

STATE OF THE ART: CONCISE REVIEW

The Cancer Stem-Cell Hypothesis

Its Emerging Role in Lung Cancer Biology and Its Relevance for Future Therapy

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The cancer stem-cell (CSC) hypothesis suggests that there is a small subset of cancer cells that are responsible for tumor initiation and growth, possessing properties such as indefinite self-renewal, slow replication, intrinsic resistance to chemotherapy and radiotherapy, and an ability to give rise to differentiated progeny. Through the use of xenotransplantation assays, putative CSCs have been identified in many cancers, often identified by markers usually expressed in normal stem cells. This is also the case in lung cancer, and the accumulated data on side population cells, CD133, CD166, CD44 and ALDH1 are beginning to clarify the true phenotype of the lung cancer stem cell. Furthermore, it is now clear that many of the pathways of normal stem cells, which guide cellular proliferation, differentiation, and apoptosis are also prominent in CSCs; the Hedgehog (Hh), Notch, and Wnt signaling pathways being notable examples. The CSC hypothesis suggests that there is a small reservoir of cells within the tumor, which are resistant to many standard therapies, and can give rise to new tumors in the form of metastases or relapses after apparent tumor regression. Therapeutic interventions that target CSC pathways are still in their infancy and clinical data of their efficacy remain limited. However Smoothed inhibitors, gamma-secretase inhibitors, anti-DLL4 antagonists, Wnt antagonists, and CBP/ β -catenin inhibitors have all shown promising anticancer effects in early studies. The evidence to support the emerging picture of a lung cancer CSC phenotype and the development of novel therapeutic strategies to target CSCs are described in this review.

Key Words: Cancer stem cell, Non-small-cell lung cancer, Small-cell lung cancer, Tumor-initiating cell, Embryonic stem cell, Hedgehog, Notch, Wnt, Side population, Aldehyde dehydrogenase, CD133, CD44, CD166, Beta-catenin, *KRAS*.

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The cancer stem-cell (CSC) hypothesis is a concept that has received a great deal of recent attention in recent years. Normal stem cells are characterized by a number of peculiar properties; multipotency, that is, the ability to differentiate into different cell types; self-renewal; and the ability to proliferate. These properties clearly have important parallels in oncogenesis and malignancy. Indeed, the concept that tumors may be derived from a rare population of *embryo-like* cells was discussed by Virchow¹ as early as the mid-nineteenth century. Other physicians of the time postulated that cancers may arise from dormant embryonic remnants in the body, which become activated to form tumors.^{2,3} However, it is only in recent years that putative CSCs have been identified. CSCs can be defined as a rare population of stem-like cancer cells that define the clinical phenotype of tumors, with important roles in initiation, progression, and maintenance of tumors. An important concept in this model is that tumors are heterogeneous, composed of neoplastic cells, vasculature, immune cells, and stromal elements. According to this model, tumors may be regarded as abnormal organs that contain a hierarchy of cells including self-renewing stem cells and highly proliferative progenitor cells that in turn give rise to the differentiated cells comprising the bulk of the tumor.

It is important to note that although the CSCs may share many characteristics with normal stem cells, it is not certain that all CSCs in all cancer subtypes are derived from normal stem cells. It is also possible that cancer stem cells may arise from committed progenitor cells, which acquire stem-like characteristics. There is now evidence in many cancer subtypes that CSCs may arise from both normal stem cells^{4–9} and differentiated progenitor cells.^{10–13} Importantly, one study in breast cancer has shown that undifferentiated estrogen receptor (ER) negative and poorly differentiated ER positive tumors arise from mammary stem cells whereas less-aggressive ER positive tumors arise from ER positive intermediate progenitor cells.¹⁴ Therefore, it is possible that the origin of CSCs may vary considerably among different cancers, among subtypes of individual cancers, and even among different stages of the same malignancy. This issue of the likely origin of CSCs has been reviewed in detail elsewhere.¹⁵

The experimental demonstration of the capacity for self-renewal, and the production of differentiated progeny, demonstrated by xenotransplantation models of metastasis,

has succeeded in identifying putative CSCs in many cancer subtypes such as cancers of the brain, breast, lung, and of the hematopoietic system.^{16–21} Many of these putative stem cells also exhibit resistance to standard forms of treatment such as chemotherapy and radiotherapy.^{22–25} This property is also shared by normal stem cells, often mediated by the overexpression of adenosine triphosphate-binding cassette (ABC) transporters that efflux drugs out of the cell, and conservation in CSCs is one of the likely causes of chemoresistance.²⁶ Therefore, CSCs can be the source of all the malignant cells in a primary tumor, can compose the small subset of drug-resistant cells that are responsible for relapse after a chemotherapy-induced remission, and can give rise to distant metastases (Fig. 1). Given that metastatic and relapsed tumors are responsible for the majority of cancer deaths, it may be reasonable to suppose that therapies that specifically target CSCs may help to improve outcomes.

LUNG CANCER CSC PHENOTYPE

In recent years, there has been an increasing amount of evidence to support a CSC phenotype in human lung cancer.^{19–21} Many of these markers have also been found in other tumors and indeed in normal stem cells. One such phenotype is the so-called *side population* (SP) cells, which are capable of excluding Hoechst 33342 dye by ABC transporters. In addition, cells expressing the cell surface markers CD133 and CD166, cells with elevated nuclear β -catenin and elevated aldehyde dehydrogenase activity have also been shown to be indicative of a stem-cell-like population (Table 1).

Side Population

SP cells are now widely regarded to be stem cells in a number of malignancies, such as lung, breast, and

glioblastomas as well as in normal hematopoietic cells.^{25,27–30} They are characterized by the ability to efflux Hoechst 33342 dye from within the cell, and this particular subpopulation of cells can be isolated using fluorescence-activating cell sorting (FACS). SP cells have been shown to exhibit many of the required characteristics of stem cells such as self-renewal, production of differentiated progenitor cells, and the capacity to form tumors in non-obese diabetic/severe combined immunodeficiency mice. SP cells have been shown to express ABC transporters such as ABCG2, MDR1, ABCA2 etc., which have important roles in chemoresistance by active efflux of the drug from within the cell.²⁸ SP cells have been successfully identified in both non-small-cell and small-cell lung cancer cell lines.^{19,31} Ho et al.¹⁹ examined the SP fraction in six non-small-cell lung cancer (NSCLC) cell lines and a small number of clinical samples. They found that the SP fraction was the tumorigenic population in a xenotransplantation model requiring far fewer cells to initiate a tumor than the non-SP fraction. Subsequent analysis of the SP-derived tumors also showed their differentiation into both SP and non-SP cells. This repopulation ability was also confirmed in vitro. ABC transporters such as ABCG2, ABCA2, and MDR1 were significantly up-regulated in the SP fraction, and the SP fraction demonstrated increased resistance to a panel of seven different chemotherapy drugs. In addition, human telomerase reverse transcriptase expression was higher in the SP, suggesting that this fraction may represent a reservoir with unlimited proliferative potential for generating cancer cells. Salcido et al.³¹ similarly examined the SP fraction in a number of small-cell lung cancer (SCLC) cell lines. The cell lines examined contained SP cells at a rate of less than 1% of the total population. Again SP cells were much more tumorigenic than non-SP cells with as few as 50 to 100 SP cells successfully forming tumors in immunodeficient mice, and again the xenograft

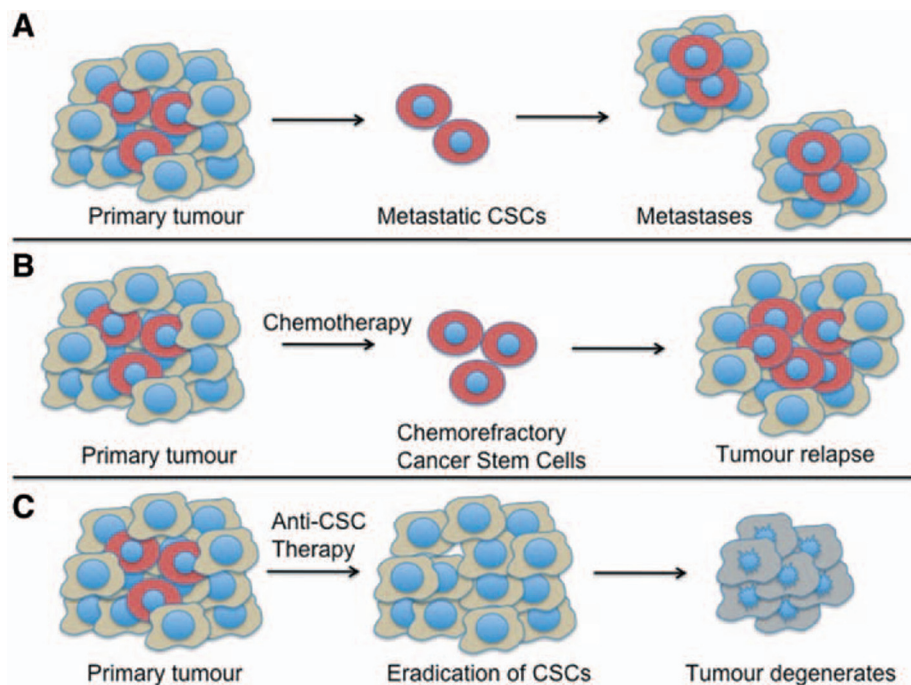


FIGURE 1. CSC hypothesis—potential implications. **A**, CSCs are thought to be the subset of cancer cells that are capable of forming new metastases and are capable of forming the full range of differentiated cells that comprise the tumor. **B**, CSCs are often resistant to standard chemotherapy and radiotherapy. Treatments that fail to eradicate the CSC subpopulation are likely to lead to relapse of disease. **C**, Successful CSC-directed therapies may improve clinical outcomes by reducing the portion of tumor cells most likely to persist through standard therapies and most likely to cause relapse or metastasis. CSC, cancer stem cell.

TABLE 1. Putative Cancer Stem Cell Markers in NSCLC and SCLC

Type of Cancer	CSC Marker	References
NSCLC	SP	Ho et al. ¹⁹
	CD 133	Eramo et al. ²⁰
		Bertolini et al. ³⁷
	ALDH	Jiang et al. ²¹
		Ucar et al. ⁵³
	CD166	Zhang et al. ⁶⁵
	CD 44	Leung et al. ⁶⁹
Nuclear β -catenin	Giangreco et al. ⁷⁵	
SCLC	SP	Salcido et al. ³¹
	CD 133	Eramo et al. ²⁰

NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer; SP, side population; ALDH, aldehyde dehydrogenase.

tumors subsequently regenerated both SP and non-SP cells. In addition, the neuroendocrine markers CD56 and CD90, characteristic of SCLC, were expressed significantly less in SP fraction cells than in the non-SP fraction, consistent with the primitive nature of SP cells. SP fraction cells had up-regulated genes that are involved in pathways modulating stemness, including MYC, FGF1, OCT4, KLF4, NOTCH2, WNT, and ABCG2. These data strongly suggest that the SP population is composed of highly undifferentiated cells with stem cell-like characteristics and resistance to standard chemotherapy.

CD133

The CD133 antigen, also sometimes referred to as Prominin 1 (PROM1), is a 120 kDa five transmembrane glycoprotein. Its function is currently not known but its expression on the cell surface has been demonstrated to be a specific marker for CSCs in a number of malignancies including central nervous system tumors, colon, breast, prostate, and ovarian cancers.^{16,17,32-35} There is now also considerable evidence to suggest that CD133 expression on a subpopulation of lung cancer cells also identifies CSCs.^{20,36-38} Eramo et al.²⁰ demonstrated that there is a rare population of CD133 positive cells in SCLC and in all subtypes of NSCLC. Lung cancer cells dissociated from primary tumors and grown in serum-free media containing epidermal growth factor and fibroblast growth factor formed spheroid bodies, which became enriched for CD133 positive cells and could be maintained indefinitely. In contrast, CD133 negative cells did not acquire CD133 positivity and died within 2 to 3 weeks of culture. Upon the exposure of CD133 positive cells to serum-containing media, the lung cancer spheres adhered to the plastic and acquired the typical morphological appearance of differentiated cells. In the process of differentiation CD133 expression was lost, confirming its specificity for undifferentiated cells. CD133 positive cells were also found to express BCRP1/ABCG2 ABC transporter and were found to be relatively chemoresistant to cisplatin, etoposide, paclitaxel, and gemcitabine. Xenograft experiments also established that the CD133 positive population was highly tumorigenic, with as few as 10⁴ CD133+ cells

consistently generating tumors in NOD/SCID mice, whereas 10 times that amount of CD133- cells were not tumorigenic. Subsequent histological examination of xenograft tumors confirmed the generation of a differentiated cell population with a similar number of CD133+ cells as the parent tumor. These results were largely replicated in the work of Bertolini et al.³⁷ They also found a rare subpopulation of lung cancer cells expressing CD133 with a much lower level of positivity in normal lung tissue. Similarly, they also confirmed the increased tumor-initiating capacity of CD133+ cells in xenograft models. CD133+ cells isolated from established xenografts, primary tumor specimens, and cell lines by FACS were found to be considerably more tumorigenic upon injection into NOD/SCID mice. Gene-expression analysis showed that genes associated with maintenance of stemness such as OCT4 and NANOG, and adhesion and motility genes such as α -6 integrin and CXCR4, were up-regulated in CD133+ cells. In addition, the expression of ABC transporter genes associated with the multidrug-resistance phenotype such as ABCC1 and ABCG2 were also found to increase in the CD133+ fraction. Chemoresistance to cisplatin of the CD133+ fraction was demonstrated in both in vitro and in vivo models. In vitro, exposure of lung cancer cell line A549 to cisplatin resulted in an eightfold increase in the number of CD133+ cells. In vivo, mice with six different lung cancer xenografts were treated with weekly cisplatin. Mice were killed at 7 days after the last treatment and at the time of tumor regrowth. FACS analysis of the resected tumors revealed marked enrichment of CD133+ cells shortly after chemotherapy but this reverted to original levels at time of tumor regrowth. These findings suggest that CD133+ cells persist in exposure to chemotherapy and are subsequently able to re-establish a tumor that had previously responded to treatment. Levels of CD133 expression in primary tumors as determined by immunohistochemistry were compared with clinical outcomes in a small cohort of advanced-stage patients undergoing platinum-based chemotherapy. There was a trend toward decreased progression-free survival in those patients found to express CD133 in their primary tumors, which is consistent with the above data regarding the relative chemoresistance of CD133+ cells. Interestingly, Zhu et al.³⁹ have reported that in a murine intestinal model, PROM1/CD133 marks an adult solid tissue stem cell that is susceptible to neoplastic transformation, supporting a model of a Prom1/CD133+ cancer stem cell. Using tamoxifen-induced Cre to activate fluorescence in CD133+ cells the investigators showed that PROM1/CD133 positivity successfully identifies normal stem cells in the intestine, giving rise to all differentiated cell types of the intestinal epithelium. In addition, activation of endogenous Wnt signaling in mice containing a Cre-dependent mutant allele of β -Catenin resulted in neoplastic transformation of PROM1/CD133+ cells in the intestine. These data suggest that CD133+ normal stem cells may be the cell of origin in certain cancers. Furthermore, in SCLC, it has been suggested that the neuroendocrine-regulating transcription factor, achaete-scute complex homologue 1 (ASCL1) may be an important regulator of stem-cell markers such as CD133 and aldehyde dehydrogenase (ALDH). Jiang et al.³⁸ showed that ASCL1 caused induction of both CD133 and ALDH1A1, and that using siRNA transfection

to repress ASCL1 caused reduced growth and inhibited soft agar clonogenicity in cultured SCLC. It was also shown that SCLC direct xenograft tumors that were enriched for CD133 positivity had greatly increased tumorigenicity. In addition, the knockdown of ASCL1 in these xenografts using ASCL1 shRNA caused a marked decrease in their tumor-initiating potential when compared with controls. However, there have been some conflicting data on the role of CD133 and CSCs. Meng et al.⁴⁰ found that CD133 status in A549 and H446 cell lines was not significantly related to proliferative capacity, invasiveness, drug resistance, or tumorigenic ability in xenograft models. Salnikov et al.⁴¹ also demonstrated that CD133 expression in NSCLC was not prognostic. Therefore, although there are some conflicting data in this area, the weight of the available data strongly suggests an important role for CD133 in correctly identifying lung CSCs.

Aldehyde Dehydrogenase

ALDH enzyme activity has also emerged as a promising marker of CSCs and indeed of normal stem cells. It has been known for some time that ALDH is highly expressed in normal hematopoietic stem cells,^{42,43} and in addition to being a putative stem-cell marker, ALDH activity also has a known role in drug resistance.⁴⁴⁻⁴⁷ ALDH activity has been used as a basis for an FACS method to sort viable hematopoietic stem cells from mixed cell populations for further study (Aldefluor assay, Stem Cell Technologies, Vancouver, BC, V5Z 1B3, Canada). This method has been subsequently applied in many malignancies and ALDH activity has identified potential CSCs in leukemia, breast cancer, brain cancer, head and neck squamous cell cancers, colon cancer, and also now lung cancers.⁴⁸⁻⁵³ Patel et al.⁵⁴ demonstrated that ALDH1A1 and ALDH3A1 were overexpressed in both squamous carcinoma and adenocarcinoma of lung compared with normal pneumocytes whereas there was low expression of ALDH seen in small-cell lung tumors. ALDH levels were seen to be higher in smokers than in nonsmokers and were seen to increase as premalignant lesions compared with normal cells suggesting an important role in tumorigenesis. Furthermore, Jiang et al.²¹ demonstrated that lung cancer cells with relatively high ALDH1 activity displayed *in vitro* features of CSCs, including capacities for proliferation, self-renewal, and differentiation, resistance to chemotherapy, and expressing the previously discussed CSC surface marker CD133. Six lung cancer cell lines were analyzed using the Aldefluor assay and FACS and were found to have a rare population of cells with high ALDH1 activity at a rate of 0.6% to 2.9% in NSCLC cell lines, whereas the two SCLC cell lines did not exhibit any ALDH activity. ALDH+ cells were found to proliferate faster and produce a mixed population of ALDH+ and ALDH- cells in culture, whereas ALDH- cells could only generate more ALDH- cells thus suggesting that it is the ALDH+ fraction that has the capacity for self-renewal and subsequent production of a heterogeneous tumor cell population. The authors also showed that the majority (64%) of ALDH1 positive cells also stained positively for CD133 and that they displayed increased resistance to cisplatin, gemcitabine, doxorubicin, daunorubicin, vinorelbine, and docetaxel. In addition, ALDH+ H353

and H125 cells were shown to be highly tumorigenic in xenograft experiments. ALDH1 cells readily produced tumors in the NOD/SCID mice whereas those cells without ALDH activity could only do so in one instance. Importantly in this study, high levels of ALDH1 protein expression were shown to correlate with clinical outcomes, with high ALDH activity indicating poor patient prognosis and a more advanced stage of disease. Ucar et al.⁵⁵ also examined ALDH activity as a potential stem cell marker using the H522 lung cancer cell line as a model. ALDH+ cells exhibited capacity for differentiation and self-renewal, giving rise to both ALDH+ and ALDH- cells whereas ALDH- cells in culture produced only ALDH- cells. In contrast to the findings by Jiang et al., however, cells with high ALDH activity were seen to grow slower *in vitro* than ALDH- cells did. In addition in their xenograft models, both ALDH+ and ALDH- cells were capable of forming tumors in NOD/SCID mice and the initial rate of tumor growth was actually faster in ALDH- cells. However, with the further transfer of tumor cells into secondary and tertiary recipient animals, ALDH+ cells eventually showed faster growth, whereas the tumor-initiating capacity of ALDH- cells decreased with each successive engraftment, thus reinforcing the hypothesis that ALDH+ is indicative of the lung CSC phenotype.

CD166

CD166, also known as activated leukocyte cell adhesion molecule, is a membrane glycoprotein that has been implicated as a potential marker of CSCs. It has a variety of functions in normal tissues, such as intravasation of leukocytes into the central nervous system,⁵⁶ migration of monocytes across endothelia,⁵⁷ and T-cell activation.⁵⁸ It has also been shown to be present on normal mesenchymal stem cells and hematopoietic progenitor cells.⁵⁹⁻⁶¹ In addition, CD166 has been shown to be an indicator of poor prognosis in a variety of cancers⁶²⁻⁶⁵ and has been demonstrated to identify a CSC phenotype using murine xenotransplantation models in colorectal cancer.⁶⁶ Recently, CD166 has also been identified as a marker for CSCs in NSCLC.⁶⁷ In this study, Zhang et al. took cells from resected primary NSCLC tumors and injected them subcutaneously into NOD/SCID mice. Unsorted cells had a low rate of xenograft formation with an approximate rate of tumor-initiating cells (TICs) of 1 in 400,000. Having excluded hematopoietic and endothelial cells, the cells were sorted according to CD166, CD133, CD44, and EpCAM expression to assess whether any of these markers would enrich for the CSC population. It was found that CD166 positive cells were far more likely to form tumors than CD166 negative cells or any of the other markers investigated. In fact, it was observed that 100-fold fewer CD166 positive cells were needed for xenograft formation compared with unsorted cells. Examination of the xenograft tumors using hematoxylin-eosin staining and immunohistochemistry showed that CD166 positive cells replicated the histological morphology of the parent tumors. It was also found that only CD166 positive cells were capable of forming tumor spheres, an often-used *in vitro* assay, to assess self-renewal capacity. Furthermore, when CD166 positive tumor spheres were dissociated into single cells, as few as 1 to 5 single cells

consistently initiated xenografts. Interestingly, this study found very little difference in the xenograft-initiating capacity of the CD133 positive fraction of tumor cells in comparison to CD133 negative cells, which stands in contrast to other studies that have identified CD133 as a marker of CSCs.^{20,37} In addition, this study sought to obtain a molecular signature for lung TICs by performing genome-wide transcriptome analysis on CD166+ and CD166- tumor cell populations. It was found that glycine decarboxylase (GLDC) and the oncogenic stem-cell factor LIN28B were particularly associated with TICs as opposed to non-TICs. Furthermore, the knockdown of GLDC and LIN28B in lung tumor spheres using shRNAs demonstrated that GLDC and LIN28B were necessary for cellular proliferation and tumorigenicity as measured by soft agar colony formation. It was also found that GLDC expression in NSCLC patient samples formed a subset of the CD166+ population and was prognostic, with high GLDC levels predicting shorter overall survival. This development of a molecular and metabolic profile of CSCs may ultimately deliver important new therapeutic targets.

CD44

CD44 is a cell membrane glycoprotein, which in normal cells has important roles in cell to cell adhesion, interactions with the extracellular matrix and cell migration. It has also been shown to be an important identifier of CSCs in a variety of cancers, most notably perhaps in breast cancer,¹⁷ but also in prostate, pancreatic, and head and neck cancers.^{68–70} A recent study by Leung et al.⁷¹ suggests that CD44 may also have a role in identifying lung cancer CSCs. The investigators analyzed the effect of CD44 positivity in a range of NSCLC cell lines. It was found that CD44 positive cells had a higher rate of tumor spheroid formation in vitro, higher rates of resistance to cisplatin chemotherapy, and increased metastatic potential in a murine xenotransplantation model. The CD44 positive cells also had a higher rate of expression of the stem-cell markers OCT4 and NANOG in addition to epithelial-mesenchymal-transition markers such as SNAI1, CDH2, and VIM. Furthermore, high rates of CD44 expression in clinical tumor samples as analyzed by immunohistochemistry were prognostic in adenocarcinomas although not in squamous cell carcinomas.

Wnt/ β -Catenin

The Wnt/ β -Catenin signaling pathway is known to play key roles in controlling cellular proliferation and cellular differentiation in both embryogenesis and in regulating homeostasis in normal adult tissues. Ordinarily, β -Catenin levels are maintained at a low level in the cytoplasm, but the activation of the Wnt/ β -Catenin pathway causes the translocation and accumulation of β -Catenin in the nucleus, thereby promoting the transcription of Wnt target genes. The Wnt/ β -Catenin pathway has been demonstrated to play a crucial role in the maintenance and regulation of normal stem cells in a number of organ systems, for example intestinal mucosa,⁷² skin,⁷³ and bone.⁷⁴ In addition, Wnt/ β -Catenin signaling has been shown to be of importance in CSCs in a number of malignancies, such as colon cancer,⁵ cutaneous squamous cell carcinoma,⁷⁵

and chronic myeloid leukemia.⁷⁶ More recently, there have been some studies to suggest a role for aberrant β -Catenin signaling in lung cancer also. Gianfreco et al.⁷⁷ investigated β -Catenin signaling in various preinvasive lung squamous cell carcinomas, using immunohistochemistry to localize β -Catenin activity. Normal and metaplastic lung specimens exhibited membranous β -Catenin only, whereas severely dysplastic specimens and carcinoma in situ frequently exhibited abundant nuclear β -Catenin levels, thereby suggesting a role for Wnt/ β -Catenin signaling in lung tumorigenesis. In addition, work by Levina et al.⁷⁸ has suggested correlation between high nuclear β -Catenin levels and lung cancer CSCs. On the basis of previous observations of intrinsic chemoresistance of CSCs, the authors treated lung (H460), ovarian (OVCAR-3), and breast (MCF-7) cell lines with chemotherapy (cisplatin, etoposide, doxorubicin) to create drug-surviving cells (DSCs) which were then investigated for their potential as CSCs. Lung cancer DSCs thus created were shown to be enriched for CD133 positivity, had higher expression of the embryonic stem-cell markers TRA-1–81, SSEA-3, and OCT4 and had higher expression of nuclear β -Catenin when compared with parent cells, all indicative of a stem-cell like phenotype. DSCs were also shown to have intrinsic capacity for tumor-sphere formation, and a high metastatic potential when injected into NOD/SCID mice compared with parent cells, further emphasizing their status as CSCs. This study also reinforces the hypothesis that CSCs form the pool of drug-resistant cells that cause relapse of the disease after chemotherapy.

KRAS

KRAS mutations are frequently encountered in human lung cancers. Previous models using oncogenic *KRAS* transgenic mice, have shown markedly high rates of lung cancer formation.⁷⁹ More recently, it has been shown that certain subtypes of lung epithelial cells become hyperplastic in response to oncogenic *KRAS*, with bronchioalveolar stem cells (BASCs) and type II alveolar cells identified as putative cells of origin in *KRAS* induced lung carcinomas.^{4,80} Work by Kim et al.⁴ has shown that *KRAS* mutation may be a key event in the formation of lung cancers arising from normal BASCs. The authors developed a “Lox-stop-lox” *KRAS* conditional mouse strain in which expression of oncogenic *KRAS* is spatially and temporally controlled by a removable transcriptional termination (*stop*) element. Infection of the mice with recombinant adenoviral Cre (AdCre) results in deletion of that stop element, producing the Lox-*KRAS* allele that expresses oncogenic *KRAS*. The authors showed that AdCre-induced activation of the *KRAS* allele increases the abundance of BASCs that are found at the bronchioalveolar duct junction. In addition, coadministration of naphthalene and AdCre infection showed significantly higher rates of tumor formation in Lox-*KRAS* mice compared with normogenic controls. These data support the hypothesis that BASCs may be the cell of origin for many lung adenocarcinomas, and that *KRAS* may have an important role in the malignant transformation of these normal stem cells during tumorigenesis. Regala et al.⁸¹ refined this hypothesis by examining the effect of matrix metalloproteinase-10 (MMP-10) on *KRAS* mediated lung cancer initiation. Using a similar

mouse model, *Lox-KRAS* transgenic mice were crossed with MMP-10 knockout mice to create a bitransgenic mouse model. They showed that urethane-initiated lung tumors were far fewer in MMP-10 deficient animals compared with controls, suggesting that MMP-10 may be an important cofactor in *KRAS* mediated BASC transformation and tumorigenesis. Furthermore, recent work by Xu et al.⁸⁰ suggests that type II alveolar cells may also be cells of origin in certain *KRAS* induced lung adenocarcinoma. In this study, a similar mouse model was used, where two *knock-in* Cre-ER alleles were used to inducibly express oncogenic *KRAS*-G12D in Clara cell antigen 10 positive epithelial cells and surfactant protein C positive type II alveolar cells in murine lung tissue. It was shown that *KRAS* induction caused lung hyperplasia with type II cells, Clara cells, and BASCs, all possible as cells of origin. However, it seemed that only type II alveolar cells progressed to adenocarcinoma in response to oncogenic *KRAS*. Therefore, the data in this area clearly show that *KRAS* mutation causes formation of lung carcinomas but further studies are necessary to further elucidate the likely cell or cells of origin in these tumors.

ESC Signature

The properties of embryonic stem cells (ESCs) have obvious parallels with cancer cells, such as self-renewal, multilineage differentiation, and proliferative capacity. ESC lines were first identified in 1998 and many studies have examined their molecular profiles, and determined panels of genes that are consistently over- or underexpressed compared with differentiated cells.^{82,83}

NANOG, OCT4, Sox2, c-Myc, Polycomb, and their targets are all crucially important in the regulation of ESC pathways and known to be involved in several cancer subtypes. Ben-Porath et al.⁸⁴ demonstrated that in various human cancers, increased expression in an ESC signature and decreased expression of the Polycomb target genes correlated with poorly differentiated tumors and worse prognosis. These findings were seen in gliomas, breast cancer, and bladder cancer.

Hassan et al.⁸⁵ applied the same methodology to NSCLC in both adenocarcinomas and squamous cell carcinomas. Increased expression of a ESC gene set and decreased expression of Polycomb gene set identified poorly differentiated, poor-prognosis adenocarcinoma tumors. This correlation was not seen in squamous cell cancers. In a similar study, Stevenson et al.⁸⁶ also found that an ESC signature in NSCLC correlated with a poor prognosis and resistance to cisplatin. It is important to note however, that these studies did not seek to identify a marker of a subpopulation of cancer stem cells within tumors but instead looked at the expression of ESC-associated genes in whole tumors and how this affects the clinical behavior of the cancer.

CSCS AS A POTENTIAL THERAPEUTIC TARGET

As previously discussed, the CSC hypothesis suggests that there is a small subset of cancer cells that are responsible for tumor initiation and growth, possessing properties such as indefinite self-renewal, slow replication, intrinsic resistance to chemotherapy and radiotherapy, and an ability to give rise to

differentiated progeny. In this model, CSCs may comprise just a small proportion of a tumor, but give rise to variably differentiated progenitor cells with limited proliferative potential, which comprise the bulk of the tumor.

If the CSC hypothesis is correct, this has some critical implications for cancer therapeutics. Traditionally, the efficacy of treatments such as chemotherapy and radiotherapy has been measured by assessing the degree of shrinkage of the tumor in response to therapy, either radiologically, or by clinical examination. However, it is clear that many patients are intrinsically resistant to conventional therapies, and even in patients in whom a complete response to therapy is observed, all too often there are subsequent relapses of disease. Indeed, even in patients who have undergone tumor resection and adjuvant therapies, large numbers of patients have subsequent recurrence of disease, often long after initial diagnosis. This suggests that to improve outcomes in these situations it may be necessary to specifically target the CSC population, which are theorized to comprise the small pool of cells that are resistant to therapy and can cause relapse and metastasis, even after periods of apparent dormancy after seemingly effective treatment. A key aspect of CSCs that has been identified to date is their intrinsic resistance to chemotherapy and radiotherapy.²²⁻²⁵ In the case of chemotherapy resistance this is often a result of the presence on CSCs of drug-efflux mechanisms such as ABC transporters.²⁶ In other cases, increased DNA repair capacity or resistance to reactive oxygen species cause intrinsic resistance of CSCs to radiation.^{22,87} The elucidation of a CSC phenotype has revealed a range of cellular pathways that are relatively specific to stem cells and are potential targets for drug therapy. Sonic Hedgehog, Notch, and Wnt signaling pathways all have important roles in regulating control of self-renewal and developmental pathways in normal stem cells,⁸⁸⁻⁹⁰ and have been shown to have important roles in CSC also.⁹¹⁻⁹⁵ These pathways have received recent attention as potential therapeutic targets that may successfully target CSCs (Fig. 2).

Hedgehog

Hedgehog (Hh) signaling is an important cell-signaling pathway in ESCs.^{88,96} Hh ligands act through the cell surface protein Patched and henceforth through the G-protein coupled receptor SMO, thereby activating downstream transcription factors (Fig. 2). The binding of Hh ligands to cell surface protein Patched causes cell membrane localization of SMO and the initiation of a signaling cascade leading to the activation of the glioma-associated (Gli) family of transcription factors. There is evidence that the Hedgehog pathway is important in several cancer subtypes^{97,98} and specific evidence of the importance of Hh signaling in SCLC,^{99,100} including data that shows that blocking Hh signaling can have an anti-tumor effect.¹⁰¹ This has led to the development of a number of new Hh antagonists that are under investigation, some of which are now in clinical trials.¹⁰² The archetypal Hh specific inhibitor is cyclopamine, a plant-derived SMO antagonist. Cyclopamine was first identified as a cause of severe congenital defects such as cyclopia in animals,¹⁰³ and subsequently its mode of action as a SMO inhibitor was elucidated.^{104,105}

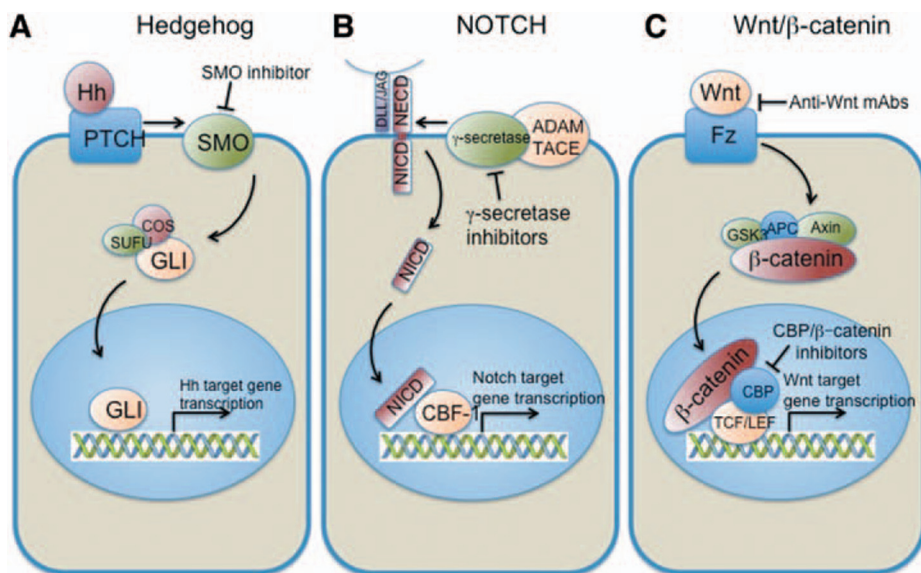


FIGURE 2. A, Hh signaling pathway. Hh ligand binding to the transmembrane receptor PTCH causes membrane localization of SMO. SMO activation causes liberation and nuclear translocation of glioma-associated family of transcription factors, thereby inducing transcription of Hh target genes. B, NOTCH signaling pathway. DLL of JAG ligands on adjacent cells bind to the transmembrane receptor Notch. Ligand binding causes a conformational change in Notch thus causing enzymatic cleavage of the receptor by ADAM/TACE and gamma-secretase. This liberates the intracellular portion of NOTCH (NICD), which then translocates to the nucleus. NICD complexes with core-binding factor-1 and initiates transcription of NOTCH target genes. C, Canonical Wnt signaling pathway. Wnt ligands bind to the transmembrane receptor Fz. Activation of Fz causes the inhibition of the action of GSK-3, APC, and Axin on b-catenin, thereby increasing cellular levels of b-catenin. b-catenin then translocates to the nucleus where it complexes with transcriptional c-factors such as TCF/LEF and CBP thereby causing the transcription of Wnt target genes. Hh, Hedgehog; a disintegrin and metalloproteinase/TACE, a disintegrin and metalloproteinase/tumor necrosis factor- α -converting enzyme; NICD, notch intracellular domain; Fz, Frizzled; GSK, glycogen synthase kinase; APC, anaphase promoting complex; TCF/LEF, T-cell factor/lymphoid enhancer factor; CBP, CREB-binding protein.

More recently, novel small molecule SMO antagonists, GDC-0449, IPI-926, and BMS-833923/XL139 have entered clinical trials. Of these compounds, IPI-926 has some evidence for efficacy in SCLC in a primary xenograft model.¹⁰⁶ Initial studies with GDC-0449 have shown promising results in basal cell carcinoma¹⁰⁷ and medulloblastoma.¹⁰⁸ GDC-0449 is now being evaluated in SCLC in the form of a phase II clinical trial (Eastern Cooperative Oncology Group 1508) in combination with cisplatin and etoposide. IPI-926 and BMS-833923/XL139 are in phase I studies in SCLC in combination with standard chemotherapy. Clinical data from these studies are not yet available and are eagerly awaited.

Notch

The Notch signaling pathway is a cell to cell communication system, which is known to play a critical role in regulating cellular proliferation and differentiation during embryogenesis and in normal adult stem cells.^{109,110} Notch pathways are known to be abnormal in several cancer subtypes including NSCLC and SCLC.^{31,111–115} There are four mammalian Notch receptors (Notch 1–4), each comprising an extracellular domain, a transmembrane domain and an intracellular domain (NICD). Notch receptors bind to two distinct families of Notch ligands, Delta-like (DLL1, DLL3, DLL4) and Jagged-like (JAG1, JAG2). Ligand-receptor binding causes the Notch receptor to undergo a conformational change, thereby exposing a previously hidden

portion to enzymatic cleavage. NICD is cleaved by a disintegrin and metalloproteinase metalloproteinase/tumor necrosis factor- α -converting enzyme, and thereafter by gamma-secretase, releasing NICD from the cell membrane. NICD then translocates to the nucleus and binds to the transcription initiation complex and core-binding factor-1 (CBF-1) thus inducing transcription of Notch target genes¹¹⁶ (Fig. 2). Gamma-secretase inhibitors which block the enzymatic cleavage and consequent activation of Notch, are potential therapeutic agents to target CSCs. Two such gamma-secretase inhibitors, RO4929097 and MK0752, are now in early clinical development with clinical trials now underway in a number of cancers including in NSCLC.^{117–119} A further approach has been to target DLL4. DLL4 is a Notch ligand known to be involved in angiogenesis and anti-DLL4 therapies such as the monoclonal antibody OMP-21M18 have been developed. There is preclinical and clinical evidence that this approach decreases the incidence of CSCs in colon cancer,¹²⁰ and OMP-21M18 is now also under investigation in lung cancer.

Wnt

As previously discussed, the Wnt signaling pathway is known to play key roles in controlling cellular proliferation and cellular differentiation in both embryogenesis¹²¹ and in regulating stem cells in normal adult tissues.^{72–74} Abnormal or deregulated Wnt signaling has also been observed in several

cancer subtypes including lung cancer.^{6,75–78,122,123} Wnt proteins are a family of 19 glycoproteins that act as ligands for the Frizzled (Fz) transmembrane receptor. The binding of Wnt ligands to Fz receptors activates two distinct signal transduction pathways, known as the canonical and noncanonical Wnt pathways. The canonical pathway causes an accumulation of β -catenin in the nucleus and consequent transcription of Wnt target genes. (Fig. 2) A number of strategies to inhibit Wnt/ β -catenin signaling to target CSCs have been investigated. For example, monoclonal antibody antagonists to Wnt-1 and Wnt-2 have been developed with some early evidence of anti-tumor efficacy in a number of cancers, including NSCLC.^{124–127} Another technique has been the use of small molecules to antagonize the binding of β -catenin to the transcriptional cofactor cyclic AMP response element binding protein (CBP). One such molecule, ICG-001, has shown in vitro anticancer efficacy in a colon cancer model¹²⁸ whereas another CBP/ β -catenin antagonist, PRI-724, has entered early clinical trials.¹²⁹ Furthermore, inhibitors of Disheveled (Dsh), a key protein in the Wnt signaling pathway, have also shown some preclinical antitumor activity.¹³⁰

DISCUSSION

The CSC hypothesis now seems increasingly well established in a wide range of malignancies. Through the use of xenotransplantation assays, putative CSCs have been identified in many cancers, often identified by markers that are held in common with normal adult or ESC. This is also now the case in lung cancer, and the accumulated data on SP cells, CD133, CD166, CD44, and ALDH1 are beginning to clarify the true phenotype of the lung cancer *stem cell*. Furthermore, the signaling pathways that are characteristic of CSCs are becoming more clearly understood. It is now clear that many of the pathways of normal stem cells, which guide cellular proliferation, differentiation, and apoptosis are also prominent in CSCs, the Hedgehog (Hh), Notch, and Wnt signaling pathways being notable examples. This gives rise to many notable potential targets for new anticancer therapies and indeed the prospect of specific anti-CSC therapies. As previously stated, the CSC hypothesis has some critically important implications for how we view cancer chemotherapy and how we assess efficacy of treatments. The CSC hypothesis suggests that there is a small reservoir of cells within the tumor, which are resistant to many standard therapies, and can give rise to new tumors in the form of metastases or relapses after apparent tumor regression. It is possible therefore that the more important issue when assessing an anti-CSC therapy may not be measuring how much of the tumor bulk it reduces but which type of cells it targets and whether it can successfully eradicate the CSC subpopulation. Therapeutic interventions that target CSC pathways are still in their infancy and clinical data of their efficacy are extremely limited as yet. However SMO inhibitors, gamma-secretase inhibitors, anti-DLL4 antagonists, Wnt antagonists, and CBP/ β -catenin inhibitors have all shown some promising results in preclinical studies and in early clinical trials. Several examples of these drugs have now entered early-stage clinical trials in lung cancer. It is also important to remember that many CSC pathways are replicated in normal adult stem cells.

Therefore there may be unforeseen toxicities associated with anti-CSC therapies which may only become clear with more extensive clinical use of these drugs. Although it is important to maintain this note of caution, our better understanding of the nature of CSCs gives rise to some tantalizing prospects of new therapies which may help to eradicate tumors more effectively, reduce risk of relapse and metastasis, and improve clinical outcomes for patients with lung cancer.

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