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Cancer Stem Cells and their Role in Metastasis

Yusuke Shiozawa¹, Biao Nie^{1,2}, Kenneth J. Pienta³, Todd M. Morgan³, and Russell S. Taichman¹

¹Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, MI, USA

² Guangdong Provincial Key Laboratory of Gastroenterology, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, China.

³Departments of Urology and Internal Medicine, University of Michigan Health, Ann Arbor, MI USA

Abstract

Cancer stem cells (CSCs), which comprise a small fraction of cancer cells, are believed to constitute the origin of most human tumors. Considerable effort has been focused on identifying CSCs in multiple tumor types and identifying genetic signatures that distinguish CSCs from normal tissue stem cells. Many studies also suggest that CSCs serve as the basis of metastases. Yet, experimental evidence that CSCs are the basis of disseminated metastases has lagged behind the conceptual construct of CSCs. Recent work, however, has demonstrated that CSCs may directly or indirectly contribute to the generation of metastasis. Moreover, CSC heterogeneity may be largely responsible for the considerable complexity and organ specificity of metastases. In this review, we discuss the role of CSCs in metastasis and their potential as therapeutic targets.

Keywords

Cancer stem cells; molecular marker; metastasis; epithelial to mesenchymal transition; circulating tumor cells; cancer therapeutic target

1. Introduction and cancer stem cells (CSCs)

The hypothesis that a small fraction of cancer cells, described as cancer stem cells (CSCs), are the basis of most tumors was first proposed in 1994. This work demonstrated that a small fraction of human leukemia cells could generate leukemia in severe combined immune deficient (SCID) mice, whereas the majority of tumor cells failed to engraft and establish disease (Lapidot *et al.*, 1994). Further studies in hematopoietic malignancies suggested that CSCs, like normal stem cells (NSCs), reside at the apices of a hierarchical cluster of mature

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Corresponding authors: Russell S. Taichman D.M.D., D.M.Sc. Department of Periodontics and Oral Medicine. 3307 University of Michigan School of Dentistry. 1011 North University Ave. Ann Arbor, MI, 48109-1078. rtaich@umich.edu Yusuke Shiozawa M.D., Ph.D. Department of Periodontics and Oral Medicine. 3307 University of Michigan School of Dentistry. 1011 North University Ave. Ann Arbor, MI, 48109-1078. shiozawa@umich.edu.

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cells, possessing the ability to self-renew and generate multiple 'mature' progeny. Subsequent critical studies identified CSC activities in numerous solid tumors, including prostate (Collins *et al.*, 2005a), colon (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007), head and neck (Prince *et al.*, 2007), melanoma (Schatten *et al.*, 2008), lung (Eramo *et al.*, 2008), liver (Yang *et al.*, 2008), breast (Al Hajj *et al.*, 2003), brain (Singh *et al.*, 2004), pancreas (Adikrisna *et al.*, 2012; Li *et al.*, 2007) (Hermann *et al.*, 2007), ovary (Curley *et al.*, 2009) and mesenchymal carcinomas (Wu *et al.*, 2007).

Yet despite the convergence on the concept of CSCs, the definition and identification of these cells in most tumor types remains elusive and whether or not CSCs exist in all human tumors remains an open question. For example, the most striking example of a tumor that may not follow a stem cell model is melanoma. In this malignancy, a highly enriched CD271(+) population may have some CSC activity as demonstrated in one study showing CD271(+) cells able to develop tumors in Rag22/2cc2/2 mice while CD271 (-) cells did not (Boiko *et al.*, 2010). However, other groups have shown that both the CD271(-) and CD271(+) cells obtained from patients with melanoma can generate tumors when implanted into non-obese diabetic (NOD)/SCID IL2R γ ^{null} mice (Quintana *et al.*, 2008; Quintana *et al.*, 2010). Given these and other data, it has been proposed that melanomas follow a stochastic model rather than a hierarchical model of local tumor growth and distant spread (Quintana *et al.*, 2008; Quintana *et al.*, 2010).

In vivo studies, while central to the definition of what a CSC is, have fueled debate as to whether *in vivo* evidence of CSC characteristics requires specialized animal hosts with increasing immune deficiencies (Kelly *et al.*, 2007; Nguyen *et al.*, 2012). Yet, defining CSC activities based upon *in vitro* methods is also complex, and many groups have defined CSC activities based upon the ability to generate spheroids (Marsden *et al.*, 2009; Suva *et al.*, 2009). Others have suggested that the ability to exclude the dye Hoechst 33342 and/or the expression of the adenosine triphosphate-binding cassette (ABC) transporter ABCG2 characterizes a CSC (An & Ongkeko, 2009; Ding *et al.*, 2010; Shien *et al.*, 2012; Zenzmaier *et al.*, 2008).

Elevated levels of aldehyde dehydrogenase (ALDH) (Storms *et al.*, 1999) have also been proposed as a method to identify CSCs; however, ALDH does not appear to be a CSC marker in all tumor types (Yu *et al.*, 2011a). Others have used a variety of cell surface markers to define CSCs from primary tumors and cell lines in a number of tumors, most notably CD133, CD44, CD24, and CD166 (Curley *et al.*, 2009; Horst *et al.*, 2009; Miki *et al.*, 2007; Patel *et al.*, 2010; Vermeulen *et al.*, 2008; Zhang *et al.*, 2012). Importantly, none of these studies has clearly defined CSCs as a single universal entity, suggesting that the CSC phenotype may vary substantially across different tumors.

There are a number of additional challenging aspects to the identification and characterization of CSCs. As mentioned previously, part of the controversy surrounding the CSC concept stems from the requirement that subpopulations of cell lines or cells from tumor samples form tumors *in vivo*. That differences in CSC activities can be traced to the model system employed should not be surprising, considering that differences in NSC activities have also been attributed to the animal hosts in which the test population is evaluated (Ito *et al.*, 2012; Lepus *et al.*, 2009; Notta *et al.*, 2010). Each mouse model is likely to have differences in the expression of growth factors, cytokines/chemokines, adhesion molecules, and other environmental components which may be required for the successful establishment of xenotransplanted NSCs and CSCs (Kelly *et al.*, 2007). Yet because of the diversity of responses in different model systems, defining universal characteristics of CSCs has proven illusive. In fact, attempts to define CSCs as a single

entity or as a single developmental state may be a major source of the difficulty (Kelly *et al.*, 2007; McCulloch & Till, 2005; Nguyen *et al.*, 2012; Trosko, 2009).

In addition to the conceptual framework of CSCs, the origin of CSCs—and even the terminology pertaining to CSCs—remains controversial. There is evidence that some CSCs may be derived from NSCs. These may result from genomic mutations in NSCs which ultimately generate neoplastic growth (Massard *et al.*, 2006). Other examples have demonstrated that when mesenchymal stem cells (MSCs) are inoculated into a breast cancer model, more metastases are observed, suggesting that transplantation of NSCs may promote tumorigenesis and metastasis and possibly the generation of CSCs (Karnoub *et al.*, 2007). Likewise, neuronal stem/progenitor cells may participate in the generation of human glioma and provide the first example of a donor-derived brain tumor, suggesting that unregulated NSCs may produce tumors (Amariglio *et al.*, 2009). Yet, it may not be true that CSCs are derived from NSCs that have undergone a multiple-hit oncogenesis (Nguyen *et al.*, 2012). In fact, emerging data suggest that CSCs may be derived from progenitor, more ‘mature’ tissue cells (Hermann *et al.*, 2007; Kelly *et al.*, 2007; Li *et al.*, 2010; McCulloch & Till, 2005; Nguyen *et al.*, 2012; Trosko, 2009) or even cell fusions (Lu & Kang, 2009) (Figure 1). As a result, investigators have advocated for the term “tumor-initiating cells” to be utilized instead of CSCs, in order to avoid the implication that these cells are necessarily derived from NSCs (Neuzil *et al.*, 2007).

One of the key underlying hypotheses of the CSC model proposes that CSCs are the basis of metastases. Yet, experimental evidence that CSCs produce disseminated metastases has lagged behind the conceptual construct of CSCs. In this review, we will discuss evidence supporting the role of CSCs in metastasis, their characteristics in the metastatic niche, and their potential as therapeutic targets in order to prevent the development of clinically overt metastatic disease.

2. Molecular features of CSCs that are linked to metastasis

Genetic signatures in CSCs are thought to predict tumor recurrence and metastases, providing some support for the concept that CSCs may be metastatic precursors (Fig1). For example, expression of the CSC marker CD133 in glioblastoma and lung adenocarcinoma is correlated with both the proliferation marker Ki67 and poorer clinical outcomes (Pallini *et al.*, 2008; Woo *et al.*, 2010). CD133 antigen expression has also been shown to correlate with patient survival in high-grade oligodendroglial tumors (Beier *et al.*, 2008), rectal cancer (Wang *et al.*, 2009), gastric adenocarcinoma (Zhao *et al.*, 2010), and non-small cell lung cancer (Shien *et al.*, 2012). Additionally, in patients with colorectal carcinoma, the combination of CD133, CD44, and CD166 can successfully identify patients at low-, intermediate-, and high-risk of recurrence and metastasis (Horst *et al.*, 2009). Likewise, methylation of Wnt-target-gene promoters are also strong predictors for recurrence in colorectal cancer, suggesting that CSC gene signatures, rather than reflecting CSC numbers, may reflect differentiation status of the malignant tissue and the risk for dissemination (de Sousa *et al.*, 2011).

One of the key steps in the metastatic cascade is the migration of tumor cells away from the primary tumor, and CSCs likely facilitate this migration. The ability of cells to migrate from one location to another is a critical normal process in development, and tumor cells appear to capitalize on the underlying physiologic mechanisms. Most adult tissues maintain some aspect of this migratory capacity through the ability to generate an epithelial to mesenchymal transition (EMT)-like process during wound healing, tissue regeneration and organ fibrosis. It has been hypothesized that CSCs may also activate their migration through the process of EMT (Figure 1). In fact, the mesenchymal phenotype marker Zeb1 may

facilitate the acquisition of stem cell-like properties (Peter, 2010). Similarly, untransformed immortalized human mammary epithelial cells are capable of undergoing an EMT-like state by expressing FoxC2, Zeb factors, and N-cadherin, all of which have been linked to a CSC state (Morel *et al.*, 2008). Likewise, by over-expressing Ras or Her2/neu, a stem-like subpopulation of CD44^{high}/CD24^{low} cells with an enhanced EMT phenotype has been identified (Radisky & LaBarge, 2008). It is possible that the EMT process is a critical aspect of CSCs and may not be entirely dependent on outside signals as data suggest that tumor stroma which promote metastasis, may not induce a EMT in tumor cells (Karnoub *et al.*, 2007). This suggests that the EMT process occurs independently in carcinoma cells. On the other hand, the acquisition of an EMT may not be solely a cell-autonomous feature of cancer cells but may be regulated by signals from the microenvironment or niche. Along these lines, tumor- or cancer- associated fibroblasts have been shown to enhance the metastatic potential of tumors by promoting migration and extravasation through an EMT process as well as the establishment of a CSC-like state (Aktas *et al.*, 2009; Armstrong *et al.*, 2011; Gregory *et al.*, 2008; Kalikin *et al.*, 2003; Martin *et al.*, 2010; van der, 2011).

Other factors are also thought to participate in the establishment of a metastatic phenotype by CSCs. During the final stages of cell division, each daughter cell must lose contact with each other to generate independent progeny. The final step in this process occurs within a tube or bridge that connecting the daughter cells. A protein structure called midbody is essential for the process of separating the two cells. Cancer cells accumulate midbody derivatives which enhance the tumorigenicity of cancer cells (Kuo *et al.*, 2011). Moreover, several microRNAs (miRNAs) may also participate in the activation of CSC-like activities, where loss of miR-124, which regulates proliferation, has been shown to promote glioma formation (Cheung & Miller, 2001). KLF4, which is used extensively to 'reprogram' somatic cells into stem-like states, has potent oncogenic activities in mammary tumorigenesis and is likely involved in maintaining stem cell-like features in tumor cells (Yu *et al.*, 2011b). Expression of phosphatase and tensin homolog (PTEN) is also thought to be critical for the establishment of a stem-like state (Zhou *et al.*, 2007), and its loss correlates with prostate cancer metastasis and progression. Recent studies linking the RAS/MAPK pathway with PTEN deletion demonstrated the development of an EMT state in the prostates of experimental animals with 100% penetrance of metastasis (Mulholland *et al.*, 2012). Here, a novel stem/progenitor subpopulation of EpCAM^{low}/CD24^{low} cells with mesenchymal characteristics was found to regenerate the primary lesions upon orthotopic transplantation and to be highly metastatic (Mulholland *et al.*, 2012).

3. Circulating Tumor Cells (CTCs) and CSCs

A number of studies have linked circulating tumor cells (CTCs) to tumor progression in a variety of solid tumors, and CTC enumeration has begun to be utilized as a prognostic tool in patients with metastatic breast (Cristofanilli *et al.*, 2004), colon (Cohen *et al.*, 2008) and prostate cancer (Danila *et al.*, 2007). However, whether CTCs are simply associated with disease worsening or whether they directly contribute to metastatic progression remains to be determined. Potentially, a fraction of CTCs have CSC activity, and it is hypothesized that CSCs in a primary tumor which enter the circulation become circulating CSCs and remain so until they lodge or home to a target organ. If true, then stem-like CTCs may be a critical subset of CTCs with the capacity to form distant metastases. Recently, CTCs isolated from patients with melanomas have been found to generate metastases in xenotransplantation models (Ma *et al.*, 2010). Further characterization of these cells may offer critical insight into the mechanisms underlying tumor spread and the contribution of CSCs.

For example, if indeed the spread of CSCs leads to metastasis then it would be expected that some CTCs would express stem cell markers (Aktas *et al.*, 2009; Kasimir-Bauer *et al.*,

2012). So far, defining the CSCs in a population of CTCs has proven extremely challenging given current limitations in the capture of CTCs (Monteiro & Fodde, 2010). Nevertheless, a recent study demonstrated that 66.7% of breast cancer patients demonstrate a putative stem cell/progenitor phenotype (35.2% CD44 (+)/CD24 (-/low) or 17.7% ALDH1 (high)/CD24 (-/low)) in CTCs (Theodoropoulos *et al.*, 2010). A second line of evidence has shown that CTCs obtained from patients with Dukes' B and C colon cancers express CD133, carcinoembryonic antigen (CEA) and cytokeratin. Prognosis among these patients is significantly poorer due to metastasis than those individuals who were found not to express these markers in their CTCs (Hou *et al.*, 2012; Pantel & Alix-Panabieres, 2007).

4. CSCs and the metastatic niche

Similar to NSCs, CSCs are thought to reside in a relative stable microenvironment, or niche, in order to retain an undifferentiated state and give rise to more differentiated progenitor cells (Calabrese *et al.*, 2007). From the perspective of metastasis, it has been reported that VEGFR1 expressing marrow cells participate in the process by establishing "pre-metastatic niches" (Kaplan *et al.*, 2005b).

Observations into the earliest steps of metastasis preceding the influx of tumor cells into distant organs form the basis for the pre-metastatic niche hypothesis. Flow cytometry and immunofluorescence studies coupled with cells labeled with green fluorescent protein (GFP), demonstrate that cell clusters of bone marrow derived cells in the parenchyma of metastatic organs precede the arrival of fluorescently labeled tumor cells. It has been gleaned that the bone marrow-derived cells express VEGFR1 and several other hematopoietic markers including CD34, CD11b, c-kit, and Sca-1, defining the cells as early hematopoietic progenitors cells engaged with the parenchyma of the distant organ (Kaplan *et al.*, 2005b; Kaplan *et al.*, 2006; Kaplan *et al.*, 2007; Wels *et al.*, 2008). The progenitors also express the VLA-4 ($\alpha_4\beta_1$), thus priming them to sites rich in fibronectin to establish the clusters in preparation for metastasis (Kaplan *et al.*, 2005a; Kaplan *et al.*, 2005b). It is thought that these cells may become educated within tumors to ultimately hunt out and lay the foundations for distant metastasis. Alternatively, the cells may become activated locally or in the circulation due to chemokines secreted by tumor. One molecule which has been implicated in this regard is placental growth factor (PIGF), a ligand for VEGFR1. PIGF secretion may activate resident organ fibroblasts to synthesize fibronectin, which facilitates the binding of VLA-4 expressing hematopoietic progenitor cells (Peinado *et al.*, 2012). Exosomes are another class of tumor derived products which may prime metastases. Exosomes from highly metastatic melanomas have been demonstrated to increase the establishment of metastases by 'educating' marrow hematopoietic progenitors to express the receptor tyrosine kinase MET (Peinado *et al.*, 2012). Consequently, activation of MET by hepatocyte growth factor (HGF) renders the marrow cells more migratory and capable of establishing pre-metastatic niches (Peinado *et al.*, 2012). Similar results have been observed following the release of microvesicles from human renal CSCs which stimulate angiogenesis and the formation of pre-metastatic niches in lungs (Atala, 2012; Grange *et al.*, 2011). In addition to directly activating the bone marrow hematopoietic components, melanoma-derived exosomes also induce leakiness of the vasculature are pre-metastatic sites setting in motion the arrival of marrow progenitors (Peinado *et al.*, 2012).

Yet, the work which most directly implicates CSCs as metastatic seeds in conjunction with a metastatic niche is work on tenascin C (TNC) expression in CSCs. It appears that the ability to express TNC is a central feature of the ability of CSCs to establish metastasis in the lungs of experimental animals (Oskarsson *et al.*, 2011). The cancer-derived TNC enhances the expression of musashi homolog 1 (MSI1) and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) (Oskarsson *et al.*, 2011), both of which have stem cell

modulating activities. MSI1 regulates NOTCH signaling, and LGR5 is a target of the WNT pathway.

Each of these pathways have been shown to be critical for the metastasis and in stem cell biology (de Sousa *et al.*, 2011; Duncan *et al.*, 2005; Malanchi *et al.*, 2012; Reya & Clevers, 2005). It is however important to note that TNC did not alter the antigen profile of breast CSCs, suggesting that CSC activities were established prior to metastasis, and TNC may be critical for maintenance of stem cell functions (Oskarsson *et al.*, 2011).

5. CSCs as niche parasites

Stephen Paget's observation that tumor "seeds" seek out "fertile soil" represents a well-known paradigm for why certain cancers localize to preferred sites with a high propensity (Paget, 1889). The bone marrow microenvironment has been well established as a regulatory site for hematopoietic function. In the marrow, hematopoietic stem cells (HSCs) are believed to localize to the specific microenvironment, or the niches, to engage in a quiescent state to preserve their self-renewal capacity, or divide and differentiate to populate their corresponding lineages (Haylock & Nilsson, 2005; Taichman, 2005; Zhu & Emerson, 2004). Two such niches have been described: a vascular niche and an endosteal niche. The majority of HSCs as detected by immunohistochemistry appear to be in the vascular niche, richly compromised of endothelial cells. In the vascular niche, it is thought that HSCs are engaged in active surveillance of hematopoietic need, facilitating a rapid response to hematopoietic demands (Kopp *et al.*, 1920; Li & Li, 2006). HSCs in the endosteal niche are thought to be more quiescent or represent a reserve population (Calvi *et al.*, 2003; Nishikawa *et al.*, 2008; Patt *et al.*, 1980; Zhang *et al.*, 2003). The principal regulatory cells of the endosteal HSC niche are thought to be osteoblasts, adipocytes, mesenchymal stem cells and CXCL12-abundant reticular or CAR cells (Shiozawa *et al.*, 2008b; Sugiyama *et al.*, 2006; Taichman *et al.*, 1996). Osteoblasts appear to be major regulators of endosteal niche function, as they have been shown to express a number of cytokines, growth factors, and adhesion molecules critical to HSC regulation.

Parallel to the concept of the HSC niche, growing evidence have suggested that disseminated tumor cell (DTCs) also localize to and reside in the bone marrow niche (Shiozawa *et al.*, 2008b). In fact, prostate cancer DTCs use similar mechanisms as HSCs in order to gain access to the niche. This includes expression of the stromal derived factor-1 (SDF-1 or CXCL12) pathway as well as adhesion molecules utilized by HSCs, facilitating successful survival of these cells within the marrow microenvironment (Shiozawa *et al.*, 2008a; Sun *et al.*, 2005; Sun *et al.*, 2008; Taichman RS *et al.*, 2002) (Figure 2). Further work from our group has recently demonstrated that DTCs target and displace HSCs out of their niche, and establish metastatic foci within the niche space (Shiozawa *et al.*, 2011a) (Figure 2). This was demonstrated using an *in vivo* micrometastasis model in which DTCs were introduced into immunodeficient mice and permitted to take up residence in the bone marrow (Havens *et al.*, 2008). Thereafter, the mice were given bone marrow transplantations to determine the ability of HSCs to engraft into their marrow niches (Shiozawa *et al.*, 2011a). Significantly fewer HSCs engrafted into the mice harboring tumor cells in their marrow, suggesting that the tumor cells were occupying these niches and preventing HSC localization and engraftment. Importantly, a competitive engraftment was performed to determine if DTCs directly out-compete HSCs for niche space (Shiozawa *et al.*, 2011a). In this experiment, existing niches were cleared by lethally irradiating mice. HSCs were given to all mice via transplant to rescue the mice from the lethal irradiation, and in some groups (prostate cancer) PCa cells were also implanted. Mice with tumors cells showed markedly less engraftment and thus decreased survival, indicating that PCa cells out-competed HSCs

for their niches (Shiozawa *et al.*, 2011a). These findings have led to the concept of DTCs as “parasites” of the HSC niche (Shiozawa *et al.*, 2011b; Shiozawa *et al.*, 2011c).

One of the central questions that arises from these studies is whether stem-like characteristics of some of these DTCs explains the phenomenon of these cells targeting the bone marrow niche. Evidence again from our group has shown that when DTCs engage the niche they become quiescent. We demonstrate that the binding of PCa to annexin II induces the expression of the growth arrest specific-6 (**GAS6**) receptors AXL, Sky, and Mer which in the hematopoietic system induce dormancy (Dormady *et al.*, 2000). In addition, GAS6 produced by osteoblasts prevents PCa proliferation and protects PCa from chemotherapy-induced apoptosis. Our results suggest that the activation of GAS6 receptors on PCa cells in the bone marrow environment may play a critical role as a molecular switch establishing metastatic tumor cell dormancy.

Additionally, this hypothesis may elucidate critical aspects of tumor tropism, a still unexplained phenomenon observed in all malignancies. Using *in vivo* murine models of human PCa cell metastasis to bone, it has been noted that the majority of skeletal metastasis are located in either the spine or the hindlimbs ((Jung *et al.*, 2012b). Much less prevalent are lesions found in the bones of the forelimb, and normalizing tumor prevalence data to account for the number of PCa cells arriving following intravascular injection, marrow cellularity and number of hematopoietic stem cell (HSC) niches does not explain this difference. However, an analysis of differential gene and protein levels lead us to identify GAS6 expression as significantly greater in the forelimb compared to the hindlimb bone marrow (Jung *et al.*, 2012a). When murine RM1 prostate cancer cells were implanted into *s.c.* spaces in immune competent animals, tumor growth in the *GAS6*^{-/-} animals was greater than in *GAS6*^{+/+} animals. In an osseous environment, the human PC3 cell line grew significantly better in vertebral body transplants (vossicles) derived from *GAS6*^{-/-} animals than in vossicles derived from GAS6 wild-type animals. These data suggest that the differences in tumor prevalence following intravascular inoculation may be a useful model to study the molecular basis of tumor dormancy (Jung *et al.*, 2012a). Combined, our work on the niche suggests, that the ability to establish dormancy may be a central feature of CSCs. If true, then perhaps the ability of the niche to establish dormancy could be used as a way to define CSCs.

The CSC hypothesis suggests that most carcinomas are organized in a hierarchical pattern in which CSCs generate a population of non-renewing cells that form the bulk of the tumor. An alternative hypothesis proposes that non-stem cell-like CTCs are induced to generate CSC-like cells in the new microenvironment. This is supported by several reports suggesting that the niche can induce non-tumorigenic cells to assume a CSC-like phenotype (de Sousa E Melo *et al.*, 2011; de Sousa *et al.*, 2011; Vermeulen *et al.*, 2008). For example, HGF-producing myofibroblasts are able to alter non-tumorigenic cells into tumor propagating cells by reactivating the Wnt pathway. These “dedifferentiated” cancer cells display all the characteristics of CSCs, including expression of stem cell-associated genes (e.g. LGR5), and have high tumorigenic potential (de Sousa E Melo *et al.*, 2011; de Sousa *et al.*, 2011; Vermeulen *et al.*, 2008). These and other data suggest that NSC and CSC-like cells can arise *de novo* as well as from more differentiated cell types following expression of transcription factors such as Oct4, Sox2, c-Myc, Nanog and KLF4 (Ben Porath *et al.*, 2008; Chiou *et al.*, 2010; Collins *et al.*, 2005b; King *et al.*, 2011; Li *et al.*, 2011; Miyoshi *et al.*, 2010; Takahashi & Yamanaka, 2006). Thus, the hierarchical model of stem cells should take into account bidirectional conversions between both stem and non-stem cell compartments (Reviewed in Li (Li & Laterra, 2012)).

6. CSCs and metastasis

While the hypothesis that CSCs directly contribute to the initiation of metastases has gained increasing acceptance, most of the supporting evidence comes from studies demonstrating associations between CSC markers and cellular phenotype. Examples include reports demonstrating that CD44 expression by breast (Al Hajj *et al.*, 2003) and pancreatic (Hermann *et al.*, 2007) tumor cells have enhanced metastatic capabilities. Similarly, expression of the chemokine receptor CXCR4 by breast (Muller *et al.*, 2001) and prostate cancers (Taichman RS *et al.*, 2002) have been shown to impart critical stem cell functions on these cells (Kucia *et al.*, 2005; Lee *et al.*, 2012; Rhim *et al.*, 2011). Importantly, however, the expression of markers such as CD44 or CD24^{-/low} by tumor cells alone does not prove that these cells can generate metastases—or even that they are necessarily CSCs. Similarly, identification of CSCs within metastatic lesions or in circulating or disseminated tumor cell populations (Balic *et al.*, 2006) does not necessarily mean these cells are capable of establishing disseminated lesions. There remains no direct experimental evidence that CSCs shed from a primary lesion can generate tumors at distant sites. The corollary also remains unproven—that targeted blockage of CSCs in the blood or lymphatic systems can prevent metastases from forming. Demonstrating these proposed characteristics of CSCs has, to date, proven extremely difficult due to the insensitivity of current metastatic. Additionally, it is possible that several CSC phenotypes may coexist within tumors, each with unique chemosensitivities and metastatic potential, and therefore a full understanding of CSC characteristics within a given tumor remains elusive (Kang *et al.*, 2003; Minn *et al.*, 2005).

Some of the most direct evidence that CSCs establish metastases comes from the demonstration that breast cancer CSCs isolated based upon the putative stem cell markers CD44⁺ and CD24^{-/low}, are able to generate primary tumors in an orthotopic site and subsequently produce lung metastases (Liu *et al.*, 2010). Likewise, orthotopic implantation of pancreatic cancer cells expressing a CSC phenotype generate distant liver metastases (Hermann *et al.*, 2007). Interestingly, different populations of CSCs may be responsible for primary vs. secondary tumor sites, implicating CSC heterogeneity as a critical component of this model. Recent work by Dieter *et al.* has demonstrated this heterogeneity within the CSC compartments, reporting at least three phenotypically distinct CSCs in a human colon cancer animal model (Dieter *et al.*, 2011). To track the contribution of tumor-initiating cell clones, the group generated tumorigenic cells from cancer specimens, marked them with lentiviral vectors, and then sequenced the integration sites in serially transplanted tumors. A population of CSCs was identified as tumor transient amplifying cells (T-TACs) which had limited self-renewal capacity but did form tumors in primary transplants. A second population of CSCs exhibiting extensive self-renewing long-term tumor initiating cells (LT-TICs) were able to generate tumors in serial xenotransplants. A third population described as rare delayed contributing TICs (DC-TICs) were exclusively active in secondary or tertiary mice. Interestingly, the marrow could serve as an major source of LT-TICs, however metastasis formation was predominantly driven by self-renewing LT-TICs (Dieter *et al.*, 2011). This study therefore provides an exquisite evaluation of the clonal contribution to metastasis and tumor formation from individual CSC clones. The study also confirms the heterogeneity among CSCs in colon cancers and provides perhaps the most direct evidence to date that CSCs may contribute to metastasis (Dieter *et al.*, 2011).

7. Therapeutic targeting of metastatic CSCs

The CSC hypothesis has fundamental and profound implications for cancer therapies, as CSCs are believed to be more resistant to chemotherapy than more differentiated cancer cells. To directly target CSCs, it will be critical to identify novel and unique pathways that are active in this subset of tumor cells. One example of this is the use of an activating

monoclonal antibody directed at CD44, which resulted in significant reductions in the levels of acute myeloid leukemia in one *in vivo* study (Jin *et al.*, 2006). Importantly, CD44 is a regulator of several miRNAs known to maintain CSCs. In fact, when CD44 expression in PCa cells is down-regulated, miR-34a levels increase leading to reduced tumor regeneration and metastasis in xenografts (Liu *et al.*, 2011). Similarly targeting of salinomycin in breast cancer inhibits tumor growth, induces epithelial differentiation of tumor cells, and down-regulates CSC genes in tumor cells (Gupta *et al.*, 2009). However, whether these strategies will work in other tumor types remains to be determined, and how they can be used to limit or irradiate metastasis is unknown.

The ability of CSCs to expand in a rapid fashion after an exaggerated period of quiescence is thought to represent one of the most daunting challenges for the development of therapeutic interventions in cancer, particularly in the metastatic setting. Combination therapies therefore are likely to be required to target both proliferating cells and CSCs and will likely be integral to this approach. Recently, two interesting studies provide promising evidence for the combination of CSC targeting therapy and conventional chemotherapy. The first study demonstrated that a neutralizing antibody against a membrane-associated NOTCH ligand inhibits tumor growth and CSC self-renewal in human colon cancer implanted mice (Hoey *et al.*, 2009). Likewise, the second study revealed that the administration of an anti-CD123 antibody prevents the engraftment of serially transplanted acute myeloid leukemia into the animals, suggesting this antibody impedes the stem-like characteristics of leukemia cells (Jin *et al.*, 2009). In both cases, the inhibitory abilities of the antibodies were enhanced when animals were treated in combination with chemotherapy (Hoey *et al.*, 2009; Jin *et al.*, 2009) (Figure 3).

Another proposed therapeutic approach is to stimulate differentiation of CSCs such that they lose their capacity for self-renewal and resistance to chemotherapeutic agents (Nguyen *et al.*, 2012) (Figure 3). This is particularly critical where CSCs are widely distributed at low density, making conventional interventions challenging. Thus far, the most well developed therapeutic agent is vitamin A and its analogues (retinoid acid) for the treatment of acute promyelocytic leukemia (APL). These agents enhance tumor differentiation and reverse malignant progression by modulation of signal transduction networks regulated by nuclear retinoid receptors. In patients with APL, a 90% remission rate and a 70% cure rate with all-trans-retinoic acid therapy followed by chemotherapy has been observed (Burnett *et al.*, 2010). *In vitro*, retinoid acid can also induce differentiation in embryonic cells, teratocarcinomas and melanomas (Rohwedel *et al.*, 1999).

As stem cells are often dependent on bone morphogenetic protein (BMP) signaling, a number of therapeutic strategies are being sought to target this pathway (Joseph *et al.*, 2012; Zhang *et al.*, 2003; Zhu & Emerson, 2004). Piccirillo *et al.* reported that the BMP-BMPR signaling system - which controls the activity of normal brain stem cells - may also act as a key inhibitory regulator of tumor-initiating, stem-like cells from glioblastoma by a reduction in proliferation and increased expression of markers of neural differentiation (Piccirillo *et al.*, 2006). The reduction in the size of the CD133(+) population and the growth kinetics of the glioblastoma cells suggest that targeted BMP-pathway therapeutics are worth pursuing (Massard *et al.*, 2006; Piccirillo *et al.*, 2006) (Figure 3). In addition, the histone deacetylase inhibitor valproic acid, commonly used to treat epilepsy, has recently been found to have anti-cancer activity that may target CSCs (Blaheta *et al.*, 2005). Valproic acid induces the terminal differentiation of cancer cells by increasing the DNA binding of activating protein-1 transcription factor, decreasing protein kinase C (PKC) activity, inhibiting the WNT signaling pathways, and activating the peroxisome proliferator-activated receptors, in addition to blocking histone deacetylase (Blaheta *et al.*, 2005). Although further study is

clearly needed, these classes of agents may be useful for reducing CSC activities both in the local setting and in the context of disseminated CSCs.

However, the challenges of targeting disseminated CSCs may be even more pronounced, as the distant microenvironment may help protect these cells from therapeutic insults (Hovinga *et al.*, 2010). In particular, the vasculature likely plays an important role in forming stem and progenitor cell niches and has been suggested to regulate many tumor microenvironments (Bautch, 2011). Therefore, damaging the CSC niche environment may impact the survival and tumor-initiating properties of CSCs (Folkens *et al.*, 2007). The impact of angiogenesis inhibitors such as bevacizumab, thalidomide, sorafenib, sunitinib, pazopanib may be in part related to their effects on the vascular niche and disruption of the CSC microenvironment (Tonini *et al.*, 2003).

Other niche regulating targets are also emerging. Periostin is a major component of the extracellular matrix, expressed by fibroblasts in the normal tissue and in the stroma of many tumors. Recent work has shown that the ability of infiltrating tumor cells to induce stromal production of periostin at secondary target organs impacts colonization and stem cell maintenance. Importantly, blocking periostin function may prevent metastasis by preventing the recruitment and activation of the WNT signaling in CSCs (Malanchi *et al.*, 2012).

A second and interesting aspect of therapies designed to target the microenvironment is the possibilities that CSCs may be released from the microenvironment or niche (Figure 3). If CSCs utilize the niche to maintain their stemness, become dormant and therefore acquire the chemoresistance, this therapeutic strategy could enhance the susceptibility of CSCs to chemotherapy. Indeed, when acute myeloid leukemia cells (Nervi *et al.*, 2009) and multiple myeloma cells (Azab *et al.*, 2009) are treated with a C-X-C chemokine receptor type 4 (CXCR4) inhibitor, known to prevent the lodging of cancer cells into select microenvironments prior to chemotherapy, the chemosensitivity of these cells was dramatically enhanced. In part, release from the protection of the microenvironmental niche could sensitize CSCs to chemotherapeutics. Additionally, it is possible that disruption of CXCL12/CXCR4 signaling may activate CSC cycling which in turn could sensitize CSCs to agents targeting proliferating cells. For targeting solitary and potentially dormant DTCs in patients with no clinically apparent distant disease, this strategy has distinct advantages as an adjuvant therapy to prevent metastasis.

8. Future prospects

The conceptual framework of CSCs is likely to be integral to furthering our understanding of the origin of tumors and identifying targets for curative therapies. However, not only is targeting CSCs not likely to be easy, but targeting only CSCs may not be enough to prevent metastasis or relapse due to metastasis. Continued development of combination therapies with multiple targets (e.g. targeting CSCs, combination of chemotherapy, differentiation therapy, targeting microenvironment) will remain essential in order to improve outcomes of individuals with solid tumors. Nevertheless, site-specific dissemination and activation of CSCs provides a mechanistic explanation for the evolution of many metastases, as well as for metastatic inefficiency (Paget, 1889). In fact, given the growth limitations known to restrict the uncontrolled growth of tumor cells, most occult metastases may require months and even years before they can be identified and treated (Kendal, 2010). As most neoplasms are identified after they have reached a critical mass, the ability to block the reactivation of dormant CSCs at distant sites of metastases is a critical area of research. Therapies based on this concept are currently not at hand and essential aspects of the current dormancy model must be validated. However these data support the need for further research directed at

preventing metastases from developing by targeting CSCs within primary and disseminated sites and to slow or limit CSC growth when necessary.

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Abbreviations

Abbreviation Definition

ABC	Adenosine triphosphate-binding cassette
ALDH	Aldehyde dehydrogenase
APL	Acute promyelocytic leukemia
BMP	Bone morphogenic protein
CEA	Carcinoembryonic antigen
CSC	Cancer Stem cell
CTCs	Circulating tumor cells
CXCL12	Stromal derived factor-1 (SDF-1 or CXCL12)
DTC	Disseminated tumor cells
EMT	Epithelial to mesenchymal transition
GAS6	Growth arrest specific-6
HSC	Hematopoietic stem cell
miRNA	microRNAs
(NOD)/SCID IL2Rγ^{null} mice	Non-obese diabetic SCID mice lacking the IL-2 receptor-mice-gamma
NSC	Normal stem cells
PCa	Prostate cancer
PKC	Protein kinase C
PTEN	Phosphatase and tensin homolog
SCID	Severe combined immune deficient mice
TNC	Tenacin C

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Fig 1. Multiple hypotheses examining the origin of CSCs and metastases

- A. Only the CSCs in primary tumors can home and metastasize to distant tissues directly.
- B. CSCs are generated in primary tumors that ultimately undergo an EMT to generate CTCs. The CTCs home to distant tissues and undergo mesenchymal to epithelial transition establishing the disseminated CSCs.
- C. In some specific tumor types, all the cancer cells including CSCs demonstrate no significant differences in the ability to generate tumors or establish metastases distant sites in animal models.
- D. Non-stem-like cancer cells shed as CTCs from primary tumors circulate and home to distant tissues. Once in the distant site, the cancer cells became CSCs through de-differentiation.

Fig 2. Competition and engagement of the HSC niche and its role in CSC maintenance and dormancy

A. CSCs compete with HSCs for engagement of the osteoblastic HSC niche which regulates quiescence and proliferation of HSCs. HSCs which are displaced from the niche are found in the peripheral circulation.

B. PCa cells which enter the niche as either CSCs or more mature cells are "dedifferentiated" into CSCs and or maintained as stem cells.

C. Once in the HSC niche, CSCs may undergo either growth arrest or exit the cell cycle in G_0 and become dormant

D. After asymmetric cell division, progeny of CSCs may either return to the niche as a CSCs or may terminally differentiate following additional proliferative activities which result in the establishment of metastatic bone lesions.

Fig 3. Potential therapeutic strategies to specifically target metastatic CSCs

E. CSCs are eradicated with compounds that selectively target the CSC phenotype.

F. CSCs spontaneously undergo apoptosis when the nutritional support from the surrounding vasculature is withdrawn.

G. CSCs can be terminally differentiated to avoid proliferation and dedifferentiation.

H. Once CSCs reside within the niche, they are thought to become dormant. If CSCs are displaced from the niche, they are activated and therefore become susceptible to cytotoxic treatment.