MSC secreted extracellular vesicles carrying TGF-beta upregulate Smad 6 expression and promote the regrowth of neurons in spinal cord injured rats

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Research Article

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Abstract

Mesenchymal stem cells (MSCs) are a promising therapy for spinal cord injury (SCI) as they can provide a favorable environment for the regrowth of neurons and axons by inhibiting receptor-regulated Smads (R-Smads) in endogenous neural stem cells (NSCs). However, their mechanism of action and effect on the expression of inhibitory Smads (I-Smads) remains unclear. Here, we demonstrated that Extracellular vesicles (EVs) from MSCs were able to upregulate the Smad 6 expression by carrying TGF-β. Smad 6 knockdown in NSCs partly weakened the BMSC-EVs-induced effect on neural differentiation. In spinal cord injured rats, we found that in the acute phase of injury, the Smad 6 expression was not reduced by the treatment of TGF-β type I receptor kinase inhibitor SB431542, indicating that the Smad 6 expression was not only mediated TGF-β, the inflammatory factors and BMPs were also involved. However, in the later phase of SCI, the Smad 6 expression was reduced by the addition of SB 431542, suggesting in this phase, TGF-β played a key role on the mediation of Smad 6 expression. In addition, by immunohistochemistry staining, Hematoxylin-eosin staining and BBB scores, we revealed that the early inhibition of TGF-β did not increase the regrowth of neurons. Instead, it increased the volume of cavity and the Caspase-3 expression at 24h post-injury, leading to a wore functional outcome. In contrast the later treatment of the TGF-β inhibitor promoted the regrowth of neurons around the cavity, resulting into a better neurological outcome. Together all these results indicated that Smad 6 acts as a feedback regulator to prevents over-differentiation of NSCs to astrocytes and BMSC-EVs can upregulate Smad 6 expression by the carring TGF-β.

Introduction

Spinal cord injury (SCI) is often caused by primary mechanical injury to the spinal cord, followed by a series of molecular and cellular interactions. It results in necrosis, degeneration, and demyelination of axons, as well as neuronal apoptosis that causes a permanent impairment of neurological functions [1, 2]. In the early phase of the injury, endogenous neural stem cells (eNSCs) are spontaneously activated and migrate into the injured cores[3]. These activated eNSCs were long thought to contribute to self-recovery by replacing the lost nerve cells[4, 5]. However, emerging studies have found that most of these cells differentiate into astrocytes rather than into neurons and oligodendrocytes [3]. Glial scars, comprising mainly astrocytes, have proved to be advantageous for limiting the spread of inflammation in the acute phase of SCIs, protecting the surviving nerve cells around the injured lesion[6–8]. However, excessive scar growth around the injured lesion is the main reason for the failure of the neural circuit to reorganize [9].

Smads, ligand-activated receptors, have been shown to be closely associated with scar formation. They can be directly induced by members of the transforming growth factor β (TGF-β) family and are classified into three types: receptor-activated Smads (R-Smads), which include Smad 1/5/8 and Smad 2/3; the common Smad (co-Smad), Smad 4; and inhibitory Smads (I-Smads), Smad 6 and Smad 7[10]. In response to ligand stimulation, R-Smads form a heterotrimeric complex with Smad 4. These complexes are then translocated to the nucleus and induce the expression of a number of genes[11]. This, in turn,

promotes astroglial generation[5, 11, 12]. I-Smads, mainly localized in the nucleus of most cells, can be upregulated by TGF-β, bone morphogenetic proteins (BMPs), UV irradiation, and some pro-inflammatory cytokines[13–17]. Activated I-Smads work as transcriptional regulators in the nucleus and inhibit intracellular activation through interaction with R-Smads. Smad 6, one of the I-Smads, is able to bind directly to BMP type I receptors and prevent the downstream phosphorylation of Smads by BMP. Smad 6 has also been shown to form a complex with activated Smad 1, preventing it from forming a complex with Smad 4[18, 19][18, 19]. In addition, Smad 6 could accelerate the degradation of BMP-induced Smads. It has been demonstrated that Smad 6 are able to recruit Smurf1, which forms a complex with BMP-induced Smads and enhances their degradation[19]. Furthermore, Smad 6, together with histone deacetylases and transcription factors, interferes with BMP/Smads-induced gene expression [20, 21]. Through these mechanisms, Smad 6, activated by BMP or TGF-β, acts as a negative feedback regulator in TGF-β superfamily-mediated signaling. Therefore, upregulation of I-Smads is considered to be effective in preventing excessive glial scar formation.

Transplantation of mesenchymal stem cells (MSCs) is a promising therapy for SCI [22]. MSCs, which were first isolated from bone marrow [23], have the ability to differentiate into three main types of nerve cells. Researchers have used MSCs to transplant to injured spinal cords in an attempt to promote their differentiation into neurons and oligodendrocytes that replace lost nerve cells[24, 25]. However, emerging evidence suggests that MSCs promote neurological recovery by providing a favorable environment for axon regrowth and protecting surviving nerve cells from apoptosis, rather than by directly replacing the lost nerve cells[26]. Recent studies indicate that MSCs inhibit Smad 1/5/8 phosphorylation which, in turn, prevents glial scars from overgrowing. However, few studies have been conducted on assessing whether MSCs can regulate the expression of I-Smads, which mediate the action of R-Smads and the differentiation of NSCs in SCIs[5].

In this study, we focused on bone marrow mesenchymal stem cells (BMSCs) and their possible mechanisms on the regulation of Smad 6 expression. We established that BMSC-conditioned medium (BMSC-EVs) was able to upregulate Smad 6 expression in NSCs; blocking TGF-β diminished the BMSC-EVs -related upregulation of Smad 6 expression, suggesting that BMSC-EVs mediated Smad 6 expression through the secretion of TGF-β. Moreover, Smad 6 knockdown in NSCs partly weakened the BMSC-EVs-mediated effect on neural differentiation of NSCs. This indicates that Smad 6 may act as a negative regulator to prevent the over-production of astrocytes. In addition, the addition of the TGF-β type I receptor kinase inhibitor to BMSC-EVs treated rats only reduced the Smad 6 expression in the later phase of injury, indicating that the upreagulation of Smad 6 was closely associated with BMSC-EVs treatment in SCI rats.

Methods

1. **Cultivation, differentiation, and transfection of NSCs**

NSCs were cultured as described in our previous studies [27, 28]. Cells were obtained from the subventricular zone of SD rats. The isolated cells were cultured as suspended neurospheres in

DMEM/F12 (Gibco, USA) using 20 ng/ml epidermal growth factor (EGF) (Gibco, USA), 2% B27 (Gibco, USA), and 10 ng/mL basic fibroblast growth factor (bFGF) (Gibco, USA) for seven days. The medium was changed every three days.

To knockdown Smad 6 in NSCs, siRNAs (sense, 5- $GAUUCUACAUUGUCUUACA$ - 3; antisense, 5 - $UGU\forall GAC\forall UGUAG\forall UC$ - 3) were transfected into passage 2 NSCs using Lipofectamine 2000 (Invitrogen) for 24 hours. PCR was used to confirm the effect of Smad 6 knockdown in NSCs; non-targeting siRNA was used as a negative control.

Passage 2 NSCs or the Smad 6-knockdown NSCs were dissociated and reseeded on glass coverslips in 5% FBS-DMEM/F12 for 24 h. The medium was then switched to 5% FBS-DMEM/F12 supplemented with one of the following: BMSC- EVs or 10 ng/ml TGF-β (R&D Systems); BMSC-EVs + 10 μM SB431542 (Sigma); BMSC-EVs + 20 ng BMP4; 10 ng/ml TGF-β + 10 μM SB431542; or 10 ng/ml TGF-β + 20 ng BMP4 (R&D Systems). The medium was changed every three days. The cells were cultured for 7 days and then prepared for immunohistochemistry and protein collection.

2. **Mesenchymal stem cell culture and the preparation of EVs**

MSCs were cultured following the procedure described in our previous studies[27, 28]. The cells were isolated from the bone marrow of Fischer 344 rats and were cultured in DMEM (low glucose, Hyclone) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% antibiotic solution at a density of 1×10^6 cells/cm^2. After 24 h of culture, the medium, along with any non-adherent cells, was removed. The residual adherent cells were reseeded at a density of 8000 cells/cm^2 in 10% FBS-DMEM. The medium was changed every three days and was passaged when 90% confluence was reached.

When the passage 3 BMSCs reached a 90% confluence, we collected the supernatant (detail described in previous studies[27, 29]). To remove the cell debris, the collected conditioned medium was centrifugated at 300 g for 10 mins, then at 2000g for 20 mins, and followed by a centrifugation at 10000 g for 30 mins at 4°C. After that, EVs were collected by a centrifugation at 100000 g for 60 mins at 4°C. A transmission electron microscopy, Western blot analysis (the antibodies were used as follows:1:1000 CD 63, 1:2000 CD 9 and 1:1000 TSG 101) and Detection of the diameter of EVs (by dynamic light scattering) were used to identify EVs. The harvested EVs were dissolved in 100 μL PBS and stored at −80°C.

3. **ELISA**

The level of TGF-β in BMSC-EVs was detected using an ELISA kit (Sigma), as the manufacturer's protocol.

4. **Animal protocols (spinal cord treatment)**

Animal procedures were approved by the Ethics Committee of Anhui Medical University (No. 20191064), in accordance with the guidelines of the Declaration of Helsinki, revised in Edinburgh in 2000. The procedure details are outlined in our previous studies[27]. Briefly, a laminectomy was performed at the

T10 level on female Wistar rats (6–8 weeks old, weighing 200–250 g). Rats were randomly classified into sham, SCI (control, treated with DMEM/F12), BMSC-EVs treated, BMSC-EVs + $SB431542_{day0}$-treated and BMSC-EVs + $SB431542_{day3}$-treated groups. Power analysis was used to determine the sample sizes. The Infinite Horizons Spinal Cord Impactor (IH-0400) was used to induce a direct weight drop injury. A mini osmotic pump (Alzet 1007D, USA) filled with either DMEM/F12 (control), BMSC-EVs, or BMSC-EVs + $SB431542_{day0}$ was linked to a soft catheter and implanted under the dura. The medium in the pump was released at a rate of μl/h, and the pump was removed after three days (for details, please refer to the manufacturer's protocols and Franzen et al's study[29]). For the BMSC-EVs + the $SB431542_{day3}$ treated groups, we injected SB431542 through the residual catheter when removing the pump on day 3 following the onset of SCI. The motor function of the lower extremity of SCI rats was evaluated blindly by two independent individuals according to the Basso, Beattie, and Bresnahan (BBB) open-field test[30] at different time points (days 1, 4, 7, 14, 17, 21, 24, and 28).

5. **Tissue processing and immunohistochemistry**

Spinal cords were removed from 4-week-post-trauma rats and fixed in 4% paraformaldehyde for 30 minutes. A 3 mm-length of the spinal cord, centered on the injured, was cut into 35 μm-thick sections using a Leica RM2135 microtome. The sections were prepared for immunohistochemistry as described[31]. The primary antibodies used included mouse anti-Map-2 for neurons (1:500; Abcam, UK) and rabbit anti-GFAP for astroglia (1:1000; Abcam, UK); the secondary antibodies used included Alexa Fluor 488 (green, 1:1000; Molecular Probes, Germany) and Cy5 (red, 1:500; Dianova, Germany). The sections were observed and photographed using a DM-6B fluorescent microscope (Leica, Germany) connected to a computer screen.

6. **RNA extraction and quantitative PCR**

Total RNA was extracted from NSCs and tissues using Trizol (Gibco), as per the manufacturer's instructions, and cDNA was synthesized using the Superscript III RT Reaction Mix (Invitrogen). Quantitative PCR was performed using the RealPlex2 Mastercycler (Eppendorf) and SYBR Green master mix (Applied Biosystems) with the following cycling parameters: 95°C, 15 s; and 60°C, 60 s for 40 cycles. The following gene-specific primers were used: Smad 6: 5-$CT\mathbb{C}$, 5-TGGTCGTACACCGCATAGAG-3; Id2: 5-TTTCCTCCTACGAGCAGCAT-3, 5-CCAGTTCCTTGAGCTTGGAG-3; GAPDH□5-ACAACTTTGGCATTGTGGAA-3□ 5-GATGCAGGGATGATGTTCTG-3.

7. **Western blot assay**

A 20 mm portion of injured spinal cord tissue, centered on the injured epicenter, was lysed in RIPA + PMSF (at a 100:1 ratio of RIPA:PMSF) buffer on ice. The collected proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and incubated overnight with primary antibodies at 4°C (Map-2, 1:2000, Abcam, UK; GFAP, 1:2000, Abcam, UK). This was followed by incubation with a secondary antibody (Santa Cruz Biotechnology; 1:2000 in blocking

solution) for 1 h at room temperature. The blots were then visualized with the SuperSignal West Pico enhanced chemiluminescence reagent (Thermo Scientific) and quantified using Image J software.

8. **Statistical analysis**

Data were presented as mean ± standard error of the mean. Statistical analysis was carried out using SPSS 16.0 software (Chicago, IL, USA). The Student's t-test (two groups) or the one-way analysis of variance (ANOVA) (more than two groups) with Tukey's post-hoc method was used to test the statistical significance. Data with p values < 0.05 were considered statistically significant. For cell counting, 10–15 fields, each containing a total of 500–1000 cells, were randomly selected. The number of positive cells was quantitated by two different blinded individuals.

Results

1. **BMSC-EVs affects the expression of Smad 6 in NSCs**

In order to determine whether BMSC-EVs was able to regulate the expression of I-Smads following SCI, we first examined the expression of Smad 6 in NSCs after adding BMSC-EVs. The level of Smad 6 mRNA increased as early as 1 h after BMSC-EVs was added to NSCs. It reached a peak at 12 h and dropped to the control level at 48 h (Fig.1A). WB analysis also confirmed this result: the NSCs that co-cultured with EVs had a higher expression at 1,6 and 12 h post-co-culture (Fig.1 B). This suggests that Smad 6 expression in NSCs was activated by BMSC-EVs.

Studies have reported that Smad 6 is regulated in response to various factors, including BMPs, TGF-β, and NF-κB signaling. BMSC has been shown to be a negative regulator of BMP-Smad 1/5/8 signaling[5]; it was also found to repress the production of pro-inflammatory cytokines[32, 33], which are considered to be triggers that activate NF-κB and BMP signaling [34]. Therefore, of the three signaling mechanisms, TGF-β may be the factor through which BMSC-EVs regulates Smad 6 expression.

2. **BMSC-EVs alters Smad 6 expression through secretion of TGF-β**

To identify whether the BMSC-EVs-induced alteration of Smad 6 expression was caused by TGF-β, we measured the expression of TGF-β in BMSC-EVs. ELISA assays of all five samples of EVs showed that TGF-β in EVs had an average concentration of approximately 590 (587 ± 115) pg/ml (Fig.2 D). To determine whether TGF-β was able to regulate Smad 6 expression in NSCs, we examined Smad 6 mRNA levels in the TGF-β-treated NSCs at different time points by RT-PCR. In agreement with previous results [35], TGF-β addition markedly increased Smad 6 expression in NSCs. Moreover, this TGF-β-induced alteration could be nullified by the TGF-β type I receptor kinase inhibitor SB431542 (Fig.2 E).

Next, to determine whether the BMSC-EVs-induced in vitro alteration of Smad 6 expression was via TGF-β signaling, we added SB431542 to NSCs in the presence of BMSC-EVs and assessed Smad 6 mRNA levels at 1, 6, and 12 h. As expected, the BMSC-EVs-induced upregulation of Smad 6 in NSCs was

suppressed by the addition of SB431542, indicating that BMSCs secrete TGF-β, thereby elevating Smad 6 expression (Fig.2 F).

3. **BMSC-EVs promotes NSC differentiation into neurons partly via the upregulation of Smad 6**

Studies have reported that BMSCs promote the differentiation of NSCs from astrocytes into neurons. Here, we studied whether Smad 6 was involved in this BMSC-EVs-induced differentiation. We directly added SB431542 in the presence of BMSC-EVs to block TGF-β signaling and then measured the proportion of astrocytes and neurons. The exposure of NSCs to BMSC-EVs for 7 days resulted in an increase in microtubule-associated protein 2 (Map-2)-positive neurons and a decrease in glial fibrillary acidic protein (GFAP)-positive astrocytes (Fig.3). Surprisingly, although the pre-treatment with SB431542 reduced Smad 6 expression, it did not increase the proportion of astrocytes, as expected. Instead, the addition of SB431542 to NSCs in the presence of BMSC-EVs led to a higher proportion of neurons and a lower proportion of astrocytes compared to the BMSC-EVs-treated NSCs (Fig.3). This could be explained by the fact that TGF-β itself can inhibit neurogenesis and promote gliosis in the central nervous system (CNS). Blocking TGF-β signaling not only reduced Smad 6 expression but also abolished mediation by TGF-β on NSC differentiation. Smad 6, as a feedback regulator, had a weaker mediation effect on the differentiation of NSCs compared to TGF-β signaling. Therefore, inhibiting TGF-β signaling led to a reduction in the number of astrocytes and an increase in the number of neurons.

To downregulate Smad 6 expression without inhibiting TGF-β signaling, we used siRNA to knockdown Smad 6 in NSCs. The addition of BMSC-EVs was still able to promote Smad 6 knockdown NSC differentiation into neurons, compared to the control groups (Fig.4 A). However, compared to normal NSCs that received BMSC-EVs treatment, the Smad 6 knockdown NSCs had a lower proportion of neurons and a higher proportion of astrocytes after co-culture with BMSC-EVs for 7 days (Fig. 4 A). These results indicated that Smad 6 was partly associated with the BMSC-EVs-induced effects on the differentiation of NSCs.

4. **Smad 6 knockdown attenuates the BMSC-EVs-induced inhibitory effect on BMP signaling**

To demonstrate that Smad 6 knockdown could affect BMSC-EVs-mediated BMP signaling, we chose to detect the expression of Id2. Id2 is one of the members of the basic helix–loop–helix (bHLH) transcription factor family, which is upregulated by BMP/Smad 1/5/8 signaling [36]. In addition, Id2 is considered to be an important transcription factor for BMP signaling in the differentiation of NSCs. It is able to mediate the differentiation of NSCs via the sequestering of oligodendrogenic transcription factors[37]. Studies have reported that overexpression of Id2 is able to enhance astrocytic differentiation, leading to an increase in astrocytes and a reduction in neurons and oligodendrocytes[37, 38]. Therefore, by evaluating Id2 expression, we could study whether the alteration in the differentiation of NSCs was associated with BMP signaling.

As expected, the BMSC-EVs treatment markedly reduced Id2 expression in NSCs in the presence of BMP4. Although the Id2 expression in Smad 6 knockdown NSCs that received BMSC-EVs was higher than that in

control groups, it was partly reduced compared to the BMSC-EVs groups (Fig.4 C). This indicated that the BMSC-EVs-induced effect on Id2 expression was partly attenuated by Smad 6 knockdown. In summary, BMSC-EVs-induced upregulation of Smad 6 inhibited the astrocytic differentiation of NSCs by repressing the BMP signaling pathway.

5. **TGF-β secreted from BMSCs upregulates the expression of Smad 6 in the later period of SCI**

To identify whether BMSC-EVs was able to mediate Smad 6 expression in vivo, we assessed the expression of Smad 6 at different time points in SCI rats, with or without BMSC-EVs treatment. Unexpectedly, rats that received BMSC-EVs treatment had a lower Smad 6 expression in the early period (days 1 and 3) of the SCI compared to rats that were not treated with BMSC-EVs. However, a marked increase in Smad 6 expression was observed on day 7 following SCI (Fig.5 A). To test whether this BMSC-EVs-induced Smad 6 expression was associated with TGF-β, we treated the SCI rats with SB431542 on day 0 (immediately following the SCI and together with the BMSC-EVs treatment) and on day 3. The results showed that the addition of SB431542 to the BMSC-EVs-treated SCI rats at day 0 did not significantly reduce Smad 6 expression in the early period of SCI (days 1 and 3) ($p > 0.05$). A mild downregulation of Smad 6 expression was also observed on day 7 ($p < 0.05$) (Fig.5 B). However, the expression of Smad 6 on day 7 was significantly lower in rats treated with SB431542 at day 3 ($p < 0.05$), compared to rats that received only BMSC-EVs treatment (Fig.5 C). All these results indicate that the expression of Smad 6 in the later period of SCI (day 7) is closely related to BMSC-EVs treatment.

6. **Treatment with SB431542 in a different phase of the SCI exerts a distinct outcome**

To further evaluate the relationship between the expression of Smad 6 and neurological outcome in SCI rats, we treated SCI rats with SB431542 in different phases (day 0 and days post-injury) in the presence of BMSC-EVs. Immunostaining at week 4 following injury was used to explore the expression of GFAP and Map-2, revealing the number of astrocytes and neurons, respectively, in the injured lesion site. BBB scores were used to assess the neurological outcome of SCI rats at different time points. The histological results showed that in the control SCI rats, a large number of $GFAP^+$ astrocytes surrounded the cavity that comprised a scar boundary. Few $Map\text{-}2^+$ neurons were found in this scar boundary (Fig.6 A). In contrast, clear neurite outgrowth and extension into the scar tissues and a thin scar boundary were found in rats that received BMSC-EVs treatment (Fig.6 A).

Based on the in vitro results, blocking TGF-β signaling should promote the generation of neurons in the injured lesion. However, the addition of SB431542 with BMSC-EVs did not increase the number of neurons but repressed the generation of $Map\text{-}2^+$ neurons and their neurite outgrowth into the scar boundary, compared to rats that received only BMSC-EVs treatment (Fig.6 A). In addition, the later treatment of SB431542 increased the Map-2 expression around the cavity. Consistent with the histology results, the later treatment with SB431542 appeared to provide a better neurological functional outcome (compared to rats that received BMSC-EVs), and early treatment with SB431542 appeared to slightly attenuate the BMSC-EVs-induced improvements in SCI rats (Fig.6 B).

TGF-β was considered as an anti-inflammatory cytokine. Although the inhibition of TGF-β was able to promote the generation of neurons, the inhibition of TGF-β in the early phase might lead to a destructive inflammation, resulting in more apoptosis of neurons at the adjacent injury lesion. It explained why the early addition of SB431542 reduce rather than promote the generation of neurons around the cavity. To confirm this hypothesis, we assessed the volume of cavity at 4 weeks post-injury (Fig.7 A) and the expression of apoptosis protein (Caspase-3) at 24 h post-injury (Fig.7 B). The results showed that the BMSC-EVs treated rats significantly decrease the volume of cavity and the Caspase-3 expression. Moreover, the early addition of SB431542 with BMSC-EVs partly reduce the EVs-induced effects. However, the later addition of SB 431542 did not increase the volume compared to the rats that received EVs treatment.

7. **The expression of Smad 6 was affected by the addition of IL-6 or BMP4**

There is an unexpected result that although the BMSC-EVs treatment increased the Smad 6 expression in vitro, it did not increase the Smad 6 expression in the early phase of SCI rats. As we have mentioned above, the Smad 6 expression is not only mediated by the TGF-β, but also by the BMPs and NF-κB signaling, which could be repressed by BMSCs. Therefore, we hypothesized that it was the BMSC-EVs-induced inhibitory effects on BMPs and NF-κB signaling that neutralized the increasing Smad 6 expression induced by TGF-β in the early phase of injury. To confirm it, we first added IL-6 or BMP4 to NSCs respectively with or without the NF-κB inhibitor (JSH-23) or BMP4 inhibitor (Noggin) and detected the expression of Smad 6 in NSCs. As expected, both of IL-6 and BMP4 were able to increase the Smad 6 expression. Moreover, these IL-6-induced or BMP4-induced effects were abolished by the addition of their inhibitors (Fig.7 A, B).

Surprisingly, the expression of Smad 6 in NSCs was not reduced by the addition of EVs in the presence of IL-6 or BMP4. Instead, the EVs increased the Smad 6 expression in NSCs compared to the IL-6- or BMP4-treated NSCs (Fig.7 A, B). It might because that the EVs carrying TGF-β had a stronger effect on the mediation of Smad 6 expression than that of IL-6 or BMP4. Therefore, although the IL-6 or BMP4-induced increasing effect was abolished, EVs was still able to increase the Smad 6 expression by TGF-β. To prove it we next added the NF-κB or BMP4 inhibitor to NSCs, it showed that the addition of these inhibitors could not reduce the expression of Smad 6. Whereas the addition of SB 431542 significantly reduced the Smad 6 expression in NSCs which culture with EVs, and IL-6 or BMP4(Fig.7 A, B).

Discussion

Following an SCI, endogenous NSCs around the injury lesion are activated and rapidly migrate into the lesion site. However, in the unfavorable microenvironment, most of these activated NSCs do not differentiate into neurons or axons; instead, they differentiate into astrocytes that form a glial scar around the injured cavity [3]. BMPs have been reported to play a key role in the promotion of gliosis[39]. Their levels increase and they accumulate in the injury lesion, contributing to the glial differentiation of endogenous NSCs following an SCI[5]. During the acute phase of the injury, the scar boundary formed

around the injured core is crucial in preventing the spread of early inflammation and protecting the adjacent surviving neural cells from destructive inflammation (Fig 9). The glial scar was long thought to be the main cause of the failure of the neurons and axons to regenerate and remodel [9, 40]. However, recent studies show that preventing glial scar formation in SCIs did not result in greater axon regeneration and better neurological recovery in SCI[9, 41]. With appropriate growth factor supplementation, axons were able to regrow along the scar boundary after CNS injury[42]. Moreover, it has been demonstrated in SCI models that axon regeneration could be improved by grafting astrocytes [43-45]. Although astrocytes may aid axon regeneration, the over-generation of glial scars is still considered to hinder remodeling or regeneration of axons, especially in the chronic phase of SCI. Therefore, the regulation of astrocytes and scar formation is critical for neurological improvement in SCIs.

BMP signaling is shown to play an important role in the formation of astrocyte scars following the onset of SCI. The upregulation of BMPs in the acute phase of SCI could aid in the rapid formation of the scar boundary[5],which is helpful for limiting inflammation and protecting the surviving adjacent cells [6-8, 46]. However, the expression levels of these factors remain high after the early phase of SCI, resulting in the over-generation of astrocyte scars around the injured cores. Therefore, I-Smads act as a negative feedback regulator of BMP signaling, which might be effective in limiting the over-expression of astrocytes. Consistent with the present study, the knockdown of Smad 6 expression in NCSs resulted in an increase in the proportion of astrocytes, a reduction in the number of neurons, and a decrease in the expression of Id2, indicating that Smad 6 was able to antagonize the BMP-induced effect and promote the differentiation of NSCs to neurons. Therefore, the balance between R-Smads and I-Smads was critical for mediating scar formation.

It is generally thought that MSCs exert their biological effects in different models by secreting a large variety of factors and molecules [47-50]. In SCI rats, MSCs were able to improve neurological outcomes by promoting the regeneration of neurons and axon regrowth [51-53] through the inhibition of BMP/Smads signaling[5, 27]. In the present study, it was found that the addition of BMSC-EVs to NSCs increased the expression of Smad 6. Moreover, the BMSC-EVs-induced effect on Id2 expression and differentiation of NSCs was partially abolished by Smad 6 knockdown, which suggested that BMSCs mediated the differentiation of NSCs not only by inhibiting BMP/Smad signaling but also in part by upregulating the expression of Smad 6.

HGF, which is released by MSCs, proved to be a key factor in the BMSC-EVs-associated mediation of BMP/R-Smad signaling[27, 54, 55]. It was previously seen that HGF was not able to affect the expression of I-Smads[56]. The present study indicated that TGF-β might be crucial in the up-regulation of Smad 6 in NSCs. First, in accordance with previous studies[29, 57], TGF-β was found in the secreted medium, proving that it was released by BMSCs. Second, the BMSC-EVs-induced up-regulation of Smad 6 was abolished by the addition of the TGF-β inhibitor SB431542. Finally, treatment with SB431542 along with BMSC-EVs was able to reduce Smad 6 expression in the later phase of SCI.

The results revealed that in vitro, BMSC-EVs was able to increase the expression of Smad 6 in NSCs. However, the in vivo results showed that treatment with BMSC-EVs did not affect the expression of Smad 6 in SCI rats. In the early period of SCI, the expression of Smad 6 was markedly reduced by treatment with BMSC-EVs. This may be explained by the fact that Smad 6 expression is not regulated solely by TGF-β, but BMP signaling, and inflammatory cytokines are also involved. In the early phase of SCI, inflammatory cells are recruited to the injured lesion, which induces a rapid increase of pro-inflammatory cytokines[58, 59] and BMP expression[5]. MSCs are shown to repress both the production of pro-inflammatory cytokines and BMP signaling[5, 27, 32]. Therefore, it is possible that the BMSC-EVs-induced repressing effects on pro-inflammatory cytokines and BMP signaling caused reduced Smad 6 expression in the early phase of SCI. This hypothesis also could explain why SB431542 treatment immediately following SCI did not markedly alter Smad 6 expression in the early period.

Although Smad 6 was not altered by SB431542 treatment in the early period of SCI, it was significantly reduced by SB431542 treatment in the later phase of SCI. TGF-β, produced by inflammatory cells, is able to repress the destructive inflammatory process by inhibiting the activation of NF-κB[60] or by directly suppressing T_H1 cells[61]. TGF-β expression begins to increase 24 h after SCI and reaches a relatively high level at about 1 week[62]. In contrast, the expression of pro-inflammatory cytokines reached a high level in a short time; it gradually decreased after 3 days of injury and dropped to normal levels after 7–14 days[27, 32, 62]. Similarly, levels of BMPs produced by inflammatory cells increase rapidly following the onset of SCI and drop gradually after that[5, 27]. Therefore, in the later phase of SCI, the expression of pro-inflammatory cytokines and BMPs restores to a normal level, while TGF-β expression remains at a high level. This indicates that, in this phase, the relatively high expression of TGF-β compared to pro-inflammatory cytokines and BMPs plays a key role in the upregulation of Smad 6. This explains why the expression of Smad 6 in the later phase of SCI was reduced by SB431542 treatment.

Another notable result is that the addition of SB431542 to BMSC-EVs treatment of SCI rats in a different phase of the SCI generated a distinct outcome. Compared to the BMSC-EVs-treated rats, rats that received both SB431542 and BMSC-EVs immediately following the onset of SCI showed a worse histology result and lower BBB scores. In contrast, rats that received BMSC-EVs in the early phase and SB431542 at day 3 following the injury had a higher proportion of neurons and a thinner scar boundary around the injured lesion, as well as higher BBB scores, which could be explained by the following reasons. First, TGF-β acts as an anti-inflammatory cytokine. It plays an important role in mediating the inflammatory process[63, 64]. The inhibition of TGF-β in the early phase led to over-activation of inflammation-associated signaling, resulting in the apoptosis of neurons around the injury lesion. Second, TGF-β has the ability to promote gliosis in the CNS[65]. The early inhibition of TGF-β might lead to a dysfunction in the scar formation process, which would then attenuate the effect on the limitation of inflammation. These two points were confirmed by detecting the volume of cavity and the Caspase-3 expression. These two indexes were increased by the early treatment of SB 431542, indicating a worse destructive inflammation in lesion site. It explained why early blocking of TGF-β induced a worse histological result and neurological outcome. Finally, in the later phase of SCI, the inflammation is nearly stable, and neurons

begin to regenerate. In this phase, inhibition of TGF-β may not cause an increase in inflammation. Conversely, it was able to promote neurogenesis in the injured lesion in the later phase of injury. This explains why, in the later phase, the addition of SB431542 was able to increase neuron expression and promote a functional neurological outcome.

In the present study, we have provided evidence that Smad 6 is able to prevent NSCs from over-differentiating into astrocytes in vitro. However, due to the lack of a direct inhibitor of Smad 6 and the Smad 6 knockout in mice (which may possibly affect the development of the CNS), we could not directly counter Smad 6 effects in SCI rats. Hence, we downregulated Smad 6 expression in vivo indirectly by inhibiting TGF-β signaling, which mediates NSC differentiation. This, combined with the in vitro results, indirectly highlights the role of Smad 6 in mediating the differentiation of NSCs in SCI rats, though further in vivo studies are required.

In conclusion, BMSC-EVs could upregulate Smad 6 expression through TGF-β. Smad 6 acts as a negative feedback regulator that inhibits BMP/Smad 1/5/8 signaling and promotes the differentiation of NSCs into neurons. These results indicate that Smad 6 could be a potential therapeutic target in the treatment of spinal cord injuries.

Abbreviations

SCI: Spinal cord injury; MSCs: Mesenchymal stem cells; BMSCs: Bone marrow- derived mesenchymal stem cells; NSCs: Neural stem cells; eNSCs: Endogenous neural stem cells; TGF-β: Transforming growth factor β; CNS: Central nervous system; CM: Conditioned medium; BMPs: Bone morphogenetic proteins; GFAP: Glial fibrillary acidic protein; Map-2: Microtubule-associated protein 2; SB: SB 431542.

Declarations

Authors' contributions

Cailiang Shen and Tianyu Han contributed to the research design. Peiwen Song contributed to the manuscript writing. Cailiang Shen contributed to the manuscript editing. Tianyu Han and Yang Niu contributed to the spinal cord injury, tissue processing, and immunohistochemistry. Xiang Xia and Yang Niu contributed to the cell culturing, Western-blot, and ELISA arrays. Ying Wang and Huang Fang contributed to the quantification and statistical analysis. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

An ethical permit was obtained from the Ethics Committee of Anhui Medical University (guidelines of the Declaration of Helsinki).

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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Figures

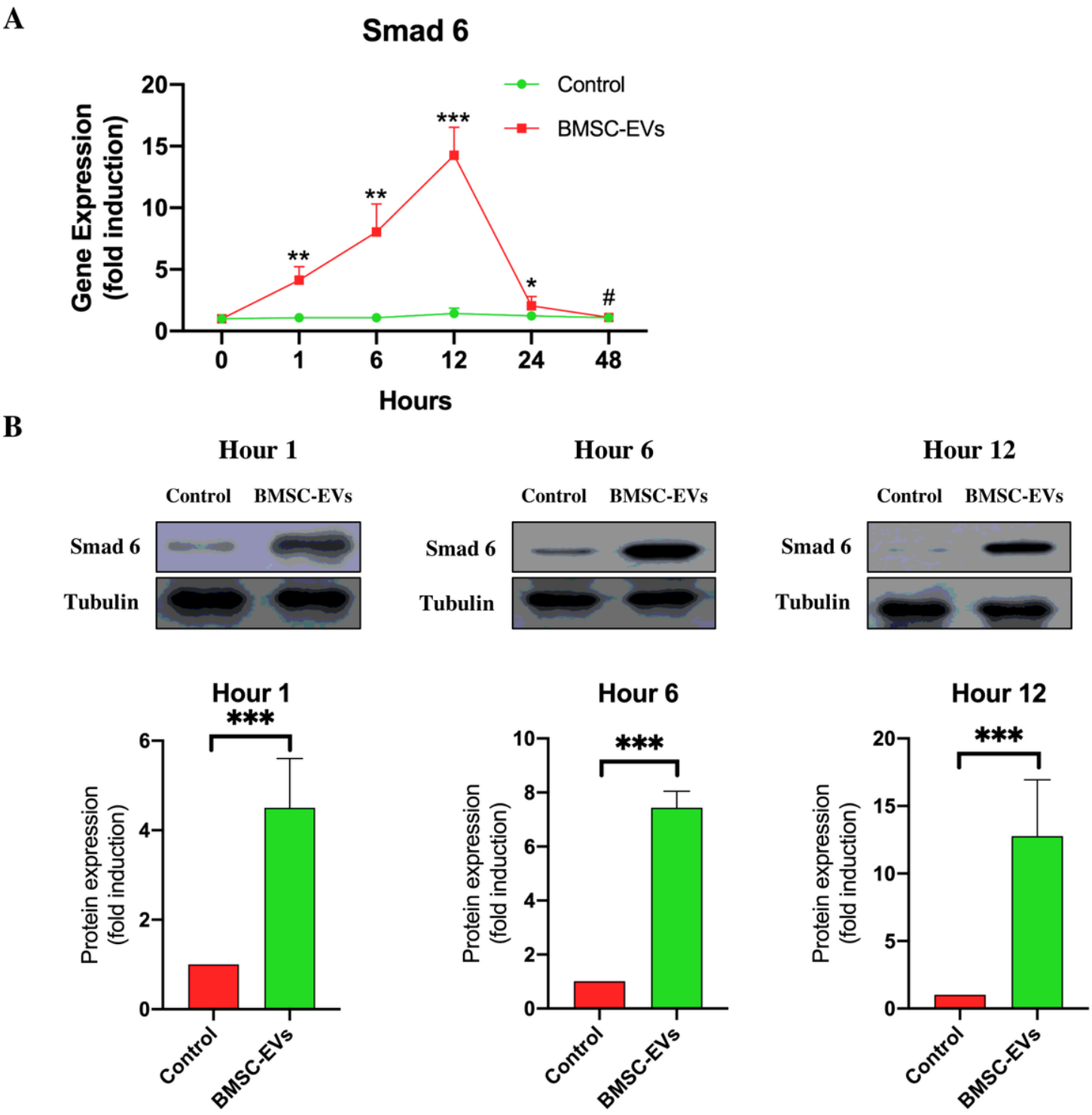

Figure 1

BMSC-EVs upregulate the expression of Smad 6 in NSCs. A. Smad 6 expression in NSCs was increased by the addition of BMSC-CM and reached a peak level at 12 h (n = 5, compared with control at each time point, Student's t-test, the data was presented as fold change to control cells at hour 1). B. Western Blots analysis confirmed that the Smad 6 expression in NSCs was up-regulated by the treatment of BMSC-EVs at different time points (n=5, the data was presented as fold change to control NSCs).

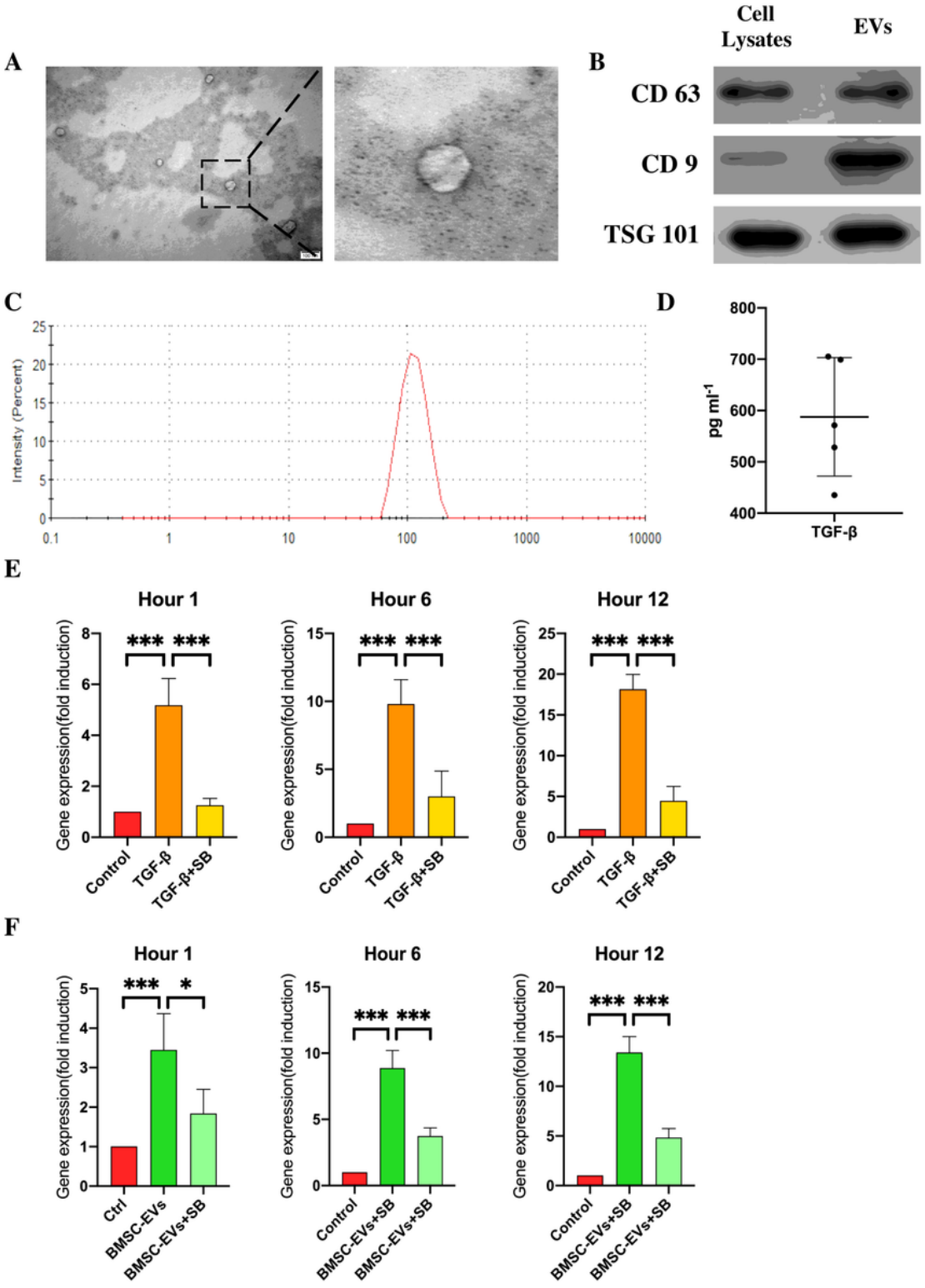

Figure 2

BMSC-EVs upregulated Smad 6 expression in NSCs via the secretion of TGF-β. A. Identification of EVs by transmission electron microscopy. B. Detection of EVs diameter by dynamic light scattering. C. Analysis of expression of CD9, CD63 and TSG101 expression by Wsetern blots. D. ELISA from five individual samples confirmed the presence of TGF-β in BMSC-EVs. E. The addition of TGF-β increased Smad 6 expression in NSCs; this TGF-β-induced alteration could be abolished by SB431542 (n = 5; the data was

revealed as fold change to control NSCs, Student's t-test). F. BMSC-EVs-induced upregulation of Smad 6 in NSCs was significantly diminished by the addition of SB431542 at different early time points (n = 5, the data was revealed as fold change to control NSCs, Student's t-test). Smad 6 mRNA expression was normalized to GAPDH mRNA; data were shown as mean ± s.d; *p < 0.05, **p < 0.01, ***p<0.001, #p > 0.05.

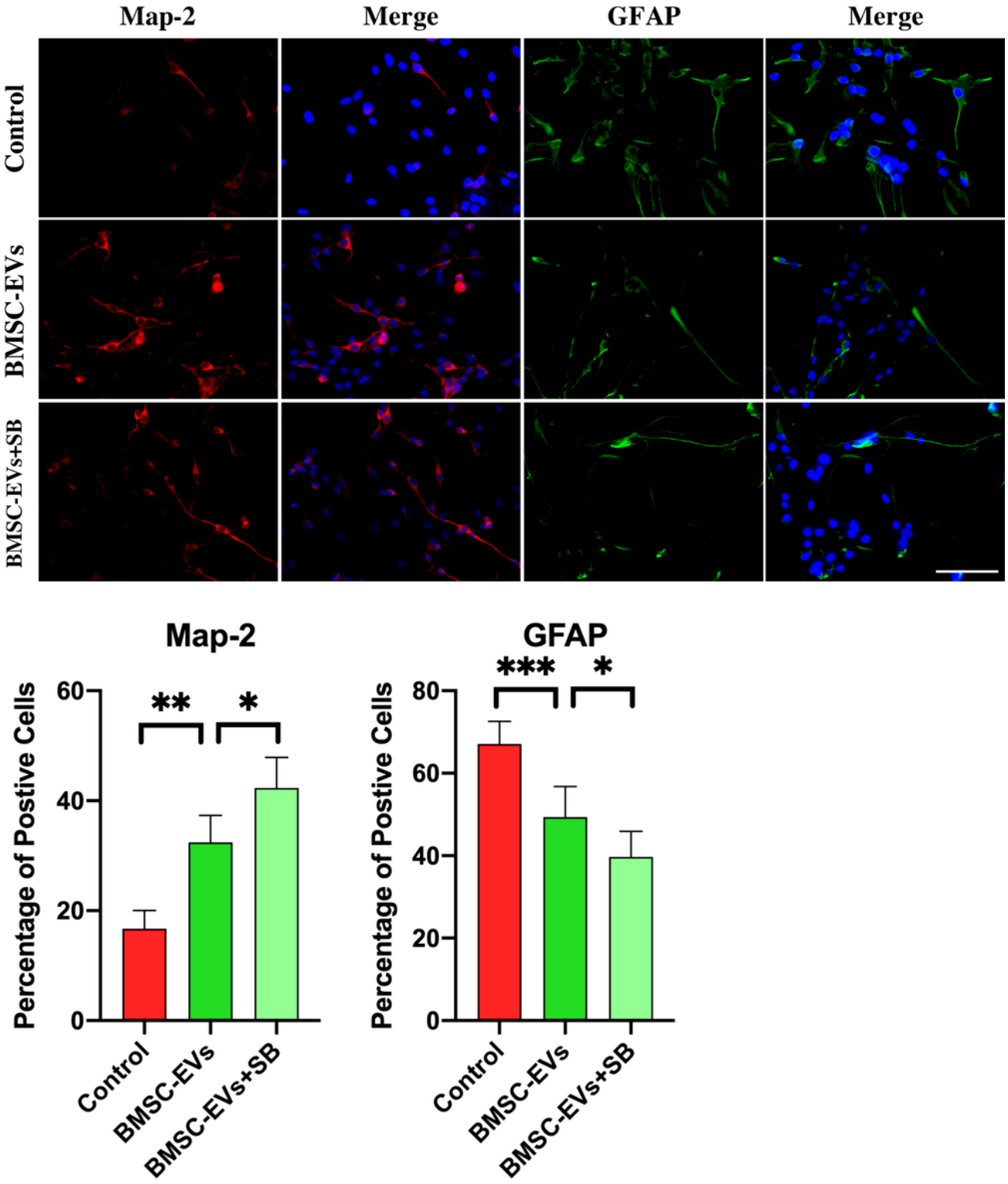

Figure 3

The inhibition of TGF-β promoted the differentiation of NSCs into neurons in the presence of BMSC-EVs A. BMSC-EVs-treated NSCs had a higher proportion of Map-2+ cells and a lower proportion of GFAP+ cells than NSCs that received only DMEM/F12 treatment (control group) after 7 days of co-culture (n = 6). The addition of SB 431542 increased the proportion of neurons and decreased the percentage of astrocytes (n=6). Data were shown as mean ± s.d; Student's t-test was used for comparison, *p < 0.05, **p < 0.01, ***p<0.001, #p > 0.05; scale bars, 100 μm. SB 431542 was abbreviated as SB.

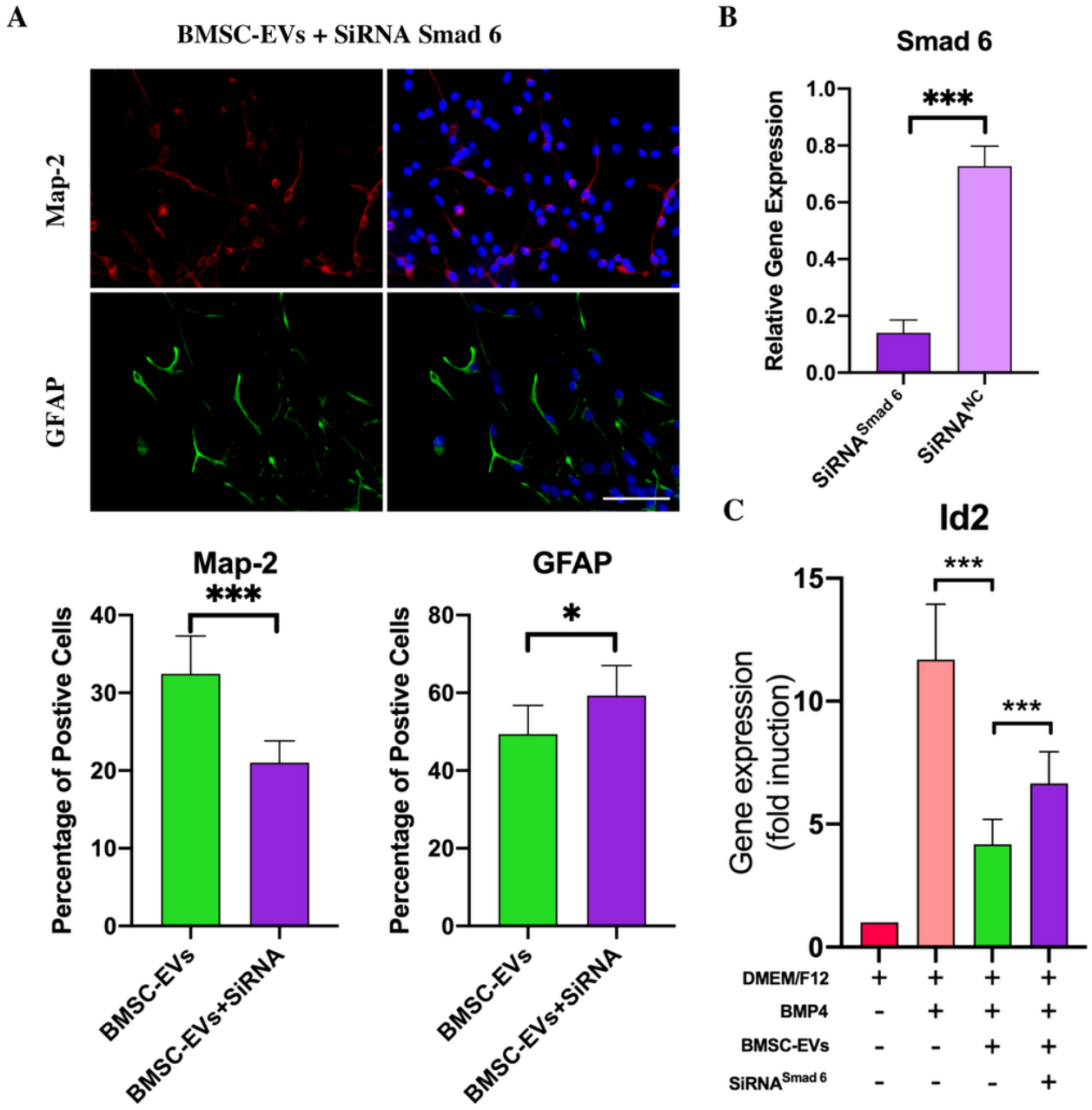

Figure 4

BMSC-EVs promoted the differentiation of NSCs into neurons partly via upregulation of Smad 6. A. Smad 6 knockdown partly abolished the BMSC-EVs-induced effect on the differentiation of NSCs, leading to a slightly lower proportion of neurons (n = 6). B. PCR results indicated that the expression of Smad 6 in NSCs was markedly reduced by Smad 6 knockdown (n = 3). C. The BMSC-EVs-induced effect on Id2

mediation was weakened by the Smad 6 knockdown in NSCs (n = 5, Id2 mRNA expression was normalized to GAPDH mRNA, and the results were revealed as fold change to control groups). Data were shown as mean ± s.d; Student's t-test was used for comparison, *p < 0.05, **p < 0.01, ***p<0.001, #p > 0.05; scale bars, 100 μm.

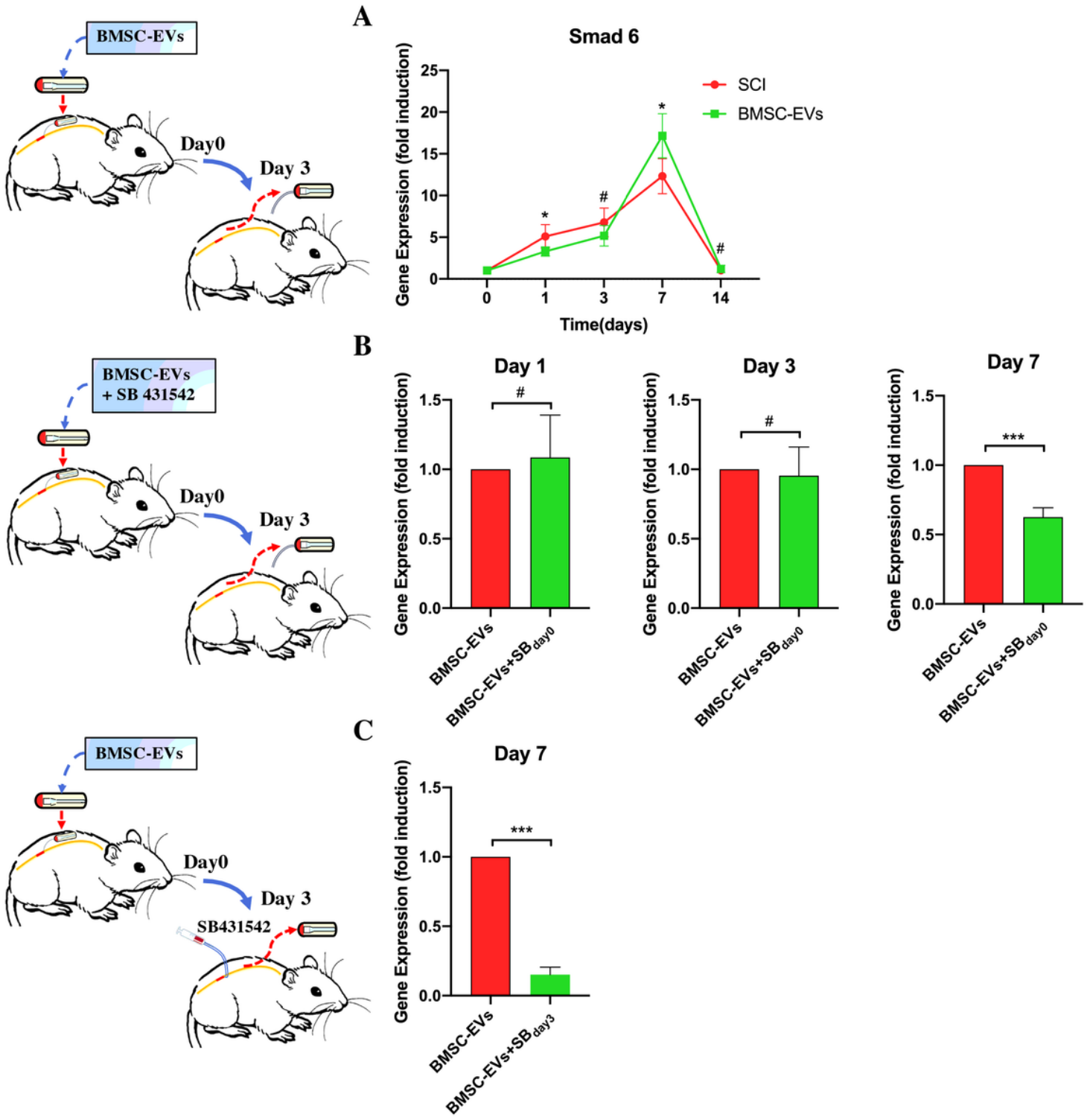

Figure 5

BMSC-EVs upregulated Smad 6 expression in the later phase of SCI. A. Treatment with BMSC-EVs significantly increased Smad 6 expression on day 7 following the onset of SCI, and reduced Smad 6 expression on day 1 (n = 5). B, C. The expression of Smad 6 on days 1 and 3 following SCI (day 1 and day 3) was not altered by early treatment with SB431542. However, the expression of Smad 6 on day 7 following SCI could be reduced by both early and later treatment of SB431542 (n = 5). Student's t-test was used for comparisons, *p < 0.05, **p < 0.01, ***p<0.001, #p > 0.05; SBday0, SB431542 treatment immediately following the SCI; SBday3, SB431542 treatment at day 3 following the onset of the SCI.

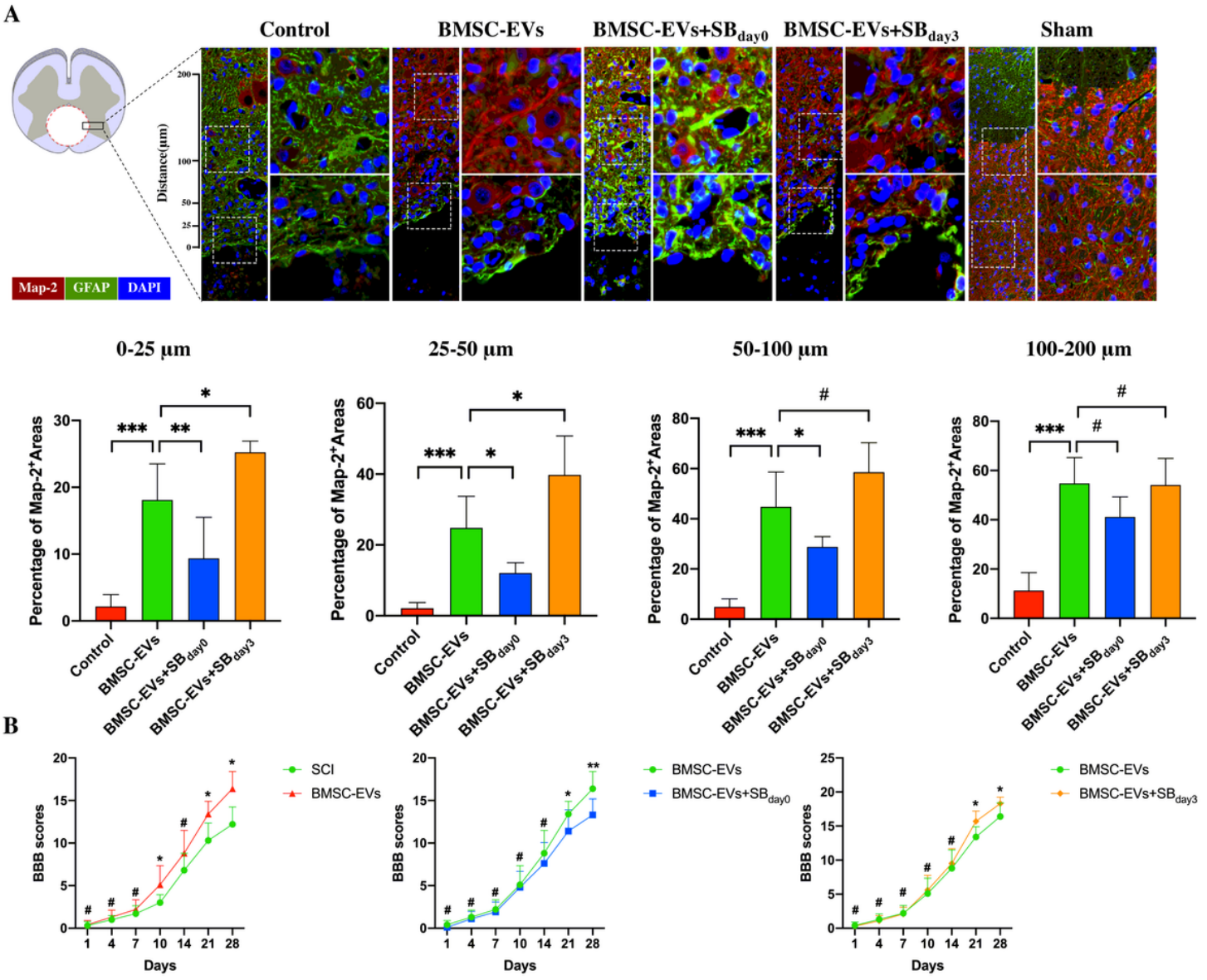

Figure 6

Inhibiting TGF-β in a different phase of SCI led to a distinct outcome. A. Area occupied by Map-2-postive neurons and GFAP-positive scar-forming astrocytes within 200 μm from the edge of cavity at 4 weeks post-SCI (n=5, Student's t-test was used for comparisons). B. BBB score of each group (n=10, Mann-Whitney was used for comparisons). *p < 0.05, **p < 0.01, ***p<0.001, #p > 0.05; SBday0, SB431542

treatment immediately following the SCI; SBday3, SB431542 treatment at day 3 following the onset of the SCI.

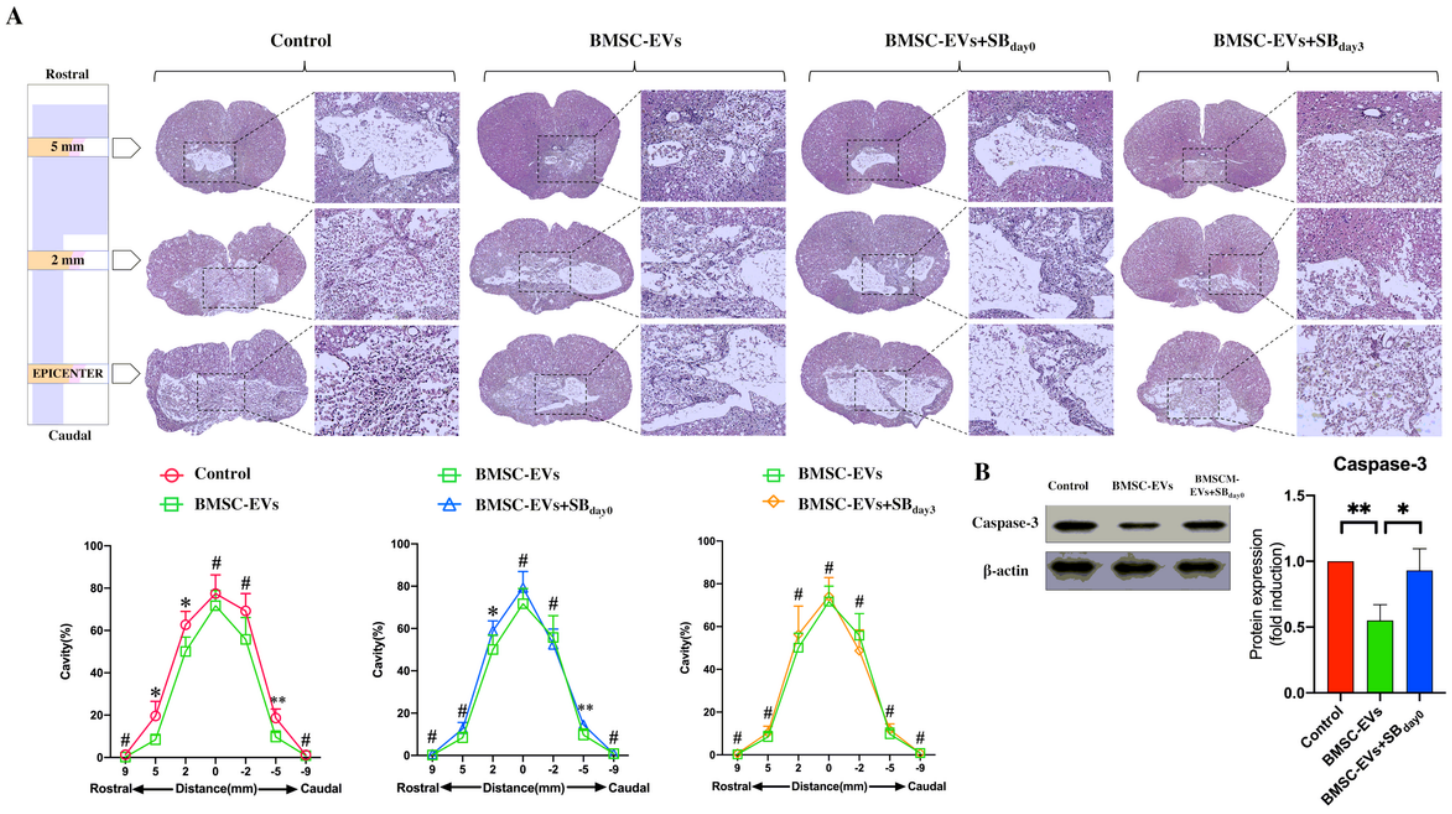

Figure 7

Inhibiting TGF-β in early phase of SCI increased the volume of cavity and the expression of Caspase-3. A. Hematoxylin-eosin staining revealed that the volume of cavity was increased by the early treatment of the SB431542, but no alteration of the volume was induced by the later treatment of the SB 431542 (n=5). B. The early treatment of the SB 431542 increased the expression of Caspase-3 after 24h post-injury (n=3). Student's t-test was used for comparisons, *p < 0.05, **p < 0.01, ***p<0.001, #p > 0.05.

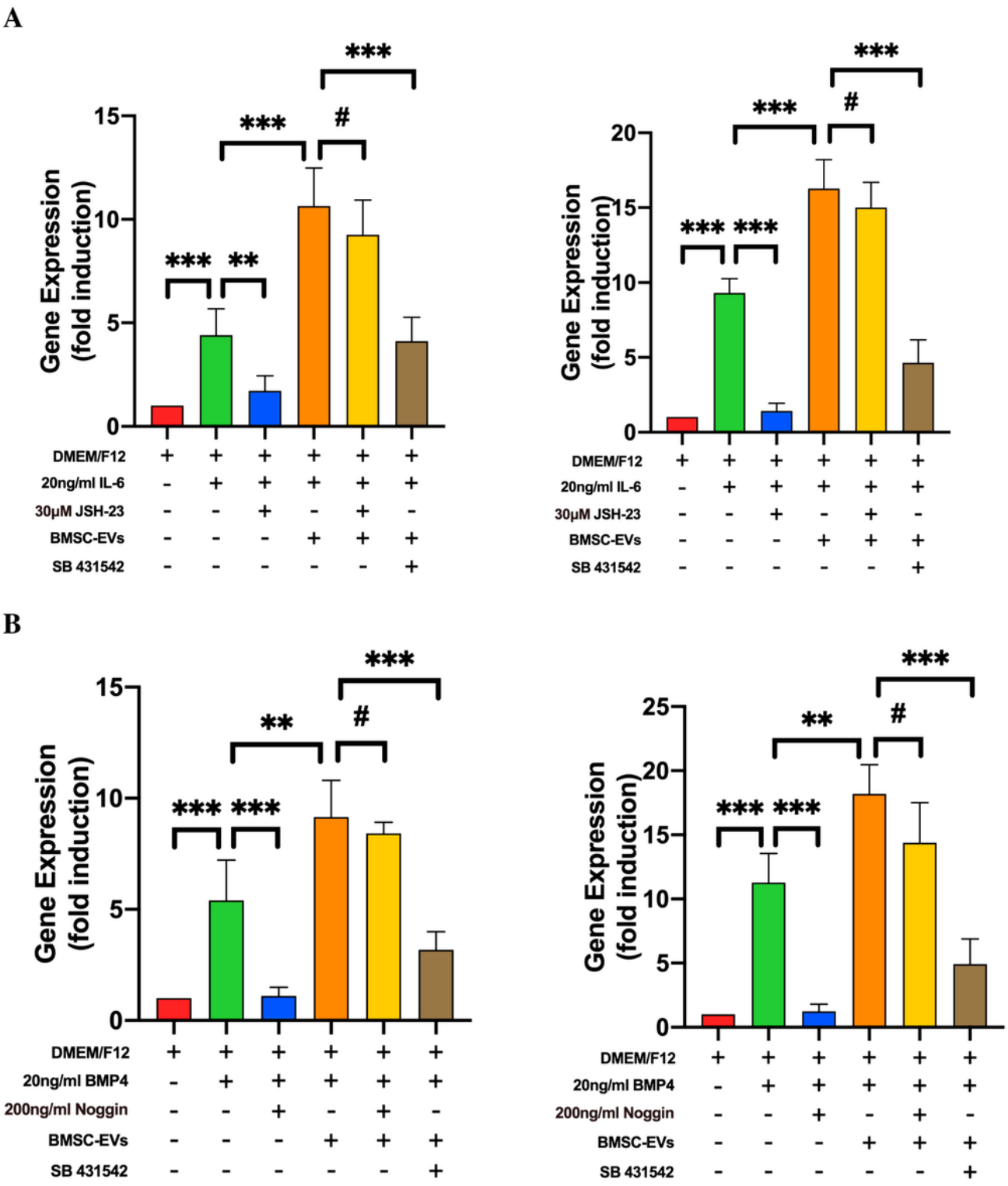

Figure 8

Expression of Smad 6 in different groups. Student's t-test was used for comparisons, *p < 0.05, **p < 0.01, ***p<0.001, #p > 0.05.

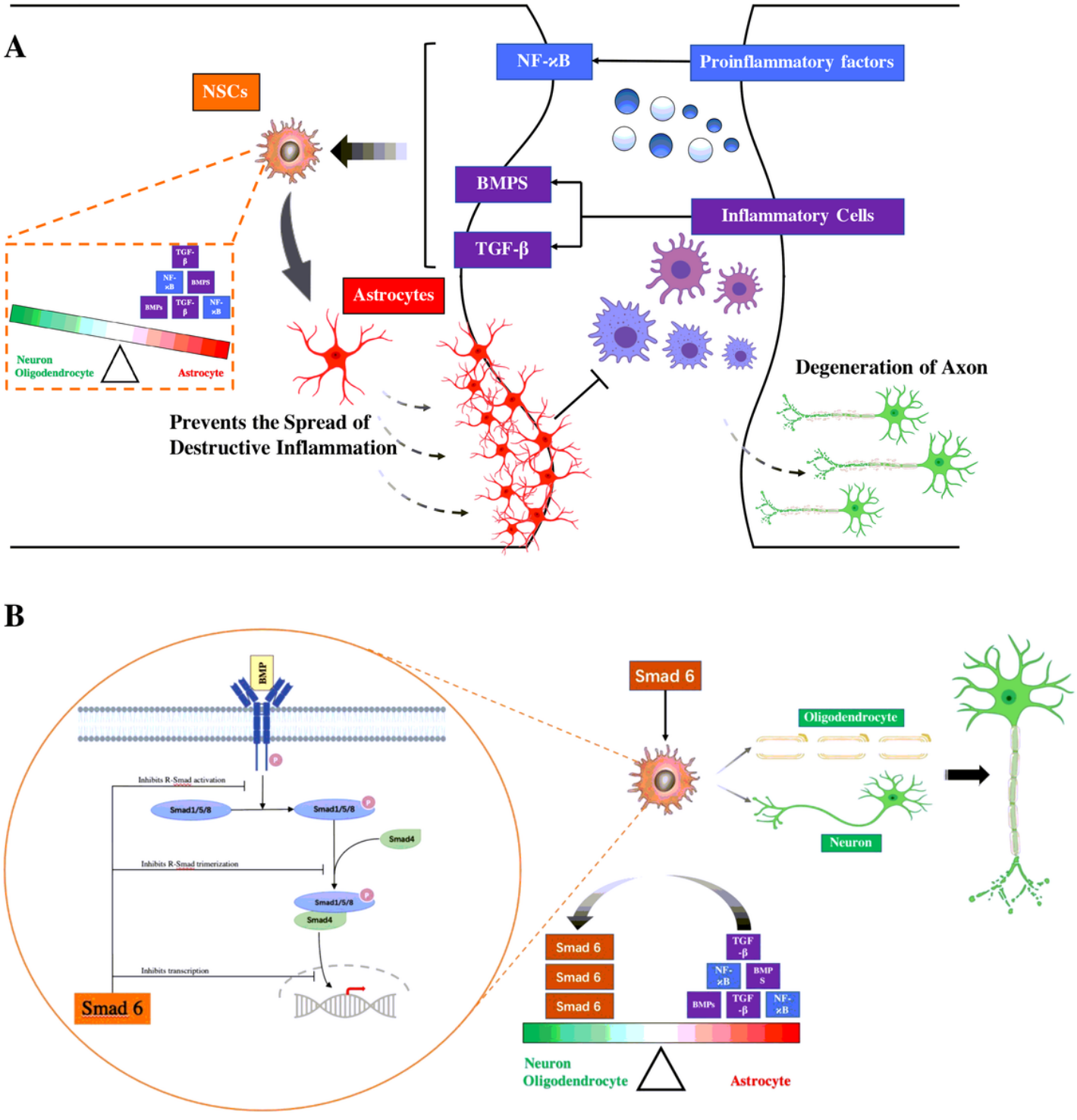

Figure 9

Various factors accumulate in the injury lesion and induce the differentiation of NSCs into astrocytes, which contribute to prevent the spread of destructive inflammation and protect the adjacent neural cells from apoptosis. Meanwhile, these factors also upregulate the expression of Smad 6, which act as a negative feedback regulator of astrocytic differentiation and prevents the over-formation of glial scar.

Supplementary Files

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- GraphicalAbstract.tif