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# Mesenchymal differentiation of glioblastoma stem cells

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Glioblastoma multiforme is a severe form of cancer most likely arising from the transformation of stem or progenitor cells resident in the brain. Although the tumorigenic population in glioblastoma is defined as composed by cancer stem cells (CSCs), the cellular target of the transformation hit remains to be identified. Glioma stem cells (SCs) are thought to have a differentiation potential restricted to the neural lineage. However, using orthotopic *versus* heterotopic xenograft models and *in vitro* differentiation assays, we found that a subset of glioblastomas contained CSCs with both neural and mesenchymal potential. Subcutaneous injection of CSCs or single CSC clones from two of seven patients produced tumor xenografts containing osteo-chondrogenic areas in the context of glioblastoma-like tumor lesions. Moreover, CSC clones from four of seven cases generated both neural and chondrogenic cells *in vitro*. Interestingly, mesenchymal differentiation of the tumor xenografts was associated with reduction of both growth rate and mitotic index. These findings suggest that in a subclass of glioblastomas the tumorigenic hit occurs on a multipotent stem cell, which may reveal its plasticity under specific environmental stimuli. The discovery of such biological properties might provide considerable information to the development of new therapeutic strategies aimed at forcing glioblastoma stem cell differentiation.
*Cell Death and Differentiation* (2008) **15,** 1491–1498; doi:10.1038/cdd.2008.72; published online 23 May 2008

According to the multistep carcinogenesis model, the progenitor cell for glioblastoma is a mature astrocyte that undergoes a series of molecular events leading to its neoplastic transformation.[1–5] On clinical grounds, this concept was validated by cases of low-grade astrocytoma that over several years progress to anaplastic astrocytoma and, eventually, to the highly malignant glioblastoma.[5] However, recent studies have demonstrated that glioblastomas are generated and maintained by a small subset of undifferentiated cells endowed with self-renewal potential.[6–8] Such a tumorigenic population expresses CD133, a marker of normal neural stem and progenitor cells.[6,9] Thus, based on the pioneer work on leukemia,[10] the current view postulates that glioblastomas arise through the neoplastic transformation of stem or progenitor cells resident in the central nervous system, commonly defined as cancer stem cells (CSCs).[11–13] Although normal neural progenitors are devoid of self-renewal activity and unable to undergo unlimited proliferation, oncogenic mutations targeting primitive cells in the brain may confer self-renewal potential to premalignant neural progenitors, which may become fully malignant following further tumorigenic mutations.[6,7,9,14]

Neural stem cells display a differentiation potential that is not restricted to tissues of ectodermal origin but include muscle and endothelial lineages.[15,16] Such mesenchymal plasticity is lost during the transition from stem to progenitor cells, and not regained during terminal differentiation. Differently from normal neural stem cells, glioblastoma stem cells (SCs) seem to be able to generate only neural cells. *In vitro,* they differentiate both into GFAP (glial fibrillary acidic protein)-positive astrocyte-like cells and into neurofilament expressing neuron-like cells, often generating double-positive cells. *In vivo,* glioblastoma SCs develop tumor xenografts with histological features confined to the malignant astrocytic phenotype.[7,8] Such a limited differentiation potential is in line with the hypothesis that the tumorigenic hit occurs in a neural progenitor, which is unable to generate mesenchymal cells. However, it is possible that the cell of origin for glioblastoma is a stem cell which has undergone a transformation event that limits the differentiation potential of its progeny.[14] Moreover, the restricted differentiation of glioblastoma SCs may result from the unavailability of a specific microenvironment that drives the formation of mesenchymal cells.

Here, we used orthotopic *versus* heterotopic xenograft mouse models and *in vitro* differentiation assays to demonstrate that the CSCs arising from a subset of glioblastoma exhibit chondro-osteogenic differentiation in response to environmental stimuli. Besides its interest in gliomagenesis, this finding may have clinical impact in the development

of new therapeutic strategies aimed at exploiting CSC differentiation.[17]

## Results

**Isolation and characterization of cancer stem cells from human glioblastoma.** Permanent CSC cultures established from seven glioblastoma patients were used (Table 1). The stem cell phenotype of CSCs was validated according to the following criteria: (1) formation of primary spheres in vitro; (2) capacity of self-renewal on clonogenic and population analysis; (3) ability to differentiate under serum stimulation both into GFAP-positive astrocyte-like cells and into neurofilament expressing neuron-like cells; (4) generation of tumors upon orthotopic (intracerebral) transplantation in immunodeficient mice; (5) maintenance of the chromosomal aberrations of the parental tumor (Figure 1). In response to removal of mitogens and serum stimulation, a large proportion of CSCs adhered to flasks and underwent differentiation. After exposing dissociated glioblastoma spheres for 14 days to 5% serum, they differentiated into cells that showed glial morphology and expressed both the astrocytic marker GFAP and the neuronal marker neurofilament (Figure 1). In general, CSCs and their progeny maintained the p53 status, GFAP and EGFR expression typical of the parental tumors (Table 1 and Figure 1). Interphase fluorescence in situ hybridization (FISH) analysis demonstrated that the stem-like cells isolated from glioblastoma tissue specimens were actual tumor cells and did not represent normal neural stem cells migrated in brain areas infiltrated by the tumor. FISH analysis also provided evidence that the in vitro manipulation did not induce major additional chromosomal changes, and that the tumor xenografts did arise from human cancer cells (Figure 1 and Table 1). Twenty weeks after intracranial implantation CSCs routinely generated highly infiltrating tumors with a diameter of $2.7 \pm 1.9$ mm, which closely resembled the human anaplastic astrocytoma (Figure 1). To further characterize the tumor phenotype of the xenografts, we performed immunohistochemistry of each transplanted tumor

and compared the immunophenotype of the xenografts and the parental tumor (Figure 1). Brain tumor xenografts replicated the immunohistochemical profile of the parental neoplasm, including expression of GFAP, EGFR and p53.

**Differentiation of glioblastoma stem cells in subcutaneous xenografts.** To determine whether glioblastoma SCs might give rise to a progeny different from neural cells, we used the subcutaneous injection of glioblastoma spheres in immunodeficient mice as a heterotopic xenograft model that favors the mesenchymal differentiation of multipotent cells. Overall, the subcutaneous xeno-grafts showed histological features that mimicked the cytoarchitecture of the parental tumor (Table 2 and Figure 2a). However, mixed glioblastoma–sarcoma tumors with areas of chondroblastic and osteoblastic differentiation developed upon subcutaneous grafting of CSCs derived from cases 2 and 4 (Table 1 and Figure 2a). Human chromosome 10 labeling of cartilaginous areas confirmed the human origin of the mesenchymal tissue derived by CSC xenografting (Figure 2b). Notably, when the cell lines 2 and 4 had been grafted into the brain, the chondro- and osteo-sarcomatous components did not develop in the context of tumor xenografts. Thus, the site of injection appears to affect the differentiation pattern of glioblastoma SCs in the in vivo condition. Interestingly, the growth rate of those subcutaneous xenografts that showed mesenchymal differentiation was much lower than the xenografts devoid of mesenchymal components (Figure 2c, top). By 16 weeks after grafting, tumor diameter was $4.1 \pm 2.1$ and $12.7 \pm 2.5$ mm in mixed glioblastoma–sarcoma and glioblastoma xenografts, respectively (mean $\pm$ S.D., $n = 31$; $P < 0.0001$, Student's t-test). Overall, the mitotic index was significantly lower in mixed glioblastoma–sarcoma xenografts as compared with the glioblastoma xenografts (mitotic index of chondro-osteosarcoma xenografts $2.2 \pm 2.1$ versus mitotic index of glioblastoma xenografts $7.1 \pm 2.2$; $P < 0.0001$, mean $\pm$ S.D., $n = 22$; Student's t-test) (Figure 2c, bottom). In the context of mixed glioblastoma–sarcoma xenografts, the mitotic index of regions with mesenchymal differentiation

**Table 1** Immunophenotype and chromosomal changes in parental tumors and cultured cancer stem cells

| Case code | Age/sex | Parental tumor | | Cancer stem cells | |
|---|---|---|---|---|---|
| | | **Phenotype** | **FISH** | **Phenotype** | **FISH** |
| 1 | 40/M | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Trisomy 10, 19, 22 | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Trisomy 10, 19, 22 |
| 2 | 77/M | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Normal 10, 19, 22 | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Normal 10, 19, 22 |
| 3 | 72/M | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Monosomy 10 | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Monosomy 10 |
| 4 | 44/M | GFAP+, p53−, EGFR+, YKL40−, CD133+ | Normal 10, 19, 22 | GFAP+, p53−, EGFR+, YKL40−, CD133+ | Normal 10, 19, 22 |
| 5 | 67/F | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Monosomy 10, 19, 22 | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Monosomy 10, 19, 22 |
| 6 | 70/F | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Monosomy 19 Normal 10, 22 | GFAP+, p53+, EGFR+, YKL40−, CD133+ | Monosomy 19 Normal 10, 22 |
| 7 | 48/F | GFAP+, p53+, EGFR−, YKL40−, CD133+ | Monosomy 19 Normal 10, 22 | GFAP+, p53+, EGFR-, YKL40−, CD133+ | Monosomy 19 Normal 10, 22 |

EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; GFAP, glial fibrillary acid protein
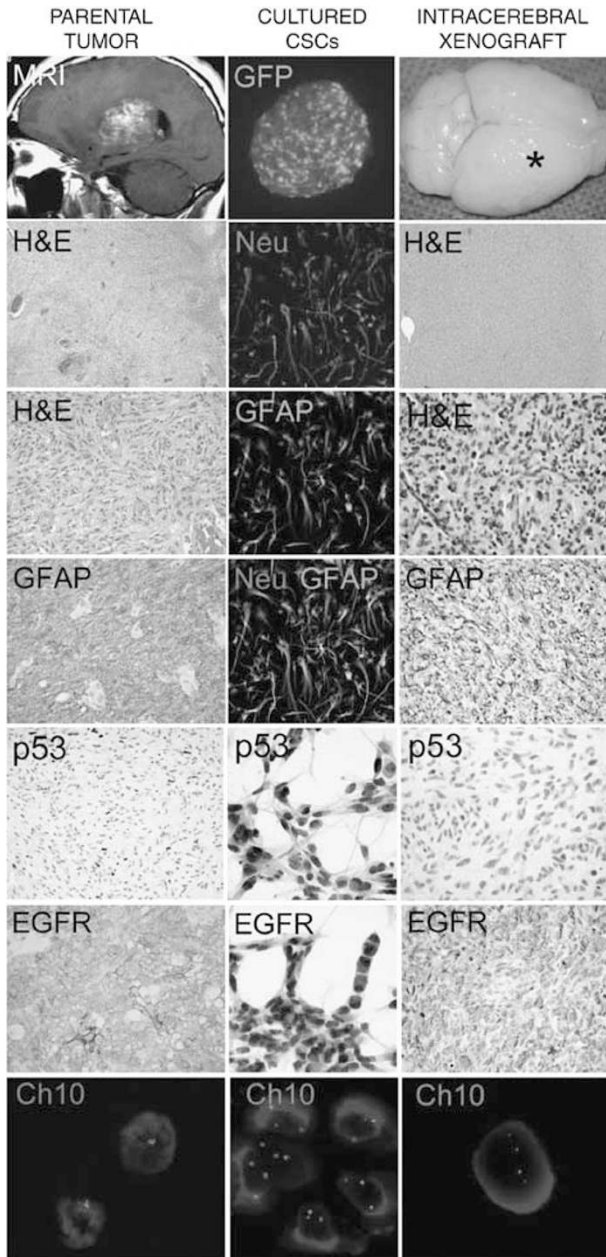
**Figure 1** Characterization of glioblastoma cancer stem cells. Representative picture showing the type of analyses performed on parental tumors, cultured glioblastoma stem cells, and intracranial tumor xenografts. In this illustrative case (case 1), the patient's tumor (left column) was located in the posterior temporal region, as defined on contrast enhanced T1-weighted magnetic resonance images (MRI). The tumor shows histological features of glioblastoma, like infiltrative growth pattern, foci of necrosis, and neoangiogenesis (H&E). On immunohistochemistry, the tumor expresses the astrocytic marker GFAP (brown), mutant p53 protein, and epidermal growth factor receptor (EGFR). Polisomy of chromosomes 10 (Ch 10, red) is demonstrated by FISH analysis on nuclei extracted from paraffin sections. The cultured stem cells (middle column) grow as spheres in vitro. Serum-differentiated cells express both neuronal (neurofilament, Neu) and astrocytic (GFAP) differentiation markers. They also maintain the p53 status, EGFR expression profile, and chromosomal changes of the parent tumor. Intracerebral xenograft tumors (right column) closely mimic diffusely infiltrative anaplastic astrocytoma (H&E). Immunophenotyping (GFAP, p53, and EGFR) and FISH analysis demonstrate similar features with the parental tumor. Star symbol indicates the site of injection. A colour version of this Figure is available online

was 5.8-fold lower than adjacent areas of astrocytic tumor tissue.

***In vitro* analysis of glioblastoma stem cells mesenchymal potential.** To confirm that a subset of glioblastoma SCs exhibited mesenchymal plasticity, we set up an *in vitro* model by using commercial human mesenchymal stem cell chondrogenic differentiation medium. Glioblastoma SC lines derived from cases 2, 4, 5, and 6 differentiated into cartilage in defined medium, as assessed by alcian blue-PAS (Periodic Acid Shiff) staining and immunoreaction with anti-YKL40 antibody, while a minority of GFAP + cells were generated in this condition (Figure 3a and Table 2). Mesenchymal differentiation was not detected in any glioblastoma SC line exposed to serum-induced differentiation (Figure 3b). To further verify the chondrogenic differentiation of CSCs, we evaluated the expression of collagen II and SOX9, which are early and specific markers of chondrocyte differentiation, together with the cartilaginous extracellular matrix component aggrecan.[18–20] RT-PCR analysis showed high level expression of collagen type II, SOX 9 and aggrecan in differentiated CSC lines 2 and 4, whereas these genes were not observed in CSC line 1 and control adult neural progenitor cells (Figure 3c). These data indicate that a significant number of glioblastomas contain populations of multipotent stem cells with different degrees of chondro-osteogenic potential.

**Single tumorigenic glioblastoma stem cells are able to produce both neural and mesenchymal cells.** The ability of glioma neurospheres from four of seven tumors to generate mesenchymal cells could be due to a mixed population of cells able to generate either mesenchymal or neural stem cells. To determine whether a single glioblastoma SC may generate a progeny of both ectodermal and mesodermal lineages, we produced several clones from each of the seven CSC lines included in the study. The clonogenic potential of the glioblastoma neurospheres obtained from the different tumors did not show a significant variability (range, 15–20%). All the clones derived by single-cell plating were analyzed upon serum-driven differentiation to confirm the neural potential of every single clone (Figure 4a and data not shown). We next evaluated the mesenchymal potential of the different clones by exposing the cell pellets to the chondrogenic medium. While the clones generated from case 1, 3 and 7 showed a limited differentiation exclusively confined to the neural lineage, the clones obtained from the other four cases (2, 4, 5 and 6) produced chondrocytes with dense irregular nuclei and ample vesicular cytoplasm enclosed in a myxoid matrix (Figure 4b). To determine the tumorigenic activity and the *in vivo* plasticity, single clones from case 1, 2 and 4 were injected subcutaneously in immunocompromised mice. All the different clones examined were able to generate heterotopic tumor xenografts. However, while the tumors growing from case 1 clones were histologically very similar to the original tumor, clones from case 4 generated glioblastoma tumor xenografts with extensive chondro-osteogenic areas (Figure 4c). A similar chondrogenic differentiation was observed in subcutaneous xenografts
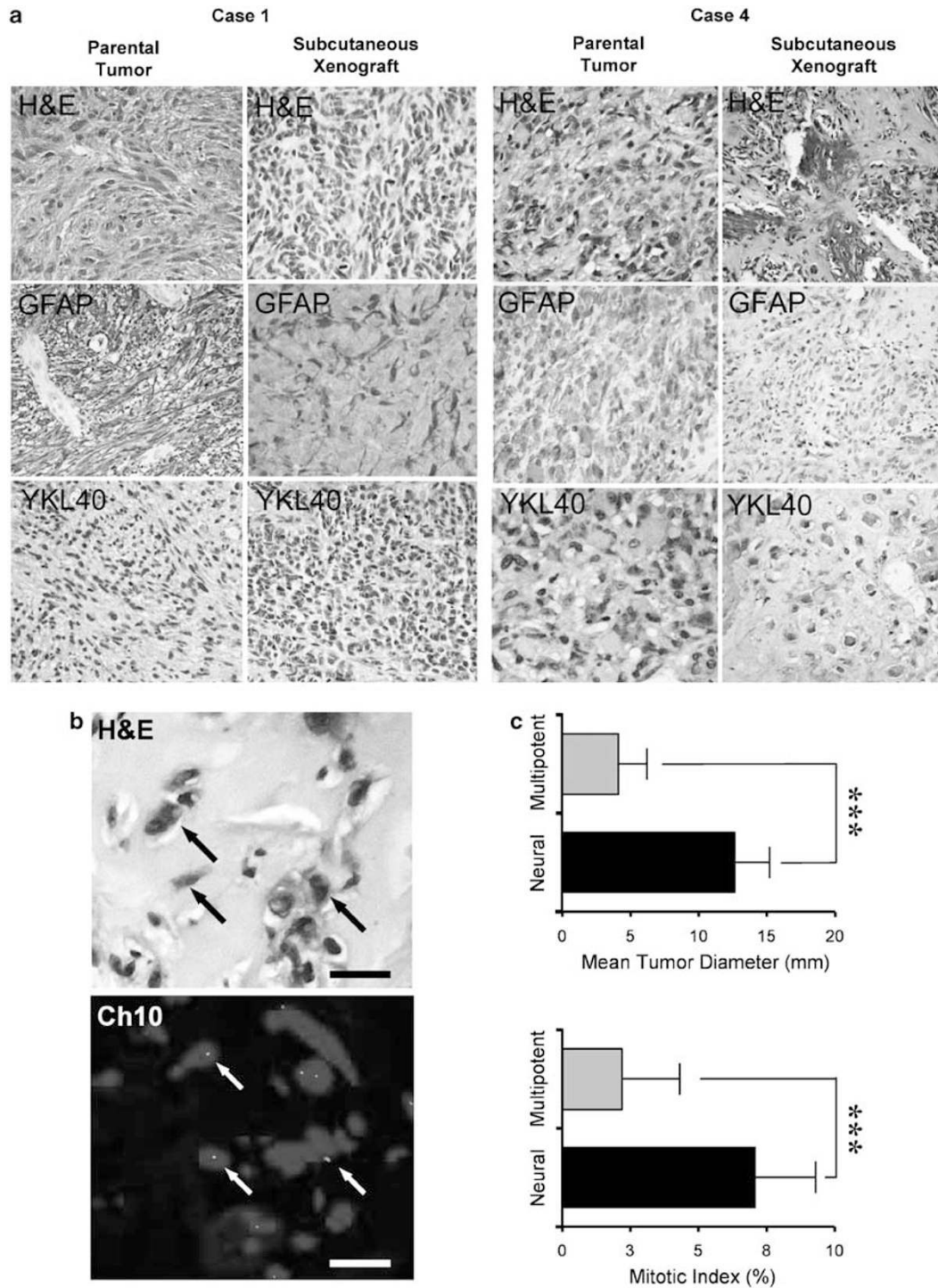
**Figure 2** Mesenchymal differentiation of subcutaneous tumor xenografts. (**a**) Comparative analysis of subcutaneous xenografts derived from two representative of seven CSC lines and their parental tumors. The parental tumors showed histological features of glioblastoma (H&E). Immunophenotyping revealed GFAP expression and absent or low expression ($<6\%$ of cells) of YKL40 in parental tumors. Upon subcutaneous grafting (subcutaneous xenograft), glioblastoma like tumors are generated by stem cells isolated from case 1, 3, 5, 6 and 7, whereas a mixed tumor developed by case 2 and 4 derived stem cells. In the mixed tumor from case 4, chondro- and osteosarcomatous components coexist with glioblastoma tissue (H&E). The mixed nature of these xenograft tumors is demonstrated by the presence of GFAP-positive cells interspersed among areas of chondrogenic differentiation (YKL40). (**b**) Area of cartilaginous differentiation in the context of a subcutaneous tumor xenograft. The arrows point out the nuclei of chondrogenic cells (H&E, scale bar 50 μm). Interphase FISH analysis of adjacent tissue section using locus-specific probe for the centromere of human chromosome 10 (Ch 10, scale bar 50 μm). (**c**) Graphs showing the diameter (top) and mitotic index (bottom) of the tumor xenografts at 16 weeks after subcutaneous implantation. Subcutaneous xenografts generated by CSC lines 2 and 4 (multipotent) were significantly smaller and proliferate less frequently than the xenografts generated by the remaining CSC lines (neural, $P<0.0001$)

**Table 2** Mesenchymal differentiation *in vitro* and in subcutaneous xenografts

| Case code | *In vitro* Assay | Subcutaneous xenografts | |
| | | Histology | Phenotype |
| --- | --- | --- | --- |
| 1 | Cartilage−, bone− | GBM | GFAP+, p53+, EGFR+, CD133+,YKL40− |
| 2 | Cartilage+, bone− | Osteo-chondrogenic GBM, AA | GFAP+/−, p53+/−, EGFR+, CD133+,YKL40+ |
| 3 | Cartilage−, bone− | GBM, AA | GFAP+, p53−, EGFR+, CD133−, YKL40− |
| 4 | Cartilage+, bone+ | Osteo-chondrogenic GBM | GFAP+/−, p53−, EGFR+, CD133+, YKL40+ |
| 5 | Cartilage+, bone− | GBM | GFAP+, p53+, EGFR+, CD133+, YKL40− |
| 6 | Cartilage+, bone− | AA | GFAP+, p53+/−, EGFR+, CD133+, YKL40− |
| 7 | Cartilage−, bone− | AA | GFAP+, p53+, EGFR−, CD133+, YKL40− |

AA, anaplastic astrocytoma; EGFR, epidermal growth factor receptor; GBM, glioblastoma; GFAP, glial fibrillary acid protein

obtained with clones derived from case 2 (data not shown). Such a clear plasticity of single cell clones suggest that a considerable percentage of glioblastomas are generated by neural stem cells that during the tumorigenic transformation retain both neural and mesenchymal potential.

## Discussion

The development of sarcomatous areas in the context of glioblastoma tumors has long been recognized by pathologists. [21,22] In some instances, this phenomenon may be so prominent that the tumors are categorized as gliosarcomas, which account for approximately 2% of all glioblastomas.[23] According to the classical view, the areas of sarcomatous transformation in glioblastoma are interpreted as foci of pronounced endothelial proliferation that have acquired neoplastic features.[22,24] However, glioblastoma cases have been described where the sarcomatous components show osteo-chondrogenic or rhabdoid differentiation.[25–27] In such cases, cytogenetic analysis demonstrated that both the glioblastoma and mesenchymal component share identical abnormalities, suggesting a common clonal origin.[25] In addition, recent studies have shown that a subclass of glioblastoma exhibits molecular and phenotypic mesenchymal features, raising the question about the cellular origin of these tumors.[28,29]

Differently from previous reports showing that glioblastoma-derived CSCs generate only neural cells,[7,8,30] we found that the CSCs arising from glioblastoma tumors exhibit a potential for mesenchymal differentiation. Chondro-osteogenic potential was expressed both *in vitro* under specific culture conditions and *in vivo* upon heterotopic grafting in mice. Conversely, mesenchymal differentiation of glioblastoma CSCs did not occur *in vitro* under serum-induced stimulation and in orthotopic xenografts, indicating the crucial role of environmental factors in driving glioblastoma stem cell specification. On the basis of their differentiation potential, we were able to distinguish two major categories of glioblastoma SCs. A subset of tumors whose CSCs exhibit both neural and chondrogenic potential, and another subset with a differentiation potential limited to the neural lineage. Such difference may reflect different stages at which the ancestral cell has undergone its neoplastic transformation. In some glioblastomas, the tumorigenic hit may occur in a multipotent stem cell that under appropriate environmental conditions can express its mesenchymal potential. In glioblastoma where CSC differentiation is restricted towards the neural phenotype, the ancestral cell might be either a transient amplifying precursor cell or a more differentiated astrocytic precursor cell that has retained the stem cell phenotype.

Multipotent glioblastoma SCs exhibit different requirements to undergo mesenchymal specification. While only about 2% of glioblastomas show mesenchymal differentiation in the brain, glioblastoma CSCs are able to differentiate as chondrocytic cells in subcutaneous xenografts and in specific culture conditions at rates of 28 and 57%, respectively. We propose that such differences may represent a variable propensity toward the mesenchymal lineages. Thus, the rare CSCs that contribute to gliosarcoma formation in the brain may have very low requirements for mesenchymal differentiation, probably lower than those CSCs unable to differentiate in mesenchymal cells in the brain, but readily producing chondrocytic cells in subcutaneous xenografts, where the presence of host-derived stromal cells may favor this process. Another subcategory of CSCs is able to produce mesenchymal cells only after defined cytokine conditioning, easily obtained *in vitro* using a chondrocytic culture medium.

Beside its interest in gliomagenesis, the demonstration that the CSCs arising from a subset of glioblastoma exhibit mesenchymal potential may have considerable clinical implications. Heterotopic tumor xenografts with mesenchymal components grew slower and proliferate less frequently than the xenografts where mesenchymal differentiation did not occur. Furthermore, in the foci of mesenchymal differentiation the mitotic index declined by a factor 5.8 relative to adjacent areas of glioblastoma tissue. Thus, the mesenchymal differentiation of glioblastoma xenografts is associated with a significant reduction in cell proliferation. It is conceivable that environmental factors might favor mesenchymal differentiation of multipotent CSCs by changing the balance between proliferation and differentiation. A recent study has demonstrated that treating glioblastoma SCs with bone morphogenic proteins (BMPs) reduces tumorigenicity of these cells in brain xenografts.[17] BMPs are thought to induce the astrocytic differentiation of CSCs, which parallels reduction in cell proliferation. Since glioblastoma SCs are highly resistant to radio- and chemotherapy,[31–33] the demonstration that pro-differentiation stimuli reduce the growth kinetic of these cells may provide a new therapeutic option for highly malignant gliomas.

*In vitro* expanded CSCs are successfully used for generating experimental models of several other tumors, such as breast, colon and lung cancer.[34–36] The ability of different host tissues to modify cancer cell proliferation and differentiation may impact considerably on tumor cell growth and survival.
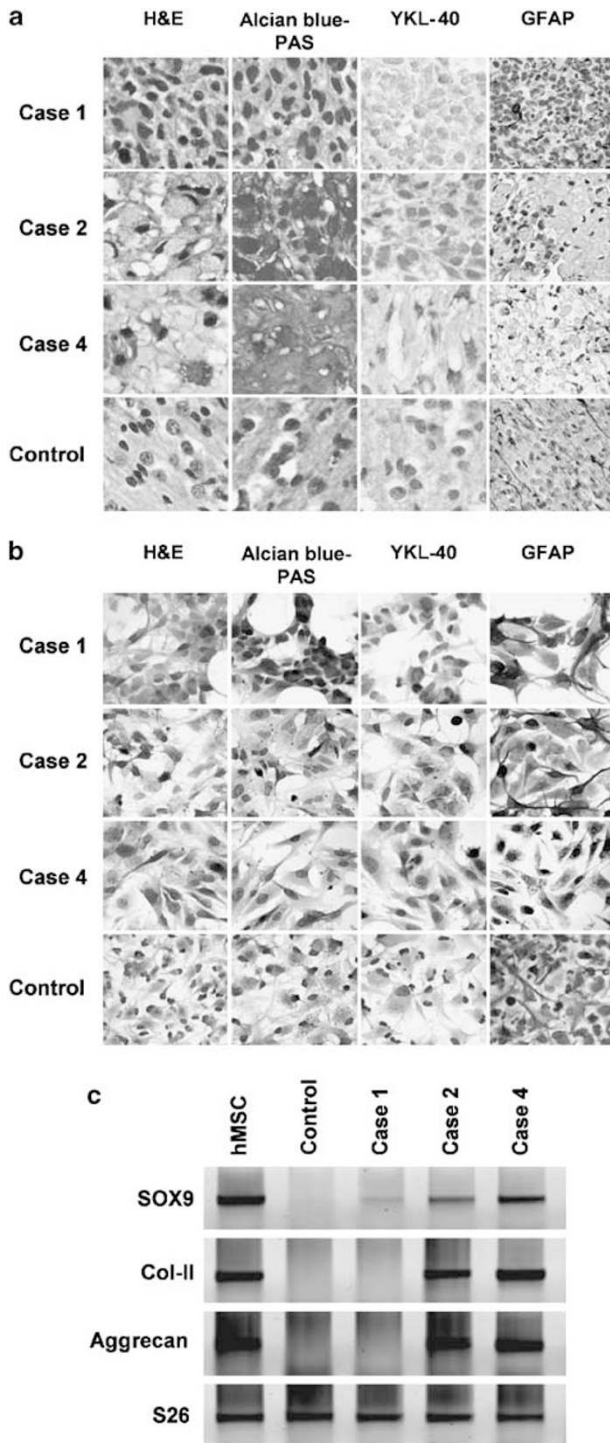
**Figure 3** *In vitro* differentiation of GBM cancer stem cells. Spheres of glioblastoma stem cell lines 1, 2, and 4 were exposed to defined medium for chondrogenic differentiation (**a**) or serum (**b**). After 28 days, cells were stained with H&E and alcian blue-PAS, and immunoreacted with antibody to YKL-40/cartilage glycoprotein and GFAP. Chondrogenic differentiation is noted in Cases 2 and 4 while it is absent in Case 1 and in control neural progenitor cells generated by human adult olfactory bulb. Cells grown in chondrogenic medium were analysed for their expression of SOX9, Col-II, and aggrecan by RT-PCR. RNA from human mesenchymal stem cells (hMSC) exposed to chondrogenesis culture condition, was used as positive control (**c**)

Our findings are in line with the hypothesis that orthotopic and heterotopic injection could be exploited to investigate the role of CSCs and the surrounding microenvironment in tumor development, histotype, metastasis formation, and response to therapy.

## Materials and Methods

**Glioblastoma stem cell cultures and cloning.** Glioblastoma stem cells were isolated from surgical samples of seven adult patients who had undergone craniotomy at the Institute of Neurosurgery, Catholic University School of Medicine in Rome (Table 1). Informed consent was obtained before surgery according to the protocols approved at the Catholic University. The diagnosis of glioblastoma was established on histological examination according to the WHO classification of tumors of the nervous system.[23,37] Cells were obtained through mechanical dissociation of the tumor tissue and cultured in a serum-free medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) as elsewhere described.[7] Clones from glioblastoma neurospheres were obtained by plating single cells into a 96-well plate. After 4 weeks, single clones were mechanically dissociated and replated to expand the culture.

**Immunophenotyping of glioblastoma stem cells.** For immuno-staining of undifferentiated glioblastoma stem cells, dissociated spheroid were plated onto poly-lysine-coated round glass coverslips in 2% serum-containing medium for 12 h to allow the attachment of the cells. Cells were then fixed with 4% paraformaldehyde and stained with antibody directed against nestin (Chemicon, Temecula, CA, USA), CD133 (Santa Cruz Biotecnology, Santa Cruz, CA, USA), GFAP (Dako, Glostrup, Denmark), β tubulin III (Chemicon), and neurofilament (Chemicon). Appropriate secondary antibodies (affinity-purified goat anti-mouse IgG TRITC-conjugated, Chemicon; goat anti-rabbit IgG FITC-conjugated, Chemicon) were used. For neural differentiation assay cells were plated on a poly-lysine-coated round glass coverslips in the absence of EGF and bFGF and in the presence of 5% serum. After 14 days of culture, cells were analyzed by immunofluorescence and immunohistochemistry.

**Intracranial and subcutaneous implantation of glioblastoma stem cells in immunodeficient mice.** The studies involving animals were approved by the Ethical Committee of the Catholic University School of Medicine in Rome. Severe combined immunodeficient mice (both sexes, 4 weeks; SCID, Charles River, Lecco, Italy) and nude athymic mice (male, 4–6 weeks of age; HDS-athymic nude mice, Harlan, Udine, Italy) were used for intracranial and subcutaneous grafting, respectively. For intracranial grafting, a range of $2–50 \times 10^4$ glioma neurospheres were resuspended in 4 $\mu$l of serum-free DMEM. The mice were anesthetized with intraperitoneal diazepam (2 mg/100 g) followed by intramuscular ketamine (4 mg/100 g). The animal skulls were immobilized in a stereotactic head frame, and a burr hole was made 2 mm right of the midline and 1 mm behind the coronal suture. The tip of a 10 $\mu$l-Hamilton microsyringe was placed at a depth of 3.5 mm from the dura and the cells were slowly injected. For subcutaneous grafting, a range of $2–200 \times 10^4$ stem cells were resuspended in 0.1 ml of cold PBS, and the suspension was mixed with an equal volume of cold Matrigel (BD Bioscience, Bedford, MA, USA). After grafting, mice were kept under pathogen-free conditions in positive-pressure cabinets (Tecniplast Gazzada, Varese, Italy), and observed weekly for neurological status and appearance of subcutaneous nodules at injection site. Mice were killed with an overdose of barbiturate by 16–20 weeks after grafting. The whole brain or subcutaneous tumor was removed, fixed in 4% paraformaldehyde, embedded into paraffin, cut in 5 $\mu$m-thick sections, and stained with Hematoxylin and Eosin (H&E). Immunohistochemical analysis was performed using the antibodies listed below. The material was studied under bright field illumination and images were captured with a Leitz microscope equipped with a Nikon Coolpix 990 camera.

**Immunohistochemistry of glioblastoma tumor specimens and glioblastoma xenografts.** Immunohistochemistry was performed on deparaffinized sections using the avidin–biotin–peroxidase complex methods (ABC-Elite kit, Vector Laboratories, Burlingame, CA, USA) with freshly made diaminobenzidine as a chromogen.[38] The expression of the astrocytic marker GFAP was assessed with the rabbit polyclonal antibody anti-GFAP (Ylem, Avezzano, Italy). The expression of p53 was detected with the monoclonal antibody DO-7 (Dakopatts Corporation, Santa Barbara, CA, USA), which recognizes a
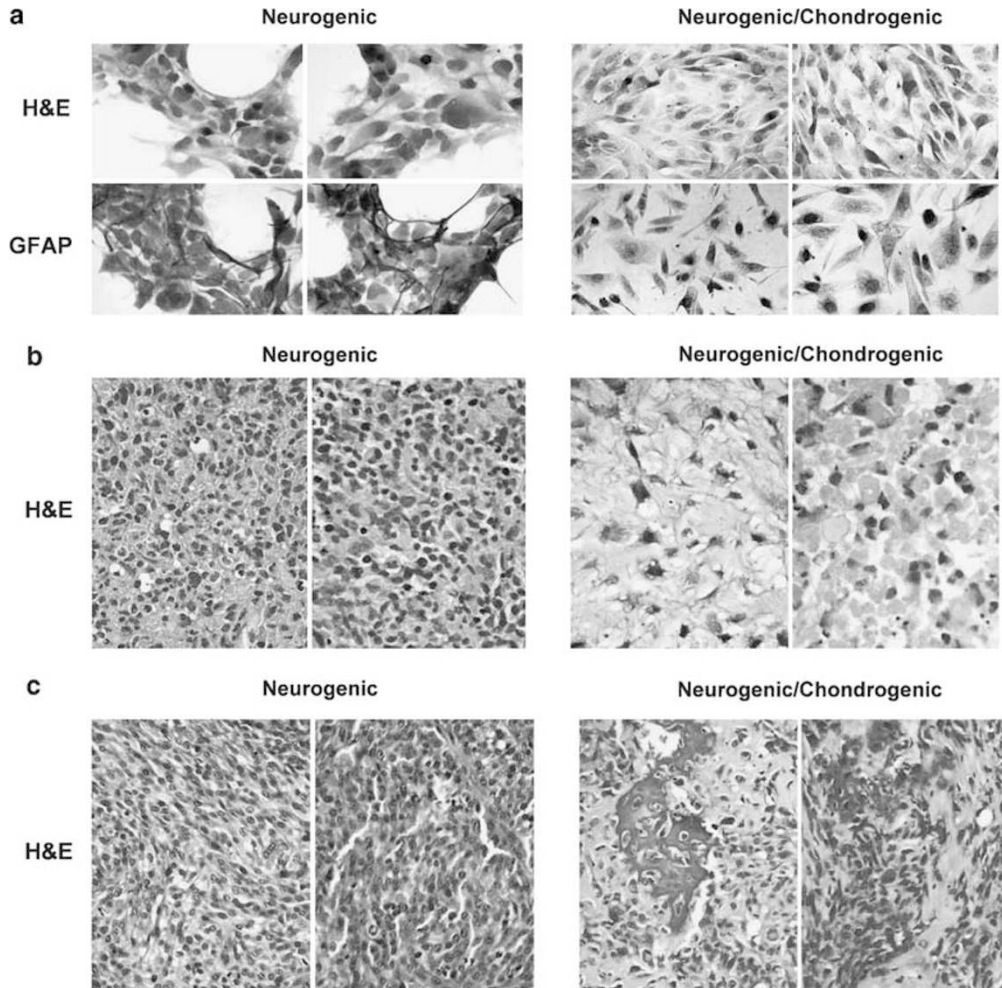
**Figure 4** *In vitro* and *in vivo* differentiation of clonally derived glioblastoma SCs. (**a**) Clones from neurogenic (case 1, 3 and 7) and neurogenic/chondrogenic (case 2, 4, 5 and 6) CSCs were exposed to serum for 14 days and analyzed by hematoxylin-eosin (H&E) or GFAP staining. (**b**) Pellets of the same clones were cultivated in chondrogenic medium and stained by H&E. (**c**) H&E staining of subcutaneous tumor xenografts generated by the injection of aforementioned of the CSC clones. Data show representative results obtained with four clones from two cases (case 1 and 4)

determinant of wild-type and mutant p53 protein in formalin-fixed sections.[39] The expression of EGFR was assessed with the monoclonal antibody anti-EGFR that recognizes a cytoplasmic domain of EGFR (Ylem). The expression of CD133 was assessed with the monoclonal antibody anti-CD133, which stains a transmembrane glycoprotein expressed on neural stem and progenitor cells (Miltenyi Biotec, Bergisch, Germany). Chondrogenic differentiation was assessed with the rabbit polyclonal antibody to YKL40/cartilage glycoprotein-39 (Quidel Corp). Endogenous biotin was saturated by biotin-blocking kit (Vector Laboratories). For antigen retrieval, paraffin sections were microwave-treated in 0.01 M citric acid buffer at pH 6.0 for 10 min. Glioblastomas were classified as GFAP-positive when immunostaining for GFAP labelled more than 30% of cells.[40] If immunoreaction for the p53 protein stained the nuclei of at least 5% of cells, the tumor was considered p53-deficient.[41,42] Tumors demonstrating moderate-to-strong immunostaining for EGFR in more than 20% of cells were considered EGFR-positive.[43] Specimens showing more than 1% of cells that stained for CD133 were classified as CD133-positive. Tumors were classified as YKL40-positive if more than 6% of the cells were stained.[29]

**Fluorescence *in situ* hybridization.** Single- and dual-probe FISH was performed on cell nuclei extracted from paraffin-embedded sections of the parent tumor, on cultured CSCs, or on histological sections of the xenografts. Locus-specific probes for the centromere of chromosome 10 (CEP 10), for the telomere of chromosome 19 (tel 19q), and for locus-specific probe on chromosome 22 (breakpoint cluster region locus q11.2; LSI22) were used (Vysis Inc., Abbot

Laboratories SA, Downers Grove, IL, USA). Standard FISH protocols for pretreatment, hybridization, and analyses were followed according to the manufacturer's instructions. Briefly, for nuclei extraction, 40 μm-thick sections were dewaxed with xylene, manually disaggregated, and digested with 0.005% proteinase K in 0.05 M TRIS pH 7 for 30 min at 37°C. The nuclear suspension was fixed in a solution of methanol and acetic acid (3 : 1). Eight microliters of nuclear suspension was placed on a slide and treated in a microwave oven for 10 min in citrate buffer pH 6 followed by enzymatic digestion with 4 mg/ml of pepsin in NaCl 0.9% pH 1.5 for 20 min at 37°C.[44] Cultured CSCs were previously fixed in a solution of methanol and acetic acid (3 : 1) for 10 min. Histological 4-μm thick paraffin sections were dewaxed with xylene and digested with proteinase K 1 μg/ml in 0.002 M TBS for 20 min at RT. Samples were then dehydrated in a graded ethanol series and subjected to FISH analysis. After specimen/probe denaturation at 73°C for 5 min, the probe (10 μl to slide) were applied to the slides and subsequently incubated overnight at 42°C for CEP 10, and at 37°C for 10–16 h for LSI22/tel 19q. Post-hybridization procedure included subsequent washing in 50% formamide/2 × SSC (30 min at 46°C) and 2 × SSC 0,1% NP40 (5 min at RT). Nuclei were counterstained with DAPI (Vectashield mounting medium with Dapi, Vector Laboratories). The slides were studied with an Axioplan fluorescence microscope (Karl Zeiss, Gottingen, Germany) that was equipped with the appropriate filter sets (Vysis Inc.). Images were captured using a high-resolution black and white CCD microscope camera AxioCam MRm REV 2 (Karl Zeiss). The resulting images were reconstructed with green (FITC), orange and blue (DAPI) pseudocolor using AxioVision 4 multichannel

fluorescence basic workstation (Karl Zeiss) according to the manufacturer's instruction.

**Mesenchymal differentiation assay of glioblastoma stem cells.** Mesenchymal differentiation of glioblastoma stem cells was obtained by using commercial Human Mesenchymal Stem Cell Chondrogenic Differentiation BulletKit (Cambrex Corporation, Milan, Italy) according to the manufacturer's protocols. Briefly, $2.5 \times 10^5$ glioblastoma neurospheres were washed two times with chondrogenic medium and then resuspended in chondrogenic medium supplemented with TGF-β3 (10 ng/ml). The cell suspension was placed into 15 ml polypropylene culture tubes, centrifuged at $150 \times g$ for 5 min at room temperature, and incubated at 37°C in a humidified atmosphere of 5% $CO_2$. The cell pellets were fed every 2–3 days by completely replacing the medium. After 28 days in culture, pellets were formalin-fixed and paraffin-embedded for histology. Chondrogenic differentiation was assayed by alcian blue-PAS staining and anti-YKL-40/cartilage glycoprotein-39 immunoreaction (Quidel Corp. San Diego, CA, USA). As control, spheres of neural progenitor cells from human embryos or adult human olfactory bulb were used.[45]

**RNA Isolation and RT-PCR.** Total RNA was extracted from the cell pellets after 28 days of differentiation under chondrogenic condition using the TRIzol® Reagent (Invitrogen SRL, Milan, Italy). One microgram of RNA was reverse transcribed in a 20 μl reaction mix using M-MLV Reverse Transcriptase (Invitrogen). The resulting cDNA was used in PCR amplification of SOX9, Collagen II, Aggrecan and S26 mRNA. PCR oligonucleotide primers, annealing temperature and optimized cycle number have been previously described.[46]

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