

Embryonic Stem Cell Markers Distinguishing Cancer Stem Cells From Normal Human Neuronal Stem Cell Populations in Malignant Glioma Patients

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Glioblastoma multiforme (GBM) is the most common and the most aggressive type of brain tumor, responsible for 18.5% of all primary central nervous system tumors.¹ Treatment of these tumors remains a difficult clinical challenge and requires a multimodal approach.² Despite obvious benefits, surgery alone or in combination with radiation therapy does not provide prolonged remissions, yielding median survivals of 20 and 36 weeks, respectively, for GBM patients.³⁻⁶ Median survival times may be increased to up to nearly 15 months if >98% of the tumor is removed⁷ or if chemotherapy is integrated with surgery and radiation.^{8,9} Standard chemotherapy plus fractionated radiation therapy and surgery yields a median survival between 50 and 60 weeks.^{8,9} Unfortunately, there has been little improvement in survival relative to the original average span of 44 to 52 weeks documented >80 years ago.¹⁰ The presence of a blood-brain barrier^{11,12} and the remarkable degree of molecular heterogeneity within malignant glial cells^{13,14} limit the therapeutic effect of chemotherapy and make patient prognosis poor and recurrence rates reach close to 100%. Tumor stem (TS) cells are believed to be a major component of resistance to the existing therapies for GBM.

The heterogeneity of human glioma tumors, and particularly the existence of a subpopulation of TS cells, is believed to be critical to the tumorigenic process.^{1,15,16} Previous studies have suggested that these TS cells, identified as being positive for the surface marker CD133, within GBM tumors are able to give rise to new tumors after transplantation into nude mice.¹⁵⁻¹⁹ Interestingly, transplantation of CD133-negative cancer cells does not appear to form tumors on transplantation.¹⁸ CD133-positive cancer stem cells have been compared with human neural stem cells on both growth properties and gene expression.^{5,17,18} However, many of these comparative studies have been carried out using fetal neural stem cells rather than endogenous adult neural stem cells.²⁰ All studies that cite CD133 positivity to be an adult neural stem cell marker refer to research on fetal or embryonic stem cell-derived neural stem cells.²¹⁻²⁴ This distinction may be important because nonfetal

adult neural stem cells, at least in the subventricular zone, do not express CD133 and have not been as well characterized.²⁵

Previous comparative studies of malignant glioma tumor cell heterogeneity have failed to provide valuable information as to the similarity of cancer stem cells to adult neural stem cells. Many studies have cited CD133 positivity to be a TS cell marker even though CD133 positivity has also been established as a marker for normal neuronal stem (NS) cells.^{16,20,23,24} Thus, the use of CD133 as a surrogate marker to identify TS cells within a GBM may not be clinically useful because glioblastomas contain both differentiated cancer cells and cancer stem cells in addition to normal adult neural stem cells that migrate into the tumor.²⁶⁻²⁸ Both NS cells and glial progenitor cells have been found throughout the healthy normal adult brain.²⁹⁻³⁶ NS cells travel with tumor cells migrating through the parenchyma of the central nervous system.²⁶ In fact, NS cells appear in the area adjacent to glioma implants 5 days after injection in mice.²⁸ This migratory phenomenon, which is also observed in brain injury,³⁷ has been proposed as a means of anticancer gene delivery.^{38,39} If stem cells are to be a viable vehicle for tumor therapies, then more detailed identification is needed to prevent the accidental implantation of cancer stem cells. Thus, identifying a specific TS cell marker to distinguish neoplastic stem cells from NS cells is crucial for not only understanding the biology of TS cells but also developing effective therapy for GBM.

The ability of TS cells to undergo tumorigenesis, combined with their resistance to chemotherapy and radiation therapy,⁴⁰⁻⁴² is of particular clinical importance given the propensity of gliomas to reemerge after surgery and therapy. As a result, TS cells may represent a primary therapeutic target to achieve complete eradication of the tumor. Although CD133-positive TS cells have been compared with human NS cells on both growth properties and gene expression,^{15,17,18} definitive TS cell-specific markers that relate to TS biology have not been found. To help address this problem, this study aimed to characterize different cell populations on the basis of growth properties and to show a distinct population of cells with cancer stem cell properties based on specific cell marker expression patterns.

MATERIALS AND METHODS

Cell Culture and Isolation

Human glioblastoma tumor masses were removed from patients undergoing craniotomy for primary resection of newly diagnosed tumor identified by magnetic resonance imaging. All patients provided Institutional Review Board-approved informed consent for the study preoperatively. Surgically removed tumor specimens were washed, minced, dissociated, and then placed, within an hour of surgery, inside a 75-cm² flask containing resuspension medium of Dulbecco modified Eagle medium/F12 supplemented with 10% fetal bovine serum. After an initial expansion in a monolayer, the tumor cells were switched to a defined serum-free neuronal stem cell (NSC) media consisting of Dulbecco modified Eagle medium/F12 supplemented with 20 ng/mL basic fibroblast growth factor-2 and 20 ng/mL epidermal growth factor to generate neural sphere formation at different time points. Specifically, cells were placed directly in neural stem cell media or switched at 24, 48, or 72 hours. This culturing system generated cells with 2 distinct growth properties, adherent cells and floating sphere-forming cells. Adherent cells are likely differentiated tumor cells with limited proliferative potential. Floating neural spheres contain multipotent stem cells. Cells were analyzed with quantitative real-time polymerase chain reaction (PCR) for the expression of neural stem cell genes, stem cell transcription factors, tumor cell markers, and genes associated with neural and glial differentiation. Additional characterization included differentiation in serum-supplemented media and antibody staining with neural and stem cell markers. If NSCs can be isolated from the brain by culturing serum free in epidermal growth factor/fibroblast growth factor, this culture system could potentially identify stem cells in brain tumors. Several groups studying human brain tumors have identified small numbers of cells with clonogenic potential based on the neurosphere assay.^{15,17-19,43-46}

RNA Isolation and Quantitative Real-Time PCR:

RNA Isolation

Cell culture media was removed from cells, and total RNA was extracted from cells with Trizol Reagent (Invitrogen) in accordance with the manufacturer's protocol. Briefly, cells were spun down in a centrifuge tube at low speed to pellet. Media was removed, and 1 mL Trizol was added to the cells and incubated at room temperature for 5 minutes. After 5 minutes, 0.2 mL chloroform per 1 mL Trizol was added to the tubes. The tubes were shaken vigorously for 15 seconds and incubated at room temperature for 2 minutes. Tubes were then centrifuged at no more than 12000g for 15 minutes at 2°C to 8°C. Next, the aqueous phase of the samples was removed and transferred into a fresh tube. To precipitate the RNA, 0.5 mL isopropyl alcohol was added to each tube, and the tube was lightly mixed back and forth for 15 seconds. Samples were then incubated at room

temperature for 10 minutes and centrifuged at no more than 12 000g for 10 minutes at 2°C to 8°C. The supernatant was removed, and the RNA pellet was washed once with 1 mL 75% ethanol per 1 mL Trizol. Samples were mixed by vortexing and centrifuged at 7500g for 5 minutes at 2°C to 8°C. The RNA pellet was air dried for approximately 10 minutes. Next, 100 µL microbiology-grade water was added to each tube, and tubes were incubated for 10 minutes at 55°C. RNA concentration was measured with spectrophotometry. For spectrophotometer readings, a 1:50 dilution was created by adding 4 µL RNA solution and 196 µL microbiology-grade water. Blanking solutions consisted of only 200 µL microbiology-grade water. The spectrophotometer was calibrated with the blanking solutions, and readings of the RNA solution were taken at A280.

cDNA Synthesis

RNA was reverse transcribed with reverse-transcription PCR (Eppendorff) with iScript cDNA Synthesis Kit (Biorad) to form cDNA. Each tube contained 5 µL 5 × iScript Buffer, 1 µL reverse-transcription enzyme × µL of RNA calculated from the spectrophotometer readings, and × µL water, depending on the amount of RNA, for a total reaction volume of 20 µL. The cDNA reaction took place under the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes and held at 4°C.

Real-Time PCR

Gene expression was measured by quantitative real-time PCR using gene specific primers. Real-time PCR was performed with the MyiQ SuperCycler Real Time PCR Detector System (Biorad) using iQ Supermix with SYBR Green (Biorad) for detection. Each reaction tube contained 12.5 µL SYBR Green, 8 µL microbiology-grade water, 1 µL forward primer, 1 µL reverse primer, and 2.5 µL cDNA, for a total reaction volume of 25 µL. Primers used for real-time PCR were as follows: TERT: forward, 5'-CGGAAGAGTGTCTGGAGCAA-3'; reverse, 5'-GGATGAAGCGGAGTCTGGA-3'; CD133: forward, 5'-CAGAGTACAACGCCAAACCA-3'; reverse, 5'-AAATCACGATGAGGGTACAGC-3'; Nanog: forward, 5'-ACAACCTGGCCGAAGAATAGC-3'; R-AGTGTCCAGGAGTGGTTGC-3'; Sox2: forward, 5'-CGGTACCCGGGGATCCCCGCATGTACAA-CATGATGG-3'; reverse, 5'-CATAATGGCCGTCGACCA-CATGTGTGAGAGGGGCA-3'; Oct4: forward, 5'-ATAGACCGGTAATGGCGGGACACCTGGC-3'; reverse, 5'-CAT AATGGCCGTCGACCAGTTTGAATGCATGGGAGA-3'; and β-actin: forward, 5'-CTCTTCCAGCCTTCCTTCCT-3'; reverse, 5'-AGCACTGTGTTGGCGTACAG-3'. The real-time PCR reaction consisted of the following cycles: cycle 1, 1 repeat at 94°C for 5 minutes; cycle 2, 45 repeats of step 1 at 94°C for 30 seconds, step 2 at a gradient from 56.2°C to 61.0°C for 30 seconds, and step 3 at 72°C for 45 seconds; cycle 3, 1 repeat at 72°C for 5 minutes; cycle 4, 1 repeat at 90°C for 1 minute; cycle 5, 90 repeats at 50°C for 10 seconds with an increase in

temperature of 0.5°C after each cycle starting with cycle 2; and cycle 6, 1 repeat at 25°C for 5 minutes. Data collection was enabled at the end of cycle 2, and melting curve data collection was enabled at cycle 5.

Immunohistochemical Staining

Immunohistochemical staining was performed using a primary and fluorescent-conjugated secondary antibody protocol. Briefly, cultured cells were washed in phosphate-buffered saline (PBS) and fixed in a 4% paraformaldehyde fixative solution for 20 minutes at room temperature. When staining for BrdU incorporation, samples were treated with 2N hydrochloric acid for 20 minutes and then washed 3 times with PBS.

Samples were then incubated at room temperature in a blocking solution of PBS supplemented with 5% donkey serum and 0.2% triton X for 1 hour. Sections were subsequently transferred to a blocking solution containing the primary

antibodies and incubated overnight at 4°C. Primary antibodies for the following targets were used: Nanog, Oct4, Sox2, BrdU, β III-tubulin, and glial fibrillary acidic protein (GFAP). The following morning, sections were washed and incubated in a fluorescence-conjugated secondary antibody (1:500 dilution) for 2 hours in the dark at room temperature. Stained sections were then washed 3 times with PBS and mounted on a glass slide using a mounting medium containing DAPI to label the nuclei of the cells.

RESULTS

Tissue culture isolation of GBM patient brain tissue using a defined serum-free NSC media identified 2 general groups of cells that form from these tissues. The first group consisted of a spherical mass of cells, similar to neurospheres formed by normal neural stem cells in culture (Figure 1A and 1B); the

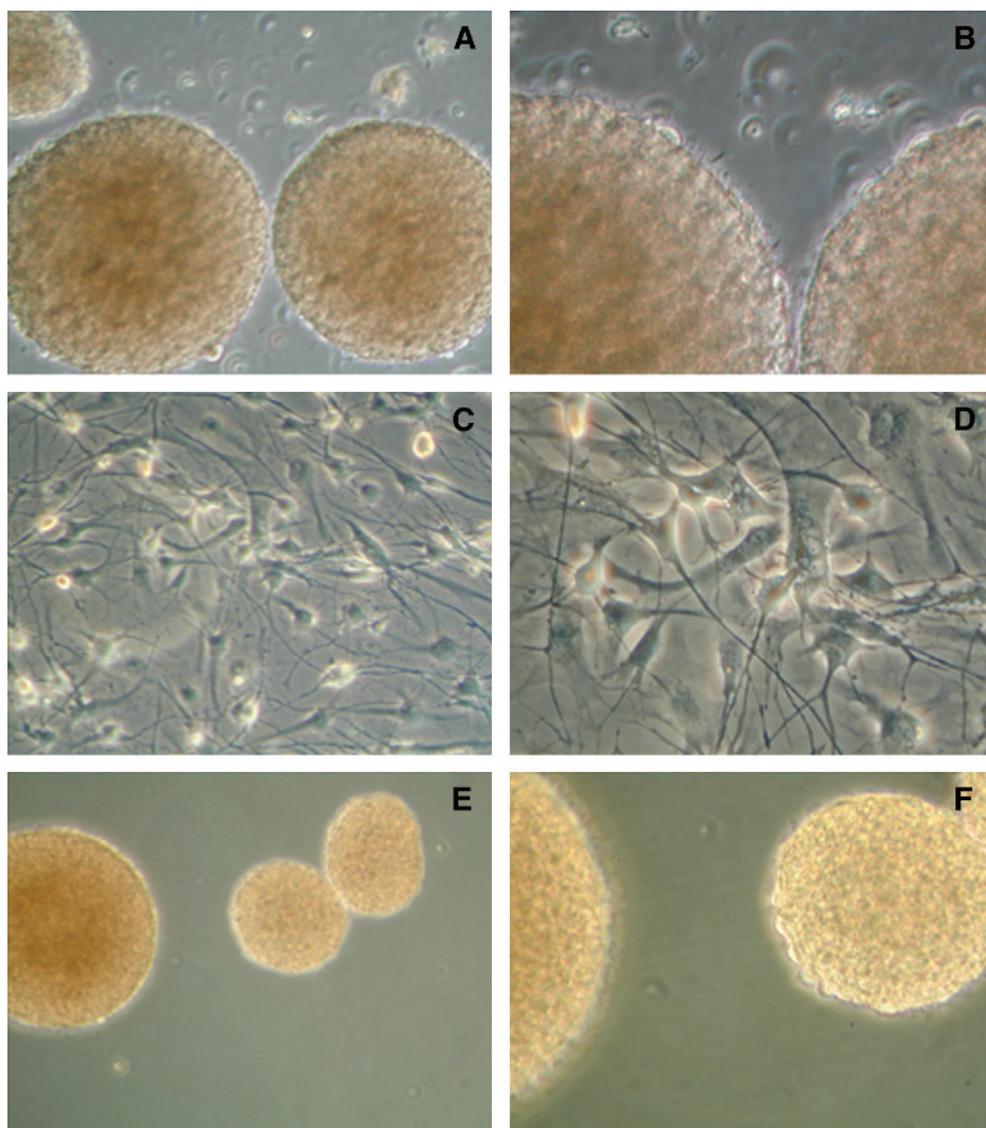


FIGURE 1. GBM patient tumor tissue grown in culture containing a group of cells that form a spherical mass of cells (A and B), which is similar to neurospheres that are formed by normal neuronal stem cells in culture, and a second group of cells that form a traditional monolayer in cell culture (C & D). Panels E and F show normal human fetal cerebral cortex forming neurospheres as a reference.

second group consisted of cells forming a monolayer in culture (Figure 1C and 1D).

Some of the cells in the spherical masses derived from GBM that histologically look like neurospheres were CD133 positive (green); proliferating cells within the masses were also identified by BrdU incorporation (red) (Figure 2). This would suggest the existence of TS cells within the spheres. When media was changed to deprive growth factors such as fibroblast growth factor and epidermal growth factor, some of the cells differentiated and others did not (Figure 3). The cells isolated from the GBM-derived neurospheres were resistant to differentiation (Figure 3A and 3B), whereas fetal control normal neural stem cells were completely differentiated into neurons and astrocytes under the same conditions (Figure 3C). Previous studies found that the sphere-forming population resided in the fraction of primary brain tumor cells that express the NSC/precursor cell surface marker CD133.^{17,18,24} We also found the cells in the sphere expressed CD133 while adherent cells did not. On the other hand, GBM cell lines have been established in monolayer cells (adherent cells).⁴⁷ CD133 is currently the established marker for neural stem cells and cancer stem cells. In evaluations of Sox-2 expression, immunoreactivity was found in the neurosphere cells resistant to differentiation and the differentiating tumor cells (Figure 4A). However, differentiating cells coming off from the neurospheres did not express Nanog, whereas cells that stay in the neurosphere that are not differentiating (resistant to differentiation) continued to strongly express NANOG (Figure 4B). These same Nanog-expressing cells in the neurosphere, which were resistant to differentiation, also expressed Oct4, SSEA4, and CD133 (Figure 5).

Differentiation analysis of >300 clones revealed 2 distinctive characteristic cells in neurospheres derived from GBM patients' brain tissue. An example is clone 80303, which, after being placed in conditions allowing for differentiation, shows MAP2 and/or β III-tubulin and S100-and/or GFAP-immunopositive cells, indicating neuronal and astroglial differentiation (Figure 6). Before differentiation, these clones, like 80303, expressed Sox2 but did not express embryonic stem cell gene TRA1-60 (Figure 6C). The second clone population, including clone 80427, did not show nearly the same degree of β III-tubulin and GFAP immunoreactivity, but unlike the first clone population, it was strongly positive for both Sox2 and TRA1-60 immunoreactivity (Figure 7). This secondary clone population also maintained spherical formation in culture as seen in clone 80721 and remained both Oct4- and CD133-immunopositive (Figure 7C).

Comparison of Gene Expression Levels Between Neurospheres and Monolayer Cells Derived From GBM Brain Tissue

PCR analysis of clones resistant to differentiation showed that Nanog and Oct4 expression was markedly

elevated, but not expression of Sox2 or Tert (Table 1), whereas in normal NS cell controls, Nanog and Oct4 expression levels were virtually identical to β -actin. Similarly, in clones not resistant to differentiation, Nanog expression and Oct4 expression were comparable to those of the normal NS cells.

Real-time PCR amplification analysis of Nanog gene expression for the various clones created before clone differentiation using cycle threshold (CT) values confirmed the findings of the immunohistochemical analysis. The Nanog CT values of the clones were then compared with the CT values for control normal fetal NS cells (Table 2). CT values are the number of PCR cycles required for a fluorescent signal to cross the threshold and exceed the background fluorescent signal, thus creating a positive reaction. CT levels are inversely proportional to the amount of target gene in the sample. Therefore, the more genes expressed in a clone, the lower the CT value. As seen in Table 2, clones 80517 and 80821 have 64 times less expression of Nanog compared with clone 80616. This indicates that some neurosphere cells within GBM tissues express just normal fetal stem cell concentrations of NANOG. These are likely normal adult neural stem cells within a GBM tumor, whereas GBM neurosphere clones 80616, 80229, 80802, 80808, and 80301, which express higher levels of Nanog, are likely actual TS cells. Like clone 80803, clones 80517 and 80821 differentiate into normal cells.

In our study, the average CT value for high-expressing Nanog clones was 24.0277667. The average CT value for low-expressing Nanog clones was 27.485, and the fetal neural stem cell clone had a mean Nanog CT value of 27.73333. High-expressing clones were identified by having >2 times the amount of Nanog detected. Thus, high-expressing Nanog

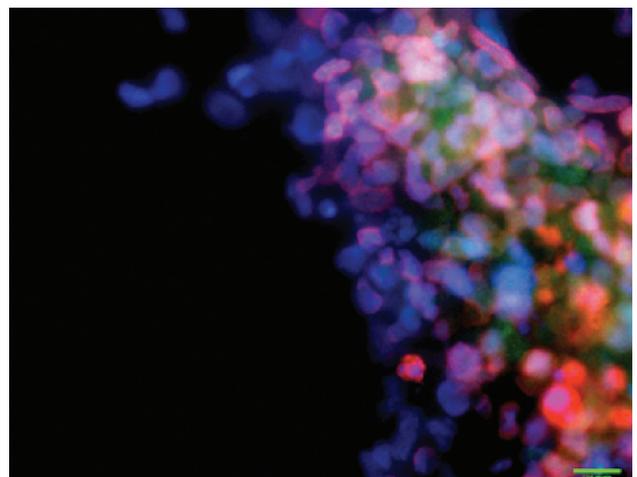


FIGURE 2. Immunohistochemistry staining of GBM derived neurosphere cells. CD133 positive cells (green). Proliferating cell marker BrdU (red). All nuclei are counter stained by DAPI (blue).

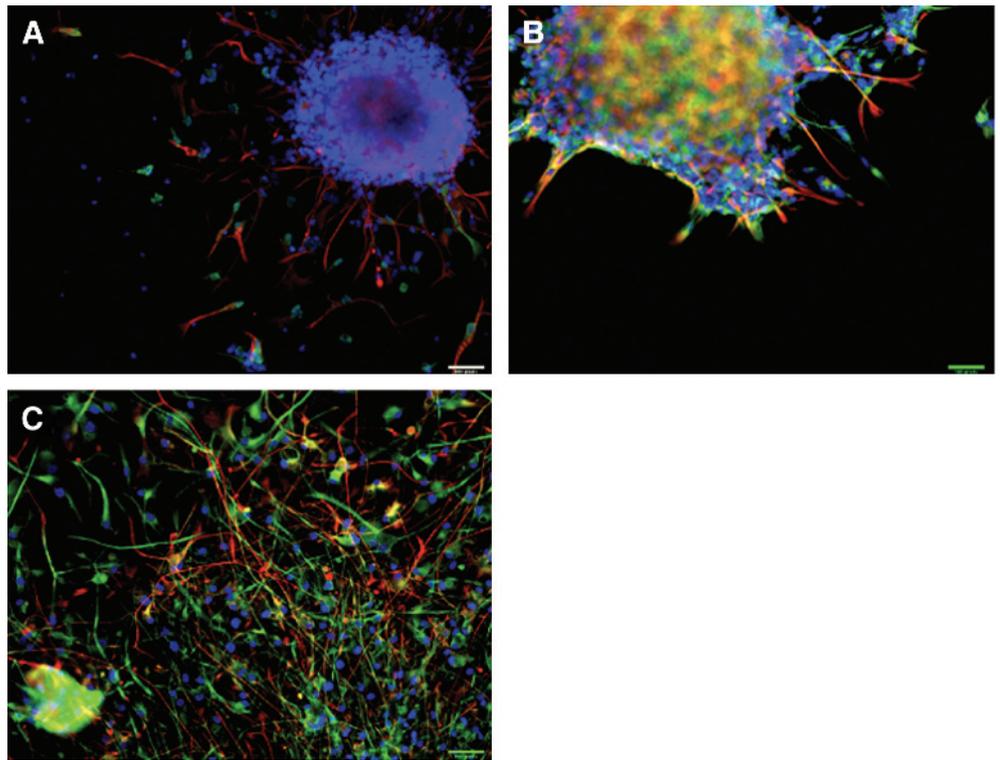


FIGURE 3. When media is changed to deprive growth factors, some GBM neurosphere cells differentiate but not others. Identical conditions result in the differentiation of normal neuronal stem cells into neural and glial lines as seen in C. A and B, non-differentiating neurospheres from GBM tissue. C, neurosphere from fetal brain neuronal cells. Betall-tubulin (green), and GFAP (red) indicate neural and glial differentiation of the cells, respectively. All nuclei were counterstained by DAPI.

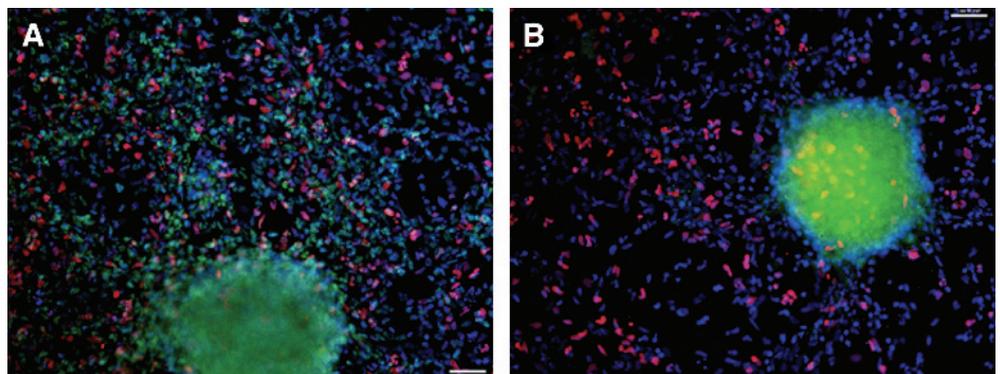
clones likely represent glioblastoma TS cells, whereas low-expressing Nanog clones, with expression values approximating fetal neural stem cells, likely represent normal NS cells that have migrated into the GBM tissue.

DISCUSSION

When a culture technique is used to isolate and purify normal neural stem cells, a spherical floating cell mass forms, which is similar to neurospheres from GBM tissue. Malignant glioma tumors are made up of various cell types, including TS cells and normal NS cells. Some of these cells are

immunopositive for CD133, which is reported to be a TS cell marker. Our differentiation pattern analysis indicates that the tumor spheroid may contain at least 2 different types of cells. One is very similar to a normal neural stem cell, which differentiates into neurons and glia; the other type is resistant to differentiation and does not form neurons or glia. Gene expression analysis revealed that tumor spheroids had significantly higher levels of embryonic stem cell genes, including Nanog, Oct4, and Sox2. Although Sox2 is required for neural stem cell maintenance, the expression of Nanog and Oct4 was unexpected because previous reports have suggested that they are not expressed in neural stem cells. Of the 2 cell

FIGURE 4. BrdU immunoreactivity indicates proliferating cells (red). All nuclei are counterstained with DAPI (blue). A, Sox2 expression (green) is seen in differentiating tumor cells outside the neurosphere as well as cells resistant to differentiation that are within the neurosphere. B, however, Nanog expression (green) is strongly expressed in neurosphere cells and not in the differentiated cells outside the neurosphere.



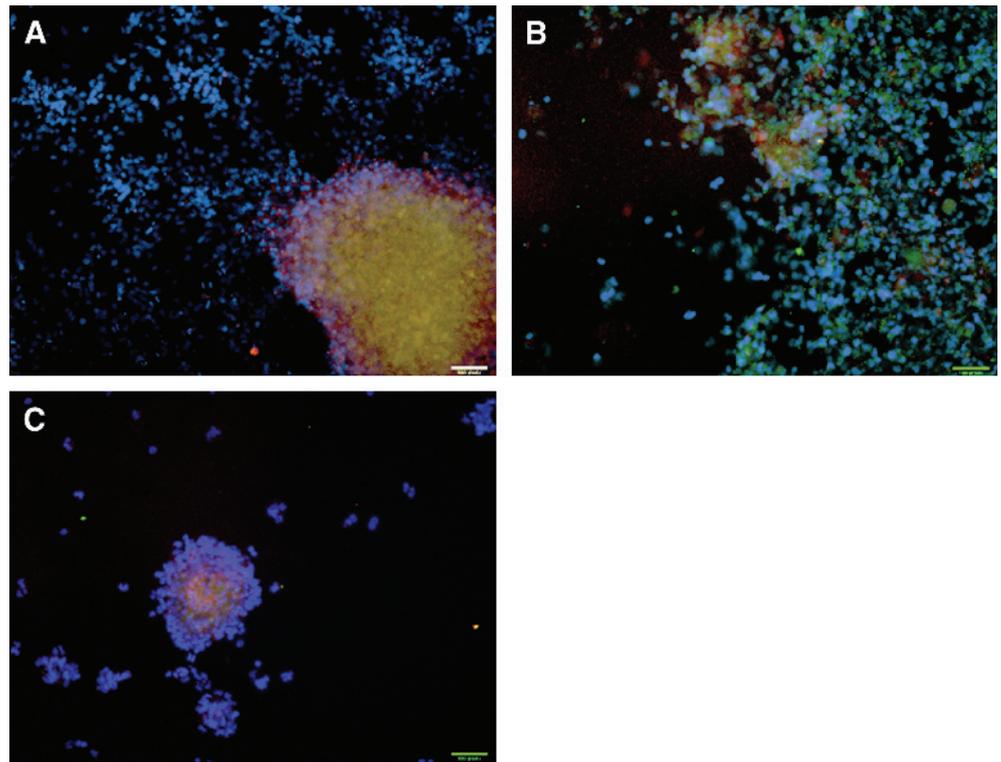


FIGURE 5. All nuclei are counter stained with DAPI (blue). A, cells in the neurosphere resistant to differentiation that express Nanog also express Oct4 (red); and B, SSEA4 (red). Nanog (green). C, cells expressing Oct4 (red) in the neurosphere also express CD133(green).

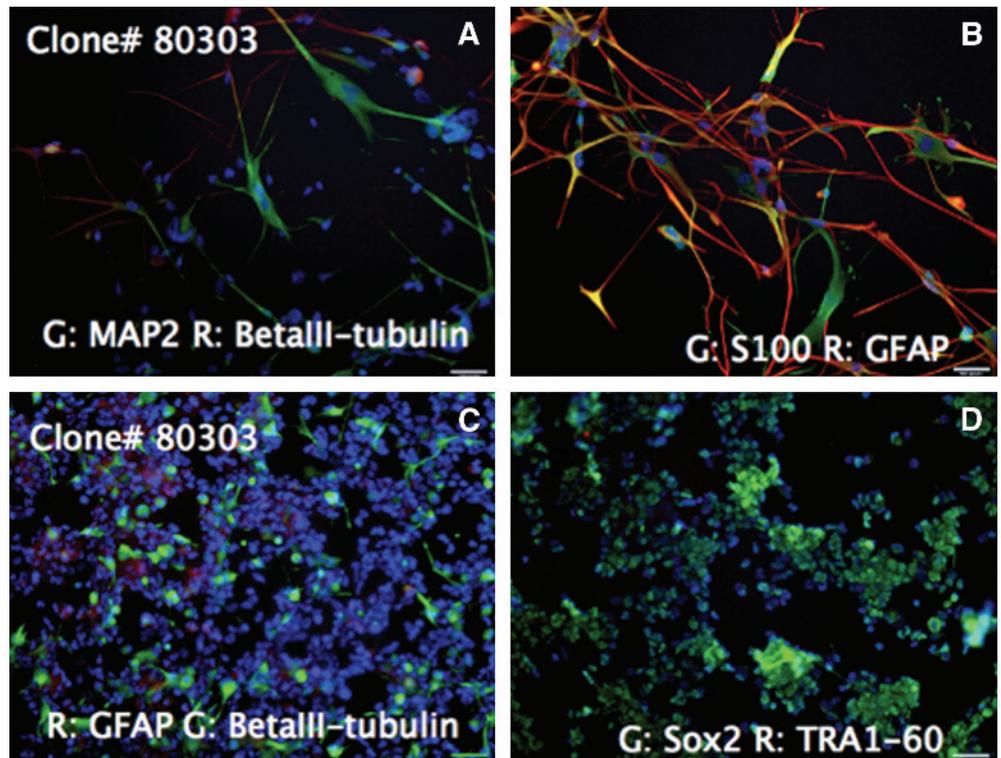


FIGURE 6. Cells that appeared differentiating outside the neurosphere stained positive for: A, neuronal markers MAP2 (green) and betaIII-tubulin (red) as well as (B) astroglial markers S100 (green) and GFAP (red). These same cells (C) with both neuronal and astroglial differentiation were also positive for Sox2 (green) but not TRA1-60 (red) as seen in D.

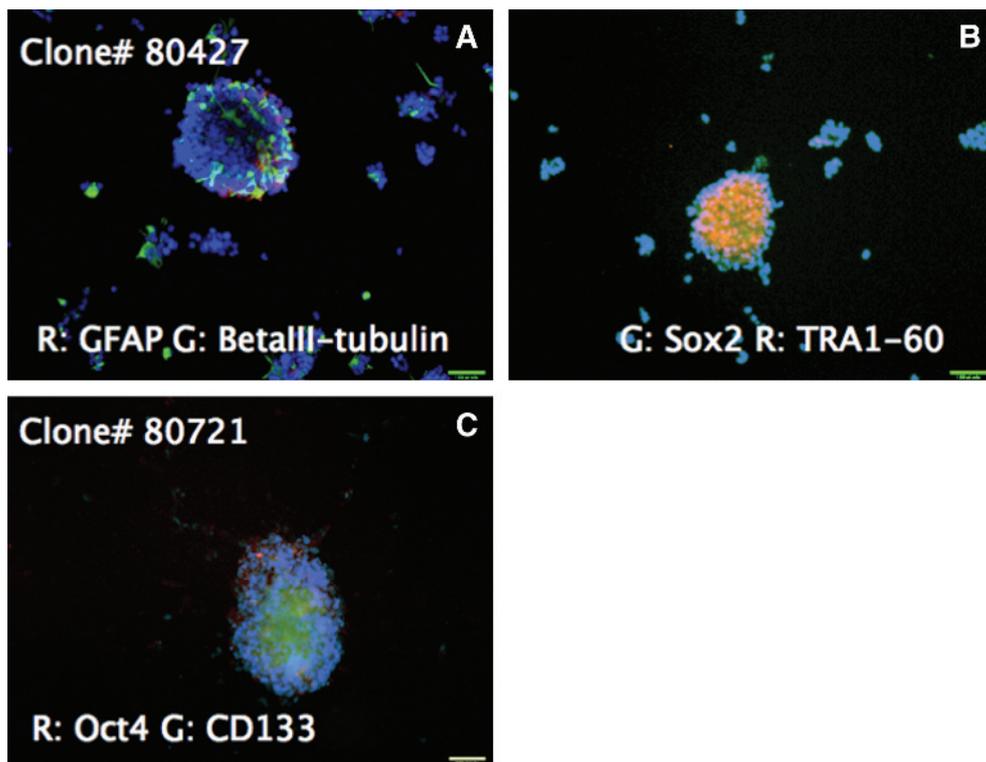


FIGURE 7. Cells maintaining a neurosphere showed very little expression for: A, neuronal or astroglial markers betalll-tubulin (green) and GFAP (red). B, however, both Sox2 (green) and TRA1-60 (red) showed significant immunoreactivity as well as (C) stem cell markers CD133 (green) and Oct4 (red) within the neurosphere.

types within the tumor spheroids, those resistant to differentiation had much higher levels of Nanog expression than those that proceeded to differentiate into neural and astroglial lines. This suggests that the high-expressing Nanog cells may represent true TS cells and the low-expressing Nanog cells represent infiltrating normal NS cells within the tumor tissue.

CONCLUSIONS

Because TS cells and normal NS cells look identical histologically and both express CD133, neither is a reliable way to differentiate tumor from non-TS cells within a GBM tumor. Our results suggest that the tumor spheroids derived from GBM

brain tissue contain normal neural stem cells and TS cells, which can be distinguished by the level of embryonic stem cell gene expression. Nanog, a transcription factor critically involved with self-renewal of undifferentiated stem cells, seems to be the most differentially expressed in these glioblastoma stem cells. Its levels in normal NS cells and differentiated tumor cells are negligible. From our results, it seems that Nanog might be a better TS cell marker than the previously described TS cell marker CD133. Nanog is thought to play a key role in maintaining pluripotency. Loss of Nanog function causes differentiation of embryonic stem cells into other cell types, and Nanog overexpression enables stem cell propagation for

TABLE 1. PCR Analysis of Clones Resistant to Differentiation Relative to Normal Tumor Non-Neurosphere Cells: Gene Expression Levels are Normalized Against Beta-Actin Gene Expression Level

Gene	Expression
Nanog	142.51797
Sox2	25.0198286
Oct4	2069.40456
Tert	11.7533491
β-Actin	1

TABLE 2. Nanog CT Values for GBM Tumor Clones

Clone	Cycle Threshold Value
80616	22.1433
80229	22.8033
80802	23.09
80808	24.6133
80301	25.5267
80709	25.99
80517	27.603333
80821	27.366667
Fetal human neuronal stem cell	27.73333

multiple passages during which they remain pluripotent. Interestingly, p53 binds to the Nanog promoter and suppresses its expression, resulting in stem cell differentiation.

Because Nanog is known to prevent differentiation of embryonic stem cells, a similar mechanism may be preventing differentiation of the TS cells. Future research studying the role of Nanog in regulating GBM TS cell differentiation and ways to block the effects of Nanog on these cells may allow the development of therapies that enhance our ability to successfully treat patients with GBM tumors.

Disclosure

The authors have no personal financial or institutional interest in any of the drugs, materials, or devices discussed in this article.

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