[Frontiers in Bioscience E4, 1528-1541, January 1, 2012]

The role of cancer stem cells in relapse of solid tumors

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TABLE OF CONTENTS

1. ABSTRACT

Recurrence at secondary locations, often years after removal of the primary tumor, accounts for most of the mortality associated with solid tumors. Metastasis, resistance to chemo- and radiotherapy, and eventual relapse have been attributed to a distinct tumor subpopulation known as cancer stem cells (CSCs). In this review, we consider the properties of CSCs that lead to these outcomes, in particular the relation between epithelial-to-mesenchymal transition, stemness, and tumor initiation. We compare recent clinical and laboratory studies of breast cancer, glioblastoma, and melanoma that illustrate how most current anticancer regimens select for cells with mesenchymal and CSC properties and therefore sow the seeds of relapse. Finally, we discuss the emerging paradigm of combined therapy that targets both CSC and non-CSC tumor components.

2. INTRODUCTION

Cancer recurrence refers to the return of cancer after a period of time during which it was undetectable, either at the same location as the primary tumor or at a distant one. The odds of a cancer recurring depend on many histopathological and clinical criteria, including the primary organ site (Table 1, 1-7), the grade and stage of the cancer, as well as patient lifestyle choices such as tobacco use and obesity (8).

Over the past two decades, several developments have led to the view that the cells responsible for recurrence constitute a discrete subpopulation of the tumor. Originally termed tumor-initiating cells (TIC), they were later found to share many properties with normal tissue stem cells, such as multipotency and expression of stem cell markers (9, 10). Thus, they are now more frequently

Table 1. Relapse rate of different types of cancer

Types of Cancer	Relapse Rate
Glioblastoma	90% (1)
Breast cancer (luminal)	16% (2)
Breast cancer (triple negative)	53% (2)
Prostate cancer	75% (3)
Osteosarcoma	30%-50% (4)
Bladder Cancer	40%-50% (5)
Melanoma (head and neck, local) Melanoma, metastatic	2.8%-12.5% (6) 80% (7)

referred to as cancer stem cells (CSCs) (11). We consider the advantages afforded to the tumor cell by the stem cell program below. We have selected three solid tumor types of epithelial, neural, or neuroendocrine origin to illustrate how the role of CSCs in tumorigenesis and relapse varies with the tissue type.

There are several hypotheses concerning the origin of CSCs. The first is that they are derived from stem cells or their immediate descendants, early progenitor cells. This could explain heterogeneity of differentiation markers within tumors. A competing view is that tumor cells arising from more differentiated cells may reactivate the stem cell program, including pluripotency (12). Evidence for this view emerged when Mani *et al.* demonstrated that epithelial-to-mesenchymal transition (EMT) conferred stem-like properties on non-stem populations (12). The overlap between CSC and EMT profiles has now been demonstrated for a variety of solid tumors (13-16). A more nuanced picture has begun to emerge with the demonstration in breast and melanoma that a dynamic equilibrium seems to exist between CSCs and non-CSCs, such that either one may be converted to the other (17). On the other hand, glioblastomas appear to arise directly from neural stem cells rather by de-differentiation (18). The issue of CSC origin is discussed in more detail in sections 4 and 5.

The link between CSCs and metastasis is less clear. Since metastatic tumors recapitulate the heterogeneity of the primary tumor, it is reasonable to suppose that these tumors are founded by CSCs. However, metastatic tumors may also contain mutations that were absent from the primary tumor. For example, metastasis suppressors such as nonmetastatic 23 (nm23), maspin, kangai1 (Kai1), N-myc downstream regulated gene-1 (NDRG1), and kisspeptin-1 (Kiss1) may be mutated or additional oncogenes may be activated (19-24). In addition, there is now solid evidence for the role of tumor-associated macrophages and regulatory T cells as potentiating factors in metastasis (25, 26). How the latter factors can be reconciled with the CSC hypothesis remains to be resolved. The link between CSCs and metastasis is covered in sections 3 and 4.

3. PROPERTIES OF CANCER STEM CELLS THAT PROMOTE CANCER RELAPSE

The cancer stem cell hypothesis posits that only a small subpopulation of tumor cells is responsible for the formation and maintenance of the bulk of the tumor. This hypothesis emerged in 1994 when Lapidot and colleagues showed evidence that only a small percentage of acute myeloid leukemia cells had the capability to initiate leukemia in mice (27). These cells were shown to express similar cell surface markers (CD34+/CD38-) to normal hematopoietic stem cells (27). Following this, cancer stem cells were identified not only in hematological cancers but also in a wide range of solid tumors including breast, brain, skin, head and neck, prostate, and glioblastoma. Several properties of stem cells that contribute to cancer recurrence are outlined below and elaborated upon in section 4.

3.1. Tumorigenicity

Tumorigenicity describes the ability of cells to generate a tumor when injected into an immunocompromised experimental animal. As mentioned above, only a small percentage of cells, that is, cancer stem cells, in a tumor are tumorigenic and capable of maintaining the bulk tumor or giving rise to a new one in a distant location (28).

In vitro, tumorigenicity can be determined by a tumorsphere formation assay. Stem-like cells are anoikis-resistant and able to form spheres in methylcellulose, while non-tumorigenic cells cannot (29).

Multiple studies show that cancer stem cells isolated from cancer cell lines are resistant to various kinds of chemotherapy and radiation (Figure 1; reviewed in ref. 30). Several mechanisms may account for this.

One is the lower proliferation rate of cancer stem cells. Most conventional therapies target rapidly proliferating tumor cells, while more slowly dividing stem-like cells escape (13).

Another is the presence of multi-drug resistance ATP-binding cassette transporters, principally ABCG2 but also MRP1 (multidrug-resistance-associated protein 1) and ABCB5 in some cancers that can efflux drugs across the plasma membrane (31, 32). Inhibitors of these transporters increase the sensitivity of cancer stem cells to anti-cancer drugs (33). The ability to exclude compounds such as Hoechst dye has been used as a basis for identifying and isolating both normal and cancer stem cells by flow cytometry (34). These cells have been termed the "side population." The existence of side populations with CSC properties has been demonstrated in cancers of the breast, lung, brain, skin, and many others (35).

Not all CSCs express these proteins however. Working with glioma, breast, and prostate cell lines, Dean Tang's group isolated side populations and found that they were indeed more tumorigenic than the rest of the population (36). These cells expressed ABCG2. However, they also found an ABCG2-negative population that was also tumorigenic but grew more slowly. ABCG2+ cells could give rise to ABCG2- cells and vice versa. The authors concluded that ABCG2 identifies fast cycling progenitor cells, while more primitive slow-cycling cells lack ABCG2. Nor is expression of such transporters limited to CSCs. Normal kidney expresses high levels of ABC

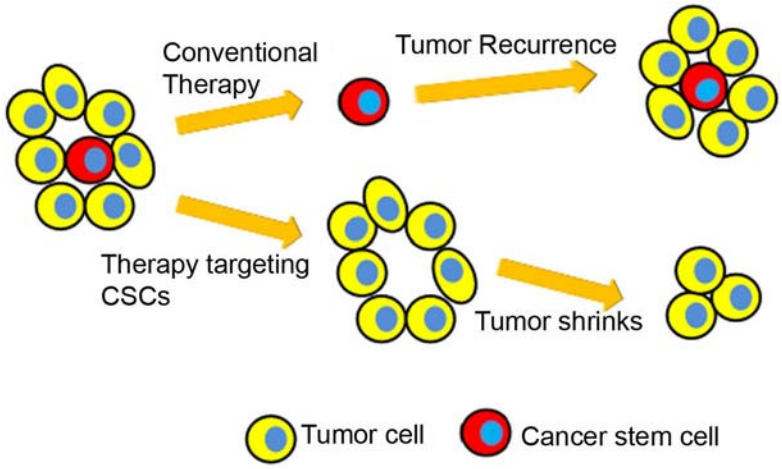

Figure 1. A simple model of CSCs and tumor recurrence. Conventional therapies eliminate non-CSCs but not CSCs, thus allowing or even promoting tumor recurrence. Therapies that target CSCs should eliminate the tumor by attrition or allow it to be eliminated by chemo- or radiotherapy. This model does not take into account recent findings that non-CSCs may give rise to CSCs.

transporters, and renal carcinomas maintain this expression in all tumor cells (31).

In addition, activation of mesenchymal transcription factors and signaling via Hedgehog (Hh), Notch, Her2, Wnt and other pathways contribute to therapy-resistance (37, 38). This topic is covered in section 5.2. The role of Hh in CSCs was shown by Varmat *et al.* (39). That found that Hh was preferentially active in normal stem cells and CSCs of the colon and that colon carcinomas acquired a Hh signature coincident with metastasis. They further demonstrated that Hh induced EMT in colon cancer cells and was required for their growth, recurrence, and metastasis. Thus, Hh in colon is tightly linked to stem cell properties. Similar conclusions have been reached for breast cancer (40).

Richard Clarke and coworkers demonstrated the importance of Notch signaling in breast ductal carcinoma *in situ* (41). Notch is upregulated in CD44+/CD24- cells and in often in DCIS. Tumor tissue that was positive for Notch 1 intracellular domain gave rise to increased mammospheres, and gamma secretase inhibitor suppressed mammosphere formation. High Notch 1 expression predicted early recurrence. For an excellent review on Notch signaling in CSCs, see Rizzo *et al.* (42).

The role of Her2 in CSCs of breast has been demonstrated by several laboratories. Korkaya *et al.* found that expression of the stem cell marker ALDH (acetaldehyde dehydrogenase) in breast tumors correlates with Her2 and that the ALDH-positive population can be reduced by treatment with the Her2 inhibitor lapatinib (43). Ectopic expression of Her2 increased the CSC population along with invasion and metastasis.

The role of Wnt in breast cancer and CSCs has been reviewed by others (44, 45). One of the most compelling studies showed that a single breast stem cell from an MMTV-Wnt mouse can give rise to a complete mammary gland, demonstrating its promotion of stem cell properties (46). Wnt signaling promotes EMT by upregulating expression of mesenchymal transcription factors Snail, Slug, and Twist, which repress E-cadherin and a host of other epithelial proteins (47-49). Moreover, when compared with the primary tumor, early lung metastases overexpress components of the Wnt pathway, and their downregulation inhibits metastasis of orthotopic xenografts (50). Rosen's group has demonstrated that Wnt is required for radiation-resistance of mammary epithelial progenitor cells from mice with activated Wnt signaling in breast cancer cells (51).

3.3. Self-renewal ability

Stem cells are perpetuated in the undifferentiated state by asymmetric replication, during which one of the daughter cells of the CSC remains undifferentiated while the other becomes more specialized (52). This process ensures the stability and multipotency of the stem cell population. However, stem cells can also divide symmetrically under stress to rapidly increase their number. This process is regulated by p53, as p53 mutation confers symmetric division on normal stem cells (53). CSCs circumvent this mechanism by mutating or otherwise downregulating p53. This ability may come into play after chemoradiation therapy, allowing rapid re-population.

3.4. Migratory ability

In normal development and regeneration, the ability of stem cells to migrate, invade and colonize distant spaces is essential. Proponents of CSC theory posit that CSCs retain or coopt this ability, exploiting it to the same end (37). These metastatic colonies are largely responsible for tumor recurrence (12). The evidence that CSCs are more migratory and invasive than nonCSCs stems from the observation that CSC and EMT gene expression profiles substantially overlap, and that cells that have undergone

EMT are functionally identical to CSCs (54). This topic is discussed in section 4.1.

4. EVIDENCE FOR CANCER STEM CELLS IN CANCER RELAPSE

4.1. Breast cancer

Breast CSCs were first described in 2003 by Clarke and co-workers (10). They found that flow-sorted CD44high/CD24low cells from disrupted tumors were highly tumorigenic in immunocompromised mice and gave rise to the same diversity of cell types found in the original tumor, whereas CD44low/ CD24high cells lacked these properties. Subsequent work showed that these cells were anoikis-resistant and so could be cultured without anchorage, giving rise to spherical colonies on nonadherent surfaces or when suspended in methylcellulose (55, 56). The ability to propagate these cells *in vitro* allowed their transcriptional signature to be identified.

At the same time, the advent of cDNA microarray and other transcriptional profiling technologies allowed comparison between histopathological characteristics and the molecular profiles of tumors. This led to the discovery within breast cancer that there are distinct molecular subtypes that are highly predictive of the course of the disease (57). These were initially divided, based on their similarities to cells in normal breast, into luminal A, luminal B, Her2+, and basal (58). While the first three classes respond to various targeted therapies, basal or triple-negative breast cancer has an especially bleak prognosis (57) due to metastasis and relapse. In general, basal breast cancers and cell lines have low expression of markers of differentiated breast such as E-cadherin and other junctional proteins (59). Recently, an additional subtype was parsed from within the basal group that is even more mesenchymal-like, termed claudin-low (60). Claudin-low cell lines are enriched in CSCs and are associated with therapy-resistance (61, 62).

Diehn *et al.* found that, similar to normal stem cells, some breast tumor CSCs have lower levels of reactive oxygen species (ROS) compared to other cells from the same tumor (63). As ROS is a mediator of tumor cell killing in response to therapeutic ionizing radiation (IR), the CSCs sustained less DNA damage and mortality in response to radiation. Similarly, Philips *et al.* found that mammospheres cultured from MCF7 and MDA-MB-231 cells survived IR better than cells grown as attached monolayers (64). Woodward *et al.* also found that MCF7 CSCs were radioresistant; in addition, they showed that progenitor cells from mouse mammary tumors had this same property (51). The stem cell niche in breast is thought to be located in the basal epithelial layer, and it has long been known that IR produces basal tumors with poor prognosis (65). To determine whether the basal layer responds differently to IR from luminal cells, Coates *et al.* xenografted normal human breast tissue into mice, treated with IR, and measured kinetics and intensity of DNA damage response by IHC. They found that basal cells induce p53, p21, and repair machinery much faster than do luminal cells (65).

A matter of controversy is whether CSCs derive from normal tissue stem/progenitor cells or from cancer cells that have reactivated the stem cell program. A recent body of work sheds light on this issue, showing that forcing a transition from an epithelial to a mesenchymal program, EMT, bestows the properties of CSCs (54).

Transition between epithelial and mesenchymal states is a normal feature of epithelial behavior during development and wound-healing (66). Mesenchymal cells share many of the properties of CSCs, such as migratory behavior, resistance to DNA-damaging agents, and ability to form colonies when suspended in nonadherent media or implanted into immunocompatible mice (54, 67). The mesenchymal phenotype is maintained by a network of transcription factors such as Snail, Twist, and others that repress epithelial proteins such as E-cadherin and activate mesenchymal functions (68, 69). EMT induces cytoskeletal changes, expression of vimentin and fibronectin, and loss of apico-basal polarity, allowing the cell to divorce from its neighbors and move independently (Figure 2; 70).

Clinical studies have shown that Snail is spontaneously upregulated in recurrent tumors *in vivo* and that recurrence is accompanied by EMT (71, 72). Ectopic expression of Snail and Twist in cell lines triggers EMT and resistance to chemotherapy (73, 74). Accordingly, a high level of Snail expression predicts a high rate of relapse and low survival of breast cancer patients (75, 76).

Weinberg's group found that ectopic expression of EMT transcription factors in transformed epithelial cells conferred not only expression of mesenchymal markers but also enriched for stem cell markers and behaviors such as mammosphere formation and tumorigenicity (54). The congruency between these two phenotypes was further underlined by the demonstration that sorting immortalized HMEC (human mammary epithelial cells) or tumor cells for stem cell markers also enriches for mesenchymal markers. Other workers showed that breast cancer cell lines with a mesenchymal profile were also greatly enriched for CSC markers and behaviors (61). Thus, a mutation that triggered EMT in a tumor would also confer CSC properties.

The same group then used similar techniques to generate large numbers of stem-like cells, explore their drug-sensitivity, and test the hypothesis that current therapies select for CSCs (75). They used knockdown of E-cadherin to drive EMT both in immortalized (HMLE) and Ras-transformed (HMLER) cell lines. This resulted in a tenfold increase in the CD44high/CD24low population and a 100-fold increase in both mammosphere- and tumor-forming ability. Treating these cells with two of the drugs most frequently used to de-bulk tumors, paclitaxel and doxorubicin, yielded increases in IC50 of twentyfold and fivefold, respectively. To simulate a tumor undergoing chemotherapy, the knockdown cells were mixed with twentyfold excess of control cells and treated with paclitaxel. This resulted in fourfold increase in knockdown cells over a four day period.

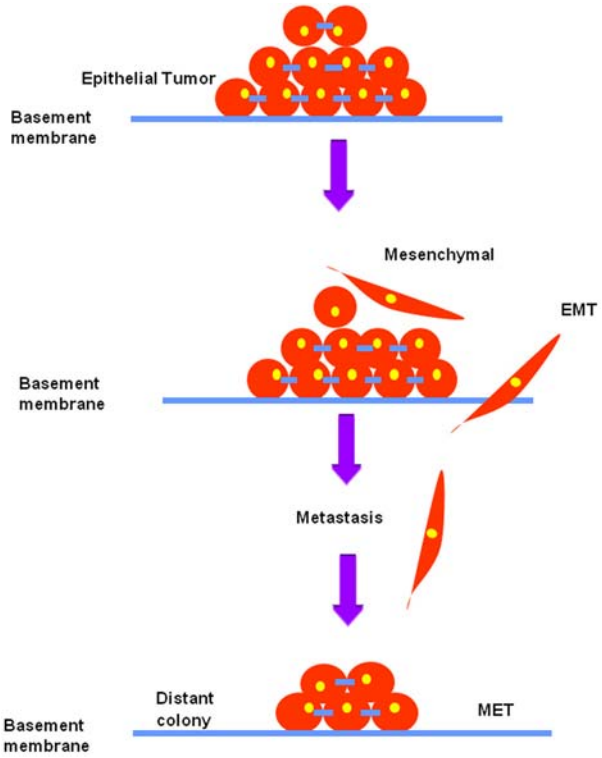

Figure 2. EMT generates cells with stem cell traits. Epithelial cells lose their cell-cell junctions and acquire mesenchymal morphology and behavior. Cytoskeletal changes and metalloprotease secretion facilitate breach of the basement membrane, escape into the circulation, and extravasation at distant sites. Once there, reversion to an epithelial program facilitates colony formation.

They then exploited these capabilities to screen drug libraries for CSC-specific cytocidal agents (75). Of 16,000 drugs tested, only salinomycin, etoposide, abamectin, and nigericin were differentially toxic to HMLE bearing the knockdown compared to control. Of these four, only salinomycin was differentially toxic to CSC-enriched HMLER. Except for etoposide, all affect transmembrane ion currents.

The Struhl group arrived at some similar conclusions using the mammary epithelial cell line MCF10A, which they had rendered conditionally transformable by tamoxifen-regulated Src (76). Based on similar expression profiles between cancer cells and cells from diabetics, they tested the AMPK inhibitor and diabetes drug metformin on this cell line and on autochthonous breast cancer cell lines. Metformin suppressed MCF10A transformation by Src and inhibited mammosphere formation by cancer cell lines. In combination with doxorubicin, it reduced tumor growth in mouse xenografts much more effectively than either drug alone.

The concept that standard chemotherapy selects for a pernicious subpopulation that drives recurrence has now been tested clinically. Li *et al.* analyzed core biopsies from breast cancer patients before and after chemotherapy and found a several-fold increase in CSC markers and mammosphere-forming ability after neoadjuvant therapy (77). In follow-up studies, they compared the gene expression signature of the post-chemotherapy (doxorubicin or letrozole) population to those of the various breast cancer subtypes (78). The surviving cells most resembled claudin-low tumors, a subtype enriched in CSC and mesenchymal characteristics (60, 62).

But is there more than a teleological link between CSCs and metastasis? The 2011 meeting of the American Association for Cancer Research (Orlando, April 2-6) included a forum to debate whether metastasis requires EMT and, by implication, CSCs. Evidence was presented from the field of breast cancer that, while the bulk of a tumor may express epithelial markers, the invasive front often expresses mesenchymal and stem cell markers. This phenomenon has also been observed in prostate cancer (15). Others have found that metastatic breast cell lines are enriched in stem cell markers (79). In clinical samples of human pancreatic cancer, Hermann and co-workers demonstrated a population of tumorigenic cells that were positive for stem cell marker CD133 and resistant to chemotherapy (80). A subset of CD133+ cells that expressed an additional marker, CXCR4, was found at the advancing front of tumors, and depletion of these cells from the population abolished metastasis without affecting tumorigenicity. The authors concluded that a subpopulation of CSCs is solely responsible for metastasis in pancreatic cancer. It would be interesting to profile this population for expression of previously established metastasis promoters

and loss of metastasis suppressors. While the weight of evidence now favors a leading role for EMT and CSCs in metastasis of several solid tumor types, this may not be universally true (81). Further studies are needed to settle this question.

4.2. Glioblastoma

Glioblastoma Multiforme (GBM) has one of the highest recurrence rates and lowest survival times of any solid tumor. It is difficult to treat because of its location, aggressiveness, and ability to infiltrate distant areas of the brain. The blood-brain barrier limits chemotherapy to lipophilic agents or drugs that can be introduced intrathecally or directly into the surgical cavity. However, even with refinements in surgical technique, targeting of radiotherapy, and novel alkylating agents such as temozolomide, median survival is only 14-15 months (82). The interval between tumor excision and recurrence is usually short, suggesting that some tumorigenic cells within the tumor are treatment-resistant.

The existence of CSCs in GBM was first shown by Singh and coworkers, who demonstrated that the only the CD133-positive subpopulation from sorted tumor cells could form tumorspheres *in vitro* and tumors in immunocompromised mice (83). The tumors developed from as few as 100 xenografted cells and faithfully reproduced the range of cell types and surface antigens present in the original surgical specimens. CSCs could also be isolated by culturing GBM explants as tumorspheres (82). These cells were positive for CD133 and another stem cell marker nestin, and they quickly formed tumors in mice. They also displayed remarkable resistance to a wide range of chemotherapeutics when compared with tumor cells from several other organ sites (82). Increased survival was apparently not due to increased drug efflux, as doxorubicin efflux was no more efficient in these cells than in chemosensitive tumor cells. Even more direct evidence for a role of CD133+ cells came from a study by Pallini and coworkers who found that in recurrent glioblastoma, the percentage of CD133+ cells was increased by 4.6-fold compared to primary tumor (84). Interestingly however, the presence of more CD133+ cells indicated a greater chance of survival. The authors found that 20-60% of these cells were normal neural stem cells derived from adjacent tissue.

The cause of radio-resistance of GBM CSCs was addressed by a succeeding study (85). The authors found that the proportion of CD133+ cells in tumors increased following radiation. CSCs activated DNA damage response and repair pathways faster than CD133- cells and repaired DNA more effectively. Excitingly, this advantage could be reversed by inhibitors of Chk1 and Chk2, protein kinases that signal DNA damage by phosphorylating p53 and other responders.

4.3. Melanoma

Melanoma is a malignant tumor of melanocytes, a nonepithelial cell type derived from the neural crest. Prior to metastasis, it is easily treated by local cell ablative techniques. In stark contrast, metastatic melanoma rivals GBM in bleakness of prognosis. While melanoma accounts for only a small percentage of skin cancers, it causes 75% of deaths associated with skin cancer (86). Median survival is about 6 months and 5 year survival is 5-15% (87). The most active agent against metastatic melanoma, dacarbazine, has a response rate of only 15-25% time to progression of five to six months (87). Recurrent disease is even less responsive.

Because the profile of this disease includes early metastasis, resistance to chemoradiation, and rapid recurrence, the presence of CSCs was predicted. Whether this model applies to melanoma is a matter of controversy however.

As in other systems, sorting for classical stem cell markers such as ABCG2, nestin, and CD133 enriched for capacity to form tumorspheres *in vitro* and tumors in mice (88). Enriched expression of these markers has been detected in circulating melanoma cells by Fusi (89). Another member of the ABC transporter family, ABCB5, which confers resistance to anthracyclines, was suggested to be a better marker for melanoma CSCs (32).

However, other findings are inconsistent with the CSC model, and most scientists in this field now prefer the term melanoma-initiating cells (MIC). Melanomas have an inherent penchant for metastasis due to the expression of mesenchymal transcription factors in melanocytes (90). Thus, melanocytes transformed by Ras and SV40 T antigen metastasize much more readily than similarly transformed fibroblasts or breast epithelial cells. The capacity of isolated melanoma cells to initiate tumors in mice is also much higher. Quintana found that about 25% of unselected melanoma cells from 12 patients could form tumors in immunocompromised NOD/SCID mice bearing a deletion of the IL2 receptor (91). Furthermore, injection of unselected single cells produced tumors 27% of the time, in contrast to breast cancer in which 100 or more cells may be required even after cell sorting for CSC markers. The tumorigenic cells could be serially transplanted repeatedly without loss of tumorigenicity irrespective of whether they expressed any of the candidate CSC markers (92). Indeed, cells appeared to readily switch marker expression, suggesting a dynamic interconversion between cell types.

A study by Held and coworkers suggests that cells may be tumorigenic without having other CSC properties (93). After sorting for CD34 and p75, they injected single mouse melanoma cells into mice and measured tumor formation. All CD34+/p75- cells formed tumors, as did most CD34-/p75- cells. However, the latter were only capable of self-renewal, while the former could also regenerate tumor heterogeneity. The CD34-/p75+ cells were infrequently tumorigenic and more sensitive to chemotherapy than either class of tumorigenic cell.

Herlyn's group has explored the role of the H3K4 demethylase JARID1B in melanoma propagation (94). They found a small pool of cells positive for JARID1B that divided very slowly, only once or so per month, while progeny of these cells divided rapidly. The ability of a slowly dividing cell type to give rise to a rapidly dividing

Table 2. Partial list of CSC markers for different types of cancer

Cancer type	Cancer Stem Cell Markers
CNS	CD133+
Head and Neck	CD44+
Breast	$CD44^{high}/CD24^{low}$, CXCR1
Melanoma	ABCB5+, CD34, JARID1B
Bone Marrow	CD34+, c-kit+, Gr1(int)
Lung	CD133+
Liver	CD133+, CD13+
Pancreas	CD44+, CD24+, ESA+
Colon	CD133+

one is consistent with a multipotent CSC phenotype. Moreover, knockdown of JARID1B led to eventual loss of tumor-propagating ability. However, inconsistent with a classic hierarchical model, JARID1B expression can be lost, and negative cells can become positive. The authors concluded that the responsibility for maintaining tumor heterogeneity and propagation rotates among the population in a dynamic process. These characteristics help to explain why metastatic melanoma is so difficult to eradicate.

5. THERAPIES TARGETING CANCER STEM CELLS

With evidence mounting for a causative role of CSCs in recurrence of at least some cancers, there is great interest in discovery of drugs to target this critical subpopulation, either to kill them or coerce their differentiation. A classical empirical library screening approach, described in section 4.1, has already yielded several candidate drugs for breast CSCs (75). A second approach, based on similarities between breast cancer and diabetes expression profiles, yielded the candidate drug metformin (76). Here we describe other anti-CSC strategies based on burgeoning knowledge of CSCs revealed by laboratory studies.

5.1. Stem cell markers as a target for therapy

Since the 1990s, isolation and characterization of CSCs has relied upon the ability to sort them out of mixed populations using cell surface proteins that have come to be called stem cell markers (Table 2). These markers vary with the tissue type (95).

Some of these markers play a vital role in the function of those stem cells and so are logical targets for therapy. They can be receptors for cell-cell for cell-matrix communication, or they can contribute to drug resistance, such as ABCB5 in melanoma (29). In breast, the classical stem cell marker CD44 serves as a receptor for the extracellular matrix component hyaluronic acid (96). It also complexes with Met and Her family growth factor receptors and enhances their signaling, thus downregulating growth-arrest and apoptotic pathways (96). CD44 opposes the tumor-suppressive activities of p53, and it is in turn repressed by p53 (97). Ligation of CD44 receptor activates Nanog and other stem cell functions (98). These are logical targets for inhibitors.

CD133/prominin-1 is a surface molecule present on CSCs from several tissue types (Table 2). CD133 is under study as a therapeutic target in metastatic melanoma, and hepatocellular and gastric cancers (99, 100). Another candidate target in liver is CD13 (85). Combining a CD13 inhibitor with 5-FU was synergistic in reducing growth of mouse xenografts (101).

While these approaches show promise, in other cases CSC markers may not be critical for cell survival or may lack specificity. In the case of dynamic turnover of marker expression as in melanoma, it may be necessary to target multiple markers or combine this approach with cytocidal or differentiation therapies.

5.2. Therapies targeting CSC signaling pathways

Several signal-transduction pathways with previously well established roles in development and tumor progression have turned out to be critical for the generation and differentiation of CSCs, as well as their drug-resistance (102). Among these are the Notch, Hedgehog, Wnt, Her2, and IL-6 and -8 signaling pathways.

Although both Notch1 and Notch4 have been implicated in breast cancer, isolated CSCs are enriched for Notch4 and deficient in Notch1 (103). Pharmacological and genetic inhibition of Notch4 inhibited mammosphere formation *in vitro* and tumor formation in xenografts. Notch signaling depends on proteolytic cleavage by gamma secretase, and and inhibitors of this enzyme are in clinical trials (104). However, their lack of specificity for individual family members is problematic.

Wnt signaling is also critical for stem cell regulation and survival and is implicated in residual disease after radiotherapy (105, 51). Two polyphenols derived from food products, curcumin and piperine, have been reported to inhibit Wnt signaling (106,107). Both had previously been found effective as preventative agents against a broad range of cancers (108-110). In combination, piperine greatly increases the bioavailability and efficacy of curcumin, possibly due to synergistic inhibition of ABC transporters (110-112). Max Wicha's group reported that the drugs suppressed Wnt signaling as well as mammosphere formation by breast cancer cell lines and reduced the percentage of ALDH+ cells. As expected, they were more effective in combination (106). The same group reported that a nutriceutical derived from broccoli, sulforaphane, could decrease the ALDH+ cell population of breast cancer cell lines by 65%-80% by blocking Wnt signaling (113).

Hedgehog is another embryonic signaling pathway that figures in stem cell biology, EMT, and tumor progression (114, 115). Ulasov and coworkers found that that both Hedgehog and Notch are upregulated upon treatment of GBM with temozolomide. Moreover, inhibition of Notch and Hedgehog pathways enhanced sensitivity of CD133+ glioma stem cells to temozolomide therapy (39). The Hedgehog inhibitor cyclopamine was first identified as a natural teratogen (116). This drug and several derivatives are now in clinical trials (117,118).

The receptors for cytokines IL-8 and IL-6 have been targeted on CSCs from breast and other cancers.

CXCR1 is the receptor for IL-8, which regulates the self-renewal of breast CSCs (119). Blockade of this receptor using either a CXCR1-specific blocking antibody or repertaxin, a small-molecule CXCR1 inhibitor, specifically depleted the CSC population in breast cancer cell lines SUM159 and HCC1954. Repertaxin was also effective in reducing growth of xenografts (119). In light of the newly uncovered role of IL-6 in maintaining the breast CSC pool, its receptor is also a promising target (120). Inhibitors of IL-6 are already under trial for multiple myeloma (121).

In addition to embryonic and inflammatory signaling pathways, growth factor signaling is also a relevant target. In breast cancer, Her2-overexpressing tumors historically have had a dim prognosis typified by chemoresistance and early recurrence. Several reports indicate that Her2+ tumors have amplified pools of CSCs, as assessed by mammosphere and tumor-initiation assays and sorting for ALDH+ cells (53, 43). Her2 signaling induces PI3K-AKT signaling, leading to downregulation of p53 and induction of Nanog. This permits an increase in symmetric divisions and thus self-renewal of CSCs (53). These effects can be reversed by restoration of p53 (53) or inhibition of Her2 by trastuzumab or lapatinib (122). Resistance to trastuzumab or early relapse is associated with secondary mutations that ramp up AKT signaling (43, 122).

6. CONCLUSIONS

For breast and some other cancers, the weight of evidence now favors the view that CSCs originate from non-CSCs that re-activate stem cell programming, while glioblastomas seem to follow different rules. Here, we have presented the argument that, whatever their origin and whether stable or transient, the entity known as cancer stem cell or tumor-initiating cell is a major factor in therapy-resistance and cancer recurrence. The ability to isolate and characterize these cells has led on the one hand to new drugs that target CSCs and on the other, to the realization that some drugs already in hand owe their efficacy to their effect on CSCs. Unsurprisingly, the therapies most successful in preventing relapse are those that combine debulking agents with anti-CSCs, e.g., paclitaxel with trastuzumab. The weight of the evidence is now such that one may question the value of any new clinical trial that does not include an anti-CSC agent. We may expect that within a few years, all cancer therapy protocols will incorporate an appropriate anti-CSC approach.

It has long been recognized that cancer is caused by the loss of ability to maintain tissue differentiation. It is worth noting that several of the anti-CSC drugs discovered thus far, including metformin and salinomycin, restore differentiation rather than killing masses of cells. Continued investigation of epithelial differentiation processes may reveal additional low-toxicity tools for coercing CSCs back into the fold.

7. ACKNOWLEDGMENTS

This work was supported by NIH grant #1R15CA151094-01 and Department of Defense grants BC097686 and BC097675 to RCE.

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Key Words: Cancer Stem Cells, Cancer Relapse, Breast Cancer, Glioblastoma, Melanoma, Review

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