Laboratory Investigation (2008) 88, 459–463

PATHOBIOLOGY IN FOCUS

The cancer stem cell hypothesis: in search of definitions, markers, and relevance

Michail Shipitsin and Kornelia Polyak

Cancer is a disease of genes. Inherited or somatic alterations in genes are what make a normal cell ignore growth-controlling signals and form a tumor that eventually leads to the destruction of the organism. Based on accumulated knowledge on the genetic composition of cancer cells, the clonal evolution model of tumorigenesis was established, which explains multiple aspects of human disease and clinical observations. However, the recently popularized cancer stem cell hypothesis questions that all or most tumor cells can participate in tumor evolution and restricts this property to a subset of them defined as 'cancer stem cells' due to their stem cell-like characteristics. Enthusiasm surrounding this area of investigation and its presumed clinical implications led to a spurt of studies in various cancer types and model systems. Rigorous study design and critical data interpretation have to be employed to test the scientific and clinical relevance of the cancer stem cell hypothesis and its relationship to the clonal evolution model.
Laboratory Investigation (2008) **88,** 459–463; doi:10.1038/labinvest.2008.14; published online 31 March 2008

KEYWORDS: tumor progression; clonal evolution; selection; cancer stem cell; heterogeneity

THE ROAD FROM HISTOLOGICAL OBSERVATIONS TO LEUKEMIC STEM CELLS

Populations of stem cells with unique properties exist in essentially all tissues of multicellular adult organisms. These cells are capable of self-renewal and can give rise to all cell lineages of the tissue they reside in. Stem cells are long-lived, being present throughout the entire life span of organisms; a characteristic that made them attractive targets of tumor-initiating transforming events that require years to accumulate.[1] According to the 'stem cell origin of cancer' hypothesis, stem cells or cells that acquired the ability to self-renew accumulate genetic changes over long periods of time, escape from the control of their environment, and give rise to cancerous growth. This distinct cell of origin may explain the well-known inter-tumoral heterogeneity of breast and other carcinomas (Figure 1). However, most tumors are largely composed of cells with some degree of differentiation, based on which the tissue of origin can be determined even in the case of distant metastases. One of the postulations of the 'cancer stem cell' hypothesis is that a population of cells with stem cell-like features exists in tumors and this population gives rise to the bulk of the tumor cells with more differentiated phenotypes.[2,3] The question is, if a cancer cell looks like a stem cell, does this necessarily mean that it is functionally a stem cell? What are the similarities and differences between stem cell-like cancer cells and normal stem cells, and can these characteristics change as tumors evolve?

Pathologists have noted the resemblance between stem cells and cancer long time ago. The ability of a single tumor cell to generate a new tumor was demonstrated in leukemias and ascites tumors using animal models long before FACS machines and antibodies were available.[4] Pathologists have also been classifying tumors as well or poorly differentiated, and these characteristics were shown to have prognostic relevance. Differentiation therapy was also successfully introduced and has been used for the treatment of certain hematopoietic malignancies including acute promyelocytic leukemia.

In the past decades, our understanding of hematopoietic stem cell biology significantly improved, and markers for cells with different degrees of differentiation were identified. Normal human hematopoietic stem cells were also shown to reconstitute normal hematopoiesis when transplanted into irradiated NOD-SCID mice. Based on this knowledge, hematopoietic malignancies were classified and researchers started to search for stem-like cells in leukemias by testing the ability of various purified populations to form leukemia in

Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA
Correspondence: Dr K Polyak, MD, PhD, Department of Medical Oncology, Dana-Farber Cancer Institute, 44 Binney Street, D740C, Boston, MA 02115, USA.
E-mail: Kornelia_polyak@dfci.harvard.edu

Received 31 October 2007; revised 08 January 2008; accepted 13 January 2008

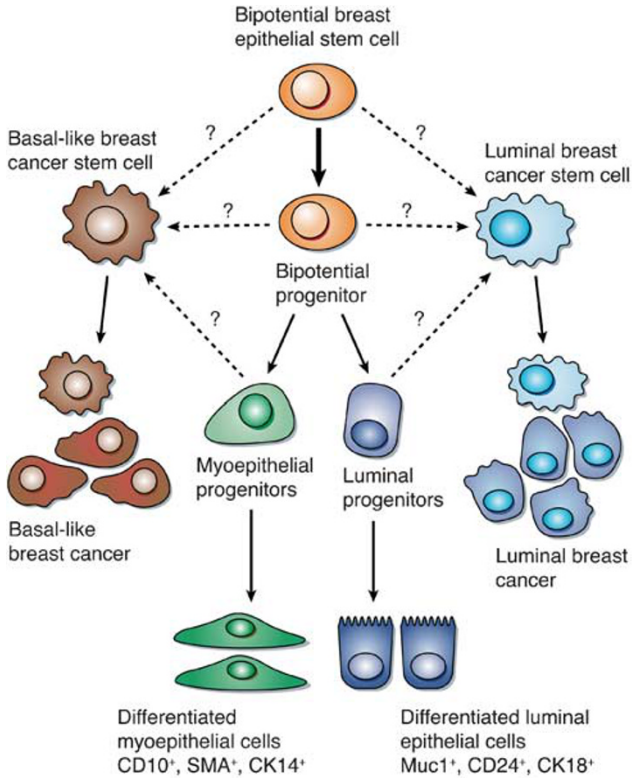

Figure 1 Hypothetical model of solid tumor cancer stem cells using breast cancer as an example. In the middle is a hypothetical differentiation pathway for normal mammary epithelial cells. Bipotential stem cells give rise to bipotential progenitors that can become committed myoepithelial or luminal progenitors differentiating into mature myoepithelial and luminal epithelial cells, respectively. During the differentiation process the self-renewal capacity of the cells gradually decreases. Cancer stem cells could potentially be derived from bipotential stem cells or from more differentiated cells that acquired self-renewal capabilities. Cancer stem cells, however, have restricted differentiation potential and only produce tumors with features of a particular lineage (eg, luminal or basal like).

NOD-SCID mice. Using this approach, injection of leukemic cells with primitive hematopoietic progenitor phenotype resulted in leukemias that could be serially transplanted into secondary recipients, whereas injection of more differentiated leukemic cells did not.[5] Further work demonstrated that certain leukemias maintain a differentiation hierarchy that may originate from cells with primitive hematopoietic phenotype.[5–7]

IDENTIFICATION OF CANCER STEM CELLS IN SOLID TUMORS

Putative cancer stem cells in leukemias were isolated and characterized on the basis of their phenotypical similarities to normal hematopoietic stem cells. In solid tumors, significant similarities have also been uncovered between putative cancer stem cells and normal progenitor/stem cells, although normal stem cells, their differentiation hierarchy, and markers that identify them are not well characterized in most solid organs. Despite this rudimentary knowledge, following the example of leukemia, researchers have succeeded in isolating subpopulations of tumor cells with stem cell-like features and with tumorigenic capabilities from many solid tumor types. One of the first such studies was conducted on breast cancer. In 2003, Al-Hajj *et al*[8] reported that $CD24^{-/low}/CD44^{+}$ fractions from metastatic pleural effusions and a primary invasive breast tumor had significantly higher tumorigenic potential when injected into the mammary fat pad of female NOD/SCID mice than $CD24^{+}/CD44^{+/-}$ cell fractions. This initial study was rapidly followed by similar findings in a wide variety of tumors, including brain tumors, prostate, colon, pancreatic, and hepatic carcinomas, melanoma, and a few other tumor types.[2,3] The overall procedure for the isolation of cancer stem cells was rather similar across the studies: fractionation of tumor cells using cell-surface markers characteristic of stem cells, followed by their implantation into NOD-SCID mice to assess xenograft growth and cellular composition. The CD133 cell-surface marker was used to purify putative cancer stem cells in several tumor types, with the exception of breast,[8] prostate,[9] and head and neck carcinomas[10] where CD44 was utilized instead. CD133 (prominin-1) was discovered as a marker of normal hematopoietic stem cells and later was found to mark stem/progenitor cells from a wide variety of tissues.[11]

WHAT MAKES A CANCER CELL A CANCER STEM CELL?

In vitro culture in unattached conditions where cells grow in round balls called 'spheres' is routinely used for enrichment and propagation of stem cells.[12] In the case of brain tumors, $CD133^{+}$ cells could successfully grow in unattached, neurosphere-like formations, whereas $CD133^{-}$ cells could not.[13] When cells from glioblastomas were cultured in neurosphere conditions, 'tumor spheres' expressed a number of neuronal stem cell markers and were also highly tumorigenic and resistant to radiation.[14] Similar findings have been obtained from breast cancer and other tumor types. Despite the apparent success of suspension cultures to enrich for stem cell-like cells, the assay raises several questions. Most importantly, the function of normal tissue stem cells (eg, self-renewal, multipotency, proliferation, and differentiation) is regulated by direct and paracrine interactions with supporting cells and the extracellular matrix (ECM) that form the so-called stem cell niche. For example, endothelial cells were shown to be critical for maintenance of neural stem cells and it appears that $CD133^{+}$ brain tumor stem cells may also require such niche.[15–17] Based on these data demonstrating the necessity of the niche for maintenance of both normal and cancer stem cells, the question is, how is it possible that in 'sphere cultures' cells alone without any ECM attachment display a stem cell phenotype? Previous studies have also described that cells in suspension upregulate many pro-survival pathways, increase their antioxidant levels, and as a consequence become resistant to apoptosis. Therefore, radiation and drug sensitivity assays performed in sphere cultures have to be

carefully designed and interpreted. Comparing cells growing in adherent and suspension cultures may yield differences irrespective of cellular differentiation status.

To gain a better understanding of putative breast cancer stem cells at the molecular level, Shipitsin *et al*[18] performed SAGE (serial analysis of gene expression) profiling of $CD24^{-/low}/CD44^{+}$ and $CD24^{+}/CD44^{+/-}$ cell populations from normal and neoplastic human breast tissue. The expression profiles of stem-like cells from normal and neoplastic breast tissue were highly similar and both expressed numerous stem cells markers, whereas both normal and breast cancer $CD24^{+}/CD44^{+/-}$ cells had features of luminal differentiation. Because almost all the tumors in this and in the Al-Hajj study were luminal subtypes, these findings as well as the use of the CD24 and CD44 cell surface markers for the identification of stem-like cells have to be further validated.

While there are many similarities, there also are differences between cancer and normal stem cells. Unlike normal stem cells that represent a very small population of cells in the tissue they reside in, putative cancer stem cells can make a rather significant portion of tumor cells. In breast cancer, putative cancer stem cells with $CD24^{-/low}/CD44^{+}$ phenotype constituted 12–60% of the tumor cells, whereas in colon cancer, CD133+ putative cancer stem cells ranged from 3.8 to 24.6% of total tumor cells;[8,12,19] thus, it is possible that in poorly differentiated tumors, cancer stem cells constitute the majority of tumor cells. In fact, based on recent mathematical calculations determining the probability of tumor growth after treatment, the assumption that cancer stem cells represent a small fraction of all cancer cells in an advanced stage tumor does not appear to be correct.[20]

The cancer stem cell hypothesis largely ignores the inherent properties of malignant cells: genomic instability and the ability to undergo rapid evolutionary changes. For example, when 'tumor spheres' derived from highly vascular glioblastomas were transplanted into mice, the initial tumors demonstrated low-grade glioma phenotype without any sign of angiogenesis. However, upon serial transplantation *in vivo*, the tumor cells developed a highly malignant phenotype with extensive angiogenesis and necrosis being present in the tumors.[21] This finding highlights a well-established fact that tumor cells evolve and if more malignant and less differentiated cancer cells have growth advantage, they will be selected for and expand in the tumor. Therefore, as tumors progress, the line between cancer stem cells and the rest of tumor cells might gradually become blurry and can even disappear (Figure 2). This possibility is well illustrated by the existence of highly tumorigenic cell lines that can efficiently form xenograft tumors without any obvious stem cell-like subpopulations.[22] The recent finding that, depending on the tumor analyzed, glioblastoma cancer stem cells can be $CD133^{+}$ or $CD133^{-}$ cells, also emphasizes that either we do not have good markers for cancer stem cells or that all tumor cells are tumorigenic just at varying degree.[23] Finally, the occasional genetic divergence of $CD24^{-/low}/CD44^{+}$ and $CD24^{+}/CD44^{+/-}$ breast cancer cells re-emphasizes the notion that tumors are genetically heterogeneous and there is a continuous selection for the survival of the fittest.[18] Thus, cancer stem cell characteristics may just be a phenotype responsible for or associated with tumor progression driving evolutionary advantages.

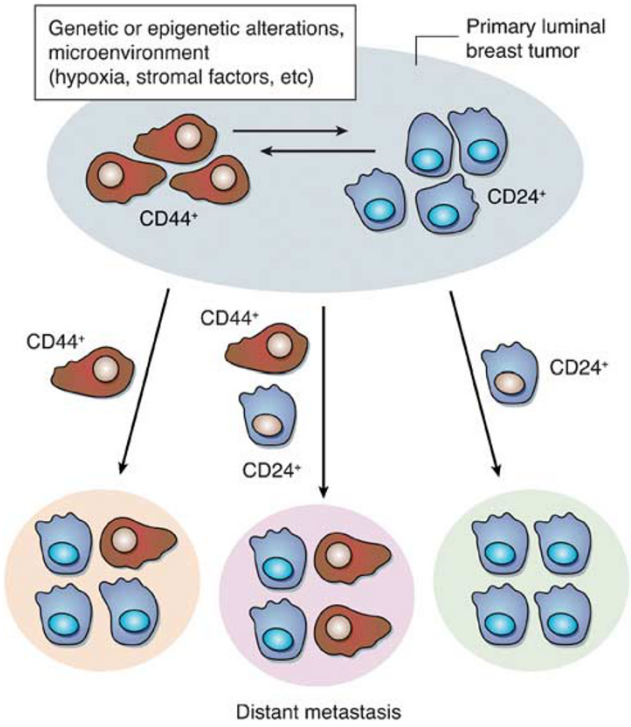

Figure 2 Hypothetical model of tumor progression to metastatic disease in breast cancer. Primary luminal breast tumors consist of $CD44^{+}$ stem-like breast cancer cells with high invasive and angiogenic capacity, and more differentiated $CD24^{+}$ breast cancer cells. Distant metastases can be initiated by invasive $CD44^{+}$ breast cancer cells that later can switch to a more differentiated $CD24^{+}$ cell phenotype due to microenvironmental conditions or selection due to therapeutic interventions (left). Alternatively, during tumor progression $CD24^{+}$ can become more invasive and initiate metastases themselves (right), or the two cell types ($CD24^{+}$ and $CD44^{+}$ breast cancer cells) may initiate metastases together (middle). Furthermore, $CD24^{+}$ cells can change to $CD44^{+}$ stem cell-like cells due to acquisition of genetic, epigenetic changes, or environmental factors such as hypoxia.

INCONSISTENCIES AND QUESTIONS TO BE ANSWERED

Xenotransplant assays have been employed to prove cancer 'stemness'. An important question is how realistically tumor xenograft models in immunodeficient mice recapitulate what is happening in human patients. For successful xenograft tumor growth, human cancer cells have to adapt to the mouse microenvironment. This brings up the issue as to whether the ability of cells to adapt in new conditions rather than their 'absolute' tumorigenic capabilities plays a major role in xenograft growth. As pointed out in a recent study by Kelly *et al*[24] using a transgenic mouse myc-induced

B-lymphoma model, both cells with or without stem cells-like phenotype were able to cause lymphomas in recipient mice. Similar results were obtained for N-Ras-induced T-lymphoma and PU.1-knockout acute myeloid leukemia (AML) mouse models. These results suggest that xenograft models might underestimate the number of tumor-initiating cells. In the case of solid tumors, this underestimation may be even more pronounced, as solid tumors require a whole supporting infrastructure of endothelial cells and fibroblasts forming intra-tumoral blood vessels and providing paracrine factors. Thus, xenotransplanted human cancer cells have to recruit normal mouse endothelial and stromal cells for their successful growth. It has been shown that the same tumor cell fraction injected into different sites of the same mouse (eg, subcutaneous, renal capsule) can form a tumor at one site but not the other. Furthermore, supplementing the transplanted tumor cells with tumor stromal and endothelial cells or mesenchymal stem cells greatly enhances tumorigenicity. The question then arises as to which one of these results reflects 'real' tumorigenicity? Another inherent problem with the use of immunodeficient mice is the lack of lymphocytes and macrophages that are known to play an important role in tumor growth, angiogenesis, and metastasis. Recent attempts to rescue these mice with human bone marrow may resolve some of these problems. However, it is unlikely that rapidly growing xenografts will ever fully reproduce the complexity of human tumors that take years to decades to evolve.

According to the cancer stem cell hypothesis, only cancer stem cells have self-renewal ability and the rest of the tumor cells would die out without being replenished from the cancer stem cells. One of the predictions of this hypothesis is that tumor progression-driving genetic events may only accumulate in cancer stem cells, since other cells are presumed to be evolutionary 'dead ends'. A major problem with this hypothesis is that it assumes stability within the tumor and does not consider the possibility that the cancer stem cell phenotype can be acquired. However, several studies have demonstrated that more differentiated cancer cells can acquire mutation (eg, β-catenin mutation) or activate a transcription factor (eg, FOXC2 or some other stem cell phenotype-inducing transcription factor) and become cancer stem cells (Figure 2). Thus, essentially we are back to where we were and what we knew for decades: tumors are diverse, genetically unstable, and evolve due to the intra-tumoral diversity of cellular genotypes and phenotypes.[25] Recent papers by several groups have shown that even a normal human differentiated cell can be converted to a functional pluripotent embryonic stem cell just by expressing the right combination of transcription factors in them.[26] Thus, the question is what would prevent a 'differentiated' cancer cell from acquiring similar changes and become a cancer stem cell?

Another inconsistency related to the cancer stem cell model is its explanation of therapeutic resistance. Based on the hypothesis, and some rudimentary data, cancer stem cells are inherently resistant to treatment and only their progeny are successfully eliminated by current therapeutic interventions. However, this would predict that the progeny of the cancer stem cell should remain sensitive to the same treatment over time and acquired therapeutic resistance should not arise. But this is clearly not the case. Proponents of the cancer stem cell hypothesis suggested that cancer stem cell can also evolve and acquire additional genetic events and can be clonally selected under some environmental pressure. However, if cancer stem cell is the dominant clone in the tumor that has selective advantage over other cancer cells due to its phenotypic characteristics, then what additional information the terminology 'cancer stem cell' really adds to the clonal evolution model?

CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

One of the most exciting and emphasized implications of cancer stem cell hypothesis is its perceived potential of improving cancer prevention, diagnosis, and treatment. Liu *et al*[27] and Shipitsin *et al*[18] independently profiled $CD24^{-/low}$/$CD44^{+}$ and $CD24^{+}$/$CD44^{+/-}$ cell populations from multiple breast tumors and concluded that a gene expression signature characteristic of breast cancer stem cells is associated with shorter distant metastasis-free and overall survival. These findings strongly suggest that the presence and frequency of $CD24^{-/low}CD44^{+}$ tumorigenic breast cancer cell populations in tumors have prognostic relevance.

The idea that cancers could be effectively treated by selectively targeting tumor stem cells populations attracted the most clinical interest, but has the least amount of supporting data in human patients. Recently, Jin and co-workers demonstrated that treatment of mice transplanted with human AML cells with activating anti-CD44 antibody markedly reduced leukemic repopulation. The major mechanism responsible for this eradication involved interference with AML stem cells engraftment and repopulation capabilities. As future studies will characterize tumor stem cells in greater details and define their distinctive properties, therapies specifically targeting cancer stem cells in solid tumors might become a reality. However, the demonstration that distant metastases are largely composed of $CD24^{+}$ more differentiated cells in patients refractory to treatment highlights the fact that 'non-stem cell-like' cancer cells can kill the patients.[18] Furthermore, as discussed above, due to inherent genomic instability cancer cells can evolve and more differentiated cancer cells may acquire stem cell phenotypes. Thus, eradication of tumors will likely be achieved by the successful targeting of all cancer cells using a cocktail of drugs effective against all cancer cell sub-populations.

In conclusion, it is well established that cancer cells with stem cell-like features exist in the majority, if not all, of tumor types. Based on xenograft assays, these subpopulations demonstrate high tumorigenicity potential, but it is currently unknown how this relates to their behavior in patients. Initial

studies suggest that the presence of these stem-like cancer cells in tumors have prognostic relevance and influences therapeutic response. However, many questions will remain to be answered before the role of stem-like cancer cells in tumor initiation and progression is fully understood. Most importantly, the numerous inconsistencies within the model have to be resolved and the hypothesis has to be tested in light of the known genetic heterogeneity and genomic instability of human cancer. Thus, it remains to be seen if incorporating cancer stem cells in our view of tumorigenesis adds to our scientific knowledge and helps cancer treatment.

1. Reya T, Morrison SJ, Clarke MF, *et al.* Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–111.
2. Dalerba P, Cho RW, Clarke MF. Cancer stem cells: models and concepts. Annu Rev Med 2007;58:267–284.
3. Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea—a paradigm shift. Cancer Res 2006;66:1883–1890, discussion 1895–1886.
4. Polyak K, Hahn WC. Roots and stems: stem cells in cancer. Nat Med 2006;12:296–300.
5. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730–737.
6. Lapidot T, Sirard C, Vormoor J, *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 1994;367:645–648.
7. Dick JE. Complexity of the human acute myeloid leukemia stem cell compartment: implications for therapy. Biol Blood Marrow Transplant 2005;11(2 Suppl 2):9–11.
8. Al-Hajj M, Wicha MS, Benito-Hernandez A, *et al.* Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100:3983–3988.
9. Collins AT, Berry PA, Hyde C, *et al.* Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65: 10946–10951.
10. Prince ME, Sivanandan R, Kaczorowski A, *et al.* Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci USA 2007;104: 973–978.
11. Shmelkov SV, St Clair R, Lyden D, *et al.* AC133/CD133/Prominin-1. Int J Biochem Cell Biol 2005;37:715–719.
12. Jensen JB, Parmar M. Strengths and limitations of the neurosphere culture system. Mol Neurobiol 2006;34:153–161.
13. Singh SK, Clarke ID, Terasaki M, *et al.* Identification of a cancer stem cell in human brain tumors. Cancer Res 2003;63:5821–5828.
14. Lee J, Kotliarova S, Kotliarov Y, *et al.* Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006;9:391–403.
15. Shen Q, Goderie SK, Jin L, *et al.* Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 2004;304: 1338–1340.
16. Ramirez-Castillejo C, Sanchez-Sanchez F, Andreu-Agullo C, *et al.* Pigment epithelium-derived factor is a niche signal for neural stem cell renewal. Nat Neurosci 2006;9:331–339.
17. Calabrese C, Poppleton H, Kocak M, *et al.* A perivascular niche for brain tumor stem cells. Cancer Cell 2007;11:69–82.
18. Shipitsin M, Campbell LL, Argani P, *et al.* Molecular definition of breast tumor heterogeneity. Cancer Cell 2007;11:259–273.
19. O'Brien CA, Pollett A, Gallinger S, *et al.* A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106–110.
20. Kern SE, Shibata D. The fuzzy math of solid tumor stem cells: a perspective. Cancer Res 2007;67:8985–8988.
21. Sakariassen PO, Prestegarden L, Wang J, *et al.* Angiogenesis-independent tumor growth mediated by stem-like cancer cells. Proc Natl Acad Sci USA 2006;103:16466–16471.
22. Zheng X, Shen G, Yang X, *et al.* Most C6 cells are cancer stem cells: evidence from clonal and population analyses. Cancer Res 2007;67:3691–3697.
23. Beier D, Hau P, Proescholdt M, *et al.* CD133(+) and CD133(−) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res 2007;67:4010–4015.
24. Kelly PN, Dakic A, Adams JM, *et al.* Tumor growth need not be driven by rare cancer stem cells. Science 2007;317:337.
25. Campbell LL, Polyak K. Breast tumor heterogeneity: cancer stem cells or clonal evolution? Cell Cycle 2007;6:2332–2338.
26. Yu J, Vodyanik MA, Smuga-Otto K, *et al.* Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318:1917–1920.
27. Liu R, Wang X, Chen GY, *et al.* The prognostic role of a gene signature from tumorigenic breast-cancer cells. N Engl J Med 2007;356:217–226.