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Lung Cancer Stem Cells: Molecular features and Therapeutic Targets

Sandeep Singh, Ph.D.[1] and **Srikumar Chellappan, Ph.D.**[*]

[1]National Institute of Biomedical Genomics (NIBMG), TB Hospital Building, 2nd floor, Kalyani, West Bengal, INDIA

Department of Tumor Biology, H. Lee Moffitt cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612

Abstract

Lung cancers are highly heterogeneous and resistant to available therapeutic agents, with a five year survival rate of less than 15%. Despite significant advances in our knowledge of the genetic alterations and aberrations in signaling pathways, it has been difficult to determine the basis of lung cancer heterogeneity and drug ressitance. Cancer stem cell model has attracted a significant amount of attention in recent years as a viable explanation for the heterogeneity, drug resistance, dormancy and recurrence and metastasis of various tumors. At the same time, cancer stem cells have been relatively less characetrized in lung cancers. This review summarizes the current understanding of lung cancer stem cells, including their molecular features and signaling pathways that drive their stemness. This review also discusses the potential startegies to inhibit the signaling pathways driving stemness, in an effort to eradicate these cells to combat lung cancer.

1. Introduction: Stem cell model of cancer

Lung cancers cause maximum number of cancer-related deaths worldwide and is highly correlated with smoking (Proctor, 2001) (Parkin et al., 2005) (Siegel et al., 2012). The risk of lung cancer remains significantly high for long-term heavy smokers even after smoking cessation. Fifty percent of new lung cancer patients are former smokers and many of them stopped smoking five years or more prior to diagnosis (Halpern and Warner, 1993; Tong et al., 1996). According to an estimate made by the World Health Organization (WHO), lung cancer will cause about 2.5 million deaths per year by the year 2030 (Proctor, 2001). In the United States, approximately 85% of the patients diagnosed with lung cancer die of this disease within five years and this rate has not changed significantly since 1970s (Jemal et al., 2008a; Jemal et al., 2008b). Despite significant advances in our knowledge about cancer, our ability to develop effective therapies to combat lung cancer has been limiting (Hanahan

[*]Corresponding Author Phone: 813-907-1250, Fax: 813-745-6748, Srikumar.Chellappan@moffitt.org..
Note: Both authors contributed equally to this paper.

and Weinberg, 2011). Treatment of the primary lesions rarely prevents the development of the distant metastases, which is the major cause for fatality (Jemal et al., 2008b). These facts highlight a need for better understanding the cellular and molecular events underlying the genesis and metastasis of this disease for designing novel therapeutic strategies. In this context, a school of thought has emerged in the recent years that suggest that tumors arise from a subset of cancer cells, called cancer stem cells, which may remain dormant, have the capacity to evade therapeutic drugs and metastasize. This concept is different from the prevailing theory where all the cancer cells have equal and similar proliferative capacity and opportunity for initiating tumor growth and spread (Nowell, 1976; Visvader and Lindeman, 2008). Further, the cancer stem cell model suggests that cancers are organized into aberrant cell hierarchies which are driven by a subset of cells that have the ability to self-renew themselves and generate heterogeneous lineages of other cell types that comprise the tumor (Figure 1)(Bonnet and Dick, 1997; Clarke et al., 2006). Thus, in principle, agents that can eliminate such cancer stem cells or tumorinitiating cells might be highly effective as anti-cancer agents.

First experimental evidences for the existence of cancer stem cells came in the year 1997, with the identification of leukemia stem cells (Bonnet and Dick, 1997; Lapidot et al., 1994). Later, in the year 2003, the first evidence for hierarchical stem cell origin of solid tumor was experimentally demonstrated in breast cancer (Al-Hajj et al., 2003). However the *de novo* existence of cancer stem cells within solid tumors had remained controversial until very recently (For review, see (Medema, 2013)). In these recent studies using mouse models of brain (Chen et al., 2012), skin (Driessens et al., 2012) and intestinal (Schepers et al., 2012) tumors, three independent groups have provided convincing evidence that cancer stem cells do exist and are responsible for maintaining tumor growth in intact organs.

Self-renewal is a characteristic property of stem cells that allows them to maintain their numbers through symmetric or asymmetric mitotic cell division (Morrison and Kimble, 2006). During asymmetric division, each stem cell generates one daughter cell with stem cell fate (self-renewal) and one daughter cell (progenitor cell) that is destined to differentiate (Clevers, 2005). However, upon injuries or when stem cell pool has to be developed during development, stem cells undergo symmetric cell division where all the divided cells have stem cell fate (Morrison and Kimble, 2006). The fine balance between symmetric and asymmetric modes of division maintains the number of stem cells and its differentiated progeny depending on the developmental signals (Morrison and Kimble, 2006). It is believed that oncogenic transformation of the normal stem cells or the progenitor cells that were derived from normal stem cells results in deregulated self-renewal and give rise to cancer stem cells (Fisher et al., 2001; Hochedlinger et al., 2005; Lo Celso et al., 2004; Smalley and Ashworth, 2003). Aberrant symmetric or asymmetric cell division maintains the number of cancer stem cells within the tumor, whereas its descendent progeny constitute the bulk of the heterogeneous tumor (Clevers, 2005; Morrison and Kimble, 2006; Pardal et al., 2003; Visvader and Lindeman, 2008). These cells eventually undergo different stages of differentiation and are unable to initiate the formation of tumors. Being the only cell type with cancer initiating and driving ability and endowed with extensive replicative potential, the stem-like cancer cells must act as unit of selection during the progression of cancer

(Greaves and Maley, 2012). Therefore, it is important to note that the clonal evolution model may co-exist with the cancer stem cell model. Cancer stem cells may themselves undergo "clonal evolution" by acquiring further mutations to undergo more aggressive self-renewal and growth as demonstrated for leukemia stem cells (Passegue et al., 2003; Visvader and Lindeman, 2008). Adding to the complexity, small number of recent studies now suggests that the differentiation of cancer stem cells may be reversible in certain cancers, and non-tumorigenic cells may also acquire cancer stem cell-like tumorigenic ability under specific circumstances (Chaffer et al., 2011; Chaffer et al., 2013; Gupta et al., 2011; Roesch et al., 2010); however the efficiency of this process under *in vivo* conditions is not clear.

2. Stem Cells in Lung cancer

Comparatively less is known about the biology of lung cancer stem cells compared to stem cell progenitors of other types of tumors. This is in part due to the complexity of the disease in terms of its phenotypic diversity and anatomically distinct sites of origin in pulmonary airways. Lung cancer can be subdivided into small cell lung cancer (SCLC), and three types of non-small cell lung cancer (NSCLC), which are squamous cells carcinoma (SCC), adenocarcinoma and large cell carcinoma. SCLC and SCC occur in the proximal region of the respiratory tract whereas adenocarcinoma originates in the distal airway (Giangreco et al., 2007). The presence of a diverse pool of self-renewing lung epithelial stem cells present in different regions of the respiratory tract has been thought to be responsible for this regional diversity. In support of this hypothesis, originating sites of SCLC, SCC, and adeno-/broncheoalviolar carcinomas appear to coincide with airway stem cell niches in murine models (Giangreco et al., 2009; Rawlins et al., 2009).

Induction of lung cancer in animal models using genetic modification suggests that lung cancer originates from resident stem cells. Classically, genetic modifications utilized comprise of transgenic expression of oncogenes or knockout of tumor suppressor genes, alone or in combination, under the control of lung epithelial cell-specific promoters (Dutt and Wong, 2006). These genetic modifications generate identical mutations throughout the large proportion of the lung and therefore should be capable of generating cancers throughout the entire lung. However, the most originating sites for various lung cancers appear to coincide with identified airway stem cell niches, and therefore suggest their stem cell origin (Rawlins and Hogan, 2006).

SCLC has very poor prognosis and high rate of metastatic dissemination (Jemal et al., 2008b). Human SCLCs predominately localize to mid-level bronchioles and typically express a range of neuroendocrine cell markers, including CGRP and other markers generally expressed within pulmonary Neuroendocrine Cells (PNECs), while markers associated with other cell types are not expressed (Gazdar et al., 1985). The loss of Retinoblastoma (Rb) and p53 functions are closely associated with human SCLC (Meuwissen and Berns, 2005). Despite Rb gene deletion throughout the airways, hyperplasia of PNECs was exclusive in a lung-specific conditional Rb inactivation model (Meuwissen et al., 2003). Similarly, deletion of both Rb and p53 in adult mice resulted in progressive epithelial hyperplasia that was restricted to the neuroepithelial bodies (NEB) microenvironment (Meuwissen et al., 2003; Minna et al., 2003). Importantly, these lesions

progressed to form metastatic tumors resembling human SCLC (Meuwissen et al., 2003). On the basis of these observations, SCLCs are proposed to originate from PNECs. In a recent report, a direct approach has been taken to identify the cell of origin for SCLCs using a cell-type specific Cre-loxP expression model in adult mouse lung (Sutherland et al.). Depletion of Rb and p53 specifically in tracheal and bronchial Clara cells, BASCs, AT-2 cells and PNECs suggested that PNECs are indeed the predominant cancer-initiating population in SCLC. In addition, data also supported the presence of a SPC-positive progenitor cell population that could give rise to SCLC following loss of p53 and Rb, although with a lower efficiency (Sutherland et al.).

Since genetically modified mouse models of human lung-SCC do not exist, SCC is generally induced in mice using chemical carcinogenesis (Henry et al., 1981; Wang et al., 2004; Yoshimoto et al., *1*977; Yoshimoto et al., 1980). Carcinogen induced murine SCCs generally occur in the proximal airways down to the second or third bifurcation and are rarely observed distally, which coincide with the suggested niche for basal stem/progenitor cells (Borthwick et al., 2001; Evans et al., 2001). Histologically, carcinogen induced SCCs are found to be initiated with generalized basal cell hyperplasia (Jeremy George et al., 2007). Additionally, hyperproliferative and preneoplastic lesions in SCC were also found to be basal stem cells specific Keratin-14-positive (Barth et al., 2000). Although direct approaches are needed to confirm the cell of origin for SCCs, current data support a direct relationship between proximal airway basal progenitors and SCC in murine models.

Bronchiolar adenocarcinomas and bronchioalveolar cell carcinomas are the most common lung cancer types among smokers as well as non-smokers (Jemal et al., 2008b). In murine models, adenocarcinomas arise from the junction between the terminal bronchiole and the alveolus termed the “bronchioalveolar duct junction” (BADJ) (Kim et al., 2005). Murine as well as human adenocarcinoma cells express Clara Cell Secretory Protein (CCSP) and/or Surfactant Protein-C (SP-C) markers suggesting that Clara or type II alveolar cells (AT-2) cells function as the originating cells for adenocarcinomas (Fisher et al., 2001; Kim et al., 2005). Additionally, in mouse models where CCSP or alveolar-SP-C promoters were utilized to express oncogenic proteins like mutated epidermal growth factor (EGF) receptor (Ji et al., 2006; Politi et al., 2006), active K-Ras (G12D) (Kim et al., 2005; Xu et al., 2012), dominant negative transforming growth factor-β (Bottinger et al., 1997), or large T antigen (DeMayo et al., 1991; Wikenheiser et al., 1992), all resulted in tumors closely resembling human bronchioalveolar carcinoma. As described above, napthalene resistant, NEB-independent, CCSP and SP-C expressing, Sca1-positive, BASCs were discovered in BADJ region of napthalene injured mouse lung (Kim et al., 2005). In the same study, using the cell-type specific Cre-loxP expression model, Kim and colleagues have demonstrated selective, dose-dependent BASC expansion and bronchioalveolar carcinoma induction after introducing K-Ras (G12D) mutation (Kim et al., 2005). In contrary, recent studies have shown that depletion of K-Ras and p53 or sustained EGFR signaling in SP-C^+ cells led to the development of alveolar tumors without the involvement of BASCs (Lin et al., 2012). Whereas, another study suggested that expression of K-Ras in Clara cells and BASCs results in lung hyperplasia; whereas, its expression in AT-2 cells resulted to lung-adenocarcinoma (Xu et al., 2012).

The above findings provide evidence that normal airway stem cells can act as originating cells for lung cancers. However, in an interesting study, specific expression of H-Ras to pulmonary PNECs using a calcitonin gene-related peptide (CGRP) promoter-driven transgenic construct resulted in the formation of bronchial adenocarcinomas but not SCLCs (Sunday et al., 1999). Similarly, in recent studies, introduction of an activated H-Ras (V12) could convert neuroendocrine type SCLC cells into a completely changed phenotype more closely resembling NSCLC adenocarcinomas (Calbo et al.). These results suggest that the physiological effects of different genetic lesions can also drive the same target cells into divergent differentiation paths. Moreover, it should also be mentioned that the cell of origin is described for those self-renewing stem cells that acquire first oncogenic mutation to destabilize its growth. However, CSC may also arise from restricted progenitors, which acquires the self-renewing properties through genetic or epigenetic mechanisms. For lung tumorigenesis, this hypothesis proposal is supported by a recent report where Sca-1-positive-BASCs were originally proposed as cell of origin for K-Ras (G12D) driven bronchioalveolar carcinoma. However, within the tumors, both Sca-1-positive as well as negative cells acquired cancer stem cells properties, as demonstrated by their ability to initiate secondary tumors when implanted in recipient mice (Curtis et al.). Therefore, cancer stem cells may show more distinct markers than their proposed cell of origin, which represents the major challenge in identification and isolation of these Cancer stem cells from tumors.

3. Detection and association of human lung cancer stem cells with disease progression

The first experimental evidence for the existence of a stem-like clonogenic subpopulation in lung cancer was demonstrated in 1980s (Carney et al., 1982; Carney et al., 1980). In these pioneering studies, only a small proportion of SCLC and lung adenocarcinoma cells from patient samples demonstrated the ability to generate colonies in soft agar (Carney et al., 1982; Carney et al., 1980). Transplantation of these colony forming cells displayed tumorigenic potential in athymic nude mice (Carney et al., 1982; Carney et al., 1980). Since then, researchers have attempted to define lung cancer stem cells by various experimental approaches. Presently, these cells are isolated by flow cytometry using the presence of stem cell specific cell surface markers or by their functional properties, as described below.

The side population phenotype is a specific functional property described to isolate normal human hematopoietic stem cells from bone marrow population (Goodell et al., 1996). It is based on the ability of drug transporters to efflux the Hoechst 33342 dye via the ATP-binding cassette (ABC) family of transporter proteins, mainly ABCB1 (P-glycoprotein, MDR1), ABCC1-5 (multidrug-resistant proteins, MRP1-5), and ABCG2 (breast cancer resistance protein, BRCP1). These proteins are specifically expressed within the cell membrane of stem cell populations (Golebiewska et al.; Zhou et al., 2001). Hoechst 33342 dye excluding cells, termed ‘Side Population’ cells (SP cells), have been described in a variety of tumor types, where they have been shown to display increased capacity for self-renewal and tumorigenicity when transplanted into immunocompromised mice (Golebiewska et al.; Wu and Alman, 2008). SP cells were detected in various human

NSCLC cancer cell lines and had several *in vitro* properties typical of stem cells, including clonogenic proliferation, invasive phenotypes, multi-drug resistance, and increased telomerase (hTERT) as well as lower levels of DNA replication associated protein MCM7 as compared to rest of the non-SP (main population, MP) cells (Ho et al., 2007). Similarly, SP cells were detected in primary tumors obtained from lung cancer patients. Like NSCLC cell lines, SCLC cell lines such as NCI-H82, H146 and H526 also demonstrated the presence of SP cells with tumorigenic potential. Gene expression profiling revealed the upregulation of several pluripotency associated genes such as Klf4, Nanog, Numb, Oct4 and Notch1 in SP cells (Salcido et al.). Our recent studies also showed a subset of SP cells in lung cancer cell lines and lung cancer specimens (Singh et al., 2013; Singh et al., 2012). SP cells were found to be expressing higher levels of ABCG2, Oct4, Sox2, Nanog, Twist, Snail, Slug, Vimentin and Fibronectin (Singh et al., 2012). Further, SP cells were mostly accumulated in the G0/G1 phase of cell cycle and were able to trans-differentiate into angiogenic tubules (Singh et al., 2013). Using systems biology approach for the differentially expressed genes among SP and MP cells, we obtained the signature of five genes which is able to predict the prognosis of NSCLCs (Perumal et al., 2012). These lines of evidence support the notion that the side population assay selects for cancer stem cells in lung tumors; however, experimental variables such as incubation time, dye concentration, cell concentrations, and gating variability may produce different frequency of SP cells from the similar type of samples (Golebiewska et al.) Therefore, a common experimental procedure needs to be proposed to avoid the variability from one laboratory to other.

Another method for identifying and isolating stem cell population is based on the high aldehyde dehydrogenase activity of stem cells. Aldehyde dehydrogenase (ALDH) is a family of intracellular enzymes that participates in cellular detoxification, differentiation and drug resistance in stem cells (Moreb et al., 1996). ALDH activity is found to regulate the self-renewal of hematopoietic stem cells by inhibiting the endogenous retinoic acid biosynthesis (Chute et al., 2006). Flow cytometry has been used to detect and isolate cells with elevated ALDH activity; this technique has led to the successful isolation of putative cancer stem cells from a variety of human cancers (Alison et al.; Moreb, 2008). Evidence for ALDH as a relevant cancer stem cell marker for the lung came with the discovery of elevated levels of ALDH-isoforms, ALDH1A1 and ALDH3A1 protein expression in SCC and adenocarcinoma patients (Jiang et al., 2009a; Patel et al., 2008). Additionally, higher expression of these isoforms of ALDH was also detected in putative lung stem cell niche for adenocarcinoma (Patel et al., 2008). In another study, more than 200 NSCLC tumor samples were analyzed for the expression of ALDH-isoforms and high expression of ALDH1A1 was strongly associated with reduced patient survival for Stage I NSCLC (Okudela et al., 2012; Sullivan et al.). Our study has demonstrated the higher ALDH activity in SP cells in NSCLC cell lines (Singh et al., 2013). NSCLC cells with high ALDH activity showed more tumorigenic and clonogenic activity as compared to the cells with low its activity, supporting high ALDH activity as a putative lung cancer stem cell phenotype (Sullivan et al.). However in a recent study with 268 cases of NSCLCs, authors have reported an inverse relationship between ALDH1A1 expression and tumor aggressiveness (Okudela et al., 2013). ALDH1A1 expression was found to be decreased among smokers and least expressed among poorly differentiated adenocarcinomas and large cell carcinomas (Okudela et al.,

2013). These observations suggest that the functional properties may not be sufficient as an independent marker for the cells with stem cells characteristics. Similar to ALDH, glycine decarboxylase (GLDC) is proposed as another metabolic enzyme driving tumorigenesis and stem-cell characteristics in NSCLCs (Zhang et al., 2012). Higher expression of GLDC was found in lung cancer stem cells isolated from primary NSCLC tumors with stages I-III and its aberrant expression was associated with poor survival of lung cancer patients (Zhang et al., 2012).

Identification and isolation of putative cancer stem cells is also commonly carried out based on stem cell specific cell surface phenotypic markers. One of such markers successfully used to isolate lung cancer stem cell is CD133 *(Prom1)*, either alone or in combination with other markers; it is a cell surface glycoprotein that consists of five transmembrane domains and two large glycosylated extracellular loops (Mizrak et al., 2008). CD133 and its glycosylated epitope, AC133, have been useful in the selection of normal human hematopoietic and neural stem cells as well as for brain, colon and pancreatic cancer stem cells (Keysar and Jimeno, 2010). Highly tumorigenic, self-renewing $CD133^+$ cells in both NSCLC and SCLC specimens were isolated from single cell suspensions of whole tumor (Eramo et al., 2008). Alternatively, through retrospective approach, all the tumor forming cells from both SCLC and NSCLC were allowed to grow in serum free, stem cell selective media in a non-adherent condition. This strict culture condition allows the expansion of only self-renewing stem cells as spheres. All the sphere forming cells displayed CD133 expression, self-renewal and differentiation to specific lineage as well as recapitulated the hetrogeneous tumor in recipient mice (Eramo et al., 2008). 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. CD133-expressing stem-like cells isolated from NSCLC patients were found to be resistant to Cisplatin treatment, suggesting the drug resistant phenotype of cancer stem cells (Bertolini et al., 2009). However, the existence of variable CD133 isoforms and CD133 glycosylation states are also reported, which complicates the detection of CD133 and AC133 in whole tumor as well at single cell level (Bidlingmaier et al., 2008; Mizrak et al., 2008). Clinical significance of CD133 expression in human lung cancer still needs to be completely validated (Salnikov et al., 2010). Few recent studies have explored CD133 expression as prognostic markers for NSCLC. Immunohistochemical study using NSCLC tumors reveled that high CD133 expression correlates with poor prognosis in NSCLCs (Mizugaki et al., 2013; Okudela et al., 2012; Shien et al., 2012). In contrast to these findings, a study done on 133 stage I/II NSCLC patients did not find any significant prognostic correlation for the stem cell markers CD133, ABCG2 or CD117 (Herpel et al., 2011). Owing to the inter- and intra-tumor heterogeneity in cancer, these results suggest that the independent stem cell markers may not have the prognostic value in all types of lung cancer. This notion is supported by the observation that the expression of CD133 and ALDH1A1 is correlated with shortest recurrence free survival and overall survival among 205 stage-I NSCLC patients following surgical resection (Alamgeer et al., 2013). Similarly, CD133 coexpression with Oct4A transcription factor is associated with shortest disease free intervals among 64 adenocarcinoma patients (Cortes-Dericks et al., 2012).

4. Molecular targeting of lung cancer stem cells

In the stem cell model of cancer, the initiation and progression of cancer depends on the deregulated self-renewal of cancer stem cells. A number of genes like *notch, wnt and shh*, which are involved in maintenance and self-renewal of normal tissue stem cells, are found to be oncogenes in various cancers. These observations suggested that the pathways that govern normal stem cell self-renewal could also govern stem cell self-renewal in cancer as well. Therefore, identification of the developmental pathways involved in self-renewal of cancer stem cells for specific tumors has become an appealing strategy for finding the suitable target for treatment (Dalerba et al., 2007). There is only a handful of information available for the mechanism of self-renewal of normal or cancer stem cells for lung, which is summarized below.

The Wnt protein-mediated activation of Frizzled receptors leads to β-catenin accumulation and nuclear translocation. This Wnt/β-catenin pathway regulates the self-renewal of hematopoietic stem cells (Kirstetter et al., 2006; Reya et al., 2003); however, its role in lung epithelial stem cells is less understood (Stripp and Reynolds, 2008). Recently, activated Wnt/β-catenin signaling has been correlated with lung epithelium regeneration. Expression of constitutively active form of β-catenin, specifically in Clara cells revealed the expansion of BASCs upon naphthalene injury for lung epithelium regeneration (Reynolds et al., 2008). However in another study, deletion of β-catenin specifically in Clara cells had no impact on repair of naphthalene-injured airways and BASC expansion (Zemke et al., 2009). These contrasting studies suggested a context dependent role of Wnt/β-catenin signaling in lung stem cell self-renewal and need further analysis. Higher expression of Wnt proteins and aberrant Wnt signaling has been reported in lung cancer progression (Lemjabbar-Alaoui et al., 2006; Uematsu et al., 2003a; Uematsu et al., 2003b). Inhibition of Wnt signaling by a Wnt-2 monoclonal antibody induced cell death in NSCLC cells (You et al., 2004). However, mRNAs analysis for multiple Wnt in NSCLC cell lines and primary lung tumors revealed markedly decreased expression of Wnt-7a, compared to normal bronchial epithelial cell lines and normal lung tissue. Ectopic expression of Wnt-7a in NSCLC cell lines reversed cellular transformation, decreased anchorage-independent growth, and induced epithelial differentiation in a subset of the NSCLC cell lines, suggesting a tumor suppressor role of Wnt-7a (Winn et al., 2005; Winn et al., 2006). The possible association of Wnt signaling with stem cell self-renewal and lung tumorigenesis suggests its importance; however, further studies will be necessary to confirm the involvement of aberrant Wnt/β-catenin signaling in lung cancer stem cell self-renewal (Daniel et al., 2006; Reya and Clevers, 2005). Further, in a study using 200 lung adenocarcinoma samples, the coexpression of insulin-like growth factor I receptor (IGF-IR) with β -catenin and POU5F1 was found to be associated with poor prognosis. IGF-IR mediated signal induction resulted in activation of PI3K/AKT/GSK3β signaling, leading to the co-localization of β-catenin, Sox2 and POU5F1 with increased frequency of CD133 and ALDH-positive cells (Xu et al., 2013).

The Hedgehog (Hh) signaling pathway is a key developmental pathway during embryogenesis (Litingtung et al., 1998). The Hh signaling pathway is activated by sonic hedgehog (shh), a mammalian Hh ligand involved in pulmonary cell fate determination and branching morphogenesis (Bellusci et al., 1997; Pepicelli et al., 1998). In response to

naphthalene injury, activated Hh signaling was observed in airway repair and epithelial regeneration and increased the numbers of neuroendocrine cells in PNECs niches (Watkins et al., 2003). Aberrations in expression and activation of this pathway also led to the development SCLCs (Goodrich and Scott, 1998; Nilsson et al., 2000; Taipale and Beachy, 2001). Suppression of aberrant Hh signaling in some SCLCs resulted in a dramatic drop in cell viability and tumorigenicity, therefore representing a suitable therapeutic target against SCLC (Vestergaard et al., 2006). However, a recent study did not find any significant correlation between Hh signaling pathway proteins with recurrence free or overall survival in 248 cases of early stage NSCLCs (Raz et al., 2012). Thus, there appears to be a certain amount of ambiguity regarding the contribution of the Hh pathway to the stemness of NSCLC initiating cells and the utility of targeting this pathway to combat this disease.

The Notch signaling pathway is one of the important cell fate determinants during tissue homeostasis. Upon the binding of Notch ligands to receptors on adjacent cells, the intracellular domain of the receptor is cleaved by a γ-secretase, allowing for the activation of downstream targets, such as the inhibitory basic helix-loop-helix transcription factor Hes1 (Artavanis-Tsakonas et al., 1999). Notch signaling appears to be required for determining proximal and distal lung epithelial cell fates during lung development (Collins et al., 2004). The indirect effect of Notch signaling has been demonstrated during lung development in a Hes1 knockout mouse model where inhibition of Notch signaling resulted in premature differentiation of pulmonary neuroendocrine stem cells (Ito et al., 2000). In other studies, activation of Notch signaling, either through the ectopic expression of intracellular Notch domains or through γ-secretase activation resulted in an increased number of distal airway stem cells, through reduced differentiation of neuroendocrine and alveolar stem cells (Dang et al., 2003; Guseh et al., 2009; Tsao et al., 2008). These results suggested that activated Notch signaling preserves the undifferentiated state of pulmonary stem cells. In lung cancer, while elevated Notch signaling transcripts have been described in NSCLC, the role of Notch in tumor maintenance remains poorly understood. Utilizing a Notch-GFP-reporter construct, a subset of cells with higher expression of Notch (GFP bright) is found to demonstrate stem-cell like properties in NSCLC cell lines (Hassan et al., 2013). Poor clinical outcome was observed for lung-adenocarcinoma patients with higher Notch ligand expression (Hassan et al., 2013). Suppression of Notch signaling in some NSCLC cells by treatment with a γ-secretase inhibitor induced cell death and decreased tumor growth in mice (Haruki et al., 2005; Konishi et al., 2007). Recently, elevated expression of Notch signaling responsive transcripts was reported in putative lung cancer stem cells with high ALDH activity. Using a γ-secretase inhibitor to suppress Notch signaling or ShRNA-mediated knockdown of NOTCH3 in lung cancer cells led to a significant reduction in $ALDH^+$ cells (Sullivan et al., 2010). It suggests that Notch signaling may be activated in putative lung cancer stem cell populations is required for tumor initiation capacity (Bertolini et al., 2009; Jiang et al., 2009b; Levina et al., 2008). Activation of Notch signaling is also associated with resistance against platinum based chemotherapy and concurrent increase in CD133-positive cells in cell line based studies (Liu et al., 2013). Both γ-secretase inhibitor and shRNA against Notch1 remarkably reduced cisplatin-induced enhancement of CD133-positive cells and increased the sensitivity against doxorubicin and paclitaxel in lung cancer (Liu et al., 2013).

Transcription factors Oct4, Sox2 and Nanog have been identified as core transcription factors that maintain embryonic stem cell self-renewal (Kim et al., 2008). Several lines of evidences suggest the involvement of Sox2 in normal lung development (Gontan et al., 2008; Que et al., 2009). Sox2 depletion in developing lung result in significant decrease in basal, ciliated and Clara cells as well as increased numbers of mucus-secreting cells, suggesting its role in normal differentiation during embryonic lung development (Tompkins et al., 2009). In adult lung, Sox2 expression was found to be crucial for proper repair of airway epithelium upon SO_2 induced injury (Tompkins et al., 2009). Sox2 depletion in basal stem cells resulted in suppressed undifferentiated proliferation *in vitro*, suggesting the role of Sox2 in self-renewal of basal stem cells in lung (Tompkins et al., 2009). Several studies have demonstrated the amplification of Sox2 in SCCs of lung (Bass et al., 2009). Further, high Sox2 expression was correlated with *in vitro* cell proliferation and anchorage independent growth of SCC cell lines, signifying its role as an oncogene (Bass et al., 2009). The oncogenic role of Sox2 was demonstrated using Sox2 overexpressing *in vivo* mouse model of lung cancer (Lu et al., 2010). Sox2 was specifically expressed in SP-C-positive developing lung or *scgb1a1*-positive adult lung airway stem cells which resulted in epithelial hyperplasia and adenocarcinoma development (Lu et al., 2010). Further, immunohistochemical study on stage-I lung adenocarcinoma patient samples revealed a strong prognostic correlation (Sholl et al., 2010). Higher Sox2 expression was significantly associated with decreased overall survival for both male and female patients (Sholl et al., 2010). These studies strongly suggest the role of Sox2 transcription factor for normal lung stem cells maintenance and lung development as well as correlated with the lung cancer progression. Studies from our laboratory had found a role for Sox2 in lung cancer stem cells maintenance and self-renewal (Singh et al., 2012). We observed that that SP cells from NSCLC cell lines express higher levels of *Sox2*. Depletion of Sox2 resulted in decreased side population frequency, indicating its direct role in maintaining the self-renewal of SP cells in NSCLCs (Singh et al., 2012). Under hypoxic conditions, Oct4 and Sox2 induced by HIF1α and HIF2α was found to up-regulate CD133 promoter in NSCLC cell lines (Iida et al., 2012) and overexpression of Oct4 and Nanog in NSCLC cell lines induces stem cell properties like self-renewal, tumorigenesis, invasion and metastasis (Chiou et al., 2010). Astudy using 64 tumor and non-tumor biopsies of lung-adenocarcinoma patients found short disease free survival correlating with high expression of Oct4A and CD133 (Cortes-Dericks et al., 2012). Depletion of Oct4 in $CD133^+$ cells resulted in decreased self-renewal of these cells (Chen et al., 2008). Immunohistochemical studies support the role of Oct4 and Nanog in lung adenocarcinoma progression. The high levels of Oct4 and Nanog was positively associated with moderate and poorly differentiated grade of adenocarcinoma as well as poor overall survival of the patients (Chiou et al., 2010). However, lack of Oct4 and Nanog expression was reported in low grade as well as lower stage adenocarcinoma (Chiou et al., 2010), whereas Sox2 was positively expressed irrespective of the stage or grade of lung cancer (Sholl et al., 2010). These observations strongly raise the possibility that Sox2 may regulate self-renewal of cancer stem cells independently of Oct4 and Nanog in lung cancer.

Resistance to chemotherapy is partly correlated with the presence of stem-like cancer cells. It has been suggested that prolonged exposure of cisplatin resulted in stem cell like characters including decreased proliferation, accumulation of resistant cells in G0/G1 phase

of cell cycle, enhanced colony formation ability, higher ALDH activity, increased frequency of CD133 and CD44 positive cells and higher expression of Oct4/ Sox2/ Nanog and EMT markers like C-Met and β-catenin (Barr et al., 2013). Exposure to 5-FU was also found to enrich stem-like cancer cells in lung adenocarcinoma cells (Shi et al., 2013). Resistance against DNA damaging agents was tested by examining the alteration of DNA-damage repair proteins in stem-like cells from a panel of NSCLC cell lines (Lundholm et al., 2013). Increased basal γH2AX (H2A histone family, member X) expression and diminished DNA damage-induced phosphorylation of DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia-mutated (ATM), Krüppel-associated protein 1 (KAP1) and monoubiquitination of Fanconi anemia, complementation group D2 (FANCD2) after Irradiation or Cisplatin treatment (Lundholm et al., 2013).

Aberrant oncogenic signaling responsible for deregulated self-renewal growth may offer effective therapy specific to stem-like cancer cells. We have found that the self-renewal of SP cells among NSCLCs is regulated through activated EGFR/Src/Akt oncogenic Signaling (Singh et al., 2012). Treatment with Erlotinib, Gefitinib, Dasatinib and PI3K inhibitor LY294002 resulted in marked decrease in self-renewal of SP cells in our cell line based study (Singh et al., 2012). Recently, resistance to anti-EGFR therapy is correlated with enrichment of stem-like cancer cells with overexpression of ALDH1, increase in SP cells and enhanced self-renewal growth (Shien et al., 2013). However, these resistant cells were found to be sensitive against histone deacetylase and proteasome inhibitors (Shien et al., 2013). Similarly, in our study we also have found that higher levels of Mcl-1 were expressed in SP cells compared to MP cells at both transcriptional and translational levels. Irrespective to the EGFR-inhibitor sensitivity or resistance, Obatoclax, a pharmacological inhibitor of Mcl-1, could effectively prevent the self-renewal of SP cells among NSCLCs (Singh et al., 2013). Interestingly, it has been reported recently that endogenous inhibitor of angiogenesis known as tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) can modulate SP phenotype and function, and suggests that TIMP-2 may act as an endogenous suppressor of the SP cells in human lung cancer cells, independent of its activity to inhibit matrix metalloproteinases (Han et al., 2013). Thus, it appears that there are mutliple signaling molecules and pathways that contribute to the functioning of lung cancer stem cells, and many of these might be targeted to combat this disease.

5. Conclusions

The cancer stem cell model has gained considerable support recently in context of lung cancers and stem-like cells are associated with aggressive cancer behavior, metastatic progression, resistance to therapy and relapse. These stem-like cells have been characterized from primary tumors, human-tumor xenografts in mouse or established cell lines using several diverse markers. This ambiguity in the nature of specific stem-cell markers for non-small cell lung cancer might be due to cellular inter- as well as intratumoral heterogeneity among stem-like cancer cells, depending on the histological subtype of NSCLC. This lack of specific identification markers for the diverse pool of stem-like cancer cells may represent a major hurdle for translating the cancer stem cell concept into improving the therapeutic strategies against lung cancer. Further, approaches have been taken to target stem-like cancer cells by controlling developmental pathways involved in self-renewal of cancer stem

cells, even though these pathways are not exclusive to only stem-like cancer cells. There are not many studies yet which describe a specific mutation driving an oncogenic signaling pathway is correlated with aberrant developmental signaling and self-renewal growth of these cancer stem cells. Thus, further understanding drivermutation driven cellular signaling in the context of aberrant self-renewal and differentiation of cancer stem like cells would facilitate targeting of these cells for cancer therapy. Moreover, since lung cancer stem cells are thought to consist of a heterogeneous population depending on the histology and site of tumors, multiple signaling pathways might have to be targeted to effectively eliminate them for therapeutic benefit. It can be imagined that the multidisciplinary efforts currently under way to characterize and target stem-like cells in lung cancer will reap significant therapeutic benefits in the future.

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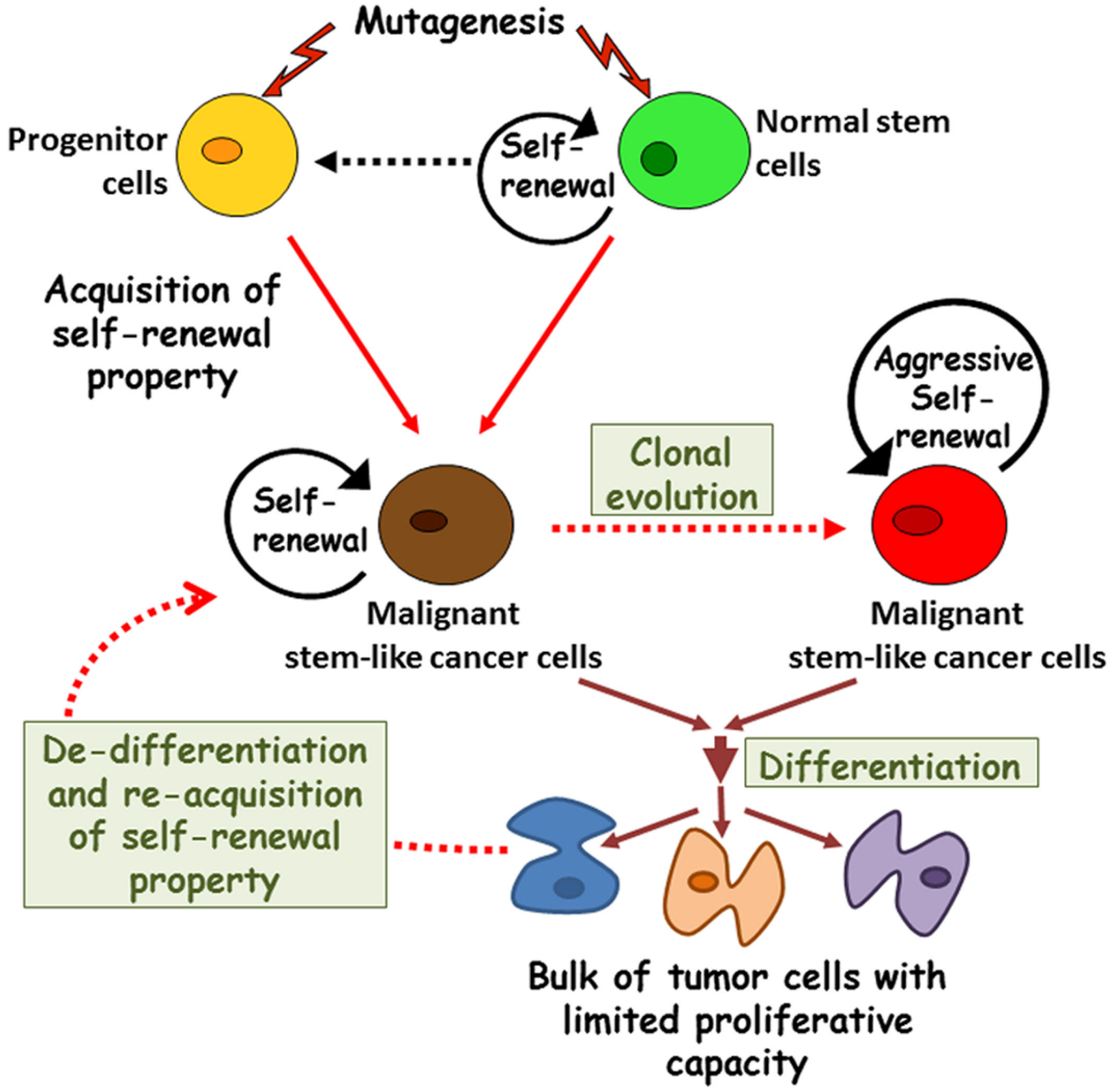

Figure 1. Origin of heterogeneity among stem-like cancer cells

This diagram depicts our current understanding of stem cell model of cancer. Normal tissue stem cells with intrinsic properties of selfrenew and multi-lineage differentiation acquire oncogenic mutations which results in their deregulated self-renewal and give rise to stem-like cancer cells. Additionally, mutations might also cause restricted progenitor cells to acquire self-renewal property and become malignant stem-like cancer cells. These cells self-renew themselves as well as differentiate to generate phenotypically diverse cancer cells, which constitute the bulk of the heterogeneous tumor. During cancer progression stem-like cancer cells may evolve and change in genotype and phenotype to produce subclonal

heterogeneity. Recent evidence also suggests the potential for reversal of mature cancer to re-acquire the stem-like properties through de-differentiation.