Am J Cancer Res 2022;12(3):1129-1142
www.ajcr.us /ISSN:2156-6976/ajcr0137384

Original Article

Stat5b inhibition blocks proliferation and tumorigenicity of glioblastoma stem cells derived from a *de novo* murine brain cancer model

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Received July 15, 2021; Accepted October 19, 2021; Epub March 15, 2022; Published March 30, 2022

Abstract: Glioblastoma (GBM) is the most common and malignant type of brain cancer in adults with poor prognosis. GBM stem cells (GSCs) reside within niches in GBM tissues and contribute to recurrence and therapy resistance. Previous studies have shown that expression of leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), a Wnt pathway-related stem cell marker, correlates with a poor prognosis in GBM, and its knockdown in GSCs induces apoptosis accompanied with downregulation of signal transducer and activator of transcription 5b (Stat5b). Here, we show that Stat5b co-localizes with Lgr5 in hypoxia-inducible factor 2α (Hif2α)-positive regions in GBM tissues. Functional analyses using GSCs derived from a murine *de novo* GBM model induced by oncogenic genes transduction using the *Sleeping-Beauty* transposon system revealed that expression of Stat5b was induced by culturing under hypoxia together with Lgr5, repressed by Hif2α knockdown, and reduced by Lgr5 knockdown or a Wnt/β-catenin signaling inhibitor ICG-001 treatment. Stat5b inhibition in the GSCs induced apoptosis and caused downregulation of *Cyclin E2* resulted in blockade of entry into S-phase in the cell cycle. Disruption of Stat5b in an orthotopic transplantation model significantly prolongs event-free survival. These results suggest that Stat5b, regulated by hypoxia and the Wnt pathway, plays an important role in the maintenance and tumorigenicity of GSCs and may be a promising therapeutic molecular target to attack GSCs.

Keywords: Stat5b, Lgr5, glioblastoma stem cells, Hif2α, hypoxia

Introduction

Glioblastoma multiforme (GBM) is the most common malignant brain tumor in adults and has an extremely poor prognosis, with median survival of approximately 15 months despite intensive combination therapies [1, 2]. Previous studies have identified stem cells in GBM tissue [3-7]. These GBM stem cells (GSCs) markedly resist to both chemotherapy [8] and radiation therapy [9] and contribute to disease recurrence [10]. Therefore, it is necessary to identify molecular therapeutic targets in the GSC populations.

Cancer stem cells reside in so-called stem cell niches and contribute to therapy-resistance in various types of cancer [11]. Activation of hypoxia-responsive signaling in hypoxic stem cell niches has also been shown to play an important role in therapy resistance and in the maintenance of GSCs [12-14].

Lgr5 is a member of the Wnt/β-catenin pathway and contributes to the maintenance of stem cells by boosting the Wnt signal [15]. Previously, Lgr5 has been reported as a stem cell marker in various normal tissues, including intestinal epithelium, stomach, skin, kidney, and mammary gland [16]. In addition, Lgr5 has been reported as a cancer stem cell marker in colorectal, gastric, esophageal, and cervical cancers, mainly by antibody staining [16, 17]. A previous report has shown that Lgr5 is highly expressed in GSCs derived from human clinical samples and is an essential factor for the survival of

GSCs. Moreover, Lgr5 protein levels correlate with a poor prognosis in the GBM cohorts [18]. GBM tissue-derived and antibody-enriched Lgr5-expressing cells exerted the GSC properties [19]. In addition, an *Lgr5-GFP* knock-in GBM mouse model has demonstrated the usefulness of Lgr5 as a GSC marker [20]. However, the precise mechanism by which Lgr5 enhances GSCs is unclear.

Stat5b, one of seven Stat family members [21], is activated by cytokines, hormones, and growth factors such as Interleukin-6, epidermal growth factor (EGF), and growth hormones [22-24]. Jak/Stat signaling is involved in the pathogenesis of multiple solid cancers and hematopoietic malignancies; in particular, constitutive activation of Stat3/5 is involved in progression in multiple cancers [25]. Stat3 has been reported to play an important role in the progression of malignant tumors derived from central nervous system tissues [26], while Stat5b has been shown to be involved in the survival, proliferation, and differentiation of immune cells and hematopoietic cells [27]. Stat5b shows 94% homology to Stat5a and has a unique molecular function in hematopoietic malignant cells [28]. Recently, mutations have been reported that cause constitutive activation of STAT5b in aggressive T-cell leukemia/lymphoma, including Stat5b^{N642H} [29]. However, a functional role for Stat5b in the regulation of GSCs is unknown.

A murine *de novo* GBM model has been established, employing the *Sleeping-Beauty* transposon system to induce *shTP53*, *EGFRvIII*, *NRasG12V in vivo* [30]. Injection of these oncogene cocktails into the lateral ventricle of neonatal mice induces the gene mutations in neural stem cells that reside in the periventricular zone. Brain tumors with the histological properties of GBM, such as micronecrosis with pseudopalisadings, develop within 2-3 months, providing a versatile platform for studying GBM biology [31]. Recently, we have purified Lgr5-expressing GSCs from the tumor tissues of this model and established Lgr5-expressing GSC neurospheres that exhibit tumorigenicity upon transplantation of from 100 to 1000 cells [20]. Given that knockdown of Lgr5 in human primary GSCs has been shown to induce apoptosis and result in downregulation of Stat5b expression [18], we hypothesized that Stat5b could play a role in the regulation of GSCs downstream of Lgr5. In the present study, we carried out functional analyses of Stat5b and investigate the mechanisms underlying the regulation of Stat5b expression in mouse GSCs.

Materials and methods

Animal experiments

C57BL/6 and BALB/c mice were obtained from the Oriental Bioservice (Kyoto, Japan). Animals were handled in the Bioscience Research Center at Kyoto Pharmaceutical University as per the Institutional Animal Care and Use Committee-approved protocol.

Glioblastoma induction

The procedure was performed as described previously [30]. Briefly, to induce *Sleeping-Beauty* transposon-mediated *de novo* glioblastoma, neonatal mice under hypothermia anesthesia were placed in a stereotaxic instrument (51730D; Stoelting Co., Wood Dale, IL, USA) and injected with 2 μl DNA/polyethylenimine complex into the right lateral cerebral ventricle at 1 μl/min with a 10 μl Hamilton syringe equipped with a 30-gauge needle and an automated infusion system (Legato130; KD Scientific, Holliston, MA, USA). The coordinates for injection were +1.5 AP, 0.7 ML, and -1.5 DV from λ. An *in vivo*-compatible DNA transfection reagent In vivo-JetPEI (Polyplus Transfection, New York, NY, USA) was used. The following DNA plasmids were used: pT2/C-Luc//PGK-SB13 (0.2 μg), pT/CAGGS-NRASV12 (0.4 μg), pT3.5/CMV-EGFRvIII (0.4 μg), and pT2/shP53 (0.4 μg).

Cell culture

GSC cultures were established using the neurosphere method as described previously [20]. Briefly, tumor tissues were minced with scalpels and digested with accutase (Innovative Cell Technologies, San Diego, CA, USA) at 37°C for 20-30 min, and then washed and incubated with serum-free neural stem cell culture medium consisting of Neurobasal Medium supplemented with B27, N2 (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), and 10 ng/ml EGF and bFGF (R&D Systems, Minneapolis, MN, USA). The neurospheres were dissociated by accutase (Innovative Cell Technologies) and maintained in the neural stem cell culture medium. The procedure used to establish

adherent glioblastoma cell lines from the murine glioblastomas was described previously [31] and the resulting cell lines and the immortalized mouse astrocyte line C8D30 (ATCC, Manassas, VA, USA) were maintained with DMEM supplemented with 10% FBS (HyClone, South Logan, UT, USA). All cells were maintained at 37°C in a 5% CO_2 atmosphere. Culturing under hypoxic conditions was performed using CO_2 Incubator 9000EX (WakenBtech, Kyoto, Japan).

Flow cytometric detection of Lgr5 expression

After hypoxic stimulation, cells were fixed with 4% formaldehyde for 10 min at room temperature and blocked with 0.5% bovine serum albumin and 2% fetal bovine serum in PBS for 30 min on ice. After being washed in PBS, the cells were incubated with Lgr5 antibodies (2.5 μg/1×10^6 cells; MAB8240; R&D Systems) or Isotype control (MAB0061; R&D Systems) for 30 min on ice, and with secondary antibodies (1:2000; Alexa Fluor 555, ab150154; Abcam, Cambridge, UK) for 30 min on ice. The stained cells were analyzed using a BD LSRFortessa X-20 cell analyzer (BD Biosciences, San Diego, CA, USA). At least 10,000 cells per sample were analyzed.

Reagents

The reagents IQDMA (ab141192; Abcam) and ICG-001 (#10-4378; Focus Biomolecules, Plymouth Meeting, PA, USA) were dissolved in Dimethyl Sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan) and added to the medium at the indicated concentrations.

Transplantation assay

1×10^3 murine GSCs were suspended in 2 μl PBS and injected with a Hamilton syringe into the 2.0 mm right lateral and 3.0 mm ventral from bregma of the sex-matched recipient mice (6-week-old) placed in a stereotaxic instrument (51730D). Injection was performed at 1 μl/min with an infusion system (Legato130). The syringe was left in position for 2 min after each injection and slowly withdrawn from the brain. To monitor the tumor formation, D-Luciferin (Wako Pure Chemical Industries, Osaka, Japan) was injected intraperitoneally at 150 mg/kg, and after 10 min, bioluminescent intensity was determined using the IVIS Lumina XR imaging system (Summit Pharmaceuticals International, Tokyo, Japan). For IQDMA treatment, event-free survival of mice was defined as death, *in vivo* bioluminescent intensity (BLI) >1×10^4, or weight loss of more than 20% from the day of transplantation. For knockdown of Stat5b, event-free survival of mice was defined as death, *in vivo* BLI >5×10^5, or weight loss of more than 20% from the day of transplantation.

Cell proliferation assay

A trypan blue dye exclusion test was performed using 0.4% trypan blue solution (Wako) and a Countess II automated cell counter (Thermo Fisher Scientific). The concentration that inhibited cell growth by 50% (IC_{50}) was determined using CalcuSyn 2.11 (Biosoft, Ferguson, MO, USA).

RT-qPCR

Total RNA was extracted from cells lysed with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and purified with the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). qRT-PCR analysis was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO) using the Light Cycler 96 System (Roche Diagnostic, Indianapolis, IN, USA). Gene expression levels were normalized to the housekeeping gene mTubulin-b1. Specific primers were purchased from Eurofins Genomics (Tokyo, Japan): mTubulin-b1, 5'-GCAGTGCGGCAACCAGAT-3' (forward) and 5'-AGTGGGATCAATGCCATGCT-3' (reverse); mStat5b, 5'-CTCTGGTGGGGCAGAACGAG-3' (forward) and 5'-TTGAGTCCCAGGCTTGGCTTT-3' (reverse); mLgr5, 5'-GAGTCAACCCAAGCCTTAGTATCC-3' (forward) and 5'-CATGGGACAAATGCAACTGAAG-3' (reverse); mCyclin E2, 5'-CTGCTGCCGCCTTATGTCAT-3' (forward) and 5'-TACACACTGGTGACAGCTGC-3' (reverse).

Expression profiling

The GSCs were treated with control-shRNA (Control) or Stat5b-shRNA (Stat5b-KD) for 48 h. Total RNA was extracted using the RNeasy mini kit (Qiagen). The global gene expression profile was obtained using SurePrint G3 Mouse Gene Expression 8×60K v2 (Agilent Technologies, Palo Alto, CA, USA) and analyzed with the Gene Spring ver14.9.1 software (Silicon Genetics,

Redwood City, CA). Raw data were normalized by the 75 percentile method. Genes downregulated by >2-fold by Stat5b knockdown was assessed by pathway analysis using WikiPathway (https://www.wikipathways.org) [32]. Raw microarray data have been deposited in the Gene Expression Omnibus (GEO) database (GSE185873).

Knockdown of Stat5b, Lgr5, and Hif2a

The procedure was performed as described previously [18]. Briefly, RNAi clones targeting Stat5b, Lgr5, and Hif2α were purchased from Sigma-Aldrich (Stat5b: TRCN00000125-54, Lgr5: TRCN0000028904, Hif2α: TRCN00-00082306; St. Louis, MO, USA). Lentiviral transduction with non-targeting shRNA (Control), Stat5b shRNA (Stat5b-KD), Lgr5 shRNA (Lgr5-KD), and Hif2α shRNA (Hif2α-KD) was performed at a multiplicity of infection of 10. For the transplantation assay, knockdown cells were selected by puromycin for 48 h prior to transplantation, and mice were injected with 1×10^3 cells using the same procedure as described above.

Western blot analysis

Total proteins were extracted with 1% SDS buffer supplemented with a protease inhibitor cocktail mix (Nacalai Tesque) and PhosSTOP EASYpack (Roche), separated by SDS-PAGE, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline with 0.05% Tween20 (TBST) or Blocking One-P (Nacalai Tesque), and then incubated with primary and secondary antibodies. Proteins were visualized using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA) or ChemiLumi One Super (Nacalai Tesque). Chemiluminescence was detected using the ChemiDoc XRS Plus system (Bio-Rad). The following antibodies were used: Stat5b (1:1000; ab178941, Abcam), Lgr5 (1:500; bs-1117R; Bioss, Woburn, MA, USA), Hif2α (1:500; NB 100-122; Novus Biologicals, Centennial, CO, USA), β-tubulin (1:200; T4026, Sigma-Aldrich), GAPDH (1:1000; 016-25523, Wako), Oct4 (1:1000; ab19875, Abcam), Survivin (1:1000; ab182132, Abcam), Cyclin E2 (1:500; ab32103, Abcam), Phospho-Rb (1:1000; #8516, Cell Signaling Technology [CST], Danvers, MA, USA), Phospho-Stat5 (1:1000; ab32364, Abcam), Caspase3 (1:1000; #9665, CST), PARP (1:1000; #9542, CST), β-Catenin (1:1000; #9587, CST).

BrdU incorporation assay

After Stat5b-KD, ICG-001 or IQDMA treatment, the percentage of cells undergoing active DNA synthesis was examined using an APC BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions. At least 10,000 cells were analyzed for each experiment.

Apoptosis assay

After Stat5b-KD, ICG-001 or IQDMA treatment, apoptosis induction was assessed using the MEBCYTO Apoptosis Kit (MBL, Nagoya, Japan). Annexin V-positive and propidium iodide (PI)-negative cells in the early phase of apoptosis were detected by flow cytometry using a BD LSRFortessa X-20 cell analyzer (BD Biosciences). At least 10,000 cells were analyzed for each experiment.

Caspase-3/7 activity assay

The astrocytes and GSCs (1×10^5) were seeded in 6-well plates and trated with IQDMA 1 μM for 24 h. Next, 100 μl of culture medium containing 1×10^4 cells was seeded in 96-well plates and 100 μl of Caspase-Glo 3/7 reagent (Promega, Madison, WI, USA) was added to each well. After incubating for 1 h at room temperature, luminescence was measured with a Synergy HT Microplate Reader (Bio Tek, Winooski, VT, USA) to assess caspase-3/7 activity.

Immunohistochemical analysis

This study has been approved by the ethical committee of Kindai University Faculty of Medicine, and the written informed consent was obtained from the GBM patients who provided surgical specimens. Paraffin-embedded sections (4 μm) were deparaffinized in xylene and rehydrated through descending concentrations of ethanol. Antigen retrieval was performed using citrate buffer (10 mM, pH 6.0) or TE buffer (50 mM Tris, 0.2 mM EDTA, pH 9.0) with heating in a steam cooker for 40 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. Normal goat serum (20%) was used for blocking.

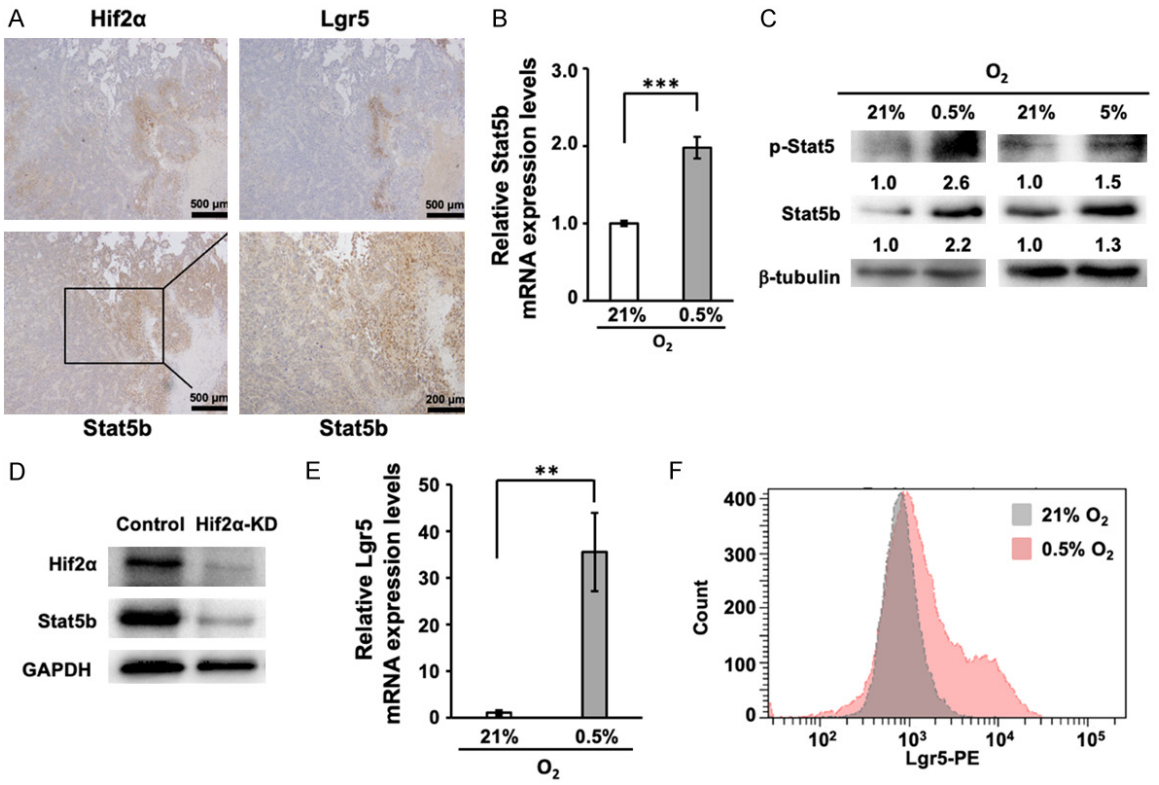

Figure 1. Lgr5 and Stat5b expression are induced by hypoxia in GSCs. A. Representative images of immunohistochemical staining for Hif2α, Lgr5, and Stat5b in human GBM tissue. B. Stat5b mRNA levels were analyzed by qRT-PCR in GSCs cultured in hypoxic conditions for 2 days (0.5% O_2; n=3; $^{***}P<0.001$). C. Western blot analysis of phospho-Stat5 and Stat5b in GSCs cultured in hypoxic conditions (0.5% O_2 for 4 days, 5% O_2 for 3 days). β-tubulin is shown as a loading control. D. Western blot analysis of Hif2α and Stat5b in GSCs after treatment with control-shRNA (Control) or Hif2α-shRNA (Hif2α-KD) for 4 days. GAPDH is shown as a loading control. E. Lgr5 mRNA expression levels were analyzed by qRT-PCR in GSCs after 2 days in hypoxic conditions (0.5% O_2; n=3; $^{**}P<0.01$). F. Lgr5 expression levels were analyzed by a flow cytometry in the GSCs after 2 days in hypoxic conditions (0.5% O_2).

Sections were incubated in primary antibodies overnight at 4°C. Signals were detected using the Envision + System and AEC (Dako, Carpinteria, CA, USA). Nuclei were counterstained with hematoxylin. The following antibodies were used: human Hif2α (1:500; MAB3472, Sigma-Aldrich), mouse Hif2α (1:200; NB100-132, Novus Biologicals), human Lgr5 (1:50; LS-A1232; LSBio, WA, USA), mouse Lgr5 (1:200; bs-1117R, Bioss), human Stat5b (1:50; sc-1656; Santa Cruz, CA, USA), mouse Stat5b (1:500; ab178941, Abcam), Ki-67 (1:800; #12202, CST).

Statistical analysis

Each assay was performed at least three times independently. The statistical significance between two groups was determined by the two tailed Student's t-test. Log-rank tests were used to determine significant differences in survival among groups. Data are expressed as the mean ± SD unless otherwise indicated. All data were analyzed in Excel. Differences were considered statistically significant at the following *P*-values: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Results

Stat5b and Lgr5 expression is induced under hypoxic conditions

Previous microarray analyses identified *Stat5b* as a gene that is downregulated upon knockdown of the stem cell marker Lgr5 in GSCs *in vitro* [18]. Therefore, we performed immunohistochemical analyses of Stat5b and Lgr5 in the tissues of human GBM and in a mouse GBM model to study the relationship between these factors *in vivo*. We found that Stat5b co-localized with Lgr5 in hypoxic regions marked by Hif2α (**Figure 1A** and Supplementary Figure 1),

which is a key factor in the response to hypoxia and plays a pivotal role in the regulation of GSCs [12-14]. These findings suggest that Stat5b is expressed in Lgr5-positive GBM in hypoxic regions and that Stat5b may be regulated by hypoxia. We hypothesized that Stat5b expression would be regulated by hypoxia. To test this hypothesis, we performed western blotting for Stat5b in the GSCs derived from our mouse GBM model [20] cultured under hypoxic conditions. The phosphorylation of Stat5 and the expression levels of Stat5b were increased in both mRNA and protein and when the GSCs were cultured under hypoxic conditions (**Figure 1B** and **1C**). Furthermore, knockdown of Hif2α using lentiviral-mediated shRNA transduction in the GSCs significantly reduced Stat5b protein levels (**Figure 1D**). These results suggest that Stat5b expression is regulated by hypoxia-responsive signaling in GSCs. In addition, we found that Lgr5 expression was induced by hypoxia at the mRNA (**Figure 1E** and Supplementary Figure 2A) and protein levels (**Figure 1F** and Supplementary Figure 2B). These results suggest that expressions of both Stat5b and Lgr5 are enhanced by hypoxic stimuli in GSCs.

Stat5b expression is downregulated by inhibition of the Wnt pathway

Knockdown of Lgr5 has been shown to reduce Stat5b expression in human GSCs [18]. In our mouse GBM model, we observed similar findings that Lgr5 knockdown leads to a reduction in Stat5b protein in GSCs and, of note, a reduction in β-Catenin, which is an important transcriptional regulator downstream of the Wnt signaling pathway (**Figure 2A**). Therefore, we hypothesized that Stat5b expression could be regulated by the Wnt pathway. To examine this hypothesis, we analyzed the effects of the Wnt/β-Catenin signaling inhibitor ICG-001 on the Stat5b expression. The efficacy of ICG-001 on colon cancer cells and its safety has been reported [33]. ICG-001 treatment significantly suppressed GSC proliferation in a concentration-dependent manner (**Figure 2B**). BrdU incorporation assays showed that ICG-001 treatment significantly reduced the proportion of BrdU-positive cells (**Figure 2C** and **2D**). In addition, ICG-001 treatment partially induced apoptotic cell death in the GSCs (**Figure 2E** and **2F**). Moreover, ICG-001 reduced Stat5b protein levels, along with well-established Wnt pathway targets OCT4 and Survivin (**Figure 2G**). These results suggest that Stat5b expression is regulated by the Wnt pathway, which is important for the survival of GSCs.

Knockdown of Stat5b suppresses GSC proliferation

To analyze the functions of Stat5b on GSC proliferation and survival, we next performed knockdown of Stat5b using lentiviral-mediated shRNA transduction. The knockdown efficiency was confirmed by qRT-PCR and western blotting (**Figure 3A** and **3B**). Stat5b knockdown markedly suppressed the GSC proliferation (**Figure 3C**). Annexin V/PI staining of the GSCs revealed that Stat5b knockdown significantly induced apoptotic cell death in the GSCs (**Figure 3D** and **3E**). Moreover, we performed the gene expression analysis of Stat5b knockdown in GSCs by microarray analysis. Pathway analysis of genes downregulated by Stat5b knockdown (fold change >2) showed that the G1 to S cell cycle pathway was significantly enriched (Supplementary Figure 3). We identified Cyclin E2 gene significantly downregulated by Stat5b knockdown (Supplementary Figure 4). We confirmed that mRNA levels of Cyclin E2 were significantly reduced upon Stat5b knockdown in GSCs (Supplementary Figure 5A). Moreover, Stat5b knockdown reduced Cyclin E2 protein levels, resulting in a decrease in the phosphorylation of Rb, an important cell-cycle regulator (Supplementary Figure 5B). Consistent with these results, a BrdU incorporation assay demonstrated that Stat5b knockdown significantly decreased the percentage of BrdU-positive cells that had entered into the DNA synthesis phase (Supplementary Figure 5C and 5D). These results suggest that Stat5b is a factor that promotes the survival and proliferation of GSCs.

Pharmacological inhibition of Stat5 suppresses proliferation of GSCs

Next, we sought to evaluate the effect of pharmacological inhibition of Stat5 on the GSCs. Treatment with IQDMA (N'-(11H-indolo[3,2-c]quinolin-6-yl)-N,N-dimethylethane-1,2-diamine), a Stat5 inhibitor [34], significantly suppressed the proliferation of the GSCs in a concentration-dependent manner (**Figure 4A**). The IC_{50} of IQDMA for GSCs and noncancerous

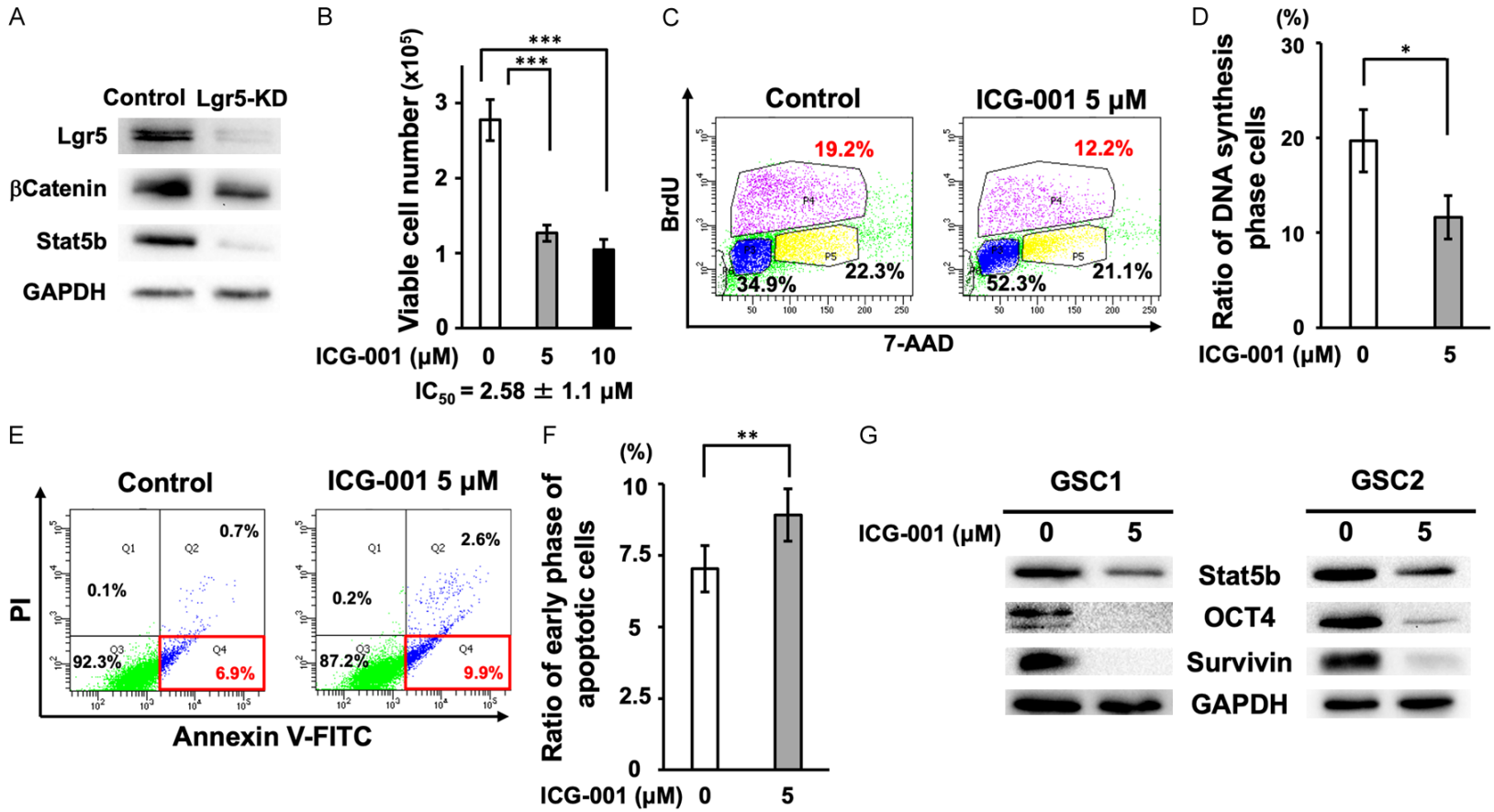

Figure 2. Stat5b expression in GSCs regulated by Wnt pathway. A. GSCs were treated with control-shRNA (Control) or Lgr5-shRNA (Lgr5-KD) for 5 days, and then Lgr5, β-Catenin, and Stat5b were analyzed by western blot. GAPDH is shown as a loading control. B. GSCs were treated with ICG-001 for 48 h, and the number of viable cells was assessed using a trypan blue dye exclusion test (n=4; $^{***}P<0.001$). C, D. The percentage of BrdU-incorporated cells treated with DMSO (Control) or ICG-001 (5 μM) for 48 h was determined by flow cytometry (n=3; $^{*}P<0.05$). E, F. Flow cytometric analysis of Annexin V/PI staining for the detection of apoptosis in GSCs treated with DMSO (Control) or ICG-001 (5 μM) for 48 h (n=6; $^{**}P<0.001$). G. Western blot analyses of Stat5b, OCT4, and Survivin in independent GSC lines treated with ICG-001 for 48 h. GAPDH is shown as a loading control.

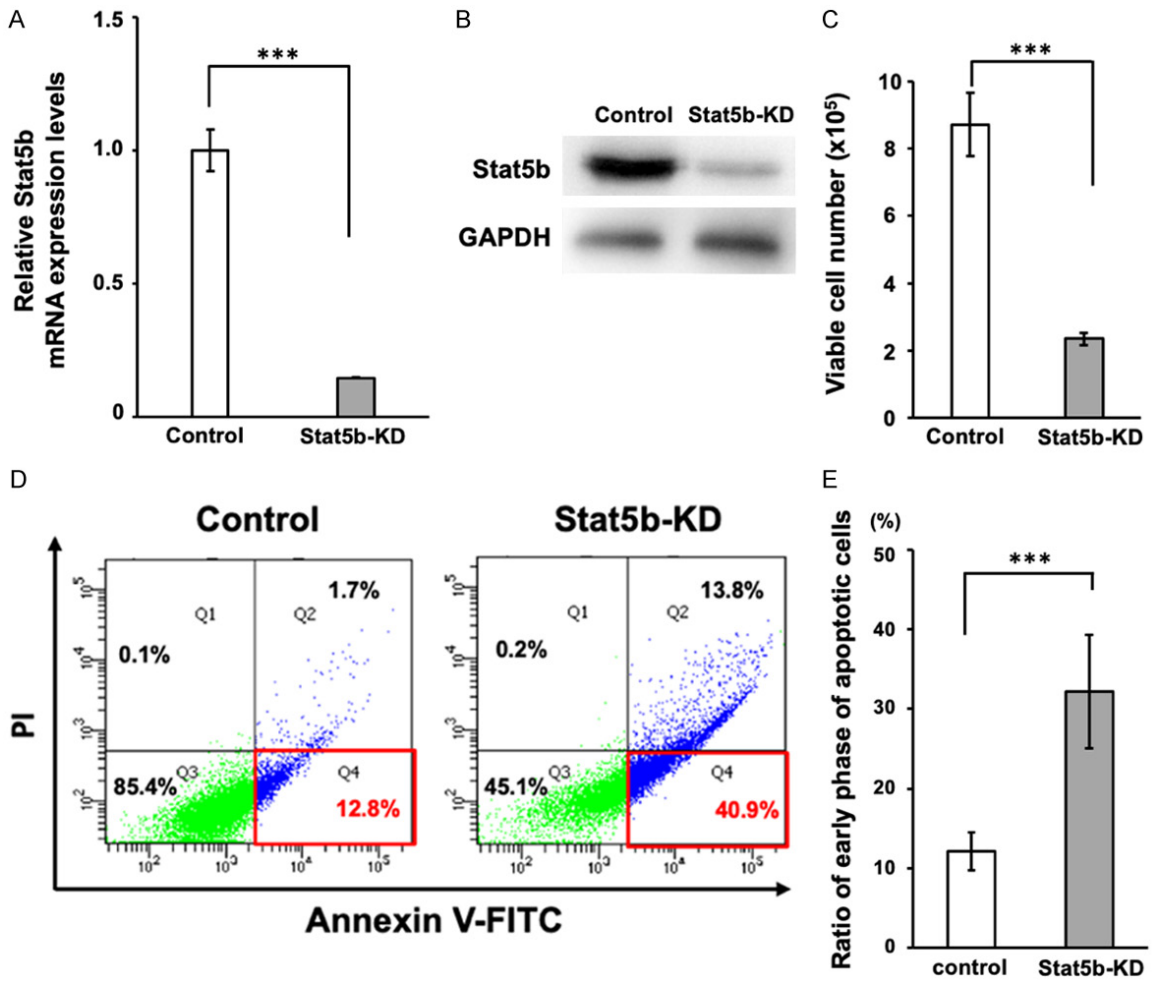

Figure 3. Knockdown of Stat5b inhibits proliferation of GSCs. A, B. qRT-PCR and Western blot analysis of Stat5b expression in GSCs transduced lentivirally with control-shRNA (Control) or Stat5b-shRNA (Stat5b-KD). GAPDH is shown as a loading control. C. GSCs were treated with control-shRNA (Control) or Stat5b-shRNA (Stat5b-KD) for 3 days, and the number of viable cells was determined (n=4; ***P<0.001). D, E. GSCs were treated with control-shRNA (Control) or Stat5b-shRNA (Stat5b-KD) for 3 days, and Annexin V-positive PI-negative apoptotic cells were detected by flow cytometry (n=6; ***P<0.001).

astrocytes are 1.02±0.31 μM and 1.91±0.34 μM, respectively. Comparing the proliferation inhibitory effects of IQDMA on between astrocytes and GSCs, proliferation was more evidently suppressed in GSCs (Supplementary Figure 6A). Treatment of IQDMA 1 μM with astrocytes and GSCs for 24 h caused significant activation of Caspase-3/7 only in the GSCs (Supplementary Figure 6B). BrdU incorporation assays showed that IQDMA treatment significantly reduced the proportion of BrdU-positive cells (**Figure 4B** and **4C**), which is consistent with the results for the Stat5b knockdown (Supplementary Figure 5C and 5D). In addition, IQDMA treatment induced apoptotic cell death in the GSCs (**Figure 4D** and **4E**). Moreover, IQDMA treatment induced the cleavage of Caspase 3 and PARP, markers of apoptosis, and reduced Stat5 phosphorylation (**Figure 4F**). These results suggest Stat5 as a therapeutic target of GSCs and IQDMA as a possible chemotherapeutic agent.

IQDMA suppresses GBM progression and Stat5b knockdown inhibits tumorigenicity of the GSCs in vivo

To assess the antitumor effects of Stat5b inhibition *in vivo*, IQDMA was intraperitoneally administered (15 mg/kg, 3 days a week) into a mouse model in which GSCs were orthotopically transplanted. We observed no body weight

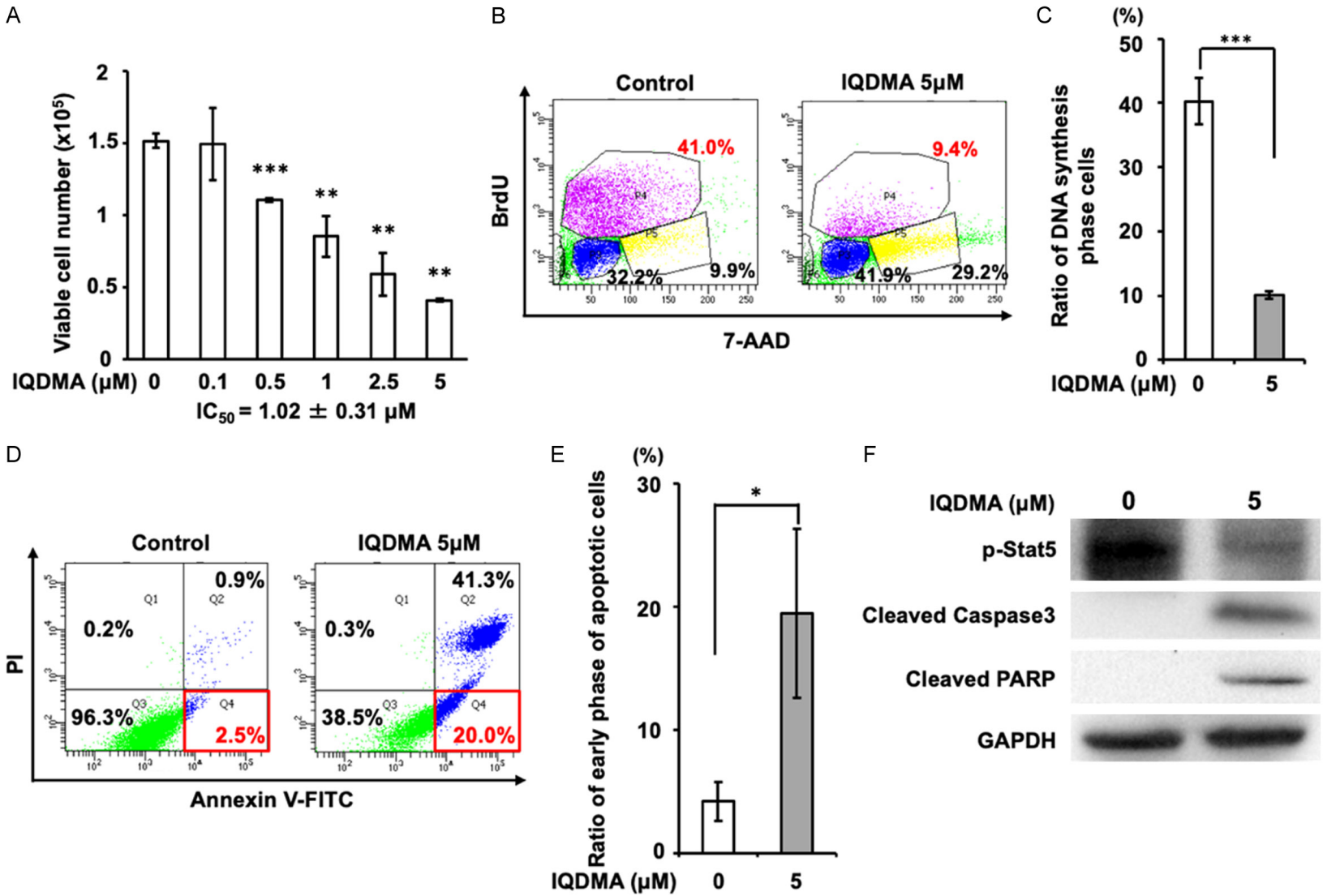

Figure 4. Treatment with the Stat5 inhibitor IQDMA suppresses GSC proliferation and induces apoptosis. A. GSCs were treated with DMSO (Control) or IQDMA (0.1, 0.5, 1, 2.5, and 5 μM) for 24 h, and the number of viable cells was counted (n=3; $^{**}P<0.01$; $^{***}P<0.001$). B, C. The percentage of BrdU-incorporated cells treated with DMSO (Control) or IQDMA (5 μM) for 24 h was determined by flow cytometry (n=3; $^{***}P<0.001$). D, E. Flow cytometric analysis of Annexin V/PI staining for the detection of apoptosis in GSCs treated with DMSO (Control) or IQDMA (5 μM) for 24 h (n=4; $^{*}P<0.05$). F. Western blot analysis of phospho-Stat5b, Caspase 3, and PARP in GSCs treated with DMSO or IQDMA (5 μM) for 24 h. GAPDH is shown as a loading control.

change or evident serious adverse effects in the IQDMA-treated mice. This treatment did, however, significantly extend event-free survival compared with the controls treated with DMSO (**Figure 5A** and **5B**). To confirm these results, *in vivo* knockdown of Stat5b by the specific shRNA was applied to the GSC-transplanted mice. The knockdown of Stat5b significantly suppressed tumor growth and extended event-free survival compared with a non-target shRNA control (**Figure 5C** and **5D**). Immunohistochemical analyses of the brain tumor tissues demonstrated that the Stat5b knockdown reduced the fraction of cells positive for the proliferation marker Ki-67 compared with the control (**Figure 5E** and **5F**). These results suggest that Stat5b plays a role in the tumorigenicity of GSCs and therefore may be a promising molecular target of GSC.

Discussion

There is increasing evidence to suggest that Lgr5 is significant in the biology of GBM [17]. In high grade human glioma tissue, particularly GBM, Lgr5 is highly expressed [18, 19]. Knockdown of Lgr5 in GBM cell lines, such as U87MG, suppresses transplanted tumor formation in mice [19, 35, 36]. Lgr5 is expressed in the stem cell fraction of low-grade glioma [37], and Lgr5 knockdown in primary GSCs derived from clinical samples suppresses their tumorigenicity [38, 39]. Recently, analyses using *Lgr5-GFP* knock-in GBM mice demonstrated that Lgr5-positive GSCs express an effector transcription factor of the hedgehog signaling pathway, Gli2, which regulates Lgr5 expression and the tumorigenicity of murine GSCs [20]. However, the responsible molecule that is downstream of Lgr5 and is essential for GSC tumorigenicity remained unknown.

In this study, we found that Stat5b co-localizes with Lgr5 in Hif2α-positive hypoxic regions of the GBM tissue. Previous reports have shown that Hif2α plays an important role in the regulation of GSCs and is expressed in GSC niches, such as peripheral areas of micro-necrosis and perivascular regions [12-14]. In our histochemical analyses, nuclear accumulation of Stat5b was similarly observed in these regions. Furthermore, our *in vitro* analyses confirmed that Stat5b expression was induced by hypoxic conditions and decreased by Hif2α knockdown in GSCs. Indeed, previous studies have shown that hypoxia stimulates Stat5 expression in vascular endothelial cells [40] and hippocampal neurons [41], resulting in the promotion of cell survival. These findings support the hypothesis that the induction of Stat5b by hypoxic conditions is adaptive in GSCs. In addition, we report here for the first time that Lgr5 is upregulated by hypoxia in GSCs at both the mRNA and protein levels. Of note, it has been reported that hypoxia suppresses Lgr5 expression through miR-215 in colon cancer cells [42]. While the reason for this discrepancy is unknown, it is possible that there are tissue-specific mechanisms that regulate Lgr5 expression. Our data suggest that Lgr5-expressing cells in GBM may represent hypoxia-adaptive GSCs, which make use of Stat5b to expand their own population.

The expression of Stat5b was decreased by the treatment with the Wnt inhibitor ICG-001 and the knockdown of Lgr5. Lgr5 knockdown in GSCs has previously been shown to inhibit the Wnt pathway [19]. These findings suggest that the expression of Stat5b is regulated by both hypoxia and the Wnt pathway. In addition, hypoxia-responsive signaling and the Wnt pathway contribute to stem cell regulation in a coordinated manner [43]. It is possible that Stat5b is similarly regulated by cooperation between these pathways. Microarray analyses identified *Stat5b* as a significantly downregulated gene upon Lgr5 knockdown [18], together with established hypoxia-related genes, including VEGFa and CA9, and key genes for GSC regulation, such as PFKFB4 [44], the Wnt target L1CAM [45], and the Wnt-regulating factor NDRG1 [46]. These findings suggest that Stat5b suppression by either Lgr5 knockdown or the inhibition of the Wnt pathway may be conserved in human and mouse GSCs and that both the hypoxia and Wnt pathways may be involved in the regulation of Stat5b expression.

Stat5b knockdown in the GSCs suppressed their proliferation and induced apoptosis. These results suggest that Stat5b plays an important role in the survival and maintenance of GSCs. In addition, the knockdown of Stat5b in the GSCs reduced expression of the cell-cycle regulator *Cyclin E2*, at both mRNA and protein levels, and the dephosphorylation of Rb

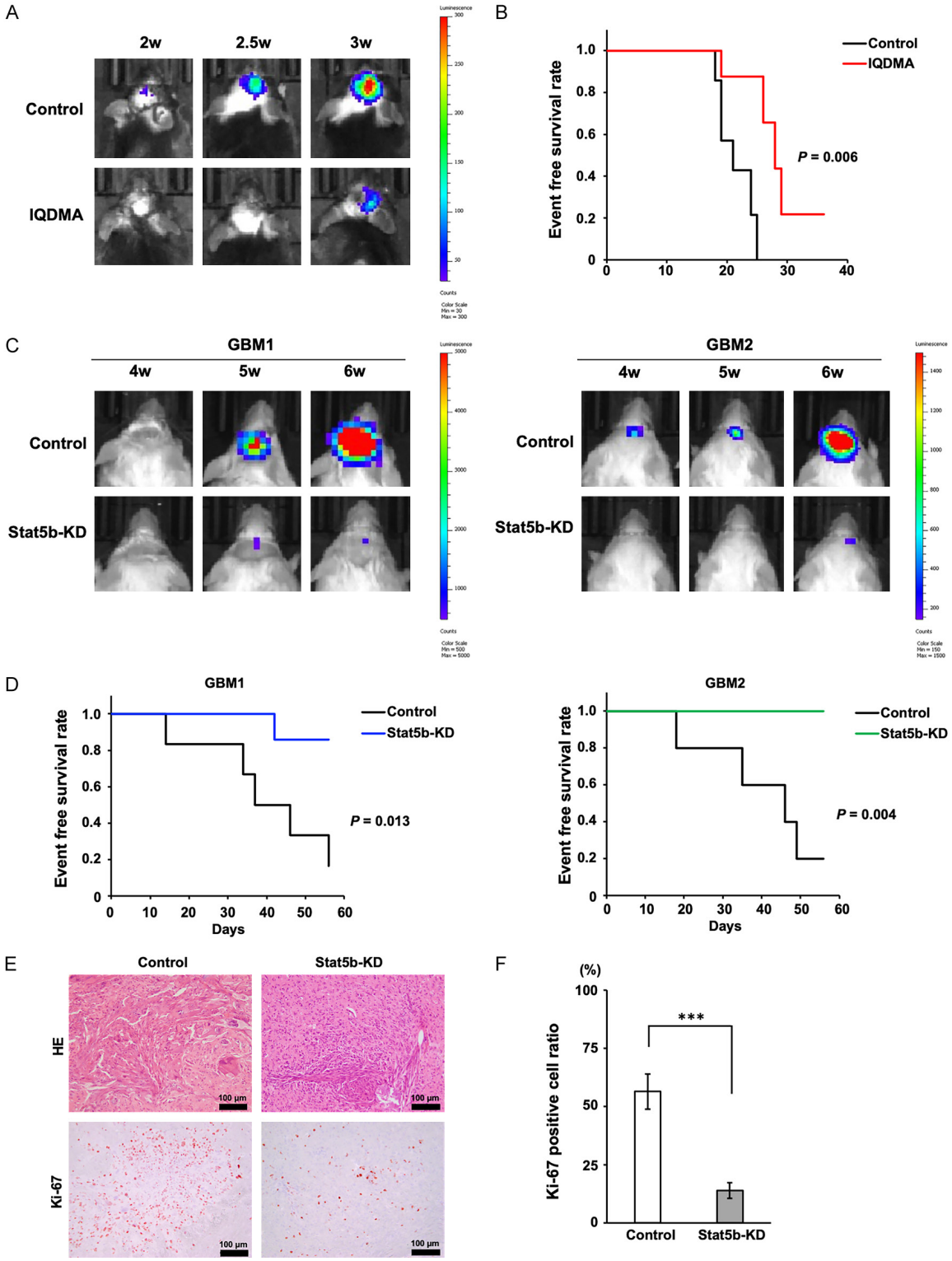

Figure 5. Inhibition of Stat5b suppresses tumorigenicity of GSCs *in vivo*. A. Representative bioluminescent images from Control and IQDMA treatment groups at 2, 2.5 and 3 weeks after the GSC transplantation. B. Event-free survival of mice with GBM xenografts treated with DMSO (Control; n=7) or IQDMA (15 mg/kg; n=8) was analyzed by the Kaplan-Meier method. C, D. Representative bioluminescent images and event-free survival of mice transplanted with GSCs transduced with control-shRNA (control) or Stat5b-shRNA (Stat5b-KD) was analyzed by the Kaplan-Meier method. Two independent lines of GSCs, GBM1 (Control; n=6, Stat5b-KD; n=7), and GBM2 (Control; n=5, Stat5b-KD; n=7) were analyzed. E. Hematoxylin and eosin staining and Ki67 immunostaining of xenograft tissue are shown. F. Quantitative analysis of Ki-67-positive cells (Control; n=4, Stat5b-KD; n=3, $^{***}P<0.001$).

protein, resulting in cell-cycle arrest. Similarly, it has been shown that knockdown of Stat5b in human GBM cell lines caused the cell cycle to arrest in G1 [47]. These findings support that Stat5b promotes the proliferation of GSCs.

It has been reported that IQDMA, a Stat5 inhibitor, binds to the Stat5 protein and inhibits its function [34]. We tested the efficacy of IQDMA against GSCs and found, consistent with the data obtained with Stat5b knockdown, that IQDMA significantly blocked cell-cycle progression and induced apoptosis. The administration of IQDMA into the GBM transplantation mouse model led to the significant suppression of tumor growth and prolonged event-free survival of the mice without evident adverse effects. Although previous studies showed reductions in the secretion of pituitary growth hormone and the body growth in Stat5b knockout mice [48] and a suppression of hematopoiesis in Stat5a/b double knockout mice [49], our results suggest that the inhibition of Stat5 by IQDMA may be useful as a chemotherapeutic agent to treat GBM. Moreover, transplantation of Stat5b-depleted GSCs exhibited suppressed tumorigenicity compared with the control cells. The histological analyses also demonstrated significant inhibition of cellular proliferation as assessed by Ki-67 staining in the Stat5b knockdown group. These findings suggest that Stat5b is essential for the promotion of GSC tumorigenicity *in vivo*.

Taken together, our data demonstrate that Stat5b promotes survival, proliferation, and tumorigenicity in a murine GSC model. We showed that Stat5b expression is regulated both by the cellular response to hypoxia and the Wnt pathway in the GSCs. Moreover, our *in vivo* analyses demonstrated that Stat5b inhibition suppresses the growth of transplanted GBM. These results suggest that Stat5b may be a useful target for the development of novel GBM therapeutics with selectivity for GSCs.

Acknowledgements

We would like to thank Noriko Saito, Satomi Toma, Hitoshi Iwasaki, Yukina Kono, Haruka Katsuma, Tomoko Fujii, Yumiko Hori, and Fumi Taniguchi for their experimental supports. We would like to thank Dr. Ah-Mee Park for providing the immortalized mouse astrocyte line. This work was supported by the Japan Society for the Promotion of Science, grant numbers 20K07623, 18K09012, 16K08722, 254604-88 for Nakata S and Fujita M, and 20K09378 for Fujita M; the Ministry of Education, Culture, Sports, Science and Technology-Supported Program for the Strategic Research Foundation at Private Universities 2015-2019 for Nakata S; the Takeda Science Foundation for Ii H; Kyoto Pharmaceutical University Fund for the Promotion of Scientific Research for Nakata S; and the Kyoto Pharmaceutical University Fund for the Promotion of Collaborative Research for Nakata S.

Disclosure of conflict of interest

None.

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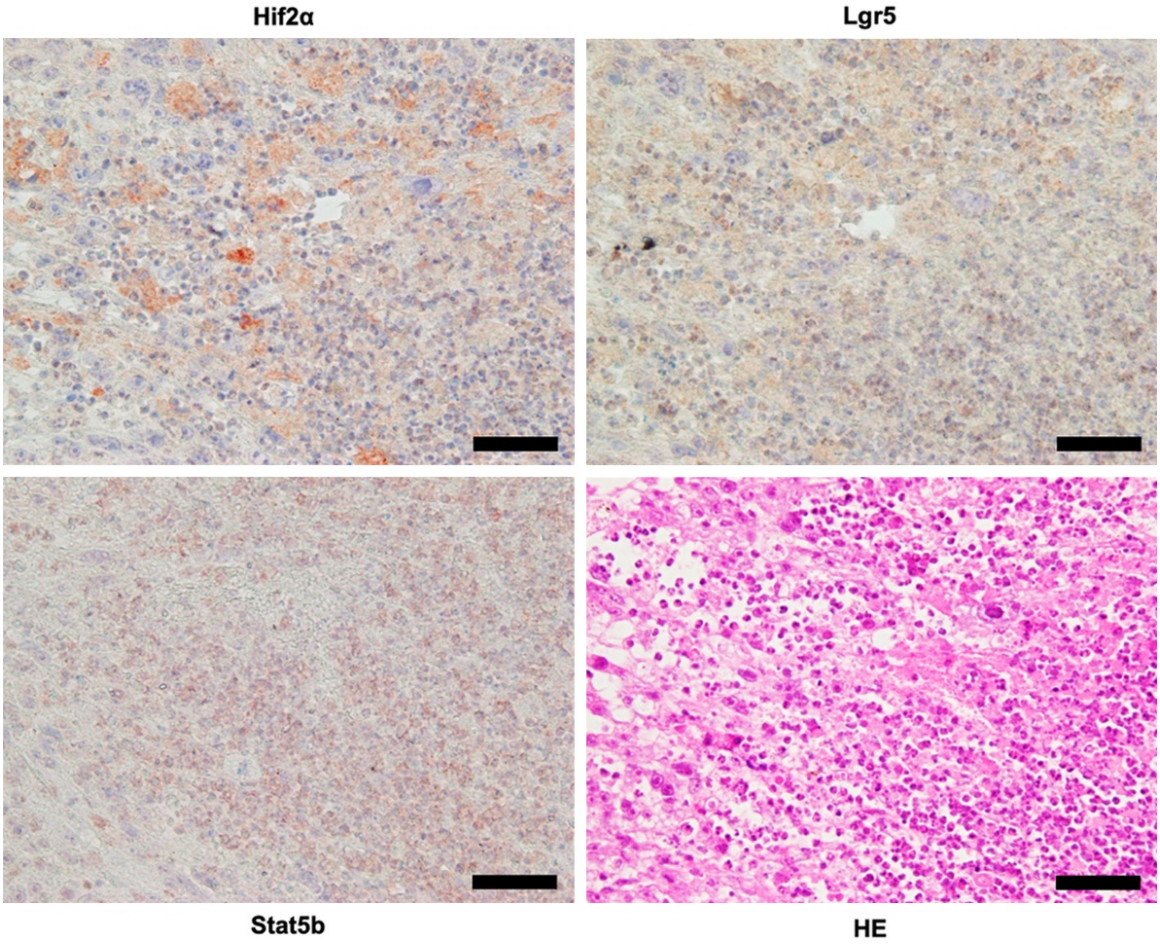
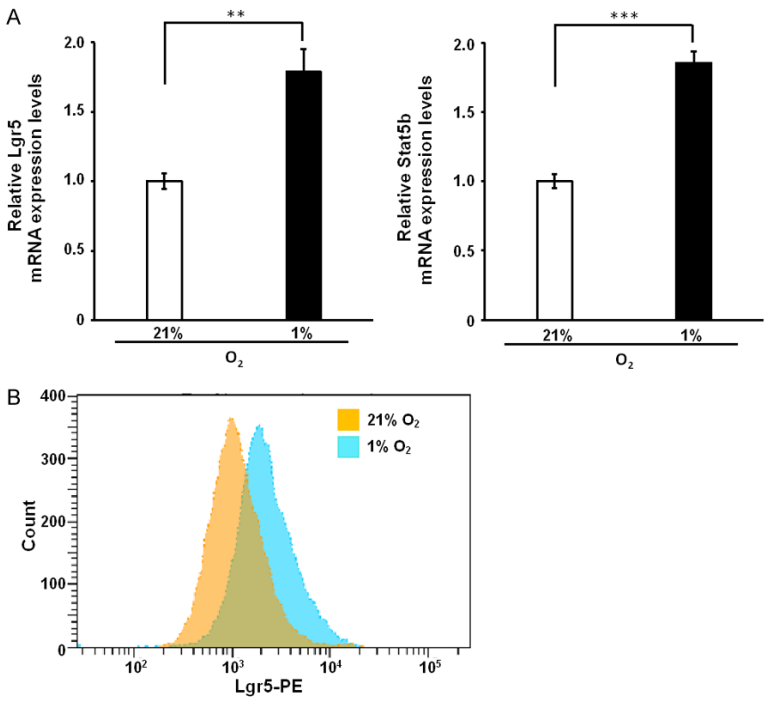
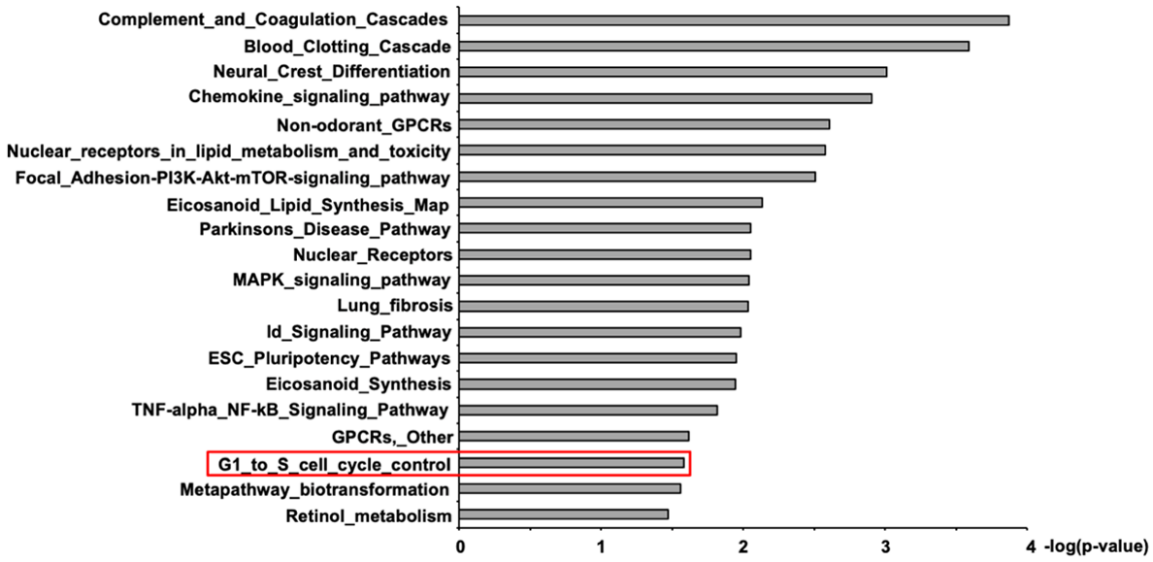


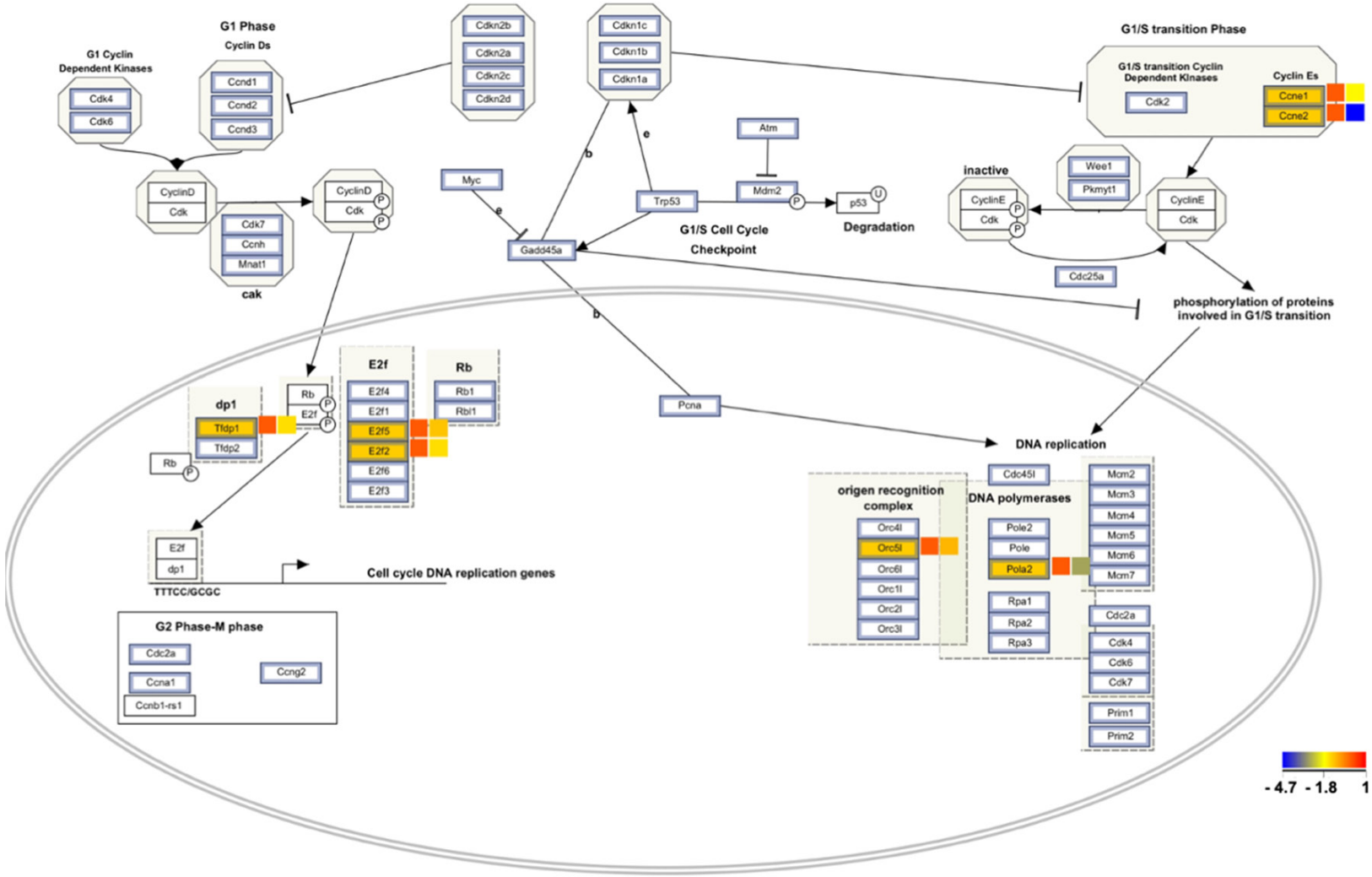
Supplementary Figure 1. Stat5b co-localizes with Lgr5 in Hif2α-positive hypoxic regions in murine GBM tissue. Representative images of immunohistochemical staining for Hif2α, Lgr5, and Stat5b in murine GBM tissue, and corresponding hematoxylin and eosin staining, are shown. Scale bar, 50 μm.



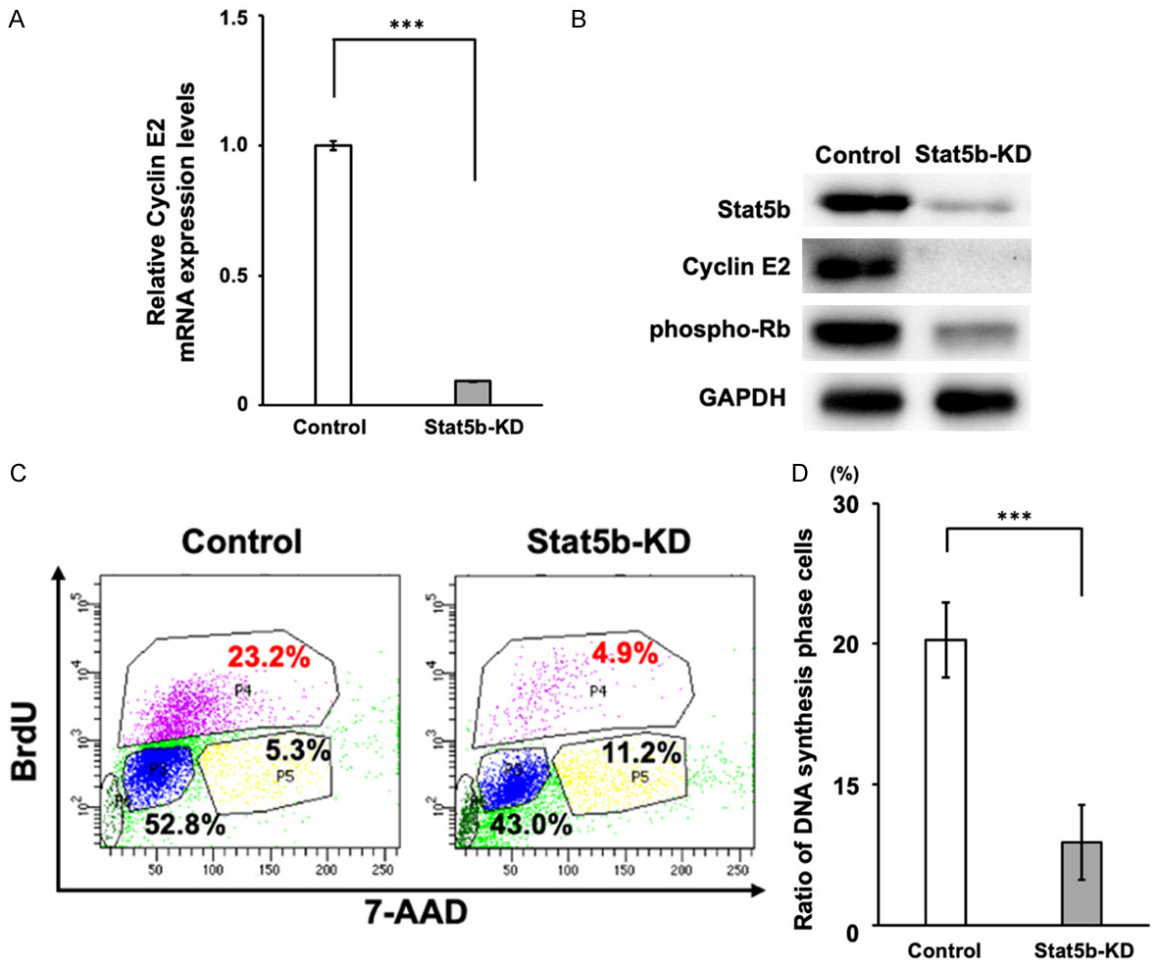
Supplementary Figure 2. Expression of Lgr5 and Stat5b is induced by hypoxia in adherent GBM cells. A. Lgr5 and Stat5b mRNA expression was analyzed by qRT-PCR in adherent GBM cells after 3 days of hypoxic stimulation (1% O_2) (n=3; $**P<0.01$, $***P<0.001$). B. Lgr5 expression levels were analyzed by flow cytometry in adherent GBM cells after 3 days of hypoxic stimulation (1% O_2).



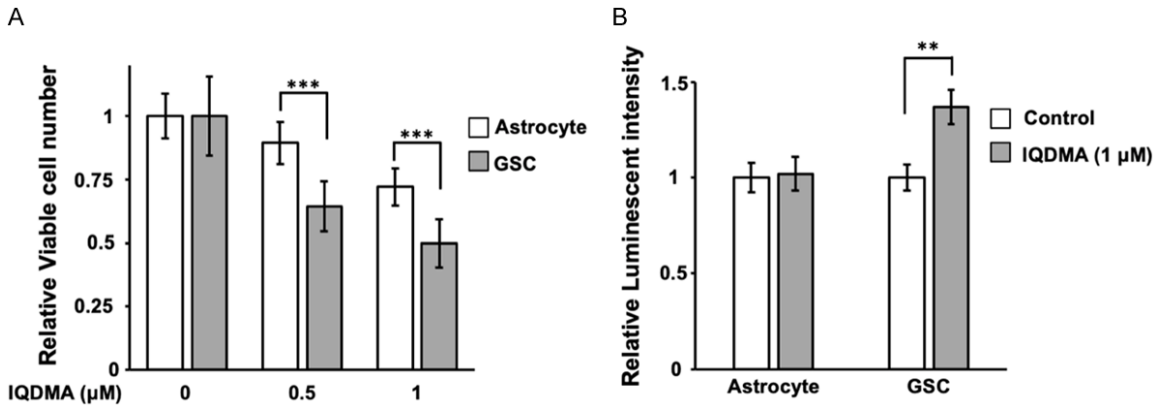
Supplementary Figure 3. Enriched pathways of the downregulated genes by Stat5b knockdown in GSCs. Pathway analysis of genes downregulated by Stat5b knockdown (fold change >2) is shown. The G1 to S cell cycle pathway is detected among top 20 regulated pathways (P=0.026).



Supplementary Figure 4. Signal diagram of G1 to S cell cycle pathway. Expression of Cyclin E2 is markedly downregulated by Stat5b knockdown among the cell cycle regulators. control-shRNA (Control), left; Stat5b-shRNA (Stat5b-KD), right.



Supplementary Figure 5. Stat5b knockdown downregulates the expression of Cyclin E2 and blocks cell-cycle progression in GSCs. A. GSCs were treated with control-shRNA (Control) or Stat5b-shRNA (Stat5b-KD) for 2 days, and Cyclin E2 expression levels were analyzed by qRT-PCR (n=3; ***P<0.001). B. Western blot analysis of Stat5b, Cyclin E2, and Phospho-Rb in GSCs treated with shRNA for 3 days. GAPDH is shown as a loading control. C, D. GSCs were treated with the shRNA for 3 days and the ratio of the BrdU incorporating cells was assessed by flow cytometry (n=4; ***P<0.001).



Supplementary Figure 6. Selective cytotoxicity of IQDMA on GSCs. A. Non-cancerous astrocytes and GSCs were treated with IQDMA (0.5, 1 μM) for 24 h, and the number of viable cells was counted (n=9; ***P<0.001). B. Astrocytes and GSCs were treated with IQDMA (1 μM) for 24 h, and Caspase 3/7 enzymatic activity was measured (n=3; **P<0.01).