

Cancer Stem Cells as Mediators of Treatment Resistance in Brain Tumors: Status and Controversies¹

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Abstract

Malignant primary brain tumors are characterized by a short median survival and an almost 100% tumor-related mortality. Despite the addition of new chemotherapy regimes, the overall survival has improved marginally, and radiotherapy is only transiently effective, illustrating the profound impact of treatment resistance on prognosis. Recent studies suggest that a small subpopulation of cancer stem cells (CSCs) has the capacity to repopulate tumors and drive malignant progression and mediate radio- and chemoresistance. This implies that future therapies should turn from the elimination of the rapidly dividing, but differentiated tumor cells, to specifically targeting the minority of tumor cells that repopulate the tumor. Although there exists some support for the CSC hypothesis, there remain many uncertainties regarding theoretical, technical, and interpretational aspects of the data supporting it. If correct, the CSC hypothesis could have profound implications for the way tumors are classified and treated. In this review of the literature, we provide original data and hypotheses supporting alternative explanations and outline some of the therapeutic implications that can be derived.

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Keywords: Neural stem cell, CD133, glioblastoma, chemoresistance, radioresistance.

Introduction

Glial neoplasms are the most frequent primary intracranial neoplasms in man accounting for more than 60% of all primary brain tumors [1]. The most malignant of these, the glioblastoma multiforme (GBM), is characterized by resistance to chemo- and radiotherapy and by a short median survival [2]. Despite intensive investigation, the origins of human brain tumors remain unresolved. Recent studies have proposed that the neural stem cell (NSC) antigen CD133 is a marker for brain tumor–initiating cells and linked it to treatment resistance. There has been a general concern, however, that defining brain tumor stem cells based exclusively on CD133 expression is too restrictive. Recent publications show that CD133-negative tumor cells are

capable of self-renewal and can give rise to tumors [3,4]. It is conceivable, therefore, that the mechanisms that allow a tumor cell to manifest as a cancer stem cell (CSC) vary depending on the procedure adopted for defining *stemness* [5]. We would like to propose that CSCs are highly plastic and opportunistic tumor cells that are capable of adapting and exploiting their microenvironment regardless of whether they express CD133 or not. Heterogeneity in these properties is inevitable. The CSC hypothesis places too great an emphasis on the intrinsic tumorigenic capacity of the tumor cells and neglects the contribution of the microenvironment. Herein, we review the literature on the subject and try to outline some of the therapeutic implications that can be derived.

Somatic Mutations Hypothesis

Malignant brain tumors have traditionally been classified by the World Health Organization (WHO) classification system [6] that seeks to define the cell of origin by the morphological similarities of the tumor cells to the nonneoplastic counterpart [7]. This predominant theory resembles a stochastic model, which predicts that gliomas result from somatic mutations in terminally differentiated astrocytes or oligodendrocytes that subsequently undergo a series of transformations to a less differentiated phenotype [8,9]. Glial neoplasms comprise a wide range of tumors that are subdivided into four grades of malignancy (WHO grades I–IV). The primary objectives of the WHO classification system are: first, to place the tumor in a category reflecting the cell of origin or line of differentiation, i.e., derivation from astrocytes, oligodendrocytes, or the choroid plexus [1]. Second, to assign within that category a grading system

Abbreviations: BMP, bone morphogenic protein; CNS, central nervous system; CSC, cancer stem cell; GBM, glioblastoma multiforme; GFAP, glial fibrillary acidic protein; HDAC, histone deacetylase; NOD/SCID, nonobese diabetic/severe combine immunodeficient mouse strain; NSC, neural stem cell

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that reflects the regional heterogeneity of histological features within the tumor compared to those of putative cells of origin. Third, to accurately predict the implications for clinical prognosis and the neoplasm's responsiveness to particular forms of treatment. The WHO grading system is based on areas showing the highest degree of nuclear atypia, mitotic activity, cellularity, vascular proliferation, and necrosis on the assumption that the tumor cell population in these areas eventually determine the course of the disease. Nevertheless, an intense debate about the type of cell that undergoes malignant transformation to produce each specific brain tumor has arisen, partially due to the apparent weaknesses of the WHO classification system (see Table 1).

Stem Cell Hypothesis

An alternative hypothesis proposes that the organization of tumors resembles the cellular hierarchy of the organ in which the tumor arose. Neoplastic transformation of tissue-specific stem/precursor cells as the initial event in cancer was proposed years ago [10]. This model proposes that tumors are driven and maintained by a minority of transformed stem/precursor cells. These CSCs have the exclusive capacity to self-renew (i.e., give rise to progeny with similar properties as themselves) and to generate the more differentiated and diverse progeny (asymmetric cell division), which make up the bulk of a tumor [11] (Figure 1). However, the terms CSCs, tumorigenic cancer cells, or tumor-initiating cells are also used as a reference to any cell population that can self-renew, initiate a tumor, and give rise to a heterogeneous progeny without assumptions about the cell of origin.

How Solid is the Evidence for CSCs in Solid Tumors?

Experiments to test the CSC hypothesis in solid tumors have been hampered by difficulties in phenotypic identification, isolation, and manipulation of CSCs. Studies of malignant transformation that leads to glioma development have also been limited by the lack of biological systems that represent early stages of this disease in adult animals [12].

Arguments for the Stem Cell Hypothesis

Significant numbers of multipotent stem/progenitor cells exist in the adult rodent, primate [13–16], and human brain [17–22]. Bromodeoxyuridine labeling and ³H-thymidine incorporation techniques have demonstrated that these stem cells are located in the subependymal and ventricular zones of the lateral ventricles, the hippocampus, substantia nigra, and postnatal cerebellum and that they continue to replicate throughout the adult life [22,23]. Experiments showed that proliferating early progenitor cells located in the subventricular zones of rodents could be transformed with ethylnitrosourea and that those could give rise to various histological types of brain tumors [24]. Further support for the CSC hypothesis comes from the observation that normal and CSCs share several markers, respond to the same external cues, share the capacity for self-renewal and differentiation, and exhibit telomerase activity, apoptosis resistance, and increased membrane transporter activity. They share similar activation of canonical signaling pathways that regulate self-renewal and stem cell properties such as Wnt, Notch, and Hedgehog. The possibility that multipotent cells rather than mature glial cells may be the target for central nervous system (CNS) carcinogenesis is also consistent with the

Table 1. WHO Classification System: Properties, Strengths, and Weaknesses.

WHO Properties	Strengths	Weaknesses	References
–Names tumors according to the cell and tissue types they resemble in the brain	–Widely recognized system –Established in 1970, revised 1993 and 2000. –Presence of tumors corresponding to normal tissue such as oligodendrogliomas, astrocytomas, and ependymomas	–Tumors contain morphologically diverse atypical cells and do not resemble normal cell types –Relies on immunocytochemical techniques but few epitopes exclusively identify specific cell lineages, e.g., GFAP –Genetic instability may induce expression of uncharacteristic antigens –Fails to explain the existence of <i>mixed</i> glial tumors, e.g., mixed oligoastrocytoma	[7,10]
–Grading system assigns differentiated astrocytoma grade II, anaplastic astrocytoma to grade III, and GBM to grade IV based on the presence of nuclear atypia, mitotic activity, necrosis, and microvascular proliferation	–Grading based on histopathological features predicts survival in most cases –High interobserver correlation	–Inconsistent when low-grade astrocytomas, exhibiting endothelial proliferation and conspicuous cellular pleomorphism, are not classified as glioblastoma (e.g., pilocytic and xanthoastrocytomas)	[91–93]
–Assumes cell of origin originates from somatic mutations in differentiated mature astrocytes, oligodendrocytes, or ependymal cells that undergo a series of transformations to a less differentiated phenotype	–Astrocytes more numerous in the adult brain than neural stem cells and astrocytic tumors are the majority of malignant primary brain tumors	–Brain tumors of apparently comparable histological structure exhibit vast differences in biological behavior –Cell of origin not identified –Mature, postmitotic cells are less vulnerable to mutagenic transformation. –Normal glia are morphologically homogenous, whereas malignant gliomas of clonal lineage display striking cellular heterogeneity –Individuals with comparable malignant glioma show marked heterogeneity in response to current treatment	[8]

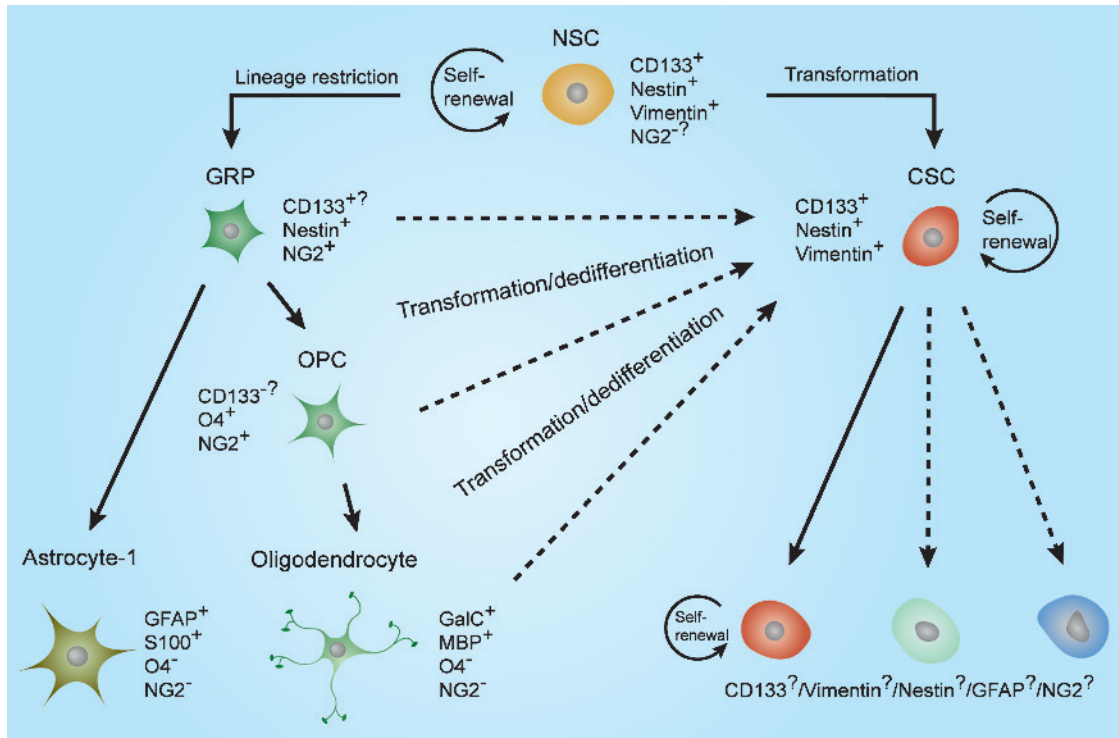


Figure 1. Cellular origins of glioblastoma. The cancer stem cell is believed to arise from a transformed NSC or a transformed precursor in the CNS. Whether the transformation event happens before or after dedifferentiation is uncertain. This would give rise to tumor cells with various potentials for self-renewal, which express a variety of markers associated with both progenitor and mature cell types. Transformation of mature cells (astrocytes and oligodendrocytes) by specific mutations may be equally permissive for tumorigenesis, resulting in clones with a self-renewing phenotype. Abbreviations: NSC, neural stem cell; GRP, glial-restricted progenitor; CSC, cancer stem cell; GalC, galactocerebroside; MBP, myelin basic protein; NG2, neuron–glia 2; O4, monoclonal antibody that recognizes the sulfatides; S100, calcium-binding protein specific for mature astrocytes; GFAP, glial fibrillary acidic protein; OPC, oligodendrocyte precursor cell.

existence of histologically mixed glial tumors. The diffuse infiltration of glial tumors trans corpus callosum to the contralateral brain parenchyma is also consistent with this view. This suggests that tumor cells in the CNS may retain the ability of their cell of origin to migrate through the parenchyma, a property that may be important in maintaining the balance of mature cells in the normal brain [25].

Empirical evidence for the existence of CSCs originally came from studies of acute myelogenous leukemia. A subset of leukemic cells ($CD34^+/CD38^-$) was identified able to propagate the disease onto immunodeficient mice and display a similar cell surface immunophenotype to normal hematopoietic stem cells [26]. Less than 1 in 10,000 $CD34^+/CD38^-$ leukemia cells was required to transfer the human leukemia into nonobese diabetic/severe combine immunodeficient mouse strain (NOD/SCID), whereas the inoculation of many thousand-fold higher numbers of cells not bearing this phenotype did not. In similar studies of multiple myeloma, a syndecan-1–negative ($CD138^-$; plasma cell differentiation marker) subpopulation of cells was identified and proven to be exclusively clonal *in vitro* and tumorigenic in NOD/SCID mice [27]. Similarly, in breast cancer, as few as 200 $CD44^+/CD24^{-/low}$ cells (which comprised between 1% and 10% of the total cell population) consistently formed tumors in immunodeficient mice, whereas injection of 20,000 cells of the remaining population did not form tumors [28]. The tumorigenic population gave rise to additional $CD44^+/CD24^{-/low}$ epithelial tumors, which could be serially pas-

saged *in vivo* and which also gave rise to nontumorigenic breast cancer cells [28]. Others showed that these tumors could be propagated *in vitro* as mammospheres, i.e., a property previously described for mammary stem cells [29]. In brain tumors (GBMs and medulloblastomas) [30] and colon cancers [31,32], CSCs have been identified based on the surface expression of CD133. In brain tumors, as few as 100 cells representing 5% to 30% of total tumor cells formed tumors when injected intracranially into NOD/SCID mice [30,33]. In contrast, 100,000 $CD133^-$ cells did not produce tumors. In addition, the cells formed tumor spheres, which were highly enriched in long-term, self-renewing multipotent cells *in vitro*, that independently gave rise to new tumors in immunodeficient mice [30]. When xenografted, they recapitulated the phenotypic heterogeneity of the initial tumor. In prostate cancer, a subpopulation of cells characterized by $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ with stem cell properties was identified. As few as 500 cells with this phenotype, constituting only 0.1% of total tumor cells, formed tumors in NOD/SCID mice. In contrast, 500,000 inoculated $CD44^-$ cells failed to form tumors (Table 2).

Arguments Against the Stem Cell Hypothesis

Although stem cells and cancer cells display many overlapping features, these similarities only provide circumstantial evidence for involvement of stem cells in the development of solid cancers. There is a tendency to interpret lineage derivation of CSCs from normal stem cells or the early stages

Table 2. Tumorigenicity of Xenografted Cancer Stem Cells.

Cancer Type	Stem Cell Marker	No. of CSCs/Total Implanted	Model	Reference
Acute Myeloid Leukemia	CD34 ⁺ CD38 ⁻ Lin ^{neg}	1 / 10,000	NOD/SCID	[26]
Multiple Myeloma	CD138 ⁻	1–10 × 10 ⁶	NOD/SCID	[27]
Breast	CD44 ⁺ CD24 ^{-/low}	200 / 20,000	NOD/SCID	[28]
Glioblastoma	CD133 ⁺	100 / 50–100,000	NOD/SCID	[33]
Medulloblastoma	CD133 ⁺	100 / 50–100,000	NOD/SCID	[33]
Bone sarcomas	Stro-1 ⁺	Self-renewal, colony formation and multilineage differentiation	<i>In vitro</i>	[94]
Lung	CD105 ⁺ CD44 ⁺ Sca-1 ⁺ CD45 ⁻			[88]
Melanomas	CD20 ⁺		SCID	[95]
Prostate	CD44 ⁺ a2b1 ^{hi} /CD133 ⁺ Sca-1 ⁺	500 / 500,000	NOD/SCID	[96,97]

SCID, severe combined immunodeficient mouse strain (lack B and T lymphocytes); NOD/SCID, nonobese diabetic/severe combine immunodeficient mouse strain (lacks B, T, and NK lymphocytes); Lin^{neg}, negative for lineage-specific differentiation antigens.

of their progeny. It is fully conceivable that more differentiated cells can, through multiple mutagenic events, acquire the self-renewal characteristic of CSCs (Figure 1). Ink4a/Arf^{-/-} transduction in NSCs and/or mature astrocytes with constitutively active epidermal growth factor receptor both induced a GBM in both cell types, indicating that it may be the deregulation of a specific genetic pathway, rather than the cell of origin that dictates the emergence of the GBM [34]. Conversely, the term *tumor-initiating cell* can also be misleading because the ability of these cells to initiate tumors is also setting-dependent. The cell that initiates a xenograft tumor does not necessarily have to be the same one that received the first oncogenic insult in the patient because the CSCs capable of forming a tumor at one point might change during the course of tumor progression [11]. Similarities between normal stem cells and CSCs in the activation of *developmental* signaling pathways are often cited as supporting the CSC hypothesis. However, deregulation of these signaling pathways in cancer can also be interpreted as the loss of control of *stemness* [5].

Another paradox is that, in many of the brain, breast, and colon tumor studies, the cells expressing CSC markers appear to represent nearly 30% of the total cells in the tumors studied (Table 2) contradicting the assumption that CSCs are a rare cell population in solid tumors. In addition, the surface proteins that are used for identifying CSCs have not been shown to be necessary nor sufficient for conferring stem cell-like properties. This emphasizes the need for additional functional assays to identify cell populations with CSCs characteristics [35]. Given the number of CSCs needed to initiate tumors in rodent models (generally in the range of hundreds to thousands), it is possible that only a subset of cells within these populations will turn out to be true CSCs. The presence of early progenitors within the CSC population could also contribute to tumorigenesis such that results might also reflect the properties of the contaminating cells rather than exclusively CSCs as determined by a particular surface immunophenotype. Another point is that all CSC populations have been identified using severely immune-deficient animals. Under these conditions, one would expect more cell types to exhibit tumorigenicity. Use of animals with a less compromised immune defense might show how tumorigenic CSC really are in terms of initiating/repopulating

the tumor. Ideally, a model should reflect the disease situation in humans. Conclusions from studies using established cell lines must be viewed with caution given the effects of selection for growth in tissue culture. CSC-like subpopulations within established cell lines might not necessarily display the same cell surface immunophenotypes or functional characteristics as CSCs within tumors in their primary site [35]. The identification of CSCs from solid tumors has so far required 1) the dissociation of the tissue into a single cell suspension by proteolytic enzymes, 2) FACS sorting of the cells based on expression or lack of surface markers or exclusion of Hoechst 33342 dye (for side population), and 3) subsequent transplantation into immunodeficient animals. The cell recovery after such procedures can be quite variable, creating concerns about the representative nature of the recovered cell population [36]. The making of the cell suspension disrupts the cellular microenvironment, which may change the properties of the cells, especially their surface proteins. We have seen in tissue sections from adult and fetal normal brain that only ependymal cells at the surface toward the ventricle show a clear membrane expression of CD133. Neuroepithelial cells in the brain parenchyma do not have a free surface because there is either an adjacent cell, extracellular matrix, or basement membrane of a vessel. The few CD133-positive cells found in the brain parenchyma show a perinuclear cytoplasmic staining (subependymal zone in adult and fetal brain) (Figure 2, A and B). At the ependymal layer, CD133 expression on the cell surface is evident in both fetal and adult tissues (Figure 2, A and B). In a single cell suspension, all cells will have a surface and might therefore be able to express CD133 on their plasma membranes.

Trypsin digestion during preparation of cell suspensions, in addition, might modify the surface expression of proteins such as CD133, thereby affecting how the cells are sorted and the ability of these molecules to play a role in the early stages of tumor growth following xenotransplantation. Trypsin is predicted to cleave the 865–amino acid–long CD133 sequence at 79 different sites (Figure 3A) and many of these cleavage sites are within the glycosylated extracellular loops. This could result in the underestimation of the size of the original CSC pool because those with cytoplasmic CD133

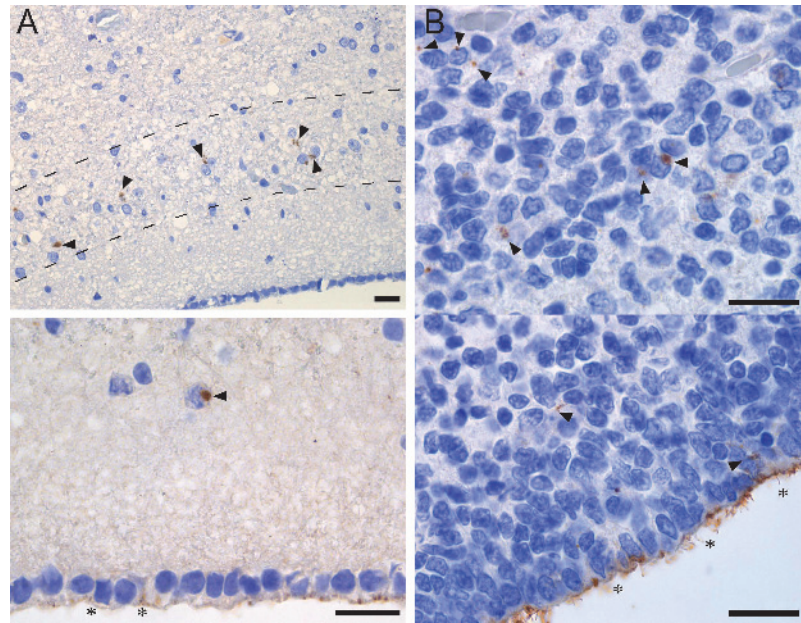


Figure 2. CD133 in normal adult and fetal brain. (A) Adult human brain, subependymal zone (near hippocampus), showing a band of few CD133-positive cells. (B) Fetal human brain, subependymal zone. Note the perinuclear cytoplasmic staining in some cells in the brain parenchyma (▼) and the surface/membrane staining in ependymal cells (*).

location will be refractory to cell sorting. Only those with abundant CD133 might be able to relocate it quickly to the cell surface and be captured by FACS. Simply injecting the cells into a new location is unlikely to recapitulate the niche environment experienced by the tumor cells (e.g., presence of hypoxia, stimulatory growth factors, and extracellular matrix molecules) in the original tumor. Studies from our laboratory using real-time quantitative polymerase chain reaction (qPCR) show that CD133 mRNA is upregulated up to 20 times in some GBM patient samples compared with normal brain (Figure 4A). However, CD133 analysis by antibody-based assays, such as immunohistochemistry (Figure 4, D and E) or flow cytometry in the same tumor material, detect much lower levels. Immunohistochemical staining of brain tumor sections has revealed a cytoplasmic staining pattern rather than membrane staining, questioning the reliability of flow cytometry based on surface protein expression. Colon carcinomas (epithelial tumor) exhibit endoluminal CD133 staining on tumor cells in malignant tubular structures (Figure 4D). Gastrointestinal stromal tumors (mesenchymal tumors) exhibit both cytoplasmic and membrane CD133 location with the same antibody (Figure 4E). This raises the question of whether this discrepancy is due to methodological flaws, such as lack of good antibody specificity or inadequate tissue processing, or whether the difference can be due to the different CD133 expression in epithelial and mesenchymal tissues. Alternatively, the differences may be explained by posttranslational modification. The AC133 antibody (Miltenyi, Bergisch Gladbach, Germany) used in most CSC studies recognizes a glycosylated form of epitope 1 of CD133, suggesting that nonglycosylated forms may not be detected by this antibody. There exist 28 alternative splice variants of CD133 [37] and current commercially available antibodies detect CD133 proteins with

molecular weight differences. They do not take into account the existence of splice variants (Figure 3B). Because the physiological function of CD133 is not yet elucidated, it is conceivable that the different locations of the antigen as well as the different splice variants may influence its function. It is not known whether the cell surface location of CD133 is required for its proposed *stemness* properties.

The influence of the microenvironment in tumor development, demonstrated both by clinical and experimental studies, is generally underplayed by the CSC hypothesis [38,39]. Studies have shown that combining mammary tumor cells with normal fibroblasts increases the latency of tumor initiation compared with mixing with cancer-associated fibroblasts, which has the opposite effect. Combining endothelial cells with medulloblastoma cells increased tumor initiation and growth *in vivo* [39]. This would suggest that cells putatively identified as CSCs by transplantation procedures might either be individually capable of making sufficient autocrine growth factors or can effectively interact with and obtain such stimulation from the new microenvironment into which they are implanted.

Are CD133⁺ Cells Cancer Stem Cells All and the Only Tumor-Initiating Cells?

Given the presence of early progenitors within the CSC population and the difficulties distinguishing cells based on genealogical derivation or relationship, it is conceivable that other immature cells, apart from CD133⁺ cells, are also capable of being tumor-initiating cells in the brain. A highly abundant progenitor cell population exists in the adult CNS that has the ability to divide [40–43] and expand in response to CNS injury [44–46]. These postnatal CNS progenitors are characterized by cell surface neuron–glial 2 (NG2) expression, a 300-kDa membrane spanning chondroitin sulphate proteoglycan whose function in these cells is not completely

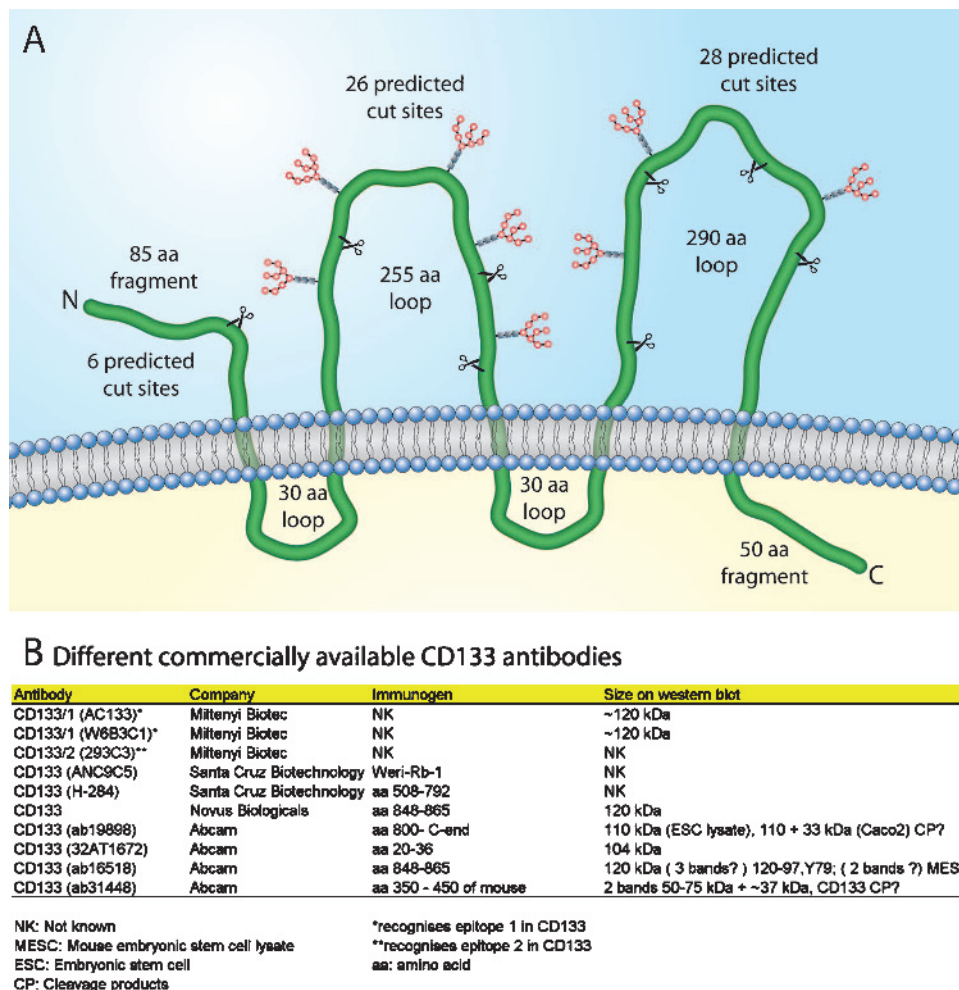


Figure 3. Schematic diagram of CD133 structure and trypsin cleavage sites. (A) The five transmembrane structure of the CD133 molecule including the amino acid lengths of the intra- and extracellular loops and the number of predicted trypsin cleavage sites, <http://www.expasy.ch/tools/peptidecutter/>. Glycosylation sites are illustrated; the actual numbers and positions are as yet unknown. (B) Examples of commercially available CD133 antibodies and suppliers showing the variation in sizes of the detected protein on Western blot analysis.

elucidated. In the developing and adult CNS, NG2 has been used as a marker for glial progenitors committed to the oligodendrocytic lineage [45–48]. Their even distribution in the white and gray matter of the entire CNS, as well as their unique features [49–51], suggests that NG2 is, in addition, expressed outside the oligodendrocyte lineage [52]. NG2⁺ cells are multipotent *in vivo*, differentiating into neurons (excitable GABAergic with functional synaptic inputs) and astrocytes [53,54] and *in vitro* differentiating in all CNS lineages [55] and pericytes [56]. Recently, NG2⁺/C-type natriuretic peptide–positive (CNP⁺) cells in the postnatal hippocampus and subventricular zones have been shown to share properties of NSCs [54,57,58].

The fact that NG2⁺ cells represent a major population of multipotent neural progenitor cells [59,60] makes them plausible candidates for transformation into tumor-initiating cells (Figure 1). Similar to CNS stem cells, NG2⁺ progenitors persist and slowly proliferate throughout adult life [61,62]. During CNS development, the well-characterized anatomical distribution of NG2⁺ progenitors [63] overlaps with areas where totipotent NSCs reside and ongoing neurogenesis occurs [64,65]. These cells represent the largest pool of postnatal

proliferative progenitors scattered throughout neurogenic as well as nonneurogenic areas of the CNS [52,66], but their possible stem cell potential and the necessity of NG2 for the function as a progenitor cell has not yet been explored.

We have previously identified NG2⁺ cells in gliomas *in vitro* and showed that they were more proliferative, but less invasive than their NG2-negative counterparts [67]. NG2 was expressed in various histological subtypes of human brain tumors. Expression was higher in the most malignant tumors, both on the tumor cells and on their associated vasculature. Ki-67 labeling showed that NG2 was more abundant in the main tumor mass, especially in areas of high cellular proliferation. We also observed that the exogenous expression of NG2 increased tumor initiation, growth rates, and neovascularization, predisposing the animals to a poorer survival. Barnett et al. [68] demonstrated that transformation of oligodendrocyte-type 2–astrocyte (O-2A) progenitor cells with overexpression of *c-myc* and *H-ras* oncogenes generated tumors strikingly similar to human glioblastomas when implanted into the adult rat brain. Using astrocyte-conditioned medium, Noble et al. [69] produced a GBM cell line derived from the O-2A lineage (Hu-O-2A/Gb1), which had the same

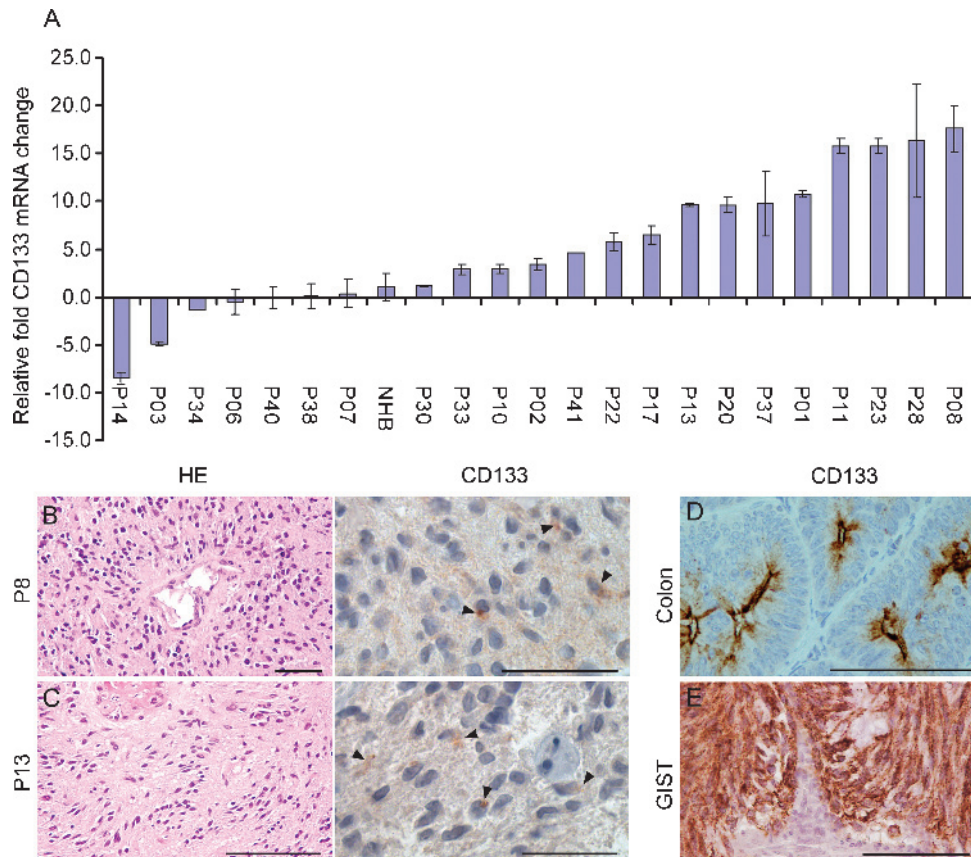


Figure 4. CD133 expression is highly variable in patient GBM biopsies. (A) Real-time qPCR of tissue from normal human brain (NHB; $n = 4$) and patient GBM biopsies ($n = 22$). The expression level of CD133 in the NHB was used as a reference (NHB = 1). Data show mean \pm SEM from two independent experiments. (B and C) The hematoxylin and eosin-stained histological sections and cytoplasmic CD133 staining from two representative patient GBMs with high real-time qPCR values. (D) Positive control using colon adenocarcinoma tissue indicating high endoluminal CD133 positivity in the malignant tubular structures. (E) Positive control using gastrointestinal stromal tumor tissue indicating high surface and cytoplasmic CD133 location. Scale bar, 100 μ m.

proton nuclear magnetic resonance metabolic spectrum as perinatal O-2A progenitor cells and responded to growth factors *in vitro* in a similar way to O-2A progenitor cells. Hu-O-2A/Gb1 expressed all the appropriate O-2A lineage antigens and could be manipulated to differentiate into oligodendrocytic or more astrocytic phenotypes. Like most human GBMs [70–72], Hu-O-2A/Gb1 demonstrated clonal chromosomal aberrations on chromosome 10 [73] and, similar to GBM, was invasive when confronted with the neuropil.

Other authors have characterized the cell types that undergo oncogenic transformation and give rise to brain tumors using the *N*-methylnitrosourea (MNU) or *N*-ethylnitrosourea models for induction of brain tumors in rats [12]. Exposure to MNU yields a series of phenotypes ranging from normal to malignant. These studies demonstrated that the target cells for MNU were the NG2⁺/nestin⁺ progenitor cells that did not express the differentiated cell markers, glial fibrillary acidic protein (GFAP) and galactocerebrosidase (GalC), unless stimulated to differentiate into astrocytes by growth factors and/or db-cyclic AMP [12]. Further validation of NG2-positive cells in glioma biopsies using the sphere formation assay and serial transplantation [74] would establish whether these cells exhibit the properties of CSCs as well. Human brain tumors may contain several distinct cell populations each with the capacity for tumor initiation and progression. This

highlights the dangers of confining the CSC phenotype to a particular antigen expression. Future identification of CSCs should focus on functional assays rather than a strict adherence to the use of specific markers.

What is the Biological Significance of Cancer Stem Cells in Tumor Progression?

If cancer is maintained by CSCs, cells that are characterized by low rates of division and proliferation, it is clear that therapies such as chemotherapy or radiation, which target actively cycling cells, are unlikely effective [74,75]. Conventional treatments may cause the tumor to shrink temporarily, but the cells withstanding these regimens will inevitably lead to a tumor recurrence. Recent studies have shown that CD133⁺ within gliomas mediate resistance to radiation, at least in part due to elevated DNA damage response and more rapid repair of the damaged DNA [76]. The percentage of CD133⁺ cells increased following high dose radiation in established glioma cell lines, short-term cultures of biopsy material, and xenograft tumor-bearing mice, resulting in more aggressive tumors on serial transplantation. It is not clear why radiation should stimulate CD133⁺ cell division. Very high radiation killed some CD133-expressing cells, reflecting perhaps a degree of heterogeneity. Others have reported that CSC-like cells from breast cancer cell lines are

more radioresistant compared with the remainder of breast cancer cells [77]. These nonadherent subpopulations were grown as spheroids in mammosphere media and contained a larger fraction of cells with the CD44⁺/CD24^{-/low} phenotype [28]. When these cultures were irradiated *in vitro*, the mammospheres were radioresistant and there was a concomitant increase in the percentage of CD44⁺/CD24^{-/low} cells, suggesting that the relative radioresistance of this subset may lead to their expansion during a course of radiotherapy. Other studies showed that CD133⁺ cells isolated from GBM short-term cultures overexpressed drug resistance genes, such as breast cancer resistance protein-1 (*BCRP1*), DNA-mismatch repair genes such as *O*-6-methylguanine-DNA methyltransferase (*MGMT*), as well as showing higher levels of antiapoptotic gene expression [78]. These CD133⁺ cells were also significantly more resistant to chemotherapeutic agents including temozolomide, carboplatin, paclitaxel (Taxol), and etoposide (VP16) compared with CD133-negative cells. In addition, it has been shown that CD133⁺ CSCs isolated from GBMs were resistant to ionizing radiation because they were more efficient at repairing damaged DNA than the bulk of the tumor cells [76]. Caveats to this study include the fact that radiation was done on single cells separated from each other and their stromal components. It is not known how similar the response of these cells would be if they were in their natural setting *in vivo*. The radiation response of cells under normoxic conditions in culture used in this paper may not reflect the response of cells in the hypoxic environment of a tumor [79]. Similar studies in breast cancer cell lines *in vitro* showed that CD44⁺/CD24⁻ were more radioresistant and that this correlated to differences in DNA damage response [77]. Recently, Blazek et al. [80] demonstrated that CD133⁺ cells sorted from medulloblastoma cell lines were more radioresistant and this effect could be enriched by hypoxia. This is contrary to the clinical findings that medulloblastomas are much more radiosensitive than GBMs, with cure rates of 70% obtained in children old enough to tolerate it. That CD133⁺ has been proposed to be the cell of origin for both tumors illustrates the problems faced by the CSC hypothesis that focuses too much on the tumor cell and ignores the contribution of the microenvironment. The differences in treatment response may be explained by age; that is, a microenvironment from younger patients (medulloblastoma) *versus* microenvironment from older patients (GBMs). Nevertheless, these shortfalls, the studies add, support the observation that CSCs are important mediators of treatment resistance. The questions that remain to be answered are whether radiation resistance is a general property of CSCs in all tumors, and if their presence in human tumors is predictive of radiosensitivity.

Can Cancer Stem Cells Be Targeted Therapeutically?

Although the chemo- and radioresistance of CSCs presents a therapeutic challenge, their similarity with normal stem cells may, at the same time, provide a therapeutic target. It is intriguing to ask whether it might be possible to develop radio/chemosensitizers that will preferentially sensitize CSCs [35]. Because the differentiation of normal stem cells is

accompanied by a reduced cell proliferation, it is conceivable that differentiating agents could suppress cancer cell division in a similar way. Recently, it has been shown that treatment of CD133⁺ CSCs derived from human GBMs with bone morphogenic proteins (BMPs) reduced cell proliferation *in vitro* and induced differentiation into astrocytes. The BMP treatment reduced the size and invasive capacity of the tumors engrafted into mice and prolonged their overall survival. Because some mice still developed tumors and died 3 months after BMP treatment, it seems that some CSCs escape the differentiating effects of BMP treatment. Other strategies employ drugs that target posttranslational modifiers such as histone deacetylases (HDACs) that catalyze the removal of acetyl groups on the amino-terminal lysine residues of core nucleosomal histones. The HDAC inhibitor, suberoylanilide hydroxamic acid, inhibits proliferation and induces cell cycle arrest at G1 and G2-M check points in breast cancer cell lines [81] with subsequent differentiation. Suberoylanilide hydroxamic acid and other HDAC inhibitors are currently in phase 1 clinical trials.

Signaling pathways regulating self-renewal of normal stem cells (e.g., polycomb gene *Bmi-1*, Notch, Wnt, and Hedgehog (Hh)) may be possible targets. For example, treatment of mice with the Hh pathway inhibitor cyclopamine inhibited the growth of medulloblastoma [82]. Similarly, inhibition of Notch with specific gamma secretase inhibitors attenuated CSC self-renewal and tumor growth [83]. Although toxicity is an obvious concern, there are several differences between cancer and normal stem cells that may provide a therapeutic window. Normal stem cells have regulated cell cycle checkpoints that are likely to protect them from cellular damage. Studies showed that both CD133⁺ and CD133-negative cells could be rendered less resistant by treatment with the Chk1 and Chk2 kinase inhibitor, debromohymenialdisine. The authors did not verify further whether these tumors lost the ability subsequently to initiate tumors *in vivo*. Radiation toxicity that could ensue from lack of tumor specificity is a major concern. There has already been a move from whole brain radiation to more focussed beam radiation delivered with the gamma knife to minimize radiation of the normal brain. It is unlikely that debromohymenialdisine could be useful as a sensitizing agent in the treatment of patients due to its lack of specificity for CD133⁺ cells. The presence of CSCs in solid tumors based on the current markers to date have not been shown to have a prognostic significance in patients.

Can Normal Stem Cells Be Used to Target Brain Cancer Stem Cells?

Several studies have reported that normal NSCs exhibit tropism for tumor cells in the CNS [84,85]. Implanted NSCs have been shown to surround the expanding tumor mass, even seeking out and attaching to the distantly infiltrating tumor cells. It has been shown that this homing property of NSCs can be exploited in experimental brain tumors to deliver various therapeutic substances [86–88]. Transplantation of normal stem cells might prove useful not only for the site-specific delivery of cytotoxic agents or virally mediated genetic elements but also for the widespread release of

molecules that regulate proliferation, differentiation, and migration of brain CSCs [89]. Applying this approach to the treatment of patients raises some major concerns. First, because these NSCs are immortalized with oncogenes, there is an inherent risk that they will undergo spontaneous transformation. Even though initial implantations in animals have not shown that they are tumorigenic, this does not rule out the possibility of subsequent genetic hits induced by an unstable/hostile tumor microenvironment that may result in their tumorigenicity. Second, the possibility of an evoked immune reaction against the graft may at best destroy the exogenous cells, or worse, induce a deleterious immune response in the patient. Even if the initial transplantation does not provoke an immune response, there will be an accumulated risk with subsequent treatments. Thus, agents delivered by stem cells may be best suited as a single-dose treatment. Engineering of autologous NSCs is not an option because these may be themselves indolent potential CSCs harboring mutations capable of activating self-renewal programs at a later point. Finally, combination therapy with small molecule inhibitors of signaling machineries disrupted in cancer might be negated because the grafted NSCs might be forced to differentiate or be adversely affected by the treatment.

Conclusions

Malignant gliomas are among the deadliest of cancers. Insight into the biology of brain tumors, so far, has not been translated into improved treatment and outcome for the patients. The CSC hypothesis and emerging data on the functional properties of these cells have obvious therapeutic implications and raise the question of whether we are targeting the right cells. Some data also suggest alternative strategies for attacking CSCs in solid tumors, whereas animal studies suggest that CSCs can be induced to stop proliferating in a way that resembles differentiation of normal stem cells. Anticancer approaches aimed at the CSCs may not be suitable for all clinical settings. In GBM tumors, treatment resistance is common to most cells within the cancer cell pool so that even proliferating cells survive the conventional treatments. In these cases, efficient control of the proliferating cells should be the primary objective, with targeting CSCs being a secondary aim. Although many uncertainties remain regarding the nature of CSCs in the CNS, accumulating data have demonstrated that there is a functional heterogeneity among the cancerous cells constituting a brain tumor. The focus of future research may therefore be to develop treatments that are modified according to this heterogeneity.

Materials and Methods

Patient Biopsy Tissue

The biopsy material was obtained from the Department of Neurosurgery, Haukeland University Hospital, Bergen, Norway. All the biopsies used in these data were confirmed GBM under routine diagnoses by neuropathologists at the Haukeland University Hospital. Normal human adult and fetal brain

(third trimester) tissues were obtained from diagnostic post-mortem material used as controls for immunohistochemistry. The regional ethical committee (REK Vest) (which is affiliated to Haukeland University Hospital and The University Bergen) as well as The Data Inspectorate Norway approved the collection of tumor tissue. All patients gave their informed consent to the collection of biopsy tissue for research purposes.

Isolation of Total RNA and Real-Time qPCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Briefly, snap-frozen tissue from patient biopsies was crushed in liquid nitrogen, dissolved in lysis buffer, and homogenized using an 18-G syringe. The remaining procedure was performed according to the manufacturers instructions. Real-time qPCR was subsequently performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The following parameters were used for the qPCR reaction: 45 cycles of 30 seconds at 95°C, 20 seconds at 60°C, and 20 seconds at 72°C using iCycler Thermal Cycler fitted with iCycler Optical Module (Bio-Rad Laboratories). Amplicon purity and size were verified by melt curve analysis and gel electrophoresis. Primers directed against 18S were used as an internal control and have been described elsewhere [90]. Primers directed against CD133 were designed using Oligo 6.67 Primer Analysis Software (Molecular Biology Insights, Inc., Cascade, CO) spanned an intron and yielded only one product as verified by gel electrophoresis. Primer sequences were: forward 5-ACCAGGTAAGAACCCGGATCAA-3, reverse 5-CAAGAATTCCGCCTCCTAGCACT-3.

Immunohistochemistry

Paraffin sections were rehydrated for 2 × 5 minutes in xylene, 2 × 3 minutes in 100% ethanol, 2 × 3 minutes in 96% ethanol, and finally in ddH₂O for 5 minutes. Heat-induced epitope retrieval was performed at 95°C for 45 minutes. The sections were incubated with the CD133/1 clone AC133 antibody (Miltenyi) diluted 1:25 in buffer (25 mM Tris-HCl, 75 mM NaCl, and 1% (v/v) BSA, pH 7.4) for 1 hour at room temperature, washed 3 × 5 minutes with 0.05% (v/v) TBS-Tween, and blocked in 3% (v/v) H₂O₂ for 10 minutes. CD133 was then detected using the MACH 3 system (Biocare Medical, Concord, CA) according to the manufacturer's instructions. After four washes, the sections were developed with DAB+ (Dako, Glostrup, Denmark) for 5 minutes, dehydrated, counterstained with hematoxylin and finally mounted in a mounting medium (Entellan; Merck KGaA, Darmstadt, Germany). Hematoxylin and eosin staining was performed using standard procedures.

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