Cancer Stem Cells: A New Theory Regarding a Timeless Disease

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Cancer Stem Cells: A New Theory Regarding a Timeless Disease

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1. Introduction

Although cancer has been described in early medical texts from antiquity, it remains the second leading cause of death in the United States. Technological improvements in screening modalities have increased detection of smaller tumors, yet current therapies for most types of cancer often fail. Cancer death is usually attributable to the development of metastatic disease. Chemotherapeutic regimens target all proliferating cells based on the principle that tumor cells proliferate at a faster rate than normal cells, resulting in differential cytotoxicity. The increased proliferative capacity of cancer cells is the result of accumulated genetic insults to various cellular pathways. These mutations include those that enhance cellular proliferation or suppress normal growth inhibitory mechanisms and apoptosis. Still other mutations allow tumor cells to evade surveillance and removal by the immune system. Cumulatively, these mutations result in neoplastic tumor growth and subsequent distant metastasis. The tumor microenvironment has also emerged as a critical component in the development of cancer from benign neoplasia, both in secreted factors that modulate tumor cells and in three-dimensional interactions with extracellular matrix proteins.

A paradigm shift in cancer biologists' thinking about solid organ tumors may provide a new understanding of cancer development and progression and has implications for how we think about developing therapies to treat cancer patients. In normal tissues and organs, stem cells reside at the apex of a hierarchical scheme that drives organogenesis. The realization that tumors themselves function like complex organs birthed the theory that cancer cells with the properties of stem cells may be the key drivers of the complex machinery behind tumorigenesis. The cancer stem cell theory is based on the finding that a small number of highly tumorigenic cells within a tumor can produce the heterogeneous populations found within an entire tumor when transplanted into immunocompromised mice. Thus, it is believed that cancer stem cells (CSCs) may be responsible for tumor formation based upon their capacity for self-renewal and differentiation. These capacities together permit asymmetric division of the stem cell, resulting in maintenance of the parental stem cell population in addition to production of differentiated progeny (Figure 1). The CSC population can be serially transplanted without loss of tumorigenicity due to its ability to undergo self-renewal.

Given these findings, many have hypothesized that CSCs arise from genetic mutations that occur in normal stem cells. Normal stem cells possess the longest life span among mammalian cells, and it is thought that these cells are more likely to accumulate mutations over time and ultimately assume a malignant phenotype. Mutation of normal stem cells may create a population of CSCs that cause tumor growth as a result of altered self-renewal mechanisms, although specific mutations have yet to be demonstrated. Recent data supports such a model for colon cancer development. Barker et al. utilized a mouse model where the adenomatous polyposis coli (APC) gene of the Wnt signaling pathway was conditionally inactivated in the intestinal stem cell.[1] These stem cells are located at the bottom of the intestinal crypt in both the small intestine and colon and become transformed with APC deletion within days. The transformed stem cells go on to form adenomas, in contrast to APC deletion in the progeny of the stem cells where adenomas rarely form.

Alternatively, some studies suggest that CSCs may arise from mutated progenitor cells called transit amplifying cells that develop the capacity for unregulated self-renewal.[2,3] Yet in other models, CSCs may arise from differentiated cells that assume a stem-cell phenotype. For example, in genetically engineered mouse models of pancreatic cancer, one study has suggested that the cell of origin of pancreatic cancer is an acinar or centroacinar cell.[4] Additional work is being performed in several laboratories to verify this finding.

Gene profiling experiments comparing CSCs and non-CSCs have revealed upregulation of other signaling pathways that are also found to be abnormal in human tumor specimens. These include Wnt/β-catenin, Notch, PI3Kinase-Akt-mTOR, as well as the Hedgehog signaling pathway. For example, c-Myc, a downstream target of the β-catenin signaling pathway, was found to be upregulated in glioma

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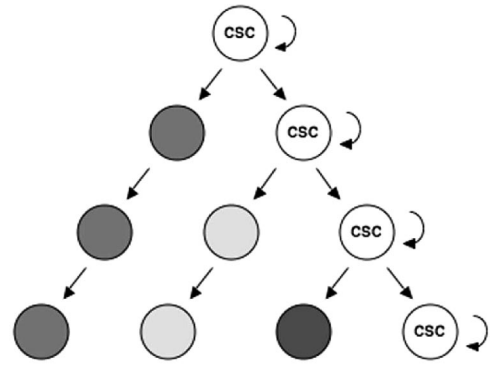

Figure 1. Cancer stem cell model. A cellular hierarchy exists within a tumor with cancer stem cells at the apex of the hierarchy. The cancer stem cells maintain their population through self-renewal and also differentiate to produce tumor heterogeneity. Adapted by permission from Macmillan Publishers Ltd.: Nature 2001, 414 (6859), 105−111.

CSCs relative to nonstem glioma cells.[5] Knockdown of c-Myc in glioma CSCs caused cell-cycle arrest and increased apoptosis, as well as decreased tumor formation in the brains of immunocompromised mice, highlighting the critical role of this molecule in glioma CSC function.

The first CSCs discovered were those responsible for myeloid leukemia by Dick et al. over a decade ago.[6] This groundbreaking work utilized fluorescence-activated cell sorting (FACS) and a tumor xenograft model to identify the CSC surface protein marker expression pattern of leukemic cells able to engraft human leukemia in nonobese diabetic severe combined deficiency (NOD-SCID) mice. These techniques have subsequently been adapted by tumor biologists studying solid malignancies, which will be the focus of this review. The first epithelial solid organ cancer stem cell was identified in breast cancer by Al-Hajj et al.[7] They reported a phenotypically distinct and rare population of highly tumorigenic cells with the cell surface marker expression of $CD44^{+}CD24^{-/low}ESA^{+}$ that, when injected in low numbers, possessed the ability to form tumors in immunocompromised mice that recapitulated the original tumor from the human patient. The ability to serially passage primary human tumors as xenografts remains the gold standard assay for CSC self-renewal capacity, allowing study of the heterogeneity reflective of actual patient tumors. However, this approach is limited by altered immune system and host−tumor interactions since this assay requires the implantation of human cells into immunodeficient mice. Presumably, technical limitations as well as differences in the microenvironment contribute to the variations in tumor initiating frequency among different tumor types and differences observed with orthotopic versus subcutaneous implantation. The use of transgenic animal models of carcinogenesis will be useful in examining CSC function in the setting of an intact tumor microenvironment and immune system.

Further work characterizing CSCs from various solid malignancies identified another trait that has proved useful for researchers. CSCs exhibit the ability to form multicellular spheres when grown using nonadherent culture conditions.

Table 1. Solid Epithelial Tumors and Associated Cancer Stem Cell (CSC) Marker Patterns

tumor type	CSC markers	CSC number for tumor formation[a]
brain	$CD133^+$	100
breast	$CD44^+CD24^-ESA^+$, $CD133^+$	100
liver (HCC)	$CD44^+CD90^+$	5000
pancreas	$CD44^+CD24^+ESA^+$, $CD133^+$	100−500
colon	ESACD44+, CD133+,	100
prostate	CD44+, CD133+	1000

[a] The minimum number of cancer stem cells injected into immunocompromised mice to produce a xenograft tumor is indicated.

This trait can be exploited to assay various tumor cell populations for self-renewal capacity following FACS. The spheres can be subsequently isolated, dissociated, and injected into mice to form xenograft tumors. In some tumors, spheres can also be passaged serially without loss of sphere-forming ability or tumorigenesis. The ease of such an *in vitro* assay allows for screening of potential surface markers identifying CSCs and testing drug efficacy in a cost-efficient and timely manner. While the sphere assay is a useful *in vitro* model, findings using the sphere assay should be validated with *in vivo* model systems.

Both normal stem cells and CSC have also been identified using biochemical activity rather than cell surface markers. Aldehyde dehydrogenase (ALDH) enzymes regulate retinoic acid synthesis and oxidize toxic aldehydes. These enzymatic functions are thought to protect stem cells from oxidative injury and can be assayed using a fluorescent ALDH substrate, bodipy-aminoacetaldehyde, commonly known by the commercial name Aldefluor. When processed by ALDH enzymes, Aldefluor becomes charged and remains within the cell, which can then be analyzed by flow cytometry. This strategy has been used to identify normal stem cell populations in the hematopoietic and neuronal systems,[8,9] as well as cancer stem cell populations in the breast, colon, and pancreas.[10−12] Aldefluor activity has been ascribed to the ALDH1 isoform Aldh1a1, which is expressed at higher levels in mouse and human hematopoietic stem cells compared with differentiated progenitor cells in gene profiling experiments.[13,14] Surprisingly, in a recent study *Aldh1a1*-deficient mice did not display altered Aldefluor staining or altered function of hematopoietic and neuronal stem cells.[15] The authors concluded that other ALDH family members were responsible for Aldefluor activity and that Aldh1a1 did not regulate stem cell function under physiologic conditions. These results remain to be validated in other model systems.

2. Solid Epithelial Tumors

The following sections will briefly summarize the solid malignancies for which CSCs have been identified utilizing primary tumor samples rather than cultured cell lines. Extensive passaging of cells *in vitro* creates selection for cancer cells that may be biologically different from the original parental cells and, as such, may not represent an optimal model system for study of CSCs. It is beyond the scope of this review to discuss every type of cancer in which CSCs have been described, so those that have been well studied will be highlighted (Table 1).

2.1. Brain

The identification of CSCs responsible for human brain tumors was based on the neurosphere assay developed to study normal neural stem cells.[16] Both normal brain stem cells and brain cancer stem cells will form neurospheres when placed in a suspension culture in the absence of serum. The tumor cells found within the neurospheres express neuronal and astroglial proteins when exposed to serum, demonstrating the ability of the CSC population to differentiate. A heterogeneous population of cells is found in these spheres, with a small subset of CSCs expressing the neural precursor markers nestin and CD133. Still other more differentiated cancer cells express markers representing more mature central nervous system (CNS) cells. The majority of the population, however, corresponds to immature progenitors, which can further differentiate but lack self-renewal capacity. As such, these cells will form spheres but not after serial passaging and are therefore distinguishable from the actual stem cells within the sphere cultures. Sphere-forming ability varies among the different types of brain tumors, although it is most evident with glioblastoma.[16]

In accordance with the tumor sphere assays, $CD133^+$ brain cancer cells demonstrated enhanced tumorigenicity *in vivo* and the ability to undergo serial passaging, characteristics reflective of self-renewal capacity. In a study by Singh and colleagues, $CD133^+$ glioblastoma cells were found to generate tumors with injection of as few as 100 cells when transplanted into the brains of immunodeficient mice, whereas $CD133^-$ cells did not form tumors, even when injected in significantly higher cell quantities.[17] The $CD133^-$ cells did, however, survive in small clusters near the injection site, suggesting that the enhanced tumorigenicity that was observed was an intrinsic characteristic of the implanted cells, rather than decreased viability of $CD133^-$ cells following transplantation into a different microenvironment. The $CD133^+$ CSCs generated tumor xenografts that exhibited cellular heterogeneity and histological phenotypes similar to their parental human tumors. This was true of the $CD133^+$ brain cancer cell populations isolated from either adult or pediatric tumors.

Interestingly, more recent studies by some groups have shown that $CD133^-$ cells derived from glioblastoma tumors also possess tumorigenic capacity.[18,19] While this finding has been ascribed to technical issues related to the preparation of cell suspensions from dissociated tumors, as well as differences in tumor subtype, it should also be considered that some tumors may follow a CSC model while others do not or, alternatively, that CD133 is not an optimal marker for CSC identification in all brain tumors. Continuing work in the field will need to focus on improving our understanding of the relevance of particular CSC cell surface markers in individual patient settings.

From a therapeutic standpoint, the understanding of CSC function may improve therapeutic options for patients with brain cancers. In one study, $CD133^+$ brain CSCs were found to differentiate to an astroglial cell type when exposed to bone morphogenetic protein (BMP), which also promotes maturation of normal neural precursor cells.[20] The CSC population was reduced by 50% following treatment with BMP4 *in vitro*. BMP treatment of $CD133^+$ CSCs in culture inhibited development of tumors when the treated tumor cells were implanted into the brains of immunodeficient mice. Most importantly, *in vivo* BMP4 administration to established intracerebral xenografts resulted in smaller tumors and prolonged animal survival. Thus, BMP, or other agents that induce differentiation of brain CSCs, may be used in

conjunction with conventional therapy to prevent relapse and decrease mortality.

2.2. Breast

Breast cancer was the first solid malignancy for which a cancer stem cell population was identified.[7] Al Haji and colleagues reported that cells with the surface marker expression of $CD44^+CD24^{-/low}ESA^+$ were highly tumorigenic, with tumor development after implantation of as few as 100 cells. The tumors exhibited cellular heterogeneity similar to the parental tumor and could be serially transplanted following FACS isolation of the CSC population. In contrast, breast cancer cells not expressing this cell surface marker pattern failed to form tumors with the injection of tens of thousands of cells into mice.

Dontu and colleagues have developed an *in vitro* culture system for human mammary epithelial stem and progenitor cells similar to that of neuronal and brain stem cells.[21] Cells isolated from human reduction mammoplasty specimens grown in nonadherent culture conditions generated spherical colonies termed "mammospheres." Mammosphere-initiating cells had stem cell properties and were able to self-renew *in vitro* and differentiate into all three cell lineages found in the mammary gland. Similarly, $CD44^+CD24^{-/low}ESA^+$ breast CSCs formed spheres *in vitro* and retained their *in vivo* tumorigenicity when implanted into immunodeficient mice.[22] By microarray analysis, the Hedgehog pathway was found to be upregulated in the normal breast stem cell population and decreased when the cells were differentiated by attachment to a collagen substrate. This pattern was paralleled in the breast $CD44^+CD24^{-/low}ESA^+$ CSCs, implicating Hedgehog signaling as a critical pathway in breast CSC function.

2.3. Liver

Until recently, the liver was thought to lack normal stem cells, given the extremely low mitotic index in comparison to other epithelial organs.[23] The remarkable regenerative capacity of this organ in response to injury has been thought not to be due to differentiation from stem cells but rather to proliferation of mature hepatocytes. This restorative effect is observed with different types of tissue injury. For example, 70% partial hepatectomy causes rapid proliferation of mature hepatocytes that restores the volume of resected tissue within a week. Similarly, carbon tetrachloride treatment results in centrolobular necrosis with subsequent division of the midlobular hepatocytes adjacent to the injury. These mature hepatocytes repopulate the necrotic zones rapidly within several days. In either injury model, the accelerated mature hepatocyte proliferation subsides with the return of normal liver function once regeneration is complete. Therefore, it appears that the majority of differentiated parenchymal hepatocytes are able to proliferate and differentiate, in contrast to other types of terminally differentiated parenchymal cells.

The age of the animal and the type of model system appears to influence the cellular response to injury. Interestingly, the age of the animal also appears to affect the histologic phenotype of liver tumors that develop in different liver tumorigenesis models. For example, young mice exposed to chemical carcinogens develop hepatoblastomas that are less differentiated than those induced in adult mice, suggesting that the cell of origin of liver cancer loses it differentiation potential with aging. In support of this notion, adult mice typically develop differentiated hepatocellular carcinoma (HCC) irrespective of the carcinogen utilized. In humans, poorly differentiated hepatoblastomas are only seen in children under the age of five, in agreement with the findings in the mouse models. One can theorize that these various types of liver cancer seen in humans and in rodent models of chemical carcinogenesis can be explained by transformation of a tissue stem or progenitor at various stages of differentiation.

Recently, Yang et al. identified CSCs expressing the cell surface marker $CD90^+$ in HCC cell lines, primary human liver cancer specimens, and blood samples from patients with liver cancer.[24] The $CD90^+$ cells, but not the $CD90^-$ population from established HCC cell lines, were tumorigenic when implanted in immunodeficient mice. The authors examined this marker in combination with other surface marker proteins that have been used for various other CSC types, including CD44. They found that the majority of $CD90^+$ cells also expressed CD44 on their cell surface. HCC cells expressing both CD90 and CD44 defined a highly tumorigenic population with CSC function, based on both *in vivo* tumorigenicity assays and serial transplantation (up to three passages). The $CD90^+CD44^+$ liver cancer cells were also more biologically aggressive than the $CD90^+CD44^-$ controls and formed metastatic lesions in the lung of implanted animals. Treatment with an antibody against CD44 induced apoptosis of $CD90^+CD44^+$ cells *in vitro* and blocked formation of both primary liver and metastatic tumor nodules *in vivo*. The authors also noted that >90% of blood samples from HCC cancer patients contained a different $CD45^-CD90^+$ population, which was able to generate tumor nodules in immunodeficient mice, thus defining a circulating pool of liver CSCs.

2.4. Pancreas

Recent work in our laboratory lead to the identification of pancreatic CSCs.[25] Primary human pancreatic adenocarcinoma samples were used to establish xenografts in NOD-SCID mice. FACS analysis of freshly sorted primary tumors or tumors expanded as low passage xenografts in NOD-SCID mice using the surface markers CD44, CD24, and ESA, studied alone or in combination, was performed to identify a potential CSC population in pancreatic cancer. These sorted cancer cell populations were implanted into mice to assess their tumorigenic potential. Initial observations using the marker ESA^+ in combination with either $CD44^+$ or $CD24^+$ revealed a population of cancer cells that had a tumor initiation rate of 1 in 4 animals with as few as 100 viable human cells injected into the mice. When all three markers were tested in various combinations, a population of $CD44^+CD24^+ESA^+$ cells was identified, which comprised only 0.2−0.8% of all human pancreatic cancer cells in the 10 human pancreatic cancer specimens studied. Of these 10 specimens, eight were obtained from primary tumors and two were isolated from metastatic lesions. As few as 100 $CD44^+CD24^+ESA^+$ cells injected in NOD-SCID mice were able to generate tumors in 50% of the animals, while conversely $CD44^-CD24^-ESA^-$ cells did not form tumors unless at least 10 000 cells were injected. Even with injection of 10 000 $CD44^-CD24^-ESA^-$ cancer cells, only one of 12 animals formed a tumor, reflecting at least a 100-fold greater tumor-initiating potential in the $CD44^+CD24^+ESA^+$ CSC population. Additionally, FACS analysis of $CD44^+CD24^+ESA^+$ derived tumors revealed a resultant

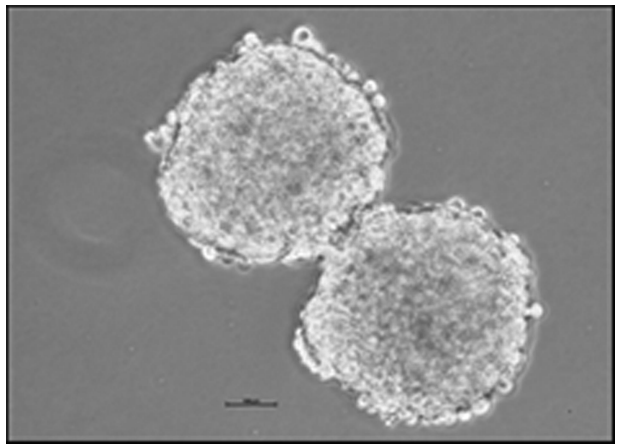

Figure 2. Pancreatic tumor spheres. Representative photograph of pancreatic tumor spheres 72 h after placing $CD44^+CD24^+ESA^+$ primary pancreatic cancer cells into a serum-free media under low-attachment conditions.

tumor heterogeneity that mimicked that observed in the primary tumor, demonstrating that the pancreatic CSC population had the ability to both self-renew and generate differentiated progeny.

We also found that single plated $CD44^+CD24^+ESA^+$ cells formed pancreatic tumor spheres similar in appearance to both mammospheres and gliomaspheres, while $CD44^-CD24^-ESA^-$ failed to form spheres in nonadherent culture (Figure 2). $CD44^+CD24^+ESA^+$ tumor spheres could be maintained in culture for greater than 30 self-renewing passages. Additionally, FACS analysis of dissociated pancreatic tumor spheres following several days in culture revealed that the spheres contained both CSC and marker negative cell populations. This demonstrates that the CSCs are able to self-renew *in vitro*, as well as produce differentiated progeny.

CD133 is a glycoprotein, which in the pancreas is normally expressed on the apical surface of pancreatic ductal epithelial cells and has been reported to distinguish cells with stem cell-like properties in embryonic mouse pancreas.[26] Hermann et al. found that $CD133^+$ cells from both primary human pancreatic cancers and established pancreatic cancer cell lines possessed tumorigenic capacity.[27] The authors reported that $CD133^+$ cells comprised 1−3% of pancreatic tumor cells, and injection of 500 $CD133^+$ cells was sufficient to form xenograft tumors that recapitulated the primary human tumor, while injection of a similar number of $CD133^-$ pancreatic cancer cells did not form tumors. Histological staining of primary tumor sections with antibodies directed against CD133 localized these cells to the leading edge of the tumor. The authors also reported an overlap of 14% between $CD44^+CD24^+ESA^+$ and $CD133^+$ pancreatic cancer cells. Further *in vivo* tumorigenicity experiments are needed to determine whether $CD44^+CD24^+ESA^+$ and $CD133^+$ pancreatic cancer cells represent distinct CSC populations or whether a combination of all four markers provides enhanced enrichment for an even more highly tumorigenic CSC population.

To further support the notion that $CD44^+CD24^+ESA^+$ pancreatic cancer cells function as CSCs, we examined whether pancreatic CSCs had upregulation of sonic hedgehog (SHH) and BMI-1, key components of developmental signaling pathways. RNA was isolated from FACS sorted $CD44^+CD24^+ESA^+$ cells along with marker negative cells, and quantitative RT-PCR was performed to assess SHH expression. We found that the expression of the SHH transcript was upregulated over 40-fold compared with nontumorigenic, marker negative cancer cells and normal pancreatic epithelial cells. We have also identified a 5-fold increase in BMI-1 expression in $CD44^+CD24^+ESA^+$ cells compared with the nontumorigenic population (unpublished observations). BMI-1 has been found to be an important regulatory molecule that participates in self-renewal in many stem cell systems.[28]

2.5. Colon

Although many of the key molecular mutations involved in colon cancer were elucidated by the Vogelstein group decades ago,[29] the identification of colon CSCs occurred only recently. The same strategy used to identify CSCs in brain and breast cancers identified a $CD133^+$ colon cancer population as being highly tumorigenic, producing xenograft tumors that possessed a similar cellular hierarchy as the primary human tumor.[30] The $CD133^+$ colon CSCs also displayed growth for greater than 1 year in tumor sphere cultures, while the $CD133^-$ cells did not, nor did $CD133^-$ colon cancer cells readily form tumors *in vivo*. Similar results were reported independently by O'Brien et al. who found the $CD133^+$ colon cancer population to be less than 1% of all colon cancer cells and highly tumorigenic when transplanted under the kidney capsule of NOD/SCID mice compared with $CD133^-$ colon cancer cells.[31] The percentage of $CD133^+$ colon CSC population within a tumor was maintained with serial passaging.

In another report, Dalerba and colleagues found that $ESA^{hi}/CD44^+$ colon cancer cells identified a CSC population,[32] with the frequency in human tumors ranging from 0.03−38% with a mean value of 5.4%. This cell surface marker combination identified a highly tumorigenic population, with injection of as few as 200 cells sufficient to generate subcutaneous tumors in immunodeficient mice. In this report, the findings differed from those reported by O'Brien[31] in that the expression of CD133 was found to be variable among the primary specimens and even absent in one cancer sample. Further supporting evidence for a central role of CD44 in colon CSCs was published by Du et al.[33] They reported that a single $CD44^+$ colon cancer cell was sufficient to generate tumor spheres *in vitro*, which retained the ability to form tumor xenografts. Knockdown of CD44 diminished tumorigenicity, whereas knockdown of CD133 had no effect on colon CSC function. This data points to a functional role for CD44, similar to that seen in HCC, in contrast to CD133, which may serve solely as a marker for a CSC population.

2.6. Prostate

Similar to other malignancies, prostate cancer has a dismal prognosis once recurrence occurs following surgical resection and hormonal therapy. Study of the normal prostate stem cell has aided identification of the prostate CSC. The normal prostate stem cell resides in the basal epithelium, expresses the $\alpha_2\beta_1$ integrin, and lacks the androgen receptor. Collins et al. were able to culture normal prostate stem cells *in vitro* and form acini in a xenograft model that had a similar appearance to normal prostate epithelium.[34] Subsequent work has revealed that the normal prostate stem cell population expresses CD133.[35]

Investigation of normal prostate stem cells ultimately lead to the identification of prostate CSCs characterized by the

expression of the cell surface markers $CD133^+/\alpha_2\beta_1$-integrin^{high}/CD44 from biopsies of human tumors.[36] These cells were basal in phenotype and displayed an invasive behavior much greater than the most malignant prostate cell lines. Identification of the prostate CSC reveals that they possess a unique gene expression signature that correlates with the clinical Gleason grade and worse patient outcome.[37] Prostate CSCs possessed a longer life span when compared with nontumorigenic cells from prostate tissue. Other groups have shown that the prostate CSC population can be isolated from prostate cancer cell lines and xenograft tumors, thus recreating the tumor heterogeneity observed in human samples.[38,39] Interestingly, unlike primary prostate CSCs, cells from established prostate cancer cell lines, when injected into immunodeficient mice, do not seem to have the same ability to generate tumor xenografts with tumor heterogeneity reflective of primary prostate cancers.

2.7. Melanoma

Malignant melanoma differs from the other types of epithelial tumors described above in that melanocytes are derived from neural crest cells. The embryonic neural crest contains pluripotent stem cells that give rise to a wide array of lineages, including neurosecretory cells, peripheral neurons, glia, and the cephalic mesenchyme, which develops into bone and cartilage. Melanocytes are specialized skin cells that produce the pigment melanin, which is responsible for skin color and protects the deeper layers of the skin from damaging ultraviolet radiation from sun exposure. If detected early and excised, melanoma has a good prognosis. The prognosis changes drastically once the tumor has invaded into the dermal vessels and lymphatics. Although it is not the most common type of skin cancer, melanoma accounts for approximately 75% of skin cancer deaths.

Two independent groups examining different aspects of melanoma cancer biology initially described CSC populations derived from melanoma tumors and cell lines. Fang et al. dissociated 17 human melanoma samples and cultured the cells under stem cell or standard melanoma conditions and media. The former protocol resulted in the formation of pigmented spheres that could be passaged for greater than eight months and were highly positive for the melanoma markers chondroitin sulfate proteoglycan (CSPG), β3 integrin, and melanoma cell adhesion molecule (MCAM).[40] The melanoma spheres were clonally expanded and found to be tumorigenic for pigmented xenograft tumors. In addition to being able to self-renew, the melanoma spheres were also able to undergo melanocytic as well as mesenchymal differentiation. This finding suggests that the melanoma CSC population possesses some features of the neural crest. The authors were also able to derive melanoma spheres from established melanoma cell lines when cultured under stem cell conditions.

The second group was examining chemotherapy resistance in melanoma cells mediated by the ATP-binding cassette (ABC) family of membrane transport proteins. They identified that the ABCB5 protein mediated doxorubicin resistance in a subset of melanoma cells and was also expressed in primary human melanocytes.[41] These cells were also positive for CD133 expression as were select areas of primary human melanomas by immunohistochemistry, suggesting that ABCB5 may mark primitive melanoma cells. The double positive population could generate $ABCB5^-$ cells in culture. The authors then went on to publish gene expression profiles of the various stages of melanoma from benign nevi to metastatic lesions,[42] where ABCB5 expression correlated with disease progression. ABCB5 expression also overlapped with expression of factors associated with stem cells, including nestin, CD144, and BMPR1A. $ABCB5^+$ cells from either clinical melanoma specimens or cultured cell lines formed xenograft tumors in NOD/SCID mice that were able to recapitulate the heterogeneity of the original tumor. Xenografts were passaged serially, demonstrating the ability of self-renewal as populations of $ABCB5^+$ cells were maintained. Lineage tracing using differentially labeled populations was performed to formally prove that the $ABCB5^+$ could self-renew as well as produce $ABCB5^-$ progeny. Treatment with a monoclonal antibody to ABCB5 inhibited xenograft tumor initiation as well as growth of established tumors..

A recent study reported generation of xenograft tumors from a single melanoma cell.[43] The authors utilized NOD/SCID *Il2rg*$^{-/-}$ mice, which differ from the NOD/SCID mice used in the previous studies by having deficient natural-killer cell activity due to lack of the interleukin-2 γ receptor. This strain had been previously used to study leukemia and demonstrated a more efficient engraftment of human leukemic cancer cells. Limiting dilution analysis was used to demonstrate that approximately 25% of unselected melanoma cells from 12 patients formed tumors using the more permissive NOD/SCID *Il2rg*$^{-/-}$ strain. Because CSC populations are thought to be rare within the tumor, this study questions the actual percentage of the tumorigenic population within a given cancer. The authors ascribe the observed effect to an increased xenogenic immune response in the traditional NOD/SCID mice and were unable to define a CSC population in melanomas based on cell surface markers with differential tumor forming capacity. Interestingly, melanoma is one of the few human cancers that responds to immune modulation by cytokine therapy, specifically interferon-α and interleukin-2, with response rates in approximately 10−20% of patients. Therefore, it is not surprising that NOD/SCID *Il2rg*$^{-/-}$ mice, which are further immunocompromised than NOD/SCID mice, had higher rates of melanoma engraftment. Whether the findings reported for melanoma will be similar for other epithelial cancers remains to be seen. Moreover, it remains a question whether results obtained in the more immunodeficient NOD/SCID *Il2rg*$^{-/-}$ mice are more reflective of what may be observed in humans. Complementary studies in transgenic mouse cancer models of human cancers that possess an intact tumor microenvironment will be important in validating the CSC hypothesis if different tumor-initiating potential effects are observed in the NOD/SCID *Il2rg*$^{-/-}$ and NOD/SCID mouse models in individual cancer types.

3. Cancer Stem Cells and Metastasis

Since the majority of cancer deaths are due to metastatic disease, intense focus has been placed on determining what regulates this critical aspect of tumor biology. One hypothesis is that CSCs are the only cells within a tumor with the capacity to metastasize. Recent work in the study of epithelial to mesenchymal transition (EMT), a key step in the metastatic process, may provide some clues as to how CSCs are involved in this process.

EMT is a cellular process that is characterized by down-regulation of cellular adhesion proteins, such as E-cadherin, resulting in loss of cell−cell junction connections, with an

overall increase in cell motility.[44] EMT normally occurs during the development of tissues and organs in embryogenesis as well as wound healing, but this phenomenon has also been found to be critical in the progression of cancer. Many primary cancers and cancer cell lines exhibit upregulation of a number of important transcription factors that regulate EMT, including Twist, Snail, and Slug.[45−47] These transcription factors suppress E-cadherin expression and mediate the transition to highly motile cells.

The relationship between EMT and the cancer stem cell populations has yet to be defined, but studies by Mani et al. in normal epithelial cells that are induced to undergo EMT may provide some insight into the role of EMT in CSC function.[48] The authors found that ectopic expression of either Twist or Snail in normal immortalized human mammary epithelial cells resulted in EMT. Subsequent FACS analysis of these EMT-induced cells showed that the vast majority displayed a $CD44^{high}/CD24^{low}$ expression pattern similar to that observed in breast cancer stem cells. EMT-induced breast epithelial cells also demonstrated a 30 fold higher rate of mammosphere formation compared with normal epithelial cells that had not undergone EMT. Finally, overexpression of Twist or Snail in transformed mammary epithelial cells resulted in greater tumor initiation in xenograft models. These data suggest that CSCs may represent a cell population with enhanced migratory and metastatic potential since they possess features similar to cells that have undergone EMT. Critical experiments to determine whether induction of EMT in nontumorigenic cancer cell populations will be successful in inducing these cells to adopt a CSC phenotype have yet to be performed.

Evidence from a pancreatic cancer xenograft model supports the premise that pancreatic CSCs are responsible for metastasis. Hermann et al. isolated a $CD133^+$ pancreatic CSC population from an aggressive pancreatic cancer cell line that demonstrated expression of CD133 and CXCR4, a chemokine receptor for SDF-1.[27] This metastatic pancreatic cancer cell line, L3.6pl, was then further divided into populations of $CD133^+/CXCR4^+$ and $CD133^+/CXCR4^-$ cells, which were then implanted in the pancreas of immunedeficient animals. Although both populations were able to form tumors, only $CD133^+/CXCR4^+$ tumor cells were found circulating in blood following implantation. The tumors established from $CD133^+/CXCR4^+$ cells in these animals produced metastases in the liver, surrounding lymph nodes, and spleen, whereas the $CD133^+/CXCR4^-$ negative tumors remained restricted to the site of injection. Finally, pharmacological blockade of CXCR4 significantly reduced the ability of these cells to metastasize in this tumor model, supporting the notion that the $CD133^+/CXCR4^+$ CSC subpopulation is responsible for metastasis.

Similar findings were reported by Yang et al. in their examination of CSCs derived from HCC.[24] The population of $CD90^+CD44^+$ cells isolated from HCC specimens, but not $CD90^+CD44^-$ controls, formed metastatic lesions in the lung of implanted animals. Formation of metastatic nodules as well as primary tumors was inhibited by treatment with an antibody against CD44, suggesting that expression of CD44 in HCC CSCs was responsible for the ability of liver CSCs to metastasize.

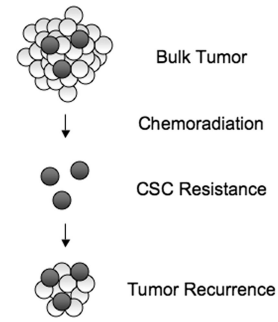

Figure 3. Model for tumor recurrence. Bulk tumor, with cancer stem cells represented as darker cells, is subjected to conventional therapy (chemotherapy or radiation). This treatment kills the majority of differentiated tumor cells, but cancer stem cells survive and subsequently proliferate to cause tumor recurrence.

4. Cancer Stem Cells and Resistance to Treatment

Cancer stem cells have been hypothesized to be resistant to conventional chemotherapy and radiation therapy and are thought to be the culprit behind cancer recurrence after clinical remission (Figure 3). Proposed mechanisms of resistance include enhanced expression of multidrug resistance transporters, antiapoptotic factors, or increased levels of DNA damage repair proteins. In hematopoietic malignancies, a subpopulation of human leukemia stem cells have been shown to be resistant to the Abl tyrosine kinase inhibitor imatinib, with proven effectiveness against differentiated leukemic cells.[49] The leukemic stem cells that survived imatinib treatment regenerated the tumor, providing further evidence in support of the important role played by cancer stem cells in tumorigenesis. Evidence of the resistance of brain CSCs to standard therapies was also shown in glioblastoma CSCs. A glioblastoma CSC population expressing $CD133^+$ in both primary tumors and xenografts increased 2−4-fold following ionizing radiation.[50] This enrichment of $CD133^+$ CSC was due to a preferential activation of DNA damage response, rendering these cells resistant to the DNA damaging effects of radiation. In a report examining therapeutic resistance in colon cancer, $CD133^+$ colon cancer stem cells were resistant to cell death induced by the chemotherapeutic agents oxaliplatin and 5-fluorouracil (5-FU), with resistance mediated by expression of IL-4 by the $CD133^+$ colon CSCs. IL-4 inhibition enhanced the antitumor efficacy of these chemotherapeutic drugs through selective sensitization of $CD133^+$ cells.[51] In a study using pancreatic cancer cell lines, Hermann and colleagues found that $CD133^+$ populations in the L3.6p pancreatic cancer cell line were enriched after exposure to gemcitabine.[27] Our group has also observed that treatment with ionizing radiation and the chemotherapeutic agent gemcitabine results in enrichment of the $CD44^+CD24^+ESA^+$ population in human primary pancreatic cancer xenografts (unpublished observations). An improved understanding of mechanisms of CSC resistance is an active area of investigation and may allow development of sensitizing treatment strategies that allow clinicians to take maximal advantage of standard therapies that are already in use.

Preclinical studies are beginning to emerge in which CSC-targeting agents are being tested. The PI3Kinase/Akt signaling pathway mediates several cellular functions such as growth, metabolism, and survival that are altered in cancer. The phosphatase and tensin homologue (PTEN) is a negative regulator of the pathway and functions as a tumor suppressor.

As such, it is frequently deleted or inactivated in human cancers. Deletion of PTEN in hematopoietic cells in mice results in proliferation of the normal hematopoietic stem cells, followed by depletion and the eventual development of myeloproferative disorder, and finally transplantable leukemia.[52] Treatment with rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), which is downstream of PTEN in the signaling pathway, restored the population of normal hematopoietic stem cells and inhibited the formation of leukemic CSCs. Rapamycin, and more stable derivatives, are currently in clinical trials for the treatment of leukemia.

As mentioned earlier, several pathways are upregulated in the CSC populations of various tumors. The Notch pathway is upregulated in both breast and brain CSCs and γ-secretase inhibitors have been used to inhibit xenograft tumor growth.[53,54] The National Cancer Institute is currently recruiting patients to participate in a phase I clinical trial utilizing a Notch pathway inhibitor for patients with advanced breast cancer. Similar trials targeting the Hedgehog pathway in colon and pancreatic CSCs are likely to occur in the near future given the findings that Hedgehog inhibition was effective against several epithelial cancers.[55]

5. Conclusions

The discovery of CSCs in several types of solid tumors over the past few years represents a major paradigm shift in the field of oncology and is likely to change our understanding of the process of tumorigenesis. The existence of cancer stem cells also has direct therapeutic implications. Most current systemic therapies have been found ineffective in the treatment of solid tumors, and this may be due, at least in part, to increased resistance of the cancer stem cells. Selective pressure provided by treatment of the tumor with chemotherapy or ionizing radiation may allow for the survival and enrichment of a resistant CSC population, with subsequent reconstitution of the primary tumor with cells that will not be responsive to further treatment cycles. It will be important to understand how cancer stem cells are different from the rest of the tumor cell population in order to develop effective targeted therapeutics to this resistant cancer cell population, with the goal of improvement in patient outcomes.

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