

Cancer stem cells in nervous system tumors

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Most current research on human brain tumors is focused on the molecular and cellular analysis of the bulk tumor mass. However, evidence in leukemia and more recently in solid tumors such as breast cancer suggests that the tumor cell population is heterogeneous with respect to proliferation and differentiation. Recently, several groups have described the existence of a cancer stem cell population in human brain tumors of different phenotypes from both children and adults. The finding of brain tumor stem cells (BTSCs) has been made by applying the principles for cell culture and analysis of normal neural stem cells (NSCs) to brain tumor cell populations and by identification of cell surface markers that allow for isolation of distinct tumor cell populations that can then be studied *in vitro* and *in vivo*. A population of brain tumor cells can be enriched for BTSCs by cell sorting of dissociated suspensions of tumor cells for the NSC marker CD133. These CD133+ cells, which also expressed the NSC marker nestin, but not differentiated neural lineage markers, represent a minority fraction of the entire brain tumor cell population, and exclusively generate clonal tumor spheres in suspension culture and exhibit increased self-renewal capacity. BTSCs can be induced to differentiate *in vitro* into tumor cells that phenotypically resembled the tumor from the patient. Here, we discuss the evidence for and implications of the discovery of a cancer stem cell in human brain tumors. The identification of a BTSC provides a powerful tool to investigate the tumorigenic process in the central nervous system and to develop therapies targeted to the BTSC. Specific genetic and molecular analyses of the BTSC will further our understanding of the mechanisms of brain tumor growth, reinforcing parallels between normal neurogenesis and brain tumorigenesis.

Oncogene (2004) 23, 7267–7273. doi:10.1038/sj.onc.1207946

Keywords: brain tumor stem cell; neural stem cell; flow cytometry; CD133

Introduction

Brain tumors are typically comprised of morphologically diverse cells that express a variety of neural lineage

markers. Study of brain tumors by traditional histopathology has only yielded a limited amount of knowledge of the clinical behavior of the tumor. It is recognized that tumors with vastly different histology have a different prognosis, but often brain tumors that share similar morphology and phenotype can have a very different prognosis and response to treatment. Brain tumors of the same histologic type can also have a very different behavior in patients of different ages. Although major advances have been made in the understanding of the molecular genetic alterations of some types of brain tumors (Maher *et al.*, 2001; Wechsler-Reya and Scott, 2001; Zhu and Parada, 2002; Gilbertson, 2004), particularly medulloblastomas and malignant gliomas, and some of these identified alterations are now beginning to guide treatment, it is not clear whether all the tumor cells are equivalent in their ability to maintain the growth of the tumor. Until recently, we lacked a functional assay of the brain tumor cells that could determine which of the morphologically diverse tumor cells are capable of maintaining the growth of the tumor. The cancer stem cell hypothesis suggests that not all the cells in the tumor have the same ability to proliferate and maintain the growth of the tumor. Only a relatively small fraction of cells in the tumor, termed cancer stem cells, possess the ability to proliferate and self-renew extensively. Most of the tumor cells lose the ability to proliferate and self-renew and they differentiate into tumor cells that become the phenotypic signature of the tumor. Finding the key cells in the brain tumor population that are able to maintain the tumor will give insight into the mechanism of brain tumorigenesis and will allow us to trace back to the cell of origin in the normal brain.

Relationship between neural stem cells (NSCs) and cancer stem cells

Stem cells are functionally defined as self-renewing, multipotent cells that exhibit multilineage differentiation (Fuchs and Segre, 2000; Weissman, 2000; Reya *et al.*, 2001). Somatic stem cells are thought to self-renew to generate all the mature cell types of a particular tissue through proliferative expansion of progenitor cells followed by differentiation into mature cell types. So far, rigorous identification and isolation of tissue-specific

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stem cells has been prospectively accomplished in only a few organ systems (Reya *et al.*, 2001). The discovery that multipotential, self-renewing NSCs exist throughout life in the adult mammalian brain has only re-emerged in the past decade (Gage, 2000; Temple, 2001; Gage, 2002), reflecting a rediscovery of 1960s evidence that suggested that neurogenesis was occurring in the adult brain (Gross, 2000). The neurosphere culture system and analysis first used by Reynolds and Weiss (1992) to identify NSCs has permitted *in vitro* characterization of NSCs, but in a retrospective manner, as the multipotential floating clusters of cells (the neurospheres) are inferred to have been derived from clonal expansion of a single NSC. Prospective study of this cell has been previously limited by lack of cell surface markers necessary for its isolation, until recent reports of NSC enrichment using antibodies to the cell surface protein CD133 (Uchida *et al.*, 2000). Also, neural crest stem cells can be prospectively based on cell surface markers (Morrison *et al.*, 1999).

The concept of the cancer stem cell arose from the observation of striking similarities between the self-renewal mechanisms of stem cells and cancer cells (Reya *et al.*, 2001; Pardal *et al.*, 2003). In hematologic malignancies such as leukemia (Blair *et al.*, 1997; Bonnet and Dick, 1997) and multiple myeloma (Matsui *et al.*, 2004), and in solid tumors such as breast cancer (Al-Hajj *et al.*, 2003), rare cells were isolated with a remarkable potential for self-renewal, and these cells alone were found to drive the formation and growth of tumors. Since normal somatic stem cells must self-renew and maintain a relative balance between self-renewal and differentiation, cancer can be contextualized as a disease of unregulated self-renewal (Reya *et al.*, 2001). NSCs possess a self-renewal machinery that is primed and can be harnessed to create a cancer cell, and their longevity targets them for the accumulation of genetic mutations. Therefore, NSCs and also probably their closely related downstream rapidly proliferating progenitors should be further investigated as possible targets of transformation in the formation of brain tumors.

Do brain tumors arise from a transformed NSC: what is the evidence?

The traditional hypothesis has been that brain tumors arise from the dedifferentiation of a mature brain cell in response to genetic alterations. This hypothesis prevailed because it was felt that the postnatal brain had no proliferation. It has also been considered for some time that brain tumors may arise from a transformation event in a resident immature brain cell. With the discovery of adult NSCs (early 1990s) (Reynolds and Weiss, 1992; Kilpatrick and Bartlett, 1993; Lois and Alvarez-Buylla, 1993), it became conceivable that a normal NSC or progenitor cell that resides in the brain may be the target for transformation leading to a brain tumor.

The potential that an NSC may be transformed into a brain tumor has been considered based on the

observations of tumors occurring in the brain's putative stem cell or proliferative zones in a number of older experimental systems. The 'subependymal plate' or subventricular zone was suspected many years ago to contain 'embryonal rests' that were thought to give rise to brain tumors, particularly those occurring in the brain adjacent to the walls of the ventricular system (Globus and Kuhlenbeck, 1944). This idea was then considered further after the identification of mitotic cells in the subependymal regions of adult rodents and primates in the 1960s (Smart, 1961; Altman, 1963; Lewis, 1968). In the 1970s, periventricular tumors were then demonstrated to occur in the subventricular region after intraventricular inoculation with avian sarcoma viruses, with a much higher rate of tumors occurring in neonatal rats versus adult rats (Copeland *et al.*, 1975; Copeland and Bigner, 1977; Vick *et al.*, 1977). The incidence of brain tumors of a variety of types in mice inoculated with a pellet of carcinogen was much greater if the pellet was placed in the subventricular region versus the peripheral cortex (Hopewell and Wright, 1969). A single dose of ethylnitrosourea administration to pregnant rats also induced periventricular tumors in the offspring (Koestner *et al.*, 1971).

The primitive cytoarchitecture and embryonic features of many malignant brain tumors have also suggested that these tumors may arise from transformed neural stem or progenitor cells (Bailey and Cushing, 1926; Rubinstein, 1972). Childhood medulloblastoma, in particular, and other solid pediatric malignancies such as retinoblastoma and neuroblastoma, have been considered as developmental processes gone awry, with suspected origin from progenitors or 'blast cells' in the tissue of origin (Maris and Denny, 2002). Since medulloblastoma cells often resemble proliferating external granule cells of the postnatal cerebellum, this tumor has long been thought to arise from a primitive pluripotential cerebellar stem cell or precursor cell (Kadin *et al.*, 1970; Rubinstein, 1972).

The cell origin of brain tumors remains a subject of ongoing debate in the current scientific literature: do brain tumors arise from the dedifferentiation of a normal brain cell or from the transformation of a normal NSC or progenitor cell (Figure 1)? Several further lines of evidence suggest that brain tumors arise from the transformation of a normal NSC or progenitor cell, all of which rely on the recognition of the many functional and genetic similarities shared by somatic stem cells and cancer cells (Pardal *et al.*, 2003). Histological studies of brain tumors note the absence of expression of differentiated cell markers in primitive tumors, as well as the presence of immunostaining for nestin (Dahlstrand *et al.*, 1992; Tohyama *et al.*, 1992), a marker of NSCs (Lendahl *et al.*, 1990). Brain tumors can be very heterogeneous, being comprised of cells expressing phenotypes of more than one neural lineage, implicating a multipotential cell of origin. Microarray analysis of human medulloblastomas suggests a similarity of gene expression with normal developing brain cells (Pomeroy *et al.*, 2002). In a recent study, transfection of a medulloblastoma cell line with

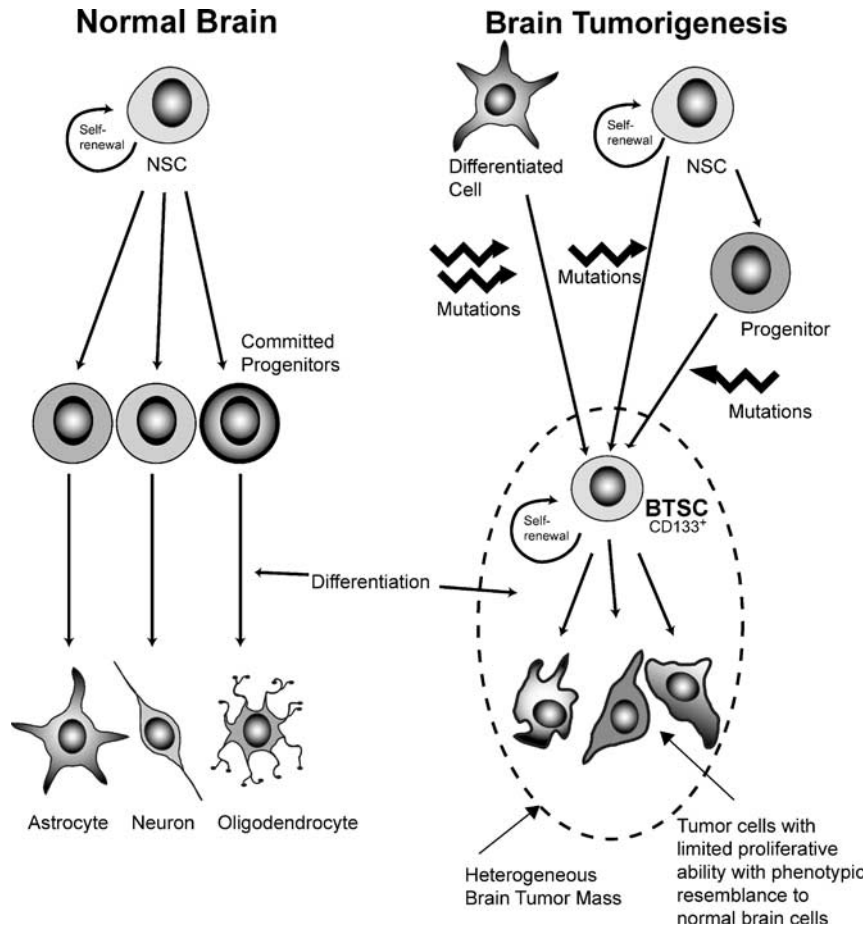


Figure 1 On the left, normal NSCs in the brain undergo highly regulated self-renewing divisions to regenerate themselves and they also generate lineage-committed progenitor cells and then differentiate into the three main neural lineages, neurons, astrocytes and oligodendrocytes. On the right, multiple mutational events target a stem cell, progenitor cell or mature brain cell results in the formation of a BTSC. The BTSC has enhanced self-renewal and proliferative capacity and also differentiates aberrantly into other cells in the tumor, which then have a limited proliferative ability

transcription factors specified a factor-dependent adoption of either neuronal or glial cell fates (Buzanska *et al.*, 2001). Finally, mutations that dysregulate the pathways that control normal stem cell self-renewal (such as the sonic hedgehog pathway) cause brain tumors, emphasizing the mechanistic similarities between normal stem cell and cancer cell self-renewal (Dahmane *et al.*, 2001; Ruiz i Altaba *et al.*, 2002; Pardal *et al.*, 2003).

Recent experiments in mice further suggest that neural progenitors may be transformed into brain tumors. By investigating the mechanisms underlying gliomagenesis, Holland and co-workers have found that undifferentiated neural precursor cells may be more sensitive to transformation than differentiated cells (Holland *et al.*, 1998; Holland *et al.*, 2000). Using a retroviral system that allows for gene expression in apparently different brain cell populations driven by different promoters, they directed the expression of oncogenes to brain cells expressing glial fibrillary acidic protein (GFAP, presumed to be expressed by differentiated astrocytes) or cells expressing nestin (presumed to mark neural stem or progenitor cells), and they found

that malignant glial tumors arise most efficiently after oncogene transfer to nestin-expressing brain cells. The interpretation of cell of origin remains unclear as a GFAP-positive cell in the subventricular zone has recently been suggested to be the adult NSC (Doetsch *et al.*, 1999). The same researchers also suggest that a differentiated neural cell such as an astrocyte may be equally permissive to transformation as a progenitor, if it has a key genetic alteration such as loss of tumor suppressor Ink4a-Arf (Bachoo *et al.*, 2002; Uhrbom *et al.*, 2002). These data support a view that dysregulation of specific genetic pathways, rather than cell of origin, may dictate the emergence and phenotype of high-grade gliomas.

Although brain tumors may arise from a dedifferentiated cell that has accumulated a series of oncogenic mutations, an NSC may be seen as a more permissive and likely compartment for transformation, since it already has the self-renewal machinery primed and it has a long lifespan favoring the accumulation of mutations. A progenitor cell is also a possible target if the genetic alteration allows it to reacquire the ability to

self-renew. A mutational event occurring in a progenitor may not be as dangerous as in a stem cell, as this cell normally has limited self-renewal ability and it typically becomes clonally exhausted as it generates differentiated cells. Whether the transforming event of a brain tumor occurs in an NSC or in a more differentiated cell type that has reacquired stem cell characteristics remains to be proven. It is conceivable that an astrocytoma arises from an oncogenic event in an astrocyte-restricted progenitor or mature astrocyte, leading to an astrocyte phenotype. However, it is also possible that the oncogenic event occurred in an NSC and this event causes a block in differentiation (Pereira *et al.*, 1998) that allows the transformed cell to differentiate down astrocytic lineages but not neuronal or oligodendrocyte lineages. The cell of origin may come down to a question of probability, any normal brain cell can give rise to a tumor, but the number and potency of the genetic alteration may determine the ease at which the cell becomes transformed. The structural organization of the brain, the integrity of which is demanded to preserve function, suggests that differentiated and morphologically complex cells such as neurons must be exceedingly resistant to proliferation.

Prospective identification of a cancer stem cell from human brain tumors

Regardless of the possible cell of origin, recent evidence suggests that brain tumors contain small numbers of cells with NSC properties. These cells have the ability to self-renew, proliferate and differentiate, and uniquely maintain the tumor growth. Although qualitative reports of sphere formation in cultured brain tumor cells exist (Mackillop *et al.*, 1985) and stem-like cells have been observed in brain tumor cultures (Ignatova *et al.*, 2002), a prospectively isolated population of cancer stem cells from brain tumors had not been described until recently (Singh *et al.*, 2003). The existence of cancer stem cells, first proven in the context of acute myeloid leukemia (AML), required sorting by surface markers to distinguish leukemic stem cells from the remaining AML cells, which had limited proliferative potential (Blair *et al.*, 1997; Bonnet and Dick, 1997). This established a hierarchy of clonally derived populations within the cancer, originating from malignant cancer stem cells, which have extensive proliferative potential, to differentiated cancer cells, which have limited proliferative potential. The relative paucity of good NSC markers presented an obstacle to the establishment of this type of hierarchy within brain tumors. Nestin, the best CNS stem cell marker to date, was a nonspecific cytoplasmic intermediate filament, and the nature of cell sorting required a cell surface marker. An excellent candidate was found in CD133, a novel 120 kDa five-transmembrane cell surface protein originally shown to be a hematopoietic stem cell marker, and recently found to be a marker of normal human NSCs (Corbeil *et al.*, 1998; Uchida *et al.*, 2000; Tamaki *et al.*, 2002).

The first prospective *in vitro* identification and characterization of a cancer stem cell from human brain tumors of different phenotypes was reported based on cell sorting for CD133 on acutely dissociated brain tumor cell populations (Figure 2) (Singh *et al.*, 2003). The brain tumor stem cell (BTSC) represented a fraction of the total cells comprising the tumor and was isolated from low- and high-grade tumors from both children and adults. The BTSC was exclusively isolated with the cell fraction expressing the NSC surface marker CD133. There are three pieces of evidence that support that these cells are BTSCs: (1) they generate clusters of clonally derived cells resembling neurospheres, (2) they self-renew and proliferate, and (3) they differentiate to recapitulate the phenotype of the tumor from which they were derived. In defining a class of BTSCs that can be prospectively isolated from a wide range of brain tumors, we support the application of principles of leukemogenesis to solid tumors: namely, the principle that only a small subset of cancer stem cells is enriched for clonogenic capacity, and that these cells alone are capable of tumor propagation. Importantly, we have recent unpublished data that support the fact that BTSCs can also initiate tumors *in vivo*.

Another recent report by Hemmati *et al.* (2003) substantiated the finding of cancer stem cells in pediatric brain tumors. They found that pediatric brain tumors contained neural stem-like cells, termed 'tumor-derived progenitors', that showed the capacity for sphere formation, self-renewal and multipotential differentiation. In addition, these cells were shown by RT-PCR to express many genes characteristic of NSCs, including *musashi-1*, *Sox2*, *bmi-1* and CD133. Further reports are emerging documenting the presence of BTSCs in human adult brain tumors. The description of BTSCs from a number of groups describes a class of cells that may drive tumorigenesis in an increasing number of brain tumors.

The application of principles for the study of normal NSCs to brain tumor cell populations establishes a link between normal neurogenesis and brain tumorigenesis. Brain tumors are not only phenotypically heterogeneous but are also functionally heterogeneous. Brain tumors exhibit phenotypic heterogeneity, being composed of cells expressing both undifferentiated and differentiated markers. *In vitro* analysis of brain tumor cells sorted for CD133 expression indicates that the capacity for tumor cell self-renewal and proliferation exclusively resides in the minority CD133+ cell fraction. These results suggest that CD133 represents a new cell surface marker of the BTSC – a novel cell type with increased potential for self-renewal that drives brain tumorigenesis. The data also suggest that brain tumors are comprised of populations of proliferating tumor stem cells that are differentiating into the more mature cell types that characterize the tumor. Purification of CD133+ cells in brain tumors implies that a hierarchy may exist in the tumor cell population, as not all tumor cells were capable of maintaining the tumor in culture. This apparent hierarchy may be functionally elucidated as more surface markers for NSCs emerge and further tumor subpopulations identified. As normal NSCs are

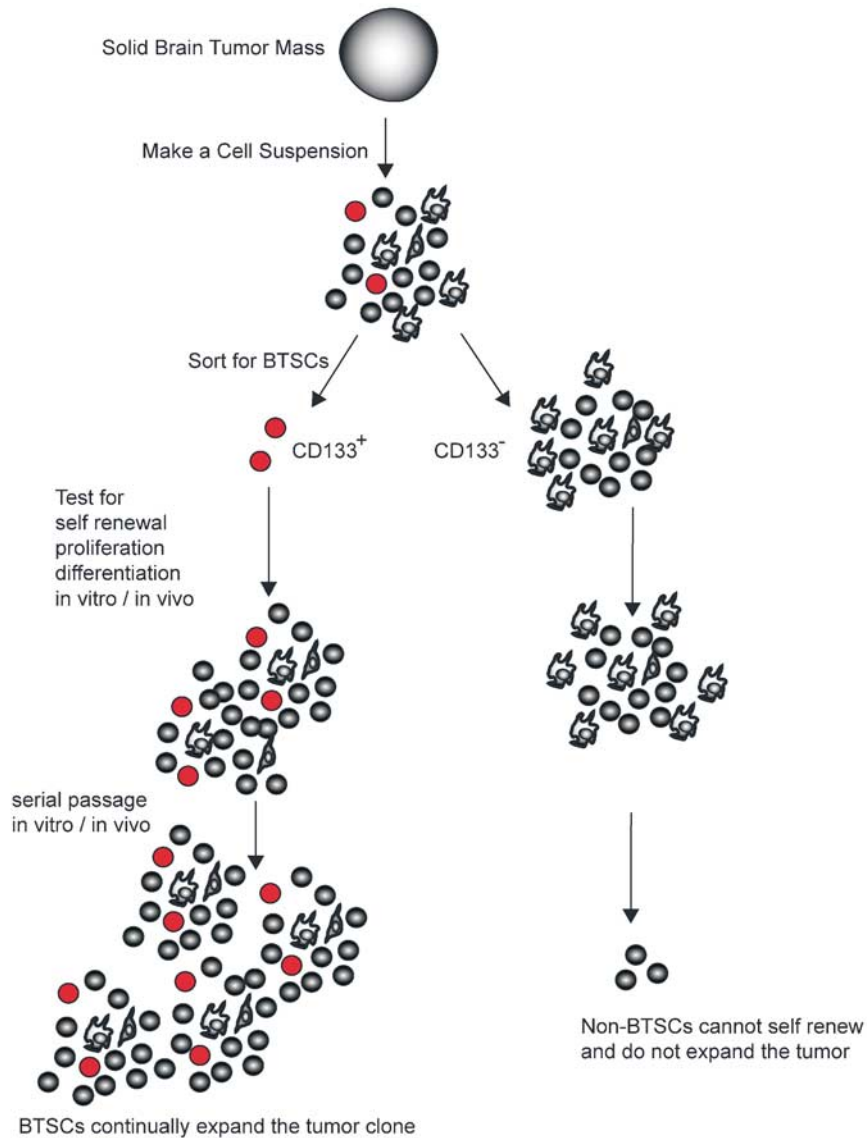


Figure 2 Cancer stem cells are identified from solid human brain tumors after acute dissociation of the solid tumor mass and then cell sorting for surface markers. CD133 identified a small subpopulation of the brain tumor cells that was uniquely able to propagate the tumor in stem cell and tumorigenic assays. CD133⁻ cells cannot expand the tumor population because they lack the ability to self-renew

also found in the CD133 population of the normal human fetal brain, and that tumors of all phenotypes studied contained nestin⁺/CD133⁺/lineage⁻ cells, it suggests that the cell of origin for a brain tumor may be a normal NSC. Future investigations of the BTSC may lead to further insight of this possibility. Much further work is required to determine the ability of BTSCs to initiate and maintain tumor growth *in vivo*, and to determine how these cells determine brain tumor phenotype.

BTSC: a new target for future brain tumor therapy

Current diagnostic and prognostic criteria for brain tumors rely on the histopathological and molecular

features of the global tumor population, and may not be sufficient to determine the key molecular alterations in a rarer tumor stem cell fraction. Molecular analysis of the BTSC population may lead to the identification of novel pathways important for the proliferation, self-renewal and differentiation of these cells that opens up new targets for therapy. It will likely be important to study the effect of activation of oncogenes or inactivation of tumor suppressor genes in the stem cell as opposed to other brain cells, as these alterations are predicted to have different effects in stem cells versus differentiated cells. Rapid prospective purification of the BTSC may allow clinicians to pinpoint the transformed cell within a lineage hierarchy, and target it with appropriately tailored drug and molecular therapies. The cancer stem cell hypothesis predicts that even if a brain tumor shows

substantial decrease in size in response to a therapy, if the cancer stem cells are spared, the tumor will regrow and the patient will have a clinical relapse.

A functional analysis of the BTSC using *in vitro* clonogenic assays and *in vivo* transplantation assays may also provide a novel means for testing of new treatment strategies that focus on the eradication of the tumor maintaining BTSC. The functional analysis of the BTSCs may give new insight into patient prognosis that may then guide the aggressiveness of treatment. For example, it was observed that malignant brain tumors had a higher stem cell (CD133+) index than low-grade tumors (Singh *et al.*, 2003), and tumors that had the same histology could have very different stem cell indices, suggesting that the stem cell index may be predictive of prognosis, even if the tumors are otherwise histologically indistinguishable. Furthermore, tumors that shared the same stem cell index could have stem cells that have different self renewal abilities *in vitro* (Singh *et al.*, 2003), suggesting further distinction between individual patient's tumors.

The fact that BTSCs can be differentiated into cells that express more mature markers supports that further exploration of the dynamic tumor differentiation process may lead to differentiation therapy. The presence of a BTSC will also have important implications for understanding brain tumor dissemination if these are the cells that migrate and establish CNS

metastasis. The identification of a BTSC provides a powerful tool to investigate the tumorigenic process in the central nervous system and further reinforces the link between normal neurogenesis and brain tumorigenesis.

Note added to proof

Brain tumor cell lines also contain cancer stem cells. Study of a 'side population' of the C6 rat glioma cell line, based on the abilities of cells to exclude the fluorescent dye Hoechst 33342, demonstrates that this minority fraction of cells in this cell line exclusively have neural stem cell-like properties *in vitro*, and tumorigenic ability *in vivo* following transplantation into the peritoneal cavity of nude mice (Kondo *et al.*, 2004).

Acknowledgements

Dr Dirks laboratory is supported by the National Cancer Institute of Canada, the Hospital for Sick Children Research Institute and the Arthur and Sonia Labatt Brain Tumor Research Center and from donations to his laboratory from the Jack Baker family and the Jessica Durigon family. Dr Singh's fellowship is supported by the Neurosurgery Research and Education Foundation with funds from the American Brain Tumor Association. We apologize to investigators of important research that was not cited in this review.

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