

Six3 promoting the migration and neural differentiation of BMSCs via Btg2-mediated Akt signaling pathway activation: A therapeutic strategy for stroke

ZHENQIANG FU^{1*}, ZHIXIN LI^{2*}, LIMIN ZHANG¹, WENTAO HU¹,
HECHENG YANG¹ and JINGTAO WANG¹

¹Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China;

²Department of Neurointervention, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China

Received February 5, 2026; Accepted September 21, 2026

DOI: 10.3892/ijmm.2026.6013

Abstract. Bone marrow-derived mesenchymal stem cell (BMSC) transplantation holds therapeutic potential for stroke; however, but its effectiveness requires optimization. The transcription factor Sine Oculis Homeobox Homolog 3 (Six3) regulates processes crucial for BMSC therapy, such as neural cell proliferation and differentiation. However, whether Six3-modified BMSCs exert enhanced therapeutic effects on stroke remains unknown. In the present study, mouse BMSCs were isolated and identified using surface markers. Six3-overexpressing BMSCs exhibited enhanced proliferative, migratory and neural differentiation capacities. RNA-sequencing analysis comparing the gene expression profiles of BMSCs with and without Six3 overexpression identified BTG Anti-Proliferation Factor 2 (Btg2) as an upregulated gene ($\log_2FC=1.45$, $P<0.05$) in Six3-overexpressing cells. Further investigations revealed that Six3 directly binds to the TGATACTGTCT sequence within the Btg2 promoter and activates its transcription. Btg2 knockdown abolished the effects of Six3 overexpression. Mechanistically, Six3 promoted Akt phosphorylation via Btg2, enhancing BMSC viability and neural differentiation. A mouse model of stroke was established using transient middle cerebral artery occlusion (MCAO, 1.5-h occlusion and 24-h reperfusion). BMSCs were administered via tail vein injection at the onset of reperfusion. GFP expression detected in brain tissue at 24 h post-reperfusion confirmed successful the homing of BMSCs

to the brain. The transplantation of Six3-overexpressing BMSCs ameliorated brain pathology, reducing apoptosis and cerebral infarct volume. Notably, Six3-modified BMSCs demonstrated a significantly greater therapeutic efficacy than unmodified BMSCs. Collectively, these findings demonstrate that Six3 enhances the therapeutic properties of BMSCs by transcriptionally activating Btg2, which promotes Akt phosphorylation. These findings provide novel mechanistic insight into the optimization of stem cell therapy in stroke.

Introduction

Stroke is one of the leading causes of mortality and disability worldwide (1). Of note, 85% of stroke cases are caused by ischemia, and ischemic stroke is mainly an acute attack of local brain dysfunction caused by interrupted blood flow, manifested as a focal infarction (2). The mortality rate for ischemic stroke is ~25% (3). Stem cell transplantation is currently recognized as an effective treatment and is increasingly used in the clinical treatment of a variety of human diseases and injuries, including stroke (4-7).

Mesenchymal stem cells (MSCs) are stromal precursor cells of mesoblastic cells, and these cells can be isolated from almost all connective tissues. Bone marrow is the most commonly used source for obtaining MSCs. MSCs have the advantages of easy access, abundant sources, no obvious transplant rejection and easy introduction of foreign genes, and have immense application prospects in the treatment of nervous system diseases (8,9). However, the therapeutic efficacy of transplantation is often unsatisfactory as the survival and homing capabilities of MSCs are limited (10). Gene modification is a promising approach (11).

Sine Oculis Homeobox Homolog 3 (Six3) is a transcription factor that promotes or inhibits transcription. Six3 plays a crucial role in brain and nervous system development, promoting nerve/neuroblast proliferation, migration and differentiation (12-16). It has been reported that Six3 expression is increased in brain tissue following the treatment of experimental cerebral ischemia with ischemic post-conditioning, suggesting that Six3 may be involved in the neuroprotective effects (17). However, the role of Six3 in MSCs remains unclear.

Correspondence to: Dr Zhenqiang Fu, Department of Neurology, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe East Road, Zhengzhou, Henan 450052, P.R. China
E-mail: fccfuzq@zzu.edu.cn

*Contributed equally

Key words: sine oculis homeobox homolog 3, btg anti-proliferation factor 2, bone marrow-derived mesenchymal stem cells, stroke, middle cerebral artery occlusion

BTG Anti-Proliferation Factor 2 (Btg2) gene encodes a member of the BTH/Tob family, which has anti-proliferation functions (18). Btg2 has been reported to be involved in the regulation of cell proliferation, differentiation and DNA repair, particularly in the migration and differentiation of neural precursor cells (19-21). It has been reported that Btg2 is highly expressed in the peri-infarct and infarcted border zones of brain tissue following cerebral ischemia and infarction and is involved in the survival and differentiation of nerve cells, and plays a neuroprotective role (22,23). It has been demonstrated that Btg2 promotes the proliferation and neural differentiation of bone marrow-derived mesenchymal stem cell (BMSC) (24). In the present study, the transcriptome of Six3-overexpressing BMSCs was analyzed, and the results revealed that the expression of Btg2 was upregulated ($\log_2FC=1.45$, $P<0.05$). The prediction results from the JASPAR database (<https://jaspar.elixir.no/>) indicate that there are binding sites for Six3 protein and Btg2 promoter regions. Therefore, it was hypothesized that Six3 promoted the transcription of Btg2 and regulated the phenotype of BMSCs.

Materials and methods

Isolation of BMSCs. A total of 130 C57BL/6J mice (half male and half female), aged 6 to 8 weeks, were euthanized by cervical dislocation following the inhalation of 5% isoflurane. Subsequently, the muscles were removed to expose and collect the femur and tibia. The isolation of mouse BMSCs was performed as previously described (25). In brief, bone marrow was obtained by flushing the marrow cavity using DMEM (Wuhan Servicebio Technology Co., Ltd.) containing 15% fetal bovine serum (FBS). The collected cell suspension was filtered using a 70- μ m filter to remove bone spurs, muscle and cell masses. Red cell lysis buffer (cat. no. R1010, Beijing Solarbio Science & Technology Co., Ltd.) was added to the cells and centrifuged at 500 $\times$ g for 5 min to collect the precipitated cells. The cells were cultured in DMEM supplemented with 15% FBS and 1% penicillin-streptomycin for 24 h, after which the medium was replaced with fresh medium. The cells at passage three were collected for use in subsequent experiments.

Characterization of BMSCs. BMSCs at passage three were used for characterization. As previously reported (26), BMSCs were cultured for 3 weeks with conditional media from osteoblasts [complete medium + 100 nM dexamethasone (Shanghai Aladdin Biochemical Technology Co., Ltd.) + 50 μ g/ml L-ascorbic acid (Macklin) + 10 mM β -sodium glycerophosphate (Macklin)], lipoblasts (complete medium + 100 nM dexamethasone + 50 μ g/ml indomethacin (Macklin) + 50 μ g/ml L-ascorbic acid) and chondroblasts [(complete medium + 1% insulin-transferrin-selenium additive (ITS) (iCell) + 10^{-7} M dexamethasone + 100 μ M L-ascorbic acid + 10 ng/ml TGF- β 1 (Sino Biological)], respectively, and stained with Alizarin red, Oil Red O and toluidine blue, respectively, and then observed for osteogenic, lipogenic and chondrogenic differentiation. The cell suspensions were incubated with CD44 (cat. no. E-AB-F1100D), CD90 (cat. no. E-AB-F1283D), CD31 (cat. no. E-AB-F1180D) and CD45 (cat. no. E-AB-F1136D) antibodies (Elabscience Biotechnology, Inc.) conjugated to

the fluorescent protein Phycoerythrin (PE), and the expression of cell surface markers was analyzed by flow cytometry. The established MSC markers CD44 and CD90 exhibited strong positivity, whereas the endothelial cell marker, CD31, and the hematopoietic cell marker, CD45, exhibited minimal positivity, indicating that the isolation of BMSCs was successful (27).

Lentivirus infection. The present study employed the 2nd generation lentiviral systems. The coding sequence (CDS) of Six3 (NM_011381: 407-1408 bp) was cloned into the pCDH-CMV-MCS-EF1-GFP-T2A-puro plasmid (cat. no. BR318; Hunan Fenghui Biotechnology Co., Ltd.) (restriction enzyme cutting site, XbaI/NheI) for the construction of a Six3 overexpression plasmid. The CDS of Btg2 (NM_007570: 103-579 bp) was cloned into the pLVX-IRES-puro plasmid (cat. no. BR025; Hunan Fenghui Biotechnology Co., Ltd.) (restriction enzyme cutting site, EcoRI/BamHI), and the short hairpin RNA sequence targeting Btg2 (5'-ATGGCCGTCTCCAGCTAGATA-3') was cloned into the pLKO.1-EGFP-puro plasmid (cat. no. BR524, Hunan Fenghui Biotechnology Co., Ltd.) (restriction enzyme cutting site, AgeI/EcoRI). The empty plasmids without any foreign fragments were used as a negative control. The 14 μ g recombinant shuttle-plasmid, together with the 10.5 μ g pSPAX2 and 3.5 μ g pMD2.G plasmids (cat. no. BR036 and BR037, Hunan Fenghui Biotechnology Co., Ltd.), were transfected into 293T cells (iCell) using Lipofectamine 3000 (cat. no. L3000015; Thermo Fisher Scientific, Inc.) to package the infectious lentiviral particles. The 293T cells were cultured in DMEM (Wuhan Servicebio Technology Co., Ltd.) supplemented with 10% FBS at 37°C and 5% CO₂, and the cell supernatant was collected and filtered through a 0.45- μ m filter at 48 and 72 h following transfection. BMSCs were cultured in DMEM supplemented with 15% FBS and 1% penicillin-streptomycin at 37°C and 5% CO₂. When the confluency of BMSCs reached 60%, they were infected with lentivirus (MOI=20) for 48 h and then observed under a fluorescence microscope. Cell activity was measured using MTT assay.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cells or tissues were incubated in TRIpure lysis buffer (5~10 $\times$ 10⁶ cells/ml; cat. no. RP1001; BioTeke Corporation), and the mixture was extracted using chloroform. The water phase was collected, and isopropyl alcohol and ethanol were added to it to precipitate the total RNA. The RNA concentration was determined using an ultraviolet spectrophotometer. cDNA was obtained by reverse transcription using All-in-One First-Strand SuperMix according to the manufacturer's instructions. RT-qPCR analysis was performed on a fluorescence quantifier (Bioneer Corporation) in a system containing cDNA, primers and SYBR-Green Master Mix (Beijing Solarbio Science & Technology Co., Ltd.). The thermal cycling conditions are as follows: 95°C, for 5 min (pre-degeneration); 95°C, for 10 sec; 60°C, for 10 sec; 72°C, for 15 sec. A total of 40 cycles were conducted. Actin beta (Actb) was regarded as a regulatory gene. The primer sequences were as follows: Btg2 forward, 5'-GCACTGACCGATCAT TACAAA-3' and reverse, 5'-GCAGGTGAGGAGCCCAT-3'; Six3 forward, 5'-GCTGTCGTCGCCTTCCA-3' and reverse, 5'-CGCACGCGGTACTTGTC-3'; Actb forward, 5'-CATCCG

TAAAGACCTCTATGCC-3' and reverse, 5'-ATGGAGCCA CCGATCCACA3'. Relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method (28).

Western blot analysis. The cells or tissues were lysed in a lysate buffer (cat. no. P0013; Beyotime Institute of Biotechnology) supplemented with PMSF, and the mixture was centrifuged (10,000 × g) at 4°C for 5 min to separate the supernatant. The protein content in the supernatant was quantified using a BCA protein concentration assay kit and diluted to a uniform concentration using a PBS buffer. The protein solution was mixed with a 5X Loading Buffer and boiled to denature the protein. The prepared protein samples were loaded onto polyacrylamide gel (~30 µg protein per lane) and separated by SDS-PAGE (5% spacer gel and 12% separation gel). The protein bands were transferred to PVDF membranes and incubated in the blocking solution (cat. no. P0023; Beyotime Institute of Biotechnology) for 1 h at room temperature (~26°C). PVDF membranes were then incubated with primary antibodies for 12 h at 4°C and secondary antibodies for 1 h at room temperature and then visualized using ECL chemiluminescence reagents (cat. no. P0018; Beyotime Institute of Biotechnology). The optical density values of the blots were analyzed using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc.). Antibody information is provided in Table SI.

Wound healing test assay. The cells were cultured until fused, treated with mitomycin C (MilliporeSigma) for 1 h under serum-free conditions, and then scratched using a pipette tip. The cell surface was cleaned using serum-free medium and then images were captured under a light microscope. Cell migration was observed following 24 h of culture.

Immunofluorescence. Tissues or cell proliferating on glass coverslips were fixed in 4% paraformaldehyde for 24 h or 15 min at room temperature. The tissues were embedded in OCT embedding agent and pre-cooled, then cut into 10-µm-thick slices. The prepared samples were then incubated with primary and fluorescently labeled secondary antibodies. DAPI (1 mg/ml) was used for nuclear staining. The staining results were observed under a fluorescence microscope. Antibody information is provided in Table SI.

Enzyme-linked immunosorbent assay (ELISA). The levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the cell supernatant were determined using corresponding ELISA kits (cat. nos. EM0020 and EM0148; FineBio; <http://www.finebio.cn/>) according to the manufacturer's instructions.

Transcriptomic analysis. Total RNA was extracted using the TRNzol universal reagent (cat. no. DP424, Tiangen Biotech Co., Ltd.), and mRNA was enriched from total RNA using Oligo (dT) magnetic beads (cat. no. N403-01; Vazyme Biotech Co., Ltd.), and then fragmented in fragmentation buffers. cDNA was synthesized from mRNA template and purified using AMPure XP beads. Following terminal repair, PolyA tail addition, and sequencing aptamer connection, the purified DNA fragments were screened using AMPure XP beads. PCR was performed to construct the DNA library,

and the qPCR program was used to examine the concentration of DNA libraries. The original concentration of the library was ~4 µM. The integrity of the genomic DNA was analyzed using an Agilent 5400 fragment analyzer (Agilent Technologies, Inc.). The qualified DNA libraries were sequenced (nucleotide length: 150 bp; paired-end) using NovaSeq 6000 high-throughput sequencing platform (Illumina Inc.). The loading volume was 5 µl and the concentration was ~20 pM. Data filtering was performed using the fastp software (version 0.23.1; HaploX), and alignment with the reference genome was conducted using the HISAT2 software (version 2.2.1, Johns Hopkins University) (29). The HTSeq software (version 2.0.2, European Molecular Biology Laboratory) (30) was used for analyzing gene expression, and the R package DESeq2 (version 1.24.0, European Molecular Biology Laboratory) (31) was used for analyzing differentially expressed genes.

Double luciferase report assay. The nucleotide sequence containing the Btg2 promoter region was cloned into the pGL3-Basic plasmid vector (restriction enzyme cutting site, NheI/HindIII) and co-transfected into 293T cells with the Six3 overexpression plasmid using Lipofectamine 3000 (cat. no. L3000015; Thermo Fisher Scientific, Inc.). Following 48 h, the luciferase activity was detected using a dual luciferase assay kit (cat. no. KGE3302; Nanjing KeyGen Biotech Co., Ltd.). The ratio of Firefly luciferase activity to sea kidney luciferase activity was calculated.

Chromatin immunoprecipitation (ChIP)-PCR. The cells were incubated in formaldehyde at 37°C for 10 min to cross-link proteins and DNA. After washing, the cells were re-suspended in an SDS Lysis Buffer and incubated on ice for 10 min to be fully lysed. The lysate was ultrasound treated to cut the DNA. The lysates were incubated with Six3 antibody at 4°C overnight and then with Protein A+G Agarose/Salmon Sperm DNA at 4°C for 1 h. The DNA-protein complex was eluted from Protein A+G Agarose/Salmon Sperm DNA and was used for PCR. The primer sequences designed for the potential binding sites of Six3 in the Btg2 promoter region were as follows: Forward, 5'-GGTACTCCGAGTTAAAGA-3' and reverse, 5'-CTGACCAGCAAGACATT-3'. PCR products were examined using agarose gel electrophoresis.

DNA pull-down assay. DNA pulldown kit (cat. no. Bes5004; Bersinbio) was used for the DNA pull-down assay according to the method provided by the manufacturer. The biotinylated DNA probes were added to streptavidin magnetic beads and incubated in the DNA beads buffer at 25°C for 30 min to prepare the DNA probe-bead complex. Agarose beads (40 µl) were added to the protein samples for pre-cleaning and then incubated with DNA probe-bead complex in 500 µl binding buffer, 5 µl poly (dI-dC), 5 µl protease inhibitor, 5 µl DTT, 9 µl EDTA and 4.5 µl EGTA at 4°C for 1 h. Magnetic beads were then collected using a magnetic separation device and washed in 0.8 ml binding buffer and 4 µl DTT. The DNA-protein complex was eluted using 60 µl Protein Elution buffer and 0.6 µl DTT at 37°C for 2 h. After removing the magnetic beads, the supernatant was obtained, which was used for western blot analysis.

MCAO/reperfusion (MCAO/R) modeling and BMSC transplantation. Male C57BL/6J mice (22–25 g), aged 10 to 12 weeks, were housed in a 12-h light/dark cycle environment with a temperature of $22\pm1^{\circ}\text{C}$ and a humidity of 45–55%. They were also allowed free access to food and water. The mice were anesthetized with 5% isoflurane for induction and 1.0% isoflurane for maintenance. The mice were subjected to MCAO according to the previously reported methods (32), maintained for 90 min and followed by reperfusion for 24 h. At the same time as the initiation of reperfusion, 0.3 ml PBS containing 1×10^5 BMSCs was injected into the tail vein once (33). The mice in the sham-operated group underwent the same surgical procedure, but without the insertion of sutures. During the operation, throughout the recovery period, and within 24 h after reperfusion, a constant-temperature heating pad was used to maintain the animal's body temperature. Moist feed was placed at the bottom of the cage to facilitate feeding. After mice underwent MCAO, continuous observation was conducted throughout the recovery period until they regained spontaneous breathing and basic activities. Subsequently, the animals' spontaneous breathing, righting reflex and bleeding were checked every 30 min. Following reperfusion, continuous monitoring continued until the mice regained consciousness, with assessments every 30 min during the first 0–2 h, every hour from 2 to 6 h, and every 4 h from 6 to 24 h. The ischemic nerve defect score was assessed following reperfusion (33). Two weeks after the MCAO procedure, the mice underwent the cylinder test (34). For mice subjected to long-term behavioral assessments, monitoring was performed every 12 h starting 24 h after reperfusion, focusing primarily on body weight, food and water intake, activity, and reactivity. Subsequently, the animals were euthanized by cervical dislocation following the inhalation of 5% isoflurane, and brain tissues were collected for use in further experiments. The humane termination criteria were determined earlier, including persistent or recurrent convulsions that do not subside, coma unresponsive to stimulation, respiratory distress, and weight loss exceeding 20%. Once any of these criteria are met, euthanasia should be performed immediately. Death was confirmed by the absence of a heartbeat (assessed via chest palpation), cessation of spontaneous respiratory movements for more than 30 sec, and the absence of both the corneal reflex and response to a vigorous toe pinch. The animal experiments were approved by the Animal Ethics Committee of the First Affiliated Hospital of Zhengzhou University (approval no. 2025-KY-1279; Zhengzhou, China).

A total of 256 male C57BL/6 mice were used in the stroke study, of which 244 underwent MCAO surgery, and 12 mice underwent sham surgery. During the establishment of the MCAO model, 39 mice succumbed within 24 h after reperfusion (found dead), and the similar mortality rates have been reported before (35). The primary causes of death were intraoperative bleeding or severe brain oedema following reperfusion. The remaining 205 mice that underwent MCAO survived until 24 h after reperfusion. Based on the neurological deficit scores, 166 models were confirmed as successful (scores ≥ 1) and used for subsequent experiments, while 39 models (scores < 1) were classified as unsuccessful and euthanized. Among them, 156 MCAO mice and 12 Sham mice were used to generate valid research data. Before the formal experiment, 10 spare mice

were used to explore the antibody incubation conditions and to optimize the cylinder test setup.

Triphenyl-tetrazolium chloride (TTC) staining. Fresh brain tissue was frozen at -20°C and cut into six consecutive slices at a thickness of 1-mm, and then incubated in TTC solution for 15 min. Following staining, the ischemic area appears grayish white, and the normal tissue appears dark red.

Nissl staining. The ischemic penumbral cortex tissue was isolated, placed in OCT embedding agent, pre-cooled, and then cut into $10\text{-}\mu\text{m}$ -thick slices. The slices were covered with cresol purple solution (Sinopharm Chemical Regent Co., Ltd.) and incubated for 10 min, then placed in ethanol solution containing 0.25% acetic acid to remove the excess dye. The results of neuron staining were observed under a light microscope.

Statistical analysis. The data were analyzed using GraphPad Prism software (version 9.3; Dotmatics). The data is presented as the mean $\pm$ standard deviation. A one-way ANOVA followed by Tukey's multiple comparisons test was employed to assess differences between groups. Data that do not conform to the rules of ANOVA were analyzed using unpaired t-tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Isolation and identification of mouse BMSCs. BMSCs were isolated from mouse bone marrow and cultured to the third passage for use in the following experiments (Fig. 1A). Flow cytometry was used to detect the expression of cell surface marker proteins, including CD44, CD90, CD31 and CD45. The results show revealed that the expression levels of CD44 and CD90 on the cell surface were increased, while the expression levels of CD31 and CD45 were decreased (Fig. 1B). Following the induced differentiation culture (Fig. 1C), Alizarin red, Oil Red O and toluidine blue staining yielded strongly positive results, indicating that the cells had the potential to differentiate into osteogenesis, adipogenesis and chondrogenesis (Fig. 1D). These findings indicated that BMSCs were successfully isolated.

Overexpression of Six3 enhances the proliferation, migration and neural differentiation of BMSCs. As shown in Figs. S1 and S2, the Gene Ontology (GO) enrichment analyses of the differentially expressed genes (DEGs) provided by the GSE8590 and GSE150710 datasets were analyzed. The results revealed that they were generally involved in biological processes such as neural development, stem cell differentiation and neuronal regeneration. Of note, four potential target genes were screened by analyzing the GSE8590 and GSE150710 data sets (Fig. 2). GSE8590 indicated that the expression of Six3 was elevated in neural progenitor cells derived from hESCs. Notably, Six3 exhibited the largest upregulation in expression among the four genes, suggesting that Six3 may be involved in the neural differentiation of MSCs. GSE150710 also revealed that the expression of Six3 was enhanced in the host receiving human neural stem cells (hNSCs), and it was also increased in the transplanted hNSCs, which further emphasized the role of Six3 in stem cell transplantation for the treatment of stroke.

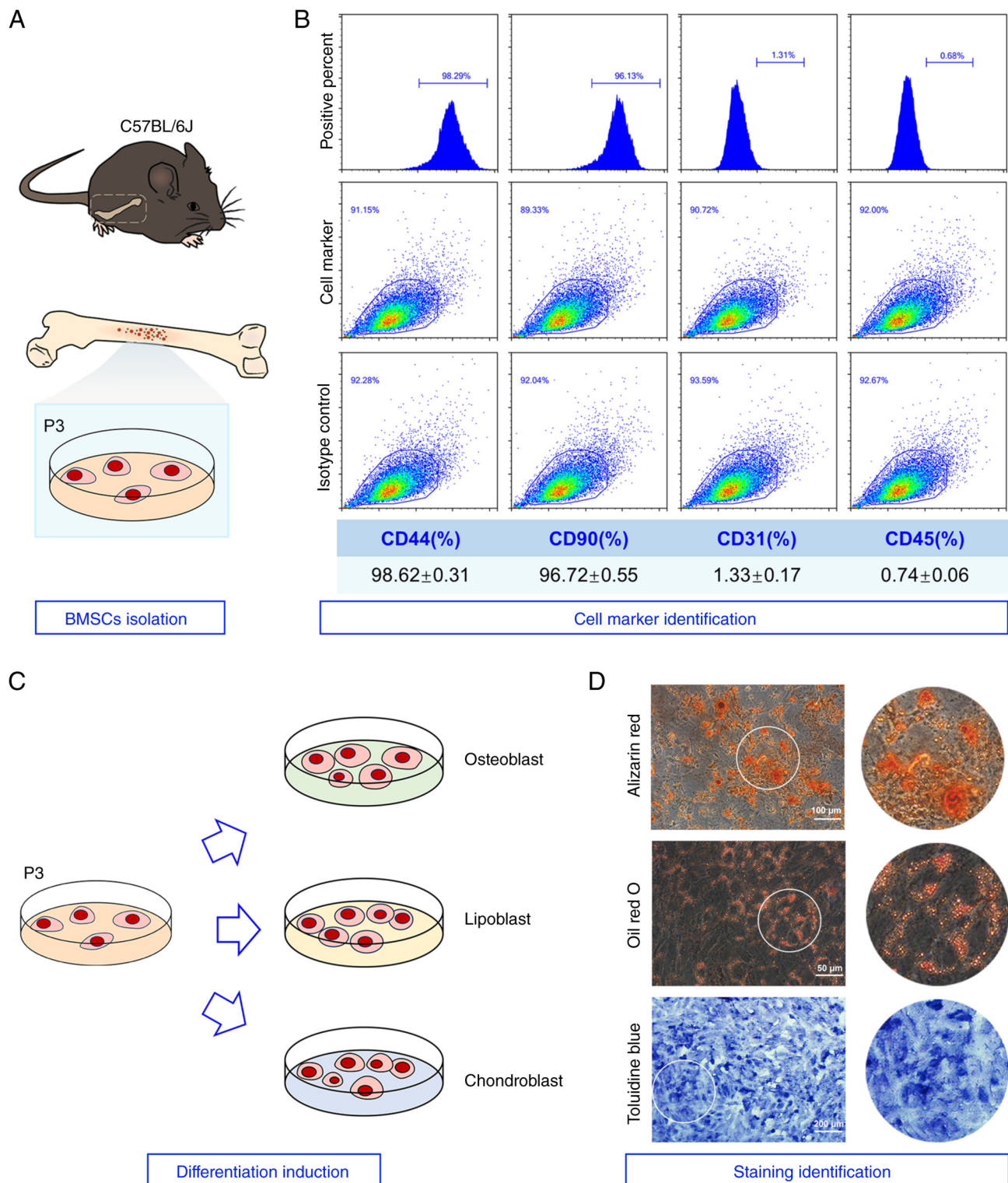


Figure 1. Isolation and identification of mouse BMSCs. (A) BMSCs were isolated from mouse bone marrow and cultured to the third passage for the following tests. (B) The expression of cell surface markers was examined by flow cytometry. (C) The cells were induced to differentiate into osteoblast, lipoblast and chondrocyte. (D) Detection of cell differentiation ability (n=3). BMSCs, bone marrow mesenchymal stem cells.

The present study aimed to modify BMSCs through a lentivirus-mediated Six3 overexpression system and then explore the role of Six3 in BMSCs (Fig. 3A). Following 48 h of infection, the successful infection by the lentivirus was observed under a fluorescence microscope (Fig. 3B). Additionally, it was observed that the overexpression of Six3 enhanced the proliferation of BMSCs. RT-qPCR and western blot analysis

revealed that Six3 was successfully overexpressed in BMSCs (Fig. 3C and D). The overexpression of Six3 enhanced BMSC viability (Fig. 3E) and promoted BMSC migration *in vitro* (Fig. 3F). According to the representative immunofluorescence images of neuronal marker proteins, the overexpression of Six3 increased the expression of Nestin (Fig. 3G), microtubule associated protein 2 (MAP2) (Fig. 3H) and neuronal nuclei

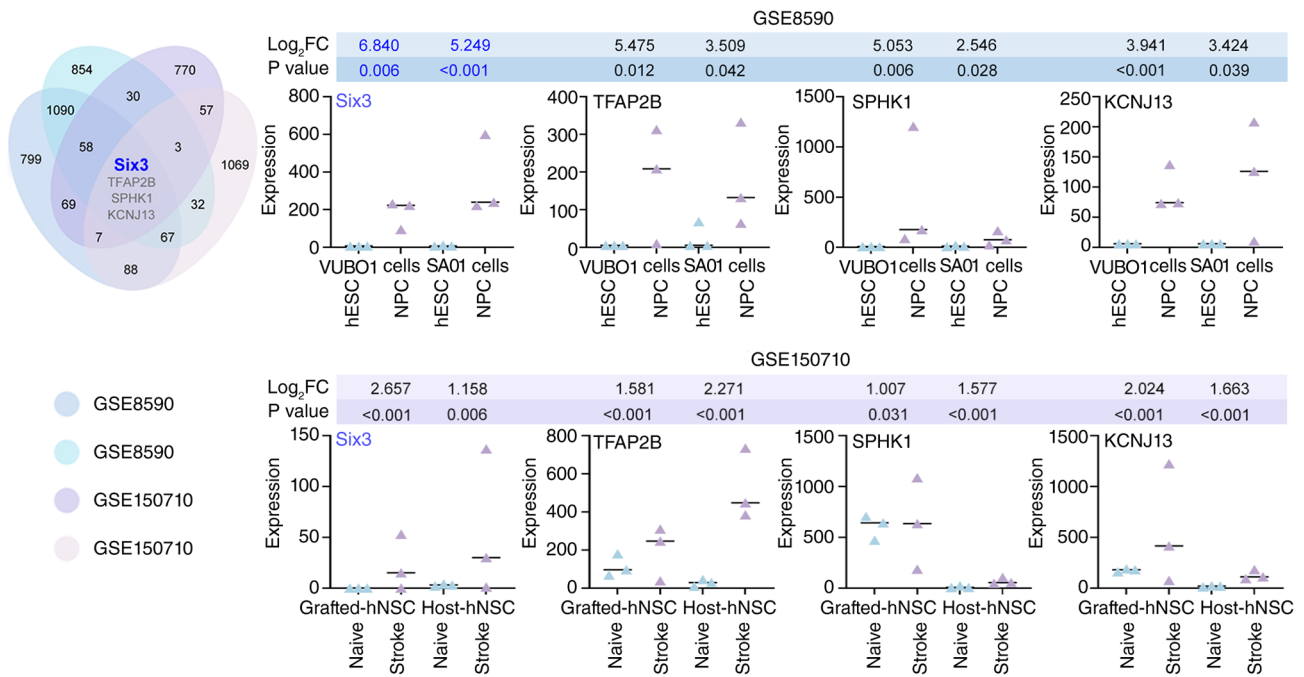


Figure 2. Bioinformatics analysis. Six3 was focused through a comprehensive analysis of up-regulated genes in the GSE8590 and GSE150710 datasets. Six3, Sine Oculis Homeobox Homolog 3; hNSC, human neural stem cells.

(NeuN) (Fig. 3I) in BMSCs. The levels of the neuronal growth factors, BDNF and NGF, in the cell supernatant were increased by the overexpression of Six3 (Fig. 3J). These results demonstrate the potential of Six3 to promote the differentiation of BMSCs into neurons.

Six3 overexpression activates Btg2 transcription and promotes its expression in BMSCs. As demonstrated in Fig. 4A, the results revealed a significant separation of BMSCs and BMSCS-Six3^{GFP} samples for transcriptomic analysis, and the favorable homogeneity of samples within the groups. The findings depicted in Fig. 4B also demonstrated that the overexpression of Six3 significantly altered the transcriptomes of BMSCs, including 150 downregulated genes and 323 upregulated genes ($|\text{Log}_2\text{FC}| > 1$, $P < 0.05$); among these, the expression of Six3 was significantly increased ($\text{Log}_2\text{FC} = 4.99$, $P < 0.05$), indicating that Six3 was successfully overexpressed (Fig. 4C). Through GO annotation and enrichment analysis of the aforementioned DEGs, it was observed that Six3 overexpression targets the genes related to neuronal death (Fig. 4D), including five downregulated genes and 26 upregulated genes (Fig. 4E). In order to further explore the mechanisms of Six3 overexpression, the upregulated or downregulated genes in the results of RNA-sequencing and DEGs in the GSE8590 dataset were comprehensively analyzed (Fig. 4F), and 13 shared genes were obtained. Among these, three genes were found to be enriched in terms related to neuronal death, including Btg2, Igfbp5 and Lrp2. It was observed that Btg2 was significantly increased following the overexpression of Six3 and had the highest basal expression in BMSCs. Therefore, Btg2 was selected for further analyses.

RT-qPCR and western blot analysis further confirmed that the overexpression of Six3 increased the expression of Btg2 (Fig. 5A and B). Dual luciferase report assay revealed that Six3

enhanced Btg2 promoter activity (Fig. 5C), and the ChIP-PCR results indicated that Six3 bound to the Btg2 promoter region (Fig. 5D). The Btg2 promoter binding protein was obtained through DNA pull-down assay, and the binding of Six3 and Btg2 promoter was further demonstrated by western blot analysis (Fig. 5E).

Six3 promotes BMSC viability and neural differentiation by promoting Btg2 expression. RT-qPCR and western blot analysis demonstrated that Btg2 expression in BMSCs was upregulated by the overexpression of Six3 and downregulated by the Btg2-targeting lentiviral system (Fig. 5F and G). Consistent with the aforementioned findings, the overexpression of Six3 increased the cell viability of BMSCs (Fig. 5H), the expression of neuronal marker proteins (MAP2 and Nestin) (Fig. 5I), and the secretion of BDNF and NGF (Fig. 5J). However, Btg2 knockdown reversed the effects of Six3; these findings indicated that Btg2 mediated the promoting effects of Six3 on the neural differentiation of BMSCs.

Six3 overexpression improves the therapeutic effects of BMSC transplantation on stroke. Mice subjected to MCAO exhibited moderate to severe neurological deficits (Fig. 6A), such as circling to the contralateral side. Mice transplanted with BMSCs exhibited mild neurological deficits. Representative TTC staining images revealed that BMSC transplantation alleviated cerebral ischemic injury in mice subjected to MCAO (Fig. 6B). Specifically, BMSC transplantation reduced brain swelling and cerebral infarction caused by MCAO. By observing the distribution of GFP in brain tissue, it was also observed that BMSCs were transplanted into the brain tissue (Fig. 6C). The 200-fold magnification images demonstrated GFP expression in the ischemic penumbra, demonstrating the migration of BMSCs to the injured area.

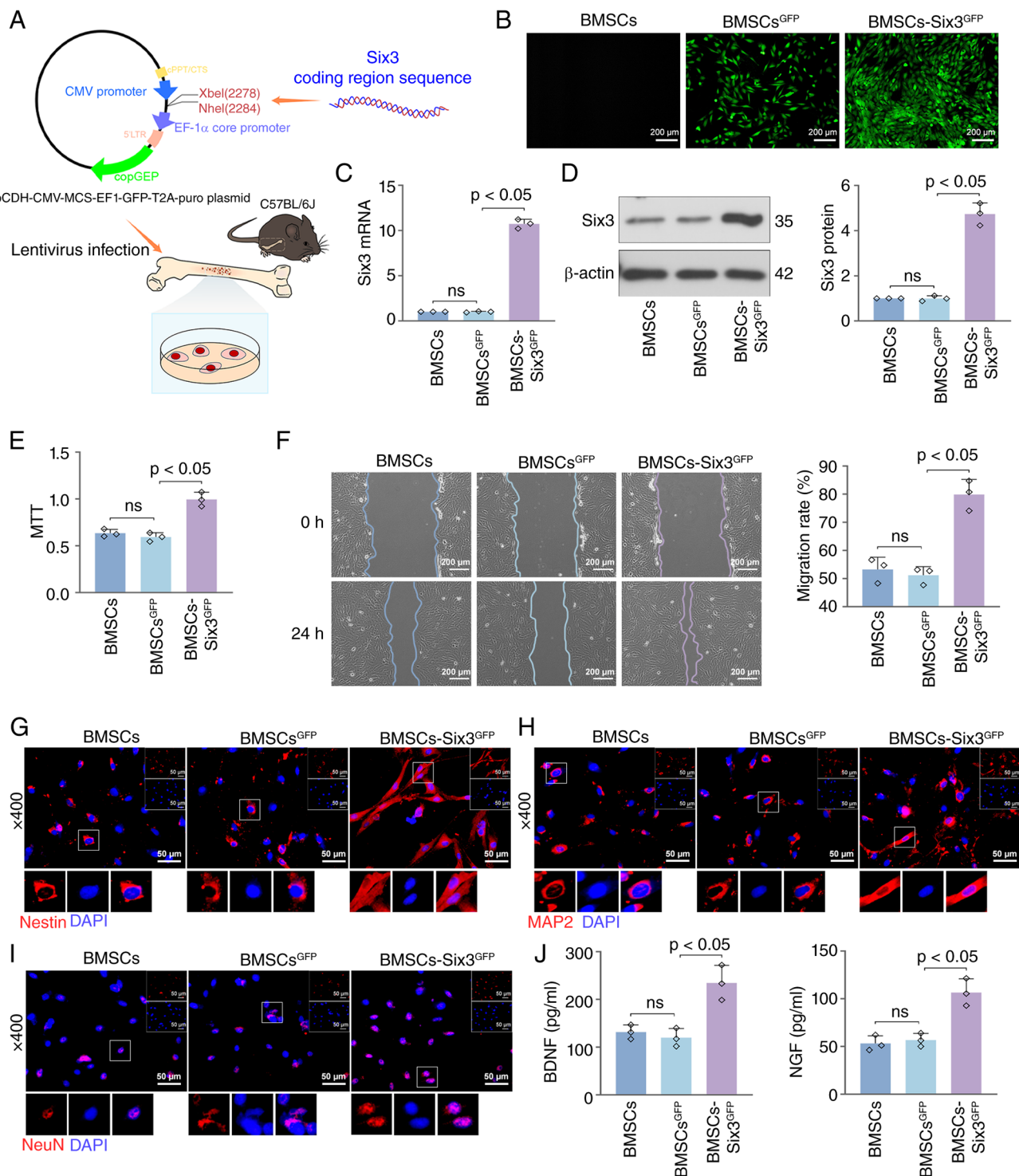


Figure 3. Six3-overexpressing BMSCs exhibits enhanced proliferation, migration and neural differentiation. (A) Six3 gene was overexpressed in the third-passage BMSCs via the lentiviral system. (B) Expression of GFP in BMSCs. (C and D) Examination of Six3 overexpression. (E) The cell viability of BMSCs was measured using MTT assay. (F) Cell migration was examined using wound healing test. Six3-overexpressing BMSCs were cultured using neural differentiation induction medium for 1 week and then the following analysis was performed. (G-I) Nerve cell markers were detected using immunofluorescent staining. (J) Levels of BDNF and NGF were detected using enzyme-linked immunosorbent assay. N=3. Six3, Sixe Oculis Homeobox Homolog 3; BMSCs, bone marrow mesenchymal stem cells; GFP, green fluorescent proteins; MAP2, microtubule associated protein 2; NeuN, neuronal nuclei; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor.

As demonstrated in Fig. 6D, the neural deficits were further improved in the mice subjected to MCAO and transplanted with Six3-overexpressing BMSCs. The infusion of BMSCs reduced the limb asymmetry in stroke-stricken mice. Mice infused with Six3 overexpressed BMSCs exhibited more

balanced limb use (Fig. 6E). The TTC staining results revealed that compared with BMSC transplantation, the transplantation of Six3-overexpressing BMSCs had a more prominent reducing effect on brain swelling and cerebral infarction (Fig. 6F).

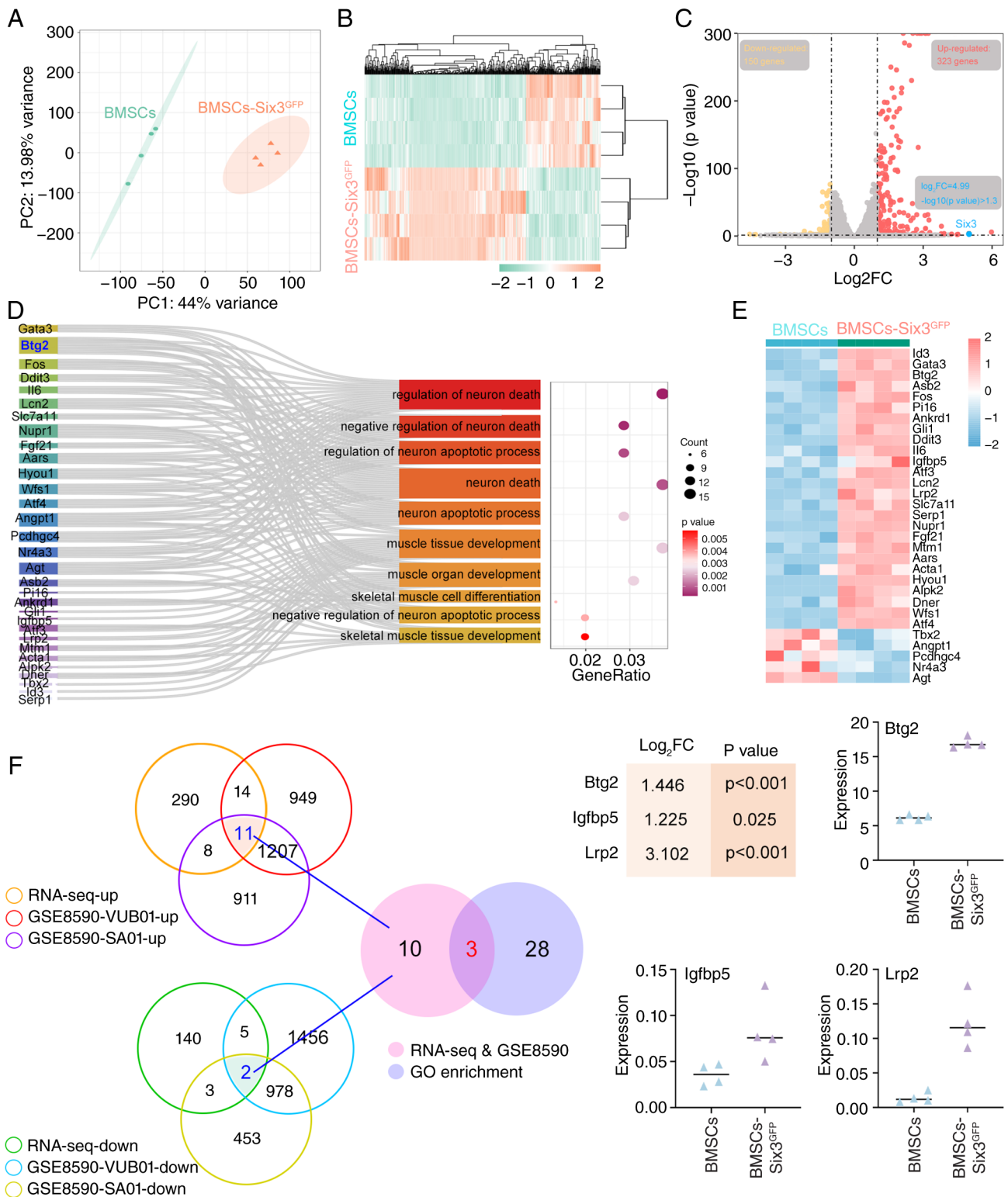


Figure 4. Target gene of Six3 is investigated through RNA-sequencing. (A) Principal components analysis. (B) Cluster analysis diagram and (C) volcano plot of DEGs. (D) GO enrichment analysis of DEGs. (E) Heatmap of gene expression covered by GO terms. (F) Btg2 was identified by conducting a comprehensive analysis of the DEGs from RNA-sequencing and GSE8590, as well as the genes included in the GO terms ($n=4$). Six3, Six3, Six3, Six3; Btg2, BTG anti-proliferation factor 2; GO Gene Ontology; DEGs, differentially expressed genes.

Neuronal atrophy and loss were detected in the brain tissue of mice subjected to MCAO, which was reduced by BMSC transplantation (Fig. 7A). The transplantation of Six3-overexpressing BMSCs further protected the morphology of neurons in the brain tissue of mice subjected

to MCAO and increased the number of neurons. The expression of Six3 and GFP in brain tissue is illustrated in Fig. 7B. The colocalization of Six3 and GFP expression was observed in the ischemic penumbra of mice transplanted with BMSCs. The high expression of Six3 in BMSCs was observed in the

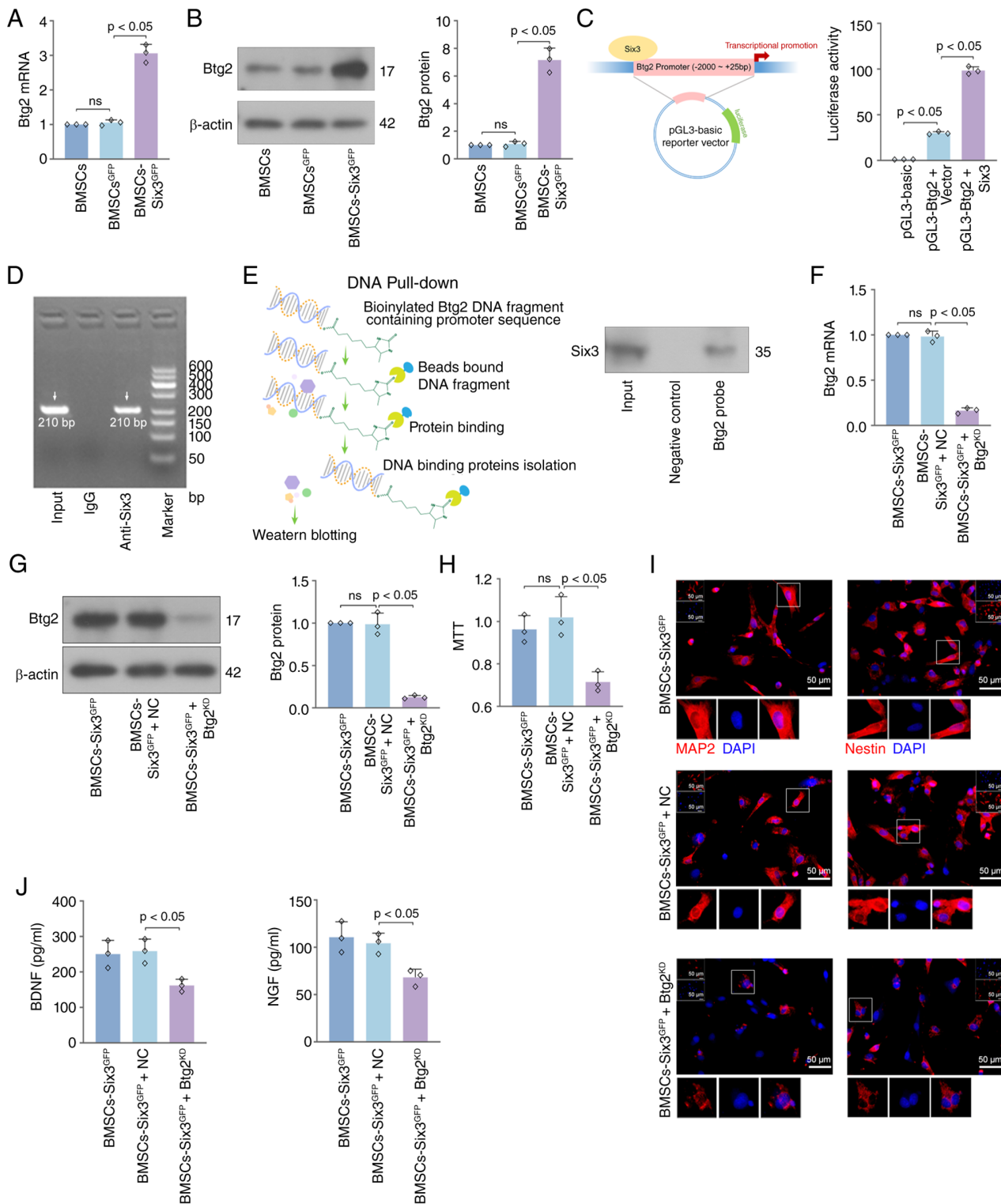


Figure 5. Six3 promotes BMSCs proliferation and neural differentiation by promoting Btg2 transcription. (A and B) Expression of Btg2 in BMSCs. (C) The enhancement of Btg2 promoter activity by Six3 was examined using a dual luciferase reporting system. (D) Binding of the Six3 and Btg2 promoter region was verified using RIP-PCR. (E) The Btg2 promoter binding protein was obtained using DNA pull-down, and then the expression of Six3 was detected using western blotting. Lentiviral vector system was used to mediate overexpression or interference of Six3 or Btg2 in third-passage BMSCs, and the following experiments were performed 48 h later. (F and G) Expression of Btg2 in BMSCs. (H) The cell viability of BMSCs was measured using MTT assay. (I) Nerve cell markers were detected using immunofluorescent staining. (J) Levels of BDNF and NGF were detected using enzyme-linked immunosorbent assay ($n=3$). Six3, Sine Oculis Homeobox Homolog 3; Btg2, BTG anti-proliferation factor 2; BMSCs, bone marrow mesenchymal stem cells; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; MAP2, microtubule associated protein 2.

brain tissues of mice in the MCAO + BMSCs-Six3 group. The expression of GFP is illustrated in Fig. 7C. Compared with the MCAO + BMSCs group, the overexpression of Six3

promoted the migration of BMSCs to the infarct area in brain tissue. As demonstrated in Fig. 7D and E, the expression of the neuronal markers, NeuN and MAP2, was increased in the

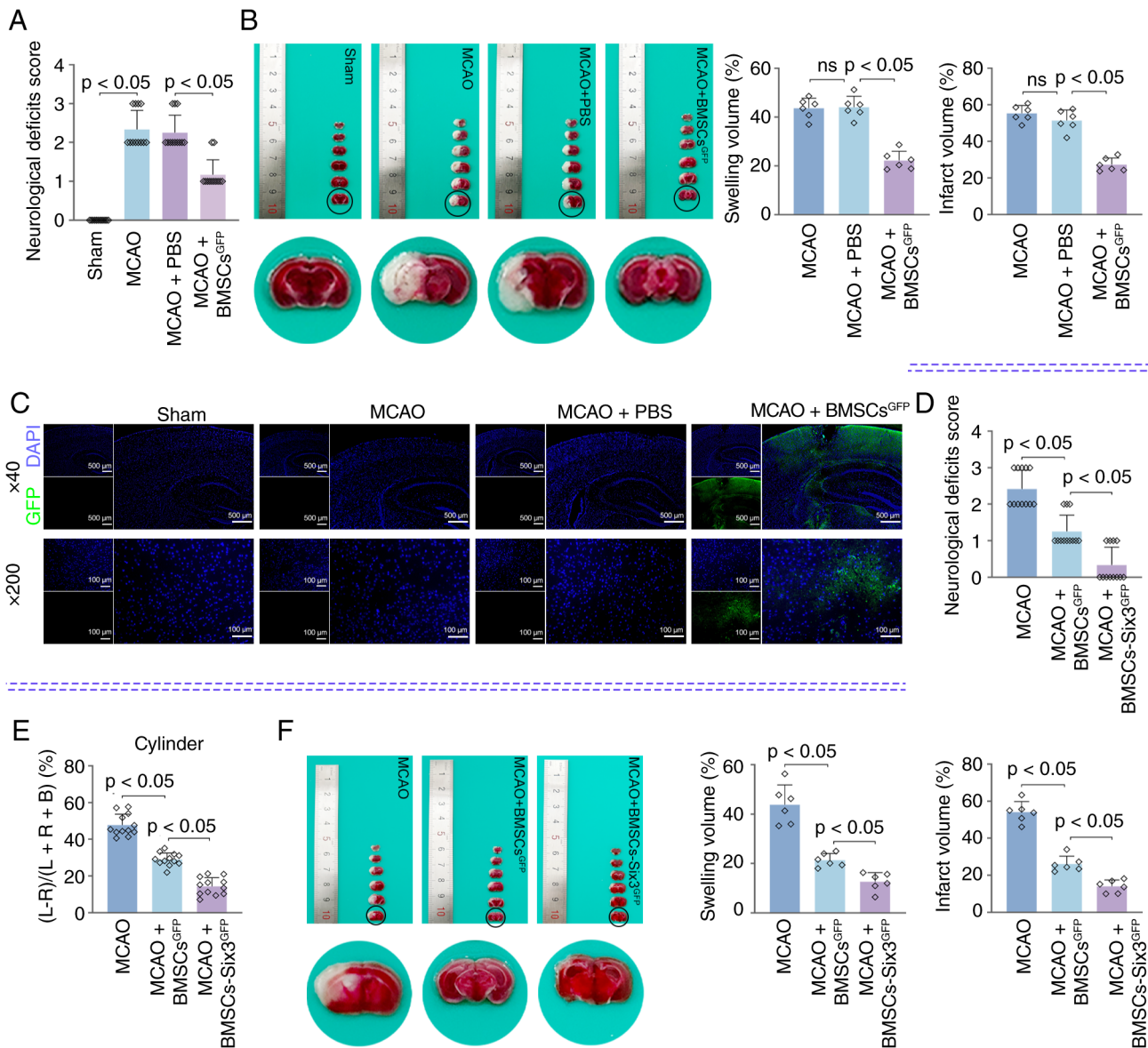


Figure 6. Overexpression of Six3 enhances the efficacy of BMSCs in the treatment of cerebral ischemic injury. Mice underwent reperfusion 1.5 h after MCAO and were injected with BMSCs through tail vein. The following experiments were performed 24 h later. (A) Neurological deficits score (n=12). (B) TTC staining of brain tissue, and quantification of infarct volume and swelling volume (n=6). (C) Expression of GFP in brain tissue (n=6). Mice underwent reperfusion 1.5 h after MCAO and were injected with Six3-overexpressing BMSCs through tail vein. The following experiments were performed 24 h later. (D) Neurological deficits score (n=12). (E) Cylinder test (n=12). (F) TTC staining of brain tissue, and quantification of infarct volume and swelling volume (n=6). BMSCs, bone marrow mesenchymal stem cells; MCAO, middle cerebral artery occlusion; GFP, green fluorescent proteins; TTC, triphenyl-tetrazolium chloride.

BMSCs within the host. Notably, the overexpression of Six3 in BMSCs enhanced the expression of these neuronal markers in BMSCs within the host brain tissue. These findings suggest that the overexpression of Six3 in BMSCs enhances their homing ability and neural differentiation in the damaged brain tissue of mice subjected to MCAO. Additionally, it was observed that the expression of Btg2 in the brain tissues of mice with stroke was increased following the transplantation of Six3-overexpressing BMSCs (Fig. 7F). As demonstrated in Fig. 7G, Btg2 was widely expressed in the brain tissues of mice subjected to MCAO. The colocalization of Btg2 and GFP was detected in the brain tissue of mice transplanted with BMSCs. The increased expression of Btg2 was observed in the Six3-overexpressing BMSCs within the injured brain tissue.

The aforementioned evidence indicates that the promoting effect of the Six3-Btg2 axis on the migration and neural differentiation of BMSCs *in vitro* is also observed in the brain tissues of mice subjected to MCAO that received BMSC transplantation.

Btg2 silencing reverses the effects of Six3 overexpression on BMSCs *in vivo*. The *in vivo* rescue experiments demonstrated that Btg2 mediated the enhancing effects of Six3 on the therapeutic efficacy of infused BMSCs for the treatment of stroke. The silencing of Btg2 led to an increase in the neurological deficit score (Fig. 8A), an increase in the asymmetry of limb use (Fig. 8B), an increase in cerebral infarction (Fig. 8C) and an increase in brain swelling (Fig. 8D). The double-stained

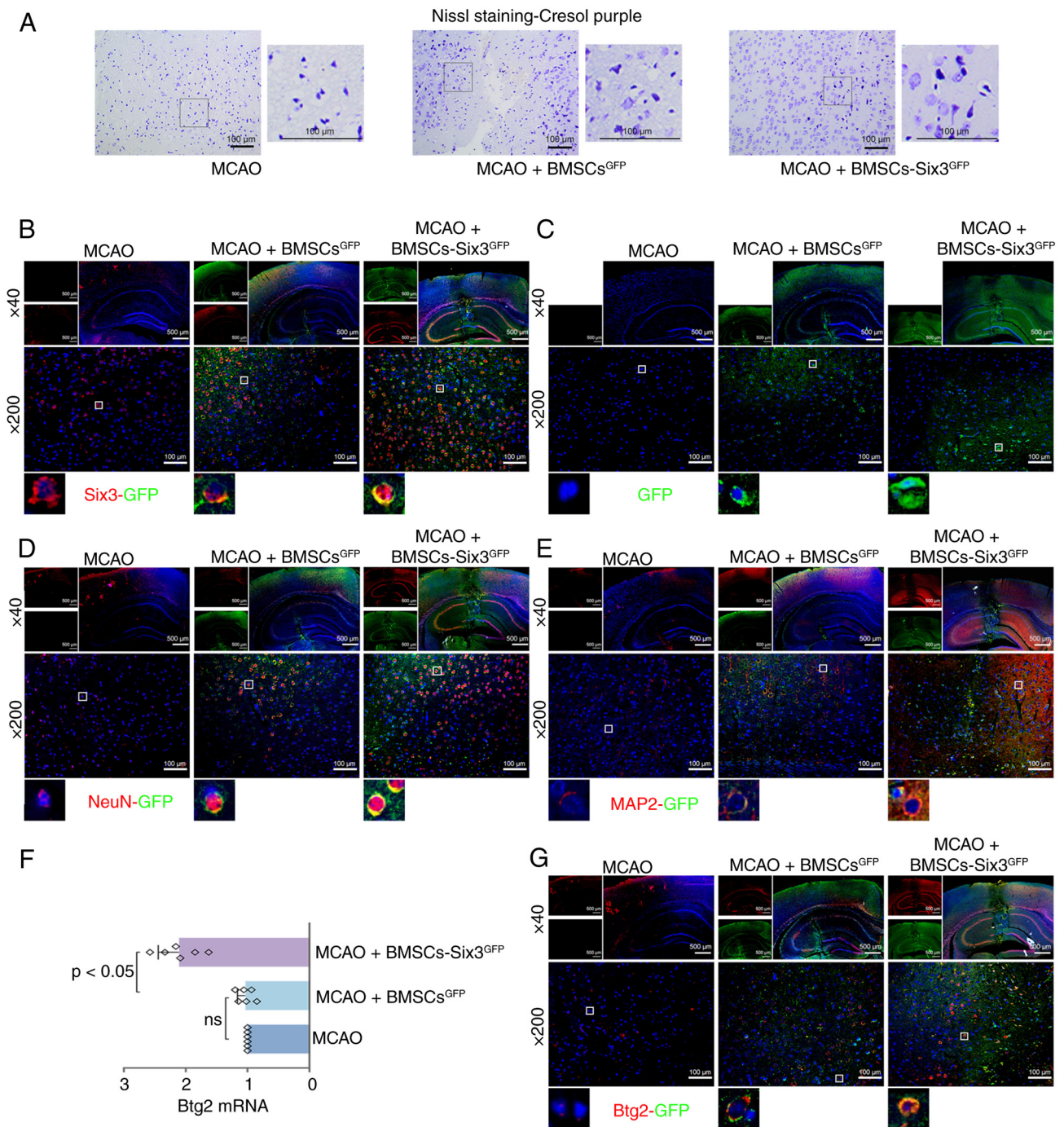
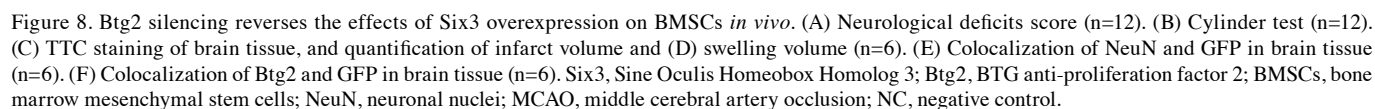


Figure 7. Overexpression of Six3 enhances the neural differentiation of BMSCs in cerebral ischemic injury, involving the transcriptional promotion of Btg2. (A) Nissl staining in brain tissue. (B) Colocalization of Six3 and GFP in brain tissue. (C) Expression of GFP in brain tissue. (D) Colocalization of NeuN and GFP in brain tissue. (E) Colocalization of MAP2 and GFP in brain tissue. (F) Expression of Btg2 in brain tissue. (G) Colocalization of Btg2 and GFP in brain tissue (n=6). Six3, Sine Oculis Homeobox Homolog 3; Btg2, BTG anti-proliferation factor 2; GFP, green fluorescent proteins; NeuN, neuronal nuclei; MAP2, microtubule associated protein 2; BMSCs, bone marrow mesenchymal stem cells; MCAO, middle cerebral artery occlusion; TTC, triphenyl-tetrazolium chloride.

immunofluorescence images confirmed the reduction of Btg2 expression in BMSCs (Fig. 8E). Btg2 knockdown abolished the promoting effects of Six3 on the neural differentiation of infused BMSCs (Fig. 8F).

Overexpression of Six3 enhances the neural differentiation of BMSCs by activating the Btg2-mediated Akt signaling pathway. Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the upregulated DEGs that were identified

through RNA sequencing was performed to investigate the regulatory effects of Six3 on downstream signaling pathways. The results revealed that the overexpression of Six3 primarily affected the PI3K-Akt signaling pathway (Fig. 9A). Elevated levels of Akt phosphorylation were observed in brain tissues of mice transplanted with Six3-overexpressing BMSCs (Fig. 9B). The silencing of Btg2 partially suppressed Akt phosphorylation in BMSCs induced by Six3 overexpression (Fig. 9C). It was demonstrated that the Akt signaling pathway was directly



Through the comprehensive analysis of GSE8590 and GSE150710 databases, the shared genes, *Six3*, *TFAP2B*, *SPHK1* and *KCNJ13*, were obtained. Through a review of the literature, it was found that the role of *Six3* in cerebral ischemia has not been revealed. According to current reports, *Six3* may play a critical important role in neuronal differentiation. *Six3* has been reported to promote neuronal differentiation by inhibiting *Wnt5a* expression in neural stem cells during early differentiation (12). It has been shown that the conditional deletion of *Six3* blocks the differentiation of precursor cells of medium spiny neurons, and the conditional overexpression of *Six3* promotes the differentiation of precursor cells in lateral ganglionic eminence (14). Although previous studies (12,14) have suggested the potential of *Six3* in promoting neuronal differentiation, the role of *Six3* on BMSCs remains unclear. Based on this, the present study used the *Six3*-overexpressing lentivirus system to enforce *Six3* expression in mouse BMSCs. The overexpression of *Six3* enhanced the viability and migration of BMSCs, a finding which was consistent with that of

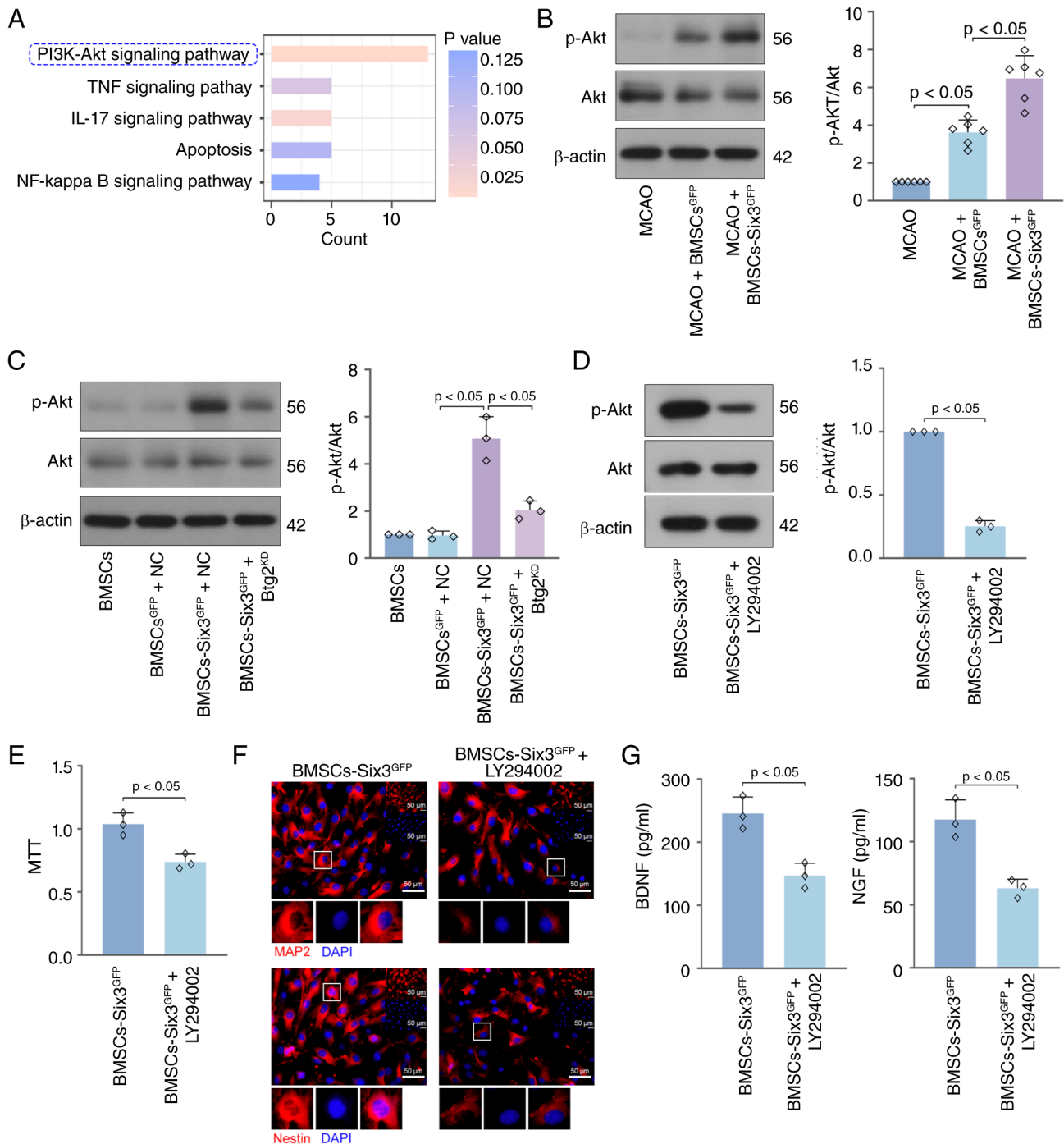


Figure 9. Overexpression of Six3 enhances neural differentiation of BMSCs activating the Btg2-mediated Akt signaling pathway. (A) Kyoto Encyclopedia of Genes and Genomes enrichment analysis of upregulated differentially expressed genes in Six3-overexpressed BMSCs. (B-D) Expression of p-Akt and Akt in (B) brain tissue (n=6) and (C and D) BMSCs (n=3). BMSCs overexpressing Six3 were treated with the PI3K inhibitor LY294002 for 24 h before subsequent experiments. (E) The cell viability of BMSCs was measured using MTT assay (n=3). (F) Nerve cell markers were detected using immunofluorescent staining (n=3). (G) Levels of BDNF and NGF were detected using enzyme-linked immunosorbent assay (n=3). Six3, Sine Oculis Homeobox Homolog 3; BMSCs, bone marrow mesenchymal stem cells; GFP, green fluorescent proteins; MAP2, microtubule associated protein 2; NC, negative control.

a previous study (13). The overexpression of Six3 promoted the expression of Nestin, MAP2 and NeuN in BMSCs, and increased the production of BDNF and NGF. The increase in these markers indicates that BMSCs differentiate into nerve cells, which can be found consistently in previously reported models of MCAO (33,37). This elucidated that the overexpression of Six3 promoted the differentiation of BMSCs into neurons.

In the present study, in order to further explore the downstream molecular mechanisms of Six3 regulating the therapeutic effects of BMSCs, the target genes of Six3 were analyzed using transcriptomics. The bioinformatics analysis results revealed three candidate genes, Btg2, Igfbp5 and Lrp2. Among these, Btg2 with a high basal expression was significantly upregulated in the genes of interest and may be involved in the regulation of neuronal cell death. During the

extensive literature review process, it was found that Btg2 promotes neuronal differentiation and inhibits the apoptosis of terminally differentiated PC12 nerve cells (21). Btg2 is highly expressed in the ischemic penumbra of the brain, participating in the survival and differentiation of nerve cells and exerting neuroprotective effects (22). Btg2 knockout has been shown to hinder the activation of the Akt signaling pathway in mouse embryonic fibroblasts and bone marrow progenitor cells (38). In addition, the activation of the Akt signaling pathway has been reported to promote the neural differentiation and migration of BMSCs (39,40). The aforementioned evidence prompted the authors to pay attention to Btg2 as a target gene for Six3 in regulating the neural differentiation and migration of BMSCs. The dual luciferase reporter and ChIP assays demonstrated that Six3 directly binds to the Btg2 promoter and activates Btg2 transcription. The rescue experiments revealed that Btg2 knockdown inhibited BMSC viability and neuronal differentiation, which reversed the effects of Six overexpression. The present study reported that the knockdown of Btg2 inhibited the Akt signaling pathway, consistent with its previously reported function. Therefore, it can be concluded that activation of the Six3/Btg2/Akt axis is an effective strategy for improving the therapeutic efficacy of BMSC transplantation in experimental stroke.

However, the significant critical role of the candidate genes other than Btg2 in the nervous system cannot be overlooked. IGFBP5 has been reported to be upregulated in the nerves of patients with diabetic neuropathy and to lead to neurodegenerative changes (41). It has been demonstrated that Igfbp5 knockdown reduces methamphetamine-induced neurobehavioral disorders and dopaminergic neuronal apoptosis (42). It has been reported that the absence of Lrp2 in the developing central nervous system of mice leads to impaired closure of the lateral cranial nerve canal and a reduction in folic acid uptake (43).

Furthermore, in the present study, the therapeutic effects of Six3-overexpressing BMSC transplantation on stroke were examined in mice subjected to MCAO. The intravenous infusion of Six3-overexpressing BMSCs demonstrated superior efficacy in the treatment of experimental ischemic injury than BMSCs alone, as evidenced by neuronal protection, infarction inhibition and a reduction in nerve defects, which was consistent with the results of the *in vitro* experiments and attributed to the improvement of the cell viability and migratory ability of BMSCs by Six3. The silencing of Btg2 reversed the effects of Six3 *in vivo*. This evidence indicates that the overexpression of Six3 is an effective genetic modification method for optimizing the therapeutic effects of BMSCs on stroke, by promoting the transcription of Btg2.

The present study adopted the administration route of tail vein injection, which is a common administration method due to its low invasiveness, simple operation and high repeatability (33,44). It is particularly suitable for non-invasive administration during the acute phase to avoid damage caused by surgery. Although tail vein injection requires BMSCs to pass through systemic veins and then cross the blood-brain barrier, this limits the number of BMSCs that can reach the ischemic brain tissues. However, the blood-brain barrier in MCAO mice is severely damaged, and this pathological condition facilitates the delivery of BMSCs to the injured

area through tail vein injection. Additionally, the present study aims to promote cell homing through genetic modification rather than seeking to achieve the maximum homing efficiency through drug administration. Therefore, a safe and easy-to-operate intravenous administration method was adopted, and the Six3 gene was overexpressed in BMSCs to enhance their homing efficiency. This not only improved the efficacy of BMSCs delivery *in vivo* but also ensured the safety and repeatability of the administration.

The present study serves as a preclinical experiment, with the core objective being to elucidate the regulatory role and molecular mechanisms of Six3 overexpression on the migration ability and neural differentiation potential of BMSCs. Therefore, the currently most mature and efficient lentiviral vector system, and the easy-to-operate tail vein injection were selected for this purpose. However, the authors are fully aware that the potential off-target effects caused by the random integration characteristics of lentiviral vectors are the main bottleneck restricting their future clinical application. In clinical transformation work, whole-genome sequencing for high-precision single-copy level analysis of integration sites may be an effective means to assess off-target effects. Moreover, it is necessary to consider that different administration routes, such as intranasal delivery, may also be suitable for BMSC gene modification and clinical use.

As regards the tumorigenicity and long-term effects of Six3 overexpression, although no malignant transformation phenotype was observed in the short-term *in vivo* experiments of the present study, due to the current experimental period and sample size, long-term tumorigenicity tests in immunodeficient mice or the tracking of the clonal dynamics of transplanted cells in the host body were not conducted. This constitutes a limitation of the present study. The current experimental evidence indicates that MSCs have a low tumorigenicity on their own (45,46). In addition, previous research has discovered safe gene-modified lentiviral vectors encoding herpes simplex virus thymidine kinase containing a point mutation that results in an alanine to histidine substitution at residue 168 [HSV-TK(A168H)], and it has been indicated that this vector would not cause the uncontrolled proliferation of human MSCs (47). This provides support for the future clinical transformation of gene modification.

Given these limitations, it is considered that before moving towards clinical application, further safe and controllable gene delivery strategies should be developed. Future research is required to focus on: i) Achieving the transient and safe expression of exogenous genes using non-integrative vectors (such as free plasmids, mRNA electroporation); ii) or using the CRISPR/Cas9-mediated site-specific integration technology to precisely insert therapeutic genes into the genome, fundamentally avoiding long-term risks associated with random insertion mutations and persistent overexpression.

In conclusion, the present study demonstrated that the overexpression of Six3 promoted the viability, migration and differentiation of BMSCs by activating Btg2 transcription, thereby enhancing the therapeutic effects of BMSC transplantation on stroke. The present study provides preclinical evidence for lentiviral system-mediated gene modification to improve the efficacy of BMSC transplantation for stroke.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Key Project of Provincial and Ministerial Co-construction for Medical Science and Technology Research in Henan (grant no. SBGJ202102141) and the Key Scientific Research Projects of Higher Education Institutions in Henan (grant no. 22A320047).

Availability of data and materials

The data generated in the present study may be found in the Gene Expression Omnibus under accession numbers GSE344610 or at the following URL: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE344610>.

Authors' contributions

ZF and ZL acquired funding, conceptualized the study, developed methodology, provided resources, conducted formal analysis, curated data and wrote the original draft. LZ and WH conducted investigation, formal analysis and data curation. HY and JW performed validation, formal analysis, visualization and data curation. HY and JW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Animal Ethics Committee of the First Affiliated Hospital of Zhengzhou University (approval no. 2025-KY-1279; Zhengzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- GBD 2016 Stroke Collaborators: Global, regional, and national burden of stroke, 1990-2016: A systematic analysis for the global burden of disease study 2016. *Lancet Neurol* 18: 439-458, 2019.
- Feigin VL and Krishnamurthi R: Stroke is largely preventable across the globe: Where to next? *Lancet* 388: 733-734, 2016.
- Andersen KK, Olsen TS, Dehlendorff C and Kammersgaard LP: Hemorrhagic and ischemic strokes compared: Stroke severity, mortality, and risk factors. *Stroke* 40: 2068-2072, 2009.
- Namiot ED, Niemi JVL, Chubarev VN, Tarasov VV and Schiöth HB: Stem cells in clinical trials on neurological disorders: Trends in stem cells origins, indications, and status of the clinical trials. *Int J Mol Sci* 23: 11453, 2022.
- Salehi MS, Safari A, Pandamooz S, Jurek B, Hooshmandi E, Owjifard M, Bayat M, Zafarmand SS, Miyan JA and Borhani-Haghighi A: The beneficial potential of genetically modified stem cells in the treatment of stroke: A review. *Stem Cell Rev Rep* 18: 412-440, 2022.
- Anthony S, Cabantan D, Monsour M and Borlongan CV: Neuroinflammation, stem cells, and stroke. *Stroke* 53: 1460-1472, 2022.
- Rawat M, Ng C, Khan E, Shah RA, Ashfaq S, Bahader GA and Shah ZA: Use of mesenchymal stem cell therapy in COVID-19 related strokes. *Neural Regen Res* 18: 1881-1883, 2023.
- Isaković J, Šerger K, Barišić B and Mitrežić D: Mesenchymal stem cell therapy for neurological disorders: The light or the dark side of the force? *Front Bioeng Biotechnol* 11: 1139359, 2023.
- Asgari Taei A, Khodabakhsh P, Nasoohi S, Farahmandfar M and Dargahi L: Paracrine effects of mesenchymal stem cells in ischemic stroke: Opportunities and challenges. *Mol Neurobiol* 59: 6281-6306, 2022.
- Li Y, Zhong W and Tang X: Strategies to improve the efficiency of transplantation with mesenchymal stem cells for the treatment of ischemic stroke: A review of recent progress. *Stem Cells Int* 2021: 9929128, 2021.
- Zhang Q, Zhou M, Wu X, Li Z, Liu B, Gao W, Yue J and Liu T: Promoting therapeutic angiogenesis of focal cerebral ischemia using thrombospondin-4 (TSP4) gene-modified bone marrow stromal cells (BMSCs) in a rat model. *J Transl Med* 17: 111, 2019.
- Shrestha P, Jaganathan A, Huilgol D, Ballon C, Hwangbo Y and Mills AA: Chd5 regulates the transcription factor Six3 to promote neuronal differentiation. *Stem Cells* 41: 242-251, 2023.
- Pandolfi EC, Hoffmann HM, Schoeller EL, Gorman MR and Mellon PL: Haploinsufficiency of SIX3 abolishes male reproductive behavior through disrupted olfactory development, and impairs female fertility through disrupted GnRH neuron migration. *Mol Neurobiol* 55: 8709-8727, 2018.
- Song X, Chen H, Shang Z, Du H, Li Z, Wen Y, Liu G, Qi D, You Y, Yang Z, *et al*: Homeobox gene Six3 is required for the differentiation of D2-type medium spiny neurons. *Neurosci Bull* 37: 985-998, 2021.
- Su Z, Wang Z, Lindtner S, Yang L, Shang Z, Tian Y, Guo R, You Y, Zhou W, Rubenstein JL, *et al*: Dlx1/2-dependent expression of Meis2 promotes neuronal fate determination in the mammalian striatum. *Development* 149: dev200035, 2022.
- Xu Z, Liang Q, Song X, Zhang Z, Lindtner S, Li Z, Wen Y, Liu G, Guo T, Qi D, *et al*: SP8 and SP9 coordinately promote D2-type medium spiny neuron production by activating Six3 expression. *Development* 145: dev165456, 2018.
- Zhao L, Tan S, Liao Q, Li X, Ke T and Li S: The neuroprotective effect and RNA-sequence analysis of postconditioning on the ischemic stroke with diabetes mellitus tree shrew model. *Brain Behav* 11: e2354, 2021.
- Matsuda S, Rouault J, Magaud J and Berthet C: In search of a function for the TIS21/PC3/BTG1/TOB family. *FEBS Lett* 497: 67-72, 2001.
- Tirone F: The gene PC3(TIS21/BTG2), prototype member of the PC3/BTG/TOB family: Regulator in control of cell growth, differentiation, and DNA repair? *J Cell Physiol* 187: 155-165, 2001.
- Micheli L, Ceccarelli M, Farioli-Vecchioli S and Tirone F: Control of the normal and pathological development of neural stem and progenitor cells by the PC3/Tis21/Btg2 and Btg1 genes. *J Cell Physiol* 230: 2881-2890, 2015.
- el-Ghissassi F, Valsesia-Wittmann S, Falette N, Duriez C, Walden PD and Puisieux A: BTG2(TIS21/PC3) induces neuronal differentiation and prevents apoptosis of terminally differentiated PC12 cells. *Oncogene* 21: 6772-6778, 2002.
- Slevin M, Sanfeliu C, Turu MM, Grau-Olivares M, Ferrer I, Boluda S, Marti-Fabregas J, Kumar S, Kumar P and Krupinski J: B-cell translocation gene 2 is over-expressed in peri-infarct neurons after ischaemic stroke. *Pathobiology* 76: 129-135, 2009.
- Lai JS, Cheng CW, Lo A, Sung TY and Hsu WL: Lipid exposure prediction enhances the inference of rotational angles of transmembrane helices. *BMC Bioinformatics* 14: 304, 2013.
- Li M, Zhao W, Gao Y, Hao P, Shang J, Duan H, Yang Z and Li X: Differentiation of bone marrow mesenchymal stem cells into neural lineage cells induced by bFGF-chitosan controlled release system. *Biomed Res Int* 2019: 5086297, 2019.
- Soleimani M and Nadri S: A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. *Nat Protoc* 4: 102-106, 2009.
- Zhang T, Lee YW, Rui YF, Cheng TY, Jiang XH and Li G: Bone marrow-derived mesenchymal stem cells promote growth and angiogenesis of breast and prostate tumors. *Stem Cell Res Ther* 4: 70, 2013.
- Huang S, Xu L, Sun Y, Wu T, Wang K and Li G: An improved protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. *J Orthop Translat* 3: 26-33, 2014.

28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
29. Kim D, Langmead B and Salzberg SL: HISAT: A fast spliced aligner with low memory requirements. *Nature Methods* 12: 357-360, 2015.
30. Anders S, Pyl PT and Huber W: HTSeq-a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31: 166-169, 2015.
31. Love MI, Huber W and Anders S: Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15: 550, 2014.
32. Sampei K, Goto S, Alkayed NJ, Crain BJ, Korach KS, Traystman RJ, Demas GE, Nelson RJ and Hurn PD: Stroke in estrogen receptor-alpha-deficient mice. *Stroke* 31: 738-744, 2000.
33. Ikegame Y, Yamashita K, Hayashi S, Mizuno H, Tawada M, You F, Yamada K, Tanaka Y, Egashira Y, Nakashima S, *et al*: Comparison of mesenchymal stem cells from adipose tissue and bone marrow for ischemic stroke therapy. *Cytotherapy* 13: 675-685, 2011.
34. Chang YS, Mu D, Wendland M, Sheldon RA, Vexler ZS, McQuillen PS and Ferriero DM: Erythropoietin improves functional and histological outcome in neonatal stroke. *Pediatr Res* 58: 106-111, 2005.
35. Shvedova M, Islam MR, Armondas AA, Anfinogenova ND, Wrann CD and Atochin DN: Modified middle cerebral artery occlusion model provides detailed intraoperative cerebral blood flow registration and improves neurobehavioral evaluation. *J Neurosci Methods* 358: 109179, 2021.
36. Shan H, Gao L, Zhao S, Dou Z and Pan Y: Bone marrow mesenchymal stem cells with PTBP1 knockdown protect against cerebral ischemia-reperfusion injury by inhibiting ferroptosis via the JNK/P38 pathway in rats. *Neuroscience* 560: 130-142, 2024.
37. Shichinohe H, Ishihara T, Takahashi K, Tanaka Y, Miyamoto M, Yamauchi T, Saito H, Takemoto H, Houkin K and Kuroda S: Bone marrow stromal cells rescue ischemic brain by trophic effects and phenotypic change toward neural cells. *Neurorehabil Neural Repair* 29: 80-89, 2015.
38. Kim BC, Ryu MS, Oh SP and Lim IK: TIS21/(BTG2) negatively regulates estradiol-stimulated expansion of hematopoietic stem cells by derepressing Akt phosphorylation and inhibiting mTOR signal transduction. *Stem Cells* 26: 2339-2348, 2008.
39. Lan XY, Liang XS, Cao MX, Qin HM, Chu CY, Boltze J and Li S: NCAM mimetic peptide P2 synergizes with bone marrow mesenchymal stem cells in promoting functional recovery after stroke. *J Cereb Blood Flow Metab* 44: 1128-1144, 2024.
40. Bing W, Pang X, Qu Q, Bai X, Yang W, Bi Y and Bi X: Simvastatin improves the homing of BMSCs via the PI3K/AKT/miR-9 pathway. *J Cell Mol Med* 20: 949-961, 2016.
41. Simon CM, Rauskolb S, Gunnersen JM, Holtmann B, Drepper C, Dombert B, Braga M, Wiese S, Jablonka S, Pühringer D, *et al*: Dysregulated IGFBP5 expression causes axon degeneration and motoneuron loss in diabetic neuropathy. *Acta Neuropathol* 130: 373-387, 2015.
42. Qiao D, Xu J, Le C, Huang E, Liu C, Qiu P, Lin Z, Xie WB and Wang H: Insulin-like growth factor binding protein 5 (IGFBP5) mediates methamphetamine-induced dopaminergic neuron apoptosis. *Toxicol Lett* 230: 444-453, 2014.
43. Kur E, Mecklenburg N, Cabrera RM, Willnow TE and Hammes A: LRP2 mediates folate uptake in the developing neural tube. *J Cell Sci* 127: 2261-2268, 2014.
44. Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M and Chopp M: Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 32: 1005-1011, 2001.
45. Wang Y, Yi H and Song Y: The safety of MSC therapy over the past 15 years: A meta-analysis. *Stem Cell Res Ther* 12: 545, 2021.
46. Chin SP, Ng WC, Tai L, Mohd MA, Then KY and Cheong SK: Umbilical cord-derived mesenchymal stem cells (CLV-100) infusion in healthy subjects: A 5-year follow-up study on safety and immunomodulatory effect. *Regenerative Eng Transl Med* 11: 723-736, 2025.
47. Bashyal N, Lee TY, Chang DY, Jung JH, Kim MG, Acharya R, Kim SS, Oh IH and Suh-Kim H: Improving the safety of mesenchymal stem cell-based ex vivo therapy using herpes simplex virus thymidine kinase. *Mol Cells* 45: 479-494, 2022.



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