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Cancer Stem Cells and Self-renewal

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Abstract

The cancer stem cell (CSC) or cancer-initiating cancer (C-IC) model has garnered considerable attention over the past several years since Dick and colleagues published a seminal report showing that a hierarchy exists among leukemic cells. In more recent years, a similar hierarchical organization, at the apex of which exists the CSC, has been identified in a variety of solid tumors. Human CSCs are defined by their ability to: (i) generate a xenograft that histologically resembles the parent tumor from which it was derived, (ii) be serially transplanted in a xenograft assay thereby showing the ability to self-renew (regenerate), and (iii) generate daughter cells that possess some proliferative capacity but are unable to initiate or maintain the cancer because they lack intrinsic regenerative potential. The emerging complexity of the CSC phenotype and function is at times daunting and has led to some confusion in the field. However, at its core, the CSC model is about identifying and characterizing the cancer cells that possess the greatest capacity to regenerate all aspects of the tumor. It is becoming clear that cancer cells evolve as a result of their ability to hijack normal self-renewal pathways, a process that can drive malignant transformation. Studying self-renewal in the context of cancer and CSC maintenance will lead to a better understanding of the mechanisms driving tumor growth. This review will address some of the main controversies in the CSC field and emphasize the importance of focusing first and foremost on the defining feature of CSCs: dysregulated self-renewal capacity. *Clin Cancer Res; 16(12); 3113–20. ©2010 AACR.*

Evidence for the Existence of Cancer Stem Cells

The initial publication in acute myeloid leukemia showed that only a small subset of $CD34^{+}CD38^{-}$ cells harbored serial leukemic transplantation potential, whereas the bulk of leukemic cells did not (1, 2). This discovery revealed for the first time, that a defined subset of leukemia cells was solely responsible for propagating the disease. Of equal importance, this finding argued against the conventional stochastic model of cancer that predicted that all cells within a cancer have equal potential to propagate the malignancy (3–6). It is important to appreciate that both models are predicted on only a small subset of cancer cells being capable of maintaining the tumor, the main difference being that in the cancer stem cell (CSC) model these cells could be prospectively isolated on the basis of a specific cell surface phenotype. In contrast, according to the stochastic model the cancer cells capable of maintaining the tumor are governed by entry into the cell cycle, a low probability stochastic event that renders it impossible to prospectively identify the tumorigenic subset (3–6). A decade following the initial prospective isolation of leukemia stem cells, Al-Hajj and colleagues showed that human breast cancers also adhere to the hierarchical or CSC model (Fig. 1; ref. 7). The initial publications in leukemia and breast cancer were followed by reports showing the prospective isolation of CSCs in numerous malignancies including: brain (8), colon (9–11), head and neck (12), pancreatic (13, 14), melanoma (15), mesenchymal (16), hepatic (17), lung (18), prostate (19), and ovarian (20) tumors (Table 1). It is essential to appreciate that the field of solid tumor CSC research remains at a nascent stage compared with the leukemia stem cell (LSC) field, and, therefore, our understanding of solid tumor CSCs and their significance is a work in progress. Preliminary evidence suggests that some, but not all, cancers are organized in a hierarchical manner (21, 22). However, there are a number of caveats of the CSC model that need to be addressed before the concept of CSCs and the

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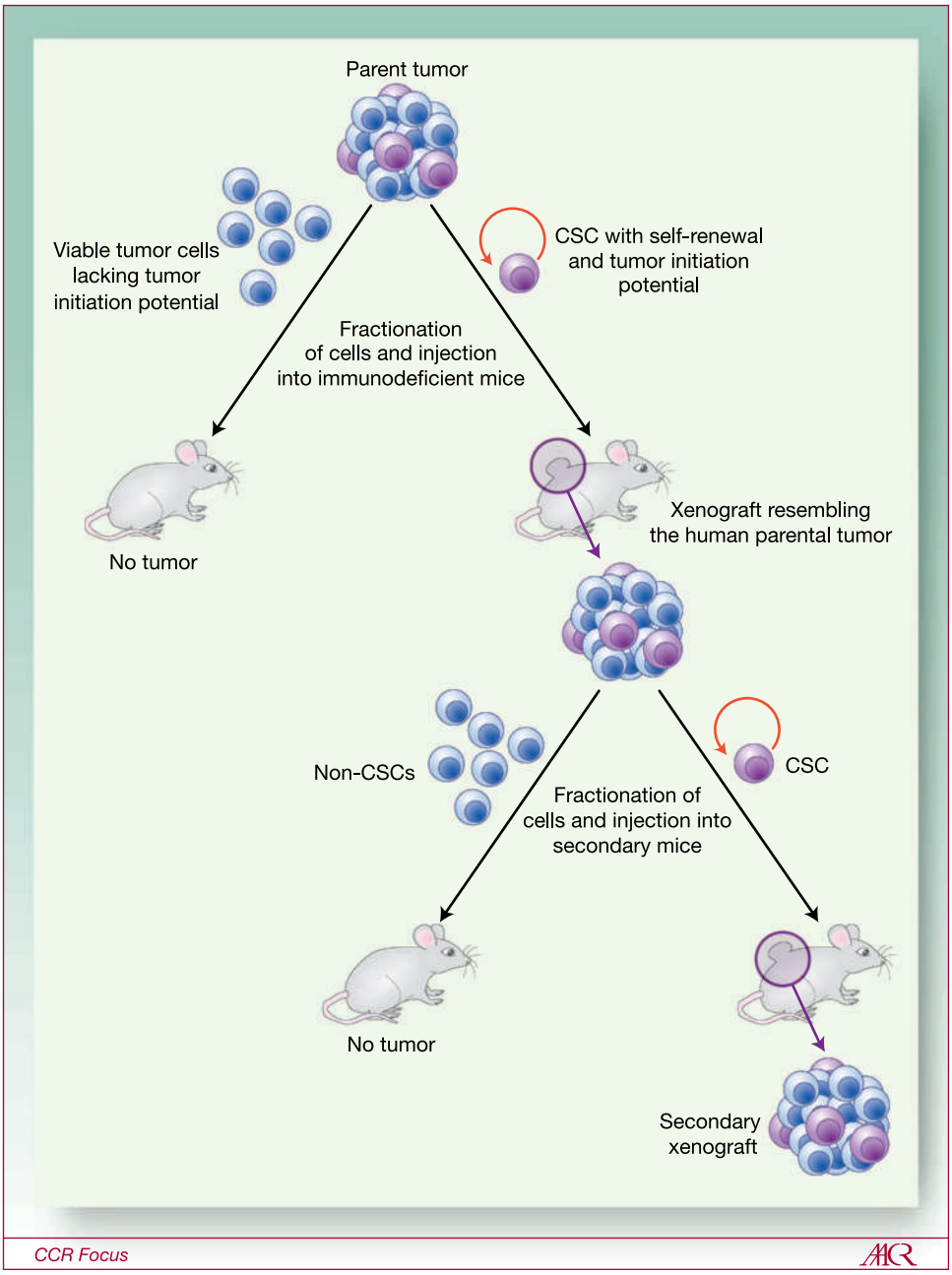

Fig. 1. Features of human CSCs as assayed in immunodeficient mice. Hierarchically organized tumors possess CSCs (in purple) that can be fractionated from the bulk non-CSC population (in blue) and then injected into immunocompromised mice to assess xenograft formation. Injection of CSCs yields tumors, whereas injection of viable tumor cells that lack the properties of CSCs will not produce a significant tumor mass. To determine whether the xenograft has reestablished a hierarchy, it is necessary to separate the CSCs from the bulk of the xenograft and reinject the cells into secondary recipients. Because only the CSC possesses long-term self-renewal capacity, it will regenerate the tumor, whereas injection of non-CSCs will not reinitiate tumor growth.

hierarchical organization of cancer can be widely accepted as a biologically and clinically relevant entity.

Assay Parameters and Experimental Methods

In order to prove that a particular marker enriches for CSC activity, *in vivo* limiting dilution assays (LDA) must be done with both the tumor-initiating and non-tumor-initiating fractions; additional attention must be paid to the latter in order to ensure that the injected cells are viable tumor cells (23). This step is critical because if only 10% of the non-tumor-initiating cells injected are malignant cells and the remainder represent contaminating fibroblasts or hematopoietic cells, it would be difficult

to draw any conclusions about tumor-initiating capacity (22, 24). The main purpose of an LDA is to estimate the active cell frequency, and in recent years it has been commonly used to estimate CSC frequencies. An equally important aim of an LDA is to confirm the validity of the single-hit hypothesis; by applying a goodness-of-fit test to the data set the LDA determines if there are any cooperating effects between tumor cells. If a data set fails the goodness-of-fit test, it suggests that multiple cells, as opposed to a single CSC, are required to generate a tumor thereby making it impossible to calculate an active cell frequency (23). To determine the goodness of fit of the data, an LDA should include a wide range of dilutions, with a moderate to large number of replicates per dose. In addition, an LDA should ideally include doses that have both positive and negative results. Surprisingly, this type of thorough *in vivo* LDA testing has been carried out for relatively few solid tumors (23). The field is becoming further complicated by the increased use of sphere-forming *in vitro* LDAs as a surrogate for the gold standard *in vivo* LDA (25, 26). Notably, the sphere assay can complement, but does not replace an *in vivo* LDA, a standard requirement in CSC research.

A thorough review of statistical methods is outside the scope of this review; however, a recent publication by Hu and colleagues addresses the use of LDAs in stem cell research and some of the common misconceptions and limitations of the assay system. Furthermore, they describe a web-based tool "ELDA" (extreme limiting dilution analysis), which is available on the Walter and Eliza Hall Institute of Medical Research web site, and can be used by researchers to calculate LDA frequencies (23). Traditional LDA statistical programs were not designed to compare subpopulations that are depleted and enriched for active cells; in contrast, ELDA has the capacity to calculate frequencies for subpopulations that produce 0% or 100% positive results, a tool that is invaluable in CSC research (23). Understanding statistical approaches and experimental design is an essential aspect of critically assessing CSC research; programs such as ELDA make these tools available to all researchers and should be used as a standard in the field.

Another limitation of the field is that even studies that have been designed and executed using proper techniques typically study relatively few tumor samples. This has led to some controversy because as an increasing number of tumor subtypes and cell surface markers are being tested it is evident that the CSC phenotype is becoming increasingly complex (21, 22). For example, initial work in brain tumors identified CD133 as a robust marker of CSCs (8, 27). However, additional studies have shown that CD133 identifies CSCs in some specific brain tumor subtypes rather than all subtypes (28, 29). Similar results are emerging with colon cancer in which combinations and permutations of CD133, CD44, CD166, as well as aldehyde dehydrogenase-1 (ALDH1) have been published with conflicting results about which marker or combination thereof best identifies the CSC population (9–11, 30, 31). Although these results have led to some consternation in the field, they should be viewed more as an indication of the complexity of the system and how early we are on the road to understanding which cancers are organized as a hierarchy and thus, correspond to the CSC model.

Table 1. Identification of CSCs in tumors using various markers

Tumor Type	Marker(s) Used to Enrich for CSCs	Reference
Acute myeloid leukemia	$CD34^+CD38^-$	1, 2
Breast	$CD44^+$ $CD24^-$	7
Breast	$ALDH1^+$	39
Brain	$CD133^+$	8
Prostate	$CD44^+$ $\alpha_2\beta_1^{high}$ $CD133^+$	19
Head and neck	$CD44^+$	12
Colon	$CD133^+$	9, 10
Colon	$EpCAM^{high}$ $CD44^+$	11
Colon	$ALDH1^+$	31
Pancreas	ESA^+CD44^+ $CD24^+$	13
Pancreas	$CD133^+$	14
Mesenchymal	Side population	16
Lung	$CD133^+$	18
Liver	$CD90^+$	17
Melanoma	$ABCB5^+$	15
Ovarian	$CD133^+$	20

Immunodeficient Mouse Models and Cancer Stem Cell Research

To date most of the publications in the CSC field have focused on phenotypic marker identification. More recently the role of the level of immunodeficiency of the xenograft model system commonly used in CSC work has come into focus (24). Traditionally CSC work has been carried out using severe combined immunodeficient (SCID) or nonobese diabetic SCID (NOD/SCID) mice. However, recent work by Quintana and colleagues showed that, at least in the case of melanoma, use of NOD/SCID mice can lead to an underestimation of the frequency of human cancer cells with tumorigenic potential (24). By carrying out thorough LDAs, they determined that although only approximately 1 in 1,000,000 melanoma cells can generate xenografts in NOD/SCID mice, 1 in 4 generate xenografts when injected together with matrigel into NOD/SCID interleukin-2 receptor gamma chain null (IL2Rγnull) lacking T, B, and NK cells. This work suggests that transplantation of less immunodeficient NOD/SCID mice underestimates CSC frequency and that not all cancers adhere to a CSC hierarchy (24). The inverse relationship between the CSC frequency and the immunocompetency of the murine xenograft model has led

critics of the research to question whether CSC work is simply selecting for a cell subset that is capable of surviving in immune-compromised mice. One method that researchers are using to address this criticism is by identifying CSC fractions in transgenic mouse models of cancer. These models allow for syngeneic transplantation of specific cell subsets and therefore eliminate the cross-species barriers to engraftment.

In addition to studying CSCs in syngeneic murine systems, there is also a need to understand the role of immune surveillance in the context of the xenograft system, a concept that was addressed in two recently published reports by Jaiswal and colleagues and Majeti and colleagues (32, 33). They showed that human CSCs transplanted into immunocompromised mice could evade detection and eradication by the innate immune system through CD47 overexpression, underscoring the importance of both innate and adaptive immunity in eradication of tumor propagating cells (32, 33). However, these studies may also imply that immune cells play an essential role in defining CSCs. It is well established that the immune system plays a pivotal role in a number of solid tumors (34); whether this role includes enabling or inhibiting the capacity of a CSC to self-renew remains to be determined.

Cancer Stem Cells and Murine Models of Cancer

The use of syngeneic transplantation studies carried out in murine models of leukemia and lymphoma has provided some important insights into the CSC field. Strasser and colleagues reported that as few as 10 cells derived from three separate Eμ-myc transgenic mice could transplant lymphoma within 35 days suggesting that high myc expression abrogates the CSC hierarchy and endows a high proportion of cells with lymphoma-initiating capacity (35). In contrast, other murine leukemia models do show a hierarchical organization, such as the MOZ-TIF retroviral transduction-transplantation model, in which the LSC frequency was calculated to be 1 in 1×10^4 (36). Similar results were obtained in a PTEN deletion model of acute myelogenous leukemia in which only 1 in 6×10^5 leukemic cells could maintain the clone (37). These murine studies involved syngeneic transplants and therefore eliminated any immunological or microenvironmental barriers. The results support the notion that whereas some cancers are organized as a hierarchy others are not.

There is emerging evidence in solid tumors that a similar CSC hierarchy exists in some murine models. One such example is a Patched-1-deficient mouse model that preferentially gives rise to medulloblastomas. In this model the normal neural stem cell surface antigen CD15 enriches for the *in vitro* proliferative and *in vivo* tumorigenic fraction from primary murine medulloblastoma cells. Using a syngeneic orthotopic injection model, a dose of 10^4 $CD15^-$ or unsorted cells both failed to generate xenografts, whereas 10^4 $CD15^+$ cells yielded five xenografts out of six injections (38).

Three genetically distinct murine models of mammary cancer have been tested to determine if they subscribe to the CSC model. In the MMTV-Wnt1 murine model a $THY1^+CD24^+$ cancer cell population, representing 1 to 4% of total tumor cells, was found to be highly enriched for tumorigenic activity in comparison to the $THY1^-CD24^-$ population. It was estimated that 1 in every 200 $THY1^+CD24^+$ cancer cells represented a CSC, defined by its ability to histologically recapitulate the parent tumor and be serially passaged in an orthotopic syngeneic model system (39). The TRP53-null mammary tumor model was also found to be organized in a hierarchical fashion, and could be fractionated into CSC and non-CSC subsets on the basis of the expression of $\beta1integrin^{hi}CD24^+$ (40). Interestingly, a CSC subset could not be identified in the MMTV-ErBB2 mouse, despite having tested multiple markers (41). These tumors are typically characterized by their homogenous appearance, and LDAs carried out on bulk cancer cells indicate that the frequency of cancer cells capable of self-renewal is very high at approximately 1 in 100 (41). These results show that some but not all transgenic mouse models generate hierarchically organized cancers (21). However, some researchers question how applicable transgenic murine models of cancer are to the actual human disease. There is one published study that used a chemical carcinogen (DMBA-TPA) to generate cutaneous tumors in mice, thereby avoiding any concern associated with transgenic murine models. They identified that the cell surface antigen CD34 could enrich 100 fold for CSC activity in an orthotopic syngeneic transplant model (42). The above examples show the valuable contribution murine models have already made to supporting the existence of CSCs. Moving forward their role will be essential as we try to understand the factors governing how CSCs interact with the microenvironment and immune system.

Cancer Stem Cells and Self-renewal Pathways

It is becoming evident that the identification of CSC cell surface phenotypes can only take the field so far. Only through achieving a better understanding of the self-renewal pathways fueling CSC propagation will we start to grasp the functional nature of these cells (Table 2). Although a number of mouse transgenic studies have shown the importance of self-renewal pathway activation for CSC maintenance (37, 43), few studies have shown this explicitly using human CSC xenograft models. Jamieson and colleagues were one of the first groups to show the importance of a self-renewal pathway in maintaining LSCs (44, 45). They identified aberrant Wnt/β-catenin self-renewal pathway activation to be the driving force in human blast crisis LSC propagation (44, 45). More recently, increased Wnt/β-catenin signaling has also been implicated in the maintenance of breast CSCs (26). The authors showed that the genetic knockdown of PTEN both enriches for

Table 2. Pathways involved in CSC Self-renewal

Pathway	Cancer	Reference
WNT	Breast cancer	26
	CML, AML	37, 38, 47
Hedgehog	Breast cancer	25
	Pancreatic cancer	13
	Glioblastoma	45, 46
	CML	43, 44
	Colon cancer	47
Notch	Colon cancer	49
	Breast cancer	49, 50
	Glioblastoma	51
BMI1	Murine acute myeloid leukemia	35
	Breast cancer	25
	Head and neck squamous cell cancer	12
	Glioblastoma	56
	Acute myeloid leukemia	55
PTEN	Murine leukemia	36
	Breast cancer	26
BMP	Glioblastoma	53
TGF-β	Glioblastoma	52

Abbreviation: BMP, bone morphogenic protein.

breast CSC markers and increases tumorigenicity in a xenograft model. The effect of PTEN knockdown on CSCs ($ALDH1^+$) was mediated by activation of Akt signaling, which resulted in an increase in Wnt/β-catenin activity (26, 46). This work also exemplifies the potential cooperative effect between distinct self-renewal pathways, such as PTEN and Wnt. It is plausible and likely probable that multiple dysregulated self-renewal pathways are functioning to maintain the CSC subset. Our understanding of Wnt activation in the context of CSCs remains at an early stage, however, it is evident from preliminary work that the Wnt pathway plays a critical role in the initiation and maintenance of CSCs (26, 44, 45, 47).

Another known regulator of self-renewal in the context of embryogenesis is the sonic hedgehog (Hh) signaling pathway. Yet little is known about its role in adult stem cells and CSCs (48–50). The preferential expression of Hh in CSCs was first published in a pancreatic cancer xenograft model (13). Recently, the Hh pathway has also been implicated in maintaining human LSCs (51, 52). Loss of the Hh pathway component, smoothened (Smo), resulted in depletion of the chronic myeloid leukemia (CML) stem cell subset. Moreover, the constitutive activation of Smo resulted in an increased number of CML stem cells and acceleration of the disease (52). There is emerging evidence that the Hh pathway has been aberrantly activated in a number of solid tumor CSC models including: breast (25), glioblastoma (53, 54), and colon (55), providing the impetus for a plethora of early phase clinical trials aimed at expunging CSCs.

The Notch pathway is also known to play a critical role in stem cell growth and differentiation (56). Recent work by Hoey and colleagues showed that the Notch pathway is also activated in the colon CSC subset (57). Using antibodies targeting Delta-like 4 ligand (DLL4), an important component of the Notch pathway, they were able to inhibit the growth of human colon cancer xenografts. One of the mechanisms by which the DLL4 antibody inhibited tumor growth was by directly modulating Notch signaling in the CSC-enriched population (57). Notch pathway activation has also been identified in breast (57, 58) and glioblastoma (59) CSC models.

A myriad of additional pathways such as transforming growth factor β (TFG-β; ref. 60) and bone morphogenetic protein (61) have been shown to influence CSC initiation and maintenance. Another example is the polycomb group member B lymphoma Mo-MLV insertion region-1 (BMI-1), which has a well established role in self-renewal (62). BMI-1 is preferentially expressed in head and neck CSCs (12) and the genetic knockdown of BMI-1 has been shown to impair CSC self-renewal capacity in hematopoietic (43, 63), breast (25), and brain (64) xenograft models.

Cancer Stem Cells, Self-renewal, and Therapeutics

Identifying and understanding the role of individual self-renewal pathways in maintaining CSCs is the first step. However, the eventual goal is to generate targeted therapeutics that inhibit these essential pathways in the CSC fraction. The targeting of these pathways will likely be complicated by the fact that the same pathways are also pivotal in normal stem cell function. There is preliminary evidence in leukemia models to suggest that there may be subtle differences between how these pathways are operating in malignant versus normal stem cells. One such example is the compound parthenolide, an agent that selectively targets LSCs, and has no known deleterious effect on the normal hematopoietic stem cells (HSC; refs. 65, 66). Rapamycin was also found to selectively target LSCs in a Pten deletion murine model of leukemia while at the same time improving normal HSC function (37). Similarly, targeted small molecule inhibition of the sonic hedgehog pathway combined with BCR-ABL inhibition has been shown to markedly reduce CML stem cell propagation (51, 52). These studies emphasize the importance of defining the cell type and context-specific effects of self-renewal pathway inhibition to eliminate CSCs while limiting the effect on normal stem cells.

Cancer Stem Cells and the Microenvironment

Identifying the relevant self-renewal pathways driving CSCs will also enable us to better understand the role of the microenvironment in initiating and maintaining

CSCs. More than 100 years ago Paget proposed his seed and soil hypothesis to explain why particular cancers preferentially metastasize to certain organs, such as colon cancer metastasizing to the liver (67–69). It is plausible that the receptor-ligand interactions between the tumor and local microenvironment govern the activation of specific self-renewal pathways in a particular CSC, thereby allowing it to initiate tumor growth at a distant site (70). Heeschen and colleagues published work showing that for a xenograft model of human pancreatic cancer the phenotype for the CSC subset that could give rise to a metastatic deposit differed from that which gave rise to tumor at the orthotopic site, $CD133^+$ $CXCR4^+$, and $CD133^+$, respectively (14). This shows that the microenvironment, in part, determines which cancer cell possesses the capacity to self-renew. Furthermore, this work illustrates that much remains to be learned about the signals that a CSC receives from the microenvironment and the role it plays in driving CSC self-renewal (Fig. 2).

The importance of the microenvironment and in particular tumor-associated stromal cells is best illustrated in elegant studies carried out by Yauch and colleagues on the Hh pathway (71). They showed that inhibition of Hh signaling in pancreatic cancer-associated stromal cells resulted in the suppression of tumor growth. In contrast, inhibition of Hh signaling in the pancreatic cancer cells themselves did not affect the tumor (71). This suggests that in some tumors paracrine, as opposed to autocrine or endogenous Hh signaling, is essential for maintaining tumor growth. The exact mechanism by which Hh pathway inhibition in the stromal microenvironment suppresses tumor growth remains to be determined. Adding further complexity to the field, there is some evidence to suggest that the inhibition of the Hh pathway in stromal cells can lead to changes in Wnt pathway components (71). Recent publications have established that Hh signaling is activated in leukemic (51, 52), breast (25), brain (53, 54), and colon (55) CSCs, however, the role of cancer associated stromal cells in initiating and maintaining these CSCs remains to be determined. One hypothesis is that a cancer cell's ability to function as a CSC depends on whether it possesses the ligand-receptor required to respond to the self-renewal signals being emitted by the surrounding stroma. If the possession of this ligand-receptor is proven to be the case, it may help to explain the predilection of CSCs for specific metastatic sites, because the self-renewal pathways that are used by an individual CSC will depend on the microenvironment of the organ in which it exists.

Summary

Compelling evidence in both xenograft and murine models supports the existence of CSCs in some but not all cancers. Furthermore, it has become apparent that the current understanding of CSCs is rather simplistic and much work is required to fully appreciate the complexity

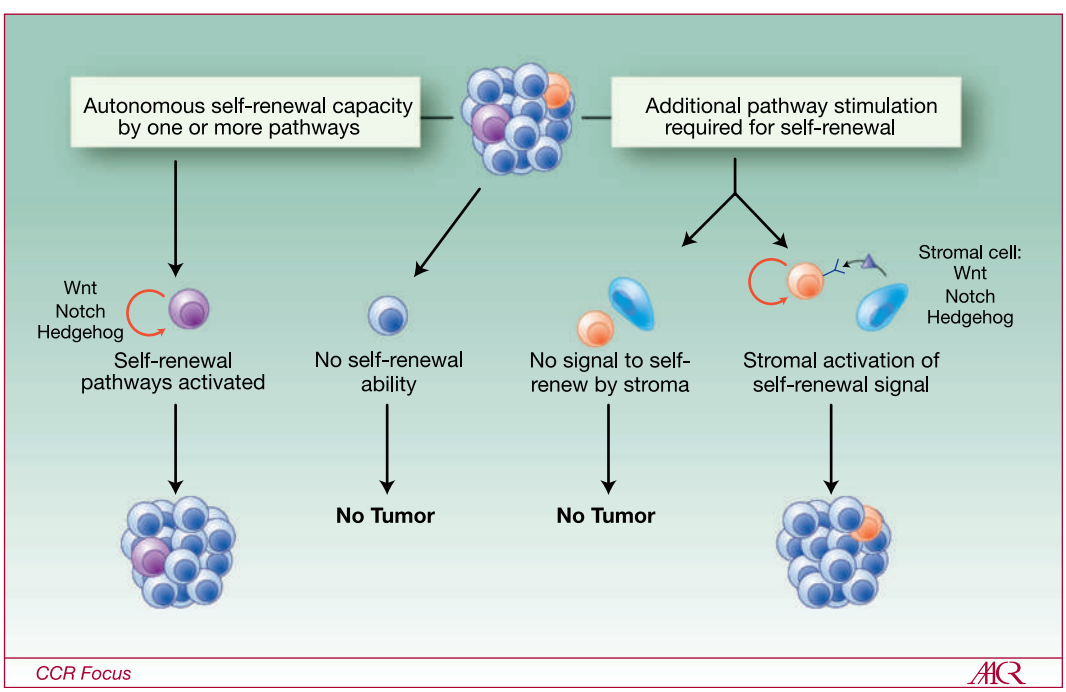

Fig. 2. Multiple facets to CSC self-renewal. Increasing evidence is emerging to support the notion that CSC self-renewal decisions can be guided by the activation of several pathways, including Wnt, Notch, Hedgehog, and others. A CSC may autonomously trigger the appropriate signaling cascade to maintain self-renewal with minimal niche support. It is likely that some CSCs need the appropriate microenvironment to provide the stimuli for uncontrolled self-renewal. Finally, some cancer cells have lost the capacity to self-renew regardless of stimulating molecules, and hence cannot initiate a tumor.

of the hierarchical organization of some cancers. Studying the functional biology of CSCs and more specifically the self-renewal pathways driving CSC regeneration is essential because it will provide insight into how these cells initiate and maintain tumor growth. A better understanding of the functional biology of these cells will also require studying the microenvironment in which they exist and the role of this interaction in maintaining and possibly defining which cancer cells can function as CSCs.

The CSC concept has generated a great deal of interest because of the potential clinical implications of these cells. The CSC model suggests that the route to eradicating a tumor will require agents that expunge the root cause of the cancer: CSCs (72, 73). This route will likely prove to be challenging because the same self-renewal pathways driving CSCs are also essential in maintaining normal stem cells. However, there is preliminary evidence in LSCs that indicates there are subtle differences in how CSCs and normal stem cells use the same pathways (37, 66). If CSCs are to be successfully targeted in the clinical setting, it will require a thorough understanding of how these pathways function in both normal and malignant cells and the development of targeted agents to exploit these differences.

Disclosure of Potential Conflicts of Interest

C. Jamieson, research grants from Pfizer, Bristol-Myers Squibb, Coronado Biosciences, Celgene. The other authors disclosed no potential conflicts of interest.

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