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Evidence for self-renewing lung cancer stem cells and their implications in tumor initiation, progression, and targeted therapy

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Abstract

The discovery of rare tumor cells with stem cell features first in leukemia and later in solid tumors has emerged as an important area in cancer research. It has been determined that these stem-like tumor cells, termed cancer stem cells, are the primary cellular component within a tumor that drives disease progression and metastasis. In addition to their stem-like ability to self-renew and differentiate, cancer stem cells are also enriched in cells resistant to conventional radiation therapy and to chemotherapy. The immediate implications of this new tumor growth paradigm not only require a re-evaluation of how tumors are initiated, but also on how tumors should be monitored and treated. However, despite the relatively rapid pace of cancer stem cell research in solid tumors such as breast, brain, and colon cancers, similar progress in lung cancer remains hampered in part due to an incomplete understanding of lung epithelial stem cell hierarchy and the complex heterogeneity of the disease. In this review, we provide a critical summary of what is known about the role of normal and malignant lung stem cells in tumor development, the progress in characterizing lung cancer stem cells and the potential for therapeutically targeting pathways of lung cancer stem cell self-renewal.

Keywords

Cancer stem cell; Self-renewal; Lung stem cell; Lung cancer

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1 The cancer stem cell model for tumor initiation and progression

Cancer has been described as a "developmental disease," in which normal developmental pathways have been co-opted by oncogenic processes in cancer pathogenesis. In this context, the development of a tumor is analogous to the development of an aberrant organ. During normal organogenesis, the self-renewal and differentiation of stem cells is carefully orchestrated to produce a functional organ with heterogeneous cellular phenotypes. In tumorigenesis, the processes of self-renewal and differentiation become deregulated resulting in the production of hyper-proliferative, aberrantly differentiated cancerous tissue. In normal development and in cancer initiation, stem cell processes of self-renewal and differentiation play a conspicuous role in the formation and homeostatic maintenance of a normal organ and a tumor. In adult tissues, these processes are reserved only for a small population of resident organ stem and progenitor cells. However, where and how tumors have co-opted the capacity for self-renewal and differentiation has been a subject of ongoing intense debate.

The cancer stem cell hypothesis provides explanations for the origins of tumor self-renewal and heterogeneity [1–3]. One component of the cancer stem cell hypothesis is that cancers arise from stem cells that have acquired sufficient oncogenic mutations for transformation. Therefore, the tumor cell of origin, referred to as a tumor-initiating cell, is a stem or stem progenitor cell that already is capable of self-renewal and differentiation. This concept has been demonstrated in myeloproliferative disorders where the selective oncogenic mutagenesis (for example, loss of the tumor suppressor gene *JunB* in mice) of primitive hematopoietic stem cells gives rise to chronic myeloid leukemia blast crisis, whereas the same mutation in mature hematopoietic cell populations does not [4]. Furthermore, since tumor cells and stem cells share similar but not identical attributes such as the capacity to self-renew, the expression of human telomere reverse transcriptase (hTERT), and the expression of primitive cell markers, add credence to the explanation that these stem cell traits found in tumors may be inherited from a transformed stem cell [5].

Another component of the cancer stem cell hypothesis is that tumor progression is driven by a subpopulation of self-renewing tumor cells. This view comes from the well-documented observation that most tumors are comprised of functionally heterogeneous cell subpopulations, including a population that differ in their ability for limitless proliferative potential and repopulation ability. Among the first experimental reports of a stem cell hierarchy in cancer came in 1997 when Bonnet and coworkers observed that only primitive $CD34^{+}CD38^{-}$ leukemia blasts isolated from mice with acute myeloid leukemia possessed the capacity to transfer the disease, whereas the majority of leukemic blasts could not propagate the disease in recipient mice [6]. This capacity for sustained neoplastic growth in $CD34^{+}CD38^{-}$ leukemia blasts is due to the leukemia stem cell's ability to self-renew. This stem cell feature is considered the key discriminating difference between cancer stem cells and noncancer stem cells. The clinical implications of a tumorigenic hierarchy within a cancer become apparent when considering that therapies selected for their rapid reduction of tumor size are not selected for their discriminatory ability to treat tumor-initiating cell subpopulations. Therefore, if a therapy fails to eliminate all self-renewing cancer stem cells, residual surviving cancer stem cells are able to repopulate the disease, causing tumor relapse. This problem is compounded by the fact that $CD34^{+}CD38^{-}$ leukemia cancer stem cells for example, are relatively resistant to conventional chemo-therapies and express drug effluxing pumps such as MDR-1 and ABCG2 [7–9].

Since the discovery of cancer stem cells in human hematopoietic cancer, solid tumor putative cancer stem cells have been identified in brain [10], breast [11], prostate [12], colon [13,14], pancreatic [15], and most recently, in lung cancers [16]. Although lung cancer is among the most commonly lethal forms of cancer in the world [17], comparatively less is known about

the biology of lung cancer stem cells compared with other solid tumor stem cells. Lung cancer remains the most lethal form of cancer in both men and women in the USA, and improvements in standard chemotherapy have been mostly palliative with a one-year survival of only 35% [18,19]. Consequently, there is a pressing need for the development of new therapeutic agents that better manage the progression of highly aggressive lung cancer cells. However the methods to identify and isolate self-renewing lung cancer stem cells are still being developed. This is due in part to the complexity of the disease in terms of its phenotypically diverse and regionally distinct types of neoplasia. Lung cancers are comprised of four major histological types: small-cell lung cancer (SCLC) and three types of non-small-cell lung cancer (NSCLC) including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. In humans, SCLC and squamous cell carcinomas occur in the proximal region of the respiratory tract whereas adenocarcinomas are distally located. The histological and regional diversity found in lung cancer may partly be due to the presence of diverse pools of self-renewing stem cells in the adult lung epithelium.

2 Sites of self-renewal in lung epithelium

The lung epithelium consists of a large variety of morphologically and functionally different cell types, whose roles include facilitating gas exchange, balancing fluids in the lung, detoxifying and clearing foreign agents, and the activation of inflammation due to injury [20, 21]. The varieties of lung epithelial cell lineages are organized along the pulmonary tree to facilitate the specialized role in each region of the lung. The proximal airway of the respiratory tract includes the pharynx, larynx, trachea, and bronchi. The tracheal airway epithelium consists primarily of ciliated columnar and mucus-secreting goblet cells that work to lubricate the surface of the proximal airway epithelium and to trap and clear foreign particulates. As the pulmonary tract of the lung branches into the smaller bronchioles and alveoli of the distal airway, the columnar epithelial cells transition into a more cuboidal morphology. Here the lung epithelium is lined with Clara cells which serve to protect and detoxify the bronchiolar epithelium. The most distal regions of the lung contain the terminal bronchioles and alveoli where gas exchange from the alveolar sacs into capillary beds occurs. The alveoli consist of type 1 and type 2 pneumocytes. The flat morphology of type 1 cells aids in diffusion of gas into the adjacent capillary beds. Type II pneumocytes are more cuboidal, and secrete surfactant proteins that serve to regulate lung fluid balance and provide elasticity to the lung epithelium [20].

Unlike tissues with rapid cell turnover such as the blood, gastrointestinal, tract and skin, turnover of the lung epithelium is less frequent, occurring every 30 to 50 days [22–24]. However during injury, cell turnover is accelerated in distinct regions in the lung where it is believed that self-renewing stem cells reside [25]. Although the existence of regional stem cell populations in adult lung epithelium has long been accepted, controversy remains as to the exact cellular identity and the capacity for self-renewal and lineage differentiation of some putative lung stem cells [26].

In the epithelium of the trachea and bronchi, cell turnover is thought to be driven by a class of lung stem cells known as basal cells. Unlike the more differentiated columnar cells of the proximal airway, basal cells reside along the basement membrane and do not extend to the apical surface of the epithelium [27]. The deposition of basal cells in rodents is primarily restricted to the trachea, whereas in human lungs, basal cells are ubiquitous in the conducting airway epithelium [28,29]. Basal cells can be discriminated by their expression of cytokeratins 5 and 14 (KRT5/14), as well as their expression of the transcription factor *Trp-63* (p63) [30].

The first clear evidence for the role of basal cells in the steady-state maintenance of airway epithelium came from pulse-chase experiments using tritiated thymidine (^{3}H-TdR), where it

was found that basal cells and Clara cells could divide and give rise to ciliated cell progeny [22,23,31,32]. However, these studies did not differentiate between the roles of these two progenitor populations in renewing ciliated airway epithelium. Tracing discrete airway cell types has been aided by the improvement and utilization of transgenic lineage reporter mice and cell sorting technology [33]. Using KRT14-creER mice, it was found that after naphthalene ablation of both ciliated cells and Clara cells, basal cells became activated, expanded, and subsequently differentiated into secretory and ciliated cells [34,35]. The regenerative properties of basal cells was also confirmed when isolated basal cells from rat and human trachea were found to repopulate a fully differentiated, pseudostratified airway epithelium *in vitro* as well as in denuded rat tracheas [36,37]. These findings suggest that basal cells are enriched multi-potent progenitor cells, capable of airway epithelial renewal in steady-state maintenance and lung injury (Fig. 1a). In support of this, mouse KRT5-GFP$^+$ and human p63$^+$ basal cells possess the ability to self-renew and well as differentiate *in vitro* [38]. Therefore, the discovery that basal cells possess the capacity for multi-potent differentiation as well as self-renewal suggests these cells may be more "stem-like" than "progenitor-like." According to the cancer stem cell model for tumor initiation, basal cells may be a candidate population for the tumor-initiating cells. The expansion of KRT14 expressing basal cells during tracheal hyperplasia in squamous cell carcinoma support this model; however, more stringent lineage tracing experiments will be needed to confirm the connection between basal stem cells and tumor initiation (Fig. 1b)[39].

Clara cells, named after Max Clara the researcher who made their discovery, are domed-shaped secretary cells that are found throughout the airway epithelium, but are more concentrated in the bronchioles [40]. Clara cells are discriminated in rodent lung epithelia by their expression of the secretoglobin, *scgb1a1*, also known as CCSP or CC10 [30]. Clara cells also possess multi-potent capacity for differentiation; however it is believed that most Clara cells do not possess the ability to self-renew. An exception to the rule has been found in a subpopulation of naphthalene-resistant Clara cells, referred to as variant Clara cells. Variant Clara cells lack the expression of the cytochrome P450 2F2 isozyme (CYP2F2) that is responsible for the generation of toxic metabolites of naphthalene [41]. Both variant Clara cells and nearby pulmonary neuroendocrine cells (PNECs) in the bifurcation zone of the bronchioles have been shown to proliferate and participate in rodent airway epithelium renewal after naphthalene ablation [42–45]. However when airway epithelial regeneration was measured after the selective ablation of variant Clara cells in mice lungs, PNEC expansion failed to reconstitute the full component of stratified lung epithelia [46]. This indicates that while variant Clara cells and PNECs possess the ability to expand and self-renew, only variant Clara cells have the capacity for multi-potent differentiation (Fig. 1a), however this observation has not been confirmed in human lung epithelia. SCLC is a particularly aggressive and highly metastatic form of lung cancer, accounting for approximately 15% of total lung cancer cases [47]. Furthermore, SCLC is generally intractable to therapy after relapse, which attributes to its dismal prognosis [48]. Similar to PNECs, SCLC exhibit primitive neuroendocrine features, such as the expression of calcitonin gene-related peptide, and commonly develop in the midlevel bronchioles. For these reasons, it is hypothesized that malignant self-renewing PNECs may be the origin of SCLC (Fig. 1b)[49,50].

In the most distal region of the lung, the terminal bronchioles transition into sac-like alveoli. The alveolar epithelium is comprised of a thin layer of flattened type 1 pneumocytes and cuboidal type 2 pneumocytes. Type 1 pneumocytes are terminally differentiated cells that facilitate gas exchange between the alveoli and the adjacent capillary bed. Interspersed among the type 1 pneumocytes are the cuboidal, type 2 pneumocytes whose roles include the secretion of surfactants that modulate surface tensions. Early pulse-chase experiments using ^{3}H-TdR in injured rodent lungs revealed type 2 pneumocytes may also have the capacity to differentiate into type 1 pneumocytes [51,52]. Further investigation of isolated type 2 pneumocytes from

injured rat lungs revealed the existence of up to four phenotypically different type 2 pneumocyte populations, each expressing differing markers and telomerase activity [53,54]. This suggests that only some type 2 pneumocytes are progenitor cells with uni-potency, although the exact marker identity of these cells remains to be elucidated.

Another class of lung stem cells was recently discovered in the putative stem cell niche of the bronchioalveolar duct junction (BADJ) of mice. After naphthalene-induced alveolar ablation, pollutant resistant, BADJ-associated cells expressing the Clara cell marker CCSP were observed to expand and regenerate the alveoli. Unlike the variant Clara stem cells, these BADJ-associated Clara-like cells were not in proximity to the PNEC stem cell niche [55]. The capacity of the BADJ associate stem cells, now termed bronchioalveolar stem cells (BASCs) were shown to self-renew after naphthalene-induced injury (Fig. 1a)[56]. Importantly, BASC outgrowth driven by the Kras oncogene was also observed to contribute to the formation of atypical adenomous hyperplasia, a precursor lesion for adenocarcinoma of the lung [56,57]. Bronchiolar adenocarcinomas and progressed bronchioloalveolar cell carcinomas of the peripheral airways are among the most common types of lung cancer in the world presently [17]. In murine models of lung adenocarcinomas, tumors arise from the BADJ and typically exhibit alveolar differentiation [58,59]. Taken together these studies strongly suggest that the tumor-initiating cell of murine adeno- and bronchioloalveolar carcinomas are self-renewing BASCs, however this has yet to be determined in human lungs [50].

3 Stem cells in lung cancer

The cancer stem cell model for tumor progression indicates that only a subpopulation of cancer cells possess the ability to proliferate indefinitely. Furthermore in xenotransplantation studies, only cancer stem cells are believed to have the capacity to generate secondary and tertiary tumors that reproduce the heterogeneity of the primary disease. The presence of a stem-like clonogenic subpopulation in lung cancer was demonstrated almost 30 years ago, when Carney and colleagues observed that only a very small proportion (<1.5%) of SCLC and lung adenocarcinoma cells from patient samples could generate colonies in soft agar [60,61]. When inoculated into athymic nude mice, these soft agar colonies generated SCLCs and lung adenocarcinomas similar to their primary lesions, suggesting that these rare, clonogenic tumor cells were enriched with lung cancer stem cells [61,62]. Since then, researchers have attempted to prospectively isolate human lung cancer stem cells using markers that discriminate between intra-tumoral cell heterogeneity. This strategy has proven successful in several solid tumor types, such as breast, brain, and colon cancers where cancer stem cells can be identified and isolated via FACS by their expression of stem cell specific cell surface markers [63]. In lieu of adequate and selectable markers for human lung stem cells, researchers have also turned to other FACS based stem cell isolation techniques to identify and isolate lung cancer stem cells.

The Side Population assay, first described by Goodell and colleagues to select for human hematopoietic stem cells [64], relies on the ability of ABC transporters expressed in stem cell populations [65], to efflux the fluorescent Hoechst 33342 dye. Hoechst 33342 dye excluding cells, termed Side Population cells (SP cells), have been described in a variety of tumor types as being enriched in stem-like properties [66]. In acute myeloid leukemia, neuroblastoma, glioma, and ovarian cancer, isolated SP cells transplanted into recipient immunodeprived mice display a significantly greater capacity for tumorigenic growth than bulk non-SP cells, implying that SP cells are enriched in cancer stem cells [8,67–70]. In human lung cancer as few as 1,000 isolated SP cells from lung cancer cell lines produce robust xenografts in mice, whereas non-SP cells failed to generate tumors with similar numbers of SP cells [71]. SP cells were also found to self-renew and express elevated levels of hTERT (telomerase), compared with bulk non-SP cells [71]. These lines of evidence support the notion that the side population assay selects for cancer stem cells in lung tumors, however several criticisms have been raised

regarding the use of Hoechst 33342 dye to isolate stem-like population cells. For example, because Hoechst 33342 is a DNA binding dye, cells that are unable to efflux the dye may suffer from its cytotoxic effects. In the breast cancer cell line MCF7 and ovarian cancer cell line SK-OV3, treatment with Hoechst 33342 dye resulted in a decrease in the ability of these cell lines to form clones, suggesting the cytotoxicity of Hoechst 33342 dye could be a biasing variable in the Side Population assay [72]. Furthermore, because of the assay's sensitivity to experimental variables such as incubation time, dye concentration, cell concentrations, and gating variability, SP phenotypes often vary between experiments [73,74]. These observations throw into question the nature of lung cancer SP cells and suggest that further stringent experimentation is needed to determine the ability of this assay to isolate cancer stem cell populations [75].

Another common strategy for isolating human cancer stem cell populations in solid tumors has been the use of flow cytometry to sort tumor cells for the extracellular portions of surface stem cell markers. CD133 (*Prom1*) is a cell surface glycoprotein that consists of five transmembrane domains and two large glycosylated extracellular loops [76]. CD133 and its glycosylated epitope, AC133, have been useful markers in the selection of both human hematopoietic stem cells and neural stem cells [77,78]. Similarly, expression of CD133 and AC133 has been reported to select for brain [10], colon [13,14], pancreatic [15] and most recently, lung cancer stem cells [16]. In 2008, Eramo and colleagues identified rare, highly tumorigenic, self-renewing $CD133^+$ cells in both NSCLC and SCLC specimens [16]. Similar to other cancer stem cell studies, some of these patient lung tumor derived $CD133^+$ cells could be expanded *in vitro* as floating tumor spheres cultured in defined serum free media [11,16,79,80]. These $CD133^+$ enriched spheres were refractory to short term chemotherapy, suggesting that putative $CD133^+$ lung cancer stem cells are resistant to conventional chemotherapy. This drug resistant feature of $CD133^+$ lung cancer cells was confirmed in experiments that revealed long term chemotherapy *in vitro* as well as *in vivo* could enrich for $CD133^+$ lung cancer cells [81,82]. The discovery of putative $CD133^+$ lung cancer stem cells in both SCLC and NSCLC indicate that CD133 may serve as a pan-lung cancer stem cell marker. However, several lines of evidence suggest that the ability of CD133 expression to discriminate lung cancer stem cells may have been overstated. For example, some CD133-negative lung cancer cells also possess the ability to self-renew and generate robust xenografts outgrowth [83]. Also, unlike gliomas where CD133 is a more established cancer stem cell marker, CD133 expression in lung cancer is not associated with patient prognosis [84–86]. Finally, in many lung cancer samples, CD133 is not detected [81,85,86]. Recently, some scientists have questioned the use of CD133 as a selective cancer stem cell marker in other solid tumor types, citing cases where CD133 negative cells also possess the capacity for self-renewal and cancer initiation [87,88]. Furthermore, it has been suggested that the existence of variable CD133 isoforms, extrinsic environmental pressures on CD133 expression and the states of CD133 glycosylation, complicate the use of CD133 and AC133 as a pan-cancer stem cell marker [76,89].

Another method for identifying and selecting stem cell populations is based on aldehyde dehydrogenase activity. Aldehyde dehydrogenase (ALDH) enzymes are a family of intracellular enzymes that participate in cellular detoxification, differentiation and drug resistance through the oxidation of cellular aldehydes [90]. In hematopoietic stem cells, ALDH activity is thought to preserve an undifferentiated cellular state by interfering with endogenous retinoic acid biosynthesis [91,92]. Using flow cytometry to detect and isolate cells with elevated ALDH activity, hematopoietic stem cells and their leukemic counterparts have been identified in both human and murine samples [93–95]. ALDH activity has also been useful in isolating putative human brain [96], breast [97], colon [98], and head and neck squamous cancer stem cell populations [99]. Evidence for ALDH as a relevant lung cancer stem cell marker came in 2008 with the discovery of elevated levels of ALDH protein expression in putative lung stem cell niches during malignant transformation [100]. Also, $ALDH^+$ cells isolated from

NCI-H358 and NCI-H125 lung cancer cell lines are enriched in tumorigenic, $CD133^+$ cancer cells [101]. Importantly, in a limited study high levels of ALDH1 protein expression correlates with poor patient prognosis, a finding consistent with the hypothesis that $ALDH^+$ lung tumor cells are enriched in lung cancer stem cells [101]. Further evidence to support ALDH as a functional marker for lung cancer stem cells is the demonstration that siRNA-mediated knockdown of ALDH proteins results in a decrease in lung cancer proliferation, migration, and ALDH activity [102,103]. Taken together, these reports support that ALDH is a promising functional marker for stem cells in lung cancer. However, additional studies are necessary to determine the context and utility of this marker as a putative lung cancer stem cell marker.

4 Targeting pathways of self-renewal in lung cancer

Stem cell self-renewal is a tightly controlled process that is governed by both signals from the stem cell niche as well as deliberate and regulated control of key developmental pathways such as the Wnt, Hedgehog, and Notch signaling pathways (Fig. 2). Tumor stem cells also undergo self-renewal, however unlike self-renewal in organogenesis, self-renewal in tumorigenesis is also thought to be achieved in part by the deregulation of these key pathways. Cancer stem cells comprise the self-renewing component of tumors; therefore it is hypothesized that the same pathways that govern normal stem cell self-renewal could also govern cancer stem cell self-renewal [1]. For this reason, the prospect of targeting these developmental pathways in tumors has become an appealing strategy for treating tumors that are often intractable to conventional therapy alone [3,104]. The Wnt/β-catenin pathway plays an important role in the regulation of hematopoietic stem cell self-renewal [105,106], however the role of Wnt signaling in lung epithelial stem cells is less well understood [107]. Recently, it was discovered that activated Wnt/β-catenin signaling in the developing lung coincided with an expansion of BASCs and attenuated bronchiolar differentiation [108]. Conversely, conditional Cre-mediated deletion of *Catnb* had no appreciable effect on the repair and maintenance of the bronchiolar epithelium, suggesting the role of Wnt/β-catenin signaling in lung stem cell self-renewal may be niche specific [109]. In lung cancers, evidence of activated Wnt signaling in lung tumors suggests aberrant Wnt signaling may be important for tumorigenesis [110–112]. Recently, inhibition of Wnt signaling by a Wnt-2 monoclonal antibody resulted in the induction of apoptosis in NSCLC cells [113], as well as in other tumor types [114,115]. The prospect of Wnt signaling as a driver of lung tumorigenesis and stem cell self-renewal make the Wnt signaling pathway an appealing target for therapy, however further studies will be necessary to define the context of activated Wnt signaling in lung cancer stem cells, as well as in different types of lung cancer [116,117].

The Hedgehog (Hh) signaling pathway is activated when one of three extracellular Hh ligands (in mammals there are three) sonic hedgehog, desert hedgehog, and Indian hedgehog (ligands) binds to and inactivates its receptor patched (PTCH) (Fig. 2). This binding event relieves the repressive function of PTCH on the downstream protein smoothened (SMO), allowing SMO to activate downstream targets through the GLI transcriptional effectors. The Hh signaling pathway is a key developmental pathway required for proper embryogenesis [118]. In the developing lungs, activated Hh signaling is involved in pulmonary cell fate determination and branching morphogenesis [119,120]. Aberrations in expression and activation of this pathway lead to deformations in development as well as to contribute to tumorigenesis [121–123]. During lung epithelial regeneration after injury, activated Hh signaling is observed in regions of repair and in pulmonary neuroendocrine stem cell niches [49]. In the same study, cyclopamine-mediated suppression of aberrantly active Hh signaling in some SCLCs resulted in a dramatic drop in cell viability and tumorigenicity [49]. These findings provide evidence that SCLC is not only a malignancy that arises from a population of self-renewing PNECs that retain active Hh signaling as well as primitive neuroendocrine features, but that therapeutic targeting of the Hh signaling pathway may suppress stem-like tumor cell self-renewal [49,

124,125]. Recently, activated Hh signaling has been implicated in the self-renewal of myeloid leukemia [126,127], glioblastoma [96], and breast cancer stem cells [128]. The mounting evidence for the role of Hh signaling in tumor cell maintenance and cancer stem cell self-renewal has prompted the development of better and more specific inhibitors of the Hh pathway, some of which are currently in clinical trials for SCLC [129–131].

The Notch signaling pathway is involved in cell fate determination, and during organogenesis and tissue homeostasis Notch-mediated cell-cell interactions dictates the preservation or differentiation of stem cells [132]. Activation of Notch signaling begins when membrane bound Notch ligands bind to receptors on adjacent cells. Upon binding, the intracellular domain of the receptor is cleaved by a gamma-secretase, allowing for the activation of downstream targets, such as the inhibitory basic helix-loop-helix transcription factor Hes1 (Fig. 2)[132]. In the developing lung, Notch signaling appears to be required for determining proximal and distal lung epithelial cell fates [133]. In transgenic Hes1 knockout mice, suppression of Notch signaling at the transcriptional level results in premature and promiscuous neuroendocrine differentiation during lung development [134]. This may be due to the alleviation of Hes1-mediated suppression of Achaete-scute homolog like-1 expression, a basic helix-loop-helix transcription factor required for proneural differentiation [135]. Studies forcing the activation of Notch signaling in the developing lung tissue, either through the ectopic expression of intracellular Notch domains or through gamma-secretase activation, result in the accumulation of distal airway stem cells and a reduction in neuroendocrine and alveolar cell differentiation [136–138]. This suggests that in some lung stem cells activated Notch signaling functions to preserve a primitive and undifferentiated state. In lung cancer, elevated Notch signaling transcripts have been described in NSCLC, however the role of Notch in tumor maintenance remains poorly understood. Suppression of Notch signaling in some NSCLC cells by treatment with a gamma-secretase inhibitor induces cell death and decreased tumor growth in mice [139,140]. Paradoxically, activation of Notch signaling in A549 cells through overexpression of Notch1 leads to a decrease in proliferation and tumorigenic growth in mice [141]. However the apparent discrepancy between these results may be due to the perturbations of Notch signaling through different Notch receptors. Thus, the Notch pathway may be a potent therapeutic target for some cancer stem cell populations. For example, in a study of early passage human glioma specimens, the suppression of Notch signaling by treatment with a gamma-secretase inhibitor reduced the capacity for xenograft growth in mice, and dramatically reduced the proportion of $CD133^+$ glioma stem cells [142]. Using a human specific DLL4 blocking antibody (anti-hDLL4) to suppress Notch signaling in human breast cancer xenografts, Hoey and coworkers observed a dramatic reduction in tumor growth as well as a significant decrease in $CD44^+$ breast cancer stem cells [143]. In addition, when host-derived DLL4 was blocked with a separate monoclonal antibody (anti-mDLL4), tumor growth and tumor vasculature were dramatically reduced, suggesting a dual role for Notch signaling in tumor cell self-renewal and tumor angiogenesis [143]. Although it is not yet clear if lung cancer stem cells require Notch signaling for self-renewal, several reports suggest Notch signaling components are expressed in putative lung cancer stem cell populations and are required for tumor initiation capacity [81,82,144].

5 Challenges to the cancer stem cell model

As the cancer stem cell model has gained acceptance, debate as to the properties that define *bona fide* cancer stem cells has intensified. Indeed, some have argued that inconsistencies in putative cancer stem cell population phenotypes suggest the current cancer stem cell model is a result of misinterpreting experimental results [145,146]. The most stringent functional assay for a cancer stem cell has been the generation of a tumor from a very low dilution (1–1,000 cells) of isolated tumor cells; the idea being that clonal expansion of a tumor is driven solely through rare cancer stem cells. Typically, this entails the injection of small numbers of isolated

human tumor cells into immunocompromised mice, such as non-obese diabetic severe combined immunodeficient (NOD/ SCID) mice, and assaying for the presentation of tumorigenic outgrowth. In 2007, experiments published by Kelly and coworkers challenged the credibility of this assay, when as few as ten unsorted mouse lymphoma cells were reported to transplant the disease in congenic recipient mice [147]. These findings led Kelly and colleagues to conclude that infrequent tumor cell engraftment in previously reported xenogeneic models may be a result of selective microenvironment pressures and therefore this phenomenon may be underestimating the population of tumorigenic cells in some cancers [147]. The validity of these conclusions was extended to the study of solid tumors the following year when Quintana and colleagues demonstrated that modified xenotransplantation assay conditions, such as the use of a more highly immunocompromised variety of NOD/SCID mice lacking the interleukin-2 gamma receptor (NOD/SCID$^{Il2rg-/-}$), resulted in the efficient generation of melanoma tumors from a single human melanoma cell [148]. While it could be argued that in some cancers, such as lymphoma and melanoma, tumorigenic cancer stem cells are not rare, the potential for extrinsic factors to influence tumor "stemness" in other cancer types remains a possibility. Other factors such as tumor hypoxia, stromal derived cytokines and tumor vasculature have also been proposed to alter the presence of cancer stem cells [63, 149–151]. In addition, the process of tumor cell epithelial-to-mesenchymal transition has been suggested as a mechanism for noncancer stem cells to acquire cancer stem cell properties [152,153]. A better understanding of how these factors contribute to tumorigenesis, tumor progression, and tumor heterogeneity will ultimately advance or marginalize the cancer stem cell model.

6 Conclusions and perspective

The cancer stem cell model proposes that tumor progression, metastasis and relapse after therapy may be driven by a subset of tumor cells that possess stem cell capacity to self-renew. Where and how tumor cells have acquired the ability to self-renewal has many researchers investigating the possibility of stem cells as a likely origin for tumorigenesis in many cancers. This premise has been demonstrated in the initiation of lung tumors in transgenic mice. Furthermore, mounting evidence now supports the existence of a subpopulation of self-renewing tumor cells in human lung cancer. These and other reports have begun to build a rationale for targeting pathways of aberrant self-renewal in the treatment of many cancer types. However in the case of lung cancer, translating these findings into new therapies and diagnostic tools can only be accomplished if human lung cancer stem cells can be accurately identified and characterized.

6.1 Key unanswered questions

Currently little is known about the biology of lung cancer stem cells: including their distinguishing properties and proportions in different types of lung cancer, how they evade and resist therapy, and importantly, what drives their self-renewal. In addition, it is not fully understood how cues from the tumor microenvironment might support or determine the cancer stem cell phenotype in lung cancer. Finally, if targeting pathways of self-renewal is to be a viable strategy for treating cancer, then a better understanding of the role these pathways in normal and malignant stem cells should be a priority for future research.

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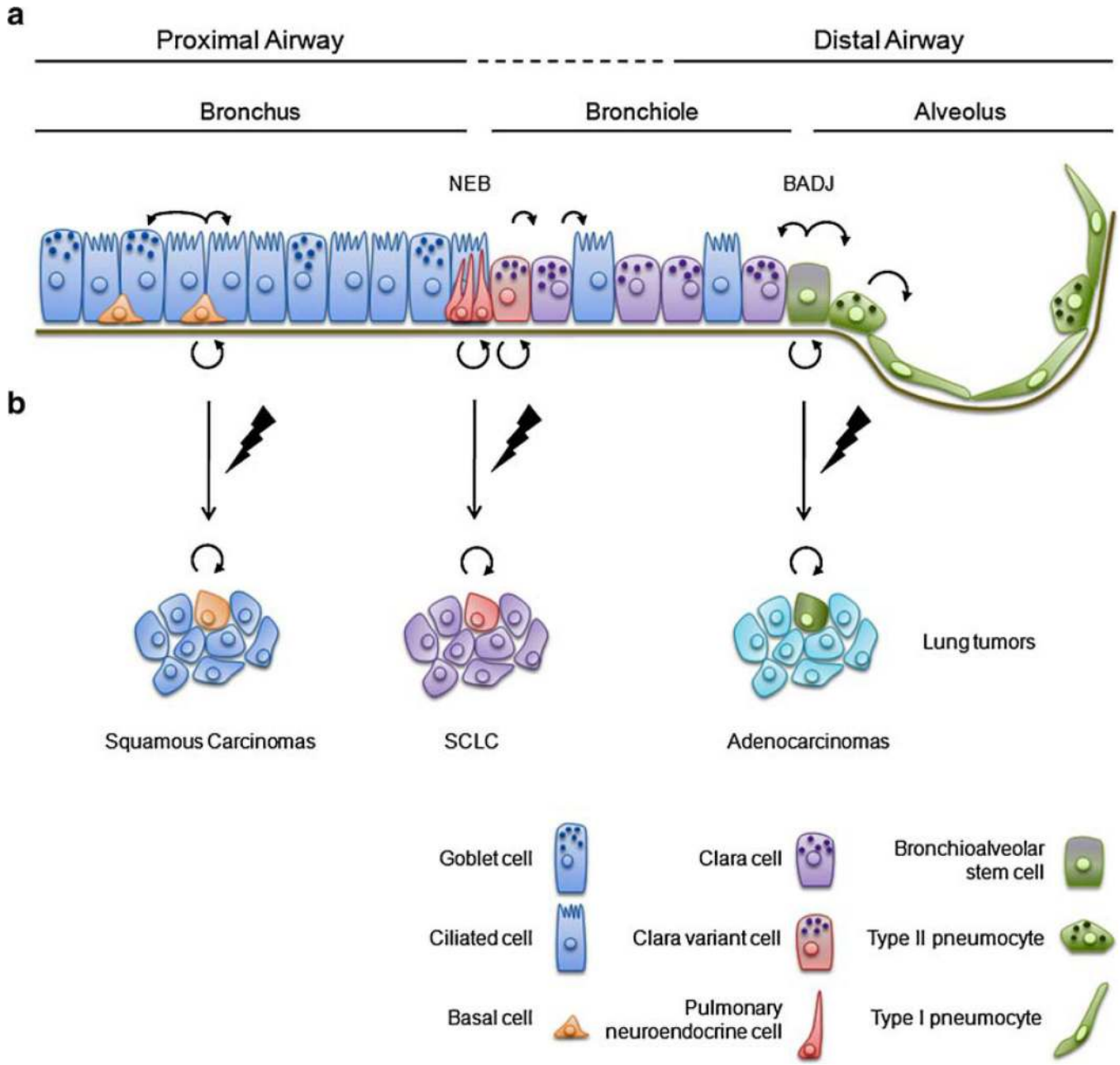

Fig. 1.

Proposed sites of self-renewal and tumor initiation in lung epithelia. Basal cells of the proximal airway, pulmonary neuroendocrine cells which colonize as neuroendocrine bodies (*NEBs*), naphthalene-resistant variant Clara cells, and bronchioalveolar stem cells of the bronchioalveolar duct junction (*BADJ*), are all proposed to be self-renewing stem cell populations in the lung epithelium (**a**). The accumulation of oncogenic mutations (represented by *jagged arrows*) in different stem cell compartments of the lung are thought to give rise to histologically different tumor types in the lung. Within each of these different lung tumor types, a distinct subpopulation of self-renewing tumor cell (shaded tumor cells with *circular arrows* above) is hypothesized to populate and progress the disease (**b**)

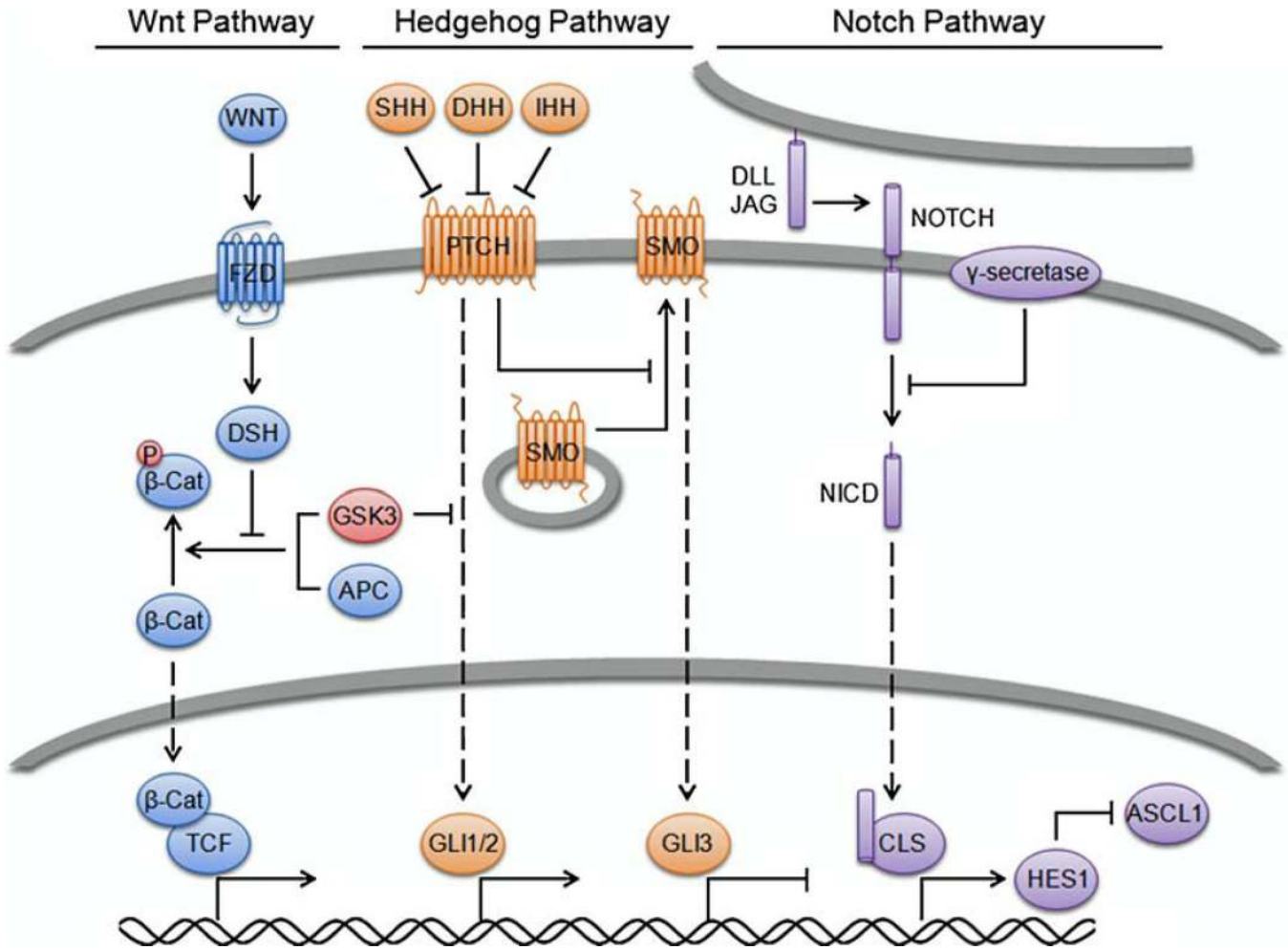

Fig. 2.

The Wnt, Hedgehog and Notch self-renewal signaling pathways. Activation of the Wnt signaling cascade begins when secreted Wnt ligands bind to Frizzled (*FZD*) receptors resulting in downstream stabilization and nuclear translocation of β-catenin. In the absence of Wnt ligand, GSK3-mediated phosphorylation of β-catenin leads to the ubiquitinilation and β-catenin degradation. The binding of the Hedgehog receptor Patched (*PTCH*) to secreted ligands (*SHH, DHH*, and *IHH*) alleviates PTCH repression of membrane translocation and activation of Smoothened (*SMO*). Activated SMO leads to the nuclear translocation of GLI transcriptions factors (*GLI1* and *GLI2*). In the absence Hh ligands, GSK3-mediated phosphorylation of GLI1 and GLI2 lead to ubiquitinilation and degradation, whereas nuclear translocated GLI3 functions to repress target gene transcription. The binding of Notch receptors to membrane bound Notch ligands (DLL and JAG) activates a series of receptor-ligand cleavage events, such as γ-secretase-mediated cleavage of the Notch intracellular domain (*NICD*). The free cytoplasmic NICD is translocated to the nucleus and binds to the CLS family transcription factor complex to activate target gene transcription. Notch signaling target genes include the Hes1 transcriptional repressor which functions to suppress Achaete-scute homolog like-1 (*ASCL1*) expression