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Isolation and Characterization of Tumorigenic, Stem-like Neural Precursors from Human Glioblastoma

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ABSTRACT

Transformed stem cells have been isolated from some human cancers. We report that, unlike other brain cancers, the lethal glioblastoma multiforme contains neural precursors endowed with all of the critical features expected from neural stem cells. Similar, yet not identical, to their normal neural stem cell counterpart, these precursors emerge as unipotent (astroglial) *in vivo* and multipotent (neuronal-astroglial-oligodendroglial) in culture. More importantly, these cells can act as tumor-founding cells down to the clonal level and can establish tumors that closely resemble the main histologic, cytologic, and architectural features of the human disease, even when challenged through serial transplantation. Thus, cells possessing all of the characteristics expected from tumor neural stem cells seem to be involved in the growth and recurrence of adult human glioblastomas multiforme.

INTRODUCTION

Cancer arises from a series of mutations occurring in few or single founder cells. From a therapeutic standpoint, a critical issue regards the elucidation of the identity and physiology of the cell(s) responsible for tumor formation, progression, and recurrence (1). With some exceptions (2), the identity of tumorigenic cells within many cancers remains unclear, but recent observations point to the involvement of somatic stem cells in oncogenesis (3). Embryonic and fetal stem cells may accumulate mutations during their expansion/growth phase throughout development and may form full-blown tumors in adulthood (3), whereas adult stem cells and their progeny may represent the direct target of oncogenic transformation in mature tissues (1). Accordingly, the involvement of cells displaying a stem cell phenotype in cancer formation and progression (1) has been confirmed in acute myeloid leukemia (4–6) and breast cancer (2).

If stem cells and/or their progeny represent the main target of neoplastic transformation, it is then reasonable to infer that the main foci of malignant transformation ought to coincide with those tissue regions that embody somatic stem cells. Indeed, the main areas of distribution of certain brain tumors overlap specific regions of the central nervous system (CNS; refs. 7 and 8) that comprise glia-like neural stem cells and their progeny (9, 10). Accordingly, deregulation of specific proto-oncogenes such as Ink4A/Arf, epidermal growth factor (EGF) receptor, and c-myc (11–13) or expression of SV40 T antigen (14) in glial fibrillary acidic protein (GFAP)- or nestin-expressing neural cells induces high-grade gliomas in brain areas that contain neural stem cells.

Recently, transformed, stem-like neural progenitors have been isolated and cultured from human brain tumor tissues (15–17), which raises fundamental issues concerning their actual ability to drive cancer growth and recurrence (18).

Here, we report the initial finding that glioblastoma multiforme, the most malignant among the adult human CNS tumors, comprises transformed precursors that bear the full complement of functional characteristics expected from stem cells (19), including the capacity for tumor generation. In fact, these cells can establish tumors with all of the classical features of human glioblastomas multiforme, even upon serial transplantation and, therefore, can be identified as tumor neural stem cells (20).

MATERIALS AND METHODS

Sample Classification. All of the samples were classified according to World Health Organization guidelines. Lines 0627, 0821, 0913, 1022, and 1205 were obtained from patients with diagnosis of primary glioblastoma; line 1030 was derived from a patient suffering from secondary glioblastoma. Patients from whom lines 0627, 0821, 0913, and 1022 were established underwent recurrence after 6 to 12 months post-surgery.

Primary Culture, Culture Propagation, Population Analysis, and Cloning. Tumor samples, either derived from patients or from orthotopic transplantation of glioblastoma multiforme–derived cell lines, were processed as by Gritti *et al*. (21). Primary cells were plated in 25 cm^2 tissue culture flasks plated at clonal density (2500–5000 cells/cm^2) in Dulbecco's modified Eagle's medium/F-12 medium containing 20 ng/mL of both EGF and fibroblast growth factor (FGF2; Peprotech, Rocky Hill, NY). As reference cell lines, normal human fetal neural stem cells (22) and the glioblastoma cell line U87MG were used in all of the assays. Population and serial subclonogenic analysis were performed as by Galli *et al*. (23).

Differentiation of Stem Cell Progeny and Immunocytochemistry. To assess for multipotency, cells were plated at a density of 2.5×10^4 cells/cm^2 onto Matrigel-coated glass coverslips (12 mm diameter) in the presence of leukemia inhibitory factor (10 ng/mL; ref. 24) for 7 to 10 days. Multiple immunofluorescence assays for neural antigens were performed as described by Gritti *et al*. (21) and Galli *et al*. (23).

Evaluation of Tumorigenicity by Subcutaneous Injection and by Orthotopic Injection. Tumorigenicity was determined by injecting glioblastoma multiforme–derived neural stem cells, U87, and human fetal neural stem cells, either subcutaneously (s.c.) or orthotopically. Cells (3×10^6) in 100 μL of PBS were s.c. injected into the right flank of *Scid/bg* mice while 2 μL of a 1×10^8 cells/mL cell suspension in PBS were delivered into the right striatum (0.2 μL/min) by stereotactic injection through a glass electrode connected to a Hamilton syringe. The following coordinates were used: antero-posterior = 0; medio-lateral = +2.5 mm; dorso-ventral = −3.5 mm. Mice were sacrificed at different times between 1 and 10 weeks post-injection, according to the cell line originally injected. Hematoxylin and eosin staining and immunohistochemistry were performed on 15-μm-thick cryostat sections. Sections were processed as by Vescovi *et al*. (22). Antibodies/antisera used were as follows: mouse antivimentin (1:200; Chemicon, Temecula, CA), mouse antihuman mitochondria (1:20; Chemicon), mouse antihuman nuclei (1:20; Chemicon), mouse anti-Galactocerebroside C (Gal C; 1:20; Chemicon), mouse antineuronal class III β-tubulin (Tuj1; 1:500; Babco, Richmond, CA), rabbit anti-GFAP (1:1000; Dako Corporation, Carpinteria, CA), rabbit antilaminin (1:200; Sigma, St. Louis, MO), and rabbit anti-Ki67 (1:1000; NovoCastra, Newcastle, UK).

Chromosome Analysis. Cells were treated with medium containing 10 μg/mL Colcemid (Irvine Scientific, Santa Ana, CA) for 1 to 2 hours and resuspended in hypotonic 1% sodium citrate at room temperature for 30

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minutes. The cells were then washed in methanol-acetic acid (3:1, v/v) fixative solution for 30 minutes and spread onto clean dry slides. Q-banding staining was then performed, and 10 metaphases were analyzed for each sample.

Telomeric Repeat Amplification Protocol and Telomere Length Assessment. Telomerase activity was detected using the PCR-based TRAPeze Telomerase Detection kit (Intergen, Purchase, NY), according to the manufacturer's instructions. Total proteins (150 ng) were loaded into each lane. For the telomere length assay, the length of the telomeres was determined using TeloTAGGG Telomere Length Assay (Roche, Gipf-Oberfrick, Switzerland) according to the manufacturer's instructions. Two μg of genomic DNA of each sample were digested with a *Hin*fI/*Rsa*I mixture for 2 hours at 37°C and then loaded into a 0.8% agarose gel.

Molecular Analysis. One μg of total RNA from tumor neural stem cells, U87, and human fetal neural stem cells, extracted using the RNeasy Mini kit (Qiagen, Chatsworth, CA), was primed with oligo(dT) for cDNA synthesis and reverse-transcribed by using Superscript RNase H$^-$ Reverse Transcriptase (Life Technologies, Rockville, MD). All cDNAs used as templates were previously normalized throughout a β-Actin reverse transcriptase-PCR. Sequence of primers and PCR conditions are available on request.

RESULTS

Selection and Expansion of Neural Precursors from Adult Human Brain Tumors. To assess for the presence of neural precursors within human brain tumors, cells from post-surgery specimens of low-grade gliomas, glioblastoma multiformes, or high-grade medulloblastomas were plated at clonal density (21) in serum-free medium, containing EGF and FGF2. This provides a stringent, low-density system ($<5 \times 10^3$ cells/cm^2), which selects away differentiated/differentiating cells in primary CNS cultures, leaving neural stem cells free to proliferate and expand exponentially (25, 26).

Twenty to 40 days after plating, phase-bright clones resembling the classical neurospheres formed *in vitro* by normal neural stem cells (ref. 27; Fig. 1*A* and *B*) were detected in all of the cultures established from high-grade glioblastoma multiformes and medulloblastomas. Clonal frequency was between 0.5 and 31% of the total cells plated for glioblastomas multiforme ($n = 12$) and 50 and 80% for medulloblastomas ($n = 4$). Conversely, clone formation was never observed in cultures from either World Health Organization grade I gliomas or World Health Organization grade II oligodendrogliomas ($n = 5$; up to 90 days *in vitro*). These data confirm and extend previous findings (15–17), which show that EGF/FGF2-responsive precursors are specifically found in glioblastomas multiforme and medulloblastomas.

Glioblastomas Multiforme Contain Multipotent Precursors Endowed with Long-term Self-renewal. The generation of clones from disaggregated primary cancers provides an index of the cancer clonogenicity (20). However, the sole formation of neurospheres in cultured CNS tumors does not provide, *per se*, the demonstration of

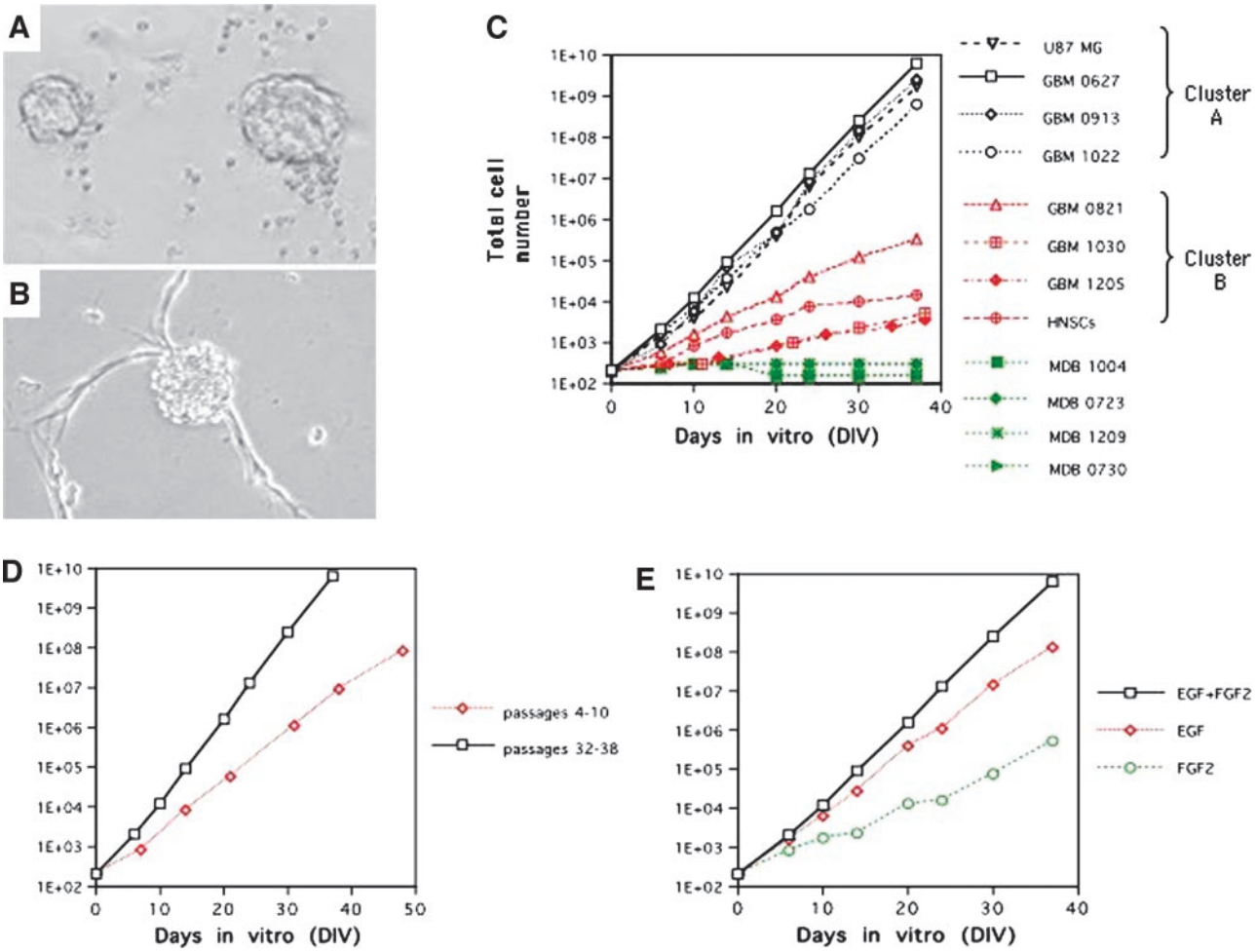


Fig. 1. Analysis of long-term proliferation in glioblastoma multiforme–derived tumor neural stem cells. Phase-bright microphotographs showing examples of glioblastoma multiforme–derived (*A*) and medulloblastoma-derived (*B*) neurospheres; ×20 objective. *C.* Intrinsic differences in the growth rate were observed among cluster A, consisting of faster-growing tumor neural stem cells such as 0627, 0913, and 1022 (*black lines*), and cluster B, comprising slower-expanding glioblastoma multiforme (*GBM*) cell lines 0821, 1030, and 1205 (*red lines*). Although medulloblastoma (*MDB*) tissues displayed the highest content of clonogenic cells (up to 80%), they never established tumor neural stem cells lines (*green lines*). *D.* Differently from normal human fetal neural stem cells (22), glioblastoma multiforme–derived tumor neural stem cells displayed different rates of amplification at low- and high-subculturing stages, as shown for cell line 0627. *E.* The exposure of cell line 0627 to EGF or FGF2 influenced the proliferation rate, which was higher in the presence of both mitogens and lower when using only one factor, as shown previously for normal human fetal neural stem cells (22).

the presence of tumor neural stem cells therein. Transiently amplifying precursors are also known to produce neurospheres in this system, and they can even undergo a limited number of passages in culture (20, 28). In addition, neurosphere formation may also result from spontaneous cell aggregation, usually as the consequence of excessive plating density (29).

Hence, we determined whether the clonogenic cells in our primary glioblastoma multiforme and medulloblastoma cultures represented transient clonogens as generated by short-term proliferating, transit amplifying cells or possessed the cardinal properties expected from cultured tumor neural stem cells (20). To this end, we assessed their capacity for long-term proliferation, self-renewal, multipotency (the ability to generate the three major neural cell types, *i.e.*, neurons, astroglia, and oligodendroglia) and their tumorigenicity (27, 30–32), by combining cloning, subcloning, and population analysis followed by orthotopic, heterotopic, and serial transplantation assays.

One of the primary consequences of dealing with candidate stem cells endowed with extensive self-renewal capacity is the establishment of steadily expanding, stable lines (19, 28). Surprisingly, although medulloblastoma cultures displayed the highest clonogenic capacity, they failed in establishing long-term cell lines (four of four specimens) under the conditions normally used for neural stem cells expansion. In agreement with previous findings (16, 17), medulloblastoma cultures could be propagated for a few passages (Fig. 1*C*), although in our hands, they could only generate Tuj1-immunoreactive cells when triggered to differentiate (data not shown). Conversely, under identical culture conditions, one-half of the 12 cultures established from glioblastomas multiforme comprised multipotent precursors, which established long-term expanding cultures. These six different glioblastoma multiforme–derived lines were expanded for more than 80 passages, with an average doubling time of 3 to 4 days (*i.e.*, 180–280 days *in vitro*), and were characterized as tumor neural stem cells through the comparison with normal human fetal neural stem cells (22) and with the well-characterized human glioblastoma cell line U87MG.

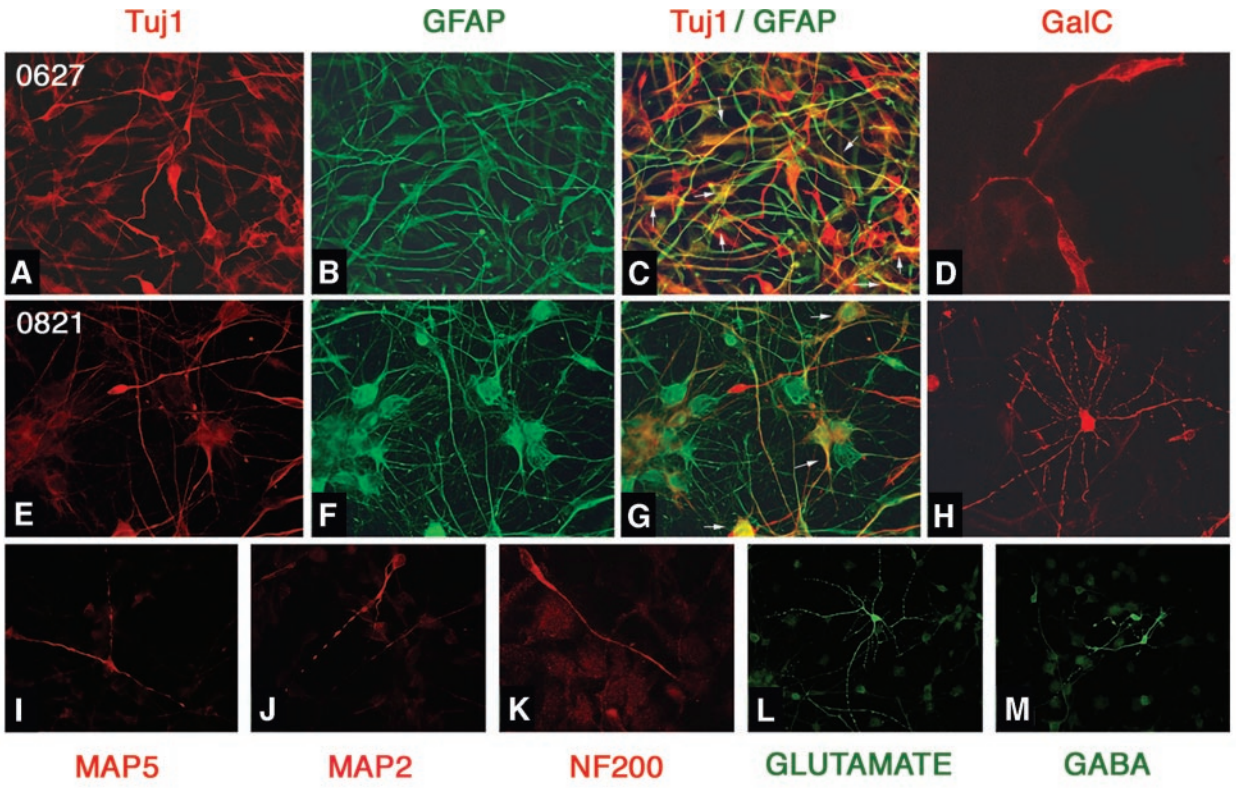
Human glioblastoma multiforme cell lines were named after the date of surgery: 0627, 0821, 0913, 1022, 1030, and 1205. When their growth rate was assessed, significant differences between specific lines emerged (Fig. 1*C*). Nonetheless, despite the fact that the kinetics of expansion was characteristic for each tumor cell line and clearly depended upon the specimen of origin, all of the lines exponentially expanded in number, similarly to normal human fetal neural stem cells and U87 cells and as opposed to their medulloblastoma counterpart (Fig. 1*C*). Based on growth properties, two distinct clusters emerged: cluster A, comprising faster-growing lines 0627, 0913, and 1022; and cluster B, consisting of slower-expanding lines 0821, 1030, and 1205 (Fig. 1*C*). Of note, whereas normal human fetal neural stem cells displayed steady growth capacity over long-term culturing (22, 33), the proliferative activity of glioblastoma multiforme–derived cell lines increased at very late subculturing stages (120–150 days *in vitro*) compared with earlier passages (15–35 days *in vitro*; Fig. 1*D*, in reference to line 0627). This may reflect their progressive enrichment in cells endowed with aberrant proliferative advantage, the tumor cell adaptation to the culture conditions, or the intrinsic, genetic fluctuation expected from tumor cells. Nonetheless, this phenomenon was the only hint as to a possible, latent instability of glioblastoma multiforme–derived cell lines, which displayed an otherwise remarkably stable cellular and molecular phenotype and karyotype (see next sections).

Additional proof that tumor neural stem cells were the likely founders of glioblastoma multiforme–derived cell lines came from the observation that single glioblastoma multiforme cells could give rise to clonal cell lines, which behaved identically to their parental (*i.e.*, bulk culture) cell lines (not shown). These clonal tumor neural stem cells were used in all of the experiments described from now on.

We continued by analyzing the response of our candidate tumor neural stem cells to mitogens that regulate proliferation in normal neural stem cells (22, 34, 35). As described for adult rodent neural stem cells (34), glioblastoma multiforme–derived cell lines expanded slowly in the presence of the sole FGF2, switched to a faster growth mode when exposed to EGF alone, and expanded even faster when exposed to both mitogens simultaneously (Fig. 1*E*, cell line 0627). Thus, glioblastoma multiforme cells respond to the same mitogenic cues that control the fate of normal neural stem cells.

We then assessed tumor neural stem cell multipotency by determining the ability of clonal glioblastoma multiforme–derived cells to generate neurons, astrocytes, and oligodendrocytes. Early passage (passages 10–15) clonally derived glioblastoma multiforme cells underwent terminal differentiation upon removal of mitogens (34, 36) and the subsequent addition of leukemia inhibitory factor to the medium, as shown previously for human fetal neural stem cells (24). Similar to normal neural stem cells (34), all of the glioblastoma multiforme clonal lines differentiated into GFAP-positive astrocyte-like cells (Fig. 2*B* and *F*) but also into neuron-like cells, which were immunoreactive for Tuj1 (Fig. 2*A* and *E*), MAP5 (Fig. 2*I*), MAP2 (Fig. 2*J*), neurofilament M_r 200,000 (NF200; Fig. 2*K*), glutamate (Fig. 2*L*), and γ-aminobutyric acid (Fig. 2*M*) and into GalC-immunoreactive oligodendrocyte-like cells (Fig. 2*D* and *H*). Conversely, U87 cells never gave rise to cells immunoreactive for any of the above pan-neural antigens, thus demonstrating that multipotency is a unique property of glioblastoma multiforme–derived cells. Such multipotency was maintained unaltered even after extensive culturing, up to passage 80 (not shown). Notably, glioblastoma multiforme–derived, differentiated cultures comprised a fraction of cells, which colabeled, promiscuously, with neuronal and glial markers (Fig. 2*C* and *G*). This abnormal type of cells was never observed in normal human fetal neural stem cells and likely reflects the activation of an aberrant differentiation program in tumor neural stem cells, as expected from developmentally deregulated tumor cells. Intriguingly, this same fraction of promiscuously double-labeled cells varied between each cell line and correlated with the proliferation capacity index, with cluster A glioblastoma multiforme cells (fast-growing), showing the highest content of abnormal end cells, and cluster B lines (slow-growing), displaying a smaller proportion and a behavior somewhat reminiscent of normal human fetal neural stem cells (Fig. 2, *table*).

Ultimately, the self-renewal capacity of glioblastoma multiforme cells was assessed directly, using subcloning experiments by which the number of stem cells in a given clonal neurosphere can be determined by assessing the number of secondary neurospheres generated after dissociation of the clone itself (23, 28, 34). This measure also provides both an estimate of the relative frequencies between symmetric proliferative divisions and symmetric differentiative divisions and an indirect index of the capacity of the stem cell population for size expansion (23, 28, 37). The generation of multiple secondary clones could be observed in all of the lines tested (line 0627, 68.0 ± 5.0; line 0913, 55.3 ± 4.0; line 1022, 85.0 ± 5.0; line 0821, 28.0 ± 8.0; line 1030, 26.1 ± 2.2; line 1205, 63.8 ± 4.0; human fetal neural stem cells, 37.9 ± 6.0; U87, 90.0 ± 5.0; means ± *SEM*, $n = 4$, Student's *t* test, $* = P < 0.001$). When the six different cell lines were compared with each other, a significantly lower frequency of proliferative symmetric divisions was detected in some of the slow-growing cell lines (0821 and 1030) compared with the other lines. This is indicative of an inherent difference in the self-renewal capacity between the different glioblastoma multiforme–derived tumor neural stem cell lines, which strictly correlates with their clonogenic ability and their growth profile.



	βtub-IR cells (%)	GFAP-IR cells (%)	βtub/GFAP-IR cells (%)	GalC-IR cells (%)
0627	16 ± 2.7	25 ± 2.8	58 ± 2.4	4.8 ± 0.1
0913	18 ± 0.9	34 ± 3.3	41 ± 1.3	1.7 ± 0.1
1022	21 ± 1.5	29 ± 2.7	37 ± 0.9	2.7 ± 0.2
0821	18 ± 1.2	60 ± 0.9	22 ± 1.5	2.1 ± 0.1
1030	9 ± 0.9	21 ± 0.6	27 ± 0.9	1.3 ± 0.3
1205	5.5 ± 1.0	47 ± 1.4	44 ± 0.8	1.3 ± 0.6
HNSCs	29 ± 2.6	12.4 ± 2.1	negative	1.8 ± 0.7
U87	negative	negative	negative	negative

Fig. 2. Differentiation potential of fast- and slow-expanding tumor neural stem cells. Analysis of the multipotency of the fast-growing line 0627 (representative of cluster A, passage 13; *A–D*) and slow-growing line 0821, (representative of cluster B, passage 15; *E–H*) by immunofluorescence for neuronal (*Tuj1*; *red* in *A*, *C*, *E*, and *G*), glial (*GFAP*; *green* in *B*, *C*, *F*, and *G*) and oligodendroglial (*GalC*; *red* in *D* and *H*) markers. Additional markers of neuronal differentiation were also detected [line *0821*, *MAP5*, *MAP2*, and *NF200*, *red* in *I*, *J*, and *K*; glutamate and γ-aminobutyric acid (*GABA*), *green* in *L* and *M*]. Cells that colabeled promiscuously with both neuronal and glial markers (*arrows* in *C* and *G*) could be observed in all of the cell lines established; magnification, ×20. The table shows a quantitative analysis of the differentiation capacity of the various glioblastoma multiforme–derived tumor neural stem cell lines, normal human fetal neural stem cells, and U87 cells with respect to their ability to generate cells of the three major neural lineages and to cells that display aberrant, promiscuous neuronal/astroglial colabeling.

These findings show that glioblastoma multiformes contain multipotent, long-term self-renewing, population-expanding cells that satisfy all of the defining criteria expected from tumor neural stem cells *in vitro*. Nonetheless, because the most critical attribute of tumor stem cells is their capacity to generate and perpetuate their tumor of origin (20), we sought to verify whether our glioblastoma multiforme–derived tumor neural stem cells could function as tumor-founding cells by assessing their actual ability to give rise to tumors *in vivo*.

The *In vivo* Tumorigenic Potential of Tumor Neural Stem Cells. When injected into immunosuppressed animals, both s.c. and intracranially (i.c.), all of the six tumor neural stem cell lines reproducibly established tumors, with a take efficiency of 50% s.c and 100% orthotopically, whereas normal human fetal neural stem cells always failed. When compared with classical U87-derived tumors, tumor neural stem cell–derived gliomas developed at a much slower rate. The first appearance of well-defined tumor masses could be observed after 1 week s.c. and 3 weeks i.c. in U87-treated animals, whereas it took at least 6 weeks s.c. and 7 weeks i.c. to observe the same extent of tumorigenesis in glioblastoma multiforme–derived, tumor neural stem cell–transplanted animals.

Histopathologic analysis of s.c. generated tumor neural stem cell–derived tumors demonstrated an unprecedented, striking glioblastoma-like tissue pattern. This was characterized by the presence of (1) several areas of necrosis surrounded by typical pseudo-palisade structures (Fig. 3*A*), (2) elevated vascular proliferation as demonstrated by laminin staining (Fig. 3*B*), (3) GFAP immunoreactivity (Fig. 3*C*), and (4) many mitotic figures, as measured by Ki67 (not shown), which were significantly higher than U87-derived tumors (3.68 ± 0.58% for line 0627-derived tumor *versus* 1.38 ± 0.13% for U87-derived tumor; $n = 4$, $P < 0.01$, Student's t test). Notably, tumors derived from U87 implantation never displayed any glioblastoma-specific feature. Rather, they were characterized by the presence of cells with globoid

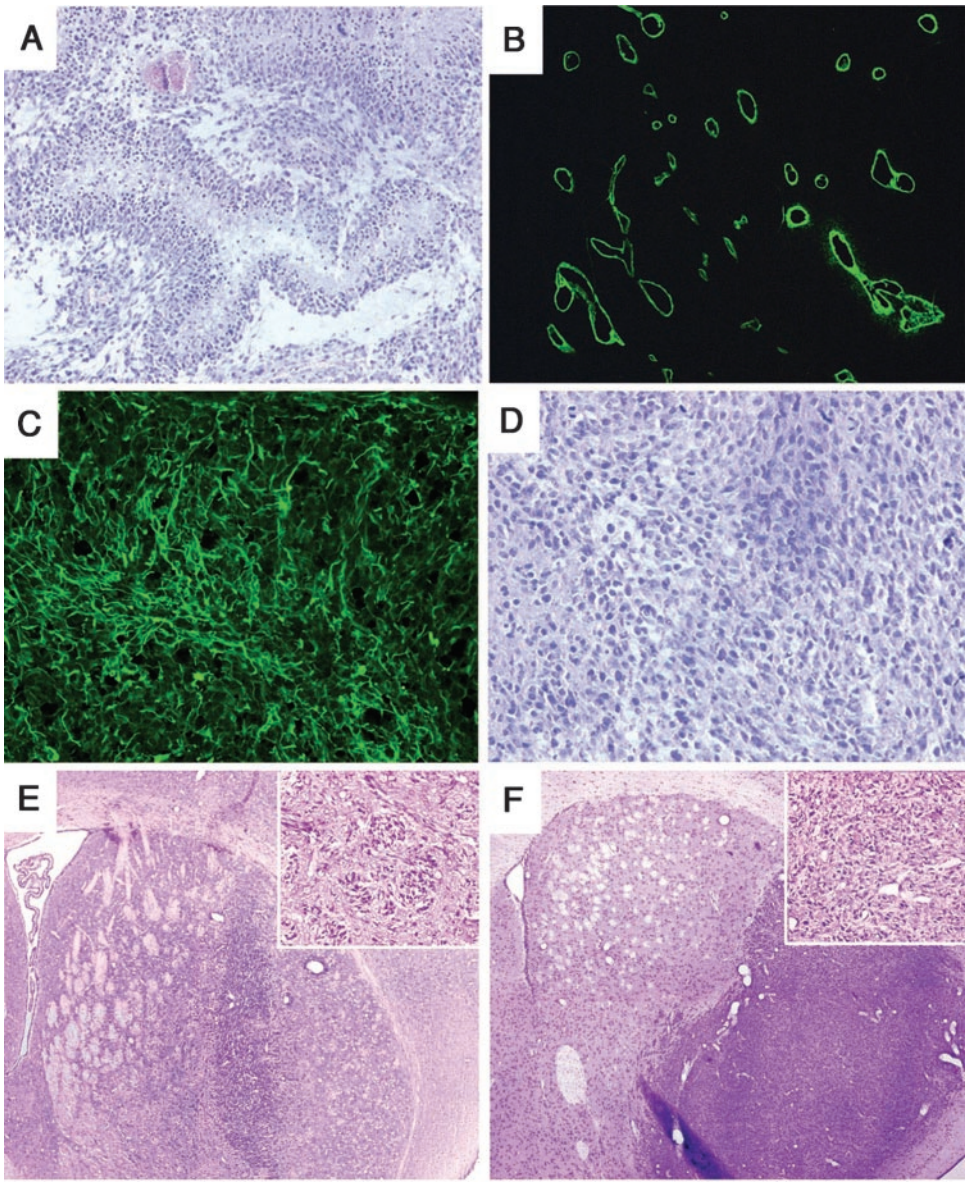


Fig. 3. Histopathologic features of tumor neural stem cell–derived s.c. and i.c. tumors. *A*. Large areas of necrosis surrounded by highly packed tumor cells ("pseudopalisade") were identified within the tumors generated by s.c. transplantation of tumor neural stem cells into *scid/bg* mice. Extensive neovascularization of the tumor tissue by laminin staining (*B*; ×10 objective) as well as widespread and intense GFAP immunoreactivity (*C*; ×20 objective) could be retrieved. *D*. U87-derived tumors did not display any of the peculiar histomorphologic features, typical of highly malignant gliomas. Tumor neural stem cell–generated i.c. tumors were characterized by a peculiar morphology (*E*; ×4 objective), and nest-like formations, reminiscent of mitotically active anomalous glial cells, could be identified in the core of the lesion (*inset* in *E*), as opposed to U87-derived tumors (*F*), which consisted of highly packed undifferentiated small rounded single mitotic elements (*inset* in *F*).

cytoplasm, which lacked fibrillary structures and hyperchromatic nuclei, the typical hallmarks of highly malignant gliomas (Fig. 3*D*).

Strikingly, when transplanted orthotopically, tumor neural stem cells were able to form i.c. tumors, which displayed an elevated extent of proliferation and an exacerbated migratory and infiltration capability, distinctive of human glioblastomas multiforme. As shown by hematoxylin and eosin staining, tumor neural stem cells generated highly anaplastic tumors, characterized by marked nuclear atypia and high mitotic index (hematoxylin and eosin in Fig. 3*E*). Conversely, unlike any typical glioblastoma, U87 cells gave rise to enormous but well-delimitated tumor masses, which developed strictly within the area of injection (hematoxylin and eosin in Fig. 3*F*). On the same line, the i.c. tumors derived from tumor neural stem cells displayed a very peculiar histomorphology. Six weeks after implantation, peculiar nest-like structures, composed of highly mitotic polymorphic cell nuclei, identified as aberrant glial elements, could be retrieved especially in the center of the lesion (Fig. 3*E*, *inset*). Due to the physical constraints imposed by the walls of the skull, the maximum level of growth reached by i.c. tumor neural stem cell–derived tumors did not allow the formation of clear areas of necrosis (pseudo-palisades), as opposed to s.c. tumors, which were allowed to grow up to 5 cm^3. In agreement with findings on s.c. tumors, also orthotopic U87-derived lesions did not show any cyto-architectural similarity with the actual human disease (Fig. 3*F*, *inset*).

Most importantly, by staining with human-specific antimitochondria, tumor neural stem cell–derived tumor cells were shown to escape the site of transplantation, infiltrating both the adjacent parenchyma and the corpus callosum, even extending into the contralateral hemisphere, as bipolar migrating cells (Fig. 4*A*). Consistent with our *in vitro* findings and with the study of Hemmati *et al*. (17), when passage seventh medulloblastoma-derived cells were transplanted orthotopically, after 6 weeks from transplantation, no sign of tumor formation could be observed, and the transplanted cells did not migrate significantly from the graft (Fig. 4*B*). The same noninfiltrative behavior could be observed in U87-derived tumors (Fig. 4*C*).

Notably, when i.c. tumors generated by glioblastoma multiforme–derived tumor neural stem cells were characterized for the expression of specific antigens, they displayed an intense mitotic activity as shown by Ki67 staining (Fig. 4*D*, double-labeled cells in *yellow*), whereas the graft established by medulloblastoma cells contained fewer proliferating cells (Fig. 4*E*). Moreover, the tumors established by glioblastoma multiforme–derived tumor neural stem cells were

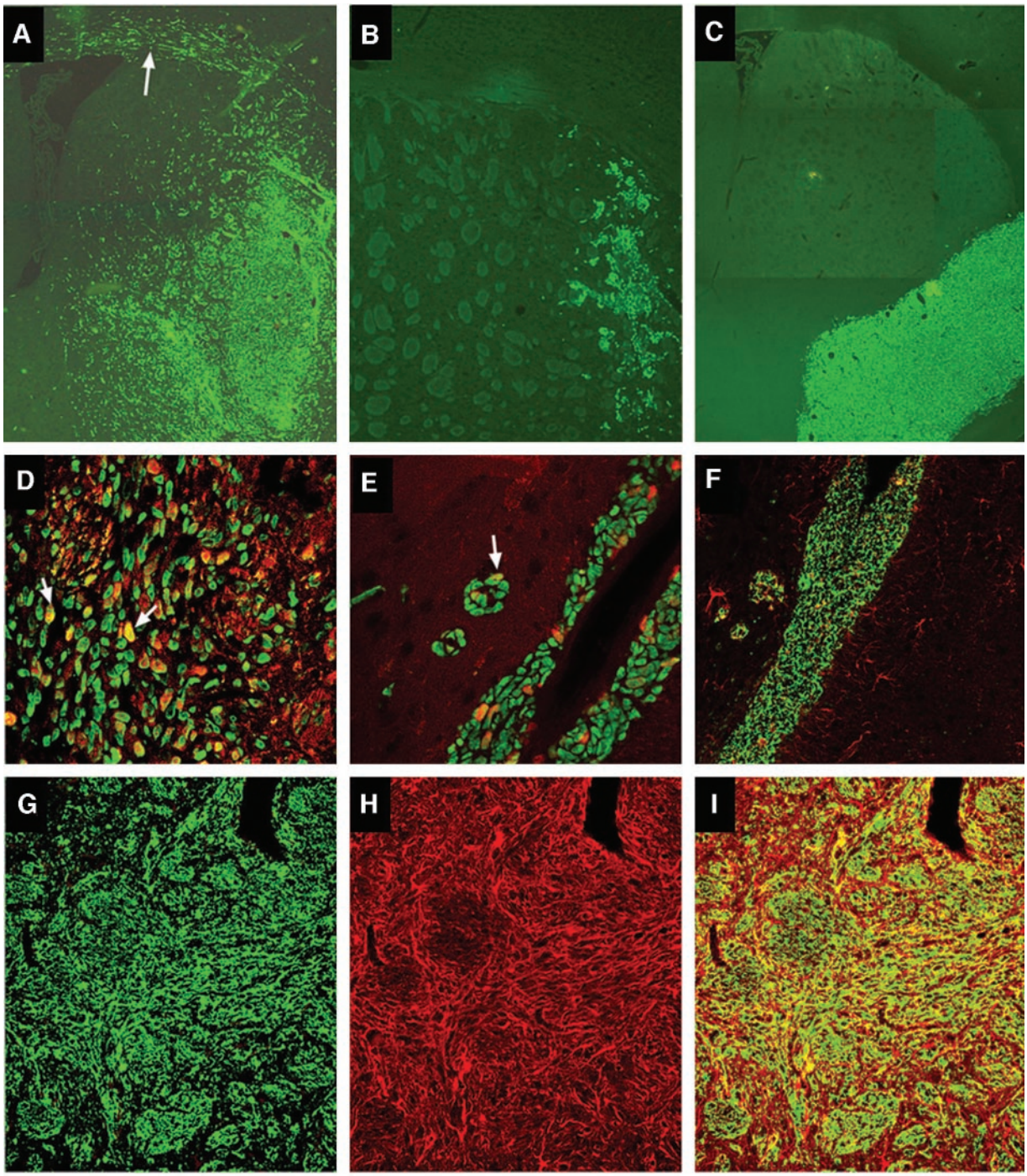


Fig. 4. Tumor neural stem cells xenografted i.c. generate tumors, which reproduce the intraparenchymal invasion pattern, typical of human glioblastoma multiformes. Six weeks after orthotopic implantation, tumor neural stem cell–derived tumors (*A*; human-specific antimitochondria staining in *green*) were characterized by an expanding area of growth, located in the center of the tumor mass and by a surrounding "penumbra," from which highly migratory cells departed, infiltrating the adjacent parenchyma and white matter (*A*, *arrow*; ×4 objective). At the same time point, medulloblastoma–derived grafts (*B*; human-specific antinuclei staining in *green*; ×10 objective) exhibited very poor capacity for migration, spreading, and invasion, if any. Notably, U87-derived tumors (*C*; human-specific antimitochondria staining in *green*; ×4 objective) were quite large in size, but cells did not exhibit any migratory capacity, so that these tumors consisted of a progressively enlarging, well-defined cell mass, which remained confined to the site of injection. An elevated mitotic index was exhibited by i.c. tumor neural stem cell tumors (*D*; Ki67 in *red* and human nuclei in *green*; ×40 objective; *arrows* point to examples of double-labeled cells), whereas very few cells within the medulloblastoma-derived grafts were indeed proliferating (*E*; Ki67 in *red* and human nuclei in *green*; ×40 objective; *arrow* pointing to a double-labeled cell). In medulloblastoma grafts, we could never find cells that expressed astroglial markers [*F*; GFAP in *red* and human mitochondria in *green*; ×20 objective; note that GFAP immunoreactivity (*red*) is confined outside the graft mass (*green*)]. Conversely, some cells in glioblastoma multiforme–derived tumor neural stem cell–generated tumors were found to label with the anti-GFAP antibody (*G*, human mitochondria in *green*; *H*, anti-GFAP in *red*; merged confocal image in *I*; ×20 objective).

immunoreactive for the astroglial-specific marker GFAP (Fig. 4*G--I*) and vimentin (not shown) and negative for neuronal (Tuj1) and oligodendroglial antigens (GalC), just like typical human glioblastomas multiforme (not shown). Similar to grafted U87 cells, medulloblastoma implants did not display immunoreactivity for astroglial (Fig. 4*F*), neuronal, or oligodendroglial antigens (not shown).

Serial Transplantation of Tumor Neural Stem Cells. To conclusively demonstrate the stemness of glioblastoma multiforme–

derived tumor neural stem cells, we performed sequential transplantation experiments. This was done in analogy with the classical hemopoietic serial repopulation paradigm used to identify true hemopoietic stem cells (38). Cultured tumor neural stem cells (primary tumor neural stem cells) were transplanted into the mouse CNS to establish a tumor from which secondary tumor neural stem cells were recultured and then transplanted into new recipients. The successful, sequential generation of brain tumors provided evidence that we had isolated *bona fide* tumor neural stem cells.

Tumor neural stem cells, isolated from post-surgery specimens, were expanded, cloned, and injected orthotopically, giving rise to brain tumors. At the first sign of neurologic impairment (8–10 weeks after transplantation), as produced by the expanding tumor mass (Fig. 5*A* and *E*), animals were sacrificed. The tumor was explanted by careful dissection, which excluded the periventricular region of the forebrain, highly enriched for stem cells and progenitors (ref. 9; Fig. 5*B–D*), and the cells were cultured under the same conditions used to establish the original tumor neural stem cell cultures. Clonal neurospheres were readily detected in these secondary cultures, which exclusively contained cells that labeled positive with human-specific markers and that were named post-transplantation–tumor neural stem cells (Fig. 5*F*). Generation of primary spheres from tumor neural stem cell–induced tumors occurred much faster than parental, primary spheres, established from the original patient's specimen (3 days for post-transplantation–tumor neural stem cells compared with 20–40 days for primary, parental tumor neural stem cell lines). Cell lines established from the post-transplantation–tumor neural stem cells also expanded at a faster rate than their parental tumor neural stem cells

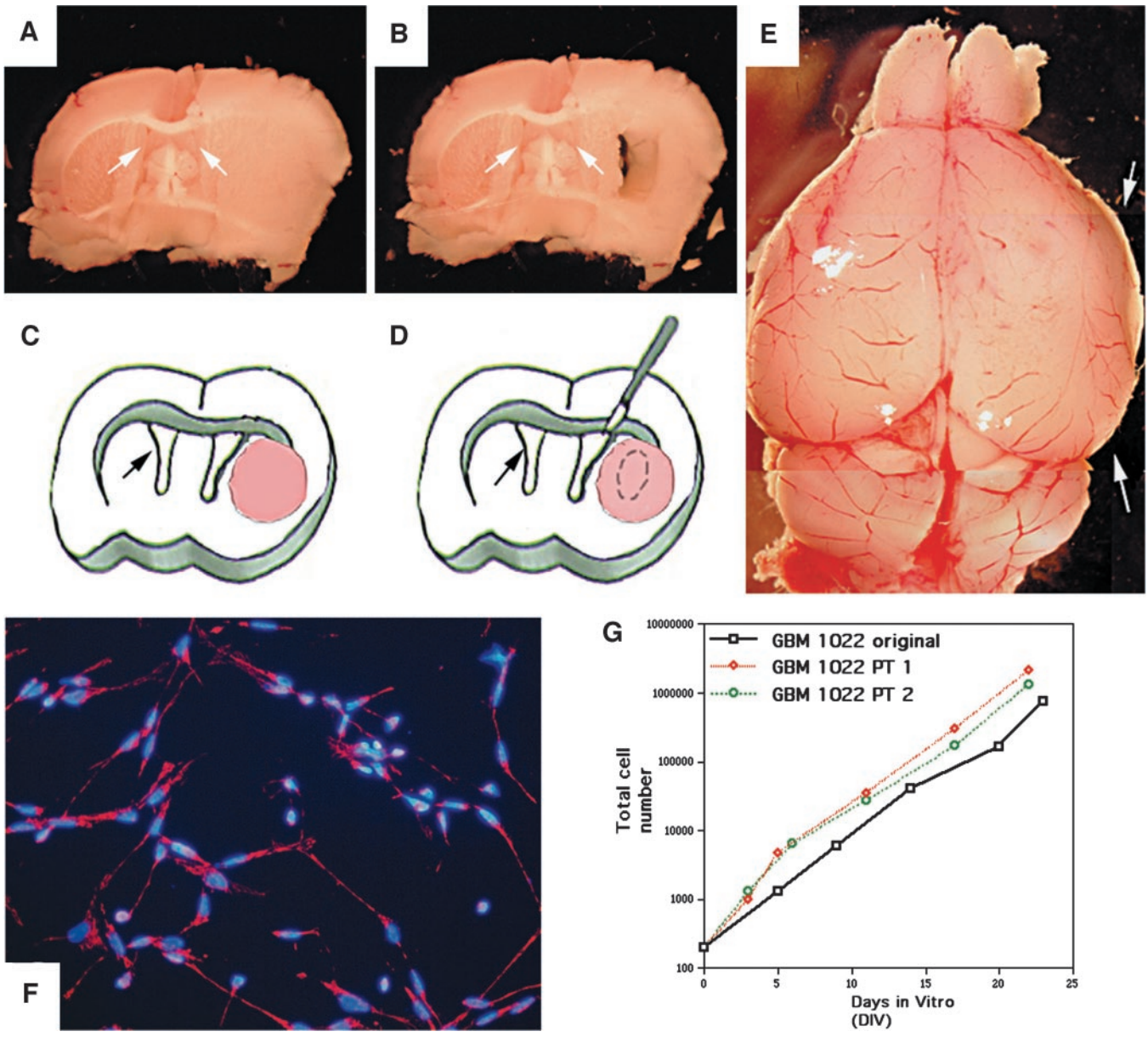


Fig. 5. Tumor neural stem cells self-renew *in vivo*, upon serial transplantation. The tumor mass established from the transplanted human tumor neural stem cells was dissected out at the first sign of neurologic impairment, taking particular care of not including the stem cell–enriched periventricular region (*arrows* in *A–D*). This procedure was facilitated by the large size of the in-growing tumor mass, which produced a macroscopic enlargement of the injected cerebral hemisphere (*E*, *arrows*). The tumor cells were then dissociated and placed in culture under conditions identical to those used to establish tumor neural stem cell lines from the original human specimen. This resulted in the establishment of secondary tumor neural stem cell (*PT-TNSCs*) lines, which contained exclusively cells of human origin, with no sign of rodent cell contamination (*F*; human-specific mitochondria staining in *red* and nuclear 4′,6-diamidino-2-phenylindole staining in *blue*; ×20 objective). *G*, Post-transplantation–tumor neural stem cells were able to long-term expand in culture, even faster than the original, parental tumor neural stem cell line. *GBM*, glioblastoma multiforme.

(line glioblastoma multiforme 1022 as reference; Fig. 5*G*). This suggests that the malignancy of serially transplanted post-transplantation–tumor neural stem cells might have been exacerbated by the exposure to the *in vivo* environment or that particularly aggressive cells had been selected upon transplantation, tumor growth, and reculturing.

When post-transplantation–tumor neural stem cell lines were differentiated under the same conditions of their parental ones, all of the three cell types could be detected. Thus, post-transplantation–tumor neural stem cells retained multipotency after transplantation and reculturing (20 ± 2.1% of Tuj1-IR cells; 52 ± 5.4% of GFAP-IR cells; 24 ± 0.7% of Tuj1/GFAP cells, $n = 10$, two different secondary glioblastoma multiforme 1022 cell lines analyzed). Furthermore, post-transplantation–tumor neural stem cells maintained the same karyotypic features (not shown) and molecular signature of their parental lines (see Fig. 6*D*, line 1022 and 1022PT), and when retransplanted orthotopically, they were still capable of giving rise to new glioblastoma multiforme–like tumors, with characteristics identical to those generated by primary tumor neural stem cells (not shown). In conclusion, tumor neural stem cells satisfy all of the critical criteria to be defined as multipotent tumor neural stem cell lines, both *in vitro* and *in vivo* (20).

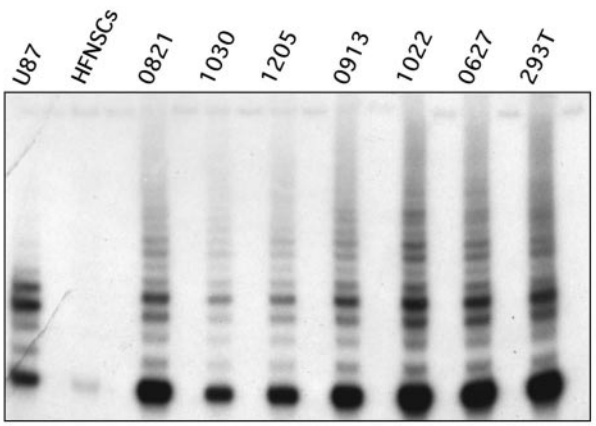
Tumor Neural Stem Cells Display Tumor-Specific Properties. In addition to the exacerbated growth rate displayed by most of the glioblastoma multiforme–derived lines when compared with normal human fetal neural stem cells (Fig. 1*C*), tumor neural stem cells were also expected to bear tumor-specific features, such as highly unbalanced karyotypes and telomerase reactivation.

Karyotypic analysis was performed on all of the six lines, on U87 cells and on human fetal neural stem cells (Fig. 6*A*). Many different numerical and structural chromosomal aberrations could be detected in tumor neural stem cells and in U87 but not in human fetal neural stem cells, which revealed a normal complement of chromosomes and no genetic abnormalities. Intriguingly, aneuploidy of specific autosomes and sex chromosomes (monosomy 1, trisomy 7 and 22, and disomy X) was retrieved most frequently in the three tumor neural stem cell lines with the highest expansion potential (cluster A), suggesting that peculiar aberrations may confer a certain degree of

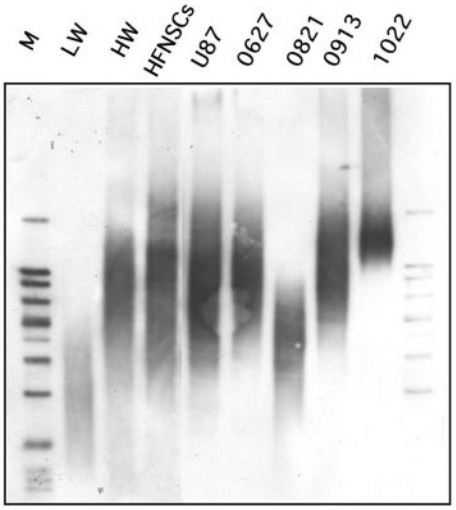
A

Cell line	Losses	Gains	Markers
010627	1, 2, 13 and 14	7, 20, 22 and XX	3
020913	1, 13 and 14	7, 19, 20, 22 and XX	5
021022	1, 2, 13 and 15	7, 19, 20, 22 and XX	3
020821	9, 15	3, 17, 18 and 20	3
021030	1, 4, 9, 10, 13, 14 and 22	21	7
021205	6, 10, 14, and 22	7	2
U87	1, 9,10, 11, 12, 14, 13, 16,19, 20,22 and X	negative	11
HFNSCs	negative	negative	0

B



C



D

Gene	Controls		Cluster A				Cluster B		
	U87	HFNSCs	0627	0913	1022	1022PT	0821	1030	1205
Emx2	-	+	+	+	+	+	-	-	-
HoxA10	-	-	-	-	-	-	+	+	+
Notch	+	+	+	+	+	+	+	+	+
Smoothened	-	+	+	+	+	+	-	+	+
Patched	-	+	+	+	-	+	+	-	-
Shh	-	-	-	-	-	-	-	-	-
Gli2	+	+	+	+	+	+	+	+	+
Wnt1	-	-	-	-	-	-	-	-	-
Wnt3A	+	-	-	+	-	-	-	+	+
DCC	+	+	+	+	+	+	+	+	+
Pax6	+	+	+	-	+	+	-	+	+
PTEN	+	+	+	+	+	+	+	+	+
DMBT1	+	-	+	-	-	-	-	+	-
EGFR	+	+	+	+	+	+	-	-	+
p15	-	+	+	+	+	+	-	-	-
p16	-	+	+	+	+	+	-	-	-
p21	+	+	+	+	+	+	+	+	+
p27	+	+	+	+	+	+	+	+	-
p53	+	+	+	+	+	+	+	+	+
MDR1	+	+	+	+	+	+	+	+	+
MRP1	+	+	+	+	+	+	+	+	+

Fig. 6. Karyotypic imbalance, telomerase reactivation, and molecular fingerprint characterize all glioblastoma multiforme–derived tumor neural stem cells. *A*, summary of the chromosomal aberrations found in all of the different tumor neural stem cell lines. *HFNSC*, human fetal neural stem cell. *B*, the enzymatic activity of the RNA catalytic subunit of telomerase as assessed by telomeric repeat amplification protocol assay in the different cell lines, normal human neural stem cells, and U87 glioma cells; *C*, telomere length, as measured by Southern blotting in glioblastoma multiforme cell lines, normal human neural stem cells, and U87 glioma cells. *M*, marker; *LW*, low-weight standard; *HW*, high-weight standard. *D*, a preliminary gene expression profile on tumor neural stem cells and control cell lines by semiquantitative reverse transcription-PCR. Tumor neural stem cell lines are displayed as clustered into fast-growing (*cluster A*) or slow-growing (*cluster B*) cell lines.

proliferation advantage to tumor neural stem cells. Conversely, slow-growing lines (cluster B) showed line-specific, chromosomal aberrations. Notably, long-term culturing did not affect the karyotypic signature of tumor neural stem cells, which remained constant even at high passages in culture (not shown).

Malignant transformation entails the acquisition of an immortal phenotype, which requires active telomere-maintenance mechanisms (39). We tested whether the catalytic subunit of the human ribonucleoprotein enzyme telomerase reverse transcriptase, which provides tumor cells with unlimited proliferation capacity by telomere lengthening, was at work in glioblastoma multiforme–derived cell lines. By means of the telomeric repeat amplification protocol assay, high human telomerase reverse transcriptase activity was retrieved in all of the glioblastoma multiforme–derived cell lines and in the corresponding original tumor specimens (not shown), whereas normal human fetal neural stem cells were telomerase negative (Fig. 6*B*), as shown previously (40). We also assessed telomere length and detected significant differences among the various tumor neural stem cell lines, with lines 0821 and 1030 displaying very short telomeres, even shorter than human fetal neural stem cells (Fig. 6*C*). Our observations suggest that tumor neural stem cells, isolated from glioblastoma multiformes, although displaying typical stem cell features, possess line-intrinsic, tumor-related functional properties.

Molecular Phenotyping of Tumor Neural Stem Cells. To assess whether the heterogeneity detected at the cellular and biochemical level in various tumor neural stem cells could also be retrieved at the molecular level (Fig. 6*D*), the gene expression profiles of the six different lines were analyzed by semiquantitative reverse transcription-PCR. The candidate genes for reverse transcription-PCR were selected and clustered taking into account their restricted pattern of expression in (1) normal neural progenitors; (2) brain tumors; (3) CNS development. Normal human fetal neural stem cells, the glioblastoma cell line U87MG, and human fetal whole brain tissue were used as specimens of reference for the above gene clusters 1, 2, and 3, respectively.

As expected, based on their cellular phenotype, the genetic profiles of the different tumor neural stem cells could readily be clustered based on the expression of specific gene sets. The fast-growing cell lines 0627, 0913, and 1022 were characterized by similar gene expression patterns, whereas each one of the slow-growing lines 0821, 1030, and 1205 displayed a unique molecular signature. Nonetheless, fast-growing lines (0627, 0913, and 1022) also showed cell-line–specific variations in their gene expression profile, confirming that despite the evidence for overlapping cellular and molecular characteristics, each tumor neural stem cell line was ultimately, inherently unique and phenotypically different from all of the others.

Intriguingly, the widest body of heterogeneity in terms of gene expression occurred at the level of developmentally regulated genes, which displayed line-specific, modulation patterns. For instance, the homeobox gene *Emx2*, a regulator of self-renewal in neural stem cells (23), also implicated in cortical development (41, 42), and its repressor *HOXA10* (43) were transcriptionally regulated in a reciprocal fashion, with lines 0627, 0913, and 1022 (cluster A) displaying high levels of Emx2 transcript and no detectable *HOXA10* mRNA and lines 0821, 1030, and 1205 (cluster B) showing the opposite trend. Similarly, genes such as *Patched* and *Pax6* demonstrated a scattered distribution across the different tumor neural stem cell lines, although not correlated to any clear functional clustering. Conversely, a higher degree of homogeneity between lines was retrieved when analyzing tumor-related genes such as *PTEN*, *p21*, *p27*, *p53*, and *MDR1*. In perspective, the line-specific molecular signature and the identification of genes that play a key role in a given type of tumor neural stem cell may be exploited to develop custom-fit therapies, by taking into account the unique genetic background of the specific tumor cells found in the glioblastoma multiforme of a specific patient.

DISCUSSION

This work reports on the isolation and identification of tumor neural stem cells from human adult glioblastoma multiforme, which possess the capacity to establish, sustain, and expand these tumors, even under the challenging settings posed by serial transplantation experiments. These data identify tumor neural stem cells as one of the cell lineages involved in establishing the malignant, aggressive profile of this lethal brain tumor.

Glioblastoma multiforme represents one of the most frequent brain cancers and either may develop from lower grade astrocytic tumors (secondary or progressive glioblastoma multiforme) or may arise very rapidly *de novo* (primary glioblastoma multiforme; ref. 44). In both cases, neither chemotherapy nor radiotherapy has been effective in managing these cancers (45). Glioblastomas multiforme display a rather heterogeneous cellular composition, as indicated by the term "multiforme," with some of the tumor cells bearing significant migratory capacity. This results in invasive spread, in the formation of multiple secondary foci, and in frequent, recurrent growth. Therefore, the identification of the cell type(s) involved in some or all of these phenomena is critical from both a scientific and therapeutic standpoint.

To date, three studies have indicated the presence of undifferentiated neural precursors within pediatric or adult human brain tumors. The initial study by Ignatova *et al.* (15) described the isolation of neurosphere-forming, bipotent (neuronal/astroglial) precursors from glioblastomas multiforme and was subsequently extended by Singh *et al.* (16), who also demonstrated that bipotent progenitors from CNS tumors (mostly medulloblastomas) displayed short-term self-renewal (three passages in culture). The latter work also proposed that oligodendrocytes might have been generated from tumor cells, but the use of a promiscuous marker such as platelet-derived growth factor receptor α, which simultaneously labels both oligodendrocytes and neurons in culture (46), precluded any conclusion in this direction. Similar findings were later reported by Hemmati *et al.* (17), who described the absence of oligodendrocytes and the lack of tumorigenic ability after intracerebral transplantation of tumor-derived cells.

Collectively, these studies show that cells endowed with some of the characteristics expected from stem cells can be found within brain tumors, thus raising some intriguing issues. First and foremost, can aberrant neural precursors be involved in the establishment, expansion, and recurrence of CNS cancers (*i.e.*, are they tumorigenic?) or, rather, do they emerge as the byproduct of the uncontrolled proliferation of the actual tumorigenic cells, which are developmentally deranged? Second, if these precursors are tumorigenic, do they possess all of the features to qualify as tumor neural stem cells (20)?

The data presented here provide answers to these questions. They describe the isolation and identification of human glioblastoma multiforme cells, which possess all of the defining features of somatic stem cells, including *ex vivo* multipotency (*i.e.*, the potential to simultaneously generate cells displaying antigenic reactivity for neuronal, astroglial, and oligodendroglial markers in culture; refs. 47–49) and the ability to establish and expand glioblastoma multiforme–like tumors. The latter contain exclusively cells expressing astroglial markers, as in typical human glioblastomas multiforme. Notably, these cells can perpetuate glioblastoma multiforme–like tumors even under the critical settings imposed by serial transplantation, thus satisfying the most rigorous criteria to be classified as tumor neural stem cells (20).

These findings were highly reproducible when analyzing tumor

neural stem cells established from different specimens, regardless of significant functional, karyotypic, and molecular differences between the various tumor neural stem cells. This may account for the presence within different glioblastoma multiforme specimens of tumor neural stem cells with common general stem cell properties that, however, are also characterized by unique, specimen-related, specific functional and molecular attributes.

Our data seem to suggest that tumor neural stem cells are found mainly within glioblastomas multiforme, although such a conclusion should be taken with caution. In fact, although our findings are consistent with those from Hemmati *et al.* (17) and show that cells from medulloblastomas expand poorly *in vitro* and do not establish tumors upon intracerebral grafting, they should not be construed as evidence that tumor neural stem cells are not present in medulloblastomas. It is possible that medulloblastomas contain cells that elicit their full stem cell potential under different settings than those used to date. Thus, it is intriguing that Kondo *et al.* (50) have reported on the presence of tumorigenic stem-like cells in a rodent glioma cell line (C6) that require different growth factors than those used in previous studies to expand in culture.

The knowledge that cells with stem cell characteristics may be involved in glioblastoma multiforme tumorigenesis is of paramount importance for our understanding of how these tumors form and expand and may provide an indication as to the key cellular and molecular mechanism to be investigated and tackled for diagnostic and therapeutic purposes. Unfortunately, it remains unclear whether tumor neural stem cells in glioblastomas multiforme derive from the transformation of normal human neural stem cells or whether their presence therein reflects the result of the transformation and de-differentiation of a more mature brain cell, which brought about otherwise silent/latent stem cell properties (5). Nonetheless, the demonstration of the existence within glioblastomas multiforme of tumor neural stem cells with the capacity of establishing this type of tumor, together with previous reports showing the existence within similar cancers of bipotent, stem-like cells (15–17), lends to the hypothesis that a hierarchical model of cell genesis may be at work within malignant brain cancers.

To date, only a few glioblastoma multiforme cell lines have been established (51–53) and used for studies on basic glioma biology or to perfect therapy for glioblastoma multiforme (54–57). The tumor neural stem cells described here can be used to routinely establish adult human glioblastoma multiforme cell lines in a quick and reproducible fashion. Of note, these cells reproduce the overall behavior of glioblastomas multiforme closer than any other previously available cell line. For example, when transplanted into immunosuppressed mice, the most widely used human glioblastoma multiforme cell line, the U87MG, gave rise to tumors that did not mimic the typical glioblastoma multiforme characteristics. Conversely, upon orthotopic transplant, all of the glioblastoma multiforme–derived, tumor neural stem cell lines formed tumors bearing all of the primary traits of human glioblastomas multiforme. Most importantly, cells in tumor neural stem cell–founded tumors displayed, *in vivo*, the typical migratory capacity expected from glioblastoma multiforme malignant cells, which was not observed when using U87, and, most importantly, retained their distinctive, line-specific proliferation and differentiation attributes. Thus, the identification of tumor neural stem cells also makes available cell lines of glioblastoma multiforme–initiating cells, which may retain specimen-specific and, thus, patient-specific traits. This may open new avenues to identify novel tumor cell markers for therapeutic and diagnostic purposes and to develop patient-tailored pharmacologic approaches for the cure of glioblastomas multiforme, aiming to target what may very well be the most aggressive cell type found in glioblastomas multiforme, to date, which is the tumor neural stem cell.

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Isolation and Characterization of Tumorigenic, Stem-like Neural Precursors from Human Glioblastoma

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