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Myb Repression Mediates Stat5b-knockdown-induced Apoptosis and Inhibits Proliferation of Glioblastoma Stem Cells

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Abstract. *Background/Aim: Glioblastoma is the most common and aggressive malignant brain tumor in adults, and glioblastoma stem cells (GSCs) contribute to treatment resistance and recurrence. Inhibition of Stat5b in GSCs suppresses cell proliferation and induces apoptosis. Herein, we investigated the mechanisms of growth inhibition by Stat5b knockdown (KD) in GSCs. Materials and Methods: GSCs were established from a murine glioblastoma model in which shRNA-p53 and EGFR/Ras mutants were induced in vivo using a Sleeping Beauty transposon system. Microarray analyses were performed on Stat5b-KD GSCs to identify genes that are differentially expressed downstream of Stat5b. RT-qPCR and western blot analyses were used to determine Myb levels in GSCs. Myb-overexpressing GSCs were induced by electroporation. Proliferation and apoptosis were evaluated by a trypan blue dye exclusion test and annexin-V staining, respectively. Results: MYB, which is involved in the Wnt pathway, was identified as a novel gene whose expression was down-regulated by Stat5b-KD in GSCs. Both MYB mRNA and protein levels were down-regulated by Stat5b-KD. Overexpression of Myb rescued cell proliferation that was suppressed by Stat5b-KD. Furthermore, Stat5b-KD-induced apoptosis in GSCs was significantly inhibited by Myb overexpression. Conclusion: Down-regulation of Myb mediates Stat5b-KD-induced inhibition of proliferation and induction of apoptosis in GSCs. This may represent a promising novel therapeutic strategy against glioblastoma.*

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Glioblastoma multiforme is the most common and aggressive malignant brain tumor in adults, with a median patient survival of approximately 15 months despite intensive combination therapies (1). Glioblastoma stem cells (GSCs) have been identified in glioblastoma multiforme tissues (2, 3) and are involved in resistance to both chemotherapy (4) and radiotherapy (5), thus contributing to cancer recurrence (6). Therefore, GSCs may serve as potential targets to tackle this devastating disease (7).

Stat5b is activated by cytokines, growth factors, and hormones (*e.g.*, interleukin-6, epidermal growth factor, and growth hormone) (8-10) and regulates the proliferation and survival of various cells (*e.g.*, hematopoietic cells) (11). A previous study identified *STAT5b* as a gene that was down-regulated by knockdown of Lgr5, a Wnt-related stem cell marker, in GSCs (12). Knockdown and pharmacological inhibition of Stat5b suppress GSC proliferation both *in vitro* and *in vivo* by inducing apoptosis (13). However, the detailed mechanism underlying GSC growth inhibition by Stat5b blockade remains unclear.

Vertebrate c-*MYB* encodes a transcription factor related to the *v-MYB* oncogene from the avian myeloblastosis virus that causes myeloblastic leukemia in birds (14). The proto-oncogene *MYB* is mainly expressed in hematopoietic cells (15). Myb protein functions as a transcription factor and synergistically regulates target gene expression with other transcription factors (16). Three Myb oncogenic alterations have been shown: overexpression, fusion with partner genes, and ectopic binding of Myb oncoproteins to enhancer sequences caused by somatic mutations (17). Amplification and overexpression of the *MYB* gene have been observed in acute myeloid leukemia, non-Hodgkin's lymphoma, colorectal cancer, and breast cancer (14). Fusion with partner genes is mainly observed in solid tumors. In brain tumors, up-regulation of Myb (18) and the MYB-QKI fusion gene contribute to promotion of tumorigenicity in pediatric low-grade gliomas (19). However, the functional role of Myb in glioblastoma is largely unknown. In this study, we examined the functional roles of Myb, which was down-regulated by Stat5b-knockdown (KD), leading to suppressed proliferation and induced apoptosis in GSCs.

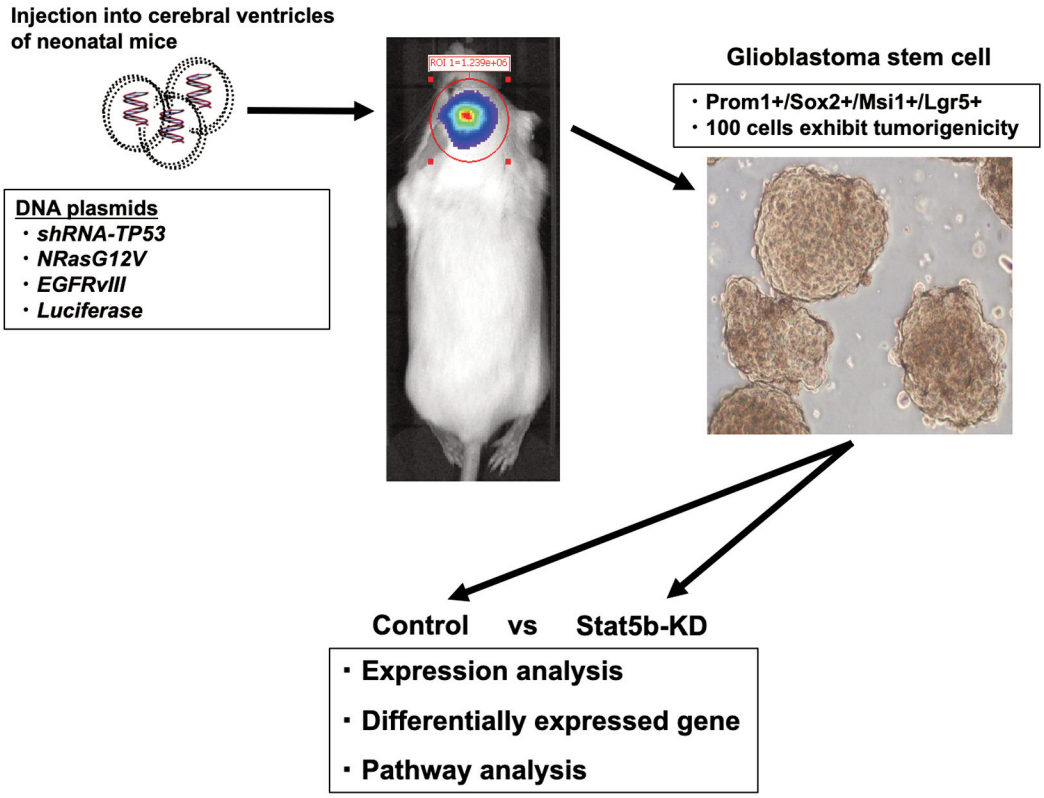

Figure 1. *Experimental workflow of the study. Glioblastoma stem cells (GSCs) were isolated from tumor tissues derived from the glioblastoma model using the neurosphere culture method. Gene expression profiling of GSCs with or without Stat5b-KD was performed.*

Materials and Methods

Glioblastoma induction. The procedure was performed as previously described (20). Briefly, two independent neonatal mice were set in a stereotaxic instrument (51730D; Stoelting Co., Wood Dale, IL, USA) and injected using an automated infusion system (Legato130; KD Scientific, Holliston, MA, USA) with 2 μl DNA/polyethylenimine complex into the right lateral ventricle at 1 μl/min using a 10 μl Hamilton syringe and a 30-gauge needle to generate *Sleeping Beauty* transposon-mediated *de novo* glioblastoma. The injection coordinates were +1.5AP, 0.7ML, and −1.5DV from λ. The DNA plasmids 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) and the DNA transfection reagent in vivo-JetPEI (Polyplus Transfection, New York, NY, USA) were used.

Cell culture. GSC cultures were established as previously described (21). Briefly, murine glioblastoma tissues were minced with scalpels, and dissociated by accutase (Innovative Cell Technologies, San Diego, CA, USA) for 20-30 min at 37˚C. The cells were incubated with serum-free neurobasal medium supplemented with B27, N2 (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), 10 ng/ml of epidermal growth factor and basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA). The formed neurospheres were dissociated using accutase (Innovative Cell Technologies) and passaged.

Knockdown of Stat5b. The procedure was performed as previously described (12). Briefly, RNAi clones (Stat5b-sh1: TRCN00 00012554 and Stat5b-sh2: TRCN0000012557) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Transduction with non-targeting shRNA, Stat5b-sh1, and Stat5b-sh2 was carried out at a multiplicity of infection of 5 or 10.

Expression profiling. The procedure was previously described (13). Briefly, global gene expression analyses (n=2) were performed with the SurePrint G3 Mouse Gene Expression 8´60K v2 microarrays system (Agilent Technologies, Palo Alto, CA, USA). The data were analyzed with the Gene Spring ver14.9.1 software (Silicon Genetics, Redwood City, CA, USA). Briefly, Raw data were normalized by the 75th percentile method. Genes down-regulated by >2-fold by Stat5b-KD were tested with the pathway analysis using Wiki-Pathway database (22). Raw data were deposited in the Gene Expression Omnibus database (GSE185873).

RT-qPCR. The cells were lysed with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was purified using the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized with the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). RT-qPCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the Light Cycler 96 System (Roche Diagnostic, Indianapolis, IN, USA) (n=3). mTubulin-b1 was used as an internal

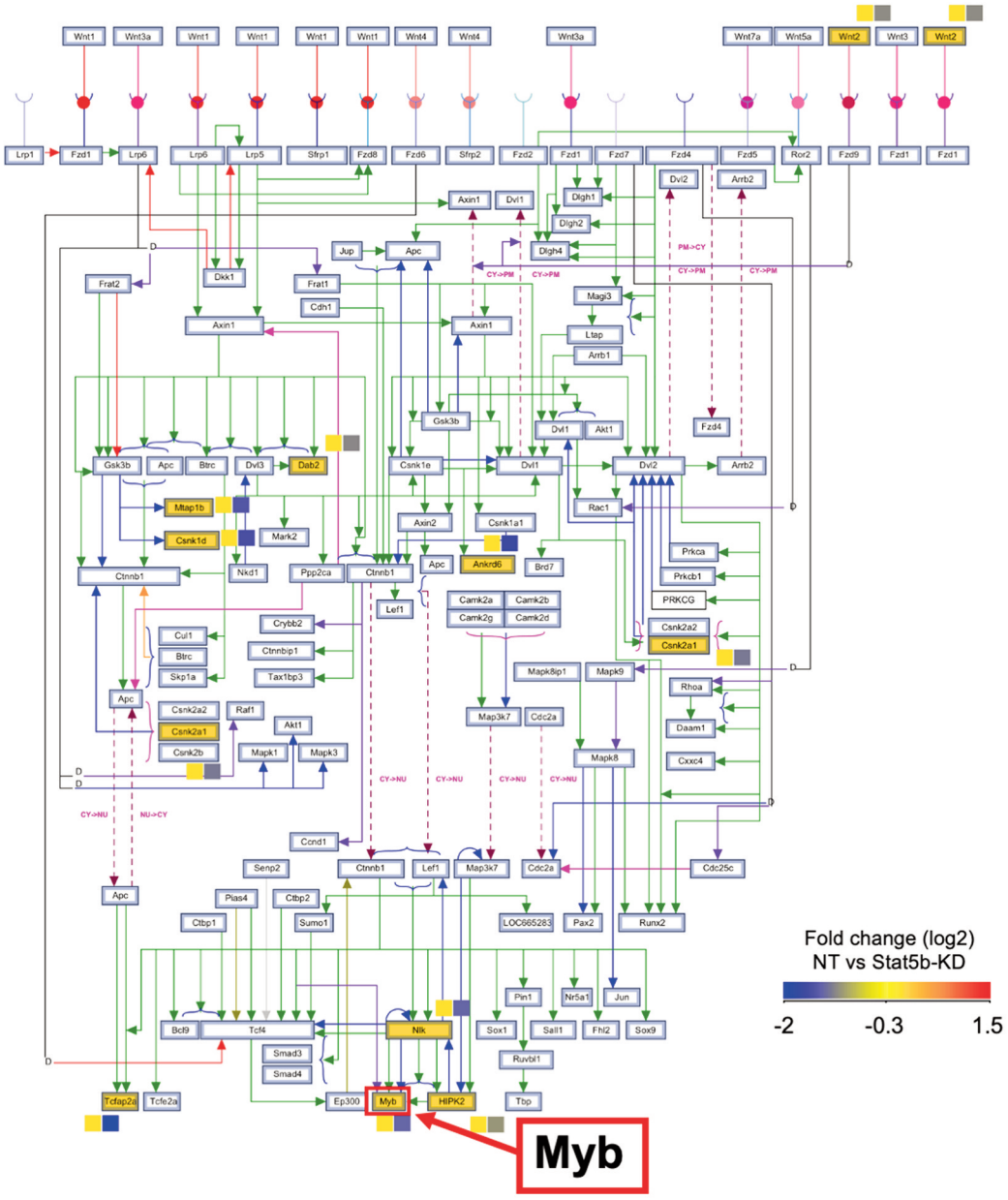

Figure 2. *Stat5b knockdown (KD) significantly down-regulates factors in the Wnt pathway including Myb. Results of the pathway analysis on down-regulated genes by Stat5b-KD (fold change >2.0), highlighted in yellow color, in the Wnt pathway (p=0.036) are shown. Transcripts, including Myb, pinpointed with yellow, in the Wnt pathway that are significantly affected by Stat5b-KD are shown.*

control. The following specific primers (Eurofins Genomics, Tokyo, Japan) were used (13): mStat5b, 5'-CTCTGGTGGGGCAGAACGAG-3' (forward) and 5'-TTGAGTCCCAGGCTTGGCTTT-3' (reverse); mMyb, 5'-ATTGTGGACCAGACCAGACC-3' (forward) and 5'-GCTGGTGAGGCACTTTCTTC-3' (reverse); mTubulin-b1, 5'-GCAG TGCGGCAACCAGAT-3' (forward) and 5'-AGTGGGATCAATG CCATG CT-3' (reverse).

Western blot analysis. The cells were lysed with 1% SDS buffer with a protease inhibitor cocktail mix (Nacalai Tesque) and PhosSTOP EASYpack (Roche). The proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Blocking was done with 3% or 5% dried milk in Tris-buffered saline with 0.05% Tween20. The membranes were incubated with primary and secondary antibodies. Protein levels were analyzed using Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). Chemiluminescence signal was detected using the ChemiDoc XRS Plus system (Bio-Rad). The following antibodies were used: Stat5b (1:1,000; ab178941, Abcam), MYB (1:1,000; PAB18191, Abnova, Taipei, Taiwan), c-Myb (1:1,000; 17800-1-AP, Proteintech, Rosemont,

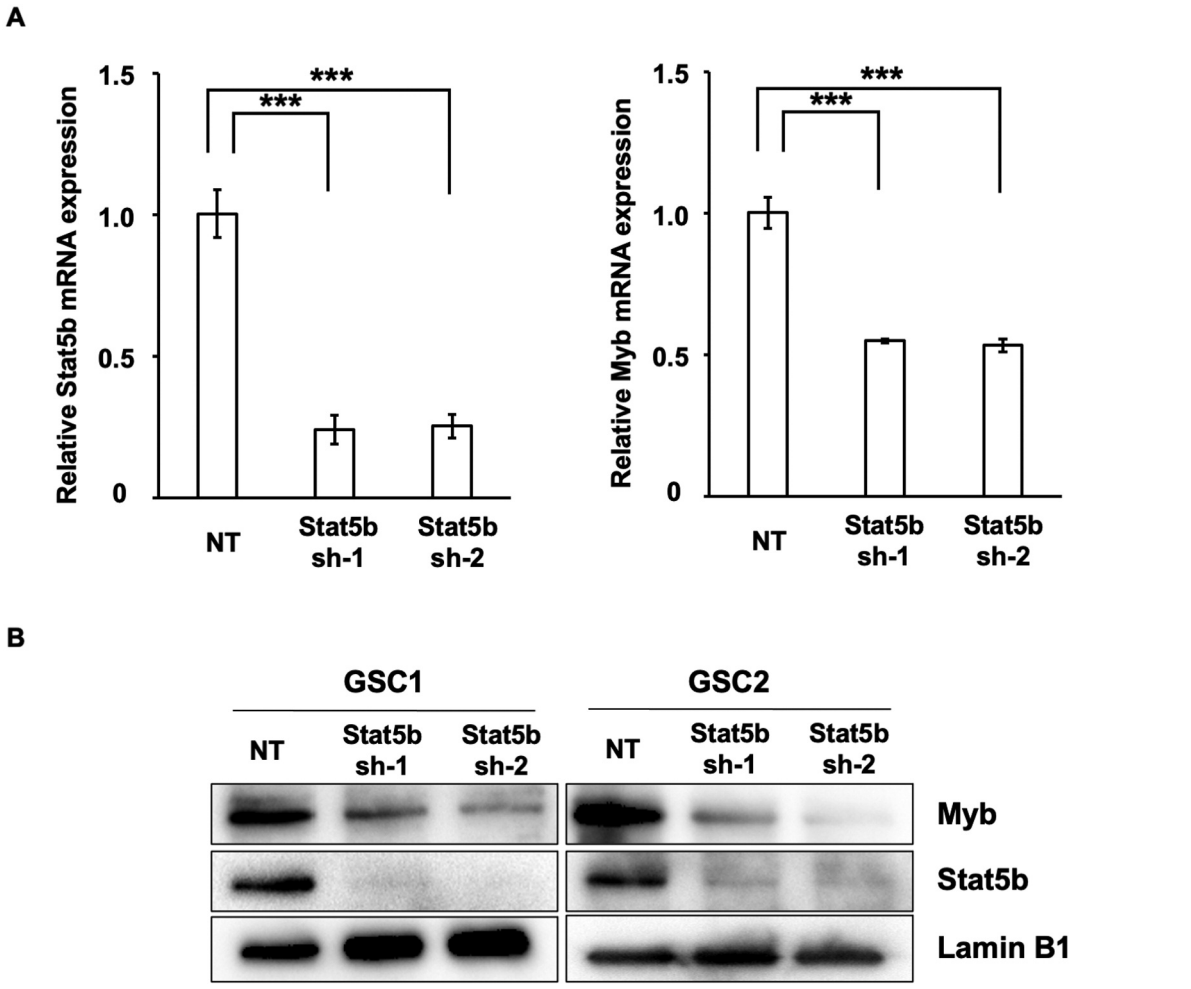

Figure 3. *Stat5b knockdown (KD) reduces Myb levels in glioblastoma stem cells (GSCs). (A) qPCR analysis of MYB mRNA expression in two independent GSC lines transduced with non-targeting shRNA (NT) or Stat5b-shRNA (Stat5b-sh1 and Stat5b-sh2) for 2 days. (B) Western blot analysis of Myb protein expression in nuclear fractions of two independent GSC lines transduced with NT or Stat5b-shRNA (Stat5b-sh1 and Stat5b-sh2) for 3 or 4 days. Lamin B1 was used as a loading control. Results of n=3 independent experiments are shown. ***p<0.001 by Dunnett's multiple comparison test.*

IL, USA), lamin B1 (1:2,000; 12987-1-AP, Proteintech), and vinculin (1:2,000; 66305-1-Ig, Proteintech).

Fractionation of nuclear/cytoplasmic proteins. Stat5b knockdown was performed and incubated for 3 or 4 days, and then cellular proteins were separated into cytoplasmic and nuclear fractions with the LysoPure nuclear and cytoplasmic extractor kit (Wako Pure Chemical Industries, Osaka, Japan).

Myb overexpression. The procedure followed was previously described (23). Briefly, Myb 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). The Myb (#MR 209649, Origene Technology, Rockville, MD, USA) and an empty vector (#PS100001, Origene) were transfected, and selection was done using G418 (10 μg/ml; Nacalai Tesque).

Cell growth assay. Cell proliferation was assessed with a 0.4% trypan blue dye exclusion test (Wako) using a Countess II automated cell counter (Thermo Fisher Scientific) (n=3).

Apoptosis assay. Apoptosis was detected by the MEBCYTO Apoptosis Kit (MBL, Nagoya, Japan). Cells in the early phase of apoptosis (Annexin-V-positive and propidium-iodide-negative) were quantified by flow cytometry using a BD LSRFortessa X-20 cell analyzer (BD Biosciences). At least 10,000 cells for each experiment were analyzed (n=3).

Statistical analysis. Data from at least three independent experiments were expressed as the mean±SD. One-way ANOVA with Dunnett's Multiple Comparison test or two-way repeated-measures ANOVA was performed using BellCurve software for Excel (Social Survey Research Information Co., Ltd. Tokyo, Japan). $p<0.05$ was considered statistically significant.

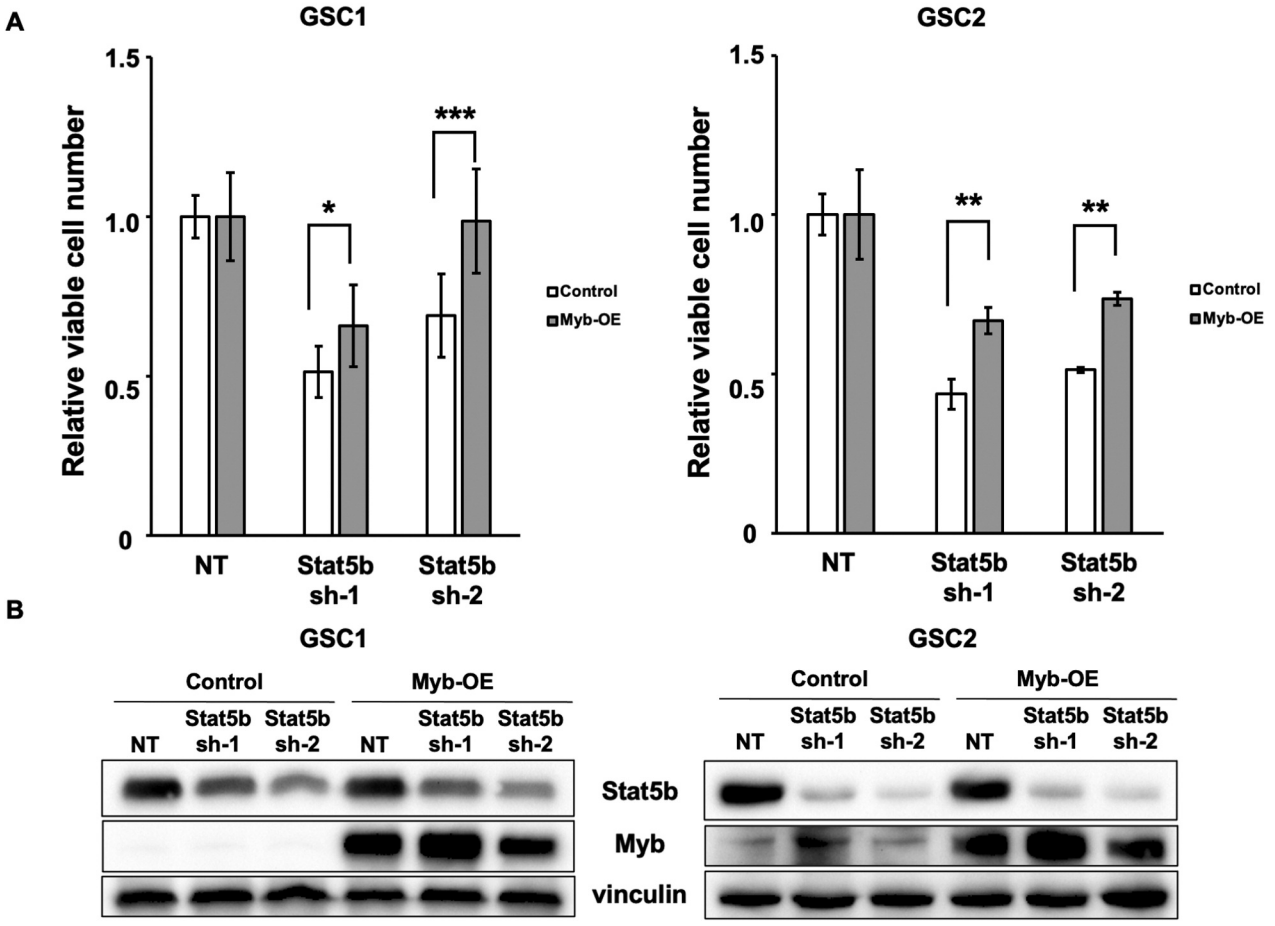

Figure 4. *Myb contributes to Stat5b-KD-induced growth suppression in glioblastoma stem cells (GSCs). (A) Two independent GSC lines (GSC1 and GSC2) derived from the different mice were transfected with Myb overexpression vector (Myb-OE) or empty vector (Control) and transduced with non-targeting shRNA (NT) or Stat5b-shRNA (Stat5b-sh1 and Stat5b-sh2) for 3 or 4 days. The relative number of trypan-blue-negative viable cells is shown. (B) The expression levels of Myb and Stat5b were analyzed by western blotting. Vinculin was used as the loading control. Results of n=3 independent experiments are shown. *p<0.01, **p<0.01, and ***p<0.001 by two-way repeated-measures ANOVA.*

Results

Stat5b-KD represses Myb levels in GSCs. Glioblastoma stem cells were established using the neurosphere culture method from tumor tissues derived from a mouse glioblastoma model established by the *Sleeping Beauty* transposon system (Figure 1). Microarray analysis of gene expression profiles revealed that the Wnt signaling pathway was significantly enriched by the gene set suppressed by Stat5b-KD (p=0.036), and that expression levels of Myb, which is involved in this pathway, were repressed (fold change=0.410) (Figure 2). With qPCR, we confirmed that Stat5b-KD significantly reduced *MYB* mRNA levels (Figure 3A). Myb functions as a transcription factor, and we confirmed that nuclear Myb protein levels were significantly suppressed by Stat5b-KD (Figure 3B). These results indicate that Stat5b-KD represses Myb levels in GSCs.

Myb overexpression rescued GSC proliferation suppressed by Stat5b-KD. To clarify whether Myb is involved in Stat5b-KD-induced suppression of cell proliferation, we established Myb-overexpressing GSCs. Myb overexpression rescued cell proliferation that was suppressed by Stat5b-KD (Figure 4A). Knockdown efficiency and Myb expression levels were confirmed by western blotting (Figure 4B). This finding indicates that decreased Myb expression mediates Stat5b-KD-induced suppression of GSC proliferation.

Myb overexpression decreased Stat5b-KD-induced apoptosis. We previously reported that knockdown or pharmacological

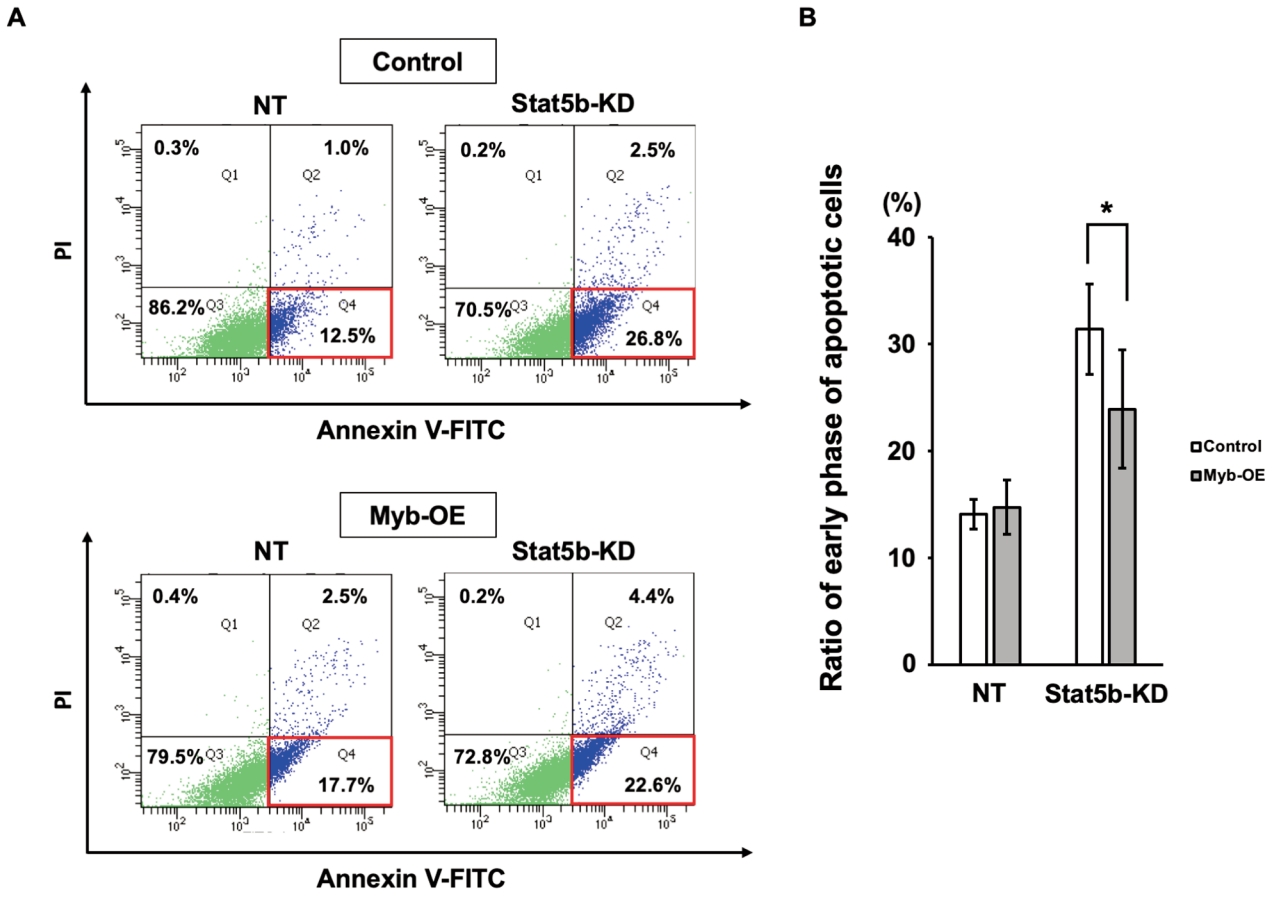

Figure 5. *Myb overexpression inhibits Stat5b-KD-induced apoptosis in glioblastoma stem cells (GSCs). (A) Myb-overexpressing (Myb-OE) or empty vector-transfected (Control) GSCs were transduced with non-targeting shRNA (NT) or Stat5b-shRNA (Stat5b-KD) for 3 days. Annexin-V-positive propidium-iodide-negative apoptotic cells were detected by flow cytometry. (B) The ratio of early-phase apoptotic cells is shown. Results of n=3 independent experiments are shown. *p<0.01 by two-way repeated-measures ANOVA.*

inhibition of Stat5b induces apoptosis in GSCs (13). Therefore, we hypothesized that decreased Myb levels mediate the induction of apoptosis by Stat5b inhibition. We found that Stat5b-KD-induced apoptosis was significantly decreased by Myb overexpression (Figure 5A and B). These results suggest that decreased Myb levels mediate Stat5b-KD-induced apoptosis in GSCs.

Discussion

To improve the clinical outcomes of glioblastoma, it is imperative to identify novel target molecules that are effective in inhibiting GSCs. Previous reports have shown that Stat5b inhibition suppresses proliferation and induces apoptosis in GSCs; however, the detailed mechanisms underlying how Stat5b inhibition inhibits cell growth remain unclear. Our study is the first to identify Myb as a downstream factor of Stat5b by global gene expression analysis. In GSCs, Stat5b-KD-induced decreases in Myb levels were confirmed by multiple methods at the mRNA and protein levels. Moreover, we demonstrated that Myb overexpression significantly rescued the suppressed proliferation and induced apoptosis by Stat5b-KD. This indicates that Myb plays a role downstream of Stat5b, promoting GSC proliferation and survival.

A previous study showed that c-Myb knockdown causes increased annexin-V-positive cell numbers and caspase-3 cleavage in Jurkat and K562 cells (24). The anti-apoptotic factors Survivin (24) and Bcl2, which is also regulated by Stat5b (25), are targets of Myb. In a previous study, we also found that ICG-001, an inhibitor of the Wnt/β-catenin signaling pathway, suppresses the expression levels of both Stat5b and survivin (13). Myb may be involved in inducing apoptosis by regulating the expression of these genes downstream of the Wnt signaling pathway in GSCs. It was also reported that JAK2 inhibitors suppress tumor growth *via*

suppressing JAK/STAT3/c-MYB signaling in Burkitt lymphoma cells (26). Thus, these findings support the hypothesis that Myb may contribute to cancer cell survival downstream of the JAK/STAT signaling pathway.

In conclusion, Myb down-regulation mediates the suppression of proliferation and induction of apoptosis in GSCs due to Stat5b inhibition. This study sheds new light on the mechanisms underlying the suppression of GSC proliferation by Stat5b inhibition, which may be useful for the development of novel therapeutic strategies against glioblastoma.

Conflicts of Interest

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

Authors' Contributions

CM and HO performed the experiments and drafted the manuscript. HI and MF designed and supervised the study. SN designed and supervised the study and wrote the manuscript.

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