BMSC-CM prevents over-differentiation of NSCs to astrocytes by upregulating Smad 6 expression

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Research

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Abstract

**Background:** 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.

**Method:** Conditioned medium (CM) was collected from bone marrow MSCs (BMSCs) isolated from rats with SCIs, and its effect on the regulation of Smad 6 expression was tested in vitro (in NSCs) and in vivo (SCI rats). Western blot analysis and immunohistochemistry staining were used to investigate the proportion of neurons and astrocytes in vitro and in vivo. BBB scores were used to assess the neurological outcome of SCI rats at different time points.

**Results:** BMSC-CM could upregulate Smad 6 expression in vitro. BMSC-CM-induced upregulation was suppressed by pre-treatment with the TGF-β type I receptor kinase inhibitor SB431542. BMSC-CM was able to promote the differentiation of NSCs to neurons; Smad 6 knockdown in NSCs partly weakened this effect on neural differentiation. In vivo, Smad 6 expression in the later phase of injury was closely associated with BMSC-CM treatment.

**Conclusion:** BMSC-CM can upregulate Smad 6 expression by the secretion of TGF-β. It promotes the differentiation of NSCs into neurons, partly through upregulation of Smad 6.

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-\(\beta\), 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-\(\beta\), acts as a negative feedback regulator in TGF-\(\beta\) 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-CM) was able to upregulate Smad 6 expression in NSCs; blocking TGF-\(\beta\) diminished the BMSC-CM-related upregulation of Smad 6 expression, suggesting that BMSC-CM mediated Smad 6 expression through the secretion of TGF-\(\beta\). Moreover, Smad 6 knockdown in NSCs partly weakened the BMSC-CM-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-\(\beta\) type I receptor kinase inhibitor to BMSC-CM treated rats only reduced the Smad 6 expression in the later phase of injury, indicating that the upregulation of Smad 6 was closely associated with BMSC-CM 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'-GAUUCUACAUUGUCUUAAC-3'; antisense, 5'-UGU∀GAC∀UGUAG∀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: 1.5 ml BMSC-CM or 10 ng/ml TGF-β (R&D Systems); 1.5 ml BMSC-CM + 10 µM SB431542 (Sigma); 1.5 ml BMSC-CM + 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 conditioned medium

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.

The CM was prepared from a 90% confluence of passage 3 BMSCs, as described in previous studies[27, 29]. Briefly, confluent BMSCs were cultured with a serum-free DMEM/F12 (Gibco, USA) medium for 48 h. The CM was harvested from different flasks. A 10 kDa MW filter unit (Millipore, USA) was used to concentrate the collected CM by centrifugation at 4000 g for 15 minutes. The harvested CM was filtered using a 0.22 mm filter (Millipore, USA) and stored at −80 °C.

3. ELISA

The level of TGF-β in BMSC-CM was detected using an ELISA kit (Sigma), as per 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-CM treated, BMSC-CM + SB431542_{day0}-treated and
BMSC-CM + SB431542\textsubscript{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-CM, or BMSC-CM + SB431542\textsubscript{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-CM + the SB431542\textsubscript{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: 5ʹ-CTCGGTTGAVTCTCAGATG-3ʹ, 5ʹ-TGGTGCACGCATAGAG-3ʹ; Id2: 5ʹ-TCACACGAGCGCATAGATC-3ʹ, 5ʹ-CAGTCGCTGAGCTGGAG-3ʹ; GAPDH: 5ʹ-ACCTAGGGCATGATGATG-3ʹ, 5ʹ-GATGCCAGGATGATGATG-3ʹ.

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-CM affects the expression of Smad 6 in NSCs

In order to determine whether BMSC-CM was able to regulate the expression of I-Smads following SCI, we first examined the expression of Smad 6 in NSCs after adding BMSC-CM. The level of Smad 6 mRNA increased as early as 1 h after BSCM-CM was added to NSCs. It reached a peak at 12 h and dropped to the control level at 48 h (Fig. 1A). This suggests that Smad 6 expression in NSCs was activated by BMSC-CM.

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-CM regulates Smad 6 expression.

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

To identify whether the BMSC-CM-induced alteration of Smad 6 expression was caused by TGF-β, we measured the expression of TGF-β in BMSC-CM. ELISA assays of all five samples of concentrated CM showed that TGF-β in CM had an average concentration of approximately 590 (587 ± 115) pg/ml (Fig. 1B). 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. 1C).

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

3. BMSC-CM 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-CM-induced differentiation. We directly added SB431542 in the presence of BMSC-CM to block TGF-β signaling and then measured the proportion of astrocytes and neurons. The exposure of NSCs to BMSC-CM 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. 2A). 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-CM led to a higher proportion of neurons and a lower proportion of astrocytes compared to the BMSC-CM-treated NSCs (Fig. 2B). 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-CM was still able to promote Smad 6 knockdown NSC differentiation into neurons, compared to the control groups (Fig. 2C). However, compared to normal NSCs that received BMSC-CM treatment, the Smad 6 knockdown NSCs had a lower proportion of neurons and a higher proportion of astrocytes after co-culture with BMSC-CM for 7 days (Fig. 2C). These results indicated that Smad 6 was partly associated with the BMSC-CM-induced effects on the differentiation of NSCs.

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

To demonstrate that Smad 6 knockdown could affect BMSC-CM-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-CM treatment markedly reduced Id2 expression in NSCs in the presence of BMP4. Although the Id2 expression in Smad 6 knockdown NSCs that received BMSC-CM was higher than that in control groups, it was partly reduced compared to the BMSC-CM groups (Fig. 2E). This indicated that the BMSC-CM-induced effect on Id2 expression was partly attenuated by Smad 6 knockdown. In summary, BMSC-CM-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-CM 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-CM treatment. Unexpectedly, rats that received BMSC-CM 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-CM. However, a marked increase in Smad 6 expression was observed on day 7 following SCI (Fig. 3.A). To test whether this BMSC-CM-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-CM treatment) and on day 3. The results showed that the addition of SB431542 to the BMSC-CM-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. 3B). 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-CM treatment (Fig. 3.C). All these results indicate that the expression of Smad 6 in the later period of SCI (day 7) is closely related to BMSC-CM 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-CM. 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-2+ neurons were found in this scar boundary (Fig. 3D). In contrast, clear neurite outgrowth and extension into the scar tissues and a thin scar boundary were found in rats that received BMSC-CM treatment (Fig. 3D).

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-CM did not increase the number of neurons, but repressed the generation of Map-2+ neurons and their neurite outgrowth into the scar boundary, compared to rats that received only BMSC-CM treatment (Fig. 3D). This result was also confirmed by Western blotting (Fig. 3E). A reduction in Map-2 expression was noted in the BMSC-CM + SB431542_{day0}-treated rats compared to BMSC-CM-treated rats. BMSC-CM + SB431542_{day3}-treated rats, which received SB431542 on day 3 post-SCI, had a higher expression of Map-2 in the neurons around the cavity compared to rats that received only BMSC-CM treatment (Fig. 3D). Consistent with the histology results, the later treatment with SB431542 appeared to provide a better neurological functional outcome (compared to rats that received BMSC-CM), and early treatment with SB431542 appeared to slightly attenuate the BMSC-CM-induced improvements in SCI rats (Fig. 3F).

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 are not differentiated 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. 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-CM to NSCs increased the expression of Smad 6. Moreover, the BMSC-CM-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-CM-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 conditioned medium, proving that it was released by BMSCs. Second, the BMSC-CM-induced up-regulation of Smad 6 was abolished by the addition of the TGF-β inhibitor SB431542. Finally, treatment with SB431542 along with BMSC-CM was able to reduce Smad 6 expression in the later phase of SCI.

The results revealed that in vitro, BMSC-CM was able to increase the expression of Smad 6 in NSCs. However, the in vivo results showed that treatment with BMSC-CM 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-CM. 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 expression[5]. Therefore, it is possible that the BMSC-CM-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\textsubscript{H}1 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-CM treatment of SCI rats in a different phase of the SCI generated a distinct outcome. Compared to the BMSC-CM-treated rats, rats that received both SB431542 and BMSC-CM immediately following the onset of SCI showed a worse histology result and lower BBB scores. In contrast, rats that received BMSC-CM 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 explain 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, upregulation of TGF-β might promote gliosis, which inhibits 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-CM 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

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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