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Desert Hedgehog Down-regulation Mediates Inhibition of Proliferation by γ-Glutamylcyclotransferase Knockdown in Murine Glioblastoma Stem Cells

MASAYA MORI[1], HIROMI II[1], MITSUGU FUJITA[2], KOZUE NOSE[1], AYAKO SHIMADA[1], RISA SHIRAKI[1], YUHI SONE[1], CHIAMI MOYAMA[1*], KEIKO TANIGUCHI[3] and SUSUMU NAKATA[1]

[1]*Laboratory of Clinical Oncology, Kyoto Pharmaceutical University, Kyoto, Japan;*
[2]*Center for Medical Education and Clinical Training, Kindai University Faculty of Medicine, Osaka-Sayama, Japan;*
[3]*Department of Drug Discovery Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan*

Abstract. *Background/Aim: Glioblastoma is the most frequent type of adult-onset malignant brain tumor and has a very poor prognosis. Glioblastoma stem cells have been shown to be one of the mechanisms by which glioblastoma acquires therapy resistance. Therefore, there is a need to establish novel therapeutic strategies useful for inhibiting this cell population. γ-Glutamylcyclotransferase (GGCT) is an enzyme involved in the synthesis and metabolism of glutathione, which is highly expressed in a wide range of cancer types, including glioblastoma, and inhibition of its expression has been reported to have antitumor effects on various cancer types. The aim of this study was to clarify the function of GGCT in glioblastoma stem cells. Materials and Methods: We searched for pathways affected by GGCT overexpression in mouse embryonic fibroblasts NIH-3T3 by comprehensive gene expression analysis. Knockdown of GGCT and overexpression of desert hedgehog (DHH), a representative ligand of the pathway, were performed in glioblastoma stem cells derived from a mouse glioblastoma model. Results: GGCT overexpression activated the hedgehog pathway. Knockdown of GGCT inhibited proliferation of glioblastoma stem cells and reduced expression of DHH and the downstream target GLI family zinc finger 1 (GLI1). DHH overexpression significantly restored the growth-suppressive effect of GGCT knockdown. Conclusion: High GGCT expression is important for expression of DHH and activation of the hedgehog pathway, which is required to maintain glioblastoma stem cell proliferation. Therefore, inhibition of GGCT function may be useful in suppressing stemness of glioblastoma stem cells accompanied by activation of the hedgehog pathway.*

**Current address:* Department of Pharmacy, Shiga University of Medical Science Hospital, Shiga, Japan.

Correspondence to: Susumu Nakata, MD, Ph.D., Laboratory of Clinical Oncology, Kyoto Pharmaceutical University, Misasagi-Nakauchicho 5, Yamashina-ku, Kyoto 607-8414, Japan. Tel: +81 755954719, e-mail: snakata@mb.kyoto-phu.ac.jp

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Glioblastoma is the most frequent histological type of adult-onset malignant brain tumor and has a poor prognosis (1). One of the reasons for this is its development of resistance to drug and radiation therapy. Recently, the presence of cancer stem cells has been implicated as a cause of cancer recurrence (2). Glioblastoma stem cells are self-renewing and multipotent cells that are highly resistant to chemotherapy and radiotherapy, contributing to recurrence of glioblastoma (3). Therefore, there is a need to establish new therapeutic strategies that are useful for inhibiting glioblastoma stem cells.

γ-Glutamylcyclotransferase (GGCT) was first reported as C7orf24, a protein with unknown function that is highly expressed in urological carcinomas compared to normal tissues (4). Subsequent studies revealed that GGCT is an enzyme that catalyzes the reaction that generates 5-oxoproline and free amino acids from γ-glutamyl dipeptide in the γ-glutamyl cycle, a circuit involved in glutathione synthesis and metabolism (5, 6). Thus, GGCT may be involved in glutathione metabolism and redox homeostasis. We and other groups have reported that GGCT inhibition has antitumor effects against various cancer types (7-9). GGCT is highly expressed in glioblastomas (10). However, the function of GGCT in glioblastoma stem cells is unknown.

The hedgehog pathway has important roles in embryonic development and tissue regeneration (11). In humans, three types of hedgehog proteins, namely desert hedgehog (DHH), Indian hedgehog (IHH), and sonic hedgehog (SHH), act as ligands (12). When the ligand binds to the Patched receptor, inhibition of the regulatory factor Smoothened is released; Smoothened transduces signals into the cell and promotes the release of the transcription factors GLI family zinc finger 1 (GLI1) and -2, which are translocated into the nucleus and regulate the expression of target genes of the hedgehog pathway. Downstream target genes of the hedgehog pathway include *GLI1* itself (11). Abnormalities in the hedgehog pathway cause carcinogenesis, such as basal cell carcinoma and medulloblastoma (13).

DHH is expressed in Schwann cells, and its expression is upregulated in response to nerve injury and promotes the formation of the connective tissue sheath surrounding the peripheral nerve (14). Thus, DHH plays an important function in the proliferation of nervous system cells and may be important for the growth of brain tumor cells including glioblastoma cells. In addition, DHH expression has been reported to correlate with the proliferation rate of uterine cancer (15, 16), but no association with glioblastoma or GGCT has been reported as far as we are aware.

In this study, we hypothesized that *GGCT* knockdown inhibits glioblastoma stem cell proliferation *via* suppression of the hedgehog pathway. To test this, we used glioblastoma stem cells derived from our originally established mouse glioblastoma tissues (17, 18). In our previous study, we reported that these glioblastoma stem cells showed activation of the hedgehog pathway with high expression of GLI1/2, which correlated with the expression of the cancer stem cell marker leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) (17). The aim of this study was to demonstrate that inhibition of cell proliferation by GGCT knockdown in glioblastoma stem cells is mediated by the Hedgehog signaling pathway, including DHH protein.

Materials and Methods

GGCT overexpression in NIH-3T3 cells. Mouse embryonic fibroblast NIH-3T3 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and *GGCT* was overexpressed using pCX4bsr vector as described in (4, 8). NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% or 2% fetal bovine serum and 25 mM HEPES. Cells were cultured in a humidified incubator at 37˚C with 5% CO_2.

Microarray analysis. Total RNA was extracted from *GGCT*-overexpressing NIH-3T3 cells and empty vector-transfected control cells using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and an RNeasy Mini Kit (Qiagen, Hilden, Germany). Expression profiling was performed using the Whole Mouse Genome SurePrint G3 Mouse Gene Expression 8x60K v2 platform (Agilent Technologies, Santa Clara, CA, USA). The data were analyzed with GeneSpring software (version 14.9.1; Silicon Genetics, Redwood City, CA, USA), and raw data were normalized using the 75th percentile method. Genes significantly upregulated (n=3, fold change >2.0 and $p<0.05$ with unpaired Welch's *t*-test) due to *GGCT* overexpression were subjected to pathway analysis using the Wiki-Pathway database (19). The raw microarray data have been deposited in the Gene Expression Omnibus database (GSE266154, GSE154592).

Glioblastoma induction. C57BL/6J mice were obtained from Charles River Laboratories Japan Inc. (Kanagawa, Japan). Animals were bred and handled in the Bioscience Research Center, Kyoto Pharmaceutical University as per an Institutional Animal Care and Use Committee-approved protocol (A21-011). Two neonatal mice were placed in a stereotaxic instrument (51730D; Stoelting Co., Wood Dale, IL, USA). Using an automated infusion system (Legato130; KD Scientific, Holliston, MA, USA), the following DNA plasmids were used in this study: the pT2/C-Luc//PGK-SB13 plasmid construct containing the firefly luciferase gene (*Luc*) under the control of the chicken beta-actin promoter (*CAGGS*) and the SB13 transposase gene driven by the phosphoglycerate kinase (*PGK*) promoter; the pT/CAGGS-NRASV12 plasmid containing the mutant *NRAS* gene (NRASV12) under the control of the chicken beta-actin promoter (*CAGGS*); the pT3.5/CMV-EGFRvIII plasmid construct containing the *EGFR* variant III (EGFRvIII) gene under the control of the cytomegalovirus (CMV) promoter; and finally, the pT2/shP53 plasmid containing a short hairpin RNA (shRNA) sequence targeting the *p53* gene, driven by the *U6* promoter. These plasmids, combined with 2 μl of *in vivo*-JetPEI (Polyplus Transfection, New York, NY, USA), were injected into the right lateral ventricle to generate *Sleeping Beauty* transposon-mediated *de novo* glioblastoma The injection coordinates were +1.5AP, 0.7ML, and −1.5DV from λ.

Glioblastoma cell culture. Glioblastoma stem cell cultures were established using the neurosphere method. Briefly, tumor tissue, from the mouse glioblastoma developed in the mouse cerebrum as described above, was minced with scalpels and digested with accutase (Innovative Cell Technologies, San Diego, CA, USA) at 37˚C for 20-30 min, and then incubated with serum-free neural stem cell culture medium consisting of Neurobasal Medium supplemented with B27 and N2 (Gibco/Thermo Fisher Scientific) and 10 ng/ml of epidermal growth factor and basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA).

DHH overexpression. *DHH* overexpression was performed using the Mouse Neural Stem Cell Nucleofector kit (#VPG-1004; Lonza, Tokyo, Japan) and with the A-033 program optimized for mouse neural stem cells of the Nucleofector 2b device (#AAB-1001; Lonza, Basel, Switzerland). Glioblastoma stem cells (5×10^6) were suspended in a solution of Nucleofector Solution (82 μl) and Supplement (18 μl). Cell suspensions with 10 μg of empty vector (#J4910HE310-3; GenScript, Piscataway, NJ, USA) or DHH-expressing vector (#J4910HE310-1; GenScript) were transferred to the attached cuvette for electroporation. One day after the gene transfer, the medium was replaced with serum-free neural stem cell culture medium supplemented with G418 (10 μg/ml; Nacalai Tesque, Kyoto, Japan) for selection of transduced cells.

Western blot analysis. NIH-3T3 cells with GGCT-OE, and glioblastoma stem cell lines with *GGCT* knockdown/*DHH* overexpression were

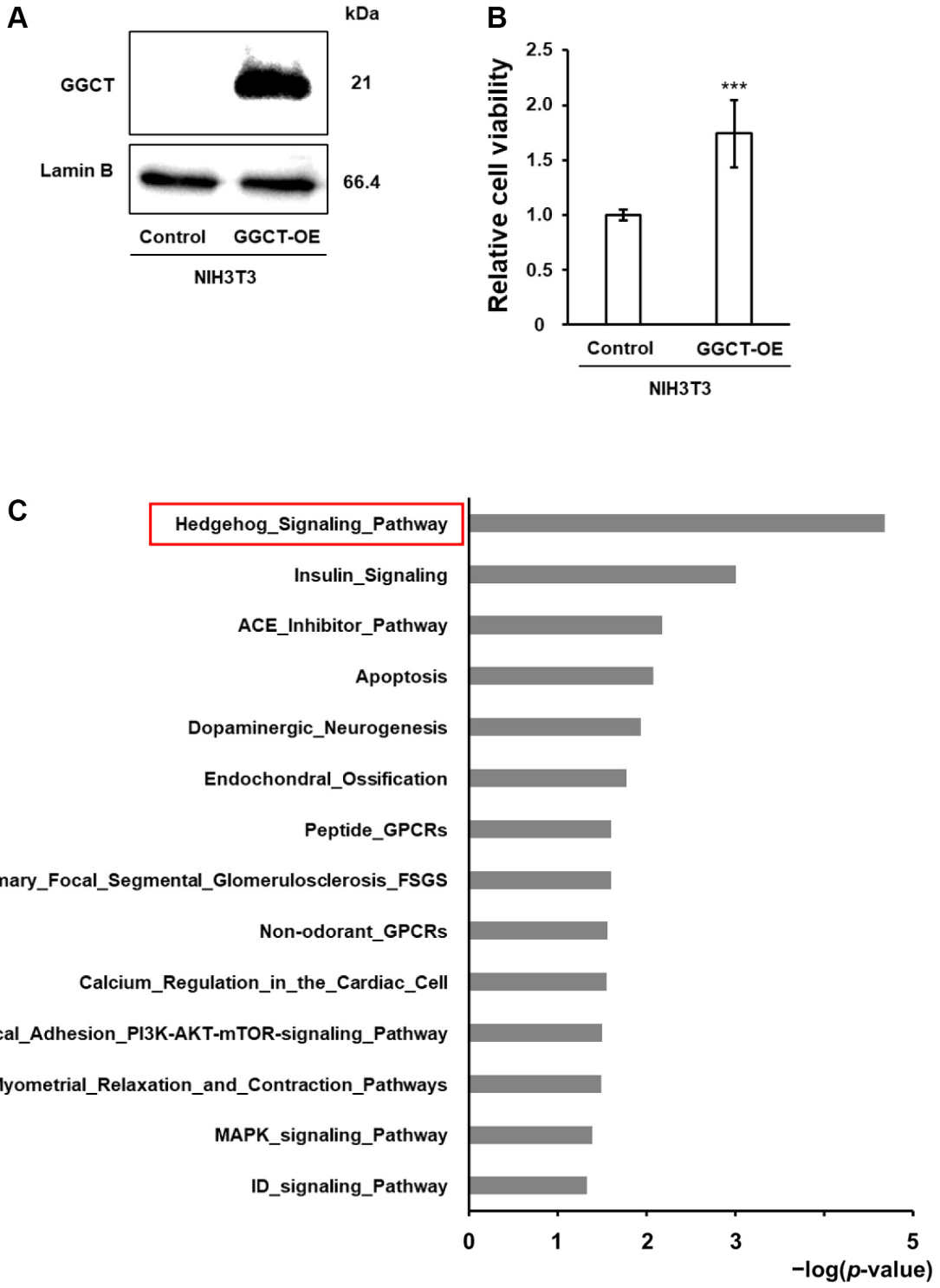

Figure 1. *γ-Glutamylcyclotransferase (GGCT) overexpression promotes mouse embryonic fibroblasts NIH-3T3 proliferation and the hedgehog pathway. (A) GGCT expression levels were compared by western blotting in NIH-3T3 cells cultured in Dulbecco's modified Eagle's medium with 2% serum 72 h after inducing GGCT overexpression. Lamin B was used as a loading control. (B) Relative cell viability was compared in NIH-3T3 cells with GGCT overexpression (n=10). ***Significantly different at p<0.001 by two-tailed Student's t-test. (C) Microarray pathway analysis was performed on genes in NIH-3T3 cells (fold change >2.0) up-regulated by GGCT overexpression. The most significantly affected pathways are shown. GGCT, γ-Glutamylcyclotransferase; OE, overexpression; ACE, angiotensin-converting enzyme; GPCRs, G protein-coupled receptors; MAPK, mitogen-activated protein kinase; ID, inhibitor of differentiation.*

lysed with sodium dodecyl sulfate lysis buffer (50 mM Tris-HCl, 1% sodium dodecyl sulfate) with a protease inhibitor cocktail mix (Nacalai Tesque) and PhosSTOP EASYpack (Roche, Basel, Switzerland). Protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific). The proteins (20-40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 7%, 10%, or 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Blocking was done for 1 h at room temperature with PVDF Blocking Reagent for Can Get Signal (TOYOBO, Osaka, Japan) for detection of DHH, with 5% dried milk in Tris-buffered saline with 0.05% Tween20 for detection of lamin B and GLI1, and with 3% bovine serum albumin in Tris-buffered saline with 0.05% Tween20 for detection of GGCT. The membranes were incubated with primary antibodies against DHH (sc-271168, 1:500; Santa Cruz Biotechnology, Dallas, TX, USA), GGCT (1:500; 16257-1-AP; Proteintech Group, Rosemont, IL, USA), GLI1 (L42B10, 1:1,000; Proteintech) and lamin B (12987-1-AP, 1:2,000; Proteintech) and secondary antibodies used were Horse anti-mouse IgG-horseradish peroxidase (1:5,000; PI-2000; Vector Laboratories, Burlingame, CA, USA) and horseradish peroxidase-linked goat anti-rabbit IgG (1:2,000; 7074; Cell Signaling Technology, Danvers, MA, USA). Protein levels were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) or ChemiLumi One Super (Nacalai Tesque). Chemiluminescence signal was detected using the ChemiDoc XRS Plus system (Bio-Rad).

Quantitative real-time polymerase chain reaction (RT-qPCR). Murine glioblastoma stem cells cultured for 4 days from electroporation were collected and lysed with TRIzol (500 μl; Thermo Fisher Scientific). Total RNA was purified using the RNeasy mini kit (Qiagen). cDNA was synthesized with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). RT-qPCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and a Light Cycler 96 System (Roche Diagnostic, Indianapolis, IN, USA) (n=3). Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method with the mRNA expression level of β-actin as an internal control. The following specific primers (Eurofins Genomics, Tokyo, Japan) were used: mGLI1, 5'-CCGACGGAGGTCTCTTTGTC-3' (forward) and 5'-AACATGGCGTCTCAGGGAAG-3' (reverse); β-actin, 5'-CACTGTC GAGTCGCGTCC-3' (forward) and 5'-TCATCCATGGCGAACT GGTG-3' (reverse).

Lentiviral vector-mediated short hairpin RNA (shRNA) interference. *GGCT* knockdown was performed as described in (20). RNA interference (RNAi) clones targeting *GGCT* with the sequence ACAGGAATATCAAGAGAAATT were purchased from Sigma-Aldrich (St Louis, MO, USA) (MISSION pLKO.1-puro). The lentiviral particles were produced according to the manufacturer's instruction. Transduction of murine glioblastoma stem cells was performed at a multiplicity of infection of 10.

Cell proliferation assay. Murine glioblastoma stem cells were transfected with shRNA, and after 3 days of culture, neurospheres were dissociated with accutase and a cell suspension was prepared. After adding an equal volume of 0.4% trypan blue (Wako Pure Chemical Industries), 10 μl of the suspension was applied to a dedicated slide chamber and cells were counted using a Countess II automated cell counter (Thermo Fisher Scientific) (n=3). Cells that were transparent were counted as live cells, and cells stained with trypan blue were counted as dead cells.

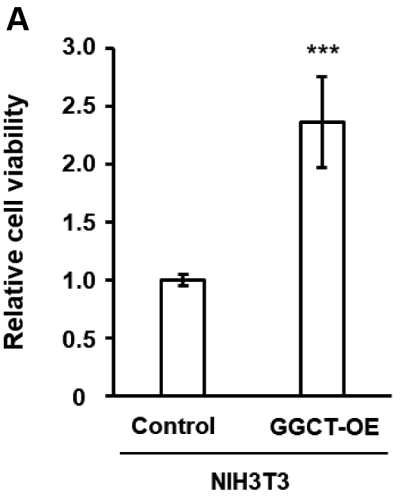

Figure 2. *Continued*

Statistical analysis. All data were obtained from at least three independent experiments and expressed as the mean±S.D. Statistical analysis was performed using two-tailed Student's *t*-test, one-way analysis of variance with Dunnett test or two-way analysis of variance with Bonferroni test with BellCurve for Excel (Social Survey Research Information Co., Ltd. Tokyo, Japan). A value $p<0.05$ was deemed significant.

Results

Overexpression of GGCT in NIH-3T3 mouse embryonic fibroblasts has a promoting effect on the hedgehog pathway. We previously reported that forced expression of GGCT in mouse embryonic fibroblasts NIH-3T3 enhanced proliferation and suppressed autophagy under serum deprivation (8). In the present study, we found that forced expression of GGCT under identical conditions of serum deprivation (Figure 1A) significantly promoted cell proliferation (Figure 1B). Similar results were also obtained under conditions with 10% serum (Figure 2A). Aiming to elucidate this growth-promoting mechanism, we performed a pathway analysis using comprehensive expression analysis and found that the hedgehog pathway was significantly affected by *GGCT* overexpression (Figure 1C). Similar results detecting members of the hedgehog pathway were obtained under conditions with 10% serum (Figure 2B and Figure 3). The full picture of this pathway was accompanied by increased expression of ligands, including DHH and the downstream target GLI1 (Figure 4). These results suggest that the promotion of cell proliferation by *GGCT* overexpression may be mediated by the promotion of the hedgehog signaling pathway.

Knockdown of GGCT in mouse glioblastoma stem cells suppresses their growth and reduces DHH expression. A previous study has shown that the hedgehog pathway plays an

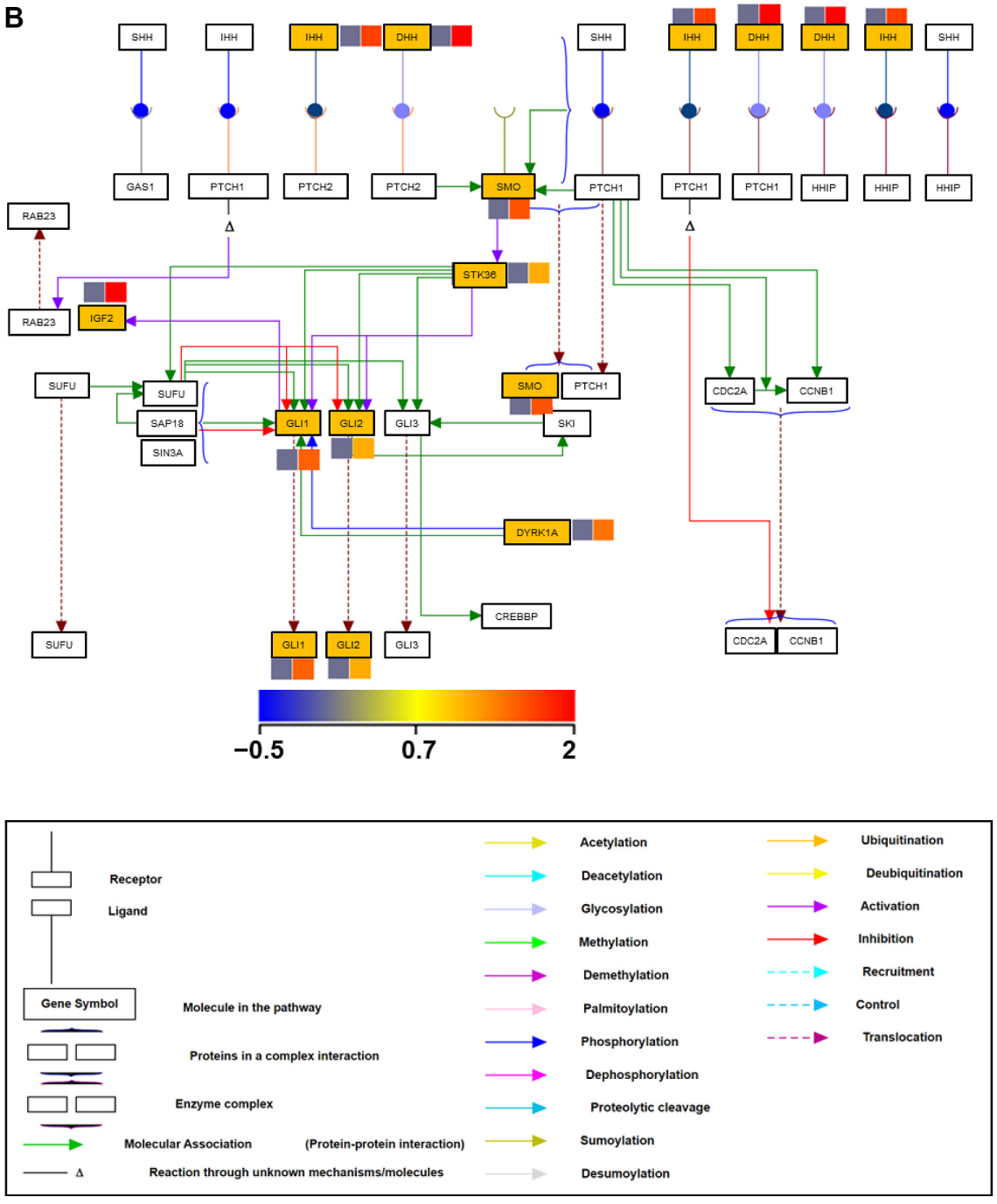

Figure 2. *γ-Glutamylcyclotransferase (GGCT) overexpression promotes NIH-3T3 cell proliferation and has a facilitative effect on the hedgehog pathway. (A) Relative cell viability was compared in NIH-3T3 cells cultured in Dulbecco's modified Eagle's medium with 10% serum 72 h after GGCT overexpression (n=12). ***Significantly different at p<0.001 by two-tailed Student's t-test. (B) The pathway diagram illustrates the key components and interactions within the hedgehog signaling pathway that are affected by GGCT overexpression. In the accompanying heat map, the expression levels of these genes are visually compared between control cells (non-targeting control, NT) on the left and GGCT-OE cells on the right. The heat map uses color coding to represent gene expression levels, with warmer colors (e.g., red) indicating higher expression levels and cooler colors (e.g., blue) indicating lower expression levels. SHH, Sonic hedgehog; IHH, Indian hedgehog; DHH, desert hedgehog; GAS1, growth arrest-specific 1; PTCH, patched; SMO, smoothened; HHIP, hedgehog interacting protein; RAB23, Ras-related protein Rab-23; STK36, serine/threonine kinase 36; IGF2, insulin-like growth factor 2; SUFU, suppressor of fused; CDC2A, cell division cycle 2 kinase A; CCNB1, cyclin B1; SAP18, sin3-associated polypeptide 18; GLI, GLI family zinc finger; SKI, Sloan-Kettering virus; SIN3A, SIN3 transcription regulator family member A; DYRK1A, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A; CREBBP, CREB-binding protein.*

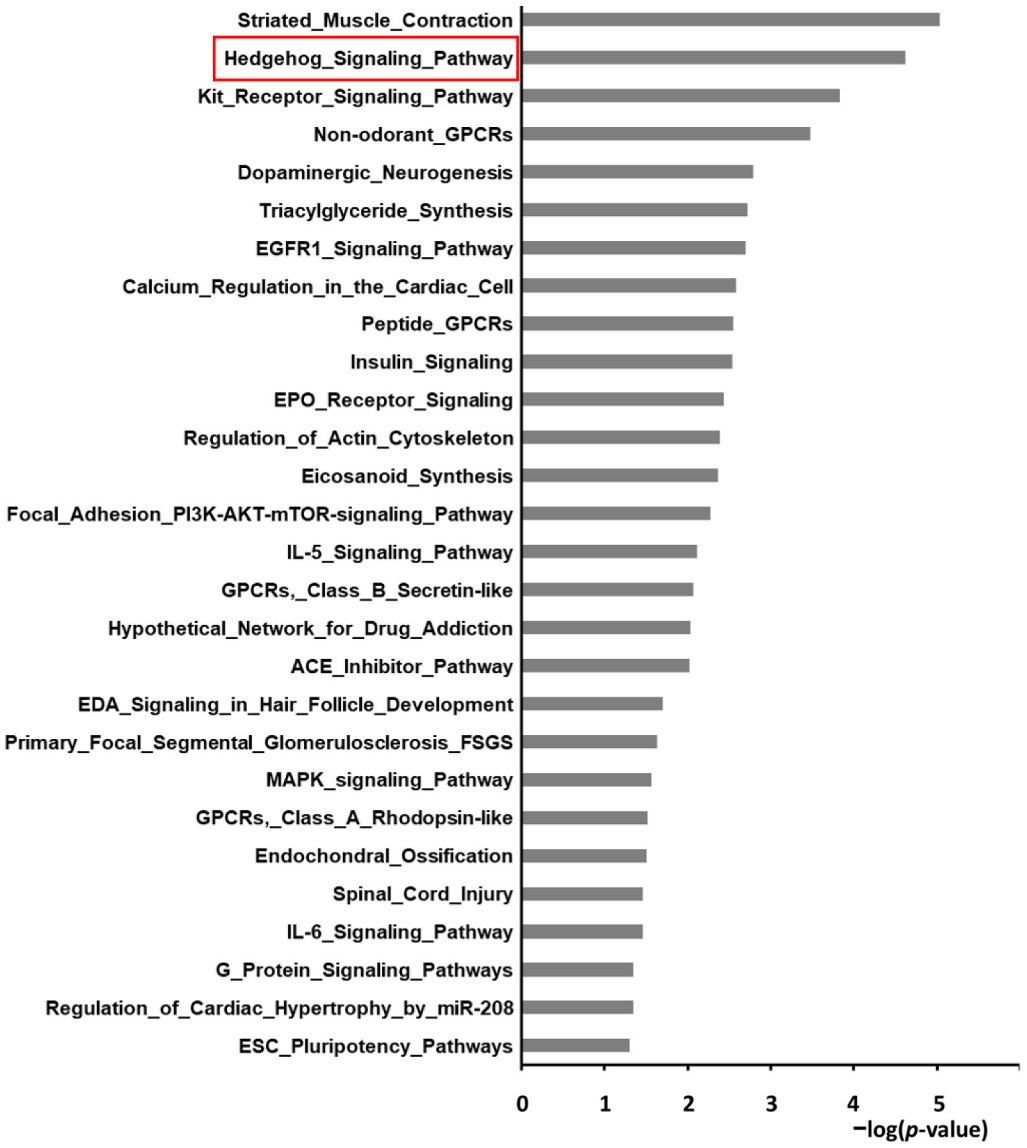

Figure 3. *γ-Glutamylcyclotransferase (GGCT) overexpression in NIH-3T3 cells cultured in Dulbecco's modified Eagle's medium with 10% serum affects the hedgehog pathway. Microarray pathway analysis was performed on genes up-regulated (fold change >2.0) in NIH-3T3 cells with GGCT overexpression. Significantly affected pathways are shown. GGCT, γ-Glutamylcyclotransferase; OE, overexpression; SHH, Sonic hedgehog; IHH, Indian hedgehog; DHH, desert hedgehog; GAS1, growth arrest-specific 1; PTCH, patched; SMO, smoothened; HHIP, hedgehog interacting protein; RAB23, Ras-related protein Rab-23; STK36, serine/threonine kinase 36; IGF2, insulin-like growth factor 2; SUFU, suppressor of fused; CDC2A, cell division cycle 2 kinase A; CCNB1, cyclin B1; SAP18, sin3-associated polypeptide 18; GLI, GLI family zinc finger; SKI, Sloan-Kettering virus; SIN3A, SIN3 transcription regulator family member A; DYRK1A, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A; CREBBP, CREB-binding protein.*

important role in the proliferation of glioblastoma stem cells (17). Our mouse glioblastoma stem cells were established from a glioblastoma model in which normal neural stem cells were transfected *in vivo* with carcinogenic genetic abnormalities: shRNA against p53, mutant NRasG12V, and mutant EGFRvIII. Sphere-forming glioblastoma stem cells express high levels of various stem cell markers, including prominin-1 (PROM1) and LGR5, and retain high tumorigenic potential to form glioblastomas by transplantation of 100 cells into another mouse (17, 18). Therefore, we performed *GGCT* knockdown in two independent lines of glioblastoma stem cells (Figure 5A). As a result, the proliferation of glioblastoma stem cells was significantly suppressed by *GGCT* knockdown (Figure 5B). These results indicate that GGCT may play an important role in glioblastoma stem cell proliferation. Further analysis by western blotting revealed that the expression of *DHH*, a

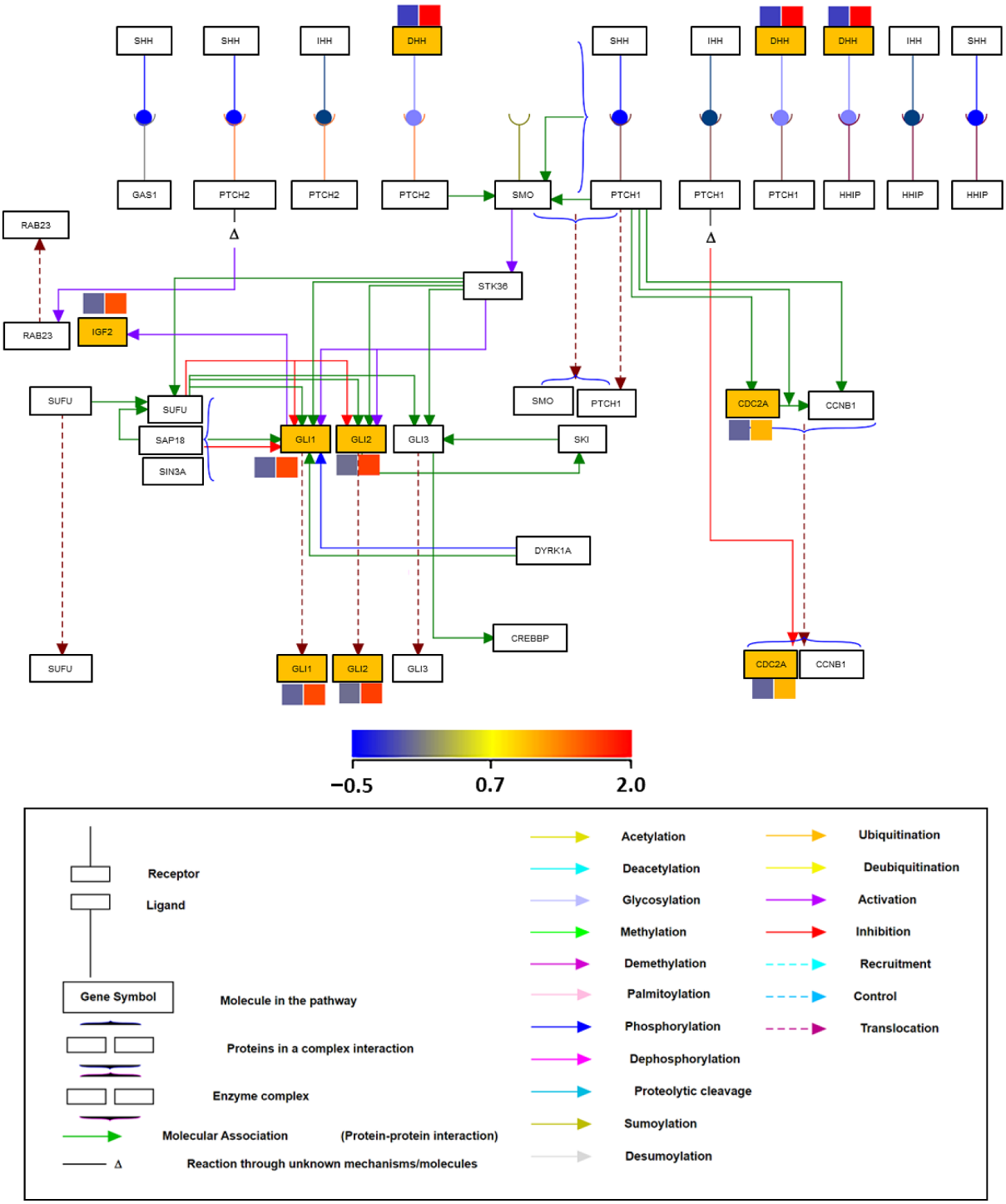

Figure 4. *γ-Glutamylcyclotransferase (GGCT) overexpression in mouse embryonic fibroblasts NIH-3T3 facilitates the expression of hedgehog pathway-related genes in NIH-3T3 cells cultured in Dulbecco's modified Eagle's medium with 2% serum 72 h after GGCT overexpression. This figure provides an overview of the hedgehog signaling pathway as determined by microarray analysis in NIH-3T3 cells with GGCT overexpression (GGCT-OE). The pathway diagram illustrates the key components and interactions within the hedgehog signaling pathway that are affected by GGCT overexpression. In the accompanying heat map, the expression levels of these genes are visually compared between control cells (non-targeting control, NT) on the left and GGCT-OE cells on the right. The heat map uses color coding to represent gene expression levels, with warmer colors (e.g., red) indicating higher expression levels and cooler colors (e.g., blue) indicating lower expression levels. GPCRs, G protein-coupled receptors; EGFR1, epidermal growth factor receptor 1; EPO receptor, erythropoietin receptor; IL, interleukin; ACE, angiotensin-converting enzyme; EDA signaling, ectodysplasin A signaling; MAPK, mitogen-activated protein kinase; miR-208, microRNA-208; ESC pluripotency, embryonic stem cell pluripotency.*

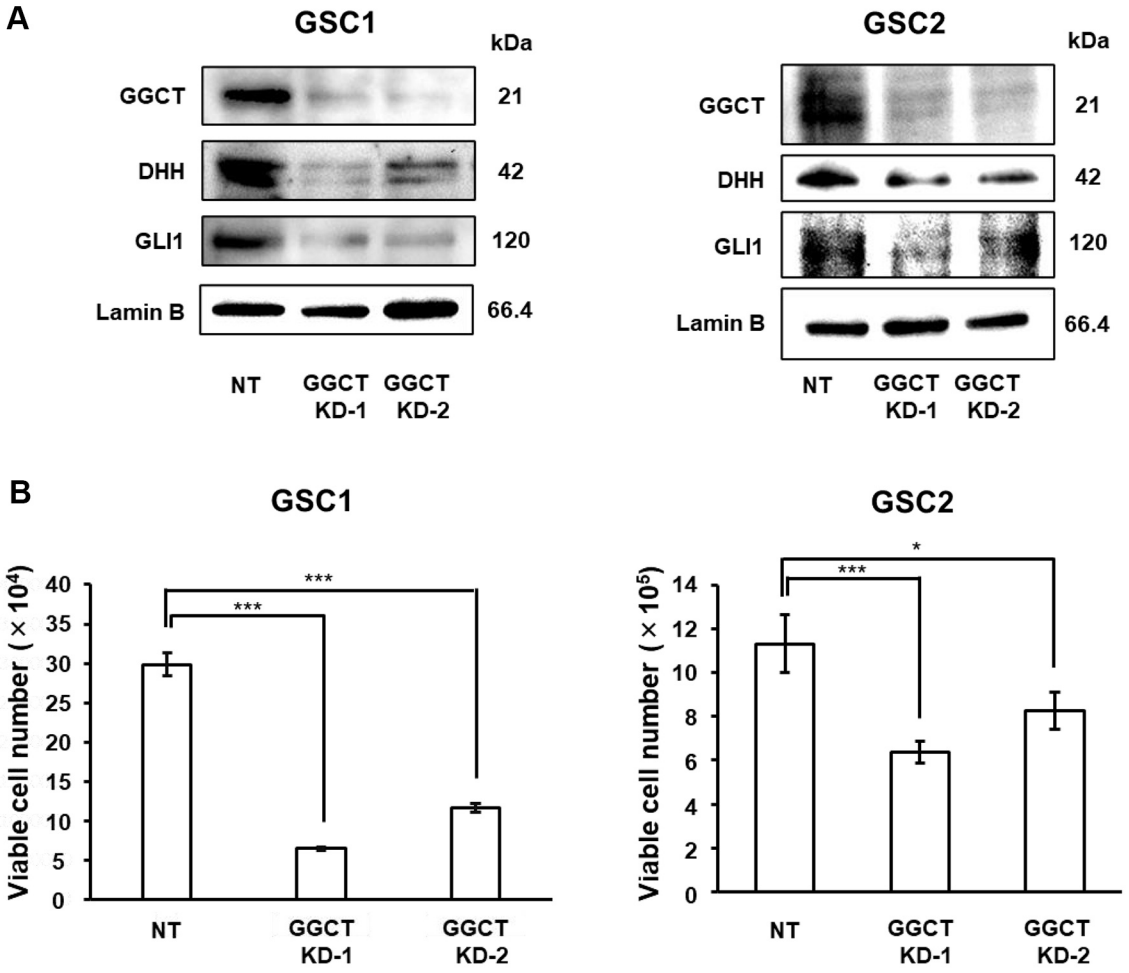

Figure 5. *γ-Glutamylcyclotransferase (GGCT) knockdown with short hairpin RNA (shRNA) suppresses glioblastoma stem cell proliferation and expression of hedgehog pathway factors. (A) Non targeting (NT) and two GGCT (GGCT-KD1 and GGCT-KD2) shRNAs were transfected into two independent of murine glioblastoma stem cell lines (GSC1 and GSC2) and after 3 days, the expression levels of GGCT, desert hedgehog (DHH) and GLI family zinc finger 1 (GLI1) were compared by western blotting (A) and glioblastoma stem cell proliferation was compared (n=3) (B). Lamin B was used as a loading control. Significantly different at *p<0.05 and ***p<0.001 by one-way analysis of variance with Dunnett test. GSC, Glioblastoma stem cells; GGCT, γ-glutamylcyclotransferase; DHH, desert hedgehog; GLI, GLI family zinc finger; KD, knockdown.*

representative ligand of the hedgehog pathway, and *GLI1*, a downstream target gene, were suppressed (Figure 5A). The protein expression of IHH and SHH, members of the hedgehog family, was also assessed, but was unchanged (data not shown). These results indicate that GGCT inhibition may inhibit glioblastoma growth *via* suppression of the hedgehog pathway.

Inhibition of proliferation of glioblastoma stem cells by GGCT knockdown was significantly ameliorated by forced expression of DHH. We next established glioblastoma stem cells with forced expression of *DHH* to analyze the contribution of the DHH reduction in the suppression of glioblastoma stem cell growth by *GGCT* knockdown (Figure 6A). Knockdown of *GGCT* was performed on the established *DHH*-overexpressing glioblastoma stem cells, and it was confirmed that the expression of *GGCT* was efficiently reduced (Figure 6B). Using this model and evaluating the viable cell number 3 days after shRNA transfection, *GGCT* knockdown had a growth-inhibitory effect that significantly reduced the viable cell number to approximately 40.5% ($p<0.001$). On the other hand, forced expression of DHH significantly ameliorated the growth-inhibitory effect of *GGCT* knockdown ($p<0.01$) (Figure 6C), even though forced expression of DHH alone had no significant effect on cell numbers. Microscopic examination of the cells 3 days after shRNA transfection showed that *GGCT* knockdown reduced sphere-formation capacity, but this effect was counteracted by forced expression of DHH (Figure 6D). Moreover, the

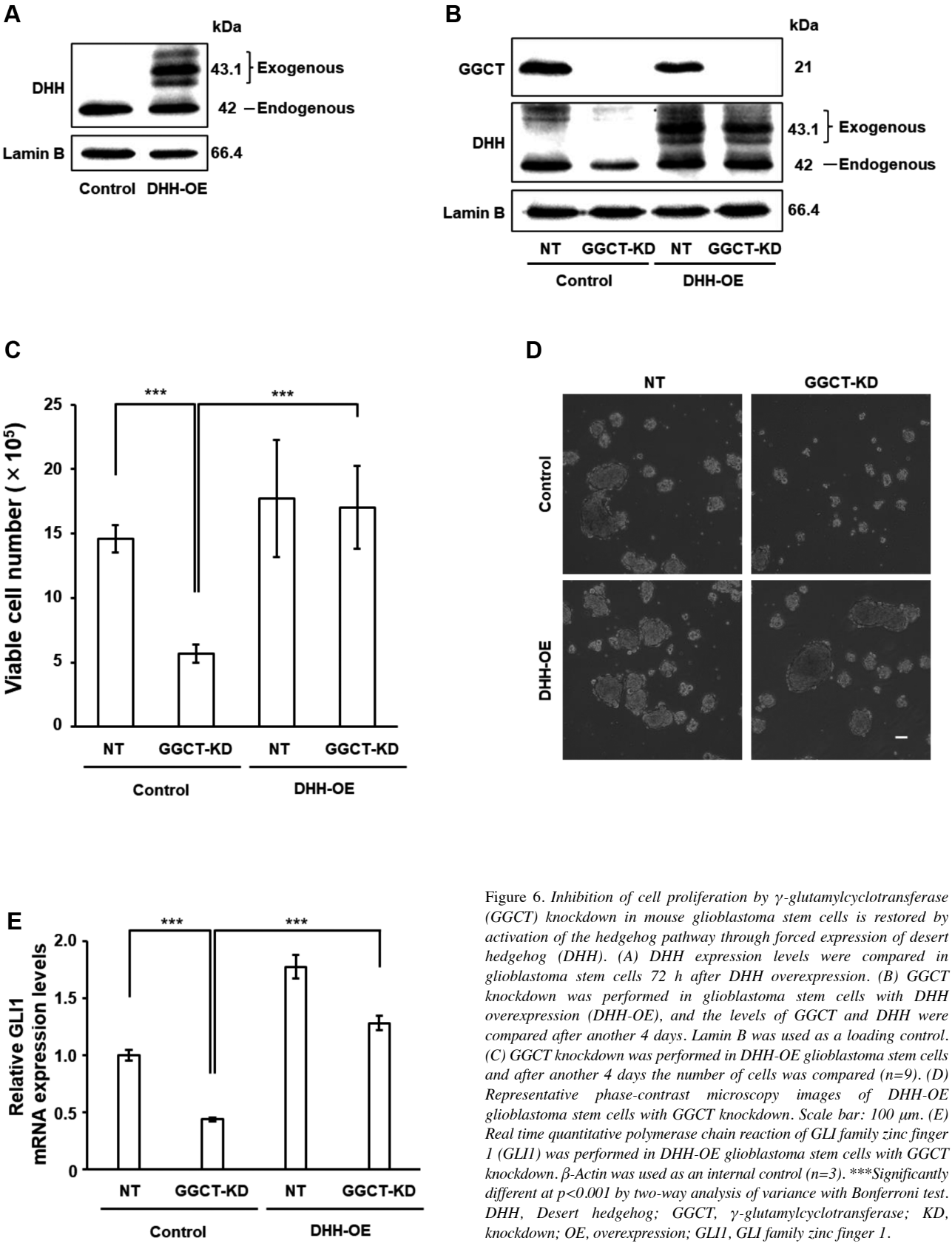

Figure 6. *Inhibition of cell proliferation by γ-glutamylcyclotransferase (GGCT) knockdown in mouse glioblastoma stem cells is restored by activation of the hedgehog pathway through forced expression of desert hedgehog (DHH). (A) DHH expression levels were compared in glioblastoma stem cells 72 h after DHH overexpression. (B) GGCT knockdown was performed in glioblastoma stem cells with DHH overexpression (DHH-OE), and the levels of GGCT and DHH were compared after another 4 days. Lamin B was used as a loading control. (C) GGCT knockdown was performed in DHH-OE glioblastoma stem cells and after another 4 days the number of cells was compared (n=9). (D) Representative phase-contrast microscopy images of DHH-OE glioblastoma stem cells with GGCT knockdown. Scale bar: 100 μm. (E) Real time quantitative polymerase chain reaction of GLI family zinc finger 1 (GLI1) was performed in DHH-OE glioblastoma stem cells with GGCT knockdown. β-Actin was used as an internal control (n=3). ***Significantly different at p<0.001 by two-way analysis of variance with Bonferroni test. DHH, Desert hedgehog; GGCT, γ-glutamylcyclotransferase; KD, knockdown; OE, overexpression; GLI1, GLI family zinc finger 1.*

expression level of *GLI1*, a downstream target factor of the hedgehog pathway, was suppressed by *GGCT* knockdown, and its expression was promoted in *DHH*-overexpressing cells (Figure 6E). These results suggest that the suppression of glioblastoma stem cell proliferation by *GGCT* knockdown may involve the suppression of the hedgehog pathway *via* downregulation of DHH expression.

Discussion

A major clinical problem is that glioblastomas are difficult to treat surgically due to their highly invasive nature. Therefore, drug therapy is important, but it has been reported that there are glioblastoma stem cells that contribute to drug resistance (21). The recurrence of glioblastoma with current therapies is partly due to the persistence of a subpopulation of glioblastoma stem cells that are highly resistant to drugs (22). Therefore, identification of novel therapeutic targets to attack glioblastoma stem cells is needed.

GGCT is highly expressed in a wide range of cancer types, including glioblastoma, and its high expression has been reported in human glioblastoma tissues and cultured cell lines. Most previous studies have analyzed the function of GGCT by knockdown or knockout in cancer cells (7-9), but not enough has been done to analyze the function of GGCT by forced expression in normal cells. Therefore, we first confirmed the function of GGCT in promoting cell proliferation by forcing expression of GGCT in mouse embryonic fibroblasts NIH-3T3. To elucidate the mechanism, by performing a comprehensive gene-expression analysis using microarrays, we demonstrated that forced expression of *GGCT* had a promotory effect on the hedgehog pathway. We focused on the fact that forced expression of *GGCT* strongly upregulated the ligands of the hedgehog pathway.

Previous studies have reported that the hedgehog pathway plays an important role in glioblastoma (23, 24). However, the function of GGCT in regulating the stemness of glioblastoma stem cells was completely unknown. Therefore, we performed *GGCT* knockdown in glioblastoma stem cells established from a mouse glioblastoma model and confirmed that GGCT suppressed cell proliferation. Furthermore, we observed that *GGCT* knockdown in glioblastoma stem cells reduced the expression of DHH, a representative ligand of the hedgehog pathway, and its downstream factor, GLI1. These experimental results led us to hypothesize that the regulation of glioblastoma cell proliferation and stemness by GGCT may be mediated by the hedgehog pathway. Therefore, we established glioblastoma stem cells with forced expression of *DHH* and performed *GGCT* knockdown. As hypothesized, forced expression of DHH significantly restored the inhibition of cell proliferation by *GGCT* knockdown through promoting the hedgehog pathway. These results suggest that GGCT has a promotory effect on DHH expression and that promotion of the hedgehog pathway plays an important role in cell proliferation and maintenance of stemness.

In summary, the results of this study demonstrate that the suppression of glioblastoma stem cell proliferation by *GGCT* knockdown involves the suppression of the hedgehog pathway *via* down-regulation of DHH expression. These findings suggest that functional inhibition of GGCT may be useful in suppressing stemness of glioblastoma stem cells through activation of the hedgehog pathway.

Conflicts of Interest

The Authors declare no conflicts of interest pertaining to the present study.

Authors' Contributions

MM, KN, AS, RS and YS performed the experiments. MM drafted the manuscript. HI, CM, KT, and MF designed and supervised the study. SN designed and supervised the study and wrote the article.

Acknowledgements

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