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Original Articles

Ligand-dependent EphB4 activation serves as an anchoring signal in glioma cells

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ABSTRACT

Of the erythropoietin-producing human hepatocellular receptors (Ephs), EphB4 has recently emerged as a potential target in several cancers due to its roles in modified cell migration and invasion. As little is known about the roles of EphB4 in glioma, we sought to investigate its function in glioma by *in vitro* cell migration and invasion assays, immunoblotting and immunostaining. EphB4 was expressed in glioma cell lines and stem-like cell lines. The stimulation of glioma cells with ephrin-B2, the sole ligand of EphB4, conducted EphB4 phosphorylation and suppressed migration and invasion that downregulation of EphB4 using small interfering RNA abrogated. The stimulation also suppressed the phosphorylation of Akt. We confirmed by immunostaining that EphB4-positive cells existing only in the tumor core, whereas ephrin-B2-positive cells widespread in both the tumor core and the invasive area signifying that EphB4-ephrin-B2 reaction occurred only at the tumor core. Taken together, our data suggest that ephrin-B2-dependent EphB4 phosphorylation acts as an anchoring signal to reduce the malignancy by inhibiting Akt phosphorylation in the glioma core, whereas the scarcity of signaling in the tumor periphery promotes invasion into the surrounding brain.

1. Introduction

Glioblastoma (GBM) is the most common and fatal brain tumor. Despite undergoing combined treatment strategies, which include surgery, radiotherapy, and chemotherapy, most patients die within 15–18 months of diagnosis [1]. Its infiltrative spread into neighboring brain structures is a defining feature of GBM [2]. GBM cell migration and invasion of healthy surrounding areas of the brain are driving causes of the dismal overall survival rate. The inhibition of glioma cell migration and invasion is a hypothetically promising strategy for treatment. However, anti-invasive therapies are not yet available.

The mechanisms that promote the invasiveness of GBM rely on microenvironmental influences, such as cell-cell interactions, cell-matrix interactions, cell motility, and remodeling of the extracellular matrix [3,4]. Recently, it has become increasingly clear that receptor tyrosine kinases (RTKs), especially erythropoietin-producing human hepatocellular carcinoma (Eph) RTKs that are activated by cell-cell contact [5], play important roles in the malignancy of several types of tumors [3,6,7].

The Eph receptors comprise the largest RTK family [8], which is divided into two groups: EphA (A1-A8, A10) and EphB (B1-4, B6). Eph receptor activation takes place upon cell contact-dependent interaction with their ephrin ligands, which comprise the glycosylphosphatidylinositol-linked A-ephrins and the transmembrane B-ephrins [8]. The binding to cell surface ephrins [5] induces phosphorylation of the Eph intracellular tyrosine kinase domain, thereby activating or repressing downstream signaling cascades [3,6]. This process is known as ligand-dependent Eph signaling.

Abbreviations: Eph, erythropoietin-producing human hepatocellular receptor; Fc, crystallizable fragment; GBM, glioblastoma; Ig, immunoglobulin; RTK, receptor tyrosine kinase; SEM, standard error of the mean; si, small interfering; si-control, control siRNA; TBS-T, Tris-buffered saline with TWEEN® 20

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The EphB4 receptor is highly expressed in many types of malignant human tumor cells, including malignant glioma [9–11], breast cancer [12–14], ovarian cancer [15], prostate cancer [16], lung cancer [17], gastric cancer [18], and colon cancer [19] cells. EphB4 signaling is involved with tumor progression [12,13] and angiogenesis [20], making it a molecular target for therapeutic applications [21,22]. Although the roles of the Eph receptors in malignant glioma are gradually becoming clear, the role of EphB4 in glioma has rarely been investigated [6,23]. Clarifying the function of EphB4 is imperative for the development of anti-cancer therapies that target EphB4 [24]. We investigated ligand-dependent EphB4 signaling to characterize the functional role of EphB4 in glioma cells.

2. Material and methods

2.1. Cell culture

The human glioma cell lines T98, U87, U138, A172 and SNB19 (European Collection of Cell Cultures) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin-streptomycin. Human patient-derived GBM stem-like cell lines, KGS01 and KGS06, established at Kanazawa University were cultured as described previously (Supplementary Fig. 1) [25]. KGS01 has already been confirmed as tumor-initiating cell [25].

2.2. Antibodies and reagents

The anti-phosphotyrosine monoclonal antibody (clone 9411) for immunoprecipitation was purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal antibody against EphB1 (Clone #3980S) for immunoblot analysis was purchased from Cell Signaling Technology. Rabbit polyclonal antibody against EphB2, mouse monoclonal antibody against EphB3 (Clone 4A122D1) and EphB6 (2A6B9) for immune blot analysis were purchased from GeneTax (San Antonio, TX, USA). The mouse monoclonal antibody against EphB4 (clone 5B8F7) and the rabbit polyclonal antibody against ephrin-B2 for immunostaining were purchased from GeneTex (San Antonio, TX, USA) and Abcam (Cambridge, MA, USA), respectively. Phospho- (p-) EphB4 (Tyr987) polyclonal antibody (PA5-64792) for immunostaining was purchased from Invitrogen (Carlsbad, CA, USA). Ephrin-B2/Fc chimeras were purchased from R&D Systems (Minneapolis, MN, USA). The control mouse immunoglobulin (Ig) G and mouse IgG Fc were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and Jackson ImmunoResearch (West Grove, PA, USA), respectively. Protein G Sepharose 4 Fast Flow was purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). The mouse anti-β-actin monoclonal antibody was obtained from Sigma-Aldrich (Milwaukee, WI, USA). The antibodies against Akt and p-Akt^{S473} to analyze downstream signaling were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.3. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as previously described [26]. Equivalent amounts of protein (200 μg) were precleared, then immunoprecipitated from the lysates. To detect the phosphorylation of EphB4 or signal alteration, the cells were stimulated with ephrin-B2/Fc for 1 h at 37 °C before cell lysate extraction.

2.4. RNA interference-mediated downregulation of EphB4

We purchased 2 target sequences for EphB4-specific small interfering (si)RNAs from Qiagen: CACGAGCTCCCTGGGAGGAAA (si-EphB4 #1) and CCCAGCCAATAGCCACTCTAA (si-EphB4 #2). The nucleotide sequence of the control siRNA (si-control), a random sequence that was not related to EphB4, was also purchased from Qiagen. The EphB4 knockdown siRNAs and control siRNA were transfected into U87 and SNB19 cells using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA), per the manufacturer's protocol. The transfected cells were cultured for 72 h before use and the downregulation of EphB4 was confirmed by western blotting.

2.5. Cell migration and invasion assays

The ability of cell migration was measured by scratch migration assay. Cells were seeded at > 90% confluency and grown until they formed a monolayer. The monolayer was scratched using a sterile 1000-μl pipette tip, then carefully washed twice with phosphate-buffered saline to remove cell debris and detached cells. We added fresh complete medium containing ephrin-B2/Fc or Fc alone (2 or 4 μg/ml) to the monolayers. Images were taken at 0, 8, 16, and 24 h post-scratching and assessed as previously described [27].

Alternatively, transwell system (Corning, USA) was performed for migration and invasion assay using 8-μm pore size membrane and Matrigel-coated membrane as previously described [25]. The cells on the filter were counted in 6 randomly selected fields.

2.6. Cell proliferation assay

We measured the proliferation of EphB4-expressing glioma cells, siRNA-transfected glioma cells and patient-derived GBM stem-like cell lines after treatment with the indicated concentrations of ephrin-B2/Fc with a fluorescence plate reader in the presence of alamarBlue (MyBioSource, San. Diego, CA, USA) as previously described [10].

2.7. Glioma samples

Glioma specimens were collected from the mice 60 days after transplanting KGS06 cells into the brain. All animal experiments followed the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University that covers the national guideline. Glioma specimens were also collected from patients with primary gliomas who underwent surgery at Kanazawa University Hospital and provided informed consent at the time of the initial diagnosis in compliance with the guidelines of Kanazawa University and in accordance with the Declaration of Helsinki. Tumor samples were harvested at the time of surgery and fixed in 4% paraformaldehyde. This study was performed according to the guidelines of the Internal Review Board of the university and was approved by the university medical ethics committee (No. 2784).

2.8. Immunohistochemistry

Paraffin-embedded sections were cut 4 μm thick, then deparaffinized and rehydrated. EphB4 and ephrin-B2 were detected by immunohistochemical staining as previously described [27].

2.9. Immunofluorescence staining

4-μm-thick sections were incubated overnight at 4 °C with the anti-Eph4 mouse monoclonal antibody (1:300), p-EphB4 rabbit polyclonal antibody (1:100) and p-Akt rabbit polyclonal antibody (1:50). After they were rinsed in TBS-T, the sections were incubated at room temperature with biotinylated anti-mouse antibody (1:50) for 30 min (VECTASTAIN®, Vector Laboratories, Burlingame, CA, USA). After they were rinsed in TBS-T, the sections were incubated with DyLight® 594 Streptavidin (1:1000; Vector Laboratories). After they were again rinsed in TBS-T, the sections were incubated with anti-ephrin-B2 rabbit monoclonal antibody (1:400) at room temperature for 1 h, then with a donkey anti-rabbit IgG secondary antibody conjugated to Alexa Fluor® 488 (1:1000; Molecular Probes, Eugene, OR, USA) at room temperature for 1 h. Finally, the sections were mounted with VECTASHIELD® MOUNTING MEDIUM with DAPI (Vector Laboratories) and

photographed using a Biorevo BZ-X700 All-in-One Fluorescence Microscope and analyzer software (Keyence, Osaka, Japan).

2.10. Statistical analysis

Each experiment was performed independently 3 or 4 times and the data are presented as the mean ± standard error of mean (SEM) or standard deviation. The statistical significance of the differences among means was evaluated by one-way analysis of variance with Bonferroni's or Dunnett's post hoc test. Differences for which $P < 0.05$ were considered significant. All statistical calculations were performed using SPSS statistics version 24.0.

3. Results

3.1. EphB4 expression and ephrin-B2/Fc-stimulated phosphorylation in glioma cell lines

We analyzed the levels of EphB4 protein in the 5 types of glioma cell lines by western blotting. The glioma cell lines expressed EphB4 at varying levels. The levels were higher in U87 and SNB19 than in the other cell lines (Fig. 1A). We next examined if EphB4 is phosphorylated in response to its ligand. Since EphB4 selectively binds ephrin-B2 [28], we stimulated endogenous EphB4 with ephrin-B2/Fc. Very low levels of p-EphB4 were detected in cells grown in serum-replete conditions; the levels markedly increased in a concentration-dependent manner upon exposure to ephrin-B2/Fc. Moreover, total EphB4 protein levels were also higher after treatment with ephrin-B2/Fc (Fig. 1B).

3.2. Ephrin-B2/Fc suppressed the migration and invasion of U87 and SNB19 cells

To investigate the role of EphB4 in glioma cell migration, invasion, and proliferation, we performed assays with U87 and SNB19 cells, which highly express EphB4. Scratch migration assays revealed that ephrin-B2/Fc decreased the migratory distance of U87 and SNB19 cells in a concentration-dependent fashion (Fig. 2A, upper). Migratory distances after 24 h were significantly shorter for U87 (2 μg/ml, $P = 0.035$; 4 μg/ml, $P = 0.001$) and SNB19 (2 μg/ml, $P < 0.05$; 4 μg/ml, $P = 0.001$) cells exposed to ephrin-B2/Fc relative to those for cells treated with Fc alone (Fig. 2A, lower). The results of transwell migration assays were similar to scratch migration assays. Ephrin-B2/Fc significantly suppressed the migration of U87 (2 μg/ml, $P = 0.045$; 4 μg/ml, $P = 0.005$) and SNB19 (2 μg/ml, $P = 0.035$; 4 μg/ml, $P = 0.007$) cells (Fig. 2B).

Similarly, ephrin-B2/Fc significantly suppressed the invasion of U87 (2 μg/ml, $P = 0.046$; 4 μg/ml, $P = 0.001$) and SNB19 (2 μg/ml, $P = 0.049$; 4 μg/ml, $P = 0.001$) cells (Fig. 2C). No differences were observed in the proliferation of U87 or SNB19 cells upon stimulation with ephrin-B2/Fc (Supplementary Fig. 2). These data suggested that ligand-dependent EphB4 signaling suppressed migration and invasion, but not proliferation, of U87 and SNB19 cells.

3.3. siRNA-mediated EphB4 suppression reversed the decreased migration and invasion caused by ephrin-B2/Fc

We next investigated if the effects we observed on migration and invasiveness depended on EphB4 alone as ephrin-B2 can also bind EphB1, 2, 3, 6, and A4 [29]. We transfected U87 and SNB19 cells with si-control and siRNAs for EphB4 (si-EphB4 #1 and si-EphB4 #2) to

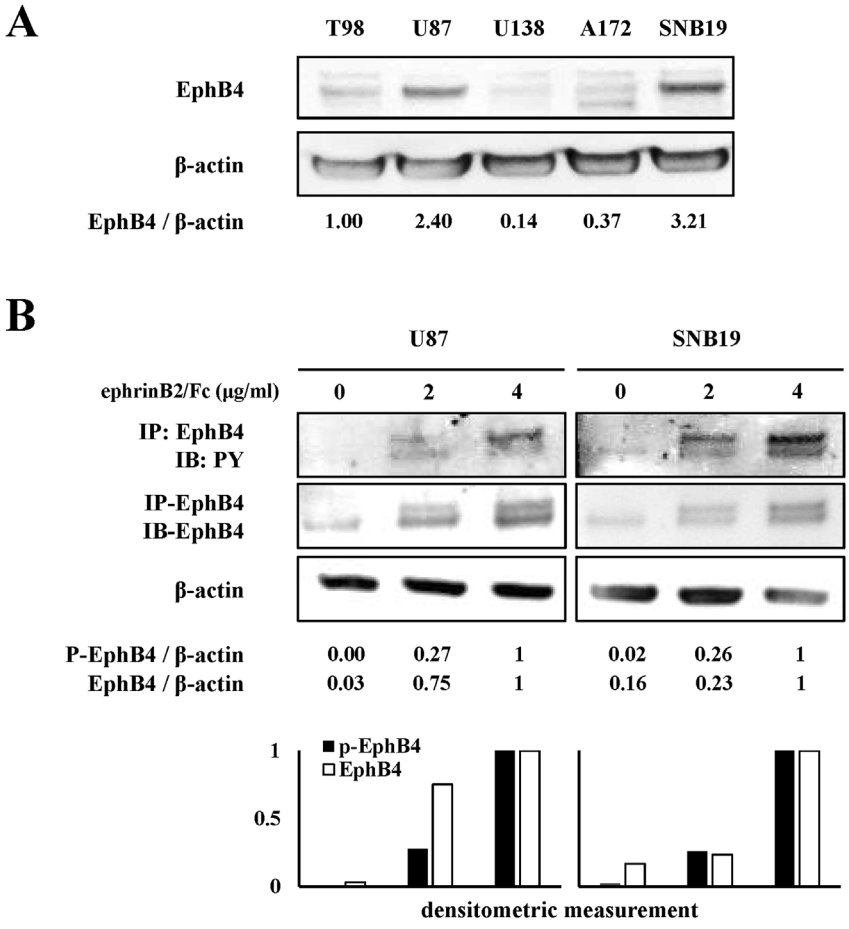

Fig. 1. Characterization of EphB4 expression in human glioma cell lines and confirmation of EphB4 phosphorylation. (A) The expression of EphB4 in 5 glioma cell lines was analyzed by western blotting. U87 and SNB19 expressed the highest levels of EphB4 among the cell lines. The densitometric measurements were normalized to the signal in T98 glioma cell line. The values were shown below blots. (B) We immunoprecipitated (IP) proteins from equal amounts of total U87 and SNB19 cell lysates after stimulation with ephrin-B2/Fc (2 or 4 μg/ml) or Fc alone (0 μg/ml) for 60 min with an anti-EphB4 antibody. The immunoprecipitates were probed by immunoblotting (IB) with the indicated antibody. The phosphorylated and total EphB4 protein levels increased in an ephrin-B2/Fc concentration-dependent manner. Equal quantities of whole cell lysates were also immunoblotted for the loading control β-actin. PY; phosphotyrosine. The densitometric measurements were normalized to the signal in the cells treated with 4 μg/ml ephrin-B2/Fc.

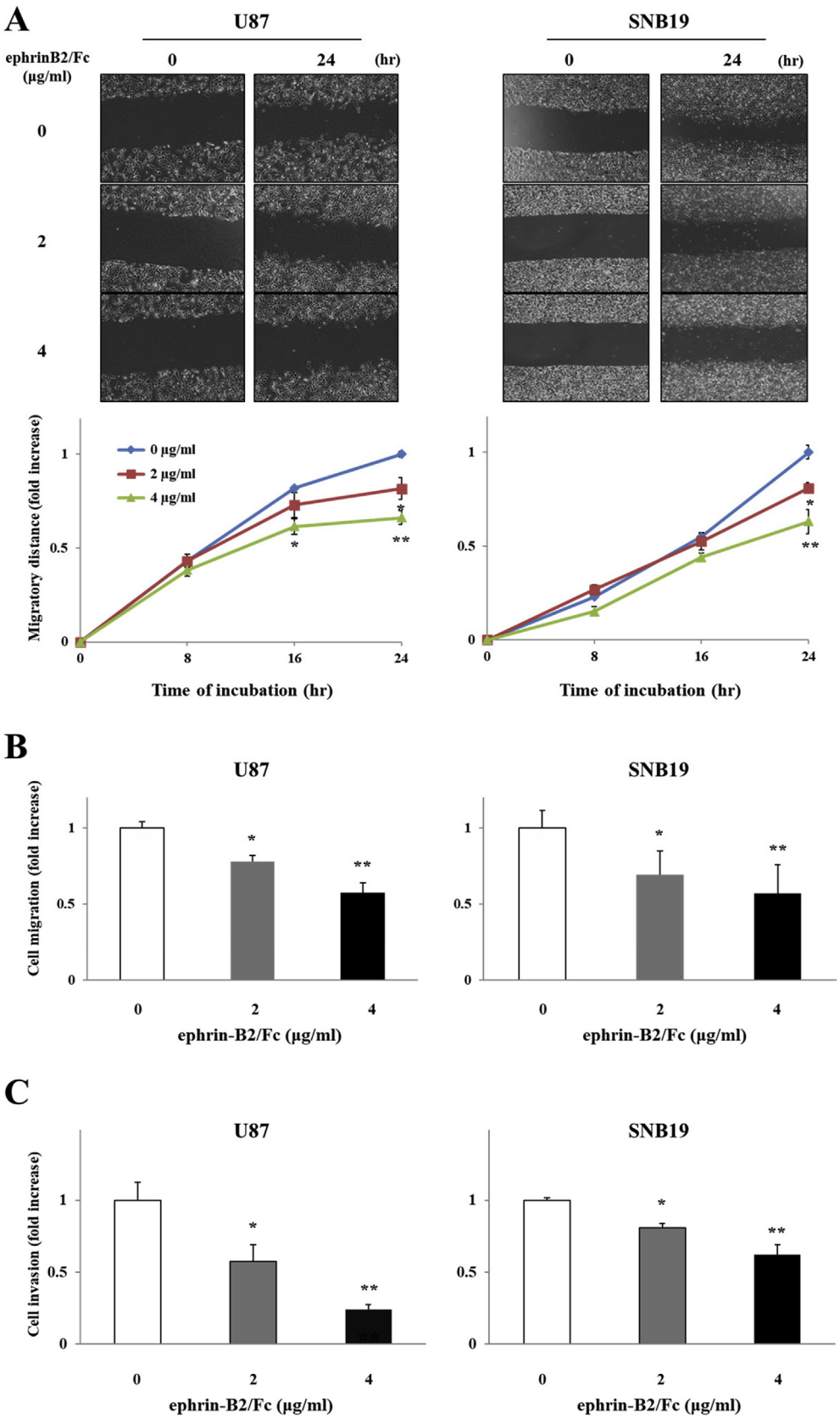

Fig. 2. Cell migration and invasion analyses of EphB4-expressing glioma cell lines stimulated with ephrin-B2/Fc.
Cells were treated with ephrin-B2/Fc (2 or 4 μg/ml) or Fc alone (0 μg/ml) before use in the assays. (A) Upper: representative time course of cell migration in the scratch assay after treatment with ephrin-B2/Fc (2 or 4 μg/ml) or Fc alone (0 μg/ml). The assay was performed by scratching confluent cells then monitoring the cells at the same reference points at 0 and 24 h under a phase-contrast microscope. Lower: the relative widths of the wounds at the indicated times after treatment with ephrin-B2/Fc or Fc alone (expressed as a percentage of the mean migratory distances of the cells treated with Fc alone at 24 h). Error bars indicate SEM. * represents $P < 0.05$ and ** represents $P < 0.01$ compared to the cells treated with Fc alone at each time point. (B) Transwell migration and (C) Transwell invasion assays. Columns display the mean cell counts from at least 6 fields. The mean value of the U87 or SNB19 cells treated with Fc alone was normalized as 1. Error bars indicate SEM ($n = 4$). * represents $P < 0.05$ and ** represents $P < 0.01$ compared to the cells treated with Fc alone. Error bars indicate SEM.

decrease the basal level of EphB4 (Fig. 3A). We verified that the siRNAs did not affect the expression of the other EphB family members by immunoblotting (Supplementary Fig. 3).

We performed the migration and invasion assays with the si-EphB4-transfected cells after treatment with ephrin-B2/Fc. We found that the migratory distance after 24 h was significantly shorter after exposure of control U87 (4 μg/ml; $P = 0.023$) and SNB19 (4 μg/ml; $P = 0.036$) cells to ephrin-B2/Fc relative to that after treatment with Fc alone. By

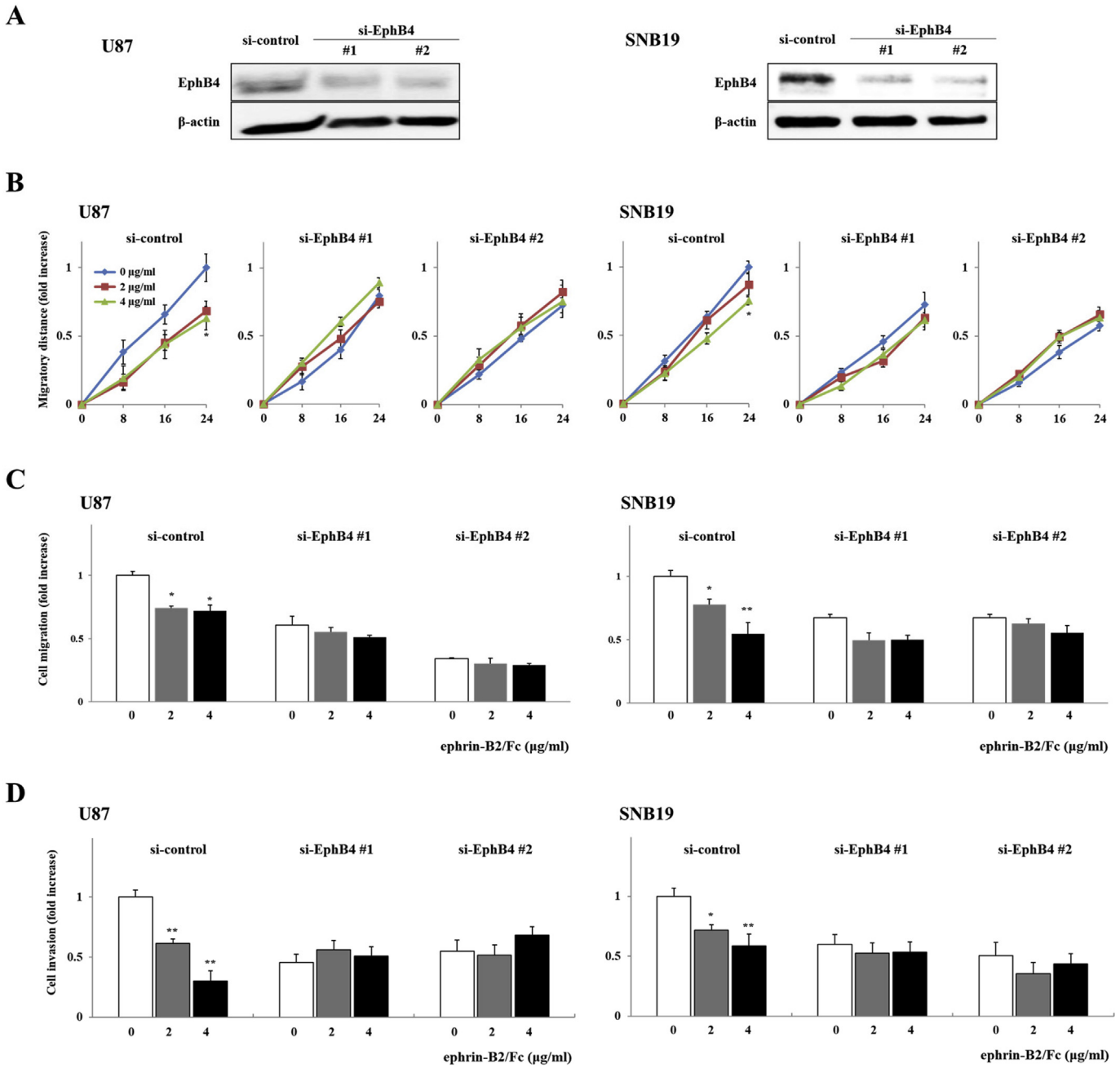

Fig. 3. Cell migration and invasion analyses of EphB4-expressing and si-EphB4-transfected glioma cell lines stimulated with ephrin-B2/Fc.
(A) Analysis of EphB4 protein expression following siRNA transfection. β-actin was used as a protein loading control. (B) Scratch migration assay. Data are presented as the mean migratory distance relative to the initial time point and normalized to the mean migratory distance at 24 h of the si-control-treated cells exposed to Fc alone. Error bars indicate SEM. * represents $P < 0.05$ and ** represents $P < 0.01$ compared to the cells treated with Fc alone. (C) Transwell migration assay and (D) Transwell invasion assay. Columns show mean cell counts from at least 6 fields. The mean value from the si-control-transfected U87 or SNB19 cells treated with Fc alone was normalized as 1. Error bars indicate SEM (n = 3; migration, 4; invasion assay). * represents $P < 0.05$ and ** represents $P < 0.01$ compared to the cells treated with Fc alone.

contrast, treatment with ephrin-B2/Fc did not affect the migratory distance of the si-EphB4 #1- and #2-transfected cells (Fig. 3B). The results of transwell migration assays were similar to that of scratch migration assays. The cell number was significantly lower after exposure of control U87 (2 μg/ml, P = 0.019; 4 μg/ml, P = 0.013) and SNB19 (2 μg/ml, P = 0.014; 4 μg/ml, $P < 0.001$) cells. By contrast, treatment with ephrin-B2/Fc did not affect the migratory distance of the si-EphB4 #1- and #2-transfected cells (Fig. 3C). Furthermore, the cell migration tended to be shorter for the si-EphB4-transfected cells than for si-control-treated cells that received Fc alone (Supplementary Figs. 4A–D).

Similar findings were observed in the invasion assay. As shown in Fig. 3D, ephrin-B2/Fc significantly suppressed the invasion of control U87 (2 μg/ml, P = 0.006; 4 μg/ml, $P < 0.001$) and SNB19 (2 μg/ml, P = 0.026; 4 μg/ml, P = 0.002) cells compared to that of the cells treated with Fc alone. However, we did not observe significantly different invasion of the ephrin-B2/Fc- and Fc alone-treated si-EphB4-transfected cell lines. These results indicated that the downregulation of EphB4 negated the effect of ephrin-B2 treatment. Thus, ephrin-B2 appeared to stimulate EphB4 ligand-dependent signaling to suppress

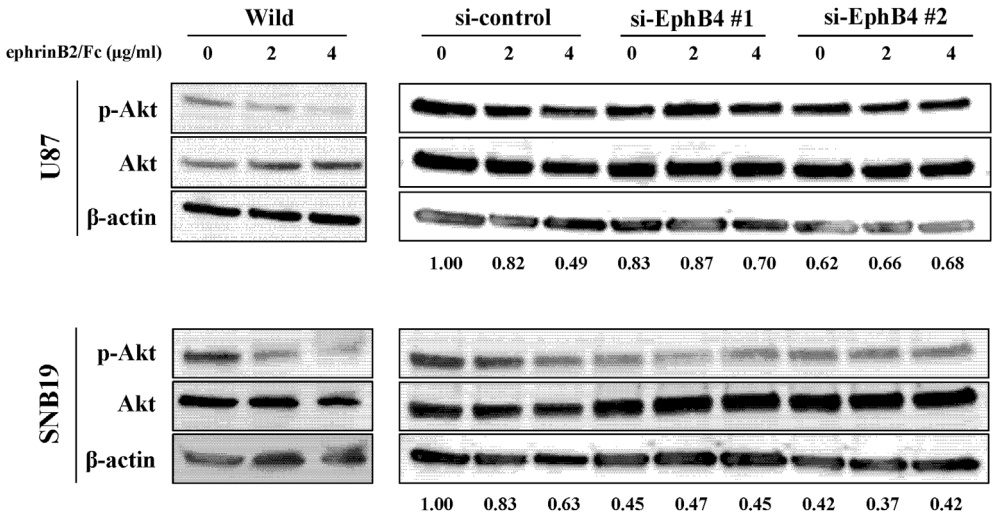

Fig. 4. Ephrin-B2/Fc inhibited Akt phosphorylation in U87 and SNB19 cells in a concentration-dependent manner.
Akt phosphorylation in U87 and SNB19 cells was assessed by western blotting. The densitometric measurements (below blots) were normalized to the signal in the si-control-transfected cells treated with Fc alone.

migration and invasion.

Moreover, cell invasion was reduced after the downregulation of EphB4 compared to after si-control treatment in the presence of Fc alone (Supplementary Figs. 4E and F). This result suggested that EphB4 itself, independent of ligand binding, plays a role in promoting migration and invasion.

3.4. Ligand-dependent EphB4 signaling by ephrin-B2/Fc blocked Akt signaling

To investigate the downstream EphB4 signaling mechanism, we investigated the levels of p-Akt after ephrin-B2/Fc treatment for 24 h. We found that ephrin-B2/Fc inhibited p-Akt levels in the U87 and SNB19 cells in a concentration-dependent manner (Fig. 4, left). The si-control-treated U87 and SNB19 cells demonstrated a similar concentration-dependent tendency for lower p-Akt levels after ephrin-B2/Fc treatment. In contrast, the p-Akt levels were not altered by ephrin-B2/Fc in the si-EphB4 #1- and #2-transfected cells. Densitometric measurements confirmed that the p-Akt levels were significantly lower in U87 and SNB19 cells exposed to ephrin-B2/Fc relative to the levels in cells treated with Fc alone, whereas the levels in the si-EphB4-transfected cell lines were unaltered after ephrin-B2/Fc treatment. In addition, the p-Akt levels were lower in the si-EphB4-transfected cells than in the control cells treated with Fc alone (Fig. 4, right).

Thus, the Akt phosphorylation responses of the glioma cells to ephrin-B2/Fc corresponded with their migration and invasion behavior.

3.5. Ephrin-B2/Fc suppressed the migration and invasion of patient-derived GBM stem-like cell lines

We performed the transwell migration and invasion assays using the glioma stem cell lines, KGS01 and KGS06 expressing EphB4 (Fig. 5A). Ephrin-B2/Fc significantly suppressed the migration of KGS01 (4 μg/ml, $P = 0.02$) and KGS06 (4 μg/ml, $P = 0.005$) compared to that of cell treated with Fc alone (Fig. 5B). Ephrin-B2/Fc also suppressed invasion of KGS01 (4 μg/ml, $P = 0.03$) and KGS06 (4 μg/ml, $P = 0.02$) (Fig. 5C). No differences were observed in the proliferation of KGS01 and KGS06 cells upon stimulation with ephrin-B2/Fc (data not shown).

3.6. Immunohistochemistry and immunofluorescent staining of gliomas with antibodies against EphB4 and ephrin-B2

We examined the localization of EphB4- or ephrin-B2-expressing cells in glioma samples to identify the site of EphB4 action. Firstly, we immunostained for EphB4 and ephrin-B2 in mouse brain tumor that KGS06 was transplanted. We confirmed that both EphB4 and ephrin-B2 were found within the tumors, whereas ephrin-B2 but not EphB4 was found in the invasive area (Fig. 5D). Subsequently, we examined 40 human GBM specimens. Thirty (75%) cases were EphB4 positive and 40 (100%) cases were ephrin-B2 positive. Among them, 20 cases included invasive area. A similar trend was observed in all EphB4 positive specimens (Supplementary Fig. 5).

Secondly, we conducted double immunofluorescence staining for EphB4 and ephrin-B2 (Fig. 6A). EphB4-positive glioma cells were observed in the tumor core (Fig. 6A–b), whereas ephrin-B2-positive cells were found in both the invasive area and the tumor core (Fig. 6A–c). Double immunofluorescence staining for EphB4 and ephrin-B2 showed that they are co-expressed in glioma cell membranes in the center of the tumor and confirmed the immunohistochemical finding that EphB4 was not expressed in the invasive area (Fig. 6A–d,e,f). In the core area, EphB4 and ephrin-B2 were positive at rates of 83.4% and 88.2%, respectively. By contrast, EphB4 and ephrin-B2 were positive at rates of 5.03% and 76.7% at the invasive area (Fig. 6B). Immunostaining for p-EphB4 showed that p-EphB4-positive cells were located in similar places as EphB4-positive/ephrin-B2-positive cells (Supplementary Fig. 6). These data suggested that EphB4 signaling occurs exclusively in the tumor core, whereas this signaling is unleased in invading tumor cells.

We additionally performed immunohistochemistry with p-Akt antibody to confirm that the area of EphB4 and ephrin-B2 co-expression have low phosphorylated Akt. The immunohistochemistry data with tumor core that are diffusely ephrin-B2 positive area demonstrated that EphB4-positive cells tended to be p-Akt-negative, and conversely p-Akt positive cells tended to be EphB4-negative (Supplementary Fig. 7). The data suggest that EphB4 and ephrin-B2 co-expression have low phosphorylated Akt.

4. Discussion

This study confirmed that glioma cell lines, glioma stem-like cell lines and GBM tissues express EphB4. The assays revealed that ligand-dependent EphB4 signaling, activated by ephrin-B2, suppresses glioma cell migration and invasion. Moreover, EphB4 expression is upregulated by ephrin-B2 and downregulation of EphB4 inhibits migration and invasiveness. EphB4 signaling inhibits Akt phosphorylation in a manner that closely corresponds to its suppression of glioma cell line migration

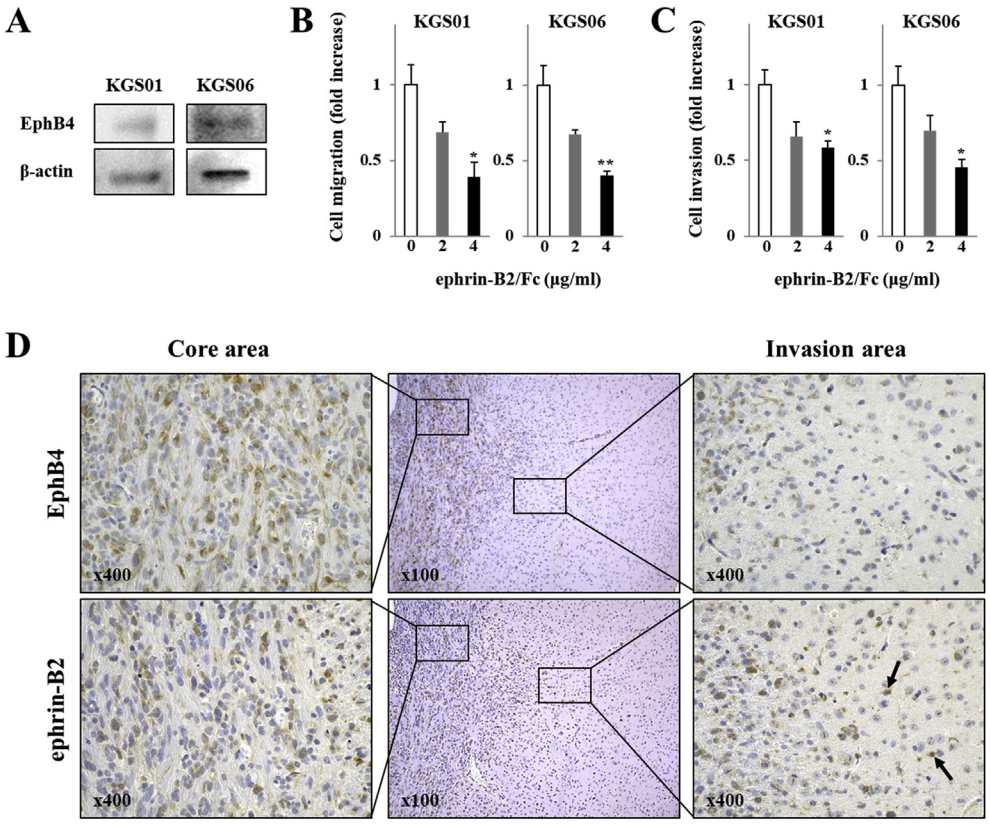

Fig. 5. The results of experiments using glioma stem-like cell lines, KGS01 and KGS06.
(A) The expression of EphB4 was analyzed by western blotting. (B) Transwell migration and (C) Transwell invasion assays. The mean value of the glioma stem-like cell treated with Fc alone was normalized as 1. Error bars indicate SEM. * represents $P < 0.05$ and ** represents $P < 0.01$ compared to the cells treated with Fc alone. (D) Immunostaining of the mouse brain section transplanted with KGS06 using antibodies against EphB4 and ephrin-B2. Strong EphB4 signals were detected within the tumor core, whereas strong ephrin-B2 signals were detected in both the tumor core and the invasive area.

and invasion. Analysis of the primary glioma tissues indicated that EphB4 and ephrin-B2 are co-expressed at the tumor core, whereas little EphB4 is expressed in the invasive area. These data indicated that ephrin-B2-dependent EphB4 signaling anchors cells in the tumor by attenuating migration and invasion by blocking Akt phosphorylation and promoting a positive feedback loop that increases EphB4 expression. Furthermore, interruption of EphB4 signaling may contribute to tumor cell spread.

EphB4 can undergo both ephrin-B2-dependent and ligand-independent activation [30–33]. In this study, we first focused on ephrin-

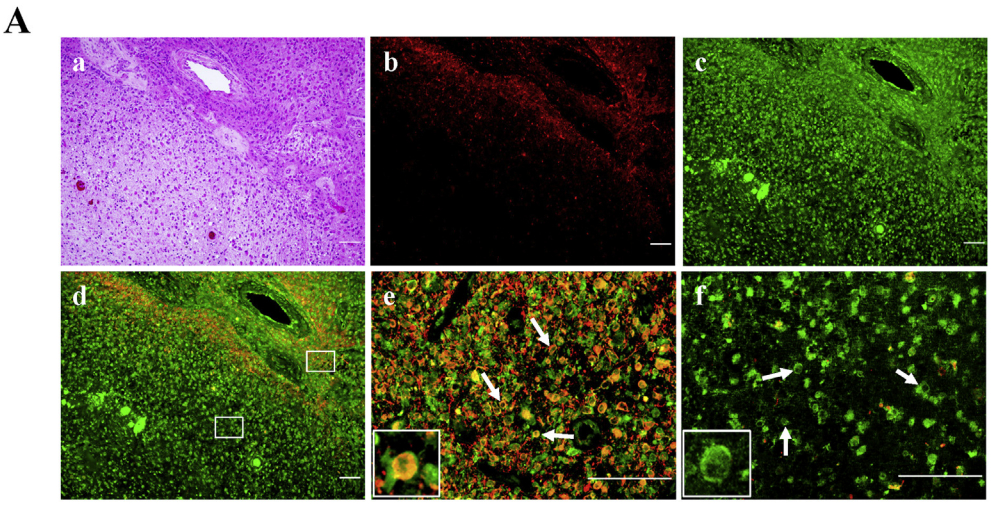

Fig. 6. mmunofluorescent staining of gliomas with antibodies against EphB4 and ephrin-B2.
(A) The upper right region of each image shows the tumor core. The lower left area shows the invasion area. The images depict H&E staining (a), EphB4-positive cells (b; red), ephrin-B2-positive cells (c; green), and double-positive cells (d; orange). The insets from panel d show the double-positive cells (e) and invasive area containing EphB4-negative/ephrin-B2-positive cells (f). Scale bar = 100 μm. (B) Columns represented EphB4 and ephrin-B2 positive cell rates at core and invasion area. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

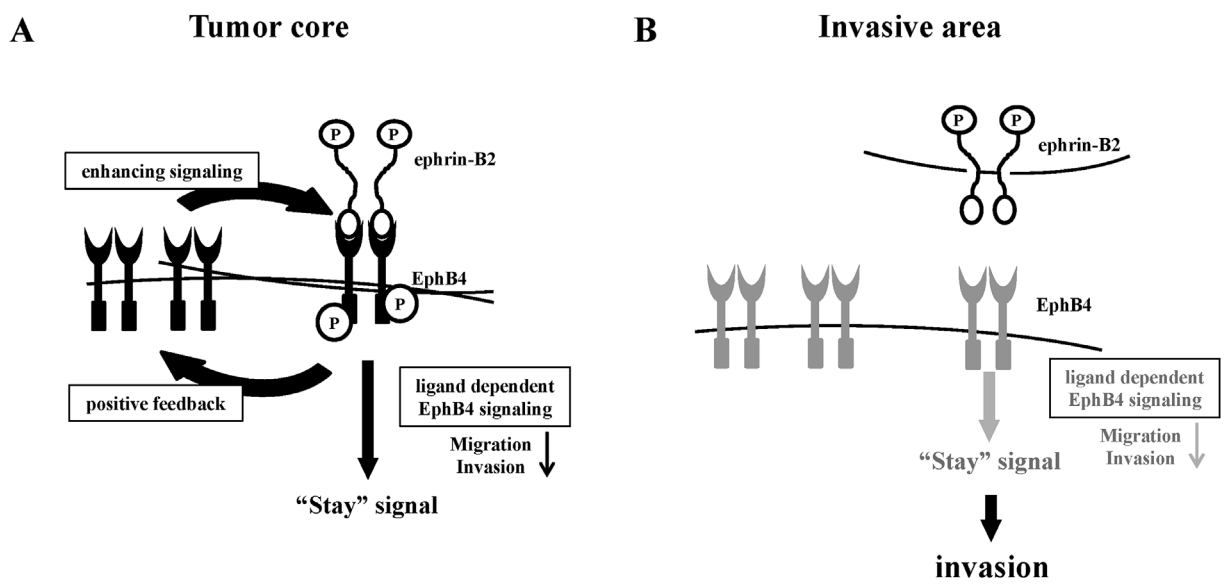

Fig. 7. Schematic diagram of the putative model of EphB4/ephrinB2 signaling.
(A) In the tumor core, ligand-dependent EphB4 signaling suppresses glioma invasion and migration. The interaction between EphB4/ephrin-B2 promotes the expression of EphB4, which enhances signaling in the presence of ephrin-B2. (B) In the invasive area, limited cell-cell contact prevents robust EphB4 signaling and EphB4 expression decreases due to the lack of positive feedback.

B2-dependent EphB4 signaling. We confirmed the effects of ephrin-B2 stimulation on EphB4-expressing and EphB4-suppressed cells because ephrin-B2 can signal through other receptors [29]. The results suggested that ephrin-B2-dependent EphB4 signaling is tumor suppressive. This suppressive function corresponds with its roles in other cancers, such as malignant melanoma [20] and prostate and breast cancer cells [12,30]. This is the first demonstration of the concentration-dependent anti-glioma effects of ligand-dependent EphB4 signaling.

Typical *in vitro* culture conditions yield little phosphorylated EphB4. Therefore, we were able to investigate ligand-independent EphB4 activation by comparing EphB4-expressing and EphB4-suppressed cells *in vitro* [14–16,33]. We found that ligand-independent, in contrast to ligand-dependent, EphB4 activation might promote glioma cell function. Previous reports also indicated that ligand-independent EphB4 activation promotes tumor function in breast cancer, mesothelioma, and ovarian cancer [33–36]. Chen et al. described an association between EphB4 expression and glioma cell tumorigenesis [9], but they did not examine Eph phosphorylation to distinguish between ligand-dependent and ligand-independent signaling. Our data complement their findings.

Intriguingly, we found that ephrin-B2 stimulates the upregulation of EphB4. An ephrin-Eph signaling feedback system has been reported for EphA2 [37,38], but never previously for EphB4. Analysis of this ephrin-B2-EphB4 positive feedback system in glioma cells is ongoing in our laboratory.

Our experiments revealed that the Akt phosphorylation levels corresponded to glioma migratory and invasive behavior after both ligand-dependent and ligand-independent activation. A previous report revealed that EphB4 receptor activation by ephrin-B2 in subchondral bone osteoblasts significantly inhibits Akt phosphorylation [39]. Other studies showed that EphB4 knockdown inhibits Akt phosphorylation in mesothelioma [36], ovarian cancer [34] and leukemia [40]. Akt regulates several critical cellular functions in cancers, including cell migration, invasion, survival, and angiogenesis [41] and the inhibition of Akt prevents glioma cell line growth [42]. In this study, we found that both ligand-dependent and -independent EphB4 activation are associated with glioma malignancy via Akt phosphorylation.

Ligand-dependent EphB4 signaling would likely function well in a cell-dense environment where cell-cell contact could be easily achieved. Both EphB4- and ephrin-B2-positive cells resided in the central, cell-dense area of the tumor. In addition, they are co-expressed in glioma tissue. Co-expression facilitates effective interaction between ligand and receptor [36,43]. Therefore, ligand-dependent EphB4 signaling is likely effective in the tumor core, a cell-dense environment where the ligand and receptor are co-expressed. Conversely, ligand-dependent EphB4 signaling may occur inefficiently in areas of low cellularity where cell-cell contact is rare. The ephrin-B2-EphB4 positive feedback system is similarly likely to function poorly in areas of low cellularity. A failure of the positive feedback system could be the cause for the minor EphB4 expression at the low-cellular density margins of the tumor. Consistently we previously reported EphB4 mRNA expression in glioma cell lines at the core and rim in *in vitro* migration assay. Four out of 5 glioma cell lines showed the expression level of EphB4 was higher at the core compared with rim [44]. This is the first report to show the alteration of EphB4 expression depending on the location of the tumor *in vivo*.

U87 and SNB 19 cells are long-term cultured differentiated cell lines that may not represent the biology of GBM cells in patients. Therefore, we added the experiments using glioma stem-like cell lines and obtained similar results. Ephrin-B2-EphB4 system is likely to function similarly at any stage of differentiation of glioma cells.

Our data support the hypothesis that ligand-dependent EphB4 signaling inhibits migration and invasion in highly cellular areas via the Akt pathway. A positive feedback system appears to enhance the effects of the signaling (Fig. 7A), whereas the dilution of the suppressive signaling in the low-cell density invasive area likely promotes location-specific tumor cell release (Fig. 7B). Thus, glioma cells may receive minimal migration and invasion signals in the tumor core, but encounter increased signals at the tumor border from where they are released into the normal brain tissue. Thus, EphB4 signaling retains glioma cells in the central portion of the tumor via a positive feedback loop and the liberation of the cells from EphB4 signaling may be responsible for their invasion into brain.

5. Conclusions

This study provides evidence that EphB4 receptor activation by ephrin-B2 suppresses glioma migration and invasion via the suppression of Akt phosphorylation. The results also indicate that EphB4 signaling occurs at the tumor core and that release from this signaling may be partially responsible for the spread of glioma cells into the periphery.

Author contributions

Author contributions to the study and manuscript preparation include the following. Conception and design: MN. Acquisition of data: YK, TF, HS, DY, MO. Analysis and interpretation of data: MN, YK. Drafting article: MN, YK. Critically revising the article: all authors. Reviewed final version of the manuscript and approved it for submission: all authors. Study supervision: MN.

Conflicts of interest statement

The authors declare that they have no conflict of interest.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.021.

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