CANCER DRUG DISCOVERY AND DEVELOPMENT

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Stem Cells and Cancer

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Humana Press

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ISBN: 978-1-60327-932-1 e-ISBN: 978-1-60327-933-8
DOI: 10.1007/978-1-60327-933-8
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2008944031

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Preface

The recent surge in stem cell research has ignited a field of discovery into many human diseases including diabetes, neuropathologies, and cancer. Stem cell therapy is a promising approach to the treatment of many debilitating diseases to replace specific differentiated cells that have been lost or died. Although stem cells may provide therapeutic benefit under certain conditions, stem cells are often implicated in the initiation, progression, and therapeutic resistance of malignant disease.

This first edition of *Stem Cells and Cancer* is intended to give a current perspective on the role of stem cells in cancer and strategies for novel therapies directed toward tumor stem cells. Cancer stem cells remain a controversial topic and the criteria that define cancer stem cells are continuing to evolve. The current cancer stem cell hypothesis is presented in several chapters with distinctions made between the hierarchical and stochastic models of tumor cell development. "Stemness," self-renewal, pluripotency, clonality, and tumorigenicity are important concepts applied toward defining cancer stem cells. Signaling pathways such as Wnt, Sonic Hedgehog, Notch, and Bmi-1 that are involved in differentiation, proliferation, and survival are implicated in the malignant process. Additional chapters address the identification of cancer stem cell populations through the evaluation of molecular markers such as CD133, CD44, and CD24, for example, or by Hoechst dye exclusion to recognize "side populations." Mesenchymal and hematopoietic stem cells are described as well as mouse models that are employed to elucidate the properties and functionality of stem cells in cancer and the stem cell niche. This book encompasses a wide variety of human cancers that include but are not limited to leukemia, gliomas, breast, and prostate cancers. Resistance to conventional therapies, genetic vs. epigenetic changes that affect therapeutic response, and strategies to prevent disease recurrence are challenges that have been incorporated into this volume. *Stem Cells and Cancer* represents a compendium of cutting edge research by experts in the field and will be instrumental in the study of this intriguing line of investigation for many years to come.

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I Introduction to Cancer Stem Cells

1 The Cancer Stem Cell Hypothesis

Kimberly E. Foreman, Paola Rizzo, Clodia Osipo, and Lucio Miele

Abstract

The "cancer stem cell" hypothesis is receiving increasing interest and has become the object of considerable debate among cancer biologists and clinicians. This ongoing debate is focusing attention on the very definition of stemness and its significance in the context of a malignancy. From a therapeutic standpoint, the cancer stem cell hypothesis emphasizes the cellular heterogeneity in cancers, and the need to specifically target small cell populations that resemble tissue stem cells and are phenotypically different from the majority of cancer cells. Regardless of their origin, these cells divide slowly, have the ability to undergo asymmetric cell division and are highly resistant to conventional chemotherapeutics. These characteristics make them prime suspects as potential causes of disease recurrence and metastasis, which are the main causes of morbidity and mortality in oncology. This chapter provides an introduction to the cancer stem cell hypothesis, briefly summarizes the evidence supporting this theory and the aspects that remain controversial. Finally, we present a brief discussion of the possible therapeutic significance of cancer stem cells and the current efforts to target developmental pathways on which these cells depend.

Key Words: Cancer stem cells, Tumor-initiating cells, Stem cell niche, Targeted therapies

THE CANCER STEM CELL MODEL OF CARCINOGENESIS

For decades, the prevailing theory of cancer initiation and progression has been that cancers derive from the serial acquisition of genetic mutations by normal somatic cells. These mutations resulted in enhanced proliferation, inhibition of differentiation, and reduced capacity to undergo apoptosis. Each mutation would result in progressive "dedifferentiation" so that the tumor cells would continually lose their mature, tissue-specific attributes, and regress to a more primitive phenotype. As differentiated cells have limited life spans, it would be difficult for any given cell to acquire all the mutations necessary to become transformed, thus explaining the relatively uncommon occurrence of transformation. However, if initial mutations led to unrestrained proliferation, this would generate more cells that could potentially be affected by further oncogenic mutations. Once transformed, cancer cells would proliferate indefinitely and form a tumor where each viable tumor cell was in principle equally capable of forming a new tumor.

Recent findings suggest that this model may be overly simplistic. The "cancer stem cell hypothesis" has gained considerable interest in recent years *(1–3)*. This theory states that cells in a tumor are organized as a hierarchy similar to that of normal tissues, and are maintained by a small subset of

From: *Cancer Drug Discovery and Development: Stem Cells and Cancer,*
Edited by: R.G. Bagley and B.A. Teicher, DOI: 10.1007/978-1-60327-933-8_1,
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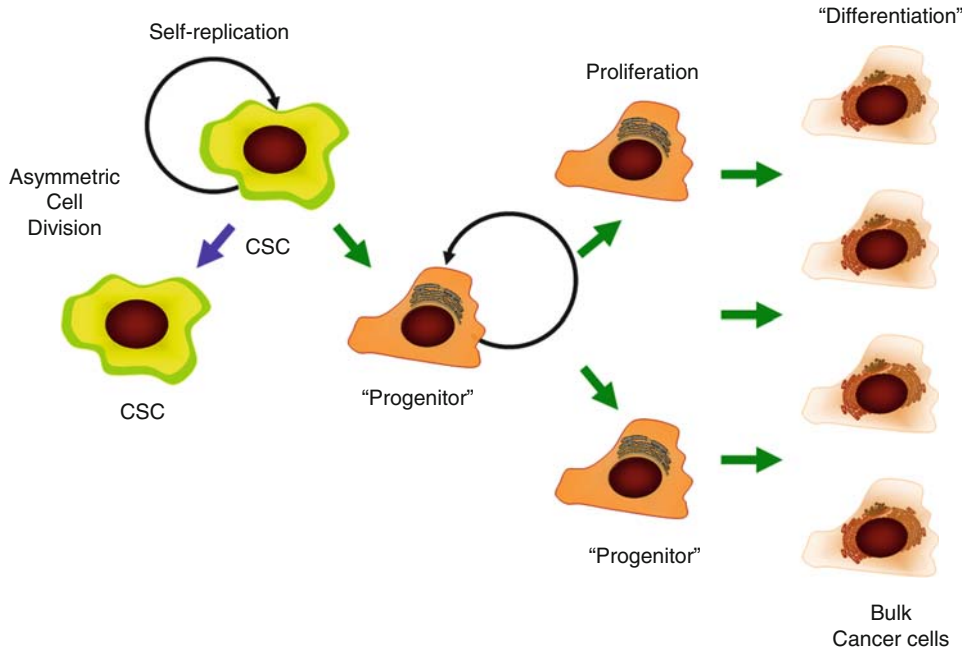

Fig. 1. The CSC hypothesis. CSCs are thought to maintain their numbers by slow self-replication, and produce other tumor cells by asymmetric cell division. In this process, cell division of a CSC generates a CSC and a transformed "progenitor-like" cell, which has limited self-renewal ability but are highly proliferative, similar to a transit-amplifying population in normal tissue. These progenitors give rise to more or less partially differentiated bulk tumor cells through a combination of proliferation and abortive differentiation.

tumor cells that are ultimately responsible for tumor formation and growth. These cells, defined as "cancer stem cells" (CSCs) or tumor initiating cells (TICs), possess several key properties of normal tissue stem cells including self-renewal (i.e., the ability of a cell to renew itself indefinitely in an undifferentiated state), unlimited proliferative potential, infrequent or slow replication, resistance to toxic xenobiotics, high DNA repair capacity, and the ability to give rise to daughter cells that differentiate. However, unlike highly regulated tissue stem cells, CSCs demonstrate dysregulated self-renewal/differentiation programs and produce daughter cells that arrest at various stages of differentiation. The daughter cells make up the bulk of the tumor and are characterized by rapid replication, limited proliferative potential, and the inability to form a new tumor. Only the CSC is able to initiate tumor formation as it is solely capable of self-renewal. A diagrammatic representation of the CSC hypothesis is shown in Fig. 1.

The strongest evidence for the CSC theory comes from studies in acute myelogenous leukemia (AML). Landmark studies by Dick and colleagues demonstrated that only rare cells in AML were able to initiate leukemia in murine models, and serial transplantation studies revealed these cells had a high self-renewal capacity *(4, 5)*. The cell responsible for tumor initiation was identified as having a $CD34^+CD38^-$ phenotype, which was particularly interesting as bulk AML samples tend to be $CD34^-$. Furthermore, $CD34^+CD38^-$ is a phenotype characteristic of normal hematopoietic stem cells (HSC) indicating the putative CSCs may have a primitive phenotype. Bonnet et al. found that as few as 5×10^3 $CD34^+CD38^-$ cells could engraft an immunocompromised mouse, while 100 times more $CD34^-$ or $CD34^+CD38^+$ cells from the same donor could not *(5)*. Importantly, the tumors derived from injection of the $CD34^+CD38^-$ cells was heterogeneous and composed of a mixture of tumorigenic and nontumorigenic cells similar to the donor sample *(5)*. Since then, stem-like cells have been identified in a variety

of human malignancies including other leukemias and solid tumors such as breast, colon, brain, head and neck, lung, pancreatic, nasopharyngeal cancers, and melanomas *(4–18)*. In many cases, a tumorigenic subset of cells could be reproducibly identified and isolated based on a distinct set of cell markers separating it from the nontumorigenic subset *(19)*. Attempts to isolate CSCs from other malignancies are underway in laboratories worldwide, and this list is likely to grow. Remarkably, even established cancer cell lines that have been grown in vitro for many years appear to contain CSC-like populations that can be isolated and are highly tumorigenic *(20, 21)*. The surface markers of CSCs from different tumor types are diverse, suggesting that their biological behaviors may be different as well.

One reason the CSC theory has generated such enthusiasm is that it may help explain long-standing problems in cancer biology. It is well-recognized that tumors are heterogeneous in terms of both functional heterogeneity and cellular composition. Functional heterogeneity refers to the observation that only a small portion of tumor cells can give rise to colonies in clonogenic assays in vitro or tumors in vivo. Under the traditional theory of tumor formation (also called the stochastic model), every tumor cell should be equally capable of forming a tumor. As tens to hundreds of thousands of tumor cells are needed to reproducibly initiate tumors in animal models, investigators concluded that the process was inefficient. However, with the CSC theory, the number of cells needed to form a tumor would simply be determined by the relative frequency of CSC in the tumor population. A sufficient number of CSCs must be present in the inoculum, since most cells in the line are proliferative but nontumorigenic. The phenotypic heterogeneity of tumors is also more easily explained by the CSC theory. Mutations in the CSC would be passed on to each daughter cell, and as the daughter cell differentiates, it may arrest at any one of numerous points prior to full maturation. In the stochastic model, the tumor cell would need to dedifferentiate to different degrees to form a phenotypically heterogeneous but genetically clonal population. Although the genomic instability associated with cancer clearly makes this possible, it is easier to envision an abortive version of the normal hierarchical differentiation program in a tissue as opposed to a random back-differentiation process affecting individual cells to different extents.

It has also been postulated that the CSC theory may explain why it is so difficult to treat cancer. If this model is correct, then directing cancer therapeutics at the bulk of rapidly replicating tumor cells is not likely to achieve tumor eradication, unless the CSCs are eliminated. This could explain the vexing problem faced by oncologists worldwide, who often can achieve complete clinical and pathological remissions of cancers with chemotherapy, only to see the cancers recur, often in metastatic and ultimately lethal forms. This clinical phenomenon implies that very small numbers of cells, sometimes undetectable even by sophisticated molecular diagnostic tools, are capable of causing tumor relapses. Standard chemotherapeutic strategies using mitotic poisons, DNA-damaging agents, antimetabolites, or even modern "targeted" agents such as growth factor receptor kinase inhibitors often are aimed at actively proliferating cells resulting in growth arrest and/or cell death. This strategy efficiently kills the daughter cells, but is much less effective against CSCs, which can remain quiescent for extended periods of time. Thus, tumors may shrink in response to traditional chemotherapy, even to the point where they are undetectable, yet CSCs often persist and eventually cause relapsed and metastatic disease *(2)*. Furthermore, when CSCs are exposed to and escape from chemotherapy-induced death, they may become more resistant to these insults and pass this on to their daughter cells. This may explain why recurrent cancers are often more resistant to treatment than primary disease. An additional characteristic of CSCs that makes them more difficult to eradicate than "bulk" cancer cells is their high level expression of ABC family transporters, which catalyze the ATP-dependent transport of toxic chemicals from the cell *(22)*. These molecules were originally identified as one of the main cause of multidrug resistance in cancers *(23)*. Evolutionarily, it is plausible that normal tissue stem cells would be particularly well protected against toxic insults, because of their fundamental role

in tissue regeneration. Unfortunately, this property also makes the neoplastic counterparts of tissue stem cells highly resistant to many common chemotherapeutic drugs. Indeed, one of the most popular ways of isolating putative CSC population takes advantage of their ability to rapidly efflux DNA-binding fluorescent dyes such as Hoechst or 7-AAD, which is due to high level expression of ABC transporters. Cells that retain less dye appear as a "side population" (SP) in flow cytometry experiments. In several cases, SP cells have been shown to be enriched in putative CSCs *(24–26)*.

WHERE DO CANCER STEM CELLS COME FROM?

While the CSC theory has offered possible new explanations for several key aspects of tumor biology, it has also raised new questions. Perhaps one of the most interesting, and yet difficult to answer, is what is the origin of the CSC? The answer to this question depends on our understanding of the stem cell differentiation process in normal tissues. If tissue stem cell differentiation is a "one way only" process, and partially differentiated cells cannot return to a "stem-like" program even when transformed, then the most obvious candidate precursor of the CSC is the tissue-specific stem cell that normally functions to replace dead and injured cells in tissues. Several points support this possibility. First, normal stem cells are already capable of indefinite self-renewal and generate more differentiated progenitors, most likely through asymmetric cell division. Even slightly more differentiated progenitor cells would have lost this ability and would have to reacquire self-renewal through mutations – a potentially complicated process. Second, tissue stem cells are long-lived and would be capable of accumulating the serial mutations necessary for transformation over the lifetime of the cell. Acquisition of multiple mutations would be more difficult for a short-lived cell. Finally, CSCs isolated from tumors tend to possess a primitive phenotype. As already mentioned, the putative CSC in AML has a $CD34^+CD38^-$ phenotype, which is the same as the HSC, while more differentiated cells ($CD34^+CD38^+$) could not initiate tumor formation *(4, 5)*. Similarly, CSCs derived from various primary tumors or cultured cell lines routinely express other markers of normal tissue stem cells including CD133, nestin, c-kit, sox2, oct4, and musashi-1 *(7, 8, 11, 27, 28)*. Clearly, it is simpler to conclude that CSCs derived from stem cells continue to express stem cell markers than to envision a more mature cell specifically regaining the ability to express these markers as a consequence of a random dedifferentiation event.

Nevertheless, formal proof that CSCs can only derive from normal tissue stem cells has yet to be obtained. At least theoretically, it is conceivable that the process of transformation puts a strong selective pressure on differentiated cells so that only cells that undergo the epigenetic changes necessary to restore "stemness" are capable of surviving transformation. In this model, reversion to a stem-like state is part of the transformation process. This is essentially a modified restatement of classical transformation models in which loss of differentiation results from a process of selection in a population of genomically unstable cells.

The feasibility of cloning organisms from somatic cell nuclei shows that under some circumstances the nucleus of a somatic cell can be reprogrammed all the way back to totipotency, generating a viable embryo and a complete organism. In fact, the recent demonstration that cells equivalent to human embryonic stem cells can be obtained from normal fibroblasts by transduction of specific factors supports the hypothesis that achieving stemness through dedifferentiation is possible, at least under some circumstances. Yu et al. recently showed that expression of oct4, sox2, nanog, and LIN28 in human dermal fibroblasts converts them into pluripotent cells with a phenotype virtually indistinguishable from embryonic stem cells *(29)*. In another report, Takahashi et al. *(30)* showed that expression of Oct3/4, sox2, Klf4, and c-Myc can achieve the same result. The fact that the protooncogene c-Myc can be part of the reprogramming mix of genes supports the idea that under some conditions,

the transformation process could reprogram a cell to a stem-like phenotype. It is important to note that these studies were conducted in fibroblasts and not epithelial cells. Can a similar process of reprogramming occur in common epithelial malignancies? A process of partial dedifferentiation has been known for years in epithelial cancers as epithelial-mesenchymal transition (EMT) *(31–34)*. This consists in loss of epithelial markers, such as tissue-specific cytokeratins, and adhesion molecules, such as E-cadherin, and acquisition of markers typical of mesenchymal cells, such as vimentin and N-cadherin. The process of EMT is thought to contribute to the ability of transformed epithelial cells to metastasize. In this model, cancer cells need to undergo EMT to migrate through the body, and once they seed distant metastatic sites, they can revert to a more or less "epithelial" phenotype through a process of mesenchymal-epithelial transition (MET). Several transcription factors such as Twist, Snail, or Slug and secretory proteins of the TGF-β family, including some bone morphogenetic proteins (BMPs), can induce the EMT program *(34)*. Vascular mimicry is thought to be a specialized form of EMT in which tumor cells can acquire an endothelial phenotype *(35, 36)*.

Thus, the question seems to be not whether or not differentiation plasticity is possible in epithelial cancer cells, but whether this process can go as far as generating a cell that has the functional characteristics of a stem cell. What a simple dedifferentiation model does not immediately explain is the hierarchical organization of cells in malignancies. If dedifferentiation is a secondary event that arises through selection and confers a selective advantage to less differentiated cells, why is there a hierarchical organization among neoplastic cells with a highly tumorigenic, dededifferentiated population capable of generating less tumorigenic, more differentiated cells? One possible explanation is that dedifferentiation is a highly improbable event, which produces a cell fate program that includes functional "stemness." Thus, only a few cells or even a single cell would have to undergo this process to generate a small population of CSCs. These then give rise to the rest of the cancer cell population through a process of hierarchical abortive differentiation that imperfectly recapitulates that of a normal tissue.

An intermediate possibility is that the CSC could originate not exclusively from tissue stem cells, but from a restricted number of cell populations including tissue stem cells and immature progenitor cells, which are immediately below tissue stem cells in the differentiation hierarchy and are capable of short-term self-replication. Experimental support for this hypothesis comes from several studies in leukemia where the introduction of oncogenic fusion gene products into hematopoietic progenitor cells resulted in AML in animal models. Cozzio et al. found that expression of the MLL-ENL fusion gene product in hematopoietic progenitor cells resulted in leukemia, albeit with less efficiency than when it was expressed in true hematopoietic stem cells *(37)*. Similar results were also found with the MOZ-TIF2 fusion gene product *(38)*. More recently, Somervaille and Cleary enforced MLL-AF9 expression in normal murine HSC and progenitor cells *(39)*. Using serial transplantation in mice, they discovered that the functional CSC expressed MAC-1 and Gr1, two markers associated with more mature cells *(39)*. Interestingly, the cells also expressed the stem cell marker c-kit, suggesting CSCs may express an unusual combination of cell markers *(39)*. Taken together, these studies clearly support the notion that AML may arise from either stem or progenitor cells in a mouse model; however, caution should be used in interpreting this data. Murine cells are generally easier to transform than human cells; hence, it is unclear if these findings are relevant to human disease *(40)*. A similar theory has been proposed for breast cancer *(41, 42)*. According to Dontu et al. *(41)*, the existence of ERα-negative breast cancers and ERα-positive breast cancers of variable biological aggressiveness may be explained by postulating that CSCs in these cancers originate from different cell populations. The most aggressive, undifferentiated ERα-negative cancers and poor-prognosis ERα-positive cancers would arise from the most primitive mammary stem cells, which are ERα-negative, while less aggressive ERα-positive cancers would arise from CSCs derived from intermediate progenitors that are ERα-positive. These can generate

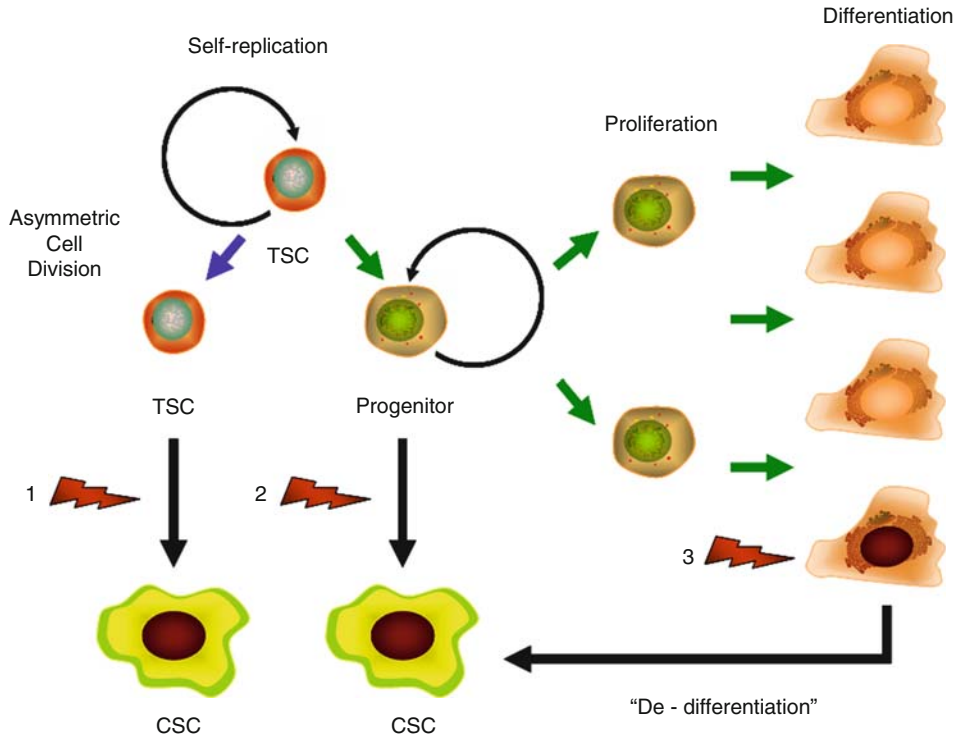

Fig. 2. Possible origins of CSCs. Three different but not mutually exclusive models are schematically presented. "Lightning" symbols indicate transforming mutations. CSCs may originate exclusively from the transformation of primitive tissue stem cells (TSC, model 1), or from the transformation of either TSC or progenitor cells (model 2). Alternatively, CSCs may originate from the transformation and dedifferentiation of more mature cells, which reacquire stem cell properties as a consequence of transforming mutations (model 3) (*see Color Plates*).

ERα-negative, rapidly proliferating "transit-amplifying" cells. This is a conceptually plausible model. However, experimentally it is difficult to distinguish it from a scenario in which all breast cancer arise from primitive, ERα-negative mammary stem cells, which lose their differentiation ability to variable degrees depending on the transforming mutations they undergo. Figure 2 represents three different, nonmutually exclusive models for the origin of CSCs.

OPEN QUESTIONS: LIMITATIONS OF THE EXPERIMENTAL EVIDENCE

An important issue that remains to be addressed is that almost all of the experimental evidence for the cancer stem cell model comes from studies in which human CSCs are transplanted into immunocompromised mice *(43, 44)*. Thus, a possible objection to the model is that selection protocols for CSCs could simply identify cells that are more adept at forming tumors in the xenogeneic microenvironment of an immunocompromised mouse. Given the limitations of current experimental models, this cannot be ruled out. However, if CSCs are essentially an artifact of xenograft models, it is not clear why human cancer cell lines of diverse tissue origins that have been grown in vitro for decades retain cell populations that exhibit stem-like characteristics very similar to CSCs isolated from primary tumors including increased expression of ABC transporters and asymmetric cell division, and are highly tumorigenic in mice. Specifically, it is not clear what selection pressure could explain the remarkable persistence of these CSC-like populations outside of the mouse microenvironment, if they

are not necessary for continued in vitro propagation of the cell line. Strasser and colleagues have proposed that the reason so many human cancer cells are needed to initiate tumor formation is that the murine microenvironment is not appropriate for development of human cancers, and only a few cells are capable of overcoming this hostile environment *(43)*. These authors have taken the approach of genetically engineered mouse cells to develop lymphoma (primary Eμ-myc lymphomas), isolating subpopulations of the tumor cells based on the murine stem cell markers Sca-1 and AA4.1 (CD93), and examining tumor formation in syngeneic naïve, immunocompetent mice *(43)*. They report identify a small subpopulation (2–5%) of cells with stem-like characteristics, but found that Sca-1^{+}AA4.1^{hi} and Sca-1^{+}AA4.1^{lo} cells were equally capable of forming tumors *(43)*. These data have been interpreted as evidence against the universal validity of the cancer stem cell model. It should be pointed out that although xenograft models are certainly artificial, transgenic mouse models of carcinogenesis have important limitations of their own, and may or may not faithfully recapitulate human carcinogenesis. Typically, in these models a very potent oncogene is overexpressed in a target cell population, and the whole process of carcinogenesis and tumor progression is dramatically accelerated compared with human disease. Mouse cells are far more susceptible to transformation than human cells, and may be able to more easily reacquire functional "stemness." It is interesting to notice that the oncogene used in this particular experimental model, c-Myc, is also one of the stemness-inducing genes that can reprogram human fibroblasts to an embryonic stem cell-like phenotype. Thus, an alternate explanation for these data is that both Sca-1^{+}AA4.1^{hi} and Sca-1^{+}AA4.1^{lo} cells in this transgenic model have acquired functional "stemness" through a process of dedifferentiation, and can behave as CSCs. More sophisticated animal models will be required to gain further insights into this issue. These models should be based on human cells, but attempt to recapitulate as much as possible the human microenvironment. Such a humanized xenograft model has been generated for the mammary gland *(45)*, and should provide valuable information on putative breast cancer stem cells.

The controversy on the human relevance of CSC data obtained in xenograft models underscores the importance of tumor microenvironment in the biology of CSCs. Tumor-stroma interactions may indeed be critical in reprogramming cancer cell developmental pathways. Transforming growth factor (TGF)-β and bone morphogenetic proteins (BMPs) can be produced by tumor stroma, as can several other mediators of intercellular communication such as Wnt, Hedgehog, and Notch ligands. There is growing interest in studying the CSC "niche" as a potential therapeutic target. Normal stem cells are well known to require signals from their immediate environment, including stromal cells, microvascular endothelial cells, and extracellular matrix for their long-term survival and self-renewal. This specialized microenvironment is commonly referred to as the stem cell niche, and it is best understood in the hematopoietic system *(46)*. There is increasing evidence that CSCs also require microenvironmental signals from specialized niches *(47–49)* (Fig. 3). Autocrine and paracrine mediators secreted by the CSCs themselves or by other tumor cells may also play an important role, at least in some malignancies *(50, 51)*. How much autocrine or paracrine interactions contribute to the CSC niche is still unclear. However, at least under some circumstances CSCs can recreate a niche-like environment in the absence of other cell types. Putative breast cancer stem cells can form spheroids called "mammospheres" in suspension culture *(52, 53)*. Other putative CSCs can also form similar spheroids. Mammospheres contain few CSCs, and mostly consist of precursors and partially differentiated cells. Mammospheres can propagate in vitro and form secondary and tertiary mammospheres, which retain the original cellular composition. This implies that at least under some culture conditions, the CSCs themselves and their immediate progeny can form a functional niche that is capable of sustaining self-renewal, asymmetric cell division and partial differentiation.

Undoubtedly, much remains to be clarified and further studies are needed. These may well reveal that the origin of the functional CSC may vary based on the cell type involved and the specific nature of the oncogenic events leading to transformation.

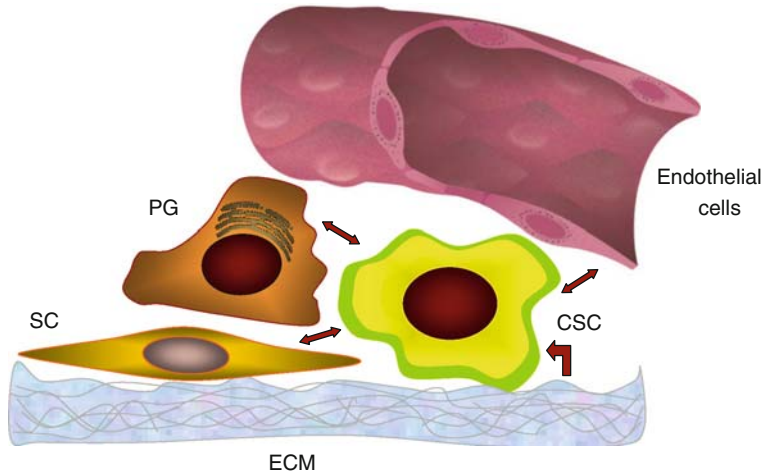

Fig. 3. The CSC "niche". In vivo, CSCs may require signals from their microenvironment to maintain their properties, as is the case for normal tissue stem cells. Microenvironmental signals may be received from endothelial cells, from various types of stromal cells (SC), such as fibroblasts, bone marrow stromal cells, or immunocytes infiltrating the tumor, from progenitor cells (PG) derived from the CSCs themselves, and/or from the extracellular matrix (ECM). It is likely that the cross-talk between CSCs and other cells is bidirectional. These signals may be therapeutically targeted to deprive CSCs of indispensable microenvironmental signals.

CLINICAL IMPLICATIONS: TARGETING CANCER STEM CELLS FOR THERAPY

Regardless of the origin of CSCs, perhaps the most important aspect of the cancer stem cell model is that it has drawn increasing attention to the hierarchical organization of malignancies. Cancers have been known for decades to be heterogeneous, but until recently the idea that the most abundant cancer cells derive from a much smaller and often elusive pool of stem-like cells was commonly accepted only in the field of leukemia. This model appears to apply to many solid tumors, and the list is growing by the day. Whatever their genesis, if human cancers do contain a small population of cells that proliferate slowly, are highly resistant to current chemotherapeutic regimens and could cause disease recurrence and metastasis, eradication of these cells may be necessary to achieve a long-lasting remission or cure. Thus, new therapies targeting the CSC must be developed if we hope to prevent or eliminate recurrent and metastatic disease. It has been proposed that identification of signaling pathways that are involved in self-renewal and are deregulated in CSCs may be an effective approach for novel target discovery *(2)*. Alternatively, the identification of proteins expressed preferentially by CSCs, such as CD96 in leukemia, could provide targets for antibody-based therapies or to modulate cell signaling and promote differentiation *(54)*. Yet another possibility is disrupting the interactions between CSCs and their niche *(48, 49)*. Several signaling pathways have been identified as playing critical roles in stem cell self-renewal including among others Notch, Wnt, and Hedgehog *(55)*. These pathways are evolutionarily ancient and have fundamental roles during development, when they control multiple cell fate decisions. They are primarily used for short-range intercellular communication utilizing secreted factors such as Hedgehog *(56)* or the Wnts *(57, 58)* or cell membrane-associated ligands such as Notch ligands Jagged and Delta *(59)*. Importantly, these pathways are involved in several of the phenomena we described above, from EMT *(60)* to CSC-niche communication *(46, 49, 51)*. Drugs that inhibit Notch signaling are in early clinical development and others are in the pipeline *(61)*, and Hedgehog inhibitors are not

far behind. Interest in using Notch inhibitors to target CSCs is growing. In glioblastomas, elevated Notch expression has been associated with high nestin levels and is linked to a poor prognosis *(62, 63)*. Furthermore, Notch inhibition reduced the ability of brain CSCs to form tumors *(64)*. In breast cancer, Notch expression and activation has been associated with a poor prognosis, and studies indicate that Notch inhibitors can kill breast cancer cells in vitro *(65–67)*. As CSCs have been identified in primary breast cancers, there has been much interest in Notch signaling in breast CSCs *(6)*. Farnie et al. recently compared mammospheres derived from normal mammary tissue and human ductal carcinoma in situ (DCIS) and reported that activated Notch-1, Notch-4, and the downstream target Hes-1 were expressed in mammospheres from DCIS samples, but not those derived from normal breast tissue *(68)*. Notch inhibition with a γ-secretase inhibitor or a neutralizing Notch-4 antibody significantly reduced the ability of DCIS derived cells to form mammospheres *(68)*. These results suggest that Notch inhibition may be able to preferentially target breast CSCs, while sparing normal mammary stem cells. Laboratories around the world, including ours, are exploring the development of therapeutic regimens including Notch, Hedgehog, or Wnt inhibitors to target CSCs. Figure 4 shows a simplified representation of pathways that have been associated with CSC maintenance.

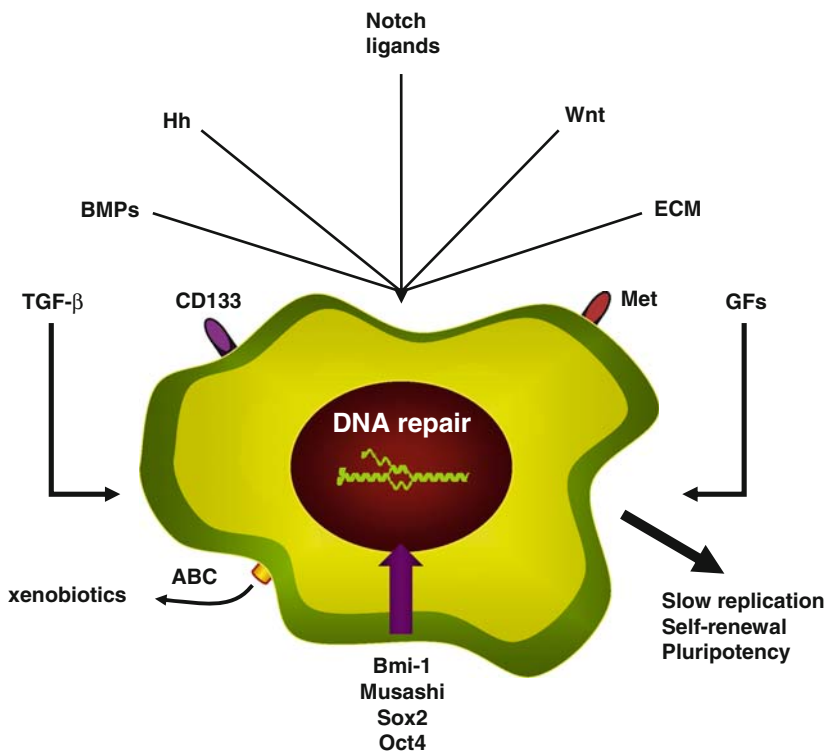

Fig. 4. Molecular pathways affecting CSCs. The figure shows a list, not meant to be all-inclusive, of pathways that have been shown to modulate the CSC phenotype. Extracellular signals delivered through the Hedgehog (Hh), Notch, Wnt pathways or through TGF-β and the related BMPs, or from ECM proteins and from growth factors such as hepatocyte growth factor (Met ligand) may all participate in regulating the maintenance, self-renewal, and differentiation of CSCs. Slow replication, ability to generate partially differentiated progenies (pluripotency) highly effective DNA repair, ability to eliminate xenobiotics through ABC family transporters (ABC), and expression of primitive membrane markers (CD133, Met) have been documented in many putative CSC populations isolated from tumors or cell lines. Transcription factors such as Bmi-1, Musashi, Sox2, Oct4, and others have been shown to be commonly expressed in putative CSCs and participate in controlling their phenotype.

CONCLUSIONS

The CSC hypothesis has sparked a tremendous increase in scientific interest in the hierarchical organization of cancer cells, the isolation of rare cellular subpopulations that may be responsible for treatment failures, and the role of microenvironmental niches in the maintenance of these populations. There are still many questions that remain unanswered, particularly surrounding the origin of CSC populations in human tumors and the interpretation of data generated by current experimental models. Yet, looking at cancers from the perspective offered by the CSC hypothesis may answer fundamental questions in tumor biology and open the way to paradigm shifts in our therapeutic approach to malignancies. Thus, it is reasonable to take the view that studying the mechanisms regulating the survival, self-renewal, and differentiation of normal and transformed stem cells could potentially lead to tremendous advances in the treatment of neoplastic diseases.

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2 Tumor Stem Cells and Malignant Cells, One and the Same

Beverly A. Teicher

Abstract

Cancer is a proliferative, invasive, and metastatic disease often caused by repeated tissue insults resulting in accumulation of genetic abnormalities that rarely produce malignant cells. The survival of mouse L1210 leukemia was determined for inoculations of 1 cell up to 10^6 cells. The survival times varied in a log-linear manner with the inoculum cell number from 19 days with 1 cell to 7 days with 10^6 cells implanted. In preclinical tumor models or in patients, tumor nodules of 10^8–10^9 cells are advanced cancer. Malignant cells frequently secrete growth modulatory substances that regulate their growth and alter growth of normal cells. Whether the metastatic malignant cell is the same or significantly different from the primary lesion malignant cell remains a topic of active investigation. Reaching a detectable lesion takes 10 years. Genetic instability produces variants in the primary tumor and metastases that are more heterogeneous than the early disease. The argument that cancer arises only from the tissue stem cell populations and that cancer stem cells comprise perhaps 1 in 100,000 or 1 in 10,000 cells within the tumor leads to the notion that agents that selectively kill cancer stem cells will not decrease the tumor mass. The cells that initiate, sustain, and populate cancers are malignant cells. Cancer stem cell notion is useful if it leads to important research questions and to better therapeutics.

Key Words: Colony forming units, Malignant cells, Genetic instability, Metastasis, L1210 leukemia

INTRODUCTION

Cancer is a proliferative, invasive, and metastatic disease that is frequently caused by repeated insults to a tissue resulting in accumulation of genetic abnormalities that, by rare chance, produce a malignant cell. Cancer cells are genetically aberrant and instable. Some cancers begin as a single clone (and a few remain clonal) and other arise from a field of repeatedly damaged cells. The search for an understanding of cancer and for the key as to how to control and ablate malignant disease often returns to the remarkable processes of normal tissue/embryo development and normal tissue repair. The "well-behaved" proliferative and self-limiting biology of wound repair, gut lining replacement, liver regeneration, skin renewal, and bone marrow generation of hematopoietic cells has taught us that cell proliferation and differentiation are a constant process in complex organisms and are well-controlled under normal circumstances.

The concept of a stem cell was put forth by Till and McCulloch to describe the ability of a single mouse bone marrow cell to produce a colony of cells in the mouse spleen and later to describe the

From: *Cancer Drug Discovery and Development: Stem Cells and Cancer,*
Edited by: R.G. Bagley and B.A. Teicher, DOI: 10.1007/978-1-60327-933-8_2,
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ability of similar single bone marrow cells to give to colonies of varied types in cell culture *(1, 2)*. A colony-forming unit (CFU) is an individual cell that is able to clone itself into a colony of identical cells. A CFU is a measure of viable bacterial numbers or a measure of viable mammalian malignant cells in a culture. In reconstituting the immune system of lethally irradiated mice, bone marrow cells from syngeneic donors are intravenously injected into the recipient animals and colonies form in the spleen. Each colony is the progeny of a pluripotent stem cell; therefore, the number of colonies is a measure of the number of stem cells. These findings led to the notion that cancer can arise from multiply insulted cells that by rare chance have aberrantly turned on genes that normally are expressed only by normal tissue "stem" cells. Thus, cancer cells have aberrantly reverted to a dedifferentiated proliferative state. These malignant cells are trying to build a tissue but they are abnormal and lethal. Indeed, an area of therapeutic investigation has a goal to discover agents that can terminally differentiate malignant cells to a quiescent nonproliferative state.

EARLY OBSERVATIONS

An interesting aspect of the current cancer stem cell debate regards the number of human tumor cells required to initiate the growth of a subcutaneous nodule in immunodeficient mice. A very large number of variables would need to be optimized to achieve reliable data from such observations. A historical perspective looking at syngeneic mouse tumors may help. The L1210 and P388 mouse leukemias were developed in 1948 and 1955, respectively *(3–5)*. L1210 and P388 leukemias were both chemically induced in a DBA/2 mouse by painting the skin with methylcholanthrene. The leukemias have been propagated in DBA/2 mice by implanting intraperitoneally 0.1 mL of a diluted ascetic fluid containing either 10^5 L1210 cells or 10^6 P388 cells. These mouse leukemias were the first tumors used for large-scale drug discovery screening programs by the national drug development program instituted in 1954 by Congress, which directed the National Cancer Institute to start a program. The Cancer Chemotherapy National Service Center (CCNSC) screen consisted of three mouse tumors: L1201 leukemia, SA-180 sarcoma, and mammary adenocarcinoma 755 *(6)*. Over the years, the primary screen varied from the original three tumors to L1210 plus two arbitrarily selected tumors to L1210 plus Walker 256 carcinosarcoma to L1210 plus P388 leukemia to L1210 plus B16 melanoma or Lewis lung carcinoma. In 1976, a change occurred in the NCI primary screen. The new screen included a panel of colon, breast, and lung tumor models (mouse and human); however, compounds were initially screened in P388 leukemia *(7)*.

Skipper and Schabel and colleagues explored the growth characteristics of the L1210 and P388 leukemias in mice *(8, 9)*. The testing was conducted in a hybrid of DBA/2 hosts. Tumor cell implant sites were intraperitoneal injection, subcutaneous implant, intravenous injection, and intracranial injection. For L1210 leukemia with an inoculum of 10^5 cells, the mean days of survival and tumor cell doubling times for these implant sites were 8.8, 9.9, 6.4, and 7.0 days and 0.34, 0.46, 0.45, and 0.37 days, respectively (Fig. 1). The mean survival times of mice implanted with L1210 cells by these various routes was determined for inoculations of 1 cell up to 10^6 cells. The survival times varied in a log-linear manner with the inoculum cell number. Thus, when the mice were implanted with 1 L1210 cell by intraperitoneal injection, they survived 19 days and when the mice were implanted with 10^6 L1210 cells intraperitoneally, they survived 7 days (Fig. 1). Similar studies were conducted with P388 leukemia. For P388 leukemia (10^6 cells), the mean days of survival and the tumor doubling times for the same implant sites were 10.3, 13.0, 8.0, and 8.0 days and 0.44, 0.52, 0.68, and 0.63 days, respectively. From these studies, it must be concluded that every L1210 and P388 cell is a cancer stem cell.

Skipper and Schabel applied similar analyses to solid tumors especially the mouse Ridgway osteogenic sarcoma *(10)*. In preclinical tumor models or in patients, tumor nodules of 10^8–10^9 cells are

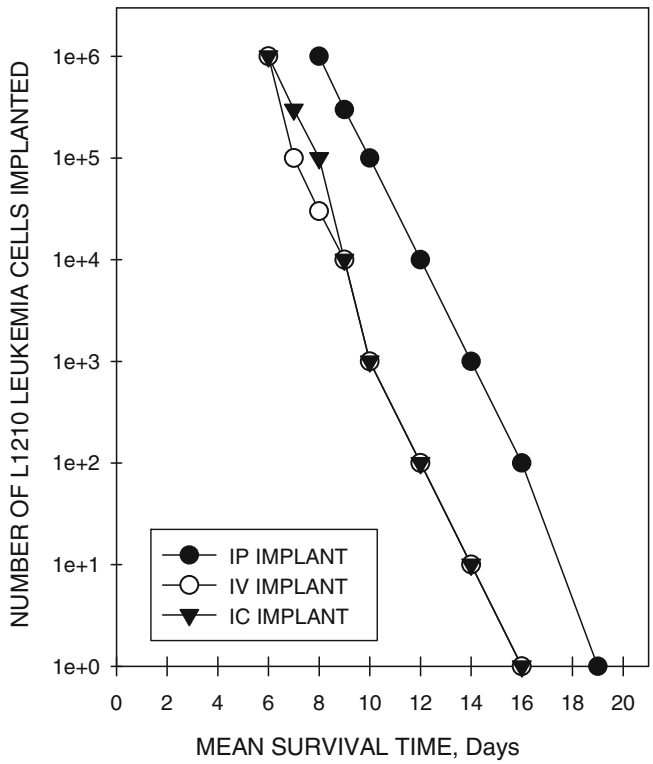

Fig. 1. Mean survival times of mice inoculated with various numbers of murine L1210 leukemia cells injected intraperitoneally, intravenously, or intracranially. These data form the basis for the in vivo bioassay method for determining the number of L1210 cells surviving after treatment of L1210 tumor-bearing mice with therapy. From these survival curves, it was determined that from: (1) intraperitoneal inoculation of L1210 cell-generation time = 0.55 days; the lethal number of L1210 cells = 1.5×10^9; (2) intravenous inoculation the L1210 cell-generation time = 0.43 days; and (3) intracranial inoculation the L1210 cell-generation time = 0.46 days *(8,9)*.

advanced cancer (Fig. 2) *(10, 11)*. One source of variability in the response of drug-sensitive tumor cells to a drug is the heterogeneity of the blood supply such that the drug does not reach the tumor cells distal from the blood supply in sufficient concentration to be lethal. Thus, the pharmacokinetics and concentration of a drug required to kill tumor cells distal from vasculature should be documented. In addition, the physiologic heterogeneity of tumor masses as a source of varied treatment response, Skipper and Schabel considered the heterogeneity of tumor stem cells, defined as cells capable of unlimited proliferative thrust, caused by the inherent genetic instability of malignant cells to be a source of variable treatment response. Skipper and Schabel considered various types of tumor stem cells that might account for fluctuation in response to chemotherapy in similarly treated individuals bearing a specific cancer, and classifications of cancers by chemotherapeutic effect. Fluctuating ratios of treatment responsive to treatment resistant stem cells, as predicted by the mutation theory, could account for one patient responding to a drug and the next not responding. Differences in tumor growth fraction and differences in tumor distribution into pharmacologic sanctuaries could also strongly influence a patient's response to therapy. Treatment resistant stem cells are primarily responsible for the failure of the best available chemotherapy to cure responsive, refractory, and very refractory experimental neoplasms. These data examined suggest that differences in the resistant to responsive stem cell ratios in different types of cancer may account for their being classified as responsive, refractory, or very refractory *(12)*.

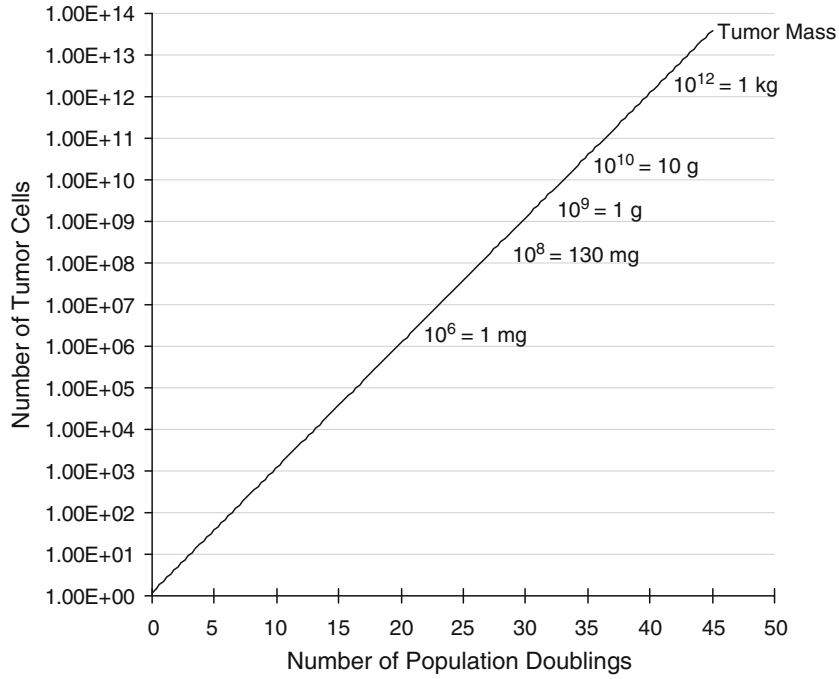

Fig. 2. Tumor cell numbers and weight of the tumor mass are shown. In patients, tumors are advanced at first presentation or at recurrence after initial noncurative therapy *(10,11)*.

TUMOR CELL HETEROGENEITY

Experience with the heterogeneous response of well-controlled preclinical tumor models grown in inbred strain of mice led investigators in the mid-1980s to believe that the assumption that there should be a common pattern of cellular heterogeneity for histologically identical types of cancer was not warranted *(13)*. Although malignant disease may develop from a single transformed cell, even in tumors where the single cell has diversified to heterogeneous cell phenotypes, evidence of a clonal origin still exists *(14)*. Although Foulds concluded that tumor evolution (progression) is characterized by permanent, irreversible changes, we recognize today that cell remain very plastic and adaptable and can often modulate their biology to changes in the microenvironment *(15)*. During molecular progression of tumors, neoplastic cells accumulate increasing genetic alterations that are generated by mutational events, genetic instability *(16, 17)*. Tumor cell genetic instability ensures that malignant disease contains heterogeneous, phenotypically diverse tumor subpopulations *(14)*. Tumor cell diversification mechanisms may be similar or identical to normal development during embryonic and postembryonic diversification and development. Tumor cell subpopulations can influence the properties of other subpopulations in the tumor including proliferation, sensitivity to drugs, immunogenicity, and metastatic potential *(14, 18–20)*.

Understanding the biology of malignant cells (cancer stem cells) that allows them to escape the constraints that normally regulate cell growth and differentiate is critical. Malignant cells frequently secrete growth modulatory substances that regulate their own growth (autocrine) and/or alter the growth of normal cells (paracrine) in the vicinity of the malignancy (Fig. 3) *(21)*. A malignant tumor whose growth depends upon the release of autocrine and paracrine growth factors may be vulnerable to treatment with specific receptor antagonists or growth factor neutralizing antibodies.

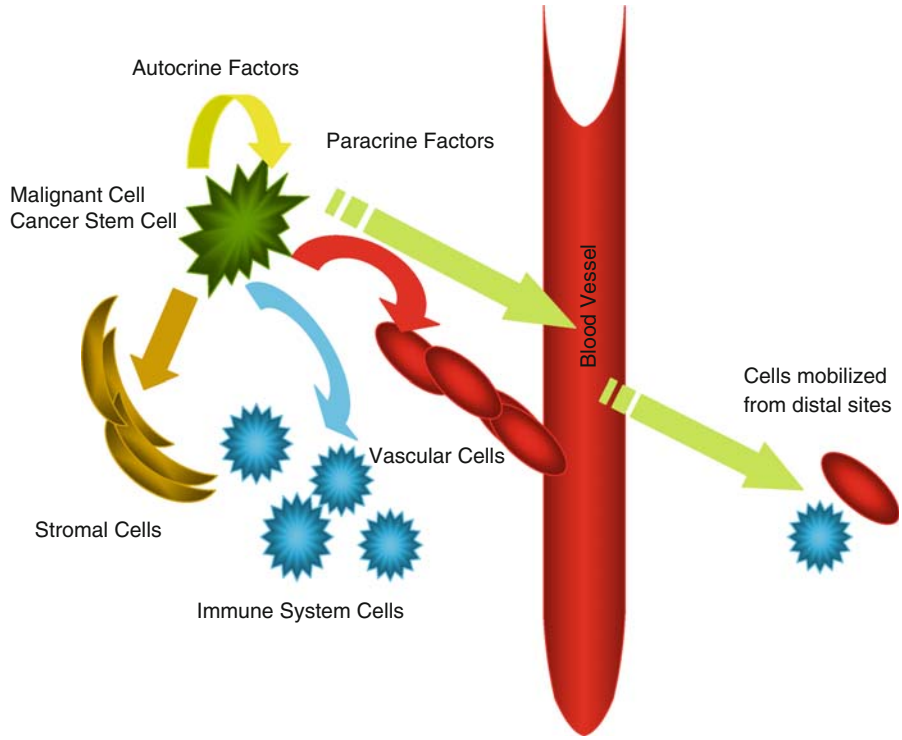

Fig. 3. Malignant cells can have abscopal effects on the host through secretion of paracrine factors and can produced autocrine factors that can sustain proliferation of the malignancy.

HEMATOPOIESIS AS A MODEL

Analogies for the development of malignancy have been sought in the processes of normal aging and in the differentiation of cells in the hematopoietic system *(22, 23)*. The incidence of many cancers increase with age because of increased probability of DNA changes that may allow occurrence of a malignant cell and because some of the alterations associated with normal aging increase the susceptibility of cells to carcinogenic events. In normal aging, there is a decrease in DNA repair capacity and a decline in cellular immune reactivity that could contribute to permitting malignant growth *(22)*. Normal hematopoiesis, the formation of the many cell types in blood, is a process of development, self-renewal through mitosis, and differentiation of hematopoietic stem cells, the source cell of all blood cell lineages *(24)*. Because most blood cells have relatively short lifespans, hematopoietic stem cells continuously replicate themselves through self-renewal to prevent depletion of the stem cell pool while simultaneously differentiating into multiple lineages of the varied blood cell types. The fate choice of hematopoietic cells to either self-renew or differentiate is controlled by intrinsic mechanisms and extrinsic signals from the environment or the stem cell niche *(25)*. In adults, the hematopoietic stem cell number is relatively constant under normal conditions. Bone marrow hematopoietic stem cells appear quiescent; however, the majority divide regularly as shown by their slow constant incorporation of radio-labeled nucleotides *(26, 27)*. There are two proposed mechanisms by which asymmetric cell division may be achieved called divisional asymmetry and environmental asymmetry. In divisional asymmetry, specific cell fate determinants in the genome, RNA, and proteins are distributed unequally during cell division. After cell division, only one daughter cell receives the determinants, thus retaining the hematopoietic stem cell fate while the other daughter differentiates.