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Commentary

Targeting cancer stem cells for more effective therapies: Taking out cancer's locomotive engine

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ABSTRACT

Novel therapies for the treatment of solid tumors have generally failed to improve patient overall survival. These therapeutic approaches are typically focused on targeting signaling pathways implicated in cell growth and/or survival in order to shrink the malignant mass and achieve an objective clinical response; however, too often these responses are followed by eventual regrowth of the tumor. This clinical conundrum could be explained by the existence of a tumorigenic cell population that is relatively resistant to these therapies and retains pluripotent status in order to repopulate the original tumor and/or contribute to distant metastasis following treatment. Compelling data from liquid tumors, and more recently from studies focused on solid tumors, now support the existence of such tumorigenic cells (i.e., cancer stem cells) as a distinct subpopulation within the total tumor cell mass. These cancer stem cells (CSCs), as compared to the non-CSC population, have the ability to reconstitute the primary tumor phenotype when transplanted into recipient animals. In addition, data are beginning to emerge demonstrating that many standard-of-care chemotherapeutics are less effective in promoting cell death or cytostasis in these putative cancer stem cells as compared to effects in the non-stem cell cancerous cells. Therefore, targeting these locomotive drivers of tumors, the cancer stem cell population, should be considered a high priority in the continued pursuit of more effective cancer therapies.

1. Introduction

An effective chemotherapeutic for most cancers remains a significant unmet medical need [1]. With a few exceptions, this need persists in spite of an increase in the number of clinical trials assessing new/novel compounds targeting the destruction of cancerous cells [2]. The relative paucity of success for new therapeutic entities in oncology has the expected components shared by other arenas e.g., poor tissue distribution and dose-limiting toxicity, but is largely attributed to the absence of sustained objective responses and non-significant changes in patient overall survival over placebo (as reviewed in [3]). The lack of a sustained effect on tumor growth reflects an aggressive tumor regrowth which is often associated with metastasis of malignant cells to remote sites, culminating in poor prognosis for overall survival [4].

The relatively poor success rate of new cancer therapeutics has not, however, slowed the frenetic pace of identifying new, innovative targets for future drug discovery programs. Cell survival and cell cycle proteins remain targets of interest joined by candidate proteins spawned by differential gene/protein expression profiling in malignant versus normal cells. These efforts have included the use of established cell lines such as the NCI-60, human primary tumor tissue and/or newly derived cell lines from such tumor tissue. All approaches have their positive attributes and have succeeded in identifying prospective target candidates for drug discovery programs. Unfortunately, target validation (i.e., efficacy of new agents in clinical trials) remains largely elusive.

The search for new targets for drug discovery programs is challenging in all therapeutic areas but is indeed hampered in oncology by both the marked cellular heterogeneity found in malignant tumors and the multitude of mutations, both 'driver' and 'bystander', which accumulate over time in malignant cells. This commentary questions not the target identification approaches being used but the cancerous tissue with which new targets are identified and emerging putative therapies are tested. A case is presented to pursue tumorigenic cells, aka cancer stem cells

Abbreviations: CSCs, cancer stem cells; CML, chronic myeloid leukemia; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; HCC, hepatocellular carcinoma; 5-FU, 5-fluorouracil; SP, side population; DCIS, ductal carcinoma in situ; PI3K, phosphatidylinositol-3-kinase; Hh, hedgehog; BMP, bone morphogenic protein; Apc, adenomatous polyposis coli; EGFR, epidermal growth factor receptor; MFE, mammosphere forming efficiency; LOPAC, library of pharmacologically active compounds; GBM, glioblastoma; MBM, medulloblastoma; GIST, gastrointestinal stromal tumor.

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(CSCs), as the primary biomass for these drug discovery efforts as it is these cells that are the primary drivers of tumor growth and harbor the greatest resistance to existing chemotherapeutic drugs. These attributes of CSCs may very well underlie the poor clinical efficacy noted to date in clinical trials for cancer.

2. Discovery of cancer stem cells

The concept that tumors may evolve from a small population of progenitor-like cells arose in the late 19th century (as reviewed in [3,5]) and was not systematically tested until nearly 100 years later by Dick et al. using circulating leukemia cells isolated from acute myeloid leukemia patients. The authors made several seminal observations including that only a small subset of blast cells was able to reconstitute the multi-lineage leukemic cell population when transplanted into immunodeficient mice [6,7]. These in vivo studies performed by Dick et al. and others [8] were fundamental in establishing the definition of a "cancer stem cell" as that of a single cell that is able to reconstitute a heterogeneous cell population in vivo. Additional studies of other hematological malignancies e.g., chronic myeloid leukemia (CML [9]), acute myeloid leukemia [6] and acute lymphoblastic leukemia [10], further supported a hierarchical model of tumorigenesis whereby CSCs have the ability to generate diverse progeny leading to the heterogeneous cell populations characteristic of liquid tumors. In many cases these CSCs from tumor-bearing animals could transfer disease when serially transplanted into recipient mice, a property that has become the gold standard for demonstrating an important 'stemness' trait, that of self-renewal [5].

Identifying putative CSCs in solid tumors has been more challenging in part due to the absence of such a facilitative in vivo model as described above with liquid tumors. However, data now exist implicating the presence of CSCs in most types of solid tumors including breast [11], brain [12], prostate [13], lung [14], liver [15], colon [16] pancreas [17], ovary [18] and head and neck [19]. Most of this work has been prospectively driven by the use of candidate surface markers (e.g., CD133 in [12]) or by exploiting the ability of CSCs to effectively efflux dyes such as Hoechst 33342 or rhodamine and thus being distinguished as the side population (SP) in flow cytometry spectra (as in [14,20]).

Collectively these studies have leant support for the hierarchical model of tumor formation which purports that only a small percentage of tumor cells are tumorigenic [4–7]. This contrasts with the stochastic model which suggests that all cells have a similar but statistically low probability of tumorigenic potential. This issue of tumor etiology remains controversial and may be settled by simple co-habitation, i.e., that each purview may be operant depending on the nature and/or stage of a given tumor. For example, CSCs may be very important in poorly differentiated tumors but a stochastic development might underlie the more homogeneous malignancies (e.g., lymphomas; [21]). It is widely accepted that the definitive data supporting the existence of CSCs remains the ability of these select cells to recapitulate the heterogeneous morphology of the parent tumor at high 'take rates' when transplanted into immunodeficient mice [3,5,15]. These data continue to accumulate and suggest that the hierarchical model has the potential to be operant in most tumor types studied. That not all tumor cells are equal in being able to recapitulate the parent tumor mass is not a new concept and was seemingly apparent in the few clinical studies examining tumor growth following autotransplantation of patient's cancer cells conducted in the 1960s (as discussed in [22]). Nonetheless, it is important to stress that the CSC hypothesis remains controversial [21] as interpretation of the serial transplantation data may be confounded by human tumor tissue in a murine environment.

Taken together, these studies have the important corollary that CSCs may be responsible for generating the regrowth of tumors following the debulking of tumor mass by surgery, radiation or chemotherapy. In this scenario, CSCs would be relatively resistant to cytocidal treatments compared to the vast majority of differentiated cells comprising the bulk of the tumor. Examining this tenet is clearly a challenge with regards to isolating the putative CSCs, and both candidate and operational approaches have been practiced. Candidate approaches pertain to isolation of putative CSCs by sorting these cells using known stem cell surface markers or by dye exclusion. Operational techniques relate to isolation of CSCs by harvesting the cells that remain following subjecting malignant specimens to known radio-chemotherapy or which exhibit stem cell behavior (e.g., sphere formation) in culture conditions.

3. Differential sensitivity of CSCs to chemotherapeutic treatments

3.1. Candidate approaches

Candidate CSCs have been isolated based on various properties including the presence of putative stem cell surface markers as well as their occupancy in the SP of FACS spectra. For example, the cell membrane protein CD133 was originally identified as a surface marker for hematopoietic stem cells but is also expressed on CSCs isolated from hematopoietic, neural and epithelial cancers [5,12,16]. Studying putative CSCs from human primary tumor tissue poses obvious problems associated with procurement, cost, tissue mass and heterogeneity, hence many groups have instead used immortalized cancer cell lines originally derived from patient tumors.

3.1.1. CD133+

Eramo et al. [23] focused on CD133+ cells for studying lung CSCs isolated from patients with small cell and non-small cell lung carcinomas (SCLC, NSCLC). CD133+ cells were found in these tissues while such cells were difficult to detect in sections from non-cancerous lung tissue. The standard-of-care chemotherapeutic agents cisplatin (5 μg/mL; approximately 20 μM), etoposide (10 μg/mL; approximately 20 μM), paclitaxel (30 ng/mL; approximately 35 nM) and gemcitabine (250 μM) had minimal cytotoxic effects on the isolated CD133+ cells from SCLC and NSCLC patients. Although no comparative culture data were presented with the CD133− population, the drug concentrations used were analogous to plasma levels achieved in the clinic.

Similarly, Ma et al. [24] surveyed responsiveness of CD133+ cells from human hepatocellular carcinoma (HCC) cell lines to doxorubicin (1–5 μg/mL; approximately 2–9 μM) and 5-fluorouracil (5-FU; 5–75 μg/mL; approximately 40–575 μM). 5-FU was slightly but significantly less effective in decreasing cell viability of the CD133+ compared to CD133− cells. However, more compelling results were reported showing a decreased sensitivity of the CD133+ population to doxorubicin [24].

Hermann et al. [25] also utilized cell surface expression of CD133 to isolate putative CSCs from a human pancreatic cancer cell line (L3.6pl). Similar to cells isolated from primary pancreatic tumors, the CD133+ cells from the cell line were quite efficient (10^3 cells) in inducing orthotopic tumor formation in recipient mice compared to CD133− cells (>10^6 cells for tumor formation). The authors reported a dramatic decrease in the efficacy of gemcitabine (100 ng/mL; approximately 380 nM), as measured by a relative absence of apoptosis in the cultured CD133+ versus CD133− cells. Interestingly, there was a marked effect of gemcitabine on cells in S-phase in both populations, suggesting that the decreased efficacy was not a function of the drug being

rapidly effluxed from the CD133+ cells. Finally, in vivo treatment of mice harboring L3.6pl xenografts with gemcitabine (biweekly 125 mg/kg i.p. for 3 weeks) resulted in tumors harboring an increased percentage of CD133+ cells, suggesting a drug-induced enrichment of CSCs.

Decreased responsiveness of CD133+ cells to a cytotoxic compound has also been reported when these cells have been isolated from primary ovarian cancer tissue, ovarian cancer cell lines or ascites fluid from ovarian cancer patients [26]. Concentration-response experiments with cisplatin demonstrated an approximate half-log decrease in potency for the cytotoxic effects of this drug in the CD133+ versus the CD133− cell population.

The most extensive evaluation of CD133+ cells to chemotherapeutic agents has been conducted following isolation of these cells from glioblastoma cell lines. The standard chemotherapeutic drugs temozolomide (100–2000 μM), etoposide (10–200 μM), carboplatin (10–200 μM) and paclitaxel (1–20 μM) were found to be less cytotoxic against CD133+ compared to CD133− cells using three different cell lines established from glioblastoma patients [27]. The reported differences were modest but consistent. However, qualitatively different results were reported in a recent study by Beier et al. [28] who showed that temozolomide (50–500 μM) was more effective in reducing the CD133+ versus the CD133− cell population in treated glioblastoma cell lines. Within the CD133+ cell population temozolomide was approximately 10-fold less effective on CD133+ cells which had high levels of O^6-methylguanine-DNA-methyltransferase (MGMT), consistent with this drug being more effective in glioblastoma tumors which have a silenced MGMT promoter. These authors did report that a lower concentration of temozolomide (5 μM), which is similar to the concentrations reached clinically in spinal fluid, was not effective in reducing the CD133+ cells which would suggest that glioblastoma CSCs are resistant to current dosing regimens of temozolomide.

3.1.2. CD44+/CD24−/low

A subpopulation of breast cancer cells, identified by high expression of the surface marker CD44 and low or negligible expression of CD24, has been found to be highly tumorigenic and able to form mammospheres in suspension culture [5]. Fillmore and Kuperwasser [29] characterized these candidate CSCs (CD44+/CD24−/low, ESA+) isolated from several breast cancer cell lines (MCF10A, MCF7, SUM149, SUM1325 and MDA.MB.231). The authors found this population of cells to be highly tumorigenic (e.g., as few as 100 cells needed to form tumors in recipient mice) and to exhibit stem cell like behaviors such as the ability to self-renew, differentiate, form mammosphere-like structures in suspension culture and have slower cell cycle kinetics. Moreover, the authors found that these candidate CSCs were less sensitive to the cytotoxic effects of either paclitaxel (10 nM) or 5-FU (1 mM). Depending on the cell line examined, the enrichment of the remaining cells with the CD44+/CD24−/low/ESA+ immunotype was between 5- and 30-fold. It is plausible that reduced sensitivity of CSCs to cell cycle cytotoxics (e.g., paclitaxel, 5-FU) could reflect slowed DNA replication kinetics. Interestingly, these authors found that a significant percentage of the resistant cells had undergone cell divisions as measured by a 'speckled' versus 'solid' appearance of bromodeoxyuridine staining; however, it is not clear if these cycling cells indeed resided in the CSC population. Nonetheless, a decreased sensitivity of CSCs to conventional chemotherapy can be demonstrated in cells isolated from either human primary breast tumor tissue or immortalized breast cancer cell lines.

Resistance of these candidate breast CSCs (CD44+/CD24−/low) to conventional chemotherapy was also demonstrated in a recent clinical study in breast cancer patients [30]. Li et al. prospectively evaluated serial biopsies from HER2-negative patients before and after 12 weeks of treatment with either docetaxel or doxorubicin plus cyclophosphamide. Treatment was associated with an expected (23%) clinical histopathological response but interestingly, the percentage of the candidate CSCs residing in tumor biopsies increased from 4.7% at baseline to 13.6% following the 12-week regimen. Seemingly consistent with this enrichment in CSCs with treatment was an increased efficiency of isolated tumor cells to form mammospheres (13–52%) in vitro and to develop tumors (29–50%) when implanted orthotopically into recipient immunocompromised mice.

3.1.3. SP cells

Steiniger et al. [20] isolated putative CSCs from two breast cancer cell lines, MCF7 and MDA-MB231, using Hoeschst 33342 dye exclusion to identify and isolate the SP cells. The authors found that these cells were more resistant to the cytotoxic effects of doxorubicin, methotrexate and 5-FU (1–100 μg/mL; approximately 8–770 μM) compared to non-side population cells. With all three drugs the SP cells retained close to 100% viability while the non-side population cells showed only 10–50% viability following treatment. In addition, the SP cells expressed surface markers characteristic of stem cells such as CD44 and c-Kit. The authors combined a SILAC (Stable Isotope Labeling with Amino Acids in Culture) approach with mass spectrometry to identify differentially expressed proteins between the two cellular populations. siRNA knockdown of one such protein shown to be up-regulated in SP cells, thymosin beta four (TB4), lowered the resistance of SP cells to the cytotoxic effects of both doxorubicin and paclitaxel.

3.2. Operational approaches

In one of the more complete analyses of chemotherapeutic effectiveness on CSCs, Eramo et al. [31] measured the ability of several drugs to induce cell death in CSCs isolated from surgical specimens of glioblastoma tumors. The biomass studied was operationally defined as enriched in CSCs due to the ability of clones to form tumor spheres enriched in tumorigenic, undifferentiated cells. There were pronounced decreases in efficacy for all drugs tested (etoposide 10 μM, camptothecin 100 ng/mL, approximately 300 nM, cisplatin 5 μM, temozolomide 250 μM, daunorubicin 1 μM, doxorubicin 1 μM, vincristine 0.1 μM and methotrexate 10 μM) in causing cell death in the glioblastoma CSC preparations. Interestingly, the lack of efficacy was not seemingly a consequence of drug extrusion from the CSCs (e.g., via drug efflux pumps) as nuclear levels of doxorubicin appeared to persist throughout the time period of the assay.

Although this paper by Eramo et al. had no comparative data for the effects of these drugs on non-CSC glioblastoma tumor cells, the authors found that CSC preparations from SCLC were sensitive to the cytotoxic effects of these agents. These results suggest that glioblastoma CSCs may be distinct in their resistance to cytotoxic compounds. However, as cited above, this same laboratory published on the relative resistance of SCLC and NSCLC CSCs to etoposide and cisplatin [23]. This apparent discrepancy may reflect differences in studying cells isolated via a more operational approach, e.g., tumor spheres enriched in putative CSCs versus a candidate approach where drugs were tested on a specific, prospectively isolated cell population (CD133+ expressing cells). This may also help explain the apparent discrepancy between the data reported by Eramo et al. [31] and Beier et al. [28].

Another operational approach for isolating putative CSCs would be recovery of the tumor cells from xenografts which remain following treatment with cytotoxic compounds. Folkins et al. [32] studied the chemosensitivity of tumor stem-like cells isolated from subcutaneous glioblastoma xenografts following treatment with various combinations of antiangiogenic and conventional

cytotoxics agents. The presence of stem-like cells was assessed by the ability of dissociated tumor cells to form spheres in suspension culture. Two weeks of treatment with the maximum tolerated dose of the cytotoxic cyclophosphamide resulted in a decrease in tumor mass; however, there was no effect on the ability of dissociated tumor cells to form tumor spheres in culture. These data imply that treatment with cyclophosphamide effectively debulked tumor mass but failed to eradicate the stem cell population.

4. Resistance to radiation therapy

For many cancers, ionizing radiation remains the most effective non-invasive therapy for an objective response although significant benefits in overall survival are often hampered by tumor regrowth [3,33]. Accelerated repopulation of cancerous tissue in patients during gaps in radiation therapy regimens obviously impacts on local control and may reflect an irradiation-resistant population of resident CSCs.

4.1. Candidate approaches

Similar to the candidate approach of Fillmore and Kuperwasser [29], Phillips et al. [34] isolated candidate CSCs (CD44+/CD24−) from breast cancer cell lines (MCF7, MDA.MB.231) to study their relative sensitivity to radiation. Mammospheres enriched in CD44+/CD24−/low cells were approximately twofold more resistant to a single dose of radiation (2 Gy) compared to non-CSCs grown in monolayer culture. In addition the CSC-enriched culture had no radiation-induced increase in the phosphorylation of the replacement histone H2AX which is an index of the repair process for breaks in double stranded DNA. Moreover, the percentage of candidate CSCs increased approximately twofold in response to fractionated irradiation (five fractions of 3 Gy), suggesting an enrichment of resistant CSCs in response to radiation.

4.2. Operational approaches

Kang et al. [35] exposed cultured glioblastoma-derived cells (GBM2, A172 and U87MG cells) to relevant doses of gamma irradiation (single dose of 30 Gy) and found that the surviving cells expressed the putative stem cell markers CD133, CD117, CD71 and CD45. These cells were capable of extensive proliferation, self-renewal, and pluripotency and were tumorigenic when implanted into immunodeficient mice. Activation of both Erk 1/2 and Akt was evident in the recovering resistant cells suggesting that inhibitors targeted to these enzymes may provide benefit to patients subsequent to radiation treatment.

Several other studies using freshly isolated human tumor tissue have shown that irradiation is less effective at killing residual CSCs compared to the differentiated, non-CSC population. Bao et al. [36] found that malignant tissues from glioblastoma patients became enriched in stem-like cells expressing CD133 following ionizing radiation in culture. The CD133+ cells were found to be highly clonogenic in vitro and tumorigenic in vivo, compared to CD133− cells, the latter showing higher rates of apoptosis in response to irradiation. The CD133+ cells also exhibited a more robust activation of DNA checkpoint proteins, as compared to CD133− cells, implicating an increased capacity for DNA repair which would be consistent with the decreased apoptosis observed in these CSCs. Finally, the irradiated CD133+ CSCs were also able to reconstitute the morphology of the original primary tumor upon transplantation into immunocompromised mice. Bar et al. [37] treated neurospheres derived from resected glioblastoma tumors with radiation and found that an increased percentage of the surviving cells expressed aldehyde hydrogenase, a marker of stem cells [38]. In addition, radiation of the glioblastoma-derived neurospheres induced a larger SP as assessed by flow cytometry. Chen et al. [39] showed that ionizing radiation and chemotherapy produced an objective response in mice with esophageal adenocarcinoma xenografts but that this was followed by tumor regrowth. Regrowth was associated with small islands of proliferating cells which likely reflects the presence of clonogenic cells (discussed under Section 5.1.3).

5. Eradication of CSCs: target identification

As the isolation of the true CSC population remains challenging so is the identification of attractive molecular targets for eradicating this tumorigenic population. Beyond the challenge of isolating bona fide CSCs, identification of CSC targets is anticipated to be further hindered by some inherent properties of stem cells, such as the existence of membrane drug transport proteins (e.g., the ATP-binding cassette proteins) and an insensitivity to cell cycle inhibitors due to slowed replication kinetics [3,5,40]. Clearly, the isolation of SP cells by FACS relies on the active efflux of Hoescht dye 33342 via extrusion pumps, and expression of membrane transport pumps has been reported in CSCs and implicated in their resistance to chemotherapy [41]. However, as stated above, the resistance of glioblastoma CSCs to doxorubicin occurred despite cellular uptake and retention of the drug [31]. Regarding the perception that stem cells are relatively quiescent in nature, resistance to cytotoxic compounds in breast CSCs was evident in spite of cells undergoing DNA replication [29]. These latter observations will need to be extended with similar studies conducted on other CSC populations to ascertain the relevance and extent of these particular attributes in order to orchestrate optimal drug discovery programs. Probing cell cycle targets may prove fruitful as resistance to irradiation-induced cell death in glioblastoma CSCs was linked to a more pronounced DNA damage repair system [36]. In this study, debromohymenialdisine, an alkaloid isolated from a marine sponge, inhibited the checkpoint kinases Chk1/Chk2 and restored radiation sensitivity to glioblastoma CSCs. Targeting DNA checkpoint control is provocative since transformation of normal cells is often tied to defects in cell cycle jurisdiction, raising the possibility of propagating mutations in non-malignant cells resulting from DNA strand breaks. Debromohymenialdisine treatment had no apparent effect on colony formation in the absence of radiation, but longer term treatment could potentially result in an increased progression of normal stem cells into CSCs.

5.1. Signaling pathways

Several signaling pathways have been identified as important for both progenitor cell differentiation during embryogenesis as well as for adult stem cell self-renewal and differentiation. These include pathways associated with Notch, phosphatidylinosital-3-kinase (PI3K)/Akt, Hedgehog, Wnt/β-catenin, and Bone Morphogenic Proteins (BMPs) [3,39,42,43]. Therefore, these pathways loom as obvious areas for mining attractive targets to eradicate CSCs and some recent reports in the literature support this strategy. However, the fact that these pathways are of importance for normal cell turnover and tissue repair may lead to narrow therapeutic windows due to dose-limiting toxicities in clinical trials.

5.1.1. Notch

The Notch pathway has been shown to be of importance in multiple cell differentiation decisions during early development as well as later in adult life [44]. Fan et al. [45] demonstrated that the Notch signaling pathway is important for sustaining growth of the tumorigenic cells resident in medulloblastoma cell lines. Using an

inhibitor of γ-secretase (GIS-18), the enzyme responsible for Notch cleavage and activation, the authors showed that treatment with GIS-18 resulted in reductions of both CD133 expressing cells and SP cells. Interestingly, the growth of more differentiated, non-tumorigenic cells was not affected by the presence of GIS-18. Implication of Notch signaling based on the use of a γ-secretase inhibitor may be tenuous due to the role of this enzyme in the signaling cascades of other type I membrane proteins [44]. However, Fan et al. did find that an index of Notch signaling (e.g., mRNA levels of Hes1) was constitutively more pronounced in the CD133+ enriched cellular fraction. In addition, Farnie et al. [46] found that CSC behavior (mammosphere formation) of ductal carcinoma in situ (DCIS) tissue from breast cancer patients was diminished following treatment with either a γ-secretase inhibitor or a neutralizing antibody to Notch.

Notch-1 activation, as determined by detecting elevated levels of the activated Notch receptor (Notch ICD), was also reported to occur in breast cancer cells which were resistant to fractionated doses of radiation [34]. It was not clear from this study, however, if the Notch activation occurred exclusively in the candidate CSC population. Notch activation associated with resistance to radiation in CSCs may be exacerbated by the hypoxic microenvironment in tumors since hypoxia inducible factors have been shown to activate the Notch signaling pathway [47].

Inhibitors of γ-secretase have been developed and are in clinical trials for solid tumor indications (Table 1; [48,49]). One Phase 1 study with MK-0752 was terminated due to adverse GI effects, perhaps reflecting the importance of Notch signaling in normal epithelial cell turnover in the gut [50]. The structures of MK-0752 and R4733 have not been divulged but Merck chemists have published on various sulfonamide analogs and Roche has filed on benzoxazepinone derivatives as inhibitors of γ-secretase [48].

5.1.2. PI3K/Akt

Aberrant signaling via the lipid kinase PI3K, and its main effector kinase Akt, is believed operant in many tumor tissues due to the published effects on promoting cell survival, proliferation, angiogenesis and invasion properties of malignant cells [51]. Eyler et al. [52] found that candidate glioblastoma CSCs (CD133+ cells) were more sensitive to the cytotoxic effects of Akt inhibition, effected by the phosphatidylinositol analog SH-6 (10–100 μM), compared to the CD133− cell population. Interestingly, the glioblastoma CSCs had lower basal levels of phosphorylated Akt, and hence Akt activity, versus CD133− cells. Similar effects were obtained with the PI3K inhibitor LY290042 (50 μM) but not with rapamycin (100 nM) which inhibits one of the downstream targets of Akt, the mammalian target of rapamycin. Mice implanted orthotopically with CSCs, which were treated in vitro with SH-6, survived longer compared to mice implanted with untreated CSCs. These are promising results in that Akt inhibition led to decreased growth in both CD133+ and CD133− cells, suggesting inhibitory effects in both the CSC and non-CSC populations. The effects observed were at times minimal (e.g., with LY294002), and obtained with rather high concentrations of the respective inhibitors, therefore additional studies will be important to further assess the involvement of the PI3K/Akt pathway in CSC survival.

In contrast to the findings of Eyler et al., Ma et al. [24] found that CD133+ cells from human HCC cell lines had increased constitutive Akt activity compared to the levels found in CD133− cells. Such differences in basal activity of Akt in candidate CSCs may be a consequence of the CSCs being isolated from different tumor types and/or cultured under different conditions. Interestingly, Ma et al. found no difference between the CD133+ and CD133− cells in the ability of an inhibitor of Akt (a different phosphatidylinositol analog than used by Eyler et al., Akt I, 10 μM) to decrease cell viability. However, the inhibitor was effective in eliminating the apparent resistance of the CD133+ cells to either doxorubicin or 5-FU.

The two studies cited above both found evidence for increased apoptosis of the CSCs following Akt inhibition, either with the inhibitor alone or in combination with a cytotoxic compound. The PI3K/Akt pathway may also be instrumental for cell fate decisions in CSCs. Vermeulen et al. [53] have reported that LY294002 markedly affected the lineage commitment of isolated colorectal CSCs in vitro. Thus the PI3K pathway may be important for survival, differentiation and evasion of apoptotic induction in CSCs. Several PI3K and Akt inhibitors (Table 1) are currently in Phase 1/2 clinical trials with some trials having been terminated due to poor exposure (e.g., XL-418; [49]), lack of efficacy or dose-limiting toxicities (fatigue, GI toxicity [49]). Inhibitors of PI3K with known

Table 1
Potential Cancer Stem Cell inhibitors in Clinical Trials[a].

Molecule	Developer	Stage (ongoing trials)	Indications[b]
Notch (γ-secretase inhibitors)			
MK-0752	Merck	P1/2 (5)	GBM, MBM, breast, solid tumor
R4733	Roche	P1/2 (1)	Solid tumors
PI3K (kinase inhibitors)			
XL-147	Exelixis	P1/2 (6)	NSCLC, lymphoma, ovarian, breast, skin, endometrial, solid tumor
GDC-0941	Genentech	P1 (2)	Solid tumor
CAL-101	Calistoga	P1/2 (3)	Leukemia, lymphoma, multiple myeloma
PX-866	Oncothyreon	P1 (1)	Solid tumor
BGT-226	Novartis	P1/2 (2)	Breast, solid tumor
BEZ-235	Novartis	P1/2 (1)	Breast, CNS, colorectal, GIST, lung, lymphoma, melanoma
AKT (kinase inhibitors)			
Perifosine	Keryx	P1–3 (23)	Leukemia, lymphoma, GIST, sarcoma, renal, multiple myeloma, ovarian, NSCLC, breast, liver, prostate, GBM, colorectal, head and neck, solid tumor
VEL-015	Velacor	P1 (1)	Prostate
Triciribine	VioQuest	P1/2 (5)	Leukemia, multiple myeloma, NSCLC, breast, colorectal, melanoma, ovarian, pancreas
RX-0201 (Archexin)	Rexahn	P2/3 (2)	Renal
Hedgehog (pathway inhibitors)			
GDC-0449	Genentech/Curis	P1/2 (5)	Skin, colorectal, ovarian, MBM, solid tumor
BMS-833923/XL-139	BMS/Exelixis	P1 (1)	Skin, solid tumor
IPI-926	Infinity	P1 (1)	Solid tumor

[a] Information accrued from [49].
[b] *Solid tumor* designates a clinical trial in an unspecified solid tumor; GBM, glioblastoma; MBM, medulloblastoma; NSCLC, non-small cell lung cancer; GIST, gastrointestinal stromal tumor.

structures include the thienopyrimidine GDC-0941, the imidazoquinazoline BEZ-235 and the semisynthetic viridian PX-866 (Table 1; [54,55]). Structural classes advanced as Akt inhibitors include the heterocyclic alkylphosphocholine derivative perifosine, the tricyclic nucleoside triciribine and the 20-mer oligonucleotide antisense molecule RX-0201 (Table 1; [49,55]).

5.1.3. Hedgehog (Hh) pathway

The Hh signaling pathway is imperative for the proliferation and differentiation of stem cells during normal embryonic development as well as for regulating cell division of tissue stem cells in the adult organism [39]. Mutations in the Sonic Hedgehog pathway, leading to abnormal activation of the pathway, have been identified in numerous cancer tissues [39]. Bar et al. [37] found that treating dispersed neurospheres, derived from resected glioblastoma tumors or glioblastoma-derived cell lines, with an inhibitor of Hh signaling (cyclopamine, 5 and 10 μM) attenuated several aspects of CSC activity. Cyclopamine treatment decreased the expression of CSC markers and led to a 50–70% blockade in the growth rate of glioblastoma-derived neurospheres. Similarly, incubation of glioblastoma-derived cell cultures with cyclopamine prevented subsequent neurosphere formation. Furthermore, cyclopamine treatment increased the sensitivity of neurospheres to irradiation and decreased the 'take rate' and growth of tumors following neurosphere injections into the brains of athymic mice. It is of interest to note that markers of Hh signaling and the stem cell marker CD133 were significantly reduced when the neurosphere cells underwent a differentiation protocol, consistent with the Hh pathway being operant primarily in the CSC population.

An activated Hh pathway was also shown to be correlated with tumor repopulation following ionizing radiation in a xenograft model of esophageal carcinoma [39]. The carcinoma cells were more sensitive to irradiation following treatment with cyclopamine (10 μM). Moreover, inhibition of Hh led to an increased sensitivity of several cancer cell lines to chemotherapeutic agents which appeared due to attenuating drug efflux via the membrane transport proteins. It was not clear whether these effects were associated with a CSC population although the authors implied that the tumor repopulation following irradiation is a consequence of surviving clonogenic cells. Nonetheless, these results suggest that resistance of CSCs to chemoradiation may be related to activated Hh signaling.

Small molecule antagonists of Hh, most of which target the transmembrane signaling molecule Smoothened, are currently in Phase 1/2 clinical trials (Table 1). These scaffolds include the derivatives of cyclopamine GDC-0449 and IPI-926; [49]. One trial in basal cell carcinoma (with G-024856) was terminated due to lack of efficacy [49].

5.1.4. Wnt/β-catenin

The canonical Wnt signaling pathway is important for self-renewal in stem cells and has been found to be dysregulated in solid and liquid neoplasms (e.g. [50,56,57]). Conditional knockout of the key Wnt mediator protein β-catenin in hematopoietic progenitor cells resulted in a significant delay (75 days vs. 45 days) in the development of CML in a bone marrow transplantation model in mice [56]. In this model, bone marrow progenitor cells from either wild type or conditional knockout animals were transfected with the BCR-ABL fusion gene and implanted into irradiated recipient mice. Interestingly, although the mice receiving the β-catenin deficient progenitor cells experienced a delayed onset of CML, they were not protected from development of an acute lymphoblastic leukemia syndrome suggesting differences in the role of Wnt/β-catenin in the development of these two leukemias. The importance of the Wnt pathway in CSCs was also suggested by Barker et al. who showed that deletion of the negative regulator of Wnt, adenomatous polyposis coli (Apc), in intestinal stem cells or crypt cells lead to transformation of these cells and eventual development of adenomas [58].

Small molecule inhibitors of Wnt signaling, e.g., ZTM000990 and PKF118-310, are undergoing preclinical evaluation [57]. In addition, PPARγ agonists, e.g., the thiazolidinediones, appear to inhibit Wnt signaling in a manner distinct from that affecting adipocyte function and may explain the reported antitumor effects of these compounds [59].

5.1.5. BMPs

BMPs are important signaling molecules for cell fate decisions of progenitor cells [42,60]. Piccirillo et al. [60] found that administration of BMP4 led to cytostatic effects on candidate CSCs (CD133+) from glioblastoma-derived cell lines or tissues in vitro. The BMP4 treated cells exhibited a more differentiated morphology compared to non-treated CSCs. Treatment of the CSCs with BMP4 prior to intrastriatal injection into immunodeficient mice resulted in focal, non-invasive lesions compared to the invasive, poorly differentiated tumors observed with untreated CSC preparations. Importantly, survival was enhanced (80% vs. 0%) in mice receiving the BMP4-treated cells. Diminished BMP signaling in glioblastoma CSCs may be related to epigenetic silencing (DNA hypermethylation) of BMP receptor expression as forced expression of the receptor leads to a loss of CSC tumorigenicity [61]. Selective DNA demethylation, as discussed below, may lead to a more differentiated phenotype of CSCs and thus depletion of this particular cellular pool.

5.1.6. Epidermal growth factor receptor (EGFR)

The EGFR family has been shown to play a critical role in the differentiation and proliferation of embryonic stem cells and has also been implicated in a number of solid tumor indications. Activation of EGFR family members leads to signal transduction through pathways involved in cell survival and proliferation. Chen et al. investigated the role of EGFR in SP cells from head and neck squamous cell carcinoma cell lines [62]. These authors demonstrated that ligand activation of EGFR increased the percentage of SP cells by as much as 56% whereas inhibition of EGFR, with the EGFR inhibitor gefitinib, decreased the SP percentage by as much as 47%. Farnie et al. [46] found that gefitinib (1 μM) reduced the stem-like behavior of DCIS tissues from breast cancer patients. In the absence of gefitinib, DCIS tissues exhibited a greater mammosphere-forming efficiency (MFE) than normal breast tissue and high grade DCIS had an elevated MFE than low grade DCIS. Treatment of high grade DCIS with gefitinib significantly reduced the MFE, suggesting that EGFR plays a role in DCIS cellular self-renewal and clonogenicity [46].

As mentioned above, Li et al. [30] found that treatment of HER2 negative breast cancer patients with either docetaxel or doxorubicin plus cyclophosphamide led to an increase in CSCs residing in the tumor biopsies. In contrast, in patients with HER2 positive breast cancer receiving the EGFR1/2 inhibitor lapatinib, the percentage of CSCs in patient biopsies decreased from 10% at baseline to 7.5% after 6 weeks of treatment. In addition, these changes in CSCs following 6 weeks of treatment with lapatinib were associated with an unexpected and significant tumor response in advanced breast cancer patients (median regression of −60.8%, $p = 0.001$) [63]. It is thus plausible that clinical efficacy of EGFR inhibitors may coincide with the resident CSCs expressing members of the EGFR signaling pathway.

5.2. CSC microenvironment

Targeting the tumor stromal environment has been pursued for anti-cancer therapeutics and represents an additional approach for eradicating CSCs. Not surprisingly, CSCs appear sensitive to

conditions that affect the local milieu including the tumor vasculature and local levels of cytokines and chemokines.

5.2.1. Angiogenesis

In culture, candidate brain CSCs (CD133+) have been shown to form more extensive contacts with human endothelial cells as compared to CD133− cells, raising the possibility that brain CSCs reside in a so-called 'vascular niche' surrounding tumor blood vessels [64]. In support of this hypothesis, it has been shown that factors secreted by endothelial cells can augment the stem-like properties of CSCs in culture conditions [32,64]. In a study by Calabrese et al. [64], the growth of orthotopically implanted tumor spheres was significantly affected by enhancing (increased growth) or depleting (decreased growth) the tumor vasculature in vivo. Folkins et al. [32] found that combining chemotherapy with antiangiogenic therapy decreased the glioma xenograft tumor mass in immunodeficient mice but also caused a pronounced decrease in the tumor sphere-forming ability of dissociated tumor cells, compared to either treatment alone. A clinical correlate may reside in the impressive efficacy of the antiangiogenic agent bevacizumab in a Phase 2 trial with recurrent, treatment-refractory glioblastoma patients [65]. Patients treated with bevacizumab experienced a median overall survival of 31 weeks. It will be of interest to examine whether the marked influence of the tumor vasculature on CSCs is most evident in brain tumors or is also found in other solid tumor types. These observations suggest that the mechanism underlying the beneficial effects of anti-angiogenic therapy, which is still debated, may be associated with inhibition of CSC growth.

5.2.2. Cytokines and chemokines

In colorectal cancer, skewing the T lymphocyte inflammatory cytokines towards a Th2 profile may be associated with a more aggressive tumor progression and resistance of colonic CSCs to chemotherapy [66]. Paracrine or autocrine production of the inflammatory cytokine IL-4 may be the culprit as blockade improves drug sensitivity in vitro and in vivo. However, this may not be specific for CSCs as blockade of IL-4 also improved the sensitivity of non-tumorigenic cancer cells to drug therapy [66].

Hermann et al. reported that pancreatic CSCs which express the chemokine receptor CXCR4 (i.e., CD133+/CXCR4+) are the highly metastatic cell population from aggressive human tumors [25]. CD133+/CXCR4+ CSCs were also evident in ascites samples from colon cancer patients. The authors showed that dosing athymic mice with the CXCR4 inhibitor AMD3100 (1.25 mg/(kg day)) did not affect tumor growth following orthotopic implantation of pancreatic cancer cells but did prevent the development of pulmonary metastases in these animals. AMD3100 is currently undergoing clinical trials primarily for liquid tumors as SDF-1/CXCR4 is important for leukocyte trafficking and the homing of hematopoietic progenitor cells [49].

5.2.3. Epigenetic targets

As mentioned above, Lee et al. found that epigenetic silencing via DNA hypermethylation may underlie diminished BMP signaling, and hence reduced differentiation, in glioblastoma CSCs [61]. In ovarian cancer cell lines, the expression of CD133 was found to be epigenetically regulated, as a higher percentage of CD133+ cells was observed following exposure to the DNA methyltransferase inhibitor decitabine [26]. Moreover, treatment of CD133− cells with either decitabine or the histone deacetylase inhibitor trichostatin A caused an increase in cell surface expression of CD133. The authors concluded that CD133+ cells have, in general, a less methylated state of promoter CpG cytosines compared to their CD133− progeny. Similar findings were reported in colorectal and glioblastoma cell lines where cells with CD133 promoter methylation had little or no cell surface expression of CD133 [67]. Taken together, these observations suggest that selective demethylation of particular promoters (e.g., BMP receptor but not CD133) may lead to depletion of the CSC pool due to enhanced differentiation of these cells.

5.2.4. Neurotransmitters

A novel approach was taken by Diamandis et al. [68] who screened approximately 1300 compounds from the LOPAC (Library of Pharmacologically Active Compounds) for their ability to inhibit normal neural stem cell self-renewal. Active 'hits' from this screen, which included compounds known to inhibit neurotransmission, were then evaluated for their ability to inhibit the proliferation of neurosphere-type cultures isolated from medulloblastoma xenografts. Some of the compounds showed a predilection for inhibiting the cancer-associated cultures versus the normal neural stem cell cultures. The brain tumor neurospheres were enriched for CD133+ cells which raises the question of whether these compounds would exhibit anti-proliferative effects on candidate CSCs from other tumor types.

6. Conclusions

Compelling data now exist to consider CSCs as important constituents for inclusion in the prosecution of new therapeutic entities for oncology indications. Pertinent to this position is that CSCs exhibit a relative lack of sensitivity to many of the standard-of-care chemo- and radiation-based treatment regimens. Moreover, clinical data are now emerging that show a positive correlation between indices of tumor cell 'stemness' and poor prognosis for survival [40,69–72], as was originally implied from early clinical experiments with autotransplantation [22].

There is no question that a looming challenge in the future development of drugs targeting CSCs will be obtaining clinical proof of concept. Moreover, obtaining this clinical goal will also be important for addressing current uncertainties and skepticism regarding the widespread role of CSC in tumorigenesis. Eradicating the CSC population may not appreciably affect tumor cell mass but may be critical for preventing aggressive growth, regrowth and metastases. In the case of targeted agents that inhibit or kill only the CSC population while sparing the non-stem population, it will be of importance to consider the appropriate tumor populations with which to conduct proof of concept studies. For example, trials with objective response as the primary end point should recruit more aggressive tumor types to enhance the likelihood that eradication of the stem cell pool will lead to tumor shrinkage. The logic for this approach is founded in the CSC hypothesis which contends that non-stem differentiated cancer cells will undergo only a limited number of cell divisions in their lifetime. Therefore, disabling the CSC pool in an aggressively dividing tumor mass should exhaust its proliferative potential and result in stasis of tumor growth.

An analogy for targeting the CSC population in a tumor would be removal of the locomotive engine from a near-endless string of box cars. Disengaging the engine would not impact the apparent size of the freight train, but would obviously cripple the train from further activity i.e., prevention of growth and metastasis. Taking out the box cars without removing the locomotive engine would allow the train to mobilize and acquire more box cars, i.e., growth and metastasis, at other stations. This analogy may also extend to the idea of driver versus bystander (or passenger) mutations in tumor tissue [73]. The many box cars are reminiscent of the numerous mutations gleaned from transcript profiling of tumor tissue, most of the mutations being bystander in nature and likely inconsequential. The important driver mutation or mutations will be associated with the locomotive engine/CSCs. The most effective cancer therapy, therefore, will consist of a regimen that disables

the locomotive engine (CSC pool) while at the same time eliminates the box cars (differentiated tumor cells). Ideally this regimen would consist of a single, targeted agent, but the complexity of CSC biology suggests that few targets will be operant in both stem and non-stem tumor cell growth and survival. Alternatively, one can envision an effective regimen consisting of a cancer stem cell therapeutic administered in an adjuvant setting following tumor resection or debulking with chemo- and/or radiotherapy. It is our position that achieving transformation success in oncology will require taking out the locomotive engines of cancers.

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References

[1] Albrecht T, McKee M, Alexe DM, Coleman MP, Martin-Moreno JM. Making progress against cancer in Europe in 2008. Eur J Cancer 2008;44:1451–6.
[2] Reichert JM, Wenger JB. Development trends for new cancer therapeutics and vaccines. Drug Discov Today 2008;13:30–7.
[3] Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. J Clin Oncol 2008;26:2839–45.
[4] Croker AK, Allan AL. Cancer stem cells: implications for the progression and treatment of metastatic disease. J Cell Mol Med 2008;12:374–90.
[5] Dalerba P, Cho RW, Clarke MF. Cancer stem cells: models and concepts. Annu Rev Med 2007;58:267–84.
[6] Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730–7.
[7] Hope KJ, Jin L, Dick JE. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nature 2004;5:718–43.
[8] Till JE, McCulloch CE. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 1961;14:213–22.
[9] Holyoake TL, Jiang X, Drummond MW, Eaves AC, Eaves CJ. Elucidating critical mechanisms of deregulated stem cell turnover in the chronic phase of chronic myeloid leukemia. Leukemia 2002;16:549–58.
[10] Cox CV, Evely RS, Oakhill A, Pamphilon DH, Goulden NJ, Blair A. Characterization of acute lymphoblastic leukemia progenitor cells. Blood 2000;104:2919–25.
[11] Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100:3983–8.
[12] Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumor initiating cells. Nature 2004;432:396–401.
[13] Collins AT, Berry PA, Hyde C, Stower MJ, Matland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946–51.
[14] Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. Cancer Res 2007;67:4827–33.
[15] Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132:2542–56.
[16] Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445:111–5.
[17] Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer Res 2007;67:1030–7.
[18] Bapat SA, Mali AM, Koppikar CB, Kurrey NK. Stem and progenitor-like cells contribute to the aggressive behavior of human epithelial ovarian cancer. Cancer Res 2005;65:3025–9.
[19] Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA 2007;104:973–8.
[20] Steiniger SCJ, Coppinger JA, Kruger JA, Yates III J, Janda KD. Quantitative mass spectrometry identifies drug targets in cancer stem cell containing side population. Stem Cells 2008;26:3037–46.
[21] Kelly PN, Dakic A, Adams JM, Nutt SL, Strasser A. Tumor growth need not be driven by rare cancer stem cells. Science 2007;317:337.
[22] Brunschwig A, Southam CM, Levin AG. Host resistance to cancer. Clinical experiments by homotransplants, autotransplants and admixture of autologous leucocytes. Ann Surg 1965;162:416–23.
[23] Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. Cell Death Differ 2008;15:504–14.
[24] Ma S, Lee TK, Zheng B-J, Chan KW, Guan X-Y. CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene 2008;27:1749–58.
[25] Hermann PC, Huber SL, Herrler TAA, Ellwart JW, Guba M, Bruns C, et al. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 2007;1:313–23.
[26] Baba T, Convery PA, Matsumura N, Whitaker RS, Kondoh E, Perry T, et al. Epigenetic regulation of CD133 and tumorigenicity of CD133+ ovarian cancer cells. Oncogene 2008 [Epub Oct 6].
[27] Liu G, Yuan X, Zeng Z, Tunici P, Ng H, Abdulkadir I, et al. Analysis of gene expression and chemoresistance of CD133+ cancer stem cells in glioblastoma. Mol Cancer 2006;5:67.
[28] Beier D, Rohrl S, Pillai D, Schwarz S, Kunz-Schughart A, Leukel P, et al. Temozolomide preferentially depletes cancer stem cells in glioblastoma. Cancer Res 2008;68:5706–15.
[29] Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res 2008 [Epub Mar 26].
[30] Li X, Lewis MT, Huang J, Cutierrez C, Osborne CK, Wu M-F, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 2008;100:672–9.
[31] Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G, et al. Chemotherapy resistance of glioblastoma stem cells. Cell Death Differ 2006;13:1238–41.
[32] Folkins C, Man S, Xu P, Shaked Y, Hicklin DJ, Kerbel RS. Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor stem-like cell fraction in glioma xenograft tumors. Cancer Res 2007;67:3560–4.
[33] Bese NS, Hendry J, Jeremic B. Effects of prolongation of overall treatment time due to unplanned interruptions during radiotherapy of different tumor sites and practical methods for compensation. Int J Radiat Oncol Biol Phys 2007;68:654–61.
[34] Phillips TM, McBride WH, Pajonk F. The response of CD24-/low/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst 2006;98:1777–85.
[35] Kang MK, Her BI, Ko MH, Kim CH, Cha SH, Kang SK. Potential identity of multipotential cancer stem-like subpopulation after radiation of cultured brain glioma. BMC Neurosci 2008;9:15.
[36] Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006;444:756–60.
[37] Bar EE, Chaudhry A, Lin A, Fan X, Schreck K, Matsui W, et al. Cyclopamine-mediated hedgehog pathway inhibition depletes stem-like cancer cells in glioblastoma. Stem Cells 2007;25:2524–33.
[38] Moreb JS. Aldehyde dehydrogenase as a marker for stem cells. Curr Stem Cell Res Ther 2008;237–46.
[39] Chen Y-J, Sims-Mourtada J, Izzo J, Clifford Chao KS. Targeting the hedgehog pathway to mitigate treatment resistance. Cell Cycle 2007;6:1826–30.
[40] Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Gen 2008;40:499–507.
[41] Mack JT, Brown CB, Tew KD. ABCA2 as a therapeutic target in cancer and nervous system disorders. Expert Opin Ther Targets 2008;12:491–504.
[42] Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 2008;132:598–611.
[43] Rowitch DH, B SJ, Lee SM, Flax JD, Snyder EY, McMahon AP. Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. J Neurosci 1999;19:8954–65.
[44] Nakayama K, Nagase H, Hiratochi M, Koh CS, Ohkawara T. Similar mechanisms regulated by gamma-secretase are involved in both directions of the bi-directional Notch-Delta signaling pathway as well as play a potential role in signaling events involving type 1 transmembrane proteins. Curr Stem Cell Res Ther 2008;3:288–302.
[45] Fan X, Matsui W, Khaki L, Stearns D, Chun J, Li Y-M, et al. Notch pathway inhibition depletes stem-like cells and blocks engraftment in embryonal brain tumors. Cancer Res 2006;66:7445–52.
[46] Farnie G, Clarke RB, Spence K, Pinnock N, Brennan K, Anderson NG, et al. Novel cell culture technique for primary ductal carcinoma in situ: role of Notch and epidermal growth factor receptor signaling pathways. J Natl Cancer Inst 2007;99:616–27.
[47] Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. Cell 2007;129:465–72.
[48] Olson RE, Albright CF. Recent progress in the medicinal chemistry of gamma-secretase inhibitors. Curr Top Med Chem 2008;8:17–33.
[49] TrialTrove™ clinical database by Citeline, Inc. (www.citeline.com; as of January 2009).
[50] Nakamura T, Tsuchiya K, Watanabe M. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decisions. J Gastronenterol 2007;42:705–10.
[51] Carracedo A, Pandolfi PP. The PTEN-PI3K pathway: of feedback and cross-talks. Oncogene 2008;27:5527–41.
[52] Eyler CE, Foo W-C, LaFiura KM, McLendon RE, Hjelmeland AB, Rich JN. Brain cancer stem cells display preferential sensitivity to Akt inhibition. Stem Cells 2008 [Epub Sept 18].
[53] Vermeulen L, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, et al. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc Natl Acad Sci USA 2008;105:13427–32.
[54] Ihle NT, Williams R, Chow S, Chew W, Berggren MI, Paine-Murrieta G, et al. Molecular pharmacology and antitumor activity of PX-866, a novel inhibitor of phosphoinositide-3-kinase signaling. Mol Cancer Ther 2004;3:763–72.
[55] Yap TA, Garrett MD, Raynaud F, de Bono JS, Workman P. Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. Curr Opin Pharmacol 2008;8:393–412.

[56] Zhao C, Blum J, Chen A, Kwon HY, Jung SH, Cook JM, et al. Loss of β-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 2007;12:528–41.
[57] Katoh M, Katoh M. Wnt signaling pathway and stem cell signaling network. Clin Cancer Res 2007;13:4042–5.
[58] Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 2008 [Epub Dec 17].
[59] Wang P-S, Chou F-S, Bloomston M, Vonau MS, Saji M, Espinosa A, et al. Thiazolidinediones downregulate Wnt/β-catenin signaling via multiple mechanisms in breast cancer cells. J Surg Res 2008 [Epub Jun 27].
[60] Piccirillo SGM, Reynolds BA, Zanetti N, Lamorte G, Binda E, Broggi G, et al. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature 2006;444:761–5.
[61] Lee J, Son MJ, Woolard K, Donin NM, Li A, Cheng CH, et al. Epigenetic-mediated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells. Cancer Cell 2008;13:69–80.
[62] Chen JS, Pardo FS, Wang-Rodriguez J, Chu TS, Lopez JP, Aguilera J, et al. EGFR regulates the side population in head and neck squamous cell carcinoma. Laryngoscope 2006;116:401–6.
[63] Chang JC, Li X, Creighton C, Decrease in tumorigenic breast cancer stem cells—final results of a neoadjuvant trial in primary breast cancer patients. 6th European Breast Cancer Conference 2008; Oral presentation: Abstract# 204.
[64] Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, et al. A perivascular niche for brain tumor stem cells. Cancer Cell 2007;11: 69–82.
[65] Kreisl TN, Kim L, Moore K, Duic P, Royce C, Stroud I, et al. Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma. J Clin Oncol 2008 [Epub Dec 29].
[66] Francipane MG, Alea MP, Lombardo Y, Todaro M, Medema JP, Stassi G. Crucial role of interleukin-4 in the survival of colon cancer stem cells. Cancer Res 2008;68:4022–5.
[67] Yi JM, Tsai H-C, Glockner SC, Lin S, Ohm JE, Easwaran H, et al. Abnormal DNA methylation of CD133 in colorectal and glioblastoma tumors. Cancer Res 2008;68:8094–103.
[68] Diamandis P, Wildenhain J, Clarke ID, Sacher AG, Graham J, Bellows DS, et al. Chemical genetics reveals a complex functional ground state of neural stem cells. Nat Chem Biol 2007;3:268–73.
[69] Glinsky GV. "Stemness" genomics law governs clinical behavior of human cancer: implications for decisions making in disease management. J Clin Oncol 2008;26:2846–53.
[70] Pallini R, Ricci-Vitiani L, Luigi Banna G, Signore M, Lombardi D, Todaro M, et al. Cancer stem cell analysis and clinical outcome in patients with glioblastoma multiforme. Clin Can Res 2008;14:8205–12.
[71] Liu R, Wang X, Chen GY, Dalerba P, Gurney A, Hoey T, et al. The prognostic role of a gene signature from tumorigenic breast-cancer cells. N Engl J Med 2007;356:217–26.
[72] Zeppernick F, Ahmadi R, Campos B, Dictus C, Helmke BM, Becker N, et al. Stem cell marker CD133 affects clinical outcome in glioma patients. Clin Cancer Res 2008;14:123–9.
[73] Torkamani A, Verkhivker G, Schork NJ. Cancer driver mutations in protein kinase genes. Cancer Lett 2008 [Epub Dec 9].