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Oct-3/4 Promotes Migration and Invasion of Glioblastoma Cells

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ABSTRACT

As a result of increased glioblastoma migration and invasion into normal brain parenchyma, treatment of local tumor recurrence following initial treatment in glioblastoma patients remains challenging. Recent studies have demonstrated increased Oct-3/4 expression, a self-renewal regulator in stem cells, in glioblastomas. However, little is known regarding the influence of Oct-3/4 in glioblastoma cell invasiveness. The present study established Oct-3/4-overexpressing glioblastoma cells, which were prepared from human glioblastoma patients, to assess migration, invasion, and mRNA expression profiles of integrins and matrix metalloproteinases (MMPs). Compared with control cells, Oct-3/4 expressing-glioblastoma cells exhibited increased migration and invasion in wound healing and Matrigel invasion assays. Oct-3/4 overexpression resulted in upregulated FAK and c-Src expression, which mediate integrin signals. Vinculin accumulated along the leading edges of Oct-3/4 expressing-glioblastoma cells and associated with membrane ruffles during cell migration. Oct-3/4 expressing-cells exhibited increased MMP-13 mRNA expression and MMP-13 knockdown by shRNA suppressed cell invasion into Matrigel and organotypic brain slices. These results suggested that Oct-3/4 enhanced degradation of surrounding extracellular matrix by increasing MMP-13 expression and altering integrin signaling. Therefore, Oct-3/4 might contribute to tumor promoting activity in glioblastomas. J. Cell. Biochem. 113: 508–517, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: GLIOBLASTOMA; OCT-3/4; INVASION; INTEGRIN; MATRIX METALLOPROTEINASE

Gliomas, the most common primary brain tumors, are the leading cause of central nervous system tumor-related deaths [Rao, 2003]. Although significant surgical and adjuvant therapy advancements have been made, glioma patient morbidity and mortality remain high. To date, the mechanisms underlying glioma genesis remain elusive, although extensive molecular and cellular analyses have been performed on tumor masses. A small population of cells has been shown to generate malignant cells in tumors; these cells have been termed "cancer stem cells (CSCs)" and possess tumor-initiating activity [Al-Hajj et al., 2003; Singh et al., 2004; Collins et al., 2005; Kim et al., 2005; O'Brien et al., 2007; Ricci-Vitiani et al., 2007]. CSCs exhibit a high self-renewal capacity and produce heterogeneous cancer cells in the same tumor mass, which plays an important role in tumor recurrence [Reya et al., 2001; Dalerba et al., 2007]. These results have demonstrated that CSCs should be a critical target for glioma treatment. Further characterization studies will help to develop more effective brain tumor therapies.

In cancer biology, the similarity between embryogenesis and tumorigenesis suggests that CSCs are a result of aberrant activation of the self-renewal process in normal stem/progenitor cells or reprogrammed-differentiated cells [Ben-Porath et al., 2008]. A recent study showed that adult fibroblasts can be reprogrammed into pluripotent embryonic stem (ES)-like cells [Takahashi et al., 2007], which raises the possibility that combined expression of stem cell-associated factors and specific oncogenes could also induce an

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undifferentiated state in cancer cells. We confirmed that Oct-3/4, Sox2, Klf-4, Nanog, and Bmi1, which are key regulators of stemness-related genes, are expressed in CSCs [Inoue et al., 2010]. These results suggest that it is worth examining expression and involvement of regulatory genes for self-renewal in tumorigenesis. Of these genes, ectopic expression of Oct-3/4, a member of the POU family of transcription factors, is sufficient to induce tumor growth in the adult mouse [Gidekel et al., 2003; Hochedlinger et al., 2005]. In addition, Oct-3/4 is expressed in various human solid tumors and cancer cell lines; increased expression correlates with tumor progression, malignancy, and prognosis [Chang et al., 1998; Chiou et al., 2008; Du et al., 2009]. Du et al. [2009] reported that Oct-3/4 expression levels positively correlate with glioma malignancy levels, which have been classified into four grades by the World Health Organization [Burger et al., 2007]. These studies suggest that aberrant Oct-3/4 expression could contribute to CSC genesis and the neoplastic glioma process.

Glioblastomas (GBs) diffusely invade surrounding normal brain tissue and induce local tumor recurrence following initial treatment and failure of curative therapies. We recently demonstrated that CSCs express stemness-related genes with high migratory and invasive activity via enzymatic activity of matrix metalloproteinase (MMP)-13 [Inoue et al., 2010]. In the present study, the relationship between Oct-3/4 expression and GB migration and invasion was analyzed. GB cells overexpressing Oct-3/4 exhibited increased migration and invasion, which was dependent on enhanced integrin signaling and MMP-13 enzymatic activity.

MATERIALS AND METHODS

HUMAN GB PRIMARY CELL CULTURE

Human GB primary cells, GB-M cells, and GB-1 cells, were prepared from surgically resected tumor specimens from two GB (grade 4) patients. The samples were obtained following informed consent, with approval from the local ethics committee at Ehime University Hospital. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako), supplemented with 10% fetal bovine serum (FBS), 4.5 g/L glucose, and a penicillin/streptomycin/amphotericin B mixture (Wako), as previously described [Inoue et al., 2010].

CONSTRUCTION OF EXPRESSION PLASMIDS AND TRANSFECTION

To generate expression plasmids encoding human Oct-3/4 and human Sox-2, full-length cDNA fragment was amplified by polymerase chain reaction (PCR) using cDNA derived from human GB tissue with the following primers: hOct-3/4; forward 5′-CTTAGATCTATGGCGGGACACCTG-3′ and reverse 5′-CAGAATTCTCAGTTTGAATGCATGGG-3′, hSox-2; forward 5′-CCAGATCTATGTACAACATGAT-3′ and reverse 5′-GGCTCGAGCATGTGTGAGAGGGGCA-3′. The PCR products were sequenced, digested with *Bgl*II and *Eco*RI, and subsequently inserted into a pEGFP-C1 vector, which was in frame with the 3′ end of enhanced green fluorescence protein (EGFP) coding sequences (Clontech). pEGFP-Oct-3/4 and pEGFP-Sox-2, which express Oct-3/4 and Sox-2, respectively, were ligated to the EGFP C-terminus. The Klf-4 expression plasmid pBRPyCAG-Klf4-IP was kindly provided by Dr. H. Niwa (Center for Developmental Biology, RIKEN Institute, Japan).

To establish GB cells that stably expressed EGFP-Oct-3/4, EGFP-Sox-2, and Klf-4, plasmids were transfected into GB cells using Lipofectamine 2000 reagent (Invitrogen) according to manufacture instructions. Stable cells were selected by culturing in medium containing G418 selection (500 μg/ml; Invitrogen) for EGFP-Oct-3/4 and EGFP-Sox-2, as well as puromycin (2 μg/ml; Sigma) for Klf-4, and were used as a mixed population of drug-resistant cells.

PREPARATION OF LENTIVIRUS WITH shRNA EXPRESSION VECTOR

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) and non-target control shRNA plasmid (pLKO.1/non-target shRNA) were purchased from Sigma. The shRNA expression lentivirus vector was prepared by transient transfection of 293T cells with a self-inactivating vector construct, VSV-G-and Rev-expressing plasmid pCMV-VSV-G-RSV-Rev, and the packaging construct pCAG-HIVgp (kind gifts from Dr. H. Miyoshi, BioResource Center, RIKEN Tsukuba Institute, Japan) using Lipofectamine 2000 reagent (Invitrogen). Lentiviral supernatant from 293T cells was harvested at 48 h post-transfection, concentrated by centrifugation, and used to infect cells. Inoculated cells were selected by culturing in medium containing puromycin.

RNA ISOLATION AND RT-PCR ANALYSIS

Total RNA was collected using ISOGEN (Nippon Gene) according to manufacturer 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. DNA fragments were amplified with GoTaq DNA polymerase (Promega). PCR conditions were as follows: 94°C for 1 min, followed by 22 (β-actin) or 30 (other genes) cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. For real-time PCR analysis, cDNA was diluted at 1:3, and 1 μl was used for triplicate quantitative PCR on a MJ mini instrument (BioRad) using Fast Start Universal SYBR Green (Roche). All gene-specific mRNA expression values were normalized to β-actin expression levels. Primer sequences are listed in Table I and previous report [Inoue et al., 2010].

WESTERN BLOT AND IMMUNOPRECIPITATION ANALYSIS

Cells were grown on poly-L-lysine-coated dishes and were subsequently lysed with Laemmli's sample solution. The lysates were electrophoresed, transferred onto nitrocellulose membrane, and immunoblotted with antibodies to Oct-3/4 (rabbit monoclonal, clone EPR2054; Epitomics), β-actin (mouse monoclonal, clone AC-15; Sigma), GFP (rabbit polyclonal; Delta Biolabs), focal adhesion kinase (FAK; mouse monoclonal, clone 77/FAK; BD Transduction Laboratories), phospho-FAK (pY397) (rabbit monoclonal, Invitrogen), Src (mouse monoclonal, clone GD-11, Millipore), phospho-Src (pY529) (rabbit polyclonal, Sigma), and MMP-13 (rabbit polyclonal; Abcam, Cambridge, UK). Following incubation with alkaline phosphatase-conjugated secondary antibody (Promega), the immunoreaction was developed using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

TABLE I. Oligonucleotide Primers Used in This Study for Real-Time PCR

Genes	Sense/antisense	Size (bp)
Integrin α1	5′-gttttgtgtggcatcacagt-3′ 5′-aatgaaccctgtaccaagga-3′	203
Integrin α2	5′-aaaatgccagcggtttatag-3′ 5′-ggtacacaccagcaccacta-3′	197
Integrin α3	5′-actctttacctttgcggatg-3′ 5′-catctgcaagttgctctcac-3′	161
Integrin α4	5′-atggctcccaatgttagtgt-3′ 5′-gtctgcattgcactcttttg-3′	164
Integrin α5	5′-aagatctgtcctcagccttg-3′ 5′-cccatttgagttctgattcc-3′	229
Integrin αV	5′-gcttaagagggcgtgaaag-3′ 5′-tccaccaagctcatcattac-3′	167
Integrin β1	5′-tttccattggagatgaggtt-3′ 5′-catgacacttgggactttca-3′	183
Integrin β3	5′-atgtaccagcatctcccagt-3′ 5′-cactctctccctttgaggaa-3′	211
Integrin β5	5′-ggagagaaattggcagagaa-3′ 5′-gatcccagactgacaactcc-3′	199

IN VITRO WOUND HEALING AND MATRIGEL INVASION ASSAY

Confluent cells were scratched to linearly remove cells using a 200-μl pipette tip in six-well culture plates, and the culture was subsequently incubated in DMEM with 10% FBS in the presence or absence of mitomycin C (10 μg/ml) (Wako). Images were collected at 0 and 14 h post-scratch using a phase-contrast microscope. The culture plate area covered by cells was measured by subtracting the value of cell-free area at 0 h from the area at 14 h using NIH image analysis software; values were expressed as a percentage of total area.

The invasion assay was performed using Boyden chambers with a 8-μm pore size Matrigel-coated membrane (Becton Dickinson). Cells (5×10^4) in serum-free DMEM containing 0.1% BSA were seeded into the upper chamber, and the lower compartment was filled with DMEM containing 10% FBS as a chemoattractant. After incubation for 24 h at 37°C in a CO_2 incubator, non-invading cells on the upper membrane surface were removed by wiping with a cotton swab. Migrated cells on the lower filter surface were fixed, stained with 0.5% crystal violet, and all cells attached to the lower surface were quantified.

ADHESION ASSAY

Adhesion assays were performed in 96-well plates. The plates were coated 1 h at room temperature with Matrigel (Becton Dickinson) at 85 μg/well, and human plasma fibronectin (Becton Dickinson) at 1 μg/well, washed twice with PBS, and blocked with 1 mg/ml BSA for 1 h. Cells were added to the non-coated or coated plate at 5×10^4 cells per well. After 30 min at 37°C in a CO_2 incubator, non-adherent cells were washed twice with PBS and adherent cells were fixed with 70% ethanol for 20 min. Cells were stained with crystal violet for 10 min and washed four times with PBS. Bound dye was extracted with 0.1 M sodium citrate, pH 4.2, for 30 min, and optical density (OD) was quantified at 570 nm using a plate reader (Flex Station3; Molecular Devices).

IMMUNOCYTOCHEMISTRY

Cells were fixed with 4% paraformaldehyde (PFA) at 6 h post-injury. The fixed cells were permeabilized and blocked with Tris-buffered saline containing 0.1% Tween-20 (TBSt) and 1 mg/ml BSA for 30 min. The cells were then incubated with rhodamine–phalloidin or antibody specific to vinculin (1:1,000; mouse monoclonal, clone hVIN-1; Sigma). After washing with TBSt, the cells were further incubated with Cy3-conjugated anti-mouse IgG secondary antibody (1:1,000; Jackson). The immunostained cells were observed with an inverted confocal laser-scanning microscopy (CLSM) (Nikon A1, Nikon).

MMP-13 ACTIVITY ASSAYS

Serum-free DMEM conditioned by confluent GBM cells for 48 h was concentrated approximately 10-fold using centrifugal filter device (Kurabo) and analyzed using SensoLyte Plus 520 MMP-13 Assay Kit (AnaSpec), according to the manufacture's instructions. The kit provides a 96-well cell culture plate coated with monoclonal anti-human MMP-13 antibody that recognizes both the latent and active forms of MMP-13. 4-Aminophenylmercuric acetate (APMA) was added to activate pro-MMP-13 for measuring total MMP-13 protein. For measuring active MMP-13 alone, this APMA activation step was omitted. Proteolytic activity of MMP-13 was measured using a fluorescence resonance energy transfer (FRET) peptide containing a quenched fluorophore (5-FAM). Upon cleavage into two separate fragments by enzymatic activity of MMP-13, the fluorescence of 5-FAM was recovered and was measured on a CytoFluor Multi-Well plate reader (Flex Station3; Molecular Device) with an excitation and emission wavelength 490 and 520 nm, respectively.

INVASION ASSAY IN A BRAIN SLICE MODEL

All animal experiments were performed in accordance with guidelines from the Ehime University Committee for Ethics of Animal Experimentation. Rat brain slice cultures for the invasion assay were prepared as previously described [Inoue et al., 2010]. In brief, whole brains from 2-day-old neonatal Wistar rats (Charles River) were sectioned into 500-μm thick coronal slices using a microslicer (Dosaka EM). The sections were then transferred onto Millicell-CM inserts with 0.4-μm pores (Millipore) in six-well culture plates. The outer well contained 1.0 ml medium (50% Eagle's minimal essential medium with HEPES, 25% Hank's balanced salt solution, 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 or "spheroids" were prepared as follows: cells (3×10^5) were seeded onto 35-mm culture dishes for suspension culture and were subsequently incubated for 12–18 h with continuous shaking at a speed of 30 rpm on a reciprocating shaker in a CO_2 incubator. Spheroids, with a diameter of 200–300 μm, formed and were placed on the corpus callosum of a brain slice and co-cultured at 37°C for 48 h. Depth and expanse of invasion by the spheroid was evaluated using CLSM (Nikon A1, Nikon).

STATISTICAL ANALYSIS

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

RESULTS

OCT-3/4 OVEREXPRESSION ENHANCES MIGRATION AND INVASION OF GLIOBLASTOMA CELLS

Human GB cells, which stably expressed EGFP-Oct-3/4 (GB/Oct-3/4), were established to analyze the role of Oct-3/4 in GB migration and invasion. Oct-3/4 stable expression was confirmed in whole cell lysates by Western blot analysis. A band of correct size corresponding to the overexpressed EGFP-Oct-3/4 protein (72 kDa) was observed in GB/Oct-3/4 cells, while endogenous Oct-3/4 protein (45 kDa) was not detectable in control cells (GB/EGFP) and GB/Oct-3/4 cells (Fig. 1A). Results from the in vitro wound-healing assay demonstrated that GB/Oct-3/4 cells migrated into the scratched area more rapidly than control GB/EGFP cells (Fig. 1B). Quantification revealed 30.89% versus 67.62% wound repair in GB-M cells and 41.14% versus 74.09% in GB-1 cells with EGFP and EGFP-Oct, respectively, at 14 h after scratch injury (Fig. 1C). To exclude the effect of Oct-3/4 overexpression on cell proliferation rather than cell migration, mitomycin C was used to inhibit cell proliferation in the assay medium. Although mitocycin C

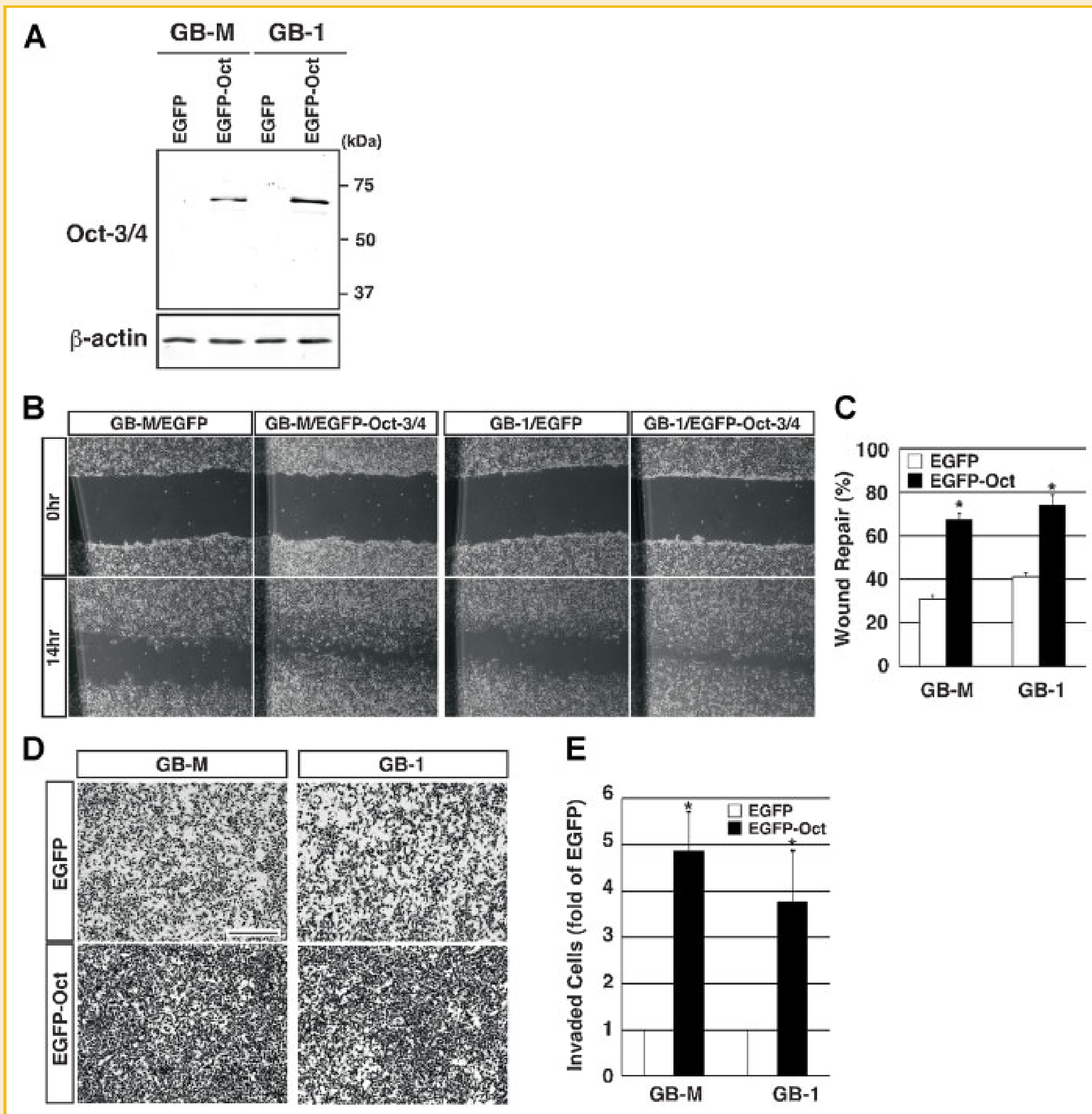

Fig. 1. Overexpression of Oct-3/4 enhances migration and invasion of GB cells. A: Western blot analysis on Oct-3/4 protein expression in GB cell lysates was performed using anti-Oct-3/4 antibody. β-Actin was used as a loading control. B: Scratch injury was created by scratching with a 200-μl pipette tip (0 h); cells were allowed to migrate into the wounded area. Phase-contrast images of migrating cells were taken at 14 h post-injury. C: The wound area was analyzed using NIH image analysis software, and the closed area was calculated by subtracting wound area at 0 h from wound area at 14 h. The column indicates percentage of wound closure at 14 h post-injury. Values represent mean ± standard deviation (SD) (n = 3); $^{*}P < 0.001$. D: Cells (5×10^4 cells) suspended in serum-free DMEM containing 0.1% BSA were seeded onto Matrigel-coated inserts placed in outer chambers containing DMEM with 10% FBS. Representative photographs of invading cells are shown. Transmigrated cells are visualized by crystal violet staining and are quantified at 24 h after seeding. Scale bar, 200 μm. Quantitative results are shown in (E). The column indicates fold increase of migrated GB/Oct-3/4 cells compared with GB/EGFP cells. Each column represents mean ± SD (n = 3); $^{*}P < 0.001$.

reduced cell migration to the scratched area by 23% in GB-M/Oct-3/4 cells, it did not abolish Oct-3/4-mediated enhancement of cell migration (data not shown).

Invasiveness, as well as cell migration, is an important hallmark of malignant gliomas. Therefore, a Matrigel invasion chamber was used to evaluate whether Oct-3/4 expression altered invasion of two GB cells. As shown in Figure 1D, Oct-3/4-expressing GB cells migrated in high numbers through the Matrigel to the lower chamber following stimulation with 10% FBS. Oct-3/4-expressing GB cells exhibited a 4.57-fold increase in invasion of GB-M cells and a 4.26-fold increase in GB-1 cells compared with the control cells (Fig. 1E). Furthermore, introduction of Oct-3/4 shRNA suppressed increased cell migration and invasion in GB/Oct-3/4 cells (data not shown). These results suggested that increased cell migration and invasion was a result of increased Oct-3/4 expression.

INTEGRINS ARE INVOLVED IN ENHANCED MIGRATION OF OCT-3/4-OVEREXPRESSING GLIOMA CELLS

Integrins and MMPs are critical factors for invasion and migration in many solid tumors [Rao, 2003; Smith and Marshall, 2010]. Therefore, to determine the mechanisms involved in increased migration as a result of Oct-3/4 overexpression, real-time PCR analysis was performed to evaluate expression profiles of integrin mRNAs in control and Oct-3/4-expressing GB cells (Fig. 2). As a result, α4, α5, αV, β1, β3, and β5 integrin mRNAs increased in GB/Oct-3/4 cells compared with control GB/EGFP cells. In contrast, α1 and α3 integrin mRNA levels were showing a tendency to downregulate in the GB/Oct-3/4 cells. Although it remains to be determined how integrin α and β subunits heterodimerize in GB/Oct-3/4 cells, previous studies have demonstrated that α5β1 and αVβ3 integrins are implicated in the malignant behavior of gliomas [Gingras et al., 1995; Rao, 2003].

Integrins play an important role in anchoring cells to the ECM, and changes in cell adhesion properties might be another possible mechanism for increased cell migration. As shown in Figure 3A, there were no apparent differences in cell adhesion on non-coated, Matrigel-coated, and fibronectin-coated culture plates between control and Oct-3/4-expressing cells. When cells adhere to ECM proteins, integrins are required for the assembly of focal contacts. Therefore, the present study analyzed expression and activation of integrin-associated signaling molecules required to form focal contacts, such as FAK and c-Src, which have been reported to enhance cell migration in gliomas [Rao, 2003]. A significant increase in FAK and phosphorylated FAK was observed in Oct-3/4-transfected GB-M cell extracts at 1 h after plating (Fig. 3B). In addition, c-Src and phosphorylated-c-Src also increased as a result of Oct-3/4 expression (Fig. 3B). At 6 h after scratch injury, morphological changes were analyzed (Fig. 3C). Phase-contrast images and F-actin staining revealed significant differences between control and Oct-3/4-expressing cells. A large number of GB/Oct-3/4 cells were polarized to the scratched region, and large motility-associated processes, such as lamellipodia and membrane protrusions, were observed (Fig. 3C). In contrast, the control cells were tightly packed and exhibited few motility-associated processes. Moreover, focal contacts were visualized by vinculin staining (Fig. 3D). Focal contacts in Oct-3/4-expressing GB cells were increased both in size and number, and were more apparently localized at membrane protrusions, which are characteristic of leading lamellae, in migratory GB/Oct-3/4 cells than in control cells.

MMP mRNA EXPRESSION INCREASES IN OCT-3/4-OVEREXPRESSING GLIOMA CELLS

Cellular invasion has been correlated with MMP enzymatic activity. Therefore, expression profiles of MMP mRNAs were measured in

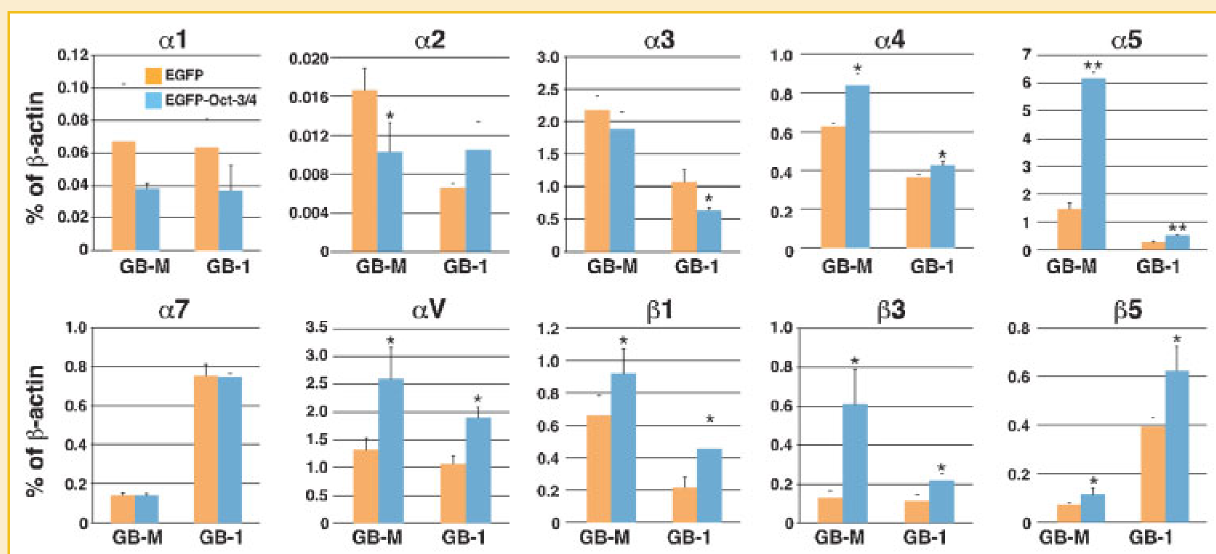

Fig. 2. Expression of integrin mRNAs in GB/EGFP or GB/Oct-3/4 cells, as determined by real-time PCR. The columns indicate as the percentage to β-actin mRNA expression level, and values represent mean ± SD (n = 3); $^{*}P < 0.05$, $^{**}P < 0.001$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

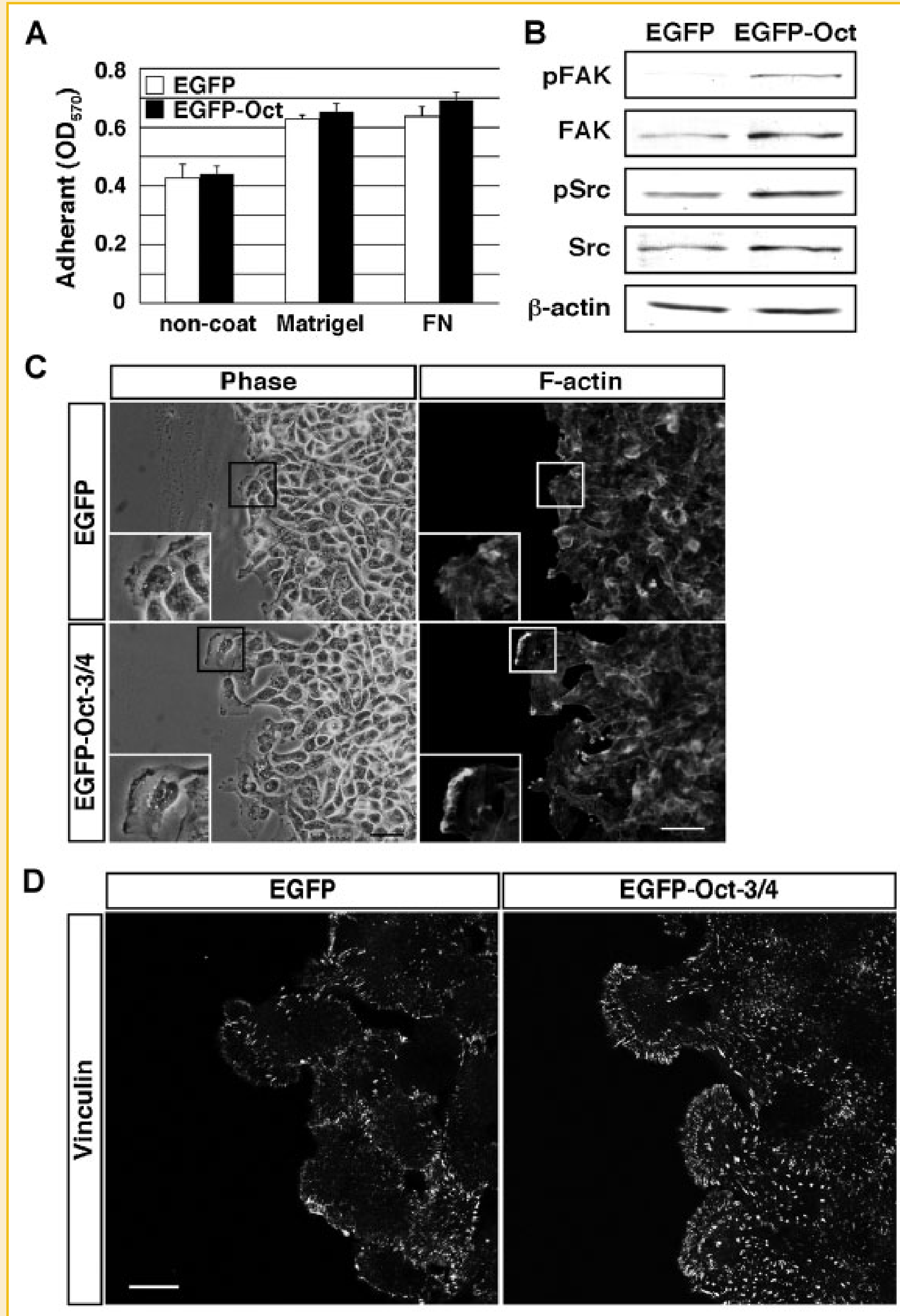

Fig. 3. Oct-3/4 promotes dynamic regulation of focal contacts and actin cytoskeleton during cell migration. A: Cell adhesion ability of GB cells was investigated. GB/EGFP cells (white column) and GB/Oct-3/4 cells (black column) were suspended in serum-free DMEM containing 0.1% BSA and were added to non-coated, Matrigel-coated, and human plasma fibronectin (FN)-coated 96-well plates for 30 min. Adherent cells were analyzed as described in the Materials and Methods Section. Values represent mean $\pm$ SD ($n = 4$). B: Western blot analysis of integrin-associated signaling molecules. GB cells were plated on culture dishes in DMEM containing 10% FBS for 1 h, and phosphorylation status of FAK and c-Src were analyzed using total cell lysates of adherent cells. β-Actin was used as a loading control. C: Phase-contrast images and F-actin staining in GB-M cells during wound healing assay at 6 h post-injury. Insets are high-magnification views of the boxed area. D: Confocal laser-scanning images of vinculin immunostaining in GB-M cells during wound healing assay at 6 h post-injury were shown. Scale bar, 20 μm.

GB/Oct-3/4 cells using real-time PCR (Fig. 4). Real-time PCR analysis revealed that MMP-13 mRNA expression was markedly increased in both Oct-3/4-expressing GB cells compared to control cells. Expressions of MMP-1, 2, 3, and 7 mRNA also have a tendency to increase in Oct-3/4-expressing GB cells. In contrast, MMP-11 mRNA expression was downregulated in GB/Oct-3/4 cells, and MMP-9 and MMP-10 mRNA were not detected in either cell type. MMP-13 protein is secreted as a latent form and converted into an activated one in the extracellular milieu. Increased expression of pro-MMP-13 by Oct-3/4 was confirmed at the protein level (Fig. 5A).

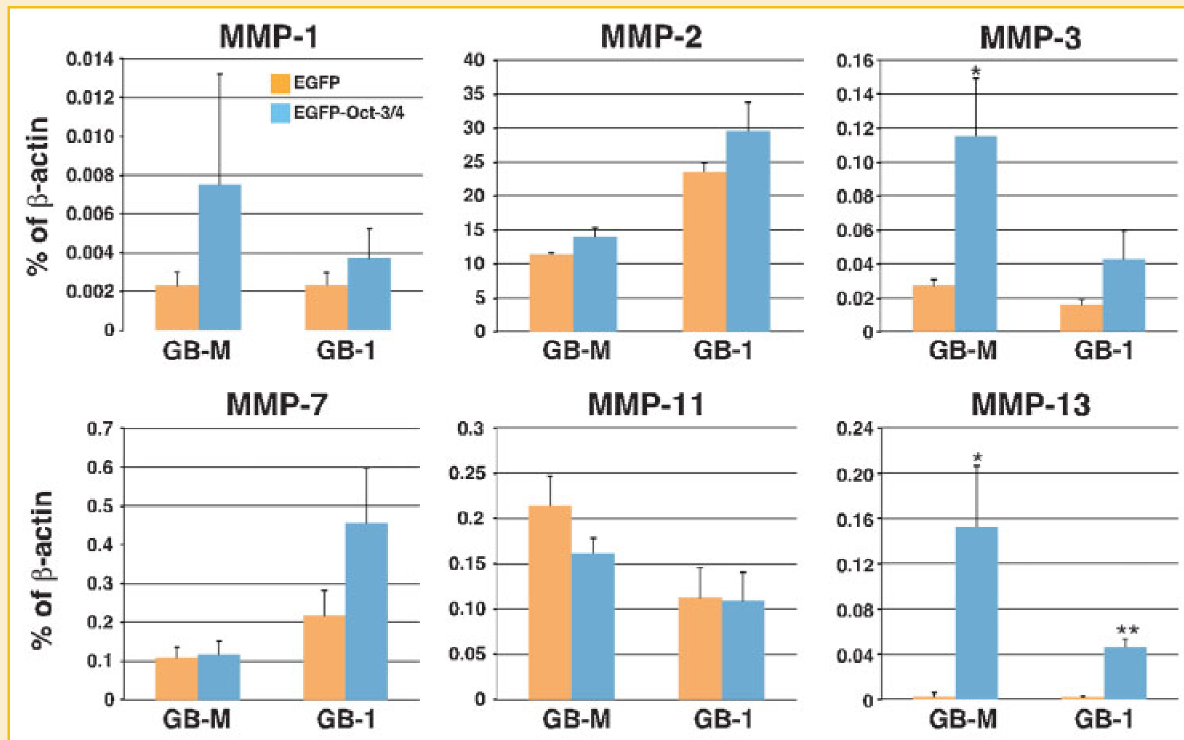

Fig. 4. Expression of MMP mRNAs in GB/EGFP or GB/Oct-3/4 cells, as determined by real-time PCR. The columns indicate as the percentage to β-actin mRNA expression level, and values represent mean $\pm$ SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.001$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

To confirm the functionality of the secreted MMP-13, enzyme-specific activity assay, in which a fluorescently quenched peptide fluoresces upon proteolytic cleavage, were employed. Secreted total MMP-13 protein was also detected following activation of latent enzyme with APMA, which is a non-proteolytic compound to induce the conformational change for activation. Fluorescence intensity in conditioned media by GB cells showed that both pro- and active-forms of MMP-13 were increased by Oct-3/4 (Fig. 5B). It was previously reported that Oct-3/4, Sox-2, and Klf-4, the major factors for reprogramming differentiated cells into induced pluripotent ES cell-like cells [Takahashi et al., 2007], are more highly expressed in CSCs than in non-CSCs, and MMP-13 is specifically expressed in CSCs [Inoue et al., 2010]. Therefore, the present study analyzed whether Sox-2 or Klf-4 expression enhanced MMP-13 mRNA expression in GB-M cells. As shown in Figure 5C, MMP-13 mRNA expression was induced by Oct-3/4, but not by Sox-2 and Klf-4.

MMP-13 shRNA EXPRESSION SUPPRESSES INVASION OF GB CELLS STABLY EXPRESSING OCT-3/4

To further determine the influence of MMP-13 expression in Oct-3/4-mediated invasion of GB cells, MMP-13 shRNA was introduced into Oct-3/4-overexpressing cells. MMP-13 shRNA suppressed MMP-13 protein expression (Fig. 6A), resulting in significantly less invading cells in the Matrigel invasion assay (Fig. 6B,C). A rat brain slice model was utilized to evaluate invasion of GB cells into brain tissue (Fig. 7); a spheroid was placed onto the corpus callosum in a brain slice, and the cells were allowed to migrate into the brain slice for 48 h. Three-dimensional migration of EGFP-Oct-3/4-expressing cells was evaluated in serial optical sections of a brain slice. Results demonstrated that migrating cells from the GB/Oct-3/4 cell spheroid scattered and invaded into the brain slice. In contrast, spheroids from the GB/Oct-3/4 cells, in which MMP-13-expression was knocked down by shRNA, did not significantly migrate on or invade into the slice, but remained in the vicinity of the spheroid.

DISCUSSION

Aberrantly high levels of Oct-3/4 expression have been measured in various tumors, including brain tumors, breast cancer, bladder cancer, and oral squamous cell carcinoma; expression has been shown to correlate with tumor progression and mortality [Chang et al., 1998; Chen et al., 2008; Chiou et al., 2008; Du et al., 2009]. Consistent with these findings, ectopic Oct-3/4 expression in a heterologous cell system has been shown to transform non-tumorigenic cells into tumorigenic cells in adult mice [Hochedlinger et al., 2005]. Although results from previous studies have suggested that aberrant Oct-3/4 expression could be responsible for tumorigenic transformation and tumor metastasis, the influence of Oct-3/4 on invasiveness of cancer cells remains poorly understood. The aim of the present study was to investigate the effects of Oct-3/4 overexpression on migration and invasion processes of GB cells; the highly invasive nature of GBs is the main reason for tumor intractability.

The present study demonstrated that Oct-3/4 overexpression induced invasion of GB cells, which is an important finding for

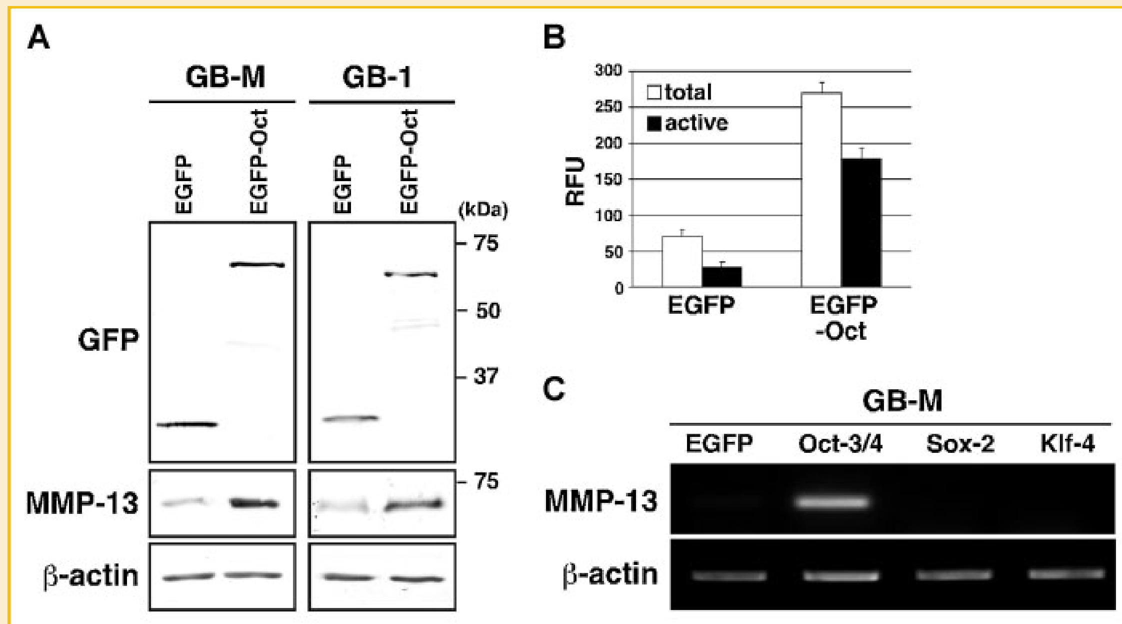

Fig. 5. Overexpression of Oct-3/4 upregulates MMP-13 expression in GB cells. A: Western blot analysis of MMP-13 protein expression in GB cell lysates. EGFP and EGFP-Oct-3/4 were detected by anti-rabbit GFP antibody. β-actin was used as a loading control. B: Proteolytic activity of MMP-13 in media conditioned by GB-M cells for 48 h was determined by protease-specific activity assay utilizing a fluorescently quenched peptide. The columns indicate relative fluorescence units (RFU), and values represent mean ± SD (n = 3). C: MMP-13 mRNA expression was analyzed by RT-PCR using cDNAs prepared from GB-M cells stably expressing EGFP, EGFP-Oct-3/4, EGFP-Sox-2, and Klf-4, respectively. Representative data are shown.

malignant glioma research. Accelerated invasion of malignant glioma cells is the greatest challenge for current treatment modalities, and complete surgical resection of diffusely invaded glioma cells is impossible without inducing severe neurological damage. The process of tumor invasion is better understood through an increased understanding of the key roles of extracellular matrix, cell adhesion molecules, and proteases [Rao, 2003; Demuth and Berens, 2004]. The present study demonstrated that Oct-3/4 overexpression altered expression profiles of integrin and MMP mRNAs in GB cells. Although only expression of secreted types of MMPs were investigated in the present study, Oct-3/4 overexpression might affect expression of other MMPs, including membrane-type MMPs. Cooperation between MMPs and integrins is an essential mechanism in tumor cell motility [Smith and Marshall, 2010], and interaction of αVβ3 integrin with MMP-2 or MMP-9 enhances tumor migration [Rolli et al., 2003]. In addition, cleavage of the integrin αV subunit precursor by membrane-type 1 MMP (MT1-MMP), also known as MMP-14, increases tumor cell migration and invasion [Deryugina et al., 2002].

Two hypoxia-inducible factor α (HIFα) proteins, HIF1α and HIF2α, are regulatory factors for the angiogenic switch to produce vascular endothelial growth factor (VEGF) in solid tumors [Pouysségur et al., 2006]. Accumulating evidence has shown that hypoxia and its master regulator HIF enhance glioma cell migration and invasion [Zagzag et al., 2000; Kaur et al., 2005; Eckerich et al., 2007; Fujiwara et al., 2007]. Interestingly, HIF2α stimulates tumor invasion via MT1-MMP activation in metastatic renal cell carcinoma [Petrella et al., 2005]. MT1-MMP, a transcriptional target of HIF2α, activates latent MMP-2 and MMP-13 at the leading edge of invasive cells [Knäuper et al., 1996]. Furthermore, HIF2α, not HIF1α, directly activates Oct-3/4, and shRNA inhibition of Oct-3/4 expression in HIF2α knock-in teratomas reduces tumor size [Covello et al., 2006]. The present study showed that the highly invasive potential of GB/Oct-3/4 cells is dependent on MMP-13 enzymatic activity; MMP-13 mRNA expression was induced by Oct-3/4 but not by the other stemness-related factors, Sox-2 and Klf-4. Therefore, increased migration and invasion of GB/Oct-3/4 cells might be exerted via αVβ3 integrin and MMP13 through MT1-MMP under control of HIF2α.

Previous studies have demonstrated that GBs, similar to other tumors, might be derived from CSCs, a discrete subpopulation of malignant cells with stem cell properties [Reya et al., 2001; Dalerba et al., 2007]. Therefore, the correlation between HIF and Oct-3/4 expression suggests the possibility of crosstalk between hypoxia and CSC signaling pathways. Hypoxia promotes normal stem cell maintenance and suppresses differentiation [Ezashi et al., 2005], suggesting that hypoxia might be a functional component of the stem cell niche, which is defined as a particular microenvironment that maintains stem cell properties of self-renewal and multi-potency. Calabrese et al. [2007] demonstrated that brain tumor microvasculature forms a niche critical for the maintenance of CD133/nestin-positive CSCs. Recently, glioma cells adjacent to blood vessels, which express HIF2α, were shown to co-express CD133, a CSC marker, in malignant gliomas [Li et al., 2009]. In addition, as described above, HIF2α directly activates Oct-3/4 [Covello et al., 2006], which suggests that Oct-3/4 is expressed in CD133-positive CSCs.

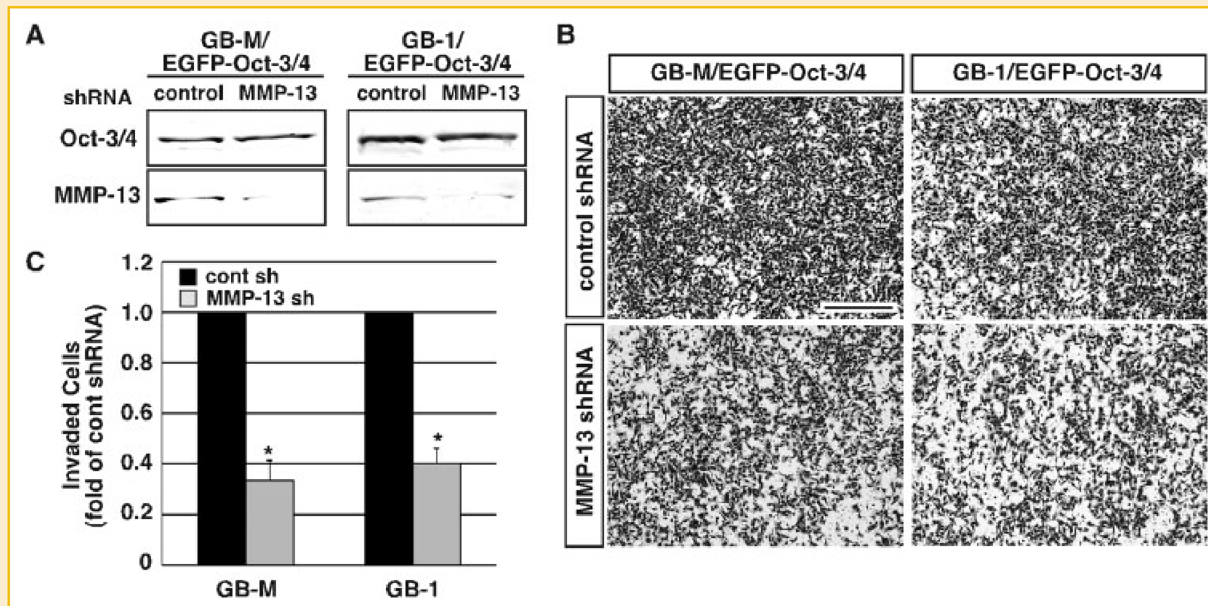

Fig. 6. Effect of MMP-13 shRNA suppression on Oct-3/4-enhanced GB cell invasion. A: MMP-13 protein expression in cell lysates of non-target controls or MMP-13 knocked-down GB/Oct-3/4 cells was detected with anti-rabbit MMP-13 antibody. B: Invasion of non-target controls or MMP-13 knocked-down GB/Oct-3/4 cells was assessed using Matrigel-coated Boyden chambers similar to methods used in Figure 1. Quantitative results are shown in (C). Each column represents mean ± SD ($n = 3$); $^{*}P < 0.001$.

Aberrant self-renewal activity is a key property of CSCs [Ben-Porath et al., 2008], which suggests that stemness-related factors, in particular Oct-3/4, might be involved in acquisition of CSC properties. Indeed, shRNA-mediated Oct-3/4 suppression in human U251 glioblastoma cells results in significant loss of sphere formation, which is characteristic for CSCs (unpublished data). In addition, knockdown of HIF1α or HIF2α in glioma stem cells results in impaired tumor sphere formation [Li et al., 2009]. These studies, together with the present findings, suggest that hypoxia could increase the stemness of CSCs via Oct-3/4 induction.

We previously reported that increased CSC invasion was a result of MMP-13 activity [Inoue et al., 2010]. These results, in combination with the present study, suggested that a hypoxic GB microenvironment could induce Oct-3/4 expression, followed by Oct-3/4-induced MMP-13 activation, which would subsequently increase migration and invasion of GBs. Although additional studies are necessary to define the molecular mechanisms of hypoxia in CSC development and tumor growth, the present findings might provide a strategy for the development of novel anti-cancer therapies via Oct-3/4 suppression.

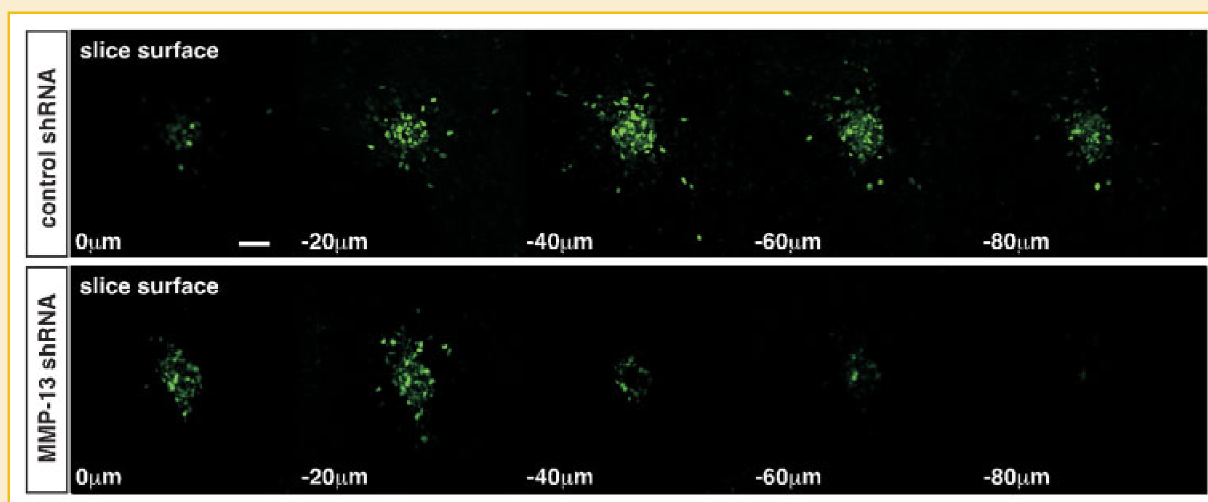

Fig. 7. Invasive behavior of non-target control or MMP-13 knocked-down GB/Oct-3/4 cells on rat brain slices. A GB-derived spheroid was placed on the corpus callosum of a brain slice and co-cultured for 48 h. Migrating and invading cells, which stably expressed EGFP-Oct-3/4, were detected under confocal laser-scanning microscopy. In each experiment, serial optical sections of the brain slices were obtained every 20 μm from the brain slice surface (0 μm) to a deeper site (80 μm). Representative photographs of invading cells are shown. Scale bar, 50 μm.

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