

Original Article

Engrailed 2 enhances neuronal differentiation of bone marrow mesenchymal stem cells to promote spinal cord injury repair

Hongpeng Ma^{1,2,3,4,†}, Yan Zhang^{1,2,3,4,†}, Shibo Zhu^{5,†}, Song Liu^{1,2,3,4}, Baicao Li^{1,2,3,4}, and Guangzhi Ning^{1,2,3,4,*}

¹Department of Orthopedics, Tianjin Medical University General Hospital, Tianjin 300052, China, ²International Science and Technology Cooperation Base of Spinal Cord Injury, Tianjin 300052, China, ³Tianjin Key Laboratory of Spine and Spinal Cord Injury, Tianjin 300052, China, ⁴Tianjin Institute of Orthopedic Innovation and Transformation, Tianjin 300052, China, and ⁵Tianjin Medical University Cancer Institute & Hospital, National Clinical Research Center for Cancer, Tianjin 300052, China

[†]These authors contributed equally to this work.

*Correspondence address. E-mail: gzning@tmu.edu.cn

Received 4 November 2025 Accepted 13 April 2026 Published 29 July 2026

Abstract

Bone marrow mesenchymal stem cells (BMSCs) hold promise for spinal cord injury (SCI) repair, yet their limited neuronal conversion efficiency restricts therapeutic outcomes. Therefore, this study investigates the role of Engrailed 2 (En2) in regulating the neuronal differentiation of BMSCs and its therapeutic potential in SCI. Here, we demonstrate that En2 markedly enhances the neuronal differentiation of BMSCs and promotes functional recovery after SCI. En2 overexpression increases TUJ1⁺ and MAP2⁺ neuronal populations *in vitro*, while transcription factor prediction and experimental validation identify ASCL1 as a key downstream target. Silencing of ASCL1 significantly impairs En2-induced neuronal differentiation and reduces nuclear β -catenin level, indicating that En2 regulates BMSC neurogenesis through the ASCL1/ β -catenin axis. *In vivo*, En2-overexpressing BMSCs delivered via HAMA hydrogel survive, differentiate into neurons, improve tissue preservation, and enhance locomotor recovery in rats with SCI, as confirmed by histology, immunofluorescence, BBB scoring, and CatWalk analysis. These findings reveal a previously unrecognized regulatory mechanism whereby En2 drives the neuronal commitment of BMSCs and highlight a promising gene-enhanced stem cell strategy for spinal cord injury repair.

Key words Engrailed 2, spinal cord injury, bone marrow mesenchymal stem cells, Achaete-scute family bHLH transcription factor 1 (ASCL1), neuronal differentiation

Introduction

Spinal cord injury (SCI) is a severe traumatic disease of the central nervous system that is typically caused by accidents, violence, or sports-related injuries [1–3]. It often leads to long-term motor dysfunction, neuropathic pain, or autonomic nervous system abnormalities [4,5]. Although current treatment methods, including pharmacotherapy, physical therapy, and surgery, can promote functional recovery to some extent, effective therapeutic approaches for SCI remain limited [6–10]. Therefore, there is an urgent need for innovative treatment strategies to promote the regeneration of neural circuits and functional recovery in SCI patients.

In recent years, stem cell transplantation has become one of the research hotspots in the field of SCI repair [11]. Autologous bone marrow mesenchymal stem cells (BMSCs) have emerged as an ideal choice for stem cell-based therapy due to their low immunogenicity and ease of acquisition [12]. BMSCs possess the abilities to differentiate and self-renew and can release cytokines to reduce inflammation at the injury site, making them widely applied in the potential treatment of various diseases [8,13–16]. In SCI treatment, transplantation has been extensively studied in clinical trials and has been shown to play a positive role in spinal cord repair, especially in improving bladder function [17,18]. However, the neuronal conversion rate of BMSCs remains low, preventing the

complete reconstruction of neural circuits and failing to achieve the desired rehabilitation outcomes for patients.

Engrailed-2 (En2) is a homeobox transcription factor that is expressed in dopaminergic neurons of the midbrain from developmental stages through adulthood, where it plays a crucial role in regulating the survival of these neurons during development [19,20]. In addition to its function as a transcription factor, En2 can also be secreted by cells and act as an axon guidance signal involved in the establishment of neural circuits. In developing axons, En2 guides directional growth through local protein synthesis mechanisms [21,22]. Previous studies have shown that En2 can influence the differentiation of progenitor cells derived from the basal ganglia, and its deletion has profound effects on hippocampal neurogenesis, forebrain projections, and the development of noradrenergic (NE) neurons in the locus coeruleus. More importantly, En2 has been shown to inhibit the proliferation of neural stem cells, promote their survival, and direct their differentiation into neurons [23].

Given the low efficiency of neuronal conversion in BMSCs, this study aims to investigate whether the regulation of En2 can effectively promote the differentiation of BMSCs into neurons and further evaluate its therapeutic potential in SCI repair through cell transplantation in a mouse model.

In this study, we utilized flow cytometry and immunofluorescence techniques to quantitatively analyze the number of neurons differentiated from BMSCs. The results indicate that En2 overexpression in BMSCs significantly increases the number of neuronal cells. Through transcription factor binding site (TFBS) database prediction, we identify *ASCL1* as a downstream target gene of En2, and silencing of *ASCL1* significantly reduces the number of neurons. Furthermore, the transplantation of En2-overexpressing BMSCs using a hydrogel carrier significantly improves the repair outcomes of spinal cord injury. These findings provide new insights into stem cell-based therapeutic strategies for SCI.

Materials and Methods

Animals

Female Wistar rats (190–210 g) were purchased from Beijing Charles River Laboratory Animal Technology Co., Ltd (Beijing, China) and housed under the following conditions: 21–23°C, 55% humidity, and a 12-h light/dark cycle with *ad libitum* access to food and water. The Institutional Review Board of Tianjin Medical University General Hospital granted approval for all animal experiments (IRB2025-DW-51). All animal protocols were conducted in accordance with the National Institutes of Health guidelines for animal research.

Plasmid construction

The En2 overexpression plasmid was constructed by inserting the coding DNA sequence (CDS) of human En2 (GenBank accession number: NM_001427) into the empty pcDNA3.1 empty vector (V790-20; Invitrogen, Carlsbad, USA), and the full-length En2 CDS fragment was synthesized by General Biological (Chuzhou, China).

For lentiviral-mediated rat En2 overexpression, the En2 CDS sequence (GenBank accession number: NM_001109214.1) was amplified and cloned into the multiple cloning site of the pCDH-CMV-MCS-EF1-GFP-Puro shuttle vector (BR318; Fenghui Biotechnology, Changsha, China) with the restriction endonucleases *NheI* and *NotI*. This shuttle vector contains a GFP (green fluorescent

protein) expression cassette driven by the *EF1* promoter, which allows the expression of GFP in infected cells, serving as a reporter for evaluating lentiviral infection efficiency. Lentiviral packaging was performed using the psPAX2 packaging vector (BR036; Fenghui Biotechnology) and pMD2.G envelope vector (BR037; Fenghui Biotechnology).

ASCL1 knockdown was performed using LV-*ASCL1*-shRNA, which was constructed on the basis of the pLVX-shRNA vector (BR004; Fenghui Biotechnology). The sequence of LV-*ASCL1*-shRNA was as follows: 5'-ccgCAACTACTCCAACGACTTGAAttcaagagaTTCAAGTCGTTGGAGTAGTTGttttt-3'; and the LV-NC-shRNA sequence was as follows: 5'-ccgTTCTCCGAACGTGTCACGTtcaaga gaACGTGACACGTTCCGAGAAttttt-3'.

Cell culture

Bone marrow cell suspension were harvested from 2-week-old Wistar rats and grown in DMEM (C11995500BT; Gibco, Boston, USA) supplemented with 10% fetal bovine serum (FBS; 26170-035; Gibco). After isolation of the bone marrow mesenchymal stem cells (BMSCs), the medium was changed every 3 days, and cell surface markers were analyzed by flow cytometry.

BMSCs under different treatment conditions were divided into four groups: Control, Diff-M, Diff-M + Veh, and Diff-M + En2-OE. BMSCs were seeded at a density of 2×10^4 cells/cm² and cultured for 24 h to allow cell adherence until reaching 70%–80% confluence. The cells were then observed for growth status, and if in good condition, the medium was aspirated, and half of the volume of fresh medium was added. The LV-*ASCL1*-shRNA lentivirus and En2-overexpressing lentivirus was then added according to the formula: Virus volume per well (μL) = MOI × Cell number / Virus titer (TU/mL) × 1000. Polybrene may be added if necessary to increase infection efficiency. After mixing, the cells were incubated at 37°C in a 5% CO₂ incubator. After 72 h of viral infection, the cells were induced to differentiate into neurons, and after 7 days, the cells were collected for further analysis. The differentiation medium consisted of DMEM/F12 (11320-033; Gibco) supplemented with 0.5% FBS, 1% penicillin-streptomycin (10378616; Gibco), 1% glutamine (25030081; Gibco), 1% N2 supplement (BL320A; Biosharp, Hefei, China), 20 ng/mL basic fibroblast growth factor (bFGF) (HY-P7091; MCE, Monmouth Junction, USA), 200 nM trichostatin A (TSA) (T129665; Aladdin, Shanghai, China), 10 μM RG-108 (Macklin, Shanghai, China), 10 μM 8-Br-cAMP (HY-12306A; MCE), and 1 μM rolipram (R129674; Aladdin).

SCI model establishment

Wistar rats were randomly divided into Sham, SCI, HAMA + Veh and HAMA + En2-OE groups. After anesthesia, SCI model was established using the Allen method by using an Impactor Model 3 device (RWD, Shenzhen, China) [24]. The impactor was positioned vertically above the exposed spinal cord, and the impact rod was slowly lowered until the indicator light turned on, indicating that the tip had contacted the spinal cord surface. The position was adjusted to ensure the impact tip was aligned with the midline of the spinal cord. The tip (20 g) was then raised, and the impact height was set to 25 cm. The impact was delivered by pressing the trigger, and the rod was immediately withdrawn afterward. Successful model establishment was confirmed by the presence of tail flicking, spasmodic retraction of the hind limbs, and hematoma formation at the injury site, without rupture of the spinal dura mater.

Cell transplantation

The Sham group only underwent laminectomy. The treatment groups (HAMA + Veh and HAMA + En2-OE groups) received an injection of 10 μ L hydrogel/cell suspension (10^5 cells/ μ L) at the center and four corners of the injury site, with 2 μ L injected at each point following SCI. The Sham and SCI groups were injected with an equal volume of saline.

Cell counting kit-8 assay

Cells were seeded into a 96-well plate at a density of 5000 cells per well and incubated for 24 h. After treatment, 10 μ L of Cell Counting Kit-8 (CCK-8) reagent (CK04; Dojindo, Tokyo, Japan) was added to each well and incubated at 37°C for 2 h, the absorbance was measured at 450 nm using a Synergy H1 microplate reader with Gen5 software (Bio-Tek, Winooski, USA).

Flow cytometry

To determine the source of rat BMSCs, cells were isolated using trypsin-EDTA and then suspended as single-cell suspensions. The cells were subsequently stained with specific antibodies against CD45 (11-0900-81, 1:200; eBioscience, San Diego, USA), CD90 (SC-18917, 1:150; Santa Cruz Biotechnology, Dallas, USA), CD34 (11-0461-80, 1:200; eBioscience), and TUJ1 (SC-80005, 1:200; Santa Cruz Biotechnology). Fluorescence-activated cell sorting (FACS) (BD LSRFortessa; BD Biosciences, San Jose, USA) was then used to assess the sorted cells.

Western blotting

Cells were lysed with RIPA buffer (P0013C; Beyotime, Shanghai, China). The supernatant was collected by centrifugation and then mixed with sample loading buffer and heated to 100°C. Gels were prepared using PAGE gel quick preparation kit (PG112, PG113; Yamei, Shanghai, China) according to a previously described procedure [25] and placed in an electrophoresis tank with running buffer, and samples were loaded after removing bubbles. Electrophoresis was performed at 60 V initially, then increased to 120 V once the samples entered the resolving gel, and stopped when the bromophenol blue dye front reached the bottom. After electrophoresis, PVDF membranes (IPVH00010; Millipore, Billerica, USA) were activated in methanol for 5 min, equilibrated with the gel in transfer buffer, and assembled for transfer. Proteins were transferred at 360 mA for 1 h. Membranes were then blocked with blocking buffer (P0240; Beyotime) at room temperature for 1 h and incubated with primary antibodies overnight at 4°C. After washing, membranes were incubated with secondary antibodies at room temperature for 1 h. Finally, signals were detected using ECL reagent (P0018M; Beyotime) and imaged with ChemiDoc XRS+ (Bio-Rad, Hercules, USA). The following primary antibodies were used: En2 antibody (A17480, 1:1000; ABclonal, Wuhan, China), β -catenin antibody (A11932, 1:1000; ABclonal), ASCL1 antibody (370024, 1:1000; Zenbio, Durham, USA), histone H3 antibody (17168-1-AP, 1:1000; Proteintech, Shanghai, China), and GAPDH antibody (60004-1-Ig, 1:50,000; Proteintech). Secondary antibodies used were HRP-conjugated goat anti-rabbit IgG(H+L) (A0208, 1:1000; Beyotime) and AP-conjugated goat anti-mouse IgG(H+L) (A0258, 1:1000; Beyotime).

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde solution and blocked

with 5% bovine serum albumin (BSA) solution (SW3015; Solarbio, Beijing, China). After overnight incubation with the primary antibody at 4°C, the cells were incubated with the appropriate secondary antibody at room temperature for 1 h. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) (D9542; Sigma, St Louis, USA). Images were captured using fluorescence microscope (LSM900; Carl Zeiss, Oberkochen, Germany). The primary antibodies used were TUJ1 antibody (A17913, 1:500; ABclonal), MAP2 antibody (17490-1-AP, 1:500; Proteintech), SYN-1 antibody (ab254349, 1:500; Abcam, Cambridge, Britain), PSD95 antibody (ab13552, 1:500; Abcam), and β -catenin antibody (A11932, 1:500; ABclonal). The secondary antibodies included FITC-conjugated goat anti-mouse antibody IgG(H+L) (A0568; Beyotime), Alexa Fluor 488-conjugated goat anti-rabbit antibody IgG(H+L) (A0423; Beyotime), Alexa Fluor 488-conjugated donkey anti-rabbit antibody IgG(H+L) (ab150075, 1:500; Abcam), and Alexa Fluor 488-conjugated donkey anti-mouse antibody IgG(H+L) (ab150105, 1:500; Abcam).

Quantitative real-time PCR

Total RNA was extracted from cells using TRIzol reagent (15596-026; Invitrogen, Waltham, USA). RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (11150ES60; Yeasen, Shanghai, China). Gene expression was detected using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Quantitative PCR was performed with an initial pre-denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Melting curve analysis was conducted to verify amplification specificity, and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. *GAPDH* was used as an internal control. Each detection was performed in triplicate. The primers used were as follows: *ASCL1*: 5'-GCCAACAAGAAGATGAGCAAG-3' (forward) and 5'-GGAGATGGTGGGCGACA-3' (reverse); *En2*: 5'-GAACCCGAACAAAGAGGAC-3' (forward) and 5'-CCGTGGCTTTCTTGATT-3' (reverse); *MAP2*: 5'-CTTTGCCGCTCCAGTTC-3' (forward) and 5'-ATCGTCGTAGGTTTCTGTTTT-3' (reverse); *TUJ1*: 5'-CCTTCA TCGCAACAGCA-3' (forward) and 5'-CCTCGTCGTCATCTTCATCA-3' (reverse); *GAPDH*: 5'-CGGCAAGTTCAACGCGACAG-3' (forward) and 5'-CGCCAGTAGACTCCACGACAT-3' (reverse). The corresponding melting curves are shown in [Supplementary Figure S1](#).

En2 binding site sequence screening

To systematically screen potential downstream target genes regulated by the transcription factor En2, bioinformatics prediction analysis was performed based on the TFBS database. The canonical binding motif of En2 was obtained from the JASPAR database (jaspar.elixir.no), and the entire protein-coding genome was scanned for potential binding sites. The promoter region, approximately -2000 bp to +500 bp relative to the transcription start site (TSS), was analyzed to identify potential binding sites that highly match the En2 binding motif. Additionally, known enhancer regions were used to further analyze distal regulatory elements that may contain En2 binding sites.

Gene Ontology functional enrichment and Sankey visualization

After obtaining the potential downstream target gene set of En2, Gene Ontology (GO) functional enrichment analysis was performed using the ClusterProfiler package in R (version 4.3.1). Enrichment

significance was calculated using the hypergeometric distribution model, and multiple testing correction was applied using the Benjamini-Hochberg method (adjusted $P < 0.05$). GO terms related to neurogenesis and neuronal differentiation were selected for further analysis. The enrichment results were visualized using bubble plots, where bubble size represents the number of enriched genes, and the color gradient reflects statistical significance. To explore the relationship between significantly enriched GO terms and their corresponding genes, a “GO term-gene” association matrix was constructed, and a Sankey diagram was generated using the R package networkD3. The Sankey diagram shows the flow relationships between GO terms and genes, with the width of the flow lines reflecting the distribution of genes across different GO terms, thus visually demonstrating the shared genes and functional overlaps between GO terms.

Basso-Beattie-Bresnahan locomotor rating scale

Basso-Beattie-Bresnahan (BBB) test was performed at 1, 7, 14, 28, and 56 days post-spinal cord injury. BBB scoring was independently evaluated by two investigators who were blinded to the experimental groups. BBB score ranges from 0 to 21, with 0 indicating complete paralysis and 21 indicating normal movement. Inter-observer consistency was evaluated prior to data analysis, and the scores showed good agreement.

Hematoxylin and eosin (H&E) staining

Spinal cord tissues were collected, fixed, dehydrated, embedded in paraffin, and sectioned into 10 μm -thick sections. The sections were stained with H&E (G1120; Solarbio, Beijing, China) according to the manufacturer's instructions. Histological images were captured using a light microscope.

Catwalk gait analysis system

The kinematics of rat movements were evaluated using the CatWalk

gait analysis system (Noldus Information Technology, Wageningen, Netherlands). The rats were placed on a glass plate of the CatWalk system in the dark. At least three independent movements were recorded and analyzed using CatWalk XT (version 10.6). The evaluation indices comprised the swing phase, stance phase, maximum contact area, and regularity index.

Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean (SEM). GraphPad Prism 8 was used for data analysis. Shapiro-Wilk test was used to evaluate the data distribution. When there were more than two sets of data, a one-way analysis of variance (ANOVA) was used, followed by the Bonferroni *post hoc* correction when variances were equal. BBB scores were analyzed using repeated measures and two-way ANOVA, followed by the Tukey honest significant difference *post hoc* test to compare differences between groups on the same day. $P < 0.05$ was considered statistically significant.

Results

En2 expression in bone marrow mesenchymal stem cells

To investigate the dynamic expression of En2 in BMSCs, BMSCs were isolated from the femurs of rats. Flow cytometry was used to identify the cells by marking CD45, CD34, and CD90 to ensure successful cell extraction (Figure 1A). Subsequently, western blotting and PCR techniques were employed to analyze the expressions of En2 protein and mRNA at different time points during culture. The results showed that En2 was expressed in BMSCs, and its expression level gradually increased with the extension of culture time (Figure 1B,C).

En2 promotes the differentiation of BMSCs into neurons

To assess the role of En2 in promoting the neuronal differentiation

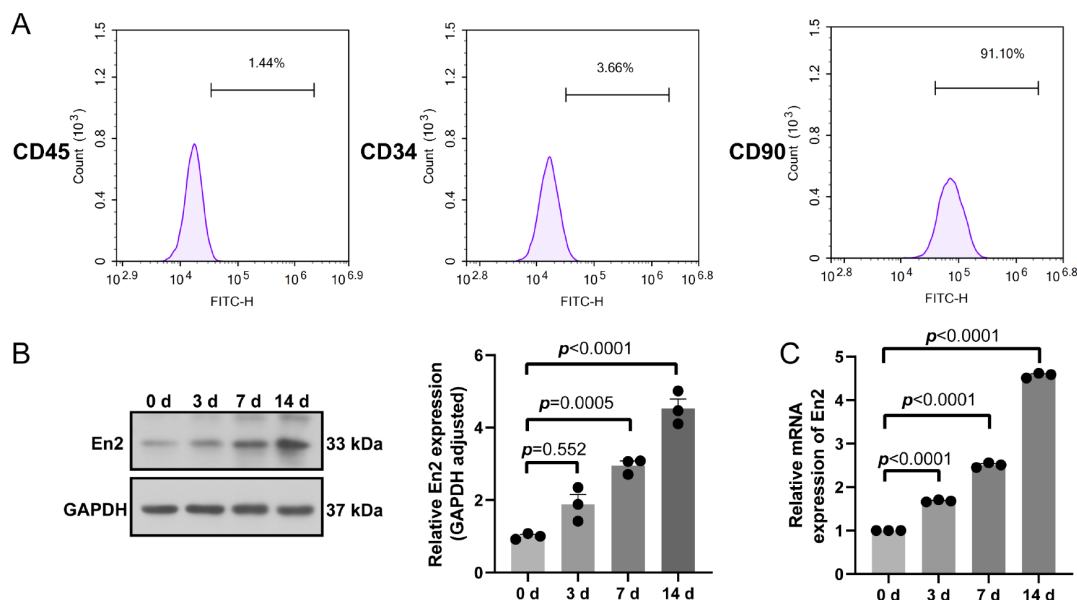


Figure 1. Surface marker expression of BMSCs and temporal changes in En2 expression *in vitro* (A) Flow cytometric analysis of BMSCs showing positive expression of CD90 and negative expression of CD45/CD34. (B) Western blotting of En2 protein expression in BMSCs at different time points, with quantification of relative protein levels ($n = 3$). (C) mRNA expression of En2 in BMSCs at different time points ($n = 3$). All data are presented as the mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni *post hoc* correction.

of BMSCs, this study used differentiation medium to induce BMSC differentiation while overexpressing the *En2* gene to observe its effects during the neuronal differentiation. First, we constructed a lentivirus that overexpressed En2 and infected BMSCs. After 72 h of infection, GFP expression was monitored to confirm that the transfection efficiency reached over 95% (Supplementary Figure S2A). qRT-PCR and western blotting were used to measure En2 expression levels. The results showed that, compared with in the Control and Vehicle groups, En2 protein and mRNA expression levels were significantly increased in the En2 overexpression group (Supplementary Figure S2B,C), validating the lentiviral transfection efficiency.

To investigate whether En2 overexpression promotes neuronal differentiation in BMSCs, cells under different treatment conditions were subjected to lentiviral infection for 72 h, followed by induction of differentiation. After 7 days, the proportion of TUJ1-positive cells was measured by flow cytometry. The results showed that the proportion of TUJ1-positive cells in the Diff-M + En2-OE group reached 92.74%, which was significantly higher than that in the Diff-M group (70.51%) and Diff-M + Veh group (71.74%) (Figure 2A), indicating that En2 overexpression effectively promotes BMSC differentiation toward neurons. Additionally, the *TUJ1* mRNA level in the Diff-M + En2-OE group was significantly higher than that in the other three groups (Figure 2C). We also assessed the mRNA expression levels of Nestin and GFAP. Nestin is a stem cell marker associated with the maintenance of stemness and multipotency, whereas GFAP is a glial marker indicative of astrocyte differentiation and glial lineage commitment. The results showed that the mRNA levels of *Nestin* and *GFAP* in the Diff-M + En2-OE group were significantly lower than those in the other groups (Figure 2B,D), suggesting that En2 overexpression reduces cell stemness, promotes differentiation, enhances neuronal differentiation, and decreases glial cell production.

Although TUJ1 detection only reflects the initiation of neuronal differentiation in BMSCs, it does not provide information on whether the cells mature further and become functional. Therefore, we also performed immunostaining for both TUJ1 and MAP2. The results showed that, compared with the Diff-M + Veh group, the proportion of TUJ1⁺ cells in the Diff-M + En2-OE group increased from 62.78% to 86.04% (Figure 2E), and the proportion of MAP2⁺ cells increased from 47.21% to 84.04% (Figure 2F), indicating that BMSCs overexpressing En2 not only acquired early neuronal identities but also initiated dendritic morphological maturation [26,27]. In addition, to evaluate whether the neuron-like cells exhibit synaptic structural characteristics, immunofluorescence staining was performed for the presynaptic marker Synapsin-1 (SYN-1) and the postsynaptic density protein PSD95. The results showed that neuron-like cells differentiated from En2-overexpressing BMSCs coexpressed SYN-1 and PSD95, suggesting their potential capacity for synapse formation and providing indirect evidence for functional maturation (Figure 2G).

In summary, lentiviral-mediated En2 overexpression enhances the neuronal differentiation ability of BMSCs, promotes differentiation toward neurons, and offers new insights for the treatment of neural injuries.

ASCL1 is a downstream target of En2 in promoting the differentiation of BMSCs into neurons

To further elucidate the transcription factors (TFs) and their

downstream target genes that may drive neuronal differentiation following En2 overexpression, this study employed a “transcription factor-target gene functional prediction” approach combined with the TFBS database. This enabled the identification of En2 transcription factor-binding sites and the prediction of its potential downstream target genes (Figure 3A). The sequence logo revealed a conserved “CAA”-centered binding motif, indicating a strong nucleotide preference of En-2 at these positions. Subsequently, we performed GO enrichment analysis to assess the functional clustering of these target genes within biological processes. The analysis revealed that En2 overexpression may trigger a range of biological effects related to neuronal differentiation, including axon guidance, cortical development, neural progenitor proliferation, and Wnt signaling activation (Figure 3B). Sankey flow diagram analysis further highlighted *ASCL1* as a key gene with high relevance in the positive regulation of neuronal differentiation (Figure 3C). Based on these findings, we validated that En2 overexpression significantly upregulated both ASCL1 protein and mRNA expression levels via western blotting and qRT-PCR (Figure 3D,E).

To confirm the role of the En2-ASCL1 axis in neuronal differentiation, we constructed an *ASCL1*-knockdown lentivirus and infected BMSCs. After 72 h of infection, ASCL1 expression was analyzed via western blotting, which showed that ASCL1 expression was significantly inhibited (Figure 4A). Compared to BMSCs overexpressing En2 alone, the group overexpressing En2 while silencing *ASCL1* showed a significant decrease in the mRNA level of TUJ1 and MAP2 (Figure 4B,C). Flow cytometry analysis also indicated that the proportion of TUJ1⁺ cells decreased from 91.04% to 66.18% upon *ASCL1* knockdown (Figure 4D), and immunofluorescence staining further confirmed this finding (Figure 4E).

Additionally, based on the results from GO enrichment analysis and Sankey flow diagram analysis (Figure 3B), we observed that the Wnt signaling pathway also plays a crucial role in this process. As the core effector molecule of the Wnt pathway, β -catenin forms a positive feedback loop with ASCL1. ASCL1, as a bHLH transcription factor, helps maintain the nuclear localization and protein stability of β -catenin, thus amplifying neuronal differentiation signals [28,29]. In BMSCs overexpressing En2, further knockdown of *ASCL1* led to a concurrent decrease in nuclear β -catenin protein level (Figure 4F,G).

These data indicate that En2 activates ASCL1 transcriptionally and, through a positive feedback mechanism, maintains the protein stability and nuclear localization of β -catenin, thereby accelerating the differentiation of BMSCs toward neurons.

En2-overexpressing BMSCs promote spinal cord injury repair *in vivo*

To evaluate the therapeutic efficacy of En2-overexpressing BMSCs *in vivo*, we used HAMA hydrogel as a cell carrier and transplanted it into rats subjected to contusive spinal cord injury. CCK-8 assay confirmed that HAMA hydrogel had no significant effect on the viability of BMSCs (Supplementary Figure S3). At 56 days post-transplantation, immunofluorescence staining for TUJ1 and MAP2 was performed at the lesion center to assess neuronal regeneration, and GFP labeling was used to trace the transplanted cells. The results showed that both the number of TUJ1⁺ and MAP2⁺ cells and the survival rate of transplanted cells were markedly higher in the En2-BMSC group than in the SCI and Veh groups (Figure 5A,B),

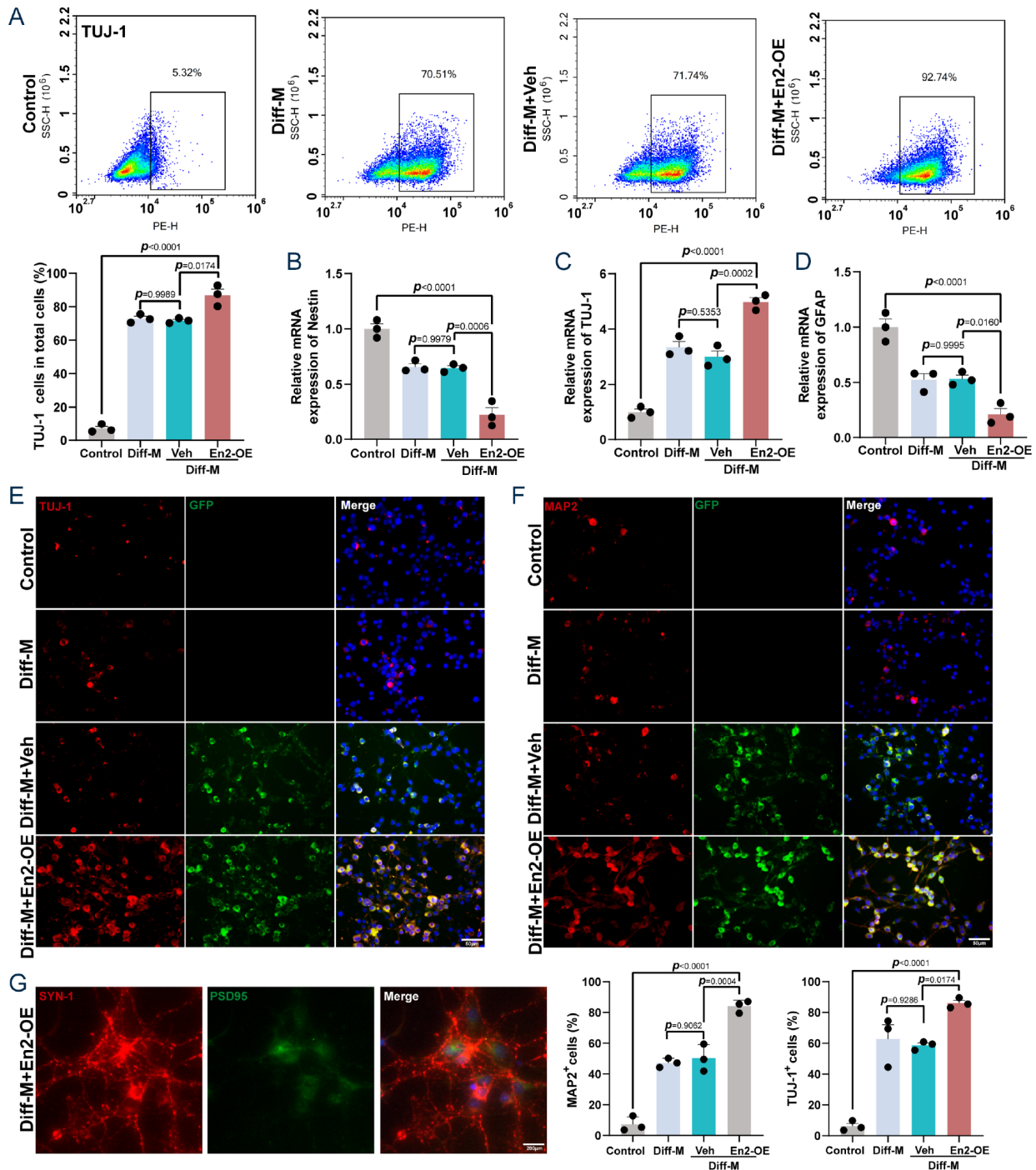


Figure 2. En2 overexpression promotes neuronal differentiation of induced BMSCs (A) Representative flow cytometry plots showing TUJ1 expression. The TUJ1-positive rate increased to 70.51% in the Diff-M group treated with neuronal induction medium alone, while En2-overexpressing cells in the Diff-M + En2 group exhibited a TUJ1-positive rate of 92.74% ($n = 3$). (B) Relative mRNA expression of Nestin in BMSCs ($n = 3$). (C) Relative mRNA expression of TUJ1 in BMSCs ($n = 3$). (D) Relative mRNA expression of GFAP in BMSCs ($n = 3$). (E) Immunofluorescence staining of TUJ1 in BMSCs from different treatment groups. Scale bar = 50 μ m. Quantification of TUJ1⁺ cells in each group ($n = 3$). (F) Immunofluorescence staining of MAP2 in BMSCs from different treatment groups. Scale bar = 50 μ m. Quantification of MAP2⁺ cells in each group ($n = 3$). (G) Immunofluorescence staining of SYN-1 and PSD95 in BMSCs. Scale bar = 200 μ m. All data are presented as the mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni *post hoc* correction.

indicating enhanced neuronal differentiation and survival capacity of En2-overexpressing BMSCs. Meanwhile, immunostaining for SYN-1 and PSD95 at the injury site revealed a higher density of

synaptic marker expression in the En2-OE BMSC group than in the BMSC transplantation group (Figure 5C), suggesting enhanced synapse formation by neurons differentiated from En2-overexpres-

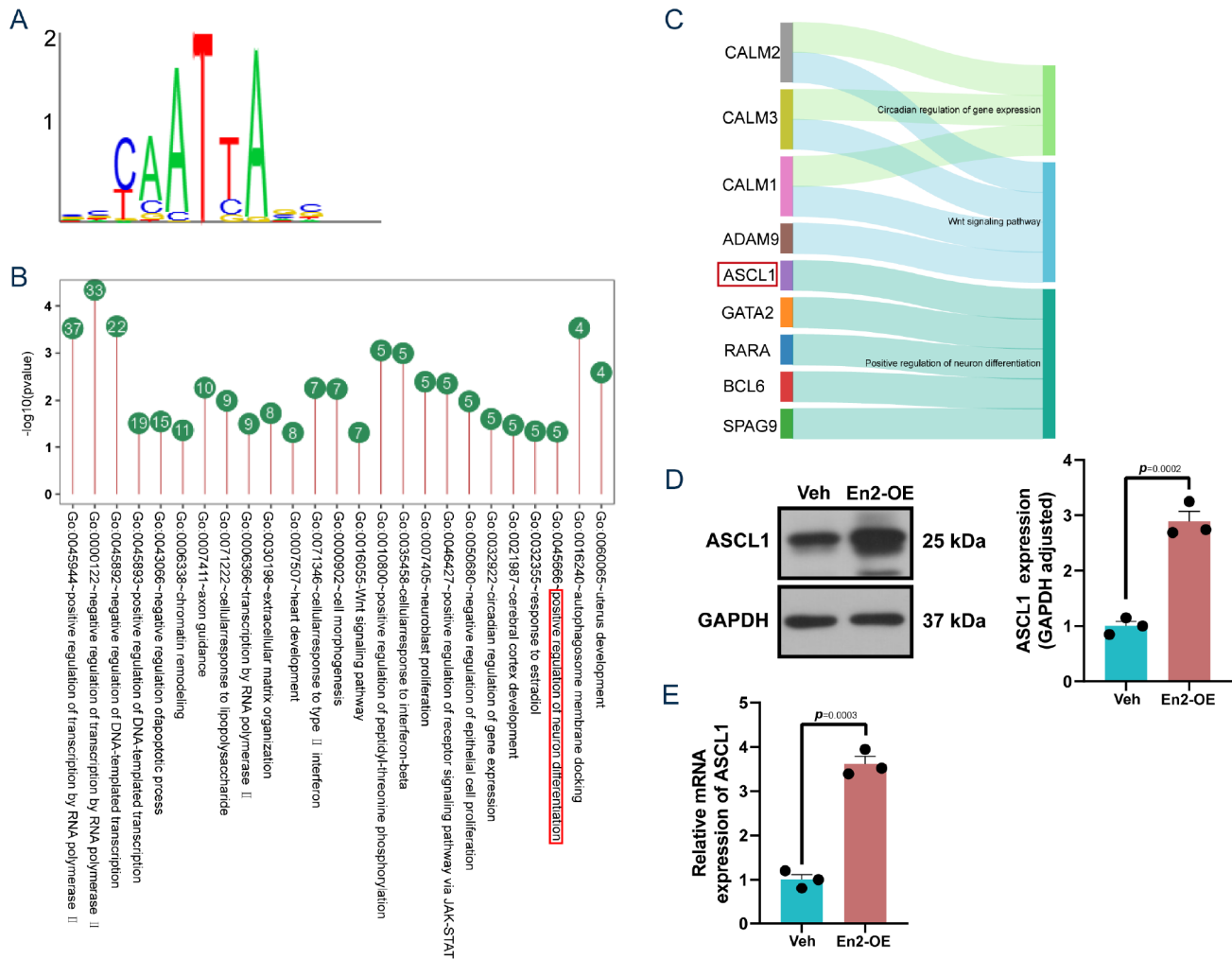


Figure 3. Overexpression of En2 reshapes the transcriptomic profile of neuroinduced cells and upregulates the key neurogenic factor ASCL1 (A) Prediction of En2 downstream targets using the TFBS database. (B) GO enrichment analysis of En2 downstream targets. (C) Sankey diagram showing the genes enriched in representative GO terms. (D) Western blotting of ASCL1 protein expression in En2-OE BMSCs. Band intensities were quantified by grayscale densitometric analysis and normalized to GAPDH ($n = 3$). (E) Relative mRNA expression of ASCL1 in BMSCs ($n = 3$). All data are presented as the mean $\pm$ SEM. Statistical analysis was performed using the Mann-Whitney test.

sing BMSCs. Furthermore, 5-HT immunostaining was performed to evaluate neural circuit reconstruction, and the En2-OE group exhibited improved neural circuit remodeling (Figure 5D). Collectively, these findings provide indirect evidence that neurons derived from En2-overexpressing BMSCs at the lesion center may contribute to functional neuronal integration. Furthermore, H&E staining revealed that tissue integrity at the lesion site was better preserved in the En2-BMSC group than in the other two groups (Figure 5E), suggesting improved histological repair following En2-BMSC transplantation.

Locomotor functional recovery was assessed using the BBB scoring system on the day prior to SCI and at 0, 1, 7, 14, 28, and 56 days post-injury. The results showed that at 56 days after SCI, rats in the HAMA + Veh group recovered to a BBB score of 14, whereas rats in the HAMA + En2-OE group exhibited significantly improved functional recovery, reaching a BBB score of 17. Additionally, since day 14, the motor function of rats in the HAMA + En2-OE group was significantly better than that of the HAMA + Veh group (Figure 6A,

B). We speculate that this difference may be related to the number of BMSCs differentiating into neurons.

Meanwhile, we evaluated the hind limb motor function of rats 8 weeks after spinal cord injury using the Catwalk gait analysis system, analyzing parameters such as hind limb swing, stance, regularity index, and maximum contact area. The regularity index represents the proportion of the total number of paw placements observed during the gait cycle relative to normal gait, while the maximum contact area refers to the footprint area as the rat transitions from braking to propulsion. The results showed that rats in the HAMA + En2-OE group had a significantly better hind limb regularity index and maximum contact area than those in the SCI and HAMA + Veh groups; additionally, the hind limb support time in the HAMA + En2-OE group was also longer (Figure 6C). These results indicate that the transplantation of En2-overexpressing BMSCs significantly enhances motor function recovery in spinal cord-injured rats, with better therapeutic effects than the transplantation of BMSCs alone.

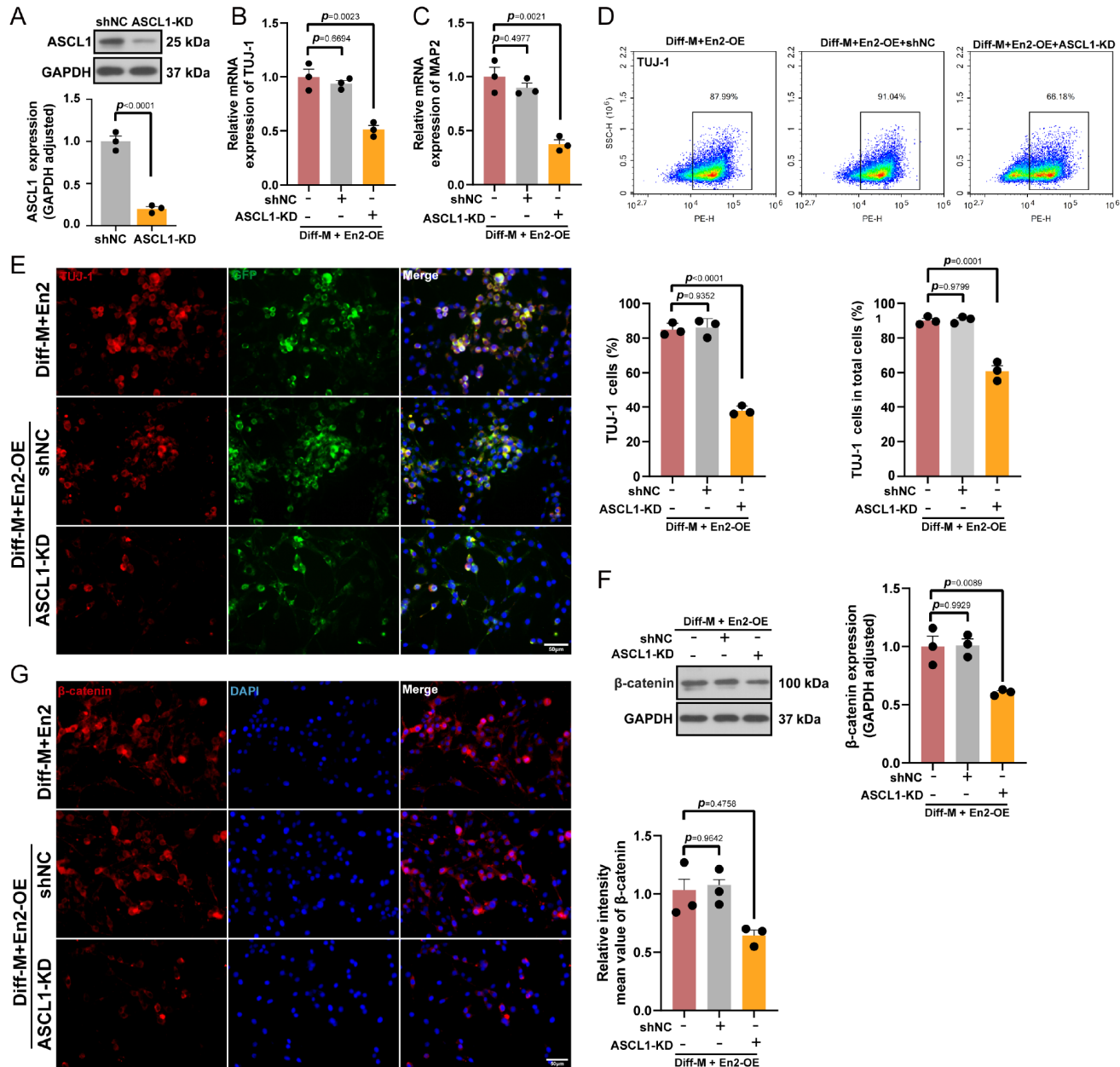


Figure 4. ASCL1 is an essential downstream effector of En2 in inducing neuronal differentiation (A) Western blotting of ASCL1 protein expression in ASCL1-KD BMSCs. Band intensities were quantified by grayscale densitometric analysis and normalized to GAPDH ($n = 3$). (B) Relative mRNA expression of TUJ1 in BMSCs under different treatment conditions ($n = 3$). (C) Relative mRNA expression of MAP2 in BMSCs under different treatment conditions ($n = 3$). (D) Flow cytometric analysis showing the TUJ1-positive rate of BMSCs under different treatments. (E) Immunofluorescence staining of TUJ1 in BMSCs from different treatment groups. Scale bar = 50 μ m. Quantification of TUJ1⁺ cells in each group ($n = 3$). (F) Western blotting of β -catenin protein expression in BMSCs. Band intensities were quantified by grayscale densitometric analysis and normalized to GAPDH ($n = 3$). (G) Immunofluorescence staining of β -catenin in BMSCs under different treatment conditions. Scale bar = 50 μ m. Quantification of β -catenin immunofluorescence intensity in each group ($n = 3$). All data are presented as the mean $\pm$ SEM. Statistical analysis was performed using the Mann-Whitney test or one-way ANOVA followed by Bonferroni *post hoc* correction.

Discussion

This study addresses the key scientific question of how to improve the neuronal conversion rate of BMSCs to enhance their therapeutic efficacy in injured spinal cord repair and systematically explores the potential role of the transcription factor En2 in regulating BMSC neuronal differentiation, as well as its application value in SCI treatment. We found that En2 not only significantly promotes the conversion of BMSCs to functional neuronal-like cells *in vitro* but

also enhances the efficiency and maturity of cell differentiation by activating the ASCL1- β -catenin positive feedback loop. Specifically, En2 overexpression significantly increased the proportion of TUJ1⁺ cells from approximately 70% to over 92%, and the proportion of MAP2⁺ cells also increased significantly, indicating that En2 not only promotes early neuronal fate commitment but also facilitates neuronal maturation.

Mechanistic studies combining TFBS prediction, GO enrichment

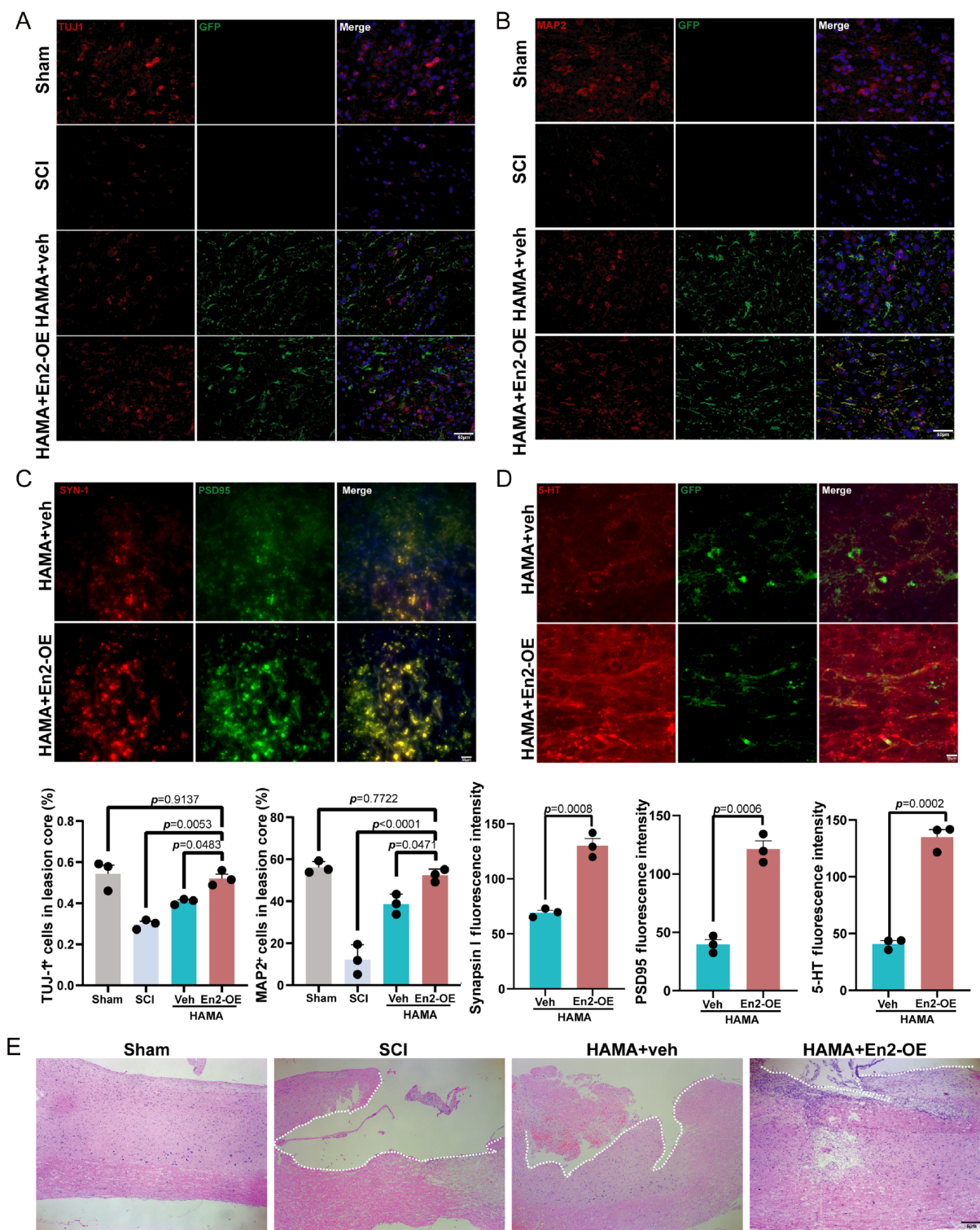


Figure 5. Transplanted En2-overexpressing BMSCs undergo neuronal differentiation and promote tissue repair at the spinal cord injury site (A) Immunofluorescence staining of TUJ1 at the lesion center, with quantification of TUJ1+ cells in different groups ($n = 3$). (B) Immunofluorescence staining of MAP2 at the lesion center, with quantification of MAP2+ cells in different groups ($n = 3$). (C) Immunofluorescence staining of SYN-1 and PSD95 at the lesion center, with quantification of fluorescence intensity in different groups ($n = 3$). (D) Immunofluorescence staining of 5-HT at the lesion center, with quantification of fluorescence intensity in different groups ($n = 3$). (E) H&E staining showing histological changes at the spinal cord injury site. Scale bar = 5 μ m. All data are presented as the mean $\pm$ SEM. Statistical analyses were performed using Student's t test for comparisons between two groups or one-way ANOVA followed by Bonferroni *post hoc* correction.

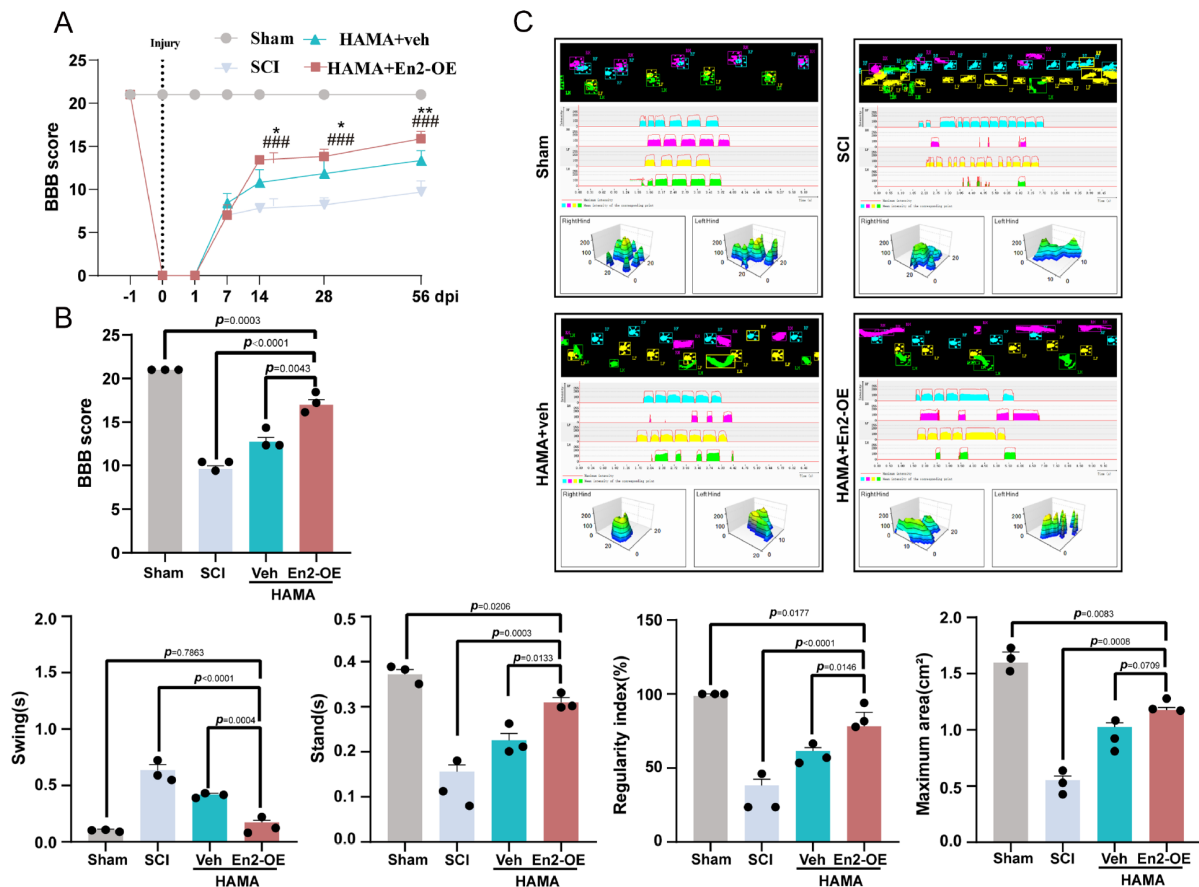


Figure 6. Behavioral assessment of functional recovery after spinal cord injury (A) BBB scores were evaluated at 1, 7, 14, 28, and 56 days post-injury to assess locomotor functional recovery ($n = 3$). (B) Comparison of BBB scores among groups at 56 days post-injury to evaluate hindlimb motor recovery. Quantitative parameters included average swing time ($n = 3$), average stance time ($n = 3$), regularity index ($n = 3$), and maximum contact area ($n = 3$). All data are presented as the mean $\pm$ SEM. Statistical analysis was performed using the Mann-Whitney test or one-way ANOVA followed by Bonferroni *post hoc* correction.

analysis, and experimental validation revealed that *ASCL1* is a direct downstream target gene of En2. *ASCL1* plays a crucial role in maintaining β -catenin nuclear localization and protein stability, forming an En2-*ASCL1*- β -catenin positive regulatory axis that further amplifies the neural differentiation signal.

En2 was initially considered a “developmental signal” that regulates the survival and axonal guidance of dopaminergic neurons. Previous studies have confirmed that En2 expression in hippocampal neural precursor cells negatively regulates cell proliferation, thereby participating in the neurogenesis process [22,30]. Further mechanistic studies have shown that En2 can directly regulate the cell cycle in neural stem cells during neurogenesis, inhibiting proliferation and promoting differentiation. Additional research found that *En2* knockout in cerebellar granule neurons (CGNs) significantly reduced their sensitivity to neurotrophic factors, while En2 overexpression in CGNs resulted in significantly enhanced neural differentiation. A similar phenomenon was observed in embryonic rat cortical precursor cells, where downregulation of En2 expression led to decreased differentiation, with more cells maintaining a progenitor-like morphology [20,31].

Based on the established role of En2 in neural cell differentiation, this study first investigated whether En2 is involved in the differentiation process of BMSCs. The results show that En2 is

expressed positively in BMSCs, and its expression level is positively correlated with culture time, suggesting that En2 may be closely associated with BMSC differentiation. However, it remains unclear whether En2 plays an active regulatory role during BMSC differentiation. To address this question, we used differentiation-inducing medium to promote BMSC differentiation. The results indicate that En2 may function as a nonactive regulatory factor during BMSC differentiation into neural cells. Further overexpression experiments aligned with the findings from neural cell studies, showing that overexpressing En2 in BMSCs not only enhanced neural differentiation ability but also promoted neuronal maturation and functional recovery. This suggests that introducing En2 into BMSCs could be akin to recapitulating the embryonic neurogenesis program, providing new theoretical insights into the mechanisms by which En2 regulates differentiation across cell types.

After the preliminary exploration of the role of En2 in the neural differentiation of BMSCs, this study further investigated the specific mechanism of En2 action. By using TFBS prediction tools, we identified the specific binding sites of En2 on DNA and further screened its downstream regulatory factor *ASCL1*. The experimental results indicate that upregulation of En2 expression can promote further expression of *ASCL1*. *ASCL1*, as a core regulatory factor in

neural development, mainly regulates cell fate determination, proliferation, and differentiation, thereby driving the generation and maturation of neural cells. Previous studies have found that overexpressing ASCL1 in neural precursor cells promotes differentiation into neurons and oligodendrocytes, while ASCL1 deficiency leads to an increase in cell cycle progenitor cells and a reduction in neuronal differentiation in human induced pluripotent stem cells (iPSCs) [32,33]. Endogenous ASCL1 expression typically precedes neuronal maturation, and forced expression of ASCL1 can accelerate differentiation. Coexpression with factors such as Brn2 and Myt1l further enhances differentiation [34–36].

Through GO enrichment analysis and Sankey flow diagrams, we further discovered that the Wnt signaling pathway also plays an important role in this process. As a key effector molecule of the Wnt pathway, β -catenin is crucial in neural induction and differentiation. Specifically, in a retinal regeneration model, the upregulation of ASCL1 was accompanied by a concurrent increase in β -catenin protein levels. Conversely, ASCL1 knockdown led to a significant decrease in nuclear β -catenin levels, suggesting that ASCL1 plays an essential role in maintaining the nuclear localization and protein stability of β -catenin. At the protein level, the bHLH domain of ASCL1 can directly bind to the β -catenin-TCF/LEF complex, enhancing its transcriptional activity [37]. In turn, β -catenin protects ASCL1 from ubiquitin-mediated degradation, forming a bidirectional stable positive feedback loop [38]. In this study, we observed that after ASCL1 knockdown, the β -catenin protein levels also decreased synchronously, a phenomenon consistent with previous reports in retinal regeneration models, further validating the critical role of ASCL1 in maintaining β -catenin activity [39].

To investigate the effect of the transplantation of En2-overexpressing BMSCs on injured spinal cord repair, this study used HAMA hydrogels loaded with stem cells. HAMA retains the natural glycosaminoglycan framework of hyaluronic acid, offering advantages such as low immunogenicity, enzymatic degradation (by hyaluronidase), and similarity to the extracellular matrix components of the spinal cord. These properties help reduce foreign body reactions, extend the retention time of BMSCs at the lesion site, and prevent the rapid loss of single-cell suspensions [40–42]. After transplantation, histological and functional assessments revealed that the En2-overexpressing BMSC group had significantly better tissue integrity than the other two groups. Additionally, BBB scores and Catwalk analysis demonstrated substantial recovery.

These results indicate that En2 is closely associated with the neural differentiation of BMSCs. En2 enhances β -catenin activity through activation of ASCL1, thereby promoting neural differentiation and maturation. However, although En2 overexpression significantly enhances the neural differentiation capacity and therapeutic efficacy of BMSCs, the safety of sustained or excessive En2 expression in BMSCs has not been fully evaluated. As a developmentally associated transcription factor with pleiotropic regulatory functions, aberrant or prolonged En2 overexpression may, under certain conditions, induce unintended differentiation trajectories and disrupt lineage stability, thereby potentially exerting adverse effects on tissue reconstruction. Second, although BMSCs possess intrinsic immunomodulatory properties and relatively low immunogenicity, En2 overexpression may upregulate costimulatory molecules such as HLA-DR and CD80/86, weakening their native “immune-privileged” status and increasing the risk of host T-cell activation and memory immune rejection. In addition,

within the complex *in vivo* microenvironment, dysregulated En2 signaling may increase the propensity of BMSCs toward immature osteogenic lineages or aberrant neuroectodermal differentiation, which in extreme cases may be associated with ectopic ossification or abnormal proliferative risks. Therefore, future studies should establish a more comprehensive safety evaluation framework, including immunogenicity assessment, long-term lineage tracing, and the application of controllable expression strategies, to more precisely define the therapeutic window of En2-modified BMSCs and facilitate their clinical translation. In addition, BMSC transplantation mainly depends on the paracrine effects and the intrinsic differentiation capacity of transplanted cells; however, their efficiency of neural differentiation and functional integration within the hostile postinjury microenvironment remains limited. In contrast, the “En2 gene-enhanced stem cell” strategy adopted in this study offers a distinct advantage by actively directing BMSCs toward a neurogenic phenotype, thereby promoting neural differentiation, neuronal maturation, and functional recovery. This gene-augmented approach may partially overcome the insufficient lineage commitment commonly observed in unmodified stem cell therapies and provides a mechanistically informed means of enhancing therapeutic efficacy. Nevertheless, this strategy also introduces technical and translational challenges, including concerns regarding the safety of genetic modification, precise control of transgene expression and regulatory hurdles associated with gene-engineered cellular products. Collectively, these considerations underscore the need to carefully balance enhanced therapeutic efficacy with biosafety and clinical translatability.

Compared with conventional stem cell-based therapies for spinal cord injury, which primarily rely on the paracrine effects or limited spontaneous differentiation of transplanted cells, the En2 gene-enhanced stem cell strategy presented in this study offers several potential advantages. First, En2 overexpression significantly promotes the neuronal differentiation propensity of BMSCs, thereby increasing the likelihood that transplanted cells acquire neuronal phenotypes rather than remaining in an undifferentiated or glial-like state. This targeted lineage bias may improve the efficiency of neuronal replacement and structural integration within the injured spinal cord. Second, En2-enhanced BMSCs exhibit improved survival and synaptic structural maturation within the hostile postinjury microenvironment, as evidenced by increased expression of neuronal and synaptic markers. In contrast to strategies that depend solely on trophic factor secretion, the present approach aims to directly reinforce intrinsic neurogenic programs of stem cells, potentially enabling more stable and sustained contributions to tissue repair and neural circuit reconstruction. Furthermore, while synaptic marker expression and neural circuit remodeling suggest enhanced structural integration, definitive evidence of functional synaptic transmission will require future studies incorporating electrophysiological assessments. Collectively, the En2 gene-enhanced stem cell strategy represents a promising yet technically demanding approach that complements existing stem cell therapies by combining cellular transplantation with targeted genetic programming to improve neuronal differentiation and integration after spinal cord injury.

Another limitation of the present study is that the *in vivo* experiments were conducted exclusively using a rat spinal cord contusion injury model. Although this model closely reflects the most prevalent clinical form of traumatic SCI and effectively

captures key pathological features, such as secondary injury cascades, inflammation, and cavity formation, it does not fully represent the diverse spectrum of SCI phenotypes. Consequently, the applicability of En2-enhanced BMSC therapy across different injury paradigms, including transection and compression models, remains to be further validated. Future studies incorporating multiple complementary spinal cord injury models will be essential to evaluate the robustness, consistency, and generalizability of this therapeutic strategy. Nonetheless, the favorable outcomes observed in the contusion model provide a clinically relevant proof of concept supporting the translational potential of En2-modified BMSCs.

Although this study verified the mechanism by which En2 promotes BMSC neural differentiation and its impact on spinal cord injury repair, the mechanistic validation only confirmed that ASCL1 depletion leads to a decrease in nuclear β -catenin levels, and no β -catenin knockout experiments were conducted. Additionally, only TUJ1/MAP2 were assessed as general neuronal markers. Furthermore, the high molecular weight HA segment exhibits natural anti-inflammatory properties, which were not considered in the context of the experiment.

Overall, the combination of hydrogel scaffolds and genetically modified BMSCs presents a potential strategy to overcome the current challenges in cell-based therapies and cell delivery for spinal cord injury treatment. The findings of this study provide valuable insights into the development of new therapeutic strategies for spinal cord injury and pave the way for future clinical applications of BMSC transplantation therapies.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

Data Availability

All data are available from the corresponding author upon reasonable request.

Author Contributions

G.N. conceived and supervised the study and acquired funding. H.M., Y.Z., and S.Z. contributed to methodology development and performed the experiments. S.L. and B.L. participated in the investigation. Y.Z. and S.Z. curated the data, while H.M. and Y.Z. conducted the formal analysis. H.M., Y.Z., and S.Z. drafted the manuscript. G.N. critically revised the manuscript. B.L. and S.L. contributed to data visualization.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (No. 82272470), the Talent Introduction and Doctoral Startup Foundation of Tianjin Medical University Cancer Institute and Hospital (No. B2417), and the Outstanding Youth Foundation of Tianjin Medical University General Hospital (No. 22ZYYJQ01).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

- Eli I, Lerner DP, Ghogawala Z. Acute traumatic spinal cord injury. *Neurologic Clin* 2021, 39: 471–488

- Tran AP, Warren PM, Silver J. The biology of regeneration failure and success after spinal cord injury. *Physiol Rev* 2018, 98: 881–917
- Badhiwala JH, Wilson JR, Fehlings MG. Global burden of traumatic brain and spinal cord injury. *Lancet Neurol* 2019, 18: 24–25
- O'Shea TM, Burda JE, Sofroniew MV. Cell biology of spinal cord injury and repair. *J Clin Invest* 2017, 127: 3259–3270
- McDonald JW, Sadowsky C. Spinal-cord injury. *Lancet* 2002, 359: 417–425
- Ahuja CS, Wilson JR, Nori S, Kotter MRN, Druschel C, Curt A, Fehlings MG. Traumatic spinal cord injury. *Nat Rev Dis Primers* 2017, 3: 17018
- Alizadeh A, Dyck SM, Karimi-Abdolrezaee S. Traumatic spinal cord injury: an overview of pathophysiology, models and acute injury mechanisms. *Front Neurol* 2019, 10: 282
- Liau LL, Looi QH, Chia WC, Subramaniam T, Ng MH, Law JX. Treatment of spinal cord injury with mesenchymal stem cells. *Cell Biosci* 2020, 10: 112
- Freyermuth-Trujillo X, Segura-Urbe JJ, Salgado-Ceballos H, Orozco-Barrios CE, Coyoy-Salgado A. Inflammation: a target for treatment in spinal cord injury. *Cells* 2022, 11: 2692
- Zha, X. Challenges and opportunities for repairing the injured spinal cord: inflammation, regeneration, and functional reconstruction. *Regenerative Medicine Reports* 2025, 2
- Cofano F, Boido M, Monticelli M, Zenga F, Ducati A, Vercelli A, Garbosa D. Mesenchymal stem cells for spinal cord injury: current options, limitations, and future of cell therapy. *Int J Mol Sci* 2019, 20: 2698
- Fu X, Liu G, Halim A, Ju Y, Luo Q, Song G. Mesenchymal stem cell migration and tissue repair. *Cells* 2019, 8: 784
- Ning G, Song W, Xu H, Zhu R, Wu Q, Wu Y, Zhu S, et al. Bone marrow mesenchymal stem cells stimulated with low-intensity pulsed ultrasound: Better choice of transplantation treatment for spinal cord injury. *CNS Neurosci Ther* 2019, 25: 496–508
- Liu W, Wang Y, Gong F, Rong Y, Luo Y, Tang P, Zhou Z, et al. Exosomes derived from bone mesenchymal stem cells repair traumatic spinal cord injury by suppressing the activation of A1 neurotoxic reactive astrocytes. *J Neurotrauma* 2019, 36: 469–484
- Wang Y, Jia H, Li WY, Guan LX, Deng L, Liu YC, Liu GB. Molecular examination of bone marrow stromal cells and chondroitinase ABC-assisted acellular nerve allograft for peripheral nerve regeneration. *Exp Ther Med* 2016, 12: 1980–1992
- Zhong H, Wang H, Huang B, Liu S, Song Z, Tang Y, J Li, et al. Current frontier technologies in spinal cord injury research: a narrative review. *Advanced Technology in Neuroscience* 2025, 2: 173–186
- Li Z, Su H, Lin G, Wang K, Huang Y, Wen Y, Luo D, et al. Transplantation of miR-28-5p-modified BMSCs promotes functional recovery after spinal cord injury. *Mol Neurobiol* 2024, 61: 2197–2214
- Ma HP, Yang B, Li Y, Xing C, Zhang Q, Ning G. Bibliometric analysis of research on spinal cord injury and mesenchymal stem cell: trends and frontiers. *World Neurosurg* 2025, 196: 123833
- Song X, Xue Y, Cai H. Down-regulation of miR-181a-5p prevents cerebral ischemic injury by upregulating En2 and activating Wnt/ β -catenin pathway. *J Stroke Cerebrovasc Dis* 2021, 30: 105485
- Genestine M, Lin L, Durens M, Yan Y, Jiang Y, Prem S, Bailoor K, et al. *Engrailed-2 (En2)* deletion produces multiple neurodevelopmental defects in monoamine systems, forebrain structures and neurogenesis and behavior. *Hum Mol Genet* 2015, 24: 5805–5827
- Song X, Hu Q, Xu X, Pan W. Protein kinase C beta relieves autism-like behavior in EN2 knockout mice via upregulation of the FTO/PGC-1 α /UCP1 axis. *J Biochem Mol Toxicol* 2023, 37: e23236
- Wallén Å, Perlmann T. Transcriptional control of dopamine neuron

- development. *Ann New York Acad Sci* 2003, 991: 48–60
23. Boschian C, Messina A, Bozza A, Castellini ME, Provenzano G, Bozzi Y, Casarosa S. Impaired neuronal differentiation of neural stem cells lacking the Engrailed-2 gene. *Neuroscience* 2018, 386: 137–149
 24. Xing C, Jia Z, Qu H, Liu S, Jiang W, Zhong H, Zhou M, *et al.* Correlation analysis between magnetic resonance imaging-based anatomical assessment and behavioral outcome in a rat contusion model of chronic thoracic spinal cord injury. *Front Neurosci* 2022, 16: 838786
 25. Huang Q, Ji D, Tian X, Ma L, Sun X. Berberine inhibits erastin-induced ferroptosis of mouse hippocampal neuronal cells possibly by activating the Nrf2-HO-1/GPX4 pathway. *Nan Fang Yi Ke Da Xue Xue Bao* 2022, 42: 937–943
 26. Wang G, Zhang D, Qin L, Liu Q, Tang W, Liu M, Xu F, *et al.* Forskolin-driven conversion of human somatic cells into induced neurons through regulation of the cAMP-CREB1-JNK signaling. *Theranostics* 2024, 14: 1701–1719
 27. Clausen M, Nakagomi T, Nakano-Doi A, Saino O, Takata M, Taguchi A, Luiten P, *et al.* Ischemia-induced neural stem/progenitor cells express pyramidal cell markers. *NeuroReport* 2011, 22: 789–794
 28. Zuo W, Yang H, Li N, Ouyang Y, Xu X, Hong J. Helicobacter pylori infection activates Wnt/ β -catenin pathway to promote the occurrence of gastritis by upregulating ASCL1 and AQP5. *Cell Death Discov* 2022, 8: 257
 29. Dey D, Shrivastava V, Joshi D, Singal CMS, Tyagi S, Bhat MA, Jaiswal P, *et al.* Hypoxia induces early neurogenesis in human fetal neural stem cells by activating the Wnt pathway. *Mol Neurobiol* 2023, 60: 2910–2921
 30. Rossman IT, Lin L, Morgan KM, DiGiovine M, Van Buskirk EK, Kamdar S, Millonig JH, *et al.* Engrailed2 modulates cerebellar granule neuron precursor proliferation, differentiation and insulin-like growth factor 1 signaling during postnatal development. *Mol Autism* 2014, 5: 9
 31. Krishnamurthy A, Lee AS, Bayin NS, Stephen DN, Nasef O, Lao Z, Joyner AL. Engrailed transcription factors direct excitatory cerebellar neuron diversity and survival. *Development* 2024, 151: dev202502
 32. Azzarelli R, Gillen S, Connor F, Lundie-Brown J, Puletti F, Drummond R, Raffaelli A, *et al.* Phospho-regulation of ASCL1-mediated chromatin opening during cellular reprogramming. *Development* 2024, 151: dev204329
 33. Marichal N, Péron S, Beltrán Arranz A, Galante C, Franco Scarante F, Wiffen R, Schuurmans C, *et al.* Reprogramming astroglia into neurons with hallmarks of fast-spiking parvalbumin-positive interneurons by phospho-site-deficient Ascl1. *Sci Adv* 2024, 10: eadl5935
 34. Ichise E, Chiyonobu T, Ishikawa M, Tanaka Y, Shibata M, Tozawa T, Taura Y, *et al.* Impaired neuronal activity and differential gene expression in *STXBP1* encephalopathy patient iPSC-derived GABAergic neurons. *Hum Mol Genet* 2021, 30: 1337–1348
 35. He B, Deng T, Zhu I, Furusawa T, Zhang S, Tang W, Postnikov Y, *et al.* Binding of HMGN proteins to cell specific enhancers stabilizes cell identity. *Nat Commun* 2018, 9: 5240
 36. Yang Y, Jiao J, Gao R, Yao H, Sun XF, Gao S. Direct conversion of adipocyte progenitors into functional neurons. *Cell Reprogram* 2013, 15: 484–489
 37. Gohlke JM, Armant O, Parham FM, Smith MV, Zimmer C, Castro DS, Nguyen L, *et al.* Characterization of the proneural gene regulatory network during mouse telencephalon development. *BMC Biol* 2008, 6: 15
 38. Shou JW, Cheung CK, Gao J, Shi WW, Shaw PC. Berberine protects C17.2 neural stem cells from oxidative damage followed by inducing neuronal differentiation. *Front Cell Neurosci* 2019, 13: 395
 39. Wohlschlegel J, Finkbeiner C, Hoffer D, Kierney F, Prieve A, Murry AD, Haugan AK, *et al.* ASCL1 induces neurogenesis in human Müller glia. *Stem Cell Rep* 2023, 18: 2400–2417
 40. Xu W, Gao W, Zhang Y, Hou G, Zhang W, Deng J, Wang K, *et al.* Injectable HAMA-CPC hydrogels loaded with high-yield 3D bioprinted adipose-derived stem cell small extracellular vesicles for increased bone repair. *J Nanobiotechnol* 2025, 23: 531
 41. Xia X, Liu Y, Lu Y, Liu J, Deng Y, Wu Y, Hou M, *et al.* Retuning mitochondrial apoptosis/mitophagy balance via SIRT3-energized and microenvironment-modulated hydrogel microspheres to impede osteoarthritis. *Adv Healthcare Mater* 2023, 12: 2302475
 42. Ma H, Liu S, Zhong H, Zhou M, Xing C, Li Y, Zhang Q, *et al.* Exploring the landscape of hydrogel therapy for spinal cord injury: a bibliometric and visual analysis (1991–2023). *World Neurosurg* 2024, 186: e95–e105