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Brain tumor stem cell: A emerging tool for investigation and inhibition of tumor genesis in brain

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ABSTRACT

Brain tumors are significant causes of morbidity and mortality. It has been hypothesized that they derive from self-renewing multipotent neural stem cells. We report here the identification and purification of a cancer stem cell from human brain tumors of different phenotypes that possess a marked capacity for proliferation, self-renewal, and differentiation. Gene expression analysis reveals that both whole tumors and tumor-derived neurospheres express many genes characteristic of neural and other stem cells, including CD133, Sox2, musashi-1, bmi-1, maternal embryonic leucine zipper kinase, and phosphoserine phosphatase, with variation from tumor to tumor. 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 BTS. Furthermore, investigators have shown these cells' ability to drive the formation and growth of the tumor. Brain tumors have also been reported to possess a subpopulation of cancer stem like cells that have the ability to proliferate, self-renew, and be multipotent.

Key words: Brain tumors cells, stem cell, tumor

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INTRODUCTION

The study of normal neural stem cells can be applied to the brain tumor cell population by establishing a link between normal neurogenesis and brain tumor genesis. Brain tumors are phenotypically heterogeneous because they are composed of cells expressing both markers of differentiation and non-differentiation. Populations of proliferating tumor stem cells differentiate into more mature cell types that characterize the tumor.

A study of leukemia that a population of neoplastic cells exhibit heterogeneity with respect to proliferation and differentiation. These stem-like cells, present within the leukemic population, possess a high capacity for self-renewal and proliferation that is not present in the majority of leukemic cells. An analysis of stem cells present in leukemia tumors demonstrated that their presence is necessary and sufficient to maintain the tumor population. Cancer stem cells associated with breast cancer and acute myelogenous leukemia have been isolated and implanted into experimental animals to form new tumors; their success following implantation demonstrates that these cells are sufficient to generate tumors.

To be considered a BTSC, a cell must be able to do the following: 1) generate clonally derived cells that form neurospheres; 2) self-renew and proliferate; 3) differentiate and express markers typical of brain cells (that is, those markers associated with astrocytes, oligodendrocytes, and neurons); and 4) generate tumors after in vivo transplantation in animal models that resemble the original tumor in donor patients.

The cellular origin of pediatric brain tumors (PBTs) is unclear. One possibility is that they arise by transformation of proliferating neural stem cells (NSCs), cells with the ability to self-renew and differentiate into neurons and glia. There are several lines of indirect evidence in support of this hypothesis. First, PBTs often contain multiple cell types, suggestive of an origin from a cell with multilineage potential. Second, many PBTs appear to arise from the ventricular zone, the location of NSCs. Third, both PBTs and NSCs express nestin, an intermediate filament characteristic of several progenitors. Fourth, PBTs often express genes that regulate proliferation and self-renewal of normal NSCs and mutations in genes that normally regulate neural stem cell proliferation are frequently found in PBTs. Finally, forced expression of oncogenes in neural stem and progenitors cells in mice produces tumors that are similar to primary human tumors.

Identification brain tumors

The identification and purification of human brain tumors of different phenotypes. These cells possess a marked capacity for proliferation, self-renewal, and differentiation. Brain tumor stem cells were isolated by selecting cells expressing the neural stem cell surface antigen CD133. The authors demonstrated that $CD133^{+}$ cells can differentiate in vitro into tumor cells that phenotypically resemble the patient's tumor. These tumor stem cells represent a fraction of cells constituting the tumor that are identified by their CD133 expression. The CD133 marker is a 120-kD five-transmembrane cell-surface protein. Previously known as a hematopoietic stem cell marker, CD133 was recently found to be a marker for normal human neural stem cells as

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well. Singh and colleagues were able to confirm the stem cell activity of $CD133^+$ tumor cells by plating BTSCs at limiting dilutions. These investigators demonstrated that the self-renewal capacity of tumor cells was only present in the fraction of cells that expressed $CD133^+$. In addition, they found that the $CD133^+$ population displayed a proliferative capacity not present in the $CD133^+$ fraction.

PBTCs contain cells with stem cell properties; an important question is whether these cells also have abnormal properties that are responsible for the aberrant and persistent growth of the tumor.

An analysis of BTSCs may also provide a novel means for testing new therapeutic strategies for brain tumors that focus on the eradication of the tumor by targeting BTSCs. In fact, the presence of BTSCs in all the tumors examined may have important implications for understanding the underlying mechanisms of brain tumor dissemination.

Generation of tumor

(i) By role of CD133 cell

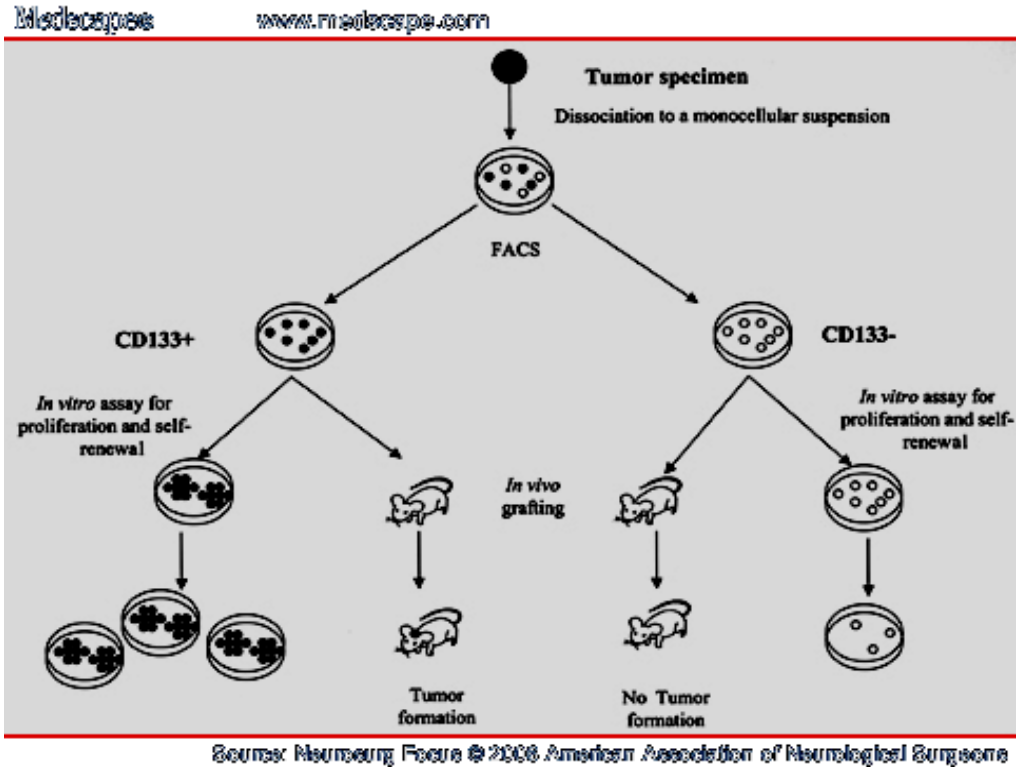

Fig 1:- Role of CD133 in tumor formation

Brain tumor stem cells were obtained after dissociation of a solid tumor specimen and were separated by cell sorting for the CD133 surface antigen. The CD133+ cells are able to

proliferate self renew, and grow as spheres. The CD133– cells lack the ability of self-renewal and are not tumorigenic in mice.

$CD133^+$ cells are able to generate tumors after having been grafted into the mouse brain, and these tumors resemble the donor patient's original tumor. An injection of only 100 $CD133^+$ cells into the NOD-SCID mouse brain led to the growth of a tumor that could be serially transplanted and was histologically identical to the tumor harbored by the patient from whom these cells were derived. In contrast, an injection of 10^5 $CD133^+$ cells did not generate tumor growth (Fig. 1). The cellular heterogeneity of brain tumors suggests that only a small fraction of cancer cells are able to regenerate a tumor and that targeting these cells could be an innovative approach to eradicating the tumor.

The mechanism by which normal neural stem cells become malignant has not yet been elucidated. Hemmati and associates demonstrated that normal and tumor-derived spheres express bmi-1, even after the withdrawal of mitogen from the culture medium. The gene bmi-1 has been demonstrated to be important for self-renewal of both leukemic and normal hematopoietic stem cells. Thus, the presence and persistent expression of bmi-1 in tumor cells could indicate a greater capacity of these cells to self-renew.

Flow chart demonstrating the establishment of a culture of neurospheres. After enzymatic dissociation of the tumor sample, the monocellular suspension was plated in serum-containing medium. After 24 to 48 hours, the medium was switched with medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF); neurospheres began to appear 5 days later. The floating neurospheres were removed and subcultured at clonal density.

Tumor-derived progenitors maintained the differentiative capacity of NSCs by examining the types of molecular markers expressed by neurospheres grown under both proliferative and differentiating conditions with immunocytochemistry.

Role of cancer stem cells play in brain tumors

Two main hypotheses have been proposed:-
(1) Brain tumors arise from the transformation of a normal stem cell or progenitor cell; and alternatively,
(2) Brain tumors arise from the dedifferentiation of a mature brain cell in response to genetic alterations.
In models of breast cancer and acute myelogenous leukemia, “cancer stem cells” have been isolated and repassaged into experimental animals to form new tumors, providing strong evidence that these cells are the root cause of the tumor. Such individual cells are capable of self-renewal, proliferation, and differentiation to create the complex heterogeneous tumor. It is unknown whether PBTCs contain such cancer stem cells, and, if so, whether such cells are derived from neural stem cells.

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(ii) By formation of culture of neurospheres

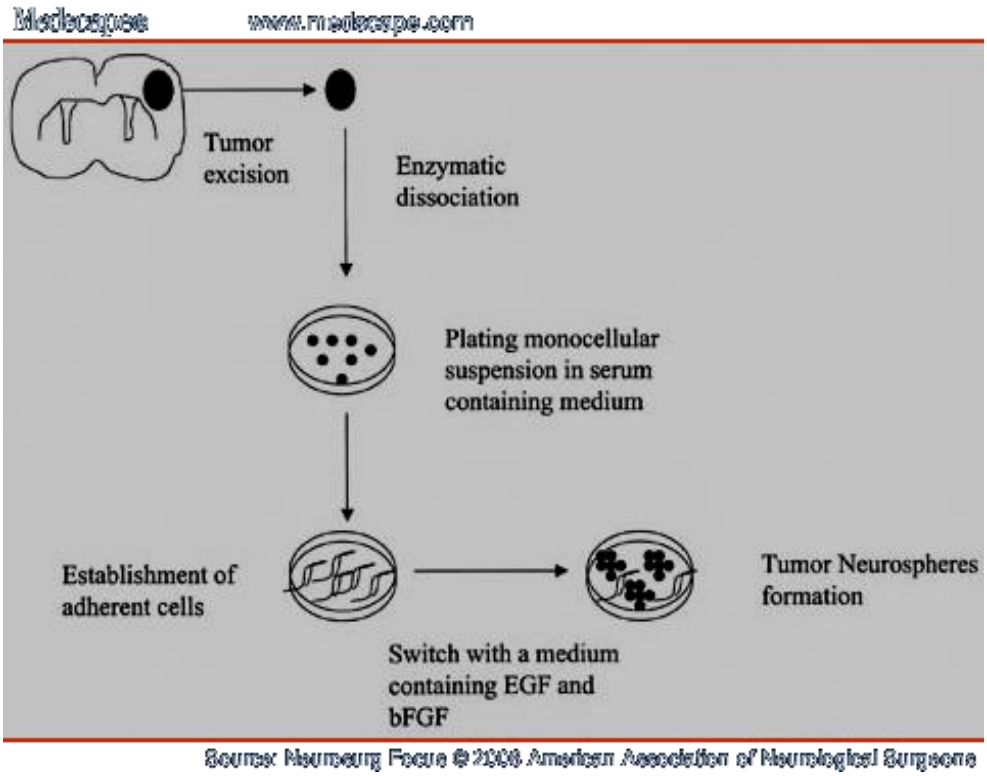

Fig 2:- Formation of culture of neurospheres.

ISOLATION METHODS

The isolation of cancer stem cells from brain tumors is important because of the implications that only through attacking these cells, will we be able to eradicate the tumors that contain them. As stated above, cancer stem cells and adult stem cells share the properties of self-renewal and multipotentiality. Several studies have demonstrated that genes known to play roles in the self-renewal of somatic stem cells also are likely to play similar roles in cancer stem cells. Some methods:-

Tissue Collection and Grading: - PBT and non tumor human brain specimens from patients undergoing neocortical resections for intractable epilepsy were obtained within 30 min of surgical resection in accordance with protocols approved by institutional review boards at University of California Los Angeles Medical Center and the California Institute of Technology. The epilepsy surgery tissue was taken from the lateral ventricular surface and immediately adjacent tissues.

Neurosphere Culture: - Tumor and neurosphere cultures were performed as according to Svendsen et al. with some modifications. A detailed protocol is found in Supporting Methods

tissue was washed, minced, digested with trypsin, dissociated, and passed through a series of cell strainers. Cells were seeded in growth medium supplemented with basic fibroblast growth factor (20 ng/ml), epidermal growth factor (20 ng/ml), and leukemia inhibitory factor (20 ng/ml) at a density of 100,000 cells per ml. Clonal cultures were plated at a density of 1,000 cells per ml in mouse neurosphere-conditioned medium, a density that has been demonstrated to produce almost entirely clonal neurospheres.

Immunocytochemistry of Neurospheres: - Immunocytochemistry of neurosphere cultures was performed as described. Differentiation of early passage (≈4 weeks) spheres was induced by plating onto coverslips precoated with poly-l-lysine (Sigma) in Neurobasal medium (GIBCO–Invitrogen) supplemented with B-27 and in the absence of added basic fibroblast growth factor or epidermal growth factor. After 7 days, the neurospheres were fixed in 4% paraformaldehyde and immunostained with rabbit anti-nestin (1:200; Chemicon) or rabbit anti-musashi (1:200; Chemicon) for neural stem and progenitor cells, mouse anti-TuJ1 (1:500; Berkeley Antibodies) or anti-Hu (1:300; Molecular Probes) for neurons, rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; DAKO) for astrocytes, and anti-O4 (1:40; Chemicon) for oligodendrocytes, followed by Alexa fluorophore-conjugated secondary antibodies (1:2,000; Molecular Probes). In some cases, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

Statistical Analysis: - Statistical analysis of cell counts was performed as described in Supporting Methods.

Immunohistochemistry of Whole Tumor Sections:- Formalin-fixed, paraffin-embedded tissue specimens from tumors BT1, -2, -3, and -5 were sectioned at 8 µm, mounted on Superfrost Plus slides (Fisher), deparaffinized, and processed for antigen retrieval by microwave heating as described.

BrdUrd-Labeling of Neurospheres: - Neurospheres were cultured in proliferative medium containing 2 µM BrdUrd (5-bromo-2′-deoxyuridine) for 14 h. The cells were fixed in 4% phosphate-buffered paraformaldehyde solution. Staining with anti-BrdUrd antibody (Becton Dickinson) was performed according to the supplier's suggestions, and fluorescent secondary antibody was used.

Semiquantitative RT-PCR: - Semiquantitative RT-PCR was performed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard as described in Supporting Methods.

Transplantation of Neurosphere Cells into Neonatal Rat Brain: - Transplantation of 50,000 cells into the neostriata of neonatal rats was performed according to the methods of with modifications as described in Supporting Methods.

Immunohistochemical Analysis of Transplanted Rat Brain: - Four weeks after transplantation, tissue was prepared for immunocytochemical analysis as described in Supporting Methods and stained with antibodies directed against human nuclei, neurons, astrocytes, and Ki-67.

Brain tumor cell membrane synthesis

Making cell membranes is part of natural cell growth activity. The tumor cells within brain tumors that are rapidly growing synthesize cell membranes at a much higher rate than do normal cells. Research in this area looks at how blocking this process in tumor cells could inhibit tumor growth.Brain tumor cell migration is the ability of brain tumor cells to infiltrate into surrounding brain tissue.The development of cancers, including brain tumors, involves abnormal alterations of the normal gene expression regulation patterns of cells

Significance for brain tumor stratification

Traditional anatomic/pathologic categorization of tumors has very limited ability to completely stratify patients into meaningful subgroups for prognosis and intervention. Protein expression by immunohistochemistry has greatly enhanced the potential link between pathologic diagnosis and prognosis. However, relative lack of useful antibodies shows the limitation of this strategy. Recently, large scale gene expression study by microarray and analyses of specific pathways have been more successful at regrouping patients within broad histologic categories. The existence of caner stem cells raises the possibility that expression profiling and molecular pathway analysis of these cells will provide further useful stratification of tumors. For example, one might isolate cancer stem cells from individual patients and then analyze gene.

Significance for brain treatment

The isolation and demonstration of brain tumor stem cells, suggests that, for those tumors that contain such cells, treatment can only be gauged as successful if the stem cell component is successfully eradicated. Since, by their nature, stem cells are heartier and more resistant to insult, this may prove to be a daunting task. There are several potential avenues to address this issue, however. First, since cancer stem cells may use specific molecular pathways to drive their self-renewal, one might use known and as-yet-to-be-discovered selective inhibitors of these pathways to attack them. In fact, as intimated above, two groups of agents, EGF receptor inhibitors and rapamycin-related compounds that are already in clinical trial and showing some promise are predicted to inhibit cancer stem cell self-renewal. Another way to attack cancer stem cells would be to use them as targets for small molecule screens, an approach that we are taking. Finally, if specific antigens are expressed by cancer stem cells, then these antigens could be used in immunotherapy , which have already shown some promise in the treatment of glioma.

2009 Standard research grants

- The study investigates the YKL-40 gene. A protein that is produced by this gene is used as a prognostic marker for GBMs (how well patients will respond to therapy).
- The STAT3 protein determines when genes are switched on or off. This study investigates STAT3's role in brain tumor growth.

- This project studies the role of cancer stem cells in resistance to radiotherapy and chemotherapy.
- This study focuses on the control of gene expression that contributes to the malignancy of GBMs. The research team will look at two particular microRNAs that control the expression of groups of genes.
- The proposed pediatric project will study the genomics of childhood brain stem cancer in the hopes of pointing to new potential therapeutic targets. Using state-of-the-art, high-resolution techniques, genetic alterations will be examined at multiple levels to provide a complete view of tumor, gene, and pathway alterations.
- The investigator developed WP1066, an orally delivered STAT3 inhibitor. STAT3 (a protein) functions to both stimulate GBM growth and inhibit the immune system that attacks these same GBM tumors.
- The goal of this study is to determine if reduction of this protein in brain tumor stem cells can cause their death and sensitivity to radiation therapy, and ultimately to test drugs in clinical trials that inhibit this protein.
- This is a gene study of chk2's role as a tumor suppressor. Chk2 is a protein that inhibits cell division and growth. The study plans to identify novel characteristics of a protein involved in the repair of DNA damage, the control of tumor growth, and response of tumors to radiation therapy.
- This study looks at the genetic makeup and expression of genes involved in the control of cell adhesion in sPNETs (supra-tentorial primitive neuroectodermal tumors).
- This is a pediatric study for patients with medulloblastoma that investigates if restoring the balance of components of the Sonic Hedgehog (Shh) signaling pathway using a particular drug will stop the tumor growth. This study hopes to lead to a rapid clinical evaluation in pediatric patients since the drug has already been approved for the treatment of leukemia in adults.
- Researchers recently found that in dividing brain cells Sonic hedgehog turns on YAP1 which can cause cells to become cancerous. Preliminary studies show that YAP1 proteins in medulloblastoma cells survive radiation and cause tumor re-growth, making it an essential target for future therapies.
- This study focuses on the significant question of how brain tumor stem cells renew themselves as well as give rise to mature tumor cells. Investigators will study a novel mechanism that controls the self-renewal activity of normal stem cells that is functioning abnormally in glioblastoma stem cells.
- This study focuses on drug resistance and attempts to increase the effect of chemotherapy. Chemotherapy is only effective for a limited time before the tumor recurs. Endoplasmic reticulum stress response (ERSR) may be a factor in drug resistance. The investigator believes that resistance to therapy is due in part to ERSR and refers to it as a potential Achilles heel for brain tumor cells.
- This study investigates the role of endoplasmic reticulum stress response (ERSR) on drug resistance. Researchers will evaluate several drugs and drug combinations that can overpower the ERSR and destroy tumor cells.

- The study focuses on a novel extracellular protein named fibulin-3, which is absent in healthy brain tissue but abundant in gliomas. Fibulin-3 has been found to play a role in the invasive nature of malignant gliomas.
- This study builds on previous NBTS-funded work that identified a potential drug molecule that blocks PI3K and kills glioma cells (other drugs block PI3K, but don't kill the glioma cells).
- This project studies how a cellular negative regulator of the highly important p53 tumor suppressor is involved in GBM development and drug resistance. The investigator discovered a cancer gene called HDMX that negatively regulates p53 signaling (involved in growth and resistance) and will study its role in tumor development and drug resistance.

2009 Advanced research grants

- This study utilizes sophisticated mouse models that highlight the altered genes and pathways that cause cancer. The models will also be used to test the idea of combination therapies directed at more than one of the altered proteins and signaling pathways.
- The objective of this project is to genetically distinguish the consequences of STAT3 activation in medulloblastoma tumor cells vs. the immune cells that invade the tumors. STAT3 activation is thought to be a critical event in the development of brain tumors and a suggested therapeutic target. However, STAT3 is also important for the abilityof the immune system to help fight tumors. Genetically engineered mice afford a new possibility to determine mechanisms by which the natural immune response changes as tumors progress from very early to later stages.

2009 Innovation research grants

- This pediatric project proposes to identify the genes that are mutated in DIPG (diffuse intrinsic pontine gliomas) using an unbiased approach to evaluate all of the genetic material in the tumors as well as a directed approach to closely examine candidate genes suspected to be mutated in this disease. These studies may identify therapeutic targets and allow comparisons between DIPG, pediatric high grade glioma arising outside the brainstem, and adult high-grade gliomas. Describing the similarities and differences among these diseases may help predict whether advances in another group of gliomas may hold promise for DIPG.
- This project will study the molecular pathways that drive AT/RT (atypical teratoid rhabdoid tumor) formation and growth. Gene expression array analysis will be performed on a large series (up to 200 samples) of AT/ RT and compared using a number of exploratory analyses. Whole genome mutational analysis in AT/RT has not been previously studied and identification of specific mutations will provide important information on focusing further efforts on specific pathways.

DISCUSSION

We asked whether BTCs contained progenitor cells with characteristics similar to those of NSCs. We have isolated and characterized multipotent, self-renewing cells from tumor samples, here referred to as “tumor-derived progenitors,” which have both similarities to and

differences from normal NSCs. Cells derived from PBTs were able to produce proliferating neurospheres that could be passaged at clonal density and differentiated into cells with defining antigenic characteristics of neurons and glia. These neurospheres expressed many genes characteristic of NSC-derived spheres. Furthermore, like normal neurospheres, tumor-derived spheres migrated and continued to proliferate when transplanted into neonatal rat brain. Unlike NSCs, however, these tumor-derived progenitors were more long-lived and often gave rise to abnormal dual-phenotype cells. Our data suggest that PBTs arise from cells with many of the characteristics of NSCs but with abnormal ability to propagate and differentiate. These studies also raise the possibility that some tumor-derived cells may be cancer stem cells with the ability to generate PBTs.

Brain tumors contain neural stem-like cells that may be responsible for their formation. Our results show that tumor-derived cells have the ability to form neurospheres and can be propagated for prolonged times in culture. This property is a general property of all of the 22 tumors examined in this study. We demonstrate that tumor-derived progenitors and NSCs express many of the same genes and proteins, and they share some common characteristics, including self-renewal and multipotency. However, the types of progeny arising from neurospheres vary from tumor to tumor and, to some extent, recapitulate the properties of their tumor of origin.

Tumors arise in organs that contain stem cell populations. The tumors in these tissues consist of heterogeneous populations of cancer cells that differ markedly in their ability to proliferate and form new tumors. This suggests that agents that target the defective self-renewal pathways in cancer cells might lead to improved outcomes in the treatment of these diseases.

Among the characteristics in common between tumor-derived spheres and normal neural stem cells is the expression of specific genes, including CD133, musashi-1, Sox2, melk, PSP, bmi-1, and nestin. Only one of the tumors we studied, a medulloblastoma (BT3), failed to express nestin in most cells in undifferentiated clonal neurospheres.One finding of our study is that the tumor-derived neurospheres tend to differentiate into an array of progeny with the same generSl profile as the parental tumor [1-11].

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