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ORIGINAL ARTICLE

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Epidermal growth factor receptor promotes glioma progression by regulating xCT and GluN2B-containing *N*-methyl-D-aspartate–sensitive glutamate receptor signaling

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Autocrine and paracrine factors, including glutamate and epidermal growth factor (EGF), are potent inducers of brain tumor cell invasion, a pathological hallmark of malignant gliomas. System xc(–) consists of xCT and CD98hc subunits and functions as a plasma membrane antiporter for the uptake of extracellular cystine in exchange for intracellular glutamate. We previously showed that the EGF receptor (EGFR) interacts with xCT and thereby promotes the activity of system xc(–) in a kinase-independent manner, resulting in enhanced glutamate release in glioma cells. However, the molecular mechanism underlying EGFR-mediated glioma progression in a glutamate-rich microenvironment has remained unclear. Here we show that the GluN2B subunit of the *N*-methyl-D-aspartate–sensitive glutamate receptor (NMDAR) is a substrate of EGFR in glioma cells. In response to EGF stimulation, EGFR phosphorylated the COOH-terminal domain of GluN2B and thereby enhanced glutamate-NMDAR signaling and consequent cell migration in EGFR-overexpressing glioma cells. Treatment with the NMDAR inhibitor MK-801 or the system xc(–) inhibitor sulfasalazine suppressed EGF-elicited glioma cell migration. The administration of sulfasalazine and MK-801 also synergistically suppressed the growth of subcutaneous tumors formed by EGFR-overexpressing glioma cells. Furthermore, shRNA-mediated knockdown of xCT and GluN2B cooperatively prolonged the survival of mice injected intracerebrally with such glioma cells. Our findings thus establish a central role for EGFR in the signaling crosstalk between xCT and GluN2B-containing NMDAR in glioma cells.

KEYWORDS
epidermal growth factor receptor, glioma, GluN2B, glutamate, xCT

Kentaro Suina and Kenji Tsuchihashi contributed equally to this work.

1 | INTRODUCTION

Expression of the epidermal growth factor receptor (EGFR) influences the malignant behavior of many types of cancer cells in manners dependent on or independent of the kinase activity of the receptor.[1,2] Amplification of the *EGFR* gene is the most common genetic alteration in malignant gliomas, with EGFR overexpression having been associated with increased tumor invasion and chemoresistance.[3-5] Infiltration of tumor cells into brain tissue is a pathological hallmark of malignant gliomas and is enhanced by autocrine and paracrine factors, including chemokines and growth factors such as epidermal growth factor (EGF).[6] Extracellular glutamate released from glioma cells has also been shown to act as an autocrine factor that promotes malignant behavior in these cells.[7,8]

Malignant gliomas that release glutamate often manifest an invasive growth pattern that is characterized by the induction of an inflammatory response in the surrounding tissue that results in neuronal death and tumor expansion.[9] System xc(–) comprises xCT and CD98hc subunits and is a major plasma membrane antiporter responsible for the cellular uptake of cystine in exchange for intracellular glutamate.[10] We previously showed that the intracellular domain of EGFR interacts with and thereby promotes the surface expression of xCT in glioma cells in a manner independent of receptor kinase activity, resulting in increased cystine uptake and glutamate release.[8] The released glutamate then acts as an autocrine factor to enhance glioma progression through interaction with glutamate receptors at the cell surface.[11,12]

Glutamate receptors include both ionotropic and metabotropic receptors and mediate excitatory neurotransmission.[13] Glutamate released from malignant gliomas can achieve excitotoxic levels and promote tumor cell proliferation and migration through activation of ionotropic receptors.[9,14] Ionotropic glutamate receptors are ligand-gated ion channels that are activated by glutamate and comprise α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR), kainate receptor, delta receptor and *N*-methyl-D-aspartate receptor (NMDAR) subtypes.[13,15,16] Inhibitors of AMPAR or NMDAR have been shown to attenuate the proliferation and migration of colon adenocarcinoma, astrocytoma, and breast and lung carcinoma cells, suggesting that these ionotropic glutamate receptors may promote malignant behavior in multiple types of cancer.[17]

Although EGFR, xCT and glutamate receptors are all thought to promote malignant behavior in glioma cells, it has remained unclear how their status affects such behavior promoted by EGF and glutamate. We have now investigated the signaling crosstalk between EGFR and glutamate receptors in glioma cells as well as its functional relevance to glioma progression.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human glioma cell lines T98G and U87MG were obtained from the ATCC (Manassas, VA, USA). U87MG cells stably expressing human EGFR (U87MG-E cells) were kindly provided by M. Nagane (Kyorin University Faculty of Medicine, Tokyo, Japan).[18] All cells were maintained under 5% CO_2 at 37°C in DMEM containing glucose at 4.5 g/L (Nacalai, Kyoto, Japan) and supplemented with 10% FBS.

2.2 | Reagents

Recombinant human EGF was obtained from PeproTech (Rocky Hill, NJ, USA), gefitinib was from Tocris Bioscience (Minneapolis, MN, USA), and sulfasalazine, MK-801, GYKI-52466 and glutamate were from Sigma-Aldrich (St. Louis, MO, USA).

2.3 | Flow cytometric analysis

Cultured cells were dissociated by exposure to cell dissociation buffer (Thermo Fisher Scientific, Tokyo, Japan), and the resulting single-cell suspensions were incubated with primary antibodies for 30 minutes at 4°C. The cells were then washed with PBS, and those that had been exposed to nonlabeled primary antibodies were incubated with fluorescently labeled secondary antibodies (Thermo Fisher Scientific) for 20 minutes at 4°C and then washed again with PBS. Apoptotic cells were excluded during flow cytometric analysis by elimination of cells positive for staining with propidium iodide. Flow cytometry was performed with a FACSCalibur instrument (BD Biosciences, Tokyo, Japan).

2.4 | Assay of glutamate

Parental U87MG (U87MG-P) or U87MG-E cells were transferred to 6-well plates (1×10^6 cells per well), cultured for 12 hours, washed with PBS, and cultured for an additional 8 hours in 2 mL of glutamate-free medium (DMEM with glucose at 4.5 g/L; Nacalai) in the absence or presence of sulfasalazine (400 μmol/L). The amount of glutamate released into culture supernatants was then measured with the use of a fluorometric glutamate assay kit (Abcam, Tokyo, Japan).

2.5 | In vitro chemotaxis assay

U87MG-P or U87MG-E cells were suspended in serum-free DMEM containing glucose at 4.5 g/L (1×10^5 cells/mL), and 200-μL portions of the cell suspension were transferred to the upper chamber of inserts with a pore size of 8.0 μm (BD Biosciences). The inserts were placed in the wells of a 24-well plate filled with 700 μL of serum-free DMEM containing glucose at 4.5 g/L and supplemented with EGF (10 ng/mL). Sulfasalazine (200 μmol/L), MK-801 (100 μmol/L) or GYKI-52466 (100 μmol/L) was added to the upper medium of the inserts, whereas glutamate (250 μmol/L) was added to both the upper and lower medium. After culture for 10 hours, cells on the upper side of the inserts were removed with cotton swabs, and those attached to the lower surface were fixed and then stained with Diff-Quick (Sysmex, Kobe, Japan). Stained cells were counted in 6 fields per

insert, and the average number was calculated. The assay was performed in triplicate.

2.6 | Measurement of intracellular Ca^{2+} concentration

Cells were transferred to 96-well plates (4×10^4 cells per well), cultured overnight, and then loaded with Fluo-4-AM (Wako, Osaka, Japan) at 1 μmol/L and Hoechst 33342 (Thermo Fisher Scientific) for 30 minutes at 37°C. The cells were then incubated at 37°C in phenol red–free DMEM containing glucose at 4.5 g/L (Thermo Fisher Scientific) and supplemented with 10% FBS and either MK-801 or gefitinib. The fluorescence intensity of Fluo-4 was measured every 5 minutes with a microplate reader and was normalized with Hoechst 33342 staining.

2.7 | Immunoprecipitation analysis

For immunoblot analysis, cells were lysed in radioimmunoprecipitation (RIPA) buffer supplemented with phosphatase and protease inhibitors (PhosSTOP and Complete; Roche Diagnostics, Tokyo, Japan), and equal amounts of lysate protein were subjected to SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and then exposed to primary antibodies. Immune complexes were detected with HRP-conjugated secondary antibodies and enhanced chemiluminescence reagents (Nacalai). For immunoprecipitation, cells were lysed on ice for 20 minutes in RIPA buffer supplemented with PhosSTOP. The lysates were then incubated on ice first for 1 hour with an isotype control antibody (IgG) or with antibodies to EGFR (Abcam) and then for 1 hour in the additional presence of protein A-conjugated Sepharose beads (GE Healthcare, Tokyo, Japan) or Dyna Beads (Thermo Fisher Scientific). The beads were isolated by centrifugation and washed 3 times with RIPA buffer, after which the bead-bound proteins were subjected to immunoblot analysis.

2.8 | Immunofluorescence analysis

U87MG-E cells were fixed with 4% paraformaldehyde and incubated overnight at 4°C with antibodies to EGFR (Abcam) or to the tyrosine-phosphorylated form of GluN2B (Sigma-Aldrich). Immune complexes were detected with Alexa Fluor 488 (or Alexa Fluor 594) conjugated secondary antibodies (Thermo Fisher Scientific), and the cells were then stained for 5 minutes with Hoechst 33342 (Thermo Fisher Scientific) to detect nuclei. Images were acquired with an FV1000-D confocal microscope (Olympus, Tokyo, Japan).

2.9 | Growth of subcutaneous tumors and drug treatment in vivo

U87MG-E cells (2×10^6) were implanted subcutaneously in the flank of nude mice. The mice were injected intraperitoneally with physiological saline, sulfasalazine (250 mg/kg), MK-801 (1 mg/kg) or both drugs once a day for 18 days beginning 3 days after cell injection. All animal experiments were performed in accordance with protocols approved by the Ethics Committee of Keio University.

2.10 | Quantitative RT-PCR analysis

Total RNA was extracted from cells with the use of an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and was subjected to RT with a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Quantitative PCR analysis was performed with the use of a Thermal Cycler Dice Real Time System (Takara Bio, Otsu, Japan). The amplification protocol comprised an initial incubation at 95°C for 2 minutes and 40 cycles of incubation at 95°C for 30 seconds and 60°C for 30 seconds, and was followed by dissociation-curve analysis to confirm specificity. The analysis was performed with human primer sets (forward and reverse, respectively) for xCT (5′-CAGGAGAAAGTGCAGCTGAA-3′ and 5′-CTCCAATGATGGTGCCAATG-3′) and GluN2B (5′-TTCCGT AATGCTCAACATCATGG-3′ and 5′-TGCTGCGGATCTTGTTTACA AA-3′). Data were normalized by the abundance of GAPDH mRNA.

2.11 | Orthotopic glioma model

Orthotopic implantation of U87MG-E cells (2.5×10^5) expressing control, xCT (Santa Cruz Biotechnology, Dallas, TX, USA) or GluN2B shRNAs (GeneCopoeia, Rockville, MD, USA) was performed as described previously.[19]

2.12 | Statistical analysis

Quantitative data are presented as means ± SEM and were compared between 2 groups with the 2-tailed unpaired Student's *t* test or among 3 or more groups by one-way ANOVA followed by Tukey's post hoc test. Survival differences were statistically assessed by the Kaplan–Meier method and log-rank test. A P-value < .05 was considered statistically significant. Statistical analysis and graph construction were performed with GraphPad Prism software (San Diego, CA, USA).

3 | RESULTS

3.1 | xCT-mediated glutamate release promotes glioma cell migration via *N*-methyl-D-aspartate–sensitive glutamate receptor signaling

To examine the possible role of glutamate release through system xc(−) in EGF-elicited glioma cell migration, we studied U87MG glioma cells stably expressing EGFR (U87MG-E cells) and parental U87MG (U87MG-P) cells.[8,20] Consistent with our previous results,[8] the abundance of the xCT subunit of system xc(−) at the cell surface was greater in U87MG-E cells than in U87MG-P cells (Figure 1A). The extent of EGF-elicited chemotaxis in U87MG-E cells was approximately 8 times that in U87MG-P cells (Figure 1B), indicating that the high level of EGFR expression in the former cells enhances their EGF-dependent

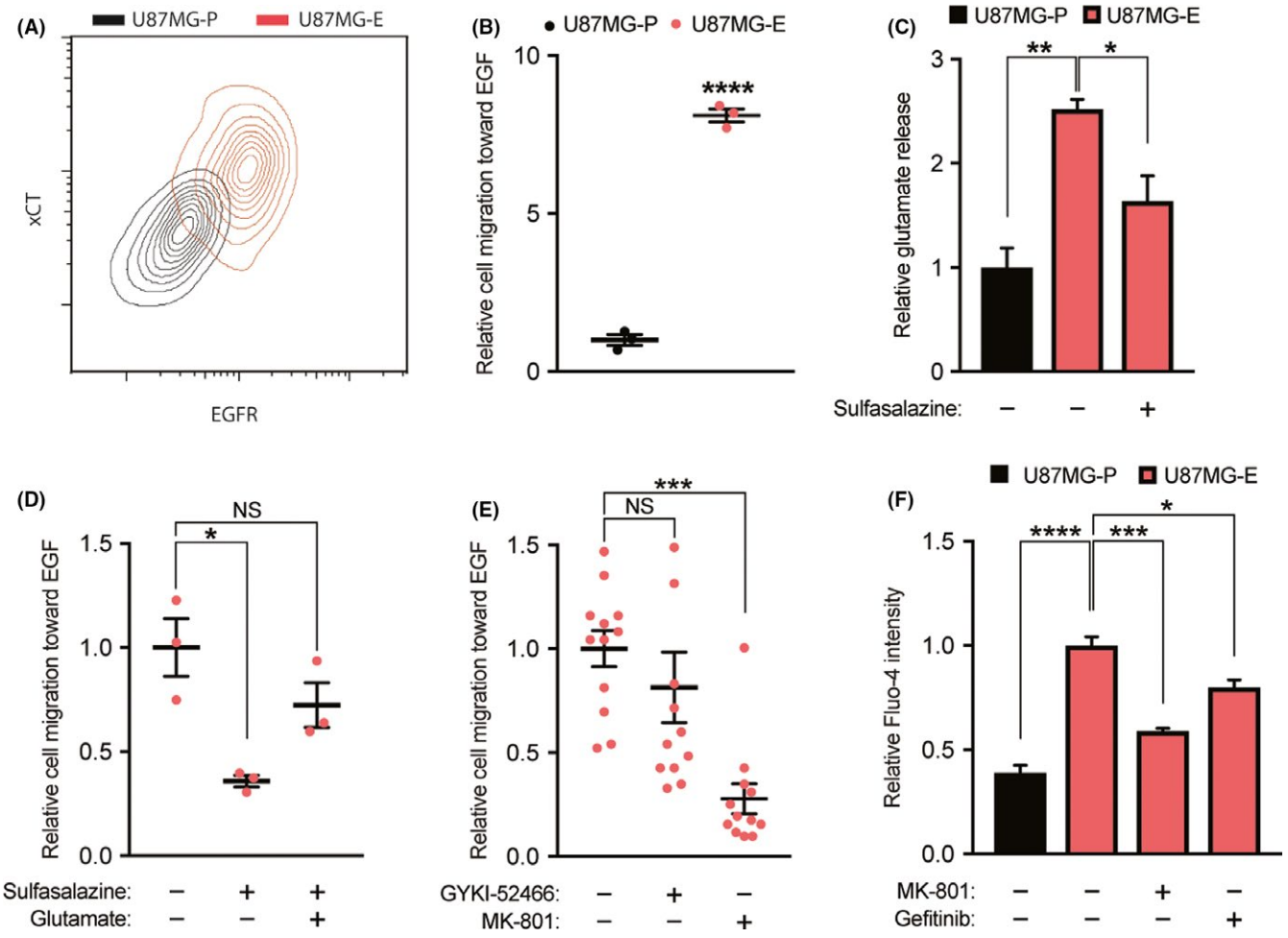

FIGURE 1 Epidermal growth factor (EGF)-elicited glioma cell migration is influenced by xCT and *N*-methyl-D-aspartate–sensitive glutamate receptor. A, Flow cytometric analysis of cell surface expression of EGF receptor and xCT in U87MG-E and U87MG-P cells. B, Assay of U87MG-P and U87MG-E cell migration toward EGF (10 ng/mL). C, Glutamate release by U87MG-P or U87MG-E cells cultured in glutamate-free medium for 8 hours in the absence or presence of 400 μmol/L sulfasalazine. D, U87MG-E cells were assayed for migration toward EGF in the absence or presence of 200 μmol/L sulfasalazine or glutamate (250 μmol/L). E, Assay of U87MG-E cell migration toward EGF in the absence or presence of 100 μmol/L GYKI-52466 or 100 μmol/L MK-801 (n = 12 for each group). F, Measurement of $[Ca^{2+}]_i$ on the basis of Fluo-4 fluorescence intensity in U87MG-P or U87MG-E cells exposed (or not) to 250 μmol/L MK-801 or 2 μmol/L gefitinib for 10 minutes. Data in (B) to (F) are expressed relative to the corresponding control and are means ± SEM of 3 independent experiments. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001; NS, not significant (2-tailed Student's *t* test [B] or 1-way ANOVA followed by Tukey's post hoc test [C-F]). ROS, reactive oxygen species

migratory activity. To examine whether glutamate release through system xc(−) contributes to the EGF-dependent migration of U87MG-E cells, we treated the cells with the xCT inhibitor sulfasalazine. The extent of glutamate release was significantly increased in U87MG-E cells compared with the parental cells, and this increase was largely abrogated in the presence of sulfasalazine (Figure 1C). Furthermore, the EGF-dependent migration of U87MG-E cells was also significantly attenuated by sulfasalazine treatment, whereas this inhibitory effect was largely prevented in the additional presence of glutamate (Figure 1D). These results suggested that glutamate release through system xc(−) plays a key role in EGF-elicited chemotaxis in glioma cells that express EGFR at a high level.

To shed light on the molecular mechanism underlying the effect of glutamate on the chemotactic response of U87MG-E cells, we examined the functional relevance of ionotropic glutamate receptors, including NMDAR and AMPAR. EGF-elicited chemotaxis in U87MG-E cells was significantly attenuated by the NMDAR inhibitor MK-801 but not by the AMPAR inhibitor GYKI-52466 (Figure 1E), implicating NMDAR, but not AMPAR, in the enhancement of glioma cell chemotaxis by glutamate. Together, our observations thus suggested that activation of NMDAR signaling by glutamate released from EGFR-overexpressing glioma cells in an xCT-dependent manner promotes EGF-elicited chemotaxis in these cells.

Given that activation of NMDAR results in the influx of extracellular Ca^{2+} and thereby promotes cell motility,[21,22] we measured the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in U87MG-P and U87MG-E cells. The fluorescence intensity of the Ca^{2+}-sensitive dye Fluo-4 in U87MG-E cells was significantly increased compared with that in U87MG-P cells and was significantly reduced by treatment with MK-801 (Figure 1F), suggesting that Ca^{2+} signaling by NMDAR

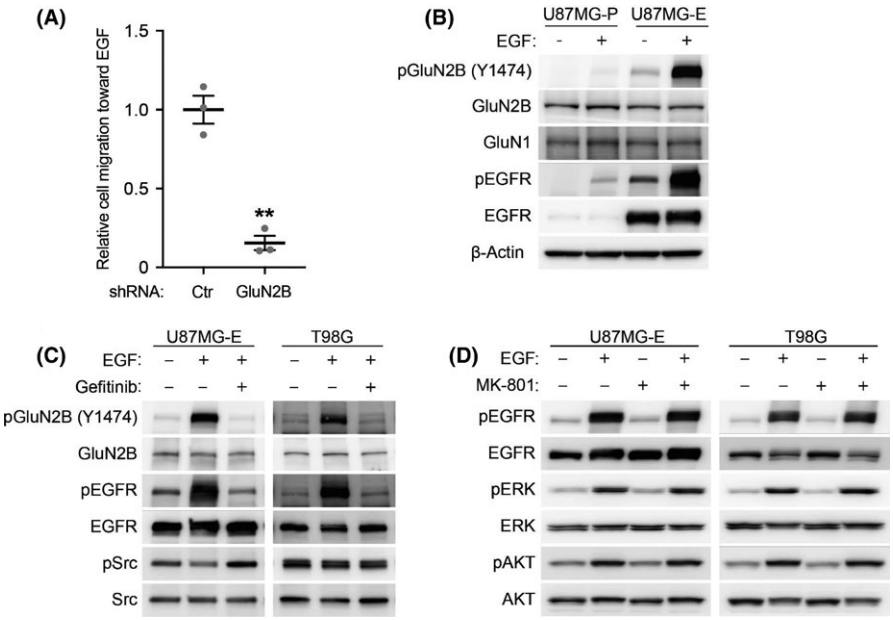

FIGURE 2 Epidermal growth factor (EGF) stimulation induces phosphorylation of the GluN2B subunit of *N*-methyl-D-aspartate–sensitive glutamate receptor (NMDAR) in EGF receptor (EGFR)-overexpressing glioma cells. A, U87MG-E cells stably expressing control (Ctr) or GluN2B shRNA were assayed for cell migration toward EGF. Data are means ± SEM of 3 independent experiments. $^{**}P < .01$ vs control shRNA (2-sided Student's *t* test). B, Immunoblot analysis of total or tyrosine-phosphorylated (pGluN2B, Y1474) forms of GluN2B, of the NMDAR subunit GluN1, and of total or phosphorylated (p) forms of EGFR in U87MG-P and U87MG-E cells that had been incubated in the absence or presence of EGF (20 ng/mL) for 20 minutes. β-Actin was examined as a loading control. C, Immunoblot analysis of total or phosphorylated forms of GluN2B, EGFR and Src (negative control) in U87MG-E and T98G cells that had been incubated in the absence or presence of EGF (20 ng/mL) or 2 μmol/L gefitinib for 20 minutes. D, Immunoblot analysis of total or phosphorylated forms of EGFR, ERK, and AKT in U87MG-E and T98G cells that had been incubated in the absence or presence of MK-801 (100 μmol/L) for 1 hour before stimulation with EGF (50 ng/mL) for 20 minutes

is activated in the EGFR-overexpressing glioma cells. To examine whether EGFR kinase activity affects such NMDAR-Ca^{2+} signaling, we treated U87MG-E cells with the EGFR kinase inhibitor gefitinib. Gefitinib significantly reduced $[Ca^{2+}]_i$ in U87MG-E cells, although this effect was less pronounced than that of MK-801 (Figure 1F). These results thus suggested that NMDAR-Ca^{2+} signaling is regulated not only by extracellular glutamate but also by EGFR kinase activity in EGFR-overexpressing glioma cells.

3.2 | Epidermal growth factor receptor activation results in tyrosine phosphorylation of the *N*-methyl-D-aspartate–sensitive glutamate receptor subunit GluN2B

Given that the expression of the GluN2B subunit of NMDAR has been associated with malignancy of multiple types of cancer, including glioma,[23] we examined whether GluN2B plays a role in EGF-dependent glioma cell migration. The shRNA-mediated knockdown of GluN2B was found to significantly attenuate EGF-elicited chemotaxis of U87MG-E cells (Figure 2A), suggesting that EGFR kinase activity might modulate GluN2B function during EGF-dependent glioma cell migration.

Given that tyrosine phosphorylation of the COOH-terminal domain of GluN2B increases receptor activity,[23,24] we next examined whether EGFR signaling might affect this phosphorylation event. EGF stimulation markedly increased the phosphorylation of GluN2B on Tyr^{1474} as well as that of EGFR in U87MG-E cells but not in U87MG-P cells (Figure 2B), suggesting that EGF induced activation of both EGFR and GluN2B selectively in U87MG-E cells. Furthermore, the EGF-induced tyrosine phosphorylation of GluN2B in U87MG-E cells as well as in T98G glioma cells, which express endogenous EGFR and xCT at high levels, was completely suppressed in the presence of gefitinib (Figure 2C), indicating that EGFR kinase activity is required for the EGF-induced tyrosine phosphorylation of GluN2B in EGFR-overexpressing glioma cells.

We also examined whether NMDAR signaling affects EGFR signaling in these glioma cells. Treatment with the NMDAR inhibitor MK-801 had no effect on the EGF-induced phosphorylation of EGFR or that of the major downstream signaling molecules ERK and AKT in U87MG-E or T98G cells (Figure 2D), suggesting that EGFR signaling is independent of NMDAR activity in these cells.

3.3 | Epidermal growth factor receptor interacts with and phosphorylates GluN2B

To provide insight into the molecular mechanism underlying EGF-induced GluN2B activation, we next examined whether GluN2B interacts with EGFR. Immunofluorescence analysis revealed the colocalization of EGFR and tyrosine-phosphorylated GluN2B in U87MG-E cells (Figure 3A). Furthermore, immunoprecipitation analysis revealed that EGFR interacts with GluN2B in U87MG-E and T98G cells, and

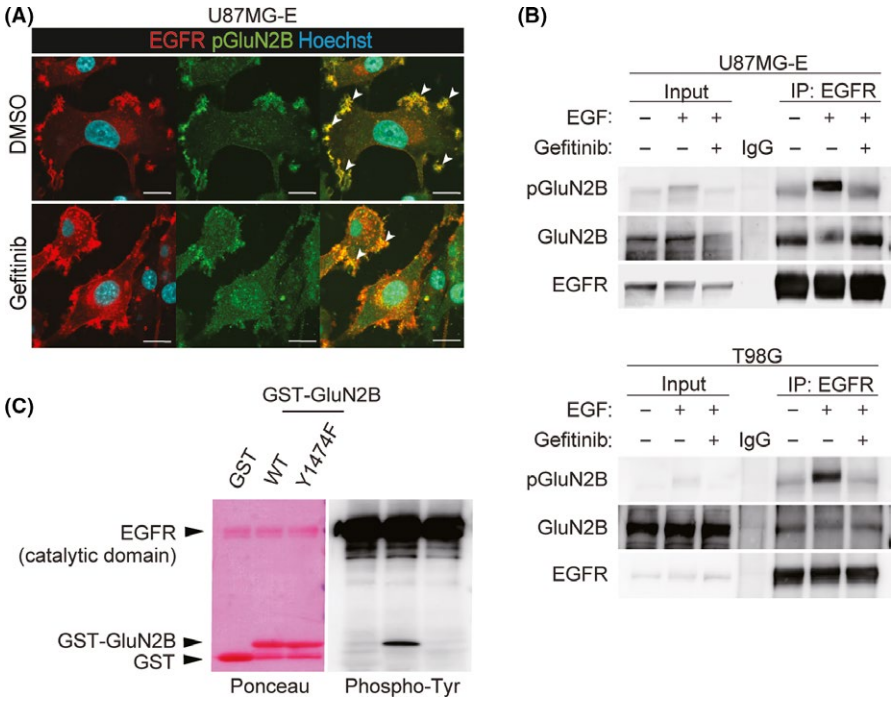

FIGURE 3 Epidermal growth factor receptor (EGFR) interacts with and phosphorylates GluN2B in glioma cells. A, Immunofluorescence analysis of EGFR (red) and tyrosine-phosphorylated GluN2B (pGluN2B, green) in U87MG-E cells that had been incubated in the presence of gefitinib (2 μmol/L) or DMSO vehicle for 2 hours. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 20 μm. Arrowheads indicate colocalization of EGFR and phosphorylated GluN2B at the cell surface. B, U87MG-E or T98G cells that had been incubated in the absence or presence of epidermal growth factor (EGF) (20 ng/mL) or gefitinib (2 μmol/L) for 20 minutes were lysed and subjected to immunoprecipitation (IP) with antibodies to EGFR or with control IgG. The resulting precipitates, as well as the original cell lysates (Input), were subjected to immunoblot analysis with antibodies to phospho-GluN2B (Y1474), to GluN2B and to EGFR. C, GST fusion protein containing the catalytic domain of EGFR was assayed for kinase activity in vitro with GST or GST fusion proteins containing WT or Y1474F mutant forms of the COOH-terminal region of GluN2B. The reaction mixtures were subjected to SDS-PAGE followed by staining with Ponceau S or by immunoblot analysis with antibodies to phosphotyrosine

that such interaction was not affected by treatment of the cells with EGF or gefitinib (Figure 3B). Together, these results thus indicated that EGFR constitutively interacts with GluN2B and may phosphorylate its COOH-terminal domain in response to EGF stimulation, resulting in NMDAR activation in EGFR-overexpressing glioma cells.

To investigate whether EGFR, indeed, directly phosphorylates GluN2B, we prepared a peptide corresponding to the COOH-terminal region of GluN2B that contains Y1474 as well as a mutant (Y1474F) form of this peptide containing phenylalanine instead of this lone tyrosine residue. An in vitro kinase assay revealed that the Y1474 peptide of GluN2B, but not the Y1474F peptide, was directly phosphorylated by EGFR (Figure 3C), suggesting that GluN2B is, indeed, a substrate of the EGFR tyrosine kinase and that phosphorylation of GluN2B at Y1474 might play a role in the EGF-elicited chemotaxis of EGFR-overexpressing glioma cells.

3.4 | Inhibition of system xc(–) and *N*-methyl-D-aspartate–sensitive glutamate receptor activity suppresses glioma growth in vivo

Finally, we evaluated the potential of xCT-targeted and NMDAR-targeted therapy for EGFR-overexpressing glioma. Administration of sulfasalazine or MK-801 significantly attenuated the growth of subcutaneous tumors formed by U87MG-E cells in nude mice, and combination therapy with both drugs inhibited tumor growth to a greater extent than either monotherapy (Figure 4A). These results thus suggested that xCT and NMDAR contribute to the growth of tumors formed by EGFR-overexpressing cells. To further examine the impact of dual inhibition of xCT and NMDAR on tumor growth, we performed the immunohistochemical analysis. Tumors formed by EGFR-expressing glioma manifest high levels of EGFR and phosphorylated GluN2B in tumor cells (Figure S1A). Combination therapy with sulfasalazine and MK-801 was found to increase the expression of cleaved caspase-3, a marker of apoptosis (Figure S1B). Together, xCT and NMDAR may play a role in EGFR-expressing glioma growth through the suppression of cell death in vivo.

To further examine whether xCT and NMDAR, indeed, are potential therapeutic targets for glioma in the brain microenvironment, we performed RNAi-mediated depletion of xCT, GluN2B, or both proteins in U87MG-E cells and evaluated the survival of mice with orthotopic tumors formed by these cells (Figure 4B). Stable depletion of xCT or GluN2B prolonged survival compared with that observed in mice implanted with control U87MG-E cells, and combined knockdown of both proteins resulted in a further extension of survival (Figure 4C). In contrast, the cell proliferation rate of U87MG-E cells in vitro was not affected by the stable depletion of xCT or GluN2B (data not shown).

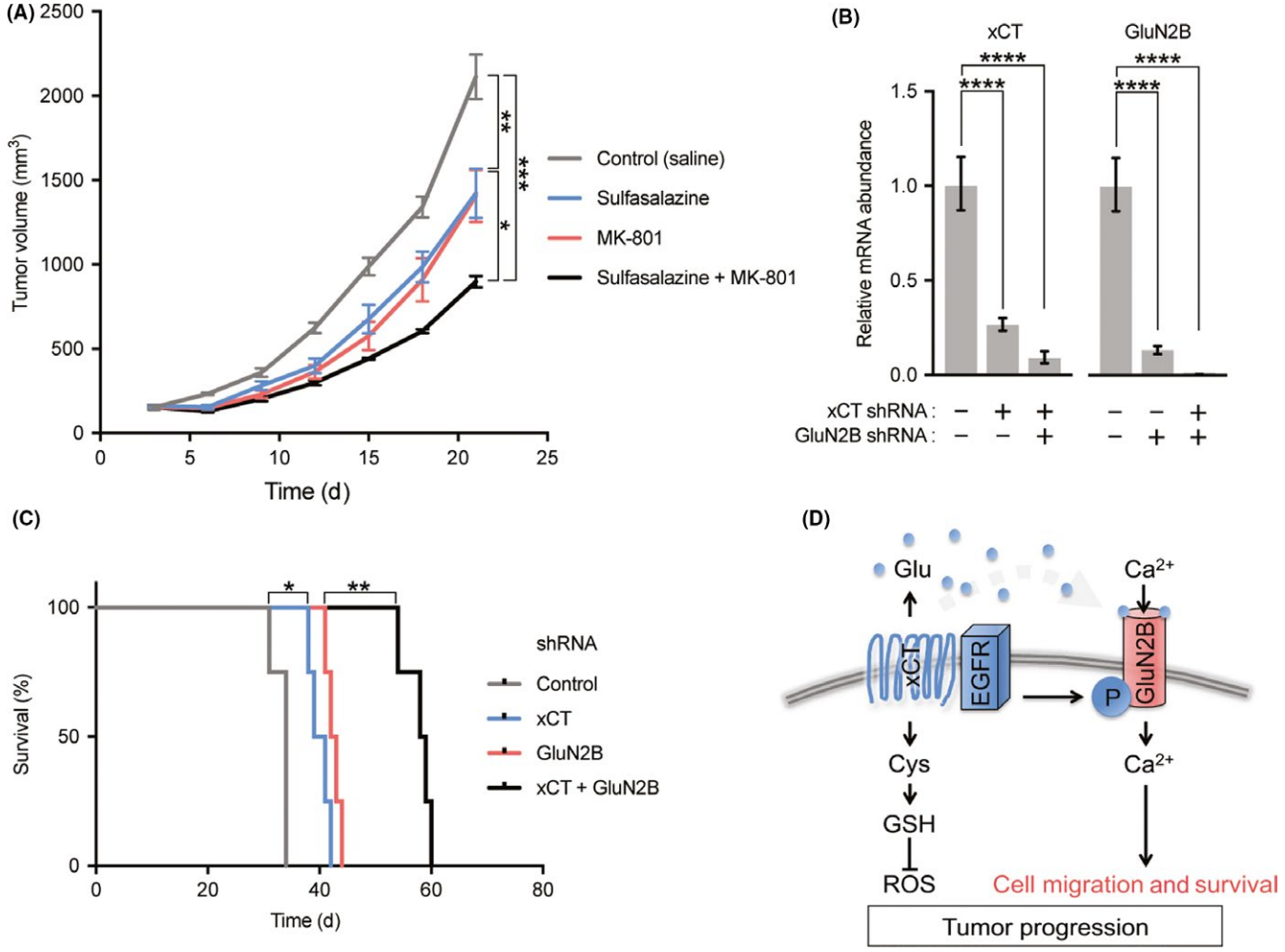

FIGURE 4 Targeting of system xc(–) and GluN2B-containing *N*-methyl-D-aspartate–sensitive glutamate receptor (NMDAR) suppresses the growth of subcutaneous and brain tumors formed by U87MG-E cells. A, Volume of subcutaneous tumors formed by U87MG-E cells in nude mice (n = 4 or 5 per group) treated daily beginning 3 days after cell injection with sulfasalazine (250 mg/kg), MK-801 (1 mg/kg) or the combination of both drugs. B, Quantitative RT-PCR analysis of xCT and GluN2B mRNA abundance in U87MG-E cells stably expressing control, xCT or GluN2B shRNA (n = 4 independent experiments). C, Kaplan-Meier survival curves for mice with brain tumors derived from implanted U87MG-E cells stably expressing control, xCT or GluN2B shRNA(n = 4 for each group). Data in (A) to (C) are means ± SEM. **P* < .05, ** *P* < .01, ****P* < .001 (1-way ANOVA followed by Tukey's post hoc test [A and B] or the log-rank test [C]). D, Model for the interaction between epidermal growth factor receptor (EGFR)-overexpressing glioma cells and free amino acids mediated by system xc(–) and NMDAR. EGFR constitutively interacts with the xCT subunit of cystine-glutamate antiporter system xc(–) in a kinase-independent manner and thereby promotes the cell surface expression of xCT and increases both the uptake of extracellular cystine and the release of intracellular glutamate. The increased cystine uptake boosts cellular defense against reactive oxygen species (ROS) and thereby promotes resistance to oxidative stress through promotion of reduced glutathione (GSH) synthesis. Activated EGFR mediates phosphorylation of the COOH-terminal region of GluN2B, resulting in enhancement of glutamate-NMDAR signaling and consequent promotion of glioma cell invasion

Together, these observations indicated that xCT and GluN2B play key roles in the malignant behavior of glioma in vivo and that both system xc(–) and NMDAR are, therefore, promising targets for cancer therapy, especially for the treatment of EGFR-overexpressing glioma.

4 | DISCUSSION

Malignant gliomas that release glutamate have been shown to provoke an inflammatory response within the surrounding tissue that leads to neuronal death and consequent tumor expansion.[9] System xc(–) has recently emerged as a key mediator of glutamate release in glioma cells.[7,25] We have now shown that EGFR interacts with, phosphorylates, and thereby activates the NMDAR subunit GluN2B in glioma cells. The $[Ca^{2+}]_i$ of EGFR-overexpressing U87MG-E cells was found to be higher than that of parental U87MG-P cells, and treatment of U87MG-E cells with the NMDAR inhibitor MK-801 or the EGFR kinase inhibitor gefitinib attenuated this increase in $[Ca^{2+}]_i$. Given that Ca^{2+} signaling plays a key role in the promotion of glioma invasion,[26] NMDAR-mediated Ca^{2+} influx may contribute to the

invasive phenotype of EGFR-overexpressing glioma cells. Together with our previous observation that EGFR activates system xc(–) in glioma cells,[8] our present results establish a role for EGFR expression in the generation of a glutamate-rich microenvironment and consequent promotion of glioma cell migration through activation of NMDAR signaling.

N-methyl-D-aspartate–sensitive glutamate receptors are heterotetramers that contain 2 GluN1 glycine-binding subunits and 2 GluN2 (GluN2A to GluN2D) or GluN3 (GluN3A and GluN3B) glutamate-binding subunits.[27,28] Src family kinases have been shown to enhance NMDAR activity in neurons through tyrosine phosphorylation of the COOH-terminal domain of GluN2A and GluN2B subunits.[29-31] The COOH-terminal domain of these GluN2 subunits contains an internalization motif, the tyrosine phosphorylation of which inhibits binding of the clathrin adapter AP-2 and thereby promotes cell surface expression of NMDAR.[32,33] In the present study, we found that EGFR constitutively interacts with GluN2B in EGFR-overexpressing glioma cells, and that it phosphorylates Tyr^{1474} in the internalization motif of GluN2B in response to EGF stimulation. Furthermore, treatment with gefitinib suppressed Tyr^{1474} phosphorylation of GluN2B without affecting the phosphorylation status of Src in U87MG-E and T98G cells (Figure 2C). The kinase responsible for Tyr^{1474} phosphorylation of GluN2B might, therefore, differ between normal brain cells and EGFR-overexpressing glioma cells as a result of the difference in EGFR expression level and activity.

We finally showed that the administration of sulfasalazine and MK-801 synergistically suppressed the growth of subcutaneous tumors formed by EGFR-overexpressing glioma cells. Furthermore, shRNA-mediated knockdown of xCT and GluN2B cooperatively prolonged the survival of mice injected intracerebrally with EGFR-overexpressing glioma cells. Our results thus implicate EGFR as a key molecule that links the functions of system xc(–) and NMDAR to malignant behavior of glioma cells (Figure 4D).

The overexpression of EGFR protein and amplification of the *EGFR* gene have often been observed in primary glioblastoma (GBM) but not in lower-grade astrocytomas.[34] The EGFR amplification has been reported to promote malignancy of glioma but effective treatments have not been established. Our study thus indicates that combination therapy with inhibitors of xCT and NMDAR may impair the invasive potential and survival of EGFR-overexpressing glioma cells and that such therapy may suppress the growth of tumors formed by these cells.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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