The evolving concept of cancer and metastasis stem cells

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The cancer stem cell (CSC) concept, which arose more than a decade ago, proposed that tumor growth is sustained by a subpopulation of highly malignant cancerous cells. These cells, termed CSCs, comprise the top of the tumor cell hierarchy and have been isolated from many leukemias and solid tumors. Recent work has discovered that this hierarchy is embedded within a genetically heterogeneous tumor, in which various related but distinct subclones compete within the tumor mass. Thus, genetically distinct CSCs exist on top of each subclone, revealing a highly complex cellular composition of tumors. The CSC concept has therefore evolved to better model the complex and highly dynamic processes of tumorigenesis, tumor relapse, and metastasis.

Introduction

Cancer remains one of the leading causes of mortality worldwide (Jemal et al., 2011). In the United States, 5-yr survival rates only increased from 50% in 1974–1976 to 68% in 1999–2006, underlining how much more progress is needed to understand and successfully treat this disease (American Cancer Society, 2011). Over the past decade, the concept of the cancer stem cell (CSC) has emerged after identification and characterization of CSC-enriched populations in several distinct cancer entities (Table 1; Lapidot et al., 1994; Reya et al., 2001; Trumpp and Wiestler, 2008). Although the concept remains controversial (Kelly et al., 2007; Quintana et al., 2010; Magee et al., 2012), new observations from clinical studies and basic research have led to a more comprehensive CSC model of tumorigenesis, tumor recurrence, and metastasis formation. The aim of this review is to illustrate the current dynamic view of CSCs to foster the development of better therapeutic approaches to target this highly complex and deadly disease.

The classical concept of CSCs

Adult regenerating tissues (such as the skin, the gastrointestinal mucosa, or the hematopoietic system) are hierarchically organized (Murphy et al., 2005; Fuchs and Nowak, 2008; van der Flier and Clevers, 2009; Seita and Weissman, 2010). At the top of the cellular organization, normal adult stem cells maintain tissues during homeostasis and facilitate their regeneration, for example in response to infection or to cell loss due to injury. These physiological stem cells are defined by their functional properties: they have the life-long capacity to self-renew (the ability to give rise to a new stem cell after cell division), are multipotent, and can reversibly enter quiescent or even dormant states and resist cytotoxic drugs (Fuchs and Nowak, 2008; Wilson et al., 2008; van der Flier and Clevers, 2009; Seita and Weissman, 2010). Similar to regenerative tissues, many tumors follow a hierarchical organization, and like physiological stem cells, CSCs are defined by a series of functional traits (Fig. 1; Reya et al., 2001; Dick, 2008; Clevers, 2011; Nguyen et al., 2012).

Universal CSC functional traits

At the helm of tumor hierarchy. First, CSCs can generate all cell types present in a tumor. Located at the top of the tumor hierarchy, CSCs can self-renew and also generate non-CSC progeny, which form the tumor bulk (differentiated progeny). Hierarchical organization of tumors, governed by CSCs, have been reported for many tumor types including germ cell cancers (Illmensee and Mintz, 1976), leukemia (Lapidot et al., 1994; Bonnet and Dick, 1997), breast cancer (Al-Hajj et al., 2003; Ginestier et al., 2007), brain cancer (Singh et al., 2004), colon cancer (Dalerba et al., 2007; O'Brien et al., 2007; Huang et al., 2009), pancreatic cancer (Hermann et al., 2007; Li et al., 2007), melanoma (Schatton et al., 2008; Boiko et al., 2010), and several others (Table 1).
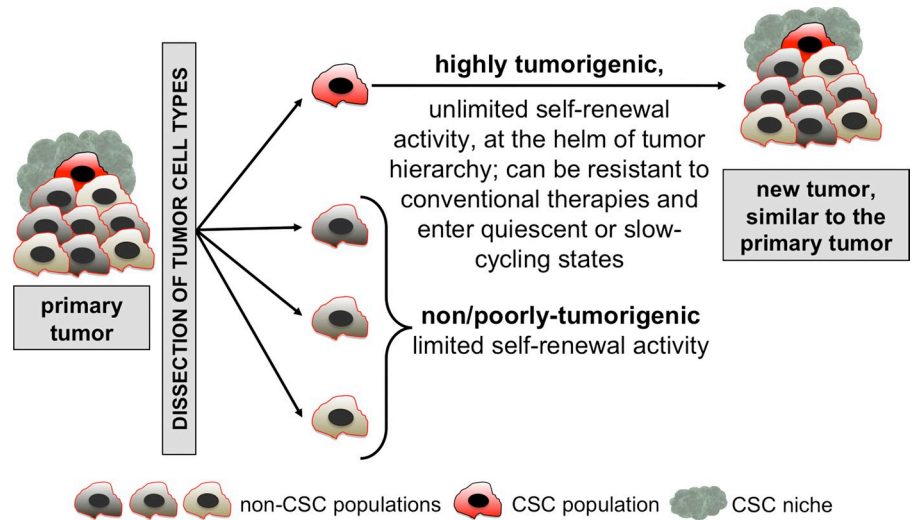
Unlimited self-renewal potential. In striking contrast with their differentiated progeny, CSCs can undergo unlimited

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Figure 1. **The classical "cancer stem cell" (CSC) concept.** Tumors are heterogeneous and hierarchically organized entities. Upon dissociation and transplantation into an immunocompromised animal, human CSCs can be functionally distinguished from non/poorly tumorigenic cell populations by their ability to reinitiate and grow a similar heterogeneous tumor in vivo.

DISSECTION OF TUMOR CELL TYPES

primary tumor

highly tumorigenic, unlimited self-renewal activity, at the helm of tumor hierarchy; can be resistant to conventional therapies and enter quiescent or slow-cycling states

non/poorly-tumorigenic limited self-renewal activity

new tumor, similar to the primary tumor

non-CSC populations   CSC population   CSC niche

self-renewing divisions. Typically, the presence of human CSCs within a cell population is experimentally addressed by serial transplantation of tumor cells into immunocompromised mice or rats (Table 1). Although considered state-of-the-art, this assay has limitations and only imperfectly recapitulates the in vivo situation found in patients. Indeed, the immunocompromised mouse models lack an adaptive immune system (neither mouse nor human) and express cytokines/chemokines and other environmental components of mouse origin, such as the tumor vasculature. Furthermore, the detection and enumeration of functional CSCs by these methods remains highly assay dependent, as several different immunocompromised mouse strains and many methods of tumor dissociation and implantation exist (Quintana et al., 2008; Ishizawa et al., 2010; Civenni et al., 2011). Nevertheless, human CSCs cannot be simply reduced to technical artifacts because of their detection in xenografts. Indeed, mouse CSCs have also been reported in syngenic mouse models of leukemia (Deshpande et al., 2006; Krivtsov et al., 2006; Yilmaz et al., 2006), breast cancer (Cho et al., 2008; Vaillant et al., 2008; Zhang et al., 2008), and skin cancer (Malanchi et al., 2008), providing strong evidence that CSCs govern many tumor types.

Other CSC functional traits
Quiescent or slow-cycling states. Although cellular quiescence does not seem to be a universal feature, some CSCs have been reported to shuttle between quiescent, slow-cycling, and active states, similar to the behavior of many adult stem cell types (Wilson et al., 2008; Essers et al., 2009; Fuchs, 2009; Tian et al., 2011). For example, the presence of quiescent leukemic stem cells (LSCs) has been reported in a mouse model for acute myeloid leukemia (AML; Ishikawa et al., 2007; Saito et al., 2010). Moreover, using clonal tracking techniques, delayed-contributing CSC clones were identified both in AML and in colon carcinoma, which suggests the existence of long-term quiescent/dormant pools of human CSCs (Hope et al., 2004; Dieter et al., 2011). In line with these findings, a study showed a strong correlation between the number of slow-cycling breast cancer cells, as measured by retention of the membrane

dye PKH26 in mammosphere cultures, and the frequency of CSCs, assessed by tumorigenicity assay in mice (Pece et al., 2010). Of major importance, quiescence or slow cycling states might render CSCs less likely to be responsive to conventional therapies, which mainly target cycling cells. Moreover, quiescent normal stem cells can reenter the cell cycle after injury to repair a tissue (Wilson et al., 2008; Essers et al., 2009; Essers and Trumpp, 2010; Saito et al., 2010; Seita and Weissman, 2010). In agreement with this stem cell feature, it has been hypothesized that potential reactivation of quiescent CSCs might induce tumor relapse, which sometimes occurs decades after completion of therapy (Aguirre-Ghiso, 2007; Pantel et al., 2009).

Increased resistance to conventional therapies. Similar to physiological stem cells, some CSCs were reported to exhibit remarkable resistance to conventional therapies. For instance, breast CSCs were found to accumulate in women with locally advanced tumors after cytotoxic chemotherapy had eliminated the bulk of the tumor cells (Li et al., 2008). Similarly, in chronic myeloid leukemia (CML), BCR-ABL–driven LSCs are resistant to tyrosine kinase inhibitors (TKIs) such as Imatinib, whereas these compounds eliminate the rest of the leukemic cells, often even achieving a complete molecular response (undetectable levels of BCR-ABL mRNA by RT-PCR; Goldman et al., 2009; Oravecz-Wilson et al., 2009; Goldman, 2010; Perrotti et al., 2010). Accordingly, during STOP trials, in which TKI treatments are discontinued, tumor relapse was observed in the majority of patients. This was most likely caused by new tumor cell production by resistant CML-LSCs, as the relapsed "non-LSCs" leukemic cells remained sensitive to the initially used TKI (Barnes and Melo, 2006; Druker et al., 2006; Ross et al., 2011). Another case of resistant CSCs was reported for myelodysplasia carrying a 5q deletion. Although the majority of tumor cells were efficiently targeted by treatment with the immunomodulator Lenalidomide, leading to a complete clinical and cytogenic remission, most patients relapsed because of the outgrowth of remaining resistant CSCs (Tehranchi et al., 2010).

CSC resistance might first of all be caused by increased drug efflux capacities, mediated by expression of multidrug

Table 1. **Identification of human primary tumor CSC biomarkers using in vivo assays**

Cancer	Animal	Type of injection	Treatment of recipient mice	Injection with Matrigel	Percentage of CSC-enriched population in tumor	Biomarkers	Minimal number of biomarker + cells to obtain a tumor	Reference
ALL (B-ALL)	NOD/SCID/IL2rγc$^{-/-}$ newborns	Intravenous	Sublethal irradiation	No	82.50%	CD34$^+$/CD19$^+$	2–6 × 10^4	Kong et al., 2008
AML	NOD/SCID	Intravenous	Sublethal irradiation	No	0.75%	CD34$^+$/CD38$^-$	2 × 10^5	Bonnet and Dick, 1997
AML	NOD/SCID, NOD/SCID/β$_2$m$^{-/-}$ and NOD/SCID/ IL2rγc$^{-/-}$	Intravenous and intrabone	IVIG of CD122 pretreatment and sublethal irradiation	No	0.076% (*)	CD34$^+$/CD38$^-$ (*) or CD34$^+$/CD38$^+$ (**) (in samples with lowest CD34$^+$/CD38$^-$ fraction)	7.5 × 10^3 (*) or 10^6 (**)	Taussig et al., 2008
AML	NOD/SCID	Intrafemoral	IVIG of CD122 pretreatment and sublethal irradiation	No	0.06–0.00009% of bulk	NA	NA	Eppert et al., 2011
Bladder	Rag2γcDKO	Intradermal	NA	Yes	3–36.3%	CD44	100	Chan et al., 2009
Breast	NOD/SCID	Mammary fat pad	VP-16, estrogen pellets	No	11–35%	ESA$^+$/CD44^{high}/CD24$^{low-neg}$	200	Al Hajj et al., 2003
Breast	NOD/SCID	Humanized mammary fat pad	Estrogen pellets	Yes	3–10%	ALDH-1$^+$	500	Ginestier et al., 2007
Brain	NOD/SCID	Intracranial	NA	No	6–29%	CD133$^+$	100	Singh et al., 2004
Colorectal	NOD/SCID	Renal capsule	Sublethal irradiation	Yes	1.8–24.5%	CD133$^+$	100	O'Brien et al., 2007
Colorectal	NOD/SCID	Subcutaneous	NA	No	2.60%	ESA^{high}/CD44$^+$	200	Dalerba et al., 2007
Colorectal	NOD/SCID	Subcutaneous	NA	Yes	3.50%	ALDH-1$^+$	25 serially passaged	Huang et al., 2009
Head and neck squamous cell carcinoma	NOD/SCID and Rag2γcDKO	Subcutaneous	NA	Yes	10–12%	CD44$^+$	5000	Prince et al., 2007
Liver	SCID	Intrahepatic	NA	No	2.50%	CD45$^-$/CD90$^+$	10^3	Yang et al., 2008
Lung	SCID and NUDE	Subcutaneous, after in vitro expansion	NA	Yes	0.4–1.5%	CD133$^+$	10^4	Eramo et al., 2008
Lung	NOD/SCID/IL2rγc$^{-/-}$	Subcutaneous	NA	Yes	Median 15%	lin-/CD166$^+$	≤500	Zhang et al., 2012
Melanoma	NOD/SCID	Subcutaneous	NA	No	1.6-20.4%	ABCB5$^+$	10^5	Schatton et al., 2008
Melanoma	Rag2γcDKO	Intradermal	NA	Yes	2.5–41%	CD271$^+$	100	Boiko et al., 2010
Melanoma	NOD/SCID/IL2rγc$^{-/-}$	Subcutaneous	NA	Yes	NA	NA	1 (in 28% of cases)	Quintana et al., 2010
Melanoma	NUDE, (NOD/SCID, NOD/SCID/IL2rγc$^{-/-}$)	Subcutaneous	NA	Yes	8–11%	CD271$^+$	1000	Civenni et al., 2011
Pancreatic	NOD/SCID	Subcutaneous and intrapancreatic	NA	Yes	0.2-0.8%	ESA$^+$/CD44$^+$/CD24$^+$	100	Li et al., 2007
Pancreatic	NUDE	Intrapancreatic	NA	No	3.6 cells per high-power field	ESA$^+$/CD133$^+$	500	Hermann et al., 2007

Studies reporting the existence of enriched human CSC populations are listed. In the first five columns, the main parameters influencing the efficiency of tumor engraftment are listed. From left to right: the tumor entity, the type of immunocompromised mouse strain used, the route of transplantation of human tumor cells, preconditioning of the recipient mice, treatment of mice during the assay, and whether the tumor cells were mixed with Matrigel upon transplantation. In the next four columns, the main results of these studies are summarized. From left to right: the frequency of the identified CSC-enriched population observed in the given tumor entity, the biomarkers identified for this CSC-enriched population, and the minimal number of tumor cells expressing these biomarkers able to give rise to a human tumor as well as the reference of the corresponding study. *, results for the CD34$^+$/CD38$^-$ population. **, results for the CD34$^+$/CD38$^+$ population.

resistance (MDR) transporters (Dean et al., 2005). Indeed, cancer cells named "side population," because of their ability to efflux the fluorescent dye Hoechst, have been found to be highly enriched for both normal cells and CSCs (Wulf et al., 2001; Ho et al., 2007; Lou and Dean, 2007; Moshaver et al., 2008). Second, aldehyde dehydrogenase 1 (ALDH-1), a cytosolic enzyme involved in the catalysis of aldehyde oxidation, was reported to be specifically active in several CSCs (Pearce et al., 2005; Ginestier et al., 2007; Huang et al., 2009; Ran et al., 2012). In a retrospective study, ALDH-1 activity was significantly higher in breast cancer metastatic cells, which developed resistance to cyclophosphamide, compared with sensitive cells (Sládek et al., 2002; Douville et al., 2009). This suggests that ALDH-1 might also play a role in cytotoxic drug resistance. Third, genotoxic treatments like ionizing radiation might be evaded by CSCs because of increased DNA damage check point response and DNA repair capacities, as observed in CD133-expressing glioblastoma CSC-enriched populations compared with CD133-negative populations (Bao et al., 2006). Last, CSCs might counterbalance the radiation-induced reactive oxygen species (ROS) production by increased expression of free radical scavengers, as reported for mouse and human breast CSCs (Diehn et al., 2009). This ability might selectively protect CSCs from ROS-mediated DNA damage and hence explain their resistance to irradiation treatments. Importantly, in vitro studies of drug-sensitive tumor cell lines suggest that cancer cells might transiently and reversibly acquire drug resistance, indicating that drug resistance might not always be a stable trait (Sharma et al., 2010).

The CSC niche

The tumor microenvironment is composed of diverse immune cells and stromal cells, as well as extracellular components (Bissell and Hines, 2011; Shiao et al., 2011; Hanahan and Coussens, 2012). CSC functional traits might be sustained by this microenvironment, termed "niche" (Cabarcas et al., 2011). For instance, vascular endothelial cells maintained self-renewal and promoted tumorigenicity of glioma CSCs in a mouse xenograft model (Calabrese et al., 2007). Hypoxia, notably via the hypoxia-inducible factor 2-α (HIF2α), increased glioma CSC self-renewal and tumorigenic capacities (Li et al., 2009b). Moreover, inflammatory molecules such as interleukin 6, secreted by infiltrating immune cells in the tumor, enhanced the proliferation of colitis-associated CSCs (Grivennikov et al., 2009).

The CSC niche might not only regulate CSC traits but might also directly provide CSC features to non-CSCs. For instance, tumor-associated myofibroblasts enhanced self-renewal of colorectal CSCs via hepatocyte growth factor (HGF) production but also strongly enhanced the in vivo tumorigenicity of non-CSCs through their secreted factors (Vermeulen et al., 2010).

In leukemia (Colmone et al., 2008) and in prostate carcinoma (Shiozawa et al., 2011), cancer cells could hijack existing physiological stem cell niches. Although physiological stem cell niches are known to play important roles for quiescence maintenance and resistance to stress-inducing treatments (Ehninger

and Trumpp, 2011; Park et al., 2012), the influence of the tumor niche on CSC function at the cellular and molecular level still remains to be elucidated.

Origin of CSCs

Importantly, the CSC concept has to be separated from the "cell of origin" question: as outlined in the previous sections, CSCs are defined by a series of functional tumor-propagating traits. In contrast, the tumor cell of origin defines the cell type, from which the disease is derived, meaning the cell type first hit by an oncogenic mutation. However, this cell of origin does not necessarily immediately acquire a CSC phenotype (Visvader, 2011). Having said this, CSCs might indeed originate from stem cells, as they already are capable of almost limitless self-renewal. For instance, embryonic stem cells can form teratomas when transplanted subcutaneously in recipient mice (Sell and Pierce, 1994).

Nevertheless, CSCs can also originate from more differentiated progenitors that acquire stemness traits by accumulation of genetic or epigenetic abnormalities. For instance, progenitors derived from stem cells that already carry initiating genetic mutations acquire further mutations during differentiation that will finally lead to transformation. This would mean that the cell of origin for the first mutation is a stem cell, but that the CSC that drives the tumorigenic clone would be a more differentiated progenitor. Such a scenario has been reported for CML. Although the BCR-ABL fusion protein is the first event and is present in hematopoietic stem cell (HSC)-like CML cells (suggesting that the "cell of origin" of the disease is a normal HSC), advanced-stage LSCs during blast crisis were found to be in a state similar to granulocyte/macrophage progenitors (GMPs; Jamieson et al., 2004). Similarly, in AML, the AML-ETO fusion protein is present in HSCs (cell of origin of the disease), but the functional LSCs were detected in a Thy1⁻ progenitor cell state by an in vitro colony forming assay (Miyamoto et al., 2000). However, in acute promyelocytic leukemia (APL), the MLL-AF9 fusion protein was not detected in HSCs (Turhan et al., 1995), but when introduced in mouse GMPs, it could induce leukemia, indicating that both the cell of origin and the LSCs were found in progenitors rather than in HSCs (Krivtsov et al., 2006).

Recent work on induced pluripotent stem cells (iPS) has demonstrated that, contrary to all expectations, the acquisition of self-renewal and pluripotency starting from any cell type can be achieved by activation of as few as four transcription factors (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). Importantly, this reprogramming process requires the transient repression of p53 and INK4a, two of the most frequently mutated tumor suppressor loci in human cancers (Li et al., 2009a; Utikal et al., 2009; Goh et al., 2011; Romagosa et al., 2011). Similarly, loss of the same tumor suppressors causes an activation of a self-renewal program in normally non–self-renewing hematopoietic progenitors; consequently, these cells start to behave as HSCs in vivo despite maintaining a progenitor phenotype. Thus, these cells do not de-differentiate, but rather acquire self-renewal potential after loss of tumor suppressors (Akala et al., 2008). These data raise the possibility

that the accumulation of mutations in certain oncogenes and tumor suppressors may lead to a process that may be described as a partial reprogramming, with the acquisition of self-renewal activity paralleling the development of CSC activity. Indeed, in vitro reprogramming of human skin fibroblasts by stable expression of hTERT, H-RasV12, and SV40 LT and ST antigens led to the generation of cells with CSC properties, able to form hierarchically organized tumors in mice (Scaffidi and Misteli, 2011). Similarly, experimentally induced expression of the depolarization-inducing transcription coactivator TAZ (Cordenonsi et al., 2011), as well as experimentally induced expression of the epithelial-to-mesenchymal transition (EMT)-inducing transcription factors TWIST or SNAIL (Mani et al., 2008), was reported to provide CSC-like properties to non-CSC cells.

Identification of CSC biomarkers

The CSC concept proposes that one of the major parameters to evaluate therapy efficacy is the quantification of remnant CSCs within minimal residual disease, and not gross measurement of tumor regression, as is typically used in clinical trials (Blagosklonny, 2006). Therefore, one big aim in the field is to identify reliable and specific CSC biomarkers for each tumor type.

Lapidot et al. (1994) first demonstrated that only a small subset of human AML cells display tumorigenic properties. Subsequently, Bonnet and Dick (1997) identified LSCs in AML as CD34$^+$/CD38$^-$ leukemic cells, closely resembling normal HSCs (Bonnet and Dick, 1997), by limiting dilution transplantations in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. However, in the meantime, examination of a large set of AML patients revealed that functional CSC clones reside within several distinct immunophenotypically defined cellular compartments, showing significant interpatient heterogeneity (Eppert et al., 2011; Gibbs et al., 2012).

For solid tumors, a first leap forward was achieved in 2003, when Michael Clarke and colleagues reported the identification of breast CSCs within the ESA$^+$/CD44$^+$/CD24$^{low-neg}$ population of mammary pleural effusion and tumor samples. As few as a hundred ESA$^+$/CD44$^+$/CD24$^{low-neg}$ cells were able to reinitiate the original tumor in NOD/SCID mice, whereas ten thousand cells with an alternate phenotype were not (Al-Hajj et al., 2003).

After these two landmark publications, CSCs were identified in many more solid and hematopoietic human tumors as summarized in Table 1. Importantly, several studies indicate the existence of CSC-enriched populations displaying different, sometimes nonoverlapping sets of markers for the same tumor type. For example, only 1% of breast cancer cells simultaneously express both reported CSC phenotypes ESA$^+$/CD44$^+$/CD24$^{low-neg}$ and ALDH-1$^+$ (Ginestier et al., 2007). This discrepancy might be due to several different factors. First, differences in methods could be responsible for these differences (Quintana et al., 2008; Civenni et al., 2011). Second, several CSC clones may coexist within primary tumors (Anderson et al., 2011; Eppert et al., 2011), and the research groups may have detected different CSC populations. Third, functional CSC clones might reside within several immunophenotypically defined cellular

compartments (Eppert et al., 2011; Gibbs et al., 2012). Last but not least, cancer entities are rarely uniform. For instance, during the last few years, various genome-wide gene expression profiling efforts combined with biomarker and clinical approaches have led to the subclassification of breast cancers into increasing numbers of molecular subtypes (Perou et al., 2000; Herschkowitz et al., 2007; Curtis et al., 2012). The mutational patterns vary between the different subtypes, and this genetic heterogeneity is likely paralleled by a heterogeneous CSC complexity. Different CSC phenotypes might thus be associated with different cancer subtypes.

Therapeutic targeting of CSCs

Recent work on AML shows that the signature of functionally validated LSCs is a good predictor for poor patient survival independent of any biomarker (Eppert et al., 2011). These data strongly suggest that therapeutic targeting of CSCs should be relevant for patients. However, many technical hurdles still need to be overcome.

Targeting CSC biomarkers. CD44, a transmembrane glycoprotein involved in cell–cell and cell–matrix adhesion, has been identified as being expressed by many tumor CSCs (Table 1; Zöller, 2011). A CD44 antibody restricted human AML LSC proliferation in xenograft studies, probably by inhibiting LSC–niche interactions (Jin et al., 2006; Krause et al., 2006). However, universal targeting of CD44, which is also expressed by many adult stem cells, might be deleterious for patients. Undesirable effects might be evaded by targeting different isoforms of CD44 specifically expressed by tumor cells, such as CD44v6 (Heider et al., 2004; Orian-Rousseau, 2010). However, severe skin toxicity has been reported in a phase I clinical trial of bevatuzumab (an anti-CD44v6 antibody) in the case of head and neck squamous cell carcinoma (Riechelmann et al., 2008).

More recently, successful eradication of AML LSC was described by targeting of CD123 (interleukin-3 [IL-3] receptor α chain) using a blocking antibody in xenografted mice (Jin et al., 2009). However, because CD123 is also expressed by normal HSCs, there is a risk of severe side effects (Taussig et al., 2005). Targeting ABC transporters expressed by CSCs has also been explored (Lou and Dean, 2007) but was put on hold after realizing that these transporters play pivotal roles in blood–brain barrier maintenance as well as in adult stem cell maintenance (Hermann et al., 2006).

Targeting CSC molecular pathways. Based on the hypothesis that cancer cells might be more dependent on the activation of oncogenic pathways than their normal counterparts, a phenomenon termed "oncogene addiction" (Weinstein, 2002), pharmaceutical companies have applied major efforts to target signaling pathways activated in CSCs and cancer cells in general. For instance, the NFκB pathway has been successful targeted and allowed selective eradication of AML and final phase (blast crisis) CML LSCs. Guzman et al. (2005) treated in vitro leukemic cells with parthenolide, a drug known to directly bind IκB kinase (IKK) as well as to modify p50 and p65 subunits. This pretreatment induced higher levels of ROS, which led to a decrease of CD34$^+$/CD38$^-$ leukemic populations

and an impaired capacity of leukemic cells to engraft in NOD/SCID mice, which suggests that mainly LSCs were targeted (Guzman et al., 2005). Similarly, inhibition of NOTCH signaling using γ-secretase inhibitors in CD133-expressing medulloblastoma CSCs led to the diminution of CD133⁺ cells and correlated with impaired tumor engraftment in vivo (Fan et al., 2006). Last but not least, inhibition of the TGFβ pathway by bone morphogenetic proteins (BMPs) was reported to lead to the differentiation of brain CSCs and successive cure of the disease in a xenograft model, providing a first proof of principle for the possible efficacy of a "differentiation therapy" (Piccirillo et al., 2006).

Sensitization to therapy. Several studies have aimed to sensitize CSCs to therapy. For instance, in colon cancer, IL-4 blockade successfully primed CSCs to chemotherapy (Francipane et al., 2008). In glioblastoma, a CHK kinase inhibitor increased the radio sensitivity of CSCs (Bao et al., 2006). Also, recent reports showing that dormant and chemotherapy-resistant normal stem cells can be activated and simultaneously sensitized to chemotherapy-mediated killing might provide a strategy for targeting dormant CSCs (Essers et al., 2009; Essers and Trumpp, 2010). Here a sequential treatment scheme would be used in which dormant CSCs would first be "primed" and activated, followed by chemotherapy, leading to complete elimination of the tumor including the initially dormant and resistant CSCs. Such a strategy was successfully applied in mouse models of AML and CML (Ito et al., 2008; Saito et al., 2010). Moreover, in a mouse model of acute promyelocytic leukemia (APL), LSCs expressing PML-RARα (pro-myelocytic leukemia-retinoic acid receptor α) could be forced out of self-renewal into differentiation by cooperative treatment of arsenic, cyclic AMP, and retinoic acid, leading to LSC clearance and impressive remission in patients (de Thé and Chen, 2010).

Novel strategies to target CSCs. Some new hope might arise from the development of bi-specific or tri-specific antibodies that are able to specifically target cell populations. For instance, T cell recognition via an anti-CD3 antibody can be combined with cancer cell recognition via an additional antigen-binding site such as EPCAM (catumaxomab) or HER2 (ertumaxomab; Shen and Zhu, 2008; Hess et al., 2012).

In addition, high-throughput screening of drugs selectively inhibiting CSCs, rather than other tumor cells, have been successfully used to uncover compounds such as salinomycin. This antibiotic drug targets putative breast CSCs (grown as mammospheres in culture), as well as CLL LSCs, probably via inhibition of the WNT pathway (Gupta et al., 2009; Lu et al., 2011). However, the toxicity of this molecule on physiological stem cells remains unexplored.

Last but not least, recent discoveries concerning specific CSC biological properties might lead to the development of novel targeting strategies: for instance, glioma stem cells were found to be mechanistically distinct from their less tumorigenic counterparts regarding production of nitric oxide via nitric oxide synthase-2 (NOS2). NOS2 inhibition successfully slowed down tumor growth in a xenograft glioma model (Eyler et al., 2011). Similarly, glycine decarboxylase activity was found to be critical for non-small cell lung CSCs, which suggests that glycine metabolism might be a novel anti-CSC target (Zhang et al., 2012). However, in both cases, general toxicity of such treatments has not yet been thoroughly investigated.

In summary, therapeutic applications deriving from CSC studies are less straightforward than was initially anticipated, notably because of the large overlap in molecules expressed by CSCs and their respective normal stem cells that has so far hampered their use in the clinic due to collateral toxicity (Deonarain et al., 2009). Nevertheless, without the efficient eradication of CSCs, a long-term cure for many cancer patients appears unreachable. Innovative efforts will therefore be required from the field to achieve this goal in the not too distant future.

Evolution toward a more dynamic view of CSCs

Given the pace of research in the field and the diverse nature of the results, the classical view of CSCs needs to be updated. In this section, we propose a more dynamic model for CSCs and integrate recent results into this model.

Multiple genetic CSC clones within one tumor. Recent studies in which several subregions of the primary tumor and metastases of pancreatic and kidney cancers were sequenced revealed an unexpected degree of intratumor heterogeneity (Campbell et al., 2010; Yachida et al., 2010; Gerlinger et al., 2012). This often highly underestimated heterogeneity may result from the fact that CSCs are genetically unstable. Indeed, in colon cancer, such genetic instability was reported, leading to the formation of new CSC clones deriving from an initial "parental" CSC clone (Odoux et al., 2008). In addition, two landmark studies in human acute lymphoblastic leukemia (ALL) studied the genetic architecture of LSCs during disease progression and after successive transplantations in xenografted mice. Both studies show that several LSC clones coexist within single patients. During disease progression, after therapy, and after serial xenotransplantation, different LSC clones can take over and initiate new tumors (Anderson et al., 2011; Notta et al., 2011). This might explain at least partially the "acquired resistance" to targeted therapy observed in some patients. That is, after successfully targeting sensitive CSC clones, already present resistant clones may take over, mimicking an acquisition of resistance to the applied treatment. As a consequence, tumors might not be faithfully represented by single-headed hierarchical structures bur rather might resemble more oligarchic structures, displaying multiple heads: if one head, i.e., a CSC clone, is cut off (by targeted therapy, for instance), another one might take over, repopulating the tumor. Thus, dynamic hierarchies might exist within a single CSC clone and its progeny (intra-CSC clone hierarchy), but also between different genetically diverse CSC clones that compete with each other (inter-CSC clone hierarchy; Fig. 2 A).

CSCs need not be rare. CSCs do not need to be rare, as shown by Kelly et al. (2007) using leukemia and lymphoma genetic mouse models. Indeed, CSC-enriched populations have been reported to represent extremely variable proportions of bulk tumor cells, ranging from 0.2% to 82.5%
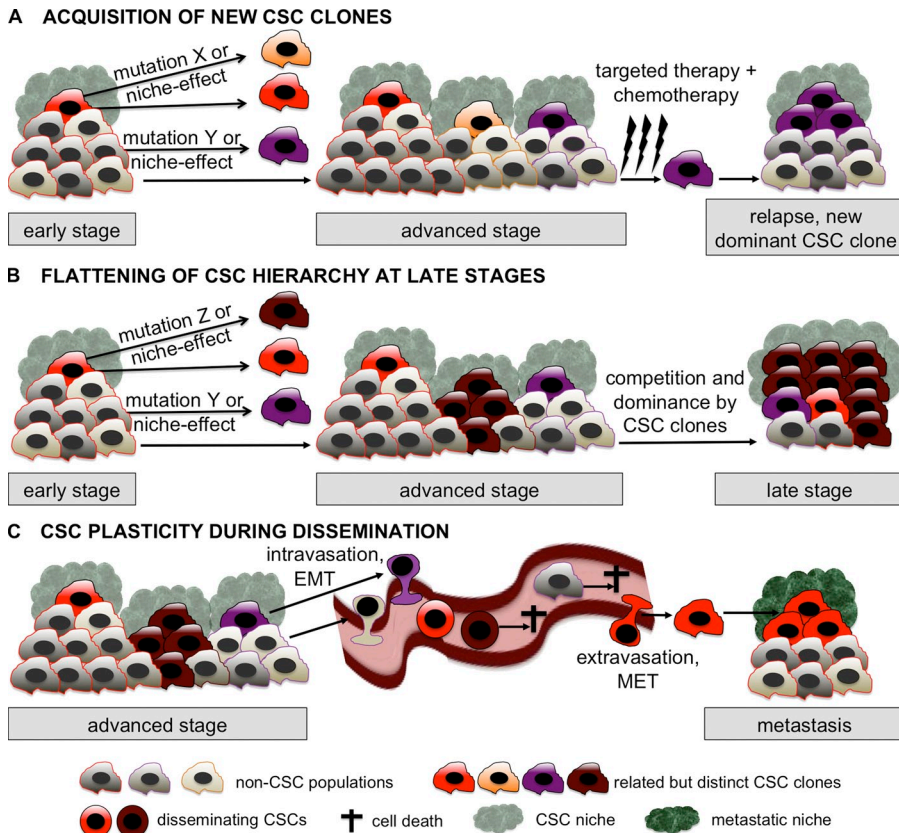
A   ACQUISITION OF NEW CSC CLONES

mutation X or niche-effect

mutation Y or niche-effect

early stage

advanced stage

targeted therapy + chemotherapy

relapse, new dominant CSC clone

B   FLATTENING OF CSC HIERARCHY AT LATE STAGES

mutation Z or niche-effect

mutation Y or niche-effect

early stage

advanced stage

competition and dominance by CSC clones

late stage

C   CSC PLASTICITY DURING DISSEMINATION

intravasation, EMT

extravasation, MET

advanced stage

metastasis

non-CSC populations

related but distinct CSC clones

disseminating CSCs   ✝ cell death   CSC niche   metastatic niche

Figure 2. **The dynamic CSC concept.** (A) Although early stage tumors might be governed by a single CSC clone, advanced stage tumors might contain several distinct but related clones, either arising from the initial CSC clone or from its differentiated progeny via mutations or via induction by the CSC niche. Targeted therapy and/or chemotherapy eliminate the tumor mass, possibly including some of the CSCs. At least one resistant CSC clone is then responsible (possibly after acquiring additional mutations) for tumor relapse. (B) Advanced stage tumors contain several distinct but related CSC clones. Some acquire enhanced self-renewal capabilities with simultaneously decreased differentiation. During tumor progression, the CSC clones compete with each other, leading to the dominance of at least one CSC clone with the subsequent loss of differentiated tumor progeny. Over time, this leads to a flattening of the hierarchical structure and to a selection of the most aggressive CSCs. Late stage tumors may thus be comprised almost exclusively of aggressive, multiresistant CSCs, a situation similar to the one proposed by the stochastic model. (C) Carcinoma CSCs display a dynamic phenotype during systemic dissemination: they are able to at least partially lose epithelial traits through EMT. It is most likely that only a subset of such disseminating CSCs are able to survive in the systemic circulation, extravasate, and reacquire epithelial features (MET) to seed in a new microenvironment and to initiate metastasis. All three scenarios illustrated in A, B, and C occur in parallel and/or during different phases of tumor progression.

(Table 1). Moreover, the frequency of CSCs might increase during tumor progression, as recently shown by in vivo limiting dilution assays of grade 1 and grade 3 breast tumors (Pece et al., 2010).

During the last few years, the idea that CSCs must be rare, which is based on our knowledge on physiological stem cells, often led to the questioning of the CSC hypothesis. For instance, it was reported that melanoma stage III–IV tumorigenic cells are very abundant, representing up to 30% of the tumor bulk. Moreover, the screening of a very high number of cell surface markers failed to distinguish these tumorigenic cells from other tumor cells (Quintana et al., 2010). Based on these results, it was proposed that melanoma does not follow a CSC model but rather a stochastic model, where tumorigenicity is a random feature distributed among all tumor cells (Gupta et al., 2005, 2011; Joseph et al., 2008).

Although certainly possible, there are also alternative explanations for these observations. The difficulty in finding any relevant biomarkers for stage III–IV tumorigenic melanoma cells might be technically explained by the temporary loss of surface proteins caused by the use of trypsin. Without using this enzyme, CD271 could be identified as a melanoma CSC marker by two other groups (Boiko et al., 2010; Civenni et al., 2011). Alternatively, late-stage melanoma might consist of several distinct CSC clones displaying different cell surface biomarkers, which are all highly malignant. These CSC clones might even differ from patient to patient, as described for ALL (Anderson et al., 2011; Notta et al., 2011). Moreover, a likely scenario is a high selection and enrichment for CSC clones, which have

retained little or no differentiation capacity. In this scenario, the initial hierarchy in the tumor is steadily decreased and flattened, leading to late-stage tumors, composed almost exclusively of heterogeneous CSC clones (Fig. 2 B).

Plasticity of CSC phenotype. In recent years, the EMT process, in which epithelial polarized adherent cells are converted into mesenchymal cell–like depolarized migratory tumor cells, has been linked to the CSC phenotype and function in breast, pancreatic, and colorectal tumors. CSCs were found to express EMT-inducing transcription factors such as TWIST, SNAIL, and SLUG and, vice versa, EMT-undergoing cells were found enriched for CSC activities compared with cells not undergoing EMT (Mani et al., 2008; Thiery et al., 2009; Wellner et al., 2009; Cordenonsi et al., 2011; Rhim et al., 2012). The process can be controlled by a ZEB1/mir200 feedback loop mechanism (maintaining stemness), itself controlled by p53 (Shimono et al., 2009; Keck and Brabletz, 2011; Schubert and Brabletz, 2011). Overall, these findings suggest that epithelial markers might be down-regulated in carcinoma CSCs and therefore would be missed in typical screens, which generally exclude cells lacking epithelial markers such as EPCAM. Furthermore, it underlines the fact that epithelial CSCs might strongly modulate their phenotype during tumor progression: for example, a CSC detected in an early stage primary tumor might have a completely different phenotype from the one of a CSC circulating in the blood (Fig. 2 C). As a consequence of this plasticity, some of the reported CSC biomarkers might be relevant in some stages of tumor progression but obsolete in others.

Generating a new dynamic concept of CSCs. These recent data and others suggest a more complex and dynamic CSC model integrating the three main additional features.

First, tumors are by definition genetically unstable entities. Therefore, the cellular composition of a tumor in an early stage disease, at relapse, or at late stages may display significant genetic differences including genetic heterogeneity. The available data suggest that in early neoplasms, only a single or very few CSC clones drive tumor growth. During disease progression, new CSC clones can arise either from existing CSC clones or from the differentiated progeny via mutation-mediated partial reprogramming. After eradication of the majority of tumor cells by chemotherapy and/or targeted approaches, one or few CSC clones may survive and expand, leading to a change in the clonal and cellular composition of the relapsing cancer (Fig. 2 A).

Second, the different genetically distinct CSC clones may also compete with each other, leading to the selection of CSC clones with high self-renewing activity and simultaneous loss of differentiation capacity. This would provide an explanation for the observed flattening of the cellular hierarchy within advanced stage tumors, which creates a situation similar to what is proposed by the stochastic model (Fig. 2 B). Third, CSC biomarkers can be unstable, as indicated by studies on EMT in solid tumors (Fig. 2 C). Thus, reported CSC biomarkers might only be relevant for a given tumor stage and therefore need to be validated for each case in conjunction with functional analyses.

Importantly, this dynamic CSC model suggests that only complex combinatorial treatments are likely to be efficacious in targeting late stage tumors or metastasis. First, mutations that are commonly present in all clones have to identified, for instance by whole genome sequencing of various regions within a single tumor mass (Yachida et al., 2010; Gerlinger et al., 2012). The associated deregulated pathways of these common mutations present in all subregions (likely including the various tumor subclones) need then to be targeted by pathway-specific strategies. Second, these strategies need to be complemented by therapies targeting additional CSC-specific features (see previous sections) to eliminate not only the non-CSC parts of each tumor clone, but also the various CSCs present in advanced stage tumors or metastasis.

CSCs and metastasis

Metastasis might be initiated by a subset of CSC clones. Systemic dissemination and metastasis is responsible for most cancer-related deaths. To date, human metastasis-initiating cells (MICs) have not yet been prospectively identified. However, several lines of evidence indicate that MICs might be found within subpopulations of CSCs. First of all, carcinoma CSCs possess tumor-initiating capacity, which is a mandatory trait for the establishment of secondary tumors in distant organs. Second, they express EMT markers (Mani et al., 2008), which suggests that they are able to migrate and which makes them likely candidates for metastasis initiating activities.

More specifically, several reports suggest that MICs might be found within CSC populations: CD44^{+} breast cancer cells (displaying a breast CSC phenotype) were proposed to have enriched metastatic activities in xenograft studies (Al-Hajj et al., 2003; Liu et al., 2010). Similarly, CD44^{+}/CXCR4^{+} cells, a subfraction of the putative pancreatic CSCs present at the invasive front of cell line–induced pancreatic tumors, were reported to be enriched for metastatic capabilities (Hermann et al., 2007). Along the same lines, CD26, in combination with the CSC marker CD133, has been proposed as a marker for colorectal MIC-enriched tumor populations in primary tumor xenograft experiments (Pang et al., 2010).

MICs might be late-stage disseminating CSC clones. MICs might disseminate early during tumor progression or might derive from late-stage disseminating CSC clones (Klein, 2009). Through mapping of the genetic evolution of both pancreatic primary tumors and metastases by next generation sequencing, two recent studies identified the genealogy of metastatic clones (Campbell et al., 2010; Yachida et al., 2010). Both studies suggest that even if both primary tumors and metastases consist of heterogeneous clones, additional driver mutations are present in the metastasis-initiating clones compared with the clones present in the primary tumors. In addition, metastatic clones were found to arise rather late within primary pancreatic tumors (Yachida et al., 2010), even if the dissemination process itself might start rather early (Rhim et al., 2012); this suggests that functional MICs typically disseminate from advanced-stage tumors rather than from early stage primary tumors.

MICs must be found among disseminating tumor cells. Because metastasis results from the successful dissemination of primary tumor cells into a distant organ, MICs have to be found among disseminating tumor cells. These include circulating tumor cells (CTCs) as well as disseminated tumor cells (DTCs) found, respectively, in the blood or in the bone marrow of carcinoma patients. In the case of breast cancer, variable proportions of CTCs and DTCs have been reported to display CSC phenotypes by immunocytochemistry (Balic et al., 2006; Theodoropoulos et al., 2010). However, the detection methods for carcinoma CTCs remain controversial. This is due to the use of EPCAM and cytokeratins (CK) as positive selection markers, as they might be down-regulated during EMT-mediated dissemination. Indeed, <40% of genetically YFP-labeled CTCs expressed CK19 or EPCAM in a mouse model for pancreatic ductal adenocarcinomas, which suggests that a large proportion of CTCs undergo EMT and lose expression of epithelial markers. Also, >40% of the detected YFP-labeled CTCs expressed the previously reported pancreatic CSC markers (CD44^{+}/CD24^{+}) and showed high clonogenicity in vitro, which suggests that at least some of the detected CTCs have self-renewing potential (Li et al., 2007; Rhim et al., 2012). Functional in vivo analyses of different subfractions of human CTCs or DTCs using xenograft assays are required to test whether phenotypic disseminating CSCs are indeed involved in metastasis initiation and, if so, which CSC subpopulation is responsible. However, these studies are technically challenging, as they require the development of optimized metastatic read-out assays starting from extremely low numbers of patient disseminating tumor cells.
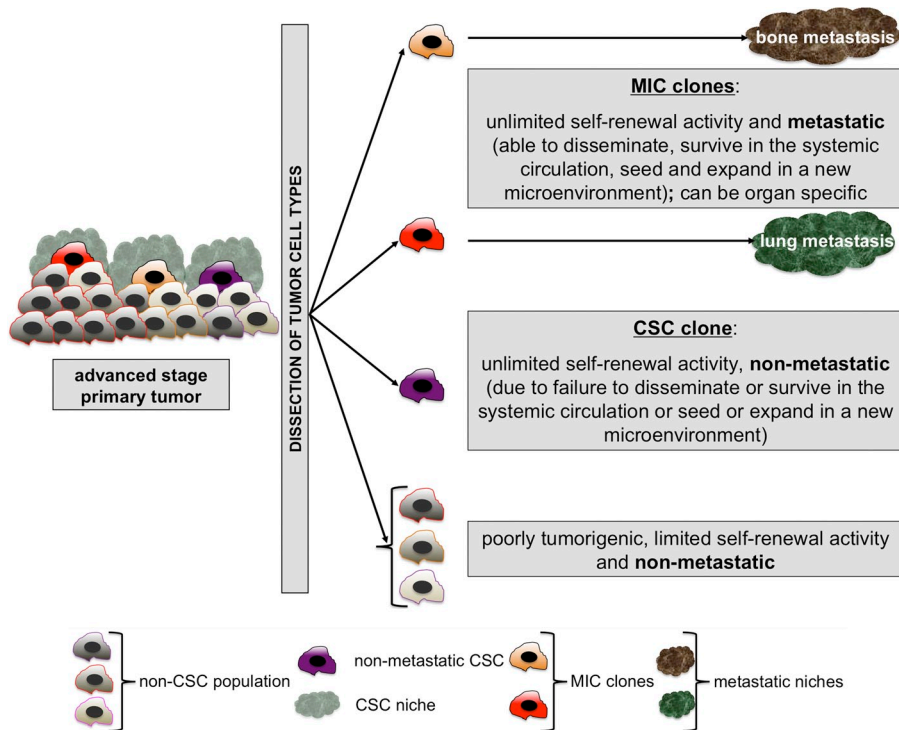
Figure 3. **The "metastasis initiating cell" (MIC) concept.** Advanced stage primary tumors are heterogeneous and multi-hierarchical structures. Upon dissociation and transplantation in immunocompromised hosts, MICs can be functionally distinguished from CSC clones by their metastatic ability in vivo (dissemination, survival in the systemic circulation, extravasation, seeding, and expansion in a new microenvironment, termed "metastatic niche"). MICs are adapted descendants and therefore subpopulations of CSCs with metastatic capacity and, possibly, organ specificity.

The metastasis initiating cell model. MICs likely arise from subpopulations of late-stage CSC clones, hence the relevance for characterizing CSCs, even in late-stage tumors. In addition to the functional capacities of CSCs, MICs have to display metastatic capacities, meaning that they must disseminate, survive in the systemic circulation, extravasate at the metastatic site, and seed and grow in the new environment. Several metastatic clones might coexist during dissemination, exhibiting different site-specific migration and seeding capacities (Fig. 3). Indeed, Massagué and colleagues have proposed that, in the context of breast cancer, different metastatic populations can be distinguished according to their capacity to metastasize to the bone, the lung, or the brain in patients with systemic disease (Kang et al., 2003; Minn et al., 2005; Bos et al., 2009). This appears to be related to their capacity to directly or indirectly generate a metastatic niche (Psaila et al., 2006-2007; Erez and Coussens, 2011; Oskarsson et al., 2011; Malanchi et al., 2012).

Conclusions

CSCs are a specific subset of transformed cells that are able to sustain primary tumor growth according to a hierarchical pattern. Strong evidence for the existence of such cells exists in many cancer types, although the model may not be appropriate for all cancer types and/or all stages. Like any model, the CSC concept needs to be constantly adapted to the currently available data and thus is steadily evolving. Recent findings uncovering high intratumor genetic heterogeneity have led to the observation that several CSC clones can coexist and compete with each other within a tumor. Furthermore, CSCs may have unstable phenotypes and genotypes, which makes it difficult to identify reliable and robust biomarkers for the development of targeted therapies. In addition, cell-extrinsic factors provided by the CSC niche might provide yet another dimension to the complexity of CSC regulation. Importantly, most cancer-related deaths are a consequence of metastasis development and not a direct consequence of primary tumor growth. Therefore, the characterization of MICs will become pivotal in the future in order to prevent and target so-far untreatable metastatic disease. Importantly, the identification of CSCs remains therapeutically relevant, even for late stages tumors, because MICs are likely a subset of these. Evolving the CSC concept will help to focus research toward developing improved therapies without risk of tumor recurrence and allow for the targeting of fatal late-stage cancers.

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