

Identification of cancer stem cells from human Glioblastomas: growth and differentiation capabilities and CD133/Prominin-1 expression

<sup>1</sup>Federica Gambelli, <sup>1</sup>Federica Sasdelli, <sup>1</sup>Ivana Manini, <sup>2</sup>Carla Gambarana, <sup>3</sup>Giuseppe Oliveri, <sup>4</sup>Clelia Miracco and <sup>1</sup>Vincenzo Sorrentino

<sup>1</sup> Molecular Medicine Section, Department of Neuroscience and Center for Stem Cell Research, University of Siena, Siena, Italy

<sup>2</sup> Pharmacology Unit, Department of Neuroscience, University of Siena, Siena, Italy

<sup>3</sup> Department of Neurosurgery, Santa Maria alle Scotte Hospital, Siena, Italy

<sup>4</sup> Section of Pathological Anatomy, Department of Human Pathology and Oncology, University of Siena, Siena, Italy

**running title:** characterization of glioblastoma CSC and CD133/Prominin-1 expression

**Key words:** glioblastoma, brain cancer stem cells, CD133, tumor stem cell marker

**Abbreviations:** 7-AAD: 7-amino-actinomycin D; APC: allophycocyanin; bFGF: basic fibroblast growth factor; BSA: bovine serum albumin; CSCs: cancer stem cells; CXCR4: chemokine (C-X-C motif) receptor 4; DAPI: 4',6-diamidino-2-phenylindole; DMEM/F12: Dulbecco's Modified Eagle's Medium: Nutrient mixture F-12; EDTA: ethylenediaminetetraacetic acid; EGF: epidermal growth factor; FACS: fluorescence-activated cell sorting; FITC: fluorescein isothiocyanate; Gal C: galactocerebroside C; GBM: glioblastoma multiforme; GFAP: glial fibrillary acidic protein; LDA: limiting dilution assay; LIF: leukemia inhibitory factor; mAb: monoclonal antibody; MSC: mesenchymal stem cell; NF-H: neurofilament heavy polypeptide; NS: neurosphere; NSCs: neural stem cells; OM: original magnification; PBMcs, Peripheral Blood Mononuclear Cells; PBS: phosphate buffered saline; PE: phycoerythrin; RT PCR: reverse transcriptase-polymerase chain reaction; SA: semi-adherent; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; S.E.M.: standard error of the mean; SOX-2: SRY-related HMG-box gene 2; SSEA-1: stage-specific embryonic antigen 1; TBS-T: Tris-buffered saline with 0.1% Tween 20.

**Correspondence to:** Vincenzo Sorrentino, MD,  
Molecular Medicine section, Department of Neuroscience,  
University of Siena, Via Aldo Moro, 53100 Siena, Italy.  
Phone: +39 0577 234 079; Fax: +39 0577 234 191; e-mail: [v.sorrentino@unisi.it](mailto:v.sorrentino@unisi.it)

**ABSTRACT**

CD133 has been proposed as a marker of tumorigenic cancer stem cells (CSCs) in human Glioblastoma multiforme (GBM), although tumorigenic CD133-negative CSCs have been also isolated. Additional evidence also indicates that CSCs from GBM could exhibit different phenotypes, rising interest into the potential significance of these different CSCs with respect to diagnosis and prognosis, and for development of novel targets for future treatment. We analyzed the expression of CD133 in freshly isolated cells from 15 human GBM specimens. Interestingly, only 4 out of 15 GBMs contained cells positive for AC133 by FACS analysis. Of note, all 4 AC133-positive tumors yielded distinct CSC lines, while only 6 CSC lines were obtained from the 11 GBM that did not contain AC133-positive cells. Of these 10 CSCs lines, we further characterized 6 CSC lines. Three CSCs grew as fast-growing neurospheres with better clonogenic ability, while the remaining 3 grew as slow-growing semi-adherent spheres with lower clonogenic ability. In addition, the former CSC lines displayed better differentiative capacities than the latter ones. Surprisingly, PCR and Western Blot analysis revealed that all 6 GBM CSC lines expressed CD133/Prominin1, suggesting that cells negative by FACS analysis may actually represent cells expressing low levels of CD133 undetected by FACS. Nevertheless, all the 6 CSC lines were tumorigenic in nude mice. In conclusion, CSCs from human primary GBMs show different phenotypes and express CD133, though at varying levels. However none of these parameters is directly correlated with the tumorigenic potentials of the cells.

## 1. Introduction

The cancer stem cell hypothesis proposes that tumors are initiated and sustained by a small subpopulation of neoplastic cells with stem cell-like features, called cancer stem cells (CSCs) (Reya et al. 2001; Clarke et al. 2006; Visvader et al. 2008). CSCs are defined by their capacity to self-renew, express stem cell markers and initiate tumors *in vivo*, recapitulating the original parental tumor. Potential CSCs have been identified in many solid tumors such as breast (Al-Hajj et al. 2003), pancreas (Li et al. 2007), lung Kim et al. 2005), colon (O'Brien et al. 2007), prostate (Collins et al. 2005), ovarian Zhang et al. 2008) and brain cancer Ignatova et al. 2002; Singh et al. 2003; Galli et al. 2004; Yuan et al. 2004). Among human malignant brain tumors, glioblastoma multiforme (GBM, WHO, grade IV) is the most aggressive, with no available curative treatment and a median survival of approximately one year, despite surgery and chemo-radiotherapies Stupp et al. 2009). The hypothesis that GBM originates from transformed neural stem/progenitor cells is supported by several clinical and experimental evidences (Oliver et al. 2004; Sanai et al. 2005); Lim et al. 2007). Indeed, CSCs can be cultured from human GBM by using conditions that promote the selection of neural stem cells (NSCs) (Ignatova et al. 2002; Singh et al. 2003; Galli et al. 2004; Yuan et al. 2004). Similarly to NSCs, these cells form clonal neurosphere-like aggregates, express neural stem/progenitor markers (e. g. nestin), and differentiate into neurons and glial cell types. While recent reports have identified specific gene expression profiles that correlate with patient's survival and outcome (Phillips et al. 2006), less is known about the identity and phenotype of tumor-initiating CSCs.

Many efforts have been aimed at identifying cell surface markers useful for prospective isolation of CSCs. Among these, much attention has been given to CD133/Prominin-1, which is currently used to identify and prospectively enrich for CSCs in brain (Singh et al. 2003; Singh et al. 2004; Hemmati et al. 2003; Piccirillo et al. 2006; Bao et al. 2006) and in other solid tumors (O'Brien et al. 2007; Monzani et al. 2007; Ricci-Vitiani et al. 2007; Curley et al. 2009). Indeed, a subpopulation of putative CSCs can be isolated from adult brain tumors using monoclonal antibodies (mAbs) directed against epitope 1 of CD133 (i.e. AC133) Singh et al. 2003; Hemmati et al. 2003). However, cells displaying properties of CSCs with comparable tumorigenic capacity *in vivo*, but negative for CD133 have also been reported, thus questioning the role of CD133 as a marker of brain CSCs (Beier et al. 2007; Günther et al. 2008; Joo et al. 2008; Wang et al. 2008; Ogden et al. 2008). These CD133-negative CSCs show often a lower proliferation index *in vitro*, but show the same tumorigenic capacity *in vivo* compared to CD133-positive cells (Beier et al. 2007). Moreover, glioma-initiating cells have been recently identified based on the expression of the stage-specific embryonic antigen 1 (SSEA-1) (Son et al. 2009) or intrinsic autofluorescence properties and morphology of these cells (Clément et al. 2010). Given the controversial results about the identity of GBM derived CSCs, we aimed at elucidating their phenotype and verifying whether stem cell-like properties such as proliferative and clonogenic abilities, multipotent differentiative capacities correlate with the expression of CD133 and tumorigenic capacity of these cells.

## 2. Materials and Methods

### 2.1 Patients and tumor specimens

Following informed consent, tumor specimens were obtained from patients diagnosed with primary GBM (WHO, grade IV) (median age: 59, range 35-78), undergoing surgical treatment at the Department of Neurosurgery of the University of Siena. GBM10 was derived from a GBM recurrence. The study was performed according to the Helsinki Declaration and was approved by the local ethics committee.

### 2.2 Establishment of tumor cell cultures

Fresh post-surgery tumor fragments were processed as described by Gritti et al. (Gritti et al. 1996) Primary cells were plated at a density of 3500 cells/cm<sup>2</sup> in culture medium (Dulbecco's modified Eagle's medium (DMEM)/ F-12 (1:1) (Sigma-Aldrich) containing 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 250 ng/mL amphotericin B, 0.6% glucose, 9.6 µg/mL putrescine, 6.3 ng/mL progesterone, 5.2 ng/mL Na-selenite, 25 µg/mL insulin, 0.1 mg/mL apotransferrin, 2 µg/mL heparin (sodium salt, grade II; Sigma-Aldrich, St. Louis, MO, USA), 20 ng/mL recombinant human Epidermal Growth Factor (hEGF) and 20 ng/mL recombinant human basic Fibroblast Growth Factor (bFGF) (PeproTech EC Ltd., London, UK). Every 7-14 days, tumor spheres were enzymatically dissociated with Accutase (Sigma-Aldrich) followed by mechanical dissociation with P1000 Gilson pipette and cells plated at 5000 cells/cm<sup>2</sup> in culture medium.

### 2.3 Limiting Dilution Assay

Dissociated cells were assayed for clonogenic capacity using a limiting dilution assay (LDA). Final cell dilutions ranged from 100 cells to 1 cell/well in 96 well plate. After 21 days in culture, the percentage of wells not containing spheres for each cell plating density was calculated. Clonogenic frequency was calculated according to the linear regression analysis method of Taswell as previously described (Reynolds et al. 2007). Briefly, the natural log of the percent negative value was plotted against the number of cells seeded in each well and a trend line was inserted. The equation for the best-fit line was used to calculate the clonogenic frequency: the cell number leading to 1/Ln (37% negative wells).  $R^2$  values, which represent the statistical power of this method, ranged from 0.9475 to 0.9983. LDA assays were carried out at least three times for each GBM cell lines and the mean number of neurosphere-forming cells per thousands cells  $\pm$  standard error of the mean (S.E.M.) was calculated.

### 2.4 Differentiation assay and immunocytochemistry

Following the dissociation of spheres, single cells were plated onto Matrigel-coated glass coverslips, at a density of 50000 cells/cm<sup>2</sup> in growth factor-free medium in the presence of 10 ng/mL Leukemia Inhibitory Factor (LIF; Chemicon, Temecula, CA, USA) for 10 days. As a control, the cells were maintained for 3 hours in culture medium to allow for cell attachment to the coverslip, as previously described (Galli et al. 2004). Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) in phosphate buffered saline (PBS) for 10 min. Cells were permeabilized with 0.3% Triton X-100 in PBS (Sigma-Aldrich) for 10 min (except for Galactocerebroside C (GalC) staining) and blocked in PBS containing 5% Normal Goat Serum (Sigma-Aldrich) and 0.2% (w/v) bovine serum albumin (BSA) for 1 hour. Primary antibodies were: mouse anti-Nestin (1:1000; Chemicon), goat anti-SRY-related HMG-box gene 2 (SOX-2) (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) rabbit anti-Glial Fibrillary Acidic Protein (GFAP; 1:2400; Dako, Milan, Italy), mouse anti- $\beta$ -tubulin III (TU20; 1:600; Chemicon), mouse anti-Neurofilament heavy

polypeptide (NF-H) (2F11; 1:1500; Dako) and mouse anti-GalC (GalC; 1:1000; Chemicon). After incubation with primary antibodies in 2% (w/v) BSA/PBS overnight at 4°C, cells were washed with PBS and appropriate secondary antibodies were added for 60 min. The following secondary antibodies were used according to the manufacturer's instructions: Cy3-conjugated goat anti-mouse, Cy3-conjugated donkey anti-goat, Cy2-conjugated goat anti-rabbit, Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Calbiochem; Merck Chemicals Ltd., Nottingham, UK). Immunofluorescence was documented with an epifluorescence Axioplan 2 imaging Microscope (Zeiss, Thornwood, NY, USA) equipped with a MicroMAX digital CCD camera (Princeton Instruments, Trenton, NY, USA) and subsequently processed with a Meta Imaging Serie 4.5 software (Universal Imaging Corporation, West Chester, PA, USA). Quantification of positive cells was carried out by counting the stained cells among at least 300 cells/cell line within about six randomly selected microscope fields.

## 2.5 Flow Cytometry

Freshly isolated or passage 0 tumor cells, as well as *in vitro* cultured CSCs, were analyzed for CD133 expression by Fluorescence-Activated Cell Sorting (FACS) using a mAb targeting the epitope 1 of CD133 (PE-conjugated CD133/1, 1:10; clone AC133; Miltenyi Biotec, Bergisch Gladbach, Germany). FACS analysis of intracellular CD133 expression was also performed in permeabilized CSCs. Established CSC lines were also analyzed up to passage 15 for the expression of selected cell surface antigens. Briefly,  $2 \times 10^5$  cells from dissociated tumor spheres were incubated for 30 min at 4°C in 2 mM EDTA/0.5% BSA/ PBS (FACS buffer) with the following mAbs: PE-conjugated CD24 (1:20; clone SN3; eBioscience, San Diego, CA, USA); PE-conjugated CD29 (1:20, clone MAR4); APC-conjugated CD34 (1:10; clone 581); PE-conjugated CD117 (1:10, clone YB5.B8; PE-conjugated CXCR<sub>4</sub> (1:10; clone 12G5), all from BD Biosciences, Franklin Lakes, NJ, USA; FITC-conjugated CD44 (1:10; clone DF1485; Dako); PE-conjugated CD105 (1:10; clone N1-3A1; Ancell, Bayport, MN, USA). Identical IgG isotypes (BD Biosciences) served as negative controls. After incubation, cells were washed in FACS buffer and non-viable cells were labeled with 7-Amino-Actinomycin D (7-AAD) (BD Biosciences). Threshold was adjusted in forward and side scatter dot plot to exclude cellular debris and a total of 30000 events were analyzed on a FACSCalibur flow cytometer running CellQuest software (BD Biosciences).

## 2.6 RT-PCR Analysis

Total RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen, Milan, Italy) and treated with DNase I (Qiagen) to eliminate genomic DNA. 1 µg RNA was reverse transcribed using oligodT (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Promega, Milan, Italy) according to manufacturer's instructions. Aliquots of cDNA were amplified in 25 µl of PCR reaction mixture containing 1X Mg-free buffer, 1.5 mM MgCl<sub>2</sub> (both from Roche Molecular Systems, NJ, USA), 0.2 mM dNTPs mix (Promega), 200 nM each primer (Invitrogen) and 0.75 U/reaction of AmpliTaq Gold Polymerase (Roche Molecular Systems). PCR conditions included an initial denaturation step of 10 min at 94°C, followed by 40 (for CD133) or 25 (for β-actin) cycles of 1 min denaturation at 94°C, 1 min at annealing temperature and 1 min extension at 72°C, with a final elongation step at 72°C for 5 min. Real-time PCR reactions were carried out using a Euroclone One Gradient PCR machine (Euroclone, Pero, MI, Italy). Amplified products were separated by electrophoresis on a 2% agarose gel (Roche-Boehringer Mannheim GmbH, Germany) and visualized by ethidium bromide staining. The following primers and

PCR conditions were used: CD133 (304 bp) forward, 5' GAGTGTGCTGAACACACACCAG-3'; reverse, 5'-ACGCAGGTTTCTCTATGATGGC-3' (40 cycles, annealing temperature: 59°C) and  $\beta$ -actin (377 bp): forward, 5'-CAACTCCATCATGAAGTGTGAC-3'; reverse, 5'-GCCATGCCAATCTCATCTTG -3' (20 cycles, annealing temperature: 58°C).

## 2.7 Western blot analysis

Dissociated tumor sphere cells were washed with PBS, lysed in RIPA buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% (w/v) Sodium Deoxycholate, 1% (v/v) Triton X-100, 0.1% (v/v) Sodium Dodecyl Sulfate), containing 10  $\mu$ g/ml each of leupeptin, aprotinin, antipain, pepstatin A and chymostatin (all from Sigma-Aldrich). Forty  $\mu$ g of protein cells lysates were separated on 7.5% SDS-PAGE and electroblotted onto nitrocellulose membranes.

Membranes were blocked in blocking solution (5% milk powder, in Tris-buffered saline, pH 7.4, 0.1% (v/v) Tween-20, TBS-T) for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C. Primary antibodies were as follows: rabbit anti-human CD133 mAb (1:1000, clone C24B9; Cell Signaling Technology, Danvers, MA, USA), mouse anti-human CD133/1 mAb (1:100, clone AC133 and clone W6B3C1, Miltenyi), rabbit polyclonal antibody anti-human CD133 (1:1000; ab19898; Abcam, Cambridge, UK) and mouse anti-human  $\beta$  actin (1:2000; Sigma), as loading control. After washing in TBS-T, blots were incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibody (Amersham Biosciences, GE Healthcare Europe GmbH, Milan, Italy) diluted 1:3000 in blocking solution for 1h at room temperature, and developed using an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences).

## 2.8 Evaluation of tumorigenicity

Tumorigenicity was assessed by orthotopic implantation. The University of Siena Ethics Committee approved all procedures.  $2 \times 10^5$  dissociated tumor cells in 2  $\mu$ l 0.0001% (v/v) DNase/DMEM were stereotactically injected into the right striatum of 6-8 week-old CD1 *nu/nu* female mice (Charles River, Calco, LC, Italy), as previously described (Galli et al. 2004). The glioma cell line U87MG was used as reference line. Mice were sacrificed at the onset of neurological symptoms. The brain was fixed in 10% buffered formalin and about 2 mm-thick coronal slices were embedded in paraffin and stained with hematoxylin and eosin according to standard histological procedure. For immunohistochemical analysis, 3  $\mu$ m-thick sections were dewaxed, rehydrated, incubated with 3% H<sub>2</sub>O<sub>2</sub> in Tris-buffered saline (TBS) to inhibit endogenous peroxidase, and processed following different methods, depending on the antibody. A panel of monoclonal antibodies anti-human antigens was tested, including: GFAP (1:100, Clone 6F2); CD34 (1:100; Clone QBEnd 10); CD105 (1:50, Clone SN6h), all from Dako. Before incubating with the anti-mouse primary Ab, sections were either pre-treated in a bath (Lab Vision PT Module) with citrate buffer (0.01 M, pH 6.0) at 95-98°C for 30 min. (for GFAP), or trypsinized (using Pronase XIV of *Streptomyces griseus*) at 37°C for 10 min. (for CD105 and CD34 antibodies). Either diaminobenzidine (Dako) or New Fuchsin (Bio-Optica, Milan, Italy) served as chromogen.

## 2.9 Statistics

Student's *t*-test was performed using a 2 tailed distribution analysis and a value of  $p < 0.05$  was considered statistically significant.

### 3. RESULTS

#### 3.1 Isolation, proliferation and multipotency of CSCs from human primary GBM

We isolated and cultured tumor cells in NSC permissive conditions from 15 post-surgery fragments of human primary GBMs. Analysis of freshly isolated tumor cells revealed that only 4 out of 15 were positive for AC133 expression, with a fraction of positive cells ranging from 1.5% to 34.6%, as determined by FACS analysis with the widely used AC133 mAb that recognizes the glycosylated epitope 1 of CD133 (Fig. 1a). From these 15 specimens, 10 yielded expandable cell populations, 4 of which were derived from the 4 tumors containing AC133-positive cells. To verify whether these GBM-derived cultures of neurospheres were enriched in CSCs, we extensively characterized 6 CSC lines, which included 2 CSC lines positive by FACS analysis for AC133 (GBM46 and GBM49) and 4 negative (GBM5, GBM9, GBM10, GBM12). These 6 CSC lines were studied for their ability to proliferate for long periods of time, to form neurospheres under clonogenic conditions, the expression of NSC markers and the capability to differentiate into cells expressing markers of neurons and glial cell types.

Between 1 to 3 weeks after plating in NSC medium, the tumor cells formed phase-bright clonal aggregates similar to neurosphere attached to the plate. In three cases (GBM9, GBM46 and GBM49) cell clusters detached from the plate and grew entirely as free-floating spheres. In contrast, GBM5, GBM10 and GBM12 cultures were comprised of attached and floating spheres interspersed with adherent single cells. The morphology of neurosphere- and semi-adherent growing cells is shown in figure 1b. To assess whether they were able to proliferate for long periods of time, CSCs were subcultured by plating at a density of 5000 cells/cm<sup>2</sup>. All 6 CSC lines proliferated for a minimum of 11 passages corresponding to at least 150 days of culture. To date, 3 established CSC lines (GBM5, GBM9 and GBM10) have been growing for more than 20 passages, with no obvious changes in their growth rate, while GBM12 lost its proliferation capacity at passage 19. Analysis of growth kinetics revealed that neurosphere-growing cells grew faster than the semi-adherent cell cultures, with an average doubling time of 5 and 18 days, respectively (Fig. 1c). The capacity to generate self-renewing neurosphere-like aggregates is a key feature of brain CSCs (Singh et al. 2003; Vescovi et al. 2006). In order to determine the frequency of repopulating cancer stem progenitor cells within the tumor-derived cell population, we performed a limiting dilution analysis. The average frequency of neurosphere-forming cells was statistically higher in neurosphere compared to semi-adherent CSC lines ( $91.4 \pm 47.3$  and  $9.9 \pm 3.2$  per thousand cells, respectively,  $p < 0.05$ ), suggesting that the former comprise a higher number of stem/progenitor cells which contribute to the *in vitro* expansion of the cell population (Fig. 1d).

Similarly to neural stem/progenitor cells, brain CSCs are characterized by the expression of nestin and NSC early transcription factor SOX-2, and by the capacity to sustain multilineage differentiation (e.g. the ability to give rise to neurons, astrocytes and oligodendrocytes) (Singh et al. 2003; Galli et al. 2004; Vescovi et al. 2006; Uchida et al. 2000). Therefore, we performed fluorescence immunocytochemistry in undifferentiated cells and after 10 days in differentiation medium. Within 3 hours of incubation in differentiation medium, most cells attached to the culture plate and, after 10 days, changes in cell morphology were observed. The majority of undifferentiated cells from all the 6 CSC lines analyzed expressed nestin (Fig. 2a). Upon induction of differentiation, the intensity of the nestin staining decreased, even though the percentage of positive cells did not change, suggesting that, in contrast to normal NSCs, CSCs partially retain a neural

stem /progenitor phenotype and/or they are poorly differentiated (Fig. 2a). Undifferentiated CSC lines expressed the transcription factor SOX-2 in a percentage ranging from 46% to 95%. After 10 days of differentiation the number of SOX-2-positive cells was only moderately reduced (Fig.2a). In order to assess the multipotency of GBM-derived cell lines, the expression of  $\beta$ -tubulin III and NF-H for neurons, GFAP for astroglia and Gal C for oligodendroglia were analyzed. In undifferentiated cells the percentage of GFAP- and  $\beta$ -tubulin III-positive cells varied from 20 to 90% and from 1.0% to 23%, respectively, and significantly increased upon induction of differentiation (Fig. 2a). The number of cells expressing the late marker of neuron differentiation NF-H was lower than  $\beta$ -tubulin III-positive cells, indicating that the cells do not differentiate toward a mature neuronal phenotype (not shown). Rare oligodendrocytes cells were observed in undifferentiated cells from all CSC lines, while their number increased upon differentiation (Fig. 2a). Aberrant co-expression of neuronal and glial markers was also observed in most differentiated cells (not shown), as expected for tumor derived CSCs (Ignatova et al. 2002; Singh et al. 2003; Galli et al. 2004; Yuan et al. 2004). A careful analysis revealed that semi-adherent CSCs showed a qualitatively lower differentiative capacity compared to neurosphere CSCs. Representative immunofluorescence analysis of undifferentiated and differentiated GBM46 (neurosphere) and GBM5 (semi-adherent) CSC lines is shown in figure 2b, *left* and *right panel*, respectively. Staining for the NSC markers nestin and SOX-2 was intense in the majority of control cells from both groups (Fig. 2b, *left panel*) and decreased after 10 days in differentiating conditions (Fig. 2b, *right panel*). A stronger signal for GFAP and  $\beta$ -tubulin III was observed in neurosphere compared to semi-adherent CSCs upon differentiation (Fig. 2b, *right panel*). Furthermore neurosphere CSCs gave rise to more typical neuronal cells with a visible cell body and long axons compared to semi-adherent CSCs (22% and 11%, respectively). A higher number of astrocytic cells with characteristic star-shape and the presence of at least four processes was also observed in differentiated neurosphere compared to semi-adherent CSCs (40% and 14% respectively), indicating that the former display a better capability to differentiate.

### 3.2 Cell surface antigen profile of GBM CSC lines

In order to further characterize the phenotype of GBM CSC lines, flow cytometric analysis of cell surface antigens expressed in neural stem/progenitor cells and/or brain cancer was performed. CXCR4 is highly expressed in human neural precursor cells (Ni et al. 2004; Reiss et al. 2002) and has been linked to glioma cell invasiveness (Ehtesham et al. 2006) and proliferation of GBM CSCs (Ehtesham et al 2009). CD24 (mucin-like adhesion molecule) has been shown to promote invasion of cancer cells (Senner et al. 1999; Baumann et al.2005), while CD117 is expressed by radio-resistant CSC subpopulations (Kang et al. 2008).

Expression of mesenchymal genes has been associated with the level of malignancy of glioma (Phillips et al. 2006; Freije et al. 2004) and immunopositivity to mesenchymal stem cell (MSC) markers has been also reported in GBM-derived cell lines (Tso et al. 2006). Therefore, to further phenotype the GBM-derived CSCs, we also analyzed the expression of the MSC markers CD29 (integrin  $\beta$ 1/fibronectin receptor), CD44 (stromal hyaluronic acid receptor) and CD105 (endoglin) in neurosphere and semi-adherent CSC lines. Furthermore, given that an increased expression of the hematopoietic cell surface antigen CD34 in glioma cells has been reported (Kim et al. 2005), we also tested whether CSC lines contained a fraction of CD34-positive cells. As shown in Table 1, a variable portion of cells expressed the analyzed antigens. A statistically significant higher number of CD29 and CD44-positive cells was observed in semi-adherent compared to neurosphere cell cultures (Student *t* test,  $p < 0.05$ ).

### 3.3 Expression of CD133 by established GBM CSC lines

FACS analysis of CD133/Prominin1 expression in GBM CSC lines was performed using the AC133 mAb (from Miltenyi). The 2 CSC lines (GBM46 and GBM49), derived from GBMs containing AC133-positive cells at isolation, maintained the AC133 positivity in a percentage of cells that was similar to that one observed in freshly isolated cells, while the other 4 remained negative (Fig. 3a). Furthermore, CD133 expressing cells did not lose their CD133 positivity by FACS during passages, neither did CD133 negative cells give rise to CD133 cells following in vitro passaging. To rule out the possibility that CD133 could be retained in the cell cytoplasm thus being undetectable by routine FACS analysis, we performed flow cytometry staining of permeabilized cells (not shown). Again no AC133 positive cells were observed in the 4 CSCs previously shown to be AC133 negative. However, RT-PCR experiments revealed the expression of CD133/Prominin1 in all GBM CSC lines, as for the amplification of a 304 bp band, confirmed by DNA sequencing to correspond to CD133/Prominin1 cDNA (Fig. 3b). To further this point, western blot analysis was then performed using a mAb (CD133/1, clone W6B3C1, Miltenyi) directed against the same CD133 epitope recognized by the AC133 mAb. As shown in Fig. 3c, the W6B3C1 mAb clearly recognized a band of approximately 120 kDa in GBM46 and GBM49 cell extracts. However, a faint band of 120 kDa was also observed in cell extracts from GBM5, GBM9 and GBM10, following prolonged film exposure. Previous reports have shown that the AC133 mAb recognizes a glycosylated epitope of CD133/Prominin1, not always present on CD133/Prominin1 protein (Florek et al. 2005). Therefore, we performed additional western blot experiments using a rabbit mAb (clone C24B9, from Cell Signaling Technology) directed against an unmodified amino acid region surrounding Asp 562 of human CD133/Prominin1. After immunoblotting with C24B9 mAb, two bands of 105/120 kDa, previously shown to correspond to differentially glycosylated forms of CD133/Prominin1 (Boivin et al. 2009) and a band of approximately 130 kDa, were detected in all GBM CSC lines. Again, the intensity of the 105/120 kDa bands was stronger in GBM 46 and GBM49 compared to those observed in GBM5, GBM9 and GBM10 cell lysates (Fig. 3d). Comparable results were obtained with a rabbit polyclonal antibody directed to the C-terminus region of CD133/Prominin1 (ab19898, from Abcam) (not shown). The specificity of this antibody was confirmed by blocking experiments (Fig. 3e). All together, these data suggest that all cell lines analyzed contain cells expressing CD133, however the levels of expression of CD133 seem to differ significantly. Accordingly, negativity in FACS analysis does not mean lack of expression or lack of localization to the plasma membrane of CD133, but more likely reflects the limits of detection of the monoclonal antibody AC133 that is certainly very selective against the CD133 protein, but is not very efficient in detecting cells expression low levels of this protein.

### 3.4 Tumorigenic potential of CSC lines upon orthotopic transplantation in nude mice

Tumorigenicity of CSC lines was assessed by orthotopic injection as described in the Materials and Methods section. In U87 MG-treated mice, tumors developed after one month forming a well-defined mass characterized by a homogenous neoplastic cell population (not shown), as previously reported (Galli et al. 2004). In contrast, GBM46, GBM12, GBM9 and GBM10 CSC lines formed tumors resembling human GBM in 3, 4, 6.5 and 7 months, respectively. On the other hands, in mice injected with GBM5 and GBM49 cells, tumors developed between 8 and 10 months, and neoplastic spread was more limited and mice brain structure quite spared. Figure 4 shows a section of a control mouse brain (Fig. 4a) and the histopathologic analysis of a tumor derived from GBM46

(Fig. 4*b-l*), which is common to all tumors, except those formed by GBM5 and GBM49 CSCs. Tumors displayed high positivity to GFAP (Fig. 4*c*), were hypercellular, hypervascularized, and highly infiltrative, recapitulating the histopathology of human GBM (Fig. 4*d-f*). There was also neoplastic diffusion into the contralateral hemisphere and neovascularization in mouse and human parental GBM was comparable, although mouse tumors did not present glomeruloid vessels that usually characterize human GBM. Areas of palisading necrosis (Fig. 4*g, 4h*) and morphological patterns of human glioma cell invasion, which are known as the secondary structures of Scherer ((Scherer et al. 1938) (i.e., perineuronal (Fig. 4*i*) and perivascular satellitosis (Fig.4*j*), invasion along white matter tracts, and subpial spread) were also observed. As also observed by FACS analysis of cultured CSCs, positivity for CD34 and CD105 was higher in glial neoplastic cells in most mice tumors (Fig. 4*k* and 4*l*) than in the corresponding parental human GBMs (not shown). Overall, we found no correlation between tumorigenic capacity of CSC lines and expression of any of the cell surface antigens analyzed, including CD133, nor patient's prognosis (data not shown).

#### 4. Discussion

Several markers have been proposed for the prospective identification of CSCs in several tumors. In brain tumors, CD133 has been proposed to identify CSCs, although the use of CD133 as a marker for the identification of cells with CSC phenotype has been controversial (Ignatova et al. 2002; Singh et al. 2003; Galli et al. 2004; Yuan et al. 2004; Beier et al. 2007; Günther et al. 2008; Joo et al. 2008; Wang et al. 2008; Ogden et al. 2008). In the present study, only 26% (4 /15) of freshly dissociated tumors contained a subpopulation of AC133-positive cells, ranging from 1.5 to 34.6% of the total cells, as determined by FACS analysis, in agreement with other reports (Singh et al. 2004; Piccirillo et al. 2006). From these 15 tumors, we established 10 CSC lines, 4 originating from the 4 tumors that contained AC133-expressing cells, while only 6 were obtained from the 11 AC133 negative tumors. From this, it would appear that CD133 expressing cells might have a higher efficiency in establishing as CSC lines *in vitro*. Of the 6 CSC lines that were further characterized, only 2 contained a population of CD133-positive cells by FACS analysis with the AC133 mAb. However, all 6 GBM CSC lines were found to express variable levels of CD133/Prominin1 by RT-PCR and western blot analyses. Indeed, the data obtained by RT-PCR were comparable with those obtained by western blot experiments, indicating a direct relationship between the levels of CD133/ prominin1 mRNA and protein. Therefore, we interpret these results as an indication that CD133-negative cells, as from FACS analysis with the AC133 mAb, may rather represent cells expressing low levels of CD133/Prominin-1 and not to reflect changes in the AC133 epitope, in contrast to what observed following differentiation of colon cancer stem cells (Kemper et al. 2010). This observation may be eventually also extended to CD133-negative GBM CSC lines reported by other groups (Beier et al. 2007; Günther et al. 2008; Wang et al. 2008).

Several reports have indicated that CSCs isolated from GBMs may have different biological and molecular properties (Ignatova et al. 2002; Singh et al. 2003; Galli et al. 2004; Yuan et al. 2004; Beier et al. 2007; Günther et al. 2008); Joo et al. 2008); Wang et al. 2008; Ogden et al. 2008). Initial work reported the isolation of GBM CSCs that could be distinguished in two clusters based on their proliferation rate in culture (Galli et al. 2004). CD133/Prominin-1 has been subsequently proposed as a marker for identification of the CSC population within the neurosphere that is able to initiate tumor formation (Singh et al. 2003; Singh et al. 2004; Piccirillo et al. 2006). However, others have reported that also CD133-negative cells with distinct phenotypes and molecular profiles can be tumorigenic *in vivo* (Beier et al. 2007; Günther et al. 2008); Joo et al. 2008); Wang et al. 2008; Ogden et al. 2008). Here we report that 3/3 CSC lines expressing low levels of CD133 grew semi-adherently, showed a lower proliferation rate and a reduced clonogenicity and multipotential capacity. In contrast, the other CSC lines, which predominantly (2/3) expressed higher levels of CD133, formed floating neurosphere and displayed a full stem-like phenotype with higher proliferation rate and better differentiation properties. Nevertheless, we observed that all CSCs formed tumors after injection in the brain, in a period of time varying between 3 and 7 months, except for GBM49 and GBM5 cells, where mice were sacrificed after 8 and 10 months, respectively. In these experiments, we injected  $2 \times 10^5$  cells, which is well within the range of  $1.5 \times 10^5$  -  $5 \times 10^5$  cells per mouse reported by other groups when using not purified GBM-derived CSC populations (Piccirillo et al. 2006; Günther et al. 2008; Kang et al. 2008). Conversely, lower numbers of tumor cells have been used mainly only when purified CD133 positive CSCs were tested (Singh et al. 2004; Bao et al. 2006). It is worth to note that the time required for tumor formation in most studies is about 3 months, although this has also been reported to require

longer period of time Günther et al. 2008). Interestingly, we found that the tumorigenicity of the CSCs was independent of CD133 levels of expression, as also reported by other groups (Beier et al. 2007; Günther et al. 2008); Joo et al. 2008); Wang et al. 2008; Ogden et al. 2008). Indeed, lack of correlation between levels of CD133 expression and the ability to form tumors is even more evident when considering that the GBM49 cell line, which contained the highest percentage of CD133 positive cells, did not develop a well-defined tumor mass before 8 months. Other than CD133 expression, the 6 cell lines of GBM-derived CSCs could also be distinguished for their efficiency to grow under clonal conditions, to grow as neurosphere or as semi-adherent cultures and to differentiate into different neural phenotypes. Surprisingly, no one of these parameters correlated with the tumorigenicity of these cells.

All CSC lines contained a variable number of cells expressing MSC markers such as CD29, CD44 and CD105. Expression of mesenchymal genes and MSC markers in glioma has been associated with poor prognosis and with the activation of stromal/angiogenic program sustaining tumor growth and malignant progression (Phillips et al. 2006; Freije et al. 2004; Tso et al. 2006). Again, in our study, we did not observe an association between expression of mesenchymal markers and malignancy. However, we found that CSCs, which grew semi-adherently, expressed significantly higher levels of CD29 and CD44 compared to neurosphere growing CSCs, in agreement with results by Günther *et al.* (Günther et al. 2008) Furthermore, we noted that expression of CD34 was higher in CSCs that grew semi-adherently compared to those that grew as neurospheres, although the difference was not statistically significant. Nevertheless, a significant higher number of CD34 positive neoplastic cells was observed in the tumors developed in mice compared to parental tumors. The hematopoietic stem cell marker CD34 is expressed transiently during early neurulation and its over expression has been reported in GBM, both by gene expression analysis and immunohistochemistry (Scrideli et al. 2008). Whether this increase in CD34 expressing cells, observed in cultured CSCs and in the tumors formed following injection in mice, has a biological significance requires further investigation.

In conclusion, our study indicates that CSC with different phenotypes can be isolated from human GBMs. However, differences in CD133/Prominin-1 expression levels and *in vitro* growth properties of CSCs established from human GBM do not correlate with the tumorigenic potentials of these cells.

### Acknowledgements

This work was supported by grants from Regione Toscana and from Fondazione Monte dei Paschi di Siena.

## References

- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A.* 2003; 100: 3983-3988.
- Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 2006;444:756-60.
- Baumann P, Cremers N, Kroese F, Orend G, Chiquet-Ehrismann R, et al. CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. *Cancer Res* 2005;65:10783-10793.
- Beier D, Hau P, Proescholdt M, Lohmeier A, Wischhusen J, Oefner PJ, et al. CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res.* 2007; 67:4010-4015.
- Boivin D, Labbé D, Fontaine N, Lamy S, Beaulieu E, Gingras D, et al. The stem cell marker CD133 (prominin-1) is phosphorylated on cytoplasmic tyrosine-828 and tyrosine-852 by Src and Fyn tyrosine kinases. *Biochemistry* 1009;48:3998-4007.
- Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, et al. Cancer stem cells-perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res* 2006;66:9339-9344.
- Clément V, Marino D, Cudalbu C, Hamou MF, Mlynarik V, de Tribolet N, et al. Marker-independent identification of glioma-initiating cells *Nat Methods* 2010;7:224-228.
- Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; 65:10946-10951.
- Curley MD, V.A. Therrien, C.L. Cummings, P.A. Sergent, C.R. Koulouris, A.M. Friel, et al. CD133 Expression Defines a Tumor Initiating Cell Population in Primary Human Ovarian Cancer. *Stem Cells* 2009;27:2875-2883.
- Ehtesham M, Mapara KY, Stevenson CB, Thompson RC. CXCR4 mediates the proliferation of glioblastoma progenitor cells. *Cancer Lett* 2009; 274:305-312.
- Ehtesham M, Winston JA, Kabos P, Thompson R. CXCR4 expression mediates glioma cell invasiveness. *Oncogene* 2006;25:2801-2806.
- Florek M, Haase M, Marzesco AM, Freund D, Ehninger G, Huttner WB, et al. Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer. *Cell Tissue Res* 1005;319:15-26.

Freije WA, Castro-Vargas FE, Fang Z, Horvath S, Cloughesy T, Liau LM, et al. Gene expression profiling of gliomas strongly predicts survival. *Cancer Res* 2004;64:6503-6510.

Galli R., Binda E., Orfanelli U., Cipelletti B., Gritti A., De Vitis S, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res*;2004 64:7011-7021.

Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, et al. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J Neurosci* 1996;16:1091-1100.

Günther HS, Schmidt NO, Phillips HS, Kemming D, Kharbanda S, Soriano R, et al. Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. *Oncogene* 2008;27:2897-2909.

Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M, et al. Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci USA* 2003;100:15178-15183.

Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* 2002;39:193-206.

Joo KM, Kim SY, Jin X, Song SY, Kong DS, Lee JI, et al. Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. *Lab Invest* 2008; 88:808-815.

Kang MK, Hur BI, Ko MH, Kim CH, Cha SH, Kang SK. Potential identity of multi-potential cancer stem-like subpopulation after radiation of cultured brain glioma. *BMC Neurosci* 2008;30:9-15.

Kemper K, Sprick MR, de Bree M, Scopelliti A, Vermeulen L, Hoek M, et al. The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation. *Cancer Research* 2010;70:719-29

Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer, *Cell* 2005; 121 823-835.

Li C, Heidt DH, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. *Cancer Res* 2007;67:1030-1037.

Lim DA, Cha S, Mayo MC, Chen MH, Keles E, Vandenberg S, et al. Relationship of glioblastoma multiforme to neural stem cell regions predicts invasive and multifocal tumor phenotype. *Neuro Oncol* 2007;9:424-429.

Monzani E, Facchetti F, Galmozzi E, Corsini E, Benetti A, Cavazzin C, et al. Melanoma contains CD133 and ABCG2 positive cells with enhanced tumorigenic potential. *Eur J Cancer* 2007;43: 935-946.

Ni HT, Hu S, Sheng WS, Olson JM, Cheeran MC, Chan AS, et al. High-level expression of functional chemokine receptor CXCR4 on human neural precursor cells. *Brain Res Dev* 2004;152:159-169.

O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007;445:106-110.

Ogden AT, Waziri AE, Lochhead RA, Fusco D, Lopez K, Ellis JA, et al. Identification of A2B5+CD133- tumor-initiating cells in adult human gliomas. *Neurosurgery* 2008;62:505-514.

Oliver TG, Wechsler-Reya RJ. Getting at the root and stem of brain tumors. *Neuron* 2004; 42:885-888.

Phillips HS, Kharbanda S, Chen R, Forrest WF, Soriano RH, Wu TD, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006;9:157-173.

Piccirillo SG, Reynolds BA, Zanetti N, Lamorte G, Binda E, Broggi G, et al. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. *Nature* 2006;444:761-765.

Reiss K, Mentlein R, Sievers J, Hartmann D. Stromal cell-derived factor 1 is secreted by meningeal cells and acts as chemotactic factor on neuronal stem cells of the cerebellar external granular layer. *Neuroscience* 2002;115:295-305.

Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414: 105-111.

Reynolds SD, Shen H, Reynolds PR, Betsuyaku T, Pilewski JM, Gambelli F, et al. Molecular and functional properties of lung SP cells. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L972-L983.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007;445:111-115.

Sanai N, Alvarez-Buylla A, Berger MS. Neural stem cells and the origin of gliomas. *N Engl J* 2005; 353:811-822.

Scherer HJ. Structural development in gliomas. *Am J Cancer* 1938;34:333-351.

Scrideli CA, Carlotti CG Jr, Okamoto OK, Andrade VS, Cortez MA, Motta FJ, et al. Gene expression profile analysis of primary glioblastomas and non-neoplastic brain tissue: identification of potential target genes by oligonucleotide microarray and real-time quantitative PCR. *J Neurooncol* 2008;88:281-291.

- Senner V, Sturm A, Baur I, Schrell UH, Distel L, Paulus W. CD24 promotes invasion of glioma cells in vivo. *J Neuropathol Exp Neurol* 1999;58:795-802.
- Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;63:5821–5828.
- Singh SK, Hawkins C, Clarke ID, Squire JA, J. Bayani, Hide T, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432:396-401.
- Son MJ, Woolard K, Nam DH, Lee J, Fine HA. SSEA-1 is an enrichment marker for tumor-initiating cells in human glioblastoma. *Cell Stem Cell* 2009;4:440-452.
- Stupp R., Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. European Organisation for Research and Treatment of Cancer Brain Tumour and Radiation Oncology Groups; National Cancer Institute of Canada Clinical Trials Group. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* 2009;10:459-466.
- Tso CL, Shintaku P, Chen J, Liu Q, Liu J, Chen Z, et al. Primary glioblastomas express mesenchymal stem-like properties. *Mol Cancer Res* 2006;4:607-619.
- Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV, et al. Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci USA* 2000;97:14720-14725.
- Vescovi AL, Galli R, Reynolds BA. Brain tumour stem cells. *Nat Rev Cancer* 2006,6:425-436.
- Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008;8: 755-768.
- Wang J, Sakariassen PØ, Tsinkalovsky O, Immervoll H, Bøe SO, Svendsen A, et al. CD133 negative glioma cells form tumors in nude rats and give rise to CD133 positive cells. *Int J Cancer* 2008; 122:761-768.
- Yuan X, Curtin J, Xiong Y, Liu G, Waschmann-Hogiu S, Farkas DL, et al. Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene* 2004;23:9392-9400.
- Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, et al. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 2008;68:4311-4320.

## Figure legends

**Figure 1.** Phenotypic characterization of stem cell-enriched cultures from glioblastoma (GBM). (a) Flow cytometric analysis showing the IgG isotype control (grey line histogram) and CD133 expression (black line histogram) in dissociated primary GBMs using the AC133 monoclonal antibody. (b) Phase contrast microscopy of representative GBM-derived cultures growing as floating (GBM9) or semi-adherent (GBM5) neurospheres. Note the elongated shape of adherent cells that mediate the attachment to the plastic surface of semi-adherent neurospheres in GBM5, but not in GBM9 cultures. Bar is 200  $\mu\text{m}$ . (c) Growth curves of faster growing neurosphere (NS) and slower growing semi-adherent (SA) CSC lines. (d) Clonogenic frequency within neurosphere (NS) and semi-adherent (SA) CSCs.

**Figure 2.** Glioblastoma (GBM)-derived cells express neural stem/progenitor markers and display multipotential differentiation capacity. (a) Quantitation of cells immunoreactive to nestin, SOX-2 and astrocytic (GFAP), neuronal ( $\beta$ -tubulin III), and oligodendroglial (GalC) markers in undifferentiated (*white columns*) and differentiated (*black columns*) neurosphere (NS) and semi-adherent (SA) GBM CSCs. Differentiation experiments were performed three times for each of the 6 CSC lines. Values are expressed as means of positive cells  $\pm$  S.E.M. ; \*  $p < 0.05$ , Student's *t* test. (b) Immunofluorescent labeling for nestin, SOX-2 and for the neural lineage markers (GFAP,  $\beta$ -tubulin III and GalC) (red) in undifferentiated (*left panel*) and differentiated (*right panel*) GBM46 (NS) and GBM5 (SA) CSC lines. Representative well-differentiated astrocyte- and neuron-like cells are indicated by an arrow and an arrowhead, respectively. Nuclei were counterstained with DAPI (blue). Scale bar = 50  $\mu\text{m}$ .

**Figure 3.** CD133 expression in glioblastoma (GBM)-derived cancer stem cells. (a) Flow cytometric analysis of CD133-positive cells using the AC133 monoclonal antibody. Data are presented as percentage mean of positive cells  $\pm$  S.E.M. from at least 5 analyses of CSCs between passage 1 and passage 15. (b) CD133 expression assessed by RT-PCR. mRNA from human Peripheral Blood Mononuclear Cells (PBMCs) was used as positive control (+). Immunoblot of CD133 detected using mouse anti-CD133/1 mAb (clone W6B3C1). (c) and rabbit anti-CD133 mAb (clone C24B9) (d).  $\beta$ -actin was used as internal loading control. (e) Blocking experiment showing the specificity of the polyclonal antibody ab19898.

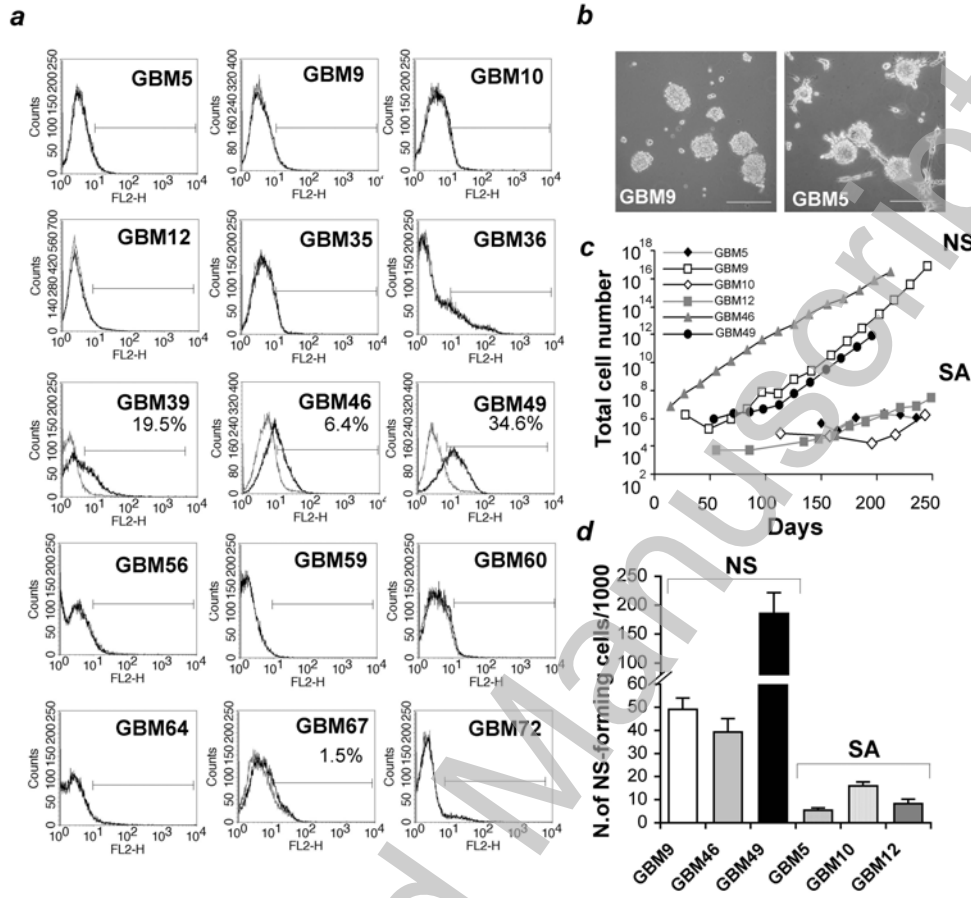
**Figure 4.** Histopathological features of tumor developed in mice after implantation of GBM46-derived CSCs. (a) Coronal section of a control mouse brain. (b) GBM (arrows) developed in a mouse, strongly positive to GFAP (c, inset). (d) Meningeal infiltration (asterisk) in a mouse small cell type GBM. Overlapping histology in mouse (e), and parental (f) GBM, showing small round and spindle cells. Palisading necrosis (asterisk) in a small cell mouse (g), and parental (h) GBM. Perineuronal (i), and perivascular (j) satellitosis (arrows) in a mouse GBM. Mouse GBM strongly immunoreactive to CD34 (the arrow indicates a giant gemistocyte) (k) and CD105 (l). *a, b*, Original Magnification (OM) x 25; inset in C, D-F, I, K, L, OM x 400; G, H, OM x 200; J, OM x 100. *a, b, d-j*, hematoxylin and eosin; *c* (chromogen, diaminobenzidine), *k, l* (chromogen, new fuchsin).

**Table 1. Cell surface antigen profile of neurosphere and semi-adherent GBM-derived CSCs.**

Antigen	Neurosphere CSCs	semi-adherent CSCs
CD24 (mucin-like adhesion molecule)	53.5±11.0	29.6±11.5
CD29 (integrin $\beta$ 1/fibronectin receptor)	74.6±5.0	91.2±2.1 <sup>*</sup>
CD34 (hematopoietic progenitor cell antigen)	21.0±3.7	52.4±15.5
CD44 (stromal hyaluronic acid receptor)	45.1±7.7	72.9±6.5 <sup>*</sup>
CD105 (endoglin)	26.2±3.3	42.9±10.1
CD117 (C-KIT)	1.6±0.4	2.2±0.6
CXCR4 (chemokine (C-X-C motif) receptor 4)	3.1±0.8	1.9±0.9

FACS analysis was performed five times on all 6 CSC lines between passage 0 and passage 15. Data are expressed as % mean of positive cells  $\pm$  standard error of the mean (S.E.M.) within neurosphere or semi-adherent CSC cultures. Comparison of antigen expression between the two groups was assessed by Student *t*-test. Asterisks denote levels of statistical significance, where <sup>\*</sup> =  $P < 0.05$ .

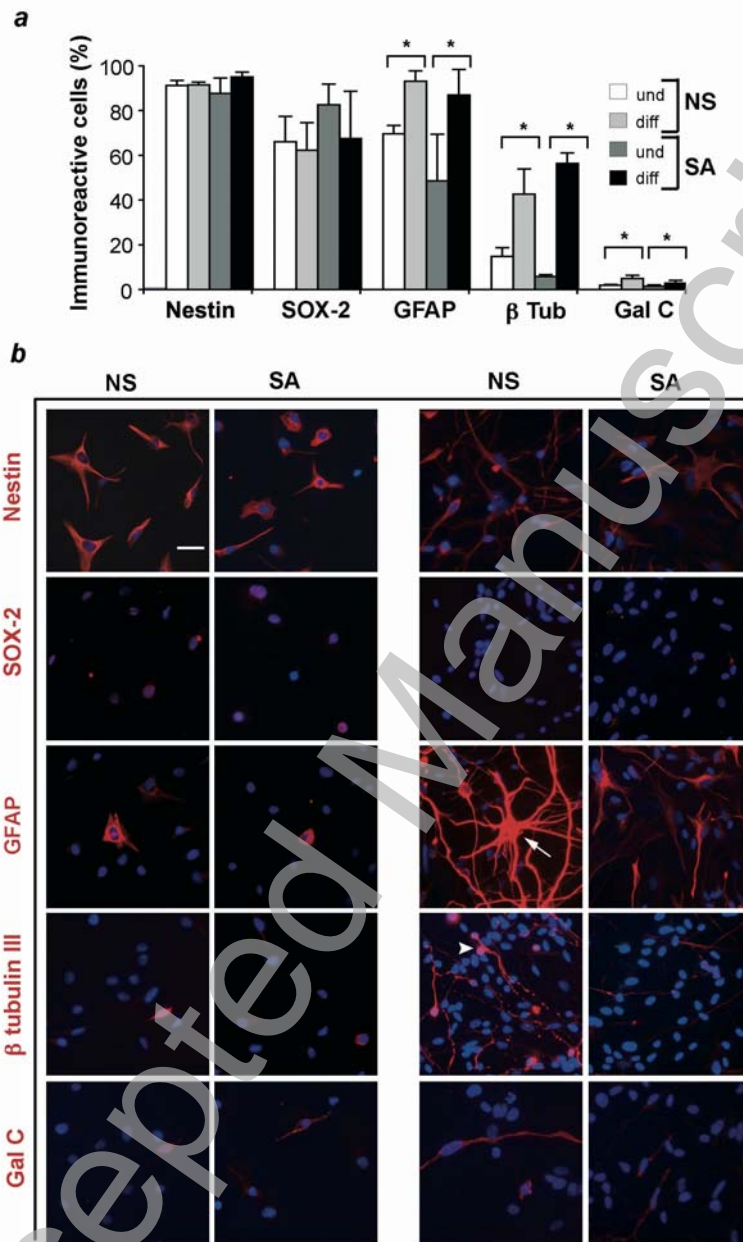
Figure 1



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CBI20110013

Accepted

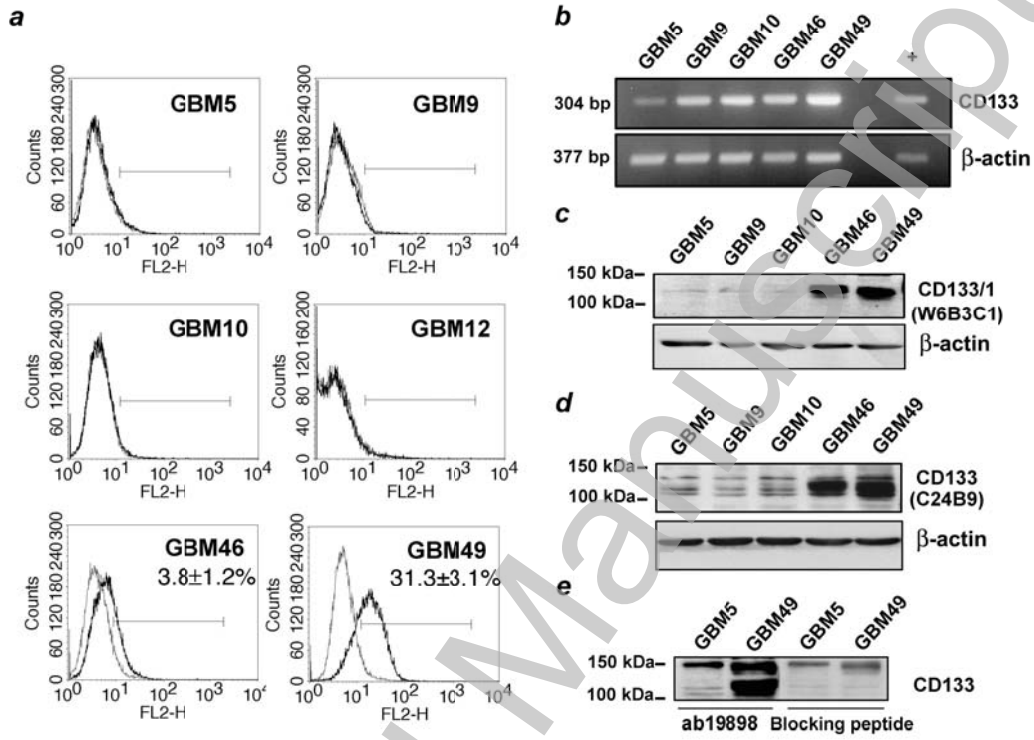
Figure 2



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CBI20110013

Accepted Manuscript

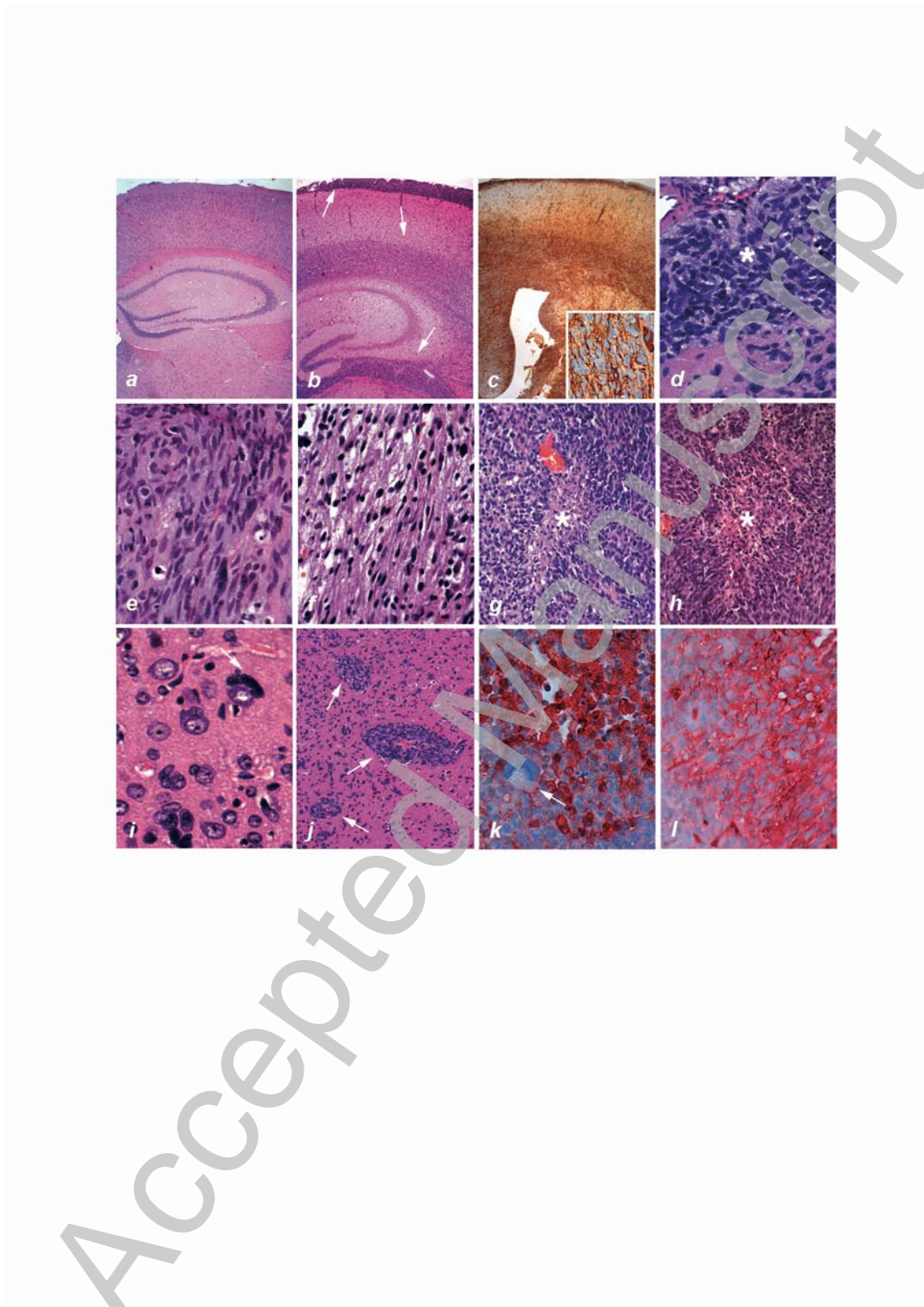
Figure 3



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CBI20110013

Accepted Manuscript

Figure 4



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CBI20110013

Accepted Manuscript