

# Effect of the STAT3 inhibitor STX-0119 on the proliferation of cancer stem-like cells derived from recurrent glioblastoma

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**Abstract.** Signal transducer and activator of transcription (STAT) 3, a member of a family of DNA-binding molecules, is a potential target in the treatment of cancer. The highly phosphorylated STAT3 in cancer cells contributes to numerous physiological and oncogenic signaling pathways. Furthermore, a significant association between STAT3 signaling and glioblastoma multiforme stem-like cell (GBM-SC) development and maintenance has been demonstrated in recent studies. Previously, we reported a novel small molecule inhibitor of STAT3 dimerization, STX-0119, as a cancer therapeutic. In the present study, we focused on cancer stem-like cells derived from recurrent GBM patients and investigated the efficacy of STX-0119. Three GBM stem cell lines showed many stem cell markers such as CD133, EGFR, Nanog, Olig2, nestin and Yamanaka factors (c-myc, KLF4, Oct3/4 and SOX2) compared with parental cell lines.

These cell lines also formed tumors *in vivo* and had similar histological to surgically resected tumors. STAT3 phosphorylation was activated more in the GBM-SC lines than serum-derived GB cell lines. The growth inhibitory effect of STX-0119 on GBM-SCs was moderate (IC<sub>50</sub> 15-44 μM) and stronger compared to that of WP1066 in two cell lines. On the other hand, the effect of temozolomide was weak in all the cell lines (IC<sub>50</sub> 53-226 μM). Notably, STX-0119 demonstrated strong inhibition of the expression of STAT3 target genes (c-myc, survivin, cyclin D1, HIF-1α and VEGF) and stem cell-associated genes (CD44, Nanog, nestin and CD133) as well as the induction of apoptosis in one stem-like cell line. Interestingly, VEGFR2 mRNA was also remarkably inhibited by STX-0119. In a model using transplantable stem-like cell lines *in vivo* GB-SCC010 and 026, STX-0119 inhibited the growth of GBM-SCs at 80 mg/kg. STX-0119, an inhibitor of STAT3, may serve as a novel therapeutic compound against GBM-SCs even in temozolomide-resistant GBM patients and has the potential for GBM-SC-specific therapeutics in combination with temozolomide plus radiation therapy.

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*Abbreviations:* STAT, signal transducer and activator of transcription; SH, Src homology; DMSO, dimethyl sulfoxide; JAK, Janus kinase; T/C, tumor/control; siRNA, small interfering RNA; shRNA, small hairpin RNA

*Key words:* STAT3, dimerization, phosphorylated STAT3, recurrent glioblastoma, cancer stem cell

## Introduction

Glioblastoma multiforme (GBM) is one of the most malignant and aggressive tumors and has a very poor prognosis with a mean survival time of <2 years even with the recent development of temozolomide-based intensive treatment (1,2). Therefore, a new therapeutic approach is urgently needed to control recurrence and overcome resistance to treatment in glioblastoma patients.

GBMs are composed of many types of cells expressing astrocytic and neuronal lineage markers and generated from multipotent stem cells. Recently, GBM stem-like cells (SC) were successfully isolated from human resected tumors using serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (3-5).

These cells share the properties of normal stem cells like self-renewal and multi-lineage differentiation. The definition according to some research groups is: i) the capability for self-renewal; ii) multi-lineage differentiation; and iii) the ability to regenerate GBM tumors histologically similar to the original tumors in xenografts (6,7). Based on these observations, it is worth attempting to develop therapeutic agents for GBM-SC that affect cell proliferation and resistance to chemo-radiation.

The activation of several signaling pathways including receptor tyrosine kinase (8), Akt (9), MAPK (10), Wnt (11) and Notch and Hedgehog (12) pathways, is involved in the progression of GBM. Importantly, constitutive activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway contributes to the tumor progression by promoting cell proliferation and the inhibition of apoptosis. The STAT protein family is a group of transcription factors that play an important role in relaying signals from growth factors and cytokines (13). STAT3 is reported to be involved in oncogenesis by upregulating the transcription of several genes that control tumor cell survival, resistance to apoptosis, cell cycle progression and angiogenesis. Targets of STAT3 include Bcl-2, Bcl-xL, c-myc, cyclin D1, vascular endothelial growth factor (VEGF) and human telomerase reverse transcriptase.

The association between STAT3 signaling and GBM-SC development has been investigated rigorously (14,15). Sherry *et al* reported that genetic knockdown of *stat3* using short hairpin RNA inhibited proliferation and the formation of neurospheres by GBM-SC, indicating that STAT3 can regulate the growth and self-renewal of GBM-SC (14).

Considering STATs are a good target for cancer stem cell therapy, several therapeutic agents including small molecules have been demonstrated to show antitumor effects through the regulation of GBM-SC. Previously, we identified a novel inhibitor of STAT3 dimerization, STX-0119, which exhibited a potent antitumor effect on a human lymphoma cell line with a highly activated STAT3 (16). In the present study, we found that STX-0119 inhibited cell proliferation and the formation of spheres in GBM-SC lines derived from human GBM tumors by regulating STAT3 target genes and inducing apoptosis and suppressed the growth of transplanted tumors of GBM-SC.

## Materials and methods

**Establishment of primary GB-stem cell lines from GBM patients.** GBM tumor samples were obtained from surgically resected materials. The clinical research using tumor tissues from GBM patients was approved by the Institutional Review Board of Shizuoka Cancer Center, Shizuoka, Japan. All patients gave written informed consent.

Tumors were dissociated by teasing with forceps to make a single cell suspension and plated in 25-cm<sup>2</sup> culture flasks in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Invitrogen), penicillin and streptomycin and gentamicin (Invitrogen) for the serum-derived GB cell line. For GBM-SC cultures, dissociated cells were plated in 6-well ultra-low attachment plates (Corning Inc., Corning, NY) in serum-free DMEM supplemented with EGF (Invitrogen) at 20 ng/ml,

bFGF (Peprotech, Rocky Hill, NJ) at 20 ng/ml, leukemia inhibitory factor (LIF, Alomone Labs Ltd., Jerusalem, Israel) at 10 ng/ml and B27 (Invitrogen), referred to herein as the stem cell medium (SCM). The cultures were passaged weekly after neurospheres formed.

U87 glioblastoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM containing 10% FBS. The U87 stem cells were cultured like the GBM-SC lines and after 20 passages in SCM were used for experiments *in vitro*.

Among the GBM-SC lines, GB-SCC026 was used for quantitative PCR and apoptosis assay after a 24-h exposure to STX-0119, while GB-SCC010 and 026 cells were utilized in animal experiments.

**Antibodies and reagents.** Antibodies against STAT3, phospho-specific STAT3 (Tyr705), cleaved caspase-3 and  $\beta$ -actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA) and Becton-Dickinson (BD) Biosciences (Franklin Lakes, NJ) for western blotting (WB). PE-labeled anti-CD133 antibody and FITC-labeled anti-CD44 antibody were purchased from BD Biosciences and used for flow cytometry.

**Chemicals.** STX-0119 and the JAK-specific inhibitor WP1066 were supplied by The Center for Drug Discovery, University of Shizuoka (Shizuoka, Japan). These compounds were suspended and diluted in a sterile 0.5% w/v methyl cellulose 400-cp solution (Wako, Tokyo, Japan) or dissolved in a mixture of 20% dimethyl sulfoxide (DMSO) (Wako) and 80% polyethylene glycol 300 (Wako) for use in animal experiments.

**Flow cytometry.** The primary GBM-SC lines or U87 stem cells were stained with the PE-labeled anti-CD133 antibody and FITC-labeled anti-CD44 antibody or isotype control antibodies at a concentration of 10  $\mu$ g/ml. Stained cells were fixed with 0.5% paraformaldehyde (Sigma-Aldrich) and analyzed on a flow cytometer (FACSCalibur, BD).

**Cell proliferation assay.** Cell proliferation was examined using the WST-1 assay (Dojin Kagaku Corp., Japan) described previously (16). Briefly,  $5 \times 10^3$ - $1 \times 10^4$  GBM-SC or U87 stem cells were seeded into each well of a 96-well micro-culture plate (Corning, NY) and compounds diluted with SCM (100-0.25  $\mu$ M) were added. After 4 days, the WST-1 substrate was added to the culture and optical density (OD) was measured at 450 and 620 nm using an immunoreader (Immuno Mini NJ-2300, Nalge Nunc International, Roskilde, Denmark). The IC<sub>50</sub> value was defined as the dose needed for a 50% reduction in OD calculated from the survival curve. Percent survival was calculated as follows: (mean OD of test wells - mean OD of background wells) / (mean OD of control wells - mean OD of background wells).

**Sphere formation assay.** GBM-SC were seeded in a 96-well micro-culture plate at 500 per well and compounds diluted with SCM (100-0.25  $\mu$ M) were added. After a 7-day incubation at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, the number of spheres with a diameter of >50  $\mu$ m was counted under a microscope.

**Quantitative polymerase chain reaction (qPCR) analysis.** The real-time PCR analysis of stem cell and neuronal markers and STAT3 target genes using the 7500 Real-Time PCR system (Applied Biosystems, Foster, CA) was performed as described previously. Briefly, all PCR primers (ABCB1, ALDH1A1, CD44, EGFR, ESA, GFAP, KLF4, NANOG, NES, OLIG2, Oct3/4, CD133, SOX2, TGFBR2, TUBB3, VIM for GB-SC markers; BCL2, Bcl-X1, Survivin, Cyclin D1, c-Myc, CXCL10, VEGFR2, MMP9, TGFB1, P53, VEGFA, VEGFC, HIF-1 $\alpha$  for STAT3 target genes) and TaqMan probes were purchased from Applied Biosystems. GB-stem cell lines or U87 stem cells were treated with STX-0119, WP1066 or DMSO for 24 h and total RNA was extracted. Complementary DNA was synthesized from 100 ng of the total RNA and quantitative PCR was carried using a TaqMan RNA-to-Ct 1-Step kit (Applied Biosystems).

**ELISA for human VEGF.** VEGF levels in the supernatant of GBM-SC lines and U87 stem cells treated with STX-0119 were measured using human VEGF-specific ELISA. Cells were plated in 96-well ultralow cluster plates (Corning Inc., Corning, NY) in SCM. After 1-day incubation, cells were treated with STX-0119, WP1066 or DMSO for 24 h. Finally, supernatants were collected and VEGF levels were measured.

**Western blotting (WB).** GBM-SC or U87 stem cells were treated with STX-0119, WP1066 or DMSO at various doses for 24-72 h in SCM. Cells were lysed using RIPA buffer (Thermo Fisher Scientific Inc., Rockford, IL) containing protease inhibitors and phosphatase inhibitors and used for western blotting as described previously. Briefly, cell lysate was subjected to SDS-PAGE with a 7.5% polyacrylamide separating gel and then transferred to PVDF membranes. After blocking, the membranes were incubated at 4°C overnight with the primary antibody against STAT3, phosphospecific STAT3, cleaved caspase-3 and  $\beta$ -actin (1:200-1:2,000) in blocking solution. After a wash, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5,000). Membranes were treated with ECL plus reagent (GE Healthcare) and analyzed on a chemiluminescence scanner (LAS-3000, Fujifilm, Tokyo, Japan).

**Animal experiments.** Immunodeficient male NOD/Shi-*Parkdc*<sup>scid</sup> (NOD-*scid*) and NOD/Shi-*scid* IL-2R $\gamma$ <sup>null</sup> (NOG) mice (5-6 weeks old) obtained from Nippon Clea (Tokyo, Japan) were used. They were housed in a separate experimental room and given sterilized food and water *ad libitum*. All animals were cared for and used humanely according to guidelines for the welfare and use of animals in cancer research, and procedures approved by the Animal Care and Use Committee of Shizuoka Cancer Center Research Institute.

The GBM-SC lines were re-suspended in RPMI-1640 medium (100  $\mu$ l) containing Matrigel (BD Biosciences) at 1x10<sup>6</sup>/ml and inoculated into the flank of NOD-*scid* and NOG mice. To evaluate the activity against the subcutaneous (s.c.) inoculated tumor cells, tumor volume was calculated based on The National Cancer Institute formula as follows: tumor volume (mm<sup>3</sup>) = length (mm) x [width (mm)]<sup>2</sup> x 1/2.

Table I. Frequency of stem cell markers in GB-SC lines.

	CD44 <sup>+</sup> (%)	CD133 <sup>+</sup> (%)	CD44 <sup>+</sup> /133 <sup>+</sup> (%)
GB-SCC010	48.3	10.6	10.4
GB-SCC026	94.6	39.2	37.5
GB-SCC028	82.8	34.4	33.7
U87 stem cell	93.8	72.4	71.6

**Immunohistochemistry.** Three tumors generated *in vivo* from GBM-SC lines were resected and fixed with formalin solution. Hematoxylin-eosin staining was performed according to the manufacturer's instructions. A pathologist compared the GBM-SC-derived tumor specimen to the surgically resected tumor and made a diagnosis regarding the similarity of the tumors.

**Statistical analysis.** The statistical analysis was performed with corrected p-values to compare with the untreated control using the Steel multiple plus Kruskal-Wallis method and Mann-Whitney's rank-sum test.

## Results

**Establishment of primary GBM-SC lines from GBM patients.** Three GBM-SC lines (GB-SCC010, 026, 028) were established from 3 GBM patient-derived tumors (Fig. 1A). Each cell line showed a neurosphere feature in SCM. On the other hand, the matched traditionally grown cell line from serum-contained cultures showed an adherent phenotype. Histological analysis of *in vivo* tumors generated from GBM-SC lines demonstrated similar findings to surgically resected tumors (Fig. 1B). All of the GBM-SC lines were shown to be positive for CD133 stain using flow cytometry analysis (Table I).

**STAT3 phosphorylation assay.** The activation (phosphorylation) of STAT3 was investigated in GBM-SC lines and matched serum-cultured cell lines derived from three GBM patients. Constitutive STAT3 phosphorylation was identified, but was stronger in the GBM-SC lines than serum-cultured cell lines (Fig. 1C).

**Expression of stem cell markers in primary GB-SC lines.** Quantitative PCR revealed stem cell-related marker gene expression in a representative GBM-SC line, GB-SCC010 (Table II), which showed changes in gene expression compared with the matched primary serum-cultured cell line. The gene expression of stem cell-related markers CD133, EGFR, MDR1, KLF4, Nanog, Nestin, Oct3/4, Olig2, Sox2, VEGFA and vimentin was upregulated >10-fold compared to the serum-cultured cell line. Impressively, the increase of MDR1 gene expression was extraordinarily high, ~200,000-fold. The stem cell-related marker gene expression in the other 2 GBM-SC lines is shown in Table II. Taking the data from all three GBM-SC lines into consideration, CD133, MDR1, Olig2, Sox2 and VEGFA were remarkably upregulated, while TGFBR2 and VEGFR2 were downregulated.

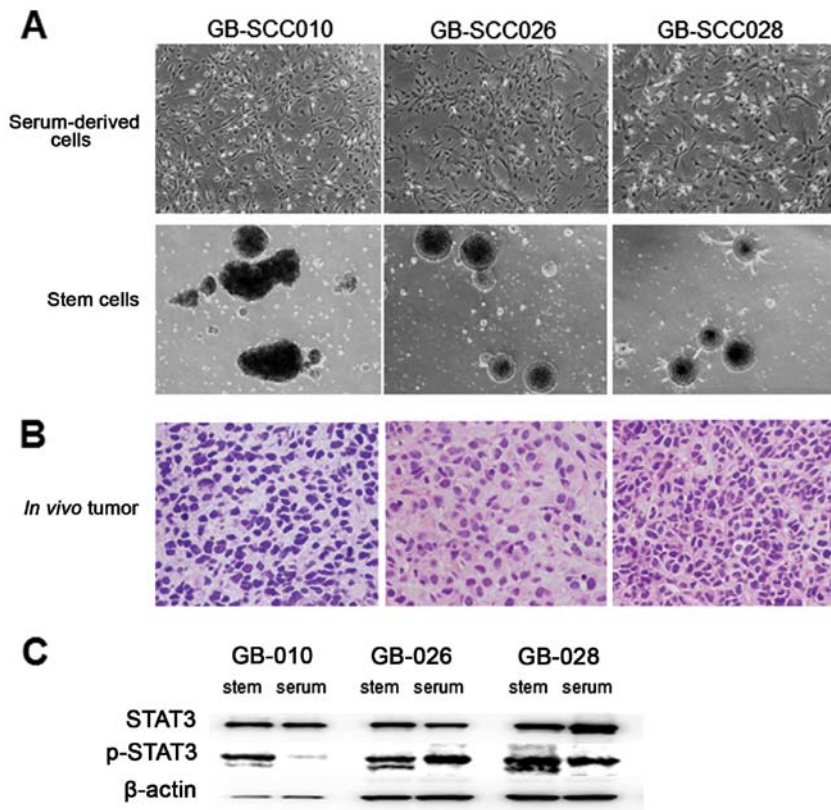


Figure 1. Morphological features and STAT3 phosphorylation of GBM-SC lines. Images of (A) matched serum-derived GBM cell lines (top) and GBM-SC lines (bottom); and (B) tumors generated from GBM-SC lines *in vivo*. Intracerebral transplanted tumors in GB-SCC010 and subcutaneous tumors in other cases (GB-SCC026 and 028 SC lines). Magnification, x100. (C) STAT3 phosphorylation in GBM-SC and matched serum-derived cell lines.

Table II. Frequency of stem cell-associated markers in GB-SC lines.

Gene	GB-010	GB-026	GB-028
ALDH1A1	ND	0.01	5.59
CD133	14.1	27985	3831
CD44	4.68	0.84	45.3
c-Myc	1.92	5.59	0.23
EGFR	48.7	0.17	25.3
ESA	11.7	0.51	49.8
GFAP	4.04	86.5	420
KLF4	27.4	1.21	4.88
MDR1	197220	18.8	783
NANOG	88.3	2.53	448
Nestin	44.7	2.65	398
Oct4/5	69.6	4.87	41.3
OLIG2	124	2035	56420
SOX2	74.3	4635	693
TGFBR2	0.03	0.02	0.06
TUBB3	20.1	17.9	139
VEGFA	77.2	89.1	329
VEGFR2	0.58	0.03	5.76
Vimentin	47.1	3.83	16.8

The quantitative PCR for GBM-stem cell markers was performed using TaqMan probes and specific primers. The expression level in serum-derived cells was rated 1 as a control and gene expression in stem cell lines was shown as fold increase to a control. ND, not detected.

**Cell proliferation assay.** The growth inhibitory effect of STX-0119 on the GBM-SC lines was moderate ( $IC_{50}$  15-44  $\mu$ M) and stronger than that of WP1066 in two cell lines (Fig. 2 and Table III). STX-0119 exhibited a stronger inhibitory effect on GB-SCC026 stem cells than the others. On the other hand, the effect of temozolomide was weak in all cell lines ( $IC_{50}$  53-226  $\mu$ M). The inhibitory effect on U87 stem cell growth did not differ between STX-0119 and WP1066. Additionally, STX-0119 inhibited sphere formation at an  $IC_{50}$  of <5  $\mu$ M and had greater inhibitory activity than WP1066.

**Effect of STX-0119 on STAT3 target gene expression and STAT3 phosphorylation.** The effect of STX-0119 on STAT3 target gene expression in a representative cell line, GB-SCC026, was analyzed using real-time PCR. STX-0119 significantly inhibited c-myc gene expression in a dose-dependent manner (Fig. 3A). In contrast, WP1066 did not downregulate c-myc expression. C-myc expression in the other GBM-SC lines was not effected by STX-0119 (data not shown). The expression of other STAT3 targets such as Bcl-xL, survivin, cyclin D1, MMP9, TGF- $\beta$ 1, VEGF was also suppressed by STX-0119 at 100  $\mu$ M (Fig. 3B). Interestingly, the expression of VEGFR2 was remarkably inhibited by STX-0119. STAT3 phosphorylation was moderately down-regulated at 100  $\mu$ M of STX-0119 in the GB-SCC026 cell line (data not shown). Furthermore, STX-0119 significantly inhibited the stem cell-associated gene expression of CD44, Nanog, nestin and CD133 (Fig. 3C).

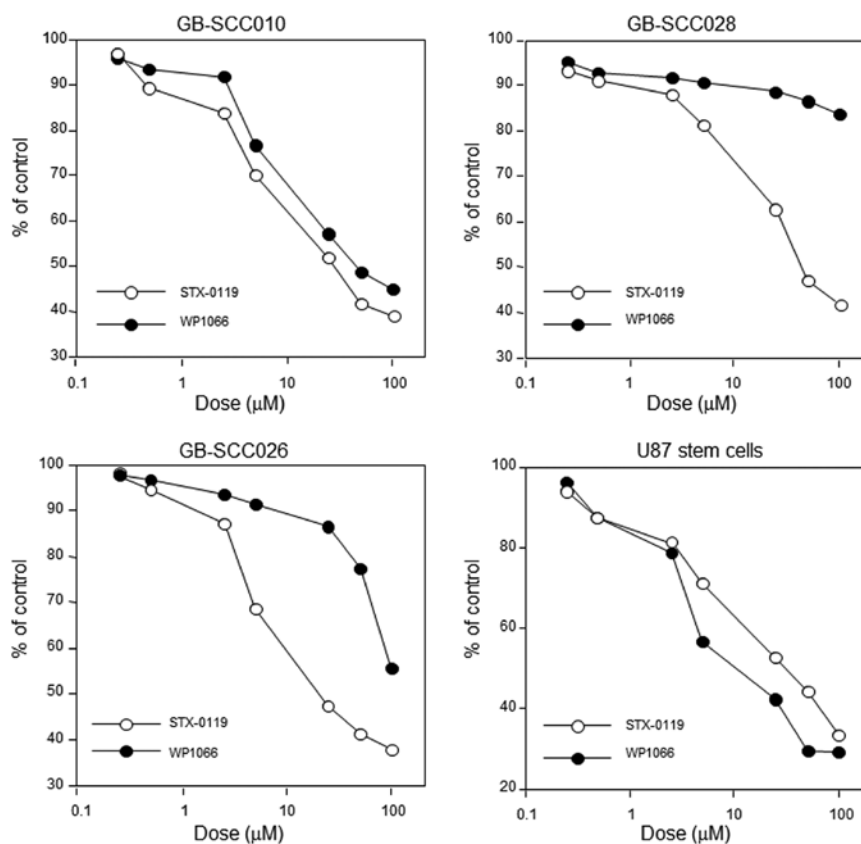


Figure 2. Inhibitory effect of STX-0119 on the proliferation of GBM-SC lines. GBM-SC or U87 stem cells ( $5 \times 10^3$ - $1 \times 10^4$ ) were seeded into each well of a 96-well micro-culture plate and various compounds diluted with SCM (100-0.25  $\mu\text{M}$ ) were added. Each point shows the mean for three samples. (○) STX-0119; (●) WP1066.

Table III. Effect of STX-0119 and WP1066 on GB-stem cell proliferation.

Cell line	Proliferation ( $\text{IC}_{50} / \mu\text{M}$ )			Sphere ( $\text{IC}_{50} / \mu\text{M}$ )	
	TMZ	STX-0119	WP1066	STX-0119	WP1066
GB-SCC010	226	36.1	44.8	3.8	7.8
GB-SCC026	53.1	15.1	>100	4.2	28.9
GB-SCC028	167	44.5	>100	5.2	50.0
U87 parental	45.2	6.6	2.1	-	-
U87 stem cell	66.7	31.4	10.6	2.4	2.2

**Induction of apoptosis by STX-0119 in GBM-SC lines.** The apoptosis induction in GB-SCC026 cell line and U87 stem cell line was investigated using caspase-3 western detection kit including the primary antibody against cleaved caspase-3 (Cell Signaling). Apoptosis was remarkably identified after a 24-h exposure by STX-0119 with the dose of  $>50 \mu\text{M}$  (Fig. 4).

**STX-0119 inhibits tumor growth in a subcutaneous model of GBM-SC lines.** Based on the growth of transplanted primary GBM-SC lines, GB-SCC010 and 026 were shown to generate well-growing tumors, used as a model of treatment with STX-0119 (Fig. 5A). STX-0119 at doses of 40 and 80 mg/kg suspended with methyl cellulose was orally administered to

NOD-*scid* and NOG mice bearing GBM tumors of  $>35 \text{ mm}^3$ . The administration of STX-0119 at 80 mg/kg for three weeks caused a  $>50\%$  inhibition of GB-SCC010-derived tumors at day 28 (Fig. 5B) and GB-SCC026-derived tumors at day 21, after the start of treatment (Fig. 5C). In contrast, WP1066 did not show an inhibitory effect on GB-SCC026 tumors. Additionally, no significant side effects including weight loss were seen in STX-0119-treated mice. Interestingly, it seemed that the vascularity around the tumor decreased after the STX-0119 administration compared to the GB-SCC026T SC-derived tumor without treatment (Fig. 6A).

**Decrease of VEGF production from GBM-SC lines treated with STX-0119.** The VEGF levels in the supernatant of GBM-SC

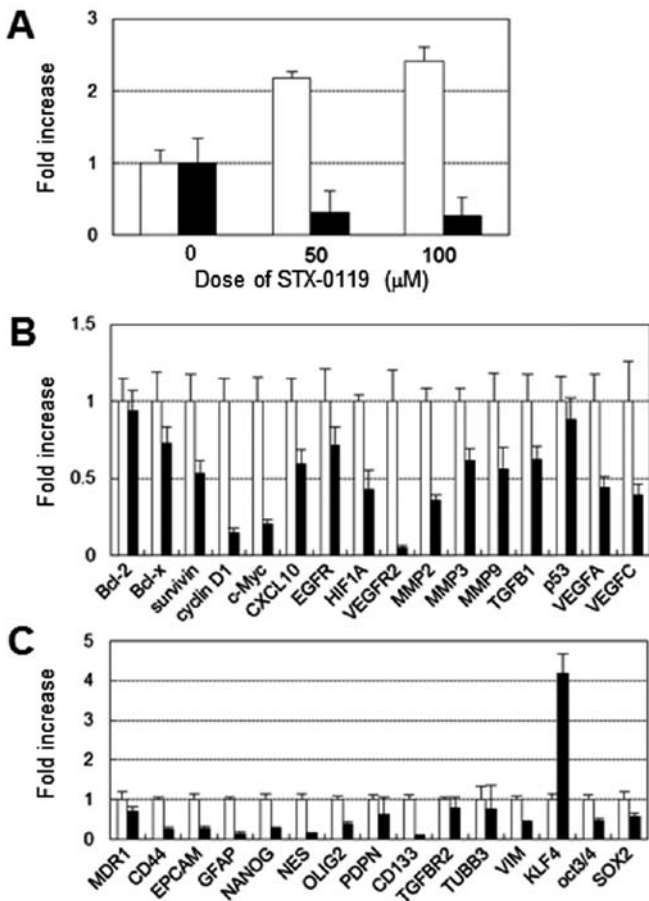


Figure 3. Downregulation of STAT3 target gene expression by STX-0119 in GB-SC lines. The changes in (A) c-myc expression, (B) STAT3 target gene expression and (C) stem cell marker gene expression. (A) GB-SCC026T stem cell line was treated with STX-0119 or WP1066. (B and C) GB-SCC026 stem cell line was treated with 100 μM of STX-0119. The gene expression level without STX-0119 was rated 1 as a control and gene expression in the stem cell line is shown as fold increase. Each column shows the mean ± SD of four determinations. (A) Open column, WP1066; closed column, STX-0119. (B and C) Open column, control; closed column, STX-0119.

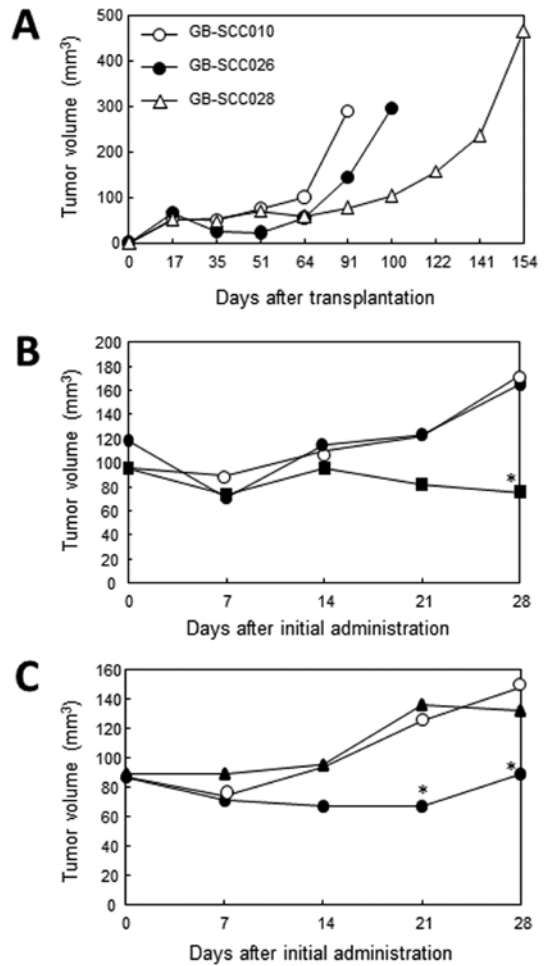


Figure 5. STX-0119 inhibits tumor growth in a subcutaneous model of GB-SC. NOG mice transplanted with human GBM-SC were used. (A) Proliferation of three GBM-SC lines in xenografts. (B) Effect of STX-0119 on GB-010 stem cell growth; (○) control; (●) STX-0119 40 mg/kg; (■) STX-0119 80 mg/kg. (C) Effect of STX-0119 on GB-026 stem cell growth; (○) control; (●) STX-0119 40 mg/kg; (▲) WP1066 40 mg/kg. Each point shows the mean value for 5 mice. \*P<0.05, statistically significant compared to control.

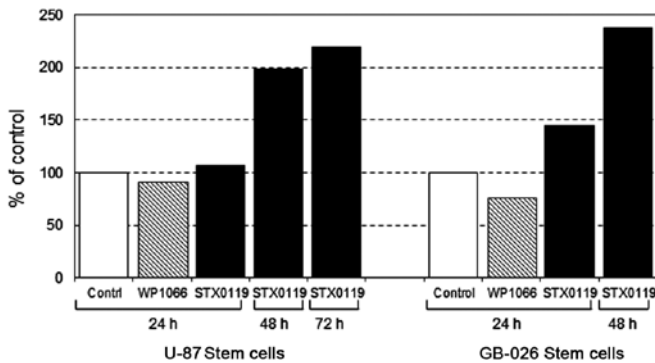


Figure 4. Induction of apoptosis in GBM SC line by STX-0119. Apoptosis was detected using a caspase-3 western detection kit containing a primary antibody against cleaved caspase-3. U87 and GB-SCC026 stem cells were treated with STX-0119 at 100 μM for up to 72 h or WP1066 at 25 μM for 24 h.

lines treated with STX-0119 significantly decreased in a dose-dependent manner (Fig. 6B). Specifically, the reduction was more remarkable in GB-SCC026T SC than O10T SC and U87 cells.

**Discussion**

Glioblastoma multiforme (GBM) is one of the most malignant and aggressive tumors with a very poor prognosis. Despite recent therapeutic advances, less than one-third of GBM patients survive more than 2 years (1,2). Thus, a novel therapeutic approach is urgently needed to control recurrence and overcome resistance to treatment. In the present study, we demonstrated that STX-0119 inhibited GBM-SC proliferation *in vitro* and *in vivo* by downregulating the gene expression of STAT3's targets like Bcl-X-L, cyclin D1, survivin, c-myc, VEGF, MMP2 and HIF-1α and inducing apoptosis. Previously we reported that STX-0119 significantly inhibited the growth of a highly STAT3-phosphorylated lymphoma cell line *in vitro* and *in vivo* by reducing expression c-myc, survivin, cyclin D1 and Bcl-xL and causing apoptosis. The IC<sub>50</sub> value, 15-44 μM, in the present study regarding GBM-SC lines was higher than that of lymphoma cell line reported previously. Importantly, we demonstrated that higher STX-0119 level than IC<sub>50</sub> value in mouse blood was obtained *in vivo* in a pharmacokinetic study previously (17).

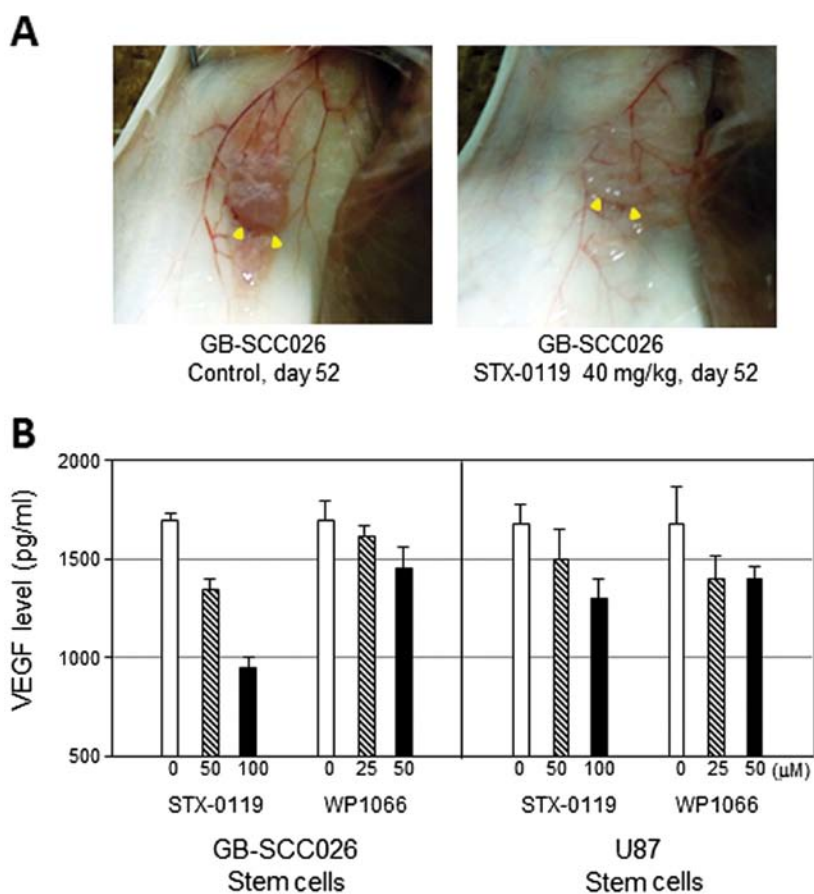


Figure 6. Inhibitory effect of STX-0119 on tumor angiogenesis. (A) The tumor viewed from the reverse side in a GB-SCC026 stem cell-transplanted mouse on day 54. STX-0119 was administered orally daily from day 0 to day 4 followed by 2 days of rest, over a total of 18 days. (B) VEGF levels in the supernatant of U87 and GB-SCC026 stem cells treated with STX-0119 or WP1066 at doses as shown for 48 h. Each column shows the mean of three determinations.

Since GBM-SC were identified as tumor-initiating cells, substantial evidence has been emerged of specific features in terms of gene signatures (6,7,18). The criteria for GBM-SC are as follows: i) isolated and expanded from GBM tumors *in vitro* as neurosphere cultures in serum-free medium containing EGF and bFGF, ii) a capacity for extended self-renewal, multilineage differentiation, iii) the ability to reproduce the histology of human GBM tumors in xenografts and iv) upregulation of drug-resistant and anti-apoptotic genes in response to radiation or chemotherapy. Our three GBM-SC lines met all these criteria. Additionally, we established matched serum-derived cell lines from the same patients. Interestingly, the expression of GBM stem cell markers was weak and the maturation markers GFAP and neuron-specific class III  $\beta$ -tubulin were upregulated. Lee *et al* reported that stem cell medium-derived GBM-SC had a specific gene expression signature that more closely resembles the tumor of origin than do serum-derived cell lines from the same tumor (7).

STAT3 is a member of a family of DNA-binding molecules, which regulate numerous physiological and oncogenic signaling pathways leading to target gene expression through STAT3-SH2 dimerization and phosphorylation, promote cell proliferation and induce anti-apoptotic activity, angiogenesis and immunological regulation. Recent studies have showed that STAT3 is constitutively activated in various

types of cancers including hematological and solid cancers. Experimental approaches for blocking STAT3 signaling using small interfering RNA (siRNA), small hairpin RNA (shRNA) and STAT3 antisense have been successful in inhibiting cell proliferation *in vitro* (19) and tumor growth *in vivo* (20).

As is the case with GB tumors, the constitutive activation of the STAT3 pathway is linked to tumor promotion and maintenance. With regard to GBM-SC signaling, the interaction between STAT3 and other pathways including EGFR, Notch, Wnt, Hedgehog, Akt, mTOR, olig2, PKC, MAPK, NF- $\kappa$ B and BMP4 was shown to regulate self-renewal (21) and astrocytic differentiation. Interestingly, STAT3 is involved in both stem cell maintenance and astrocytic differentiation, which could be important for therapeutics. Sherry *et al* (14) reported that the stat3 inhibition by shRNA induced growth arrest and inhibition of sphere formation as well as stem cell marker downregulation, which suggested that STAT3 contributes to the maintenance of stem-like characteristics. Our observation in a quantitative PCR assay that one GBM-SC line showed significant inhibition of stem cell markers and STAT3-target genes on STX-0119 treatment, but another GBM-SC line did not, support such a finding. These results might indicate that the dependency on STAT3 differs among GBM-SC lines, perhaps leading to the difference in response to the anti-STAT3 reagent STX-0119. First, it is reasonable to assume

that STX-0119-treated GBM-SC lines become sensitive to apoptotic stimuli when they have differentiated. This would be consistent with the observation that GBM-SC lines do not undergo apoptosis upon STAT3 inhibition in stem cell medium unlike serum-derived glioma cell lines. Additionally, other potential functions of STAT3 in GBM-SC lines, as a tumor suppressor and differentiation-inducing activity in astrocytes (22,23), should be considered to clarify the various therapeutic effects of STX-0119 including off target ones.

A second explanation for the heterogeneous response to the anti-STAT3 agent is activation of Yamanaka factor-associated signaling. Our observation regarding the downregulation of Yamanaka factor expression in GBM-SC lines treated with STX-0119 revealed an unexpected upregulation of KLF-4. The Oct-4 and Sox2 genes were demonstrated to be associated with the maintenance of stemness in glioma-initiating cells (24) and Oct-4 and nanog have been linked to STAT3 signaling (25). Considering that KLF-4 is located downstream of STAT3 and nanog, these observations indicate that KLF-4 signaling is linked to pathways other than STAT3 and nanog. Investigation of the cross talk between Yamanaka factor signaling and the STAT3 pathway should facilitate the development of agents against cancer stem cells. Another interesting point is that KLF-4 is reported to act as a tumor suppressor gene in colorectal cancer cells in which loss of heterozygosity (LOH) and point mutations in KLF-4 gene were identified (26). This observation suggests that KLF-4 might differ from other Yamanaka factors in terms of genetic function and upregulation of KLF-4 is one of mechanisms for the antitumor effect of STX-0119.

GBM-SC-targeted therapeutics such as small molecules and other reagents have been developed. JAK2/STAT3 inhibitors, AZD1480 (27), WP11193 (28) and LLL12 (29), were demonstrated to have inhibitory effects on GBM-SCs *in vitro* and *in vivo* mediated by downregulation of GBM stem cell marker as well as STAT3 target genes and induction of apoptosis in stem cells. Curcumin, a natural compound, was shown to suppress tumor growth *in vivo* by inducing autophagy (30). However, anti-GBM-SC agents have yet to be successfully developed. One major reason for this is the significant heterogeneity (31) and involvement of multi-pathway signaling in stem cell growth (21). Novel biomarkers specific for GBM-SC include protein phosphatase 2A (32), inhibitors of DNA binding proteins 2 and 4 (33), IL-13R $\alpha$ 2 (34), Girdin (35) and HIF-1 $\alpha$ . These markers have also been shown to be significant prognostic markers. Hypoxia in the tumor microenvironment is crucial to the maintenance of GBM-SC and HIF-1 $\alpha$  may be a good GBM stem cell marker at tumor sites (36,37). Additionally, TGF- $\beta$ 1 signaling (38) and EMT factors (39), closely involved in the growth and maintenance of GBM-SC and tumor progression, might be potential targets for treatment. Of note, HIF-1 $\alpha$  and TGF- $\beta$ 1 expression was downregulated in STX-0119-treated GBM-SC lines in our study, suggesting these markers to be STAT3 pathway-linked GBM stem cell markers. Another good prognostic marker regarding tumor recurrence in GBM patients is the absence of a mesenchymal transition gene signature (40).

In the present study, we demonstrated that the STAT3 inhibitor STX-0119 significantly inhibited stem cell proliferation *in vitro* and tumor formation *in vivo* by downregulating

the gene expression of STAT3 targets and stem cell markers. Collectively, combining STAT3 inhibitors with other reagents targeting GBM-SC-specific molecules (receptor tyrosine kinase inhibitors and anti-angiogenic agents) besides STAT3, may be the basis for the next generation of GBM treatment. It is the most important to control GBM recurrence after standard therapy and prolong the relapse-free and overall survival of GBM patients.

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