

Growth Factors from Tumor Microenvironment Possibly Promote the Proliferation of Glioblastoma-Derived Stem-like Cells *in Vitro*

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Abstract Glioblastoma multiforme is a lethal brain glial tumor characterized by low survival and high recurrence, partially attributed to the glioblastoma stem cells according to recent researches. Microenvironment or niche in tumor tissue is believed to provide essential support for the aberrant growth of tumor stem cells. In order to explore the effect of growth factors in tumor microenvironment on glioblastoma stem cells behavior, glioblastoma-derived stem-like cells (GDSCs) were isolated from adult human glioblastoma specimen with antibody against surface marker CD133 and were co-cultured with various tumor cells including U87MG cells, unsorted glioblastoma tumor cells, CD133⁻ cells and normal rat primary astrocytes. Results suggested that tumor cells could promote GDSCs proliferation while non-tumor cells could not, and several growth factors were exclusively detected in the co-culture system with tumor cells. It was concluded that growth factors derived from tumor microenvironment possibly contributed to the uncontrolled proliferation of GDSCs.

Keywords Glioblastoma stem-like cells · Proliferation · Microenvironment · Growth factors

Abbreviations

GDSCs	Glioblastoma-derived stem-like cells
BDNF	Brain-derived neurotrophic factor
VEGF	Vascular endothelial growth factor
NGF	Nerve growth factor
TGF- β	Transforming growth factor- β
NT-3	Neurotrophin-3

Introduction

Glioblastoma multiforme, ranked grade IV according to the World Health Organization classification, is widely considered as the most common and malignant glioma with a median survival of less than 12 month because of fast proliferation and high recurrence rates [1]. However, conventional anti-cancer treatments including radiation, chemotherapy and surgery showed limited or no therapeutic effect on these brain tumors [2, 3]. It has been assumed that a subpopulation of cells exists in tumor tissue, which is responsible for tumor initiation and refractory. Recently, isolation and identification of tumor stem cells in human brain tumors and C6 glioma cell line supported this hypothesis. A lot of researches demonstrated that brain tumor stem cells bear almost all features of neural stem cells in proliferation, self-renewal and differentiation capacity as well as the ability to generate new tumors that faithfully reproduce the phenotype of human disease [1]. Brain tumor stem cells have been successfully enriched using antibody against the surface protein CD133, an antigen originally found to express on primitive population such as hematopoietic stem cells [4–7].

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Mounting evidence has shown that the immediate microenvironment that stem cells reside in plays an important role in regulating their proliferation and differentiation. The stem cells niche is composed of various extracellular signals including extracellular matrix molecules, soluble factors and a number of differentiated cell types in specific location that function to maintain stem cells [8–11]. It has been suggested that the differentiated cells in niche might have protective role to stem cell in normal tissue [12, 13]. These mature cells not only communicate with stem cells directly but also release many soluble factors to keep stem cells in quiescent state. Similarly, brain tumor stem cells perhaps acquire specific signals from niche to support their uncontrolled proliferation and tumorigenesis. Bulk of tumor cells (non-stem cells) around the tumor stem cells provide direct cell contact and perhaps secrete factors influencing the tumor stem cells proliferation and differentiation. But it still remains unclear which factors contribute to the tumor stem cells proliferation and differentiation. In order to address how the microenvironment provided by tumor cells affects tumor stem cells behavior, we isolated CD133⁺ cells from human glioblastoma tissues and conformed their stem cell characteristics including proliferative and differentiation ability, further set up co-culture system for tumor stem cells with various brain tumor cells and normal cells, finally compared the growth factor expression pattern in conditioned medium from co-culture system with that from separately cultured cells. The results suggested that glioblastoma-derived stem-like cells (GDSCs) had potent proliferation and self-renewal abilities, but only differentiated into astrocyte, not neuron and oligodendrocyte. Several growth factors released from GDSCs co-culturing with various tumor cells rather than normal brain cells possibly contributed to the abnormal proliferation of GDSCs.

Materials and Methods

Primary Glioblastoma Tumor Sphere Culture and Magnetic Cell Sorting

Tumor samples were obtained from consenting adult patients with typical glioblastoma multiforme, as approved by the Research Ethics Committee at Nanjing General Hospital of Nanjing Military Command for patients and China Pharmaceutical University. As described in previous studies [6, 14], glioblastoma tissues were washed, minced and digested with trypsin-EDTA, and passed through the 70 μ m nylon cell strainer (BD Falcon, USA). A portion of the mixed cells were used for glioblastoma tumor cell culture with DMEM/F12 medium containing 10 % FBS. The rest cells were cultured in serum-free DMEM/F12 medium with B27 supplement, EGF (20 ng/ml) and FGF (20 ng/ml)

for 3 days before cell sorting [14]. Cells were dissociated and labeled with 100 μ l of CD133 microbeads, then subjected to magnetic cell sorting according to the manufacturer's protocol (Miltenyi Biotec). Both sorted CD133⁺ and CD133⁻ cell populations were resuspended and cultured at clonal density (1×10^4 cells/ml) in DMEM/F12 medium with B27, EGF (20 ng/ml) and FGF (20 ng/ml).

Primary Astrocyte and Human Astrocytoma Cell Line U87MG Culture

Primary astrocyte from Sprague–Dawley (SD) rat (P0 pups) were cultured in DMEM/F12 (1:1) supplemented with 10 % FBS according to the described method [15, 16]. The purity was about 97 % indicated by immunostaining of astrocyte marker GFAP (data not shown). Human astrocytoma cell line U87MG (a generous gift from Dr. Zhi-cheng Xiao) were maintained in DMEM medium with 10 % FBS at 37 °C incubator with 5 % CO₂.

BrdU-Incorporation Proliferation Assay

Dissociated single cells from glioblastoma-derived tumor spheres were seeded onto poly-L-lysine coated glass coverslips in 24-well plates at a density of 1×10^5 /well and cultured in serum-free DMEM/F12 medium with B27, EGF (20 ng/ml) and FGF (20 ng/ml) for 24 h. Then BrdU (10 μ g/ml, Sigma) was added into the medium and cells were cultured up to 1, 2, 5 and 7 days, respectively. Cell proliferation was indicated by BrdU incorporation rate.

MTT Assay

CD133⁺ and CD133⁻ cells were respectively plated in 96-well plates in 200 μ l of serum-free DMEM/F12 medium with B27, EGF (20 ng/ml) and FGF (20 ng/ml) at a density of 1000 cells/well. Then MTT assays were performed on day 0, 1, 3, 5, and 7, respectively. 20 μ l of MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h. 150 μ l of DMSO was added to dissolve the insoluble purple formazan product to produce a colored solution. The optical density (OD) was read at 570 nm wavelength on the multi-well scanning spectrophotometer (Bio-Rad).

Limiting Dilution Assay

Limiting dilution assay was carried out according to methods described previously [17, 18]. CD133⁺ cells and CD133⁻ cells were plated in 96-well plates in 200 μ l of serum-free DMEM/F12 medium with B27, EGF (20 ng/ml) and FGF (20 ng/ml), respectively. Final dilutions ranged from 200 cells/well to 6 cells/well in 200 μ l of medium. After 7 days in culture, the percentage of wells not

containing spheres for each cell plating density was calculated and plotted against the number of cells per well. Regression lines were plotted and x-intercept values were measured, which represent the number of cells required to form at least 1 tumor sphere in each well.

Differentiation Assay

To assess GDSCs differentiation capacity, tumor spheres were mechanically dissociated into single cells, plated onto glass coverslips pre-coated with poly-L-lysine and cultured in DMEM/F12 medium with 1 % FBS and B27. Culture medium was refreshed every 2 days, and then fixed with 4 % paraformaldehyde for immunocytochemistry after 7 days in culture.

Immunocytochemistry

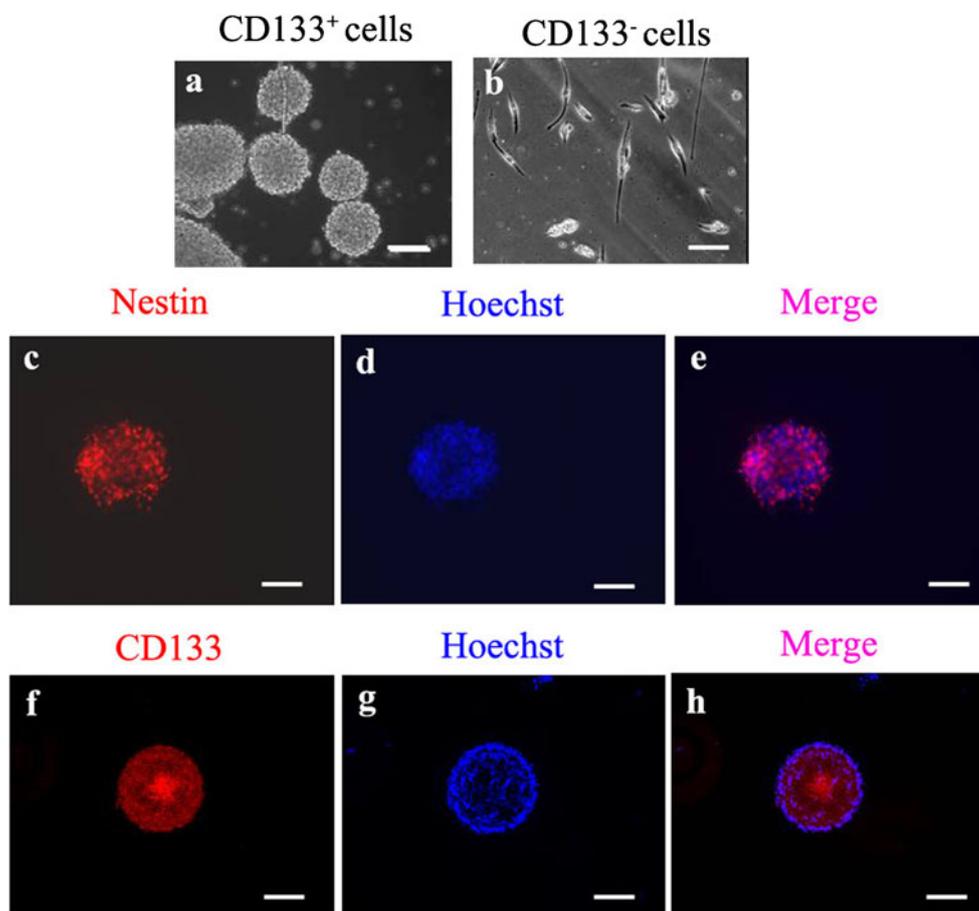
Immunocytochemistry was performed according to previously described methods [19]. Briefly, Cells on coverslips were washed with PBS, fixed with 4 % paraformaldehyde, blocked with 10 % normal goat serum in PBS, and incubated with primary antibody in 10 % normal goat serum at 4 °C overnight followed with Cy2- or Cy3-labeled secondary

antibody. To determine the stem cell feature of sorted cells, tumor sphere cells were stained with antibodies against CD133/1 (mouse monoclonal IgG1; Miltenyi Biotec) and nestin (rabbit polyclonal; Chemicon). For BrdU incorporation assay, cells were incubated in 2 M HCl for 30 min at room temperature before blocking and incubation with anti-BrdU (mouse monoclonal, Santa Cruz). To determine the differentiation of stem-like cells, cells were immunostained with antibodies against β tubulin-III (Tuj; mouse monoclonal; Stem Cell company) for neurons; glial fibrillary acidic protein (GFAP; rabbit polyclonal; DAKO) for astrocytes, and CNPase (mouse monoclonal; Sigma) for oligodendrocytes. All of the immunostaining was followed by anti-rabbit IgG antibody conjugated with Cy2 or anti-mouse IgG antibody conjugated with Cy3 (1:200; Beyotime, China), and nuclei were counterstained with Hoechst 33342 (10 μ g/ml; Molecular Probes). Cells were visualized under Olympus fluorescent microscope. At least 10 viewing fields per group were randomly selected to calculate percentages of cells.

Co-culture System

The co-culture system was set up by use of Millicell polycarbonate filter chamber (Millipore Corp., Bedford, USA)

Fig. 1 Sorted CD133⁺ cells from glioblastoma IV formed neurosphere-like colonies (a), while CD133⁻ cells did not (b). Tumor spheres were immunoreactive for typical neural stem cell marker nestin (c) and for CD133 (d). Cells were counterstained with Hoechst (d, g), the merged photographs were shown in e and h. Scale bar= 100 μ m



according to previously described methods [20]. GDSCs were co-cultured with various feeder cells including rat primary astrocytes, U87MG cells, unsorted glioblastoma tumor cells and CD133⁻ cells (sorted from the same glioblastoma), respectively. The feeder cells were plated into the apical compartment at a density of 5000 cells/millicell and cultured in DMEM/F12 medium with 10 % FBS for 2 days. Then the millicells were rinsed completely with PBS and culture medium was changed for serum-free DMEM/F12 medium with B27, EGF (20 ng/ml) and FGF (20 ng/ml). Four hours later the millicells were inserted into the 24-well plate pre-cultured with GDSCs. For GDSCs proliferation assays in co-culture system, BrdU (10 μ g/ml) was added after co-culturing for 24 h. Five days later millicells were removed from co-culture system and tumor spheres were collected, dissociated into single cells and plated on coverslips pre-coated with Poly-L-lysine and cultured for further 1 day. BrdU incorporation assay was performed and BrdU positive cells were counted under Olympus fluorescent microscope.

Dot-Blotting Assay

After GDSCs were co-cultured with feeder cells or the cells were separately cultured for 5 days, conditioned media (CM) were collected, concentrated and dotted on the nitrocellulose

membrane (2 μ l/dot), and then membranes were incubated with growth factor antibody (anti-NGF polyclonal antibody; anti-VEGF monoclonal antibody, anti-BDNF polyclonal antibody, anti-TGF- β monoclonal antibody and anti-NT-3 polyclonal antibody, 1:500, respectively, all antibodies were from Chemicon) followed by horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Sigma). Signal intensities of various growth factors were determined by densitometry, and the relative signal intensities were measured. Data were from three independent experiments.

Statistic Analysis

Data were analyzed by Student's *t* test and expressed as mean \pm SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

Results

Isolation and Identification of Glioblastoma-Derived Stem-like Cells

Cultures were established from solid primary adult human glioblastoma ($n=3$) to observe whether stem cells existed in the brain tumor samples that we obtained. Cells were

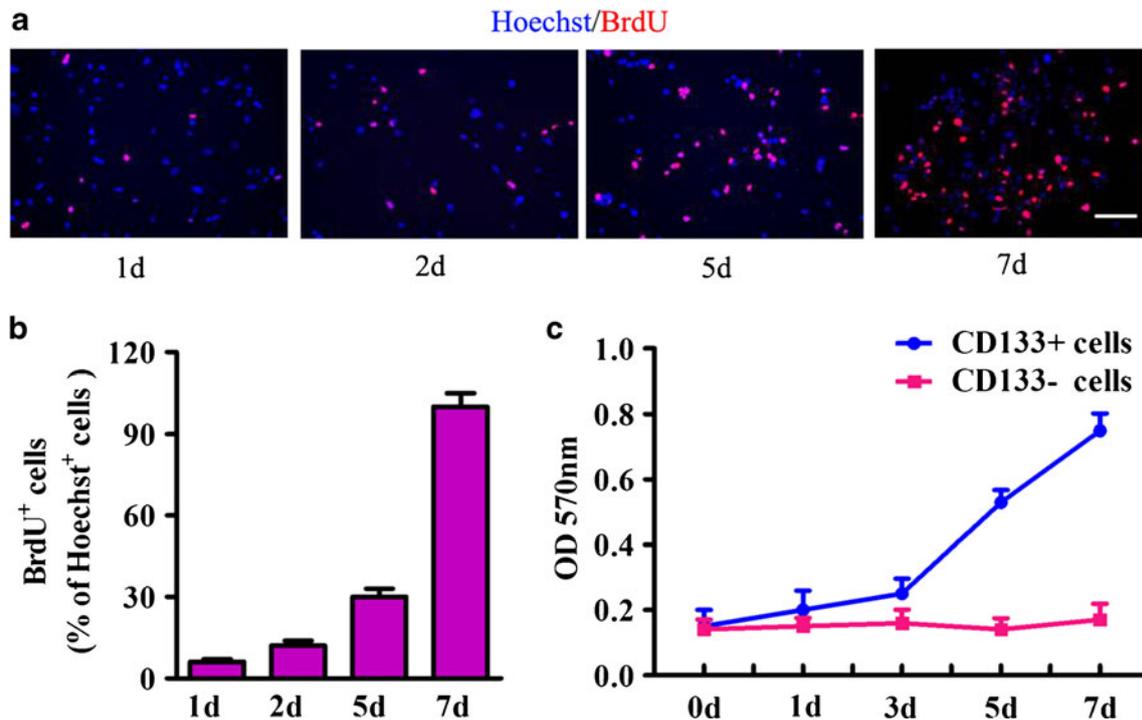


Fig. 2 Glioblastoma-derived CD133⁺ but not CD133⁻ cells could proliferate in vitro. Proliferation was determined by BrdU-incorporation assay at 1, 2, 5, 7d and by MTT assay at 0, 1, 3, 5, 7d. (a) Fluorescence overlap micrographs showed the morphology of

BrdU⁺ cells (red) and nuclei (blue). Scale bar=100 μ m. (b) Quantification of percentage of BrdU⁺ cells. Bar graph represents mean \pm SE ($n=10$). (c) CD133⁺ and CD133⁻ proliferation and survival were determined by MTT assay. Data were shown as mean \pm SE

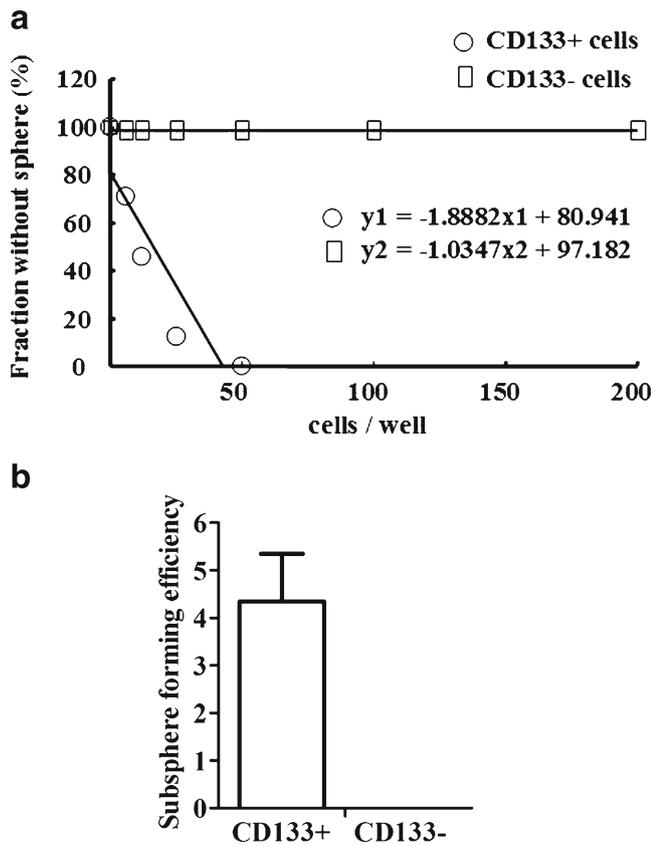


Fig. 3 Glioblastoma-derived CD133⁺ and CD133⁻ cells displayed different self-renewal potential. (a) Percentage of negative wells was plotted against the number of cells per well by limiting dilution assay. Regression line was shown in the figure. (b) Subspheres-forming efficiencies of CD133⁺ cells and CD133⁻ cells were computed through regression lines

isolated from tumor specimens, dissociated into single cells, sorted into CD133⁺ and CD133⁻ cells and cultured in favored neural stem cell growth medium which was established previously. After 7 days in culture, it was found that neurosphere-like colonies appeared only in CD133⁺ cell culture from glioblastoma, while CD133⁻ cells attached to culture plate and failed to form such colonies (Fig. 1b). The glioblastoma-derived tumor spheres were dissociated into single cells and cultured in the DMEM/F12 medium with B27, EGF and FGF. Subspheres derived from a single mother cell again appeared after being cultured for 5 days (Fig. 1a). To determine whether these tumor spheres express stem cell markers, we harvested subspheres for immunostaining. As shown in Fig. 1c-h, these tumor spheres were immunoreactive for nestin (an intermediate filament protein found in undifferentiated central nervous system cells and a characteristic neural stem/progenitor cell marker) [21] and for CD133 (a novel transmembrane cell surface protein, hematopoietic stem cell, human neurosphere and human brain tumor stem cells marker)[6, 22, 23]. The results demonstrated that glioblastoma-derived CD133⁺ cells could form neurosphere-like colonies which expressed nestin and CD133 as neural stem cells.

Glioblastoma-Derived CD133⁺ but Not CD133⁻ Cells Showed Proliferative and Long-Term Self-Renewing Abilities

Proliferation and self-renewal are critical features of stem cells. In order to further identify the stem cell feature of glioblastoma-derived CD133⁺ cells, we next tested whether these cells could proliferate and undergo long-term self-

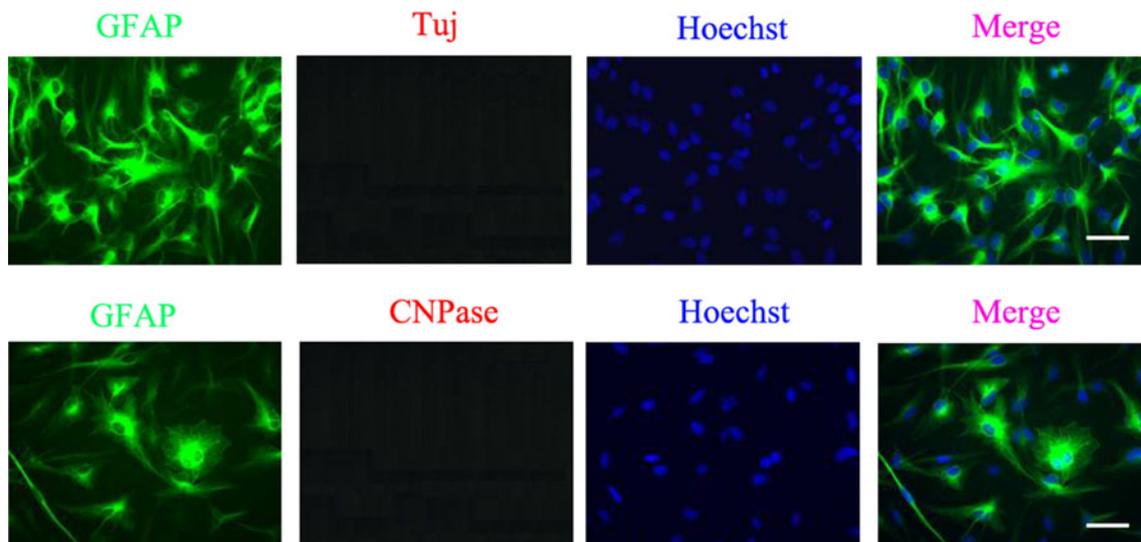


Fig. 4 Glioblastoma-derived CD133⁺ cells only differentiated into astrocytes (GFAP positive) (a, e), while staining for neuron (β -tubulin; b) and oligodendrocyte (CNPaes; f) were not observed. Cells were

counterstained with Hoechst (c, g). Merged pictures were seen in d and h, Scale bar=100 μ m

renewal in vitro. For the proliferation assay, dissociated single cells from tumor spheres were seeded on to PLL-coated coverslips and BrdU incorporation assay was performed. As shown in Fig. 2a and b, BrdU positive cell numbers increased with culturing days extending, which suggested that glioblastoma-derived CD133⁺ cells have proliferation capacity. Meanwhile, the results of MTT assay demonstrated that CD133⁺ cells displayed higher proliferation and survival than CD133⁻ cells (Fig. 2c).

We then compared the self-renewal capacity of CD133⁺ cells and CD133⁻ cells through limiting dilution assay. CD133⁺ tumor spheres were mechanically dissociated into single cells and adherent CD133⁻ cells were trypsinized before being reseeded at serial dilutions from 200 cells/well to 6 cells/well, respectively. New colonies derived from CD133⁺ mother cells appeared within 1–7 days after reseeding. However, CD133⁻ cells seemed not to form new colony. Percentage of wells without newly-formed spheres was plotted against the number of cells per well were plotted (Fig. 3a). Subsphere-forming efficiency of CD133⁺ cells and CD133⁻ cells calculated through the regression line was 4.30 ± 1.02 and 0, respectively (Fig. 3b). These results indicated that CD133⁺ cells exhibited proliferative and self-renewal ability, whereas CD133⁻ cells did not under the same condition. In our experiment, glioblastoma-derived CD133⁺ cells could be passaged multiple times through mechanical dissociation of large spheres, and could be maintained for at least 6 months if being replated in fresh proliferation medium every 1 week.

Glioblastoma-Derived CD133⁺ Cells Could Differentiate into Astrocytes

In addition to proliferation and self-renewal, differentiation also constitutes an essential characteristic of stem cells. To explore whether Glioblastoma-derived CD133⁺ cells can differentiate into various cell types, the new colonies derived from CD133⁺ mother cells were dissociated into single cells and allowed to differentiate for 7 days. Then cells were immunostained with antibodies against β tubulin-III for neurons, GFAP for astrocytes, and CNPase for oligodendrocytes. However, the results showed that CD133⁺ cells could only differentiate into astrocyte, but no neuron and oligodendrocyte (Fig. 4). Based on the above results that glioblastoma-derived CD133⁺ cells displayed marked stem cell activity including proliferation, long-term self-renewal and differentiation, we can draw the conclusion that the CD133⁺ represented a specific subpopulation of glioblastoma cells with some stem cell features, which were subsequently denoted as glioblastoma-derived stem-like cells (GDSCs).

Brain Tumor Cells Could Facilitate GDSCs Proliferation

Lines of evidence showed that the microenvironment surrounding tumor stem cells played an important role in regulating their behavior. To examine a possible influence of non-stem cells in the glioblastoma tissue on GDSCs, we established co-culture system with Millicell chamber. In our experiment, various feeder

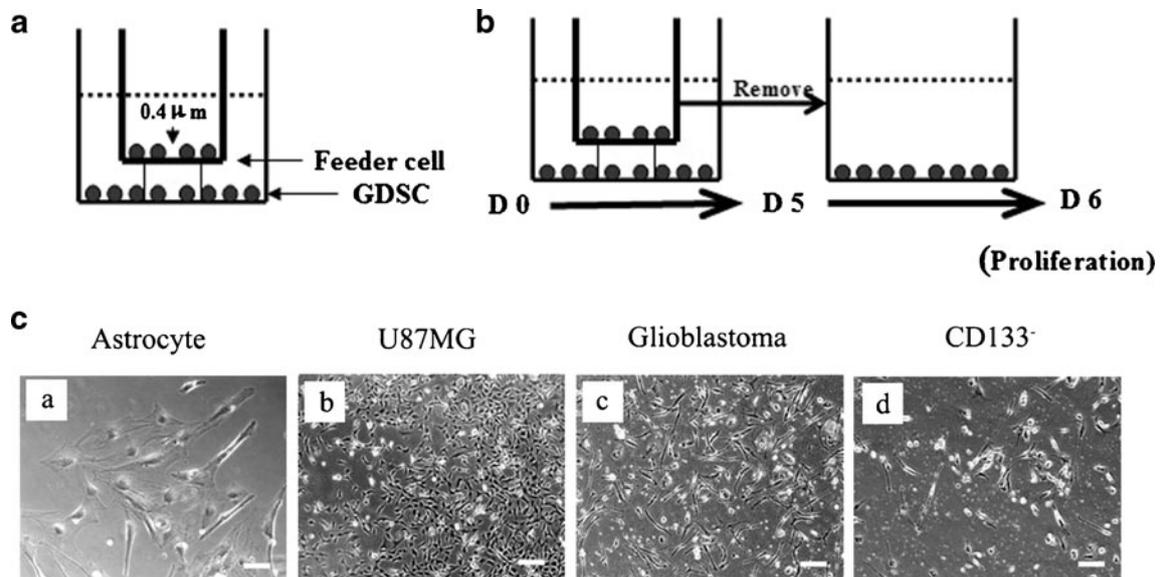


Fig. 5 Co-culture systems were established with different feeder cells. **a** and **b** are schematic show of the co-culture experiment. The feeder cells were seeded in the apical compartment of millicell, and could not migrate through the membrane pores. GDSCs were seeded in the basal compartment. After GDSCs being co-cultured with various tumor cells

for 5 days, millicell was removed, GDSCs were further cultured for 1 days and proliferation assay was carried out. **(c)** Various tumor cells were cultured and photographed with an inverted phase-contrast microscope. Scale bar=100 μ m

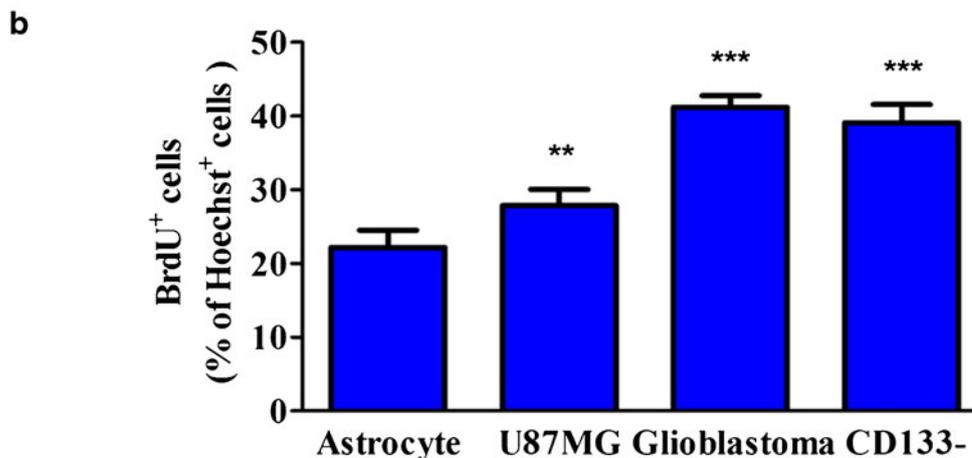
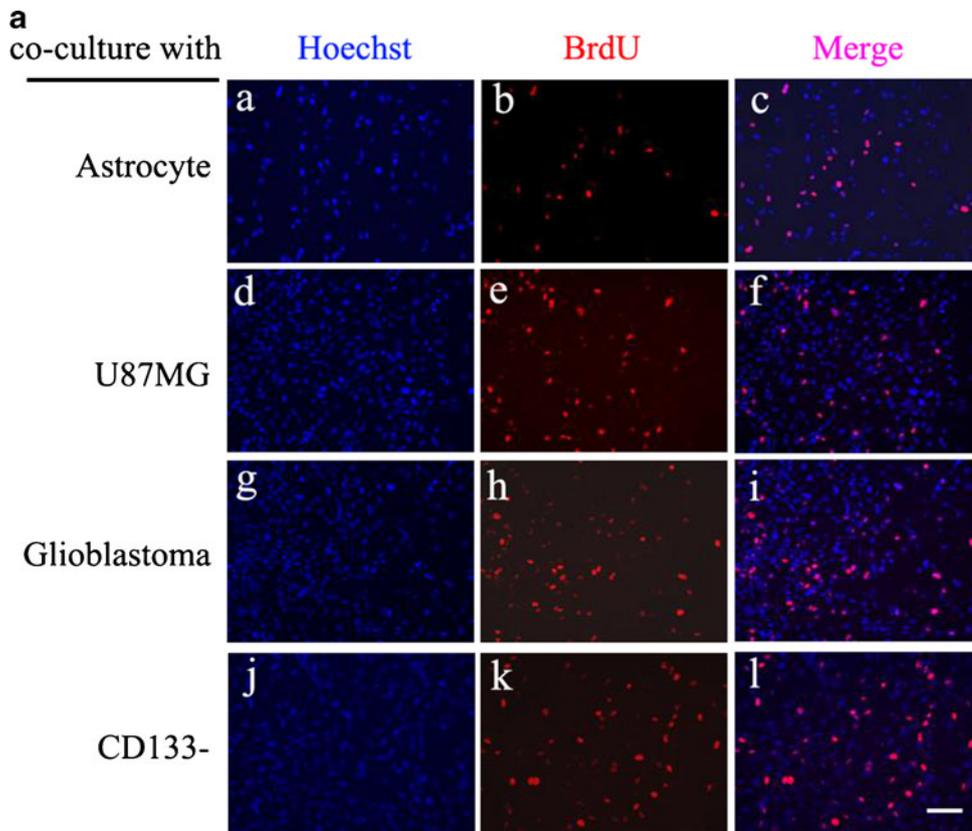
cells (non-stem cells) including primary astrocyte (Fig. 5Ca), U87MG cells (Fig. 5Cb), unsorted glioblastoma cells (Fig. 5Cc), and CD133⁻ cells sorted from glioblastoma (Fig. 5Cd) were cultured in the apical compartment respectively, while GDSCs were co-cultured in the basal compartment. Immunocytochemistry was performed to detect BrdU incorporation after 5-day co-culture (Fig. 6a) and proportions of BrdU positive cells to Hoechst 33342 positive cells were quantified (Fig. 6b). The results showed that after being co-cultured with tumor cells including U87MG, primary glioblastoma and CD133⁻ cells, GDSCs displayed more distinct proliferation than with primary

astrocyte from normal rat brain. But there were no obvious difference between the influence of mixed cell culture from glioblastoma and sorted CD133⁻ cells. These results implied that brain tumor microenvironment might facilitate GDSCs proliferation compared with non-tumor microenvironment.

Growth Factor Released in the co-culture System Possibly Promoted GDSCs Proliferation

Results above suggested that brain tumor cells could promote GDSCs proliferation. Moreover, this effect was

Fig. 6 Proliferation of GDSCs was enhanced by co-culturing with U87MG, unsorted glioblastoma tumor cells and CD133⁻ cells. **(a)** After being co-cultured for 5 days, GDSCs were immunostained with BrdU (red) for proliferative cell and Hoechst 33342 (blue) for nuclei. **(b)** Proportions of BrdU⁺ cells to Hoechst 33342⁺ cells in co-culturing system were computed with astrocyte-coculturing group as control. Data were analyzed by Student's *t* test and shown as mean ± SE. ** P<0.01, *** P<0.001 compared with control (astrocyte group). Scale bar=100 μm



probably mediated by soluble factors released from the co-cultured cells because this system excluded direct contact between feeder cells and GDSCs. Therefore, to explore potential molecular mechanisms underlying the favorable microenvironment for GDSCs proliferation, we collected conditioned medium from the co-culture system as well as separately cultured cells and detected five growth factors through dot blot assay. As shown in Fig. 7, no growth factors were detected in supernatant from GDSCs. Unsorted glioblastoma cells and CD133⁻ cells themselves only expressed low level of BDNF and NT3, similar to astrocyte, whereas all the five growth factors were found in U87MG. Interestingly, when GDSCs were co-cultured with tumor cells including U87MG, glioblastoma tumor cells and CD133⁻ cells, conditioned medium all displayed VEGF and BDNF immunoreactivity (Fig. 8). However, the level of TGF- β , NT-3 and NGF was higher in conditioned medium derived from GDSCs co-cultured with glioblastoma tumor cells and CD133⁻ cells compared with U87MG. No such growth factors were detected in conditioned medium derived from co-culture of GDSCs with primary astrocyte.

Discussion

Recent researches suggest that brain tumor stem cells are responsible for tumor initiation and the chemoresistance and radioresistance of brain tumors, making it a promising target in clinical therapy of glioblastoma [24–27].

CD133, a cell surface protein expressed in several types of stem cells, has been widely used as a specific marker in isolating tumor stem cells from glioblastoma specimens and cell lines in many studies. However, it remained controversial that only CD133⁺ cells of glioblastoma have tumor-initiating potential, indicated by the fact that a subset of primary glioblastomas might stem from previously unknown CD133⁻ tumor cells with apparent stem cell-like properties. Additionally, CD133⁻ glioma cells can give rise to CD133⁺ cells [28, 29]. Nonetheless, it should be noted that CD133⁻ glioblastomas were characterized by a lower proliferation index clinically compared with CD133⁺ glioblastomas [30]. Therefore, we focused on the study of CD133⁺ cells.

In this study, we successfully enriched CD133⁺ cells from human glioblastoma specimens and confirmed their

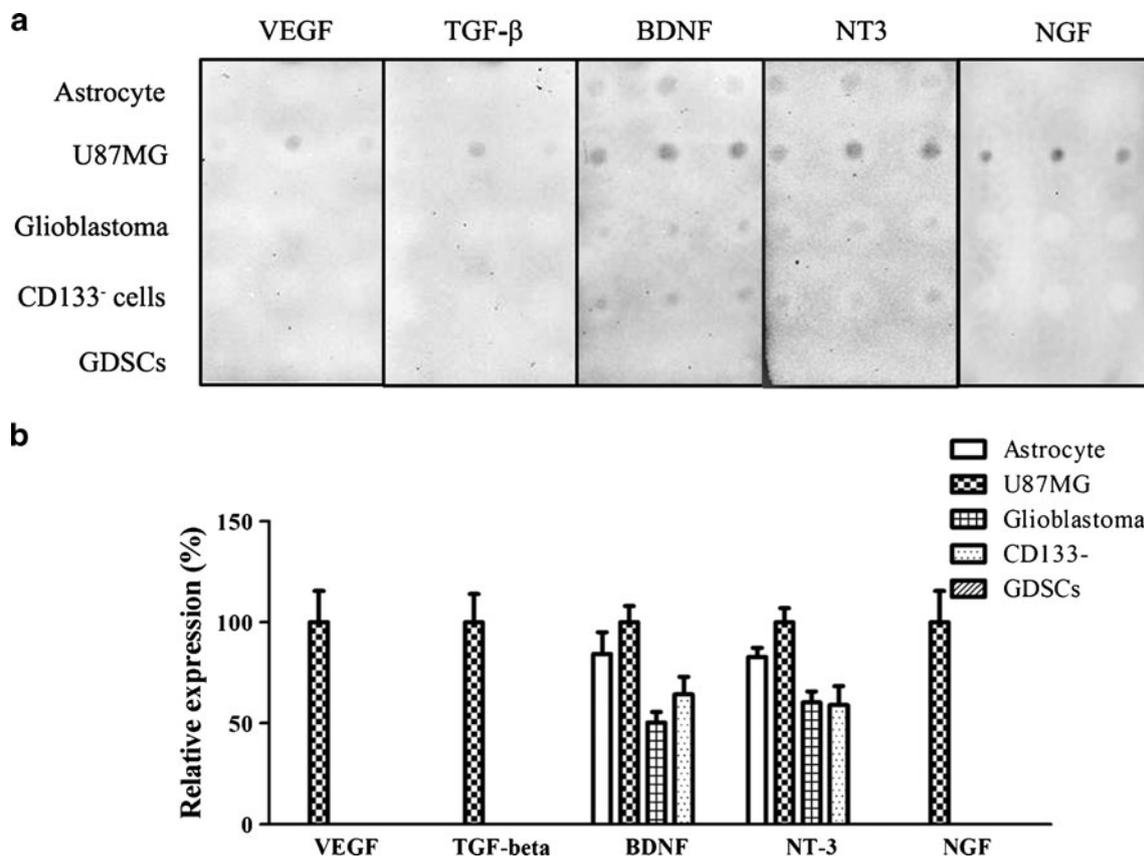


Fig. 7 Growth factors expression pattern was determined in conditioned medium from separately cultured cells. **(a)** The dot blots showed the profile of growth factors expression in various cells. **(b)** Signal intensities of growth factors were quantified by densitometry and the

relative signal intensities to U87MG group for individual growth factors were measured. Data were shown as mean \pm SE from three independent experiments

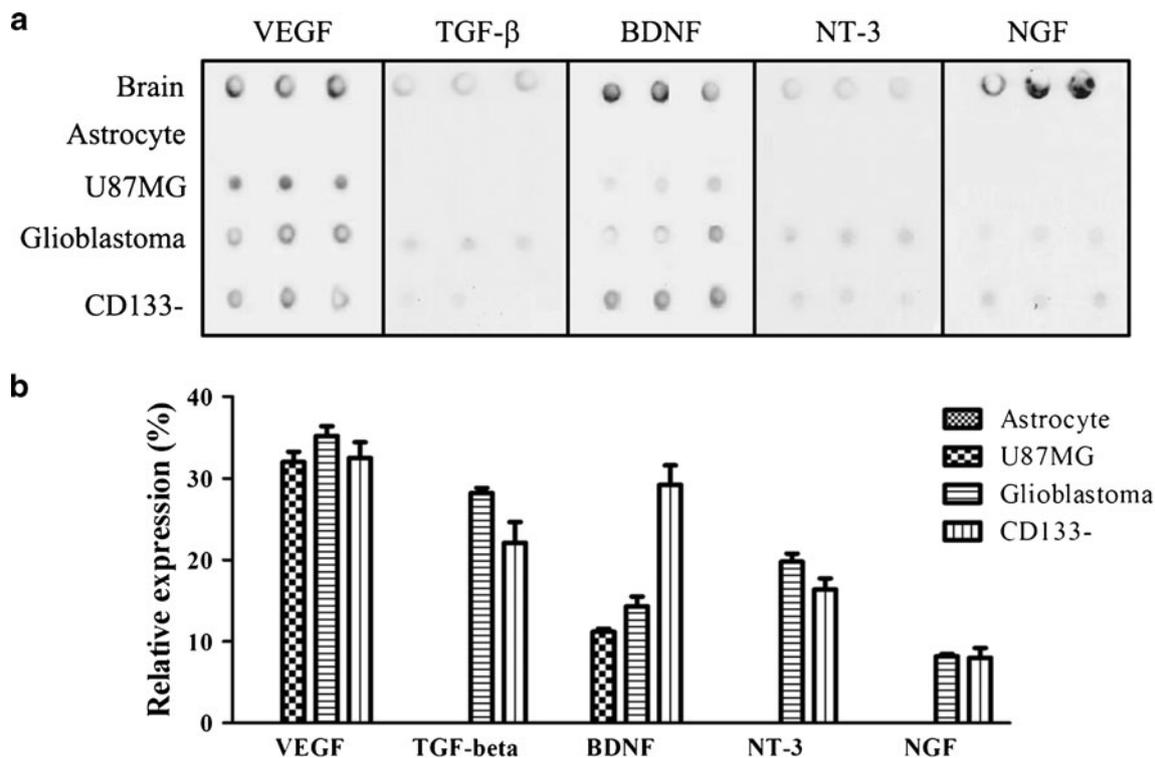


Fig. 8 Growth factors expression patterns were different in conditioned medium from GDSCs co-cultured with various cells. **(a)** The dot blots showed the profile of growth factor expression in co-culture system. **(b)** Signals intensities of various growth factors were

determined by densitometry and the relative signal intensities to brain group for individual growth factors were computed. Data were shown as mean \pm SE from three independent experiments

self-renewing, proliferative and neurosphere-forming capacity. However, upon the removal of mitogens, the CD133⁺ cells only differentiated into astrocyte but not neuron and oligodendrocyte as reported before [31]. As proposed by Buick et al. [32] and Singh et al. [6], malignant transformation might limit the differentiation capacity of normal pluripotent stem cells and tumor stem cells were probably lineage-restricted and mainly differentiated into the cell type that comprised the tumor. In addition, variation in location of specimens in the brain, sex and age of the patient might also lead to difference in some properties of tumor stem cells. Further studies are required to probe into the detailed mechanisms.

Since tumor tissue is characterized as a phenotype and functional cellular heterogeneity containing tumor initiating cells (stem cells), committed cells and other support cells, these non-stem cells inevitably have an influence on the tumor stem cells both directly and indirectly. Previous studies showed that endothelial cells interact closely with self-renewing brain tumor cells and secrete factors that maintain these cells in a stem cell-like state [33]. In this study, we explored the effect of non-stem cells in tumor tissue on GDSCs using a co-culture system. Intriguingly, GDSCs showed strong proliferation ability when co-cultured with tumor cells rather than with normal astrocytes. Moreover, unsorted cells and CD133⁻ cells from glioblastoma tissue

seemed to provide a more favorable environment for GDSCs proliferation compared with U87MG. It is noteworthy that there was no direct contact between GDSCs and the feeder cells in this co-culture system, which highlighted the possibility that soluble factors released from the co-cultured system with tumor cells promoted the proliferation of GDSCs cells.

Based on previous researches, it was assumed that tumor stem cells may arise from a microenvironment with dominant growth-promoting signals rather than growth inhibitors [34]. The neurotrophin family members BDNF, NT-3 and NGF are known to impact brain tumor cells functions including viability, proliferation and migration. Their receptors TrkB, TrkC and TrkA receptors have been identified on glioblastoma. Moreover, the low affinity receptor p75 is also involved in the metastatic potential in glioblastoma [35–38]. TGF- β signaling results in various biological effects involving growth, differentiation and morphogenesis of brain tumors. It has been proposed that the tumor promoting activity of TGF- β could be a fundamental target in human glioma cell malignancy [39]. VEGF is highly expressed in malignant glioma and has been identified as a critical regulator of angiogenesis. Studies also showed that VEGF-specific inhibitors like Bevacizumab might impact brain tumor growth by targeting both a vascular niche and the associated tumor stem cells [33].

In our experiment, growth factors mentioned above were detected in the conditioned medium from the co-cultures as well as separately cultured cells. It is interesting that the combination of GDSCs and non-stem cells during the co-culture led to a distinct variation in the growth factor expression profile of the analyzed supernatants compared to the single approaches. Since VEGF, TGF- β and NGF were not present in the separately cultured GDSCs, unsorted cells and CD133⁻ cells, the assumption of an interaction between GDSCs and the co-cultured cells could be suggested, which led to the secretion of these factors. Additionally, the high expression level of growth factors in the co-culture system with tumor cells seemed to correlate with high proliferation rate of GDSCs, suggesting that abnormal growth factor expression in tumor microenvironment probably lead to uncontrolled proliferation of tumor stem cells. Furthermore, it's likely that these factors help to maintain the complex microenvironment of brain tumors in a synergic way to facilitate tumor stem-like cells proliferation, which might result from the interaction of endocellular signaling pathways of each factor. Of note, secretion of some factors by separately cultured astrocyte and U87MG seemed to be inhibited when they were co-cultured with GDSCs. Similar results were observed in previous study [40]. However, the underlying mechanisms remained to be further elucidated.

In conclusion, the present studies demonstrated that tumor cells in microenvironment could facilitate tumor stem cells proliferation, which was partially attributed to soluble growth factors released from the microenvironment. Therefore, tumor stem cells together with its microenvironment will become an intriguing target for novel therapeutic drugs and strategies development for brain tumor therapy in the future.

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