

BMSCs promote apoptosis in glioma cells by inhibiting STAT3 phosphorylation

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Abstract: Background: Bone marrow mesenchymal stem cells (BMSCs) show promise in glioma therapy, but the underlying mechanisms remain unclear. Signal transducer and activator of transcription 3 (STAT3) is a key oncogenic driver in glioma; however, whether BMSCs exert anti-glioma effects through STAT3 modulation requires further investigation. **Objectives:** To investigate whether BMSCs promote glioma cell apoptosis by inhibiting STAT3 phosphorylation and to validate the causal role of STAT3 using a specific agonist. **Methods:** C6 glioma-bearing rats were randomized into control, model, BMSC, positive control (MS-275) and STAT3 agonist (Colivelin) groups. BMSCs (1×10^6 cells) were injected via the tail vein on days 14 and 17. Tumor growth, apoptosis, glial fibrillary acidic protein (GFAP) expression, basic fibroblast growth factor (bFGF)/vascular endothelial growth factor (VEGF) mRNA levels and STAT3/phosphorylated STAT3 (p-STAT3) expression were assessed by magnetic resonance imaging (MRI), histology, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, flow cytometry, immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. *In-vitro* validation was performed using BMSC-conditioned medium and the STAT3 inhibitor Stattic in C6 cells. **Results:** BMSC transplantation significantly reduced the tumor area and Ki-67 proliferation index, increased the number of apoptotic cells and the apoptotic rate ($P < 0.01$) and decreased p-STAT3 levels and the p-STAT3/STAT3 ratio ($P < 0.01$) without altering total STAT3 expression. Colivelin reversed BMSC-induced apoptosis. bFGF and VEGF mRNA levels were increased in the BMSC group ($P < 0.01$). *In-vitro*, BMSC-conditioned medium suppressed STAT3 phosphorylation and induced apoptosis, effects that were enhanced by Stattic. **Conclusion:** BMSCs promote apoptosis in glioma cells by inhibiting STAT3 phosphorylation. The reversal of these effects by a STAT3 agonist confirms the causal role of STAT3 inhibition. These findings identify STAT3 as a key therapeutic target for BMSC-based glioma therapy.

Keywords: Apoptosis; Bone marrow mesenchymal stem cells; Glioma; Paracrine; Rat model; STAT3

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INTRODUCTION

Glioma is a common type of malignant brain tumor with high morbidity and mortality, posing a serious threat to human health (Wang *et al.*, 2022). Although certain advances have been made in the treatment of glioma, such as the application of novel chemotherapeutic agents (Chen *et al.*, 2022; Battaglia *et al.*, 2025), there has been no significant decline in the mortality rate of glioma patients clinically (Mishchenko *et al.*, 2024). Therefore, exploring new therapeutic strategies and elucidating their underlying mechanisms have important clinical significance.

Bone marrow mesenchymal stem cells (BMSCs), as a common type of adult stem cell, have received widespread attention in tissue repair and regenerative medicine. Studies have shown that BMSC transplantation can significantly inhibit the growth of glioma cells, but the specific mechanism remains incompletely understood (Chen *et al.*, 2025). It is currently believed that the therapeutic effect of BMSCs is not achieved through differentiation into glial cells but mainly through

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paracrine mechanisms by secreting various cytokines, regulating the tumor microenvironment and affecting the proliferation and apoptosis of tumor cells (Dai *et al.*, 2024; Ababneh *et al.*, 2025). However, the specific paracrine action of BMSCs in regulating the apoptosis of glioma cells remains unclear.

Signal transducer and activator of transcription 3 (STAT3) is an important signaling molecule for various cytokines and receptors. It can be activated through phosphorylation, then enter the nucleus and bind to deoxyribonucleic acid (DNA), thereby regulating the expression of multiple genes (Chen *et al.*, 2024). STAT3 can be briefly activated under normal physiological conditions, but this activation lasts only for a few hours (Almutairy *et al.*, 2022). It is constitutively activated during tumor development and progression and its expression level is also significantly increased (Lv and Ding, 2025; Bhattacharya *et al.*, 2022) here is a correlation between STAT3 expression and apoptosis in various tumors (Stenckova *et al.*, 2022; Oze *et al.*, 2025). Reducing STAT3 expression can affect the expression of intracellular signaling proteins, thereby inhibiting apoptosis (Haidurov *et al.*, 2025; Xu *et al.*, 2024). The

STAT3 signaling pathway participates in the apoptotic process of glioma cells. Blocking the activity of the STAT3 signaling pathway can significantly inhibit STAT3 protein expression and phosphorylation (Chen *et al.*, 2025). This prevents it from interacting with cell surface receptors and entering the nucleus, thereby inhibiting cell growth and proliferation and inducing apoptosis in human glioma cells.

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are not only key angiogenic factors but also direct transcriptional targets of the STAT3 signaling pathway. Upon activation, STAT3 binds to STAT3-responsive elements in the VEGF promoter region, promoting its transcription (Hu *et al.*, 2024). In glioma, STAT3-mediated VEGF upregulation promotes tumor angiogenesis, thereby supporting tumor growth and survival (Coskun *et al.*, 2022). Therefore, detecting changes in VEGF and bFGF expression helps to understand whether BMSCs affect tumor angiogenesis and the microenvironment through regulation of the STAT3 pathway. In this study, VEGF and bFGF were used as pre-specified validation indicators to investigate the regulatory effects of BMSCs on STAT3 downstream angiogenesis-related genes.

Notably, there is a potential mechanistic link between the paracrine effects of BMSCs and the STAT3 signaling pathway. On one hand, BMSCs secrete cytokines such as interleukin-6 (IL-6), which can activate the Janus kinase (JAK)/STAT3 pathway (Jeong *et al.*, 2025, Zhang *et al.*, 2025). On the other hand, changes in STAT3 activity can affect the response of tumor cells to BMSC-derived signals. Studies have shown that STAT3 phosphorylation levels in tumor cells determine their sensitivity to mesenchymal stem cell (MSC)-derived IL-6 and STAT3 activation enhances tumor cell proliferation and drug resistance (Tu *et al.*, 2025). Furthermore, the regulation of the STAT3 signaling pathway is highly complex, involving both positive and negative regulatory factors and participates in various biological processes including immunosuppression and metabolic reprogramming in the tumor microenvironment (Hu *et al.*, 2024). STAT3 function exhibits significant cell type and microenvironment dependency, with its activation status, duration and interactions with other signaling pathways collectively determining its effects on cells. However, although BMSC transplantation has been observed to affect glioma cell apoptosis and the STAT3 signaling pathway is known to be involved in glioma progression, whether BMSCs influence glioma cell apoptosis through regulation of the STAT3 signaling pathway and the role of STAT3 in this process, have not been systematically studied in glioma models. Existing evidence is mainly based on correlative observations, lacking direct validation through gain-of-function and loss-of-function experiments. Based on this background, we hypothesized

that BMSCs may promote glioma cell apoptosis by regulating STAT3 phosphorylation levels in glioma cells, thereby affecting the expression of downstream apoptosis-related genes. To test this hypothesis, we established a C6 glioma rat model, performed BMSC transplantation, examined tumor growth, apoptosis and changes in key molecules of the STAT3 pathway and used the STAT3-specific agonist Colivelin for functional validation. This study aims to elucidate the molecular mechanism by which BMSCs regulate apoptosis in glioma cells and provide experimental evidence for STAT3-targeted glioma therapy.

MATERIALS AND METHODS

Reagents and apparatus

BMSCs (China Sangong Bioengineering Co., LTD); MS-275 (China Shanghai Sinopharm Group Co. LTD); STAT3 pathway agonist (Beijing Jiuqiang Company, China); glioma C6 cells (American BioLegend Company); hematoxylin, eosin, anhydrous alcohol solution (I and II) and xylene (I and II) (China Tianjin Fuchen Chemical Reagent Factory); glial fibrillary acidic protein (GFAP) antibody (American HyClone Company); optical microscope (Orion Company, Finland); rabbit anti-STAT3 monoclonal antibody (Cell Signaling Technology, USA, Cat# 9139S, dilution 1:1000); rabbit anti-phosphorylated STAT3 (p-STAT3) (Tyr705) monoclonal antibody (Cell Signaling Technology, USA, Cat# 9145S, dilution 1:1000); rabbit anti-GFAP polyclonal antibody (Abcam, UK, Cat# ab7260, dilution 1:800); rabbit anti-Ki-67 monoclonal antibody (Abcam, UK, Cat# ab16667, dilution 1:200); and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Proteintech, China, Cat# 60004-1-Ig, dilution 1:5000). All primary antibodies were incubated overnight at 4°C. Secondary antibodies included horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Proteintech, China, Cat# SA00001-2, dilution 1:5000) and HRP-conjugated goat anti-mouse IgG (Proteintech, China, Cat# SA00001-1, dilution 1:5000), both incubated at room temperature for 1 hour.

Experimental animals and grouping

Fifty male Sprague-Dawley (SD) rats (aged 8–10 weeks, weighing 250–300 g) were randomly divided into 5 groups (n=10 per group). The randomisation sequence was generated using a computer-generated random number table (Excel RAND function) before the start of the experiment. Group allocation was sequentially assigned to animals according to the pre-generated random list as animals became available for enrolment. The sample size of 10 rats per group was determined based on a power analysis using GPower software (version 3.1.9.7) for a one-way ANOVA with five groups. The calculation was performed using parameters derived from a pilot experiment (n=3 per group) measuring the

primary outcome of tumor area: an effect size (Cohen's f) of 0.49, a significance level (α) of 0.05, and a desired statistical power ($1-\beta$) of 0.85. This analysis indicated that a minimum of 8 animals per group would be required to detect a significant difference between groups. To account for potential dropouts, including the humane endpoint criteria, we included 10 animals per group, resulting in a 20% buffer above the calculated minimum. (1) *Control group*: received 0.5 mL normal saline via the tail vein on days 14 and 17 after modeling; (2) *Model group*: received 0.5 mL normal saline via the tail vein on days 14 and 17 after modeling; (3) *BMSC group*: received BMSC suspension (5×10^6 cells/time, dissolved in 0.5 mL normal saline) via the tail vein on days 14 and 17 after modeling, with a total of 1×10^6 cells from two injections; (4) *Positive control group*: received MS-275 (Entinostat, Sigma, USA, Cat# SML1960) via the tail vein at 5 mg/kg/d for 7 consecutive days starting from day 14 after modeling, dissolved in sterile 0.9% normal saline. MS-275 is a selective class I histone deacetylase (HDAC) inhibitor that has been shown to induce apoptosis, inhibit tumor growth (Parveen *et al.*, 2023) and affect STAT3 acetylation levels (Zhong *et al.*, 2023), thus serving as the positive control drug in this study. Regarding safety, literature reports that a single intravenous injection of 50 mg/kg MS-275 in rats is safe. In this study, the cumulative dose of 5 mg/kg/d $\times$ 7 days was 35 mg/kg, below this safety limit, further ensuring the safety of the dosing regimen (Niu *et al.*, 2023); (5) *Pathway agonist group*: received the STAT3-specific agonist Colivelin (Bvant, China, Cat# BE-10121) via intraperitoneal injection at 1 mg/kg/d for 7 consecutive days starting from day 14 after modeling, dissolved in sterile 0.9% normal saline. This dose was selected based on previous studies showing that Colivelin at this dose effectively activates the JAK2/STAT3 pathway without significant toxicity (Yang *et al.*, 2025). Colivelin is a synthetic neuroprotective peptide that activates the JAK2/STAT3 signaling pathway by binding to the glycoprotein 130 (gp130) receptor complex, inducing STAT3 phosphorylation and nuclear translocation and has been widely used to activate STAT3 signaling in glioma cell studies (Tang *et al.*, 2024). All rats were obtained from the Laboratory Animal Center of Zhejiang University (Hangzhou, China) and were specific-pathogen-free (SPF) grade. Housing and husbandry conditions: All animals were housed in the same animal facility under controlled environmental conditions. Rats were group-housed (5 per cage) in standard polycarbonate cages ($46 \times 27 \times 20$ cm) with stainless steel wire lids. Bedding consisted of autoclaved corncob bedding (thickness approximately 2–3 cm), which was changed twice weekly. The animal room was maintained at a temperature of $22 \pm 2^\circ\text{C}$, with relative humidity of $50 \pm 10\%$, and a 12/12 h light/dark cycle (lights on at 7:00 AM, off at 7:00 PM). Environmental enrichment was provided in the form of polycarbonate tunnels and wooden chew blocks within each cage.

Standard rodent chow and autoclaved drinking water were provided ad libitum.

Acclimatization: Upon arrival from the vendor, animals were subjected to a 7-day acclimatization and quarantine period in the animal facility before any experimental procedures. During this period, they were monitored daily for general appearance, activity, food and water intake, and signs of illness; only healthy animals without abnormalities were included in the study. No experimental interventions or procedures were performed during the acclimatization period. Cage positions were rotated weekly to minimise any potential position effects within the animal facility.

Postoperative housing: Following stereotaxic surgery, rats were placed on a constant temperature heating pad (37°C) until recovery from anaesthesia, then housed individually for 24 hours in clean cages with the same bedding and environmental conditions as described above to allow close observation. After the 24-hour observation period, animals were returned to their original group-housing cages (5 rats per cage).

A total of 50 rats were initially enrolled and randomly assigned to five groups ($n=10$ per group). All 50 animals were included in body weight monitoring and MRI-based tumor volume assessment. For subsequent histological, immunohistochemical, flow cytometric, RT-PCR and Western blot analyses, 5 animals per group were randomly selected from the surviving animals in each group. During the experiment, 2 rats in the model group and 1 rat in the pathway agonist group reached humane endpoints and were euthanized before the scheduled end of the study; these animals were excluded from tissue-based analyses but their baseline data were retained. No other animals were excluded.

To minimize potential confounding factors, the following measures were implemented: (1) all animals were housed in the same room under identical environmental conditions (temperature $22 \pm 2^\circ\text{C}$, humidity $50 \pm 10\%$, 12/12 h light/dark cycle) to eliminate housing location bias; (2) treatments (BMSC injection, MS-275, Colivelin, or normal saline) were administered in a randomised order across groups on each treatment day to avoid time-of-day effects; (3) all outcome measurements (MRI, tissue collection, histological staining, and molecular assays) were performed in a randomised sequence across groups, with samples from different groups processed interchangeably to prevent batch effects; and (4) cage positions were rotated weekly to minimise any potential position effects within the animal facility.

Rationale for multiple BMSC administrations: Ding K *et al.*, demonstrated in an ultra-high molecular weight polyethylene (UHMWPE) particle-induced mouse

calvarial osteolysis model that two BMSC injections resulted in more pronounced preservation of trabecular bone structure, while a single injection only slightly reduced bone resorption, confirming the advantage of multiple dosing. The license key was SCXK (Hu)2003-0003.

BMSC culture and preparation

BMSCs were purchased from China Sangong Bioengineering Co., Ltd. (Shanghai, China). Cells were expanded in low-glucose Dulbecco's Modified Eagle Medium (DMEM) (HyClone, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (HyClone, USA) in a 37°C, 5% CO₂ incubator. The medium was changed every 2-3 days. When cells reached 80-90% confluence, they were passaged using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, USA). All experiments used passages 3 to 5 to ensure phenotypic stability.

BMSC characterization

To confirm stem cell identity, flow cytometry was performed on passage 3 BMSCs. Passage 3 BMSCs were digested with 0.25% trypsin-EDTA, washed twice with phosphate-buffered saline (PBS) and adjusted to a concentration of 1×10^6 cells/mL. 100 μ L of cell suspension was incubated with fluorescein isothiocyanate (FITC)-conjugated CD29, CD90, CD105 antibodies (positive markers) and phycoerythrin (PE)-conjugated CD34, CD45 antibodies (negative markers) (all from BioLegend, USA) for 30 min at 4°C in the dark. After PBS washing, cells were resuspended in 500 μ L PBS and analyzed using a flow cytometer (BD FACSCalibur, USA), collecting 10,000 events per sample. Data were analyzed using FlowJo software, with isotype control antibodies used as negative controls to set positive thresholds. Cell identification criteria were: CD29⁺/CD90⁺/CD105⁺ positivity >95%, CD34⁺/CD45⁺ positivity <2%.

Multilineage differentiation potential: Passage 3 BMSCs were seeded in 6-well plates at 1×10^5 cells/well and cultured in osteogenic, adipogenic and chondrogenic induction media (Cyagen, China) according to the manufacturer's instructions for induction and staining. Osteogenic induction: After 21 days of induction, cells were fixed with 4% paraformaldehyde and Alizarin Red S staining was used to detect calcium nodule formation. Adipogenic induction: After 14 days of induction, cells were fixed with 4% paraformaldehyde and Oil Red O staining was used to detect lipid droplet formation. Stained cells were observed and photographed using an optical microscope (Olympus IX73, Japan).

Glioma model establishment (Lan et al., 2020)

Rats were anesthetized with isoflurane inhalation (induction concentration 5%, maintenance concentration

2%, oxygen flow rate 0.5 L/min, R500 small animal anesthesia machine, Shenzhen RWD Life Science Co., China) and fixed in a stereotaxic apparatus (68001, Shenzhen RWD Life Science Co., China). The surgical area was shaved and disinfected, a 1.5 cm midline incision was made and the bregma was exposed. According to the Paxinos-Watson rat brain stereotaxic atlas, a burr hole was drilled 1.0 mm anterior to the bregma and 3.0 mm right of the sagittal suture using a dental drill (STRONG 90, Korea). Using a microinjector (7002KH, Hamilton, USA), the needle was vertically inserted to 6.0 mm below the dura, then withdrawn 1.0 mm (final depth 5.0 mm below the dura). 10 μ L of C6 cell suspension (containing 1×10^6 cells) was slowly injected at a rate of 1 μ L/min. After injection, the needle was left in place for 3 min to prevent backflow of the cell suspension, then slowly withdrawn. The burr hole was sealed with bone wax and the scalp was sutured. After surgery, rats were placed on a constant temperature heating pad (37°C) until recovery from anaesthesia, then housed individually for 24 hours with close observation, as described in the 'Housing and husbandry conditions' section above.

Postoperative analgesia and care: All surgically treated rats received immediate subcutaneous injection of meloxicam (Metacam, Boehringer Ingelheim, Germany, 2 mg/kg) for analgesia, with additional doses at 24 and 48 hours post-surgery. The surgical incision was checked daily for 3 consecutive days for signs of infection (redness, swelling, exudate) and mupirocin ointment (Sino-American SmithKline, China) was applied topically if infection was suspected. Rats were observed daily for mental status, activity, food and water intake and body weight changes. On day 14 after injection, all rats underwent cranial magnetic resonance imaging (MRI) to confirm tumor formation. A 3.0T MRI scanner (Siemens Trio) was used to acquire T2-weighted images (T2WI) and T1-weighted images (T1WI) with and without contrast enhancement. **Scan parameters:** slice thickness 1.0 mm, interslice gap 0.5 mm, field of view (FOV) 4 cm $\times$ 4 cm, matrix 256 $\times$ 256. Tumor volume was calculated from T2WI images using the modified ellipsoid formula: Tumor volume (mm^3) = $0.5 \times \text{long diameter (mm)} \times \text{short diameter (mm)}^2$. MRI showing well-defined space-occupying lesions with hyperintensity on T2WI was considered successful modeling. The modeling success rate was 100% (40/40).

Humane endpoint criteria were set as: (1) weight loss exceeding 20% of baseline body weight; (2) persistent epileptic seizures or hind limb paralysis; (3) inability to eat or drink independently for more than 24 hours; (4) moribund state (unresponsive to external stimuli). Rats reaching humane endpoints were immediately euthanized with an overdose of sodium pentobarbital (150 mg/kg, intraperitoneal injection) and recorded as experimental

endpoints. In this study, 2 rats in the model group reached humane endpoints on day 18 and 1 rat in the pathway agonist group reached humane endpoints on day 19; no rats in other groups reached humane endpoints.

Animal euthanasia and sample collection: On day 21 after modeling, all rats were anesthetized with isoflurane inhalation (induction concentration 5%, maintenance concentration 3%) and then euthanized with an overdose of sodium pentobarbital (150 mg/kg, intraperitoneal injection). After confirming death (cessation of breathing and heartbeat, loss of pupillary light reflex), the thoracic cavity was quickly opened and cardiac perfusion was performed with pre-cooled normal saline via the left ventricle until the effluent was clear. The whole brain was rapidly removed and placed on ice. The brain was cut into 2 mm thick coronal slices and tumor areas were isolated. One portion was fixed in 4% paraformaldehyde for 48 hours for histological examination and another portion was immediately snap-frozen in liquid nitrogen and transferred to a -80°C freezer for molecular biology analysis.

All animal experiments in this study were approved by the Animal Ethics Committee of Affiliated Jinhua Hospital (Approval No.: AL-JHYY2025) and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). All surgeries were performed under isoflurane anesthesia to minimize animal suffering. During the experiment, rats were observed daily for body weight, activity, food and water intake and neurological deficit symptoms.

Brain histopathological examination

After euthanasia, the whole brain was rapidly removed and fixed in 4% paraformaldehyde for 48 h. Gradient ethanol dehydration, xylene clearance and paraffin embedding were performed. The embedded tissues were sectioned into 5 µm thick coronal slices and mounted on slides. Hematoxylin and eosin (HE) staining: Sections were routinely deparaffinized and hydrated, stained with hematoxylin for 5 min, differentiated with 1% hydrochloric acid in ethanol, blued under running water for 15 min, stained with eosin for 3 min, dehydrated through graded ethanol, cleared in xylene and mounted with neutral balsam. Tumor morphological changes, including tumor border, cell density, nuclear atypia, necrotic areas and vascular proliferation, were observed under a light microscope.

Tumor area measurement: Three non-consecutive sections containing the largest tumor cross-section were selected from each rat. Whole slide images were captured using a digital slide scanner (Pannoramic MIDI, 3DHISTECH, Hungary). Tumor boundaries were outlined using ImageJ software (NIH, USA) and the tumor cross-sectional area

(mm²) was calculated. The average value was used for statistical analysis.

Immunohistochemical staining

Paraffin sections were routinely deparaffinized and hydrated, then subjected to microwave antigen retrieval in citrate buffer (pH 6.0, ZSGB-Bio, China, Cat# ZLI-9064) at 98°C for 15 min. After natural cooling, endogenous peroxidase was blocked with 3% hydrogen peroxide at room temperature for 10 min, followed by blocking with 5% goat serum for 30 min. The following primary antibodies were applied: rabbit anti-GFAP polyclonal antibody (Abcam, UK, Cat# ab7260, dilution 1:800) and rabbit anti-Ki-67 monoclonal antibody (Abcam, UK, Cat# ab16667, dilution 1:200), incubated overnight at 4°C. The next day, HRP-conjugated goat anti-rabbit secondary antibody (Proteintech, China, Cat# SA00001-2, dilution 1:5000) was applied and incubated at 37°C for 30 min. 3,3'-Diaminobenzidine (DAB) chromogen (ZSGB-Bio, China, Cat# ZLI-9017) was applied for 3 min, followed by hematoxylin counterstaining for 30 sec, dehydration, clearing and mounting.

GFAP-positive area analysis: Five high-power fields (400×) were randomly selected from each section and the percentage of positive area was analyzed using ImageJ software. **Ki-67 proliferation index analysis:** Five high-power fields (400×) were randomly selected from each section and the percentage of Ki-67 positive nuclei relative to total nuclei was calculated.

RT-PCR

RNA extraction: Approximately 50 mg of tumor tissue from the brain of each group was collected and total ribonucleic acid (RNA) was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA purity was assessed using a NanoDrop 2000 spectrophotometer, with A260/A280 ratios between 1.8 and 2.0 considered acceptable. **Reverse transcription:** 1 µg of total RNA was reverse transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan, Cat# RR047A) under the following conditions: 37°C for 15 min, 85°C for 5 sec. The resulting complementary DNA (cDNA) was stored at -20°C until use. **Primer sequences:** Primers for target genes bFGF and VEGF and the internal control gene β-actin were synthesized by Sangon Biotech (Shanghai, China); sequences are shown in table 1. **PCR reaction:** Amplification was performed using SYBR Premix Ex Taq II (TaKaRa, Japan, Cat# RR820A) on a LightCycler 480 II real-time PCR system (Roche, Switzerland). The 20 µL reaction system contained: SYBR Premix 10 µL, forward and reverse primers 0.4 µL each (final concentration 200 nM), cDNA template 2 µL and diethyl pyrocarbonate (DEPC)-treated water added to 20 µL. Reaction conditions: 95°C for 30 sec pre-denaturation; 95°C for 5 sec, 60°C for 30 sec, for 40 cycles.

Table 1: Primer sequences.

primer	primer sequence		Primer length(bp)
β-actin	ascend	5'-CCTTAGTGGGGCACCTGTC-3'	18
	down	5'-TGAGCTGCTGTATGCTTGTG-3'	
VEGF	ascend	5'-ACTTGGGCTATTAACCTGGG-3'	23
	down	5'-TCCAGTCTCCATCCCCTCAG-3'	
bFGF	ascend	5'-GGAACCTTCCTGACCTGCCTC-3'	22
	down	5'-CCGCCATTGTAAGCCGAAA-3'	

Melting curve analysis (65°C to 95°C, fluorescence acquisition every 0.5°C) confirmed amplification specificity. The mRNA detection of bFGF and VEGF in this study was based on the following pre-specified scientific hypothesis: the STAT3 signaling pathway regulates angiogenic factor expression and BMSCs may affect the expression of these factors by modulating STAT3 activity. Therefore, this assay was a pre-specified validation analysis rather than exploratory data mining. All test indicators, group comparisons and statistical methods were determined before the experiment began.

Data analysis: Three replicate wells were set for each sample and the average cycle threshold (Ct) value was used. The relative expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method, with β-actin as the internal reference gene for normalization. The target gene expression level in the control group was set to 1.00 and other groups were expressed as fold changes relative to the control group. All experiments were repeated three times and the average values were used for statistical analysis.

Flow cytometry

Fresh brain tissue from tumor areas was collected and single-cell suspensions were prepared by digestion with 0.25% trypsin. Apoptosis was detected using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Dojindo, Japan, Cat# AD10) according to the manufacturer's instructions. 1×10^5 cells were resuspended in 500 μL binding buffer and 5 μL Annexin V-FITC and 5 μL PI were added, followed by incubation at room temperature in the dark for 15 min. Cells were analyzed using a flow cytometer (BD FACSCalibur, USA), collecting 10,000 events per sample. Apoptosis rates were analyzed using CellQuest software.

Western blot assay

Approximately 50 mg of rat brain tumor tissue was collected, lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (containing protease and phosphatase inhibitors) on ice for 30 min, centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was collected. Protein concentration was determined by bicinchoninic acid (BCA) assay. 30 μg of total protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore,

USA, Cat# IPVH00010). After blocking with 5% skim milk powder at room temperature for 1 h, primary antibodies (STAT3 and p-STAT3 diluted 1:50, GAPDH diluted 1:1000) were added and incubated overnight at 4°C. Then, secondary antibody (goat anti-rabbit diluted 1:200) was added and incubated at 37°C for 1 h. After washing with PBS, membranes were developed with enhanced chemiluminescence (ECL) reagent and images were captured. ImageJ software (NIH, USA) was used for densitometric analysis of Western blot bands. The gray values of target proteins (p-STAT3, STAT3) were compared to the gray value of the internal control GAPDH to obtain relative expression levels. Using the control group as a reference (set to 1.00), fold changes for each group were calculated. The p-STAT3/STAT3 ratio was further calculated to reflect STAT3 phosphorylation levels. Each experiment was repeated three times with three replicate wells per experiment and the average values were used for statistical analysis.

In-vitro STAT3 inhibition experiment

Logarithmically growing C6 rat glioma cells were digested with 0.25% trypsin, seeded in 6-well plates at a density of 2×10^5 cells/well and cultured overnight in DMEM containing 10% FBS to allow cell attachment. The next day, the medium was discarded, cells were washed once with PBS and treatments were added according to the following groups (total volume 2 mL per well): (1) Control group: regular DMEM (containing 10% FBS); (2) BMSC-conditioned medium (BMSC-CM) group: BMSC-CM (diluted 1:1 with fresh serum-free DMEM); (3) BMSC-CM + STAT3 inhibitor group: BMSC-CM (1:1 dilution) supplemented with the STAT3 inhibitor Stattic (Sigma, USA, Cat# SML1960) at a final concentration of 5 μM; (4) STAT3 inhibitor group: regular DMEM (containing 10% FBS) supplemented with Stattic at a final concentration of 5 μM. The dose of Stattic was selected based on literature reports showing effective inhibition of STAT3 phosphorylation without significant cytotoxicity (Feng *et al.*, 2025). Three replicate wells were set for each group and the experiment was repeated three times. After 24 h of treatment, cells were collected for subsequent assays. Treated C6 cells were lysed in RIPA lysis buffer (containing protease and phosphatase inhibitors) on ice for 30 min, centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was collected. Protein concentration was determined by BCA assay. 30 μg of total protein was subjected to SDS-PAGE and

transferred to PVDF membranes. After blocking with 5% skim milk powder at room temperature for 1 h, membranes were incubated with rabbit anti-rat p-STAT3 antibody (1:1000, CST, USA, Cat# 9145) and rabbit anti-rat STAT3 antibody (1:1000, CST, USA, Cat# 9139) overnight at 4°C. The next day, HRP-conjugated goat anti-rabbit secondary antibody (1:5000, ZSGB-Bio, China) was added and incubated at room temperature for 1 h. Chemiluminescence was developed using ECL reagent and gray value analysis was performed using ImageJ software with GAPDH as an internal control.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for apoptosis detection: Apoptosis in C6 cells was detected using the TUNEL method. C6 cells were seeded in 24-well plates (containing cell slides) at a density of 1×10^5 cells/well, allowed to adhere overnight and treated according to the groups in 2.11 for 24 h. The medium was discarded, cells were washed once with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. After three PBS washes (5 min each), cells were permeabilized with 0.1% Triton X-100 in sodium citrate buffer on ice for 2 min. After three PBS washes, 50 μ L of TUNEL reaction mixture (enzyme solution and labeling solution mixed at 1:9, Roche, Switzerland, Cat# 11684817910) was added to each well and incubated at 37°C in the dark for 60 min. After three PBS washes, 4',6-diamidino-2-phenylindole (DAPI) staining solution (1 μ g/mL) was added and incubated at room temperature in the dark for 5 min to stain nuclei. After PBS washing, slides were mounted with anti-fade mounting medium. Observation and photography were performed using a fluorescence microscope (Olympus IX73, Japan). Five high-power fields (200 $\times$) were randomly selected from each slide and the total numbers of TUNEL-positive cells (green fluorescence) and DAPI-positive cells (blue fluorescence) were counted. The apoptosis index (%) was calculated as (number of TUNEL-positive cells/total number of DAPI-positive cells) $\times$ 100%. Three replicate wells were set for each group and the experiment was repeated three times.

Observation indexes

Primary outcome measure: The primary outcome measure for hypothesis testing and sample size determination was the anti-tumor efficacy of BMSCs, assessed quantitatively by the tumor area measured from HE-stained sections. This outcome was chosen as the primary endpoint because it directly reflects tumour growth inhibition, which is the most clinically relevant measure of therapeutic efficacy in the glioma model.

Secondary outcome measures: Secondary outcome measures included: (1) tumor cell apoptosis, assessed by TUNEL staining and flow cytometry; (2) tumor cell proliferation, assessed by the Ki-67 proliferation index; (3) STAT3 pathway activity, assessed by the ratio of p-

STAT3/STAT3 protein expression (Western blotting); (4) paracrine activity, assessed by the mRNA expression of bFGF and VEGF (RT-PCR); and (5) GFAP expression (immunohistochemistry). Additional supportive parameters evaluated were body weight changes and MRI-based tumor volume. The sample size calculation was based on the primary outcome measure (tumor area), as described in the 'Experimental animals and grouping' section.

Blinding

The group allocation sequence was generated and concealed by an independent investigator who was not involved in animal handling, data collection, or outcome assessment. The investigator responsible for administering treatments (BMSC suspension, MS-275, Colivelin, or normal saline) was blinded to the group allocation; syringes were prepared by the independent investigator and labelled only with animal numbers. The experimenters performing outcome assessments (MRI volumetry, histological scoring, TUNEL counting, flow cytometry, RT-PCR, and Western blot analysis) were blinded to treatment groups until all analyses were completed. Samples were coded with numerical identifiers, and the code was broken only after data analysis was finalized and the dataset was locked. The statistician received only coded datasets without group identifiers until the final analysis was complete. Complete blinding of the surgeon performing the stereotaxic implantation of C6 cells was not feasible due to the nature of the surgical procedure; however, the surgeon had no role in subsequent treatment allocation or outcome assessment.

Statistical analysis

SPSS 26.0 software was used for statistical analysis and graphs were generated using GraphPad Prism 9.0 software. Prior to using parametric tests, the assumption of normality was assessed for all continuous data (body weight, tumor area, Ki-67 index, apoptosis rate, p-STAT3/STAT3 ratio, GFAP-positive area, and mRNA expression levels) using the Shapiro-Wilk test. Additionally, the assumption of homogeneity of variances was evaluated using Levene's test. All data met these assumptions ($P > 0.05$ for both tests), confirming the appropriateness of one-way ANOVA for analysis. Although all data in this study satisfied these assumptions, we pre-specified the following alternative procedures in the event that assumptions were not met: if normality was violated, non-parametric tests (Kruskal-Wallis test for multiple-group comparisons, followed by Mann-Whitney U test with Bonferroni correction for pairwise comparisons) would have been applied; if homogeneity of variance was violated, Welch's ANOVA with Games-Howell post-hoc test would have been used. These alternative procedures were specified in the statistical analysis plan before data analysis commenced.

Table 2: Rat body weight (mean $\pm$ SD, grams).

Grouping	Cases	1d	7d	14d	21d
control group	5	225.4 $\pm$ 12.3	305.4 $\pm$ 11.7	335.4 $\pm$ 10.9	368.4 $\pm$ 10.7
model group	5	223.3 $\pm$ 12.2	243.1 $\pm$ 11.8	273.1 $\pm$ 11.7	303.2 $\pm$ 11.5
BMSC group	5	226.4 $\pm$ 12.4	266.8 $\pm$ 12.2	316.8 $\pm$ 12.1	346.8 $\pm$ 12.3
positive control group	5	224.5 $\pm$ 12.2	264.5 $\pm$ 11.5	314.5 $\pm$ 11.9	344.5 $\pm$ 12.6
pathway agonist group	5	225.6 $\pm$ 12.5	241.6 $\pm$ 12.6	268.6 $\pm$ 11.6	298.6 $\pm$ 11.9
F/P	-	0.102/0.922	8.297/<0.001	9.384/<0.001	9.753/<0.001
<i>P</i> _{control group compared with model group}	-	0.443	<0.001	<0.001	<0.001
<i>P</i> _{control group compared with BMSCgroup}	-	0.867	<0.001	<0.001	<0.001
<i>P</i> _{control group compared with positive control group}	-	0.285	<0.001	<0.001	<0.001
<i>P</i> _{control group compared with pathway agonist group}	-	0.762	<0.001	<0.001	<0.001
<i>P</i> _{model group compared with BMSC group pathway agonist group}	-	0.872	<0.001	<0.001	<0.001
<i>P</i> _{model group compared with positive control group}	-	0.555	<0.001	<0.001	<0.001
<i>P</i> _{model group compared with pathway agonist group}	-	0.972	0.440	0.069	0.941
<i>P</i> _{BMSCgroup compared with positive control group}	-	0.968	0.389	0.483	0.915
<i>P</i> _{BMSCgroup compared with pathway agonist group}	-	0.478	<0.001	<0.001	<0.001
<i>P</i> _{positive control group compared with pathway agonist group}	-	0.138	<0.001	<0.001	<0.001

Note: The 5 cases per group listed in the table represent the animals selected for subsequent histopathological and molecular analyses at day 21; body weight data from all 10 animals per group were used for statistical analysis at each time point.

All data are expressed as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparisons among multiple groups. If significant differences were found, Dunnett's test was used for comparisons with the control group, or Tukey's honestly significant difference (HSD) test was used for multiple comparisons. Western blotting experiments were repeated three times independently and histological experiments included 5 rats per group, with 3 sections and 5 fields analyzed per rat. Bonferroni correction was applied to control for type I errors in multiple comparisons. $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered highly significant. Effect sizes for pairwise comparisons are reported as Hedges' g to correct for small sample bias. For comparisons involving three or more groups, eta-squared (η^2) is reported for the overall ANOVA effect. Data in the text and figures are presented with 95% confidence intervals (CIs) for key parameters where applicable, in addition to the mean $\pm$ SD. Symbols in figures indicate: * $P < 0.05$, ** $P < 0.01$ vs. the control group; # $P < 0.05$, ### $P < 0.01$ vs. the model group (or BMSC-CM group); ns, not significant. To minimize potential confounding factors, all rats were randomly assigned to experimental groups using a computer-generated random number sequence before the start of the experiment. Baseline characteristics, such as body weight and initial tumor volume, were confirmed to be comparable across all groups ($P > 0.05$). The BMSCs used for transplantation were from a standardized, characterized passage number (P3-P5) to ensure consistency in cell quality and phenotype. All animals within a given experimental batch were housed, handled, and fed identically to control for environmental variables. Blinding procedures are described in the 'Blinding' section above; the statistician received only coded datasets

without group identifiers until the final analysis was complete.

RESULTS

Characterization of BMSCs

To confirm the stem cell properties of the cells used in this study, flow cytometry was performed on passage 3 BMSCs. The results showed (Fig. 1A) that the cells highly expressed the mesenchymal stem cell positive markers CD29 (99.96%), CD90 (99.94%) and CD105 (99.97%), while barely expressing the hematopoietic stem cell markers CD34 (0.52%) and CD45 (0.43%). Differentiation experiments showed (Fig. 1B) that under osteogenic induction conditions, BMSCs showed abundant red mineralized nodules with Alizarin Red S staining; under adipogenic induction conditions, BMSCs showed abundant red lipid droplets with Oil Red O staining. These results confirm that the cells used in this study possess a typical BMSC immunophenotype and functional characteristics and can be used for subsequent animal experiments.

Successful establishment of glioma model and baseline assessment

Body weight changes during the experiment are summarized in table 2. At baseline (day 1), no significant differences in body weight were observed among all groups ($P > 0.05$). From day 7 to day 21, the control group showed the highest body weight gain. The model group exhibited a marked decrease in weight gain compared with the control group ($P < 0.001$). Both the BMSC group and the positive control group showed significantly improved body weight compared with the model group ($P < 0.001$).

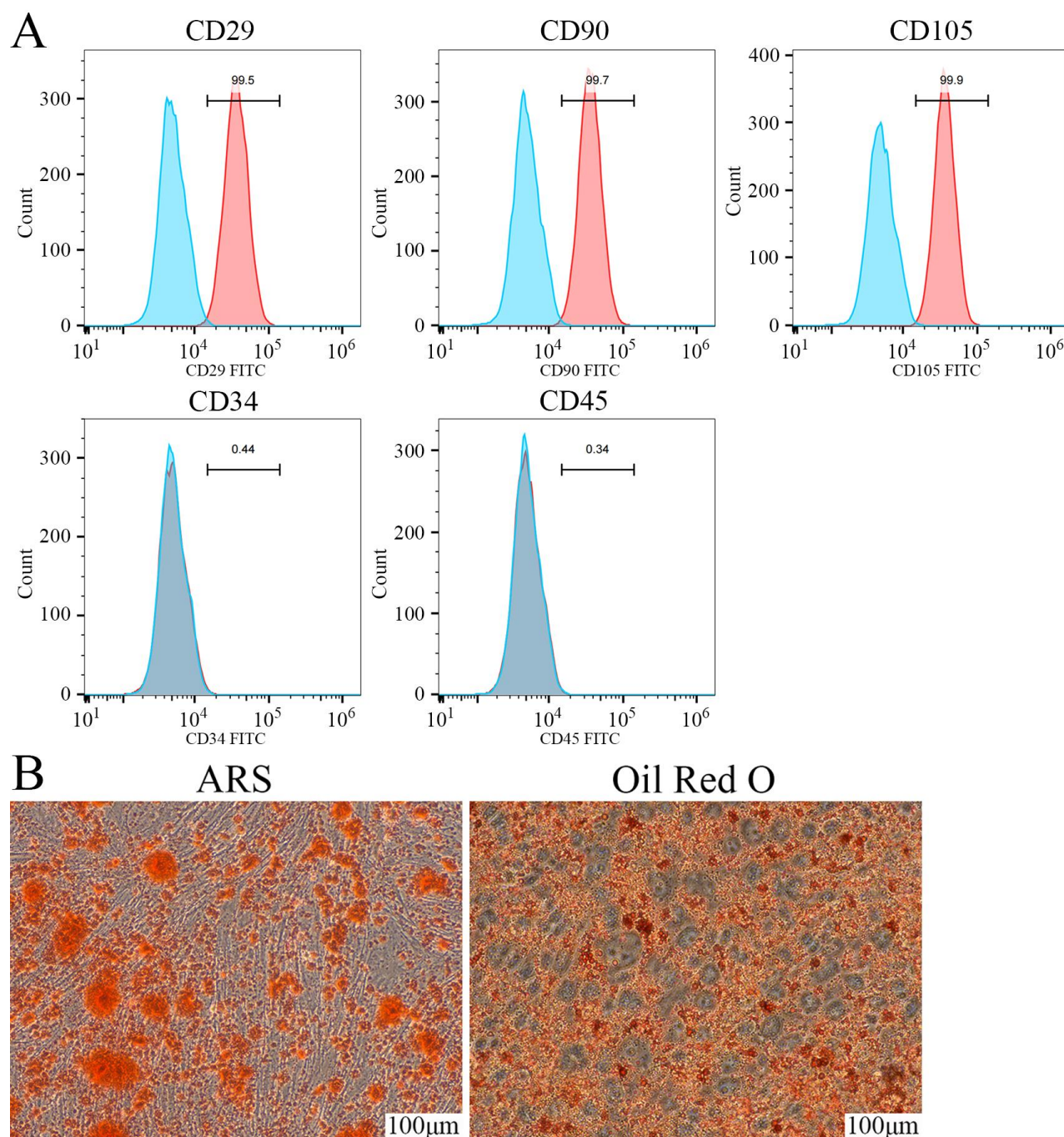


Fig. 1: Characterization of rat BMSCs

(A) Flow cytometric analysis of BMSC surface marker expression. Cells showed high expression of CD29, CD90, and CD105 and low expression of CD34 and CD45. Multilineage differentiation potential of BMSCs. Alizarin Red S staining was positive after osteogenic induction; Oil Red O staining was positive after adipogenic induction. Scale bar = 100 μ m.

However, the STAT3 pathway agonist group showed no significant improvement in body weight compared with the model group ($P > 0.05$ at day 21). On day 14 after C6 cell injection, MRI T2-weighted imaging showed well-defined round or oval hyperintense lesions in the right striatum/cortical region of all groups except the control group, confirming the successful establishment of the

glioma model (Fig. 2). Tumor morphology and signal characteristics were similar across groups, with representative images from the model group shown. Initial tumor volumes (day 14) calculated from MRI images showed no significant differences among groups ($P > 0.05$), ensuring baseline consistency for subsequent intervention experiments.

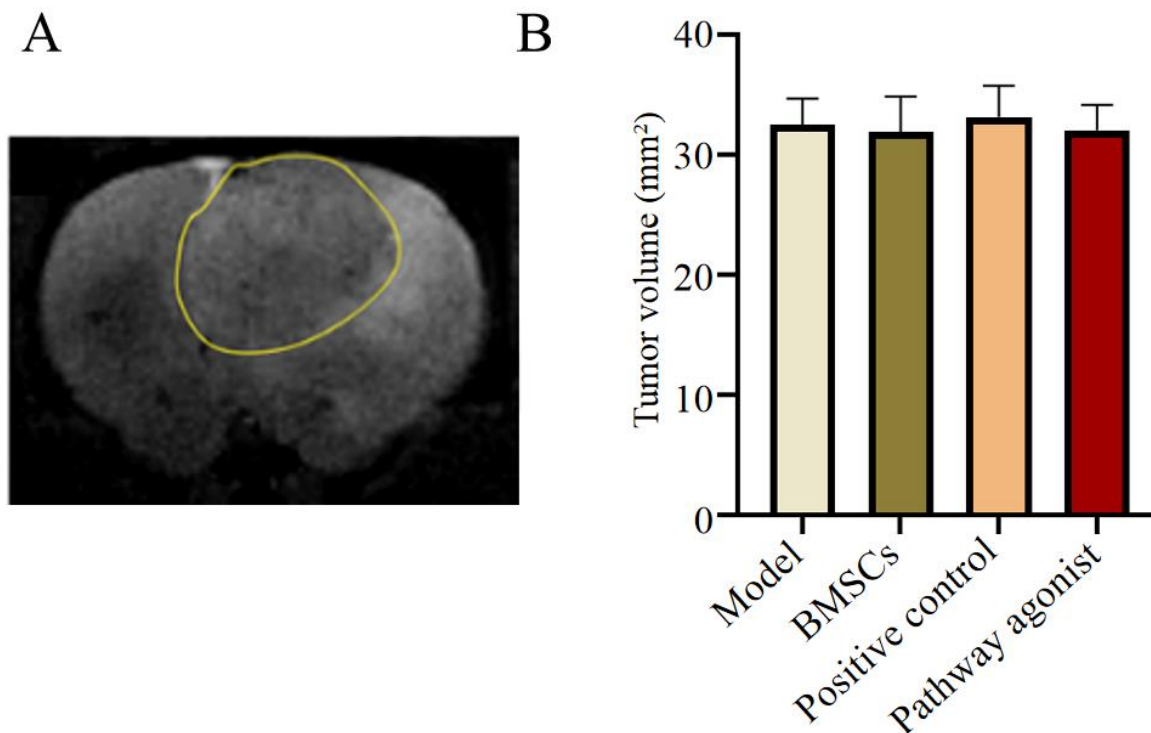


Fig. 2: MRI validation of the glioma model

(A) Representative coronal T2-weighted MRI images of rat brains on day 14 after C6 cell injection. Well-defined round or oval hyperintense lesions were observed in the right brain parenchyma of rats in the model, BMSC, positive control, and pathway agonist groups, confirming successful glioma implantation. Tumor morphology and signal characteristics were similar across groups; representative images from the model group are shown. No abnormalities were detected in the control group. Scale bar = 5 mm; (B) Quantitative analysis of initial tumor volume (day 14) in each group. Data are presented as the mean $\pm$ SD, $n=10$ per group. $P > 0.05$, no significant difference among groups (one-way ANOVA).

Tumor morphology and quantitative analysis of tumor area

HE staining showed clear brain tissue structure in the control group, with normal morphology and orderly arrangement of neurons and glial cells and intact vascular structures. In the model group, ill-defined glioma lesions were observed in the brain, with dense tumor cells, obvious nuclear atypia, an increased nuclear-to-cytoplasmic ratio, large necrotic areas (asterisks) within the lesions, thickened vascular walls, irregular lumens and lymphocytic infiltration around the lesions. The BMSC and positive control groups showed significantly reduced tumor lesions, markedly decreased necrotic areas, reduced tumor cell density, more orderly cell arrangement and reduced vascular proliferation. The pathway agonist group showed pathological features similar to the model group, with large necrotic areas and disorganized cellular structures (Fig. 3A). Quantitative analysis of the tumor area showed (Fig. 3B) that the tumor area in the model group was $32.5 \pm 4.2 \text{ mm}^2$. Compared with the model group, the tumor areas were significantly reduced in the BMSC group ($18.7 \pm 3.1 \text{ mm}^2$) and the positive control group ($17.9 \pm 3.5 \text{ mm}^2$) ($P < 0.01$); the pathway agonist group ($31.8 \pm 4.0 \text{ mm}^2$) showed no significant difference from the model group ($P > 0.05$).

Quantitative analysis of GFAP expression

GFAP immunohistochemical staining showed only a few GFAP-positive cells in the brain tissue of the control group, mainly distributed in the cytoplasm and processes of astrocytes, appearing as brown filamentous structures. In the model group, abundant GFAP-positive cells were observed within tumor areas, with diffuse brown positive signals, irregular cell morphology and thickened and elongated processes, indicating active proliferation of glioma cells. The BMSC and positive control groups showed significantly reduced GFAP-positive cells, with scattered positive signals and more normal cell morphology. The pathway agonist group showed GFAP expression patterns like the model group, with abundant brown positive cells (Fig. 4A). Quantitative analysis of the GFAP-positive area percentage showed (Fig. 4B) that the GFAP-positive area in the model group was $28.4\% \pm 3.2\%$. Compared with the model group, the GFAP-positive areas were significantly reduced in the BMSC group ($14.2\% \pm 2.5\%$) and the positive control group ($13.8\% \pm 2.8\%$) ($P < 0.01$); the pathway agonist group ($27.6\% \pm 3.5\%$) showed no significant difference from the model group ($P > 0.05$).

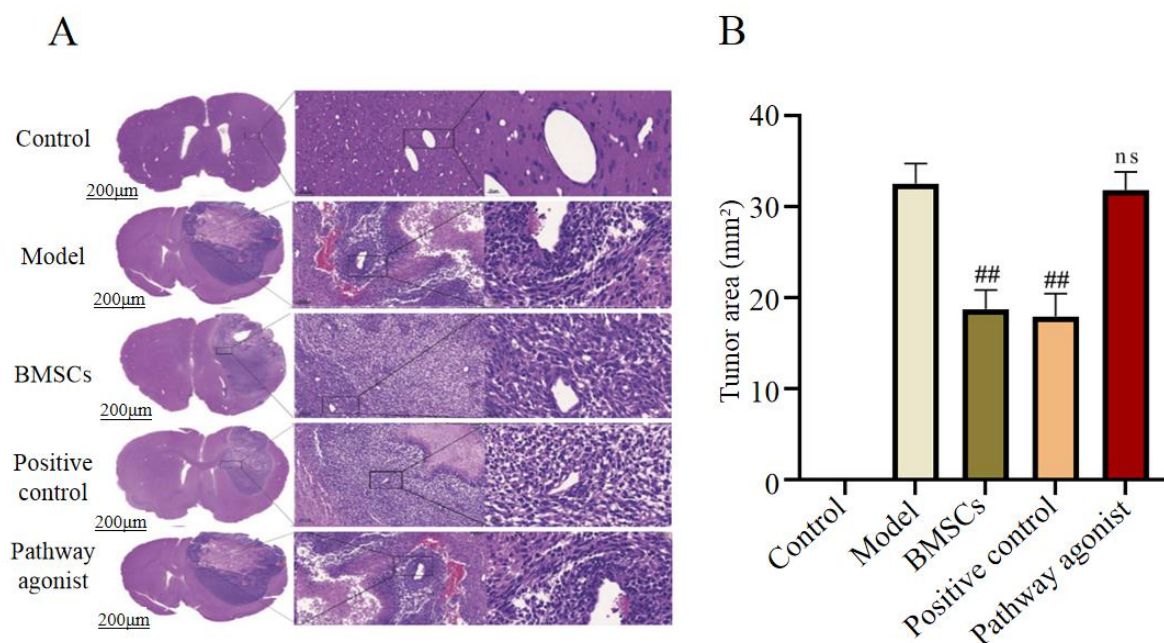


Fig. 3: Histopathological changes and quantitative analysis of tumor area in each group (A) Representative HE-stained images of brain tissue from each group. Black dashed lines outline tumor boundaries; asterisks indicate necrotic areas; arrows indicate vascular wall thickening. Scale bar = 500 μm; (B) Quantitative analysis of the tumor area. Data are presented as the mean ± SD, n=5 per group. ^{##} $P < 0.01$ vs. the model group; ns, not significant vs. the model group (one-way ANOVA followed by Dunnett's test).

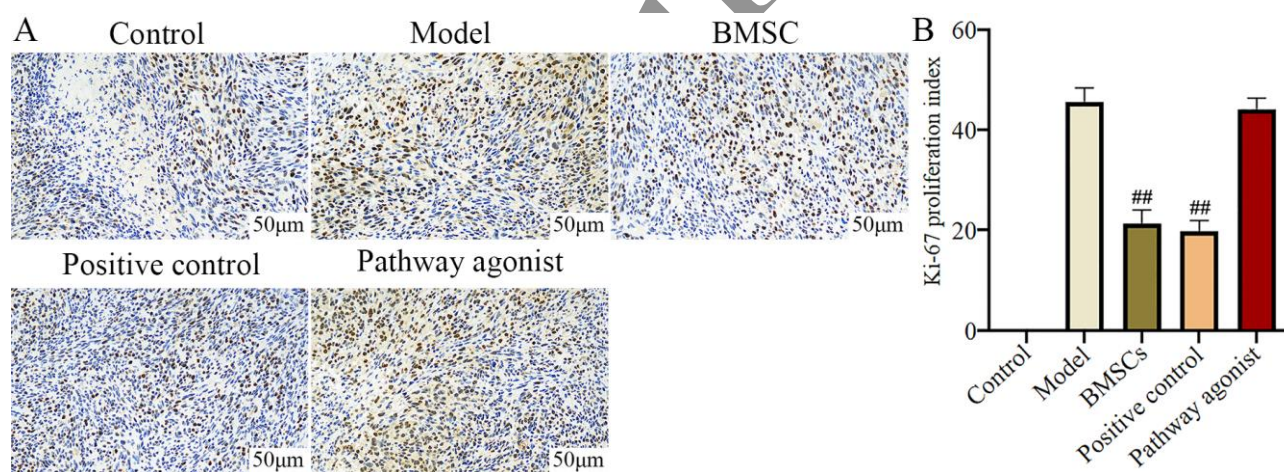


Fig. 4: GFAP expression and quantitative analysis in glioma tissues of each group (A) Representative immunohistochemical staining images of GFAP in brain tissue from each group. GFAP-positive cells appear brown. Scale bar = 100 μm; (B) Quantitative analysis of the GFAP-positive area percentage. Data are presented as the mean ± SD, n=5 per group. ^{##} $P < 0.01$ vs. the model group; ns, not significant vs. the model group (one-way ANOVA followed by Dunnett's test).

Quantitative analysis of tumor cell proliferation

Ki-67 immunohistochemical staining showed abundant brown granules in the nuclei of tumor cells in the model group, with diffuse high expression and dense distribution of positive cells, indicating active proliferation. The BMSC and positive control groups showed significantly reduced KFi-67-positive cells, with scattered positive signals and reduced staining intensity. The pathway agonist group showed Ki-67 expression patterns similar to

the model group, with abundant positive nuclei (Fig. 5A). Quantitative analysis showed (Fig. 5B) that the Ki-67 proliferation index in the model group was $45.6\% \pm 3.8\%$. Compared with the model group, the proliferation indices were significantly reduced in the BMSC group ($21.3\% \pm 2.7\%$) and the positive control group ($19.8\% \pm 3.1\%$) ($P < 0.01$); the pathway agonist group ($44.1\% \pm 4.2\%$) showed no significant difference from the model group ($P > 0.05$).

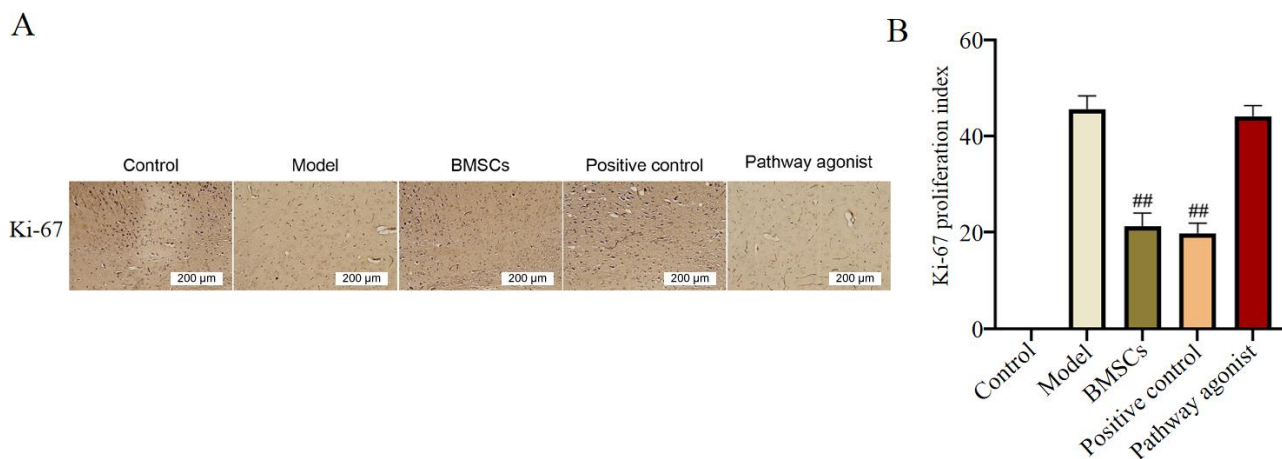


Fig. 5: Proliferation analysis of glioma cells in each group

(A) Representative immunohistochemical staining images of Ki-67 in brain tissue from each group. Ki-67-positive nuclei appear brown. Scale bar = 200 μm; (B) Quantitative analysis of Ki-67 proliferation index. Data are presented as mean ± SD, n=5 per group. ## $P < 0.01$ vs. model group; ns, not significant vs. model group. One-way ANOVA followed by Dunnett's test.

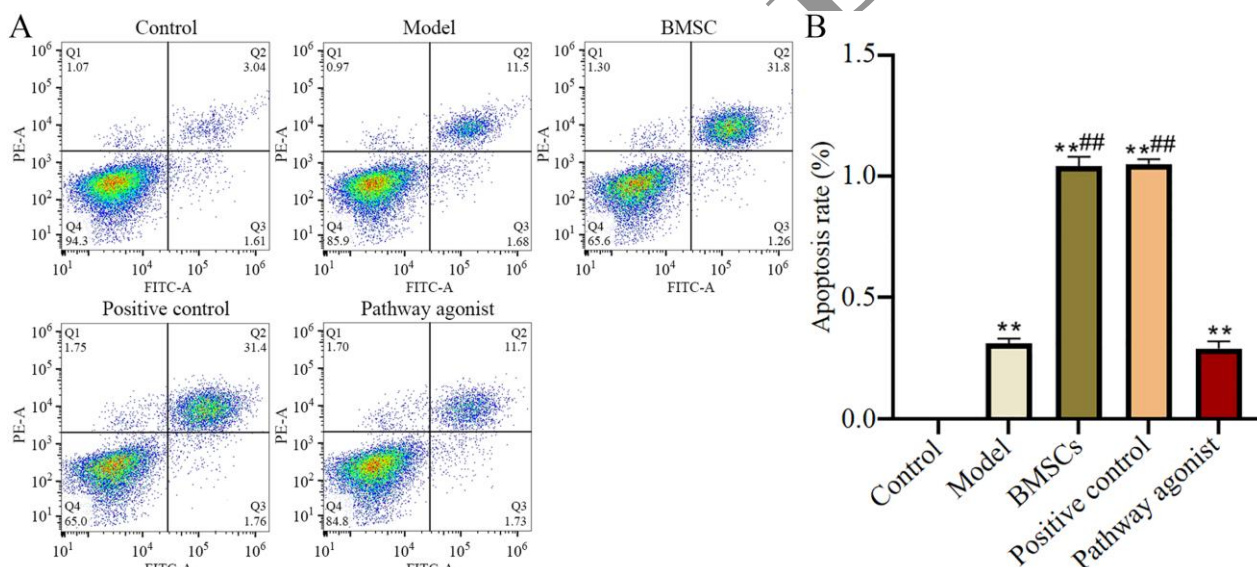


Fig. 6: Apoptosis analysis of glioma cells in each group by flow cytometry

(A) Representative flow cytometry scatter plots of Annexin V-FITC/PI staining for glioma cells from each group. Q1: necrotic cells; Q2: late apoptotic cells; Q3: viable cells; Q4: early apoptotic cells; (B) Quantitative analysis of total apoptosis rate (early + late) by flow cytometry. Data are presented as mean ± SD, n=5 per group. ## $P < 0.01$ vs. model group, ** $P < 0.01$ vs. control group. One-way ANOVA followed by Dunnett's test.

Detection of apoptosis by flow cytometry

Flow cytometry was used to further quantitatively detect the apoptosis rate of glioma cells in each group. Annexin V/PI double staining results showed (Fig. 6A) that cells in the control group were mainly distributed in the Q3 quadrant (viable cell area), with very few apoptotic cells. The model group showed a small number of cells distributed in the Q4 (early apoptosis) and Q2 (late apoptosis) quadrants. The BMSC group showed a significantly increased proportion of cells in the Q4 and

Q2 quadrants, indicating increased apoptotic cells. The positive control and pathway agonist groups showed distributions similar to the model group. Quantitative analysis showed (Fig. 6B) that the total apoptosis rate (early + late) in the model group was $0.31\% \pm 0.02\%$. The apoptosis rate was significantly increased in the BMSC group to $1.04\% \pm 0.04\%$ ($P < 0.01$) and in the positive control group to $1.05\% \pm 0.02\%$ ($P < 0.01$), while the pathway agonist group showed $0.29\% \pm 0.03\%$, with no significant difference from the model group ($P > 0.05$).

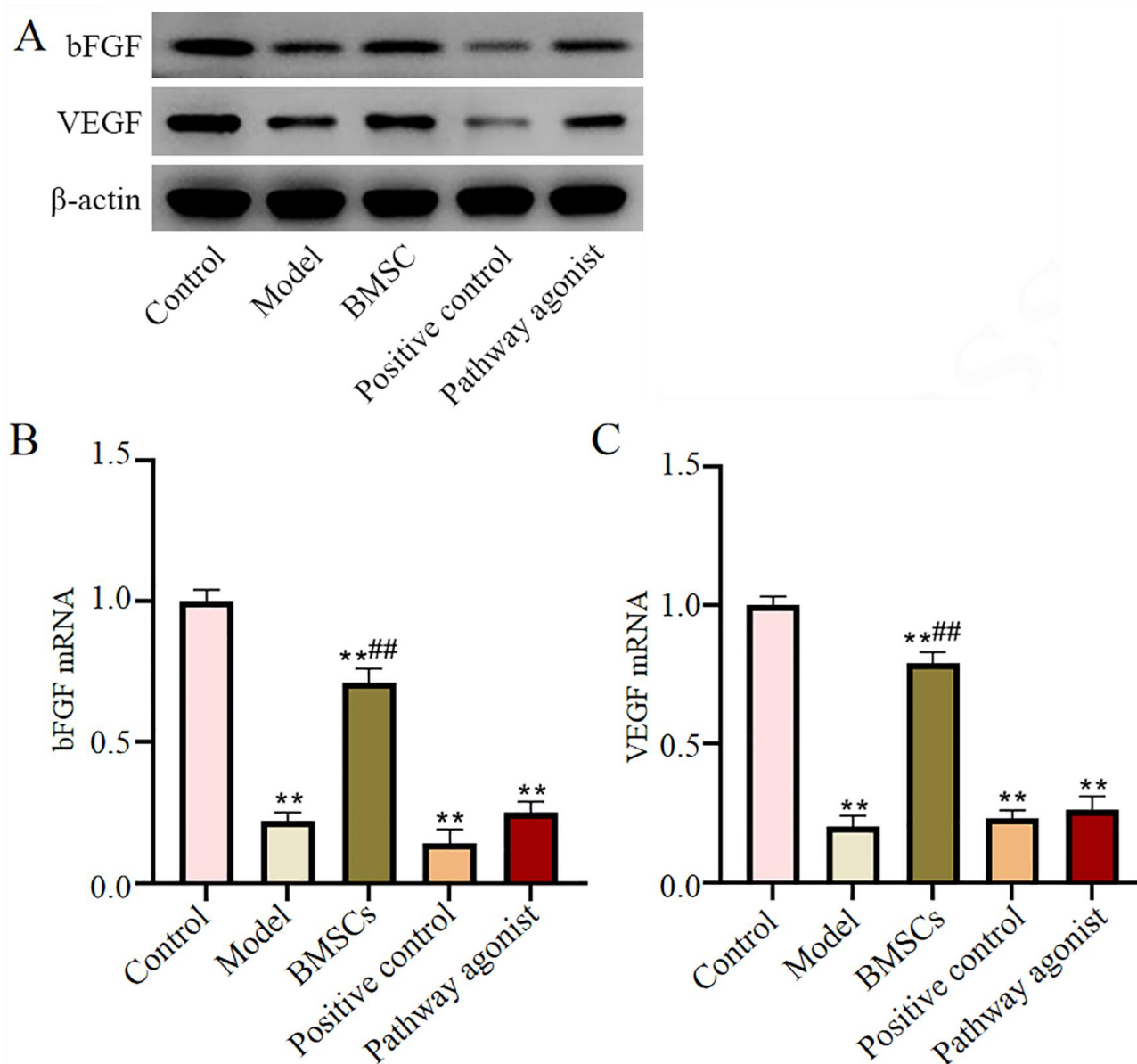


Fig. 7: bFGF and VEGF mRNA expression analysis in brain tissue of each group

(A) Representative RT-PCR electrophoresis images of bFGF, VEGF and β -actin mRNA expression in brain tissue from each group; (B) Quantitative analysis of bFGF mRNA relative expression. Using the control group as a reference (set to 1.00), calculated by the $2^{-\Delta\Delta CT}$ method, $n=5$ per group; (C) Quantitative analysis of VEGF mRNA relative expression. Using the control group as a reference (set to 1.00), $n=5$ per group. ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. model group; ns, not significant vs. model group. Data are presented as mean $\pm$ SD. One-way ANOVA followed by Dunnett's test for multiple comparisons.

BMSCs promote apoptosis in glioma cells through paracrine action

To investigate whether BMSCs exert their effects through paracrine mechanisms, RT-PCR was used to detect bFGF and VEGF mRNA expression levels in the brain tissue of each group (Fig. 7A). Quantitative analysis showed (Figs. 7B-C) that, using the control group mRNA expression level as a reference (set to 1.00), bFGF and VEGF mRNA relative expression levels were decreased in the model

group ($P < 0.01$). bFGF and VEGF mRNA expression levels were significantly increased in the BMSC group compared to the model group ($P < 0.01$). bFGF and VEGF mRNA expression levels in the positive control group showed no significant difference from the model group ($P > 0.05$). bFGF and VEGF mRNA expression levels in the pathway agonist group also showed no significant difference from the model group ($P > 0.05$).

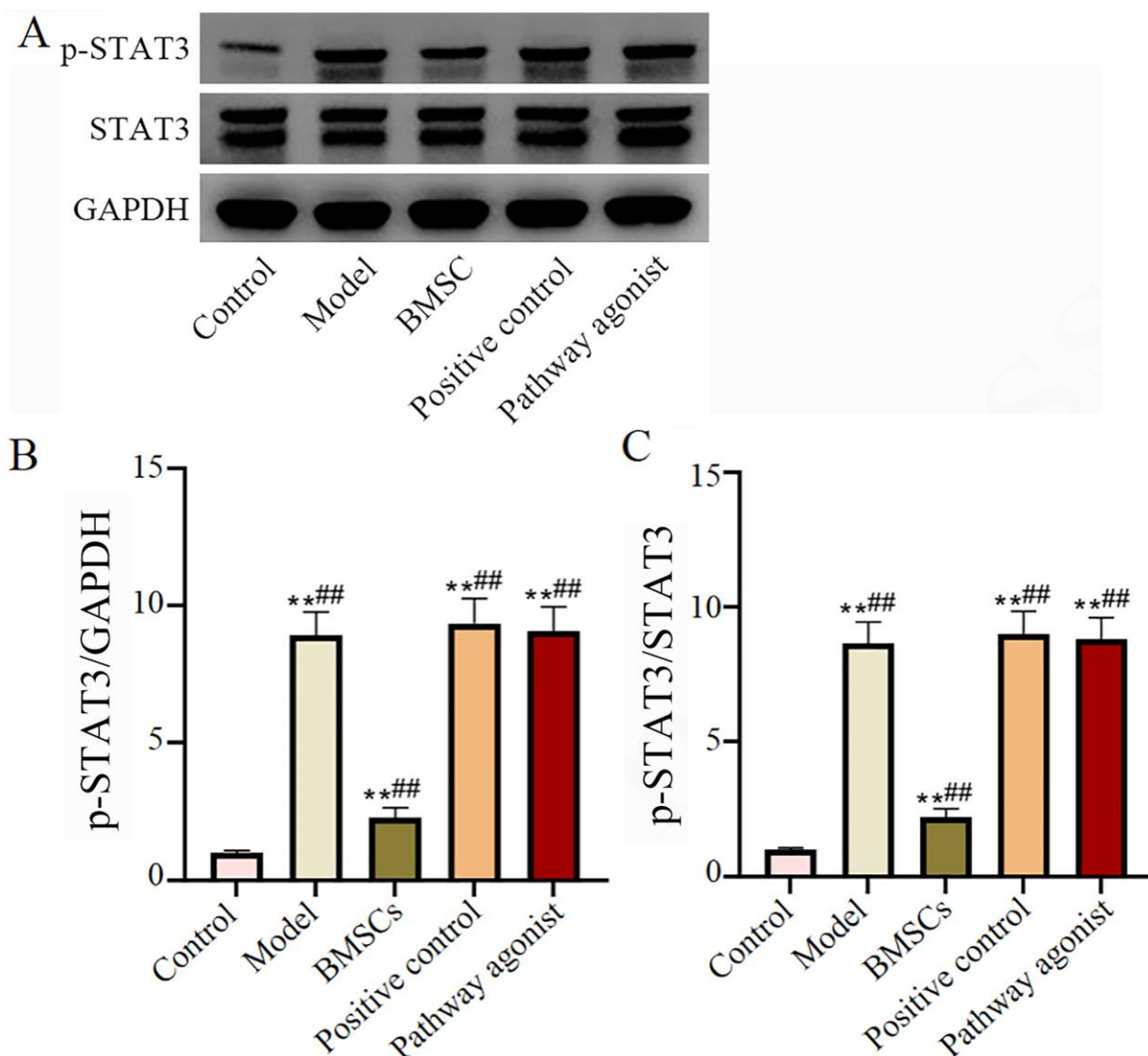


Fig. 8: STAT3 pathway-related protein expression and quantitative analysis in brain tissue of each group (A) Representative Western blot images of STAT3, p-STAT3 and GAPDH protein expression in brain tissue from each group; (B) Quantitative analysis of p-STAT3/GAPDH ratio. Using the control group as a reference (set to 1.0), n=3 independent experiments; (C) Quantitative analysis of p-STAT3/STAT3 ratio, n=3 independent experiments. ## $P < 0.01$ vs. model group, ** $P < 0.01$ vs. control group. Data are presented as mean $\pm$ SD. One-way ANOVA followed by Tukey's HSD test for multiple comparisons with Bonferroni correction.

BMSCs regulate apoptosis in glioma cells through the STAT3 pathway

Western blotting was performed to detect STAT3 and p-STAT3 protein expression levels in the brain tissue of each group. The results showed no significant differences in total STAT3 protein expression among groups ($P > 0.05$), while p-STAT3 expression levels showed significant differences (Fig. 8A). Densitometric analysis showed (Fig. 8B) that, using the control group p-STAT3/GAPDH ratio as a reference (set to 1.0), p-STAT3 relative expression was significantly increased in the model group to 8.92 ± 0.85 ($P < 0.01$); p-STAT3 relative expression in the BMSC group was 2.28 ± 0.36 , significantly lower than in the model group ($P < 0.01$) but

higher than in the control group ($P < 0.05$); the positive control group showed 9.35 ± 0.91 , with no significant difference from the model group ($P > 0.05$); the pathway agonist group showed 9.07 ± 0.88 , with no significant difference from the model group ($P > 0.05$). p-STAT3/STAT3 ratio analysis showed (Fig. 8C) that the ratio was significantly increased in the model group (8.65 ± 0.79), significantly decreased in the BMSC group (2.21 ± 0.31) and showed no significant difference from the model group in the positive control group (8.98 ± 0.86) and the pathway agonist group (8.79 ± 0.82). These results indicate that BMSCs significantly inhibit STAT3 phosphorylation.

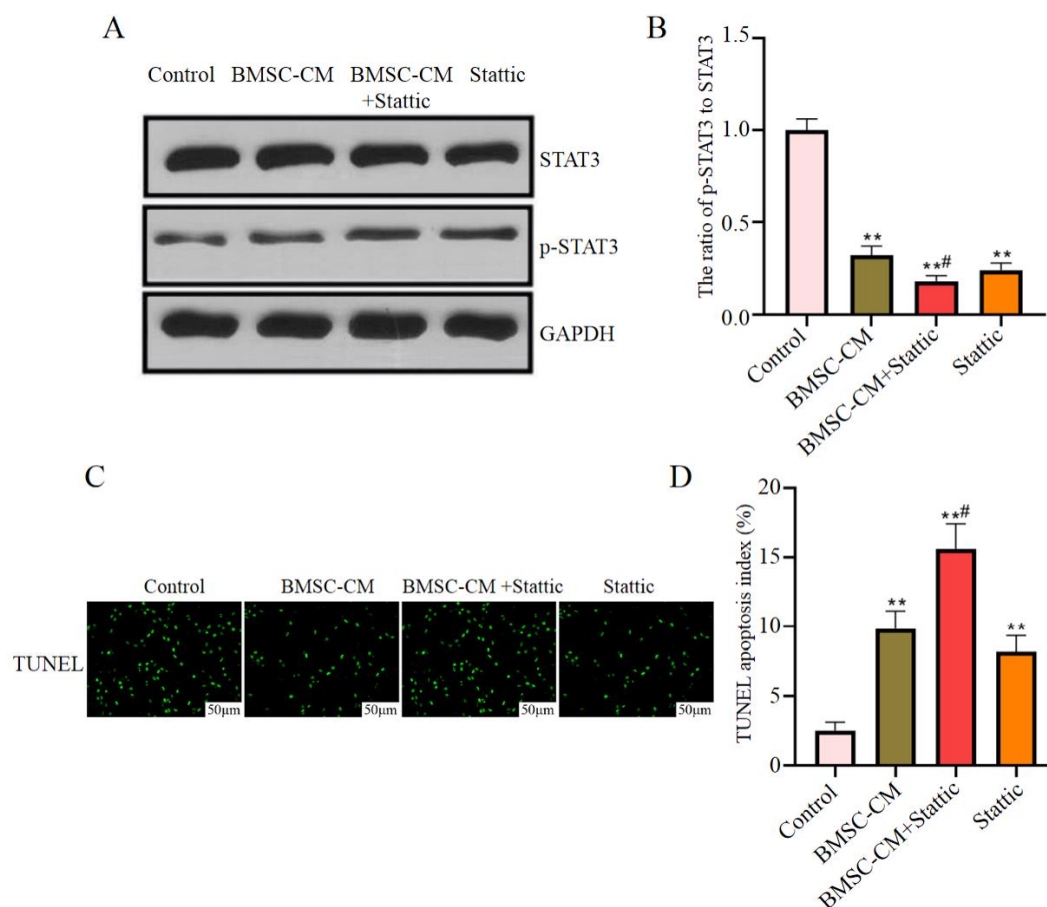


Fig. 9: *In-vitro* validation of the role of STAT3 in BMSC-mediated anti-glioma effects

(A) Representative Western blot images of p-STAT3, STAT3 and GAPDH protein expression in C6 cells from each group; (B) Quantitative analysis of p-STAT3/STAT3 ratio. Using the control group as a reference (set to 1.0), $n=3$ independent experiments. $**P < 0.01$ vs. control group; $\#P < 0.05$ vs. BMSC-CM group; (C) Representative TUNEL staining images of C6 cell apoptosis. TUNEL-positive cells show green fluorescence; nuclei are stained blue with DAPI. Scale bar = 50 μm ; (D) Quantitative analysis of TUNEL apoptosis index. Five fields were counted per coverslip, $n=3$ independent experiments. $**P < 0.01$ vs. control group; $\#P < 0.05$ vs. BMSC-CM group. Data are presented as mean $\pm$ SD. One-way ANOVA followed by Tukey's HSD test.

***In-vitro* validation of STAT3 in BMSC-mediated anti-glioma effects**

To validate the role of the STAT3 pathway in BMSC-mediated anti-glioma effects at the cellular level, *In-vitro* STAT3 inhibition experiments were performed using C6 glioma cells and p-STAT3 levels (Western blotting) and apoptosis (TUNEL staining) were examined. Western blot analysis showed (Fig. 9A) that p-STAT3 expression levels were relatively high in control C6 cells. After treatment with BMSC-CM, p-STAT3 levels were significantly reduced to 0.32 ± 0.05 times that of the control group ($P < 0.01$). After combined treatment with BMSC-CM and Stattic, p-STAT3 levels were further reduced to 0.18 ± 0.03 times, significantly lower than in the BMSC-CM alone group ($P < 0.05$). p-STAT3 levels in the Stattic alone group were reduced to 0.24 ± 0.04 times, with no significant difference from the BMSC-CM group ($P > 0.05$) (Fig. 9B). TUNEL staining showed (Fig. 9C) that only a few TUNEL-positive cells (green fluorescence)

were seen in the control group, with an apoptosis index of $2.5\% \pm 0.6\%$. After BMSC-CM treatment, TUNEL-positive cells significantly increased and the apoptosis index was significantly elevated to $9.8\% \pm 1.3\%$ ($P < 0.01$). After combined treatment with BMSC-CM and Stattic, TUNEL-positive cells further increased and the apoptosis index increased to $15.6\% \pm 1.8\%$, significantly higher than in the BMSC-CM alone group ($P < 0.05$). The apoptosis index in the Stattic alone group was $8.2\% \pm 1.1\%$, with no significant difference from the BMSC-CM group ($P > 0.05$), but significantly higher than in the control group ($P < 0.01$) (Fig. 9D).

DISCUSSION

This study aimed to investigate the mechanism by which BMSCs regulate apoptosis in glioma. A rat glioma model was established and animals were treated with BMSCs, a pathway agonist, or MS-275. Body weight, pathological changes in brain tissue, GFAP expression, apoptosis,

bFGF/VEGF expression and STAT3/p-STAT3 levels were evaluated. The BMSCs used in this study were characterized by flow cytometry, showing high expression of CD29 (99.96%), CD90 (99.94%) and CD105 (99.97%) and minimal expression of CD34 (0.52%) and CD45 (0.43%), confirming their MSC identity. They also exhibited osteogenic and adipogenic differentiation potential, further validating their stem cell properties. MRI confirmed successful tumor implantation in all groups except the control, with no significant differences in baseline tumor volume among groups ($P > 0.05$), ensuring comparability for subsequent interventions. A notable decline in body weight was observed in the model group, whereas body weight increased normally in the control group. Compared with the model group, the BMSC and positive control groups showed improved weight gain, reduced tumor area and a significantly lower Ki-67 proliferation index ($P < 0.01$), indicating that BMSCs inhibit both tumor growth and cell proliferation. Histological examination revealed distinct vascular structures and orderly cell arrangement in the control group. In contrast, the model group showed invasive glioma growth with abundant necrotic areas, thickened vascular walls, lymphocytic infiltration and disorganized cellular morphology. In the BMSC and positive control groups, necrotic areas were markedly reduced and cellular arrangement was generally more orderly, suggesting therapeutic effects of both BMSCs and the positive control drug. Notably, these effects were abrogated by treatment with the STAT3 pathway agonist, indicating that STAT3 signaling may be involved in BMSC-mediated anti-glioma effects. BMSCs have been reported to exert immunomodulatory effects in glioma by secreting cytokines that regulate T lymphocyte function (Santillán-Guajan *et al.*, 2024), highlighting the importance of exploring the mechanisms underlying BMSC-based glioma therapy.

In this study, BMSCs were administered via the tail vein and therapeutic effects on glioma were observed. Related studies have shown that BMSC-derived exosomes possess intrinsic brain tissue enrichment properties and can effectively cross the blood-brain barrier (BBB) and accumulate at brain tumor sites (Rehman *et al.*, 2022; Zhang *et al.*, 2024) further demonstrated in an orthotopic glioma model that BMSC-derived exosomes effectively cross the BBB, deliver loaded drugs to brain tumor tissue and are efficiently internalized by glioma cells. These findings provide direct evidence for peripherally administered BMSCs acting on glioma and support the feasibility of the administration route used in this study. It should be noted that MS-275 was selected as the positive control drug in this study. Although MS-275 does not directly act on STAT3, HDAC inhibitors can indirectly affect STAT signaling pathways through various mechanisms. Recent studies have shown that in lipopolysaccharide (LPS)-stimulated glial cells, MS-275

reduces tumor necrosis factor- α (TNF- α) release, alleviates neuroinflammation and may regulate pathway enrichment related to cytokine signaling and immunity through the JAK-STAT pathway (Mancino *et al.*, 2025). Furthermore, Garrett *et al.*, (2023) reported that HDAC inhibitors are the most selective compounds for inhibiting isocitrate dehydrogenase 1 (IDH1)-mutant glioma growth and among the six HDAC enzymes expressed in IDH1-mutant gliomas, HDAC1 and HDAC6 are the most essential for both *In-vitro* and *In-vivo* growth. Knockdown of these enzymes leads to more circumscribed, less invasive tumors. As a selective class I HDAC inhibitor, MS-275 effectively inhibits HDAC1 and was therefore used in this study as a positive control drug to evaluate the anti-glioma effects of BMSC therapy relative to known HDAC inhibitors. GFAP expression levels partially reflect the apoptotic status of glioma cells (Zhu *et al.*, 2023). In this study, GFAP expression was lowest in the model group and the pathway agonist group, intermediate in the BMSC and positive control groups and highest in the control group. The apoptotic rate was lowest in the model group and the pathway agonist group and highest in the BMSC and positive control groups. These results suggest that both BMSCs and the positive control drug may exert their effects through modulation of the STAT3 signaling pathway.

In this study, whole brain tissue (including both glioma and adjacent normal cells) was homogenized for molecular analyses. We acknowledge that this approach does not isolate signals purely from glioma cells. However, by comparing the control group (normal brain) with the model group (tumor-bearing brain) and further with the BMSC intervention group, we can infer relative changes attributable to the tumor and the therapeutic intervention. The data presented reflect average changes in the brain microenvironment under the influence of glioma, rather than signals derived purely from tumor cells. Future studies using laser capture microdissection (LCM) or flow cytometry sorting of labeled tumor cells are needed to validate these findings at single-cell resolution. Previous studies have suggested that BMSCs may promote apoptosis in glioma cells through paracrine mechanisms (Cesca *et al.*, 2026). In the present study, bFGF and VEGF expression was low in the model group, positive control group and pathway agonist group, but significantly higher in the BMSC group and control group. These findings support the notion that BMSCs may exert pro-apoptotic effects in glioma cells through paracrine actions involving growth factors such as bFGF and VEGF (Singh *et al.*, 2026). No significant differences in total STAT3 expression were observed among groups. However, p-STAT3 levels differed significantly across groups. p-STAT3 expression was highest in the model group, positive control group and pathway agonist group; intermediate in the BMSC group; and lowest in the control group. These results suggest that BMSCs may

interact with STAT3 pathway components by modulating bFGF and VEGF expression, thereby suppressing STAT3 phosphorylation and its subsequent nuclear translocation. Reduced binding of STAT3 to cellular DNA may lead to decreased regulation of anti-apoptotic genes in glioma cells, ultimately promoting apoptosis. These findings support the notion that the STAT3 signaling pathway may serve as a therapeutic target in glioma (Ma *et al.*, 2025, Groot *et al.*, 2022). Notably, in the STAT3 agonist (Colivelin) group, high p-STAT3 expression coexisted with low VEGF expression. This apparent dissociation may be explained by the direct mechanism of Colivelin, which activates the JAK/STAT axis independently of upstream signals such as VEGF. Additionally, sustained STAT3 activation may trigger negative feedback inhibition of VEGF transcription. Furthermore, our measurements reflect steady-state levels at day 21, which may not capture early dynamic changes in VEGF expression. These observations suggest that BMSCs may indirectly influence STAT3 signaling through paracrine factors such as VEGF, whereas the agonist directly activates the pathway. The increase in VEGF and bFGF mRNA levels in BMSC-treated tumors, despite enhanced apoptosis, may appear paradoxical. This can be explained by cell source specificity and functional plasticity. RT-PCR analysis was performed on whole tumor tissue, which includes not only tumor cells but also transplanted BMSCs and various stromal cells. Thus, the elevated VEGF/bFGF signals likely originated from the transplanted BMSCs themselves, which are known to secrete these factors. Emerging evidence suggests that BMSC-derived factors may promote vascular normalization rather than pathological angiogenesis. In an ischemic stroke model, BMSC-derived extracellular vesicles were shown to enhance tight junction protein expression (ZO-1, Occludin) in brain microvascular endothelial cells, reduce BBB permeability and protect vascular integrity through inhibition of the Caveolin-1/cluster of differentiation 147 (CD147)/VEGFR2/matrix metalloproteinase (MMP) pathway (Li *et al.*, 2024). These findings support the hypothesis that BMSC-secreted factors can remodel the tumor vasculature, improving perfusion, alleviating hypoxia and thereby indirectly sensitizing tumor cells to apoptotic signals. Furthermore, BMSCs may exert direct effects on tumor cells. Su *et al.*, (2025) demonstrated that BMSC-derived exosomal miR-29a-3p inhibits the JAK2/STAT3 pathway and promotes apoptosis in non-small cell lung cancer, which is consistent with our finding that BMSCs directly suppress STAT3 phosphorylation in glioma cells, leading to apoptosis. Thus, the coexistence of increased VEGF/bFGF expression and enhanced apoptosis in the BMSC group may reflect dual mechanisms: direct inhibition of STAT3 signaling in tumor cells and indirect modulation of the tumor microenvironment through vascular normalization. Future studies using cell sorting or in situ hybridization are needed to precisely determine

the cellular sources of VEGF and bFGF and their spatial relationship with STAT3 activity. However, this study has several limitations. The sample size was relatively small (n=5 per group), which may limit the statistical power for detecting secondary outcomes. Experiments were conducted as single batches without independent replication, which may overestimate effect sizes. The observation period was limited to 21 days, precluding the assessment of long-term therapeutic effects. Future studies should include larger sample sizes, independent replication, extended observation periods and techniques such as cell sorting or in situ hybridization to clarify the cellular sources of VEGF/bFGF and their spatial relationship with STAT3 activity. Multicenter studies are needed to validate the generalizability of the results and the exploration of multiple BMSC administrations and combination therapies with STAT3-targeted drugs should be pursued to provide more sufficient experimental evidence for clinical translation.

CONCLUSION

In conclusion, this study demonstrates that BMSCs promote apoptosis in glioma cells by inhibiting STAT3 phosphorylation. BMSC treatment reduces p-STAT3 levels, downregulates anti-apoptotic gene expression and increases apoptosis. The reversal of these effects by a STAT3 activator confirms the causal role of STAT3 inhibition. These findings identify STAT3 as a key target for BMSC-based glioma therapy.

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Authors' contributions

Hu Xu: Responsible for experimental design and data collection; Qi Tu: Statistical analysis, chart creation and the initial draft writing of the paper; Zhijian Xu: Supervision, Project Administration, Writing – Review & Editing; Mingfeng Tong: Investigation, Writing – original draft and Writing – review & editing; Xing Wan: As the corresponding author, I was responsible for the conception of the research, guidance of the experiments, provision of funding, revision of the papers and final version review. All the authors have read and agreed to the final manuscript content.

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Data availability statement

The datasets generated and/or analyzed during the current study can be obtained from the corresponding author upon reasonable request.

Ethical approval

This study was approved by the Ethics Committee of Jinhua Municipal Central Hospital (Approval No.: AL-JHYY2025). This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest

The author states that this research was conducted without any potential conflicts of interest that could be construed as commercial or financial.

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