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Isolation of cancer stem cells from adult glioblastoma multiforme

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Abstract

Glioblastoma multiforme (GBM) is the most common adult primary brain tumor and is comprised of a heterogeneous population of cells. It is unclear which cells within the tumor mass are responsible for tumor initiation and maintenance. In this study, we report that brain tumor stem cells can be identified from adult GBMs. These tumor stem cells form neurospheres, possess the capacity for self-renewal, express genes associated with neural stem cells (NSCs), generate daughter cells of different phenotypes from one mother cell, and differentiate into the phenotypically diverse populations of cells similar to those present in the initial GBM. Having a distinguishing feature from normal NSCs, these tumor stem cells can reform spheres even after the induction of differentiation. Furthermore, only these tumor stem cells were able to form tumors and generate both neurons and glial cells after in vivo implantation into nude mice. The identification of tumor stem cells within adult GBM may represent a major step forward in understanding the origin and maintenance of GBM and lead to the identification and testing of new therapeutic targets.

Keywords: cancer stem cells, adult brain tumors, glioblastoma multiforme, glioblastoma spheres, in vivo implantation

Introduction

Glioblastoma multiforme (GBM) is typically comprised of morphologically diverse cells within the tumor mass. Despite current advances in therapy, the morbidity and mortality of GBM remain very high (Surawicz et al., 1998). This is due, at least in part, to the focus of most treatments on the bulk of the tumor. However, the diverse cells within GBM may play different roles in tumorogenesis. There is increasing evidence that cancers might contain and arise from stem cells. Studies on leukemia and breast carcinoma have demonstrated that a small group of cells within the tumor mass is responsible for tumor formation and maintenance. This subpopulation of stem-like cells plays an important role in the tumorogenic process (Lapidot et al., 1994; Larochelle et al., 1996; Bonnet and Dick, 1997; Al-Hajj et al., 2003; Dick, 2003). GBMs contain cells that express neural markers as well as cells that express glial markers, indicating that there may be multipotent neural stem cell-like cells (Ignatova et al., 2002; Katsetos et al., 2002). Such mixed glioblastomas may develop from neural stem cells (NSCs) or from differentiated cell types that acquired multipotential stem cell-like

properties by either reprogramming or de-differentiating in response to oncogenic mutation (Kondo and Raff, 2000; Bachoo et al., 2002; Zhu and Parada, 2002). This plasticity of central nervous system (CNS) cells has already been reported as normal oligodendrocyte precursor cells can be induced to acquire stem cell-like properties (Kondo and Raff, 2000; Belachew et al., 2003; Nunes et al., 2003). Very recent studies show that pediatric brain tumors and a rat glioma cell line contain cancer stem cells that may be important for the malignancy (Hemmati et al., 2003; Singh et al., 2003; Kondo et al., 2004). Here, we demonstrate that human adult GBMs contain a subpopulation of cells that can form neurospheres in defined stem cell medium with growth factors. The spheres share many characteristics of stem cells, including self-renewal ability and multipotent differentiation, which can produce daughter cells of all phenotypes present in the GBM. Furthermore, we show that these spheres are different from normal neurospheres, being able to reform new spheres after the induction of differentiation. More importantly, after in vivo implantation only the isolated tumor stem cells were able to form tumors that contained both neurons and glial cells. Our findings suggest that a subpopulation of cells exist within adult GBM, which may represent a general source of cancer stem cells in adult brain tumor and need to be targeted for more effective and specific cancer therapy.

Results

Primary cultures of GBM contain 'neurospheres' forming cells that can self-renew

Phenotypically diverse cell populations exist in a tumor mass of GBM. To determine whether there is a population of cells with distinct proliferative ability characteristic of stem cells distinguishable from other cells, we studied four GBM primary cultures, GBM1–4, grown from frozen stocks of the first passage of tumor cells, and two freshly resected GBM cultures, GBM5 and 6, directly cultured without freezing. The six GBM primary cultures were grown as monolayers attached at the bottom of flasks in a medium containing FBS. We switched the GBM cells into culture conditions known to be permissive for stem cell proliferation, established previously for isolation of NSC as neurospheres (Reynolds and Weiss, 1992; Kabos et al., 2002). Neurosphere-like colonies appeared in all six GBM cultures after 1 week and reached 100–200 each after 2 weeks. We have denoted these colonies as glioblastoma spheres (Figure 1a). These glioblastoma spheres were harvested and subjected to subsphere-forming assay by limiting dilution (Kabos et al., 2002). Subspheres formed in the wells seeded with individual sphere cells with efficiency between 3 and 5% for the six GBMs (Figure 1b and c). However, sphere formation was not evident in any wells seeded with monolayer cells. When compared with normal human NSCs that were also grown from frozen stocks with the same passage number, there were <1% cells with subsphere-forming ability (Figure 1c). This observation indicates that the glioblastoma spheres have a self-renewing ability. To confirm this ability, the subspheres were harvested and a second round subsphere-forming assay was performed as above. Again, we found that individual cells formed further spheres with an efficiency similar to that of the previous assay (Figure 1c).

One mother-cell-derived glioblastoma spheres express NSC markers as well as lineage markers

The self-renewal capability of glioblastoma spheres indicates that sphere cells isolated from GBM primary cultures share this property with the NSC. We next tested whether the glioblastoma spheres

could express genes characteristic of NSC. Spheres derived from a single mother cell and isolated by subsphere-forming assay were dissociated and expanded. Two NSC markers were chosen to characterize the expanded spheres: CD133, a cell surface marker of normal human NSCs (Miraglia et al., 1997; Uchida et al., 2000), and nestin, a cytoskeletal protein associated with NSCs and progenitor cells in the developing CNS (Lendahl et al., 1990). For all six GBMs studied, daughter spheres derived from one mother cell were positive for both CD133 and nestin (Figure 2a). However, <0.5% cells of the non-sphere-forming tumor cells in the monolayer culture stained positive for CD133 and nestin. Some cells within tumor spheres were also found to be positive for lineage markers β-tubulinIII and myelin/oligodendrocyte, but no GFAP staining was detected within spheres (Figure 2a), although a few cells were positive for GFAP in dissociated sphere cells. The cells in non-sphere-forming monolayer cultures predominantly expressed the three lineage markers of CNS, β-tubulinIII (30–50%), GFAP (10–25%), and myelin/oligodendrocyte (20–40%). The pattern was clearly different from that of the glioblastoma sphere staining, it was 5–15% for β-tubulinIII, 1–5% for GFAP, and 10–20% for myelin/oligodendrocyte. An example is shown in Figure 2b and c. The observed phenotypic variation of stained cells within spheres from one mother cell indicates that the mother cell has the potential to produce the terminal cell types specific to the tumor tissue.

One mother-cell-derived glioblastoma sphere is multipotent

To test whether glioblastoma spheres derived from one mother cell have multipotent ability and produce progenies of different lineages, the spheres were subjected to a differentiation assay. Single-cell suspensions of glioblastoma spheres were differentiated and stained for various lineage markers. The results demonstrate that cells differentiated from tumor sphere were positive for β-tubulinIII, GFAP, and myelin/oligodendrocyte. Furthermore, a few cells were nestin or CD133 positive (Figure 3a). For all GBM samples, the staining pattern of the differentiated progeny was similar in profile to that of primary cultured tumor cells from which the glioblastoma spheres had originally been isolated (Figure 3b and c). However, the phenotype pattern was different from that of normal human NSC, which was also grown from frozen stock, and cultured for the same period of time as that of glioblastoma spheres. As shown in Figure 3d, the differentiated progeny of normal NSCs was characterized with 50–60% GFAP-positive cells, 20–30% β-tubulinIII-positive cells, myelin/oligodendrocyte-stained cells accounting for 10%, and less than 5% cells positive for CD133 or nestin, respectively. In contrast, glioblastoma spheres predominantly differentiated into β-tubulinIII (80%) and myelin/oligodendrocyte (25%)-positive cells that recapitulated the parental tumor phenotype (Figure 3b and c). Furthermore, we found that there were approximately 5% β-tubulinIII and GFAP double-positive-stained cells only in glioblastoma sphere-differentiated progenies. These results reveal that glioblastoma spheres are multipotent for the three neural cell types, but different from normal human neurospheres regarding the phenotype of their progenies.

The mRNA transcriptional levels are different between glioblastoma spheres and their differentiated progeny

To better define the multipotent ability, a single glioblastoma sphere derived from one mother cell was scaled up for semiquantitative RT–PCR to assess the mRNA levels of lineage marker and NSC genes. Half of the spheres were directly subjected to RNA extraction. The rest was differentiated first for 2 weeks prior to RNA extraction. As shown in Figure 4a as a representative, nestin mRNA was only detected in the glioblastoma sphere and not in the differentiated progeny. Conversely, the PCR

product of astrocyte marker GFAP was amplified in the differentiated progeny but not in the sphere. Transcripts of neuronal and oligodendrocyte-specific genes were elevated in the differentiated progenies. These results are consistent with the above findings that glioblastoma sphere derived from one mother cell was able to generate multiple-lineage daughter cells.

Glioblastoma spheres are different from normal neurospheres in their ability to regain or maintain NSC features after differentiation

The above results demonstrate that glioblastoma spheres possess a phenotype different from that of normal human neurospheres upon differentiation (Figure 3c and d). To further determine that glioblastoma spheres are cancer stem cells and not derived from contaminating normal NSC, we conducted a differentiation experiment for both glioblastoma spheres and normal human neurospheres by dissociating individual spheres into a single-cell suspension and subjecting the cells to differentiation conditions. After 2 weeks of differentiation, all cells were attached and growing as a monolayer. Cells were then switched into NSC growth medium. New spheres started to form in the GBM-differentiated monolayers after 1 week. No spheres were observed in the normal human neurosphere-differentiated monolayer even after 4 weeks. The newly formed glioblastoma spheres stained positive for nestin and CD133 (Figure 4b). We also karyotyped the glioblastoma sphere cells and identified chromosome loss, gain, and translocation events. Taken together, both cellular analysis and genetic data support our hypothesis that glioblastoma spheres isolated from primary GBM cultures are not normal NSCs migrated into the tumor mass. Rather, these glioblastoma spheres possess abnormal characteristics including enhanced self-renewal and altered differentiation abilites, which may be responsible for maintaining the tumor stem cell pool and also for generating the differentiated progeny.

Glioblastoma spheres represent the tumor stem cells and are capable of forming tumors in vivo

To address whether glioblastoma spheres and non-sphere-forming monolayer cells differ in their abilities to form tumor in vivo, we implanted the isolated glioblastoma spheres or non-sphere-forming monolayer cells into the brain of nude mice. There was no tumor detected 6 weeks after 50 000 cells per mouse implantation of non-sphere-forming monolayer cells (n=6; Figure 5c). However, all the mice developed brain tumors after the same number of glioblastoma sphere cell implantation (n=6) (Figure 5a and c). We next increased the non-sphere-forming monolayer cells to 250 000 and decreased the glioblastoma sphere cells to 5000 per mouse for the intracranial implantation. After 6 weeks, there was still no tumor detected in the non-sphere-forming monolayer cell-implanted mice (n=6), but brain tumors were detected in all mice (n=6) implanted with glioblastoma sphere cells, although the tumor size was smaller than that of the above (data not shown).

The tumors, which developed in mice brains after implantation of human glioblastoma spheres, could be stained by human-specific antibodies against nestin (Figure 5d and e) and CD133 (Figure 5g–i), the NSC-related markers. Also, the tumor cells were stained positive for anti-GFAP, which was not stained in the glioblastoma spheres in vitro (Figure 6a–c), anti-myelin/oligodendrocyte (Figure 6d–f), and anti-β-tubulinIII (Figure 6g–i), suggesting that the implanted glioblastoma spheres can generate both glial cells and neurons in vivo. Thus, the malignance of the human adult GBM apparently depends on the insolated glioblastoma spheres, the cancer stem cells.

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Discussion

The application of principles used for studying the NSCs to tumor biology indicates a link between normal neurogenesis and brain tumorigenesis (Holland et al., 1998; 2000; Holland, 2001; Kabos et al., 2002; Ehtesham et al., 2004). NSCs have the ability to generate three cell lineages: neuron, astrocyte, and oligodendrocyte. Human adult GBM also contains all the three cell types within the tumor mass. Until now, there has been no convincing evidence to show which cell type in GBM is the root for tumor growth. Is there a stem cell population present within the tumor that is responsible for the tumor growth and maintenance in GBM? Our data at least partially answered this question. Currently, there is increasing evidence that some malignant tumors, both soluble and solid, contain cancer stem cells that are responsible for the initiation and growth of malignancy (Lapidot et al., 1994; Larochelle et al., 1996; Bonnet and Dick, 1997; Al-Hajj et al., 2003; Dick, 2003). Cancer stem cells are potentially important because it is likely that the stem cell population within tumor mass plays a key role for the recurrences that occur after current treatments. Thus, it is critical for cancer therapy that treatments must target and eliminate this special population of cancer cells. Consequently, the need to identify and study cancer stem cells becomes significant.

Our data indicate that human adult GBMs contain a subpopulation of cells that can self-renew and differentiate into mature cell types, recapitulating the diverse complexity of primary GBMs. Several lines of evidence support that we have isolated cancer stem cells from adult human GBMs: (1) the isolated cells only account for a small fraction in tumor mass, form spheres that are morphologically indistinguishable from normal neurospheres, and express known NSC markers; (2) the cells can self-renew and proliferate to generate subspheres and different progenies; (3) the isolated single mother cell can differentiate into multi-lineage progenies; (4) spheres derived from a single mother cell possess a different phenotype compared with normal NSCs upon differentiation, can reform spheres after the induction of differentiation, and display genetic aberrations commonly found in brain tumor cells; (5) the isolated neurosphere-forming cells can produce brain tumors in nude mice, whereas the non-sphere-forming monolayer cells do not.

In addition to providing further evidence for the existence of cancer stem cell in solid tumor, as the presence of stem-like precursors in adult human glioblastomas has been independently reported after our work was completed (Galli et al., 2004), our finding that cancer stem cells can reform new spheres after the induction of differentiation has an important implication. It may, for example, provide a basic strategy for identifying cancer stem cells from normal NSC. Although flow cytometric isolation of subpopulations of cells from tumors has been used (Al-Hajj et al., 2003; Singh et al., 2003; Kondo et al., 2004), there is no appropriate marker for isolating cancer stem cells, especially for distinguishing cancer stem cells from NSCs. It is a fundamental step to identify and isolate cancer stem cells, so that elucidating the pathways that account for tumorigenic potential and developing effective and specific anticancer strategy may be realized. We found that glioblastoma spheres derived from a single mother cell can differentiate into the three CNS cell lineages and recapitulate the phenotypically complex property of the parental tumor. These data suggest that the presence of varied cell types within the tumor is not simply a consequence of different types of cells growing together to form tumor mass, but rather is an intrinsic property of cancer stem cells. The isolated brain tumor stem cells, not only giving rise to further stem cells but also to a diverse population of other phenotypes, may serve as a useful object for understanding the fundamental similarities and differences between normal neurogenesis and tumorigenesis in CNS.

An interesting implication arises from the ability of one glioblastoma cell to generate neurons as well as astrocytes and oligodendrocytes. These cells were isolated from adults, suggesting that there are two possibilities. On the one hand, these might be converted from normal NSC by oncogenic stimulation. They were stem cells before acquiring the tumorigenic property. On the other hand, these cells might be somehow either reprogrammed, or de-differentiated from a more differentiated cell type in response to some signal transduction cascades (Kondo and Raff, 2000; Bachoo et al., 2002; Zhu and Parada, 2002). Studying the molecular basis involved in the oncogenic conversion of stem cells or the conversion from more differentiated cells into a stem cell-like phenotype might shed light on the biology of both NSCs and cancer cells.

In conclusion, the present study highlights the importance of cancer stem cells in brain tumor research and suggests a basic strategy to identify brain cancer stem cells from normal NSCs. The study also indicates that the cancer stem cell of adult brain tumor may serve as a tool for studying the basic biology of adult stem cells.

Materials and methods

Culture of primary glioblastoma cells and spheres

Tumor specimens were obtained within half an hour of surgical resection from six adult GBM patients, as approved by the Institutional Review Boards at the Cedars-Sinai Medical Center. Tumor tissue was washed, minced, and enzymatically dissociated (Reynolds and Weiss, 1992). Tumor cells were resuspended in DMEM/F12 medium containing 10% fetal bovine serum (FBS) as growth medium and plated at a density of 2 106 live cells per 75 cm2 flask. The cells attached and grew as a monolayer in flasks. The frozen stock tumor cells, which were also subjected to the same procedure as above and cryopreserved from monolayer cultures, were recovered into the growth medium. All the six monolayer-growing adult glioblastoma cells were switched into a defined serum-free NSC medium (Reynolds and Weiss, 1992) containing 20 ng/ml of basic fibroblast growth factor (bFGF, Peprotech, Rocky Hill, NJ, USA), 20 ng/ml of epidermal growth factor (EGF, Peprotech) and 20 ng/ml leukemia inhibitory factor (LIF, Chemicon).

Subsphere-forming assay

After primary spheres formed and reached 100–200 cells each in the monolayer culture, the sphere cells were harvested, dissociated into single cells and plated into a 96-well plate for the subsphere-forming assay by limiting dilution as described previously (Kabos et al., 2002). In brief, the cells in single-cell suspension were diluted and plated at 1–2 cells/well. In parallel, the cell monolayer remaining in the flasks after harvesting of the spheres was also plated. Cells were fed by changing half of the medium every 2 days. After plating, the cells were observed and only wells containing a single cell were considered. The wells were scored for sphere formation after 14 days.

Differentiation assay of spheres derived from one mother cell

Glioblastoma sphere derived from one mother cell in the subsphere-forming assay was trypsinized into single-cell suspension and seeded into chamber slides (Lab-TekII, Nalge Nunc International) for differentiation assay. The cells were grown in medium devoid of growth factors but permissive for

differentiation for 14 days, and processed for immunocytochemistry (Kabos et al., 2002). For the new sphere-reforming assay, the cells were switched back into NSC culture medium containing growth factors after differentiation process.

Immunocytochemistry staining

To examine the expression of NSC markers and lineage markers, immunostaining was performed as described previously (Kabos et al., 2002). For staining the primary cultured tumor cells and differentiated glioblastoma sphere or normal neurosphere, the cells growing in precoated chamber slides were fixed with 2% paraformaldehyde for 15 min at room temperature, treated with 10% donkey serum, and then stained with the following antibodies: anti-CD133/1 (mouse monoclonal IgG1; 1 : 10; Milteny Biotec), anti-nestin (mouse monoclonal IgG1; 1 : 200; Chemicon), anti-β-tubulinIII (mouse monoclonal IgG1; 1 : 200; Chemicon), anti-GFAP (rabbit polyclonal; 1 : 1000; Chemicon), and anti-myelin/oligodendrocyte (mouse monoclonal; 1 : 1000; Chemicon). The primary antibodies were detected with Tex-Red or FITC-conjugated donkey anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG (1 : 200; Jackson ImmunoRescearch). The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) to identify all nuclei. For immunostaining of glioblastoma spheres, the spheres or dissociated sphere cells were freely floated in 'U'-bottom wells of a 96-well plate during the staining process. Then, the cells were resuspended into the mounting medium containing DAPI and mounted on microslides. Quantification of cells positive for a specific marker was carried out by counting all the stained cells within 20 randomly selected microscope fields per specimen, and the percentage was calculated based on the total number of nuclei counted.

RNA extraction and RT–PCR assay

Glioblastoma spheres derived from a single mother cell were expanded and differentiated as outlined previously. Spheres and differentiated progeny were respectively subjected to total RNA extraction with RNeasy kit (GIAGEN) and reverse transcripted by using a Bioscript kit (Bioline) and Olig(dT)12–18 primer (Invitrogen). The PCR was carried out in a 20 l reaction mixture that contained 1 l cDNA as template, specific oligonucleotide primer pairs (Table 1), and Accuzyme (Bioline). Each specific gene was concurrently amplified with internal control -actin in the same reaction tube. The final primer concentrations were 400 and 200 nm for specific gene and -actin, respectively. Cycle parameters for nestin, β-tubulinIII, GFAP, myelin-associated oligodendrocyte basic protein (MOBP), and -actin amplification were 30 s at 94°C, 30 s at 61°C, and 60 s at 72°C for 25 cycles. The amplified products were identified by agarose gel electrophoresis and ethidium bromide staining.

Table 1 Oligonucleotides for PCR

mRNA targets	Oligonucleotides (5′→3′)	Product size (bp)
Nestin	Forward: gagggcaaagtggtaagcagc Reverse: tagagacctccgtcgctgttga	774
β-TubulinIII	Forward: aacgaggcgctctacgacatc Reverse: ctcctcctcgtcgtcttcgta	720
MOBP	Forward: cacagtcggatgcccaaagat Reverse: tgtttcaccaccaccaccacc	512
GFAP	Forward: tggtgaagaccgtggagatgc Reverse: tcctccccttctctccttcct	380
β-Actin	Forward: gcaccacaccttctacaatgagc Reverse: ttgaaggtagtttcgtggatgcc	582

Spectral karyotype analysis of glioblastoma sphere cells

The spheres were cultured and dissociated as described above. The cultured cells were harvested within 5 days with Colemid (Invitrogen) for 2–3 h, KCL treated, and fixed with methanol : acetic acid (3 : 1). The spectral karyotype analysis was performed on fixed metaphase cells according to the manufacturer's instruction (ACI, Carlsbad, CA, USA). Spectral images were acquired and analysed.

Implantation into nude mice

Athymic nude mice (nu/nu; 6–8 weeks old; Charles River Laboratories, Wilmington, MA, USA) were anesthetized with i.p. ketamine and xylazine, and stereotactically implanted with either isolated glioblastoma sphere cells (5000; 50 000 per mouse) or non-sphere-forming monolayer cells (50 000; 250 000 per mouse) in 2.5 l of 1.2% methylcellulose/PBS in the right striatum. The experiment was repeated once with identical situations. The implanted mice were killed after 6 weeks by intracordic perfusion–fixation with 4% paraformaldehyde and examined for tumors on the brain sections as described below. All of the animals used were experimented in strict accordance with the Institutional Animal Care and Use Committee guidelines in force at the Cedars-Sinai Medical Center.

Hematoxylin–eosin and immunohistochemistry staining of brain sections

The tumor-cell-implanted mice brains were postfixed after the perfusion–fixation with 4% paraformaldehyde, and cut with a vibratome into 40 m coral sections. For hematoxylin–eosin staining, brain sections were mounted on slide and stained with Harris hematoxylin for 2 min first, and then counterstained with alcoholic eosin. To characterize the brain tissue by immunohistochemistry, free-floating sections were treated with 10% donkey serum (sigma) for 30 min at room temperature and then stained with primary antibodies for human-specific anti-nestin (mouse monoclonal IgG1; 1 : 200; Chemicon), anti-CD133/1 (mouse monoclonal IgG1; 1 : 10; Milteny Biotec), and both human and mice reactive anti-β-tubulinIII (mouse monoclonal IgG1; 1 : 200; Chemicon), anti-GFAP (rabbit polyclonal; 1 : 1000; Chemicon), and anti-myelin/oligodendrocyte (mouse monoclonal; 1 : 1000; Chemicon) antibodies. The primary antibodies were detected as described above.

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Figure Legends

Figure 1: Primary cultures of adult GBM form glioblastoma spheres that can self-renew. (a) Primary neurosphere-like colony, glioblastoma sphere, developing from monolayer tumor cells. Scale bar=50 m. (b) Subsphere derived from a single cell of primary sphere. Scale bar=50 μm. (c) Two rounds of subsphere-forming assay for glioblastoma spheres and normal human neurospheres. The data shown represent means and standard deviations of triplicate experiments

Figure 2: Single-mother-cell-derived glioblastoma spheres express NSC markers as well as lineage markers, but with different staining profile from non-sphere tumor cells. (a) Glioblastoma spheres derived from a single mother cell were stained by NSC markers CD133 (red) and Nestin (green). Lineage markers for neuron, β-tubulinIII (red) and oligodendrocyte, myelin/oligodendrocyte (green) were stained, but staining for the astrocyte marker, GFAP, was not observed in the glioblastoma sphere. Cells were located by counterstaining with DAPI (blue). Scale bar=50 μm. (b) Dissociated glioblastoma sphere cells show immunostaining for various markers. (c) Non-sphere monolayer cells show different staining profile from sphere cells. The data shown in (b) and (c) represent means and standard deviations of triplicate experiments

Figure 3: Glioblastoma sphere-differentiated progenies show a phenotype similar to the parental tumor, but different from that of normal human neurospheres. (a) Differentiated progenies of glioblastoma spheres were stained for the NSC markers, CD133 (red) and Nestin (green). The cells were also stained for lineage-specific markers of neuron (red), astrocyte (green) and oligodendrocyte (green). Scale bar=50 μm. (b) The phenotype of primary cultured parental tumor cells. (c) The phenotype of differentiated progenies from glioblastoma spheres. (d) The phenotype of differentiated progenies from human normal neurospheres. The data shown in (b–d) represent means and standard deviations of triplicate experiments

Figure 4: Semiquantitative RT–PCR and new spheres reformed from differentiated cells. (a) Semiquantitative RT–PCR showing mRNA transcription levels within undifferentiated glioblastoma

sphere and differentiated monolayer cells. Both were derived from the same single mother cell. Lane 1: 1 kb ladder (L); lanes 2 and 6: NSC marker gene, nestin (N); lanes 3 and 7: neuron marker gene, β-tubulin (T); lanes 4 and 8: oligodendrocyte marker gene, MOBP, myelin-associated oligodendrocyte basic protein (M); lanes 5 and 9: astrocyte marker gene, GFAP, glial fibrillary acidic protein (G). A representative of three experiments is shown. (b) New spheres developed again after the previous glioblastoma spheres differentiation process. The spheres were stained for CD133 (upper panel, red) and Nestin (lower panel, green). Cells were located by counterstaining with DAPI (blue). Scale bar=50 μm

Figure 5: Evidence of in vivo tumor-forming ability of glioblastoma spheres. The isolated glioblastoma spheres were able to form brain tumors in nude mice after intracranial implantations (a, b), but the non-sphere-forming tumor cells did not, even when implanted cells number reached 50 times of the glioblastoma sphere cells (c) by hematoxylin & eosin staining. The tumors developed in the mice brain could be stained by human-specific antibodies against NSC markers, nestin (d, e) and CD133 (g–i). The sections were labeled also with DAPI (blue) to identify nuclei (f, h). The merged images were also shown (i). Scale bar=1000 μm (a, d); Scale bar=250 μm (b, c, e–i)

Figure 6: Glioblastoma spheres can generate both neurons and glial cells after intracranial implantations. The tumor mass developed by intracranial implantation of glioblastoma spheres contained positive stained cells for glial cell marker, GFAP (a–c), oligodendrocyte (d-f), and neuron marker, β-tubulinIII (g–i). The sections were labeled also with DAPI (blue) to identify nuclei (b, e, h). The merged images were also shown (c, f, i). Scale bar=250 μm

Oncogene (2004) 23, 9392–9400.
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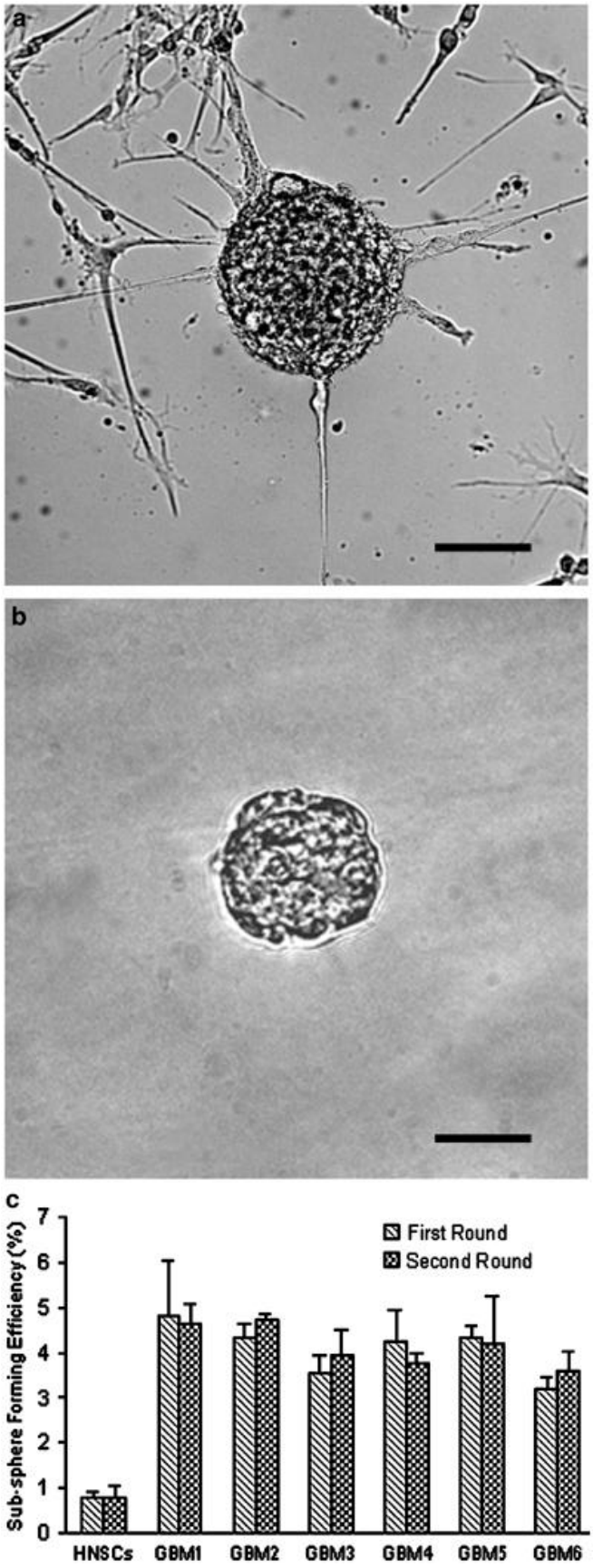
Figure 1



Oncogene (2004) 23, 9392–9400.
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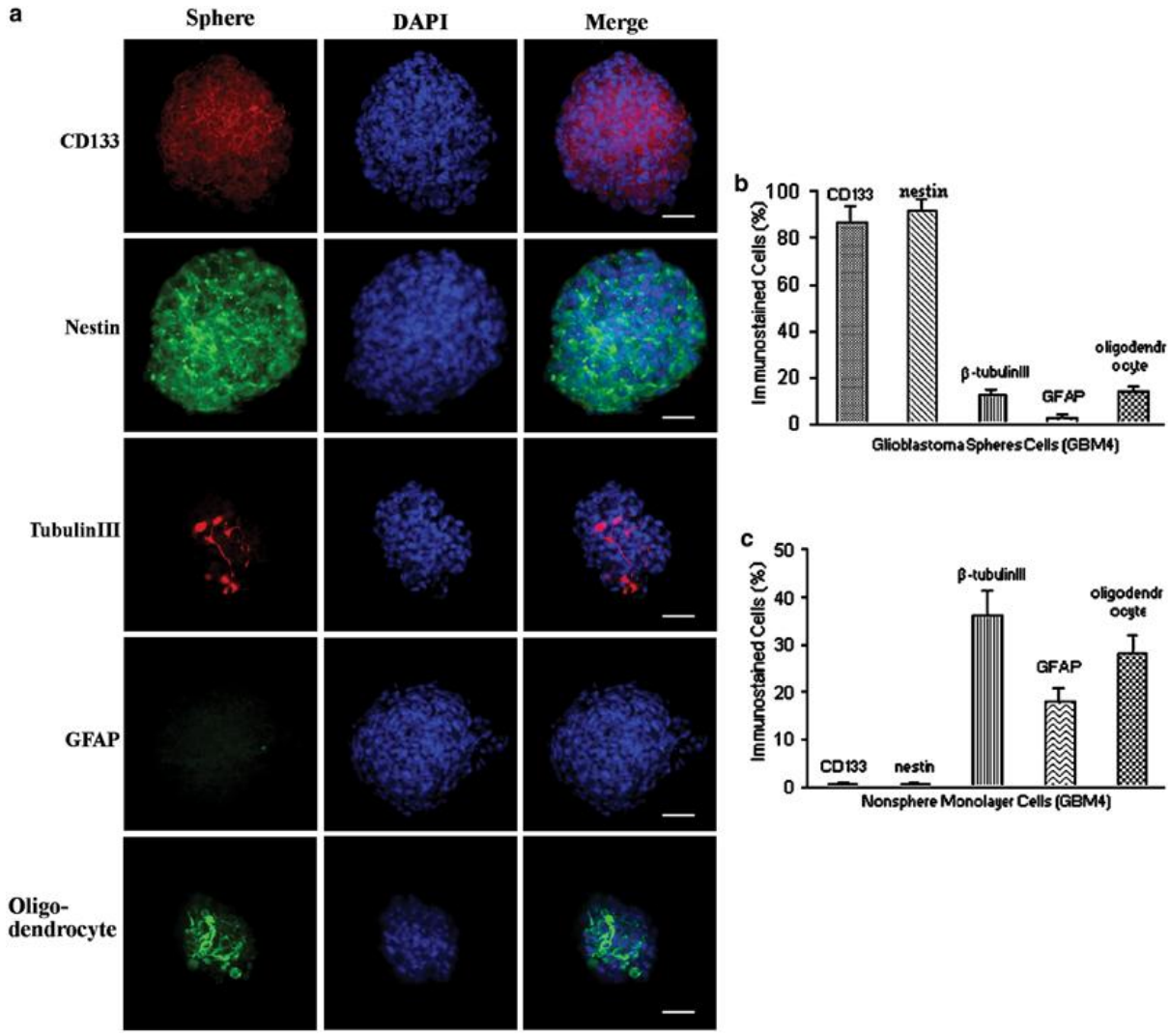
Figure 2



Oncogene (2004) 23, 9392–9400.
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Figure 3

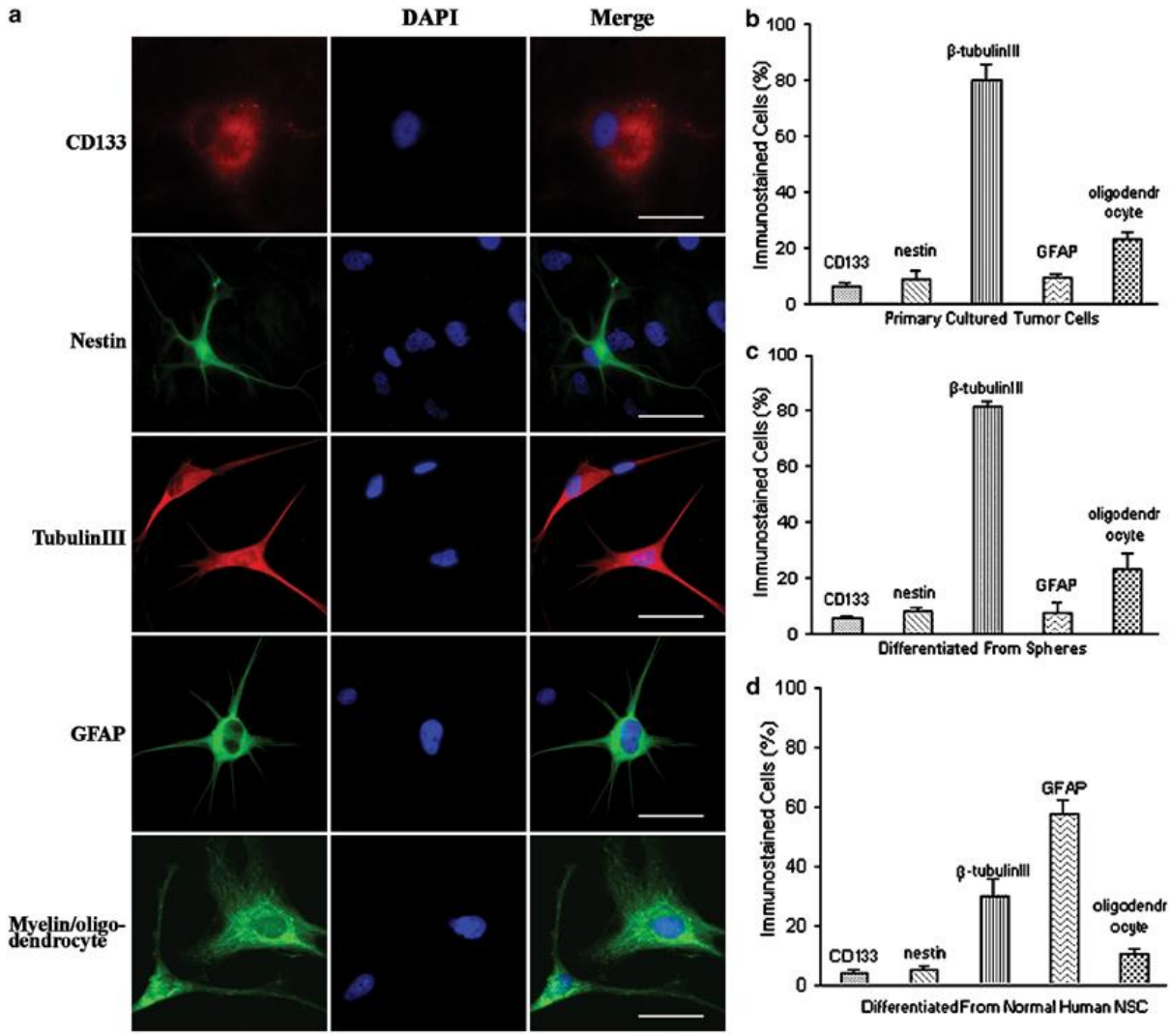

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Figure 4

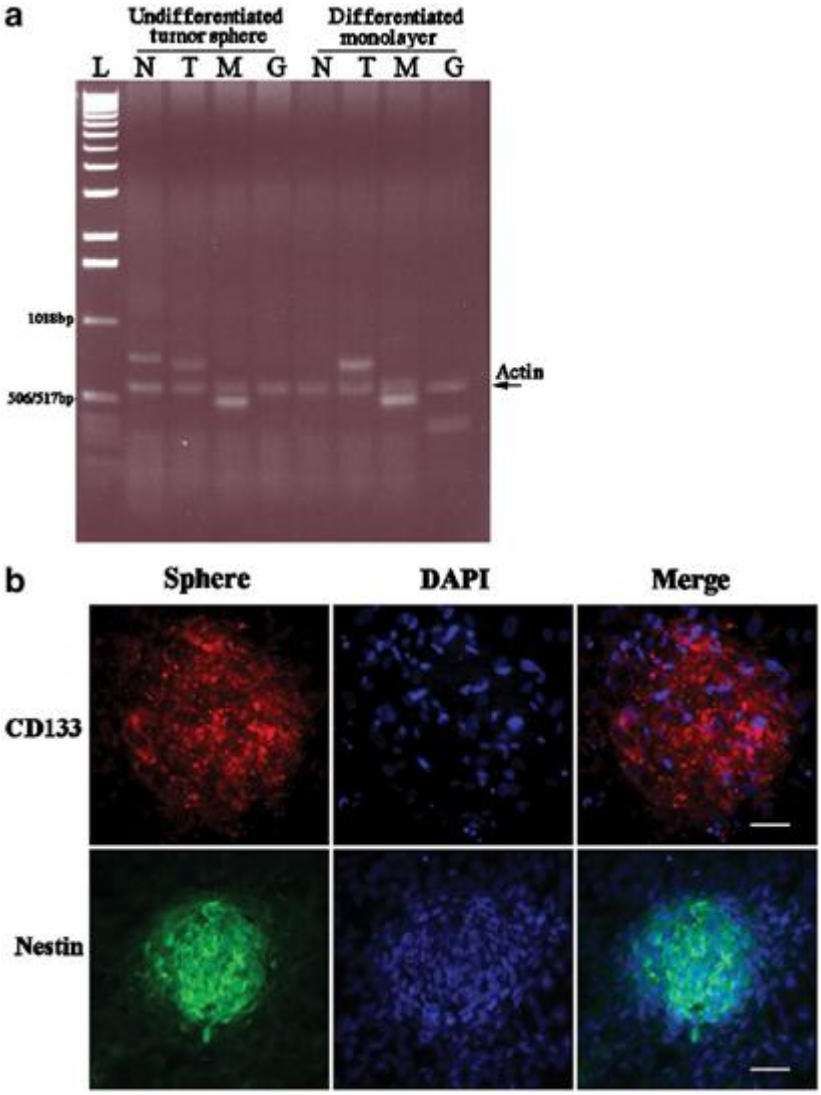

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Figure 5

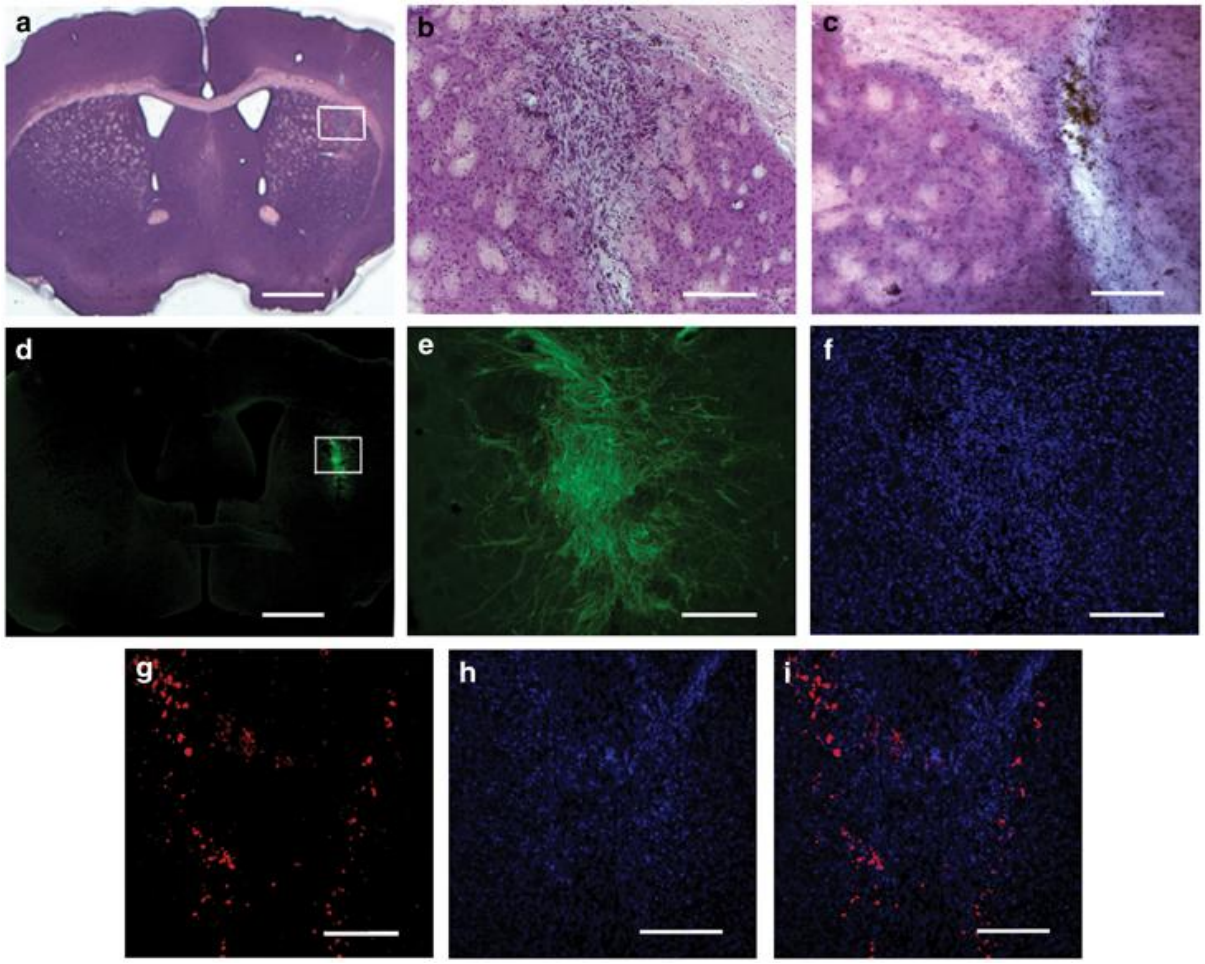

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Figure 6