Integrative Network Analysis Combined with Quantitative Phosphoproteomics Reveals Transforming Growth Factor-beta Receptor type-2 (TGFBR2) as a Novel Regulator of Glioblastoma Stem Cell Properties*\S

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Glioblastoma is one of the most malignant brain tumors with poor prognosis and their development and progression are known to be driven by glioblastoma stem cells. Although glioblastoma stem cells lose their cancer stem cell properties during cultivation in serum-containing medium, little is known about the molecular mechanisms regulating signaling alteration in relation to reduction of stem cell-like characteristics. To elucidate the global phosphorylation-related signaling events, we performed a SILAC-based quantitative phosphoproteome analysis of serum-induced dynamics in glioblastoma stem cells established from the tumor tissues of the patient. Among a total of 2876 phosphorylation sites on 1584 proteins identified in our analysis, 732 phosphorylation sites on 419 proteins were regulated through the alteration of stem cell-like characteristics. The integrative computational analyses based on the quantified phosphoproteome data revealed the relevant changes of phosphorylation levels regarding the proteins associated with cytoskeleton reorganization such as Rho family GTPase and Intermediate filament signaling, in addition to transforming growth factor-β receptor type-2 (TGFBR2) as a prominent upstream regulator involved in the serum-induced phosphoproteome regulation. The functional association of transforming growth factor-β receptor type-2 with stem cell-like properties was experimentally validated through signaling perturbation using the corresponding inhibitors, which indicated that transforming growth factor-β receptor type-2 could play an important role as a novel cell fate determinant in glioblastoma stem cell regulation. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.049999, 1017–1031, 2016.

Glioblastoma (GBM, WHO grade IV astrocytoma/glioma) is one of the most malignant brain tumors with a mean survival time of 12 to 15 months after diagnosis (1, 2). Despite the advances in surgical resection, chemotherapy, and radiation treatment, the prognosis of patients with glioblastoma remains poor. In addition to its high infiltration ability, glioblastoma possess high intratumoral heterogeneity, resulting in the complications for therapeutic intervention. Recently, increasing evidences have shown that heterogenic brain tumors originate from glioblastoma stem cells (also termed glioblastoma initiating/propagating cells) and are organized in a hierarchical manner (3, 4). Glioblastoma stem cells have similar properties to neural stem cells (NSCs)\(^1\) including the expression of the

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\(^*\) The abbreviations used are: NSC, neural stem cell; ACVR, activin receptor; AKT, RAC-alpha serine/threonine-protein kinase; ARHGEF7, Rho guanine nucleotide exchange factor 7; BAIIAP2, brain-specific angiogenesis inhibitor 1-associated protein 2; bFGF, basal fibroblast growth factor; BINGO, Biological Networks Gene Ontology tool; CXCR4, C-X-C chemokine receptor type 4; CAMK1, calcium/calmodulin-dependent protein kinase type-1; DMEM/F12, Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12; EGFR, epidermal growth factor; FASP, filter aided sample preparation; FDR, false discovery rate; GFAP, glial fibrillary acidic protein; GO, gene ontology; HRP, horseradish peroxidase; IDH1/2, isocitrate dehydrogenase 1/2; IPA, Ingenuity Pathway Analysis; ITGA4, integrin alpha-4; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LTQ, Linear Trap Quadrupole; MAPK, mitogen-activated protein kinase; MYL12A, myosin regulatory light chain 12A; PKA, protein kinase A; Rho, Ras homolog gene family; RNAi, RNA interference; s.d., standard deviation; SILAC, stable isotope labeling by amino acids in cell culture;

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markers such as Nestin, Sox2, and Musashi-1 as well as self-renewal and multilineage potential. Besides the NSC-like characteristics, glioblastoma stem cells are also defined by high tumorigenicity and resistance to the current chemotherapy and radiation treatment, contributing to glioblastoma progression and recurrence. As it has been reported that decreased stem cell-like properties can reduce the tumorigenicity and radioresistance of glioblastoma stem cells (5–7), detailed understanding of the molecular mechanisms underlying alteration of glioblastoma stem cell properties is consider to lead to novel insights into effective therapeutic strategies against glioblastoma (8).

The signal transduction through protein phosphorylation is functionally important for various cellular processes such as proliferation, migration, or apoptosis. Several cell signaling pathways including Notch, Sonic hedgehog, and Wnt have been found to maintain stem-like properties of glioblastoma stem cells, in which protein phosphorylation play important roles in cell fate determination (9). Moreover, a kinome-wide RNA interference (RNAi) screen has reported that several kinases act as self-renewal regulators of glioblastoma stem cells (10). These previous findings underline the importance of phosphorylation processes as regulators of stem cell relevant pathways in glioblastoma stem cells.

In some previous studies, serum-mediated cell alteration is used to examine stem-like characteristics of glioblastoma stem cells established from tumor tissues of glioblastoma patients (3, 11, 12). Although the previous transcriptome and proteome analysis suggested some key molecules for maintenance of glioblastoma stem cell properties, the global changes of protein phosphorylation in serum-induced alteration remain unclear (13, 14). Thus, we aimed to reveal the phosphoproteome dynamics in glioblastoma stem cells named GB2, which were established from the tumor tissues of the glioblastoma patient (15–18). GB2 cells grow as neurospheres in serum-free culture and are classified into proneural-type glioblastoma stem cells based on the transcriptional profiles of 24-signature genes suggestive of proneural characteristics (16, 19). In addition, GB2 cells express wild-type isocitrate dehydrogenase 1 and 2 (IDH1/2), which are frequently mutated in low grade glioma, while the epigenetic regulation mediated by 5-hydroxymethylcytosine was reported to be associated with the expression of glioblastomagenesis-related genes, including EGFR, AKT3, CDK6, CCND2, and BRAF (18). Our previous study showed that the cultivation in serum medium down-regulated the gene expression of the cancer stem cell marker CD133 and the NSC marker nestin in GB2 cells (16). Moreover, the transplantation of GB2 cells into the frontal lobe of immunocompromised mice showed that the cells grown in serum medium lost their high tumorigenicity. In this study, we applied a combination of stable isotope labeling by amino acids in cell culture (SILAC), TGFBR1/2, transforming growth factor-beta receptor type-1/2, phosphoproteome dynamics in glioblastoma stem cells, and computational phosphorylation network analyses to identify key molecules involved in the regulation of GB2 cells.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Anti-Sox2 (sc-17320), anti-TGFBR2 (sc-400), and Horseradish peroxidase (HRP)-conjugated anti-goat IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GFAP (20334) was from Dako (Glostrup, Denmark). Anti-α-Myc (5605), anti-Musashi-1 (5663), anti-phospho-ERK1/2 (T202/Y204) (9101), anti-phospho-Src (Y416) (2101), and anti-phospho-cofilin (Ser3) (3313) were from Cell Signaling Technology (Danvers, MA). Anti-α-Tubulin (CP06) was from Calbiochem (San Diego, CA). HRP-conjugated anti-mouse and anti-rabbit IgG were from GE healthcare (Little Chalfont, UK). Recombinant human TGF-β1 was from PEPROTECH (Rocky Hill, NJ). LY2109761 was purchased from Selleck Chemicals (Houston, TX), SB431542, epidermal growth factor (EGF), and basal fibroblast growth factor (bFGF) were from Wako (Osaka, Japan). LY2109761 and SB431542 were used as dimethyl sulfoxide (DMSO) solution.

**Cell Culture and SILAC Labeling**—GB2 cells were originally established from the tumor tissues classified as primary glioblastoma in the University of Tokyo Hospital with informed consent and approval by the Research Ethics Committee at the Institute of Medical Science, the University of Tokyo as previously reported (15–18). The cells were cultured in the serum-free condition consisting of Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12) media, B27 supplement without vitamin A (Life Technologies, Carlsbad, CA), EGF (20 ng/ml), and bFGF (20 ng/ml). For maintenance of stem cell-like properties, EGF and bFGF were added every 2–3 days. Uncoated plastic dishes were used for neurosphere culture of GB2 cells, whereas the plates were coated with Laminin (Sigma, St. Louis, MO) for at least 2 h at 10 μg/ml prior to use for adherent culture of the cells (20). To induce loss of stem-like characteristics in GB2 cells, the cells were cultured in the serum condition consisting of DMEM/F12 and 10% fetal bovine serum. The expression of Sox2 and GFAP were quantified by Western blotting to evaluate the stem cell-like properties of GB2 cells. For quantitative mass spectrometric analysis, glioblastoma stem cells were labeled with either normal arginine and lysine (Arg-0/Lys-0) or heavy isotope of the corresponding amino acids (Arg-10/Lys-8) in adherent serum-free culture until sufficient incorpo-
ration of the stable isotopes into the cell proteome was achieved (21). GB2 cells within 20 passages from the establishment were used for all the experiments. The conventional glioblastoma cell lines (U251, T98G, and U87) were cultured in DMEM/F12 and 10% fetal bovine serum.

Cell Proliferation Assay—In vitro proliferation of GB2 cells cultured in the serum-free or serum condition was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). GB2 cells maintained in serum-free medium were dissociated using Accumax (Innovenative Cell Technologies, San Diego, CA) and plated in serum-free or serum medium at a density of 2000 cells/well. The cell viability was measured and quantified by FLUOstar Optima plate reader (BMG Labtech, Durham, NC) on day 0, 1, 3, 5, and 7.

Sample Preparation—GB2 cells grown in normal medium (Arg-0/ Lys-0) were induced to lose their stem-like characteristics by replacing the medium with serum-containing DMEM/F12 and then culturing for 7 days, whereas the cells labeled with heavy stable isotopes (Arg-10/Lys-8) were maintained in serum-free medium. The cells were washed three times with PBS, harvested, and lysed in UPX Universal Protein Extraction Buffer (Protein Discovery, Knoxville, TN) containing PhosSTOP (Roche Diagnostics, Basel, Switzerland), and Benzonase (Novagen, Madison, WI). The cell lysates were centrifuged for 30 min at 15,000 rpm and the supernatants were transferred into new tubes. Each cell lysate was quantified using BCA Protein Assay Kit, Reducing Agent Compatible (Thermo Scientific, Rockford, IL), and mixed in equal ratio (250 μg : 250 μg). The proteins in the mixed cell lysate were fractionated by molecular weight with GELFREE™8100 (Protein Discovery) using a 10% cartridge and digested into peptides based on the Filter-Aided Sample Preparation (FASP) method (22). Briefly, each fractionated sample was loaded on the filter and the detergents were removed by washing two times with the buffer containing 8 M urea. The proteins were reduced with 1 mM dithiothreitol for 90 min, alkylated with 5.5 mM iodoacetamide for 30 min, and digested overnight using sequencing-grade modified trypsin (Promega, Madison, WI).

Phosphopeptides were enriched by Titansphere Phos-TiO Kit (GL Sciences, Tokyo, Japan) as recommended by the manufacturer. In short, after equilibration of the TiO2 tip column, digested peptide mixtures were applied to Spin Tip, mixed with the buffer containing 2-hydroxypropanoic acid, and centrifuged. After the column was washed, captured peptides were eluted with 5% ammonium and 5% pyrrolidine solution, successively. The enriched phosphopeptide solutions were acidified by 10% TFA, desalted by ZipTip C18 (Millipore, Billerica, MA), and evaporated down to a volume of up to 10 μl by a vacuum concentrator.

LC-MS/MS Analysis—A total of 16 Titansphere elutes prepared above were analyzed using Dina-2A nanoflow LC system (KYA Technologies, Tokyo, Japan) coupled with LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were injected into 75 μm reversed-phase C18 column at a flow rate of 10 μl/min and eluted with a linear gradient of solvent A (2% acetonitrile and 0.1% formic acid in H2O) to solvent B (40% acetonitrile and 0.1% formic acid in H2O) at 300 nl/min. The peptides were sequentially sprayed from nanoelectrospray ion source (KYA Technologies, Tokyo, Japan) and analyzed by the collision induced dissociation (CID) method. Mass spectra were acquired in data dependent mode, switching automatically MS and MS/MS acquisition. All full-scan MS spectra in the range from m/z 380 to 2000 were acquired in the FT-MS part of the mass spectrometer with a target value of 1,000,000 and a resolution of 100,000 at m/z 400. The 20 most intense ions that satisfied an ion selection threshold above 2000 were fragmented in the linear ion trap with normalized collision energy of 35% for activation time of 10 ms. For accurate mass measurement, the Orbitrap analyzer was operated with the “lock mass” option using polydimethylcyclosiloxane (m/z = 445.120025) and bis(2-ethylhexyl) phthalate ions (m/z = 391.284286) (23).

Protein Identification and Quantification—Protein identification was performed by searching MS and MS/MS data against the RefSeq (National Center for Biotechnology Information) human protein database (35,853 protein sequences as of Feb 4, 2013) using Mascot ver. 2.4.1 (Matrix Science, London, UK). Carbamidomethylation of cysteine was set as a fixed modification, whereas oxidation of methionine, protein N-terminal acetylation, pyro-glutamination for N-terminal glutamine, phosphorylation of serine, threonine, and tyrosine, and stable isotopes of arginine (15N6 and 18N4) and lysine (13C6 and 15N2) were set as variable modifications. Trypsin was defined as a proteolytic enzyme and a maximum of two missed cleavages were allowed. The mass tolerance was set to three parts per million (ppm) for peptide masses and 0.8 Da for MS/MS peaks. In the process of peptide identification, we conducted decoy database searching by Mascot and applied a filter to satisfy a false positive rate lower than 1%. The ratio of heavy/light for each spectrum was calculated by the quantitation node and the probability of phosphorylation for each Ser/Thr/Tyr site on the peptide sequence was calculated by the PhosphoRS node in Proteome Discoverer ver. 1.3 (Thermo Fisher Scientific, Bremen, Germany). Phosphorylation sites with a site probability above 75% were regarded as confidently localized. After selecting the data supported by at least two peptide matches, the heavy/light ratios of phosphopeptides with confidently localized phosphorylation sites were log2-transformed and normalized based on the median according to the previous report (24). The phosphopeptides exhibiting more than twofold changes were considered as regulated for further computational analyses. The phosphorylation status of Fyn, MAPK1/3, and Cofilin was measured by Western blotting for validation of the phosphoproteome regulation. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (25) via the PRIDE partner repository with the dataset identifier PXD002614 and 10.6019/PXD002614.

Gene Ontology Analysis—All the Gene Ontology data on the GB2 phosphoproteome were analyzed by Cytoscape (26) along with its Plugin BINGO ver. 3.0.2 (27), which is a java-based tool to determine which GO categories are statistically over-represented in a set of human genes. On the basis of the quantified phosphoproteome data, we compared the annotations of the regulated phosphoproteins with those of the unregulated ones. The hypergeometric statistical test and Benjamini and Hochberg false discovery rate correction were performed to extract over-represented GO terms (adjusted p value < 0.01).

Pathway Analysis—The statistical extraction of canonical pathways was conducted through the use of KeyMolnet (KM Data, Tokyo, Japan) (28). The list of the regulated phosphoproteins was imported into KeyMolnet and the “Interrelation search” algorithm was used to generate the network of the molecular interactions between the related proteins. The top canonical pathways associated with the generated network were extracted from the KeyMolnet knowledge base with the p values calculated by a hypergeometric test.

The other pathway analysis was also performed using Ingenuity Pathway Analysis (IPA) (Qiagen, Redwood City, CA). The regulated phosphoproteins and their log2-transformed SILAC ratios were uploaded into the IPA software and the top canonical pathways associated with the uploaded phosphoproteins were listed along with the p values calculated using a right tailed Fisher’s exact test.

Upstream Kinase/Regulator Prediction Analysis—Upstream kinase prediction based on the identified phosphorylation sites were performed using NetworKIN ver. 2.0 (29). To remove likely false-positive predictions, the predicted kinase-substrate interactions were then filtered by the criteria below; context score > 0.8, NetworKIN score > 4 and predictions for each site having a NetworKIN score of above 0.8.
80% of the highest scoring. Kinase enrichment analysis was performed with hypergeometric testing using PhosphoSiteAnalyzer, a bioinformatic platform for the NetworKIN prediction results from the phosphoproteome data (30).

The upstream regulator analysis was performed using IPA. The upstream regulators associated with the regulated phosphoproteome were listed along with the p values calculated using a right tailed Fisher’s exact test (p value < 0.05).

**Signaling Perturbation Analysis—** For clonal sphere formation assay, cells grown as spheres were dissociated with Accumax and plated in serum-free medium with each of the TGFBR inhibitors (LY2109761 or SB431542) at a density of 25 or 50 cells/well. To assess the effect of the TGFBR ligand, TGF-β1 was added to the culture at a final concentration of 100 pm after 1 h cultivation. The number of tumorspheres (> 50 μm) formed on the 96-well plates was counted 3 weeks after seeding under a phase-contrast microscope. A two-sided Student’s t test was used for calculation of the p values. For Western blotting analysis, cells were dissociated with Accumax and cultured in serum-containing medium in the presence of LY2109761 or SB431542 at a final concentration of 20 μM. After the cells were lysed in the lysis buffer (8 M Urea, 500 mM Tris-HCl, pH 8.2), the lysates were separated on SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with a primary antibody and then with an appropriate HRP-conjugated secondary antibody according to the protocol recommended by the manufacturer of each antibody. The blots were exposed to Luminata™ Forte Western HRP Substrate (Millipore) and analyzed by ChemiDoc MP imaging system (Bio-Rad, Hercules, CA).

**RNA Interference by siRNA Transfection—** TGFBR2 Stealth siRNA (siTGFB2: 5′- CAG AAT UGC AUG AGC AAC UGC A-3′), TGFBR2 Silencer Select Predesigned siRNA (siTGFB2: s14077), and Negative control stealth siRNA with medium GC content were purchased from Life Technologies. Transfection of siRNAs into serum cultured GB2 cells was performed using Lipofectamine RNAiMAX transfection reagent (Life Technologies, Carlsbad, CA). The GB2 cells were collected 3 days post-transfection for measuring the status of NSC and astroglial markers by Western blotting. To further assess the effect of TGFBR2 knockdown, cells grown as spheres were dissociated with Accumax and plated in serum-free medium with each siRNA transfected at 2000 cells/well, where the viability of GB2 cells was maintained during the transfection experiment. The number of spheres (> 50 μm) formed on the 96-well plates was counted 2 weeks after seeding under a phase-contrast microscope. A two-sided Student’s t test was used for calculation of the p values.

**RESULTS**

**Effect of Serum-containing Culture on Glioblastoma Stem Cell Properties—** GB2 cells, which were previously established from the brain tumors of the patient, maintain sphere-forming ability and express high levels of the NSC markers under the serum-free condition (15–18). In this study, GB2 cells were induced to lose stem cell-like properties by cultivation in the medium containing 10% fetal bovine serum as reported previously (5, 12). GB2 cells were grown as floating spheres in the serum-free condition, whereas serum cultured GB2 cells were morphologically changed into adherent monolayers (Fig. 1A). The status of stem cell-like characteristics was further assessed by Western blots of NSC and astroglial markers. Our result showed the expression of Sox2 and Musashi-1 (the NSC markers) was decreased, whereas that of glial fibrillary acidic protein (GFAP; an astroglial marker) was increased by cultivation in the serum-containing condition (Fig. 1B). Although GB2 cells showed decreased proliferation by the transfer from serum-free to serum containing medium (supplemental Fig. S1), the cells propagated over 10 passages in the serum condition, which was consistent with the previous studies (5, 16).

**Mass Spectrometry-based Analysis of Serum-induced Phosphoproteome Dynamics in Glioblastoma Stem Cells—** SILAC technology was applied to quantitatively analyze global protein phosphorylation changes between serum-free and serum cultured GB2 cells (Fig. 2A). To achieve sufficient incorporation of stable isotope-labeled amino acids, GB2 cells were cultured as monolayers on laminin-coated plates and adapted to the medium containing “Heavy” and “Light” forms of the arginine and lysine for at least six passages (20). The serum-free cultured cells (Heavy) and serum cultured cells (Light) were lysed and mixed at a protein ratio of 1:1. The mixed cell lysate was fractionated by molecular weight, reduced, alkylated, and digested into peptides with trypsin. After phosphopeptide enrichment with TiO2 column, the sample was subjected to nanoflow LC-MS/MS analysis. In our shotgun phosphoproteome analyses of GB2 cells, 21,456 redundant peptides corresponding to 1726 proteins were identified (false discovery rate [FDR] of < 1%), which included...
19,090 phosphopeptides derived from 1584 phosphoproteins (supplemental Table S1). This result indicated that phosphopeptide enrichment was conducted at high selectivity (89%) in our sample preparation. The 2876 phosphorylation sites, which were confidently assigned to the specific amino acid residues on each peptide by the phosphoRS algorithm, were composed of 2523 phosphorylated serine (pS), 317 pT, and 36 pY residues and their distribution was similar to the previous large-scale phosphoproteome analysis (31) (Fig. 2B). The detailed information on all the identified phosphopeptides and their phosphorylation sites are presented in supplemental Table S1.

**Quantitative Description of the Regulated Phosphoproteome**—The changes in the phosphorylation level of the identified phosphopeptides were quantitated based on SILAC-encoded precursor ions using the Proteome Discoverer platform. The quantitative reproducibility was evaluated by Pearson’s correlation coefficient between the representative quantification results, leading to high accuracy ($r = 0.93$). The 2452 unique phosphopeptides from 1139 phosphoproteins were identified by more than one peptide spectrum match and 732 phosphorylation sites on 419 phosphoproteins were found to be regulated more than twofold in the course of serum-induced alteration. Among these proteins, 372 phosphopeptides derived from 238 phosphoproteins were highly enriched in the serum-free cultured GB2 cells ($\log_2 [H/L] > 1$), whereas 362 phosphopeptides from 184 phosphoproteins were more abundant in the serum cultured cells ($\log_2 [H/L] < -1$) (Fig. 2C and supplemental Table S2). The phosphorylation dynamics of Fyn, MAPK1/3, and Cofilin in GB2 cells was further validated by Western blotting (supplemental Fig. S2).

Protein kinases are crucial for cell fate regulation through reversible phosphorylation of signaling molecules in cellular pathways and their enzymatic activities are regulated by...
phosphorylation of specific amino acid residues on each kinase. In our phosphoproteome data, 17 phosphorylation sites on 11 protein kinases were found to be differentially regulated between the serum-free and serum condition (Table I). The critical phosphorylation sites for the kinase activity of Fyn (Ser21 and Tyr420) were up-regulated in serum-free medium, whereas those of mitogen-activated protein kinase 1 (MAPK1) (Thr185/Tyr187) and MAPK3 (Tyr204) were up-regulated in the serum cultured cells. Fyn, one of the Src-family tyrosine kinases, is known to phosphorylate the cytoplasmic domain of the cancer stem cell marker CD133. Fyn-mediated phosphorylation of this molecule are known to contribute to promote self-renewal and tumor formation of glioblastoma stem cells (32). The phosphorylated Thr185/Tyr187 residues of MAPK1 and Tyr204 of MAPK3 were also reported to be up-regulated in glioblastoma stem cells after serum-mediated loss of stem-like characteristics in the previous study (14).

Gene Ontology Analysis of the Regulated Phosphoproteome—To understand what protein functions were statistically over-represented in our phosphoproteome data, GO classification was performed using a biological network GO tool (BiNGO) (27). The over-represented terms in the biological process category were found to be significantly involved with developmental process and cytoskeleton organization (Fig. 3B). The result in relation to the cellular component category showed that the regulated phosphoproteins were prominently classified into cytoskeleton, adherens junction, and cell projection, which were all related to cell morphology (Fig. 3C) (33).

Network Analysis Reveals Cytoskeletal Signaling as Regulated Pathways—To investigate which signaling pathways could contribute to regulation of stem cell-like characteristics, we further analyzed the quantified phosphoproteome data using KeyMolnet and IPA. First, we imported the list of the regulated phosphoproteins into KeyMolnet and generated their molecular network with the information on the interactions between the imported molecules by the “Interrelation search” algorithm. As a result of the statistical test based on the number of the overlapping molecular relations between the depicted network and the KeyMolnet-curated canonical pathways, the phosphoproteins related to “Intermediate filament signaling” and “Integrin signaling” were found to be significantly regulated in serum-induced alteration of GB2 cells (Fig. 4A). Regarding the “Intermediate filament signaling,” many phosphorylation sites on the NSC marker proteins, vimentin and nestin, were detected in our shotgun phosphoproteome analysis (Fig. 4B and Table II).

| Gene symbol | Description | Phosphorylation site | Log(H/L) (Mean ± S.D.) |
|-------------|-------------|----------------------|-----------------------|
| CAMK2D      | Calcium/calmodulin-dependent protein kinase type II subunit delta isoform 3 | S330 | 1.79 ± 0.186 |
|             |             | S333 | 1.466 ± 0.000 |
|             |             | T287 | -0.051 ± 0.081 |
|             |             | T331 | 1.996 ± NA    |
|             |             | T337 | 0.230 ± 0.017 |
| DCLK1       | Serine/threonine-protein kinase DCLK1 isoform 1 | S352 | -1.368 ± 0.000 |
| FYN         | Tyrosine-protein kinase Fyn isoform a | S21  | 1.155 ± NA    |
|             |             | S25  | 1.155 ± NA    |
|             |             | Y420 | 2.575 ± 0.000 |
| GRK5        | G protein-coupled receptor kinase 5 | S484 | 1.129 ± 0.000 |
| LYN         | Tyrosine-protein kinase Lyn isoform A | S13  | 1.405 ± 0.000 |
| MAPK1       | Mitogen-activated protein kinase 1 isoform 1 | T185; Y187 | -6.212 ± 0.000 |
| MAPK3       | Mitogen-activated protein kinase 3 isoform 2 | Y187 | -2.447 ± 0.000 |
| MARK1       | Serine/threonine-protein kinase MARK1 | T648 | 1.343 ± NA    |
| MARK2       | Serine/threonine-protein kinase MARK2 isoform a | S422 | 0.463 ± 0.000 |
|             |             | S452 | -0.625 ± 0.310 |
|             |             | S885 | -1.655 ± NA    |
|             |             | S87  | 0.318 ± 0.000 |
|             |             | S241 | -0.790 ± 0.518 |
| FDPK1       | 3-phosphoinositide-dependent protein kinase 1 | S245 | 1.022 ± NA    |
| PRKCB       | Protein kinase C beta type isoform 2 | S660 | 3.417 ± NA    |
|             |             | S664 | 3.417 ± NA    |
|             |             | T500 | -0.166 ± 0.030 |
|             |             | T504 | -0.122 ± NA    |

* NA: Not Available because only one phosphopeptide was quantified.
Next, we applied our phosphoproteome data to IPA to further evaluate the signaling networks regulated through serum-induced alteration. The IPA-based canonical pathway enrichment analysis indicated that “Signaling by Rho Family GTPases” was highlighted as the most significant pathway (−log [p value] = 8.73), which was known to be involved in cytoskeleton dynamics (Fig. 5 and supplemental Table S4). The strong association of some kinases in Rho family GTPase signaling with maintenance of stem cell-like properties was also reported in the previous kinome-wide RNAi screening study regarding glioblastoma stem cells (10).

Upstream Kinase/Regulator Analysis Highlights TGFBR2 as a Key Regulator in Glioblastoma Stem Cell Characteristics—To search for the signaling molecules that could function upstream of the regulated phosphoproteins, we then performed an integrated prediction analysis of upstream kinases/regulators using NetworKIN and IPA. First, the NetworKIN algorithm was applied to predict responsible kinases upstream of the regulated phosphoproteome, which was followed by a hypergeometric enrichment test using PhosphoSiteAnalyzer (30). The 4643 kinase-substrate relations between 72 kinases and 1468 phosphorylation sites were processed for statistical tests with a Benjamini-Hochberg adjusted p value < 0.05 as a criterion for significance and the phosphorylation sites in relation to TGFBR2 and activin receptor type-2 (ACVR2) were found to be over-represented in the serum-free cultured cells (Fig. 6A and supplemental Table S5).
threonine kinases, are known to function as starting points of TGF-β and activin signaling pathways.

In addition to the NetworKIN-based kinase prediction analysis, IPA-guided upstream regulator analysis was then performed to identify the factors that might act upstream of the regulated phosphoproteome. As a result, TGFBR2, a ligand of TGFBR1, was predicted as the most significant upstream regulator ($p$ value = $9.77 \times 10^{-18}$) (Fig. 6B, C and supplemental Table S6).

**Experimental Validation of TGF-β Signaling in Serum-induced Alteration of Glioblastoma Stem Cells**—In our computational analyses of upstream kinases/regulators based on the phosphoproteome data, TGFBR2 was integratively predicted to be associated with serum-induced regulation of GB2 cells. TGF-β1 is known to transmit signals through the heterodimeric complex of the transmembrane serine/threonine kinases (TGFBR1 and TGFBR2). Binding of TGF-β1 to the constitutively active TGFBR2 dimer recruits and subsequently

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**Fig. 4.** Network analysis of the regulated phosphoproteome by KeyMolnet. A, Canonical pathway analysis by KeyMolnet. The $p$ values were calculated from hypergeometric tests based on the number of the overlapping molecular relations between the generated network and the canonical pathways stored in KeyMolnet. B, Quantitative phosphorylation changes regarding “Intermediate filament signaling pathway.” The image was created by overlaying the quantitative phosphoproteome data on “Intermediate filament signaling pathway” in KeyMolnet. The colors represent the largest distribution of the log$_2$-transformed SILAC ratios regarding each phosphorylated protein as shown at the lower-right corner. The dashed circles show vimentin and nestin.
in the serum-induced phosphoproteome dynamics in GB2 cells.

Moreover, in the serum cultured monolayer cells and conventional glioblastoma cells, expression profiles of glioblastoma stem cells with those of serum cultured cells at both of the mRNA and protein levels after serum-mediated alteration showed that integrin family expression of the NSC marker (Sox2). The siRNA-based knockdown of TGFBR2 also showed the increase of Sox2 as well as the decrease of GFAP and affected sphere formation capacity of GB2 cells (supplemental Fig. S5). Collectively, these results indicate that TGFBR2 might be critically involved in promoting reduction of stem cell-like properties in GB2 cells.

**DISCUSSION**

Glioblastoma stem cells are known to possess the NSC-like capacity of self-renewal and multilineage potential as well as the characteristics of high tumorigenicity in vivo. The cells maintained in serum-free medium have been reported to keep stem cell-like properties and recapitulate the genotype and transcriptome of the original tumors, whereas the cells after cultivation in serum-containing medium lose their cancer stem cell character and exhibit the property similar to conventional glioblastoma cell lines (5). Thus, systematic understanding of glioblastoma stem cell regulation is considered to lead to develop novel therapeutic strategies against glioblastoma.

Comprehensive description of gene/protein expression profiles provides a powerful tool for analyzing complicated molecular systems to control stem-like characteristics of glioblastoma stem cells. Schulte et al. compared the global gene expression profiles of glioblastoma stem cells with those of serum cultured monolayer cells and conventional glioblastoma cells, leading to identification of C-X-C chemokine receptor type 4 (CXCR4) as a target to block the growth of invasive glioblastoma stem cells (13). The integrated analysis of gene and protein expression in glioblastoma stem cells after serum-mediated alteration showed that integrin family members and extracellular matrices, such as collagen type 4, laminin alpha 2, and fibronectin, were up-regulated in the serum cultured cells at both of the mRNA and protein levels (14). This report also revealed that interaction of integrin 5 and fibronectin functioned in the formation of the niches of glioblastoma stem cells. However, the comprehensive profile of protein phosphorylation in serum-induced alteration of glioblastoma stem cells is still not clear, although phosphorylation plays a critical role in cell fate regulation in glioblastoma stem cells (36). Here, to grasp phosphorylation-dependent cellular signaling crucial to regulation of glioblastoma stem cell properties, we performed a SILAC-based quantitative analysis of serum-induced phosphoproteome dynamics in GB2 cells.

### TABLE II

**List of the quantified phosphorylation sites regarding vimentin and nestin**

| Phosphorylation sites | Log$_2$(H/L) | S.D.$^a$ | Peptide count used for quantification |
|-----------------------|--------------|----------|--------------------------------------|
| S7                    | −0.829       | ± NA     | 1                                    |
| S10                   | −0.031       | ± 0.061  | 3                                    |
| T20                   | −0.047       | ± NA     | 1                                    |
| S22                   | 0.079        | ± 0.214  | 14                                   |
| S22; S26              | −0.795       | ± 0.000  | 3                                    |
| S25                   | −0.122       | ± 0.080  | 9                                    |
| S26                   | −0.197       | ± 0.000  | 6                                    |
| S27                   | 0.049        | ± 0.182  | 3                                    |
| S29                   | 0.048        | ± 0.301  | 2                                    |
| S34                   | 0.147        | ± 0.000  | 2                                    |
| S39                   | 1.493        | ± 0.494  | 2                                    |
| S42                   | 0.392        | ± 0.501  | 4                                    |
| S5                    | −0.829       | ± NA     | 1                                    |
| S51                   | 0.076        | ± 0.398  | 22                                   |
| S51; S56              | 0.783        | ± 0.000  | 4                                    |
| S55                   | 0.887        | ± 0.229  | 14                                   |
| S56                   | 0.910        | ± 0.208  | 31                                   |
| Y61                   | 0.820        | ± 0.221  | 10                                   |
| S72                   | −0.690       | ± 0.352  | 2                                    |
| S73                   | 0.354        | ± 0.072  | 5                                    |
| S83                   | 0.963        | ± 0.798  | 21                                   |
| Y117                  | 0.105        | ± NA     | 1                                    |
| S205                  | −0.197       | ± 0.110  | 5                                    |
| S214                  | 0.094        | ± 0.118  | 12                                   |
| S299                  | 0.318        | ± 0.000  | 2                                    |
| S325                  | 0.650        | ± 0.314  | 23                                   |
| S339                  | 0.193        | ± 0.007  | 6                                    |
| T361                  | 1.208        | ± 0.000  | 3                                    |
| S430                  | −0.484       | ± 0.047  | 14                                   |

| Peptide count used for quantification |
|--------------------------------------|
| 1                                    |
| 3                                    |
| 1                                    |
| 14                                   |

| Nestin |
|--------|
| Phosphorylation sites | Log$_2$(H/L) | S.D.$^a$ | Peptide count used for quantification |
| T315 | 1.938 | ± NA | 1 |
| S325 | 0.955 | ± 0.084 | 6 |
| S471 | −0.485 | ± 0.163 | 21 |
| S680 | −0.551 | ± 0.419 | 17 |
| S768 | −0.879 | ± 0.620 | 38 |
| S1016 | −2.016 | ± NA | 1 |
| S1347 | −0.305 | ± 0.000 | 5 |
| S1409; S1418 | −0.811 | ± 0.000 | 3 |
| S1418 | −0.550 | ± 0.325 | 13 |
| S1577 | −0.833 | ± 0.297 | 19 |

$^a$ NA: Not Available because only one phosphopeptide was quantified.
Our data showed that the SILAC ratios of 734 unique phosphopeptides from 419 phosphoproteins were altered more than twofold and the proteins related to developmental process were found to be statistically over-represented by the GO categorization, which reflected the overall effects of the serum-induced alteration on the phosphoproteome of GB2 cells.

In canonical pathway analyses using KeyMolnet and IPA, the phosphoproteins differentially regulated in serum cultured GB2 cells were found to be significantly associated with “Intermediate filament signaling,” “Signaling by Rho family GTPases,” and “Integrin signaling.” These cellular signaling pathways were all involved with cellular assembly and organization, such as cytoskeleton organization, adhesion junction, and morphogenesis, which was consistent with the GO classification. Notably, we found significant changes regarding functional phosphorylation sites on several key molecules in the Signaling by Rho family GTPases (Fig. 5B). The IPA-based description showed that the functional phosphorylation sites on RAC1 guanine nucleotide exchange factor 7...
Fig. 6. Upstream kinase/regulator analyses based on the regulated phosphoproteome data. A, Heatmap of the over-representation p values calculated for each predicted kinase using PhosphoSiteAnalyzer. The subset “serum (−)” indicates SILAC ratio > 2.0, whereas “serum (+)” shows SILAC ratio < 0.5. TGFBR2 and ACVR2A/B-specific phosphorylation sites were predicted to be significantly enriched in the “serum (−)” subset (adjusted p value < 0.05). B, Upstream regulator analysis by IPA. The top ten upstream regulators relevant to the regulated phosphoproteome are shown with the corresponding score (−log [p value]). C, IPA-based description of TGF-β1 and the target molecules in our phosphoproteome data. Red indicates increased phosphorylation in serum-free cultured GB2 cells, whereas green shows increased phosphorylation in serum cultured cells. Dashed lines represent indirect interactions caused by TGF-β1.
(ARHGEF7), brain-specific angiogenesis inhibitor1-associated protein 2 (BAIAP2), cofilin-1, and vimentin were upregulated in serum-free cultured GB2 cells. The calcium/calmodulin-dependent protein kinase type-1 (CAMK1)-mediated Ser516 phosphorylation of ARHGEF7 is known to promote synapse formation during neuronal development and in structural plasticity (37). BAIAP2 is an adaptor protein that links membrane-bound small G-proteins to cytoplasmic effector proteins. The phosphorylation of Thr340 and Thr360 on BAIAP2 promotes the binding to 14-3-3 protein, which act as a regulator of lamellipodia (38). The Ser3 phosphorylation of cofilin-1 is known to decrease its actin depolymerization activity, which leads to inhibition of cytoskeletal rearrangement associated with breakage of neural sphere during NSC development (39). Among the 29 phosphorylation sites on vimentin, Ser39 and Thr361 were regulated in serum-induced alteration of GB2 cells (Table II). The phosphorylation of vimentin at Ser39 by serine/threonine protein kinase AKT1 is known to
promote in vivo growth and metastasis of soft-tissue sarcoma cells (40). On the other hand, the functional phosphorylation sites on integrin alpha-4 (ITGA4) and myosin regulatory light chain 12A (MYL12A) were up-regulated in serum cultured GB2 cells. ITGA4 pairs with either of integrin-β1 or β7 to form the receptors for fibronectin or vascular cell adhesion protein-1. The protein kinase A (PKA)-mediated phosphorylation of Ser121 on ITGA4 leads to block the binding with paxillin, resulting in enhancement of cell spreading (41). The increase of Ser19 phosphorylation on MYL12A by adhesion stimulation are correlated with recruitment of myosin IIα and IIβ and expand lamellipodium of spreading cells (42). These results suggest that the phosphorylation status in the Signaling of Rho family GTPases should contribute to cellular assembly and organization in the serum-induced alteration process of GB2 cells. Further functional analysis is necessary to clarify the relation between these phosphorylation events and loss of glioblastoma stem cell properties in each context.

Our phosphoproteomics-based network analysis highlighted Fyn and MAPK1/3 as functionally important kinases in correlation with the regulation of serum-induced phosphoproteome dynamics in GB2 cells. Regarding the tyrosine kinase Fyn, a member of the Src family kinases, Yeo et al. reported that phosphorylation of Ser21 within the PKA recognition site and subsequent autophosphorylation of Tyr420 were critical for adhesion-mediated signaling (43). Our phosphoproteomic result indicated that the phosphorylation levels at Ser21 and Tyr420 were elevated in serum-free cultured GB2 cells, which reflected the previous evidence that the high phosphorylation level of Tyr420 on Fyn was observed in clinical samples from glioblastoma patients (44). In serum cultured GB2 cells, the functional sites for the kinase activity of MAPK1 (Thr185/Tyr187) and MAPK3 (Tyr202) were also highly phosphorylated in agreement with the previous study regarding glioblastoma stem cells (14).

The association of TGFBR2-mediated TGF-β signaling with serum-dependent alteration of GB2 cells was statistically extracted by the NetworKIN-based kinase prediction as well as the IPA-guided upstream regulator determination, which was experimentally validated by signaling perturbation using the corresponding inhibitors. There are several previous reports that TGF-β signaling plays an important role in the regulation of proliferation, migration, and apoptosis regarding glioblastoma (45). High activity of TGF-β-SMAD signaling was observed in aggressive glioblastomas and conferred poor prognosis in the patients (46). Besides the SMAD-dependent pathways, SMAD-independent signaling via MAPK1/3 was reported to be involved with high expression of Nodal and cell proliferation in glioblastoma (47). Regarding glioblastoma stem cells, autocrine production of TGF-β maintains their stem cell-like properties through the expression of Sox2 (48), whereas TGF-β is also reported to dominantly induce alteration of glioblastoma stem cells into pericytes to support tumor vessel function and growth (49). These different responses to TGF-β might contribute to the emergence of tumor heterogeneity in cancer stem cells (50). Our result indicated that inactivation of TGFBR2 led to increased self-renewal property in GB2 cells, which is in accordance with the previous report that the capacity of sphere formation and self-renewal was increased in TGFBR2-ablation mutant of neural stem cells (51). Because sphere formation does not necessarily equate to stem cell characteristics, in vivo dilution experiment will be needed to prove the alterations in stem cell properties. Although inhibition of TGFBR1 were reported to potentiate radiation responses in glioblastoma stem cells (52), our study revealed that TGFBR2 might also be a novel regulator of glioblastoma stem cell properties. The extensive analysis of the cell lines established from other patients will be required to clarify whether the results are generalizable to other tumors.

In this study, the integration of network analysis with quantitative phosphoproteomics unveiled the key signaling pathways/molecules involved in the serum-induced dynamics in glioblastoma stem cells. Notably, the combination of phosphorylation site-oriented kinase prediction and protein interaction-based upstream regulator description highlighted the association of TGFBR2-mediated signaling with alterations in stem cell-like characteristics. Our result indicated that the integrated bioinformatic analysis based on the quantitative phosphoproteome data led to systematic identification of the glioblastoma stem cell fate regulators from a statistical point of view. Further analysis of the time-resolved phosphoproteome regarding loss of glioblastoma stem cell properties will uncover multiple key signaling molecules at different stages of stem cell regulation for effective treatment against glioblastoma.

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