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REVIEW

Will cancer stem cells provide new therapeutic targets?

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This review presents a brief synopsis of recent progress in the area of cancer stem cells, with emphasis on leukemia and breast cancer, and discusses potential limitations to accomplishing the ultimate goal of eradicating residual disease in cancer.

Introduction

The concept that a rare population of tissue stem cells may be the cellular origin of cancer was proposed almost 150 years ago by pathologists such as Cohnheim and Durante (1). These scientists observed similarities between embryonic tissue and cancer with respect to their enormous capacity for proliferation and differentiation. Their observations led to the hypothesis that resting embryonic stem cells may reside in adult tissue and that upon activation these cells may acquire the ability to give rise to cancer (1). In 1937 Jacob Furth and colleagues showed that leukemia can be transmitted from one mouse to another using a single undifferentiated leukemia cell (1). This proved for the first time the existence of a leukemia cell possessing similar self-renewal properties as normal stem cells. However, this study did not show functional differences among biologically distinct tumor cells.

Approximately 30 years later, using *in vitro* colony formation assays, it was established that a rare subpopulation of acute myeloid leukemia (AML) possessed the ability to self-renew, proliferate and to give rise to new tumors (2–5). In the 1970s researchers such as Barry Pierce, Van Potter and colleagues revisited the idea and referred to cancer as 'maturation arrest of tissue-determined stem cells' or 'blocked ontogeny' (6–8). This hypothesis was formally tested *in vivo* in the 1990s, when John Dick and colleagues demonstrated the stem cell potential of leukemia cells in a non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse model (9).

In 2001 two excellent papers reviewed an expanding body of literature addressing the similarities in the biology of stem cells and cancer stem cells and proposed once again that cancers may arise from quiescent tissue stem cells (10,11). Recent technological advances in the isolation and characterization of these cells and the understanding of signaling pathways involved in their self-renewal and differentiation have led to considerable progress in this area. Furthermore, there is the very real possibility that these exciting studies may in the near future enable the development of novel therapies.

Cancer stem cells

There are two important issues that need to be addressed in the field of cancer stem cell biology. One is whether a small population of cancer cells may be identified which is capable of giving rise to new tumors. The initiating subpopulation of tumor cells are the so-called 'cancer stem cells'. The second issue is whether any cell or only the normal tissue stem cells are the cells of origin of cancer. If normal tissue stem cells are the subject of transforming events leading to the generation of tumor stem cells, then cancer stem cells and normal tissue stem cells are expected to have many similar properties. The challenge in eradicating cancer stem cells will be to first identify these cells and then to find unique pathways which may be targeted without harming normal tissue stem cells.

Cancer stem cells represent a subpopulation of cells within a tumor which is capable of initiating new tumors following a prolonged period of remission. Presumably this occurs because cancer stem cells have unique properties such as longevity, quiescence and self-renewal, similar to normal tissue stem cells. Self-renewal is the process by which a stem cell produces a similar daughter cell by symmetric division. However, asymmetric division also occurs when a stem cell divides to generate a different, more specialized daughter cell with a limited capacity for division or survival. Recently, several laboratories have made progress in the identification of this small subpopulation of highly tumorigenic, presumptive cancer stem cells in leukemia, brain and breast cancers (12).

Sell and Pierce, as proponents of the stem cell origin of cancer, originally suggested that malignancy leads to blocked differentiation and that the stem cells are the targets of a 'first-hit' mutation (6). Stem cells, as opposed to differentiated cells, are long-lived and are more likely to be the subject of mutations that are necessary for cancer initiation and progression. Furthermore, cancer stem cells, similar to normal tissue stem cells, may exist in a quiescent state for a long time and this quiescence property may make these cells resistant to conventional chemotherapeutic drugs, which only target dividing cells. However, definitive support for the stem cell origin of cancer model will require demonstration that

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCRP1, Breast Cancer Resistance Protein-1; CALLA, Acute Lymphoblastic Leukemia Common Antigen; CLP, committed lymphoid progenitor; CMP, common myeloid progenitors; EMA, Epithelial Membrane Antigen; EpCAM, epithelial cell adhesion molecule; ESA, epithelial-specific antigen; GMP, granulocytic/monocytic-restricted progenitors; Hh, Hedgehog; *Hox*, homeobox; HSCs, hematopoietic stem cells; LRP, Lung Resistance-Related Protein; LT, long-term progenitors; MEP, megakaryocytic/erythroid-restricted progenitors; *MLL-ENL*, Mixed Lineage Leukemia–Eleven Nineteen Leukemia; MMTV-Wnt-1, mouse mammary tumor virus promoter-driven Wnt-1; MUC-1, sialomucin; NOD/SCID, non-obese diabetic/severe combined immunodeficient; PDGF, Platelet-Derived Growth Factor; PTC-1, Patched-1; Sca-1, stem cell antigen-1; SL-IC, SCID leukemia-initiating cells; SP, side population; ST, short-term progenitors.

the transformation events occurred in a normal tissue stem cell and that this phenomenon is observed regardless of tumor type. The concept of cancer stem cells is still not universally accepted and an alternative stochastic model has been proposed in which any tumor cell is capable of generating a new tumor given the right microenvironment (13).

Hematopoietic cancer stem cells

The first step in eradicating cancer stem cells is their identification and further characterization. Towards this goal, John Dick and colleagues have characterized AML stem cells. These investigators demonstrated that in AML only a small subpopulation of leukemic cells was capable of initiating leukemia in mice upon serial transplantation. These cells were designated SCID leukemia-initiating cells (SL-IC). Transplantation of SL-IC recapitulated the pathology of the original human leukemia in the recipient mice (9). These leukemic cells were also found to express stem cell markers such as $CD34^+$ $CD38^-$, suggesting that the initial transformation event occurred in a stem cell and not the committed progenitor cells. Further characterization of the SL-IC showed that these cells were not homogeneous and exhibited a heterogeneous phenotype with regards to their timing of engraftment, initiation, lifespan of each graft, proliferation capacity and quiescent state (14). SL-IC with the shortest time to engraftment and tumor initiation were identified as short-lived SL-IC and the SL-IC with a delayed time to engraftment and tumor initiation were called long-term SL-IC. These properties make the therapy of cancer more challenging because they suggest that it might be necessary to target not only the short-lived SL-IC but also the more quiescent cells, which have a more delayed self-renewal capacity.

Alternatively, Irv Weissman and colleagues have proposed a slightly different model to suggest that both hematopoietic stem cells (HSCs) along with committed hematopoietic progenitors can give rise to leukemia (15). These studies employed the Mixed Lineage Leukemia–Eleven Nineteen Leukemia (*MLL-ENL*) oncogene. MLL-ENL is the protein product of a t(11;9) translocation between the *MLL* gene and *ENL*. The mechanism by which MLL-ENL causes leukemia may be linked to its ability to up-regulate the c-myc oncoprotein (16). The *MLL* gene is a common target for chromosomal translocations associated with human acute leukemia (17). MLL oncoproteins are constitutive activators of homeobox (*Hox*) genes and promote myeloid transformation via a Hox-dependent mechanism (18). These investigators first isolated HSCs as well as committed progenitor cells such as the common myeloid progenitors (CMP), granulocytic/monocytic-restricted progenitors (GMP) and megakaryocytic/erythroid-restricted progenitors (MEP). They then retrovirally transduced each isolated cell population using the *MLL-ENL* oncogene and showed that not only the HSCs but also some of the committed progenitors, CMP and GMP but not MEP, were capable of being transformed, giving rise to AML following transplantation *in vivo*. Thus, in this mouse model the cancer-initiating cells may not be limited to the stem cells and committed progenitors may also be the subject of transforming events.

A small and distinct population of bone marrow termed 'side population' (SP) cells efflux Hoechst dye. SP cells were shown to possess the potential to regenerate the entire bone marrow, establishing their functional capacity as HSCs (19). Efflux of Hoechst dye is due to the existence of Breast Cancer Resistance Protein-1 (BCRP1) transporter on SP cells. The BCRP1 pump was first identified in breast cancer cells resistant to topoisomerases and may be responsible for the mechanism of drug resistance in many types of cancer (20). Additionally, it has been reported that BCRP1-positive cells, such as stem cells or tumor cells, may have a survival advantage under hypoxic conditions, since BCRP1 binds heme and is up-regulated by hypoxia (21). To explore the leukemogenic potential and clinical significance of leukemia SP cells, bone marrow and peripheral blood cells were treated with Hoechst dye and then analyzed by FACS at two different emission wavelengths (22). Leukemic SP cells were found in the majority of leukemic patients under study. Isolated SP cells expressed the cytogenetic markers of AML in all cases of the active disease and also displayed an increased ability to efflux chemotherapeutic agents, such as daunorubicin and mitoxantrone. These studies suggested that the SP cells are frequently involved in AML and may be an important player in remission due to their special ability to efflux chemotherapeutic agents.

Thus, there is increasing evidence that leukemia stem cells may possess unique properties which make these cells more resistant to conventional chemotherapeutic agents. For example, it has been reported that human AML $CD34^+/CD38^-$ progenitor cells were more resistant to daunorubicin with respect to decreasing proliferation and the induction of apoptosis when compared with their $CD34^+/CD38^+$ counterparts (23). AML $CD34^+/CD38^-$ cells also exhibited higher expression of drug resistance proteins, such as Lung Resistance-Related Protein (LRP) and Multiple Resistance-Associated Protein (MRP). Furthermore, AML $CD34^+/CD38^-$ progenitor cells displayed lower expression of Fas/Fas-L and Fas-induced apoptosis compared with $CD34^+/CD38^+$ cells. Moreover, $CD34^+/CD38^-$ cells elicited lower immunogenicity when examined by a mixed lymphocyte reaction assay. This phenomenon was linked to lower expression of immune recognition molecules such as MHC-II, LFA-3, B7-1 and B7-2 on the $CD34^+/CD38^-$ subpopulation of leukemic cells (23). Another group of investigators have demonstrated that the majority of NOD/SCID mouse leukemia-initiating cells isolated from human leukemia are predominantly in the G_0 phase of the cell cycle (24).

The ultimate goal in eradicating cancer stem cells is to identify and target unique survival mechanisms that are active only in cancer stem cells, sparing the normal tissue stem cells. It has been reported that treatment of AML stem cells with a proteasome inhibitor, carbobenzoxyl-L-leucyl-L-leucyl-leucinal (MG-132) led to apoptosis in AML stem cells, sparing normal HSCs. Furthermore, NFκB was constitutively active in quiescent leukemic stem cells. One mechanism by which MG-132 may lead to apoptosis is by inhibiting degradation of IκBα, a negative regulator of NFκB (25). In summary, as demonstrated with AML, cancer stem cells may be identified and further characterized with respect to their unique self-renewal and survival mechanisms.

Signal transduction pathways important in HSCs

There is increasing evidence that the same molecular pathways regulating the self-renewal of HSCs may also be employed in

hematopoietic cancer stem cell propagation. The molecular pathways that have been primarily implicated in hematopoietic cancers include the Notch and Wnt pathways and, more recently, regulation by the Polycomb family member Bmi-1 (11,26). Bmi-1 is required for the self-renewal of HSCs as well as leukemic stem cells (26,27). Furthermore, Bmi-1 expression in blood is restricted to HSCs in mice and human (26,27). More importantly, functional studies revealed that Bmi-1-deficient stem cells generated a normal hematopoietic lineage but that the Bmi-1-deficient HSC could not be regrafted in a secondary transplant, which indicated that self-renewal of the HSC was impaired (26).

Julie Lessard and Guy Sauvageau have also linked Bmi-1 to the self-renewal of leukemic stem cells. They initiated leukemia by the retroviral transduction of Meis1a and Hoxa9 into Bmi-1-deficient and wild-type fetal liver cells. Even though both Bmi-1-deficient and wild-type cells showed the same phenotypic and clinical characteristics of leukemia, the Bmi-1-deficient leukemic cells lacked the ability to sustain the leukemic process in secondary transplants. Thus, Bmi-1 was required for self-renewal of the leukemic stem cells as well as normal HSCs. A potential mechanism by which Bmi-1 regulates stem cell self-renewal may be related to the regulation of survival and proliferation genes such as $p19^{Arf}$, $p16^{Ink4a}$ and a p53 target gene, *Wig1* (26). These studies provided the first functional evidence that a specific gene product, Bmi-1, played a common role in the self-renewal of normal as well as leukemic stem cells.

The Notch signal transduction pathway has also been implicated in the self-renewal of stem cells in hematopoietic, neural and germ cells. Furthermore, aberrant Notch regulation may cause certain human cancers such as breast cancer and leukemia (11). There are four mammalian Notch genes that encode a single transmembrane receptor (28). Binding of a Notch ligand (Delta-like or Jagged) results in the generation of the Notch intracellular domain (Active Notch) by a metalloprotease-dependent protease activity which cleaves the extracellular domain and a presenilin-dependent protease activity which cleaves the intracellular domain. The active Notch then enters the nucleus, binds to a ternary complex CSL (CBF1, Suppressor of Hairless or Lag-1) and mediates transcription. There are many levels of regulation following Notch activation that dictate how a cell may respond to a particular Notch-activating event. These include regulation at the level of: (i) ligand activation; (ii) receptor activation (Notch 1–4); (iii) proteolysis of the Notch receptors; (iv) ubiquitin-mediated degradation of Notch, which determines the intensity or duration of Notch activity (28–31). Therefore, it is not surprising that Notch has been suggested to act as both an oncogene and a tumor suppressor in different cell types. For example, Notch pathway activation acts as an oncogene in mammary epithelium and pre-T cells, however, it is recognized as a tumor suppressor in keratinocytes (28).

Wnts are secreted proteins that affect many cellular processes, including proliferation, survival, cell fate determination, cell adhesion and patterning during development. Wnt proteins bind to their receptors Frizzled and LRP-5/6 and cause the stabilization of β-catenin. Stabilized β-catenin translocates to the nucleus, binds to transcription factors such as lymphoid enhancer factor (LEF)/T cell factor (TCF) and mediates gene activation. This canonical Wnt signal transduction pathway has been shown to be involved in the self-renewal of several epithelial stem cells, including those in the intestine, skin, central nervous system and hematopoietic system (32). Wnt pathway activation has also been implicated in many cancers such as colon, prostate, ovary, skin and breast. These results suggest that the carcinogenic process may be related to the proliferation and accumulation of stem cells within these tissues (32). Recent studies have shown that myeloid progenitors, but not HSCs, derived from patients in a CML blast crisis were dependent on β-catenin pathway activation for self-renewal using an *in vitro* colony-forming assay. This is a nice demonstration that the cancer-initiating cells and tissue stem cells may be different and that a cell may acquire the ability to become a cancer stem cell at different stages in a differentiation pathway.

While a large body of literature has focused on finding common molecular pathways for the self-renewal of normal stem cells and cancer, a direct link between aberrant activation of these pathways and self-renewal or survival of cancer-initiating cells is limited. We are just beginning to define the cancer stem cell population for various hematopoietic and solid cancers. Once this cancer-initiating population has been identified, efforts should be aimed at characterizing unique self-renewal and survival pathways which may be targeted for selective eradication of cancer stem cells.

Breast cancer stem cells

The same methods used for the identification and characterization of hematopoietic cancer stem cells might be of value in characterizing stem cells in solid cancers. The following sections describe efforts at identifying and characterizing normal mammary gland stem cells and breast cancer stem cells.

The existence of normal mammary stem cells was established as early as 1959, when DeOme and colleagues observed that epithelium isolated from several different regions of mammary gland was able to generate fully functional mammary outgrowths containing ductal, lobuloalveolar and myoepithelial cells (33). In order to identify and characterize these cells within the mammary gland, several investigators have employed a variety of methods, including electron microscopy (34), serial transplantation using limited dilutions (35), Southern blot analysis of unique viral integration sites (35) and, most recently, flow cytometry (36). Based on these studies, it has been concluded that the entire mammary gland can develop from a multipotent stem cell clone positioned throughout the gland (35). Based on electron microscopy studies, mammary stem cells possess mitotic chromosomes, are lightly stained and do not show any structural evidence of specialized functions, such as synthesis and secretion of lipids and proteins (37). Furthermore, the mammary gland stem/progenitor cells may be isolated based upon their ability to efflux Hoechst dye as well as expression of cell surface markers such as stem cell antigen-1 (Sca-1) (36). As discussed previously, the SP profile has been demonstrated to be a characteristic of HSCs. A comparable SP population has been identified in the mammary gland and shown to give rise to mammary ducts and alveoli (36). Furthermore, the SP cells were enriched for expression of Sca-1, another marker highly expressed in primitive HSCs. Mammary gland cells deficient in Sca-1 expression exhibited loss of functional stem cell properties in reconstitution experiments (36).

Parallel studies of stem/progenitor cells performed in the human breast nicely complement many of the results described

above in rodent models. Several investigators have identified markers which may be specific for the human breast stem/progenitor cells. Olli Petersen and colleagues showed that luminal epithelial cells expressing epithelial-specific antigen (ESA) and sialomucin (MUC-1) were restricted in their differentiation repertoire, whereas a subpopulation of luminal cells that expressed ESA but lacked MUC-1 expression (ESA^+/$MUC\text{-}1^-$) was able to regenerate itself, myoepithelial cells and terminal duct lobular units (38). In independent experiments, John Stingl and Joanne Emerman used a combination of *in vitro* colony assays and flow cytometry to identify and characterize mammary epithelial progenitor cells (39). These investigators reported that cells with a luminal progenitor potential expressed the epithelial cell adhesion molecule (EpCAM, also known as ESA), α-6-integrin and MUC-1. The bipotent progenitors, cells capable of producing luminal and myoepithelial cells, were identified by expression of EpCAM, α-6-integrin, higher levels of the basal cell marker Common Acute Lymphoblastic Leukemia Antigen (CALLA), a greater ability to efflux the fluorescent dye rhodamine 123 and lower level expression of MUC-1. However, myoepithelial progenitors expressed α-6-integrin and lower levels of EpCAM.

Matt Smalley and colleagues (40) have identified SP cells in the human breast, similarly to previous studies performed in mouse mammary gland (36). Further characterization of the human breast SP cells has shown that ~40–60% of the SP cells express estrogen receptor (ER)-α (40,41). Maria Vivanco and colleagues also characterized different populations of normal human breast epithelial cells, including SP cells, and reported that the SP cells expressed low levels of CALLA and Epithelial Membrane Antigen (EMA, identical to MUC-1) as well as ERα and ERβ (42). Based on the above studies, the human breast bipotent progenitor or stem cells appear to share some common phenotypes, such as expression of surface marker, ESA (EpCAM), efflux of fluorescent dyes such as rhodamine and Hoechst and low level expression of MUC-1 (EMA). Whether or not SP cells express the ER appears controversial. In human breast cells one group reported low or no expression of ER (42), whereas two other groups report that 40–60% of SP cells express ER (40,41). Since the BCRP1 transporter, which is thought to be responsible for the SP phenotype (43), contains an apparently functional estrogen response element in its promoter, it is likely that at least a subpopulation of SP cells contain ERα.

Based upon approaches used to study neural stem cells, Max Wicha and colleagues have developed a non-adherent *in vitro* culture system that allows for the propagation of human breast epithelial stem/progenitor cells in suspension and in an undifferentiated state, also known as mammospheres (44). These investigators showed that mammospheres were enriched for mammary stem/progenitor cells capable of giving rise to all three differentiated mammary epithelial lineages, including luminal ductal, alveolar and myoepithelial cells. Mammospheres also contained an increased percentage of SP cells.

Once normal breast stem cells are identified and characterized, the next step will be to determine if cancer stem cells and normal stem cells are derived from the same cell lineage, i.e. basal cells, SP cells, Sca-1-positive cells, ESA^+/$MUC\text{-}1^-$ cells, etc. This can be demonstrated by comparing the phenotypic or genotypic profiles of these cells. As discussed below, a direct link between normal and breast cancer stem cells remains to be firmly established. Some investigators have identified highly tumorigenic breast cancer cells with stem cell/progenitor-like properties. However, the origin of these cells has not yet been determined. Recent studies have focused on the signal transduction pathway which may be misregulated in these cells resulting in the initiation of breast tumors.

Mike Clarke and colleagues were the first to identify a population of highly tumorigenic cells isolated from human breast tumors (45). This highly tumorigenic subpopulation expressed $CD44^+$ $CD24^{-/low}$ surface markers and had the capacity to form tumors following transplantation into etoposide-treated NOD/SCID mice. As few as 100 $CD44^+$ $CD24^{-/low}$ cells were able to form tumors, whereas tens of thousands of $CD44^-$ $CD24^+$ cells were not. How the $CD44^+$ $CD24^{-/low}$ population of cancer-initiating cells are related to the mammary gland stem cells/progenitors described above remains to be established. Furthermore, the mouse model used in this study may not recapitulate the human breast microenvironment necessary for the initiation and propagation of human breast cancer cells.

Charlotte Kuperwasser and colleagues in Bob Weinberg's laboratory (46) have recently developed a model in which both the stromal and epithelial components of the human breast were transplanted into the cleared fat pad of recipient NOD/SCID mice. These investigators suggested that this model provides a more realistic microenvironment for the growth of normal human breast as well as premalignant or malignant human mammary epithelial cells. The importance of the regulatory role of the mammary stroma and microenvironment in normal development as well as carcinogenesis has been known for many years (47–49). Kuperwasser *et al*. have revisited this idea and shown that the human breast stromal component should not be ignored when studying the initiation and progression of human breast cancer (50). Thus, it may be important to re-evaluate the relative tumorigenicity of different human breast cancer epithelial stem cell/progenitor populations in the appropriate microenvironment.

Signal transduction pathways important in breast cancer stem cells

There is increasing evidence that aberrant activation of the Wnt signaling pathway may result in the initiation or self-renewal of mammary tumor stem cells. Caroline Alexander and colleagues showed that mammary hyperplasias induced in mice expressing mouse mammary tumor virus promoter-driven Wnt-1 (MMTV-Wnt-1) or ΔN-β-catenin (the activated form of β-catenin) exhibited a marked increase in the percentage of mammary SP cells (51). This group also showed that ectopic activation of the Wnt pathway in short-term primary cultures of mammary epithelial cells by the addition of soluble Wnt-3a increased the fraction of SP cells. Furthermore, mammary cells taken from ΔN-β-catenin-expressing mice colonized cleared fat pads more efficiently than cells taken from wild-type mice. These results suggest, but do not prove, that the increased SP fraction in ΔN-β-catenin-expressing mice translates to increased functional stem cell activity. However, while some studies suggest that the SP population provides a surrogate mammary stem cell marker, this needs to be validated by functional transplantation studies. Of interest in the study by Alexander and her colleagues was the observation that the increase in the SP population was observed in the Wnt-1 and ΔN-β-catenin-induced hyperplasias as compared

with normal mammary gland, but was not seen in the resulting tumors. Thus, Wnt pathway activation may be important for the expansion of a progenitor population, but other still undefined oncogenic events occur in the resulting tumors.

Additional support for the existence of a progenitor cell as a cell of origin for Wnt pathway-induced mammary tumorigenesis comes from the identification of an increased mammary progenitor population, as assessed by the expression of Sca-1 and keratin 6, in both MMTV-Wnt-1 induced hyperplasias and tumors (52). Keratin 6 and Sca-1 appear to be expressed in mammary gland progenitor cells. Keratin 6 is expressed in the mammary gland anlage at embryonic day 16.5 (53). In the mature mammary gland keratin 6 is expressed in a small fraction of the body cells in the terminal end buds, but rarely in the differentiated ductal and alveolar cells (54). Sca-1 is a glycosylphosphatidylinositol (GPI)-linked protein found in HSCs and, as discussed previously, a population of mammary cells enriched for functional stem cell properties (36). Analysis for loss of heterozygosity of the tumor suppressor gene *PTEN* in hyperplasias and tumors present in bigenic MMTV-Wnt-1/PTEN heterozygous mice strongly supported the hypothesis that a bipotential progenitor cell was the cell of origin. However, mammary tumors that arose in mice expressing the *Neu*, H-*Ras* or Polyoma middle T antigen transgenes driven by the MMTV promoter did not exhibit a similar increase in the Sca-1 and keratin 6-positive progenitor cell population (52). Furthermore, a recent study suggested that the MMTV-Neu tumors arose from parity-induced mammary epithelial cells, whereas MMTV-Wnt-1 tumors originated from ductal epithelial subtypes (55). These studies demonstrated that the enrichment of a particular cell population in a hyperplasia or tumor may be highly dependent on the type of initiating event as well as the cell type in which these events occur.

Aberrant Notch regulation may cause human cancers, such as mammary tumors and leukemia (11). MMTV induction of some mammary tumors has been shown to result from integration of the MMTV proviral genome into the *Notch-4* locus, resulting in constitutive activation of Notch 4 (56). It is of interest that MMTV-induced mammary tumorigenesis does not result in the activation of Notch 2 or Notch 3 and only rarely of Notch 1. Transgenic mice expressing MMTV-Notch 4^{IC} (Notch 4 intracellular domain or active Notch 4) develop mammary tumors within 4–6 months. These mice also show retarded lobular–alveolar development and impairment of milk protein expression. Similarly, parous transgenic mice expressing the WAP-Notch 4 construct developed mammary tumors. These mice also had a block in alveolar development, but ductal morphogenesis was unaffected. The tumors in these mice were highly malignant and metastatic (56). It was speculated that the role of Notch 4 in tumorigenesis is dependent on the genetic background and the timing of Notch 4 expression with respect to the stage of mammary gland development (56). Whether activation of Notch 4 results in the expansion of specific mammary progenitors and, therefore, induction of tumorigenesis appears likely, but remains to be established. However, the development of tumors and the block in mammary gland development upon Notch 4 constitutive activation at different stages of development suggests that these two phenomena may be interdependent. Thus, it is likely that the Notch pathway will exert pleiotropic effects on both stem cell self-renewal and differentiation. Recent support for this hypothesis has come from studies of Notch signaling in mammosphere cultures by Dontu *et al*. (57).

The Hedgehog (Hh) signaling pathway is another example of pathways essential for development whose aberrant expression results in a variety of different human cancers, such as breast cancer and mammary ductal hyperplasias (11,58,59). The three mammalian Hh family ligands are Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog. Upon binding to the Hh receptor, Patched-1 (PTC-1) or Patched-2, Smoothened is activated and mediates activation of the transcription factors Gli-1, Gli-2 and Gli-3. These transcription factors then translocate to the nucleus and mediate gene transcription. Mice deficient in Gli-1 are normal, however, loss of Gli-2 or Gli-3 leads to perinatal lethality and developmental defects (60). Haploinsufficiency of PTC-1, a Hh signaling receptor, results in mammary ductal hyperplasia and dysplasias in addition to severe histological defects in ductal structure and terminal end buds (61). Preliminary results in mammosphere cultures support the importance of the Hh pathway in stem cell self-renewal (M. Wicha, personal communication).

Interestingly, haploinsufficiency of Gli-2, a transcription factor activated by the Hh pathway, results in mammary ductal hyperplasia and dysplasias as well as impaired alveolar development during pregnancy (62). However, the observed ductal hyperplasia and dysplasia appear to have resulted from aberrant signaling within the mammary gland stroma as opposed to the mammary epithelium (62). A recent study reported the expression of PTC-1, Sonic Hedgehog, and Gli-1 in a number of human breast carcinomas. This group reported that cyclopamine, an inhibitor of the Hh pathway, blocked the growth of Hh-activated breast carcinoma cells (58).

Stem/progenitor cells and breast cancer heterogeneity

It has been hypothesized that the clinical and genetic heterogeneity of breast cancer is a result of the activation of different oncogenes or loss of different tumor suppressor genes in specific stem/progenitor cells (52). Based on variations in gene expression patterns determined using DNA microarrays as well as hierarchical clustering, breast carcinomas have been classified into at least five different subtypes (basal-like, $ERBB2^+$, normal breast-like, luminal subtype A and luminal subtype B) with different clinical outcomes (63). Basal-like and $ERBB2^+$ (*HER2*/*Neu*-positive) tumors expressed nondetectable to low levels of ER, whereas the normal and luminal subtypes expressed higher levels of ER. The basal-like and $ERBB2^+$ subtypes showed the shortest overall survival and a lower relapse-free survival compared with the other subtypes. The luminal subtypes both express ERα, but differed considerably with respect to patient outcome, with subtype A displaying a better survival outcome compared with subtype B. Thus, it is conceivable that the differences in the underlying mechanisms involved in cancer initiation in stem/progenitor cells may result in the enormous heterogeneity observed in breast cancer.

If this hypothesis is correct it might be expected that the markedly different clinical outcomes observed are a reflection of differences in the cancer stem cell populations present within these subgroups. This is depicted as a hypothetical model on the stem cell origin of human breast cancer based primarily on models proposed for human hematopoietic cancers (Figure 1a and b). The heterogeneity of leukemias may be explained by the mechanisms underlying leukemia formation as well as the cell type in which the transforming events

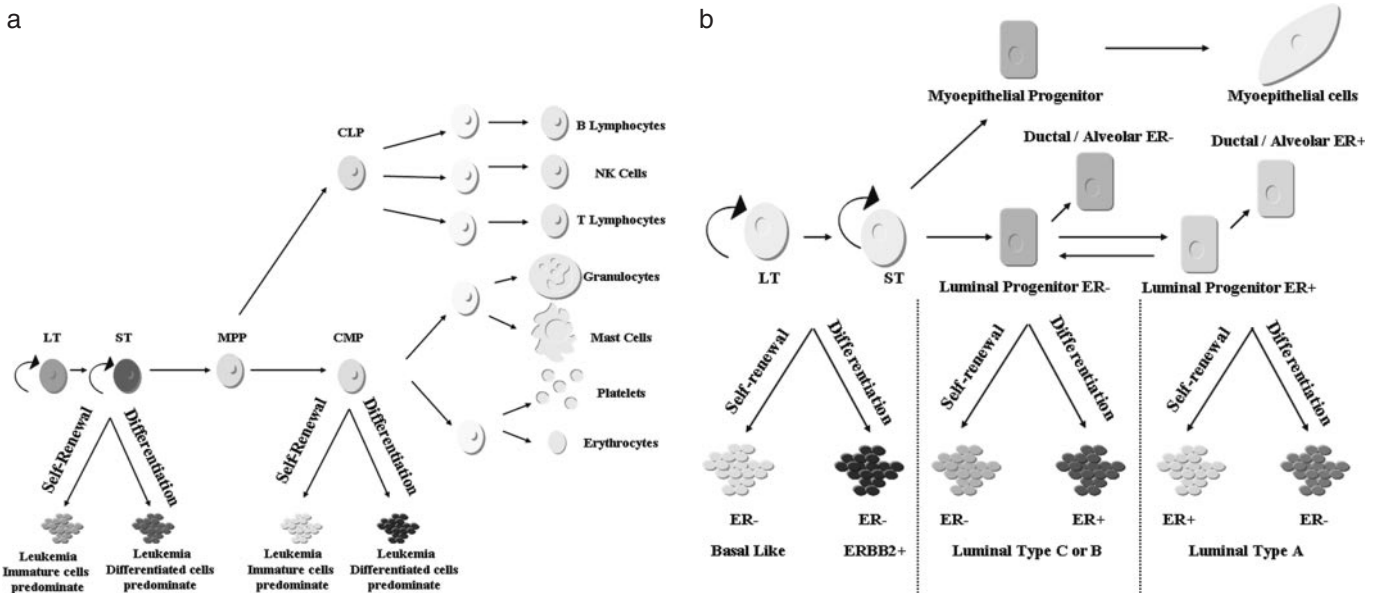

Fig. 1. (**a**) A hypothetical model for leukemogenesis. A long-term HSC (LT) is capable of giving rise to short-term (ST) or transient amplifying stem cells. STs further differentiate to multipotent progenitors (MPP), which are then capable of giving rise to the committed lymphoid (CLP) and myeloid (CMP) progenitors. The CLPs and CMPs further differentiate to generate all the different types of hematopoietic cells. Leukemia is initiated when a HSC or a progenitor cell (i.e. LT, ST, MPP, CLP or CMP) is transformed by sequential genetic mutations to a cancer initiating cell or a cancer stem cell. A cancer stem cell may then undergo self-renewal and generate leukemic cells with a similar genetic profile as the originating cell, e.g. AML where the majority of cancer cells are immature and express stem cell surface markers such as $CD34^{+}CD38^{-}$. It is likely that a leukemia stem cell may undergo differentiation to give rise to leukemic cells that express differentiation markers, as seen in CML. For simplicity, the transition from a normal stem cell to a cancer stem cell is omitted. LT, long-term stem cells; ST, short-term stem cells; MPP, multipotent progenitor cells; CLP, committed lymphoid progenitor cells; CMP, committed myeloid progenitor cells. (**b**) A hypothetical model of breast cancer development. Breast cancer is initiated when a progenitor cell acquires sequential mutations resulting in transformation. The cancer initiating cell or cancer stem cell may self-renew symmetrically giving rise to cancer cells with similar expression profiles or it may differentiate asymmetrically and generate cancer cells with different genetic profiles. For example, an ER^{-} cancer stem cell may self-renew giving rise to a population of cancer cells that are ER^{-} or it may follow a differentiation path and generate a population of cancer cells that express ER (ER^{+}) [adapted from Dontu *et al*. (64)]. The heterogeneity of breast cancer may be explained by the type of underlying genetic event resulting in transformation, e.g. in MMTV-Neu- or MMTV-Wnt-1-induced tumors, and the type of cell in which these occur. For example, a transforming event in a stem cell may give rise to a basal-like or $ERBB2^{+}$ type tumors, whereas a transforming event in a committed progenitor may give rise to luminal subtype tumors. Obviously, the tumor behavior and response to therapy may be different and dictated by the tumor initiating cells and the type of transforming event. It is conceivable that a normal differentiated cell may undergo genetic or epigenetic changes resulting in transformation, possibly dedifferentiation and self-renewal (32). For simplicity, the transition from a normal stem cell to a cancer stem cell is omitted. LT, long-term stem cells; ST, short-term stem cells or transit amplifying cells; ER^{-}, estrogen receptor negative; ER^{+}, estrogen receptor positive; $ERBB2^{+}$, *HER2*/*Neu*-positive tumors. See online supplementary material for a color version of this figure.

first occurred. Long-term progenitors (LT) undergo differentiation to short-term progenitors (ST) followed by differentiation to committed myeloid progenitors (CMP) or committed lymphoid progenitors (CLP). Tumors may arise when a specific cell, i.e. LT, ST, CMP or CLP acquires mutations promoting aberrant self-renewal or differentiation of cancer stem cells. In hematopoietic cancers it has been shown that the stem cells as well as the committed progenitors may be the target of transforming events resulting in leukemia.

Like hematopoietic cancers, breast cancer may be a result of transforming events in a stem cell or a committed progenitor cell (Figure 1b). A cancer-initiating cell or cancer stem cell may self-renew symmetrically giving rise to cancer cells with similar expression profiles or it may differentiate asymmetrically and generate cancer cells with different genetic profiles. For example an ER^{-} cancer stem cell may self-renew giving rise to a population of cancer cells that are ER^{-} or they may follow a differentiation pathway and generate a population of cancer cells that express ER (64). Likewise, a transforming event in a stem cell may give rise to basal-like or $ERBB2^{+}$ breast cancers that are ER^{-}, whereas a transforming event in a committed progenitor may give rise to the luminal subtypes of breast cancer expressing different levels of ER. Obviously, the clinical outcome and response to therapy would be quite different depending on the type of cancer stem cells which gave rise to a particular subtype of breast cancer. For example, this model predicts that the ER^{+} luminal B subtype might respond poorly to hormonal therapies because it is derived from an ER^{-} stem cell/progenitor while the luminal A subtype with a better clinical outcome may be dependent on an ER^{+} stem cell/progenitor (64). This is clearly a testable hypothesis. Interestingly, the basal-like subtype of breast cancer which has the poorest prognosis expresses several genes similar to those markers observed in the bipotential progenitors found in Wnt-1-induced mouse mammary hyperplasias and tumors (C. Perou and J. Rosen, unpublished observations).

Stem cells in brain cancer

A well-established method for the isolation and enrichment of neural stem cells has been developed over the last decade (65–67). In brief, cells derived from either embryonic or adult subventricular zone brain are isolated and cultured in serum-free medium containing epidermal growth factor and/or basic fibroblast growth factor. The neural progenitor cells divide and form proliferating clusters known as neurospheres. The majority of the neurosphere cells express neural stem cell

markers such as nestin, but do not express neural differentiation markers such as neuron-specific enolase or glial fibrillary acidic protein. Similar to the hematopoietic system, cultured neurospheres contain a higher percentage of cells that stain weakly with the DNA binding dye Hoechst 33342. In addition, the low Hoechst binding subpopulation contain cells that are able to continue to proliferate and form new neurospheres (68).

Recent progress has been made in the identification of putative cancer stem cells in brain cancers. For example, Peter Dirks and colleagues generated neurospheres from 14 solid primary pediatric brain tumors, including medulloblastoma, pilocytic astrocytoma, glioblastoma and anaplastic ependymoma (69). Brain tumor neurospheres are grown under culture conditions which favor neural stem cell growth, similarly to the neurosphere and mammosphere cultures discussed previously. The brain tumor neurospheres displayed the potential to self-renew, as well as to give rise to differentiated tumor cells similar to those present in the original tumors from which they were derived. Furthermore, the brain tumor stem cells expressed markers of undifferentiated neural stem cells (CD133 and nestin). A similar brain tumor neurosphere population with stem cell self-renewal potential was developed by Hemmati *et al*. (70). The self-renewing brain tumor stem cells possessed neural stem cell markers such as CD133, Sox2, Musashi-1 and Bmi-1. Furthermore, upon grafting into neonatal rat brains, the tumor stem cells migrated and produced neurons and glia and continued to proliferate for more than 4 weeks.

Using a different approach based upon SP profiling, a putative stem cell population from a C6 glioma cell line has been identified (71). The C6 glioma SP cells displayed a higher potential for tumor formation and invasiveness compared with glioma cells, which did not possess a SP profile. However, in these studies it was not clear that the differences observed were statistically significant between the SP versus the non-SP group. Thus, while efforts have been aimed primarily at the identification and characterization of a tumor subpopulation with self-renewal potential *in vitro*, it will be essential in future studies to show that the identified cell populations are actually more tumorigenic *in vivo* as compared with the bulk of tumor cells.

Eric Holland and colleagues have reported that combined Ras and Akt signaling can induce glioblastoma (a malignant form of brain tumor) formation only after tissue-specific activation of Ras and Akt in neural progenitor cells, but not in differentiated astrocytes in mice (72). The same group also showed that loss of the *Ink4a–Arf* locus (encoding two proteins $p16^{INK4a}$ and $p14^{ARF}$, which are cell cycle modulators) cooperated with Ras to induce glioma formation in neural progenitor cells as well as differentiated astrocytes. The resulting tumors were nestin-positive, suggesting that deletion of *Ink4a–Arf* may cause dedifferentiation of astrocytes before inducing oncogenic transformation (73). Other pathways whose misregulation may cause malignant transformation of neural cells include the Platelet-Derived Growth Factor (PDGF)-activated signal transduction pathway. Tissue-specific overexpression of PDGF and PDGF receptors in neural progenitors or differentiated astrocytes cause the formation of oligodendrogliomas in the majority of mice. Furthermore, PDGF caused the dedifferentiation of cultured astrocytes to glial progenitor cells (74). These reports suggest that gene expression alterations, such as constitutive activation of Akt and Ras signaling, loss of the *Ink4a–Arf* locus or activation of receptor tyrosine kinases such as PDGF can induce oncogenic transformation in neural progenitor cells, as well as in differentiated astrocytes. Interestingly, when the oncogenic events were induced in differentiated neural cells (astrocytes) the resulting tumors expressed neural progenitor markers, suggesting the occurrence of dedifferentiation to self-renewing progenitors prior to transformation.

Conclusions and future prospects

It has been suggested that tumors contain a small population of putative cancer stem cells with unique self-renewal and survival mechanisms (1,11,27,64,75). Residual cancer stem cells may survive in a quiescent state for many years after remission and result in later relapse and metastasis. Therefore, it is conceivable that targeting cancer stem cells will eradicate tumor-initiating cells, whereas conventional chemotherapies will only eradicate the bulk of a tumor, sparing the cancer stem or initiating cells (45). At present, cancer stem cells and normal tissue stem cells appear to utilize the same self-renewal pathways. Therefore, it will be critically important to understand the biology of normal tissue stem cells in order to better characterize any changes which might occur in cancer stem cells. In particular, recent studies have characterized the cancer stem cells in AML and have identified a common mechanism by which the hematopoietic and AML stem cells self-renew. It is still unclear whether this phenomenon is true of other hematopoietic malignancies such as acute lymphoblastic leukemia and lymphomas. The preliminary identification of cancer stem cells in solid tumors, such as brain and breast cancers, is encouraging but requires further validation. It is still not clear whether the identified cancer stem cells express the same phenotype or surface markers as the tissue stem cells from which they were derived. The challenge will now be to determine whether unique pathways are employed in cancer stem cell survival and self-renewal. The design of new therapeutic agents should be aimed at targeting these unique molecular pathways, sparing normal tissue stem or differentiated cells.

As shown in the hematopoietic system, leukemic stem cells may be heterogeneous with respect to their self-renewal potential and quiescent state. This is also likely to be true in solid tissue cancers as well. Understanding the behavior of cancer stem cells should better enable the design of therapies targeted at the short-lived as well as long-lived cancer stem cells. As demonstrated in breast cancer, gene profiling is a powerful tool in identifying different types of breast cancer with respect to response to therapy, relapse and metastatic potential. However, it may be necessary to profile the tumor stem cells from these different types of breast cancer as well in order to determine more appropriate therapeutic approaches.

Additionally, in the field of stem cell targeted therapy, appropriate preclinical models in which to determine the efficacy of cancer treatment are lacking. It may be important in future studies designed to identify human breast cancer stem cells or initiating cells to use improved mouse xenograft models which mimic the human breast microenvironment. Furthermore, with the availability of over 60 genetically engineered mouse models of breast cancer, it will be important to validate these studies performed with human cells in xenograft models using parallel studies with well-defined mouse models. For example, will it be possible to identify a subpopulation of tumorigenic cancer stem cells in mouse mammary tumor

models using any of the cell surface markers employed to characterize either normal mammary stem cells/progenitors or human breast cancer stem cells? If these experiments are successful, it should be possible to utilize the wide variety of mouse genetic models to define the critical pathways involved in stem cell/progenitor self-renewal and survival. Additionally, selective activation or knockdown of genes in stem cells using stem cell promoter/enhancer-specific elements will allow study of the role of different pathways in the maintenance or initiation of many types of cancers. This approach has particular promise with respect to the generation of new mouse models of human cancer which may more closely model the human disease. For example, there has been recent progress in generating mouse models of pancreatic cancer based upon the ability to target oncogene activation in early pancreatic progenitors (76).

Most likely there will not be a single magic bullet. Once unique pathways are identified, combination therapy may be more effective than single therapy, based on the observation that cancer stem cells may be heterogeneous with respect to their quiescence state and proliferation capacity as well as the mechanisms underlying their transformation. Therefore, the future of cancer treatment may require individualized combination therapies targeting various unique pathways that are active in cancer stem cells.

Supplementary material

Supplementary material can be found at: http://www.carcin.oupjournals.org

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