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Original Research

Hypoxia-induced phenotypic transition from highly invasive to less invasive tumors in glioma stem-like cells: Significance of CD44 and osteopontin as therapeutic targets in glioblastoma

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ABSTRACT

The poor prognosis of glioblastoma multiforme (GBM) is primarily due to highly invasive glioma stem-like cells (GSCs) in tumors. Upon GBM recurrence, GSCs with highly invasive and highly migratory activities must assume a less-motile state and proliferate to regenerate tumor mass. Elucidating the molecular mechanism underlying this transition from a highly invasive phenotype to a less-invasive, proliferative tumor could facilitate the identification of effective molecular targets for treating GBM. Here, we demonstrate that severe hypoxia (1% O_2) upregulates CD44 expression via activation of hypoxia-inducible factor (HIF-1α), inducing GSCs to assume a highly invasive tumor. In contrast, moderate hypoxia (5% O_2) upregulates osteopontin expression via activation of HIF-2α. The upregulated osteopontin inhibits CD44-promoted GSC migration and invasion and stimulates GSC proliferation, inducing GSCs to assume a less-invasive, highly proliferative tumor. These data indicate that the GSC phenotype is determined by interaction between CD44 and osteopontin. The expression of both CD44 and osteopontin is regulated by differential hypoxia levels. We found that CD44 knockdown significantly inhibited GSC migration and invasion both in vitro and in vivo. Mouse brain tumors generated from CD44-knockdown GSCs exhibited diminished invasiveness, and the mice survived significantly longer than control mice. In contrast, siRNA-mediated silencing of the osteopontin gene decreased GSC proliferation. These results suggest that interaction between CD44 and osteopontin plays a key role in tumor progression in GBM; inhibition of both CD44 and osteopontin may represent an effective therapeutic approach for suppressing tumor progression, thus resulting in a better prognosis for patients with GBM.

Introduction

Glioblastoma multiforme (GBM) is the most malignant brain tumor, characterizing by early tumor recurrence and poor prognosis of a median survival of 15 months [1]. Glioma stem-like cells (GSCs) that survive in the invasion zone of the tumor periphery are likely to be the main cause for such early progression. Munthe et al. reported that glioma cells in the tumor periphery exhibit a stem cell phenotype [2]. Angelucci et al. described significant differences between cancer stem cells from the tumor mass and those from peritumoral GBM tissue in terms of proliferation, ultrastructural features, and stemness profile [3]. We previously reported the presence of GSCs that highly express CD44 and exhibit high invasiveness in the tumor periphery in patients with highly invasive GBM [4].

Abbreviations: GBM, glioblastoma multiforme; GSC, glioma stem-like cell; OPN, osteopontin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBSt, Tris-buffered saline containing 0.1% Tween 20; BSA, bovine serum albumin; SD, standard deviation.

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CD44 is a multifunctional cell surface adhesion receptor involved in regulating the progression, invasion, and metastasis of cancer cells [5,6], and it is regarded as a cancer stem cell marker [7,8]. CD44 promotes tumor cell migration and invasion by binding to the specific ligand hyaluronic acid (HA) in various cancers, including GBM [9,10]. Other ligands in addition to HA, including collagen, matrix metalloproteinases, and osteopontin (OPN), also bind to CD44 [11–13]. Among these ligands, OPN is highly expressed in GBM and exhibits diverse functions related to proliferation, angiogenesis, tumorigenesis, and invasion [14–16]. Pietras et al. reported that interaction between OPN and CD44 promotes glioma stem cell–like phenotypes and aggressive glioma growth in vivo via enhanced hypoxia-inducible factor (HIF-2α) activity [7].

GSCs are thought to be located in a hypoxic microenvironment called the "niche." The invasion area of the tumor periphery in GBM is characterized by a lack of neovascularization and disruption of the blood-brain barrier. This hypoxic environment can generate a tumor-border niche for GSCs. Hypoxia activates HIF genes, including the genes encoding HIF-1α and HIF-2α [17,18]. HIF-1α and HIF-2α, though structurally similar, differ in several respects, including their requirement for oxygen for activation, target genes, and expressing cells [19–21]. Our previous study demonstrated that HIF-1α and CD44 are expressed at high levels in the tumor periphery in highly invasive GBM; in low-invasiveness/highly proliferative GBM, HIF-2α is expressed at higher levels and CD44 at lower levels in the tumor periphery compared with highly invasive GBM [4]. These results suggest that differences in the level of hypoxia in the GSC microenvironment at the tumor periphery may have a critical effect on the invasiveness and proliferation activity of GBM tumors through the activation of HIF-1α and HIF-2α.

In the present study, we examined the effects of hypoxia on the expression and functions of HIFs, CD44, and OPN in order to elucidate the role of these molecules in GBM tumor invasion and proliferation. In addition, we investigated the effect of hypoxia on the functional role resulting from interaction between CD44 and OPN. Aiming for clinical application, anti-tumor effects of silencing CD44 and OPN were investigated. Identification of key molecules mediating tumor progression and development of a method to effectively suppress the functions of these molecules would provide a useful therapeutic approach for treating both primary and recurrent GBM.

Materials and methods

This study was approved by the Ethics Committee for Clinical Research of Ehime University Hospital (no. 1708013). All procedures were performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Ehime University. These protocols are in accordance with the standards detailed in the National Research Council's Guide for the Care and Use of Laboratory Animals [22].

GSC culture and hypoxic treatment

Two human GSC lines, GSL-1 and GSL-2, were used in the present study. These cell lines were previously established from GBM patients and designated SFC-1 and SFC-2, respectively [4]. The cell culture was performed as described previously and is detailed in Supplementary Materials. For severe hypoxia treatment, the cells were incubated for 3 h in an atmosphere of 1% O_2, 5% CO_2, and 94% N_2 in a multi-gas incubator (APM-50D, ASTEC). For moderate hypoxia treatment, the cells were incubated for 6 h in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. The incubation time of the hypoxic treatment was determined based on effectiveness of the treatment and cytotoxicity of the hypoxia combined with transfection of siRNA in GSCs.

Treatment of cells with small interfering RNA (siRNA)

The sequences of siRNAs for HIF-1α, HIF-2α, CD44, and OPN are listed in Supplementary Table S1. As a control for each siRNA, we used a corresponding random siRNA sequence (5'-GCGCGCUUUGUAGGAUUCG dTdT-3'). GSCs were transfected with each siRNA using Lipofectamine 3000 reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. After a 24-h incubation of GSCs transfected with each siRNA, the culture medium was changed to remove the Lipofectamine, and subsequent experimentation was performed.

Establishment of stable CD44-knockdown cells

Lentiviral particles were generated using the shRNA expression vector pLKO.1-puro, which carries a shRNA sequence against CD44 (CD44 MISSION shRNA, SHCLNG-NM_000610, Sigma Aldrich) together with the MISSION Lentiviral packaging mix (SHP001, Sigma Aldrich), according to the manufacturer's instructions. Briefly, HEK 293T cells were co-transfected with the two products described above using Lipofectamine 3000 reagent (Invitrogen). The supernatant containing virus particles was harvested 48 h after transfection and used to infect GSCs. After 48 h of incubation, infected cells were selected using puromycin (0.5 μg/ml; Invitrogen).

RNA isolation and quantitative real-time–polymerase chain reaction (qRT-PCR)

Total RNA was extracted from GSCs and tissue of each tumor sample (core and periphery) using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with a gDNA remover kit (Toyobo). qPCR analysis was performed using Fast Start Universal SYBR Green Master Mix (Roche Diagnostic Japan) with an MJ mini instrument (Bio-Rad, Hercules, CA, USA). The information of primers and procedures are detailed in Supplementary Materials.

Western blot analysis

Examination of protein expressions of CD44, OPN, and HIFs by the treatment with hypoxia and siRNA/shRNA is detailed in Supplementary Materials.

Immunohistochemical analysis

Immunohistochemistry of tumor tissues and GSCs were performed as described previously [4]. The procedures are detailed in Supplementary Materials.

Measurement of OPN secreted by GSCs

When GSCs reached confluence, the culture medium was removed, and the cells were washed with PBS. After addition of serum-free, low-glucose DMEM, the cells were cultured for 24 h. The cells were then centrifuged, and the supernatant was used as conditioned medium. The concentration of OPN in the conditioned medium was determined using an OPN ELISA kit (Sigma-Aldrich) according to the manufacturer's protocol.

Cell invasion and migration assays

In vitro invasion and migration assays in GSCs were performed using chemotaxis chambers as described previously [23–25]. Falcon 24-well plates with a reconstituted basement membrane, Matrigel (Becton Dickinson Biosciences, CA, USA) were used in invasion assay and 48-well microchemotaxis chambers (Nucleopore, Pleasanton, CA, USA) were

used in migration assay. The procedures are detailed in Supplementary Materials. CD44 is a major cellular adhesion molecule for hyaluronic acid (HA) and their interaction strongly promotes cell migration and invasion. To investigate effects of OPN on the migration and invasion of GSCs, attractants with and without HA were used in the migration/invasion assays. In the study of the migration and invasion without HA, 1μg/ml of OPN (Sigma Aldrich) was added to the upper well. In the study of the migration and invasion with HA, 0.5 mg/ml of HA (Wako, Japan) was added to the lower well.

GSC growth assay

Control and CD44-knockdown or OPN-silenced cells were seeded onto 35-mm culture dishes at a density of 1×10^4 cells/dish and incubated up to 1 week at 37°C under normoxic conditions or 1% or 5% O_2 hypoxic conditions. Each day during the 1-week incubation period, each dish was rinsed with fresh culture medium and stained with Hoechst 33342. The number of cells in each of 6 random fields per dish was then determined by microscopic examination (× 40 magnification).

In vivo *xenograft experiments*

80 mice were used in this study. Control and CD44-knockdown cells (1×10^6) were suspended in 5 µl of Matrigel and injected into the brain of 6-week-old male NOD/SCID mice (CLEA Japan, Inc.; n=20 mice per group) that had been anesthetized intraperitoneally with a mixture of medetomidine (0.75 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg). MRI for animals was performed to confirm tumorigenesis before the mice were euthanized by decapitation. The procedures of animal experiments and the criteria for deciding humane euthanasia are detailed in Supplementary Materials. We performed animal experiments with fully considering all animal welfare including minimization of suffering and distress, use of analgesics or anesthetics, and facility of air conditioning in animal rooms.

Statistical analysis

Values are expressed as the mean ± standard deviation (SD), and the data were compared using the Student's t-test (unpaired). Comparisons of data for more than two groups were carried out using one-way analysis of variance with the Tukey post hoc test. Kaplan-Meier plots were generated to estimate unadjusted time-to-event variables. The log-rank test was performed to assess the statistical significance of differences between groups. Statistical significance was defined as $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***), whereas "ns" denotes $P>0.05$.

Results

Effect of hypoxia on the expression of HIFs, CD44, and OPN in GSCs

mRNA Expressions of HIF-1α, HIF-2α, CD44, and OPN in GSC lines were examined under normoxic (21% O_2) and hypoxic conditions (1% O_2 [severe] and 5% O_2 [moderate]). Expression of HIF-1α and CD44 mRNAs was up-regulated to a greater degree under the 1% O_2 condition than under normoxic or moderately hypoxic (5% O_2) conditions in both GSC lines (Fig. 1). In contrast, the expression of HIF-2α and OPN mRNAs was more strongly up-regulated in both GSC lines under moderate hypoxia than under conditions of normoxia or severe hypoxia (Fig. 1A). Analysis of protein expressions for HIF-1α and HIF-2α disclosed that expression of HIF-1α protein was elevated to a greater degree under 1% O_2 than under conditions of 5% or 21% O_2. In contrast, expression of HIF-2α protein was more strongly increased under 5% O_2 than under the normoxic condition, but the protein expression of HIF-2α was also increased under the conditions of 1% O_2 (Fig. 1B).

HIF-1α up-regulates CD44 expression in GSCs under severe hypoxia

We investigated whether HIF-1α affects the expression of CD44 under the condition of severe hypoxia (1% O_2). Silencing of the HIF-1α with the siRNA resulted in significant suppression of both the mRNA and protein expression of CD44 in both GSC lines under the condition of severe hypoxia (Fig. 2A and B). Double-labeling immunofluorescence demonstrated that CD44 and HIF-1α co-localized in GSCs subjected to severe hypoxia (Fig. 2C).

HIF-2α up-regulates OPN expression in GSCs under moderate hypoxia

Under moderate hypoxia (5% O_2), silencing of the HIF-2α gene using the siRNA resulted in significant suppression of both the mRNA and protein expressions of OPN in GSCs (Fig. 2D and E). In addition, double-labeling immunofluorescence demonstrated the co-localization of OPN and CD44 in both GSC lines, indicating that GSCs can express both CD44 and osteopontin in the same cells, regardless of the level of CD44 expression (Fig. 2F).

Expressions of OPN in GBM tissues and secreted-OPN in GSCs

Expressions of OPN mRNA were examined in the core and peripheral tissues of five resected GBM tumors. Three of these tumors were highly invasive types expressing high CD44 (HI-tumors), and the other two tumors were low-invasiveness/highly proliferative types expressing low CD44 and high VEGF (LI-tumors). The nature of these GBM types is described in Supplementary Figure S1. The LI-tumors expressed a significantly higher level of OPN in the tumor periphery than the HI-tumors (Fig. 3A). Double-labeling immunofluorescence demonstrated that OPN co-localized with Nestin in the peripheral tissues of both tumor types (Fig. 3B). In addition, OPN co-localization with Nestin was more pronounced in the LI-tumor than HI-tumor. We confirmed that both GSC lines secreted OPN in proportion to the level of OPN mRNA and protein expression. The concentration of OPN in the culture medium under normoxia was much higher in medium of GSL-2 cells than GSL-1 cells (Fig. 3C and D).

Migration and invasion are enhanced by activated CD44 in hypoxic environments and reduced by OPN

In vitro migration and invasion assays revealed that the migration and invasion of both GSC lines was markedly enhanced under the severe hypoxia condition of 1% O_2 compared with normoxia (Fig. 4A). Moderate hypoxia (5% O_2) tended to suppress the migration and invasion of GSC lines compared with normoxia, but the difference was significant only for invasion by GSL-1 cells. The enhancement of GSC migration and invasion induced by severe hypoxia was significantly inhibited by knockdown of the CD44 gene using CD44 siRNA; this also inhibited GSC migration and invasion under normoxia (Fig. 4A).

In contrast, OPN significantly inhibited CD44-promoted migration and invasion in both GSC lines (Fig. 4B). To investigate the inhibitory effects of OPN on CD44-promoted migration and invasion of GSC lines, we performed migration and invasion assays by adding HA to the lower wells of the chamber. Addition of HA to the lower wells markedly increased the migration and invasion behaviors of both GSC lines compared with the control. OPN inhibited the HA-stimulated migration and invasion of GSC lines in a dose-dependent manner (Fig. 4B).

Effects of CD44 knockdown and OPN silencing on GSC proliferation

Among five clones of bacteria maintained in glycerol stocks, clone 2 most-effectively inhibited CD44 expression in both GSC lines (Fig. S2); therefore, we used this clone to generate CD44-knockdown cells in the following experiments. Knockdown of the CD44 gene significantly reduced the expression of other stem cell markers in both GSC lines,

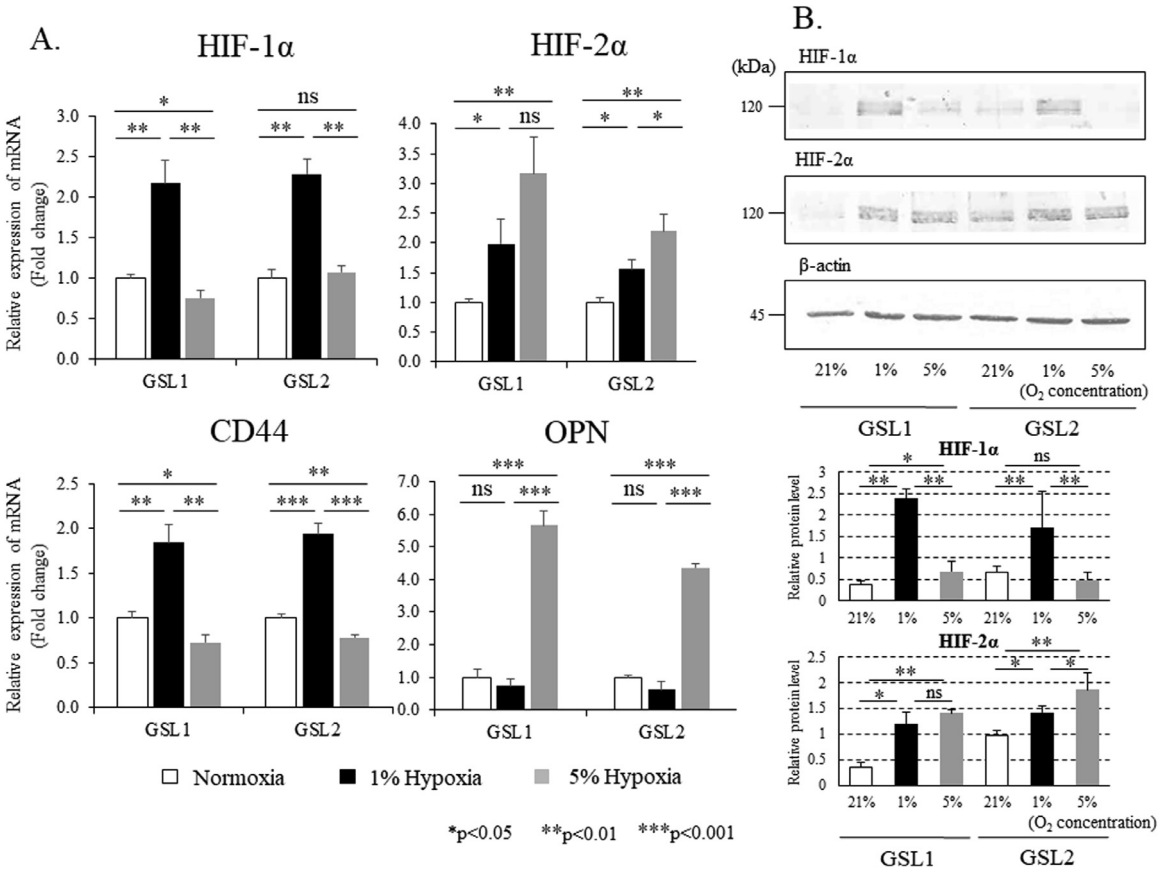

Fig. 1. Effect of hypoxia on the expression of HIFs, CD44, and OPN in GSC lines. **(A)** The mRNA expression of each molecule in both GSC lines was examined by qRT-PCR under three different oxygen concentrations, normoxia (21% O_2), severe hypoxia, (1% O_2), and moderate hypoxia (5% O_2). mRNA levels of HIF-1α, HIF-2α, CD44, and OPN were normalized to GAPDH mRNA and the values are expressed relative to those at normoxia (fold change) of each molecule, which were given a value of 1. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. HIF, hypoxia-inducible factor; GSC, glioma stem-like cell. (B) Western blot analysis demonstrating the hypoxic effects at 1% and 5% oxygen and normoxia (control) on the protein expression of HIF-1α and HIF-2α in two GSC lines. Relative protein expression levels of HIF-1α and HIF-2α to β-actin (lower panel). $^{*}P<0.05$; $^{**}P<0.01$.

including CD133, Nestin, Oct3/4, and Sox2, in addition to the angiogenesis factor VEGF (Fig. 5A). In contrast, knockdown of CD44 did not affect the proliferation of either GSC line (Fig. 5B). Silencing OPN using OPN siRNA resulted in decreased expression of CD44 and Nestin but increased expression of VEGF (VEGF expression was significantly increased only in GSL-2 cells) (Fig. 5C). Incubation of GSCs under the moderate hypoxia condition (5% O_2) markedly increased the cell growth rate, whereas culture under the severe hypoxia condition (1% O_2) resulted in a decreased growth rate (Fig. 5D). The enhancement in growth rate under moderate hypoxia of 5% O_2 was significantly suppressed by reducing OPN expression to a lower level, as compared with the growth rate under the condition of normoxia.

In vivo *study using a CD44-knockdown transplanted-GSC mouse model*

The effect of CD44 knockdown on various histologic features of tumor tissues in mouse brain and the survival of these mice were investigated using NOD/SCID mice receiving transplanted GSCs with and without CD44 knockdown. Tumors generated by GSC transplantation with CD44 knockdown exhibited less migration of tumor cells around the tumor mass and exhibited a well-demarcated boundary compared to controls (Fig. 6A). The proliferative Ki-67 staining index (SI) of tumors from mice with transplanted GSCs with CD44 knockdown was not significantly different from that of the control (maximum Ki-67 SI: 50.7% [former] vs. 50.9% [latter]; P=0.973). In contrast, positive expression of the stem cell markers Nestin and Sox2 was observed in tumors generated by transplantation of GSCs without CD44 knockdown, whereas no expression of these markers was observed in tumors generated by transplantation of GSCs with CD44 knockdown (Fig. 6B). As a result, mice bearing GSC-induced tumors with CD44 knockdown survived for significantly longer than tumor-bearing mice without CD44 knockdown (Fig. 6C).

Discussion

In the present study, we demonstrated that the highly invasive phenotype of GSCs expressing high levels of CD44 can be converted to a low-invasiveness/highly proliferative phenotype by the specified hypoxic conditions of 1% (severe) and 5% (moderate) oxygen. The severity of hypoxia for the activation of HIF-1α versus HIF-2α differed in the GSCs. Both mRNA and protein expressions of HIF-1α were up-regulated by severe hypoxia (1% O_2) but not affected by moderate hypoxia (5% O_2). In contrast, under moderate hypoxia, both mRNA and protein expressions of HIF-2α was significantly up-regulated to a much higher level than under normoxia, but even hypoxia at 1% O_2 had an enhancing effect on the expression of HIF-2α. Following up-regulation, HIF-1α activated the expression of CD44 under the severe hypoxia (HIF-1α/CD44 pathway), resulting in inducing a highly invasive phenotype in the GSCs. The increase in HIF-2α expression in turn led to a significant up-regulation of OPN expression (HIF-2α/OPN pathway). The increased OPN expression promoted the proliferation of GSCs while simultaneously inhibiting their migration and invasion, resulting in a low-invasive/highly proliferative phenotype. Consequently, the intensity of the biological functions of invasion/migration and proliferation in GSCs may be estimated

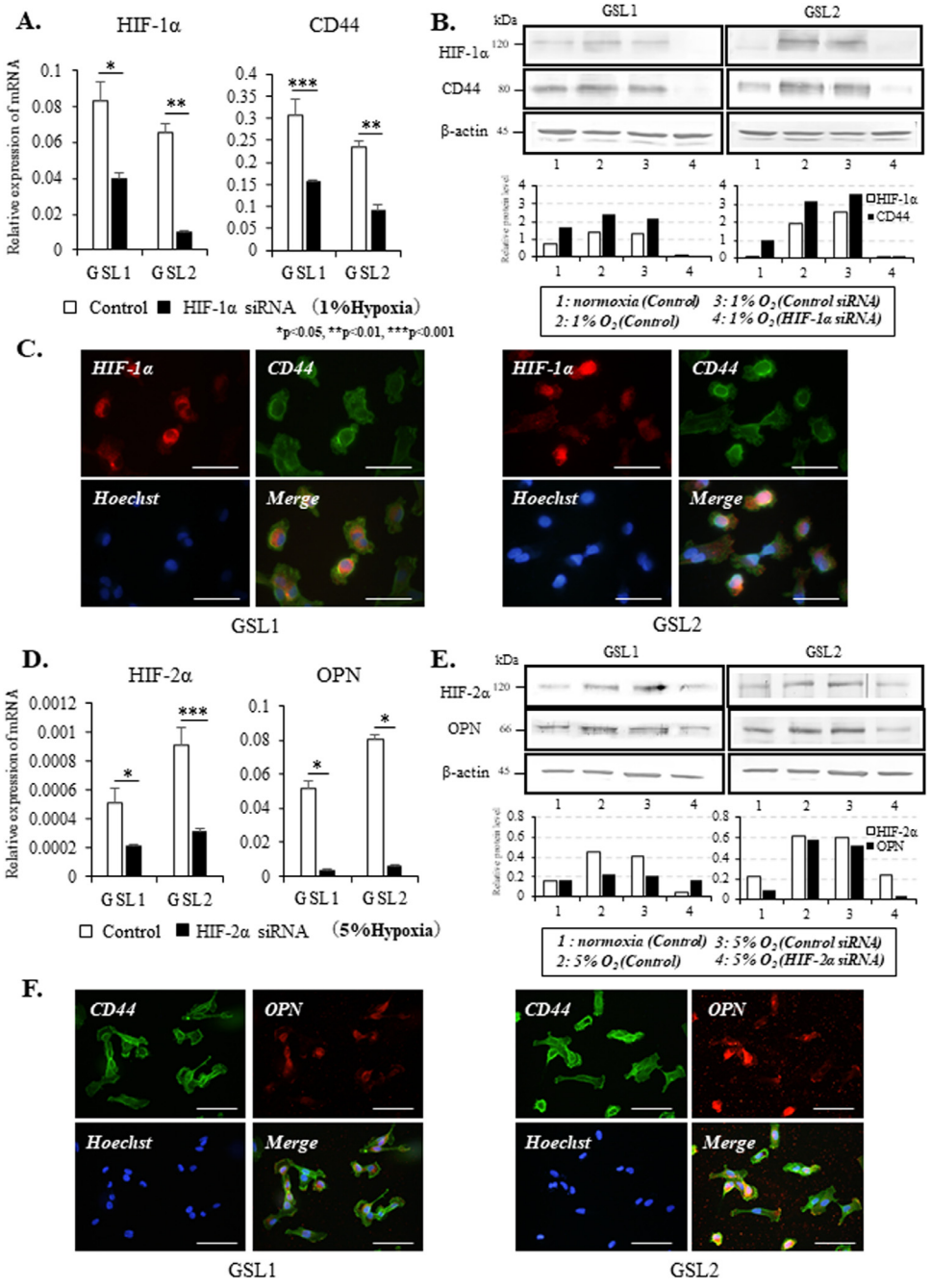

Fig. 2. Up-regulation of CD44 and OPN expression under different hypoxic conditions. (A) HIF-1α siRNA significantly inhibited the expression of CD44 in both GSL-1 and GSL-2 cells under the condition of 1% O_2 hypoxia. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (B) Western blot analysis demonstrating the silencing effect of HIF-1α siRNA on the expression of HIF-1α and CD44 in two GSC lines. Relative protein expression levels of HIF-1α and CD44 to β-actin (lower panel). (C) Double-labeling immunofluorescence demonstrating co-localization of CD44 (green) and HIF-1α (red) under the condition of 1% O_2 hypoxia in both GSC lines. Cell nuclei are labeled with Hoechst (blue) (bar = 100 μm). (D) HIF-2α siRNA significantly inhibited the expression of OPN in both GSL-1 and GSL-2 cells under the condition of 5% O_2 hypoxia. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (E) Western blot analysis demonstrating silencing of HIF-2α expression by siRNA and OPN expression in two GSC lines. Relative protein expression levels of HIF-2α and OPN to β-actin (lower panel). (F) Double-labeling immunofluorescence demonstrating co-localization of CD44 (green) and OPN (red) under the condition of 5% O_2 hypoxia in both GSC lines. Nuclei are labeled with Hoechst (blue) (bar = 100 μm).

by examining the difference in the expression levels of CD44 and OPN. Tumors exhibiting high OPN expression and low CD44 expression may represent GSCs with a low invasiveness/highly proliferative phenotype. In contrast, tumors exhibiting low OPN and high CD44 expression may be highly invasive GSCs.

CD44 is a major cellular adhesion molecule for HA, an extracellular matrix component [26]. CD44 also interacts with a variety of other ligands and plays a crucial role in many cellular processes [10,26]. These other ligands include OPN that plays a critical role in maintaining the stemness and tumorigenicity of GSCs [27–29]. To elucidate the relationship between CD44 and OPN in the progression process of highly invasive GBM tumors, we examined the expression of OPN and the functions of CD44 and OPN under severe (1% O_2) and moderate (5% O_2) hypoxia. In GBM tumor tissues, we found that OPN was expressed at considerably higher levels on the tumor stem cells in the peripheral tissues of low-invasiveness/highly proliferative tumors. However, such high expression of OPN in the tumor periphery was not commonly observed in highly invasive GBM tumors. Several studies have reported that OPN expression in gliomas varies and that the OPN expression level is correlated with glioma grade; thus, high OPN expression is correlated with poor prognosis in GBM patients [30,31]. Friedmann-Morvinski et al. reported that OPN is expressed at higher levels in the border area of GBM tumors compared with the central area and that the invasive and more-aggressive tumor cells located along the periphery of the tumors express high levels of OPN [29]. Our results were in agreement in terms of the location of tumor cells expressing high levels of OPN in low-invasiveness/demarcated-type GBM tumors. However, in our study, OPN expression was not high at the periphery of highly invasive GBM tumors.

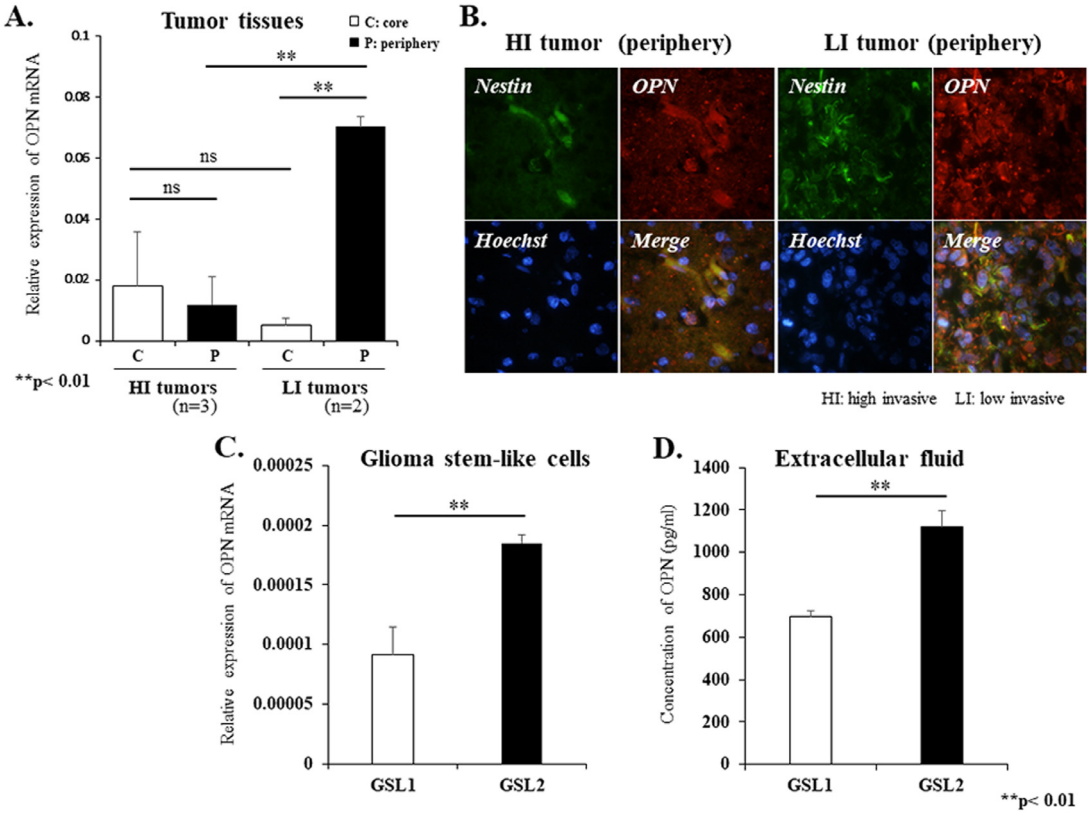

Fig. 3. Expression of OPN in GBM tissues and GSCs and secretion of OPN. (A) Both highly invasive GBM (HI tumor) and low-invasiveness GBM (LI tumor) expressed more OPN in the periphery of the tumor than in the core. The peripheral tissues of LI tumors expressed OPN at a significantly higher level than in the core of either LI or HI tumors. $^{**}P<0.01$. (B) Double-labeling immunofluorescence demonstrating co-localization of OPN (red) and Nestin (green) in the periphery of HI and LI tumors. Nuclei are labeled with Hoechst (blue) (bar = 50 μm). (C) Expression of OPN in two GSC lines under the condition of normoxia. Values are relative expression of OPN mRNA normalized to that of GAPDH mRNA. $^{**}P<0.01$. (D) Extracellular concentration of OPN in conditioned medium of two GSC lines. $^{**}P<0.01$.

We previously reported that CD44 markedly increases GSC migration and invasion under normoxic conditions [4]. In the present study, severe hypoxia (1% O_2) further enhanced GSC migration and invasion compared with the normoxic condition, and both the migration and invasiveness of GSCs were inhibited by silencing CD44 expression using siRNA. These results indicate that under severe hypoxia, activated CD44 (HIF-1α/CD44 pathway) promotes the migratory and invasive activities of GSCs, resulting in the highly invasive phenotype of GBM.

We also demonstrated that OPN inhibits GSC migration and invasion under the normoxia. Previous studies have reported that OPN promotes the tumor cell migration and invasion in GBM [16,32,33]. However, in these studies, migratory and invasive activities of OPN were examined by using differentiated non-stem glioma cells such as U87, U251 and C6. In these glioma cells, OPN increased the migration and invasion through the binding of arginine-glycine-aspartate (RGD) domain of OPN and integrin $\alpha v\beta 3$ on the glioma cells, resulting in enhancing cell motility and invasion [34]. In contrast, in glioma stem-like cells expressing high CD44 as used in the present study, another domain of OPN, CD44-binding domain binds to CD44 and promotes various cellular functions including tumor proliferation and enhancement of stemness [7,40]. We found that addition of HA in the lower well in the plate markedly increased the degree of both migration and invasion, but the inhibitory effect of OPN on HA-promoted migration and invasion was similar to the inhibition of GSC migration and invasion without HA and dose dependent. These results suggest that binding of the CD44-binding domain of OPN and CD44 may inhibit the signaling pathway from the binding of adhesion molecules such as HA to promoting the cell motility in the GSCs expressing high CD44 at the early stage. These interactions of CD44 and OPN in GSCs may be specific for glioma stem-like cells, particularly expressing CD44 at a high level. CD44 exists as isoforms of nine exon variants (CD44v2-CD44v10) inserted by alternative splicing and a standard form CD44s [35]. So, we investigated expressions of CD44 isoforms in GSCs and differentiated glioma cells. The results revealed that GSCs showed different expression patterns of CD44 variant isoforms from differentiated glioma cells, particularly in CD44v5 and CD44v6 (unpublished data). So far, it has been reported that CD44v6 binds to OPN [36], but other variant isoforms of CD44 may participate in various biological functions by interacting with OPN.

Then, to elucidate the relation of such inhibition of GSC migration and invasion by the interaction of OPN and CD44 and tumor progression, we investigated the effects of CD44 and OPN on GSC proliferation under hypoxic conditions. Knockdown of CD44 did not affect in vitro tumor proliferation. In contrast, the growth rate of GSCs was increased under moderate hypoxia, but the enhancement in growth rate was suppressed by silencing OPN using siRNA. These results indicate that CD44 does not affect in vitro proliferation of GSCs, whereas OPN promotes GSC proliferation under moderately hypoxic conditions.

It has been reported that binding of OPN to CD44 induces the intramembranous cleavage of CD44 via presenillin-dependent γ-secretase [7,37,38], resulting in the release of the CD44 intracellular domain (CD44-ICD) into the cytoplasm. Cytoplasmic CD44-ICD translocates to the nucleus and activates the transcription of several genes involved in cell survival, inflammation, tumor growth, and tumor invasion [38,39]. The CD44-ICD also stimulates HIF-2α activity, resulting in increased stemness of tumor cells, which promotes aggressive tumor growth in vivo and enhances the radiation resistance of tumors [7,40]. These interactions between OPN and CD44 suggest that inhibition of the expression

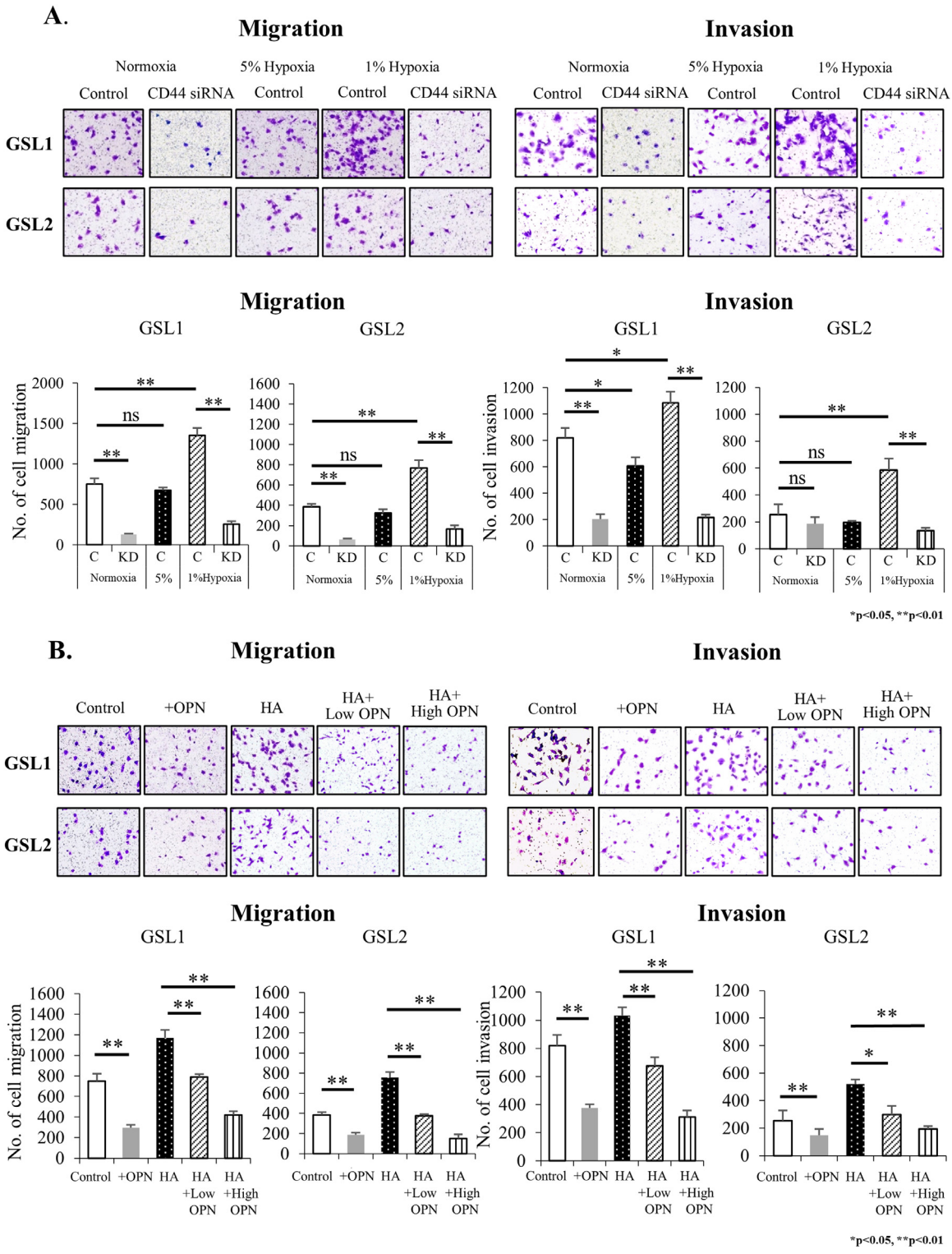

Fig. 4. Hypoxia enhances and OPN suppresses GSC migration and invasion. (A) Effects of hypoxia and CD44 on in vitro cell migration and invasion of two GSC lines. Under the condition of 1% O_2 hypoxia, the migration and invasion of both GSC lines was markedly increased. CD44 silencing via siRNA significantly inhibited the 1% O_2 hypoxia–induced increase in GSC migration and invasion as well as GSC migration and invasion under normoxia. Upper panel shows tumor cells that had migrated or invaded to the lower side of the filter membrane (stained with crystal violet). C, control; KD, knockdown with CD44 siRNA. $^{*}P<0.05$; $^{**}P<0.01$. (B) Inhibitory effect of OPN on the migration and invasion of GSC lines. OPN inhibited CD44-mediated migration and invasion in both GSC lines under the normoxic condition. Addition of HA (0.5 mg/ml) to the attractant in the lower well enhanced the migration and invasion of both GSC lines, whereas OPN administration (low: 0.2 μg/ml; high: 5.0 μg/ml) suppressed the enhancement of migration and invasion in a dose-dependent manner. $^{*}P<0.05$; $^{**}P<0.01$.

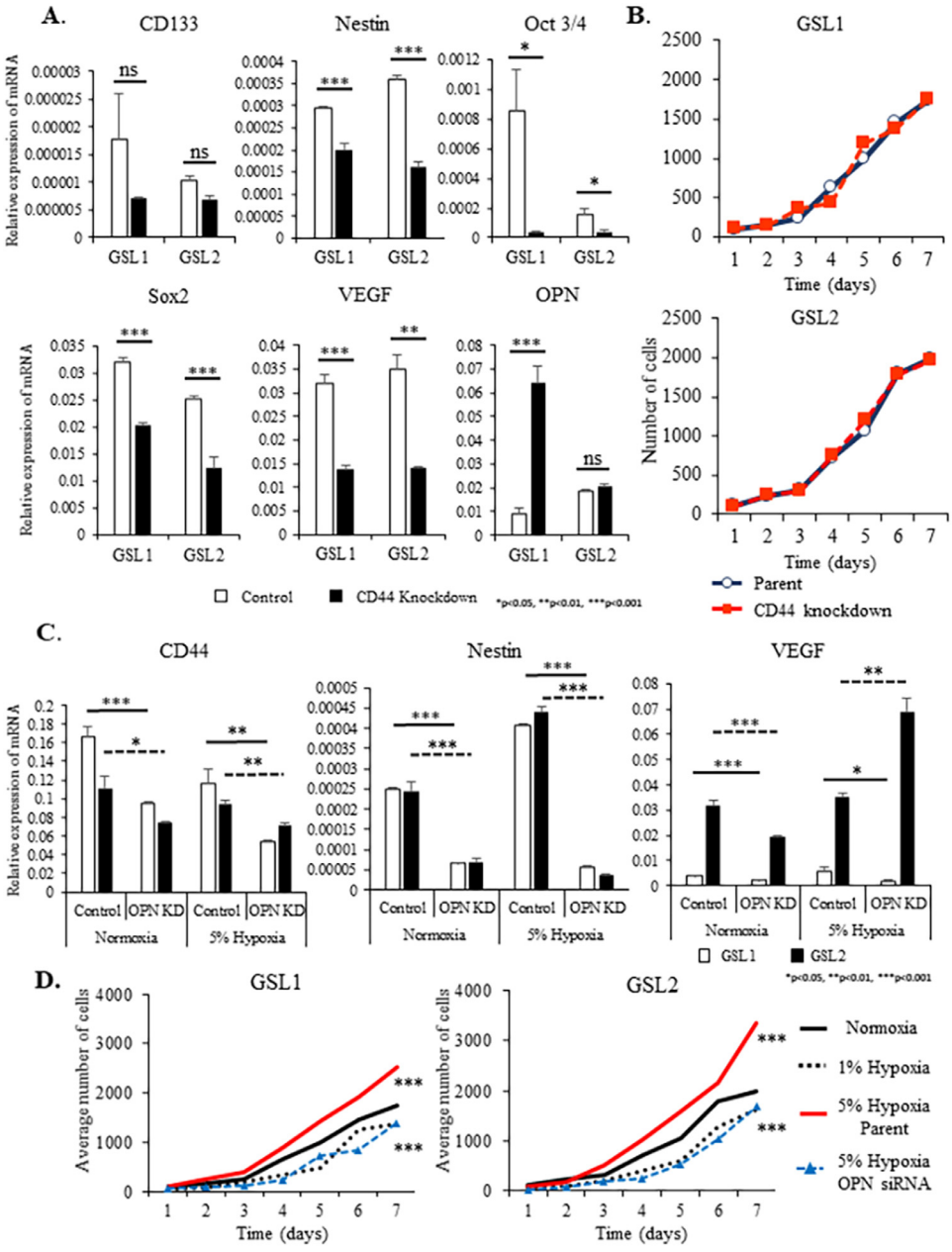

Fig. 5. Effect of CD44 or OPN knockdown on the expression of other markers and cell proliferation. (A) Effect of CD44 knockdown on the expression of neural stem cell markers and VEGF. Knockdown of CD44 using shRNA significantly decreased the expression of all stem cell markers (except CD133) examined in both GSC lines. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (B) Effect of CD44 knockdown on GSC proliferation. Graphs show growth curves of GSCs with and without CD44 knockdown. CD44 knockdown had no effect on the proliferation of the GSC lines in cell culture. (C) Effect of OPN inhibition on the expression of CD44, Nestin, and VEGF under normoxia and 5% O_2 hypoxia. Silencing of OPN expression using siRNA significantly decreased the expression of CD44 and Nestin in both GSC lines and increased the expression of VEGF only in GSL-2 cells. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. (D) Effects of hypoxia and OPN silencing on GSC proliferation. Under the condition of 5% O_2 hypoxia, the growth rate of both GSC lines increased significantly compared with GSCs cultured under the normoxic condition (control). Inhibition of OPN expression significantly decreased the growth rate of both GSC lines compared with the control. $^{***}P<0.001$.

of CD44 and/or OPN leads to suppression of both tumor cell invasion and proliferation in GBM.

When tumor cells proliferate, they would need to halt their migratory activity [41–43]. Such a shift of biological functions could occur by the interaction of CD44 and OPN whose molecules are activated at the differential level of hypoxia. When highly invasive GSCs expressing a high level of CD44 under the severe hypoxic condition are placed in the environment of moderate hypoxia, the GSCs decrease the elevated expression of CD44, resulting in reduction of migratory and invasive activities. OPN activated under the moderate hypoxic condition could bind to CD44, which may also promote the suppression of CD44-activated migration and invasion and stimulate the proliferation of the GSCs, resulting in the phenotypic shift to low-invasiveness/highly proliferative GSCs.

Highly invasive nature of GBM becomes an inevitable obstacle to completely resect the tumor. If the tumors could be rendered less invasive, they could be almost totally resected. We therefore focused on CD44 and OPN as potential therapeutic targets to identify an optimal strategy for treating GBM. CD44 knockdown in GSCs significantly decreased the mRNA expression of various stem cell markers (CD133, Nestin, Oct3/4, and Sox2) compared with the control. Lamour et al. reported that OPN knockdown decreased the expression of Sox2, Oct3/4, and Nanog in glioma-initiating cells by suppressing the release of CD44-ICD fragments, which function in activating the expression of these stem cell markers [15]. Decreased expression of stem cell markers via CD44 knockdown may also have involved a reduction in intracellular CD44-ICD levels due to the reduced availability of CD44 for binding to OPN.

GSC invasion was markedly inhibited by knockdown of CD44 both in vitro and in the in vivo mouse xenograft model. In contrast, CD44-knockdown GSCs proliferated at almost the same high rate as the parent cells in vitro, but the growth rate of brain tumors generated in mice by transplantation of CD44-knockdown GSCs was strongly suppressed, resulting in a significantly longer survival time of these mice compared with the control. PCR analyses demonstrated that the expression of VEGF mRNA in CD44-knockdown cells was markedly decreased compared with the control, but the Ki-67 LI of tumor tissue generated from

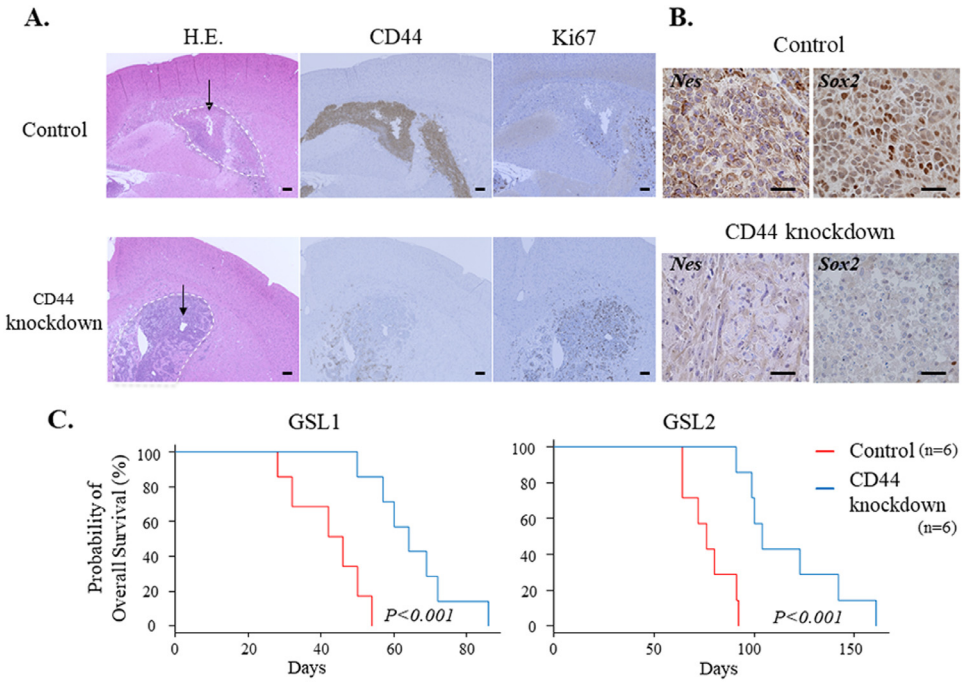

Fig. 6. Histologic features and survival curves of mice receiving transplanted CD44-knockdown GSCs. (A) Microscopic images of tumors generated in mouse brain via transplantation of GSCs with or without CD44 knockdown (× 40) (upper panel, control; lower panel, CD44 knockdown). Histologic examination of control specimens at low magnification indicated an unclear tumor margin with diffuse infiltration of tumor cells around the primary tumor mass, particularly marked invasion into the corpus callosum (H&E) (left column). In contrast, transplantation of GSCs with CD44 knockdown generated a well-demarcated tumor around the GSC injection site without extensive infiltration of tumor cells into both the corpus callosum and the surrounding brain tissues (left column). White broken lines: the border of the primary tumor mass. Arrow: needle track of tumor injection. Almost all tumor cells in the control were positively stained with anti-CD44 antibody, but almost no tumor cells stained positive for CD44 in the tumor with CD44 knockdown (middle column). The CD44-knockdown tumor exhibited positive Ki-67 staining to the same degree as the control tumor in the core area of the tumor generated around the GSC injection site (Ki-67 SI: 50.7% [CD44 knockdown], 50.9% [control]). Note that almost no Ki-67 staining was observed in the tumor exhibiting marked invasion into the corpus callosum (migrating cells do not proliferate) (right column) (bar = 100 μm). (B) Immunohistochemical analysis of stem cell markers in tumors generated by GSC transplantation (× 400). Both Nestin and Sox2 were positively immune-stained in the tumor generated by transplantation of control GSCs (upper panel), but neither marker stained positively in the tumor generated from GSCs with CD44 knockdown (lower panel) (bar = 50 μm). (C) Kaplan-Meier overall survival curves of mice that received transplantation of GSL-1 or GSL-2 cells. Mice that received transplants of GSCs with CD44 knockdown exhibited significantly longer survival than the control for both GSL-1 (P<0.001) and GSL-2 (P<0.001).

CD44-knockdown GSCs was not significantly decreased compared with the control. These findings suggest that the observed inhibition of tumor growth in the brain of these mice could have been caused by a reduction in GSC stemness via CD44 knockdown rather than suppression of angiogenesis due to decreased VEGF expression.

Several reports have indicated that inhibition of OPN expression could reduce formation of GBM tumors in vivo [16,29,44]. We investigated effects of OPN knockdown on the growth and sphere-forming capability of GSCs using spheres of GDC40 (glioblastoma-derived stem-like cells; kindly provided by Dr. Y. Kanemura, Osaka National Hospital, Japan) [45] that express OPN and VEGF at high levels and CD44 at a low level. Inhibition of OPN expression in GDC40 cells resulted in increased CD44 expression and decreased VEGF expression (Fig. S3). The GDC40 line, which exhibits characteristically low migration and invasion both in vitro and in vivo, exhibited a decrease in growth rate but induced diffuse infiltration of cells around tumor spheres after OPN silencing (Fig. S4). These results suggest that inhibition of OPN expression could lead to suppression of tumor growth both in vitro and in vivo. However, this could also carry the risk of inducing the formation of more-invasive tumors via increased CD44 expression.

In our preliminary study, we found that hyperbaric oxygen (HBO) markedly inhibited the expression of both CD44 and OPN in GSCs (Fig. S5), resulting in apoptosis of the GSCs and significant suppression of their migration and invasion (unpublished data). As HBO therapy has been safely performed clinically as an adjuvant treatment to enhance the effects of radio-chemotherapy in patients with various cancers, including GBM [46,47], a combination of targeting the expression of CD44 and OPN and HBO therapy could be a useful approach for treating GBM. There are some limitations to translate the present results into clinical practice because of a small number of GSCs used in the present study and unknown effects of other molecules activated by hypoxia. Consequently, more detailed analyses with additional GSCs will be required to elucidate the molecular mechanism underlying phenotypic transition in GBM.

Conclusions

Severe hypoxia (1% O_2) increased the expression of CD44 via activation of HIF-1α, resulting in GSCs with a highly invasive phenotype. In contrast, moderate hypoxia (5% O_2) enhanced OPN expression in GSCs via activation of HIF-2α, inducing the cells to assume a less-invasive and highly proliferative phenotype. As biological function, elevated CD44 promoted the migration and invasion of GSCs under the severe hypoxia, whereas highly secreted OPN inhibited the migration and invasion of GSCs while increasing their proliferation under the moderate hypoxia. Knockdown of CD44 expression in GSCs inhibited not only the migration/invasion but also the expressions of stem cell markers such as Nestin and Sox2 of these cells both in vitro and in vivo mouse xenograft model, thus resulting in much longer survival of tumor-bearing mice. In contrast, silencing OPN expression led to decreased GSC proliferation. These results suggest that inhibiting the expression of both CD44 and OPN would provide beneficial effects in the treatment of both primary and recurrent GBM.

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Availability of data and materials

The data used to support the findings of the study are available from the corresponding author upon request.

Consent for publication

Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

Declaration of Competing Interest

The authors declare that there are no competing interests regarding the publication of this paper.

CRediT authorship contribution statement

Masahiro Nishikawa: Investigation, Writing – original draft. **Akihiro Inoue:** Conceptualization, Methodology, Investigation, Validation, Writing – original draft. **Takanori Ohnishi:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Hajime Yano:** Methodology, Validation. **Saya Ozaki:** Investigation. **Yonehiro Kanemura:** Resources. **Satoshi Suehiro:** Validation. **Yoshihiro Ohtsuka:** Investigation. **Shohei Kohno:** Validation. **Shiro Ohue:** Validation. **Seiji Shigekawa:** Validation. **Hideaki Watanabe:** Validation. **Riko Kitazawa:** Resources. **Junya Tanaka:** Methodology, Validation. **Takeharu Kunieda:** Supervision.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101137.

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