by distribution of specific molecules within the cell that define polarity, with a major role played by the Notch-binding protein Numb (Dingli et al. 2007), though other critical, cell scaffolding molecules also contribute (Liu et al. 2014). Paracrine signaling from neighboring niche cells and the surrounding tissue can also drive distinct stem cell responses. All these aspects of stem cell biology continue to play critical roles when a normal tissue stem cell is transformed into a cancer stem cell (Dingli et al. 2007; Pardal et al. 2003).

It is hypothesized that cancers arise when the normal proliferative response to injury becomes uncontrolled due to mutations to pro-proliferative oncogenes, anti-proliferative tumor-suppressing genes, and/or alterations in the cell-supporting environment. Hyperproliferation may stay localized and relatively benign or may eventually lead to malignancy and invasion of surrounding tissues. Tumors are often comprised of a heterogeneous population of cells, within which are cells that possess stem cell-like properties and behaviors (Reya et al. 2001). Multiple studies have shown that this subpopulation does not just contribute to the growth of the tumor within its original niche, but retains the ability form de novo tumors at other locations (Pardal et al. 2003; Buzzeo et al. 2007; Glinsky 2008). Along with unrestricted self-renewal, these cells also retain the capacity for multipotent differentiation, giving rise to the heterogeneous lineages of cancer cells that comprise tumors. These distinctive properties, which are similar in many ways to the behaviors of somatic stem cells, have led them to be classified as cancer stem cells (CSCs) (Guo et al. 2006; Girouard and Murphy 2011). However, the "Cancer Stem Cell Hypothesis" extends this concept, in that the data that demonstrate stem-like properties for CSCs also support the hypothesis that they originally arise from resident tissue stem cells (Reya et al. 2001; Rahman et al. 2011). These data suggest that tumors are initiated by the uncontrolled growth of stem cells or differentiated cells that have been reprogrammed to a less differentiated state (Spike and Wahl 2011). Because each tissue in the body carries a resident stem cell population for the purposes of regeneration, it is hypothesized that each also harbors tumor-initiating cells, which produce lesions that reflect both their stem cell origin and their originating tissue (Clarke and Fuller 2006).

4.2 Role of Bronchioalveolar Stem Cells in Development, Homeostasis, and Injury Repair

In the lung, each functional compartment harbors distinct stem cell populations, each of which displays characteristic molecular signaling patterns and responses to adult tissue injury (Wansleeben et al. 2013). In developing lung, signaling via pathways critical to the embryo as a whole (WNT, Hedgehog (Hh), and Notch) are supplemented by more tissue-specific signaling patterns. Lung tissue is specified early in development in a small stem cell population originating from the laryngotracheal groove by the expression of Thyroid transcription factor 1 (Ttf-1, also termed Nkx2.1) (Cardoso and Kotton 2008; Morrisey and Hogan 2010). In murine models, this is rapidly followed by expression of a variety of signaling factors that specify the morphogenesis required to create the branched structure of the bronchial and alveolar epithelium, mesenchyme, and supporting vasculature (Metzger et al. 2008; Maeda et al. 2007; Griffiths et al. 2005; Que et al. 2009). Mature mouse and human lung diverge in morphology and corresponding epithelial stem cell populations in the upper airways, with the trachea of the mouse more closely resembling the upper airways in humans (Rock and Hogan 2011). Humans also possess respiratory bronchioles and goblet cells that are absent or rare, respectively, in murine lung. However, more distal markers and stem cell populations show some overlap (Wansleeben et al. 2013). In mice, Trp63-Krt5-positive basal stem cells of the upper airways give way to Secretoglobin family 1A, member 1 protein (SCGB1A1)-positive club cells (formerly termed CC10- or CCSP-positive Clara cells) of the lower airway (Reynolds et al. 2000), and can transiently upregulate Krt14 in response to injury (Reynolds and Malkinson 2010; Cole et al. 2010; Daniely et al. 2004; Ghosh et al. 2010; Hong et al. 2004; Rock et al. 2009). Small airways also harbor scattered, injury-responsive neuroendocrine cells (NE) (Reynolds et al. 2000; Linnoila 2006). Within alveoli, the terminal breathing structure of the distal lung, the gas exchange function of large, capillary-adjacent alveolar epithelial type 1 cells (AEC1) is supported by less common alveolar epithelial type 2 cells (AEC2). AEC2 perform the differentiated function of surfactant expression, but also exhibit a stem cell-like responsiveness to injury (Griffiths et al. 2005; Evans et al. 1973, 1975) Functional facets of the pulmonary neuroendocrine system). In vitro, multiple studies have shown differentiation of AEC2 to AEC1 and recent lineage-tracing studies following injury have shown evidence that AEC2 can serve as progenitors for AEC1 in vivo (Evans et al. 1975; Marconett et al. 2013). Recent studies have also shown that the bronchioalveolar duct junction, situated between the distal and terminal airways leading to the alveolus, harbors rare, multipotent bronchiolaveolar stem cells (BASCs). BASCs typically express both SCGB1A1 and surfactant protein (as assessed by expression of surfactant protein C (SFTPC)) (Kim et al. 2005; Kim 2007), though recently a stem-like population of bronchioalveolar duct junction cells that are SFTPC-negative and express integrin α6ß4 have been identified (Chapman et al. 2011).

The lung is considered a low turnover organ, presumably meaning a decreased likelihood of propagating the mutations that are essential for the formation of

malignancies. However, its critical function and constant exposure to the environment creates a strong drive toward homeostasis, resulting in discrete regions of proliferation that can be detected following environmental and experimental injury to the distal lung (Bowden et al. 1968; Giangreco et al. 2009; Snyder et al. 2009). The source of this proliferation has been assigned to the stem cells of the various lung compartments, including small airway club and NE cells, alveolar AEC2s and BASCs, the latter of which have been shown to be particularly responsive to the injury/regeneration signals unleashed by pneumonectomy (Nolen-Walston et al. 2008; Jackson et al. 2011; Eisenhauer et al. 2013). Likewise, the SFTPC-negative-integrin α6ß4-positive bronchioalveolar duct junction population is a label retaining cell that has been shown to respond to the distal lung injury caused by bleomycin treatment by proliferating, making it a candidate bronchiolaveolar long-term stem cell (Chapman et al. 2011).

4.3 Role of Stem Cells in Lung Cancer

Worldwide, the morbidity and mortality caused by lung cancer outranks that of all other cancers, with approximately 1.5 million deaths per year attributed to the disease (The Lancet 2013). Lung tumors arise in all areas of the lung and its variable nature reflects the specialized nature of the various compartments of the organ (Curtis et al. 2010). Thus, within a number of main categories of lung cancer, a large number of tumor subtypes are now recognized. In addition, even within lung tumors, a high degree of heterogeneity can be found, with a continuum of undifferentiated to well-differentiated cell phenotypes observed. Tumors composed of mixtures of cell types with little variation in differentiation status can also be found. These observations make it difficult to pinpoint the type of initiating cell most common to lung cancer, but instead indicate more complex tumor-initiating events, where a tumor-promoting environment may activate multiple cell types or, alternatively, in which initiating cells with a broad potential for differentiation are activated. However, both scenarios can be supported by a sequence of mutations that lead to tumor formation initiated by a select population of lung cancer stem cells (Sutherland and Berns 2010; Peacock and Watkins 2008; Sullivan et al. 2010; Giangreco et al. 2007).

Lung cancers are prevalent despite the slow rate of epithelial cell turnover and the propensity for human lung tissue to scar rather than regenerate. Over the past several decades, correlative observations have led to even stronger causative data showing that lung cancers arise due to both the self-inflicted insult of smoking and the passive insults due to environmental, especially atmospheric, pollution (which may include secondary cigarette smoke) (Samet 2013; Dresler 2013; Gomperts et al. 2011). Finally, recent evidence of epigenetic contributions to the initiation and progression of lung cancer has added an additional dimension to the etiology of lung cancers (Risch and Plass 2008; Scherf et al. 2013). Both cigarette smoke and airborne pollutants have been shown to contain toxins and carcinogens that drive the development of lung inflammation (Walser et al. 2008; Kundu and Surh 2012).

Both this change in the lung epithelial cell environment, as well as direct effects of mutagens on the resident cell populations, can be considered potential triggering events in the transformation of lung stem cells to cancer stem cells. The toxins and reactive oxygen species (ROS) generated by side stream (secondary) cigarette smoke are proven agents of lung cell DNA damage, impairment of normal epithelial cell function, and alterations in gene expression. In addition, cytokines activated during an inflammatory event often remain within the lung as a proximate, underlying cause of chronic pulmonary inflammation. As injury-responsive cells, lung stem cells are clearly vulnerable to mutagenic events and an environment that conceivably leads to an altered, cancer stem cell phenotype (Peebles et al. 2007; Kundu and Surh 2008; Gonda et al. 2009).

4.4 Bronchioalveolar Cells as Stem Cells for Lung Cancer: Data from Pathology

Cancer is identified by its originating cell, i.e., the cell that incurred the first oncogenic mutation. Lung cancers are classified into histological categories based on the initiating cell type (Davidson et al. 2013). Apart from a very small percentage (2 %) of cancers of non-defined origin, the two main groups of cancers that arise from lung cells are small cell lung cancers (SCLC), which comprise approximately 18 % of diagnosed cases, and non-small cell lung cancers (NSCLC), which account for the remaining 80 %. SCLC is the more aggressive and potentially lethal form of the disease, with a 5-year US survival rate of only 5 %. While NSCLC are more common, they metastasize at a slower rate. The NSCLC 5-year survival rate in the US is slightly better, at 15 %, but taken together, survival rates for all lung cancers remain exceedingly poor (The Lancet 2013).

The most common types of SCLC exhibit dense, neurosecretory granules, indicating a putative origin as small airway neuroendocrine cells. Bronchioalveolar cells more commonly appear to contribute to the three subcategories of NSCLC, with evidence based on both the epithelial nature of the tumor cell populations and the location of the initiating tumor at airway branch points, within the bronchioalveolar duct junctions or alveoli. These subcategories are characterized as adenocarcinoma, squamous cell carcinoma, and large cell lung carcinomas (Sakashita et al. 2014). In addition to tumors that arise from lung cells, the highly vascularized lung is also a preferential site for metastatic growth of tumor cells of extra-pulmonary origin, commonly including, but not limited to, breast cancers and melanomas.

While lung cancer is a common type of cancer, chronic lung diseases are even more common and are hypothesized to be manifestations of repeated lung epithelial cell injury. In chronic obstructive pulmonary disease (COPD), obliterative bronchitis (OB), and idiopathic pulmonary fibrosis (IPF), changes in the lung cellular environment are triggered by epithelial cell destruction. Changes in immune cell number and function lead to the release of inflammatory cytokines, activation of lung mesenchymal cell populations, and/or influx of circulating cells. The damage produced

by repeated inflammation has a significant impact on the resident cell populations responsible for the homeostatic regulation and repair of epithelium. It is hypothesized that homeostatic repair, and the necessary increase in mitosis required for restoration of homeostatic conditions within the lung, increases the probability of carcinogenic mutations occurring within injury-responsive cell populations. These mutations can therefore accumulate to the point where carcinogenic changes occur and lung cancer develops (Peacock and Watkins 2008). Histological observations have linked both COPD and IPF to development of hyperplasias that can lead to cancer, including the formation of atypical adenomatous hyperplasia's (AAH) at the periphery of lungs where fibrotic plaque formation has also occurred (Mizushima and Kobayashi 1995; Sekine et al. 2012). Though originally hypothesized to arise from AEC2s based on pathological analysis of human tissue, later studies using experimental models showed that bronchioalveolar cells may also contribute to AAH formation (Kim et al. 2005). In fact, controversy surrounding the cell of origin of these lesions, which are believed to be a precursor of adenocarcinoma, still persists.

4.5 Bronchioalveolar Cells as Stem Cells for Lung Cancer: Data from Experimental Models

Human AAH can, under conditions favorable to tumor formation, transition to adenocarcinomas. The cell of origin for these tumors is often observed to be SFTPC-positive and has long been considered to be the surfactant secretory AEC2 of the alveolus. Early transgenic mouse models utilized constitutive overexpression of the viral tumor-suppressing binding protein SV40, which inactivates both Trp53 and pRb. Expression of this potent oncogene was driven by either murine or rabbit club cell *Scgb1a1* promoters or the human *Sftpc* promoter, and each model exhibited distinct development of alveolar or bronchiolar tumors in the form of adenocarcinomas with no marker overlap, indicating singular cells of origin (Wikenheiser et al. 1992, 1993; Sandmöller et al. 1994, 1995; Magdaleno et al. 1997; Wikenheiser and Whitsett 1997). In even earlier studies, spontaneous lung cancers in the form of adenocarcinomas were found to preferentially develop when a mutated K-Ras was utilized as a transgene (Suda et al. 1987). As oncogenically mutated K-Ras can be found in approximately 25 % of human ACs, these latter studies were followed up by development of a conditional, oncogenic K-Ras transgenic model, termed K-RasG12D (Jackson et al. 2001). The earliest data from this model confirmed that the hyperplastic cells induced when mutant K-Ras was expressed were indeed SFTPC-positive. However, evidence from human pathology that bronchiolar cells could also contribute to adenocarcinoma formation proved more difficult to model.

These conflicting observations appeared to be resolved by the identification of SCGB1A1-SFTPC co-expressing BASCs identified by their response to oncogenic K-Ras overexpression in the K-RasG12D model (Kim et al. 2005). Using this model, the rapid expansion of BASCs due to K-Ras signaling led to studies that defined BASC involvement in lung cancer tumorigenesis. Similarly, studies where

the tumor suppressors p27 and PTEN were inactivated also stimulated BASC hyperproliferation and subsequent AAH and adenocarcinoma formation (Besson et al. 2007; Yanagi et al. 2007). However, as with the identity of BASCs as a stem cell progenitor for AEC2 and airway cells (Rawlins et al. 2009), the identity of BASCs as a bronchioalveolar cancer stem cell remains controversial.

A more recent study by Xu and colleagues posited that AEC2s, and not BASCs, were the initiating cell specifically for malignant adenocarcinoma formation in the lung due to mutant K-ras expression. This study used different transgenic models, where K-rasG12D was expressed via knock-in at loci for the *Sftpc* gene, the *Scgb1a1* gene or both. Comparison of these models showed formation of AEC2-like, club cell-like, or BASC-like hyperplasias in the corresponding knock-in models, but confirmed that only mice that expressed mutant K-Ras in SFTPC-positive AEC2 exhibited the ability to progress to adenocarcinoma (Xu et al. 2012). These observations were echoed by Mainardi and colleagues with data derived from a lung ubiquitous mutant K-Ras (K-RasG12V) transgenic model where malignant adenocarcinomas arose only from AEC2. Furthermore, in this model, conditional expression of K-RasG12V produced hyperplasias throughout the distal lung of both bronchiolar and bronchioalveolar origin, with only those that exhibited AEC2 markers progressing to adenocarcinoma (Mainardi et al. 2014). However, a subsequent study by Sutherland and colleagues that combined the use of conditional K-RasG12D expression, lineage tracing analyses, and knock down of Trp53 showed a more complex outcome (Sutherland et al. 2014). In these models, both SCGB1A1-positive club cells and SFTPC-positive AEC2 were capable of forming adenocarcinomas and the knock down of Trp53 was required for progression to a metastatic, invasive phenotype. These models all differ in the method used to conditionally induce K-RasG12D or K-RasG12V expression from floxed alleles (via genetically induced Cre-recombinase expression in the Xu and one portion of the Mainardi studies versus via intratracheal administration of Cre-recombinase expressing adenoviruses in the remainder of the Mainardi study and in the Sutherland approach) and it remains to be seen if underlying differences in location or transgene expression, local inflammatory response to the transducing vectors and/or hyperplastic cells, as well as penetrance and levels of mutant K-Ras expression affect the outcome in these models. However, all these results expand the pool of potential stem/progenitor-like distal lung-initiating cell types and challenge the concept that a single, bronchioalveolar cancer stem cell plays a primary role in the initiation of adenocarcinomas.

4.6 Conclusions

The stem cell-like resistance to injury and proliferative potentials of cancer-initiating cells appears to contribute to cancer growth and resistance to treatment. Further research into the validity of BASCs, club cells, and/or AEC2s as stem or progenitor sources of lung carcinogenesis is needed to properly identify these populations as lung tumor cells of origin, particularly in human lung. Identifying cancer stem cells

within bronchioalveolar lung tumors will not only illuminate the mechanistic underpinnings of some of the most common and deadly types of lung cancer, but may provide a focus for a wide range of possible treatments and therapies that specifically target stem-like cells. Several possible therapeutic targets unique to these cells include the repair or correction of dysfunctional signaling cascades including altered EGFR, Wnt, Hedgehog, and Notch pathways (Alison et al. 2009). These signaling pathways play vital roles in lung development, response to injury, and the regulation of stem cell self-renewal. They may also play a role in the initiation of tumorigenesis, where a variety of mutations have been shown to cause dysregulation of the processes of stem cell renewal and directed an appropriate differentiation. Characterizations of these mutations are currently being pursued using genome-wide analyses, which are rapidly progressing to the point of becoming useful supplements to standard pathological approaches for identification of tumor-initiating cells (Sakashita et al. 2014). The possibility of customized therapeutic approaches, as well as a rapid method for addressing therapeutic resistance, could lie in the ability of those who study and treat bronchioalveolar tumors to isolate and analyze their initiating cancer stem cells.

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