The cystine/glutamate antiporter xCT is a key regulator of EphA2 S897 phosphorylation under glucose-limited conditions

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Abstract

EphA2, which belongs to the Eph family of receptor tyrosine kinases, is overexpressed in a variety of human cancers. Serine 897 (S897) phosphorylation of EphA2 is known to promote cancer cell migration and proliferation in a ligand-independent manner. In this study, we show that glucose deprivation induces S897 phosphorylation of EphA2 in glioblastoma cells. The phosphorylation requires the activity of the cystine/glutamate antiporter xCT and reactive oxygen species (ROS)-dependent ERK and RSK activation. Furthermore, depletion of EphA2 in glioblastoma cells leads to decreased cell viability under glucose starvation. Our results suggest a role of EphA2 in glioblastoma cell viability under glucose-limited conditions.

Keywords: EphA2, xCT, amino acid transporter, RSK, glioblastoma, glucose

1. Introduction

EphA2, a member of the Eph family of receptor tyrosine kinases, is frequently overexpressed in a variety of human cancers including glioblastoma [1-5]. Its expression level correlates with the grade of glioblastoma and with poor survival of the patients [6-8]. EphA2 is known to promote cell proliferation and survival, cell migration and invasion, and the maintenance of stem-like tumor-propagating cells in glioblastoma [9-12]. Unlike ligand ephrin-induced tyrosine kinase activation of Eph receptors, these effects of EphA2 are ligand ephrin-independent and require phosphorylation of EphA2 on serine 897 (S897), a residue in the linker region between the kinase and SAM domains. Akt, RSK, and PKA have been reported to phosphorylate S897 of EphA2 in response to growth factors, cytokines, β-adrenoceptor agonists, and exposure to X-rays [9, 11-16]. In addition, overexpression of EphA2 promotes S897 phosphorylation through its tyrosine kinase activity without other stimuli [17].

Reactive oxygen species (ROS) are produced in many processes including cellular metabolism. They react with and modify a variety of biological molecules, such as proteins, lipids, and DNA. Although excessively high levels of ROS cause oxidative damage to these molecules and lead to cell death, ROS at moderate levels function as important

regulators of signal transduction pathways. They oxidize cysteine residues found in redox-sensitive enzymes, which result in reversible modification of their activities. For example, H_2O_2 can oxidize catalytic cysteine residues in protein phosphatases and inactivate their activities [18-20]. In cancer cells, ROS contribute to accelerating the accumulation of additional mutations and to amplifying the tumorigenic phenotype. Cancer cells exhibit higher levels of ROS than normal cells. Therefore, they up-regulate the antioxidant systems to avoid excessive levels of ROS and harmful oxidant stress [21, 22].

The cystine/glutamate antiporter xCT (SLC7A11) forms a complex with the heavy chain subunit 4F2 (4F2hc, Cd98, SLC3A2), and exchanges extracellular cystine for intracellular glutamate at the plasma membrane [23, 24]. The imported cystine is reduced to cysteine, which is utilized for glutathione synthesis. Glutathione is one of the most important antioxidant systems in many types of cancer including glioblastoma. Therefore, xCT expression is up-regulated in glioblastoma patients and in glioblastoma cell lines, and its expression correlates with tumor growth, drug resistance, and poor prognosis [25-30]. Treatment with pharmacological inhibitors of xCT in several types of cancer cells causes iron-dependent accumulation of lipid-based ROS and cell death termed ferroptosis [31-33]. On the other hand, recent studies have reported that inhibition of xCT in cancer cells improves

cell viability after glucose deprivation [34-37]. In this study, we show that EphA2 is phosphorylated on S897 and promotes cell survival under glucose deprivation in glioblastoma cells. Furthermore, xCT plays an important role in this process.

2. Materials and Methods

2.1. Reagents and antibodies

Inhibitors and recombinant protein were used at the following concentrations: U0126 (Merk Millipore) 20 µM; BI-D1870 (Santa Cruz Biotechnology) 10 µM; sulfasalazine (SSZ, Sigma-Aldrich), 250 µM; okadaic acid (Alomone labs), 500 nM; catalase (Sigma-Aldrich), 250 U/ml; recombinant human epidermal growth factor (EGF, Sigma-Aldrich, E9644), 100 ng/ml. We used the following antibodies in this study: rabbit monoclonal antibodies against EphA2 (D4A2), S897 phospho-EphA2 (D9A1), ERK (137F5), T202/Y204 phospho-ERK (D13.14.4E), RSK2 (D21B2), S227 phospho-RSK2 (D53A11), and xCT/SLC7A11 (D2M7A) (Cell Signaling Technology); a mouse monoclonal antibody against α-tubulin (Sigma-Aldrich, T5168); secondary antibodies conjugated to horseradish peroxidase (DAKO).

2.2. Cell culture and transfection

U251 (ECACC, No. EC09063001), A172 (the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan, No. RCB2530), T98G (the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan, No. RCB1954), and LN229 (ATCC, No. CRL-2611) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin under humidified air containing 5% CO_2 at 37ºC. The medium without glucose and amino acids was prepared by dissolving 0.2 g of $CaCl_2$, 0.1 mg of $Fe(NO_3)_3/9H_2O$, 97.67 mg of $MgSO_4$, 0.4 g of KCl, 3.7 g of $NaHCO_3$, 6 g of NaCl, 0.109 g of NaH_2PO_4, 40 ml of minimum essential medium vitamin solution (100×) liquid (Thermo Fisher Scientific) in 1 l of water. We prepared the medium without glucose by adding amino acids in the glucose- and amino acid-free medium at the following concentrations: arginine-HCl (0.4 mM), cystine-2HCl (0.2 mM), histidine-HCl-H_2O (0.2 mM), isoleucine (0.8 mM), leucine (0.8 mM), lysine-HCl (0.8 mM), methionine (0.2 mM), phenylalanine (0.4 mM), threonine (0.8 mM), tryptophan (0.08 mM), tyrosine-2Na-2H_2O (0.4 mM), valine (0.8 mM), glutamine (2 mM). We used the CRISPR/Cas9-mediated homology-independent knock-in system [38] to generate EphA2 knockout (KO) U251

cells. The single guide RNA (sgRNA) targeting EphA2 sequence (5'-cgcctgcttcgccctgctgt-3') was cloned into the sgRNA expression vector peSpCAS9(1.1)-2xsgRNA (Addgene plasmid 80768). U251 cells were seeded in two 6-cm dishes (2.5×10^5 cells/dish) and transfected using Lipofectamine 2000 (Life Technologies). Two days after transfection, the cells were placed in the medium containing 250 µg/ml G418 (Wako) to eliminate untransfected cells. Ten days after selection, colonies grown from single cells were isolated, and they were expanded and screened by immunoblotting with anti-EphA2 antibody. Control and xCT KO U251 cells were obtained as described previously [36].

2.3. Immunoblotting

Cells were seeded in 35-mm dishes (1×10^5 cells/dish). The next day, they were rinsed twice with phosphate-buffered saline, and the medium was replaced with glucose-free or glucose- and amino acid-free medium with or without 5 mM glucose, 0.2 mM cystine, 2 mM glutamine, or 0.1 mM glutamate for the indicated times. Cells were lysed with Laemmli sample buffer, and the proteins in the cell lysates were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore Corporation). The membrane was blocked with 3% low fat milk in Tris-

buffered saline, and then incubated with primary antibodies diluted with 3% low fat milk or Can Get Signal (TOYOBO). The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and ECL western blotting detection reagents (GE Healthcare Life Sciences). The signals were captured with Amersham Imager 600 (GE Healthcare Life Sciences). Densitometric analysis was performed using Amersham Imager 600 analysis software.

2.4. Glutamate release assay

U251 cells were seeded in a 48-well plate (Greiner Bio-One, No. 677180, 2.0×10^4 cells/well) and cultured for 24 h. Then they were rinsed twice with phosphate-buffered saline, and the medium was replaced with glucose- and amino acid-free medium supplemented with 0.2 mM cystine and 2 mM glutamine for 4 h. Glutamate release into the medium was measured using L-Glutamate Assay Kit YAMASA NEO (Yamasa Corporation) according to the manufacturer's instruction. The optical density was measured at 595 nm using a microplate reader (Tecan, GENious).

2.5. Lactate dehydrogenase (LDH) assay

U251 cells were seeded in a 48-well plate (1.0×10^4 cells/well) and

cultured for 24 h. Cell death was measured by LDH release assay using MTX LDH kit (Kyokuto Pharmaceutical Industrial) according to the manufacturer's instruction. The optical density was measured at 595 nm using a microplate reader (Tecan, GENious). The value of LDH release after treatment with 0.1% Tween 20 was defined as 100% cell death.

2.6. Data analysis

Data were analyzed using the analysis of variance (ANOVA) with Tukey HSD post hoc test. $p < 0.05$ was considered significant. Statistical analyses were performed using KaleidaGraph (Synergy Software).

3. Results

3.1. Glucose deprivation induces S897 phosphorylation of EphA2 in glioblastoma cells

Glucose deprivation induces ERK activation in several kinds of cancer cells including glioblastoma [39-42]. We previously reported that EphA2 is phosphorylated on S897 through ERK activation in glioblastoma cells [12,17]. Therefore, we wondered whether the level of EphA2 S897 phosphorylation changes after glucose deprivation in glioblastoma cells. To test this possibility, U251 glioblastoma cells, which highly express EphA2

[12], were exposed to medium with 5 mM glucose (+Glc) or without glucose (–Glc), and the cell lysates were analyzed by immunoblotting with an antibody against S897-phosphorylated EphA2 (pS897-EphA2). We found that glucose deprivation induced S897 phosphorylation of EphA2 in U251 cells (Fig. 1A). ERK was also phosphorylated upon glucose deprivation. It is known that S897 of EphA2 is phosphorylated upon treatment with growth factors such as EGF [9,11-14], and glucose deprivation and EGF stimulation induced S897 phosphorylation of EphA2 at similar levels (Fig. 1B). S897 phosphorylation of EphA2 in response to glucose deprivation was also observed in other glioblastoma cell lines T98G (Fig. 1C) and LN229 (Fig. 1D) cells. Since glucose deprivation leads to accumulation of ROS [36,39,42], we next examined the involvement of ROS in the glucose-deprivation-induced EphA2 S897 phosphorylation. In U251 cells, treatment with catalase, a scavenger of H_2O_2, suppressed glucose deprivation-induced EphA2 S897 phosphorylation (Fig. 1E). These results suggest that glucose deprivation induces ROS-dependent S897 phosphorylation of EphA2 in glioblastoma cells. Increased intracellular ROS can oxidize catalytic cysteine residues in protein phosphatases and inactivate their activities [18-20]. We found that treatment of U251 cells with a protein phosphatase inhibitor okadaic acid alone induced S897 phosphorylation of EphA2 (Fig. 1F).

3.2. Glucose deprivation-induced EphA2 S897 phosphorylation requires ERK and RSK activities

In U251 cells, EGF stimulation induces EphA2 S897 phosphorylation through the MEK/ERK/RSK pathway [12]. Therefore, we used specific inhibitors to examine the involvement of the kinases in glucose deprivation-induced S897 phosphorylation of EphA2. Treatment of U251 cells with the RSK inhibitor BI-D1870 and the MEK inhibitor U0126 suppressed glucose deprivation-induced S897 phosphorylation (Fig. 2A). Thus, activation of the MEK/ERK/RSK pathway is required for glucose deprivation-induced EphA2 S897 phosphorylation. In U251 cells, RSK2 is predominantly expressed among the RSK members [12], and we observed increased phosphorylation of RSK2 on serine 227 (S227), which is essential for complete activation of RSK2 [43], after glucose deprivation (Fig. 2B). Similar to EphA2 S897 phosphorylation, glucose deprivation-induced RSK2 S227 phosphorylation was suppressed by catalase (Fig. 2C), and treatment with okadaic acid alone induced RSK2 S227 phosphorylation (Fig. 2D).

3.3. The cystine/glutamate antiporter xCT is involved in glucose deprivation-induced EphA2 S897 phosphorylation

We previously reported that the cystine/glutamate antiporter xCT mediates glucose deprivation-induced ROS production [36]. To examine whether xCT is involved in glucose deprivation-induced EphA2 S897 phosphorylation, we used the xCT inhibitor sulfasalazine (SSZ) and found that SSZ completely suppressed glucose deprivation-induced EphA2 S897 phosphorylation (Fig. 3A). Similar results were obtained in T98G (Fig. 3B) and LN229 (Fig. 3C) cells. To confirm the involvement of xCT, the level of S897 phosphorylation of EphA2 was examined in xCT-deficient U251 (xCT KO) cells [36]. Although EGF stimulation induced S897 phosphorylation of Eph2 in both control and xCT KO cells (Fig. 3E), glucose deprivation did not induce the S897 phosphorylation in xCT KO cells (Fig. 3D). Phosphorylation of RSK2 on S227 was also suppressed by xCT depletion (Fig. 3D). In A172 cells, which expressed very low levels of xCT compared to other glioblastoma cells [36], the level of EphA2 S897 phosphorylation did not change after glucose deprivation (data not shown). These results suggest that high level expression of xCT is required for S897 phosphorylation of EphA2 under glucose deprivation in glioblastoma cells.

3.4. Cystine and glutamine induces EphA2 S897 phosphorylation through xCT under glucose deprivation

We examined the amino acid transport activity of xCT in U251 cells by measuring glutamate release into the glucose- and amino acid-free medium. When U251 cells were placed in the amino acid-free medium supplemented with cystine and glutamine, they showed higher glutamate release into the medium, whereas cystine alone was much less effective (Fig. 4A). Addition of glutamine alone had no effect on glutamate release. The xCT inhibitor SSZ completely suppressed the cystine- and glutamine-induced glutamate release (Fig. 4B). Thus, cystine uptake by xCT is coupled to the export of glutamate, which is supplied from glutamine [35, 44], and higher transport activity of xCT requires cystine and glutamine in the medium. We next examined whether the amino acid transport activity by xCT is sufficient for S897 phosphorylation of EphA2. Addition of cystine and glutamine in glucose- and amino acid-free medium increased the level of S897 phosphorylation of EphA2 (Fig. 4C). On the other hand, addition of glutamate did not induce EphA2 S897 phosphorylation (Fig. 4D), suggesting that glutamate export by xCT does not contribute to the phosphorylation of EphA2. Cystine- and glutamine-induced EphA2 S897 phosphorylation was also observed in T98G (Fig. 4E) and LN229 (Fig.4F) cells, but not in xCT KO U251 cells (Fig. 4G). These results suggest that cystine uptake through xCT induces S897 phosphorylation of EphA2 in glucose-limited conditions.

3.5. Deletion of EphA2 reduces cell survival under glucose deprivation

Finally, to examine whether EphA2 is involved in cell viability under glucose deprivation, we established EphA2 KO U251 cells using CRISPR/Cas9-mediated deletion of EphA2 (Fig. 5A). We used two independent EphA2 KO U251 cell lines to measure LDH release in the medium at 12 h after glucose deprivation. We found that glucose deprivation-induced cell death was significantly enhanced in EphA2 KO cells (Fig. 5B). These results suggest that EphA2 contributes to cell survival under glucose deprivation.

4. Discussion

Cancer cells alter their metabolism to maintain sustained proliferation and survival. One of the most important features of this altered metabolism is the increased glucose uptake and utilization [45,46]. Cancer cells highly depend on glucose metabolism not only to supply sufficient energy and biosynthetic intermediates, but also to generate NAPDH, which is required for glutathione and thioredoxin systems, the major antioxidant systems in cancer cells [46,47]. Therefore, to survive in glucose insufficient conditions, cancer cells need to adapt to metabolic stress. In this study, we show that in

glioblastoma cells under glucose-limited conditions, cystine uptake though xCT triggers ROS production, which induces phosphorylation and activation of RSK, leading to S897 phosphorylation of EphA2. In addition, EphA2 promotes cell survival under glucose deprivation conditions. Our results suggest that xCT serves as a key regulator for the EphA2 ligand-independent signaling in glioblastoma cells under glucose-limited conditions.

Cancer cells have a high demand for nutrients including amino acids, and therefore they up-regulate amino acid transporters including xCT [23,24]. One of the major roles of xCT in cancer cells is to supply cysteine necessary for the synthesis of the antioxidant glutathione. Previous studies have reported that inhibition of xCT activity suppresses cancer cell motility and invasiveness [29,48,49]. The mechanism is suggested to involve release of glutamate from the cell through xCT and activation of a metabotropic glutamate receptor [49]. On the other hand, we show that xCT regulates the phosphorylation status of RSK and EphA2 under glucose-limited conditions through cystine uptake-mediated ROS generation. In addition, EphA2 promotes cell survival from glucose deprivation. Thus, these studies suggest that xCT contributes to not only amino acid metabolism but also regulation of signal transduction pathways to promote cell survival and invasive behavior in cancer cells. Because xCT inhibitors or downregulation of xCT

expression improve cancer cell viability under glucose-limited conditions by regulating glutamine metabolism and ROS production [34-36], it is difficult to show clearly that xCT-mediated EphA2 phosphorylation promotes cancer cell survival under glucose deprivation. On the other hand, glucose uptake is restricted in cancer cells grown in 3D culture [47]. Therefore, it is important to investigate whether xCT and EphA2 are involved in cancer cell survival in 3D culture. Previous studies reported that downregulation of EphA2 in glioblastoma cells inhibits glioblastoma cell growth and tumorigenicity in 3D culture and in vivo [10,11]. Further studies are needed to determine whether the xCT-EphA2 axis contributes to glioblastoma cell survival in 3D culture and in vivo.

Phosphorylation of S897 of EphA2 has been reported to correlate with malignant progression of several human cancers including glioblastoma [50]. A previous study reported that exposure to X-rays induces EphA2 S897 phosphorylation and increases cell survival from X-rays in cells overexpressing EphA2 [16]. Expression of EphA2 and its S897 phosphorylation are markedly increased in lung cancer cells resistant to inhibitors of epidermal growth factor receptor, and loss of EphA2 expression reduces the viability of the cells [51]. In addition, overexpression and S897 phosphorylation of EphA2 promotes cell survival under suspension culture

conditions [52, 53]. In this study, we found that EphA2 is phosphorylated on S897 and promotes cell survival under glucose deprivation, a nutrient stress condition. Thus, EphA2 overexpression and S897 phosphorylation appear to be associated with cancer cell survival under unfavorable conditions. Glucose deprivation induces EphA2 S897 phosphorylation through ROS-dependent activation of ERK and RSK. Increased intracellular ROS can oxidize key cysteine residues in the catalytic domains of protein phosphatases and inactivate them [18-20]. Indeed, treatment of U251 cells with okadaic acid alone induces phosphorylation of ERK, RSK2, and S897 of EphA2. Therefore, it is likely that inactivation of a phosphatase responsible for ERK dephosphorylation is involved in this process. On the other hand, the mechanism by which EphA2 ligand-independent signaling promotes glioblastoma cell survival is unclear. We previously reported that S897 phosphorylation of EphA2 recruits Ephexin4, a guanine nucleotide exchange factor for the small GTPase RhoG, and that EphA2/Ephexin4-mediated RhoG activation leads to anoikis resistance [52]. Therefore, the Ephexin4-mediated RhoG activation may act downstream of EphA2 to promote cell survival under glucose-limited conditions. Further studies are required to elucidate how ligand-independent EphA2 signaling regulates glioblastoma cell survival.

5. Conclusion

In this study, we demonstrate a molecular link between the amino acid transporter xCT and EphA2 in the regulation of glioblastoma cell survival under glucose-limited conditions. Cystine uptake through xCT induces ROS production, which results in phosphorylation of S897 EphA2 through RSK, leading to promotion of cell survival. On the basis of these findings, we propose that combining inhibitors targeting cancer-specific glucose metabolism and EphA2 may offer a potential therapeutic approach for glioblastoma.

Acknowledgments

Abbreviations

S897, serine 897; ROS, reactive oxygen species; SSZ, sulfasalazine; KO,

knockout; sgRNA, single guide RNA; LDH, lactate dehydrogenase; Glc, glucose; AA, amino acid.

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Figure legends

Fig. 1. Glucose deprivation induces S897 phosphorylation of EphA2 in glioblastoma cells. A. U251 cells were placed in glucose-free medium with or without 5 mM glucose (Glc) for the indicated times, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. B. U251 cells were deprived of glucose or stimulated with EGF (100 ng/ml) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. C, D. T98G (C) and LN229 (D) cells were placed in glucose-free medium with or without 5 mM glucose for the indicated times, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. E. U251 cells were placed in glucose-free medium with or without glucose (5 mM) or catalase (250 U/ml) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. F. U251 cells were treated with okadaic acid (500 nM) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. Numbers below blots represent the ratio of pS897-EphA2/EphA2 as determined by densitometric analysis.

Fig. 2. Glucose deprivation-induced EphA2 S897 phosphorylation requires RSK activity. A. U251 cells were placed in glucose-free medium with or

without glucose (5 mM), BI-D1870 (10 µM), or U0126 (20 µM) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. Numbers below blots represent the ratio of pS897-EphA2/EphA2 as determined by densitometric analysis. B. U251 cells were placed in glucose-free medium with or without glucose (5 mM) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. The right graph represents the ratio of pS227 RSK2/RSK2, as determined by densitometric analysis. Data are the means ± SD of four independent experiments (*p<0.05, *t*-test). C. U251 cells were placed in glucose-free medium with or without glucose (5 mM) or catalase (250 U/ml) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. D. U251 cells were treated with okadaic acid (500 nM) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. Data are the means ± SD of three independent experiments (*p<0.05, *t*-test).

Fig. 3. The cystine/glutamate antiporter xCT is involved in glucose deprivation-induced EphA2 S897 phosphorylation. A-C. U251 (A), T98G (B), or LN229 (C) cells were placed in glucose-free medium with or without glucose (5 mM) or SSZ (250 µM) for 2 h, and the cell lysates were analyzed

by immunoblotting with the indicated antibodies. D. Control U-251 cells or U251 xCT KO cells placed in glucose-free medium with or without glucose (5 mM) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. E. Control U-251 cells or U251 xCT KO cells were stimulated with EGF (100 ng/ml) in normal medium for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. Numbers below blots represent the ratio of pS897-EphA2/EphA2 as determined by densitometric analysis.

Fig. 4. Cystine and glutamine induces EphA2 S897 phosphorylation through xCT under glucose deprivation. A, B. U-251 cells were placed in glucose- and amino acid-free medium (–Glc–AA) with or without cystine (0.2 mM), glutamine (2 mM), or SSZ (250 μM) for 4 h, and glutamate release into the medium was measured. Data are the means ± SD of three independent experiments (***$p<0.001$, one-way ANOVA with Tukey's post hoc test). C. U251 cells were placed in glucose-free or glucose- and amino acid-free medium with or without glucose (5 mM), cystine (0.2 mM), or glutamine (2 mM) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. D. U251 cells were placed in glucose-free or glucose- and amino acid-free medium with or without glucose (5 mM) or glutamate

(0.1 mM) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. E-G. T98G (E), LN229 (F), or control U251 and xCT KO (G) cells were placed in glucose-free or glucose- and amino acid-free medium with or without glucose (5 mM), cystine (0.2 mM), or glutamine (2 mM) for 2 h, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. Numbers below blots represent the ratio of pS897-EphA2/EphA2 as determined by densitometric analysis.

Fig. 5. Deletion of EphA2 reduces cell survival under glucose starvation. A. U251 and EphA2 KO U251 cell (#1 and #2) lysates were analyzed by immunoblotting with the indicated antibodies. B. Control and EphA2 KO #1 or #2 cells were placed in glucose-free medium with or without glucose (5 mM) for 12 h. Quantification of cell death was performed using an LDH release assay. Data are the means ± SD of three independent experiments (**$p < 0.01$, ***$p < 0.001$, one-way ANOVA with Tukey's post hoc test).
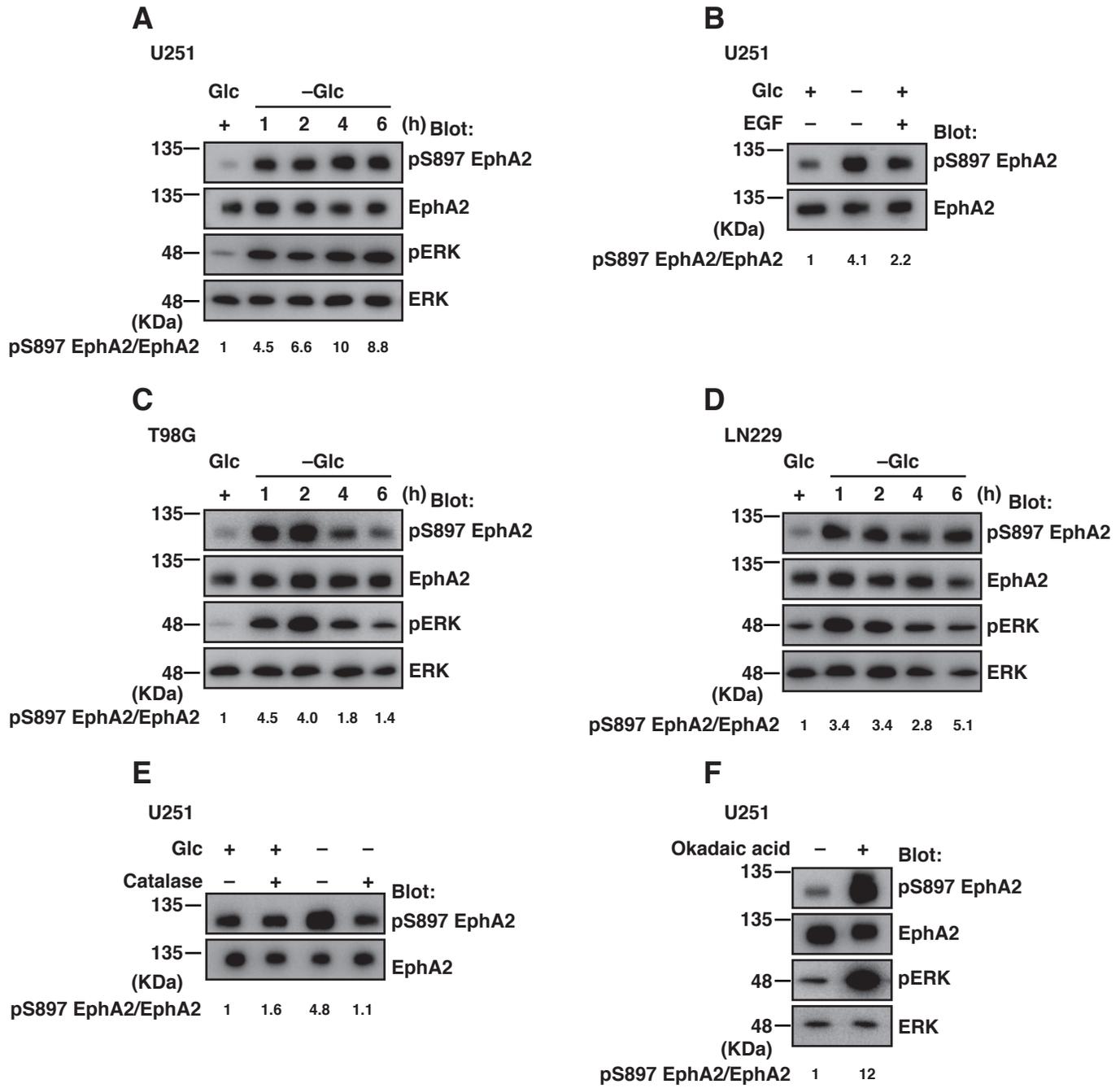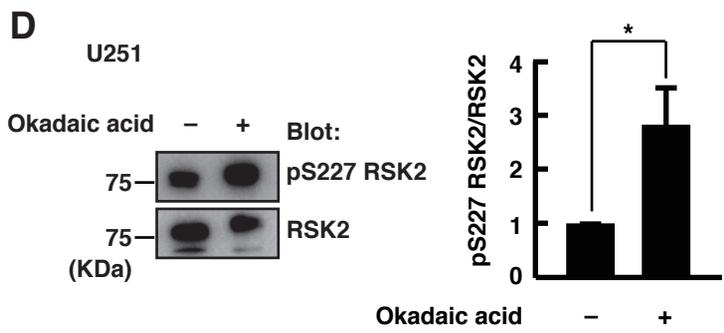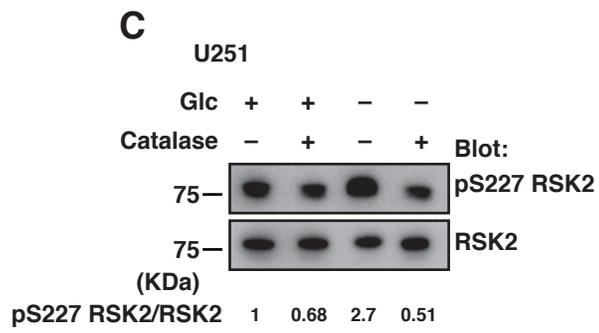
A

U251

Glc	–Glc				
+	1	2	4	6	(h) Blot:

135— pS897 EphA2
135— EphA2
48— pERK
48— ERK
(KDa)

pS897 EphA2/EphA2   1   4.5   6.6   10   8.8

B

U251

Glc	+	–	+	
EGF	–	–	+	Blot:

135— pS897 EphA2
135— EphA2
(KDa)

pS897 EphA2/EphA2   1   4.1   2.2

C

T98G

Glc	–Glc				
+	1	2	4	6	(h) Blot:

135— pS897 EphA2
135— EphA2
48— pERK
48— ERK
(KDa)

pS897 EphA2/EphA2   1   4.5   4.0   1.8   1.4

D

LN229

Glc	–Glc				
+	1	2	4	6	(h) Blot:

135— pS897 EphA2
135— EphA2
48— pERK
48— ERK
(KDa)

pS897 EphA2/EphA2   1   3.4   3.4   2.8   5.1

E

U251

Glc	+	+	–	–	
Catalase	–	+	–	+	Blot:

135— pS897 EphA2
135— EphA2
(KDa)

pS897 EphA2/EphA2   1   1.6   4.8   1.1

F

U251

Okadaic acid	–	+	Blot:

135— pS897 EphA2
135— EphA2
48— pERK
48— ERK
(KDa)

pS897 EphA2/EphA2   1   12

Figure 1

Figure 2

A

U251

Glc   +   +   −   −
SSZ   −   +   −   +   **Blot:**

135 —   pS897 EphA2

135 —   EphA2

(KDa)

pS897 EphA2/EphA2   1   0.51   1.4   0.58

B

T98G

Glc   +   +   −   −
SSZ   −   +   −   +   **Blot:**

135 —   pS897 EphA2

135 —   EphA2

(KDa)

pS897 EphA2/EphA2   1   0.53   18   1.3

C

LN229

Glc   +   +   −   −
SSZ   −   +   −   +   **Blot:**

135 —   pS897 EphA2

135 —   EphA2

(KDa)

pS897 EphA2/EphA2   1   0.20   6.8   1.9

D

U251

    Control   xCT KO

Glc   +   −   +   −   **Blot:**

135 —   pS897 EphA2

135 —   EphA2

75 —   pS227 RSK2

75 —   RSK2

35 —   xCT

(KDa)

pS897 EphA2/EphA2   1   2.7   0.72   0.34

E

U251

    Control   xCT KO

EGF   −   +   −   +   **Blot:**

135 —   pS897 EphA2

135 —   EphA2

(KDa)

pS897 EphA2/EphA2   1   3.5   0.24   2.1

Figure 3

A

U251

Glutamate release (μmol/10^5 cells)

Cystine	–	+	+	–
Glutamine	–	–	+	+

–Glc–AA

*** ***

B

U251

Glutamate release (μmol/10^5 cells)

Cystine	–	+	–	+
Glutamine	–	+	–	+
SSZ	–	–	+	+

–Glc–AA

***

C

U251

	–Glc		–Glc–AA	
Glc	+	–	–	–
Cystine	–	–	–	+
Glutamine	–	–	–	+

Blot:

135— pS897 EphA2

135— EphA2

(KDa)

pS897 EphA2/EphA2   1   2.4   0.56   1.8

D

U251

	–Glc		–Glc–AA	
Glc	+	–	–	–
Glutamate	–	–	–	+

Blot:

135— pS897 EphA2

135— EphA2

(KDa)

pS897 EphA2/EphA2   1   7.0   1.4   1.7

E

T98G

	–Glc		–Glc–AA	
Glc	+	–	–	–
Cystine	–	–	–	+
Glutamine	–	–	–	+

Blot:

135— pS897 EphA2

135— EphA2

(KDa)

pS897 EphA2/EphA2   1   14   3.5   11

F

LN229

	–Glc		–Glc–AA	
Glc	+	–	–	–
Cystine	–	–	–	+
Glutamine	–	–	–	+

Blot:

135— pS897 EphA2

135— EphA2

(KDa)

pS897 EphA2/EphA2   1   6.7   2.0   9.8

G

U251

–Glc–AA

Cystine	–	+	–	+
Glutamine	–	+	–	+

Blot:

135— pS897 EphA2

135— EphA2

(KDa)

Control   xCT KO

pS897 EphA2/EphA2   1   1.7   0.37   0.14

Figure 4

A

U251

B

U251

Figure 5