Priority Report

Glioma Cell Populations Grouped by Different Cell Type Markers Drive Brain Tumor Growth

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Abstract

Although CD133 has been proposed as a marker for brain tumor–initiating cells, studies show that a tumorigenic potential exists among CD133⁻ glioma cells as well. However, it is not established whether the ability of CD133⁻ cells to form tumors is a property confined to a small subpopulation, rather than a common trait associated with most glioma cell types. Thus, we used lentiviral vectors expressing green fluorescent protein under lineage-specific promoters to identify CD133⁻ glioma cells expressing Nestin, glial fibrillary acidic protein (GFAP), and neuron-specific enolase (NSE). Flow cytometry analysis showed the presence of CD133⁻ subpopulations expressing these markers in glioma cell lines and in primary cultures from human glioblastoma (GBM) biopsies. Moreover, analysis of cell cycle distribution showed that subgroups expressing Nestin, GFAP, and NSE uniformly contained actively cycling cells, when cultured in serum-containing medium and stem cell medium. These subpopulations were fluorescence-activated cell sorted from CD133⁻ U373 glioma cells and implanted intracerebrally in severe combined immunodeficient mice. Moreover, we implanted Nestin-, GFAP-, and NSE-positive glioma cells sorted from a human GBM biopsy, following removal of CD133-positive cells. All the CD133⁻ subpopulations produced tumors, with no significant differences in survival or tumor take rates. However, there was a trend toward lower take rates for CD133⁻ glioma subpopulations expressing GFAP and NSE. These findings suggest that the ability to form tumors may be a general trait associated with different glioma cell phenotypes, rather than a property limited to an exclusive subpopulation of glioma stem cells. *Cancer Res; 70(11); 4274–9. ©2010 AACR.*

Introduction

The central nervous system (CNS) displays a cellular hierarchy, comprising stem cells, progenitor cells, and mature cell types (1). CD133 has been identified as a marker for neural stem cells (2), and experimental data suggest that it is also a marker for cancer stem cells in brain tumors (3). Still, evidence from a growing number of studies strongly suggests that tumor-initiating cells exist among CD133⁻ cells as well (4–6). However, it is not known whether the ability of CD133⁻ human glioma cells to form tumors still reflects a property restricted to a small subpopulation, rather than a general trait associated with most glioma cell types. Thus, whereas these data specifically question the use of CD133 as a cancer

stem cell marker, they also raise concerns about the concept of cancer stem cells itself.

Malignant brain tumors often display a striking cellular heterogeneity, and glioma cells are known to express several markers other than CD133 for various cell types within the CNS (7, 8). Thus, brain tumors may contain several subpopulations with different functional properties. Fluorescence-activated cell sorting (FACS) of viable cells is usually based on cell surface epitopes, and subpopulations grouped by intracellular markers are not easily isolated. However, many of the commonly used CNS cell lineage markers are intracellular. Therefore, we developed lentiviral vectors expressing green fluorescent protein (GFP) under the cell lineage promoters regulating the expression of Nestin, neuron-specific enolase (NSE), and glial fibrillary acidic protein (GFAP) because these are markers associated with some of the main CNS cell types. This allowed us to study their expression in glioma cell cultures and to determine the proliferative and tumorigenic potential of isolated cell populations associated with these markers.

Materials and Methods

Biopsy collection

The biopsies were glioblastomas (GBM) harvested from patients treated at the Department of Neurosurgery, Haukeland University Hospital, Bergen. The Regional Ethical Committee approved the collection of tumor tissues.

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Cell culture

Patient biopsies were immediately dissociated by trypsinization and subsequently grown as monolayer cell cultures. After 1 day, viruses containing the promoter constructs were added. U87, U251, and U373 cells and patient biopsies were cultured in DMEM (Sigma-Aldrich), 10% fetal bovine serum, with NEAA, 100 units/mL Pen/Strep, and 400 mol/L L-glutamine (Cambrex). U251 and U373 cells were also cultured in stem cell medium (SCM) consisting of Neurobasal Medium (Invitrogen), with 1× B-27 (without vitamin A), 1× GlutaMax I (Invitrogen), 20 ng/mL epidermal growth factor (Sigma), 20 ng/mL FGF2 (R&D Systems), and 100 units/mL Pen/Strep (Cambrex).

Lentiviral vector production

The p232-dsRed lentiviral vector (from Steven Elledge and Frank Stegmeier, Department of Genetics, Harvard Medical School, Boston, MA) was modified by removing the fragment XbaI-EcoRI. MCS-ECFP (Clontech) was inserted in this site by cutting with Nhe1 and Mfe1. Nestin-enhanced GFP (eGFP) was cut from pNestin-eGFP (from Rohan Humphrey, Department of Pediatrics, University of California San Diego, La Jolla, CA) with Eco47III-Not1 and ligated into p232-ECFP cut with Eco47III-Not1. Cecilia Lundberg (Department of Physiological Sciences, Lund University, Lund, Sweden) provided the GFAP-GFP and NSE-GFP lentiviral plasmids. Viruses were produced as described earlier (9).

The lentiviral control vector pWPXL-eGFP, packaging plasmid psPAX.2, and envelope plasmid pMD2.G were provided by Dr. Trono's laboratory (CMU, Geneva, Switzerland). Superfect (Invitrogen) was used to triple-transfect psPAX.2, pMD2.G, and the transfer vectors into 293FT producer cells. Supernatant containing lentiviral particles was harvested 48 hours after transfection. Cells were transduced with lentiviral particles in the presence of 10 g/mL polybrene (Sigma-Aldrich). The pWPXL-eGFP vector was used to calibrate the transfection efficacy.

Immunocytochemistry

Immunocytochemistry was performed as previously described (6, 9). A detailed protocol is described in Supplementary information.

Flow cytometry and cell sorting

Flow cytometry analysis of reporter gene and CD133 expression. The cells were infected with lentiviral particles and harvested 2 weeks after infection. The cells were stained by phycoerythrin-conjugated CD133/1 clone AC133 antibody (Miltenyi) at a concentration of 1:50 in buffer with 20% FcR Blocking Reagent (Miltenyi). Cells were analyzed for GFP and CD133 expression with CellQuest on a FACSCalibur (BD Biosciences).

Protocols for cell cycle analysis and FACS are described in Supplementary information.

Animal experiments

Severe combined immunodeficient (SCID) mice were implanted with glioma cells intracerebrally and sacrificed at the onset of symptoms. Animal experiments are described in Supplementary information.

Results

Glioma cell cultures contain subpopulations expressing different cell type markers

We developed lentiviral constructs expressing the reporter gene *GFP* under the GFAP, Nestin, and NSE promoters (Fig. 1A). The overall experimental setup is shown in Fig. 1B. Using these vectors, we transduced the U87, U251, and U373 glioma cell lines and two primary cultures from human GBMs at 60% confluence. Both immunocytochemistry (Fig. 1C) and visual inspection of GFP fluorescence confirmed the expression of all the cell type markers (Fig. 1C, inset).

Expression of the various cell type markers is maintained under different culture conditions

To obtain a quantitative assessment of reporter gene expression under the various promoters, we performed flow cytometry analysis of all the glioma cell cultures (Fig. 2A and B). All markers were expressed in the glioma cell lines and primary cultures, although the rate of GFP-positive cells varied extensively. In one of the primary cultures, NSE expression was barely detectable. Because stem cells have specific growth requirements, we repeated the analysis on U373 and U251 glioma cells in SCM and in serum-containing medium (Fig. 2C). This experiment showed reporter gene expression under all the cell lineage–specific promoters, both in serum-containing medium and in SCM.

### Subpopulations expressing various cell type markers proliferate *in vitro*

To compare the proliferative potential of the different subpopulations, these subgroups were FACS sorted from the U 373 glioma cell line, and their cell cycle distributions were analyzed by flow cytometry analysis, following propidium iodide staining. Both in serum-containing medium and in SCM, all subpopulations contained cell fractions in the G_1, G_2, and S phases (Fig. 3), and the experiments were done in triplicates. The S-phase fractions varied between 15.9% and 30.95%. We also pulsed U251 cell cultures with bromodeoxyuridine (BrdUrd) and subsequently performed double staining for BrdUrd and the various cell type markers (Supplementary Fig. S1A). The immunofluorescence experiments confirmed that all subpopulations contained actively cycling cells. Moreover, there was no significant difference in the fraction of BrdUrd-positive cells between the groups (Supplementary Fig. S1B).

Subpopulations grouped by the various cell type markers produce tumors *in vivo*

Although previous reports have shown tumor development from CD133-negative glioma cells, we wanted to compare the tumorigenicity of CD133-positive and CD133-negative glioma cells in our model system. Thus, from one patient GBM
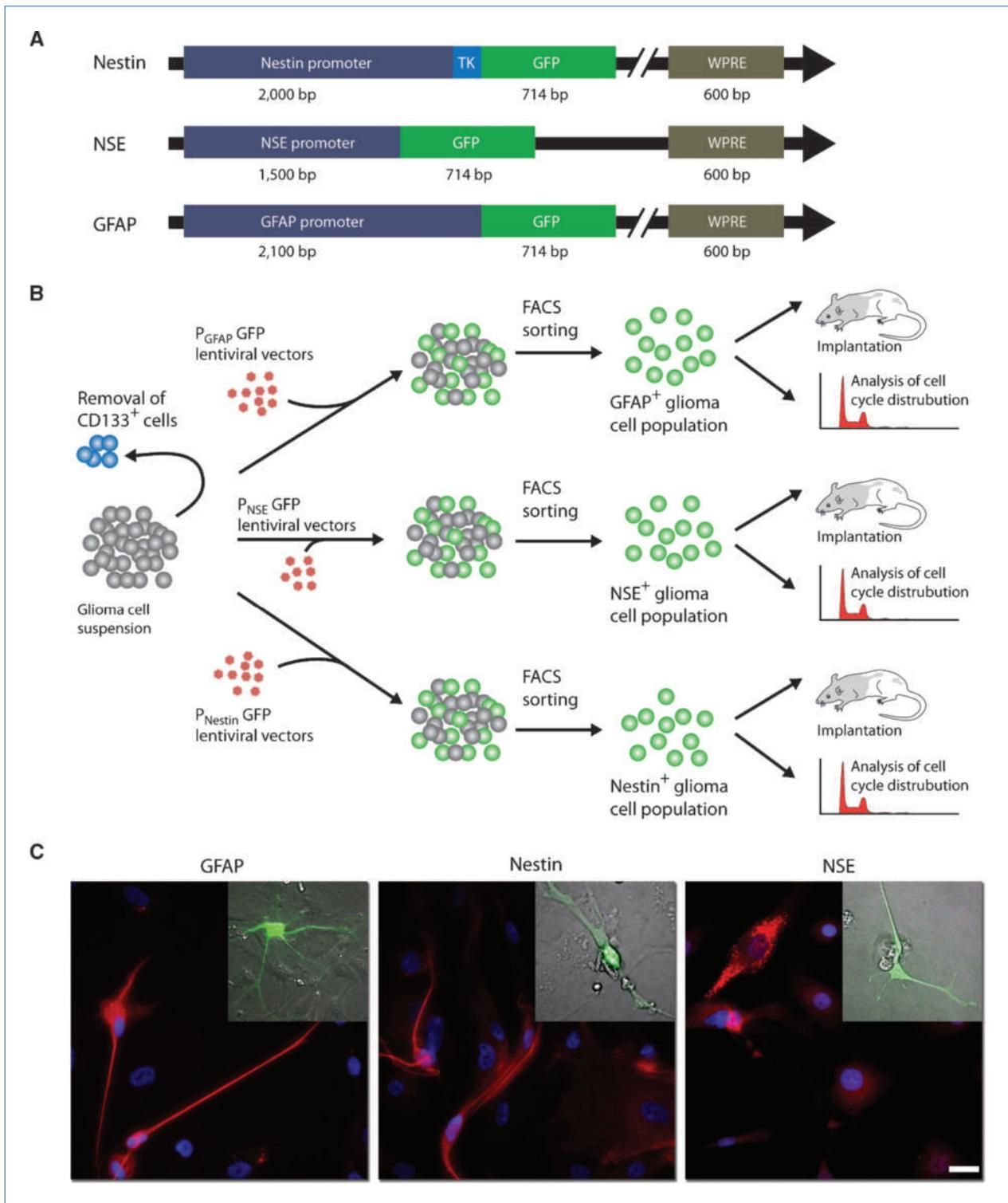
Figure 1. GFP lentiviral vectors show the expression of different cell type markers in glioma cell lines and biopsies. A, overview of the lentiviral vectors that were used. WPRE, woodchuck posttranscriptional regulatory element; TK, thymidine kinase. B, overall experimental design. CD133$^-$ glioma cells are lentivirally transduced to express GFP under lineage-specific promoters. GFP$^+$ cells are FACS sorted and analyzed for cell cycle distribution or xenografted in mice. C, immunocytochemical staining of one of the primary GBM cell cultures with antibodies against the different cell type markers as indicated (red). 4′,6-Diamidino-2-phenylindole counterstaining (blue) and GFP expression (green) under the corresponding lineage promoters (inset). Bar, 50 μm.
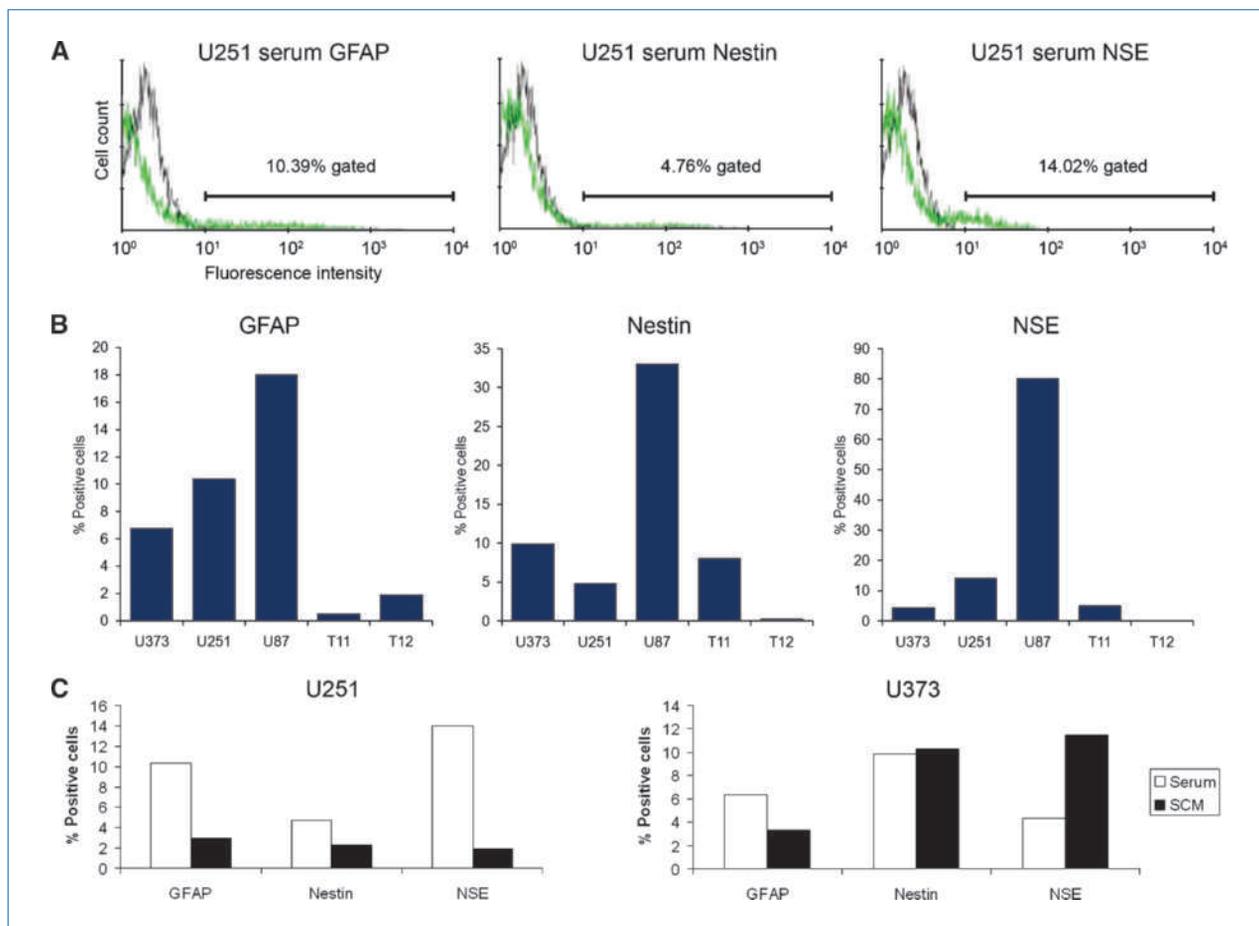
Figure 2. Expression of markers for the main CNS cell types vary with culture conditions. A, flow cytometry analysis of untransduced U251 glioma cells (black curve) and lentivirally transduced U251 cells (green curve) expressing GFP under the GFAP, Nestin, and NSE promoters (encircled in red). B, bar graph presentation of the fraction of positive cells for the markers as indicated in the cell lines and primary cultures. C, GFP expression in the U251 and U373 cell lines under the cell type promoters as indicated in serum-containing medium and in SCM.

xenograft, we isolated CD133-negative cells and CD133-positive cells that were transplanted intracerebrally in five and four mice, respectively. All mice produced tumors without significant differences in take rates or survival (Supplementary Fig. S2). Subsequently, NSE-, GFAP-, and Nestin-positive as well as Nestin-negative subpopulations were FACS sorted from the U373 glioma cell line and xenografted intracerebrally in mice in two separate experiments. In the last experiment, we FACS sorted glioma subpopulations from a patient GBM biopsy and xenografted these intracerebrally in SCID mice. All xenograft experiments were conducted after removal of CD133-positive cells, and remaining cells were confirmed CD133 negative by flow cytometry analysis and microscopy. In the U373 cell line, all subpopulations produced tumors, and the time from implantation to onset of symptoms varied from 25 to 118 days (Fig. 4A). We observed a slower disease course for the animals implanted with NSE-expressing cells than mice implanted with the other subpopulations, but there was no significant difference in survival

between the groups ($P = 0.67$). The accumulated tumor take rates for the U373 glioma subpopulations were >80% for all groups, with no significant difference between them (Fig. 4C; $P = 0.83$).

Similarly, subpopulations isolated from one of the patient biopsies uniformly produced clinically manifest tumors in SCID mice 55 to 141 days after implantation (Fig. 4B). One asymptomatic mouse implanted with Nestin^{+} cells had a small tumor when the experiment was terminated. Nestin^{+} cells and Nestin^{−} cells gave rise to tumors with 100% and 80% take rates, respectively, whereas only one of the mice in each group xenografted with GFAP[+] and NSE^{+} glioma cells developed tumors (50% and 33% take rates, respectively). There was no significant difference, neither in survival ($P = 0.24$) nor in take rates (Fig. 4B and C; $P = 0.14$). For the cell line and biopsy experiments collectively, the take rate was 93% for Nestin-expressing glioma cells, 90% for Nestin-negative cells, 75% for GFAP-positive cells, and 70% for NSE-positive cells ($P = 0.37$).
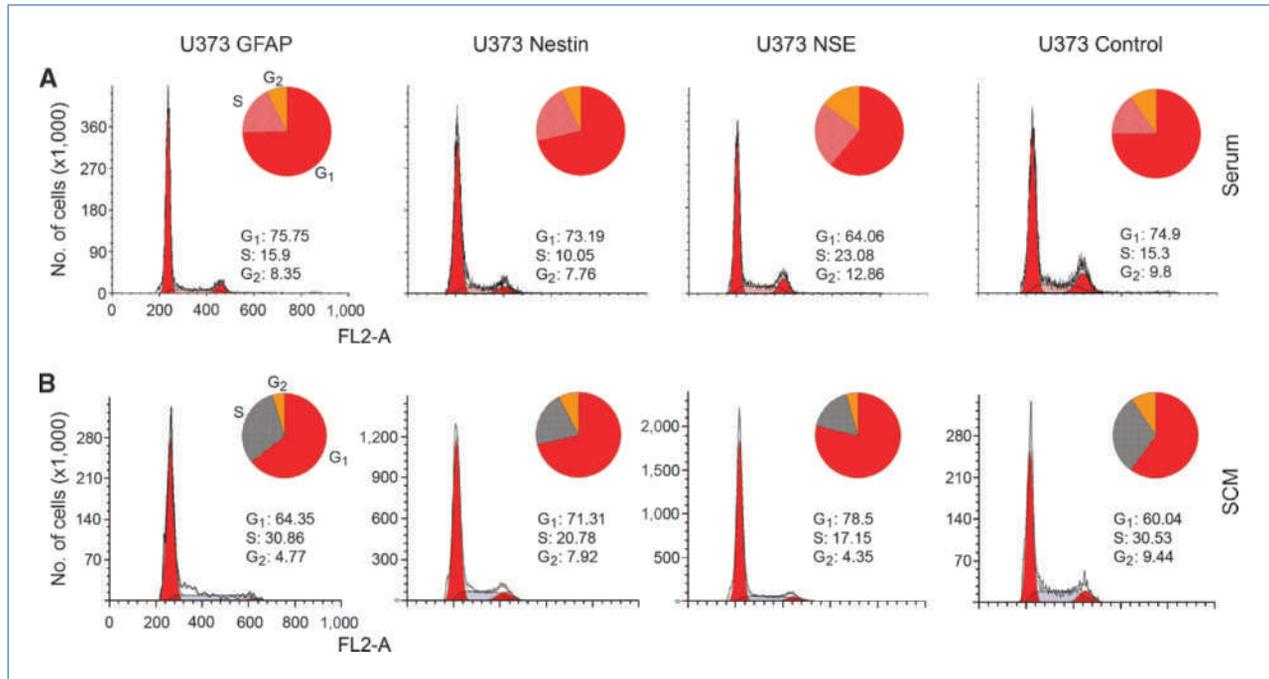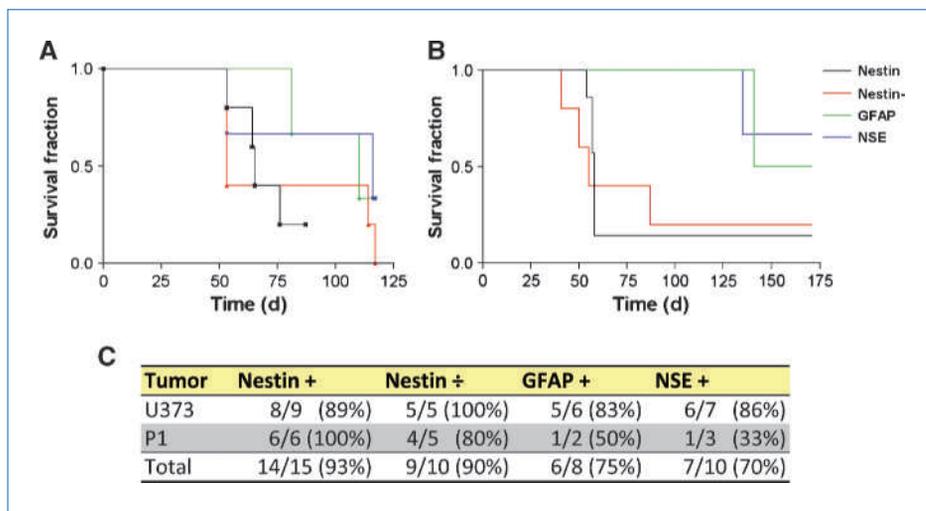
Figure 3. Glioma cell subpopulations proliferate *in vitro*. Cell cycle distribution in the U373 glioma cell line for the different subgroups as indicated in serum-containing medium (A) and in SCM (B).

Discussion

This work shows the existence of cell populations in glioma cell lines and human GBMs that express markers associated with different CNS cell types. This was a robust finding because experiments performed both in SCM and in serum-containing medium showed a stable presence of these subgroups. Moreover, cell cycle distribution analysis showed that all subgroups proliferated *in vitro*, both in SCM and in serum-containing medium. Still, it is conceivable that subpopulations grouped by such markers represent dis-

tinct functional entities that contribute to the phenotypes of human GBMs (10, 11). Thus, the heterogeneity observed in brain tumors may be paralleled by a similar diversity on a cellular level, in which different subpopulations of glioma cells are dedicated toward different functional roles. Notably, the upregulation of nestin at the periphery of the tumor bulk and in migrating glioma cells suggests a role for nestin-expressing cells in glioma invasion (12). Moreover, NG2-positive glioma cells have been shown to mediate angiogenesis and regulate vascular morphology in brain tumors (13, 14). Finally, glioma cells expressing immature markers

Figure 4. All CD133^{−} glioma cell subpopulations produce tumors *in vivo*. A, survival curves for mice xenografted with glioma cell subpopulations in one of the experiments using the U373 glioma cell line and (B) for mice xenografted with cell subpopulations from a GBM biopsy. C, accumulated tumor take rates for the two experiments with U373 glioma subpopulations (top row), for GBM biopsy–derived subpopulations (middle row), and for all experiments (bottom row).

Tumor	Nestin +	Nestin ÷	GFAP +	NSE +
U373	8/9 (89%)	5/5 (100%)	5/6 (83%)	6/7 (86%)
P1	6/6 (100%)	4/5 (80%)	1/2 (50%)	1/3 (33%)
Total	14/15 (93%)	9/10 (90%)	6/8 (75%)	7/10 (70%)

associated with stem cells and progenitors confer radioresistance and chemoresistance (15, 16), which is a typical feature in malignant gliomas.

These findings contrast to some extent those reported by Singh and colleagues, that glioma cells exhibiting proliferation and self-renewal were exclusively CD133 positive (3, 17). Yet, single-cell clonal analysis of C6 glioma cells shows that both CD133^{+} and CD133^{−} cells contain cancer stem–like cells (18). However, it is not known whether glioma cells expressing markers associated with mature cell types are tumorigenic. To address this issue, we isolated glioma cells by their expression of GFAP, Nestin, and NSE. Notably, all subgroups from the cell line as well as the human GBM biopsy produced tumors, with no significant differences in survival or tumor take rates. Although not significant, tumors developed later from glioma cells expressing GFAP and NSE, and there was a trend toward lower tumor take rates for these subpopulations. Thus, although tumorigenicity is common to different CD133^{−} subpopulations, they may display relative differences in growth kinetic parameters. Similarly, Kelly and colleagues reported a high frequency of tumorigenic leukemic cells after grafting into histocompatible mice (19). Accordingly, the low frequency of tumorigenic cells in xenotransplantations may reflect the limited ability of human tumor cells to grow in a foreign environment. Others showed that the frequency of tumorigenic melanoma cells increased in severely immunocompromised mice, also addressing the role of the environment (20). Still, our findings show the presence of different glioma cell subpopulations based on their expression of various CNS cell type markers, and investigating their biological roles should be the focus of future work.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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