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Cancer Stem Cells in Solid Organ Malignancies: Mechanisms of Treatment Resistance and Strategies for Therapeutic Targeting

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1. Introduction

It has been long recognized that tumors are composed of a heterogenous population of cells with various levels of cellular differentiation and morphologic features. Previous explanations for this phenomenon have centered around the concept of clonal evoluation, with the gradual acquisition of mutations leading to distinct tumor cell populations. While this model has validity, more recent evidence suggests that distinct tumor cell populations likely also arise from differentiation of cancer cells with stem-like properties. Termed the cancer stem cell (CSC) theory, this model posits that tumors are composed of a small population of cells possessing the characteristics of self-renewal and pluripotency, and thus the ability to initiate or support tumor growth, as well as their differentiated progeny which lose these abilities with increasing differentiation (Figure 1).

Much in the way a normal organ is supported by endogenous stem cells, the CSC theory holds that similarly-functioning cells with stem-like abilities are the driving force behind tumor initiation, progression and metastatic spread. Since they were first identified in acute myelogenous leukemia (AML) (Lapidot et al., 1994), CSCs have been indentified in a wide variety and number of malignancies, including colorectal, head and neck, pancreatic, prostate, central nervous system (CNS), lung and breast cancer.

The CSC theory has garnered a great deal of attention, in part, because it proposes a fundamental shift in the way we think about and treat cancer. Similarly to how normal tissue stem cells are resistant to traditional cytotoxic cancer therapies, CSCs have increasingly been demonstrated to be preferentially spared by such treatment. It is thought that standard chemotherapy and radiation targets the differentiated tumor cell bulk, leaving the resistance CSC behind, which can lead to recurrence even years later (Figure 2).

Along with the identification and an increasing focus on characterization of CSCs has been the search for therapies that effectively target this resistant subpopulation. While the search is still in its infancy, a number of intriguing treatment strategies have been proposed. In many cases these strategies target known resistance mechanisms employed by CSCs.

Although efficacy for these strategies has yet to be determined in phase II or III clinical trials, early preliminary evidence is encouraging. In this chapter we will discuss mechanisms of CSC treatment resistance as well as the exciting possibility of current therapeutic approaches that seek to specifically target the CSC population.

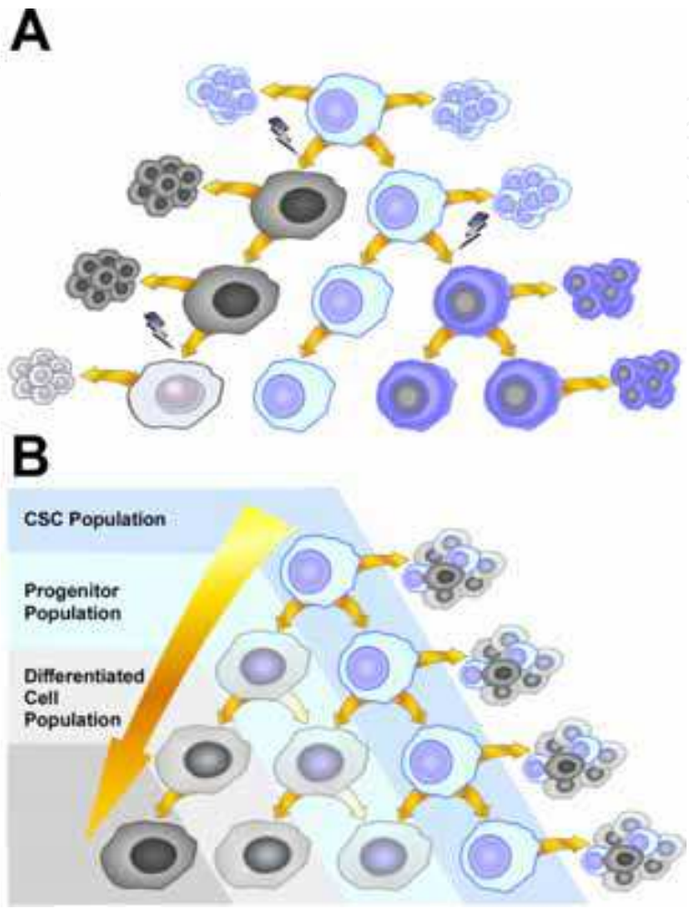

Fig. 1. Comparison of two models of tumor development and progression, (A) Traditional stochastic model of tumor progression. Each tumor cell is capable of giving rise to new tumors. Tumor heterogeneity develops from the stochastic accumulation of mutations. (B) Cancer stem cell theory. Tumor cell hierarchy with a CSC population at the hierarchical apex. Heterogeneity develops from differentiation of CSC progeny. Tumorigenic capability is lost with increasing differentiation

2. Cancer stem cells and treatment resistance

2.1 Resistance to standard cytotoxic therapy

An unfortunate number of advanced cancers recur despite an initial response to treatment. The CSC theory proposes that this phenomenon is likely due to the inability of current anti-cancer therapy to specifically target and eradicate the cells capable of "seeding" tumor growth, i.e. the CSC population. Studies in blood, brain, breast, and colon cancer have shown that identified tissue specific CSC populations exhibit decreased cell death after chemotherapy and radiation as compared to the more differentiated cancer cells (Woodward

et al., 2007). This leads to the selection of an enriched population of treatment-resistant CSCs that are capable of initiating new tumor growth (recurrence) and spread to distant organs (metastasis).

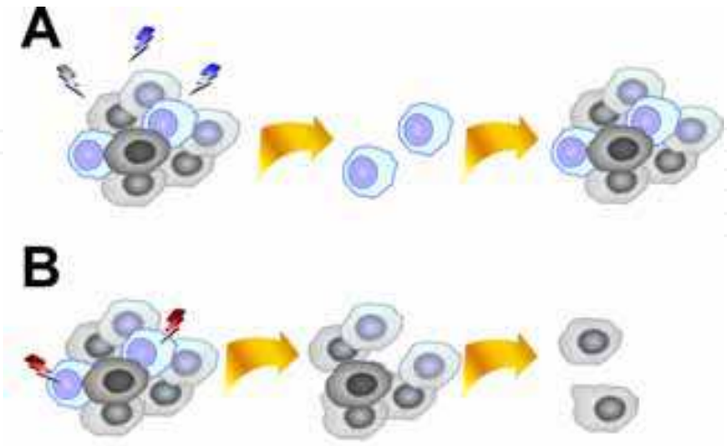

Fig. 2. Treatment Implications of CSC Theory. (A) Traditional cytotoxic therapy targets the differentiated tumor bulk, sparing the relatively more resistant CSC subpopulation (light blue cells) which can lead to tumor recurrence, (B) CSC-directed therapy kills CSC subpopulation, leading to eventual tumor eradication

These findings are supported by a number of studies using both *in vitro* methods as well as *in vivo* xenograft models. One such study by Dylla et al demonstrated that a subpopulation of highly tumorigenic colorectal cancer cells expressing the cell surface antigens $ESA^+CD44^+CD166^+$ increased by 2.2-fold following treatment with cyclophosphamide and irinotecan (Dylla et al., 2008). Serial transplantion of these chemoresistant cells gave rise to heterogeneous tumors identical to the parent tumor, demonstrating that the chemotherapy selected for a resistant population of cells able to maintain their original tumorigenic capacity. A similar effect has been described in $CD44^+$ pancreatic cancer cells following high-dose gemcitabine treatment. Interestingly, as the proportion of $CD44^+$ cells decreased in culture, tumor colonies became re-sensitized to gemcitabine treatment (Hong et al., 2009), suggesting that the progeny of the CSC do not have the drug resistant behavior. Additionally, using primary lung tumors, Bertolini et al demonstrated that a population of tumor-initiating cells expressing CD133 were enriched after treatment with platinum-based chemotherapy both *in vitro* and *in vivo*. And furthermore, on retrospective analysis of formalin-fixed tissue biopsies, tumors with increased expression of CD133 by immunohistochemistry demonstrated a shorter time to recurrence following chemotherapy than $CD133^-$ tumors (Bertolini et al., 2009), indicating that CSC enriched tumors possess a more aggressive behavior.

Similar selection of treatment-resistant CSC populations has been observed following radiotherapy in other tumor types. Glioblastomas are a uniformly lethal malignancy with a median survival of less than 12 months (Bao et al., 2006). Radiation is currently the most effective treatment for glioblastomas and can lead to significant treatment responses, although the tumor invariably recurs. Studies have shown that glioblastoma surviving radiation are enriched for $CD133^+$ cells and, as described previously, are just as efficient in recapitulating tumors in xenograft models as non-radiated $CD133^+$ cell populations (Bao et al., 2006; Eyler et al., 2008).

2.2 Mechanisms of treatment resistance in CSCs

While a comprehensive understanding of the mechanisms of CSC resistance to chemotherapy and radiation are lacking, a number of genetic and cellular adaptations that confer resistance have been observed. These include slow cell cycling kinetics, efficient DNA repair mechanisms, increased expression of multidrug-resistance transporters, protection from a specialized microenvironment and apoptotic resistance (Figure 3).

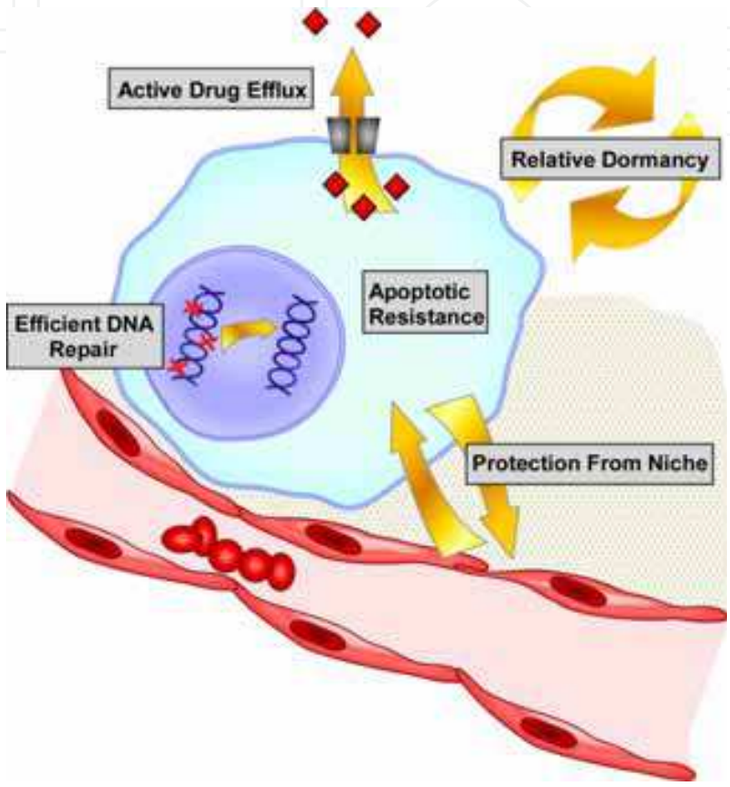

Fig. 3. Mechanisms of CSC Treatment Resistance. CSCs exhibit multiple behaviors that have been cited reasons for their resistance to current cytotoxic-based therapies. These include active drug pumps such as members of the ATP-binding cassette transporters (ABC-transporter), efficient DNA repair mechanisms, apoptotic resistance, relative dormancy due to a slowly cycling state, and protection from a specialized microenvironment (niche)

2.2.1 Slow cell cycle kinetics

Both radiation and chemotherapy target cells that are rapidly replicating and dividing. CSCs are inherently resistant to these cell cycle-dependent therapies because of their low proliferation rate. Similar to a normal stem cell, a CSC cycles significantly less often than more differentiated transit-amplifying cells. In head and neck cancer, CSCs identified by high CD44 expression displayed increased clonogenicity and spent extended time in G2, which was protective against apoptosis. Targeting G2 checkpoint proteins released the G2 blockade from these cells and made them more prone to apoptosis (Harper et al., 2010). The relative dormancy of a CSC also provides it with the opportunity to accumulate multiple mutations over time. These mutations may be passed along to the cell's progeny, creating another avenue to acquired therapeutic resistance.

2.2.2 Efficient DNA repair mechanisms

Like normal stem cells, CSCs possess a well-fortified defense system that protects against DNA damage and mutation. In a study by Eyler et al, radiation was shown to cause equal levels of damage to all cells within a tumor, but CSCs were able to repair the damage more rapidly (Eyler et al., 2008). Several mechanisms exist for detection of DNA damage as well as rapid repair. Cell cycle checkpoints, including ataxia telangiectasia mutated and checkpoint kinases (Chk1 and Chk2), are activated in response to genomic stress, halting further replication and division until the DNA damage is repaired. Chk 1/2 have been found to have higher basal and inducible activity in CSCs than in non-stem cells (Eyler et al., 2008; Morrison et al.). Inhibition of the Chk 1/2 kinases partially reverses the radioresistance of glioblastoma cells, suggesting that these checkpoints are critical to the radioresistance seen in glioblastoma (Bao et al., 2006).

The presence of certain DNA repair proteins can give cells a survival advantage as well. One specific DNA repair protein, O^6-methylguanine-DNA-methyltransferase (MGMT), has been implicated in conferring resistance to the chemotherapeutic agent temozolomide (Beier et al., 2008). Temozolomide impairs DNA replication by methylating the O^6 position of guanine, which can then be reversed by the function of MGMT. Consequently, temozolomide has little effect in tumor expressing active MGMT.

2.2.3 Multidrug transporters

CSCs exhibit a large number of drug efflux pumps that work to preserve DNA integrity by selectively removing cytotoxic chemicals, including chemotherapeutics, from the cell. Members of the ATP-binding cassette (ABC) superfamily are known to be involved in the multidrug-resistant phenotype of CSCs from many different cancers, including melanoma, lung, breast and pancreas (Bertolini et al., 2009). Of these, the ABCG2 (BCRP1) transporter appears to be exclusively expressed in stem cells and has been shown to be upregulated in multidrug resistant stem cell lines (Hong et al., 2009). ABCB1 (MDR1) has been shown to remove vinblastine and paclitaxel from stem cells, whereas ABCG2 prevents accumulation of imatinib mesylate, topotecan and methotrexate (Eyler et al., 2008). Antibodies to these protein transporters can effectively block tumor growth and increase chemosensitivity as seen in melanoma xenografts (Schatton et al., 2008). Similarly, lung cancer cells co-expressing CD133 and ABCG2 are enriched following chemotherapy, further implicating the role of ABC transporters in conferring chemoresistance and increased survival to CSCs (Bertolini et al., 2009).

In addition to an increased capacity for drug efflux, CSCs also express molecular mediators, like Aldehyde Dehyrogenase (ALDH), that are able to degrade metabolically-active byproducts of chemotherapeutic agents and render them inactive. By these means, ALDH1, 3 and 5 confer resistance to cyclophosphamide in several blood, breast and colon cancer cell lines (Dylla et al., 2008). Additionally, knockdown of ALDH1 expression in resistant colorectal CSCs has been shown to increase cell sensitivity to cyclophophamide *in vivo* (Dylla et al., 2008).

2.2.4 Wnt signaling

Expression of β-catenin, an essential component of the Wnt signaling pathway, has been shown in multiple studies to be linked to CSC survival and tumorigenesis (Taipale et al., 2001; Chen, M. S. et al., 2007; Woodward et al., 2007; Morrison et al.). Woodward et al showed that the Wnt/β-catenin pathway is also involved in CSC resistance to radiation in

mammary progenitor cells and breast cancer cell lines (Woodward et al., 2007). Irradiation of a murine mammary epithelial cell culture resulted in high levels of activated β-catenin in cells expressing stem cell antigen (Sca), while accumulation of β-catenin was not present in Sca-negative cells. Increased levels of β-catenin in irradiatiated Sca-positive cells correlated to enhanced self-renewal in mammospheres as well as upregulation of the anti-apoptotic protein Survivin. Upregulation of Survivin has also been reported in colon cancer cell lines where it seems to assist cancer cells in escaping senescene by enhanced telomerase activity (Endoh et al., 2005).

2.2.5 Specialized microenvironment (niche)

The CSC microenvironment undoubtedly influences CSC behavior. Surroundling stromal cells likely modulate CSC susceptibility to cytotoxic stress, such as radiation. Radiation-induced apoptosis depends on an oxygen-rich environment to generate free radicals capable of damaging DNA. Traditionally, CSCs were believed to preferentially reside in hypoxic microenvironments as a means of resisting radiation-induced cell death. However, CSCs are more often found next to blood vessels where they are well-oxygenated (Calabrese et al., 2007; Krishnamurthy et al.), further evidence that they require or co-opt the microenvironment for propagation and survival. Interestingly, CSCs seem to contribute to tumor angiogenesis, producing higher levels of VEGF in both normoxic and hypoxic conditions than non-CSC populations (Eyler et al., 2008). CSCs also rely upon factors secreted by the vasculature, such as leukemia inhibitory factor, brain-derived neurotrophic factor and pigment epithelial-derived factor, for normal stem cell maintenance (Eyler et al., 2008).

Similar to normal tissue stem cells, CSCs within breast and head and neck tumors have been found to exhibit increased antioxidant defenses in comparison to their non-tumorigenic progeny (Diehn et al., 2009). Lower ROS levels are the result of increased free radical scavengers that can protect the cell from radiation-induced damage and apoptosis.

Hypoxic states appear to enrich CSC populations. This effect has been described in medulloblastomas as well as endothelial-derived tumors (Blazek et al., 2007; Eyler et al., 2008). Hypoxia-induced factor (HIF)-1 may be responsible for mediating radioresistance in this situation as well as inducing the production of VEGF. Tumors derived from irradiated-CSCs are often highly vascular, indicating that HIF in radio-resistant CSCs contributes to angiogenesis and tumor growth in an irradiated environment. Furthermore, recent clinical trials have shown enhanced cell killing when antiangiogenic therapy is combined with radiation (Lee et al., 2000; Hess et al., 2001).

2.2.6 Resistance to apoptosis

CSCs may also acquire resistance to apoptosis by sustained activation of cell survival pathways or by inhibition of apoptotic pathways. Ma et al showed that cells expressing CD133 in hepatocellular carcinoma demonstrated a prolonged expression of the Akt/PKB and Bcl-2 survival pathways in response to treatment with fluorouracil and doxorubicin (Ma et al., 2008). Treatment with an Akt1-inhibitor sensitized the cells to chemo-induced apoptosis. NFkB, an anti-apoptotic transcription factor downstream of Akt, has also been implicated in the survival and progression of several cancers and may also promote EMT conversion in CSCs leading to metastasis (Sarkar et al., 2008).

Ultimately, it seems that CSCs may resist cytotoxic therapies through a combination of mechanisms that may differ among individual tumors. This emphasizes the need to develop

CSC-directed therapies to augment current anti-cancer treatments. In cancers where growth is dependent on CSCs, complete eradication of this sub-population may achieve long-term cure.

3. Therapeutic targeting of cancers cells

The frequent failure of standard cytotoxic therapies to provide a lasting cancer-free survival may be explained, in part, by the resistance of CSCs to standard chemotherapy and radiation. While traditional therapies can lead to early and often dramatic clinical responses, by failing to eradicate the tumorigenic CSC population, disease relapse can be expected. Clearly strategies that incorporate our increasing understanding of tumor cell heterogeneity with regards to treatment response are needed.

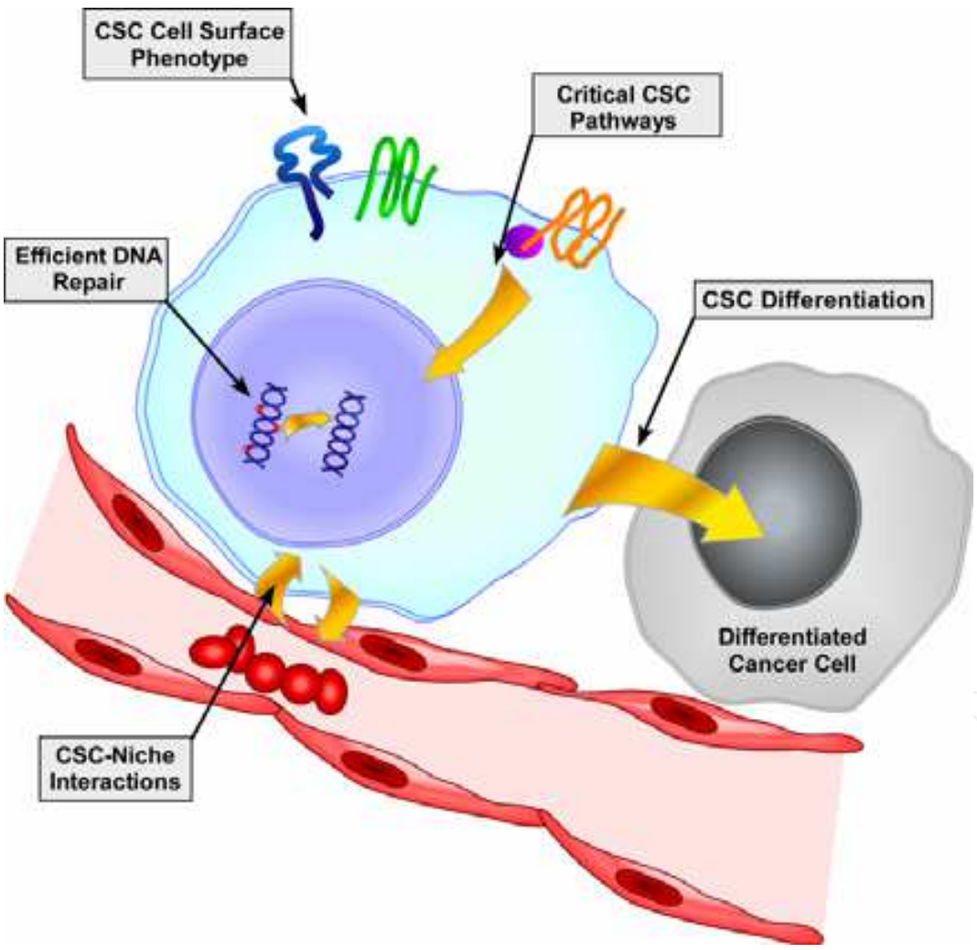

Fig. 4. Overview of Strategies for Targeting CSCs. Proposed strategies for selectively targeting CSCs include addressing their mechanisms of resistance such their efficient DNA repair mechanisms, critical survival pathways and/or specialized microenvironment. Other strategies try to take advantage of the unique cell surface phenotype that differentiates these cells from the remaining tumor bulk or force the CSC to differentiate into a more treatment-sensitive target

Given the resistance to standard cytotoxic therapy displayed by many CSCs, therapeutic targeting of this tumor cell population will likely prove to be a challenging endeavor. Several different strategies are currently being developed to selectively target CSCs. These include therapies that target the unique cell surface phenotype of CSCs and critical CSC signaling pathways as well as strategies aimed at forcing CSCs to differentiate and thereby increase their therapeutic sensitivity. Alternative strategies are aimed not at the CSC itself, but at its microenvironment (Figure 4). Most of these approaches have shown success in preclinical trials, with current early clinical phase I and II studies underway in a subset.

3.1 Targeting the cell surface phenotype

For many malignancies, distinct cell surface phenotypes have been defined which identify tumor cell populations enriched in CSCs. It is not surprising, therefore, that therapies directed against these cell surface antigens are under development. Monoclonal antibody therapy, antibody-drug conjugates and dendritic cell vaccinations are under investigation as potential methods of selectively targeting the CSC population using their cell surface phenotype (Figure 5).

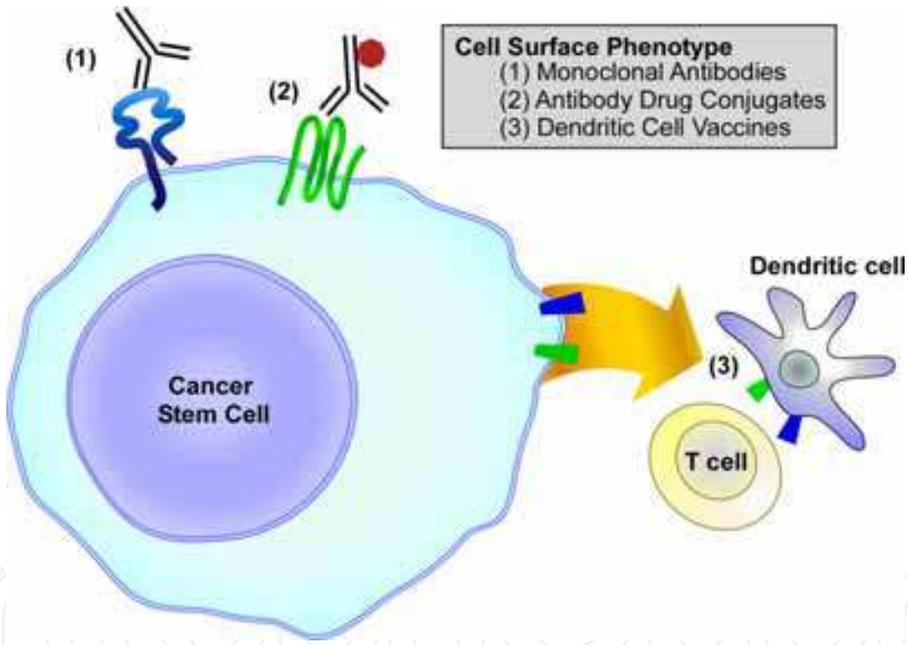

Fig. 5. Targeting the CSC Phenotype. Strategies for targeting CSC antigens include antibody-based therapy such as monoclonal antibodies and antibody-drug conjugates. Dendritic cell vaccines primed with CSC antigens are another possible targeting method under development

A wide variety of cell-surface antigens have been identified in tumor populations enriched in CSCs. Although there are some antigens that appear to mark CSCs in multiple tumor types there is also a variety of different surface antigens used to define CSCs across tumor types. In many cases, multiple cell-surface antigens have been identified that can be used to selectively enrich for a population with stem-like properties. While little is currently known about the functional role of many of these proteins, early preclinical work suggests that targeting them may have therapeutic value.

3.1.1 Antibody-based therapy

Monoclonal antibody (mAb) therapies are being used with increasing success as targeted agents in cancer therapy. They are believed to function through diverse mechanisms, including interactions with the host immune system through antibody-dependent cell cytotoxicity and complement activation, as well as the blockade of important tumor cell signaling pathways or the elimination of critical cell surface antigens (Adams et al., 2005). In addition, mAbs often display synergism when used in combination with traditional cytotoxic chemotherapy and can act as delivery vectors for more traditional cytotoxic therapies when conjugated to radioisotopes or chemotherapeutics. Increasingly, monoclonal antibodies are being shown to be a valuable addition to standard therapeutic regimens in multiple solid organ malignancies (Bonner et al., 2006; Vermorken et al., 2008; Tebbutt et al.; Ibrahim et al.).

Antibody therapies directed against CSC antigens are a logical outgrowth of the CSC theory and the increasing evidence in its support. Given the important roles in tumor development and growth displayed by tumor cells expressing CSC antigens, targeting these same antigens brings with it the hope of being able to selectively target the command center of the tumor. In preclinical testing, antibody-based therapies directed against CSC antigens has demonstrated encouraging results.

CD44: CD44 has been defined as a CSC antigen in a number of malignancies including breast, colorectal and head and neck cancer. CD44 is a large, heavily glycosylated transmembrane protein that has known functions in cell adhesion, signaling, migration and defense against reactive oxygen species (Ishimoto et al.). It undergoes complex alternative splicing resulting in functionally different isoforms with variable tissue expression. CD44 is known to interact with the CSC niche by binding to components of the extracellular matrix, most notably hyaluron as well as osteopontin, collagen and fibronectin to a lesser degree (Culty et al., 1990; Jalkanen et al., 1992; Weber et al., 1996).

Even prior to its recognition as a marker of CSCs, a variant of CD44 was recognized for its ability to promote metastatic behavior in a rat model of pancreatic cancer (Gunthert et al., 1991). Furthermore, blockade of CD44 with a mAb slowed growth of lymph node and lung metastases as well as prevented metastatic formation in this same model of pancreatic cancer, presumably through blocking of ligand interaction (Seiter et al., 1993).

Increased CD44 expression correlates with locoregional recurrence following radiation therapy for laryngeal cancer (de Jong et al.). In addition, CD44 expression has been correlated to patient prognosis in colorectal (Lugli et al.), breast (Neumeister et al., ; Zhou, L. et al.) and pancreatic cancer (Gotoda et al., 1998).

Early phase I clinical studies examined the effect of a humanized mAb to CD44v6 (Bivatuzumab) labeled with the radio isotope rhenium-186 in patients with head and neck squamous cell carcinoma (Stroomer et al., 2000; Borjesson et al., 2003). These studies demonstrated acceptable toxicity with stable disease in patients who received higher drug doses.

However, the enthusiasm for further development of CD44-based antibody therapy waned after a subsequent phase I study demonstrated unacceptable toxicity. In this dose-escalation study, bivatuzumab conjugated to the chemotherapeutic mertansine was evaluated in patients with head and neck cancer. Skin-related toxicity occurred with increasing dose and the trial had to be halted early after one patient died from toxic epidermal necrolysis (Tijink et al., 2006; Rupp et al., 2007).

CD133: CD133 is a well-recognized CSC marker in multiple malignancies, including glioblastoma, colorectal, prostate, pancreatic, ovarian and renal cancer (Hermann et al., 2007; Ricci-Vitiani et al., 2007; Baba et al., 2009). CD133 is a pentaspan transmembrane glycoprotein that localizes to cell protrusions. It has known interactions with cholesterol and is speculated to be involved in plasma membrane organization, although the exact functional properties of this molecule are not well characterized (Mizrak et al., 2008). Mutations in this gene are associated with multiple retinal diseases and it has been well-defined as a marker for hematopoetic and neural progenitor cells.

Expression of CD133 has been linked to adverse tumor behavior. CD133-positive cells have been shown to be resistant to standard chemotherapy in multiple tumor cell types, among them head and neck (Zhang, Q. et al.), pancreatic (Hermann et al., 2007), glioblastoma (Blazek et al., 2007), and colorectal cancer cells (Dallas et al., 2009). CD133 expression has also been correlated to tumor recurrence in patients with colorectal cancer treated with chemotherapy and radiation (Nagata et al.). Whether CD133 has a direct functional role or is merely a convenient marker of cells that express these abilities is, as yet, unknown.

As of yet, antibody therapy directed against CD133 has only been evaluated in limited preclinical models. Chen and colleagues demonstrated that CD133 targeting with a mAb could inhibit proliferation of colorectal cancer cell in vitro (Chen, W. et al.). Damek-Poprawa and colleagues conjugated a genetically modified cytotoxin from Aggregatibacter actinomycetemcomitans to an anti-human CD133 mAb and demonstrated its ability to selectively target $CD133^+$ head and neck cancer cells in vitro (Damek-Poprawa et al.).

The potential for success of anti-CD133 antibody therapy has been recently questioned by the discovery that CD133 expression may not be as tightly linked to CSC function as previously suggested. Chen and colleagues elegantly demonstrated that the CD133 negative cell population in neuroblastoma, a tumor in which CD133 is well-characterized as a CSC antigen, harbors a subset of cells with tumor-initiating capability (Chen, R. et al.). Clearly, a more in-depth knowledge of the correlation between cell-surface phenotype and functional activity is needed if we are to be successful in selectively targeting the CSC population.

ALCAM: Activated leukocyte cell adhesion molecule (ALCAM), also known as CD166, has been characterized as a stem cell niche marker in the colon (Levin et al.) and as a CSC marker in colorectal (Dalerba et al., 2007) and prostate cancer (Rajasekhar et al.). CD166 is a member of a subfamily of immunoglobulin receptors with five extracellular immunoglobulin-like domains, a transmembrane section and a short cytoplasmic tail (Weidle et al.). It is involved in homotypic interactions as well as heterotypic interactions with CD6.

Altered CD166 function, levels of expression and subcellular localization are all suspected to play a role in tumor biology. Functional polymorphisms of the CD166 gene that confer increased transcriptional activity have been correlated to an increased risk of the development of breast cancer (Zhou, P. et al.). Furthermore, overexpression of CD166 as compared to surrounding normal tissue has been demonstrated in papillary and medullary thyroid cancer (Micciche et al.). In pancreatic cancer, overexpression of CD166 has been associated with shorted disease-free and overall survival (Kahlert et al., 2009). However, in gastric cancer, decreased CD166 expression through microRNA and siRNA ALCAM silencing has been shown to increase cellular proliferation (Jin, Z. et al.). The conflicting reports on the role of over- or under-expression of CD166 in multiple cancers highlights our incomplete understanding of the functional role of this molecule. It is quite possible that the function of CD166 differs by malignancy type.

Rather than over- or under-expression, altered cellular localization of CD166 has been correlated with disease progression in multiple tumor types. In colorectal (Lugli et al.) head and neck (Sawhney et al., 2009), ovarian (Mezzanzanica et al., 2008) and breast cancer (Burkhardt et al., 2006) loss of membranous staining has been associated with disease progression. In many cases, loss of membranous CD166 expression (or an increase in cytoplasmic CD166) is associated with loss of cell-cell adhesion and the acquisition of a metastatic phenotype (Mezzanzanica et al., 2008). Some have speculated that CD166 functions as a sensor of cell density, which may help to explain the fact that it is strongly expressed on CSCs.

In preclinical studies, selective targeting of CD166 with a recombinant single-chain antibody inhibited breast cancer invasion *in vitro* and colorectal tumor growth in a nude mouse xenograft model (Wiiger et al.). CD166 internalization as a means for intracellular drug delivery has also been studied *in vitro*. Piazza and colleagues demonstrated that the human single-chain antibody fragment I/F8 selectively targets CD166 and induces internalization of the antibody-CD166 complex. They then developed an immunotoxin from the conjugation of I/F8 to the ribosome inhibiting protein saporin and demonstrated the ability of their antibody fragment to deliver the toxin intracellularly and selectively kill CD166 expressing cells (Piazza et al., 2005). A similar strategy targeting CD166 for intracellular delivery of liposomal drugs has been shown to have some efficacy in vitro in select prostate cancer cells (Roth et al., 2007). Given the loss of cell surface CD166 expression that has been shown to occur with disease progression in many malignancies, targeted therapy directed at extracellular CD166 epitopes may prove to have limited therapeutic efficacy in vivo.

3.1.2 Dendritic cell vaccines

In addition to antibody-based therapy, cancer stem cell antigens are also being targeted through dendritic cell vaccines. In a rat model of glioblastoma, dendritic cell vaccination using CSC antigens produced T-cell responses against CSCs but not those primed with daughter cells. Furthermore, survival was prolonged in animals receiving CSC dendritic cell vaccines as compared with non-CSC tumor cell vaccination (Xu et al., 2009). Current phase I trials are underway in patients with glioblastoma using dendritic cell vaccines primed with mRNA or whole cell lysates from CD133 positive tumor cells.

3.2 Targeting cancer stem cell signaling pathways

3.2.1 Targeting notch signaling

The Notch signaling pathway is a highly conserved pathway in multicellular organisms. There are four different Notch receptors (Notch 1-4) that are single pass transmembrane proteins with large extracellular and small intracellular domains. The two most well-characterized notch ligands are Delta-like and Jagged, which are also single pass transmembrane proteins. Upon ligand binding, the extracellular portion of Notch is cleaved by a metalloprotease called TACE (Tumor Necrosis Factor Alpha Converting Enzyme) and the ligand and notch extracellular domain are then endocytosed by the ligand-expressing cell. Subsequently, γ-secretase cleaves the intracellular notch domain, releasing it move to the nucleus and regulate gene expression (Harrison et al.). Strategies to target Notch signaling in cancer have focused on multiple points in this pathway, including γ-secretase inhibition and antibody therapy directed against notch ligands as well as the notch receptor (Figure 6).

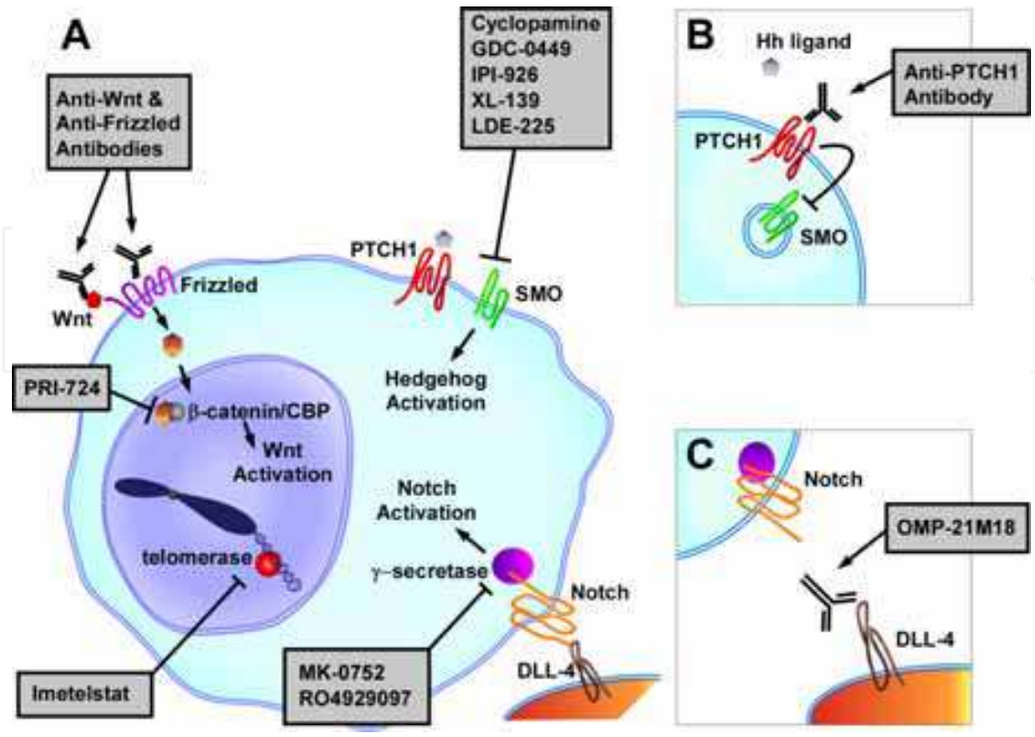

Fig. 6. Targeting critical CSC pathways

The Notch pathway is known to regulate cell fate and renewal, particularly during embryonic development. Notch signaling in cancer has been demonstrated to be a particularly important in regulating the function of the CSC tumor population. For instance, in lung adenocarcinoma CSCs, Notch signaling is important for key stem-like properties. Sullivan and colleagues demonstrated that lung adenocarcinoma CSCs identified by high Aldehyde Dehydrogenase activity had elevated expression of Notch pathway transcripts. Furthermore, when the notch pathway was targeted either through γ-secretase inhibition or expression of a shRNA against Notch3, decreased tumor cell proliferation and clonogenicity were noted (Sullivan et al.).

In glioblastoma xenograft models, γ-secretase inhibition has been shown to deplete $CD133^+$ CSCs and prolong survival (Fan et al.). In addition, when combined with temozolomide therapy, γ-secretase inhibition blocked tumor progression in 50% of mice with established xenografts (Gilbert et al.). Preclinical activity of γ-secretase inhibition has also been demonstrated in colorectal (Akiyoshi et al., 2008), breast (Han et al., 2009; Rasul et al., 2009), ovarian (Wang, M. et al.) and lung cancer (Konishi et al., 2007). These encouraging preclinical results have paved the way for currently ongoing Phase I studies. RO4929097, MK-0752 and PF-03084014 are three different γ-secretase inhibitors currently being evaluated in phase I oncology trials.

Gamma-secretase inhibition is a relatively non-specific method of decreasing Notch signaling. Although Phase I data is not yet available for us to understand treatment toxicity in human patients, early preclinical evidence suggests that dual inhibition of Notch1 and 2 through γ-secretase inhibitition may lead to intestinal morbidity through depletion of crypt-based progenitor cells (Riccio et al., 2008). In an effort to more selectively target individual Notch receptors (Notch1-4), antibodies selectively targeting Notch1 and Notch2 have been

developed. In xenograft tumor models, Notch1 blockade inhibits tumor growth through inhibition of both cancer cell growth and angiogiogenesis (Wu et al.). Based upon these encouraging early results, there may be a role for the selective targeting of individual Notch receptors as a way to minimize therapeutic morbidity.

As well as notch receptor blockade and γ-secretase inhibition, blocking notch receptor ligands is an alternative strategy to abolish notch signaling. Preclinical studies in colorectal tumor xenograft models have demonstrated the efficacy of anti-DLL4 antibodies in inhibiting tumor growth, particularly in combination therapy with irinotecan (Fischer et al.). Interestingly, in contrast to cetuximab therapy, anti-DLL4 showed efficacy in both KRAS wild-type and mutant tumors. Preclinical efficacy of anti-DLL4 therapy has also been noted in pancreatic cancer (Oishi et al.), Ewing's sarcoma (Schadler et al.). DLL4 blockade appears to work through similar mechanisms to notch receptor blockade in that it reduces CSC frequency and tumor cell growth (Hoey et al., 2009) as well as inhibits angiogenesis (Ridgway et al., 2006).

Phase I clinical studies are currently underway to evaluate the safety of a humanized mAb targeting the N-terminal epitope of DLL4 (OMP-21M18) in combination with other chemotherapeutics in colorectal, lung and pancreatic cancer. The potential for significant toxicity with anti-DLL4 therapy will be carefully evaluated given that chronic DLL4 blockade has been demonstrated to induce hepatic toxicity and, in a dose-dependent manner, lead to the development of subcutaneous vascular neoplasms in rats (Yan et al.).

3.2.2 Targeting hedgehog signaling

The hedgehog (Hh) signaling pathway is a key developmental pathway that regulates animal morphogenesis. In cells receiving Hh signaling, pathway activity is controlled at multiple levels. In the absence of Hh, Patched1 (PTCH1), a transmembrane receptor, suppresses the activity of Smoothened (Wiiger et al.) by preventing its cell surface localization. In the presence of Hh ligand, the Hh pathway is activated by PTCH1 relieving its inhibition of SMO. SMO localizes to the cell surface and initiates a signaling cascade that activates the glioma-associated (Gli) family of zinc finger transcription factors (Evangelista et al., 2006).

Dysregulation of the Hh signaling pathway has been noted in multiple types of cancer, the prototype of which is basal cell carcinoma. Inactivating mutations of PTCH1 are noted to be the cause of Gorlin syndrome, a disease characterized by the development of multiple basal cell carcinomas (BCCs) and keratocystic odontogenic tumors with increased susceptibility to the development of medullblastoma and rhabdomyosarcoma. In addition, most sporadic BCCs have been demonstrated to have inactivating PTCH1 mutations (Caro et al.). Increased Hh pathway expression has also been documented in a large number of malignancies, among them medulloblastoma (Raffel et al., 1997; Taylor et al., 2002), head and neck (Schneider et al.), pancreatic (Walter et al.) and breast cancer (ten Haaf et al., 2009).

It has been speculated that the hedgehog pathway may promote key tumor behaviors by acting predominantly on CSCs (Evangelista et al., 2006). The Hh pathway is well-known to regulate tissue growth and regeneration through its effects on normal tissue stem cells (Bhardwaj et al., 2001; Machold et al., 2003; Ahn et al., 2005; Palma et al., 2005; Plaisant et al.). Shin and colleagues have shown that in response to injury, sonic hedgehog protein expression is upregulated in bladder epithelial stem cells. This in turn elicits increased Wnt expression in the adjacent stroma, with resultant epithelial and stromal cell proliferation and restoration of urothelial function (Shin et al.).

Accumulating evidence suggests that CSCs may rely on Hh signaling in a similar manner. In gastric cancer cells, inhibition of Hh signaling selectively reduced proliferation and increased susceptibility to chemotherapy in the CSC subpopulation (Song et al.). In CD133-positive glioma CSCs, treatment with the Hh inhibitor cyclopamine increased sensitivity to temozolomide therapy. Interestingly, the combination of cyclopamine with a γ-secretase inhibitor provided an even greater increase in CD133-positive cytotoxicity with temozolomide, indicating a potential role for the simultaneous inhibition of multiple CSC signaling pathways.

Current strategies to target the Hh pathway rely mainly on SMO targeting. The naturally-occuring plant teratogen cyclopamine and subsequent synthetic derivates were first demonstrated to inhibit aberrant Hh pathway activation due to oncogenic SMO and PTCH mutations through inhibition of SMO over a decade ago (Taipale et al., 2000). Since that time the efficacy of SMO inhibition has been demonstrated in preclinical models of glioblastoma, small cell lung, gastric, pancreatic and prostate cancer (Evangelista et al., 2006). GDC0449, an oral small molecule inhibitor of SMO, is the furthest along in clinical development. Other small molecule inhibitors of SMO under development include LDE-225, BMS-833923, IPI-926 and PF-04449913 (Figure 6).

Phase I clinical trial data for GDC0449 in patients with advanced and/or metastatic solid organ malignancies has recently been reported (Lorusso et al.). An acceptable side-effect profile was observed. Furthermore, a clinical response was seen in 19 of 33 patients with basal cell carcinoma and in 1 patient with medulloblastoma, both of which are tumors known to be driven by PTCH1 and SMO mutations. Phase II trials in multiple malignancy types are now underway with this compound as well as other phase I studies evaluating other small molecule SMO inhibitors. Interestingly, the well-characterized antifungal Itraconazole was recently demonstrated to inhibit Hh pathway activation and cancer growth through SMO inhibition (Kim et al.). Undoubtedly, future studies will likely evaluate the efficacy of this relatively well studied and well-tolerated agent in a cancer setting.

Future strategies to overcome tumor dependence on Hh signaling will likely incorporate downstream targeting of Hh pathway components. Because current therapeutic approaches predominately target the transmembrane protein SMO, they would not be expected to abrogate Hh pathway activation due to overexpression of molecules further downstream in the signaling cascade. This is becomes a valid consideration given that overexpression of Gli, a downstream effector of the Hh pathway, has been documented in some tumor types, including esophageal and colorectal cancer (Rizvi et al., ; Mazumdar et al.). In addition, the acquisition of SMO mutations may interefere with the ability to target this protein. In fact, in medulloblastoma, the acquisition of a SMO mutation that disrupts the ability of GDC0449 to bind SMO has been demonstrated to confer resistance to this method of targeted Hh pathway inhibition (Yauch et al., 2009). Several small molecule anatagonists of downstream Hh pathway effectors have been discovered (Hyman et al., 2009; Mazumdar et al.) and may provide a basis for the development of future therapeutics that more comprehensively target the Hh pathway.

3.2.3 Targeting Wnt signaling

The Wnt highly conserved signaling pathway that plays a key role in maintenance of the stem cell population, proliferation, differentiation and apoptosis (de Sousa et al.). In the canonical Wnt pathway, signaling is mediated primarily through β-catenin. In the absence of Wnt ligands, β-catenin is phosphorylated which primes it for ubiquitination by a

destruction complex composed, in part, by the tumor suppressor protein APC. After ubiquitination Wnt undergoes proteosomal degredation. Upon Wnt ligand binding to the Wnt receptor Frizzled (FZD), β-catenin is no longer degraded due to dissolution of the destruction complex. β-catenin translocates to the nucleus where along with coactivators CBP and p300 it activates the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors, leading to expression of Wnt target genes.

Upregulation of Wnt signaling is a common finding in cancer (Deonarain et al., 2009). A prime example of this is colorectal cancer, in which frequent activating mutations of β-catenin or inactivating mutations of APC lead to constitutive Wnt pathway activation. Interestingly, Wnt signaling appears to be particularly important in CSC function. In colorectal cancer, higher Wnt pathway activation is noted in the cancer stem cell population adjacent to the tumor stroma as compared with the bulk of tumor cells. Furthermore, high Wnt signaling has been shown to functionally define the CSC compartment *in vitro* and *in vivo* (Vermeulen et al.). Wnt signaling has also been demonstrated to play a key role in the regulation of cancer stem cells in lung cancer (Teng et al.) and glioblastoma (Jin, X. et al.).

Therapies are under development that target multiple points in the Wnt signaling pathway, from antibodies directed against Wnt ligands and their receptor Frizzled to small molecules such as PRI-724 that inhibit the β-catenin/CBP transcription activating complex (Figure 6). Of these, PRI-724 is the furthest along in clinical development, with phase I clinical trials currently ongoing.

3.3 Telomerase inhibition

Telomerase is an enzyme that adds repeating sequences of TTAGGG to the 3' ends of DNA strands, thereby preventing loss of important DNA from chromosome ends. Telomerase activity has been implicated in the limitless self-renewal potential of CSCs, making it an attractive target for inhibition. In preclinical models, telomerase inhibition depletes the CSC tumor cell subpopulation in breast and pancreas (Joseph et al.), neural (Castelo-Branco et al.) and prostate (Marian et al.) cancer cell lines. Imetelstat (GRN163L), a synthetic oligonucleotide that targets the template region of telomerase is currently being evaluated in Phase I clinical trials.

3.4 Targeting CSC DNA repair mechanisms

Efficient DNA repair has been identified as one mechanism by which the CSC tumor subpopulation is more resistant to standard DNA-damaging therapy (Bao et al., 2006). In glioblastoma, $CD133^+$ CSCs display increased DNA damage checkpoint response to radiation and have more efficient DNA repair. Furthermore, the radioresistance of these CSCs can be reversed with inhibition of the checkpoint kinases Chk1 and Chk2 (Ropolo et al., 2009). Of the many compounds that have been identified to inhibit Chk1 and Chk2 (Garrett et al.), Ly2606368, a Chk1 inhibitor, is currently being evaluated alone and in combination with cisplatin in phase I clinical trials.

3.5 Targeting CSCs through differentiation therapy

Rather than targeting drugs to specific features of CSCs, an alternative strategy may be to make CSCs more responsive to existing chemotherapeutic agents. This may be accomplished by promoting differentiation of CSCs from their resistant, stem cell state to more responsive differentiated cells. This has already been shown to be an effective strategy

in some model systems. In chronic myeloid leukemia, primitive, quiescent CSCs are resistant to imatinib, an inhibitor of the BCR-ABL fusion kinase. However, treatment with several days of G-CSF stimulates differentiation of these CSCs, increasing sensitivity to imatinib (Jorgensen et al., 2006). Similarly, $CD133^+$ CSCs in glioblastoma are very drug resistant. Treatment with bone morphogenic proteins (BMP), particularly BMP4, effectively initiates differentiation of glioblastoma cells, thereby reducing $CD133^+$ cells, clonogenic ability, and cell proliferation in mouse xenografts (Piccirillo et al., 2006). This therapy may also make these glioblastoma cells more sensistive to other drugs, increasing the efficacy of chemotherapy for this tumor (Figure 7).

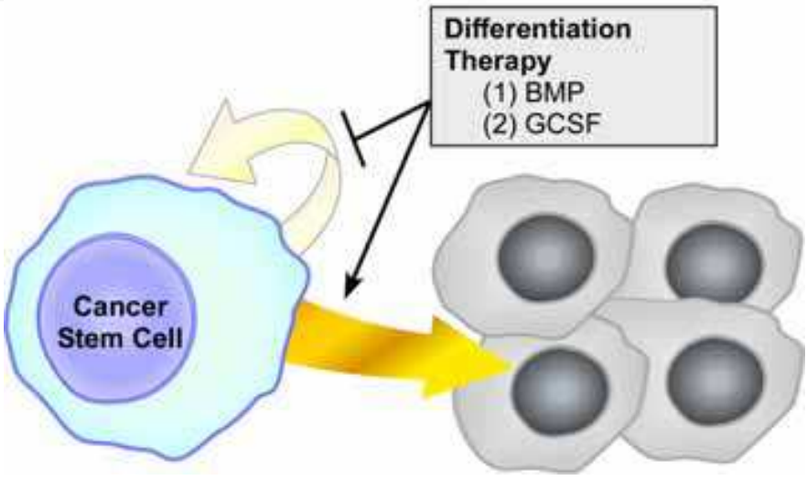

Fig. 7. Differentiation therapy. Strategies forcing CSC differentiation may increase therapeutic efficacy of traditional cytotoxic therapy

3.6 Targeting the CSC microenvironment

Rather than targeting the CSC directly, attempts to disrupt the CSC's specialized microenvironment may prove an alternative strategy for eradicating the CSC population. Much in the way normal adult tissue stem cells require a specialized microenvironment, or niche, to maintain a balance between self-renewal and differentiation, increasing evidence suggests that CSC behavior relies on similar microenvironmental cues.

Components of this specialized microenvironment include both non-tumor cells such as fibroblasts, myoepithelial cells, osteoblasts, leukocytes and endothelial cells, as well as the extracellular matrix proteins and signaling molecules they produce. The composition of the niche varies by tumor type; for example, hematopoetic stem cells reside in an osteoblastic niche (Zhang, J. et al., 2003; Arai et al., 2004), while epithelial stem cells reside in a niche composed of fibroblasts and myoepithelial cells (Ohlstein et al., 2004).

Evidence that CSCs require a similar microenvironment is mounting. Leukemic CSCs preferentially home to the niche and enjoy a growth advantage once there (Kawaguchi et al., 2001), while both glioblastoma (Charles et al.) and HNSCC (Krishnamurthy et al.) CSC's reside in a perivascular niche that is critical for their survival. The CSC-niche interaction functions to support and maintain CSCs through a variety of interactions and signaling cascades, and it has been suggested that maintaining the CSC-niche interaction is the primary role of many known CSC surface markers such as CD44 (Marhaba et al., 2008).

Targeting these physical interactions as well as niche-CSC signaling pathways hold therapeutic promise (Figure 8).

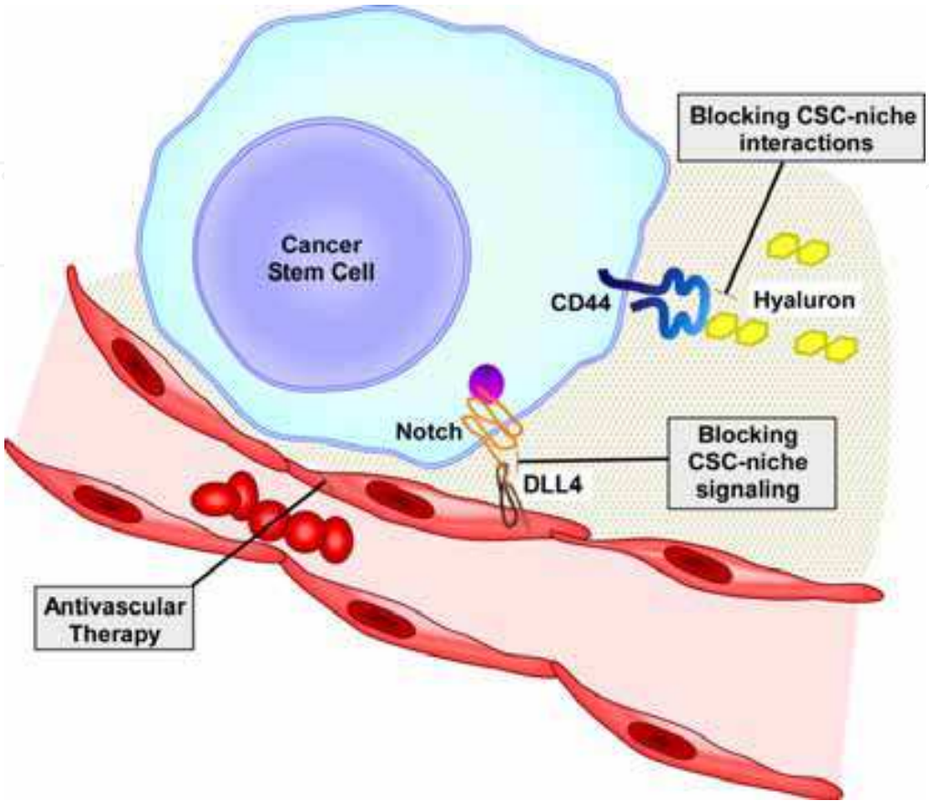

Fig. 8. Several differing strategies are currently under evaluation for targeting the CSC niche. Targeted anti-vascular therapy and anti-Notch strategies highlight the prominent role of the endothelial cell in the niche. Other therapies seek to target physical interactions between CSC proteins and non-cellular niche components, such as therapies that disrupt the interaction of CD44 with components of the extracellular matrix

This interaction appears to function both ways, as glioblastoma CSCs may create and maintain their vascular niche by differentiating into endothelial cells (Ricci-Vitiani et al., 2007). Furthermore, there is evidence that some tumors may induce creation of a niche-like environment prior to the arrival of tumor cells in metastatic spread, likely via secretion of tumor-derived growth factors (Kaplan et al., 2005). Thus inhibition of the CSC-niche interaction may be a useful strategy for elimination of CSCs. In mouse glioblastoma xenografts, inhibition of CSC-derived endothelial differentiation led to tumor reduction, likely via inhibition of the CSC niche (Ricci-Vitiani et al., 2007). These findings may help to explain the efficacy of anti-vascular therapies, such as VEGF inhibition, in select cancers.
Anti-CD44 therapy may be another strategy to disrupt the CSC niche. CD44 is well-known to interact with components of the extracellular matrix such as hyaluron, osteopontin, fibronectin and collagen. There is increasing evidence that disruption of this interaction may impact CSC survival. Current strategies using antibody therapy directed against CD44, hyaluron-chemotheraeputic conjugates, and even miR-34a are under development (Wang, S. J. et al., 2006; Li et al.; Liu et al.).

Target	Drug	Trial #	Phase	Cancer types
Hedgehog Pathway	GDC-0449 (SMO and/or PTCH1 inhibitor)	NCT01088815	II	Pancreatic
		NCT00980343	II	Brain & CNS
		NCT00957229	II	BCC
		NCT00959647	II	BCC, CRC, Ovarian
		NCT00982592	II	Gastric, Esophageal
		NCT01267955	II	Chondrosarcoma
		NCT00887159	II	Lung
		NCT00961896	II	BCC
	LDE-225 (SMO inhibitor)	NCT01125800	I	Pediatric solid malignancies
		NCT01208831	I	Solid malignancies
		NCT00880308	I	Solid malignancies
		NCT01033019	II	BCC
	BMS-833923 (XL139) (SMO inhibitor)	NCT00670189	I	Solid malignancies
		NCT00909402	I	Gastric, Esophageal
		NCT00927875	I	Small cell lung cancer
	IPI-926 (SMO inhibitor)	NCT00761696	I	Solid malignancies
		NCT01130142	I, II	Pancreatic
		NCT01310816	II	Chondrosarcoma
	PF-04449913 (SMO inhibitor)	NCT01286467	I	Solid malignancies
Notch Pathway	MK-0752 (γ-secretase inhibitor)	NCT00645333	I, II	Breast
		NCT00106145		Breast
	RO4929097 (γ-secretase inhibitor)	NCT01071564	I	Breast
		NCT01192763	I	Pancreatic
		NCT01193868	II	Lung
	PF-03084014 (γ-secretase inhibitor)	NCT00878189	I	Solid Malignancy & Leukemia
	OMP-21M18 (anti-DLL4 mAb)	NCT01189942	I	CRC
		NCT01189929	I	Pancreatic
		NCT01189968	I	Lung
Telomerase	Imetelstat (GRN163L)	NCT01137968	II	Lung
Dendritic Cell Vaccines	Dendritic cell vaccine to CD133+ CSC mRNA	NCT00846456	I, II	Glioblastoma
		NCT00890032	I	Brain & CNS
	Dendritic cell vaccine to whole CD133+ CSC lysate	NCT01171469	I	Brain & CNS
Wnt Pathway	PRI-724	NCT01302405	I, II	CRC, Pancreatic
	Resveratrol	NCT00256334	I, II	CRC

Table 1. Current clinical studies evaluating CSC-directed therapies in solid organ malignancies. BCC = Basal cell carcinoma, CRC = Colorectal carcinoma

4. Conclusions

The CSC theory, aside from the contribution to our understanding of tumor biology, has potential far-reaching clinical implications. Early preclinical success, while certainly encouraging, has yet to be confirmed in clinical studies. For many of the therapeutic strategies discussed, phase I and II clinical studies are currently ongoing and will additional evidence as to the safety and efficacy of these therapies in the near future (Table 1).

In order to specifically target CSCs while sparing somatic stems cells, it will be critical to identify unique molecules and dysregulated pathways in the CSC population when compared to the somatic stem cell population. Our understanding of the differential regulation of CSCs and normal tissue stem cells is yet in its infancy and clearly needs further exploration.

Our ability to consistently and reliably identify tumor cell populations with CSC functionality needs to be improved. It is becoming increasingly apparent that currently identified CSC antigens are insufficient to detect all cells harboring CSC functions (Chen, R. et al.). This may be due to plasticity in the CSC compartment with cells gaining and losing CSC functions in response to environmental signals. It may also be due to the possibility that current surface antigens simply are not selective enough, or the combination of surface antigens not fully refined. Part of this problem stems from our incomplete understanding of the functional aspects of these CSC markers/molecules. In many cases these antigens are used because they have been shown to conveniently mark a population of cells that happen to have stem-like properties rather than because their expression is intrinsically tied to CSC functionality.

Furthermore, if we wish to target these CSC antigens, our understanding of their expression patterns in normal tissues needs to be elucidated. A prime example of this is the case of anti-CD44v6 therapy discussed previously. Clinical trials for a CD44-drug conjugate were halted early due to excessive skin toxicity and a patient death that occurred because of targeting of CD44v6 expressed in the basal layer of the skin. A more comprehensive knowledge of CSC antigen expression patterns may help better predict and subsequently avoid treatment-related toxicity.

Preclinical studies suggest that the combination of CSC-specific and broad cytotoxic therapy holds the best chance for disease eradication. In many preclinical examples, CSC-targeted therapy appears to increase the sensitivity of the CSC subpopulation to traditional cytotoxic therapies. Furthermore, it is possible that differentiated tumor cells may provide feedback and support to their undifferentiated CSC counterparts and that removal of this population may impact CSC survival as well. As therapeutic strategies are developed to target the CSC subpopulation, consideration will need to given to appropriate combinations with traditional cytotoxic therapies.

While there are clearly many obstacles to overcome, CSC-directed therapy has the ability to revolutionize cancer treatment. By focusing on tumor subpopulation heterogeneity in treatment response and tumorigenic potential, we will undoubtedly uncover novel therapeutic targets that would have remained otherwise undiscovered. Although CSC theory is yet in its infancy, the success of early preclinical studies brings hope that it may carry with it improved treatments.

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Cancer Stem Cells - The Cutting Edge

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Over the last thirty years, the foremost inspiration for research on metastasis, cancer recurrence, and increased resistance to chemo- and radiotherapy has been the notion of cancer stem cells.The twenty-eight chapters assembled in Cancer Stem Cells - The Cutting Edge summarize the work of cancer researchers and oncologists at leading universities and hospitals around the world on every aspect of cancer stem cells, from theory and models to specific applications (glioma), from laboratory research on signal pathways to clinical trials of bio-therapies using a host of devices, from solutions to laboratory problems to speculation on cancersâ€™ stem cellsâ€™ evolution. Cancer stem cells may or may not be a subset of slowly dividing cancer cells that both disseminate cancers and defy oncotoxic drugs and radiation directed at rapidly dividing bulk cancer cells, but research on cancer stem cells has paid dividends for cancer prevention, detection, targeted treatment, and improved prognosis.

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