



Early Differentiating Mouse Astroglial Progenitors Share Common Protein Signatures with GL261 Glioma Cells

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Abstract

Neural stem cells (NSC) biology is being applied to tumor research because these cells have been shown to share key properties with cancer cells. Gliomas represent the most common brain tumor, and neural stem/progenitor cells (NSPC) or early-differentiated cell type lineages may be on its origin. Here, we identified the developmental stage of the differentiation process of NSC into astrocytes that showed the highest number of tumorigenic similarities.

NSPC were grown as neurospheres and astroglial differentiation was induced during 7 days *in vitro* (DIV). Cellular characterization was evaluated by specific neural markers at two developmental windows, i.e. NSPC/astrocyte progenitors from neurospheres until 3 DIV, and differentiating astrocytes thereafter. Predominance of immature Sox2-positive cells was verified in the first window and a prevalence of GFAP-positive cells in the second one. We then compared some tumor-related markers in GL261 glioma cells with such differentiating periods. The early progenitor cells (until 2 DIV) were those with the closest resemblances to the glioma cell line regarding BrdU incorporation, expression of microtubule-associated protein light chain 3 and of angiogenic factors (VEGF/VEGFR2), as well as S100B release. Our results suggest that early differentiating astroglial progenitors may be more susceptible to malignant transformation.

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Abbreviations: BrdU: Bromo-2'-deoxyiridine; DIV: days *in vitro*; GBM: Glioblastoma; GFAP: Glial Fibrillary Acidic Protein; GLAST: Glutamate Aspartate Transporter; LC3B: Microtubule-Associated Protein Light Chain 3; MMP: Matrix Metalloproteinases; Mrp1: Multidrug resistance-associated protein 1; NPC: Neural Progenitor cells; NSC: Neural Stem Cells; NSPC: Neural Stem/Progenitor Cells; NS: Neurospheres; Sox2: Sex determining region of Y chromosome-related high motility group box 2; VEGF: Vascular Endothelial Growth Factor; VEGFR-2: VEGF Receptor 2.

Introduction

Neural stem cells (NSC) research became prevalent in the early 1990s^[1-3] and such cells are described as a multipotent brain population with the ability to maintain a pool of neural stem-like cells. These cells can differentiate into more restricted precursor cells, such as neural precursor cells (NPC), which are able to generate the three major cell types that compose the central nervous system (CNS): neurons, astrocytes and oligodendrocytes. Since the first studies from Temple^[4], several methodologies were de-



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scribed for the isolation and expansion of NSC^[5] and among them, the neurospheres (NS) formation assay is one of the most widely used. This procedure was implemented for the first time by Reynolds and Weiss^[2], where cells from embryonic or adult CNS are isolated and propagated *in vitro* as NS and expanded in long term suspension cultures, in the presence of growth factors^[6]. These cellular structures represent three dimensional heterogeneous cell clusters, comprising stem cells, committed progenitors, as well as differentiated neural cells^[7-9]. As growth factors maintain the proliferative characteristics of NS, their withdrawal will stop the proliferating process and will start the differentiation of cells^[10], leading to an increase in markers of fully differentiated neural cell populations, such as neurons and glial cells^[7,11].

With the advances in stem cell biology, a debate has emerged regarding the origin of tumors, particularly gliomas. These are the most common type of brain tumors^[12] comprising morphologically different cells expressing an extensive variety of differentiated and undifferentiated markers^[13]. These tumors are conventionally classified according to the normal neural cell type they most resemble: astrocytomas (astrocytes), oligodendrogliomas (oligodendrocytes), or ependymal cells (ependymomas)^[14]. Some authors suggest that the neoplastic transformation of fully differentiated glia is underlying the mechanism of gliomagenesis^[14,15]. However, this hypothesis fails to explain adequately the source of some gliomas, such as the mixed oligoastrocytoma^[14]. Thus, recent discoveries have prompted more of a focus on the NSC hypothesis, where emphasis is given to early stages of gliogenesis, such as proliferating NSC, NPC and radial glia^[16]. In fact, as the mechanisms that control gliogenesis become better understood, it has been postulated that their deregulation contribute to glioma pathogenesis^[17]. As NSC and NPC share some features with tumor cells, this hypothesis is based on the neoplastic transformation of these early developmental stages. Accordingly, gliomas can originate neurosphere-forming cells that express both neuronal and glial markers, presenting a “stem-like” profile, similar to NSC from neurogenic regions of normal brain^[18-21]. In addition, many gliomas are confined to the subventricular zone of the adult brain, a niche where NSC are also localized^[14,22]. Moreover, glioma cells appear to be associated with white-matter tracts and blood-vessel basement membranes and this is also true for NSC and NPC, suggesting a common substrate for motility^[14]. Regarding some tumor-related properties, processes such as invasion, infiltration and angiogenesis that are highly frequent in high grade gliomas^[23,24], are also essential for NSC biology and for mammalian neural development^[25-27]. Furthermore, the ATP-dependent efflux transporters that mediate the characteristic resistance of tumor cells to chemotherapy^[28,29] have also been proposed to be highly expressed in NSC, playing a role in maintaining these cells in an undifferentiated state^[30,31].

However, it is important to emphasize that although neural stem/progenitor cells (NSPC) or early-differentiated cell type lineages have been suggested to be in the origin of gliomas, it is not well established which stage is more prone to glioma initiation. This study investigated which developmental phenotype, from NSPC to differentiated astrocytes, revealed the highest tumorigenic potential, by identifying the subtype most similar to the glioma phenotype, regarding diverse tumor-related factors. By using primary NS cultures and subsequent differentiation into astrocytes, we have identified the early differentiating astrocytes as the cells evidencing the closest similarities to the high grade astrocytoma cell line, suggesting that such cells may be at a higher risk for malignant transformation.

Methods

Animal procedures

Animal care followed the recommendations of European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council Directive 86/609/EEC) and National Law 1005/92 (rules for protection of experimental animals). All animal procedures were approved by the Institutional Animal Care and Use Committee.

Primary NS cultures

NS were isolated from CD1 mouse fetuses at embryonic day 15, as previously described by us^[32]. Briefly, pregnant female mice were euthanized by asphyxiation with CO₂. The fetuses were rapidly decapitated and after removal of meninges and white matter, the neocortices were collected in Hanks' balanced salt solution without Ca²⁺ and Mg²⁺ (HBSS, Invitrogen) and transferred to trypsin-EDTA (5 g/L trypsin- 2 g/L EDTA, Sigma) plus 1 U/mL DNase I solution (Sigma), for 30 min at 37° C. Following trypsinization, cells were washed three times with HBSS, resuspended in a final volume of RHB-A® stem cell culture medium (Stem-Cells, Inc., SCS-SF-NB-01) and mechanically dissociated using a Pasteur pipette. Around 1 x 10⁶ cells/mL were placed on tissue culture uncoated plates in RHB-A® medium supplemented with 10 ng/mL of murine epidermal growth factor (EGF, PeproTech), 10 ng/ml murine basic fibroblast growth factor (bFGF, PeproTech) and 1% antibiotic antimycotic solution (AB/AM, Sigma), and maintained at 37°C in a humidified atmosphere of 5% CO₂. Under these proliferating conditions, the cells grow as free-floating NS. Proliferating medium was changed after 2 days *in vitro* (DIV) by mechanical dissociation of NS and cells replated onto uncoated tissue culture plates at a density of 1 × 10⁶ cells/mL. At 4 DIV, NS were dissociated to obtain a suspension of dissociated cells. A set of these cells were then plated onto tissue culture plates, some of them containing glass coverslips [coated with poly-D-lysine (PDL, Sigma)], at 37°C, in a humidified atmosphere of 5% CO₂, during 4 h. After that, cells plated on coverslips were fixed with freshly prepared 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) for immunocytochemistry studies, while cells not plated on coverslips were lysed to obtain total cell extracts and their growth media collected. A second set of NS was maintained to further induce astroglial differentiation.

Astroglial differentiation of NS

NS were induced to differentiate into astrocytes by culturing dissociated NS in Dulbecco's modified Eagle's medium

(DMEM, Biochrom) supplemented with 11 mM sodium bicarbonate, 38.9 mM glucose, 1% AM/AB and 10% fetal bovine serum (FBS, Biochrom), onto tissue culture plates at 7.0×10^5 cells/mL. Cells were maintained in differentiating conditions during 7 DIV, without changing the culture medium. At each day of the astroglial differentiation process, cells were fixed for immunocytochemistry studies with freshly prepared 4% PFA, or lysed to obtain total cell extracts, and their growth media collected.

Characterization of NS and differentiating cells

For characterization of NS and differentiating cells, an immunofluorescent detection of NSPC, astrocytes, neurons and oligodendrocytes was performed. Previously fixed cells were incubated overnight, at 4°C, with antibodies against: the Sox2 (Sex determining region of Y chromosome-related high motility group box 2) protein (rabbit, 1:500, Merck Millipore, AB5603) and the nestin protein (mouse, 1:200, Merck Millipore, MAB353), both markers of undifferentiated/immature cells; the vimentin protein (mouse, 1:25, Santa Cruz, sc-32322), a marker for glial progenitor cells; the glial fibrillary acidic protein (GFAP) (rabbit, 1:500, Sigma, G9269), and the glutamate aspartate transporter (GLAST) (rabbit, 1:500, AbCam, AB416), both astrocytic markers; the neuronal protein HuC/D (mouse, 1:750, Molecular Probes, A-21271), an early post-mitotic neuronal marker; the microtubule-associated protein 2 (MAP2) (mouse, 1:100, Merck Millipore, MAB3418), a neuronal marker; the chondroitin sulfate proteoglycan NG2 (rabbit, 1:200, Merck Millipore, AB5320), that stains for oligodendrocyte precursor cells; and the myelin basic protein (MBP) (mouse, 1:200, Merck Millipore, MAB387), which is a marker for mature oligodendrocytes. Nuclei were stained with Hoechst dye 3328 (1:1000, Sigma). Finally, pairs of U.V. and fluorescence images of ten random microscopic fields were acquired per sample using a fluorescence microscope (AxioScope A1, Zeiss) with integrated camera (AxioCamHRm, Zeiss). Immune-positive cells for each cell type and total cells were counted to determine the percentage of positive nuclei and the resultant values were presented as percentage of positive cells for each staining. This characterization aimed to define specific developmental windows which will help to select the cell stages to be compared to gliomas cells.

GL261 mouse glioma cell line

The GL261 cell line was a kind gift from Dr. Geza Safrany, from the National Research Institute for Radiobiology and Radiohygiene, in Hungary. This cell line is representative of a carcinogen-induced mouse syngeneic glioma model, which carries point mutations in the K-ras and p53 genes^[33] and exhibit, as other glioma cell lines, populations of cells that have characteristics of cancer stem cells, such as the CD133+ cells^[34]. Furthermore, these cells present growth and invasive features similar to the high grade astrocytoma, the human glioblastoma multiforme (GBM), thus representing an important tool to study the biology of this type of human cancer, as well as for preclinical and translational research^[34,35]. GL261 cells were maintained in DMEM supplemented with 11 mM sodium bicarbonate, 38.9 mM glucose, 10% FBS and 1% penicillin/streptomycin (Biochrom), at 37°C in a 5% CO₂ conditioned atmosphere, during 5 DIV. After this period, cells were fixed for immunocytochemical studies with freshly prepared 4% PFA, or lysed to obtain total cell extracts, and their growth media was collected.

Cell proliferation assay

The assessment of the proliferative potential of NS and differentiating astrocytes, as compared to the GL261 cell line, was performed by quantification of bromo-2'-deoxyuridine (BrdU)+ cells. Cells plated on coverslips were previously incubated with 10 µM BrdU (Sigma) during 3 h, and fixed with 4% of PFA, followed by a 30 min treatment with 1N HCl at 37°C. Detection of BrdU+ cells was performed by incubation with an antibody against BrdU (mouse, 1:750, Sigma). Immune-positive cells for each cell type and total cells were counted to determine the percentage of positive nuclei.

Western blotting

Protein expression of both the multidrug resistance-associated protein 1 (Mrp1) and the microtubule-associated protein light chain 3 (LC3B) of NS and differentiating astrocytes, as compared to the GL261 cell line, was determined by Western blot analysis. Briefly, 25 - 40 µg of total protein were obtained by lysing cells in ice-cold Cell Lysis Buffer (Cell Signaling). The extracts were separated on a 8% (for Mrp1 determination) or a 12% (for LC3B evaluation) sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). After electrophoretic transfer onto a nitrocellulose membrane, and blocking [4% milk in T-TBS (0.2% Tween 20, 20 mM Tris-HCl at pH 7.5, 500 mM NaCl) for Mrp1 detection and 5% milk T-TBS for LC3B evaluation], blots were then incubated overnight at 4°C with a specific rabbit Mrp1-A23 antibody (1:750), a specific LC3B rabbit antibody (1:1000, Cell Signaling, 2775S) and β-actin mouse antibody (1:5000, Sigma, A5441). Finally, blots were incubated with horseradish peroxidase-labeled secondary antibody [anti-rabbit (1:5000, Santa Cruz, sc-2004)] for 1 h. The chemiluminescent detection was performed after membrane incubation with enhanced chemifluorescence reagent by using LumiGLO® (Cell Signaling). The relative intensities of protein bands were analyzed using the Image Lab™ analysis software, after scanning with ChemiDocXRS, both from Bio-Rad Laboratories (Hercules, CA, USA). Results were standardized with respect to the β-actin protein band to correct any loading differences, and expressed as fold change relatively to NS.

Immunocytochemistry studies

The evaluation of angiogenesis- and autophagy-related proteins in NS and differentiating astrocytes, as compared to the GL261 cell line, was determined by immunocytochemistry. Briefly, cells were incubated overnight, at 4°C, with antibodies against the vascular endothelial growth factor (VEGF) (rabbit, 1:100, Santa Cruz, sc-152), VEGF receptor 2 (VEGFR-2) (mouse, 1:100, Santa Cruz, sc-6251) and LC3B (rabbit, 1:500, Cell Signaling), followed by a species-specific fluorescent secondary antibody la-

beled with Alexa Fluor® 594 goat (anti-mouse IgG, 1:1,000, Invitrogen, A11005) or Alexa Fluor® 488 goat (anti-rabbit, 1:1,000, Invitrogen, A11008) for 90 min at room temperature. For nuclei staining, coverslips were stained with Hoechst dye 33258. Finally, pairs of U.V. and fluorescence images of ten random microscopic fields were acquired per sample with a fluorescence microscope with integrated camera. For VEGF and VEGFR-2 evaluation, the protein levels were quantified by measurement of the fluorescence intensity per number of cells, by using ImageJ software (N.I.H., USA). For LC3B, we have determined the percentage of cells with punctuate LC3B, relatively to the total number of nuclei. All the results were expressed as fold change relatively to NS values.

ELISA

The determination of the extracellular S100B levels in NS and differentiating astrocytes, was compared to the GL261 cell line values, using ELISA, as previous described^[36]. Briefly, supernatants were incubated for 2 h at 37°C on a 96-well plate previously coated with a monoclonal anti-S100B antibody (1:1000, Sigma, S2532). After, a polyclonal anti-S100B antibody (1:5000, Dako, Z0311) was added and samples were incubated for 30 min at 37°C. Finally, an anti-rabbit peroxidase-conjugated antibody (1:5000, Santa Cruz, sc-2004) was added for further 30 min at 37°C. The colorimetric reaction with Sigma Fast OPD tablets (Sigma) was measured at 492 nm, using a microplate reader. The results obtained in ng/mL were normalized to total protein concentrations, and expressed as fold change relatively to NS values.

Gelatin zymography

The determination of matrix metalloproteinases (MMP) activities of NS and differentiating astrocytes, as compared to the GL261 cell line, was performed through the gelatin zymography method, as previously described in our lab^[37]. Briefly, aliquots of culture supernatants were analyzed by SDS-PAGE zymography in 0.1 % gelatin/10% acrylamide gels under non-reducing conditions. Following electrophoresis, gels were washed for 1 h with 2.5% Triton X-100 (in 5 mM Tris pH 7.4; 5 mM CaCl₂; 1 μM ZnCl₂) to remove SDS and renature the MMP species in the gel. Then, gels were incubated overnight at 37°C, in the developing buffer (5 mM Tris pH 7.4; 5 mM CaCl₂; 1 μM ZnCl₂) to induce gelatin lysis. For enzyme activity analysis, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 (Sigma) and destained in 30 % ethanol /10% acetic acid/ H₂O. Gelatinase activity, detected as a white band on a blue background, was photographed in ChemiDocXRS and analyzed using the Image Lab™ software. Results were normalized to total protein concentration, and expressed as fold change relatively to NS.

Statistical analysis

Results of at least, three different experiments were expressed as mean ± SEM. Differences between groups were determined by one-way ANOVA with Dunnett multiple comparisons post tests, using Instat 5.01 GraphPad Software (San Diego, CA). We considered $p < 0.05$ to be statistically significant.

Results

Differentiation of NS into astrocytes outlined two different developmental windows: NSPC/astrocyte progenitors vs. mature astrocytes

We induced NS differentiation into astrocytes during 7 DIV and throughout this process we have phenotypically characterized our cells. Using specific markers for NSC, early astrocyte progenitors and fully differentiated astrocytes, the obtained results clearly delineated two different developmental windows (Figure 1). The first, from NS until 3 DIV in differentiating conditions, was defined as NSPC/astrocyte progenitors. This period is characterized by the predominance of immature phenotypes (72 - 80% Sox2+ cells), and along the differentiation process there is still an increase in both early astrocyte progenitors (1.2-fold at 3 DIV vs. NS) and differentiated astrocytes (3.1-fold at 3 DIV vs. NS, $p < 0.01$). The second developmental window, after 4 DIV differentiating cells, is associated with mature astrocytes and is characterized by a prevalence in GFAP+ cells (from 70.3% at 4 DIV to 89% at 7 DIV, $p < 0.01$ vs. NS) and a sequential reduction in immature cells, particularly regarding Sox2+ cells, where it can be observed a significant decrease (from 0.8-fold at 4 DIV to 0.4-fold at 7 DIV, all $p < 0.01$ vs. NS). Despite the presence of oligodendroglial (“**Figure S1**”) and neuronal (“**Figure S2**”) progenitors throughout differentiation, these cells were not able to further differentiate into mature cells, which were scarcely represented along the differentiation process. These first results reveal that our differentiation protocol into astrocytes was efficient and appropriate to obtain this preferential cell type. Collectively, this characterization allowed the selection of the stages NS, 1, 2, 3 (NSPC/astrocyte progenitors) and 7 DIV (mature astrocytes) as the best to be used in the following experiments, regarding the evaluation of diverse tumor-related factors. The time points 4, 5 and 6 DIV were excluded because they presented very similar expression profiles concerning both undifferentiated and astrocytic markers. Due to the high variation on the astrocytic markers from NS to 3 DIV, we decided to include these phenotypes in the next experiments. The 7 DIV differentiating cells, were also selected since it was the developmental stage with the highest percentage of mature astrocytes.

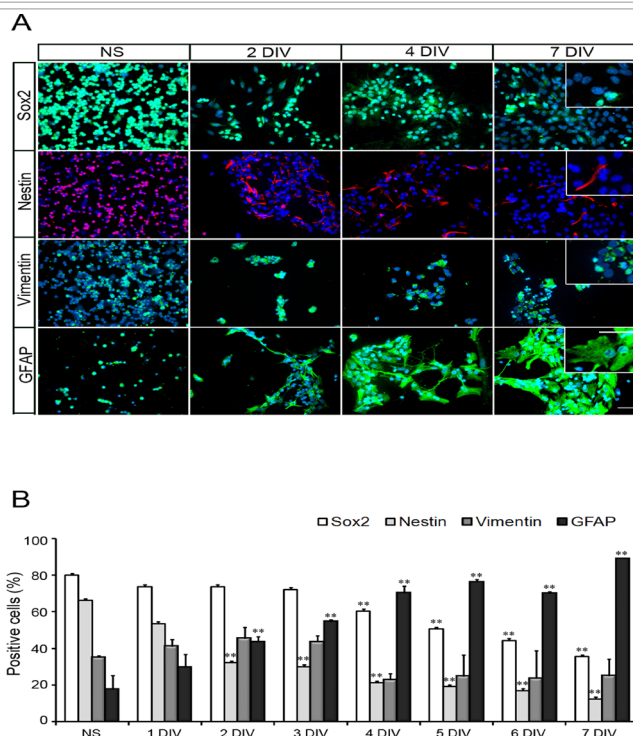


Figure 1: Expression of immature and astrocytic phenotypes during neurospheres (NS) proliferation and along differentiation. NS were induced to differentiate into astrocytes until 7 days *in vitro* (DIV). Cell markers for immature/undifferentiated cells (Sox2 and nestin), astroglial progenitors (vimentin), and astrocytes (GFAP) were analyzed by immunocytochemistry. (A) Nuclei were stained with Hoechst dye (blue) and cells were labeled for Sox2 (green), nestin (red), vimentin (green) and GFAP (green). Representative results from one experiment are shown. Detailed cellular staining for each marker are shown at 7 DIV. Scale bar represents 60 μ m. (B) Graph bars represent the percentage of cells (mean \pm SEM) positive for each neural cell marker, relatively to the total number of nuclei. Data were obtained from at least three independent experiments. ** $p < 0.01$ vs. NS.

Cells in the first developmental window presented a high level of resemblance with glioma cells regarding BrdU incorporation, expression of Mrp1, LC3 and VEGF/VEGFR2, as well as S100B release

In an attempt to explore which was the developmental phenotype with a higher risk for malignant transformation, we searched for resemblances with a glioma cell line, regarding diverse tumor-related factors.

First, we have evaluated the proliferative potential of NS, differentiating cells and GL261, through the quantification of BrdU cellular incorporation. As expected, the number of BrdU+ cells decreased along differentiation (Figure 2), corroborating the results obtained in the previous section. There was a very marked decrease from NS to 1 DIV differentiating cells (0.5-fold, $p < 0.01$), followed by a gradual reduction until 7 DIV (~0.1-fold, $p < 0.01$). In GL261 cells, we have observed that approximately 27% of the cells were BrdU+ (0.6-fold vs. NS, $p < 0.01$), and when compared with all the developmental stages, the values of 1 DIV differentiating astrocytes were the only ones with no statistical significance.

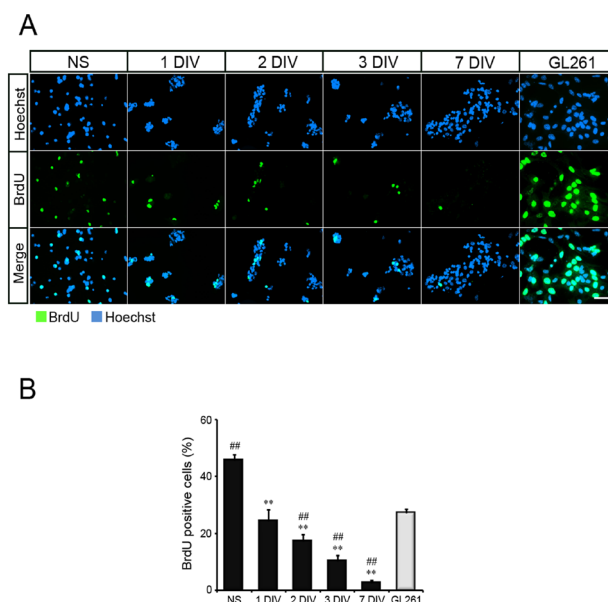


Figure 2: Number of BrdU+ cells in neurospheres (NS), differentiating astrocytes and GL261 cells. NS were induced to differentiate into astrocytes during 7 days *in vitro* (DIV). Cells were incubated with 10 μ M 5-bromo-2'-deoxyuridine (BrdU) during 3 h and its cellular incorporation was analyzed by immunocytochemistry. (A) Nuclei were stained with Hoechst dye (blue) and cells were labeled for BrdU (green). Representative results from one experiment are shown. Scale bar represents 60 μ m. (B) Graph bars represent the percentage of cells (mean \pm SEM) positive for BrdU, relatively to the total number of nuclei. Data was obtained from at least three independent experiments. ** $p < 0.01$ vs NS; and ### $p < 0.01$ vs. GL261 cells.

The multidrug resistance phenomenon of brain tumors can be mediated by several ATP-dependent transporters, such as Mrp1, which are also highly expressed in normal NSC^[30,38]. We have determined Mrp1 expression levels in NS, differentiating cells and GL261 by Western blot (Figure 3) and observed that NS highly express this efflux transporter, as compared to differentiating cells. The expression profile revealed a decrease in Mrp1 expression from NS to 3 DIV, where the lowest value was obtained (0.5-fold vs. NS, $p < 0.01$), followed by an increase until 7 DIV (0.7-fold vs. NS). The GL261 cells showed the highest expression value as compared to all the developmental stages (1.4-fold vs. NS). Overall, these data on the expression of Mrp1, demonstrate that NS are the cells that most resemble the glioma cell line, without any significant difference between them.

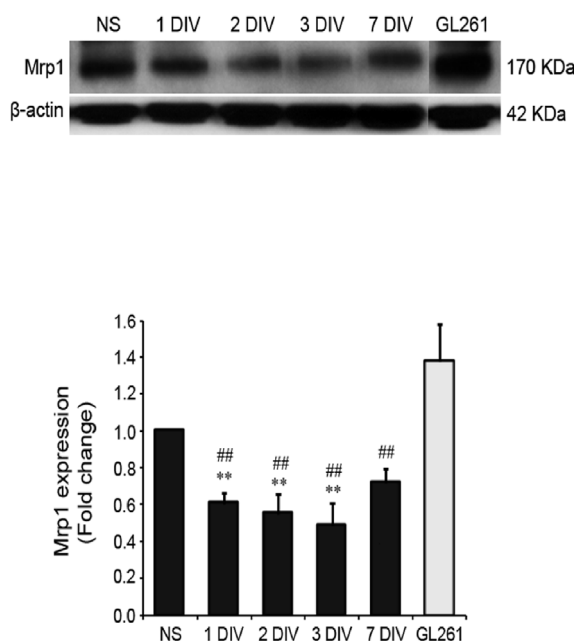


Figure 3: Multidrug resistance-associated protein 1 (Mrp1) expression levels in neurospheres (NS), differentiating astrocytes and GL261 cells. NS were induced to differentiate into astrocytes until 7 days *in vitro* (DIV). Total cell lysates were analyzed by Western blot. Representative results from one blot are shown. The intensity of the bands was quantified by scanning densitometry, standardized with respect to β -actin and expressed as mean \pm SEM fold change compared to NS. Data was obtained from at least three independent experiments. ** $p < 0.01$ vs. NS; ### $p < 0.01$ vs. GL261 cells.

The increasingly recognized importance of autophagy in tumorigenesis, tumor progression, tumor suppression and ultimately, tumor therapy turned this type of cell death process an extremely important research focus^[39]. The microtubule-associated protein LC3 is considered to be a marker of autophagy and it can be determined by both immunocytochemistry and Western blot. In this last assay, it is measured the LC3 conversion (conversion from LC3-I to LC3-II, expressed by the LC3II/I ratio), because LC3-I is cytosolic, whereas LC3-II is autophagosome membrane-associated^[40,41]. This autophagic marker was assessed in NS, differentiating cells and in the GL261 cells (Figure 4). In both assays, LC3 expression results showed a similar profile, presenting an increase from NS to 7 DIV differentiating cells (3.1 - 3.5-fold, $p < 0.01$ vs. NS). In addition, we could observe that autophagic levels of GL261 cells were only higher when compared to NS and 1 DIV differentiating cells. Combining the results from both assays, and according to the statistical differences between the different developmental stages and gliomas cells, we can consider cells from 1 and 2 DIV, as the phenotypes presenting levels of LC3 more closely resembling to those of glioma cells.

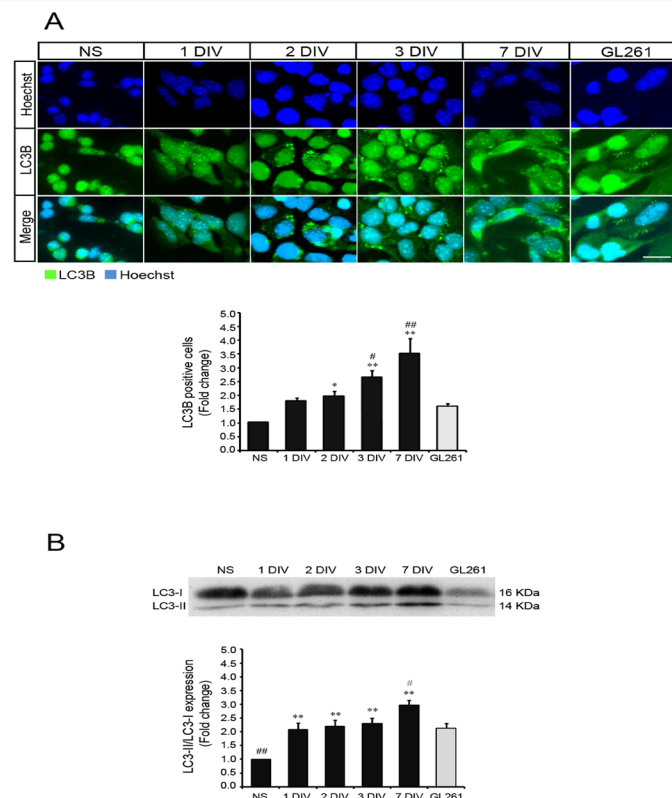


Figure 4: Microtubule-associated protein light chain 3 (LC3) expression levels in neurospheres (NS), differentiating astrocytes and GL261 cells. NS were induced to differentiate into astrocytes until 7 days *in vitro* (DIV). The expression of the autophagic marker LC3 was analyzed by immunocytochemistry and Western blot. (A) LC3-II translocated into autophagosome membranes appearing as green bright puncta. Nuclei were stained with Hoechst dye (blue). Representative results from one experiment are shown. Scale bar represents 20 μ m. Graph bars represent the fold change relatively to NS (mean \pm SEM) in the percentage of cells with punctuate LC3, relatively to the total number of nuclei. (B) LC3-II/LC3-I ratio was determined after quantification of protein expression by scanning densitometry. Results are expressed as mean \pm SEM fold change compared to NS. Data were obtained from at least three independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs. NS; # $p < 0.05$ and ## $p < 0.01$ vs. GL261 cells.

Malignant gliomas are also characterized by a marked increase in blood vessel formation (angiogenesis), which is an essential process for tumor growth and invasion in the brain^[42]. The expression of the angiogenic factor VEGF and its receptor VEGFR-2 in NS, differentiating cells and GL261 cells were determined by immunocytochemistry. As shown in Figures 5A and 5B, throughout differentiation the intensity of VEGF fluorescence peaked at 2 DIV (1.5-fold vs. NS), then decreasing until 7 DIV (0.3-fold vs. NS, $p < 0.05$). GL261 cells exhibited the highest expression levels, which were 2.4-fold higher than NS ($p < 0.01$), attesting the angiogenic potential of these cells. A similar profile was obtained for VEGFR-2, the most important receptor in the direct regulation of angiogenesis and described to be highly expressed in gliomas^[43,44], as depicted in Figures 5A and 5C. Like VEGF, the highest value of fluorescence intensity was observed at 2 DIV differentiating cells (1.6-fold vs NS), decreasing thereafter until 7 DIV (0.5 fold vs. NS). Once again, GL261 cells showed the highest values of this receptor, as compared to all differentiating stages (2.6-fold vs. NS, $p < 0.01$). Altogether, regarding these angiogenesis markers, the results indicate that 2 DIV cells are the only stage without any statistical difference when values are compared with gliomas cells.

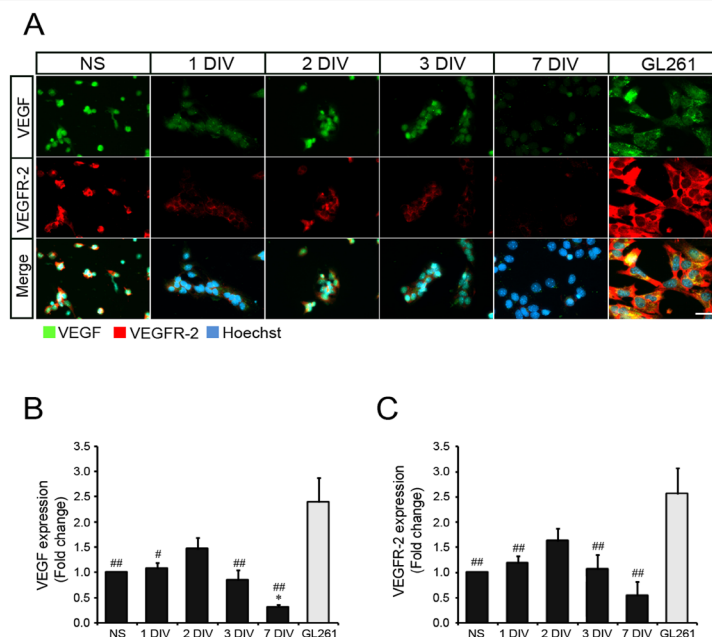


Figure 5: Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR-2) expression levels in neurospheres (NS), differentiating astrocytes and GL261 cells. NS were induced to differentiate into astrocytes until 7 days *in vitro* (DIV). The expression of VEGF and VEGFR-2 was analyzed by immunocytochemistry. (A) Nuclei were stained with Hoechst dye (blue) and cells labeled for VEGF (green) and VEGFR-2 (red). Representative results from one experiment are shown. Scale bar represents 60 μ m. (B,C) Graph bars represent the fold change relatively to NS (mean \pm SEM) in the protein levels quantified by measurement of fluorescence intensity per number of cells. Data were obtained from at least three independent experiments. * $p < 0.05$ vs. NS; # $p < 0.05$ and ### $p < 0.01$ vs. GL261 cells.

S100 proteins are known to be involved in proliferation, differentiation and migration/invasion^[45,46]. The extracellular concentration of S100B in NS, differentiating astrocytes and GL261 cells was determined by ELISA, and normalized to the respective total protein concentration (Figure 6). The release of S100B by NS was almost null (nanomolar range), highly increasing at the beginning of the differentiation process, with a secretion peak at 2 DIV ($p < 0.01$ vs. NS). In addition, the extracellular levels of S100B in all of the other developmental stages were significantly lower than those of GL261 cells. These tumor cells evidenced the highest amount of S100B released to the extracellular media, and the concentration produced by the 2 DIV cells the closer to GL261 cells.

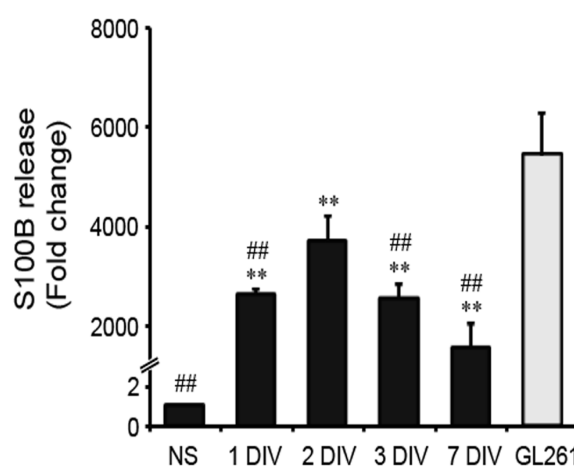


Figure 6: S100B protein levels released by neurospheres (NS), differentiating astrocytes and GL261 cells. NS were induced to differentiate into astrocytes until 7 days *in vitro* (DIV). Culture supernatants were collected for quantification of S100B concentration by ELISA assay. Graph bars represent the fold change relatively to NS (mean \pm SEM) in the extracellular concentration of S100B protein from at least three independent experiments. ** $p < 0.01$ vs. NS; ### $p < 0.01$ vs. GL261 cells.

In conclusion, looking at the comparisons made between NSC, differentiating phenotypes and gliomas cells, we can conclude that 2 DIV differentiating cells are the ones with the highest resemblances with gliomas cells in the majority of the determined tumor-related factors (LC3B, VEGF, VEGFR2 and S100B), followed by the 1 DIV differentiating phenotype, which also parallels glioma cells regarding proliferation (BrdU incorporation) and LC3B expression. Thus, these early progenitors are the stages at higher risk of malignant transformation.

MMP levels of gliomas cells presented resemblances with cells from both developmental windows

MMP have the ability to degrade macromolecules of the extracellular matrix, and were shown to play a crucial role in proliferation, migration and differentiation of neural cells during early brain development^[25]. These proteases have been also described as being responsible for tumor invasion and infiltration^[47]. The activity of extracellular MMP-9 and -2 in NS, differentiating astrocytes and GL261 cells was determined by gelatin zymography. The activity of MMP-9 (Figure 7A) evidenced maximal levels at 2 DIV (4.6-fold vs. NS, $p < 0.01$), decreasing thereafter until 7 DIV (2.1-fold vs. NS, $p < 0.05$). The lowest values for MMP-9 activity were seen in NS, which was the only stage with levels significantly different from the ones obtained in GL261 cells (3.1-fold vs. NS, $p < 0.01$). All the other phenotypes presented results in the range of those of glioma cells. Likewise, the highest level of MMP-2 activity (Figure 7B) was observed at 2 DIV (4.7-fold vs. NS, $p < 0.01$), and the lowest value was again that of NS, which significantly differed from all the other differentiating cells ($p < 0.01$) and even from glioma cells, that showed to be 3.6-fold enhanced ($p < 0.01$ vs. NS). Altogether, MMP results showed that, both temporal windows evidence levels very similar to glioma cells, indicating that this parameter is not suitable to ascertain about the phenotype more prone to malignancy.

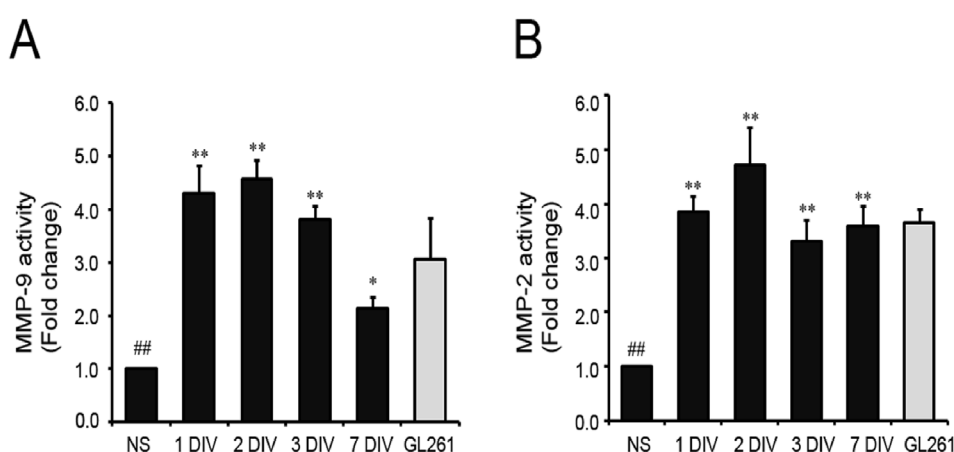


Figure 7: Matrix metalloproteinases (MMP) activity levels in neurospheres (NS), differentiating astrocytes and GL261. NS were induced to differentiate into astrocytes until 7 days *in vitro* (DIV). Culture supernatants were collected for quantification of MMP activities by zymography assay. Graph bars represent the fold change relatively to NS (mean \pm SEM) in the intensity of the bands quantified by scanning densitometry and standardized with respect to protein quantification for MMP-9 (A) and MMP-2 (B). Data were obtained from at least three independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs. NS; ### $p < 0.01$ vs. GL261 cells.

Discussion

During normal brain development, the NSC proliferation and differentiation processes are mediated by several mechanisms that are also present in tumor initiation and progression. Therefore, it was suggested that NSPC or early-differentiated cell type lineages may have a major role in gliomagenesis. In this work, NSPC were grown as NS and further induced to differentiate into astrocytes until 7 DIV. After the cellular characterization of NS and differentiating cells, tumor-related factors were evaluated and their profile compared to the one of GL261 mouse glioma cells. We found that: (i) the methodology used to promote differentiation of NS into astrocytes was effective and allowed to outline two different developmental windows, i.e. NSPC/astrocyte progenitors vs. mature astrocytes; and (ii) the first developmental window, and particularly differentiating cells at 1 and 2 DIV, were the stages that most resembled the GL261 cells phenotype, sustaining immature/early differentiated glia as the most vulnerable to malignancy.

Gliomas express a number of molecules that also regulate NSC proliferation^[20,48]. Sox2 is identified in several glioma samples^[49] and have an important role in the continuous proliferation of glioma cells^[50]. Concerning the astrocytic properties of astrocytomas, vimentin is often referred as a marker for these brain tumors^[51], and it was described that its co-expression with nestin may serve as an indicator of an astrocytoma with an enhanced motility and invasive potential^[52]. Mature astrocytic markers, such as GFAP, are also described to be expressed in glioma cells^[53-55], although Jan et al.^[51] showed that the malignant progression to a higher degree of invasiveness is associated with an increase in vimentin expression and a progressive loss of GFAP. Besides the expression of immature and astrocytic cell markers, gliomas also express neuronal proteins such as β -III-tubulin^[56,57] and MAP2^[58], both indicators of a high-grade astrocytoma^[58]. Similarly, the expression of progenitor and mature oligodendrocytes markers are found in gliomas/astrocytomas^[59,60], although MBP is minimally expressed in GBM^[59].

The multidrug resistance phenomenon is one of the main causes of treatment failure and disease progression in tumors, as it confers resistance to chemotherapeutic drugs. It is described that some multidrug transporters are functionally expressed in NSC and are downregulated during differentiation, indicating that they might be important for maintaining these cells at an undifferentiated state^[30,38]. Accordingly, our results showed that the ATP-dependent efflux transporter Mrp1 was highly expressed in NS, following by a decrease along differentiation. Regarding GL261 cells, the levels of Mrp1 expression were also very high, such as previously detected in glioma samples and in glioma cell lines^[61,62]. Since Mrp1 expression is significantly up-regulated in cancer stem-like cells (CD133+ cells), isolated from GBM^[63], and given that these undifferentiated tumor cells share some properties with

normal NSC^[48,64,65], they have been considered as the main drivers of gliomas proliferation and of the multidrug resistance process^[16]. Correspondingly, the NS and GL261 cells, besides showing the highest Mrp1 expression levels, were also the population with more BrdU+ cells, suggesting that multidrug transporters not only give resistance, but also allow and are needed for NSPC^[66] and tumor cell^[67] proliferation.

Autophagy has been implicated in normal brain development^[68,69], and an abnormality in the autophagic process is related to malignant diseases. Vazquez et al.^[70] referred that during differentiation, neuroepithelial cells undergo efficient remodeling of their cytoskeleton and shape in an energy-consuming process, and since autophagy is required to recycle cellular components and provide energy, this mechanism could fulfill these requirements, supporting differentiation. Our results are coherent with these observations since the expression levels of the autophagic marker LC3B increased along differentiation, as cells acquire a more specific fate. Regarding gliomas, Aoki et al.^[71] also showed that LC3B was expressed in *in vivo* and *in vitro* GBM cell lines. However, autophagic activity in glioma stem-like cells was shown to be significantly lower than that in NSPC^[72]. In fact, CD133+ GBM cells showed defective autophagy, which probably relates with their resistance to the classical drug temozolomide^[73], since autophagy induction can promote the differentiation of these cells and increase their susceptibility to therapy^[74,75]. Also, proliferation and autophagy are two related cellular mechanisms because in accordance with recent works, autophagy negatively regulates cancer cell proliferation^[76,77]. Correspondingly, our results showed that the phenotypes with the lowest autophagic levels were those with higher BrdU values.

The infiltration of glioma cells is mediated by a myriad of mechanisms that involve angiogenesis, neovascularization and invasion, the same pathways that are also present in normal brain development. Most of these processes require the extracellular matrix ECM remodeling through the action of MMP^[78,79], leading to the release of several pro-angiogenic factors, such as VEGF, which initiates and sustains neovascularization^[80,81]. This suggests that VEGF expression and MMP activities are correlated, in both normal neurogenesis and brain tumors. Accordingly, our results showed a peak of VEGF expression in the second day of differentiation into astrocytes, as it was also observed for MMP-2 and -9 activities. VEGF is highly expressed by embryonic NSPC in culture^[82,83] and it is believed that it contributes to some developmental processes, such as proliferation, vascularization and neurogenesis^[26], whereas the inhibition of the VEGF signaling induces differentiation^[84]. These data are consistent with our findings evidencing an increase in the expression of VEGF and its receptor from NS to the second day of differentiation, when there is still a high prevalence of immature/progenitor cells markers. Such markers decrease when the cellular profile point out to a prevalence of differentiated astrocytes. Being a key factor in the progression of malignant brain tumors, the overexpression of VEGF has been found in high grade astrocytomas^[85]. Similarly, Oka et al.^[86] showed that the injection of VEGF-expressing cancer stem cells into the mouse brain leads to the massive expansion of vascular-rich GBM. Knizetova et al.^[85] found a potential autocrine role of the VEGF-VEGFR2 interplay as contributing to malignant astrocytoma growth and radioresistance. Accordingly, we have found high levels of both VEGF and VEGFR-2 expression in GL261 cells.

S100B is highly expressed in astrocytes and in other glial cell types and its expression defines a late developmental stage after which GFAP-expressing cells lose their NSC potential and ability to form NS^[87]. At nanomolar concentrations (as the ones observed in NS), S100B exerts neurotrophic properties for normal brain development^[88,89]. However, higher levels of S100B (in the micromolar range) have shown toxic effects and have been detected in brain tumors, particularly in astrocytomas^[90]. In such levels, S100B might contribute to reduce the differentiation potential of cells of the astrocytic lineage, participating in the maintenance of a neoplastic, invasive phenotype^[45,91]. A recent study have demonstrated that the transfection of S100B promotes cell invasion and migration, which can be related with the development of brain metastasis^[92]. In fact, Vos et al.^[93] related high levels of S100B with a shorter survival in a relatively high proportion of patients with GBM. Corroborating these findings, we found a high release of S100B by GL261 cells (micromolar range). In contrast to our expectations of an increase in S100B release along differentiation, as cells lose NSC-potential and acquire a differentiated state, the highest levels were achieved in the second day of differentiation, suggesting that these progenitor cells may have a more invasive profile, most similar to glioma cells. Interestingly, along the differentiation process there is an evident similarity in the expression profiles of the tumor-related markers that are associated with an invasive and angiogenic potential, such as MMP, VEGF/VEGFR-2 and S100B.

The activity of many MMP mediate the degradation of the ECM in a variety of physiological and pathological tissue remodeling processes, including embryo implantation, tumor invasion, metastasis and angiogenesis^[47]. These proteases play an important role during development^[94], promoting proliferation, neurite extension, migration of newborn neurons, as well as neurogenesis, by providing an optimal niche for neural progenitor cells^[25]. Accordingly, our results showed an increased MMP activity in early-differentiating phenotypes, when cells are switching from a proliferating to a more differentiated state, then decreasing when cells acquire a more differentiated profile. It is also described that the levels of MMP-2 and -9 are significantly elevated in several malignant tumors^[95], and particularly present in glioma specimens, contributing for the progression, invasion and worst prognosis of these tumors^[95]. However, although GL261 cells expressed active MMP, we were expecting greater expression levels due to the characteristic invasive phenotype of the GL261 cell line^[34,35]. Consequently, the MMP levels of glioma cells were comparable to the majority of the developmental stages, making it difficult to specify a comparable phenotype.

Overall, we showed that cells from the first developmental window, more specifically early-differentiating progenitors (until 2 DIV), evidenced the closest similarities to the GL261 high grade astrocytoma cell line, suggesting that these cells may be more predisposed for malignization. These results are in accordance with the recent work of Munoz et al.^[97] regarding the differential transformation capacity of neuro-glial progenitors during development. In this study, a tumorigenic process was induced and initiated by an oncogenic stimulus (Ras-pathway activation) in neuro-glial progenitors populations at different developmental stages. It was demonstrated that this malignant transformation was sufficient to initiate tumorigenesis when targeted to early stages of radial glial development, but not at later stages, when cells are committed to a glial lineage. Similar results were described by Holland et

al.^[98], where the oncogenic stimulus (combined Ras and Akt activation) induced GBM formation after gene transfer to neural progenitors, but not after transfer to differentiated astrocytes. Thus, early progenitors may lack the regulatory mechanisms that exist at later, more lineage-restrictive, developmental time points, making these cells more vulnerable to malignancy.

The results of our work highlight that developmental differences may affect tumor initiation. Further studies should include the manipulation of these early progenitors, through the silencing of some genes that are thought to be more involved in gliomagenesis, such as PTEN and p53, and investigate the propensity of these transformed cells to initiate the tumorigenic process.

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Supplementary Material

Figure S1

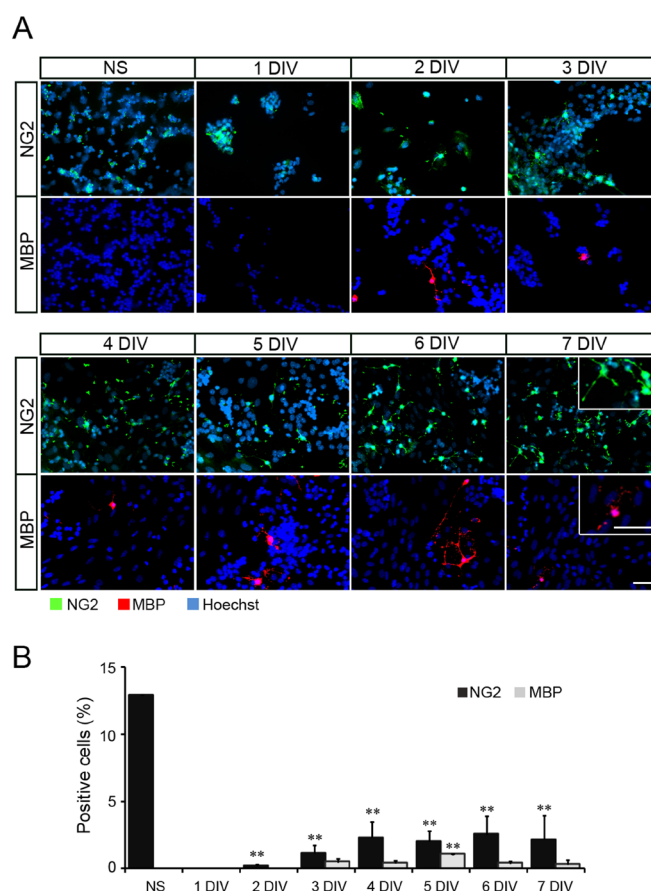


Figure S1: Expression of oligodendroglial phenotypes during neurospheres (NS) proliferation and along differentiation. NS were induced to differentiate into astrocytes until 7 days *in vitro* (DIV). NG2-expressing progenitor oligodendrocytes and MBP-expressing oligodendrocytes were determined by immunocytochemistry. (A) Nuclei were stained with Hoechst dye (blue) and cells labeled for NG2 (green) and MBP (red). Representative results from one experiment are shown. Detailed cellular staining for each marker in inset images are shown at 7 DIV. Scale bar represents 60 μ m. (B) Graph bars represent the percentage of cells (mean \pm SEM) positive for NG2 (black) and MBP (grey), relatively to the total number of nuclei. Data were obtained from at least three independent experiments. ** $p < 0.01$ vs. NS.

[“Back to Top”](#)

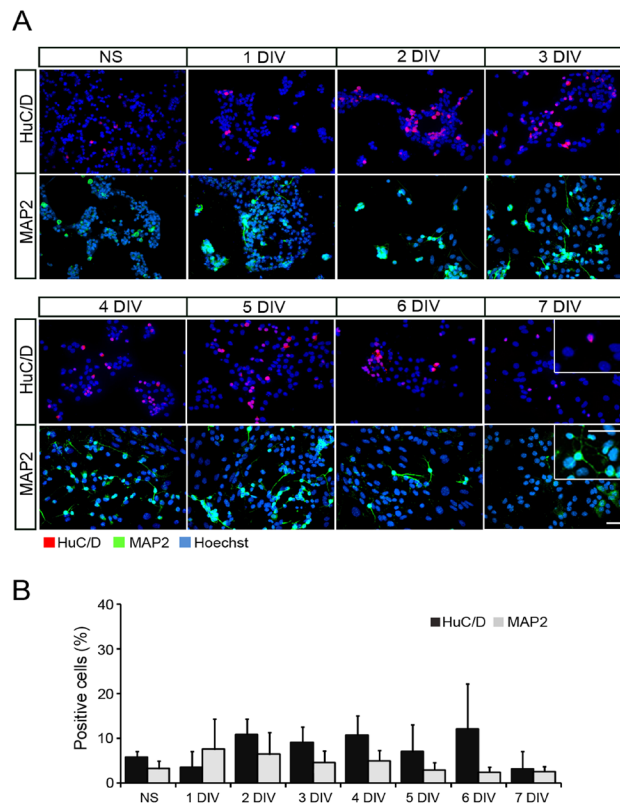
Figure S2

Figure S2: Expression of neuronal phenotypes during neurosphere (NS) proliferation and along differentiation. NS were induced to differentiate into astrocytes until 7 days *in vitro* (DIV). HuC/D-expressing early neuronal progenitors and MAP2-expressing neurons were determined by immunocytochemistry. (A) Nuclei were stained with Hoechst dye (blue) and cells labeled for HuC/D (red) and MAP2 (green). Representative results from one experiment are shown. Detailed cellular staining for each marker in inset images are shown at 7 DIV. Scale bar represents 60 μ m. (B) Graph bars represent the percentage of cells (mean \pm SEM) positive for HuC/D (black) and MAP2 (grey), relatively to the total number of nuclei. Data were obtained from at least three independent experiments.

[“Back to Top”](#)

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