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Brain Tumor Stem Cells: Identification and Concepts

Sheila Singh, MD, PhD[a,b], Peter B. Dirks, MD, PhD[a,b,*]

[a]*The Arthur and Sonia Labatt Brain Tumor Research Centre, The Hospital for Sick Children, Suite 1503, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8*

[b]*Program in Developmental and Stem Cell Biology, Division of Neurosurgery, Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada*

Brain tumors are typically composed of morphologically diverse cells that express a variety of neural lineage markers. Although major advances have been made in understanding of the molecular genetic alterations of some types of brain tumors, particularly medulloblastomas and malignant gliomas, it has remained unclear whether all the tumor cells in these presumably clonally derived populations are equivalent in their ability to maintain the growth of the tumor. Until recently, a functional assay that could determine which of the morphologically diverse tumor cells found in a human brain tumor are responsible for maintaining the growth of the tumor has been lacking.

There is increasing evidence in brain tumors and other malignancies that the tumor clone is functionally heterogeneous, existing in a cellular hierarchy based on small subpopulations of stem cells. These concepts were first definitively demonstrated in human acute myelogenous leukemia, in which regeneration of a diversely heterogeneous human leukemia cell population in a xenograft mouse model occurred only after injection of a rare relatively homogeneous population of leukemic cells that expressed hematopoietic stem cell (HSC) markers [1,2].

The cancer stem cell (CSC) hypothesis suggests that the cells comprising the heterogeneous cancer population are not created equal in terms of the ability of the cells to initiate and maintain cancer tissue growth. This tumorigenic ability only resides in a rare population of cells that have stem cell properties. The discovery of CSCs in human brain tumors could only have come on the heels of work in leukemia. In addition, through advances in understanding of normal neural stem cell biology, the use of techniques for cell purification by flow cytometry, and the development of cell functional assays in vivo, the time was made ripe for several groups to characterize brain tumor stem cells (BTSCs) [3–8].

The authors' laboratory first prospectively purified a brain tumor subpopulation that had a remarkably potent tumorigenic ability not seen in the bulk population of tumor cells. The BTSC was exclusively isolated with the cell fraction expressing the neural precursor cell surface marker CD133. The frequency of CD133+ cells in human brain tumors correlated with the known biologic aggressiveness of the tumors, with highly aggressive glioblastomas containing relatively higher fractions of CD133+ cells (10%–30%) compared with low-grade gliomas, in which the CD133 fraction was much lower (0.5%–5%). CD133+ cells were highly proliferative in vitro and potently tumorigenic in vivo, regenerating a phenocopy of the patient's original tumor after injection of a relatively small number of cells. Conversely, CD133− cells showed little in vitro proliferative capacity and, despite engraftment in vivo, no tumorigenic ability.

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* Corresponding author.
E-mail address: peter.dirks@sickkids.ca (P.B. Dirks).

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The identification of a brain tumor–initiating cell provides new insights into human brain tumor pathogenesis, giving strong support for the CSC hypothesis as the basis for solid tumors, and establishes a new cellular target for brain tumor treatment.

Lessons from leukemia: the foundation of the cancer stem cell paradigm

The CSC hypothesis is a variation on a theme first introduced more than 150 years ago by the pathologists Rudolph Virchow and Julius Cohnheim, both of whom observed histologic similarities between primitive tumors, such as teratocarcinomas, and the developing fetus [9,10]. They postulated that cancer arises from the activation of dormant embryonic rests, or tissue remnants. The tools to explore the heterogeneous potential of cancer cells to self-renew emerged a century later, when Till, McCulloch and their colleagues [11–14] made the essential discovery in studies of normal hematopoietic cells that bone marrow contained single cells that could give rise to myeloerythroid colonies in the spleen. These colonies were clonal and self-renewing as well as radioprotected and could reconstitute lethally irradiated mice. The experimental approach that first identified the HSC was first applied to cancer when Bruce and Van Der Gaag [15] showed that only 1% to 4% of murine lymphoma cells transplanted into recipient mice formed colonies in their spleens. This low clonogenic frequency was confirmed by Park and colleagues [16], who showed that only 1 in 100 to 1 in 10,000 murine myeloma cells could form colonies in vitro.

At the same time, the CSC hypothesis was applied to solid tumors in two landmark studies. In the early 1960s, Brunschwig and coworkers [17] performed a series of now ethically unfeasible autotransplantation experiments and found that tumor cells harvested from patients with disseminated malignancies and reinjected subcutaneously into the same patients produced a low frequency of tumor formation. Further clues that tumor growth may be driven by a rare fraction of tumor stem cells were forthcoming in the late 1960s and early 1970s, when in vitro analysis of hematopoietic neoplasms and solid tumors demonstrated that only 1 in 1000 to 1 in 5000 total cells isolated formed clonogenic colonies when plated in culture [18,19] but particularly when the self-renewal of cells in the primary colony was tested in secondary replating assays [20]. These studies support the theory that a small subpopulation of CSCs within a heterogeneous tumor is exclusively responsible for regenerating the tumor in vitro and in vivo.

Scientists recognized that the largest obstacle to the progression of their work was the lack of methods for prospective purification of these clonogenic cell populations. With the advent of multiparameter fluorescent-activated cell sorting and monoclonal antibodies (mAbs), the purification of HSCs and their leukemic counterparts could be achieved with prospective cell sorting combined with established in vitro clonogenic assays [21–23]. To test the hypothesis that cancers arise from the clonal expansion of a single transformed stem cell, a functional in vivo xenotransplantation model was required to identify definitively the neoplastic clone exclusively capable of indefinite self-renewal in vivo and initiation of the growth of a phenotypically heterogeneous tumor. A remarkable series of experiments performed by Bonnet and Dick [1] led to the identification and purification of leukemic stem cells capable of repopulating nonobese diabetic severe combined immunodeficient (NOD-SCID) mice. This work definitively showed that leukemia was organized as a hierarchy based on leukemic stem cells, and it laid the groundwork for the application of the CSC hypothesis to a broad range of cancers.

Prospective identification of a cancer stem cell from human brain tumors

Until recently, a lack of good neural stem cell markers has presented an obstacle to the establishment of this type of hierarchy within brain tumors. Nestin, the best central nervous system (CNS) precursor cell marker to date, was a cytoplasmic intermediate filament, and cell sorting required a cell surface marker. An excellent candidate was found in CD133.

CD133, or prominin-1, is a 120-kd five-transmembrane cell surface protein of unknown function originally and has been shown to be expressed nearly simultaneously at plasma membrane protrusions of mouse neuroepithelium (therefore called prominin and identified by the rat mAb 13A4) [24] and as a marker of a subset of CD34+ human HSCs (identified as AC133) [25,26]. The mAb identified by Buck and colleagues [25] was shown to identify a glycosylation-dependent epitope (the AC133 antigen) on the human CD133 protein. The term *AC133* should refer to this human antigen. Human and

mouse CD133/prominin-1 is 60% identical at the amino acid level [27].

Study of mouse CD133/prominin-1 expression by the 13A4 antibody demonstrates expression on apical surfaces of all three germ layers during embryonic development, and AC133 antibody staining of human embryonic tissues demonstrates expression in the apical surface of the neural tube, gut, and mesonephros [27]. In adult tissues, 13A4 staining and AC133 staining are more restricted, with expression of murine CD133/prominin-1 on the ependyma, photoreceptor cells, and kidney epithelial brush border, and with AC133 expression being extremely restricted to adult bone marrow, presumably HSCs. AC133 expression is downregulated on differentiation of several different cell types, but CD133/prominin-1 expression is not, highlighting the importance of the expression of the glycosylated epitope in undifferentiated populations [28]. CD133 has been shown to have multiple alternatively spliced forms and alternative promoters, demonstrating the increasing complexity of understanding of this gene and its products [29].

CD133/prominin-1 has been found more recently to define populations of precursor cells or stem cells in multiple tissues increasingly, particularly such cells in human beings, including the following:

- Myogenic [30] and endothelial precursors [31,32]
- Placenta and trophoblast cells [33]
- Adult renal progenitor cells [34]
- Umbilical cord blood stem cells [35]
- Developing spermatozoa in the testis [36]
- Prostatic epithelial stem cells [37]
- Normal human neural stem cells [38,39]

In addition to BTSCs, CD133/prominin-1 identifies human prostate CSCs [40]. In an attempt to discover new neural stem cell markers, Uchida and coworkers [39] selected hybridomas that produced mAbs against clonogenic neural stem cells isolated from human fetal brain. They sought mAbs that cleanly separated human fetal brain into neurosphere-forming and non–neurosphere-forming fractions. They found that the antibody they identified (mAb 5F3) identified the CD133/prominin-1 antigen (AC133) and that it could enrich highly for clonogenic capacity in vitro, identifying 95% of all neurosphere-forming cells (ie, almost no neurosphere-initiating cells found in the CD133− compartment) and representing 1% to 6% of total fetal brain cells. At best, the frequency of neurosphere-forming cells in vitro in the CD133+ compartment was 1 of 32 cells, demonstrating that further enrichment is necessary. Importantly, CD133+ human fetal brain cells injected into NOD-SCID mouse brains stably engrafted and differentiated in vivo.

Recently, human prominin-2, a 112-kd pentaspan glycoprotein structurally related to prominin-1 with similar genomic organization and characteristic membrane topology, including two large glycosylated extracellular loops, has been identified [41,42]. Prominin-2 colocalizes with prominin-1 in transfection experiments of cultured cells.

The function of prominin-1 is unknown. It has been shown to localize to cholesterol-based lipid microdomains, or ''rafts,'' related to epithelial microvilli, possibly playing a role in their maintenance [43]. Patients with a frameshift mutation in prominin-1 that causes a premature truncation of the protein have retinal degeneration, which is likely related to failure of plasma membrane localization of the abnormal protein [44]. More recently, CD133/prominin was shown to be asymmetrically localized in dividing neuroepithelial cells, consistent with its association with neural stem cells [45].

CD133 also enriches for stem cell populations in brain tumors

The authors' laboratory performed the first prospective in vitro and in vivo identification and characterization of a putative CSC from human brain tumors of different phenotypes, based on cell sorting for the neural stem cell surface marker CD133 (using an antibody to the glycosylation-dependent human epitope AC133-1) [6,7]. The BTSC represented a fraction of the total cells comprising the tumor and was isolated from low-grade and high-grade tumors from children and adults. The BTSC was exclusively isolated with the cell fraction expressing CD133, such that CD133 could be used as a cell surface marker of in vitro or in vivo BTSC activity.

In vitro, CD133+ cells initiated formation of clonogenic neurosphere colonies, proliferated, and could be induced to differentiate into mature neural cell lineages that were characteristic of the mature lineages seen in the patient's original tumor [6]. A definitive demonstration of a CSC, however, requires the ability of that cell to initiate the growth of a tumor in vivo, resulting in a mass

that demonstrates an identical phenotype to the tumor from which it was originally derived. The potency of a CSC can only be shown if tumors arise after injection of only extremely small numbers of purified cells, in comparison to the tumorigenic ability of the "nonstem cells." Transplantation into immunocompromised NOD-SCID mice revealed that as few as 100 to 1000 CD133+ cells isolated from human medulloblastoma and glioblastoma possessed the increased self-renewal and proliferative capacity necessary to regenerate the tumor, corresponding to an in vivo brain tumor–initiating cell [7]. Injection of 100,000 CD133− cells caused viable cell engraftment detectable at 4 months after transplantation but no evidence of tumor formation. Serial passage of CD133+ cells reisolated from the primary transplant and injected into secondary recipients was also shown, which is the only true demonstration of self-renewal ability of the CD133+ cells and is also required for definitive demonstration of a stem cell. The fraction of CD133+ tumor stem cells in brain tumors varies according to the phenotype of the tumor (and within single phenotypes as well), with a greater fraction of CD133+ cells in the more biologically aggressive tumors (eg, glioblastomas), suggesting that the fraction of CSCs in a tumor may have prognostic significance.

An increasing number of reports have substantiated the finding of CSCs in brain tumors. Hemmati and colleagues [4] 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 reverse transcriptase polymerase chain reaction (RT-PCR) to express many genes characteristic of neural stem cells, including the following:

- musashi-1
- *Sox2*
- *bmi-1*
- CD133

Galli and coworkers [3] used clonogenic neurosphere assays to enrich for cells from adult glial tumors that exclusively possessed the self-renewal capacity necessary to regenerate the original heterogeneous tumors after transplantation. Interestingly, in this study, the take rate of engraftment into immunodeficient mice was greater in an orthotopic location (the brain) as opposed to a subcutaneous location, suggesting the importance of the correct microenvironment to support brain tumor cell growth (an appropriate niche). Yuan and colleagues [8] also demonstrated the tumorigenic ability of adult glioblastoma cells cultured as neurospheres.

Human ependymomas, which are less aggressive but still considered malignant brain tumors, were also shown to contain a subpopulation of CD133+ cells that could initiate tumor formation in mice, a property not seen in the CD133− ependymoma cells [46]. Interestingly, tumor spheres from ependymomas expressed CD133 and the radial glial marker brain lipid-binding protein (BLBP), but medulloblastoma spheres only expressed CD133, suggesting that tumor stem cells from distinct brain tumor phenotypes may express distinct markers. Moreover, human ependymoma cells expressed genes similar to those of normal radial glial cells (putative neural stem cells) isolated from the same relative position in the neuraxis where the tumor was found, suggesting that human ependymomas arise from regionally distinct populations of radial glial cells.

The reproducible discovery of the BTSC describes a class of cells that may drive tumorigenesis in an increasing number of brain tumors. Brain tumors of a variety of different types are not only morphologically heterogeneous but are functionally heterogeneous for tumor-initiating ability. The BTSCs have distinct biologic properties from the bulk of the tumor cells, also establishing the CD133+ brain tumor cells as novel targets for treatment. Use of the neurosphere assay in vitro and orthotopic injection into NOD-SCID mice in vivo provide complementary and powerful experimental methods to study the function of BTSCs (Fig. 1). The "gold standard" analysis for analysis of a CSC remains an in vivo assay; in vitro results should be interpreted cautiously. Interpretation of functional results of putative stem cells in vitro is likely to require further validation in vivo.

Although cancer-initiating ability has been shown to reside overwhelmingly in the CD133 fraction, not every CD133+ cell can clonogenically initiate sphere formation in vitro, demonstrating that not every CD133+ cell has stem cell properties in vitro. A "truer" CSC fraction requires further purification. In addition, because CD133 identifies tumor-initiating cells in only a few types of human brain tumors so far, it is possible that other markers could prove to be useful in other types of brain tumors. These remain early days for the characterization of BTSCs and, like leukemia, the stem cell compartment may have its own

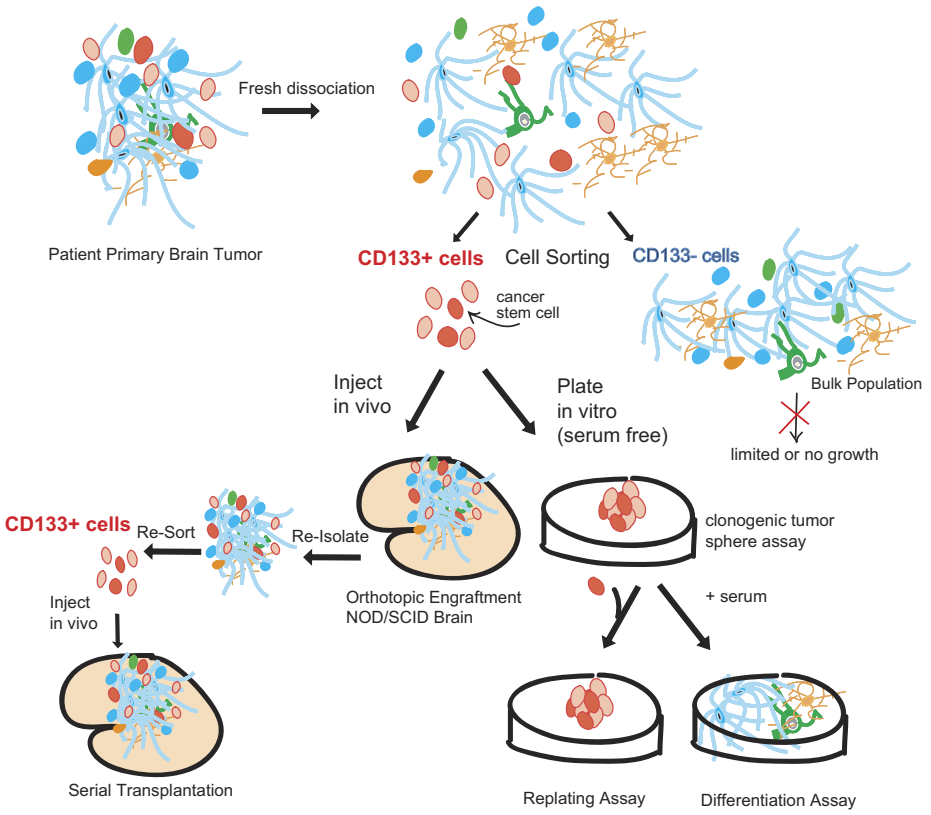

Fig. 1. Scheme for isolation and characterization of human BTSCs. Fresh solid tumor masses are immediately dissociated into single-cell suspensions. Dissociated uncultured cells are sorted using CD133. CD133+ cells are then compared with CD133− cells in functional assays in vitro and in vivo. It is known that the true CSCs (*red*) represent a small subpopulation of the CD133+ cells. In vitro, cells are assessed for proliferation and self-renewal in a clonogenic sphere formation assay, and the tumor cells are tested for their differentiation ability. In vivo, CD133+ cells are injected into the brains of NOD-SCID mice, and tumors are assessed at 12 to 16 weeks. Importantly, to demonstrate self-renewal in vivo, CD133+ cells are reisolated from primary transplants and tested for secondary engraftment ability in the serial transplantation assay.

functional hierarchy identified by different cell surface phenotypes.

The means by which CD133+ cells initiate and maintain tumorigenesis remain to be elucidated. One interesting recent study suggests that the CD133+ fraction may promote tumorigenesis by secretion of vascular endothelial growth factor (VEGF), which enhances angiogenesis [47]. It is also important to determine if CD133+ human brain tumor cells are resistant to conventional therapies, such as chemotherapy and radiation therapy.

Questions about the cell of origin

The concept of a CSC implies that the cancer-initiating event occurred in a normal stem cell. Genetic events that lead to cancer, regardless of the cell of origin, may endow the cancer cell with unregulated self-renewal ability, giving it this key stem cell property. A normal stem cell is the most attractive candidate for the cell of origin of a CSC, because these cells have primed self-renewal ability, potentially allowing fewer genetic insults to permit the cell to escape tight constraints on self-renewal. Because normal neural stem cells are also presumably long-lived, these cells may be the only ones capable of accumulating a sufficient number of mutations to become transformed. In the blood system, transformation of stem cells or progenitors can induce leukemia, and the transformed progenitors upregulate a limited repertoire of genes thought to be involved in self-renewal [48]. Although normal neural stem cells and BTSCs share expression of CD133, their common cell surface

marker does not prove that the BTSC is derived from a normal neural stem cell. In mouse models, brain tumors can be initiated in nestin-positive neural precursor compartments as well as in glial fibrillary acidic protein (GFAP)-positive compartments [49–51]. Because GFAP marks precursor and differentiated compartments in the brain, questions about the cell of origin have not yet been definitively addressed in the brain.

Implications for brain tumor research

The identification of BTSCs has important implications for how future brain tumor research is conducted. The genetic alterations seen in brain tumors must be considered in the context of the cellular hierarchy. Even if all the cells in the brain tumor share the same genetic alterations (ie, they are clonally derived), the ultimate effect of that genetic alteration depends on the type of cell in the stem cell hierarchy. In other words, the functional consequence of a genetic alteration is not equal in all cells within a tumor. Further study of the tumor hierarchy requires a fundamental change in the way fresh brain tumor tissue is handled. Generation of single-cell suspensions from fresh solid brain tumor tissue needs to become a routine practice so as to assess the distinct components of the tumor hierarchy. In addition, analysis of the expression of genes in the bulk tumor population, such as with microarray analysis, is likely to miss important gene expression changes in rare stem cell populations. The CSC hypothesis suggests that gene expression changes in the rare cells in the tumor must be studied, because these cells are the most important cells driving tumorigenesis. It is well known that cell purification is challenging and that performing gene expression analyses on rare populations of cells is difficult. We still only have a crude understanding of the tumor hierarchy, in large part, because the normal developmental hierarchy of the neural stem cell system is poorly defined; thus, we need to keep closely abreast of advances in normal neural stem cell biology. Improved purification of CSCs should lead to the identification of new biomarkers that might distinguish normal from neoplastic stem cells and become novel targets for therapy.

Implications for cancer treatment

The most obvious implications of the identification of BTSCs are for cancer treatment. Treatments that spare the tumor-maintaining CSCs are predicted to fail. It is expected that targeting a rare population within a heterogeneous tumor is going to be challenging. A major additional challenge on a more practical scale lies in applying the concept of the CSC to assessment of cancer therapy. Can we devise methods for assessment of the response of the CSCs to the treatment other than assessment of tumor size reduction, which may more reflect bulk tumor response? Because of the many similarities in the functional ability of BTSCs and normal neural stem cells, another challenge is finding selective drugs that selectively target BTSCs and spare the normal neural stem cells.

Summary

The prominent emergence of CSCs in the literature recently reflects new experimental evidence strongly supporting a hypothesis that has been discussed for decades. The implications of the discovery of BTSCs are major for future brain tumor research and treatment. Nevertheless, these are early days; much work needs to be done on further defining the functional behavior of CD133+ cells, the molecular mechanisms that regulate proliferation and self-renewal, and mechanisms that supposedly make these cells more resistant to therapy. Nevertheless, the brain tumor field has the advantage of good in vitro and in vivo assay systems based on normal neural stem cell assay systems and a start in tumor stem cell purification with CD133 sorting, which, hopefully, should make further advances not long in coming.

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