EphA4 promotes cell proliferation and migration through a novel EphA4-FGFR1 signaling pathway in the human glioma U251 cell line

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Abstract

The Eph receptor tyrosine kinases and their ephrin ligands form a unique cell-cell contact-mediated bidirectional signaling mechanism for regulating cell localization and organization. High expression of Eph receptors in a wide variety of human tumors indicates some roles in tumor progression, which makes these proteins potential targets for anticancer therapy. For this purpose, we did gene expression profiling for 47 surgical specimens of brain tumors including 32 high-grade glioma using a microarray technique. The analysis, focused on the receptor tyrosine kinases, showed that EphA4 mRNA in the tumors was 4-fold higher than in normal brain tissue. To investigate the biological significance of EphA4 overexpression in these tumors, we analyzed EphA4-induced phenotypic changes and the signaling mechanisms using human glioma U251 cells. EphA4 promoted fibroblast growth factor 2-mediated cell proliferation and migration accompanied with enhancement of fibroblast growth factor 2-triggered mitogen-activated protein kinase and Akt phosphorylation. In addition, active forms of Rac1 and Cdc42 increased in the EphA4-overexpressing cells. Furthermore, we found that EphA4 formed a heteroreceptor complex with fibroblast growth factor receptor 1 (FGFR1) in the cells and that the EphA4-FGFR1 complex potentiated FGFR-mediated downstream signaling. Thus, our results indicate that EphA4 plays an important role in malignant phenotypes of glioblastoma by enhancing cell proliferation and migration through accelerating a canonical FGFR signaling pathway. [Mol Cancer Ther 2008;7(9):2768–78]

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Introduction

The Eph receptors represent the largest family of receptor protein tyrosine kinases and interact with their ligands, ephrins. The Eph receptors and ephrins are divided into two subclasses, A and B, based on their homologies, structures, and binding affinities (1). Fourteen Eph receptors and eight ephrin ligands have been identified thus far in mammals (2–4). Ephrin-A ligands are anchored to the plasma membrane by a glycosylphosphatidylinositol modification. Ephrin-B ligands have a transmembrane domain and a short cytoplasmic tail. The Eph-ephrin system relays a direct cell-cell contact-mediated bidirectional signaling pathway (5, 6). This bidirectional signaling is fundamentally involved in developmental processes (7–9) or in the remodeling of blood vessels (10, 11). Eph-ephrin signaling mainly affects the cell shape and motility by regulating cytoskeletal organization and cell adhesion and also influences cell proliferation and cell-fate determination (3). Therefore, it is speculated that Eph signaling could play some roles in tumorigenesis as one of their possible consequences.

More recently, the genes for Eph receptors and ephrins have been recognized to be differentially expressed in various human tumors including malignant melanoma, glioma, prostate cancer, breast cancer, small cell lung cancer, endometrial cancer, esophageal cancer, gastric cancer, and colorectal cancer (12–18). Profound distortion of expression patterns could be correlated with altered tumor behavior such as increased invasiveness or increased metastatic potential and consequently with poor patient outcome. Despite the widely observed phenomenon of Eph receptor overexpression, their role has not been fully elucidated in the process of malignant phenotypes. However, recent bodies of evidence have implicated Eph involvement in the tumor progression of human cancers including malignant gliomas (19, 20).

The fibroblast growth factor receptor (FGFR) also belongs to a family of receptor protein tyrosine kinases and consists of four members, FGFR1, FGFR2, FGFR3, and FGFR4. At the present, 23 FGF ligands have been cloned and known to bind to and activate FGFRs with their different affinities (21). The FGFR-associated substrate 2α (FRS2α) is a docking

protein that plays an important role in FGFR signaling. FRS2α binds constitutively to FGFR through its PTB domain at the NH_2 terminus and six tyrosine residues at the COOH terminus are phosphorylated by activated FGFR. Once FRS2α is phosphorylated, it recruits adaptor molecules such as Grb2 and Shp2 and relays the signals to the Ras/mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase/Akt pathway (22, 23). In addition, FGFR is known to direct cytoskeletal reorganization through regulating small GTPases, although its precise signaling mechanism has not been clarified (24).

The FGF growth factor family mediates a wide range of biological activities by binding to and activating the FGFR family (25). Aberrant FGFR signaling leads to diverse human pathologies including carcinogenesis. In gliomas, malignant progression from low to high grade appears to involve up-regulation of FGFR expression (26). FGFR2 is expressed abundantly in normal white matter and in all low-grade astrocytomas but is repressed in malignant astrocytomas. Conversely, FGFR1 expression is almost absent in normal white matter but is sufficiently expressed to be detected in malignant astrocytomas (27). Glioblastomas also express an alternatively spliced form of FGFR1 containing two immunoglobulin-like disulfide loops (FGFR1β), whereas normal human adult expresses FGFR1α, a form of the receptor containing three immunoglobulin-like loops. Intermediate grades of astrocytomas exhibit a gradual loss of FGFR2 and a shift in expression from FGFR1α to FGFR1β as they progress (28). Thus, FGFR1 signaling is suggested to be associated closely with malignant progression of astrocytes.

In this article, we would like to report that our gene expression study showed aberrant expression of the EphA4 receptor in high-grade astrocytic tumors and that the overexpressed EphA4 contributed to the malignant phenotype of the tumor cells through enhancing proliferation and migration by its interaction with FGFR1.

Materials and Methods

Microarray Gene Expression Analysis

Tumor specimens were obtained from 47 patients who underwent therapeutic removal under approved protocols from the institutional review board. Histologic diagnosis was made by light microscopic evaluation of the sections stained with H&E. The classification of human brain tumors was based on the WHO criteria for tumors of the nervous system (29). The 47 brain tumors consisted of 32 high-grade glioma and 15 low-grade glioma. Total RNA was extracted from tissue samples and assessed for quality. Amplified RNA from tumor and normal brain tissue was labeled with Cy5 and Cy3 (Amersham Biosciences), respectively. Hybridization target probes were prepared from total RNA and hybridized to the CodeLink Agilent Human I Bioarray chip according to the manufacturer's instructions (Amersham Biosciences). Microarray images were processed using Gene Spring software (Silicon Genetics). Intensity values were normalized using lowess normalization (30). Average fold differences in gene expression between tumor and normal brain were calculated. Basic data visualization, data filtering, and hierarchical clustering were done. The microarray raw data and clinical features has been submitted to Gene Expression Omnibus[5] (accession no. GSE4381) in our previous report (31).

Cell Culture

GP2-293 cells (Clontech) were cultured and maintained with DMEM with 10% fetal bovine serum and used as packaging cells for pseudo-retrovirus production. RPMI 1640 supplemented with 10% fetal bovine serum was used for each culture of U251 cells (human malignant astrocytoma cell line; Japanese Collection of Research Bioresources Cell Bank).

Expression Vector Construction and Viral Production

The expression constructs of EphA4 and FGFR1 were generated as follows. The full-length cDNA fragment encoding human EphA4 or FGFR1 was obtained from the U251 cells with the reverse transcription-PCR method using the primers EphA4-P1-F (CGGATCCACCATGGCTGGGATTTTCTATTTC), EphA4-R (GAAGCTTGACGGGAACCATTCTGCCGTGCATC), FGFR1-F (CGGATCCGAAATGTGGAGCTGGTGACCCCAGCA), and FGFR1-R (GAAGCTTGCGGCGTTTGAGTCCGCCATTGGCA). The DNA fragment for EphA4 DN lacking both juxtamembrane and kinase domains was generated with the recombinant PCR method using the following primers EphA4-P1-F, EphA4-P2-R (ACTGCTTGGTTGGGATCTTCATTCAAATGTTTCTCTTCAT), EphA4-P3-F (ATGAAGAGAAACATTTGAATGAAGATCCCAACCAAGCAGT), EphA4-P4-R (GAAGCTTGTTGCTGTTCACCAGGATGTTCC). First, two DNA fragments of EphA4 were amplified using P1-F/P2-R and P3-F/P4-R primer sets. Next, the recombined DNA fragment for EphA4 DN was amplified using the two PCR products as templates and the P1-F/P4-R primer set. The DNA fragment for FGFR1 DN lacking cytoplasmic domain was amplified with the primers, FGFR1-F and FGFR1(TM)-R (CAAGCTTCTTGTAGACGATGACCGACCCCAC). The sequences of all of the PCR-amplified DNAs were confirmed by sequencing after cloning into a pCR-Blunt II-TOPO cloning vector according to the manufacturer's instructions (Invitrogen). HA and myc tag were added at the COOH-terminal ends of EphA4 and FGFR1 constructs, respectively. All DNA fragments were cut out and transferred into a pQCLIN retroviral vector (BD Biosciences Clontech) together with enhanced green fluorescent protein (EGFP) following internal ribosome entry site sequence to monitor the expression of the inserts indirectly. A pVSV-G vector (Clontech) for constitution of the viral envelope and the pQCXIX constructs were cotransfected into the GP2-293 cells using a FuGENE6 transfection reagent (Roche Diagnostics). Briefly, 80% confluent cells

[5] http://www.ncbi.nlm.nih.gov/geo

cultured on a 10 cm dish were transfected with 2 μg pVSV-G plus 6 μg pQCXIX vectors. Forty-eight hours after transfection, the culture medium was collected and the viral particles were concentrated by centrifugation at 15,000 × *g* for 3 h at 4°C. The viral pellet was resuspended in fresh RPMI 1640. The viral vector titer was calculated by counting EGFP-positive cells, which were infected by serial dilution with virus-containing medium, and the multiplicity of infection was determined. These EphA4 and FGFR1 constructs are illustrated schematically in Fig. 2A.

Antibodies and Reagents

Recombinant human FGF2 and mouse ephrin-A1/Fc chimera were purchased from R&D Systems. Antibodies used for the study were as follows: anti-MAPK rabbit polyclonal, anti-phospho-MAPK rabbit polyclonal, anti-Akt rabbit polyclonal, anti-phospho-Akt rabbit polyclonal, and horseradish peroxidase-linked second antibodies (Cell Signaling); anti-HA and anti-myc monoclonal antibodies (Roche Diagnostics); anti-FRS2 rabbit polyclonal, anti-EphA4 rabbit polyclonal, anti-FGFR1 rabbit polyclonal, and anti-RhoA mouse monoclonal (Santa Cruz); anti-phosphotyrosine mouse monoclonal and anti-Rac1 and anti-Cdc42 mouse monoclonal (BD Biosciences). Rac GTPase-specific inhibitor was purchased from Calbiochem EMD Biosciences.

Cell Proliferation Assay

Cell proliferation was assessed using a CellTiter96 Aqueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, cells (1×10^3) serum-starved overnight were seeded on 96-well plastic plates with 200 μL culture medium supplemented with or without 0.5 μg/mL ephrin-A1/Fc or 20 ng/mL FGF2. After 72 h incubation at 37°C, 40 μL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt reagent was added followed by further incubation for 1 h. The absorbance at 490 nm was measured using a microplate reader.

Cell Migration Assay

The U251 cells were cultured in 12-well plates until confluence was reached. The monolayer cells were scratched out using a fine pipette tip after serum starvation overnight. To monitor the migration, pictures were taken at 0, 12, and 24 h after addition of ephrin-A1 or FGF2 using a fluorescence microscope (Keyence Biozero). The areas occupied by the cells were calculated with NIH image software (NIH). The ratio of the increased area by cell migration after 12 and 24 h to that at 0 h was calculated to quantitate the extent of migration.

Immunoblot Analysis

Cells that were serum starved for 24 h were treated with or without ephrin-A1/Fc or FGF2. The cells were washed with cold PBS and harvested with Lysis A buffer containing 50 mmol/L HEPES buffer, 1% Triton X-100, 5 mmol/L EDTA, 50 mmol/L sodium chloride, 10 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, and protease inhibitor mix, Complete (Roche Diagnostics). The lysate was clarified by centrifugation for protein analysis. The proteins separated with SDS-PAGE were transferred onto polyvinylidene difluoride membranes. After blocking with 3% bovine serum albumin in TBS (pH 8.0) with 0.1% Tween 20, the membrane was probed with the first antibody. After rinsing twice with TBS, the membrane was incubated with the horseradish peroxidase-conjugated second antibody (Cell Signaling) followed by visualization using a ECL detection system (Amersham Biosciences).

Pull-Down Assay

GTP-bound RhoA and Rac1/Cdc42 were pulled down from the cell lysate using agarose-conjugated GST-fused Rhotekin binding domain and GST-fused PAK-1 binding domain (Upstate), respectively, followed by quantitative detection by immunoblotting. Briefly, the cells were treated with or without ephrin-A1/Fc or FGF2 and lysed in magnesium-containing lysis buffer (25 mmol/L HEPES buffer, 150 mmol/L sodium chloride, 1% Triton X-100, 10% glycerol, 25 mmol/L sodium fluoride, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). After clarification, an equal amount of lysate was incubated with 20 μg agarose-conjugated GST-fused Rhotekin binding domain or GST-fused PAK-1 binding domain at 4°C for 3 h. The agarose beads were washed three times with magnesium-containing lysis buffer and the samples were analyzed with SDS-PAGE followed by immunoblotting.

Immunoprecipitation

The total protein extracted with Lysis A buffer (as described above) was incubated with the antibody at 4°C overnight. The antibody was immobilized with protein A agarose by incubation for a further 2 h. The immunoprecipitates were collected by centrifugation at 3,000 × *g* for 30 s. After the immunoprecipitates were washed four times with Lysis A buffer, the samples were analyzed with SDS-PAGE followed by immunoblotting.

Statistics

Comparisons were made using Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

High Expression of EphA4 mRNA in Malignant Glioma

We analyzed the expression of 13,156 clones from Incyte's human cDNA library genes using Agilent human cDNA microarrays (Agilent Technologies) for 32 malignant gliomas consisting of 10 anaplastic astrocytomas and 22 glioblastomas. We did hierarchical clustering of 103 tyrosine kinases (Fig. 1A). The histograms to determine the fold difference in the measured expression levels between tumors and normal brain are shown in Fig. 1B. Among several receptors with increased expression, we found the mRNA levels of the EphA4 receptor were significantly higher in glioma tissues (>4-fold) than those in normal brain tissues. There was a difference between anaplastic astrocytomas and glioblastomas with regard to EphA4 expression. The level in glioblastomas was higher than that in anaplastic astrocytomas. In low-grade gliomas, EphA4 expression level was also elevated but lower than

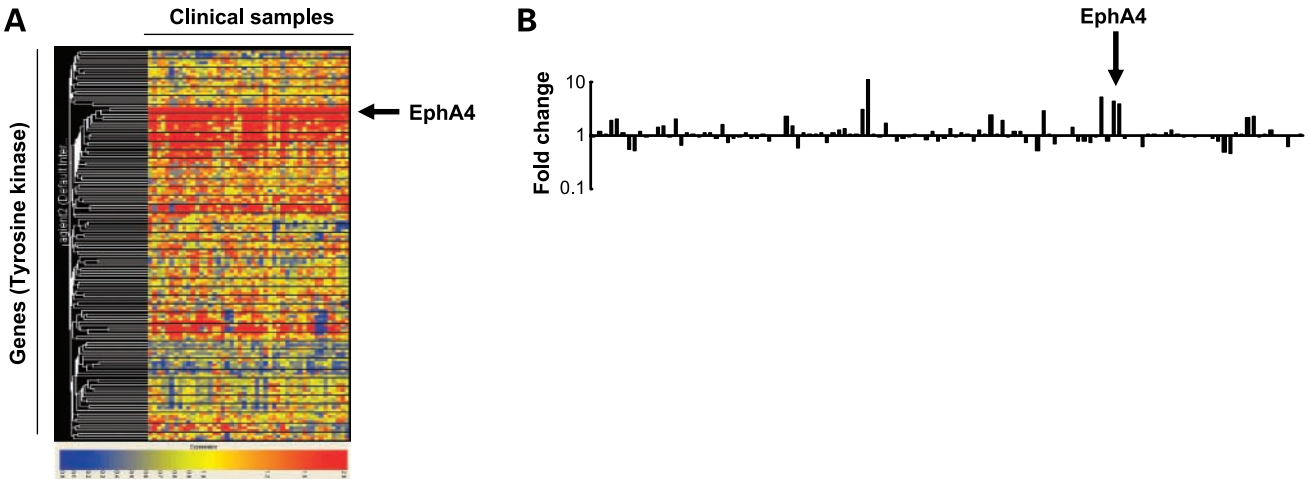

Figure 1. Profiles of 103 tyrosine kinases mRNA expression in 47 surgical samples of brain tumors. *Arrows,* EphA4 mRNA expression. **A,** hierarchical clustering of 103 tyrosine kinases. Each row and column shows a single gene and a tissue sample, respectively. The order of the clinical samples (*X axis*) corresponds to the WHO criteria from high grade to low. **B,** histogram of gene expression. The mRNA level of the EphA4 gene was significantly higher in glioma tissues (>4-fold) than in normal brain tissues.

that in high grade. EphA4 expression correlated with increasing tumor grade. The other transcripts with significantly higher expression were indicated in Supplementary Table.[6] Based on these results, we focused on the high expression level of EphA4 and examined the biological significance of EphA4 in gliomas.

Design of EphA4 and FGFR1 Constructs Used in This Study

Based on the previous evidence that EphA4 interacts with FGFR (32) and that, among the FGFR family, FGFR1 is most closely associated with the malignant progression of human glioma as described in Introduction, we sought to investigate how EphA4 affected FGFR signaling using human glioma U251 cells. For this study to explore the role of EphA4-FGFR1 interaction in glioma cell biology, it is vital to select glioma cell lines that express high levels of both EphA4 and FGFR1. However, these cell lines were not available, so we selected U251 cells, which have increased expression of FGFR1 (33), introduced EphA4 into U251 cells, and did our experiments using the transfected U251 cells.

Expression constructs of EphA4 and FGFR1 are summarized schematically in Fig. 2A. We generated a dominant-negative form of EphA4 (EphA4 DN), which lacked its juxtamembrane domain and COOH-terminal half of the kinase domain. It has been reported that EphA4 transphosphorylates FGFR through their direct interaction and that the NH_2-terminal region of the EphA4 kinase is necessary to interact with the FGFR (32). The juxtamembrane domain is postulated to close its interaction portion constitutively when EphA4 kinase is inactive. Therefore, this juxtamembrane-deleted EphA4 mutant can expose its binding site to FGFR although lacking transphosphorylation activity. In addition, this kinase-inactive mutant can also work as an EphA4 inhibitor against ephrin-ligand stimulation. On the contrary, a dominant-negative form of FGFR1 (FGFR1 DN) lacks the whole cytoplasmic domain, which inhibits the activation of intrinsic FGFR1 by FGF stimulation. These wild-type and dominant-negative constructs of both EphA4 and FGFR1 were retrovirally introduced into human glioma U251 cells and their protein expression was checked as seen in Fig. 2B. As the expression unit included an internal ribosomal entry site sequence followed by EGFP, we could monitor the expression by fluorescence microscopy in Fig. 2C.

EphA4 Promotes FGF2-Mediated Proliferation and Enhances FRS2, MAPK, and Akt Phosphorylation in U251 Cells

We evaluated the effect of EphA4 on cell proliferation of the U251 cells using the ectopic expression of EphA4 WT retrovirally. EphA4 WT did not affect cell proliferation significantly without FGF stimulation but promoted cell proliferation significantly in the presence of FGF2 at 20 ng/mL for 72 h compared with a mock control (Fig. 3A). Inversely, EphA4 DN expression as well as FGFR1 DN inhibited FGF2-triggered proliferation. FGF2-triggered proliferation of FGFR1 DN was higher than that with no ligand stimulation. The reason might be that FGFR1 DN could not completely block FGF2-mediated FGFR1 activation. However, FGF2-induced cellular responses were clearly inhibited in FGFR1 DN compared with those in the other cells. Ephrin-A1 stimulation seemed slightly to promote cell proliferation for EphA4 WT cells, FGFR1 DN-expressing cells, and mock control cells, but it was not statistically significant. These results suggested some strong correlation of EphA4 with FGFR signaling.

[6] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Then, we sought to examine whether downstream signals of the FGFR were modulated by overexpression of EphA4. First, we checked the phosphorylation level of FRS2, which was well known to relay the FGFR signals via its tyrosine phosphorylation. FRS2 phosphorylation by FGF2 stimulation was detectable in the mock control cells as shown in Fig. 3B. In EphA4 WT-introduced U251 cells, the FGF2-induced phosphorylation was more evident. Inversely, EphA4 DN, as well as FGFR1 DN, weakened FRS2 phosphorylation compared with the mock control cells. It was unlikely that EphA4 promoted FRS2 phosphorylation directly because ephrin-A1 stimulation itself did not induce FRS2 phosphorylation in any cells. This promotive effect of EphA4 on FRS2 phosphorylation was considered to occur possibly through activated FGFR following FGF2 stimulation.

Next, we checked the phosphorylation levels of MAPK and Akt, which were well known to affect cell proliferation and survival, respectively. Those were further downstream signaling molecules of FGFR and were also relayed via FRS2 phosphorylation. From time-course analysis of phosphorylated MAPK and Akt stimulated by 20 ng/mL FGF2, we found that their phosphorylation level reached a peak at around 15 min. Although MAPK was not phosphorylated by Ephrin-A1 stimulation, Akt-phosphorylation was clear and peaked at 30 min (data not shown). We therefore selected 15 and 30 min to evaluate the degree of MAPK and Akt phosphorylation by FGF2 and ephrin-A1, respectively. Phosphorylated MAPK and Akt and their total proteins are shown in Fig. 3C (*left* and *right*, respectively). FGF2 stimulation clearly phosphorylated MAPK and Akt; especially, EphA4 WT augmented FGF2-induced phosphorylation of MAPK and Akt compared with the mock control cells (Fig. 3D). On the contrary, EphA4 DN as well as FGFR1 DN attenuated FGF2-mediated MAPK and Akt phosphorylation. Notably, the baseline phosphorylation levels of both MAPK and Akt were moreover increased in the EphA4 WT-introduced U251 cells without ligand stimulation but inhibited in the EphA4 DN-introduced cells. Ephrin-A1 stimulation showed an almost undetectable increase of phospho-MAPK but a small increase of phospho-Akt. EphA4 DN

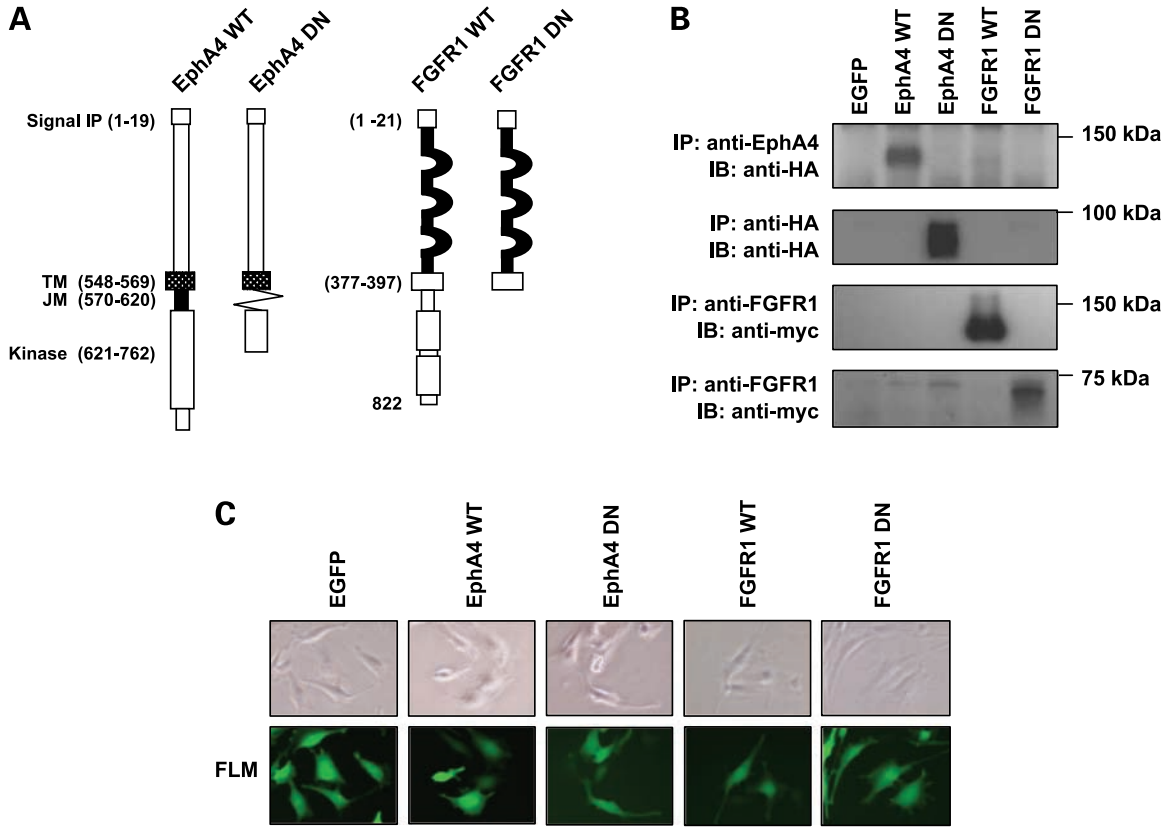

Figure 2. Construction of expression vectors for either the wild-type (*WT*) or the dominant-negative (*DN*) form of EphA4 or FGFR1. **A,** schematic representation of EphA4 and FGFR1 constructs. *Numbers,* amino acids. EphA4 DN is a deletion mutant lacking the juxtamembrane (570-620) and COOH-terminal half of EphA4 kinase domain (763-986) amino acids. FGFR1 DN is also a deletion mutant without whole cytoplasmic domain. *TM,* transmembrane domain; *JM,* juxtamembrane domain. **B,** checking the expression of EphA4 and FGFR1 constructs in U251 cells. EphA4 and FGFR1 constructs were tagged with HA and myc, respectively, at the COOH terminus. Each lysate (500 μg) was immunoprecipitated and detected by immunoblotting with the HA or myc antibody. *IB,* immunoblotting; *IP,* immunoprecipitation. **C,** microscopic imaging of EGFP coexpressing with the introduced proteins of interest. *FM,* fluorescent microscopy.

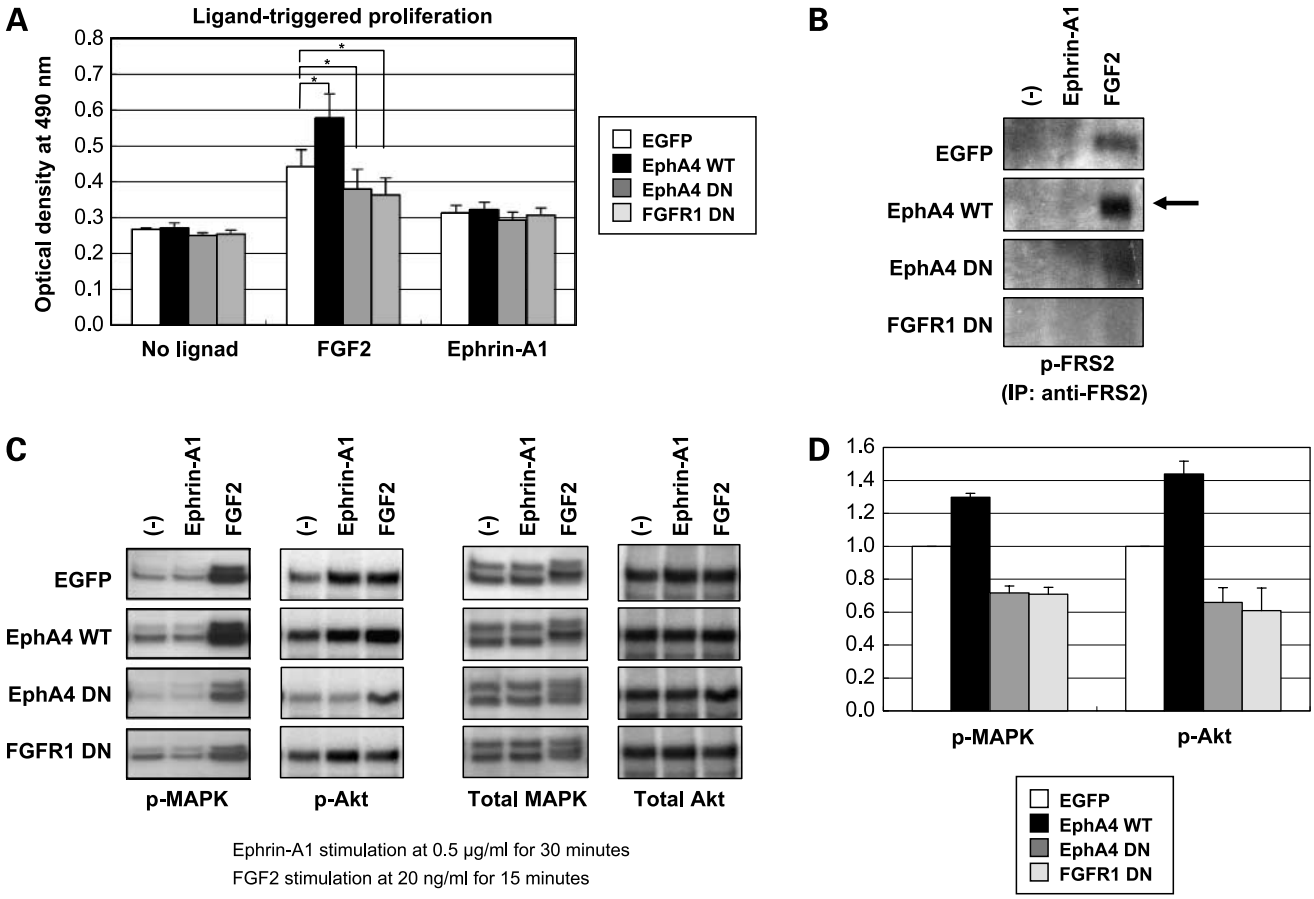

Figure 3. Effects of EphA4 WT on proliferation in the U251 cells. **A**, cell proliferation judged by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. Serum-starved cells (1×10^3) were plated on 96-well plates in the absence or presence of ligands (20 ng/mL FGF2 or 0.5 μg/mL ephrin-A1). The absorbance at 490 nm of each well was measured after 72 h incubation. *Bars,* SD. *, $P < 0.05$ versus the EGFP cells. **B**, effect of EphA4 on FRS2 phosphorylation. Serum-starved cells were treated with 20 ng/mL FGF2 for 15 min or 0.5 μg/mL ephrin-A1 for 30 min. The immunoprecipitated samples using an anti-FRS2 antibody were checked by immunoblotting with an anti-phosphotyrosine antibody. **C**, effect of EphA4 on phosphorylation of MAPK and Akt. Each cell lysate (10 μg) treated with 20 ng/mL FGF2 for 15 min or 0.5 μg/mL ephrin-A1 for 30 min was analyzed by immunoblotting. *Left,* probing with anti-phospho-MAPK and anti-phospho-Akt antibodies; *right,* reprobing with anti-total MAPK and anti-total Akt antibodies. **D**, relative densitometric units of the phospho-MAPK and phospho-Akt bands in each cell stimulated by FGF2. The densities of the EGFP bands are set arbitrarily at 1.0. *Bars,* SD.

blocked this increase, but FGFR1 DN did not, suggesting that ephrin-A1-triggered Akt phosphorylation was not mediated by FGFR1.

EphA4 Also Promotes FGF2-Mediated Migration Accompanied with Increased Active Rac1/Cdc42

To study the effect of EphA4 on cell migration, we did a scratch wound assay using the U251 cells ectopically expressing EGFP, EphA4 WT, EphA4 DN, or FGFR DN and checked the extent of their migration after scratching by monitoring EGFP for 24 h (Fig. 4A). If the incubation time is so long that migrating cells are able to proliferate, the migration rate could be affected by proliferation. Therefore, we analyzed the migration rate before proliferation might have a significant effect on this analysis (Fig. 4B). It was evident that EphA4 WT promoted FGF2-stimulated cell migration to cover the scratched area compared with the mock control and EphA4 DN (Fig. 4A, *top*). Under the same experimental condition, FGF2 stimulation caused a small increase in cell proliferation and the difference of proliferation was quite limited among the transfected cells (Supplementary Fig. S1).[6] These results imply that the wound closing process is due to cell migration activity triggered by FGF2. To evaluate this quantitatively, we assayed in triplicate and calculated the migration area (by square inches) using NIH imaging software and illustrated this graphically (Fig. 4B, *top*). EphA4 WT-expressing cells significantly migrated more than EGFP-expressing, EphA4 DN-expressing, or FGFR1 DN-expressing cells for 24 h. Interestingly, the promotion of cell migration by EphA4 WT was also observed under ephrin-A1 stimulation (Fig. 4A, *bottom*). As for ephrin-A1 treatment, cell proliferation was slightly induced in the transfected cells (Fig. 3A). Therefore, promoted wound closure means increased activity of cell migration triggered by ephrin-A1.

As Rho family GTPases have been known to play key roles in cell migration by regulating actin dynamics, we

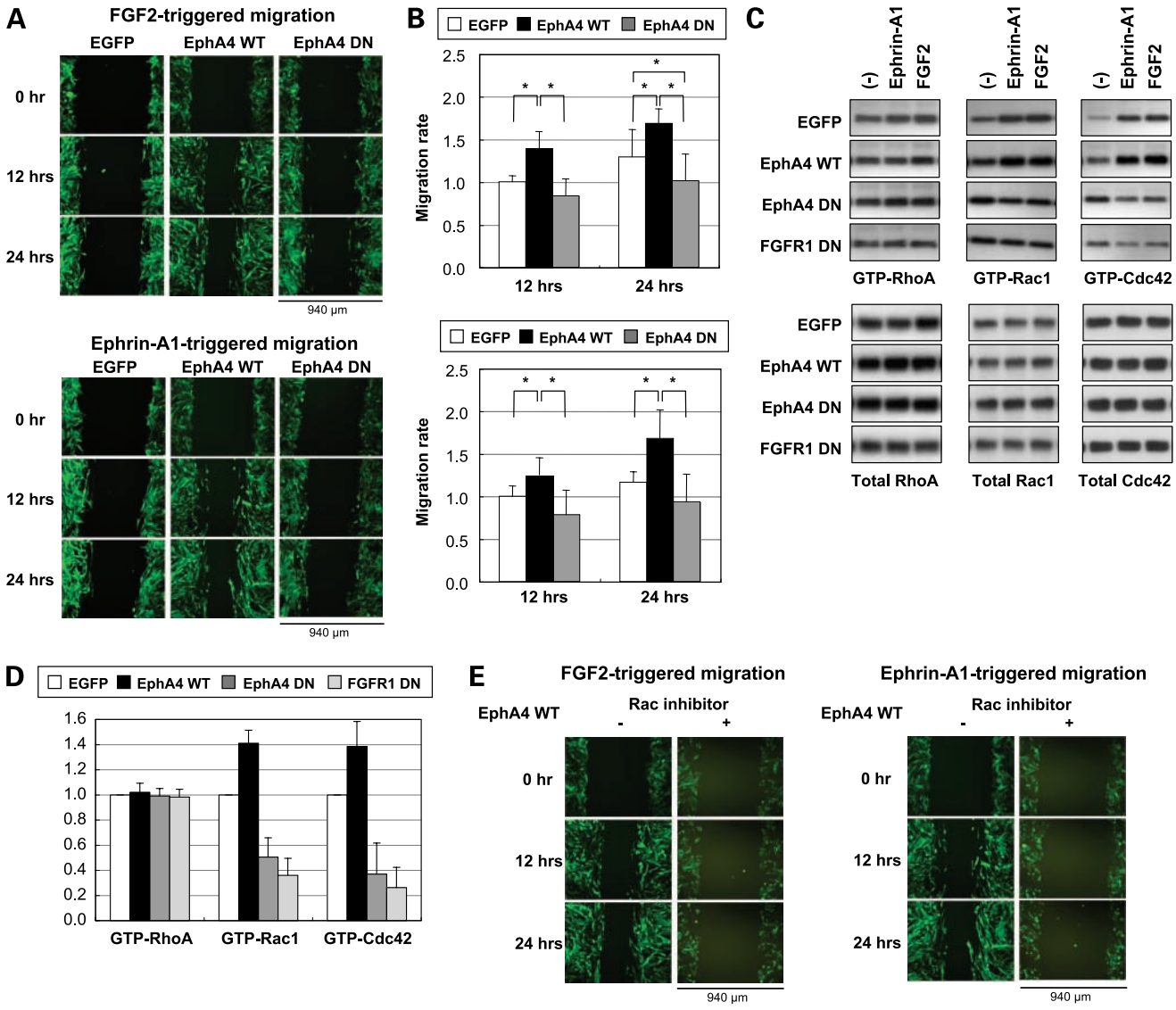

Figure 4. Effect of EphA4 WT on migration in the U251 cells. **A,** evaluation of cell migration by scratch wound assay. Serum-starved cells were cultured in the presence of 20 ng/mL FGF2 (*top*) or 0.5 μg/mL ephrin-A1 (*bottom*). The degree of migration was checked by monitoring EGFP. Pictures were shown at 12 and 24 h after ligand stimulation. **B,** extent of cell migration was quantified and shown graphically (*right*). The areas occupied by the cells were calculated with NIH image software. The ratio of the increased area after 12 and 24 h to that at 0 h was then calculated to quantitate the extent of migration. *Bars,* SD. *, $P < 0.05$ versus the EGFP cells. **C,** effects on activation of Rho GTPases. *Top,* active RhoA, Rac1, and Cdc42 of each cell stimulated by 20 ng/mL FGF2 for 15 min or 0.5 μg/mL ephrin-A1 for 30 min. The active Rho GTPases were pulled down as described in Materials and Methods and checked by immunoblotting using the indicated antibodies. *Bottom,* protein expression of three Rho GTPases using whole protein lysate. **D,** relative densitometric units of the GTP-RhoA, Rac1, and Cdc42 bands in each cell stimulated by FGF2. The densities of the EGFP bands are set arbitrarily at 1.0. *Bars,* SD. **E,** effect of Rac inhibitor on ligand-triggered migration in the transfected U251 cells of EphA4 WT. Cell migration was evaluated by scratch wound assay. Serum-starved cells were cultured in the presence of 20 ng/mL FGF2 (*left*) or 0.5 μg/mL ephrin-A1 (*right*) plus 50 μmol/L Rac inhibitor. The degree of migration was checked by monitoring EGFP. Pictures were shown at 0, 12, and 24 h after stimulation.

sought to examine how the activity of Rho GTPases would be affected by EphA4. Rho GTPase activities were evaluated by immunoblotting following a pull-down assay. FGF2 stimulation activated Rac1/Cdc42 as shown in Fig. 4B. EphA4 WT promoted activation of Rac1/Cdc42 stimulated by FGF2, whereas EphA4 DN, as well as FGFR1 DN, inhibited their activation, suggesting EphA4 promotes FGF2-mediated activation of Rac1/ Cdc42. Similarly, Rac1/Cdc42 activities stimulated by ephrin-A1 were also promoted by EphA4 WT but were inhibited by EphA4 DN, suggesting EphA4 promotes ephrin-A1-mediated activation. Importantly, the activation was inhibited by FGFR1 DN. As shown in Fig. 3A, ephrin-A1 did not induce FRS2 phosphorylation; therefore, it was suggested that the effect of EphA4 WT on ephrin-A1-triggered activation of Rac1/Cdc42 was, at least in part, through FGFR-mediated

signaling not involving FRS2. Consistent with these results, cotreatment of Rac inhibitor with FGF2 or ephrin-A1 drastically inhibited the wound closure in the scratch wound assay (Fig. 4E), showing that Rac inhibition blocked FGF2- or ephrin-A1-triggered glioma cell migration. On the other hand, neither ephrin-A1 nor FGF2 stimulation induced any RhoA activity in EGFP-expressing mock control cells and the activity was not modulated by EphA4 WT, EphA4 DN, or FGFR1 DN. These results suggest that EphA4 enhances FGF2-induced activation of Rac1/Cdc42 and promotes glioma cell migration.

EphA4-FGFR1 Heteroreceptor Complex Enhances FGF2-Triggered Phosphorylation of FGFR1

Based on the results that EphA4 promoted FGF2-mediated cell proliferation and migration as we have shown thus far, we hypothesized a functional relationship between EphA4 and FGFR1, which would affect FGFR1 activity. First, we checked their physiologic interaction by the ectopic expression of EphA4 WT and/or FGFR1 WT retrovirally using the U251 cells. FGFR1 was not detected to be coimmunoprecipitated with EphA4 in EGFP-expressing control cells (Fig. 5A). When stimulated by Ephrin-A1, coimmunoprecipitation of FGFR1 was detectable in EphA4 WT- or FGFR1-expressing U251 cells. This complex formation was most evident in EphA4- and FGFR1-coexpressing cells, meaning that activated EphA4 prefers to forming a receptor complex with FGFR1 in the U251 cells.

To clarify the functional effect of EphA4 on FGFR1 activation, we next examined the tyrosine phosphorylation level of FGFR1 in the U251 cells (Fig. 5B). Without FGF2 stimulation, neither EphA4 WT, EphA4 DN, nor FGFR1 DN affected the phosphorylation level of intrinsic FGFR1. FGFR1 was naturally phosphorylated by FGF2 stimulation in EGFP-expressing cells. This phosphorylation was further enhanced by EphA4 WT. Contrarily, EphA4 DN or FGFR1 DN inhibited FGFR1 phosphorylation by FGF2, revealing that EphA4 strengthened FGF2-mediated phosphorylation of FGFR1. Under this experimental condition, EphA4 was weakly phosphorylated by exogenous addition of FGF2 (Supplementary Fig. S3).[6] These results showed that EphA4 enhanced FGF2-triggered activation of FGFR1 through a EphA4-FGFR1 heteroreceptor complex, which possibly explained the reason that EphA4 promoted downstream signaling of FGFR and contributed to glioma cell proliferation and migration. On the other hand, ephrin-A1 stimulation did not lead to enhanced phosphorylation of FGFR1 in this experimental condition, although EphA4 was clearly phosphorylated by ephrin-A1 (Supplementary Fig. S2).[6] This might, in part, correlate to the result that FRS2 was phosphorylated by FGF2 not by ephrin-A1 (Fig. 3B).

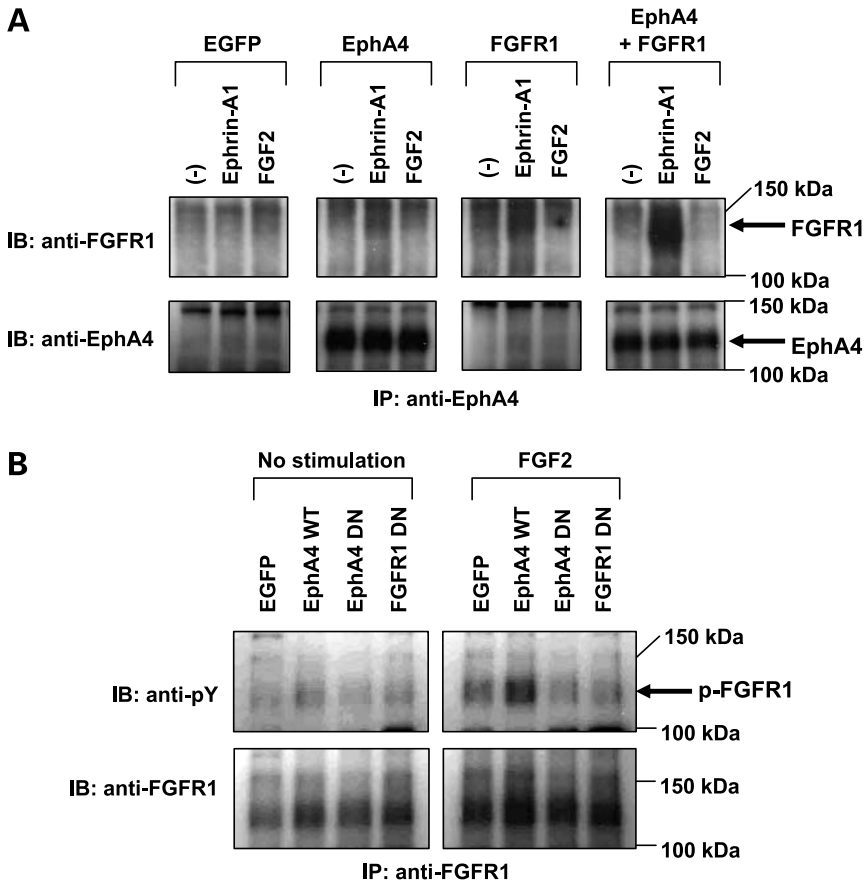

Figure 5. EphA4-FGFR1 interaction. **A,** EphA4 forms a protein complex with FGFR1. The U251 cells coexpressing EphA4 and FGFR1 were used in addition to those expressing EGFP, EphA4, and FGFR1. *Top,* EphA4 protein was immunoprecipitated from the cell lysate treated with or without 0.5 μg/mL ephrin-A1 for 30 min (ephrin-A1) or 20 ng/mL FGF2 for 15 min (FGF2) and separated by SDS-PAGE followed by immunoblotting using an anti-FGFR1 antibody. *Bottom,* reprobing with an anti-EphA4 antibody. **B,** enhanced phosphorylation of FGFR1 by EphA4. Each cell was treated with or without 40 ng/mL FGF2 for 15 min (FGF2) and the tyrosine phosphorylation level of FGFR1 was examined by probing using an anti-phosphotyrosine antibody (*top*). The total levels of precipitated FGFR1 were detected by reprobing with an anti-FGFR1 antibody (*bottom*).

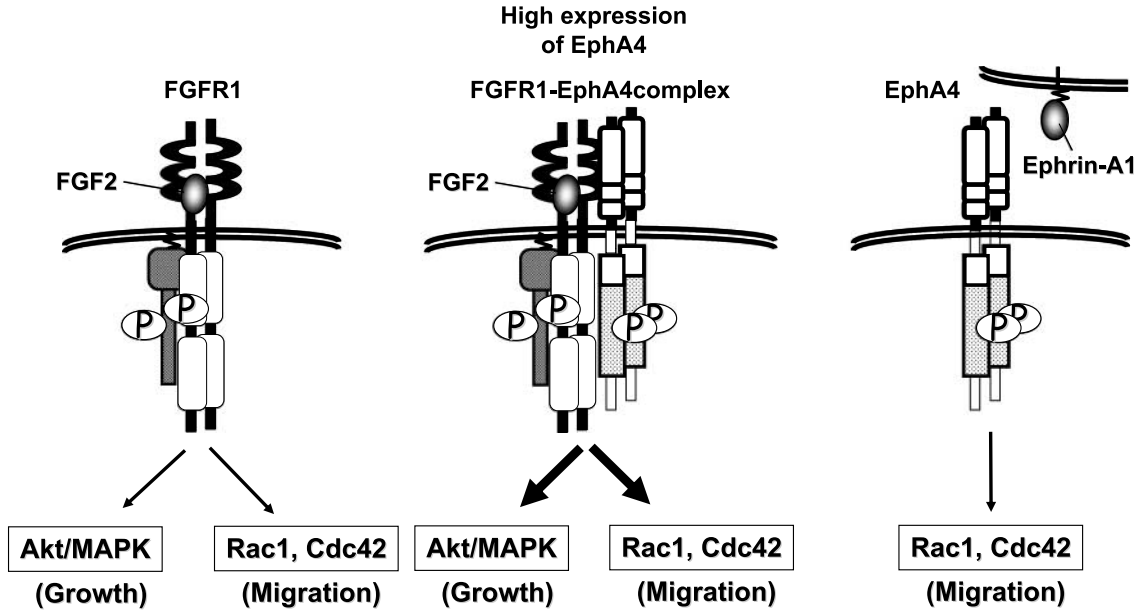

Figure 6. A diagram of the proposed mechanism of enhanced FGFR signaling by EphA4 overexpression.

Discussion

Our study revealed that highly expressed EphA4 in malignant gliomas functions as an accelerator of glioma cell proliferation and migration through promoting the FGFR1 signaling pathway as summarized in Fig. 6, suggesting that EphA4 might be a potential therapeutic target for malignant glioma.

Our microarray gene expression analysis for 32 surgical specimens of high-grade astrocytomas showed that EphA4 mRNA was expressed 4-fold higher in tumors than in normal brain tissue. Elevated expression of Eph members has been described in various human cancers (18). EphA4 expression has also been reported in cancers such as prostate, colon, and melanoma, although it has never been described in association with malignant gliomas. Among the Eph receptor family, EphA2, EphA5, and EphB2 expression has been described previously in glioblastoma cells (20, 34–36). Bruce et al. have identified aberrant EphA5 expression and showed that activation of EphA5 does not promote cell proliferation in glioma cell line (34). Nakada et al. reported phosphorylation of R-Ras associated with EphB2 effects on glioma cell adhesion, growth, and invasion (20, 35). Recently, EphA2 elevated in glioblastoma multiforme stimulated by ephrin-A1 was reported to induce an inhibitory effect on the growth and invasiveness of glioblastoma multiforme cells (36). As shown in Fig. 4A and *B* (*bottom*), the promotion of cell migration by EphA4 WT was observed under ephrin-A1 stimulation. Correspondingly, Rac1/Cdc42 activities stimulated by ephrin-A1 were promoted by EphA4 WT (Fig. 4C and *D*). Although, to the best of our knowledge, EphA4 has never been reported about glioma cell motility, these results suggest that ephrin-A1-triggered activation of EphA4 enhances Rac1/Cdc42 activation and promotes glioma cell migration (Fig. 6).

Recently, Nakada et al. revealed that ephrin-B3 promotes glioma invasion using U251 that is a high expressor cell line of ephrin-B3 and that knockdown of ephrin-B3 in U251 resulted in morphologic change and decreased migration and invasion induced by EphB2 (37). Although, in this article, EphA4 was not discussed in association with ephrin-B3, there are some previous reports about the interaction between ephrin-B3 and EphA4 (38, 39). In summary, these studies provide the evidence that EphrinB3/EphA4 has an important role in neuronal circuit formation, growth, and development. Based on this information, there is a possibility that the interaction between ephrin-B3 and EphA4 plays some role in the malignant phenotype of cancer. However, as shown in Supplementary Fig. S2A,[6] EphA4 was not phosphorylated even in the EphA4-overexpressing U251 cells without ligand stimulation, although ectopically expressing EphA4 could be activated by endogenous ephrin-B3. There was no significant difference between the mock control and the EphA4-overexpressing cells without ligand stimulation in glioma cell proliferation and migration (Figs. 3A and 4A). Together with these data, the interaction between endogenous ephrin-B3 and EphA4 did not appear to affect the results in our experimental condition.

The MAPK pathway is well known to play a major role in the tumor cell proliferation (40). There are several reports regarding the regulation of this pathway by Eph signaling (6). However, some studies report negative regulation of MAPK pathway by Eph receptor activation (41–43). Reversely, others show positive regulation of the pathway by Eph signaling, although the precise mechanism has not

been shown (44–46). In addition, Yokote et al. reported a positive-regulating mechanism of the MAPK pathway by EphA4 through direct interaction with the FGFR receptor and its activation (32). Our report also showed that FGFR1 signaling was enhanced by EphA4 through their interaction followed by an increased MAPK pathway in the U251 glioma cells. Corresponding to the increased MAPK, proliferation of glioma cells was also promoted by EphA4. It was important that this enhancement was dependent on FRS2 phosphorylation, which was suggested by the findings that either FGFR1 DN or EphA4 DN inhibited FRS2-MAPK signaling enhanced by EphA4. In addition, it may be considered that the glioma cells have little or less negatively regulating molecules for the MAPK pathway such as Ras-GAP but dominantly possess a positively regulating mechanism such as the enhancement of FGFR signaling by EphA4. From our results that ephrin-A1 stimulation alone did not affect the MAPK pathway, it is considered that the strength of these two negative and positive regulation may be different among the cell types, which may explain the discrepancy in the previous results regarding the regulation of the MAPK pathway by Eph receptors.

The FGFR-mediated phosphatidylinositol 3-kinase/Akt signaling pathway was also enhanced by EphA4, which was suggested by the findings that phospho-Akt was increased by EphA4 WT but inhibited by EphA4 DN (Fig. 3C). However, the Akt pathway was found to be activated by ephrin-A1 stimulation itself and to be inhibited by EphA4 DN. In addition, FGFR1 DN had no effect on the Akt pathway mediated by ephrin-A1 stimulation. This means that EphA4 has its own Akt activating pathway, except for the enhancement of FGFR-mediated Akt pathway in the U251 cells as reported that Akt pathway was mediated by Eph receptors (44).

Rho family GTPases are signaling molecules influencing cell motility by cytoskeletal reorganizing (46). Eph signaling modulates the balance of Rho versus Rac and Cdc42 activities (3, 6). As shown in Fig. 4, neither ephrin-A1 nor FGF2 changed the Rho activity of the U251 cells. The reason might be that the U251 glioma cells expressed little ephexin, a guanine nucleotide exchanging factor for Rho, which was identified as a direct signaling molecule relayed from EphA4 (data not shown; ref. 47). On the contrary, Rac1 and Cdc42 were both activated by FGF2 or ephrin-A1 stimulation and EphA4 further enhanced these activities. Interestingly, both Rac1 and Cdc42 activations were found to be inhibited by EphA4 DN and FGFR1 DN. This means that FGFR1 and EphA4 are both necessary to activate Rac1 and Cdc42 by either FGF2 or ephrin-A1 and a close functional association is suggested between their receptors. These results were consistent with the promoted migration by EphA4 shown in Fig. 4A and could explain why EphA4 enhanced the migration in the U251 cells. The contribution of other types of guanine nucleotide exchanging factors for Rac1 or Cdc42 might be important for the enhanced migration, but the precise mechanism remains to be elucidated.

As shown in Fig. 5, we clearly showed that EphA4 formed a complex with FGFR1 and FGFR1 activity was enhanced by EphA4. These results supported the reason why EphA4 enhanced FGFR-mediated MAPK, Akt pathway, and Rac1/Cdc42 activities. These findings were supported by the report showing the interaction between EphA4 and FGFR (32). This report also showed the direct association between two molecules and how they assembled to relay their signal. It is noteworthy that different types of receptor protein tyrosine kinases such as EphA4 and FGFR1 are able to interact with each other and enhance their functions. This means cross-talk between the receptors may exist at the plasma membrane and could produce a more diverse signaling mechanism in living cells. In the glioma cells, the EphA4-FGFR1 interaction is considered to promote proliferation and migration by acting as a enhancer for the malignant phenotype.

Long-term survival of patients with malignant gliomas has improved very little despite aggressive multimodality treatments including surgery, radiotherapy, and cytotoxic chemotherapy (48). One limitation of the conventional treatment is that these therapeutic strategies are not properly based on the malignant glioma-specific biological properties. Therefore, it is important to know the molecular-based characteristics (bioinformatics) of malignant gliomas to achieve a therapeutic breakthrough. Recently, the molecular targeting approach has been developed in treating many types of cancer (49). To realize the molecular targeting therapy in malignant gliomas, one strategy is to identify key molecules using the microarray technique (50). From this view, we analyzed gene expression in malignant gliomas using a DNA microarray and found high expression of EphA4 in malignant gliomas. We then clarified the functional significance of the overexpression of EphA4 using a human glioma cell line, U251 cells. Our collective results provide the typical strategy to analyze functionally the molecule that has been identified from the gene expression study. We expect EphA4-FGFR1 signaling may become a candidate as a potential target in therapeutic intervention for malignant gliomas and cell-based screening for an EphA4-specific inhibitor is ongoing based on our findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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