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Cancer Stem Cells in Lung Tumorigenesis

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Abstract

Although stem cells were discovered more than 50 years ago, we have only recently begun to understand their potential importance in cancer biology. Recent advances in our ability to describe, isolate, and study lung stem cell populations has led to a growing recognition of the central importance cells with stem cell-like properties may have in lung tumorigenesis. This article reviews the major studies supporting the existence and importance of cancer stem cells in lung tumorigenesis. Continued research in the field of lung cancer stem cell biology is vital, as ongoing efforts promise to yield new prognostic and therapeutic targets.

The Concept of Cancer Stem Cells

In 1855 Rudolf Virchow observed that “*omnis cellula e cellula*” or “every cell stems from another cell” [1]. This foundational tenet of cell theory was extended to the concept of stem cells by Siminovitch and colleagues [2] in the early 1960s. They observed that colony-forming cells giving rise to spleen colonies had three characteristics that now define stem cells: self-renewal, proliferative capacity, and multipotency [3].

Several similarities between stem cells and tumor cells subsequently led to the cancer stem cell model of tumorigenesis. Like organs derived from stem cells, tumors consist of rapidly proliferating cells and also represent a heterogeneous collection of cells with different phenotypes [4]. Because these heterogeneous tumor cells are clonal in origin, they are thought to arise from a self-renewing population that gives rise to terminally differentiating progenitor cells [4]. The cells that make up the self-renewing clonal population within a tumor have been termed “cancer stem cells” because of the analogous biologic processes they undergo compared with normal stem cells [4].

The pioneering work leading to the cancer stem cell hypothesis depended on the accurate identification and isolation of tumor cells with stem cell-like properties. This was accomplished by using cell surface markers, or surface molecules, that could be used not only to identify distinct populations of unique cells by immunostaining but also to isolate these distinct populations by techniques such as flow cytometry and magnetic bead isolation [5]. Classic stem cell surface markers, as well as novel surface markers, have been used to isolate populations of tumor cells with stem cell-like properties.

In leukemia for example, only a small subset of leukemia cells are able to initiate acute myeloid leukemia in nonobese diabetes/severe combined immunodeficiency mice [6]. This

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subset of leukemia cells could be identified and isolated using the stem cell surface markers cluster of differentiation (CD) 34 and CD38 [6]. Consistent with their identity as leukemia stem cells, these cells demonstrated the properties of self-renewal, proliferation, and multipotency [6].

Similar findings have been made in solid tumors in which only a small fraction of heterogeneous tumor cells are clonal. In breast cancer, for example, Al-Hajj and colleagues [7] isolated a cell population with high expression of the cell surface marker CD44 but low expression of the cell surface marker CD24. As few as 100 CD44⁺/CD22⁻ cells were able to form tumors in mice; these cells gave rise to mixed tumors that included not only additional CD44⁺/CD22⁻ cells but also thousands of cells with alternative phenotypes [7]. Importantly, none of these alternative phenotypes were able to independently form tumors in mice [7].

CD133, a cell surface marker of neural stem cells [8], is also expressed in a small subset of cells in brain [8,9] and colon [10] tumors. Similar to the unique cell population seen in epithelial breast cancer, cancer cells expressing the cell surface molecule CD133 were observed by Singh and colleagues [8,9] and O'Brien and colleagues [10] to be the only cells capable of forming new heterogeneous tumors in mice.

The relative immunity of metastatic disease to chemotherapy might also be linked to the existence of tumor cells with stem-like properties. One of the more discouraging observations about chemotherapy is that despite its success in shrinking primary tumors, it has been largely unsuccessful in curing metastatic disease [4,5]. It has been reported that normal stem cells express high levels of antiapoptotic and drug-resistance proteins such as bcl-2, human multidrug-resistance (MDR1), and the adenosine triphosphate-binding cassette transporter, making them resistant to chemotherapy and apoptosis induction [11–13].

These observations have led some to speculate that the inability of chemotherapy to cure metastatic disease might be due to the expression of drug-resistant proteins that make stem cells inherently more resistant to toxins that kill their terminally differentiated counterparts [4,5]. Thus, although chemotherapy might shrink primary tumors by killing terminally differentiated cells that form the bulk of the tumor, the tumor may subsequently be repopulated by chemoresistant cancer stem cells causing tumor recurrence [4,5].

Despite emerging evidence that cells within tumors with stem-cell markers and stem cell-like properties exist (hereafter referred to as “cancer stem cells”), their origin still remains unclear. It is not well understood, for example, whether these cancer stem cells arise from normal stem cells that acquire oncogenic mutations or from fully differentiated cells that acquire mutations causing dedifferentiation and acquisition of stem cell-like properties [5].

Some indirect evidence supports the hypothesis that normal stem cells acquire oncogenic mutations causing malignant tumors. One group, for example, has studied *Oct-4*, a stem cell marker that maintains cell pluripotency in the developing embryo [14]. When overexpressed in the somatic tissues of adult mice, constitutive *Oct-4* expression causes epithelial dysplasia by expansion of progenitor cells but does not affect fully differentiated cells [14]. These observations suggest that only stem cells, but not their fully differentiated counterparts, are targets of *Oct-4*-induced dysplasia [14].

Weaker evidence comes from the observation that continued oncogene pathway activation is necessary for epidermal tumor formation in adult transgenic mice [15]. Some interpret this observation to suggest that only clonal self-renewing cells are potential sources of malignant tumors because they alone live long enough to acquire the necessary number of mutations required for oncogenic transformation [16].

Evidence for Lung Stem Cells in Adults

The epithelium of the adult airways consists of three distinct compartments arranged along a proximal-distal axis (Table 1) [17]. Part of the difficulty in demonstrating the existence of adult lung stem cells has been due to the slow rate of turnover in the adult epithelium. In contrast to the rapidly proliferating cells present in the bone marrow or epidermis, the proliferative fraction has been estimated to be as small as 1.3% in the tracheal epithelium and 0.06% in bronchiolar epithelium [17]. Despite these difficulties, evidence now exists supporting the presence adult stem cells in each of the epithelial compartments.

The proximal airways, composed of the trachea and main bronchi, are lined by pseudostratified epithelium [17]. Keratin (K)-expressing basal cells have been shown to have capacities for self-renewal, proliferation, and multipotency, making them putative major airway stem cells [18,19,21,22]. Borthwick and colleagues [21] used an epithelial injury mouse model to demonstrate the presence of tracheal K5-expressing basal stem cells. Mouse airways were injured by intratracheal detergent or sulfur dioxide inhalation, and the proliferation and localization of bromodeoxyuridine label-retaining cells was observed in the trachea during and after injury. These label-retaining cells were found in the same niches as cells with high K5 promoter activity [21]. Transgenic mice expressing K5-promoter-driven enhanced green fluorescent protein demonstrated enhanced green fluorescent protein expression in these putative stem cell niches, confirming K5 expression in these self-renewing tracheal stem cells [22]. K5-expressing cells also demonstrated a 4.5-fold greater capacity to form colonies and a 12-fold greater capacity to form large colonies in vitro, demonstrating the proliferative potential of these self-renewing tracheal stem cells [22].

Rawlins and colleagues [23] confirmed these observations by using elegant lineage tracing experiments that support the pluripotency of tracheal K5-expressing basal cells. Hong and colleagues [18,19] used a naphthalene airway injury mouse model to demonstrate the multipotency of tracheal keratin-expressing basal cells [18,19]. In their model, injury resulted in the rapid induction of K14-expressing basal cells. Transgenic mice harboring a *K14* promoter linked to a lox-cre reporter were used to show that these rapidly proliferating K14-expressing basal cells gave rise to phenotypically heterogeneous progeny, including clonal species of the original K14-expressing basal cells, demonstrating self-renewal, proliferation, and multipotency of these tracheal stem cells [18,19].

The bronchioles of middle airways are lined by simplified columnar epithelium [17]. Two types of bronchiolar progenitor cells that proliferate in response to injury have been described. Rapidly proliferating bronchiolar progenitor cells were first reported in classic studies by Evans and colleagues [31,32] in a rat model of nitrogen oxide/ozone inhalation injury. They demonstrated that mature Clara cells could transiently dedifferentiate and give rise to phenotypically different bronchiolar epithelial cells after inhalation injury [31,32]. These Clara cells are functionally equivalent to mature differentiated epithelial cells in the quiescent steady state but have the potential to become transit-amplifying cells (Clara type A cells) that give rise to diverse progeny in response to injury [29,33]. Although originally described as “lung stem cells,” these Clara cells have recently been redesignated as “facultative progenitor cells” [29,33].

More recent studies have identified other populations of bronchiolar progenitor cells resistant to naphthalene-induced injury that likely represent true bronchial airway stem cells [34]. Because Clara cells are selectively damaged by naphthalene [35], these models allow for the study of progenitor cells in a Clara-cell depleted environment. These models have shown that neuroepithelial bodies contain two separate populations of cells that proliferate in response to naphthalene-induced injury [24]. The first population consists of calcitonin

gene-related peptide-positive pulmonary neuroendocrine cells that proliferate and form hyperplastic lesions after naphthalene injury [25]. The second population consists of Clara-cell secretory protein (CCSP)-positive cells that proliferate and repopulate bronchiole airways with phenotypically diverse progeny after naphthalene exposure [24–26]. The inability of calcitonin gene-related peptide-positive pulmonary neuroendocrine cells to independently repopulate naphthalene-injured airways implies that neuroepithelial bodies CCSP⁺ cells represent true bronchial airway stem cells [26] (Table 1).

The distal airways are composed of respiratory bronchioles and alveoli that are lined by cuboidal epithelium [17]. The bronchioalveolar duct junction (BADJ) has been identified in recent studies as a microenvironment harboring airway stem cells. In 2002 Giangreco and colleagues [36] identified a neuroepithelial bodies-independent CCSP-expressing population in the BADJ that was capable of proliferation and self-renewal after naphthalene-induced injury. These CCSP⁺ BADJ cells gave rise to populations of label-retaining cells with different mitotic rates, suggestive of multipotent potential [36]. In 2005 Kim and colleagues [38] reported that these CCSP⁺ BADJ cells coexpressed surfactant protein C (SP-C). Further characterization demonstrated that these cells displayed a Sca-1⁺/CD45[−]/platelet-endothelial cell adhesion molecule (PECAM)[−]/CD34⁺ cell-surface marker phenotype [28]. This Sca-1⁺/CD45[−]/PECAM[−]/CD34⁺ population was capable of proliferation, self-renewal, and multilineage differentiation in culture, suggesting they represented true distal airway stem cells [28].

Recently, several groups have challenged these observations. Teisanu and colleagues [29] suggested that CD45[−]/CD31[−]/CD34[−]/Sca-1^{low}/AF^{low} is a more appropriate defining phenotype of BADJ stem cells. Through lineage tracing experiments, Rawlins and colleagues [23] demonstrated that Clara cells are not able to give rise to type II alveolar cells and suggest instead that Id2⁺ is an appropriate marker of lung distal tip epithelial stem cells [30]. Research to characterize true cell marker phenotypes of distal airway stem cells is ongoing [30].

Evidence for Lung Cancer Stem Cells

Different types of human lung cancer tend to localize along the same proximal-to-distal axis as airway epithelium [37] (Table 1). Preliminary evidence points to the existence of cancer stem cells in each of these lung cancer types (extensively reviewed by Giangreco and colleagues [37]). Squamous cell carcinomas, for example, tend to be centrally located tumors [37]. Barth and colleagues [20] observed that squamous cell metaplasia was enriched in basal cells expressing K5, suggesting that the proliferating basal cells seen in squamous cell metaplasia are tracheal basal stem cells. Small cell lung carcinomas (SCLC), on the other hand, predominantly occur in the midlevel bronchioles [37]. Gutova and colleagues [27] demonstrated the existence of populations of cells that exhibited multidrug resistance, clonogenic proliferation, and the expression of the putative stem cell markers CD44 and MDR1 using the marker urokinase plasminogen activator receptor in six SCLC cell lines [27]. Lastly, human adenocarcinomas and bronchioalveolar carcinomas, commonly recognized as a subtype of adenocarcinomas, tend to occur in the distal airways [37]. Kim and colleagues [28] demonstrated that BADJ stem cells are expanded in their mouse model of adenocarcinoma after K-ras activation in a dose-dependent manner (discussed more extensively below).

Recent studies provide additional evidence supporting the existence of lung cancer stem cells. Ho and colleagues [38] isolated populations of cells that efflux Hoechst 33342 dye from six human lung cancer cell lines. These cells exhibited several properties typical of stem cells, including clonogenic proliferation, invasive phenotypes in 3D Matrigel (BD

Biosciences, San Jose, CA) culture, multi-drug resistance, and increased telomerase expression [38]. Hoechst 33342-staining cells were also found in small amounts in 16 human lung cancer specimens [38].

Eramo and colleagues [39] demonstrated a population of CD133⁺ cells with stem cell properties in human lung cancers and in naphthalene-induced injury mouse models. Specimens of human non-small cell lung cancer (NSCLC) and SCLC demonstrated enrichment of CD133⁺ cells in these tumors compared with controls with healthy lungs, a finding mirrored in naphthalene-injured murine airways compared with uninjured airways [39]. CD133⁺ cells isolated from freshly resected human lung cancer specimens could be grown indefinitely in culture and generated tumors similar in phenotypic diversity to the original tumor when injected into immunodeficient mice [39]. Bertolini and colleagues [40] independently reported similar findings using CD133⁺ cells isolated from 60 human lung cancer samples.

A third group reported that the proliferative potential, invasiveness, and chemoresistance of CD133⁺ cells isolated from human lung tumors was dependent on Oct-4 expression, consistent with the known importance of Oct-4 in embryonic stem cell development [41,42]. Together, these data support the notion that these self-renewing, multipotent CD133⁺ cells are lung cancer stem cells.

Several classic tumorigenic pathways and developmental pathways have been implicated in lung cancer stem cell development. Among the classic oncogenic pathways, one of the most important and well studied is the K-ras pathway. Activation of the K-ras pathway leads to the development of lung adenocarcinomas in humans and in mouse models [43]. In 2001 Jackson and colleagues [44] reported findings implicating the K-ras pathway in the development of lung cancer stem cells. Using a transgenic mouse with inducible *K-ras* activation in lung tissues, they observed that a small number of cells within hyperplastic lesions and in adenomas expressed CCSP as well as the type II alveolar cell marker SP-C [44]. They hypothesized that these cells expressing CCSP⁺ SP-C⁺ represented transdifferentiating Clara cells or K-ras activation in lung cancer stem cells that had the potential to develop into both Clara and type II cells [44]. In 2005 they confirmed the latter hypothesis by demonstrating that K-ras pathway activation led to BADJ stem cell expansion in a dose-dependent fashion in vivo and in vitro [28]. Exposing these mice to naphthalene, known to stimulate BADJ stem cell expansion, increased lung tumor surface area by an average of 11-fold [28].

This group recently reported that *Bmi1*, a known oncogene, is necessary for K-ras-induced tumorigenesis in their mouse model [45]. Loss of *Bmi1* in their K-ras transgenic mice decreased the prevalence and progression of lung tumors and impaired BADJ stem cell proliferation and self-renewal in vivo and in vitro [45].

In addition to the K-ras oncogenic pathway, developmental signaling pathways known to regulate normal stem cell self-renewal and proliferation have also been linked to the development of lung cancer. The phosphoinositide 3-kinases (PI3K)/phosphatase and tensin homolog (PTEN)/protein kinase B (Akt) pathway, for example, is known to be necessary for normal stem cell function [46]. Several groups have also observed that this key stem cell pathway has a tumor-suppressor function in a variety of cancers, including NSCLC [47]. Loss of PTEN expression, for example, has been reported to result in constitutively active PI3K signaling in NSCLC [48].

Recent work has linked the PI3K/PTEN/Akt pathway to lung cancer stem cells and to suppression of *K-ras* mutations [48–50]. Spontaneous lung adenocarcinomas develop in transgenic mice with inducible loss of PTEN expression in bronchioalveolar cells [50].

Before the development of these adenocarcinomas, there is an expansion of Sca-1⁺/CD45⁻/PECAM⁻/CD34⁺ BADC stem cells and population cells that exhibit Hoechst dye efflux properties [50]. Interestingly, loss of PTEN expression results in *K-ras* mutations in 33% of mice developing spontaneous adenocarcinomas [50]. In addition, loss of PTEN expression in *K-ras* mutant mice results in more advanced and extensive adenocarcinomas than in *K-ras* mutant mice alone [49]. Consistent with these observations, inhibitors of PI3K signaling block expansion of Sca-1⁺/CD45⁻/PECAM⁻/CD34⁺ BADC stem cells and also tumor growth [48,51]. Together, these data suggest that loss of PTEN expression and PI3K activation leads to increased BADC stem and side-population cells and increases the frequency of *K-ras* mutations, resulting in the subsequent development of lung adenocarcinoma.

In addition to the PI3K/PTEN/Akt pathway, two pathways recognized to be important during embryonic lung development have also been linked to lung cancer stem cells [52]. The Hedgehog (Hh) signaling pathway acts as an important regulator of stem cell fates during embryonic development and has been linked to SCLC [53,54]. The observation that intraepithelial Hh signaling is increased after naphthalene-induced airway injury suggests that progenitor cells activate Hh signaling in response to injury [53,54]. Increased Hh signaling is also observed in the lungs of PTEN-deficient mice that develop spontaneous lung adenocarcinomas in conjunction with BADC stem cell expansion, implying a causal role for Hh signaling in this process [50]. The Wnt developmental pathway is another critical regulator of embryonic lung stem cells [55,56]. In adult mice, conditional deletion of p38 α , a known downstream target of noncanonical Wnt signaling [57], leads to an expansion of CCSP⁺ SP-C⁺ stem cells, hyperproliferation, and increased sensitization to K-ras–induced tumorigenesis [58]. These recent observations suggest it is likely that we will continue to discover the importance of reactivation of these nascent embryonic stem cell pathways in lung tumorigenesis.

Summary and Future Directions

Although the concept of human stem cells was first formulated more than 50 years ago, the notion that cells with stem-like properties may have an important causal influence in cancer, and in lung cancer in particular, represents a recent advancement in our overall understanding of tumorigenesis. The studies we have reviewed lay a convincing groundwork for our ongoing understanding of the casual role of cancer stem cells in tumorigenesis; however, many questions remain unanswered. Chief among these is the influence of the microenvironment on the transformation and progression of lung cancer stem cells. Many of the studies have demonstrated stem cell-like behavior of putative stem cell populations by xenograft transplantation models or in vitro cell culture. This ignores the possibility that development of stem cell niche autonomy due to microenvironment factors and inflammatory cells/mediators may have an active role in lung cancer pathogenesis [59–62].

The importance of continued research in this area is underscored by preliminary evidence demonstrating that the acquisition of “stemness” by NSCLC tumors is a negative prognostic and predictive factor in overall survival. Loss of PTEN expression, for example, has important ramifications in lung cancer stem cells, as detailed above, and is also an independent prognostic factor in overall survival of patients with NSCLC [63]. Similarly, stage IIIB/IV NSCLC patients with tumors enriched in CD133⁺ lung cancer stem cells tend to have shorter progression-free survival after treatment with platinum-based chemotherapy [40].

A better understanding of lung cancer stem cell biology promises to lead to new approaches to treatment, especially of advanced disease. One of the paradigm shifts that may occur is a

change in our concept of metastatic tumor cells. One group has already demonstrated that most early metastatic breast cancer cells have a stem cell phenotype [64]. The acquisition of stem cell features may explain why early metastatic disease escapes immunosurveillance: these metastatic cells are disguised from the immune system by being phenotypically similar to native stem cells. These observations, combined with the previously referenced work demonstrating that stem cells are resistant to chemotherapeutics due to the antiapoptosis and drug-resistant genes they express, may mean that finding effective therapy for advanced-stage lung cancer will ultimately require isolating and specifically targeting lung cancer stem cells. Although the traditional approach of demonstrating therapeutic effectiveness by reduced tumor burden is widely accepted, it may prove meaningless if what is really important is reducing the cancer stem cell burden. In the future, the effectiveness of therapeutics for both early and advanced disease may indeed be measured in terms of their potency against lung cancer stem cell populations.

It is not unreasonable to speculate that the next major breakthrough in antitumor therapeutics will target one of the oncogenic, tumor suppressor, or developmental pathways that confer “stemness” to lung tumors. Ongoing research efforts in the field of lung cancer stem cell biology are therefore vital, as forthcoming advances in the fight against lung cancer lie on the stem cell horizon.

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Proximal-to-Distal Compartments of Lung Epithelium and Their Associated Putative Stem Cells/Human Lung Cancer Types

Table 1

Anatomy	Location	Epithelium	Putative Normal Lung Stem Cell Markers	Human Lung Cancers	Putative Lung Cancer Stem Cell Markers
Major airways	Proximal	Pseudostratified	K14 ⁺ basal cells [18,19] K5 ⁺ basal cells [21–23]	Squamous cell carcinomas	K5 ⁺ basal cells [20]
Bronchioles	Mid	Simplified columnar	NEB CCSP ⁺ cells [24–26]	SCLC/neuroendocrine tumors	UPAR ⁺ [27]
Respiratory bronchioles/alveoli	Distal	Cuboidal	Sca-1 ⁺ /CD45 ⁻ /PECAM ⁻ /CD34 ⁺ BADI cells [28] CD45 ⁻ /CD31 ⁻ /CD34 ⁻ /Sca-1 ^{low} /AF ^{low} BADI cells [29] Id2 ⁺ [30]	BAC/adenocarcinomas	Sca-1 ⁺ /CD45 ⁻ /PECAM ⁻ /CD34 ⁺ BADI cells [28]

BAC = bronchioalveolar carcinoma; BADI = bronchioalveolar duct junction; CCSP = Clara-cell secretory protein; CD = cluster of differentiation; K = keratin; NEB = neuroepithelial bodies; PECAM = platelet endothelial cell adhesion molecule; SCLC = small cell lung carcinoma.