The Difficulty of Targeting Cancer Stem Cell Niches

Mark A. LaBarge

Abstract

Normal stem cell niches typically are identified by their distinctive anatomical features and by association with tissue-specific stem cells. Identifying cancer stem cell (CSC) niches presents a special problem because there are few if any common anatomical features among tumors, and the physical phenotypes that reportedly describe the CSCs as entities may be subject to the host's microenvironment, sex, and tumor stage. Irrespective of a niche's location, the occupant's phenotype, or the precise molecular composition, all niches must do basically the same thing: maintain the activities in a stem cell that define it as such. Therefore, a potentially successful strategy, both for elaborating a molecular and cellular portrait of a CSC niche, and for therapeutically targeting them, is to identify components in the tumor microenvironment that are required for maintaining the functions of self-renewal, differentiation, and quiescence in the face of cytotoxic therapeutic regimens. Clin Cancer Res; 16(12); 3121–9. ©2010 AACR.

The cancer stem cell (CSC) hypothesis offers attractive explanations for generation of heterogeneity within tumors, metastatic dissemination, and resistance to therapy. The underlying logic is modeled on normal developmental hierarchies that are delineated for a number of adult tissues. Pools of undifferentiated stem cells give rise to less potent progenitors, which produce the most specialized cells of a given tissue. Only stem cells are thought capable of regenerating entire tissues in perpetuity. Analogously, only CSCs are thought capable of self-renewal, of initiating tumors at primary and distant locations, and of giving rise to more differentiated daughters that are incapable of reestablishing the tumor. Normal stem cell activity is maintained in niches; therefore, employing the same logic used for developmental hierarchies, niches that maintain CSCs, should also exist (see refs. 1–3 for additional reviews).

Niches are specialized microenvironments located within each tissue, wherein stem cells reside (Fig. 1; reviewed in refs. 4, 5). Microenvironments are defined as the sum total of cell-cell, -ECM, and -soluble factor interactions, and the physical states and geometric constraints that a cell may experience. Niche microenvironments can exert tremendous control over stem cell range of function. It was shown that progenitors both in skin and skeletal muscle could adopt residency in vacated stem cell niches, where they reacquired stem cell traits (6–8). Impressively, testis and neural stem cells from male mice were shown to give rise to lactating mammary glands when transplanted into the mammary fat pad (9, 10). Indeed, embryonic and adult stem and progenitor cell fate decisions are quantifiably flexible in response to combinatorial microenvironments (11–13). The ability of the niche to determine the functional spectrum of stem cell activities led us to hypothesize that stem cell niche microenvironments beget stem cell functions (14). Due to their role in maintaining stem cell activity, disrupting CSC-niche interactions may be crucial for overcoming barriers to therapeutic resistance.

At least two possibilities exist for generating CSC niches: either they are manufactured as nascent domains by tumor cells, or CSCs usurp existing tissue-specific stem cell niches. Localization of nascent CSC niches is complicated because there are no common anatomical features among tumors (Fig. 2), and consensus is continually shifting about the identity of CSCs. On the other hand, usurped niches are most likely to be observed in early stages of cancer progression when there is some semblance of the original tissue's structure (e.g., ductal hyperplasias and possibly ductal carcinomas in situ in breast).

In either scenario, the CSC niches and the CSC entities are linked like an amniotic sack and a fetus; one cannot exist without the other. Evidence suggests that the biochemical identity of CSCs is dependent on the host strain, sex, and tumor stage. Accordingly, the composition of CSC niches also will be dependent on host factors. Therefore, successful therapeutic strategies will target the functions that all CSC niches have in common:

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to mediate self-renewal and to maintain an undifferentiated state and CSC activity even in the presence of cytotoxic agents.

**Have the Residents of CSC Niches Been Identified?**

Unambiguous localization of the CSC niche will require characterization of the CSCs themselves. The basic algorithm used to identify and enrich for CSC activity is to first prepare malignant growths or tumors as single cell suspensions, which are divided into fractions by differential expression of marker proteins or by dye efflux ability. CSC activity is measured by introducing the fractions into living hosts, where their ability to reform the tumor is quantified. Frequencies of CSC within a population of tumor cells are calculated by doing limiting dilution studies, and the so-called "gold standard" for identifying a stem cell, normal or cancer, is to do a single cell transplantation experiment. Since the first characterization of human leukemia initiating cells using severe combined immunodeficient (SCID) murine hosts (15), a number of reports have characterized CSCs that generate other heme malignancies (16–19) or solid tumors (20–29). Those studies either reported on murine tumor cells implanted into syngeneic inbred hosts, or human tumor cells xenografted into strains of SCID or nonobese diabetic (NOD)/SCID mice. With some notable exceptions (discussed below), the common trend suggested that CSC were rare within the total population, but were capable of self-renewal and were resistant to therapy. However, studies also have shown that the choice of host impacts the outcome of the experiment, and that host microenvironments will select for outgrowth of successful clones, suggesting that the host microenvironment will dictate the identity of the CSC.

Human melanomas xenografted for 8 weeks into SCID mice were reportedly generated from a CSC subpopulation that comprised ~1 of 1,000,000 of the tumor cells (24). A subsequent report similarly found that melanoma CSC occurred with a frequency of ~1 of 837,000 when xenografted in SCID mice for 8 weeks, but that the frequency increased to ~1 of 111,000 when measured after 32 weeks (30). Furthermore, 4,000 melanoma cells implanted into NOD/SCID mice and into NOD/SCID Il2rg<sup>-/-</sup> (NOG) mice, which are even more immunosuppressed hosts, showed that tumor formation was three fold more efficient in NOG mice. Indeed, 27% of random single melanoma cells were capable of forming tumors in the NOG strain (Table 1). The authors also showed that out of 50 distinct melanoma fractions, identified using antibodies recognizing previously identified CSC or melanocyte markers, none showed enrichment for tumor forming in NOG mice. Heterogeneity of expression with respect to one marker, CD133, was restored both from the CD133- and CD133+ fractions (30). In a different study, a similarly high frequency (~37%) of malignant engraftment was observed when single murine lymphoma cells were introduced into syngeneic hosts (31). These reports underscore that both time and host microenvironment are essential determinants of tumor formation in the context of tumor transplant assays.

As detailed in studies done from 1937 to 1958, tumor formation from even one cell can be efficient in the contexts of particular hosts, and at particular developmental stages (Table 1). In 1958, Hauschka and Levan reported the results of a 5-year long study of tumor-forming efficiency from single cells of the Krebs-2 and Erlich Ascites tumors (32). The Krebs-2 tumor strain was derived from "either the skin or the mammary gland of a random-bred male mouse" and the notes
pertaining to the exact origin of the Elrich strain were “lost in the war.” In a total of 212 infant and adult Swiss mice injected with single cells, 16% (Krebs) and 14% (Elrich) resulted in lethal tumors. Two clones derived from Krebs-2 cells were serially passed 80 times through 564 Swiss mice (clone K2C) or passed 70 times through 411 Swiss mice (clone K2D). Clone K2D generated a unimodal kill curve with a peak of activity between 5 to 7 days, whereas clone K2C generated a bimodal curve with peaks between 5 to 7 and 14 to 15 days. A second clone was isolated from K2C and it recapitulated the bimodal kill curve in Swiss mice, suggesting that one random cell was capable of making both types of tumors. Clone K2C then was used to form tumors in outbred Swiss agouti mice of three genotypes that differed by coat color (AA, Aa, and aa), and in eight different inbred strains. The bimodal kill curve was reproduced in the outbred Swiss strains and in one inbred strain (C57BL), whereas the other seven inbreds produced unimodal curves. The authors concluded the bimodal kill curves did not result from a “stem-line” origin, but that genetically heterogeneous agouti hosts selected for outgrowth of two clone types, whereas seven of the inbred strains selected only one. They proposed that the C57BL strain had some residual heterozygosity. Additionally, the authors noted that they originally preferred to use female mice due to their softer abdominal walls, which presumably pierced more easily with a glass pipette, but that tumor forming efficiency was only 2%, so the majority of their study was done in males.

Fig. 2. Loss of predictable tissue morphology in tumors presents challenges to identifying the CSC niche. A and B, stem cell niches are clearly identifiable in normal tissues. A, a cross-section of normal human mammary gland terminal duct shows the exquisite bilayered architecture of the tissue. The stem cells are nestled inside their suprabasal niche and express both keratins (K)14 and K19 (yellow, inset). They give rise to their differentiated progeny, the K14-expressing myoepithelial (green) and K19-expressing luminal cells. B, a depiction of the Drosophila germarium stem cell niche showing the stem cells (GSC, green) adjacent to the cap cells (CC, light blue). The stem cells give rise to the more differentiated progeny at their right. C and D, normal architecture is quickly lost in early stages of tumorigenesis. C, a cross section of human mammary duct, which is stricken with ductal carcinoma in situ, a noninvasive form of breast cancer that is thought to precede more invasive forms. D, shows a tumor laden germarial tip. The tumor in this case was derived from differentiation-defective bgcn mutant stem cells that outcompeted the normal stem cells for niche occupation due to upregulated E-cadherin expression, and subsequently filled the germarium with their mutant progeny. In both tumors, it is difficult or impossible to determine which of the tumor cells are the CSCs based on tissue morphology. A was adapted with permission from Villadsen et al. (71). B and D are adapted with permission from Jin et al. (1). C is reproduced from http://commons.wikimedia.org/wiki/File:DCIS.jpg.
Because tumor forming was measured as the number of lethal "takes," the variable of time could be omitted from the authors' interpretation, thus underscoring the importance of the host microenvironment in determining tumor-forming efficiency from single cells.

The examples above suggest that the methods commonly used to identify CSC activity are subject to a high degree of influence from tissue microenvironments, which differ according to time, host strain, and even sex. Therefore, any CSC phenotypes should be viewed as a product of the host strain used in the experiment. Using genetically heterogeneous outbred or wild-type strains of mice to identify CSC will generate more robust data, but may pose a problem for identifying human CSCs due to the need of immuno-compromised hosts. Outbred hosts will likely result in identifying more than one candidate CSC phenotype from the same tumor, as observed in Swiss agouti mice (32). However, those phenotypic differences may be used to advantage to identify common proteins essential to maintaining the functions that all CSC must have in common.

### Are Niches and CSCs Tumor-Stage Specific?

In addition to maintaining the primary tumors, CSCs also are hypothesized to give rise to metastases. However, reports that identified different CSC subpopulations within primary and metastatic tumors, coupled with apparently stage-specific molecular requirements for progression, suggests that metastatic CSCs may be the children of de novo niches, which evolve during progression of the primary tumor. For instance, only the CD133<sup>hi</sup> fraction from human primary colon tumors, implanted subcutaneously (23) or under the kidney capsule (22), in NOD/SCID mice grew as xenografts. Whereas, both CD133<sup>hi</sup> and CD133<sup>lo</sup> fractions of human metastatic colon cancers isolated from livers and implanted subcutaneously in NOD/SCID mice formed

#### Table 1. Tumor formation from single cells

<table>
<thead>
<tr>
<th>Source tumor</th>
<th>Implant site</th>
<th>Host characteristics (Takes/Total animals)</th>
<th>Percent lethal takes from one cell</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2 Leukemia</td>
<td>Intravenous</td>
<td>Young mice (3 of 65)</td>
<td>5</td>
<td>Furth and Kahn (73)*</td>
</tr>
<tr>
<td>AK5</td>
<td>Intravenous</td>
<td>Young mice (2 of 32)</td>
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<td>Adult mice (1 of 50)</td>
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<td>Hauschka (32, 74, 75)</td>
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<td>Infant mice (10 of 62)</td>
<td>16</td>
<td>Hauschka (32, 74, 75)</td>
</tr>
<tr>
<td>Ehrlich near-tetraploid carcinoma</td>
<td>Intraperitoneal</td>
<td>Adult mice (1 of 50)</td>
<td>2</td>
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</tr>
<tr>
<td>Ehrlich near-tetraploid carcinoma</td>
<td>Intraperitoneal</td>
<td>Infant mice (7 of 50)</td>
<td>14</td>
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<td>Infant mice (0 of 47)</td>
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<td>63HED lymphosarcoma</td>
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<td>Adult mice (0 of 24)</td>
<td>0</td>
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<td>Infant mice (3 of 44)</td>
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<td>Klein (77)</td>
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<td>Intraperitoneal</td>
<td>Adult rats (1 of 3)</td>
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<td>Hosokawa§</td>
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<td>Yoshida (80)</td>
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<td>NOD/SCID Il2rg&lt;sup&gt;−/−&lt;/sup&gt; (69 of 254)</td>
<td>27†</td>
<td>Quintana et al (30)</td>
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<td>C57BL/6-Ly5.1+ (3 of 8)</td>
<td>33†</td>
<td>Kelly et al (31)</td>
</tr>
</tbody>
</table>

NOTE: Adapted and updated from Hauschka and Levan (32).
*Study was not evaluated by ML.
†Unpublished data.
‡Increased take-percentage was obtained through use of an improved dilution medium.
§Citations missing due to discrepancies between the listed citations (written by Hauschka and Levan as Hosokawa, Gann 1952) and the library catalog.
∥These were not lethal takes, but tumor-forming studies only.
tumors with similar efficiency (33). The tumors derived from the CD133<sup>hi</sup> fraction grew relatively faster, and those from the CD133<sup>lo</sup> fraction were the only ones to harbor both CD133<sup>+</sup> and CD133<sup>-</sup> cells. Tumor cells from both subpopulations expressed human EpCam, which verified that the tumors were of epithelial origin (33). The discrepancy between the findings in primary and metastatic colon cancers may reflect experimental differences, but may also suggest that different cells are giving rise to the primary tumors and to the metastatic ones (reviewed in ref. 34).

Examination of the stage of tumor progression at which metastasis occurs, and the molecules that were involved, provided evidence for stage-specific CSCs. In a murine model of breast cancer, it was shown that the transcription factor GATA-3, a protein essential for differentiation into the luminal lineage (35, 36), was required by the tumor during the earliest hyperplastic stages of growth, but as the tumors progressed through early and late adenoma stages GATA-3 was silenced by promoter methylation (37). Metastases were observed only during the early and late adenoma stages when GATA-3 was lost, and GATA-3 expression was not detected at metastatic sites. Exogenous expression of GATA-3 in the early and late stage adenomas led to a reverted state reminiscent of early hyperplasia with no metastases, whereas knocking down GATA-3 in the early hyperplasia cells led to their death. Because there were distinctive stage-specific functional requirements for GATA-3, it seems unlikely that the same GATA-3<sup>+</sup> CSC that established the primary tumor also established the GATA-3<sup>-</sup> null metastases. A second study also showed stage-specific requirements for growth of human and mouse breast cancer cell line-derived tumors in NOD/SCID and in syngenic immuno-competent mice, respectively. Growth of the primary tumors did not require the receptor tyrosine kinase Axl, but the protein was required for spontaneous metastasis (38). Moreover, Axl was highly expressed in patient metastases, but not by their primaries, and its high expression was found to be an independent predictor of recurrence. These reports serve as examples whereby the molecules GATA-3 and Axl are not merely markers that delineate potentially stage-specific CSCs in breast cancers, but they also play clear functional roles in the genesis of primary or metastatic tumors.

That different CSCs may give rise to primary and to metastatic tumors does not contradict the existence of CSCs <em>per se</em>, but does predict that the composition of metastatic and primary CSC niches will differ. Moreover, it suggests that either the original CSC evolves throughout tumor progression, or that there should be a mechanism to generate new metastatic CSCs <em>de novo</em>.

Microenvironments can impose specific cell fate decisions (12), thus a tumorigenic cell's location inside a tissue is a crucial determinant of its activity. Reacquisition of the stem cell phenotype has been observed in normal progenitors that populated vacant niches (6–8, 39); therefore, the possible consequence of CSCs usurping preexisting niches should be considered. In model systems of solid and heme malignancies, differentiation-defective stem cells were shown to be the etiological root of the diseases (16, 21, 40, 41). In <em>Drosophila melanogaster</em>, differentiation-defective hyperplastic stem cells outcompeted normal stem cells for occupancy of their niche (Fig. 2B and D; ref. 42). Those mutant stem cells exhibited upregulation of E-cadherin, an adherens junction receptor that mediates interaction between germinal stem cells and their niche. Malcom Steinberg's differential adhesion hypothesis (43) may explain why modulation of adherens proteins resulted in the reshuffling of CSC and normal stem cells for niche occupancy. He posited that different cell types self-organize into groups according to common levels of adherens junction proteins with an ultimate goal of reducing free-energy (43, 44). In the case of the germinarium the mutant stem cells supplanted the normal by expressing relatively more E-cadherin, causing an energetically more favorable association with the niche. Germine mutations in E-cadherin are associated with diffuse gastric cancers, and cadherin expression is frequently misregulated in breast cancers (reviewed by Knudsen; ref. 45). Perhaps pathological modulation of cadherin expression can play a role in early disease stages by repositioning differentiation-defective CSCs into normal stem cell niches, where they receive self-renewal and survival signals (Fig. 2). Cadherin modulation may occur again in later tumor stages, at least in carcinomas, when tumor cells seem to lose epithelial characteristics (e.g., loss of E-cadherin) and acquire more mesenchymal ones.

Owing to genetic instability, the tumor microenvironment is a shifting landscape that may deleteriously impose stem-like phenotypes onto malignant cells. Normally, transforming growth factor β (TGF-β) is present in the mammary stroma in an inactive form, and is cleaved into its active form following exposure to radiation, wounding, and during tumorigenesis (46). Experiments with Rous Sarcoma virus-infected cells showed that active TGF-β at wound sites was necessary to realize the full malignant potential of a predisposed cell (47). Similarly, progression to frank neurofibromatosis required mutations both in the epithelial cells and in the nearby stromal cells, which disrupted TGF-β and receptor tyrosine kinase c-Kit regulation (48, 49). A CSC-like phenotype was reportedly induced in mammary epithelial cells exposed to active TGF-β, forcing them to undergo epithelial-to-mesenchymal transition (EMT; ref. 50), which included downregulation of E-cadherin. Induction of the stem cell-like program in nonmetastatic malignant mammary epithelial cells elicited invasive metastatic behavior (50). Other mechanisms that may drive evolution of cellular subtypes may include increased activity by proteases during tumorigenesis. Increased expression of matrix metalloproteinase-3, which is frequently observed in breast cancers, led to genomic instability and EMT in mammary epithelial cells (51, 52) and tumor formation in mice (53). Thus, a number of known tumor microenvironment components are sufficient to induce a CSC phenotype, and should be considered as potential CSC niche constituents.
Fig. 3. Modulating pathways involved in maintenance of the mammary stem cell state in malignant mammary epithelial cells may effectively halt disease progression. A, malignant mammary epithelial cells, HMT3522-T4-2 (T4-2), were embedded in Matrigel and grown for 72 hours in presence of the gamma secretase inhibitor (GSIxx), which blocks activation of the entire Notch pathway. Untreated cells formed disorganized and apolar colonies that do not growth-arrest, which appear characteristically rough under phase microscopy, whereas GSIxx treated cells growth-arrest after a few divisions and form smooth acini. B, GSIxx imposed the phenotypic reversion in a dose-dependent manner. C, representative confocal images of the hemispherical optical section from acini in three-dimensional culture. Basal polarity marker, integrin $\alpha_6$ (red), is expressed on all cell surfaces in colonies of the untreated malignant cells, whereas it was only expressed at the basal surfaces of the phenotypically reverted acini grown in presence of GSIxx. D, image analysis of three-dimensional cultures using NIH ImageJ was used to generate composite images, shown as probability of distribution heat maps, of integrin $\alpha_6$ distribution that occurs most frequently among the acini in a given three-dimensional culture condition. E, left side, Integrin $\alpha_6$ distribution maps are shown for cultures of GSIxx treated T4-2 cells, as well as cultures that were infected with retroviruses expressing small hairpin RNA (shRNA) to knock down each of the four Notch receptors (Notch1-4). E, right side, Bar graphs showing the impacts of each shRNA on apoptosis, via TUNEL stain, and proliferation, via Ki67 stain. Targeting different notch receptors elicited distinct phenotypes: Notch 1 and 2 shRNAs imposed normal polarity and had modest impact on apoptosis or proliferation, whereas Notch 3 and 4 shRNAs had little impact on restoring normal polarity and had a large impact on the apoptotic and proliferative phenotypes. (For more reading on the concept of phenotypic reversion see refs. 66, 72).
Can the Elusive CSC Niche Be Therapeutically Targeted?

Because the biochemical identity of CSCs may shift as a tumor evolves, targeting specific entities for removal is unlikely to be effective. Instead, disrupting the functions that all stem cells have in common may yield better results. Accordingly, strategies that target the developmentally crucial Notch, Wnt, and Hedgehog pathways are discussed in detail in this CCR Focus Series (see refs. 54–56, respectively), as well as strategies that target unchecked self-renewal (see ref. 57). For instance, activity in the Notch pathway is required for maintenance of mammary stem cell activity (12, 58), and a number of studies reported advantageous effects of down modulating the pathway in breast cancer models (59–62). When we impaired signaling in the Notch pathway in malignant mammary epithelial cells grown in three-dimensional culture, through use of gamma secretase inhibitors or of small hairpin RNAs directed against the four Notch receptors, a growth arrested polar phenotype was imposed (Fig. 3). Other candidate CSC niche constituents and their cognate receptors, which are not covered in detail in this series, include the ECM molecule, fibronectin, and the glucosaminoglycan, hyaluronic acid. Studies designed to understand a basis for therapeutic resistance suggested that those molecules facilitated a quiescent state in some cancer cells when they were under siege from chemotherapy. Antibodies against the fibronectin receptor VLA-4 (α4β1 integrin) prevented association of tumor cells with premetastatic niches (63), and reduced the incidence of minimal residual disease in a model of acute myelomonocytic leukemia (AML; ref. 64). Treatment of malignant breast cancer cells with fibronectin-(65) or β1 integrin-(66) blocking antibodies promoted phenotypic reversion to a polar, growth-arrested state in organotypic three-dimensional cultures. Finally, hyaluronic acid-rich substrates protect hematopoietic stem cells from the cytotoxic effects of 5′ fluoro-uracil (67), and antibodies against its receptor, CD44, reduced minimal residual disease in AML models (68). Thus ECM proteins and glucosaminoglycans that impose specific behaviors on stem cells also should be considered when formulating anti-CSC therapies.

Summary

The presence of a CSC niche is dependent on the presence of a CSC. That tumors may be generated and maintained by CSCs, in a manner analogous to the developmental hierarchy of the hematopoietic system, is challenged by a number of reports that suggest the ability of any given subset of malignant cells to form tumors is determined by the host's microenvironment. Therefore, the biochemical identity of any putative CSC is likely to be host-specific to a large extent. Moreover, the ability of microenvironments to impose biochemical and functional phenotypes on cells may add additional complications, because tumors are constantly evolving by virtue of reciprocal and dynamic collaborations between patients' deteriorating genomes and twisting microenvironments (69, 70). Therefore, the cells that initiate tumor growth may differ from those involved in metastasis. However, it is clear that the functional abilities ascribed to stem cells are important to tumor progression, and it is suggested that those functions can be imposed upon malignant cells either by normal stem cell niches, or by tumor microenvironments. Accordingly, herein is presented a model whereby a malignant cell may first usurp preexisting tissue-specific stem cell niches, becoming the initial CSC that nurtures a
tumor in its early stages. The evolved tumor microenvironment may then impose CSC-like functions onto other cells, which facilitate metastatic dissemination (Fig. 4). Targeting of the microenvironment molecules that can impose stem-like functions, or targeting of the signaling pathways in cells that mediate those functions, may represent worthwhile therapeutic paradigms.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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