

# Identification of a Cancer Stem Cell in Human Brain Tumors

Sheila K. Singh, Ian D. Clarke, Mizuhiko Terasaki, Victoria E. Bonn, Cynthia Hawkins, Jeremy Squire, and Peter B. Dirks

The Arthur and Sonia Labatt Brain Tumour Research Centre, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada [S. K. S., I. D. C., M. T., V. E. B., P. B. D.], and Program in Developmental Biology [S. K. S., I. D. C., M. T., V. E. B., P. B. D.], Division of Neurosurgery [S. K. S., P. B. D.], Department of Pediatric Laboratory Medicine [C. H.], and Department of Laboratory Medicine and Pathobiology [J. S.], University of Toronto, Toronto, Ontario M5G 1X8 Canada

## ABSTRACT

Most current research on human brain tumors is focused on the molecular and cellular analysis of the bulk tumor mass. However, there is overwhelming evidence in some malignancies that the tumor clone is heterogeneous with respect to proliferation and differentiation. In human leukemia, the tumor clone is organized as a hierarchy that originates from rare leukemic stem cells that possess extensive proliferative and self-renewal potential, and are responsible for maintaining the tumor clone. We report here the identification and purification of a cancer stem cell from human brain tumors of different phenotypes that possesses a marked capacity for proliferation, self-renewal, and differentiation. The increased self-renewal capacity of the brain tumor stem cell (BTSC) was highest from the most aggressive clinical samples of medulloblastoma compared with low-grade gliomas. The BTSC was exclusively isolated with the cell fraction expressing the neural stem cell surface marker CD133. These CD133+ cells could differentiate in culture into tumor cells that phenotypically resembled the tumor from the patient. 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.

## INTRODUCTION

Brain tumors are the leading cause of cancer mortality in children and remain difficult to cure despite advances in surgery and adjuvant therapy. Most current brain tumor research is focused on the molecular and cellular analysis of the bulk tumor mass. Brain tumors are typically comprised of morphologically diverse cells that express a variety of neural lineage markers. Study of the basic morphology and phenotype of brain tumors has only yielded a limited amount of knowledge of the clinical behavior of the tumor, as brain tumors that share similar morphology and phenotype can have a very different prognosis and response to treatment. We lack 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. Determination of key cells in the 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.

There is overwhelming evidence in other malignancies, such as leukemia, that the clonal population of neoplastic cells exhibits marked heterogeneity with respect to proliferation and differentiation (1, 2). Rare stem cells within the leukemic population possess extensive proliferation and self-renewal capacity that is not found in the majority of the leukemic cells. The ability to fractionate and func-

tionally analyze leukemic stem cells led to the determination that they are necessary and sufficient to maintain the leukemia (1, 3).

We have applied the techniques used to isolate normal neural stem cells in culture (as clonally derived neurospheres, each of which arise from a single stem cell; Ref. 4) to the analysis of human pediatric brain tumors. We used assays of neurosphere cells to functionally characterize the tumor cell populations. We report the identification and purification of a cell from primary human brain tumors of different phenotypes that has marked capacity for proliferation, self-renewal, and differentiation. This cell represented a minority of the tumor cell population and was identified by expression of the cell surface marker CD133. This CD133+ cell, which we have termed the BTSC,<sup>3</sup> lacked the expression of neural differentiation markers, and was necessary for the proliferation and self-renewal of the tumor in culture. This cell was also capable of differentiating *in vitro* into cell phenotypes identical to the tumor *in situ*. The marker phenotype of the BTSC was similar to that of normal neural stem cells, in that it expressed CD133 and nestin, and was the same in patients with the same pathological type of tumor and in patients with different pathological subtypes. This suggests that brain tumors can be generated from BTSCs that share a very similar phenotype. A better understanding of brain tumor biology will come from additional cellular and molecular studies of the BTSC. Comparison of normal neural stem cells and BTSCs will aid in finding the normal brain cell that originates the tumor.

## MATERIALS AND METHODS

**Primary Tumor Sphere Culture.** Tumor samples were obtained from consenting patients, as approved by the Research Ethics Board at The Hospital for Sick Children. Tumors were washed, acutely dissociated in oxygenated artificial cerebrospinal fluid and subject to enzymatic dissociation as described previously (4). Tumor cells were then resuspended in TSM consisting of a chemically defined serum-free neural stem cell medium (4), human recombinant EGF (20 ng/ml; Sigma), bFGF (20 ng/ml; Upstate), leukemia inhibitory factor (10 ng/ml; Chemicon), Neuronal Survival Factor (NSF) (1x; Clonetics), and *N*-acetylcysteine (60 μg/ml; Sigma; Ref. 5), and plated at a density of  $3 \times 10^6$  live cells/60-mm plate. RBCs were removed using lympholyte-M (Cedarlane).

**Limiting Dilution Assay and Primary Sphere Formation Assay.** Limiting dilution assay was performed as described previously (6, 7). After primary sphere formation was noted, sphere cells were dissociated and plated in 96-well microwell plates in 0.2 ml volumes of TSM. Final cell dilutions ranged from 200 cells/well to 1 cell/well in 0.2-ml volumes. Cultures were fed 0.025 ml of TSM every 2 days until day 7, when the percentage of wells not containing spheres for each cell plating density was calculated and plotted against the number of cells per well. Regression lines were plotted and x-intercept values calculated, which represent the number of cells required to form at least 1 tumor sphere in every well. CD133-adherent tumor cells were trypsinized before collection for assays. For primary sphere formation assays, this analysis was performed on the entire acutely dissociated tumor cell population on day 0 to quantify stem cell frequency within the tumor.

<sup>3</sup> The abbreviations used are: BTSC, brain tumor stem cell; TSM, tumor sphere medium; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; PDGFR, platelet-derived growth factor receptor; SKY, spectral karyotyping; SFM, serum-free medium; GFAP, glial fibrillary acidic protein.

Received 2/6/03; revised 7/24/03; accepted 7/29/03.

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<sup>1</sup> P. D. is supported by National Cancer Institute of Canada with funds from the Terry Fox Run, the Arthur and Sonia Labatt Brain Tumour Research Center, the Hospital for Sick Children Research Institute, and a gift from the Baker Family. S. S. is supported by a fellowship from the Neurosurgery Research and Education Foundation with Funds from the American Brain Tumour Association.

<sup>2</sup> To whom requests for reprints should be addressed, at Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada, M5G 1X8. Phone: (416) 813-6426; Fax: (416) 813-4975; E-mail: peter.dirks@sickkids.ca.

**Cell Proliferation Assays.** Cells were plated in 96-well microwell plates in 0.1-ml volumes of SFM supplemented with growth factors, at a density of 1000 cells/well. Cell proliferation assays were performed on days 0, 3, 5, and 7 postplating using the Roche 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based Colorimetric Assay Cell Proliferation kit 1. Quantification of viable cells through reading of UV absorption spectrums at 575 nm was performed on a Versamax microplate reader.

**Differentiation Assay of Tumor Spheres.** Two days after primary culture, cells were plated onto glass coverslips coated in poly-L-ornithine (Sigma; Ref. 4) in medium with 10% FBS in individual wells of a 24-well culture plate. Cells were fed with FBS-supplemented medium every 2 days, and coverslips were processed 7 days after plating using immunocytochemistry.

**Immunocytochemical Staining of Tumor Stem Cells.** Immunocytochemistry was performed as described previously (7). Briefly, for immunostaining of undifferentiated tumor spheres, cells were plated onto poly-L-ornithine coated glass coverslips in SFM containing 10% FBS, for 4 h. Cells were then fixed with 4% paraformaldehyde and stained with antibodies against CD133/1 (mouse monoclonal IgG1; Miltenyi Biotec), nestin (rabbit polyclonal; Chemicon),  $\beta$ -tubulin 3 (mouse monoclonal IgG1; Chemicon), GFAP (rabbit polyclonal; DAKO), mitogen-activated protein 2 (mouse monoclonal IgG1; Chemicon), and PDGFR  $\alpha$  (rabbit polyclonal C20; Santa Cruz Biotechnology). Appropriate secondary antibodies (Texas Red donkey antirabbit; Jackson ImmunoResearch; and Alexa 488 goat antimouse; Molecular Probes) were used.

For immunostaining of differentiated tumor cells, differentiation assays were performed 2 days after primary tumor culture; 7 days after differentiation, immunocytochemistry was performed as described above. Cells were additionally immunostained with 4',6-diamidino-2-phenylindole (Sigma), to permit counting of cell nuclei in at least 5 microscopic fields per specimen. Quantification of cells stained with each antibody could then be averaged and estimated as a percentage of total nuclei counted.

**Magnetic Cell Sorting and Flow Cytometry.** Within 3 days of primary culture, cells were centrifuged at  $800 \times g$  for 5 min, triturated with a fire-narrowed Pasteur pipette, and resuspended in  $1 \times$  PBS with 0.5% BSA and 2 mM EDTA. Magnetic labeling with  $1 \mu\text{l}$  CD133/1 Microbeads/1 million cells was performed using the Miltenyi Biotec CD133 Cell Isolation kit. Ten  $\mu\text{l}$  of CD133-2-phycoerythrin (fluorochrome-conjugated mouse monoclonal IgG1; Miltenyi Biotec) was added for an additional 30 min to evaluate the efficiency of magnetic separation by flow cytometry. Magnetic separation was carried out on the autoMACS machine (Miltenyi Biotec). Positive and negative fractions were eluted with a double-sensitive mode. Aliquots of CD133+ and CD133- sorted cells were evaluated for purity by flow cytometry with a FACSCalibur machine (BD Biosciences). CD133+ and CD133- sorted cell populations were resuspended in SFM with growth hormones.

**Spectral Karyotype Analysis of Tumor Sphere Cells.** Tumor spheres were gently aspirated to disaggregate and cultured in TSM as described above. The cultures were harvested within 3–5 days with 0.1  $\mu\text{g}/\text{ml}$  Colcemid (Life Technologies, Inc.) for 2–3 h, KCl (0.075 M) -treated, and fixed in 3:1 methanol: acetic acid. SKY was performed on tumor metaphase cells according to the manufacturer's instructions (ASI, Carlsbad, CA) and as published previously (8). Spectral images were acquired and analyzed with an SD 200 Spectral Bio-imaging System (ASI Ltd., MigdalHaemek, Israel) attached to a Zeiss Axioplan 2 microscope (Carl Zeiss, Toronto, Ontario, Canada), and analyzed using SKYVIEW (ver. 1.2; ASI) software.

**Immunohistochemistry on Tumor Sections.** Formalin-fixed, paraffin-embedded tissue sections were mounted on positive charged microscope slides. Tissue sections were then baked overnight at 60°C, and treated with epitope retrieval techniques and blocked for endogenous peroxidase and biotin before the application of the primary antibody. Incubation of antihuman CD133 at a 1:10 dilution was performed overnight at room temperature. Subsequent immunodetection was performed using the Elite Vector Stain ABC System (Vector Laboratories, Burlingame, CA). Color visualization was performed using 3-3'-diaminobenzidine as the chromagen substrate (Sigma Chemical Company, St. Louis, MO).

## RESULTS

**Primary Brain Tumors of Different Phenotypes Form Neurosphere-like Colonies.** One key determinant of stem cells is the capacity for extensive proliferation. To determine whether cells with

distinct proliferative abilities were present in human brain tumors, we established cultures from 14 solid primary pediatric brain tumors (Table 1), which were acutely dissociated into individual cells. We used culture conditions that favored stem cell growth, established previously for isolation of neural stem cells as neurospheres (4). SFM allows for the maintenance of an undifferentiated stem cell state, and the addition of bFGF and EGF induced the proliferation of multipotent, self-renewing, and expandable neural stem cells (9, 10). Regardless of pathological subtype, within 24–48 h of primary culture all of the brain tumors yielded a minority fraction of cells that demonstrated growth into clonally derived neurosphere-like clusters, termed tumor spheres (Fig. 1). The frequency of the stem cell population within the tumor was determined by primary sphere formation assays performed on 6 tumors, yielding a stem cell frequency ranging from 0.3% to 25.1% (Table 2). The remaining majority of tumor cells exhibited adherence, loss of proliferation, and subsequent differentiation, whereas tumor spheres remained nonadherent, continuing to proliferate and expand the tumor cell culture over time. Tumor spheres generated from all 14 of the specimens showed immunoreactivity for nestin (an intermediate filament protein found in undifferentiated central nervous system cells and a characteristic neural stem cell marker; Ref. 11) and for CD133, a novel putative neural stem cell marker (Refs. 5, 12; Fig. 1, E–L). Furthermore, the tumor spheres did not express markers for differentiated neurons, astrocytes, or oligodendrocytes.

**Tumor Sphere Cells Exhibit Increased Potential For Self-Renewal.** Only a small proportion (<1%) of cells composing individual neurospheres from a normal brain are stem cells with the ability to self-renew and generate all neural lineages (9, 13). The remaining majority are progenitor cells with more restricted self-renewal capacity and lineage potential. Tumor spheres are defined as clonally derived nonadherent colonies of cells derived from a single tumor stem cell. To build on the analogy between neurosphere and tumor sphere, we subjected tumor spheres to stem cell assays designed to test the self-renewal, proliferation, and differentiation capacities of a putative BTSC.

The self-renewing capacity of the tumor spheres was assayed by dissociation of primary tumor spheres, and plating of cells at serial dilutions down to 1 cell/well. All of the dissociated primary tumor spheres demonstrated the capacity to form secondary tumor spheres, exhibiting an ability to self-renew. When self-renewal capacity was compared among tumor subtypes at a plating density of 100 cells/well, medulloblastomas were found to generate a greater mean number of secondary tumor spheres ( $20.27 \pm \text{SE } 5.24$ ), compared with pilocytic astrocytomas ( $5.85 \pm \text{SE } 1.96$ ) and to control sphere forming human fetal neural stem cells (Clonetics;  $2.88 \pm \text{SE } 0.25$ ; Fig. 2A).

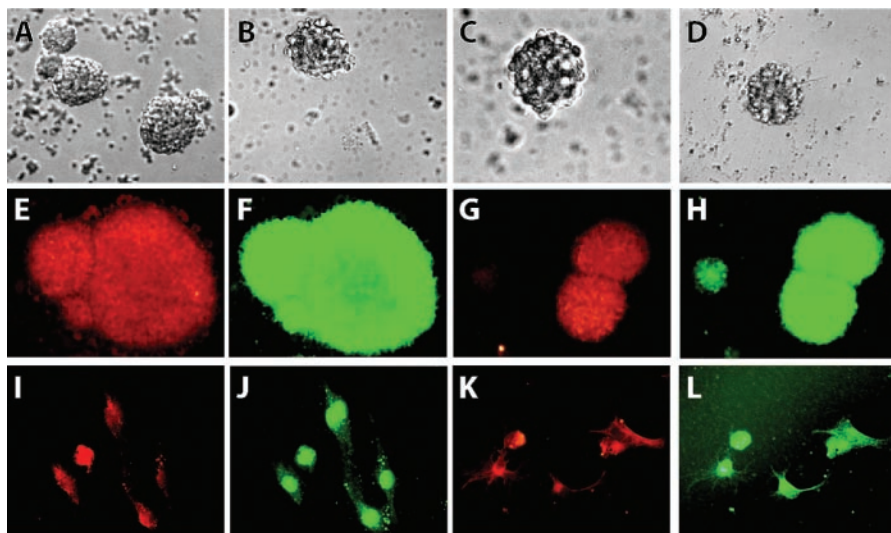
If a single tumor sphere results from the proliferation of a single

Table 1 Summary of patient population

Patient #	Sex	Age (yrs)	Tumor location	Pathological subtype
1	M	2.5	Infratentorial	Medulloblastoma
2	M	8	Infratentorial	Medulloblastoma
3	M	4	Infratentorial	Medulloblastoma
4	M	7	Infratentorial	Medulloblastoma
5	M	9	Infratentorial	Medulloblastoma
6	F	3	Infratentorial	Medulloblastoma
7	M	6	Infratentorial	Medulloblastoma
8	M	6	Infratentorial	Pilocytic astrocytoma
9	M	4	Infratentorial	Pilocytic astrocytoma
10	F	9	Infratentorial	Pilocytic astrocytoma
11	M	14	Infratentorial	Grade 2 astrocytoma
12	M	2.5	Infratentorial	Ependymoma
13	F	5	Supratentorial	Ganglioglioma
14	M	11	Infratentorial	Medulloblastoma



Fig. 1. Primary brain tumors of different phenotypes form neurosphere-like colonies. Photomicrographs of cultured brain tumor cells (magnification  $\times 20$ ) at 24–48 h after plating in TSM, containing EGF and bFGF. Each tumor subtype yielded growth of cells in neurosphere-like clusters, termed tumor spheres. Tumor spheres are shown from a medulloblastoma (A), pilocytic astrocytoma (B), ependymoma (C), and ganglioglioma (D). Undifferentiated primary tumor spheres from a medulloblastoma (E, F, I, and J) and a pilocytic astrocytoma (G, H, K, and L) are immunostained at 4 h for characteristic neural stem cell marker nestin (E and G) and for CD133 (F and H). Individual undifferentiated medulloblastoma sphere cells and astrocytoma sphere cells are also shown stained for nestin (I and K) and CD133 (J and L).



BTSC, limiting dilution analysis allows for the determination of the minimal frequency of repopulating tumor sphere cells within the cell population (6, 7). The number of cells required to generate at least 1 tumor sphere/well was calculated as  $23.5 \pm 17.0$  (SD) in the medulloblastomas,  $99.18 \pm 45.9$  (SD) in the pilocytic astrocytomas, and  $98.25 \pm 4.6$  (SD) in the control human neural stem cells (Table 3; Fig. 2B). These data reveal that the frequency at which 1 tumor sphere cell will proliferate to form a new tumor sphere varied according to tumor pathological subtype, with more aggressive medulloblastomas exhibiting increased self-renewal capacity compared with pilocytic astrocytomas ( $P = 0.004$ ) and human neural stem cell controls

( $P = 0.001$ ). Morphology of secondary tumor spheres was identical to that of primary spheres (Fig. 2C), and secondary tumor spheres retain expression of the neural stem cell markers nestin (Fig. 2D) and CD133 (Fig. 2E), whereas failing to express neural differentiation markers. Secondary spheres from two medulloblastomas (patient 7 and patient 14) were passaged to tertiary spheres, and quantified by limiting dilution at 12.5 cells/well and 20 cells/well, respectively. All of the tumors studied generated spheres with multiple passages. These data show that all of the brain tumors had a subpopulation of cells with a capacity to self-renew and that the self-renewal ability of the tumors correlated with the clinical aggressiveness of the different tumor phenotypes.

To evaluate proliferative capacity of tumor sphere cells, cells were plated at 1000 cells/well, and the number of viable cells was quantified on days 0, 3, 5, and 7 after plating by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. All of the tumor sphere cell populations assayed demonstrated increased proliferative capacity compared with human controls (Fig. 3A). The his-

Table 2 Primary sphere formation assay

Tumor type	Primary sphere (clonogenic) frequency
Medulloblastoma ( $n = 3$ )	13.8%, 14.8%, 25.1%
Pilocytic astrocytoma ( $n = 3$ )	0.3%, 1.3%, 1.5%
Glioblastoma ( $n = 1$ )	11.4%
Anaplastic ependymoma	8%

Fig. 2. Tumor sphere cells exhibit increased self-renewal potential compared with controls. A, when tumor cells were plated at a density of 100 cells/well, medulloblastomas were found to generate a greater mean number of secondary tumor spheres ( $20.27 \pm 5.24$ ) than pilocytic astrocytomas ( $5.85 \pm 1.96$ ) or control human neural stem cells ( $2.88 \pm 0.25$ ). B, cells plated at limiting dilution in 200  $\mu$ l volumes of medium showed that the frequency at which one tumor stem cell proliferates to form a secondary tumor sphere varied according to tumor pathology [representative samples of each tumor subtype shown: medulloblastoma, patient 14 ( $\blacktriangle$ ), pilocytic astrocytoma, patient 10 ( $\circ$ ), and control fetal human neural stem cells ( $\bullet$ )]. The mean x-intercept values calculated from limiting dilution analysis for each tumor subtype reveal that the number of cells required to form at least 1 tumor sphere/well was much lower in medulloblastomas ( $\circ$ ), compared with gliomas ( $\blacktriangle$ ) or human controls ( $\bullet$ ; Fig. 2B; Table 2). C, morphology of secondary tumor spheres was identical to that of primary tumor spheres (magnification  $\times 200$ ). D and E, undifferentiated secondary tumor spheres (magnification  $\times 100$ ) retain expression of neural stem cell markers nestin (with rhodamine, in red; D) and CD133 (with FITC, in green; E).

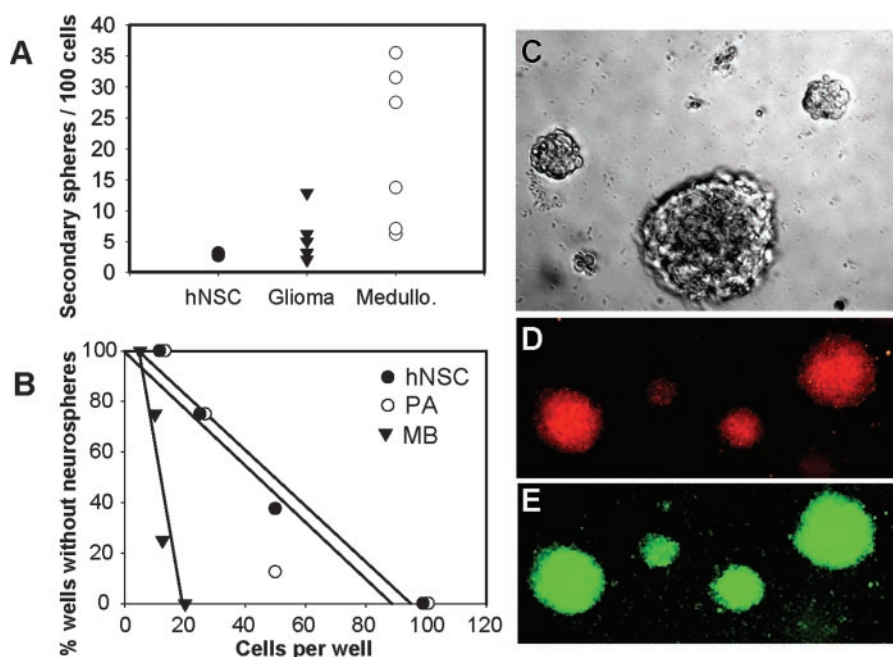


Table 3 Self renewal capacity of tumor subtypes

Sample	Mean $\times$ intercept $\pm$ SD	Sample number
hNSC <sup>a</sup>	98.25 $\pm$ 4.6	2
Medulloblastoma	23.5 $\pm$ 17.0	6
Gliomas	99.18 $\pm$ 45.9	5

<sup>a</sup> hNSC, human neural stem cells.

topathologic MIB-1 index is thought to correlate with tumor proliferation. The mean MIB-1 index of medulloblastoma and pilocytic astrocytoma tumor specimens is shown in Fig. 3B. We suggest that the differing tumor stem cell proliferation rates are responsible for the MIB-1 indices observed in various tumor subtypes.

**BTSCs Differentiate to Produce a Phenotype Identical to the Tumor *in Situ*.** We next applied conditions used for normal neurosphere differentiation to primary tumor spheres to determine whether the BTSC was capable of multilineage differentiation. After differentiation with 10% FBS for 7 days, immunocytochemistry was performed on tumor stem cells using the following antibodies: CD133, nestin,  $\beta$ -tubulin 3 ( $\beta$ -tub-3; for neurons), GFAP (for astrocytes), and PDGFR- $\alpha$  (for oligodendrocytes). Immunocytochemistry was also performed on undifferentiated primary tumor stem cells using the same panel of antibodies.

Undifferentiated tumor spheres from all of the tumor subtypes exhibited immunoreactivity for CD133 and nestin, and lack of immunoreactivity for markers of differentiated neural cell types such as GFAP for astrocytes and  $\beta$ -tubulin 3 for neurons (Fig. 4, A–D). Strikingly, dissociated tumor spheres from all of the specimens grown adherently and in serum for 7 days preferentially differentiated down the lineage that characterized the original tumor phenotype of the patient (histopathology patient data not shown). Thus, the majority of differentiated cells from a primary medulloblastoma sphere expressed  $\beta$ -tub-3 when differentiated (81.9%  $\pm$  SD 6.02), reflecting the neuronal marker expression commonly seen in medulloblastomas, whereas the majority of tumor stem cells from pilocytic astrocytomas expressed GFAP when differentiated (77.9%  $\pm$  SD 14.9), recapitulating the astrocytic lineage of the tumor (Fig. 4, A–D). All of the tumor subtypes lost expression of CD133 and nestin when subjected to differentiating conditions (Fig. 4, A–D). Although a small minority of differentiated cells from each tumor subtype expressed other differentiated cell markers, the overwhelming majority of differentiated

cells expressed markers that reflected the immunophenotype of the original tumor. Interestingly, only medulloblastomas that expressed GFAP in the primary tumor (by immunohistochemistry) were able to express GFAP after tumor sphere differentiation *in vitro*, illustrating the striking *in vitro* recapitulation of tumor phenotype within tumors of the same histopathological subtype. In addition, 2 medulloblastoma specimens (Patients 2 and 4, 20.5%  $\pm$  3.35) demonstrated costaining for  $\beta$ -tub-3 and GFAP (Fig. 4E, bottom panel), whereas the majority of differentiated medulloblastoma tumor cells (60.3%  $\pm$  SD 3.55) in these tumors stained for  $\beta$ -tub-3 alone (Fig. 4E, top panel). Immunocytochemistry was repeated on these samples for another neuronal marker, mitogen-activated protein-2, and costaining with GFAP was again evident (data not shown). This differentiated tumor stem cell immunophenotype may represent a bipotential precursor cell, such as has been identified previously by Kilpatrick and Bartlett (14) in normal neural precursor cells. These results show that the BTSC may re-establish the original tumor and demonstrate that brain tumors are heterogeneous and consist of a differentiating population that originates from a BTSC.

**CD133+ Tumor Cells Show Marked Stem Cell Activity.** To better define the BTSC, we needed a surface marker that would enable isolation. CD133 is a novel 120 kDa five-transmembrane cell surface protein originally shown to be a hematopoietic stem cell marker, and found recently to be a marker of normal human neural stem cells (5, 12, 15). We demonstrated CD133 expression by immunohistochemistry in brain tumors (Fig. 5A, medulloblastoma, patient 1), showing a plasma membrane staining pattern also characterized in other tissues. To determine whether CD133 was present in tumor sphere cultures, we analyzed the expression of CD133 using flow cytometry, and we sorted for CD133 positive and negative cell populations using magnetic bead cell sorting.

Flow cytometric quantification of CD133 expression in brain tumor cultures ranged from 3.5% to 46.3% in both medulloblastomas and pilocytic astrocytomas (Table 4). The lower-grade astrocytomas typically had a lower CD133 fraction compared with high-grade medulloblastomas. When tumor cell cultures were sorted for CD133 expression (Fig. 5B), CD133 positive and negative cell populations were collected and cultured separately, under the same conditions as unsorted BTSCs. Sorted CD133+ and CD133– aliquots from each tumor were checked by flow cytometry to evaluate the efficiency of

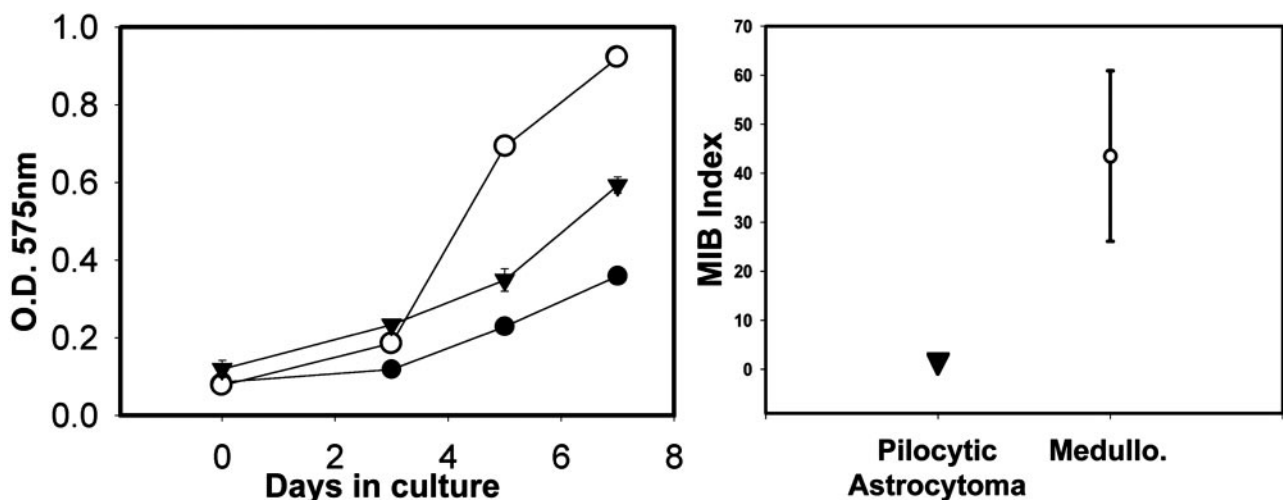


Fig. 3. Tumor sphere cells exhibit increased proliferation consistent with histopathologic tumor mitotic indices. A, tumor stem cells from dissociated medulloblastoma spheres (○) showed a greater degree of cell proliferation than pilocytic astrocytoma spheres (▲) or control human neurospheres (●). B, the higher degree of proliferation of the tumor sphere cell population is associated with an increased mitotic rate of the tumor as a whole, as reflected by mean MIB-1 values of each tumor subtype (medulloblastomas, ○; mean MIB-1 = 43.5%  $\pm$  17.4,  $n$  = 7; pilocytic astrocytoma, ▲; mean MIB-1 = 1.5%  $\pm$  0.5,  $n$  = 3).

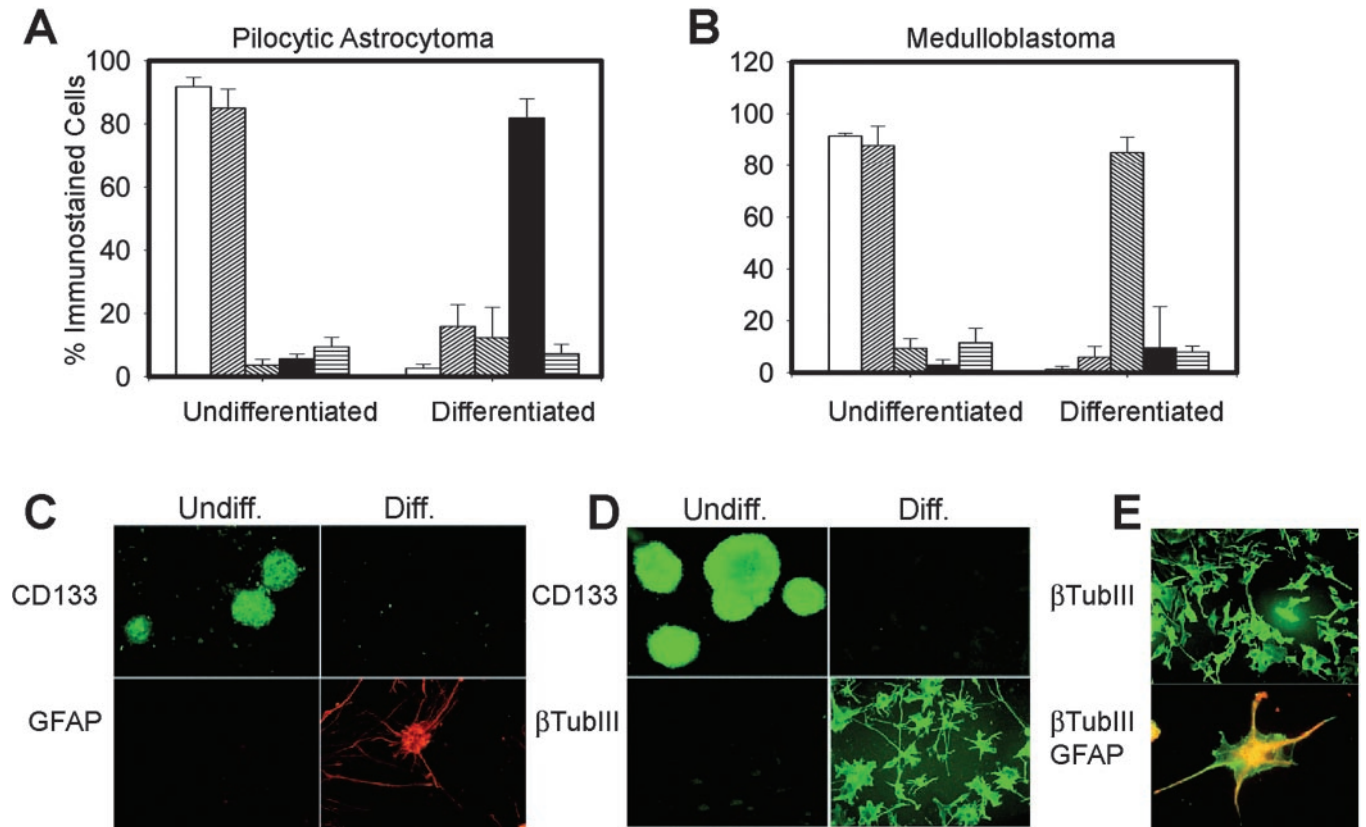


Fig. 4. Tumor spheres differentiate to express immunophenotypes similar to the primary tumor. Immunocytochemistry was performed on tumor stem cells using CD133 (□), nestin (▨),  $\beta$ -tubulin 3 (▩), GFAP (■), and PDGFR- $\alpha$  (▧). A–D, all tumor spheres lost expression of CD133 and nestin when differentiated. Dissociated tumor spheres preferentially differentiated down the lineage that characterized the original tumor phenotype (A), pilocytic astrocytoma (specimen from Patient 9); (B), medulloblastoma (specimen from Patient 7). Tumor stem cells (77.9%) from pilocytic astrocytomas expressed GFAP (A and C), whereas 81.9% of tumor stem cells from medulloblastomas expressed the early neuronal marker  $\beta$ -tubulin 3 (B and D) when differentiated. E, whereas the majority of cells within two medulloblastoma cultures (patients 2 and 4) differentiated into cells with a neuronal immunophenotype (top panel, magnification  $\times 200$ , immunostaining with  $\beta$ -tubulin-3), these cultures showed a small population of cells that costained for  $\beta$ -tubulin 3 and GFAP (bottom panel, magnification  $\times 400$ ; GFAP and rhodamine in red,  $\beta$ -tubulin 3 and ALEXA 488 in green).

sorting: purity of the CD133+ populations ranged from 46.9% to 79.8%, and purity of the CD133- populations ranged from 92.6% to 97.3%. Over a 7-day culture period in SFM and growth factors, CD133+ tumor cells demonstrated growth as nonadherent tumor spheres, which continued to expand the cell population (Fig. 5C, top panels). However, CD133- cells adhered to the culture dishes, did not proliferate, and did not form spheres (Fig. 5C, bottom panels).

The observed stem cell activity of CD133+ tumor cells was confirmed when CD133+ and CD133- tumor cells from 8 tumors (4 medulloblastomas, 3 pilocytic astrocytomas, and 1 ganglioglioma) were plated at limiting dilutions (Fig. 5D). These data show that the capacity for tumor self-renewal resides in the CD133+ fraction, and that this stem cell property is absent in the CD133- tumor cell population. Cell proliferation assays of tumor cells sorted for CD133 expression demonstrate that CD133+ cells possess proliferative capacity, whereas CD133- cells did not proliferate (Fig. 5E). Thus, CD133 identifies an exclusive subpopulation of brain tumor cells that have neural stem cell activity.

**BTSC Cultures Exhibit Abnormal Karyotypes and Are Not Migrating Normal Neural Stem Cells.** Because normal stem cells can migrate to sites of injury, and brain tumor cultures may potentially be contaminated with some normal neural stem cells, we conducted appropriate cellular and genetic analyses to demonstrate that the BTSC we isolated was indeed transformed and are not normal brain stem cells.

Cellular analyses of medulloblastoma cultures sorted for CD133 expression reveal that neither CD133+ nor CD133- cell differentiation profiles resemble the differentiation profile of a normal human

neural stem cell (Fig. 6, A and B). Normal human neural stem cells demonstrate multipotentiality by differentiating into characteristic proportions of astrocytes (50–60%), neurons (20–30%), and oligodendrocytes (5–10%; Fig. 6C). However, differentiated CD133+ cells that harbor stem cell activity and CD133- tumor cells sorted from a medulloblastoma exhibit an abnormally high proportion of cells immunostaining for  $\beta$ -III tubulin (86.5% and 83.0%, respectively), resembling the original tumor rather than normal brain phenotype (Fig. 6, A and B).

We also performed cytogenetic analysis and SKY (8) using metaphase preparations obtained directly from cultured tumor spheres from a medulloblastoma (Patient 7; Fig. 6D). After dissociation of the primary tumor and formation of primary tumor spheres, medulloblastoma cells were again dissociated and metaphase spreads were prepared. Detailed SKY analysis was possible in 8 metaphases, and all of the cells had an identical clonally abnormal karyotype. In all of the metaphases the consistent numerical alterations 45 XY, -10, -16, and +18 were present. Of these alterations, loss of chromosome 10 is one of the most characteristic findings in medulloblastoma (16, 17). We also performed interphase fluorescent *in situ* hybridization on another medulloblastoma specimen (Patient 14), from which tumor cells underwent magnetic bead cell sorting for CD133. Unsorted tumor cells, CD133+ purified tumor stem cells, and CD133- cells were probed for centromere 17 and the *p53* locus on chromosome 17p. All three cell populations (unsorted, CD133+, and CD133-) showed presence of isochromosome 17q (data not shown). Taken together, these cellular and genetic data demonstrate that the tumor-derived sphere cells possess an abnormal karyotype and are not contaminating



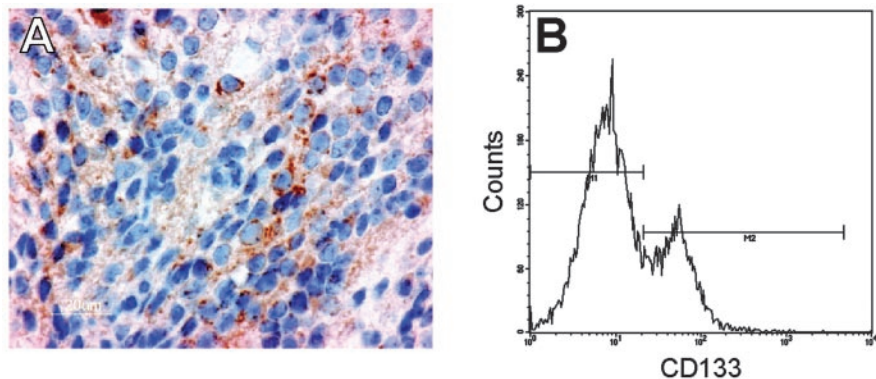
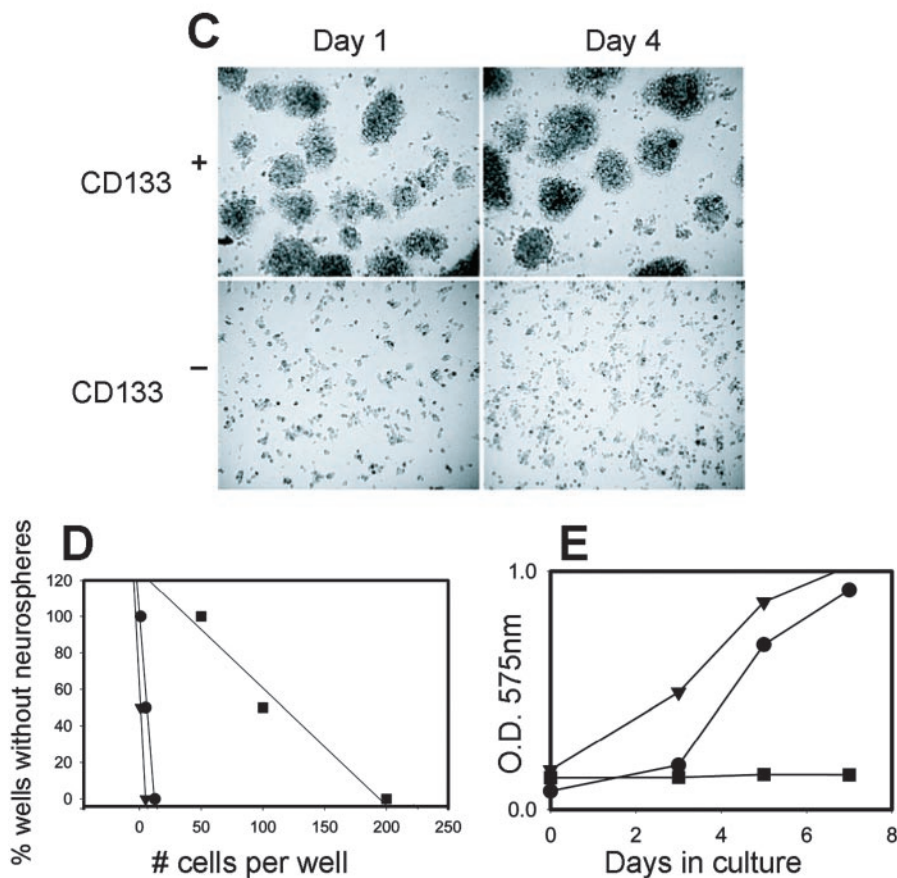


Fig. 5. CD133+ tumor cells show marked stem cell activity. *A*, immunohistochemistry for CD133 shows a plasma membrane staining pattern in scattered cells within a medulloblastoma. BTSCs from both medulloblastomas and pilocytic astrocytomas were immunostained for CD133 and subjected to flow cytometry for quantification of CD133 expression (Table 3), which varied widely in each tumor subtype. *B*, flow cytometry histogram in representative medulloblastoma tumor cells (from patient 6), with the first peak (gate M1) representing cells negative for CD133-phycoerythrin expression, and the second peak (gate M2) representing CD133 positive cells. Tumor cells were then sorted for CD133 expression by magnetic bead cell sorting. CD133+ and CD133- populations were collected, checked for purity by flow cytometry, and cultured separately in TSM for stem cell assays. Purity was found to range from 46.9 to 79.8% in CD133+ populations, and 92.6 to 97.3% in CD133- populations. *C*, CD133+ tumor cells proliferated in culture as nonadherent spheres, whereas CD133- tumor cells adhered to culture dishes, did not proliferate and did not form spheres. *D*, limiting dilution analysis showed that self-renewal capacity resides in the CD133+ tumor cell population (CD133+ cells, ▲; unsorted tumor cells, ●; CD133- cells, ■). *E*, cell proliferation assays demonstrate that CD133+ cells (▲) possess marked proliferative capacity, whereas CD133- cells do not (■; unsorted tumor cells, ●).



normal neural stem cells. Rather, these cells have undergone a transformation event, incurring the enhanced self-renewal and proliferation properties we observed *in vitro*.

## DISCUSSION

Stem cells are functionally defined as self-renewing, multipotent cells that exhibit multilineage differentiation (18, 19). Somatic stem

cells are thought to self-renew to generate all of the mature cell types of a particular tissue through differentiation, although rigorous identification and isolation of tissue-specific stem cells has been accomplished prospectively in only a few organ systems (2). The neurosphere assay has permitted rigorous *in vitro* characterization of the neural stem cell, but prospective study of this cell has been limited previously by lack of cell surface markers necessary for its isolation.

The potential that a neural stem cell may be transformed into a brain tumor has long been considered, but no prospective isolation of stem cells has been performed in brain tumors. Brain tumor cells can express the neural stem cell marker nestin (20, 21), and brain tumors are comprised of cells expressing phenotypes of more than one neural lineage. Microarray analysis of human medulloblastomas also suggests a similarity of gene expression with normal developing brain cells (22). Recent experiments in mice also suggest that neural progenitors may be transformed into brain tumors. Mouse brain cells expressing neural progenitor markers are more receptive to oncogenic transformation than differentiated brain cells (23–26). However, oth-

Table 4 CD133 expression of brain tumor stem cells

Patient #	Tumor pathology	% CD133 expression by flow cytometry
1	Medulloblastoma	45.4
2	Medulloblastoma	6.1
6	Medulloblastoma	14.2
7	Medulloblastoma	12.9
8	Pilocytic astrocytoma	3.5
9	Pilocytic astrocytoma	7.7
10	Pilocytic astrocytoma	37.1
Control	Embryonic mouse neural stem cells	45.4

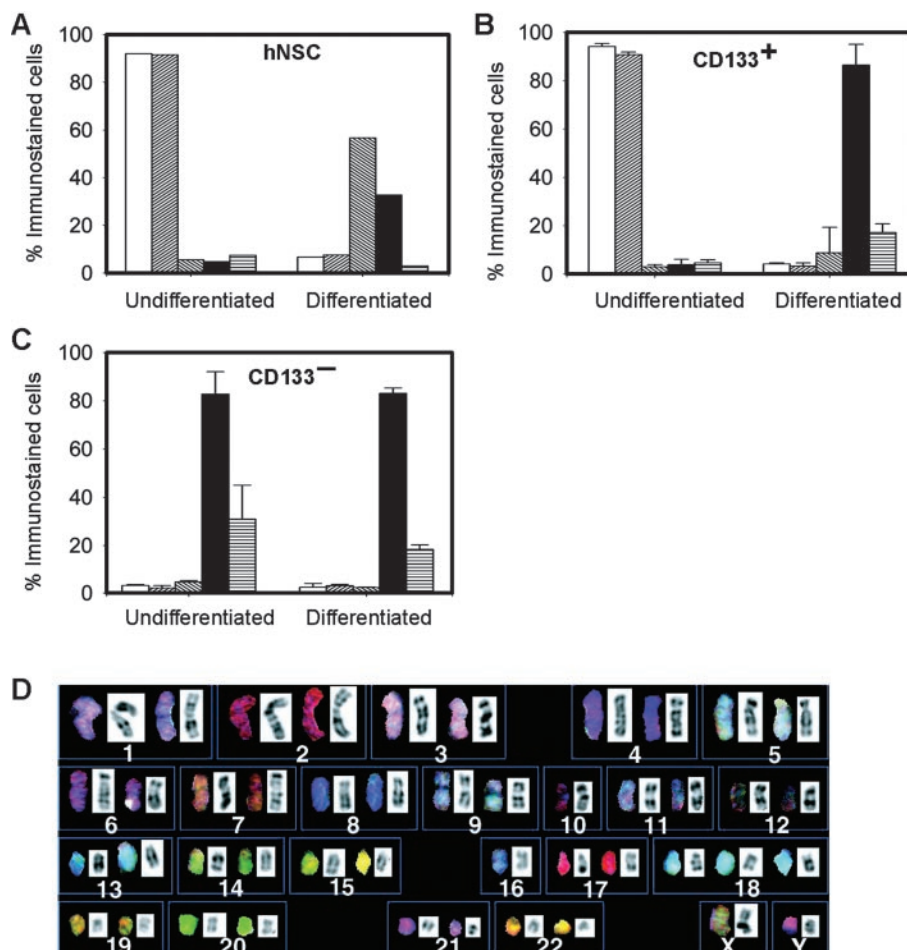


Fig. 6. Unlike normal neural stem cells, the BTSC has undergone transformation. *A*, normal human neural stem cells differentiate into characteristic proportions of astrocytes (GFAP, ▩), neurons ( $\beta$ -tubulin 3, ■) and oligodendrocytes (PDGFR- $\alpha$ , ▧) reflecting the composition of normal brain. Immunostaining for CD133 (□) and nestin (▨) is characteristically lost after differentiation. *B* and *C*, both differentiated CD133<sup>+</sup> (*B*) and CD133<sup>-</sup> (*C*) tumor cells sorted from a medulloblastoma exhibit abnormally high proportions of cells staining for  $\beta$ -tubulin 3 (■), recapitulating the original tumor phenotype. Note that CD133<sup>-</sup> cells display minimal staining for undifferentiated cell markers CD133 (□) and nestin (▨). *D*, spectral karyotype analysis performed on tumor sphere cells isolated from a medulloblastoma shows changes typical of medulloblastoma, including loss of chromosomes 10 and 16, and gain of chromosome 18.

ers suggest that a differentiated neural cell is equally permissive to transformation if it has a key genetic alteration (27).

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 (2). In malignancies such as leukemia (1), multiple myeloma (28), and most recently breast cancer (29), 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. Because 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 (2).

In this report, we have identified a new population of cancer stem cells in brain tumors of different phenotypes. These tumor stem cells represented a fraction of the total cells comprising the tumor, and they were identified by CD133 expression. There are three pieces of evidence that support that these cells are BTSCs: (*a*) they generate clusters of clonally derived cells resembling neurospheres; (*b*) they self-renew and proliferate; and (*c*) they differentiate to recapitulate the phenotype of the tumor from which they were derived.

The application of principles for study of normal neural stem cells 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. Our data suggest that brain tumors are comprised of populations of proliferating tumor stem cells that are differentiating into the more mature cell types, which characterize the tumor. Purification of

CD133<sup>+</sup> cells in brain tumors implies that a hierarchy may exist in the tumor cell population, because not all of the tumor cells were capable of maintaining the tumor in culture. This apparent hierarchy may be functionally elucidated as more surface markers for neural stem cells emerge and additional tumor subpopulations are identified. The data suggest that the tumors originate from tumor cells that express CD133, as this fraction exclusively had the ability to proliferate, self-renew, and differentiate. Because normal neural stem cells are also found in the CD133 population of the normal human fetal brain, it suggests that the cell of origin for a brain tumor may be a normal neural stem cell. Future investigations of the BTSC may lead to additional insight of this possibility, and may clarify whether the BTSC sits at the top of a lineage hierarchy, or further down as a lineage-restricted progenitor.

We recall the principles that first defined the cellular organization of proliferative blast cells in leukemia to understand the tumor-specific differentiation profile. Buick *et al.* (30) first proposed that malignant transformation might limit the differentiation capacity of normal pluripotent stem cells and cited experimental support for this "blocked differentiation" model in the culture of bone marrow from leukemia patients. Moreover, if a tumor is viewed as an aberrant organ initiated by a cancer stem cell (2), then the role of the tumor stem cell would be necessarily lineage-restricted to generate only the mature cells that comprise the tumor. It is intriguing to speculate whether specific growth factors could force lineage-restricted tumor stem cells to differentiate down a different pathway; for example, could a neuronal growth factor impose a neuronal fate on stem cells from a pilocytic astrocytoma? We do not believe that absence of multilineage

differentiation of the BTSC refutes that these cells are stem cells, because some cells differentiated into more than one lineage, and these cells uniquely had the ability to proliferate and self-renew to generate differentiated progeny that comprise the tumor. The BTSCs from the different tumor phenotypes and patient samples demonstrated little variability in marker expression, which was also expressed in normal neural stem cells. This limited variation in marker phenotype for the BTSC for different tumors suggests that normal neural stem cells as opposed to committed progenitors are the more likely targets of transformation.

In this study, we provide new insight into the brain tumorigenic process. With evidence of self-renewal, proliferation, and lineage-restricted differentiation that recapitulates the original tumor phenotype, we define a class of BTSCs that can be prospectively isolated from many brain tumors. These cells grow as neurosphere-like clusters and expressed neural stem cell markers. We also provide evidence to support the use of a novel stem cell assay, namely cell sorting for CD133 expression, for the purification of the BTSC from brain tumors. These findings 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.

The identification of the BTSC has important implications for understanding the molecular mechanisms of brain tumorigenesis, as current molecular pathological analyses of global tumor cell populations (such as is performed in tumor microarray experiments) may not be sufficient to determine the key molecular alterations in rarer tumor stem cells. The presence of a BTSC will also have important implications for understanding brain tumor dissemination if these are the cells that migrate and establish central nervous system metastasis. The functional analysis of the BTSC may also provide a novel means for testing of new treatment strategies that focus on the eradication of the tumor maintaining BTSC. The fact that we are able to differentiate BTSCs into cells that express more mature markers supports that additional exploration of the dynamic tumor differentiation process may lead to differentiation therapy. Finally, as it has been emerging that normal stem cells and cancer cells share similar phenotypic and functional properties, studies of stem cells found in brain tumors may shed additional light on the biology of normal neural stem cells.

## ACKNOWLEDGMENTS

We thank John Dick for inspiration, mentorship, and guidance; Sean Egan for invaluable help and advice; Michael Ho, Gisele Knowles, Jane Bayani, and Jana Karaskova for expert technical assistance; and Todd Mainprize and Ajay Pandita for helpful discussions. We thank Dr. Wieland Huttner for antihuman prominin antibody. We also honor the late Larry Becker for his constant devotion to the principles of our work, and his inspired efforts on our behalf.

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## Identification of a Cancer Stem Cell in Human Brain Tumors

Sheila K. Singh, Ian D. Clarke, Mizuhiko Terasaki, et al.

*Cancer Res* 2003;63:5821-5828.

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