

Lung cancer stem cells – origin, characteristics and therapy

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Abstract: Cancer stem cells (CSCs) have gained an increasing attention recently in cancer research. CSCs have ability to generate new tumor through their stem cell properties, essentially self-renewal potential and differentiation into multiple cell lineages. Extensive evidences report that CSCs are resistant to many conventional therapies and mediate tumor recurrence. CSCs of lung cancer are well recognized by their specific markers such as CD133, CD44, ABCG2 and ALDH1A1 together with the CSC characteristics including spheroid and colony formation. Targeting these surface proteins with blocking antibodies and inhibition of ABC transporters and aldehyde dehydrogenase (ALDH) enzymes with small molecules may prove useful in inhibiting tumor progression. The Hh, Notch and Wnt pathways are key signaling cascades that govern cell fate during development and have been shown to be involved in CSCs in various solid tumors. Therapeutic approaches also target these signaling pathways in repressing the tumor progression. This review will focus on stem cell origins, role of signaling pathways, stem cell markers and therapeutic approaches specific to lung cancer.

Keywords: Cancer stem cells (CSCs); CD44; CD133; Hh pathway; aldehyde dehydrogenase (ALDH); CD90

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Introduction

Lung cancer has remained the most common occurring cancer globally. Traditional methods like surgery, radiotherapy and chemotherapy are the treatment methods for lung cancer. Yet resistance to radiotherapy and chemotherapy, relapse in patients has always remained a challenging issue in lung cancer treatment. This resistance is attributed to a class of cells known as cancer stem cells (CSC) (Figure 1). CSCs constitute a unique subset of cells of tumor cells which indefinitely enable the growth of a malignant population of cells (1).

The CSC hypothesis is now the widely studied field in oncology. CSC hypothesis suggests that cell heterogeneity within a tumor is due to the presence of a small subpopulation of cells that display the properties of normal

somatic stem cells (2). Several attributes of somatic stem cells and CSCs are similar such as self-renewal capacity, the ability to differentiate and produce multi-lineage progeny that are tumorigenic and non-tumorigenic, the capacity to establish and maintain tumors, and flexibility in attaining these characteristics (3). The characteristic properties of lung CSCs are characterized by several studies as; self-renewal, multipotent differentiation, tumorigenic potential, expression of stem cell markers, increased invasiveness, proliferation as tumor spheres, chemo resistance, radio resistance to hypoxia, resistance to apoptosis, quiescence (4).

The intrinsic resistance of these CSCs to therapy is the result of increased telomere length (5), activation of anti-apoptotic pathways, increased membrane transporter activity (6) and the ability to migrate and

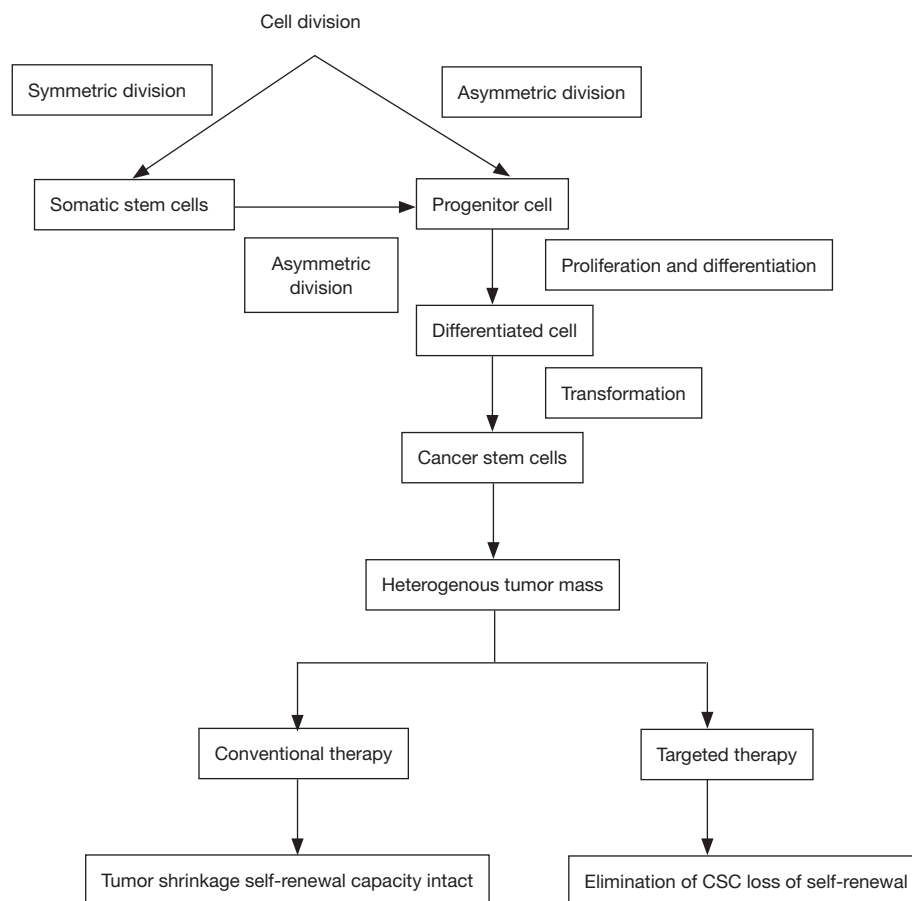


Figure 1 The origin of cancer stem cells.

metastasize (7). Lung CSCs display longer telomeres than non CSC counterparts. Serrano *et al.* (8) showed that treatment with the specific telomerase inhibitor, MST312, had a strong and preferential anti-proliferative effect on the lung CSC population *in vitro* and *in vivo*. ATP-binding cassette (ABC) transporters, such as p-glycoprotein and multidrug resistant associated protein (MRP1), are membrane transporters that can pump structurally unrelated small molecules, such as cytotoxic chemotherapeutic drugs out of the cell. Normal stem cells and lung CSCs express high levels of ABC transporters resulting in low intracellular drug concentrations (9). Studies indicate the high expression of ABCG2 gene in lung CSCs but are switched off in most terminally differentiated progeny (10).

CSCs origin

The origin of CSCs is still under question and the different theories proposed for origin of CSCs include cell fusion,

horizontal gene transfer, cell microenvironment and mutations (11,12).

Cell theory proposes that CSCs could be originating due to cell fusion between tumor cells and bone marrow derived cells that come from a tissue suffering chronic inflammation (13). This theory had been supported by findings of an *in vivo* animal model study done by Houghton *et al.* (14). The study emphasized that bone marrow-derived cells (BMDC) undergo metaplasia and dysplasia eventually leading to cancer. Mouse bone marrow cells can also undergo spontaneous cell fusion and take up the phenotype of the other cell (15). In addition, researchers suggest that cell fusion could also be the source of recurrent CSCs. These are basically the cells that originate after the first set of therapy and trigger cancer. It is also known that the tumor tissue after therapy consisted of fusogenic cells such as CSCs, tumor cells, macrophages/monocytes and BMDC (16). Similar studies on humans proved that the BMDC from donor and tumor cells of recipient

fused together thereby increasing the malignancy of the tumor (17). Moreover recent studies on human cancer demonstrated that BMDC/tumor cell hybrid creates cells that are resistant to radiotherapy and have enriched cell repair mechanisms (18). Though these studies do not directly provide evidence for origin of stem cells, it is clear that cell fusion is highly likely to give rise to CSCs.

Horizontal gene transfer (HGT) which played a major role in dispersion of antibiotic resistance has also been discovered to be involved in cancer progression (19). *In vivo* studies have proved that apoptotic cells transfer their genes to the recipient cells during the process of phagocytosis (20). Another theory arose from the fact that cell-free DNA is capable of circulating in the eukaryotes until being taken by a recipient cell. Animal model studies demonstrate colon cancer progression through horizontal gene transfer in immunocompetent mice (21,22).

CSCs can accumulate more genetic mutations that boost the cancer progression and resistance through HGT. Mutations are well known for causing cancer through inheritance, environmental carcinogens or DNA replication errors (23). Merkle *et al.* demonstrated that human stem cells are capable of taking up large copy number variants. Five out of the 140 cell lines they tested had six mutations that are dominant in Tp53 gene. Tp53 gene was responsible for suppressing tumor growth in humans. The mutant allele was observed to escalate with the passage number revealing that stem cells accept mutations that confer them with growing abilities. This also necessitates the need to genetically analyze the stem cells before using them for therapies or clinical applications (24).

Microenvironment of a cell is made of factors that influence the cell conditions and behavior either directly or indirectly. Some of these factors include the extracellular matrix (ECM), hormones, neighboring cells and forces acting as a result of movement of the host (25). The factors contributing to cancer progression are mostly common in several different cancers. The stem cell micro environmental control plays a major role in maintaining the plasticity of the cell. Any errors in the control can lead to dedifferentiation of stem cells thereby causing cancer. A study led by Kidd *et al.* showed that mesenchymal stem cells (MSC) exhibit tropism in response to a cytokine environment. Interestingly, such cytokine environments are seen in tumor growth areas which in turn allow the incorporation of MSCs in the cancerous region (26). Moreover MSCs are basically derived from bone marrow which implies that cell fusion and microenvironment could

synergistically act to develop cancers (27). Moreover it has also been reported that *Helicobacter felis* infection in mice stimulates the flow of bone marrow stem cells into the stomach. Due to lineage difference, it is suggested that this influx could lead to stomach cancer (12). Additionally studies have also proved that inflammatory microenvironments support the pre-cancerous lesion growth and tumor genesis. The cancer microenvironment is constituted of a thicker ECM than that found in the normal one. Tumor microenvironments holding different factors can also indirectly facilitate the tumor heterogeneity and resistance to chemotherapy (28,29).

Another new theory on origin of CSCs is based on autoreactive T-cells. The researchers suggested that CSCs emerge from autoreactive T-cells that are not completely killed by a weak immune system (30). This brings about a new approach in cancer therapy where we need to focus on bolstering the immune system rather than weakening it.

The origin of lung CSCs has been traced back to cells on specific anatomical sites on lungs. The basal cells of proximal airway (trachea and bronchi) associated with squamous cell carcinoma exhibiting stem cell like behavior was found to over express keratin. Clara cells and pulmonary neuroendocrine cells (PNEC) associated with small cell lung cancer exhibiting stemness had altered expression of secretoglobin and calcitonin related peptide. Lung adenocarcinoma and bronchoalveolar carcinoma has been associated with stem cells from the bronchoalveolar duct junction region. Studies on mouse models demonstrated the association of Matrixmetalloproteinase-10 (MMP10) with the maintenance and tumorigenicity of lung CSC (31,32).

Lung CSC markers

Though the current knowledge on lung CSC biology is limited, a number of CSC markers have been identified and studied. These CSC markers are associated with resistance to anti-cancer therapies. Few of them include; include aldehyde dehydrogenase (ALDH1), CD133, side population (Hoechst-negative), CD44, CD87 and CD117. The identification of unique lung CSC markers still remains challenging mainly due to the intratumoral heterogeneity and high degree of plasticity that can cause instability of the CSC phenotype and reversion of cell surface markers.

CD133

CD133 is the most frequently demonstrated CSC

marker. It is a cell surface glycoprotein that consists of five transmembrane domains and two large glycosylated extracellular loops. It was considered a stem cell marker only in the hematopoietic system and the nervous system. The membrane antigen also is a marker for tumorigenic cells in certain other solid tumors. CD133 positive cells displayed higher ability of self-renewal, tumor initiation and drug resistance (33). Chen *et al.* demonstrated that Oct-4 expression maintained cancer stem-like properties in lung cancer-derived CD133-positive Cells. Oct-4 expression is normally found in totipotent and pluripotent stem cells of pregastrulation embryos. This implicates Oct-4 play a crucial role in maintaining cancer stem-like and chemo, radioresistant properties in lung cancer derived CD133+ cells (34). Cui *et al.* investigated various human lung cancer cell lines A549, H157, H226, Calu-1, H292 and H446. The results of real-time PCR analysis after chemotherapy drug selection and the fluorescence-activated cell sorting analysis showed that CD133 only functioned as a marker in the small cell lung cancer line H446 (35).

CD90

CD90 expression was primarily identified in murine breast CSCs in liver tumourogenesis. CD90 (Thy-1) is a 25–37 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed mainly in leukocytes and is involved in cell-cell and cell-matrix interactions. Yan *et al.* explored experimentally the use of CD90 as a marker for identification of lung CSCs (36). The previous studies of these workers demonstrated stronger proliferation and self-renewal abilities, and higher levels expression of the stem cell markers Sox2 and Oct4 in A549 and H446 cell lines.

ALDH1

The ALDH family is cytosolic isoenzyme catalyzing oxidation of intracellular aldehydes. Jiang *et al.* were the first to isolate ALDH1-positive cells from human lung cancer cell lines and showed ALDH1-positive cancer cells exhibited the important CSC properties: *in vitro* self-renewal, differentiation, and multidrug resistance capacities. Furthermore, they analyzed ALDH1 expression in lung tissues from three different populations of patients with lung cancer, and reported that increased ALDH1 protein levels were positively associated with stage and grade of the tumors and inversely related to the patients' survival (37).

Another stem cell marker CD44 is a multifunctional class 1 transmembrane glycoprotein mediating complex functions is found associated with breast and prostate cancers. CD44, alone or in combination with other putative CSC markers such as CD24 is used in identification of tumors (38,39). Leung showed that CD44+ cells are enriched for tumor propagating capacity and CD44 is a potential CSC marker of NSCLC cell lines (40).

Studies report a small portion of CSC cells can be enriched in the side population cells (SP) after fluorescence activated cell sorting. The activation was due to ABC transporters such as ABCG2 activation in this group of cells which cannot be stained with Hoechst 33342 in comparison to cells treated with the pump inhibitor verapamil (41). SP cells possess higher efficiency and capacity of tumor-sphere formation. SP cells showed increased resistance to cisplatin, gemcitabine and Vinorelbine compared with the non-SP group (42-44). ABCG2 has been found to play a major role in the multidrug resistance phenotype, and elevated ABCG2 levels have been found in CSC cells in NSCLC. It has also been found that patients with the dual expression of CD133 and ABCG2 have a relative higher risk of tumor recurrence (45).

Signaling pathways

Hh pathway, Wnt pathway and Notch pathway which regulate proliferation and differentiation during embryogenesis are associated with CSC self-renewal also. The Hedgehog (HH) signaling pathway plays a key role in regulating morphogenesis, homeostasis and repair of stem cells in the adult human body. This pathway was found to be activated in both types of lung cancer—small cell and non-small cell lung cancer (46). It was discovered that HH pathway can increase the chemoresistance thereby causing failures of chemotherapy in lung cancers. Mutations caused in the HH pathway provide way to activation and advancement of tumorigenic pathway and CSCs eventually leading to cancer (47). The HH pathway involves three ligands namely Sonic Hedgehog (SHH), Desert Hedgehog (DHH) and Indian Hedgehog (IHH) that have various spatial and temporal expression levels. These ligands can also function as mitogens and promote cell division and differentiation (48). The main receptor of the ligands is the Patched receptor which is expressed near the source of the HH signals (49). The Patched receptor represses the activity of another transmembrane protein called Smoothed

(Smo) when there are no HH signals. However when one of the three ligands bind to the Patched receptor, SMO accumulates and activates the transcriptional factors of GLI family which will then move into the nucleus to activate the HH target genes .

Signaling by the Wnt pathway is exceedingly complex in mammalian cells. The canonical pathway, Wnt ligands bind to a cell surface receptor complex causing the phosphorylation of dishevelled family proteins (Dvl). The Dvl then activates glycogen synthase kinase 3 (GSK-3) and casein kinase 1 (CK1), which mediates the degradation of β -catenin molecules, resulting in the accumulation of β -catenin in the cytoplasm. Some β -catenin is able to enter the nucleus and interact with transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family transcription factors to promote specific gene expression. It has been shown that Wnt1 and Wnt2 are over expressed in NSCLC cell lines and primary tumors (50,51).

The Notch pathway is an evolutionarily conserved signaling system which plays diverse roles in normal tissue development and homeostasis. In humans the Notch pathway comprises four receptors (Notch1–4) and five ligands, JAG1, JAG2, DLL1, DLL3, and DLL4. Numerous lines of evidence link the Notch pathway to cancer, for example, activating mutations in Notch1 are detected in T-cell leukemias and components of the pathway have been linked to the progression and metastasis of solid tumors (52–54).

Therapeutic approaches with lung CSCs

Being responsible for cancer initiation and relapse, several therapies consider CSCs as key targets for cancer treatment (55). Studies suggest that since CSCs promote both primary and metastatic growth, targeting and destroying them should be the main goal for a successful therapy (56). A proper treatment of CSCs requires that perfect identification of type of CSC involved. This has been the most challenging part so far in the treatments targeting CSCs. Various surface markers for CSCs that are proteins precisely expressed on their surface, including receptors and antigens are now the targets for therapeutic studies (57). The technique used to screen for the surface markers is fluorescence assisted cell sorting (FACS) (58). By using a multiparametric cell sorter, one can also screen for other cellular features that are unique to CSCs. It can also identify rare small cell populations that are the leading cause for the aggressive form of lung cancer (59). The well-

known candidate surface markers for CSCs are CD 133, CD24 and CD44. Based on the organ where the cancer begins, these markers might be expressed differently, along with a few other markers. For instance CSCs in breast cancer are screened for CD44 and/or CD24 combined with ALDH1 (60). A recent review by Katarzyna suggests that MET tyrosine kinase receptors are potential CSC markers that can be used as effective targets as they play a major role in several types of cancers by supporting the CSCs with their versatile phenotype and therapy resistance (61). Another important cell surface marker is the CD90 that are highly expressed in lung cancer cells (62). A recent study discovered that CD90 is specifically expressed by CSCs of insulinomas which makes it an ideal therapeutic target for treatment of insulinoma (63). Another effective target could be CD47, a transmembrane protein which is seen in almost all solid tumors .Novel CSC based treatment will reach the above mentioned targets by using their respective antibodies against them (64).

Although a great progress has been made in identification of CSCs with surface markers, it is important to understand that all CSCs do not express the surface markers and in some cases the non-CSCs also express surface markers. Moreover, CSCs of same origin can express different surface markers making the isolation complicated. This implies that surface markers based identification is basically isolating the CSC-rich cell population and not exactly the individual CSCs (58). Thus, additional *in vitro* and *in vivo* assays that affirm the self-propagating and self-renewal property of CSCs might be necessary to certainly isolate the CSCs. However, CSCs exhibit phenotypic instability once they are outside the solid tissue which makes the precise identification hard to achieve (65).

Another way of identifying CSCs is through their cell signaling pathways. This approach seems to be a promising one as many types of CSCs share the same signaling pathways to initiate cancer in the host. The PI3K/Akt and Hedgehog pathways are some of the common target pathways that are used by CSCs to activate the subsistence and propagation mechanisms of tumor cells. This pathway is under constitutive expression in 30–40% of human cancers. Rather than altering the entire pathway, scientists are focused towards knocking down one important enzyme/factor in the pathway that will potentially shut down the whole process. Studies found that HH inhibitors can also suppress cancer-associated fibroblasts that regulate the function and motility of human lung CSCs (66). This could be a novel strategy towards the treatment of lung and breast

cancer (67).

One of the key factors that have become an effective target is phosphatidylinositol 3-kinase (PI3K). PI3K has been reported to acquire cancerous mutations in several types of human cancer (68). Another major target is the class of rapamycin. Rapamycin inhibitors developed by researchers can knock down the M TOR portion of the pathway which regulates the metabolism, protein translation and cell growth (69). Recent studies recommend the use of alpha fetoprotein, a product of activated PI3K/Akt pathway as a potential cancer target. This protein is reported to induce the reprogramming of liver non-CSCs and CSCs to form malignant tumors (70). Another interesting target is Wasp Interacting Protein (WIP) which is regulated by PI3K/Akt-based integrin/receptor recycling pathway. WIP is involved in driving the oncogenic activity of the mutant p53 gene, which is the major tumor suppressor gene in its wild form (71).

Effective inhibition of CSCs is also observed by targeting their efflux pumps and tumor micro environment. The major drug transporter P-glycoprotein (Pgp) is now regarded as a valuable target in cancer therapy as it is responsible for multi drug resistance in cancer. Pgp inhibitors used in combination with conventional cancer drugs seems to be a less toxic approach as Pgps are specifically expressed by cancer cells and not the normal cells (72).

Lung cancer cells displaying CD133 marker were also associated with a higher expression of Oct-4 which helps them in self renewal and metastasis. Recent works utilize small interfering RNA to knock down the Oct-4 gene expression in human lung cancer cells and showed that CD133+ cells lost their ability to form spheres and differentiated back to CD133- cells. In addition, the treatment also enhanced the apoptotic activity of caspase 3 and poly (ADP-ribose) polymerase thereby making the cancer cells more susceptible to chemotherapeutic drugs. In case of lung CSCs, the Oct-4 is predicted to act by Tcl1/Akt1 pathway that inhibits apoptosis. Thus targeting the Oct-4 can result in the programmed cell death of lung CSCs. This has been proven *in vitro* using murine Lewis lung carcinoma cells (73). An endogenous mitochondrial anti-oxidant called alpha lipoic acid was also recently found to suppress Oct-4 in human lung cancer cells. It reduced the phenotype of lung CSCs such as anchorage-independent growth and three dimensional sphere formations by diminution of the cellular proteins β -catenin and Oct-4. This was achieved by affecting the amount of

active (phosphorylated) Akt (74). Thus Oct-4 could be a potential and novel target for enhancing the treatment of lung cancer. The function of miR-34a in regulating NSCLC cell behavior has not been extensively studied. Shi *et al.* showed that transfection of synthetic miR-34a in three NSCLC cell lines, A549, H460, and H1299, inhibited tumor regeneration *in vivo*. Furthermore, the lentiviral vector-mediated overexpression of miR-34a in purified CD44^{hi} H460 cells also inhibited tumor outgrowth. On the other hand, expression of miR-34a antisense oligos in the CD44^{lo} H460 cells promoted tumor development. These studies showed that miR-34a is a negative regulator of the tumorigenic properties of NSCLC cells and CD44^{hi} lung CSCs, and establishes a strong rationale for developing miR-34a as a novel therapeutic agent against NSCLC (75).

Alternate strategy would be targeting the CSCs niche with integrin. Integrin is the primary receptor that is involved in cell-matrix adhesion and has a profound impact on the ability of CSCs to survive in specific locations. $\alpha 6 \beta 1$ integrin has been implicated in the function of breast and other CSCs yet little is known about its regulation and relationship to mechanisms involved in the genesis of CSCs. Goel *et al.* reported that a CD44(high)/CD24(low) population, enriched for CSCs, is comprised of distinct epithelial and mesenchymal populations that differ in expression of the two $\alpha 6 \alpha 6 A$ and $\alpha 6 B$. $\alpha 6 \beta 1$ expression defines the mesenchymal population and is necessary for CSC function, a function that cannot be executed by $\alpha 6 A$ integrins (76). Yet, specific integrins that is liable to promote stemness, drug resistance, and metastasis of CSCs are yet to be determined (77). Studies report a few integrin $\alpha 2$ -binding agents that have been made up to reduce the proliferation capability of CSCs (78).

Conclusions

Lung cancer therapy has become most complicated due to the relapse and recurrence of the disease. Of recent many studies are concentrated on a class of cells—CSCs, which are responsible for conferring the ability of tumor regeneration indefinitely. This review focused on the origin, characteristics of CSCs, lung CSC markers, role of the signaling pathways and novel therapeutic approaches. A great deal of knowledge is essential on the biology and gene expression of these stem cells for targeted therapy. This targeted therapy on CSCs could be incorporated along with traditional therapy which would augment the effectiveness.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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