

# Identifying Cancer Stem Cells in Solid Tumors: Case Not Proven

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## Abstract

**Building on studies of leukemia, a number of recent articles have reported data suggesting that cancer stem cells could be isolated from solid human cancers. Some of these reports have speculated that the isolation of these cells will allow the identification of the specific molecular properties that can be targeted for therapeutic purposes. Although previous work with animal model systems also suggests the presence of stem cells in solid tumors, there remain many uncertainties, both theoretical and technical, about the interpretation of the current results. The case that a small proportion of cells in solid tumors are specific cancer stem cells and that these cells can be successfully identified and isolated has not yet been proven.** (Cancer Res 2006; 66(4): 1891-6)

## Introduction

The concept of a cancer stem cell is very appealing; it is a cell that can regrow the tumor and hence is the cell that must be killed if the tumor is to be controlled by treatment. It has been known for many years that it often requires a large number of tumor cells to transplant both rodent and human tumors. Initial studies done using transplantation of cells from spontaneous tumors into nonsyngeneic animals or using cells from virally or chemically induced tumors could potentially be explained by invoking immune rejection. However, the demonstration of similar results using spontaneously arising tumors transplanted into syngeneic mice is not easily explained by this mechanism (1), and has led to the suggestion that only a fraction of tumor cells might have the ability to regrow the tumor (i.e., are cancer stem cells). This concept is supported by the observation that serial transplantation of animal tumors often leads to a reduction in the number of cells required to transplant the tumor as the number of transplant generations increases, suggesting selection for cancer stem cells (for example, see ref. 2).

Furthermore, analyses of the doses required to control tumors with radiation therapy, using information about the measured radiation sensitivity of tumor cells, suggests that not every cell in a tumor needs to be killed in order to cure the tumor (for review, see refs. 3-5). This is consistent with work reported by Hill and Milas (6), who used various spontaneous rodent tumors, particularly mammary tumors, to show that the radiation dose required to cure early generation transplants of these tumors (TCD<sub>50</sub> value) was inversely proportional to the number of cells required to transplant the tumors (the TD<sub>50</sub> value). The work also showed that the TD<sub>50</sub> value for mouse mammary tumors was inversely proportional to

the plating efficiency of the tumor cells in culture and that there was a range of more than 2 orders of magnitude in TD<sub>50</sub> value and plating efficiency between the different tumors. A similar range of plating efficiencies has been reported for human tumors plated in culture (see e.g., ref. 7). These analyses suggest that the proportion of cells in a tumor with the ability to regrow the tumor following treatment (i.e., cancer stem cells) may be very low ( $\leq 1\%$ ).

However, as pointed out by Wang and Dick (8), because individual cells are not identified in such studies, it is not possible to distinguish between whether there are varying numbers of (true) stem cells in different tumors, or whether all tumor cells in any one tumor retain a probability less than unity of being able to regrow the tumor. It is also not possible to rule out the idea that there may be varying degrees of "stemness" in cancer stem cells derived from tumors. Studies with leukemia, modeled on the work identifying specific surface markers used to isolate bone marrow stem cells (long-term reconstituting cells) have succeeded in identifying cells with specific surface markers that seem to play a cancer stem cell role in leukemias (8). Recent studies with solid human tumors using similar methodologies are being interpreted as evidence for the presence of cancer stem cells in solid tumors (9, 10). It was reported that as few as 100 to 200 cells from a specific cell fraction sorted from primary tumors (e.g., breast or brain tumors) could successfully transplant the tumors (into the mammary gland or the brain) of immune-deprived mice, respectively, whereas much larger numbers of cells ( $10^4$ - $10^5$ ) from other fractions were not able to transplant the tumors. These findings are consistent with the hypothesis that cancer stem cells exist but they suggest that only ~1% of cells in the sorted population was able to act as a "stem-like" cell under the conditions used for testing tumorigenicity (i.e., the surface markers used for sorting the cells do not really identify the cancer stem cells). Furthermore, there remain both theoretical and technical questions about whether the cells sorted are the true (and only) cancer stem cells in the tumors (see Fig. 1). From the perspective of developing new therapeutics based on the properties of the sorted cells, it is a critical question whether the cells isolated and identified by their ability to regrow a tumor in a new transplant site are necessarily the ones (or the only ones) that can regrow the tumor in its original location following treatment.

## Theoretical Issues

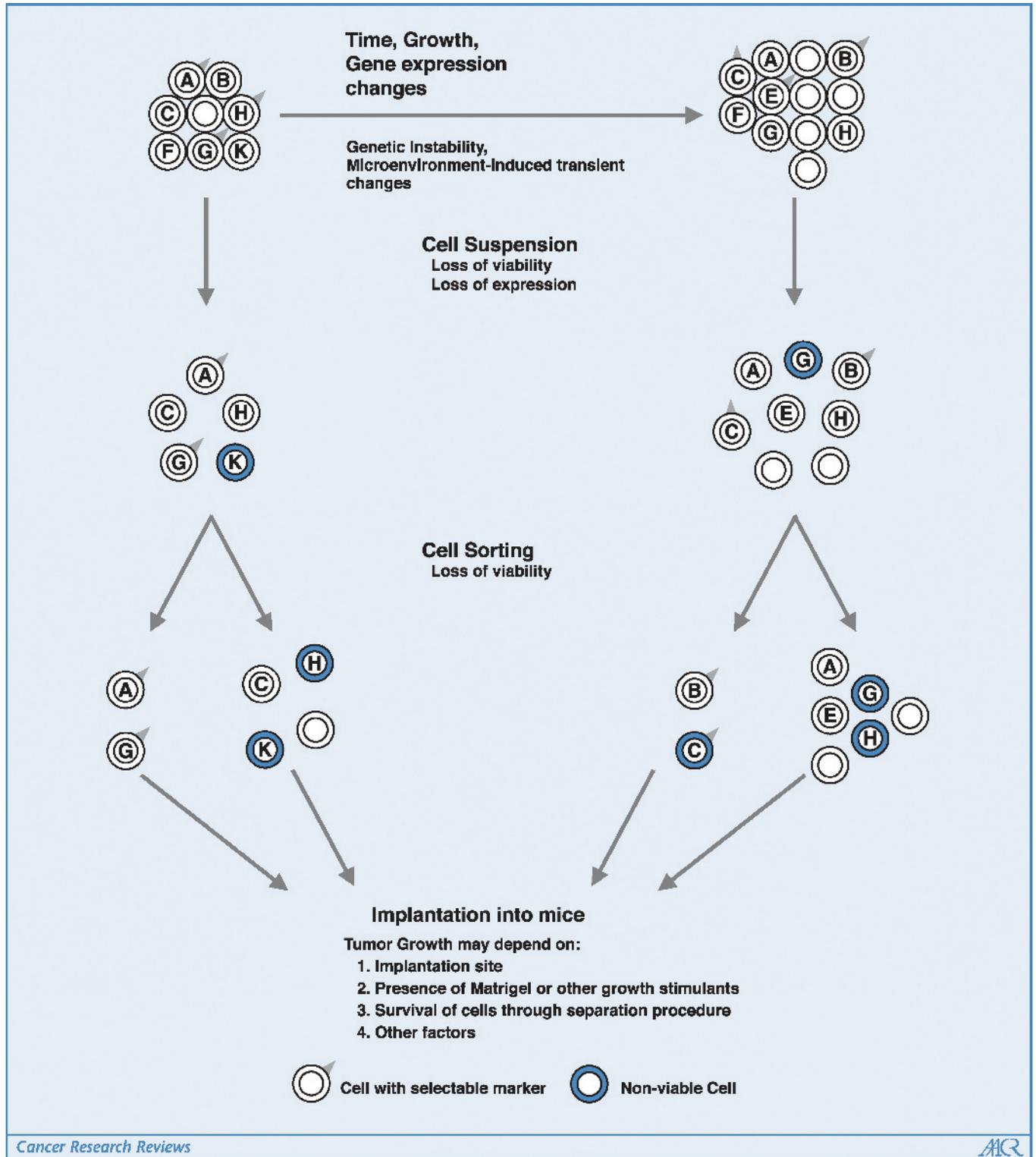
The cancer stem cell model is based on the idea, developed from studies of normal bone marrow, that stemness and maturation or differentiation are mutually exclusive. It does not easily incorporate the idea that there may be variations of stemness with time. The mutual exclusivity concept has been applied successfully to the normal development of bone marrow, but the recent observations that both bone marrow and stromal cells derived from bone marrow may have a wide range of abilities to differentiate into different cell types suggests that stemness and maturation are

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doi:10.1158/0008-5472.CAN-05-3450

influenced by the environmental stimuli that the cells experience (11). Similar results are also being reported for cells derived from a variety of tissues including muscle and brain, two tissues that classically have been regarded as highly differentiated with little proliferative capacity (12, 13). Thus, the environment to which

normal cells are exposed can affect their ability to mature and differentiate, and must be tightly controlled *in situ*. This is encapsulated in the concept of a “niche” in the bone marrow that is required to maintain the status of the bone marrow stem cells (14). Tumor cells are characterized by the fact that they do not



**Figure 1.** Schematic diagram to illustrate potential difficulties in sorting and identifying “cancer stem cells.” *Top*, theoretical concerns; *bottom*, technical concerns (discussed in the text).

respond normally to homeostatic controls and it is likely that such a lack of response extends to controls associated with stemness and maturation. Tumor cells usually contain a wide range of genetic and epigenetic alterations, and such alterations may affect the relationship between stemness and differentiation. Whether there is an environment in solid tumors that could be described as a "cancer stem cell niche" remains to be established.

Furthermore, the genome of cancer cells is known to be unstable, a factor that drives tumor development and progression, so the relationship between stemness and differentiation may change in the tumor cells during the lifetime of an individual tumor. Studies with transplantable tumors have shown high rates of genetic or epigenetic changes in tumor cells during growth ( $\sim 10^{-5}$ /cell/generation) that can affect various properties including transplantability (see e.g., refs. 15, 16). If these rates are applied to a typical breast cancer (2 cm diameter, 10% of cells in proliferative cycle, 2-3 days cycle time), this implies  $\sim 10^3$  genetic or epigenetic changes per day. Cells with mismatch repair deficiency are also known to have "mutation" rates 2 to 3 orders of magnitude above background (17), and the breast cancer susceptibility genes *BRCA1* and *BRCA2* are recognized as playing a role in DNA repair (18). Changes in gene expression can also be induced by the environment to which the cells are exposed (e.g., hypoxia) and such changes can be expressed transiently, thus, not all cells in a tumor population may be expressing such changes at the time when tumor cells are sorted for testing of their "stem cell" ability by transplantation (Fig. 1; refs. 19, 20). Moreover, such effects may be exacerbated during treatment because many anticancer agents are also mutagens.

Despite these theoretical concerns about the likelihood that true cancer stem cells occur in solid tumors, it can be argued that the findings that cells with stem-like properties can be enriched by sorting tumor cell populations indicates that such cells do exist. However, this is only true in a functional sense and it remains unclear whether or not tumor cells that have apparently lost the ability to manifest as cancer stem cells today can regain that ability next week or next month. This issue could be addressed (albeit not definitively) by establishing *in vitro* cultures from the different sorted fractions of tumor cells and examining, as a function of time, the proportion of the cells in these cultures that are capable of initiating tumor growth when injected back into animals.

## Technical Issues

Currently, the isolation and identification of cancer stem cells from human solid tumors is as much an art as it is a science. It requires the use of techniques to: (a) prepare a cell suspension, usually with proteolytic enzymes, (b) sort the cells based on the expression, or lack thereof, of surface molecular markers or exclusion of the dye, Hoechst 33342, and (c) transplant the cells back into immune-deprived animals to determine how many of the sorted cells are required to regrow a tumor (see Fig. 1). The finding that cell populations can be isolated, from which different numbers of tumor cells are required to regrow a tumor after transplantation, is fundamental to the interpretation that stem-like cells are being isolated. Studies with leukemias have supported this idea (8), but in practice, such studies are much more difficult when applied to solid tumors. It is instructive to examine the various stages of the experiments.

**The environment of the tumor cells.** A solid tumor is not a bag of tumor cells, it contains both tumor cells and a variety of normal

cells and vascular elements set in a complex extracellular matrix. Interactions between cells and the extracellular matrix play a major role in controlling their behavior and indeed may dictate whether or not a tumor develops from a genetically damaged cell. Equally, an aberrant extracellular matrix may promote cancer development (21). Furthermore, the pathophysiologic microenvironment in tumors can be very heterogeneous. Both factors can play a significant role in the expression of a wide range of genes in the tumor cells including surface markers (extensively shown for hypoxia; refs. 22, 23). The breaking of these interactions by preparing cell suspensions may well change the properties of the cells and testing these properties by simple injection of cells into a new tissue location is unlikely to recapitulate the environment experienced by the tumor cells in the original tumor. It may or may not recapitulate a niche suitable for the growth of any or all the "cancer stem cells" or may even inhibit their growth (21). Furthermore, cell recovery from solid tumors can be quite variable (it rarely exceeds 10% of the original cell number in the tumor) and tumors contain many normal cells, so that there may be concerns about the representative nature of the recovered cell populations.

**Sorting the tumor cells.** One selection technique applied for sorting the tumor cells in the prepared suspension is based on the presence or absence of surface molecular markers. In the work on breast cancers reported by Al-Hajj et al. (9), these markers (CD44<sup>+</sup>, CD24<sup>-</sup>, and ESA<sup>+</sup>) were not obviously related to stemness but rather were features of a differentiating phenotype. The absence of such markers on stem cells seems to make more sense than their presence; thus, positive expression of CD44 may be regarded as surprising. Moreover, it is well recognized that there are a number of different CD44 molecules expressed on the surface of cells, only one of which (CD44v) has been particularly associated with metastasis, which might be considered as a property of cancer stem cells (24). The evidence that the sorted cells may have stem cell properties is stronger in brain tumors in which the marker used was CD133 (25), which has previously been associated with "neural stem cells".

However, with any such molecular markers, it is a concern that the enzyme treatment used during the preparation of the cell suspension might modify expression on the cell surface, thereby affecting the population into which the cells are sorted and the ability of these molecules to play a role in the early stages of tumor growth following transplantation. Another problem may be the loss of viability of the cells during the enzyme treatment and sorting procedure. This latter issue could possibly be tested by also plating the cells in culture to test their clonogenic capacity. A final concern here is that the proportion of cells in the cell suspension which express the markers used for sorting (e.g., 11-35% CD44<sup>+</sup>, CD24<sup>-</sup> cells) in the breast cancer studies of Al-Hajj et al. (9) is too great for them all to represent true cancer stem cells, based on the work in animal tumors described above. This number can be contrasted with the finding that >10,000 cells from the unsorted populations were needed to initiate tumors, implying a stem cell fraction in the unsorted population of <0.01%. There is a clear discrepancy between these numbers and this is not accounted for even if only 1% of the sorted cell population were actually stem cells.

In this context, it might be informative if the expression of the markers used for sorting in the cell suspension could also be studied in the primary tumor mass using immunohistochemistry, so that evidence for a correlation could be sought. The work with

brain tumors used immunohistochemistry, but whether there was a correlation between the proportions of CD133<sup>+</sup> cells in the tumors and in the derived suspensions was not reported (10). During sorting, these authors also found that there was a very high proportion of putative stem cells (CD133<sup>+</sup> cells) in primary brain tumors (6-29%), although it is noteworthy that the higher numbers were mostly associated with the more aggressive tumor type. For these brain tumors, as few as 100 CD133<sup>+</sup> cells could form tumors in the brains of nonobese diabetic/severe combined immunodeficient mice, whereas 50 to 100 × 10<sup>3</sup> CD133<sup>-</sup> cells could not. The number of unsorted cells required to form tumors in the mouse brain was not reported for these tumors, although the authors indicate that others have found values of 10<sup>5</sup> to 10<sup>6</sup> cells required to transplant primary brain tumors. If such numbers apply to the brain tumors actually studied, there is again a significant discrepancy between the fraction of putative stem cells (CD133<sup>+</sup>) in the tumors and the number of sorted or unsorted cells required for successful tumor transplant. In both cases, the proportion of putative stem cells in the tumors is much higher than would be predicted from the studies with rodent tumors, and if correct, implies that it would be unnecessary to sort these cells for identification and testing of specific drugs targeted at cancer stem cells.

An alternative strategy for sorting is to identify a so-called "side population" of tumor cells based on increased efficacy for exclusion of the dye Hoechst 33342. This approach is also problematic. The technique seems to be highly variable, may require modification for each tumor cell population studied, and there are concerns about the possible toxicity of Hoechst dye in the cells that do not form part of the side-population (26). Furthermore, such side populations can be identified in many long transplanted tumor cell lines (26-28). This observation has been interpreted to indicate that such cell lines contain small fractions of stem cells, but this is unlikely, on the basis of animal studies and more likely reflects the inadequacy of the sorting technique or of the testing procedures for tumor transplantability (see below). Also, the cells in the side population express high levels of known drug-efflux proteins on their surface but why these proteins should be associated with stemness is not entirely clear. In fact, a recent article (27) has reported that it is low expression of one of the major candidate drug-efflux proteins for identifying breast stem cells (ABCG2) that is associated with stemness in certain cancer cell lines rather than high expression. These authors suggested a two-stage model for the expression of this protein in relation to stemness, suggesting that the protein was turned on and then off again during maturation of the tumor cells. The authors had to postulate that other (unidentified) drug-efflux proteins were responsible for the presence of the ABCG2-negative cells in the side-population.

**Tumor transplantation.** The test of whether a tumor cell can regrow a tumor is not simple. It is highly dependent on the environment to which the cell is exposed. Careful studies using spontaneously arising tumors in syngeneic rodents have shown that the number of cells required to transplant a tumor depends on the location of transplantation and their environment; e.g., on whether heavily irradiated tumor cells (feeder cells) were injected along with the viable tumor cells (known as the Revesz effect; ref. 29). Other biological substances, including brain extract, have been shown to have similar effects (30). The presence of a large number of heavily irradiated cells (10<sup>5</sup>-10<sup>6</sup> per injection) was shown to modify the number of cells required to transplant a tumor s.c. by

orders of magnitude and the size of this effect varied from one tumor to another (29). Even long transplanted cell populations in which virtually every cell has stem cell properties are influenced by the Revesz effect. For example, such cell lines can produce tumors from as little as one to three cells if transplanted in the presence of large numbers of heavily irradiated cells, but may require hundreds of cells to produce a tumor in the absence of such heavily irradiated cells. The location of the transplant can also be an important factor even for s.c. implants (see ref. 31 for a review of various factors that can affect TD<sub>50</sub> assays). Implantation into the kidney capsule has also been shown to be a highly receptive site to obtain tumor growth from small numbers of tumor cells or small pieces of tumor (32, 33), and it has been shown that inducing an inflammatory response at the site of injection could modify the transplantability of tumor cells.

Early work suggested that the ability of the tumor cells to remain and survive at the injection site was an important aspect of the Revesz effect (30). More recent studies identifying growth factors and angiogenic factors produced by tumor cells suggest that an important function of the feeder cells may have been to provide a suitable concentration of stimulatory growth factors for the viable tumor cells to be able to survive and proliferate. More recent work demonstrating the increased efficacy of transplantation into s.c. or orthotopic sites when the cell suspension is mixed with Matrigel (a basement membrane-like substance that contains many growth factors) prior to implantation, is consistent with this idea (34, 35). Another example is the widely reported observation that the MCF-7 breast cancer cell line transplants more efficiently and grows better in immune-deficient mice that have been separately implanted with pellets that provide a slow release of estrogen (see e.g., ref. 36). These findings suggest that the cells putatively identified as cancer stem cells by transplantation procedures might be cells that are individually capable of making sufficient growth factors to close the autocrine growth loop or those which can effectively interact with and obtain such stimulation from the (micro)environment into which they are implanted, rather than those having specific properties of stemness when in a large tumor mass. It is probable that the molecular phenotype that allows a tumor cell to manifest as a cancer stem cell on transplantation varies depending on the procedure adopted for testing stemness.

## Conclusions

The above concerns do not rule out the possibility that cancer stem cells exist in solid tumors and that they play a major role in the local regrowth of cancers following treatment and/or in the development of metastases. But it is very possible that, in cancer cells, degrees of stemness exist and that these are variably expressed depending on the environment to which the cells are exposed. There is a tenet in Scottish law that allows the court to bring down a verdict of "not proven" in a case in which the evidence is suggestive but insufficient for conviction. In my view, this is the current status of the case for identifying cancer stem cells from solid tumors.

## Acknowledgments

Received 9/25/2005; revised 11/8/2005; accepted 11/23/2005.

**Grant support:** Canadian Institutes of Health Research, the Ontario Cancer Research Network, and the National Cancer Institute of Canada with funds raised from the Terry Fox Run.

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## Response

As noted by Wicha et al. (1), the concept of cancer stem cells is not new. The recent revival of interest in this topic arises from studies of normal tissue stem cells and from experiments suggesting that cells with stem-like properties can be sorted from solid tumors based on the expression of specific surface markers. The existence and properties of normal tissue stem cells are of great interest but provide only indirect evidence for the presence of stem cells in solid cancers. Many of the issues discussed in the article by Wicha et al. (1), such as anchorage-independent growth, metastatic dormancy, and the deregulation of specific signaling pathways (Wnt, Notch, and Hedgehog) involved in normal cell differentiation, focus on the concept of cancer stem cells, but at best, they provide only circumstantial evidence of their existence. In fact, the evidence for deregulation of these signaling pathways in cancers could be interpreted to indicate that control of "stemness" is lost in cancer cells. Furthermore, the argument that transgenic models for cancer development, which use tissue-specific promoters, may not be representative of human cancers is potentially contradictory, because it also implies that transformation by known oncogenes can convert differentiated cells into cancer cells, thus undermining the concept that cancers arise from aberrant normal stem cells. In fact, in their article (1), Wicha et al. state that they have proposed the possibility that mammary tumors can arise from oncogenically

transformed early progenitor (differentiated) cells and Singh et al. (2) suggest the same possibility for brain tumors.

An important aspect of the current debate is whether the cells being sorted from tumors are truly cancer stem cells and whether they are the only cells with self-renewal capacity in the tumor. A related issue is the specificity of the markers currently being used to isolate stem-like cells from different solid cancers. Cells expressing these markers can represent up to nearly 30% of the cells in the breast and brain tumors studied. These numbers are not consistent with the expectation that "cancer stem cells" are a rare cell population in solid tumors. The data for prostate cancers from Maitland et al. [ref. (38) in Wicha et al. (1)] reporting that CD44+/ $\alpha$ 2 $\beta$ 1hi/CD133+ cells (which are 0.1% of the tumor cell population) is more consistent with expectation but these data remain unpublished at this time. The work of Glinesky et al. (3) that identified an 11-gene signature whose expression was regulated by the stem cell self-renewal gene *Bmi-1* also raises the question of the proportion of cells in the tumors expressing this signature. By its nature, the microarray technology used in these studies requires that a significant fraction of the cells in the tumor must be expressing the signature for it to be detected. One might conclude from this data that, if the current sorting techniques are truly isolating

cancer stem cells, then these cells are not a rare cell population within many solid tumors but rather are a large minority. This would certainly be a paradigm shift in thinking about cancer stem cells and would invalidate much of the argument for

specific drug development to target cancer stem cells. Such a conclusion is clearly premature, as there are other possible explanations for these results, but it illustrates the current uncertainties about identifying cancer stem cells in solid tumors.

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*Cancer Res* 2006;66:1891-1896.

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