

Original Article

Inhibition of Bmi1 suppresses tumorigenicity in glioma stem cells via the YAP/Pax3 pathway

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Abstract: Glioma stem cells (GSCs) are related to the recurrence of glioblastoma multiforme (GBM) due to their resistance to chemotherapy (CT) and radiotherapy (RT). Bmi1 is associated with self-renewal of malignant cancer stem cells (CSCs). The aim of this study was to evaluate the effect of Bmi1 downregulation in GSCs to target and eradicate CSCs in malignant tissues. A series of experiments involving Bmi1 knockdown, MTS assay, Pax3 and YAP overexpression, quantitative real-time PCR (qRT-PCR), western blotting, BrdU incorporation assay, and soft agar assay were performed in this study. Knockdown of Bmi1 notably suppressed GSC proliferation as shown in BrdU-positive, Bmi1-knockdown cells. Additionally, mRNA and protein expression levels of Pax3 were suppressed in Bmi1-knockdown cells, as shown by western blot and qRT-PCR results. Excessive Pax3 expression in Bmi1-knockdown cells attenuated the suppressive effect of Bmi1 knockdown on GSC proliferation. Pax3 expression was modulated by Bmi1 via the YAP pathway. Moreover, Pax3 modulated Bmi1 activity in GSCs. The above findings indicate that Bmi1 activity is involved in the proliferation and transformation of GSCs in GBM.

Keywords: Bmi1, GSC, Pax3, proliferation, tumorigenicity

Introduction

Glioblastoma multiforme (GBM) is the most prevalent primary cancer of the adult brain [1, 2]. The clinical outcome of GBM is poor, with a median survival rate of 14.6 months, despite therapeutic approaches such as surgery, chemotherapy (CT), and radiotherapy (RT) [3-5]. Unregulated proliferation, infiltration tendency, unstable genomes, and hypervascularization are the main contributors to the poor prognosis [6]. The resistance of glioma stem cells (GSCs) to CT and RT results in recurrence and limited overall survival [7, 8]. Consequently, innovative strategies targeting GSCs are required to meet the challenge of resistance and promote therapeutic efficiency. It is widely accepted that GSCs contribute to the proliferation, recurrence, and resistance of GBM [9, 10]. Although investigation of GSC markers with high specificity and sensitivity is ongoing, several reports have shown that EGFRvIII⁺ cells display the ability to promote malignancy [11, 12].

Bmi1 participates in the self-renewal of cancer stem cells (CSCs) via the modulation of genes

that are essential for cell cycle regulation, determination of the density of stem cells (SCs), suppression of cellular senescence, and viability modulation of various malignancies [13, 14]. Bmi1 regulates essential periods during the development of malignancy such as metastasis, invasion by regulating epithelial-to-mesenchymal transition (EMT), and resistance to drugs [15, 16]. Bmi1 expression is remarkably associated with unfavorable clinical outcomes and viability as well as increased aggression in patients with breast cancer (BC) [17-19].

Pax3 participates in the development of malignancies [20]. Pax3 expression has been found to be positively associated with the development of melanoma [21, 22]. Several reports have shown that Pax3 negatively regulates glial fibrillary acidic protein (GFAP) expression during differentiation of neural SCs (NSCs) to astrocytes [23, 24]. The aim of the present study was to elucidate the effects of Bmi1 on the role of GSCs, particularly in the regulation of proliferation and the development of malignancy, using human primary GBM cells.

Materials and methods

Derivation and culture of glioma cell lines

We harvested primary glioblastoma multiforme cells from the corresponding cases. The Papain Dissociation System (Worthington, NJ, USA) was used to digest the excised glioma specimens. Malignant cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA, USA), containing B27 minus vitamin A (1/50; Invitrogen, Carlsbad, CA, USA), 20 ng/mL endothelial growth factor (EGF; eBiosciences, CA, USA), penicillin/streptomycin (1/100; Fisher, Waltham, MA, USA), 20 ng/mL basic fibroblast growth factor (bFGF; eBiosciences, CA, USA), and glutamine (1/100; Corning, VA, USA). Growth factors, such as EGF and bFGF, were supplemented twice a week. Cells were separated and plated in DMEM/F12 containing 20% (v/v) fetal bovine serum (FBS) in preparation for culture. Accutase (Fisher, Waltham, MA, USA) was applied during cell separation for passaging. This study has been approved by the Ethics Committee of the West China Hospital, Sichuan University.

Bmi1 knockdown

Three lentivirus vector plasmids specific for target sequences contained in the Bmi1 shRNA plasmid (Santa Cruz Biotechnology, Dallas, TX, USA) were used to knockdown Bmi1 using puromycin selection genes. The cells were transfected with the Bmi1 shRNA plasmid using media devoid of antibiotics or serum supplements.

MTS assay

Cells from each distinct group were cultured in 96-well plates (2000 cells/well). MTS assay kit (Promega, Madison, WI, USA) was used to determine cell viability. The assay was performed in triplicate. For Pax3 overexpression, Bmi1 knockdown cells were seeded in 12-well plates, and 24 h later, cells were transfected with vector or Pax3 plasmid. After 24 h, cells were split into a 96-well plate. Thereafter, cell viability was determined using the MTS assay kit according to the manufacturer's instructions.

Pax3 and YAP overexpression

Plasmid vectors that overexpressed YAP and Pax3 were obtained from Addgene. The Micro-

Poly-Transfecter Cell Reagent was used for transient transfection.

Quantitative Real-Time PCR (qRT-PCR)

The purified RNA was dissolved in 30 µL of RNase-free water. cDNA was synthesized using 2 µg of total RNA with the PrimeScript™ RT reagent kit and gDNA Eraser (Takara, Dalian, Liaoning, Japan), in preparation for the subsequent qRT-PCR of Bmi1. The SYBR Premix Ex Taq II kit and an Applied Biosystems 7500 Fluorescent Quantitative PCR system was used for qRT-PCR. Expression of β-actin was used for the normalization of the expression of the target genes. The relative mRNA concentration was determined according to Ct values. All specimens were evaluated in triplicate.

Western blotting (WB)

Lysates containing total protein were acquired using the modified RIPA buffer (0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Igepal CA-630, and Tris-HCl, pH 8.0), and SDS polyacrylamide gel electrophoresis was subsequently performed. Overnight incubation with primary antibodies was performed at 4°C. Thereafter, incubation was carried out with the secondary antibodies for 1 h at room temperature.

BrdU incorporation assay

After seven-days in culture, cells were incubated for 4-6 h with 10 µmol of BrdU. Cells were fixed with 4% paraformaldehyde (PFA; Sigma, St. Louis, MI, USA) and stained with an anti-BrdU antibody (BD, Franklin Lakes, NJ, USA). Counterstaining was performed with Hoechst 33258 (Sigma, St. Louis, MI, USA). The number of cells positive for BrdU was recorded as the proportion of the total number of cells counterstained with Hoechst 33258. A Nikon DS-Ri1 microscope was used for observation. Procedures were carried out in triplicate.

Soft agar assay

Ten thousand cells were separated and resuspended in SeaPlaque agar (0.7%). Cells were added to culture medium containing the growth factors twice a week for 3 weeks. Cells were stained with nitro blue tetrazolium (NBT; Sigma, St. Louis, MI, USA) and fixed with 4% PFA. Procedures were carried out in triplicate.

Inhibition of Bmi1 suppresses tumorigenicity in GSCs

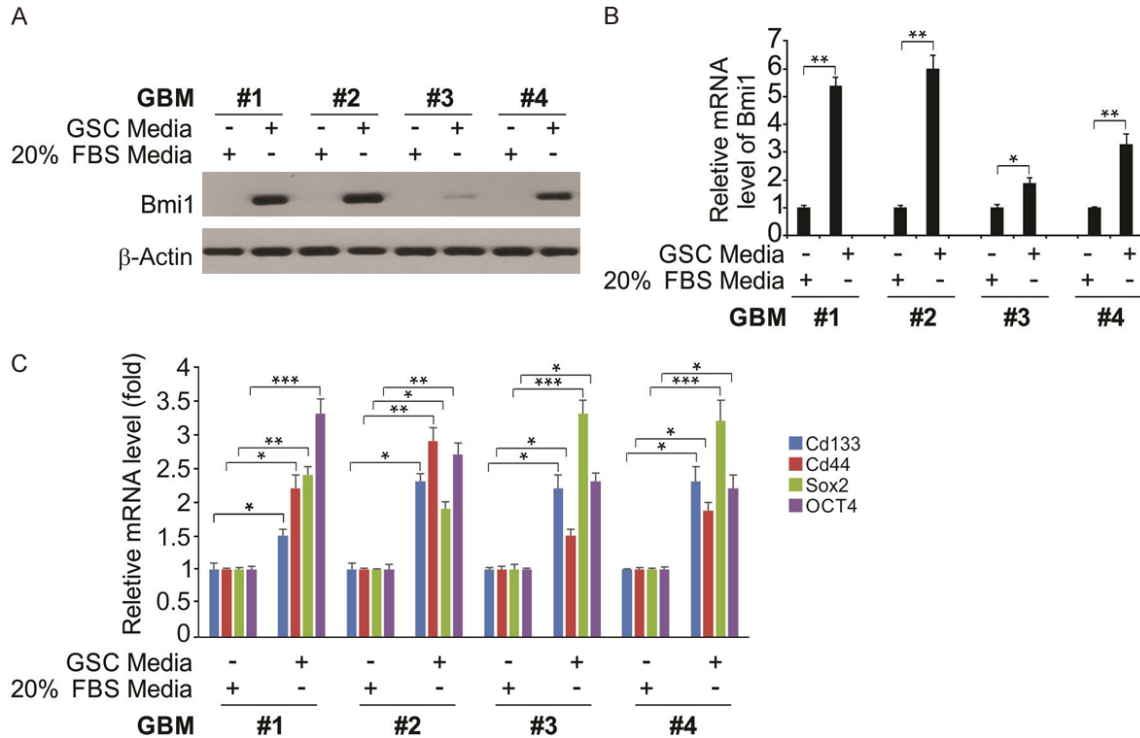


Figure 1. Bmi1 is preferentially expressed in glioma stem cells (GSCs). The level of Bmi1 was determined by western blot (A) and qRT-PCR (B) in glioblastoma multiforme (GBM) cells treated with GSCs or 20% FBS media. (C) The mRNA expression levels of CD133, CD44, Sox2, and OCT4 was analyzed by qRT-PCR. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

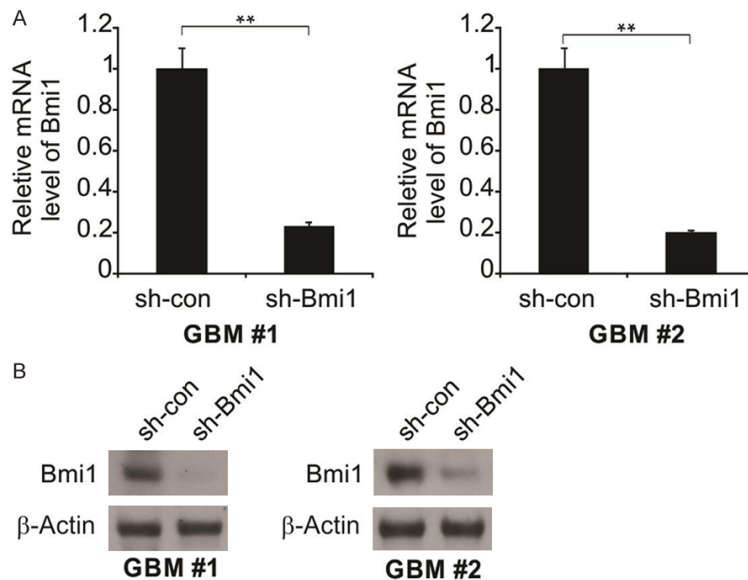


Figure 2. Bmi1 knockdown in GSCs. A. Cells were infected with lentiviruses expressing shcon or shRNAs. The mRNA level was detected by qRT-PCR. B. Protein levels were determined by western blot. ** $P < 0.01$.

Statistical analysis

The results were reported as the mean $\pm$ SD. Student's *t*-test or ANOVA was performed to

evaluate the differences between the groups. The difference was regarded as significant with $P < 0.05$.

Results

Expression of Bmi1 was promoted in GSCs

To examine the influence of Bmi1 on the preservation of the GSC state, we compared the expression of Bmi1 in SCs to that of non-SCs in malignant tissues. Cultures from freshly excised tumors were digested and plated in medium containing growth factors or serum. This resulted in the derivation and separation of matched glioma cells of typical populations of CSCs or cells after differentiation. WB was subsequently performed to evaluate Bmi1 expression in distinct cellular compartments. The protein and mRNA levels of Bmi1 were evaluated by GSC/differentiated cultivation with distinct

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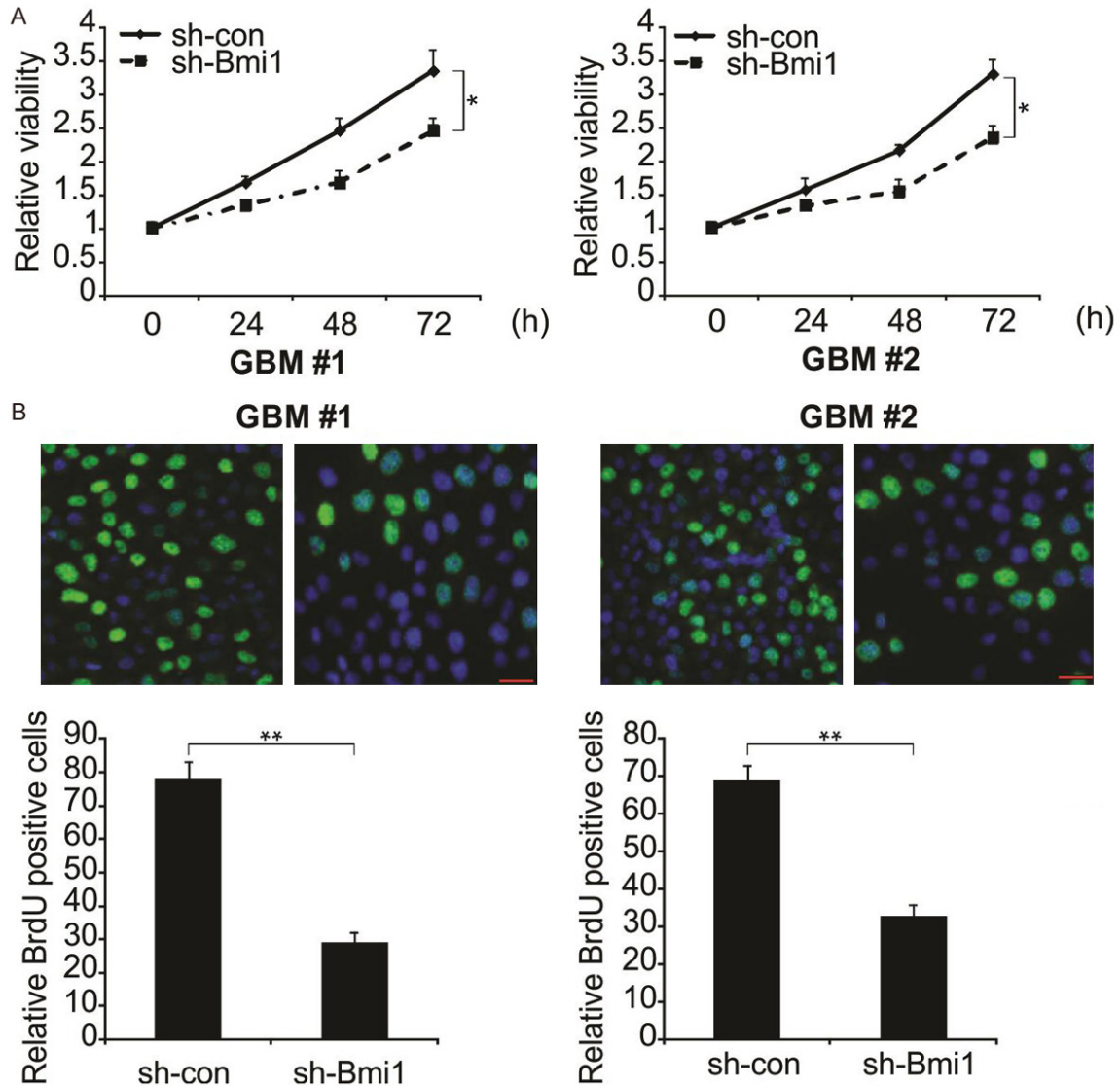


Figure 3. Bmi1 knockdown confers proliferation defects in GSCs. A. MTS analysis of sh-con and sh-Bmi1 at the indicated time points. B. Representative images and quantified values from the BrdU analysis of the GSCs. Scale bar: 50 μ m. * P <0.05; ** P <0.01.

concentrations of protein (**Figure 1A** and **1B**). Bmi1 expression increased remarkably in GSCs compared to that in the differentiated cells (**Figure 1A** and **1B**). In addition, the mRNA levels of CD133, CD44, Sox2, and OCT4 increased in the GSCs (**Figure 1C**).

Stable shRNA transfection reduced expression of Bmi1

We subsequently conducted qRT-PCR of GSCs transfected with a shRNA plasmid specific for Bmi1. Transfection with the shRNAs notably suppressed Bmi1 transcription (**Figure 2A**). A

noticeable suppression of Bmi1 expression was shown via WB (**Figure 2B**).

Bmi1 knockdown in GSCs inhibited proliferation

The MTS assay was used to quantify proliferation. Bmi1 knockdown notably suppressed proliferation (**Figure 3A**). We also carried out BrdU incorporation assays to investigate the influence of Bmi1 on the proliferation of GSCs. GSC proliferation was notably suppressed in BrdU-positive, Bmi1-knockdown cells (**Figure 3B**). These findings indicated that the expression of Bmi1 is related for the proliferation of GSCs.

Inhibition of Bmi1 suppresses tumorigenicity in GSCs

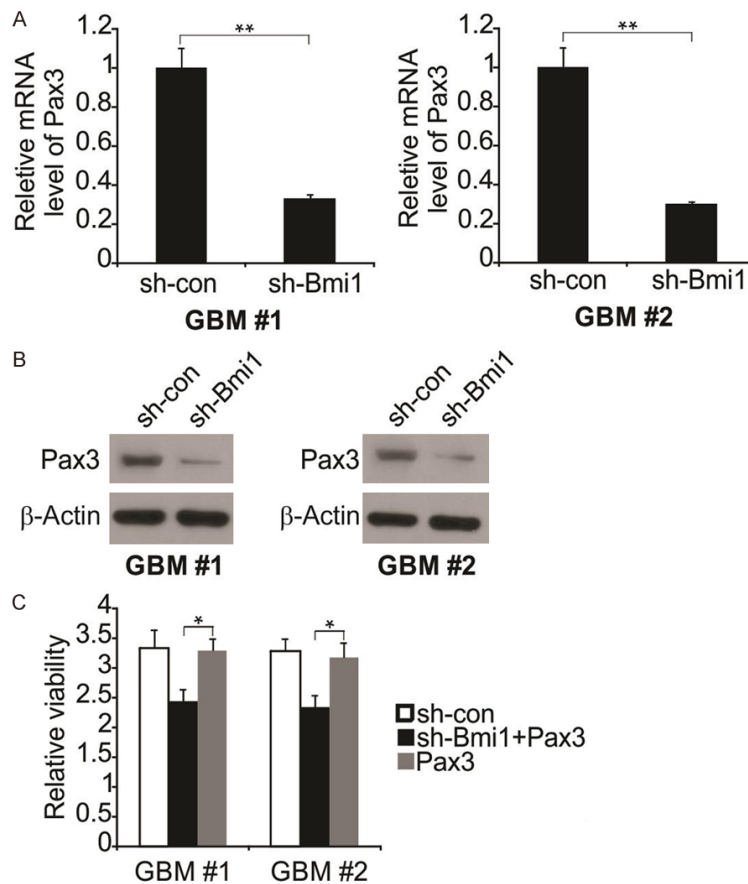


Figure 4. Bmi1 regulates Pax3 expression. A. The mRNA levels of Pax3 in Bmi1 knockdown cells were analyzed by qRT-PCR. B. The protein level of Pax3 in Bmi1 knockdown cells was analyzed by western blot. C. The cell viability of GSCs with Bmi1 knockdown and/or Pax3 overexpression. * $P < 0.05$; ** $P < 0.01$.

knockdown cells as shown by the WB and RT-PCR results (Figure 4A and 4B). Furthermore, excessive Pax3 expression in Bmi1 knockdown cells attenuated the suppressive effect of Bmi1 knockdown on proliferation (Figure 4C).

YAP modulated Bmi1-stimulated Pax3 expression

We examined the participation of YAP in Pax3 regulation via Bmi1 in GSCs. In Bmi1 knockdown cells, suppression of Pax3 expression in GSCs that excessively expressed YAP was inhibited, as presented in Figure 5.

Bmi1 knockdown suppressed tumorigenicity in GSCs

Colony formation was utilized to evaluate the anchorage-independent growth of GSCs in which Bmi1 expression was knocked down. Infected GSCs, suppressed colony growth (Figure 6A and 6B). The above findings indicated that Bmi1 is correlated to the development of tumorigenicity in GSCs.

Discussion

GSCs were first isolated from leukemic cells [25]. Reya et al. observed the expression of the same biomarkers in SCs and malignant cells. It was hypothesized that malignancies could arise from a subgroup of precursor SCs [26]. SCs were isolated from various malignancies, such as prostate and breast cancers [27]. Despite the isolation of GSCs from gliomas, our understanding of the

participation of GSCs in the development of malignancy is insufficient [28, 29]. Our research aimed to explore the influence of GSCs on the hallmarks of malignancies. In this study, our findings demonstrate that Bmi1 is essential

Bmi1 knockdown inhibited Pax3 expression

Our research subsequently examined the influence of Bmi1 knockdown on the expression of Pax3 in GSCs. Pax3 was suppressed in Bmi1

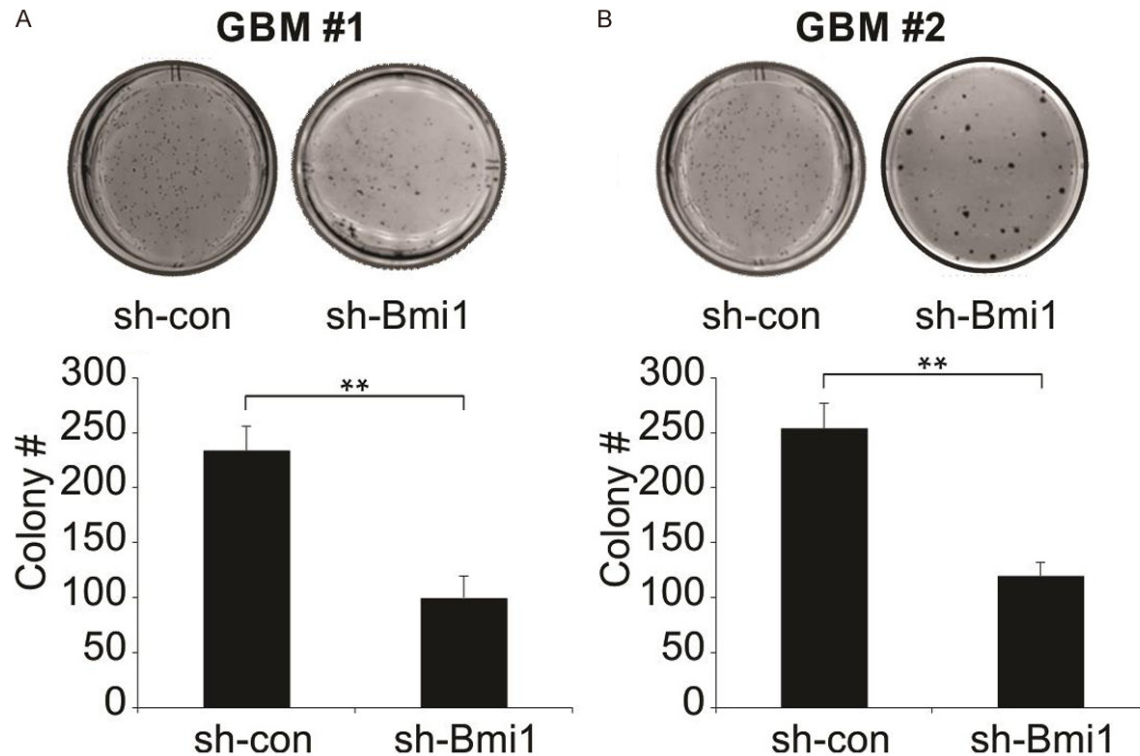


Figure 6. Bmi1 regulates the tumorigenicity of GSCs. The soft agar assay for GBM #1 (A) and GBM #2 (B). Bmi1 knockdown resulted in a significant decrease in colony formation. ** $P < 0.01$.

al for CSC modulation in gliomas. GSCs were transfected with shRNAs targeting Bmi1 to downregulate its expression. It was observed that Bmi1 transcription and translation were noticeably suppressed in transfected GSCs. Bmi1 knockdown inhibited the proliferation of GSCs. Moreover, Bmi1 knockdown resulted in reduced Pax3 expression mediated by the YAP pathway. As Pax3 induces proliferation, the above findings indicate that Bmi1 participates in the proliferation of GSCs through the YAP/Pax3 axis.

The polycomb group protein Bmi1 is overexpressed and acts as an oncogene in a variety of human tumors. The Hippo/YAP signaling pathway is a major integrator of cell surface-mediated signaling and regulates key processes in development and tumorigenesis. Previous studies have reported Bmi1-mediated YAP stabilization and the active hippo/YAP signaling pathway. In the current study, we elucidated the involvement of YAP in Bmi1-induced Pax3 upregulation. Pax3 is a developmental transcription factor that displays a regionalized expression pattern in the neural tube, neural

crest, and somites of the vertebrate embryos. As differentiation progresses, expression decreases. In the early stages of embryogenesis, Pax3 is transiently expressed in the posterior and dorsal neural tube. Pax3 is essential for the correct specification of these development tissues and cell survival, as homozygous deletion of Pax3 in mice results in severe neural crest and neural tube defects due to p53-dependent cell death. Our results indicate Pax3 mediates the function of Bmi1 in CSCs.

Various studies have examined the expression and activities of Bmi1 in CSCs [17, 30]. They have reported that Bmi1 deficiency results in the shortage of features related to CSCs [31]. Our study is the first to directly evaluate the influence of Bmi1 suppression on GSCs. Bmi1 stimulation is regarded as a trigger for the development of malignancy [32, 33]. Studies show that Bmi1 is a downstream modulator of oncogenes in BC, acute myeloid leukemia, gastric carcinoma, and GBM [32, 34, 35].

The findings of our research indicate that Bmi1 plays a role in the functions of GSCs by enhanc-

ing the development and proliferation of malignant cells. Targeting GSCs is important due to its role in recurrence and the debilitating effects of GBM. The clear distinction between SCs and non-SCs is necessary for the development of GBM treatments.

The findings of our research indicate that Bmi1 participates in the proliferation of glioma SCs and potentially contributes to the selective targeting of the Bmi1 axis stimulation in GBM.

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Disclosure of conflict of interest

None.

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