Cancer stem-like cells of glioblastoma characteristically express MMP-13 and display highly invasive activity

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Abstract. Glioblastoma is the most malignant type of primary brain tumor that has been shown to contain a small population of cancer stem cells. Recent studies have suggested that cancer stem cells cause tumor recurrence based on their resistance to radiotherapy and chemotherapy. Although the highly invasive nature of glioblastoma cells is also implicated in the failure of current therapies, it is not clear whether cancer stem cells are involved in invasiveness. In this study, we isolated tumor sphere-forming cells bearing cancer stem-like characteristics such as self-renewal, multipotency, drug-resistibility, and *in vivo* tumorigenicity, from the human glioblastoma cell line U251, under serum-free neural stem cell culture condition, and assessed their migratory and invasive ability. These cells showed enhanced migratory and invasive ability on both Matrigel and organotypic brain slices compared to parental U251 cells. The expression of matrix metalloproteinase (MMP)-13 was specifically expressed in tumor sphere-forming cells derived from U251 and primary human glioma cells. Knockdown of MMP-13 expression by shRNA suppressed the migration and invasion of these cells. The results suggest that the highly invasive potential of cancer stem cells depends on MMP-13 enzymatic activity, thus MMP-13 might be a potential therapeutic target for glioblastomas.

Introduction

Accumulating evidence indicates that a variety of malignant tumors have been found to contain their own stem cells. These cells, referred to as cancer stem cells (CSCs), have the potential for self-renewal and differentiation into multilineage cells (1). Glioblastoma (GBM) is one of the tumors from which CSCs have been isolated (2,3). CSCs of GBM have been shown to be resistant to radiotherapy due to their increased activity of the DNA damage checkpoint response (4). CSCs more strongly resist a variety of chemotherapeutic agents than non-CSCs (5). These observations suggest that, irrespective of the cause, CSCs may persist even after various intensive therapies and may be the cause of recurrence.

The extracellular matrix (ECM) is a dynamic environment that plays a pivotal role in regulating cellular functions. Tumor cells may invade the surrounding normal tissue while digesting ECM by secreting proteolytic enzymes, such as matrix metalloproteinases (MMPs) and plasminogen activator (6). Although there is little ECM in the extracellular milieu of the brain, in contrast to most other tissues, the brain parenchyma contains a relatively amorphous matrix that contains fibrillary collagen, hyaluronic acid and other fibrous proteins (6). Furthermore, ECM proteins such as interstitial collagen, fibronectin, and laminin are present in the border area between normal brain and glioma cells (7).

MMPs are a family of structurally related enzymes that play a central role in tumor invasion by degrading ECM macromolecules. MMPs can be divided into subgroups of collagenase, gelatinase, stromelysins, matrilysins, and membrane-type MMPs (8). A growing body of literature has documented a strong correlation between brain tumor malignancy and MMP activity level (9-11). MMP-2 and MMP-9 are highly expressed in glioma cells to create space necessary to invade the surrounding brain parenchyma (12,13). Yeh *et al* recently demonstrated that rat C6 glioma cells with induced expression of MMP-13, a collagenolytic MMP, had higher migratory and invasive activity *in vitro* (14). Furthermore, several MMPs including MMP-2 and MMP-9 can activate some growth factors that are implicated in GBM motility and proliferation, such as TGF-ß by releasing them from the ECM (15). Thus, understanding the mechanisms underlying the highly invasive potential of GBM cells through MMPs may lead to a radical cure for such intractable neoplasms.

Although recent literature indicates that CSCs might be responsible for tumor initiation, progression and recurrence, little is known about whether CSCs are involved in invasiveness, a hallmark of malignant gliomas. In this study, we isolated cancer stem-like cells derived from the human glioma

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cell line U251 by allowing the cells to make spherical aggregates and then evaluated their invasive potential. Compared to the parental U251 cells, the cancer stem-like cells were much more capable of migration and invasion, and this invasiveness was primarily dependent on the enzymatic activity of MMP-13 but not MMP-2 or MMP-9. These results suggest that CSCs are responsible for the highly invasive potential of glioblastomas.

Materials and methods

Cell culture. Human glioma cell line U251 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), 4.5 g/l glucose, and penicillin/streptomycin/amphotericin B mixture (Wako). To establish U251 cells that stably express enhanced green fluorescence protein (EGFP) termed GFP-U251, U251 cells were transfected with pEGFP-C1 (Clontech, Mountain View, CA) using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. Cells stably expressing EGFP were selected by culturing in medium containing G418 selection (500 μg/ml; Invitrogen) and cell sorting by FACS ARia (Becton-Dickinson, San Jose, CA). For knockdown experiments, GFP-U251 cells were transfected with MMP-13 small hairpin (sh) RNA plasmid containing a 21-bp shRNA sequence 5'-GCTCCGAGAAATGCAGTCTTT-3' of human MMP-13 (pLKO.1/MMP-13 shRNA; Sigma, St. Louis, MO) or non-target control shRNA plasmid (pLKO.1/non-target shRNA; Sigma) using Lipofectamine 2000 reagent. Cells stably expressing shRNA were selected by culturing in medium containing puromycin (2 μg/ml; Sigma).

Some subpopulations of U251, GFP-U251, non-target shRNA-U251, or MMP-13 shRNA-U251 cells formed round cell aggregates or spheres when the cells were cultured in serum-free DMEM containing 10 μg/ml bovine insulin, 5.5 μg/ml human transferrin, 6.7 ng/ml sodium selenite (ITS mixture; Invitrogen), 100 μg/ml bovine serum albumin (BSA; Sigma), the penicillin/streptomycin/amphotericin B mixture, recombinant human epidermal growth factor (EGF; 10 ng/ml), basic fibroblast growth factor (bFGF; 10 ng/ml), and platelet derived growth factor-AA (PDGF-AA; 10 ng/ml). Growth factors were purchased from Peprotech, London, UK. In this report, the serum-free medium containing the growth factors is called sphere medium.

Human GBM specimens and primary cell culture. Surgically resected tumor specimens from GBM patients were obtained after informed consent with approval by the local ethics committee at Ehime University Hospital. GBM tissues were mechanically minced, digested with 0.1% trypsin, and then triturated with a Pasteur pipette. Cells were passed through a 70-μm strainer (Becton-Dickinson Falcon), and resuspended in high glucose DMEM supplemented with 10% FBS. Sphere formation using the 3-6 passaged-cells was performed in the same manner as U251.

RNA isolation and RT-PCR. Total RNA was collected using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA was obtained from DNase I-treated RNA (3 μg) by reverse transcription using M-MLV Reverse Transcriptase (Invitrogen) and oligo(dT)$_{15}$ primer as previously described (16). DNA fragments were amplified with GoTaq DNA polymerase (Promega, Madison, WI). The PCR conditions were as follows: 94˚C for 1 min, followed by 22 (ß-actin), 35 (CD133), or 30 (other genes) cycles of 94˚C for 30 sec, 56˚C for 30 sec, and 72˚C for 1 min. The primer sequences, the accession number, and product size are listed in Table I.

Flow cytometry analysis. Tumor sphere-forming cells and U251 cells were dissociated using 0.25% trypsin, fixed with 4% paraformaldehyde (PFA) and stained with phycoerythrin (PE)-conjugated anti-CD133/2 (1:10; Milteny Biotec, Germany) or control-IgG isotype (Becton-Dickinson, Franklin Lakes, NJ) antibodies, and then analyzed by FACS Calibur flow cytometer and CellQuest software (Becton-Dickinson).

Secondary tumor sphere formation assay. Primary tumor spheres with 100-200 cells/sphere were dissociated using 0.25% trypsin and seeded at a density of one cell in 100 μl of sphere medium in 96-well plates. Cells were fed with 20 μl of sphere medium every 3 days. Five 96-well plates were generated for cells derived from either U251 cells or tumor spheres, and only the wells that contained single cells were marked and analyzed 21 days later regardless of whether secondary tumor spheres were formed. The percentage of wells that generated secondary tumor spheres was determined.

Western blot and immunoprecipitation analysis. Cells grown on poly-L-lysine coated dishes were lysed with Laemmli's sample solution. The lysates were electrophoresed, transferred onto nitrocellulose membrane, and immunoblotted with antibodies to ß-actin (mouse monoclonal; Sigma) and MMP-13 (rabbit polyclonal; Abcam, Cambridge, UK). Immunoreaction was developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

To detect secreted MMP-13, media conditioned by tumor spheres or parental U251 cells were incubated with mouse monoclonal anti-human MMP-13 antibody (Daiichi Fine Chemical, Toyama, Japan) and protein A/G agarose slurry at 4˚C overnight. The resulting immunocomplex was washed three times with lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail (Sigma); it was then subjected to Western blot analysis using rabbit polyclonal anti-human MMP-13 antibody.

In vivo xenograft experiments. U251 cells and sphere-forming cells were suspended at 5×10^2-1×10^6 cells in 100 μl 1:1 phosphate-buffered saline (PBS)/Matrigel (Becton-Dickinson), and subcutaneously implanted over the right thigh of 6-week-old male BALB/c nude mice (Charles River). Tumor growth was monitored weekly. Mice were sacrificed at 2, 3, 4, 6, and 8 weeks after implantation, and tumors were dissected out and the tumor volume and weight were measured. Tumor volume (V) was determined by the equation $V = L \times W^2 \times 0.5$, where L is the length and W is the width of the tumor. For orthotopic transplantation, cells were suspended in PBS (1×10^5 cells/μl). Six-week-old NOD-SCID (Charles River)

Table I. Oligonucleotide primers used in this study for RT-PCR.

Genes	Accession no.	Sense/antisense	Size (bp)
CD133	NM_006017	5'-TTGAGGAACTCCCTGAAAAG-3' 5'-ACAAATTCAAGGGGTCGATA-3'	384
Oct-3/4	NM_002701	5'-GACAGGGGGAGGGGAGGAGCTAGG-3' 5'-CTTCCCTCCAACCAGTTGCCCCAAAC-3'	118
Nanog	NM_024866	5'-CAGCCCCGATTCTTCCACCAGTCCC-3' 5'-CGGAAGATTCCCAGTCGGGTTCACC-3'	366
Sox-2	NM_003106	5'-GGGAAATGGGAGGGGTGCAAAAGAGG-3' 5'-TTGCGTGAGTGTGGATGGGATTGGTG-3'	125
Klf-4	NM_004235	5'-ACGATCGTGGCCCCGGAAAAGGACC-3' 5'-TGATTGTAGTGCTTTCTGGCTGGGCTCC-3'	698
Bmi1	NM_005180	5'-AGGAGAGGTTGCAGATGAAG-3' 5'-GCTGGGCATCGTAAGTATCT-3'	162
MMP-1	NM_002421	5'-ATTCTACTGATATCGGGGCTTTGA-3' 5'-ATGTCCTTGGGGTATCCGTGTAG-3'	304
MMP-2	NM_004530	5'-GCAGTTTGCTTTGTATGCAC-3' 5'-TGGAGAAGAGACTCGGTAGG-3'	185
MMP-3	NM_002422	5'-ATGCCCACTTTGATGATGATGAAC-3' 5'-CCACGCCTGAAGGAAGAGATG-3'	338
MMP-7	NM_002423	5'-GAGTGCCAGATGTTGCAGAA-3' 5'-AAATGCAGGGGGATCTCTTT-3'	168
MMP-9	NM_004994	5'-CCAGTTTCCATTCATCTTCC-3' 5'-CGAGTACTCCTTACCCAGGA-3'	214
MMP-10	NM_002425	5'-ATTGATGCAGCTGTTTCTGA-3' 5'-TCAACCTTAGGCTCAACTCC-3'	152
MMP-13	NM_002427	5'-CCCAGGAATTGGTGATAAAG-3' 5'-ATTACCCCAAATGCTCTTCA-3'	195
bcrp1	NM_004827	5'-GGCCTCAGGAAGACTTATGT-3' 5'-AAGGAGGTGGTGTAGCTGAT-3'	341
mdr1	NM_000927	5'-CACGTGGTTGGAAGCTAACC-3' 5'-GAAGGCCAGAGCATAAGATGC-3'	317
mrp1	NM_004996	5'-AAGACCAAGACGTATCAGGT-3' 5'-CAATGGTCACGTAGACGGCAA-3'	258
ß-actin	NM_031144	5'-AGAAGAGCTATGAGCTGCCTGACG-3' 5'-TACTTGCGCTCAGGAGGAGCAATG-3'	301

mice were anesthetized with pentobarbital and placed in the stereotactic frame using ear bars. A hole was made in the skull 2.0 mm lateral to the Bregma. Cell suspension (3 μl) was transplanted into the right striatum 3.0 mm below the surface of the brain using a Hamilton syringe. When mice developed neurological deficits, they were sacrificed, and fixed with 4% PFA and the dissected brain were sliced into 5-μm-thick coronal sections using a cryostat as described previously (17). Brain sections were then subjected to hematoxylin and eosin (H&E) staining. All animal experiments were performed in
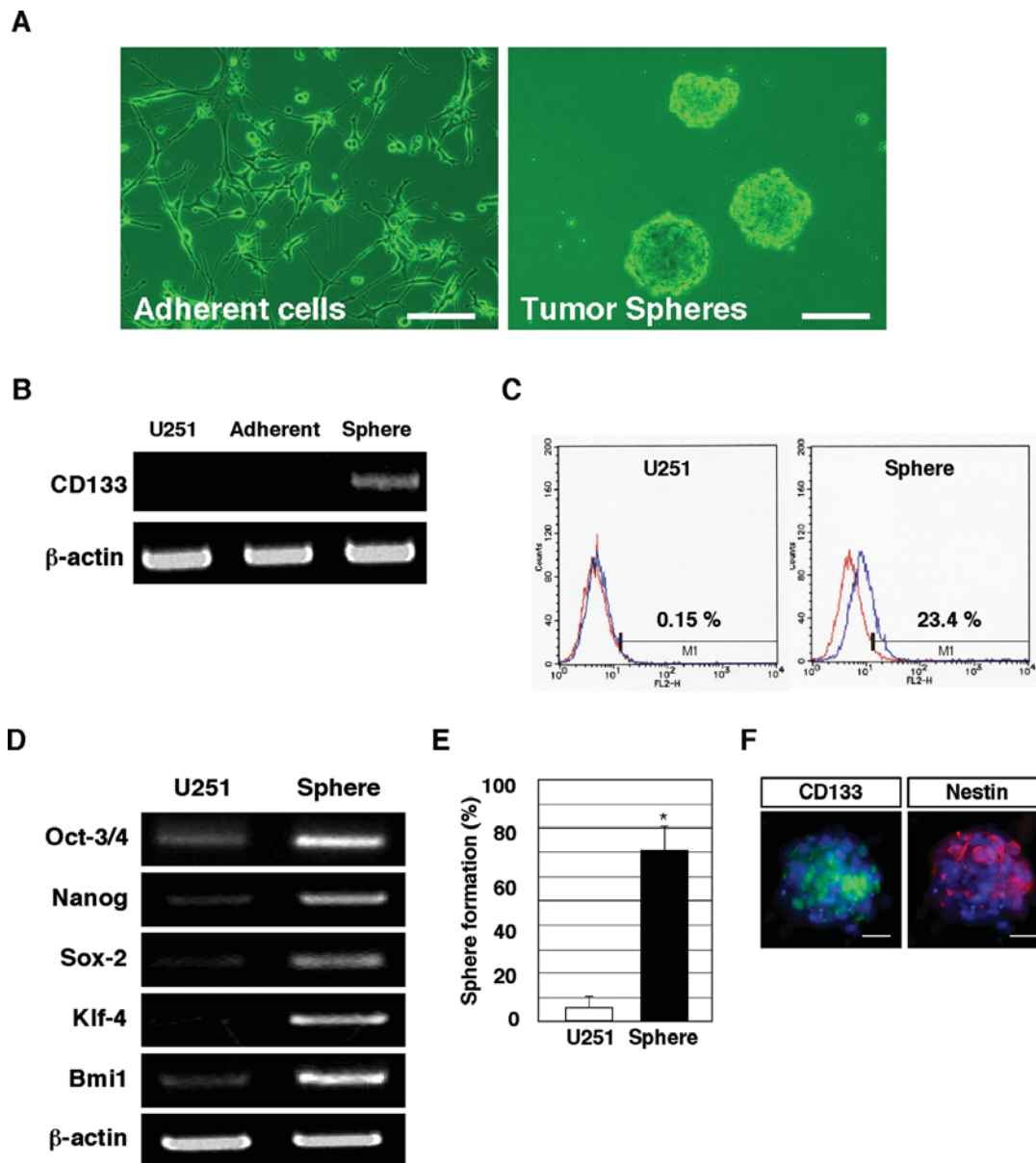
Figure 1. Isolation and characterization of U251-derived tumor spheres. (A) U251 cells were grown in sphere medium for 2 weeks. The morphology of adherent cells and developed tumor spheres are shown. Scale bar, 100 μm. (B) Expression of the stem cell marker CD133 mRNA in U251 and tumor spheres. Representative RT-PCR data are shown. Expression of ß-actin mRNA was used as an internal control. (C) Flow cytometry analysis for CD133 on U251 and tumor sphere-forming cells. Dissociated cells were stained either with isotype IgG control (red line) or anti-CD133/2 antibody (blue line) conjugated with PE before the analysis. The percentage of CD133-positive cells in the total population is shown. (D) Expression of the stemness-related gene mRNAs in U251 and tumor spheres. Representative RT-PCR data are shown. (E) Tumor spheres exhibited self-renewal potential. Dissociated-tumor spheres were seeded at a density of one cell/well in 96-well plates and cultured for 21 days in sphere medium. The percentage of wells that generated secondary tumor spheres was determined. Values are expressed as mean ± SD from triplicate experiments. *P<0.0001. (F) Secondary tumor spheres retained the expression of stem cell markers, CD133 (green) and nestin (red). Nuclei were labeled with Hoechst 33258 (blue). Scale bar, 50 μm.

accordance with the guidelines of the Ehime University Committee for Ethics of Animal Experimentation.

Invasion assay in a brain slice model. Rat brain slice cultures for an invasion assay were prepared as described previously (18,19). In brief, whole brains were quickly removed from 2-day-old neonatal Wistar rats (Charles River) and placed in Hanks' balanced salt solution (HBSS; Invitrogen). The brains were mounted on a stage of a microslicer (Dosaka EM, Kyoto, Japan), cut into 500 μm-thick coronal slices, and then transferred onto 30-mm Millicell-CM inserts with 0.4-μm pores

(Millipore) in 6-well culture plates. The outer-well contained 1.0 ml of brain slice medium (50% Eagle's minimal essential medium with HEPES, 25% HBSS, 25% FBS, 6.5 mg/ml glucose, and penicillin/streptomycin/amphotericin B mixture). Brain slices were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Cell aggregates, called spheroids in this study, distinct from the spheres consisted of cancer stem-like cells, were prepared as follows: GFP-U251 cells (2.0×10^5) were seeded to 35-mm culture dishes for suspension culture, and incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2

for 12-18 h while continuously agitating at a speed of 30 rpm on a reciprocating shaker. Then, the spheroids were formed with a diameter 200-300 μm. The spheroid or the sphere derived from GFP-U251 was placed on the corpus callosum of a brain slice, and cocultured at 37°C for 48 h. The depth and expanse of invasion by the spheroid or the sphere was evaluated with an inverted confocal laser-scanning microscope (CLSM) (Nikon A1, Nikon, Japan).

Immunohistochemical staining of spheres invading into the brain slices. MMP-13 expression of tumor spheres invading brain slices was immunohistochemically analyzed. The slices were washed with PBS and fixed in 4% PFA at 48 h after starting the invasion assay. After inhibition of endogenous peroxidase activity, the slices were incubated in Tris-buffered saline containing 0.1% Tween-20 (TBSt) containing 1 mg/ml BSA, and then incubated with a normal rabbit IgG or an anti-human MMP-13 polyclonal antibody (1:1000) at 4°C overnight. The slices were further incubated with a biotinylated goat anti-rabbit IgG antibody for 1 h at room temperature followed by incubation with streptavidin-peroxidase (Labvision, Fremont, CA) for 1 h. Immunoreaction was visualized with 3-amino-9-etylcarbazole as chromogen (Labvision).

Statistical analysis. Values are expressed as mean ± standard deviation (SD) and subjected to analysis of variance followed by a two-tailed Student's t-test (unpaired). Significance was set at P<0.05.

Results

Tumor spheres from U251 glioma cell line express CD133 and have cancer stem cell phenotypes. To determine whether a subpopulation of self-renewing cancer stem-like cells, such as SP (side population) cells, exist in the human glioma cell line U251, we cultured U251 cells in serum-free sphere medium containing EGF, bFGF, and PDGF. Under this culture condition, most cells formed floating neurosphere-like cell aggregates, termed tumor spheres, within 2 weeks (Fig. 1A, right). The remaining cells that did not form tumor spheres adhered to the bottom of the culture dish, and did not significantly proliferate (Fig. 1A, left). We next investigated the expression of CD133 antigen, a putative brain tumor stem-like cell marker (3), by tumor spheres. CD133 mRNA was only expressed by spheres, but neither by U251 nor adherent non-tumor sphere-forming cells (Fig. 1B). Furthermore, flow cytometric analysis revealed that tumor sphere-forming cells, but not parental U251 cells, significantly expressed CD133 antigen (Fig. 1C). In addition to CD133 expression, stemness-related genes, Oct-3/4, Nanog, Sox-2, Klf-4 and Bmi1, were more highly expressed by tumor sphere-forming cells than parental cells (Fig. 1D). Tumor spheres were dissociated and plated into 96-well plates as single cells per well; then, secondary spheres derived from single cells were counted to examine whether sphere-forming cells possess self-renewing ability. A total of 70.5±9.89% of single cells derived from tumor spheres formed secondary spheres, whereas 6.1±4.47% of single cells derived from parental U251 cells formed secondary spheres (Fig. 1E). The morphology of secondary tumor spheres was indistinguishable

Table II. *In vivo* tumorigenicity of U251 and tumor sphere-forming cells.

Cell	Injected cell number	Tumor formation	Latency[a] (days)
U251	1.0x10⁶	8 (n=8)	22.8±3.2
	5.0x10⁵	8 (n=8)	40.8±5.3
	1.0x10⁵	10 (n=10)	51.7±3.9
	1.0x10⁴	0 (n=10)	-
	5.0x10³	0 (n=6)	-
	1.0x10³	0 (n=6)	-
Sphere	1.0x10⁶	8 (n=8)	17.0±3.7
	5.0x10⁵	8 (n=8)	26.0±3.4
	1.0x10⁵	10 (n=10)	33.4±3.1
	1.0x10⁴	6 (n=6)	42.0±18.3
	5.0x10³	6 (n=6)	47.3±5.4
	1.0x10³	6 (n=6)	53.0±8.1
	5.0x10²	6 (n=6)	57.1±8.1

[a]The time from injection to the first appearance of a tumor with 5 mm diameter.

from that of primary spheres, and these secondary spheres were immunoreactive to antibodies to CD133 and nestin, a marker for neural stem/progenitor cells, suggesting that the U251 cell line contains stem-like cells with a self-renewing ability (Fig. 1F). We further defined that tumor sphere-forming cells possessed the other characteristics of cancer stem cells, such as multipotency, and drug-resistibility (data not shown). These data corroborate the assertion that tumor spheres generated from U251 cells are enriched with cancer stem-like cells.

Tumorigenicity of tumor spheres. Because strong tumorigenicity is one of the hallmarks of CSCs, we compared the tumorigenicity of tumor sphere-forming cells with that of parental U251 cells (Fig. 2). Subcutaneous implantation of 1x10⁶ U251 cells or tumor sphere-forming cells into the right thigh of nude mice developed tumor masses in all mice (Fig. 2A and Table II). However, the tumor masses dissected from mice implanted with sphere-forming cells (3.3±0.8 g and 2562±512 mm³) were heavier by 6.85-fold in weight and 10.1-fold larger in volume at 8 weeks than those from mice implanted with parental U251 cells (0.48±0.14 g and 253±85 mm³), regardless their comparable cell growth rate (Fig. 2B and data not shown). In contrast, adherent cells hardly developed tumors and only a small tumor was formed at 8 months after transplantation. Furthermore, tumor masses were palpable at 17.0±3.3 days after implantation with tumor spheres and 22.8±3.2 days after implantation with parental U251 cells (Table I). Only 500 tumor sphere-forming cells generated a palpable tumor mass within two months in all mice, whereas 1x10⁴ parental U251 cells were unable to generate a palpable tumor mass.
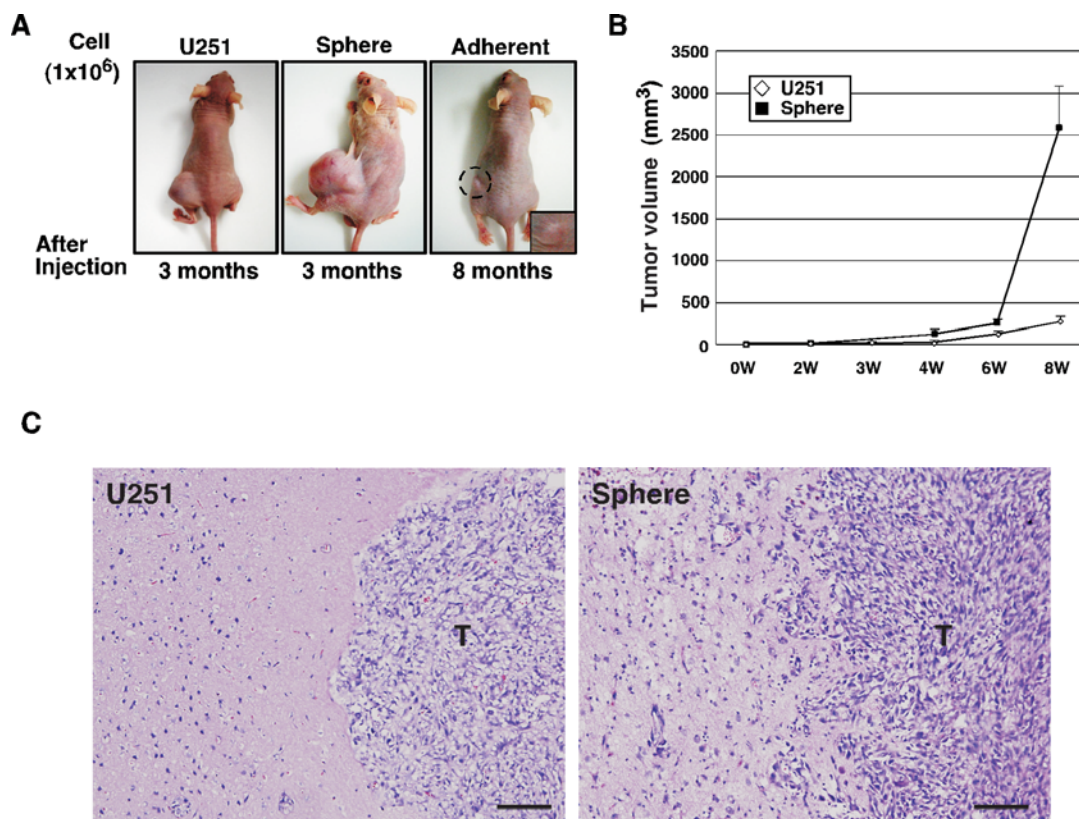
Figure 2. *In vivo* tumorigenicity of tumor sphere-forming cells. (A) U251, tumor sphere-forming cells, and adherent cells (1×10^6 cells) were subcutaneously injected into the right thigh of male nude mice. The resulting tumor xenografts at the indicated times are shown. The insert shows a high-magnification view of the circled area. (B) The volumes of tumors taken from nude mice at the indicated times are shown. Values are represented as mean ± SD (n=4). (C) U251 and tumor sphere-forming cells (3×10^5 cells) were orthotopically implanted into the brains of NOD-SCID mice. Coronal brain sections at 5 weeks after cell transplantation were stained with H&E. T indicates tumor. Scale bar, 100 μm.

To examine the phenotype of orthotopically implantation, U251 and tumor sphere-forming cells (3×10^5 cells) were implanted in the right striatum of NOD-SCID mice, and all the mice developed brain tumors by 5-6 weeks after implantation. Histopathological investigation of xenografts showed that brain tumors of parental U251 cells were clearly delineated by the normal brain tissue (Fig. 2C, left). Whereas tumors of tumor sphere-forming cells were vaguely delineated, indicating their active invasion (Fig. 2C, right). Thus, tumor sphere-forming cells possess the highly motile and invasive nature.

Invasion of glioma spheres observed in a rat brain slice model. We therefore evaluated their migratory and invasive ability of tumor sphere-forming cells, since such abilities are critical for the lethal outcome of GBMs. To examine the invasive ability into a brain tissue, a rat brain slice model was used. In this model, the invasive ability of glioma cells was evaluated through three-dimensional serial observation using CLSM. A spheroid or a tumor sphere, both of which were made of parental GFP-U251 cells, were placed on a brain slice on the corpus callosum as illustrated in Fig. 3A. As shown in Fig. 3B, some cells migrated out of the spheroid and tumor sphere, and those cells were allowed to move in the slice for 48 h. The three-dimensional movement of the EGFP-expressing cells was evaluated through serial optical sections from the surface to the bottom of the slice. The cells

from the tumor spheres intensively invaded the brain slice (maximum depth from the surface of the slice: -69±3.2 μm), and appeared to spread along the neuronal fibers of the corpus callosum. These migration patterns in the brain slices are similar to those in clinical situations called butterfly glioma, caused by migration of glioma cells to the contralateral hemisphere through the corpus callosum (20). Whereas, most cells of the spheroid remained in the spheroid and invaded the brain slice to a depth of -50 μm (maximum depth: -57±5.8 μm) (Fig. 3B and C). Fig. 3D shows the distribution of cells of the spheres and spheroids in the brain slices. Theses cells showed similar behavior with a transwell chamber assay and an *in vitro* Matrigel invasion assay (data not shown). Thus, tumor sphere-forming cells had stronger migratory and invasive ability than spheroids in all evaluated serial sections.

Involvement of MMP-13 in invasive ability of tumor spheres. The aggressive invasive ability of tumor sphere-forming cells may be correlated with MMP activity. RT-PCR analysis revealed the expression of MMP-13 mRNA in tumor sphere-forming cells (Fig. 4A). MMP-2 and MMP-7 mRNA were expressed in both tumor sphere-forming cells and parental U251, but their levels of expression did not differ. MMP-1, MMP-3, MMP-9, and MMP-10 mRNA were not detected in either type of cells. The specific expression of MMP-13 mRNA in tumor sphere-forming cells was also evident in
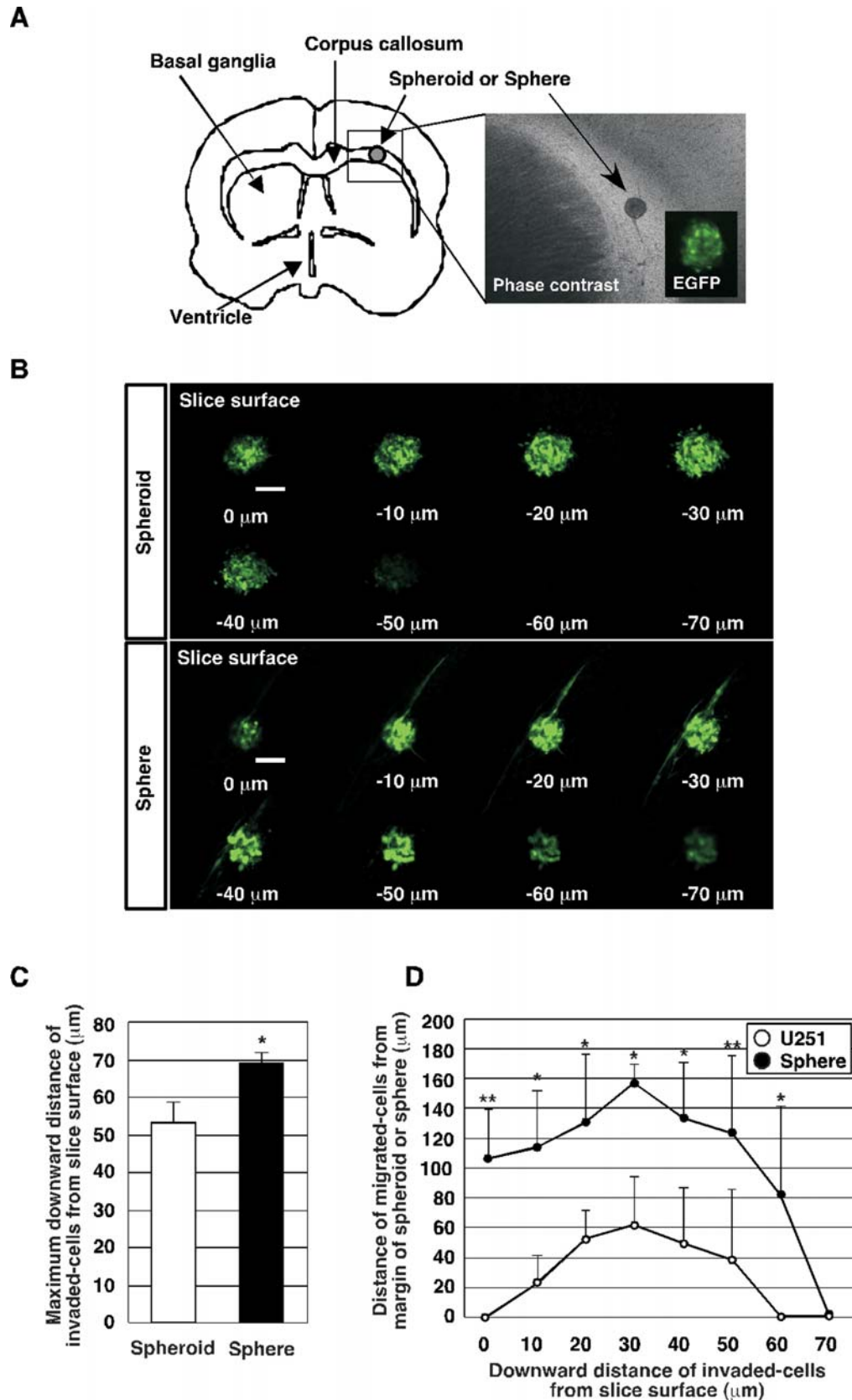
Figure 3. Invasive behavior of U251 cells and tumor spheres on rat brain slices. (A) The procedures for assessing tumor cell migration and invasion in cocultured brain slices are illustrated. A spheroid and tumor sphere derived from GFP-U251 is placed on the corpus callosum of a brain slice, and cocultured for 48 h. Phase contrast and fluorescent images are shown. (B) The migrating and invading cells, which stably expressed EGFP, were detected under CLSM. In each experiment, serial optical sections of the brain slices were obtained every 10 μm from the brain slice surface (0 μm) to a deeper site (70 μm). Representative photographs of invading cells are shown. Scale bar, 50 μm. (C) The distance from the brain slice surface was calculated. *P<0.005. (D) The maximal distance of migrating cells from the margin of the spheroid or tumor sphere in each optical-section was calculated. Each column in (C) and (D) represents the mean ± SD of three independent experiments. *P<0.05; **P<0.01.
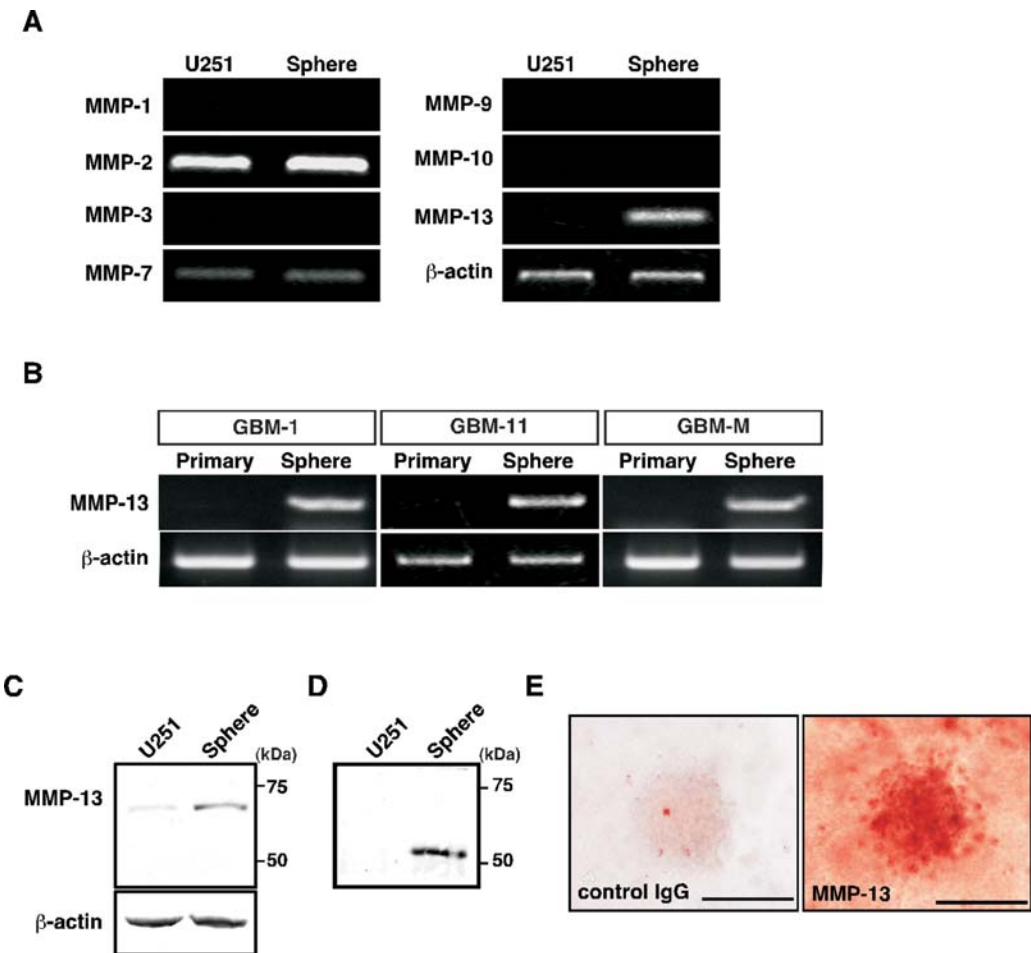
Figure 4. Expression of MMPs by tumor spheres. (A) MMP mRNA expression by U251 cells and tumor spheres was analyzed by RT-PCR. Representative data are shown. (B) MMP-13 mRNA expression by tumor spheres derived from three human GBM tissues was analyzed by RT-PCR. Representative data are shown. (C) Western blot analysis of MMP-13 protein level in cell lysates of U251 and tumor spheres. ß-actin was used as a loading control. (D) Secreted and activated MMP-13 protein was immunoprecipitated from the medium conditioned by U251 cells or tumor spheres. (E) Tumor spheres from EGFP-U251 cells cultured on rat brain slices for 48 h were immunohistochemically stained using a rabbit control IgG or a polyclonal anti-MMP-13 antibody. Representative photographs are shown. Migrated cells expressed MMP-13. Scale bar, 200 μm.
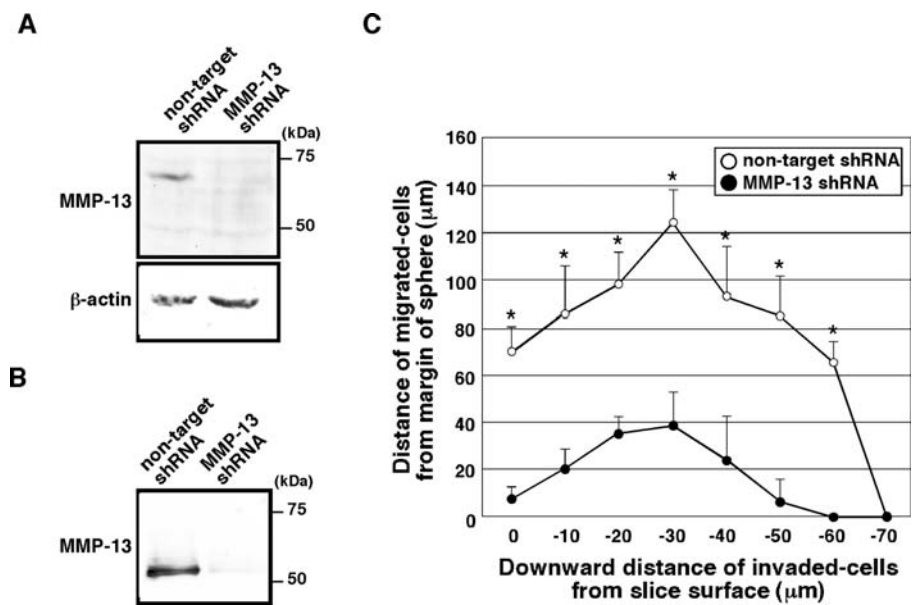

Figure 5. Effect of MMP-13 supression by shRNA on the migratory and invasive ability of tumor spheres on brain slices. (A) The protein levels of MMP-13 in cell lysates of tumor spheres transfected with non-target control or MMP-13 shRNA. (B) Secreted and activated MMP-13 protein in the medium conditioned by tumor spheres in (A) was immunoprecipitated. (C) A non-target control or MMP-13 knockdowned tumor sphere was cultured on brain slices for 48 h. The migrating and invasive cells were analyzed in the same manner as those in Fig. 3. Each column represents the mean ± SD of three independent experiments. *P<0.001.

tumor sphere-forming cells derived from three human GBM tissue (Fig. 4B). MMP-13 protein is secreted as a latent form and converted into an activated form in the extracellular milieu. Tumor spheres expressed pro-MMP-13 at a higher level than U251 cells, and the activated form was detected only in the medium conditioned by tumor spheres as revealed by immunoprecipitation using anti-MMP-13 antibody (Fig. 4C and D). To examine whether MMP-13 was expressed by invading spheres, immunohistochemical staining was performed on the brain slice after 48 h of coculture. As shown in Fig. 4E, migrating cells from the tumor sphere were immunopositive for MMP-13.

To investigate the functional involvement of MMP-13 in the highly motile and invasive nature of tumor spheres, we established tumor spheres which endogenous MMP-13 expression was knocked down by shRNA (Fig. 5A and B). The brain slice analysis showed that shRNA-mediated knockdown of MMP-13 was strongly suppressed the migration and invasion ability of tumor spheres (Fig. 5C).

Discussion

The concept that unregulated stem cells produce certain cancers is based on the similarities between CSCs and somatic stem cells, such as the capacity for self-renewal, proliferation, and differentiation into multiple cell types. This concept was firstly found in acute myeloid leukemia (21), and was then also evident in solid tumors, such as glioma, breast cancer, prostate cancer, lung cancer and colon cancer (2,3,22-26). CSCs as the CD133-positive cells, the side population (SP) cells, and the tumor sphere-forming cells have also been isolated from many established cancer cell lines such as those of glioblastoma/glioma, neuroblastoma, adenocarcinoma, hepatocellular carcinoma, and breast cancer (27-31). However, since many cancer cell lines established from primary tumors have been maintained *in vitro* for many years, the existence of a cell with stem-like properties in cell lines is still a matter of debate.

In this study, we isolated and characterized CSCs from the U251 cell line, and assessed their migratory and invasive potential. We first demonstrated that the combination of EGF, bFGF, and PDGF allows U251 to form tumor spheres, leading to isolation of cancer stem-like cells. In addition to EGF and bFGF, PDGF has been shown to contribute to glial oncogenesis (32). Therefore, PDGF was also added to the sphere medium as a mitogen to maintain cancer stem-like cells, as reported with other glioma cell lines (27). The existence of CSCs in U251 cell line was confirmed by the formation of tumor spheres with the self-renewal potential in the sphere medium. In addition to CD133 and nestin expression in tumor spheres, stemness-related genes. As the aberrant activation of self-renewal is one of the key properties in CSCs, stemness-related genes in embryonic stem (ES) cells, especially Oct-3/4, which is one of a key regulator of self-renewal (33,34), have been suggested to involve in the acquisition of CSC properties. The expression of Oct-3/4 has also been shown in various human solid tumors and cancer cell lines, and higher expression correlates with tumor progression, malignancy, and prognosis (35). Du *et al* recently reported that the expression levels of Oct-3/4

positively correlated with the grade of gliomas, and that Oct-3/4 was necessary to maintain rat C6 glioma cells in an undifferentiated state (36). Therefore, its expression may be implicated in the acquisition of stem-like properties, especially self-renewal, in tumor sphere-forming cells.

We also consider the other possibility that non-CSCs could generate CSCs in tumor spheres, that is, parental U251 cells could undergo dedifferentiation or re-programming under stem cell culture conditions. We and another group demonstrated that Bmi1 was highly expressed in tumor sphere-forming cells (31). Bmi1 is a member of the mammalian Polycomb Group family of genes and has been reported to play an important role in the self-renewal of somatic stem cells by suppressing tumor suppressor genes (37-39). Interestingly, Bachoo *et al* have reported that when terminally differentiated astrocytes with the deletions of p16^{INK4a} and p19^{ARF}, downstream target of Bmi1, were cultured in serum-free stem cell medium, they dedifferentiated into cancer stem-like cells to be the cell-of-origin of malignant glioma (40). Furthermore, additional reports indicate that Oct-3/4, Sox-2, and Klf-4 may be the major factors to reprogram differentiated cells into germline-competent-induced pluripotent stem cells (41,42). Indeed, shRNA-mediated Oct-3/4 suppression in U251 cells significantly interfered tumor sphere formation. Barely floating tumor spheres devoid of Oct-3/4 could not keep spheres and differentiated into attached glioma-like cells (unpublished data). Therefore, only U251 cells that can highly express stemness-related genes, such as Bmi1 and Oct-3/4, under serum-free culture conditions might be able to acquire stem-cell-like properties through re-programming.

Unlike most other tissue, the brain is largely free of a well-defined ECM, but ECM proteins are present in the border area between normal brain and glioma cells (7). MMPs have been strongly implicated as mediators of the invasive properties of brain tumors and tumors of other organs. In addition, we and other groups showed that glioma cells are able to secrete various MMPs (10,13). However, the expression of MMP-13 has not been well demonstrated in human gliomas to date. In this study, production and activity of MMP-13, but not of MMP-2 and MMP-9, were notably higher in tumor sphere-forming cells than in parental U251 cells. The motility and invasive activity of tumor sphere-forming cells correlated strongly with elevated MMP-13 production. It is of interest that Oct-3/4 leads to up-regulation of MMP-13 production in bladder cancer cells (43). Furthermore, CSCs are found on the invasive front of solid tumors (44). In light of these reports, the highly invasive potential of GBM cells may be due to MMP-13 expression by CSCs under the regulation of Oct-3/4.

Stem cells of various tissues are maintained within a microenvironment, so-called niche, composed of several differentiated cell types (45). Calabrese *et al* clearly demonstrated that brain tumor microvasculature forms a niche that is critical for the maintenance of CD133/nestin-positive CSCs (46). Thus, MMP-13 might be required for CSCs to detach from a perivascular niche by degrading the basement membrane of tumor microvasculature. Further studies are necessary to clarify these assumptions.

CSCs are considered responsible for tumor metastasis, escape from anticancer therapies and tumor recurrence.

Although the precise role of MMP-13 in CSCs *in vivo* remains unknown, the accelerated invasion of CSCs may be attributable to the activation of MMP-13. Therefore, CSCs with high MMP-13 activity may be the case of the highly invasive potential of GBM cells, as suggested in this study. Thus, inhibition of MMP-13 activity may provide a novel target in brain tumors.

Acknowledgements

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