

# HGFN deficiency exacerbates spinal cord injury by promoting inflammation and cell apoptosis through regulation of the PI3K/AKT signaling pathway

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## Conflict of interest

None declared

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## Abstract

**Background.** Spinal cord injury (SCI) is a devastating neurological disease characterized by neuroinflammation and neuronal apoptosis. The PI3K/AKT signaling pathway is related to the pathological process of SCI. Hematopoietic growth factor inducible neurokinin-1 type (HGFN) is a transmembrane glycoprotein that exerts neuroprotective actions in various neurodegenerative diseases. However, the potential role and mechanism of HGFN in the development of SCI are still unclear.

**Objectives.** To investigate the effect of HGFN on inflammation and neuronal apoptosis as well as the underlying mechanism in SCI.

**Materials and methods.** A rat model of SCI was established, and Basso–Beattie–Bresnahan (BBB) motor function assay was performed to detect motor function. Expression of HGFN was measured at 7 days after injury by western blot and immunofluorescence. An HGFN-shRNA-carrying lentivirus was injected into the injury site to block the expression of HGFN. The effects of HGFN on neuronal apoptosis and the PI3K/AKT pathway were analyzed by TUNEL staining and immunofluorescence. The Iba-1 expression and the levels of pro-inflammatory cytokines were measured in spinal cord tissues by immunofluorescence staining and real-time polymerase chain reaction (PCR) analysis.

**Results.** The SCI rats showed increased expression of HGFN in spinal cord tissues. The HGFN deficiency aggravated SCI lesions, as evidenced by decreased BBB scores. At 7 days post-injury, HGFN knockdown promoted neuronal apoptosis, accompanied by the increased expression level of the apoptosis effector cleaved caspase-3 and cleaved PARP, and decreased anti-apoptotic protein Bcl-2 expression. Moreover, HGFN knockdown aggravated the inflammation process, indicated by increased Iba1-positive cells. The HGFN knockdown increased the production of pro-inflammatory cytokines including IL-1 $\beta$ , TNF- $\alpha$  and IL-6. Further analysis revealed that HGFN deficiency reduced the activation of the PI3K/AKT pathway in spinal cord tissue after injury.

**Conclusions.** Lentivirus-mediated downregulation of HGFN exacerbates inflammation and neuronal apoptosis in SCI by regulating the PI3K/AKT pathway, and provides clues for developing novel therapeutic approaches and targets against SCI.

**Key words:** inflammation, PI3K/AKT pathway, apoptosis, spinal cord injury, HGFN

## Background

Spinal cord injury (SCI) is a devastating neurological disease that impairs neurological functions and leads to irreversible motor dysfunction.<sup>1</sup> The initial injury involves mechanical trauma of the spine that provokes a series of cellular and molecular events, including posttraumatic inflammation, edema, motor neuron apoptosis, and death of neurons.<sup>2,3</sup> Although considerable effort has been devoted to understanding the pathophysiology of SCI, the underlying mechanisms of the pathophysiological cascade of SCI remain elusive. Extensive pathological hallmarks, inflammation responses and neuronal apoptosis represent the major characteristics of SCI.<sup>4,5</sup> The phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is closely related to the pathological process of SCI, and the activation of the PI3K/AKT pathway delays the inflammatory response and promotes neurological function recovery in the progression of SCI.<sup>6,7</sup>

Hematopoietic growth factor inducible neurokinin-1 type (HGFIN), an endogenous type I transmembrane glycoprotein, was initially isolated from a cDNA library based on low-metastatic melanoma cells<sup>8</sup> and has been found to regulate various biological functions.<sup>9–11</sup> Notably, HGFIN exerts neuroprotective effects by protecting against neuronal apoptosis and enhancing neurogenesis through the regulation of the PI3K/AKT pathways.<sup>12</sup> In addition, HGFIN is upregulated in amyotrophic lateral sclerosis and inhibits neuron cell death.<sup>13</sup> It has been indicated that HGFIN is significantly associated with inflammatory responses and is considered a negative regulator of inflammation.<sup>14–16</sup> Hematopoietic growth factor inducible neurokinin-1 type exerts an anti-inflammatory effect in acutely injured kidneys and acute wound healing,<sup>15,17</sup> and attenuates the inflammatory response of astrocytes and lipopolysaccharide (LPS)-induced inflammation.<sup>18,19</sup> The inhibition of HGFIN suppresses pro-inflammatory cytokine expression in LPS-induced microglia.<sup>20</sup> Importantly, HGFIN expression has been observed to be significantly altered in the progression of SCI and exert a functional role in the regulation of neuronal death and neuroinflammation.<sup>21</sup> However, the regulatory role and specific mechanism involved in the effect of HGFIN on the pathophysiology of SCI are still unknown.

In the present study, we aimed to investigate the impact of HGFIN on the extent of the SCI model, including neuronal apoptosis and the inflammation process. The expression of HGFIN in spinal cord tissues after SCI was examined. In addition, the impact of HGFIN deficiency on neuronal apoptosis and the inflammatory process was explored following SCI. Moreover, the association between HGFIN and the activation of the PI3K/AKT pathway was examined. We speculated that HGFIN exerted its function through the regulation of PI3K/AKT signaling. Collectively, the target transmembrane glycoprotein,

HGFIN, might be a potential therapeutic strategy for SCI treatment.

## Objectives

This study aimed to investigate the effect of HGFIN on rat SCI model. We examined the expression level of HGFIN in the spinal cord tissues following SCI. In addition, we investigated the specific effects of HGFIN on the neuroprotective actions and inflammatory process after SCI. We evaluated the effects of HGFIN on the activation of the PI3K/AKT signaling pathway to further clarify its possible underlying mechanism.

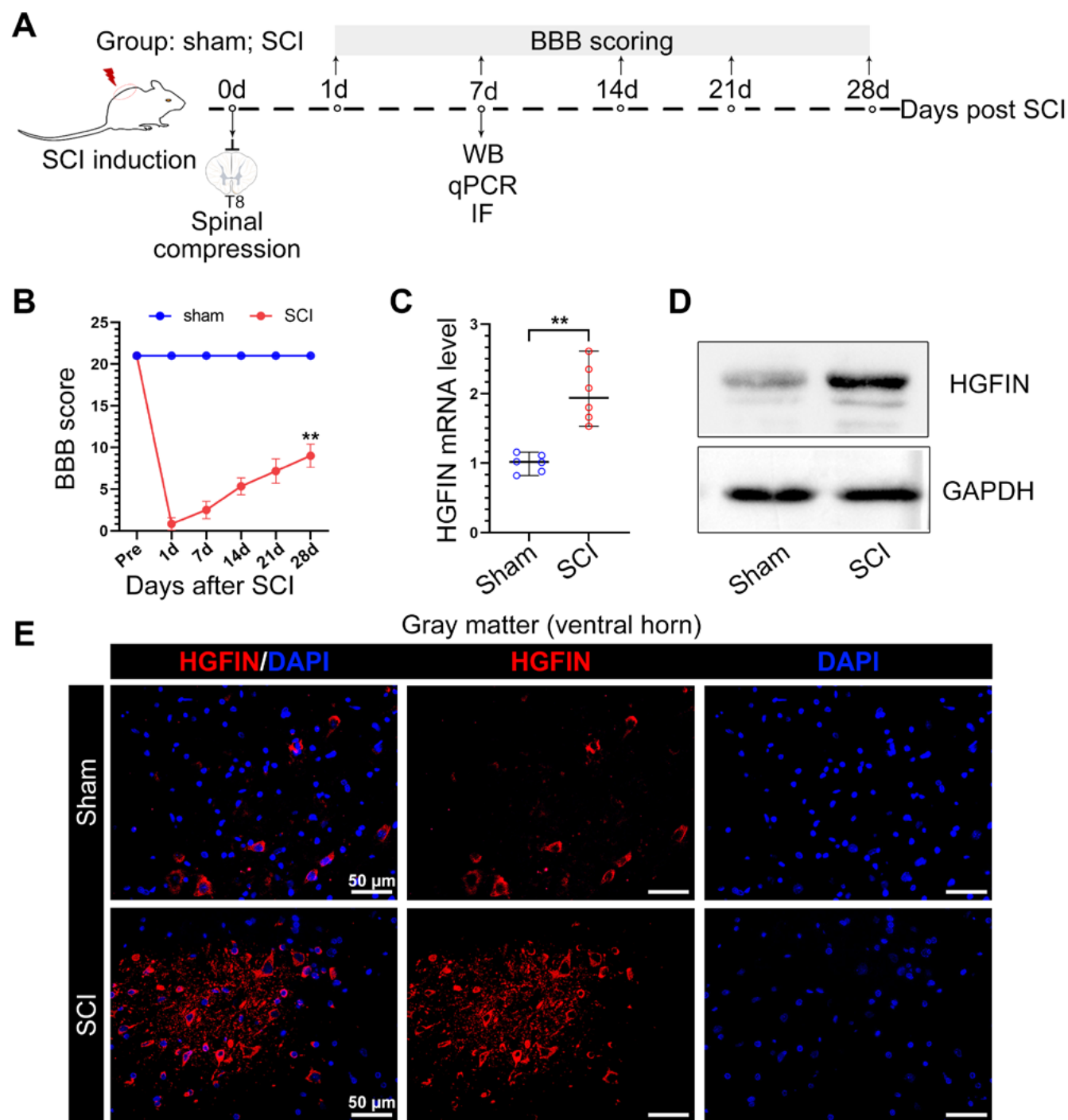
## Materials and methods

### Animals

All procedures involving the animals were approved by the Animal Care and Use Committee of the Chaohu Hospital at the Anhui Medical University (approval No. KYXM-202207-009). Male Sprague–Dawley (SD) rats (8–12 weeks old) were maintained under standard conditions (22 ± 1°C, 45–55% humidity, and 12-h light/dark cycle). The number of animals subjected to surgical treatment was calculated to be 6 per experimental group. Rats were randomized into 4 groups: 1. Sham group; 2. SCI group; 3. SCI+sh-NC group; 4. SCI+sh-HGFIN group. The assessments were shown as a schematic in Fig. 1A.

### Lentivirus construction and animal treatment

Lentiviruses containing HGFIN-shRNA (NM\_053110) were constructed and synthesized by Shaanxi YouBio Technology Co., Ltd (Changsha China). The target sequence against HGFIN was as follows: 5'-CGAAGGT-GAAAGATGTGTATG-3'. The virus titer was determined as 1 × 10<sup>9</sup> TU/mL. For the establishment of the SCI model, SD rats were anesthetized, and a T10 laminectomy was carried out after making a 4-cm longitudinal incision and careful dissection. Vascular clips were placed through the dorsal intervertebral space of T8–T9 to compress the spinal cord for 10 min in order to generate an injury. Rats in the sham group had the surgical procedure without spinal cord contusion. After surgery, the muscles and skin were sutured. After sterile analgesic treatment and ongoing monitoring, rats received bladder massage 3 times a day to prevent urological infection. Following SCI, 10 µL of lentivirus containing HGFIN-shRNA (1 × 10<sup>9</sup> TU/mL) or NC-shRNA (1 × 10<sup>9</sup> TU/mL) was locally injected into the injured site immediately using

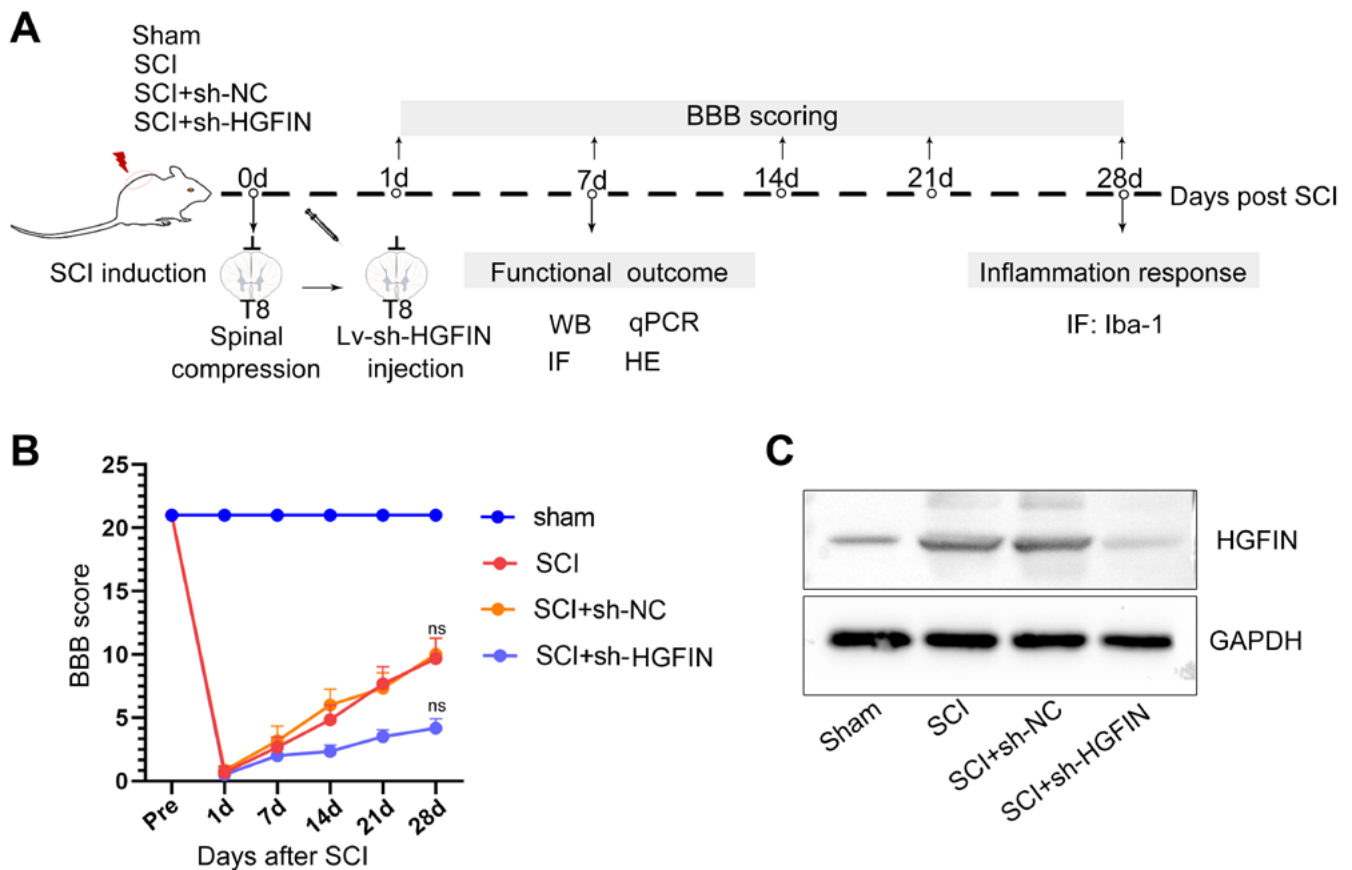


**Fig. 1.** Expression of hematopoietic growth factor inducible neurokinin-1 type (HGFIN) in the spinal cord after spinal cord injury (SCI). **A.** Experimental protocol for SCI timing and outcome assessment; **B.** Basso–Beattie–Bresnahan (BBB) exercises scored at 1, 7, 14, 21, and 28 days after injury in the sham and SCI groups ( $n = 6$ ). Differences between group means in BBB scores at 28 days after the injury were identified using the Mann–Whitney U test; **C.** HGFIN mRNA levels in spinal cord tissues after SCI were measured by real-time polymerase chain reaction (PCR). The Mann–Whitney U tests were used to calculate the significant differences; **D.** Western blot results of HGFIN protein levels after SCI; **E.** Immunofluorescence staining for the expression of HGFIN in the grey matter after SCI. Data are presented using medians (range)

\*\* $p < 0.01$  compared to the sham group; WB – western blot; IF – immunofluorescence.

a microsyringe, while the rats in the sham group received the same volume of normal saline. Behavioral testing was assessed using a well-established Basso–Beattie–Bresnahan (BBB) score assay at 1, 7, 14, 21, and 28 days. The animals were euthanized using 30% volume/min

$\text{CO}_2$ . Spinal cord tissues at the lesion sites of the cord were collected from rats on day 7 post-injury. All tissues were quickly placed in paraformaldehyde overnight and embedded in paraffin for further examinations. The assessments are shown as a schematic in Fig. 2A.



**Fig. 2.** Hematopoietic growth factor inducible neurokinin-1 type (HGFIN) deficiency aggravates injury to the spinal cord after spinal cord injury (SCI). HGFIN shRNA lentivirus vectors were locally injected into the injured site immediately after SCI. **A.** Timeline of the experimental protocol; **B.** Basso–Beattie–Bresnahan (BBB) exercises were scored at 1, 7, 14, 21, and 28 days after SCI in all groups ( $n = 6$ ). Differences between the group means in BBB scores at 28 days after the injury were identified using the Kruskal–Wallis test with Dunn’s post hoc test; **C.** HGFIN protein levels were detected by western blot assay following injection of sh-HGFIN lentivirus in SCI rats

ns – not significant, compared to the sham or SCI+sh-NC groups; WB – western blot; IF – immunofluorescence; qPCR – quantitative real-time polymerase chain reaction; HE – hematoxylin and eosin.

## Real-time polymerase chain reaction

For the real-time polymerase chain reaction (PCR) analysis, total RNA was extracted from spinal cord tissues with TRIpure™ solution (BioTeke, Beijing China). Reverse transcription of the RNA samples was carried out using a PCR system according to the protocol. Data were calculated using the  $2^{-\Delta\Delta C_t}$  method and normalized to the housekeeping gene *GAPDH*. Primers were as follows:

rat HGFIN  
 5'-TAGAAGTCAACATCATCCAGGTA-3' (forward),  
 5'-ACGGACAGGAGGCACAG-3' (reverse);  
 rat TNF- $\alpha$   
 5'-CGGAAAGCATGATCCGAGAT-3' (forward),  
 5'-AGACAGAAGAGCGTGGTGGC-3' (reverse);  
 rat IL-1 $\beta$   
 5'-TTCAAATCTCACAGCAGCAT-3' (forward),  
 5'-CACGGGCAAGACATAGGTAG-3' (reverse);  
 rat IL-6  
 5'-CAGCCACTGCCTTCCCTA-3' (forward),  
 5'-TTGCCATTGCACAACCTCTTT-3' (reverse).

## Western blot analysis

For western blot assay, total proteins were extracted from spinal cord tissues and homogenized in RIPA lysates (Solarbio, Beijing, China) supplemented with phenylmethylsulphonyl fluoride (PMSF). The protein concentration was evaluated by the BCA assay kit (Solarbio). Proteins were resolved on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to the polyvinylidene fluoride membranes (MilliporeSigma, St. Louis, USA), and blocked in the non-fat powdered milk. The membranes were then incubated with primary antibody at 4°C overnight. After incubation with HRP-conjugated secondary antibodies, the blots were developed with enhanced chemiluminescence substrate reagents (Solarbio). Primary antibodies used in the present study included HGFIN (1:5000; Proteintech Genomics, San Diego, USA; 66926-1-Ig), cleaved PARP (1:1000, AF7023; Affinity Biosciences, Cincinnati, USA), cleaved caspase-3 (1:1000, AF7022; Affinity), Bcl-2 (1:1000, AF6139; Affinity), AKT (1:3000, 10176-2-AP; ProteinTech), and p-AKT (1:2000, 66444-1-Ig; ProteinTech).

## Immunofluorescent staining

For immunofluorescence staining, spinal cord samples were embedded in paraffin. The sections were deparaffinized and blocked with 1% bovine serum albumin (BSA), followed by incubation at 4°C overnight with primary antibodies against HGFN (66926-1-Ig; ProteinTech), p-AKT (Ser473, 66444-1-Ig; ProteinTech), and ionized calcium-binding adapter molecule-1 (Iba-1, 10904-1-AP; ProteinTech). Then, the tissues were nurtured with Cy3-labeled secondary antibodies (IgG; Invitrogen, Waltham, USA) for 60 min, followed by counterstaining with 4',6-diamidyno-2-fenyloindol (DAPI; Aladdin, Shanghai, China) to stain the cell nucleus. After being mounted, the immunofluorescent images were obtained using a fluorescence microscope (Olympus Corp., Tokyo, Japan).

## TUNEL-NeuN staining

Double immunofluorescent staining with a TUNEL-NeuN assay was used to detect neuronal apoptosis. In brief, paraffin-embedded spinal cord samples were deparaffinized and permeabilized in 0.1% Triton X-100 (Beyotime, Shanghai, China). The sections were repaired in citric acid/sodium citrate solution for 10 min. Then, samples were stained with TUNEL reagent using the In Situ Cell Death Detection Kit (No. 12156792910; Roche Diagnostics, Basel, Switzerland) according to protocols. Samples were blocked in 1% BSA and incubated at 4°C overnight with a primary antibody against NeuN (ab104224; Abcam, Cambridge, USA) and visualized with FITC-labeled goat anti-mouse IgG (ab6785; Abcam). The nucleus was counterstained with DAPI and sections were sealed. TUNEL-positive cells (labeled with red fluorescence) and NeuN-positive cells (labeled with green fluorescence) in tissues were captured under a fluorescence microscope. The ratio of NeuN-TUNEL double-stained cells/NeuN-stained cells was calculated for the quantification of neuronal apoptosis.

## Hematoxylin and eosin staining

The fixed spinal cord tissues were used for hematoxylin and eosin (H&E) staining according to the routine procedure. In brief, the paraffin-embedded sections were successively deparaffinized, dehydrated and subjected to H&E staining for conventional histopathologic examination in SCI. The sections were incubated with hematoxylin for 5 min, followed by flushing with running water, and counterstained with eosin staining solution for 3 min. After dehydration, clearing and mounting, the sealed slides were captured under a light microscope.

## Statistical analyses

All calculations were performed by the GraphPad Prism software (GraphPad Software, San Diego, USA) with a probability value of  $p < 0.05$  considered significant; all values were expressed using medians (range). The small sample size limits checking test assumptions reliably. Nonparametric tests were used in the present study. A Mann–Whitney U test was performed to analyze data when comparing 2 groups. The homogeneity of variance was determined via the F-test. In the case of multiple comparisons, a Kruskal–Wallis test with Dunn's post hoc test was performed. The homogeneity of variance was determined via the Brown–Forsythe test.

In the present study, HGFN mRNA levels were analyzed using the Mann–Whitney U test. The quantification of TUNEL/NeuN, p-AKT positive cells, Iba-1 positive cells, and levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were analyzed using the Kruskal–Wallis test with a Dunn's post hoc test. Differences between group means in BBB scores were analyzed 28 days after the injury using the Kruskal–Wallis test.

## Results

### HGFN expression is increased in spinal cord tissues after SCI

An SCI animal model was successfully established. We used BBB scores to assess motor function for 28 days after the SCI. Normal motor function was scored as 21 points. As indicated in Fig. 1B, rats in the SCI groups showed lower BBB scores compared with the sham group. By the time of injury, BBB scores increased progressively and recovered to around 9 at 28 days after SCI. The expression of HGFN in the spinal cord tissues after SCI was determined by western blot and real-time PCR assay. The HGFN mRNA and protein expression levels in spinal cord tissues were significantly increased at 7 days in the SCI group compared with the sham group (Fig. 1C,D). Furthermore, immunofluorescence staining was performed to detect HGFN expression in the spinal cord tissues of SCI rats, and the results indicated that HGFN was highly expressed in the gray matter in the spinal cord tissues of SCI rats (Fig. 1E).

To further explore the regulatory role of HGFN, lentiviruses carrying HGFN shRNA particles were injected into SCI rats to block HGFN expression. Basso–Beattie–Bresnahan motor function scores were performed to assess motor function at indicated time points after SCI. Results of BBB scores indicated that HGFN deficiency aggravated SCI lesions (Fig. 2B). Basso–Beattie–Bresnahan scores in the SCI+sh-HGFN group were lower than those in the SCI+sh-NC group. Animals treated with sh-HGFN accelerated injury and as the observations continued, we noted functional recovery in the SCI+sh-HGFN group, and there were no significant differences in motor



function at the end of observation on day 28 between the SCI+sh-NC and SCI+sh-HGFIN groups. Western blot assay revealed that HGFIN protein expression was up-regulated in SCI rat tissues and when administered with HGFIN shRNA exhibited significantly lower expression of HGFIN (Fig. 2C).

### Reduced HGFIN expression promotes SCI-induced neuronal apoptosis

The impact of HGFIN deficiency on neuronal apoptosis was assessed at 7 days after SCI. Double staining of TUNEL and NeuN revealed that SCI caused an elevated number of apoptotic neurons, and treatment with sh-HGFIN further aggravated neuronal apoptosis (Fig. 3A). In addition, the ratio of NeuN-TUNEL double-stained cells/NeuN-stained cells was quantified. A larger percentage of apoptotic neurons was observed in SCI groups ( $p < 0.05$ ), and HGFIN knockdown further promoted neuronal apoptosis by increasing the percentage of apoptotic neurons (Fig. 3B). As indicated in Fig. 3C, the levels of apoptosis effector cleaved caspase-3 and cleaved PARP were found to be significantly upregulated, while the levels of anti-apoptotic protein Bcl-2 were downregulated at 7 days post-SCI. The HGFIN depletion further promoted SCI-induced neuronal apoptosis, as indicated by upregulated cleaved caspase-3, cleaved PARP and downregulated Bcl-2.

### Depletion of HGFIN activates the PI3K/AKT signaling pathway

Next, we explored whether HGFIN deficiency was implicated with PI3K/AKT pathway activation. To this end, immunofluorescent staining for p-AKT was performed at 7 days post-injury. As indicated in Fig. 4A, a reduction of p-AKT was observed in the injured spinal cord tissues of SCI rats compared to the sham rats. Knockdown of HGFIN after SCI significantly reduced p-AKT expression in the spinal cord gray and white matter ( $p < 0.05$ ; Fig. 4A,B). Western blot assays further confirmed that HGFIN deficiency decreased the protein expression of p-AKT (Fig. 4C).

### Depletion of HGFIN promotes the inflammatory response after SCI

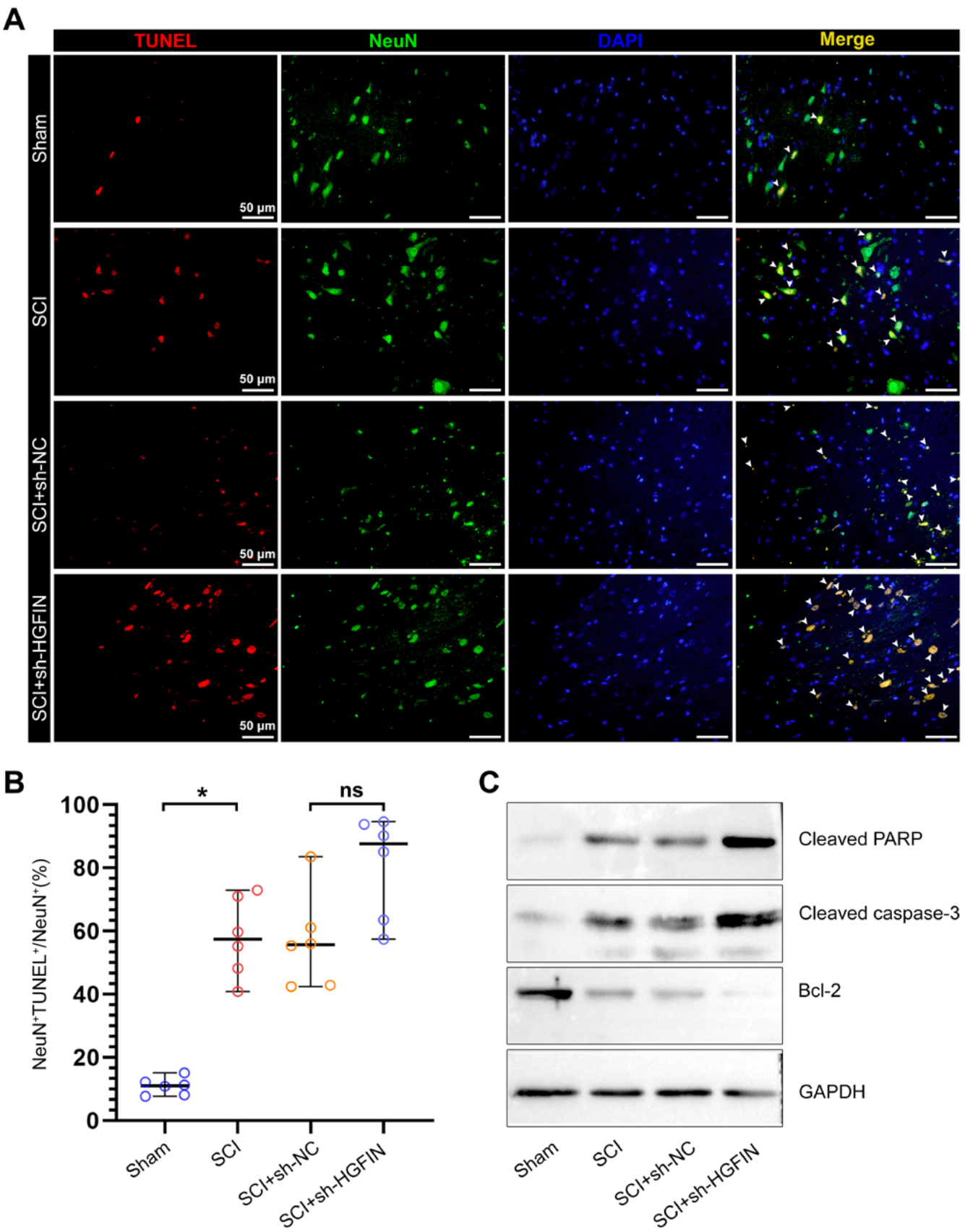
The Iba-1 immunofluorescent assays were performed to assess the reactive inflammatory responses in the injured spinal cord tissues. As shown in Fig. 5A, the number of Iba-1 positive cells in the SCI groups was markedly increased ( $p < 0.05$ ; Fig. 5B). Furthermore, SCI rats administered with HGFIN shRNA showed a larger number of Iba-1 positive cells in the spinal cord tissues at 7 days post-SCI. Hematoxylin and eosin staining was performed to assess the histopathological changes in spinal cord tissues. The results of the staining showed the infiltration

of neutrophils, congestion and structural damage of the SCI, and the injury was notably aggravated following HGFIN knockdown (Fig. 6A). In addition, the effect of HGFIN deficiency on the expression of inflammatory-associated cytokines was measured. The expressions of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$  and IL-6, were low in the sham groups and significantly increased in SCI spinal cord rats. The inhibition of HGFIN further promoted the production of pro-inflammatory cytokines ( $p < 0.05$ ; Fig. 6B).

## Discussion

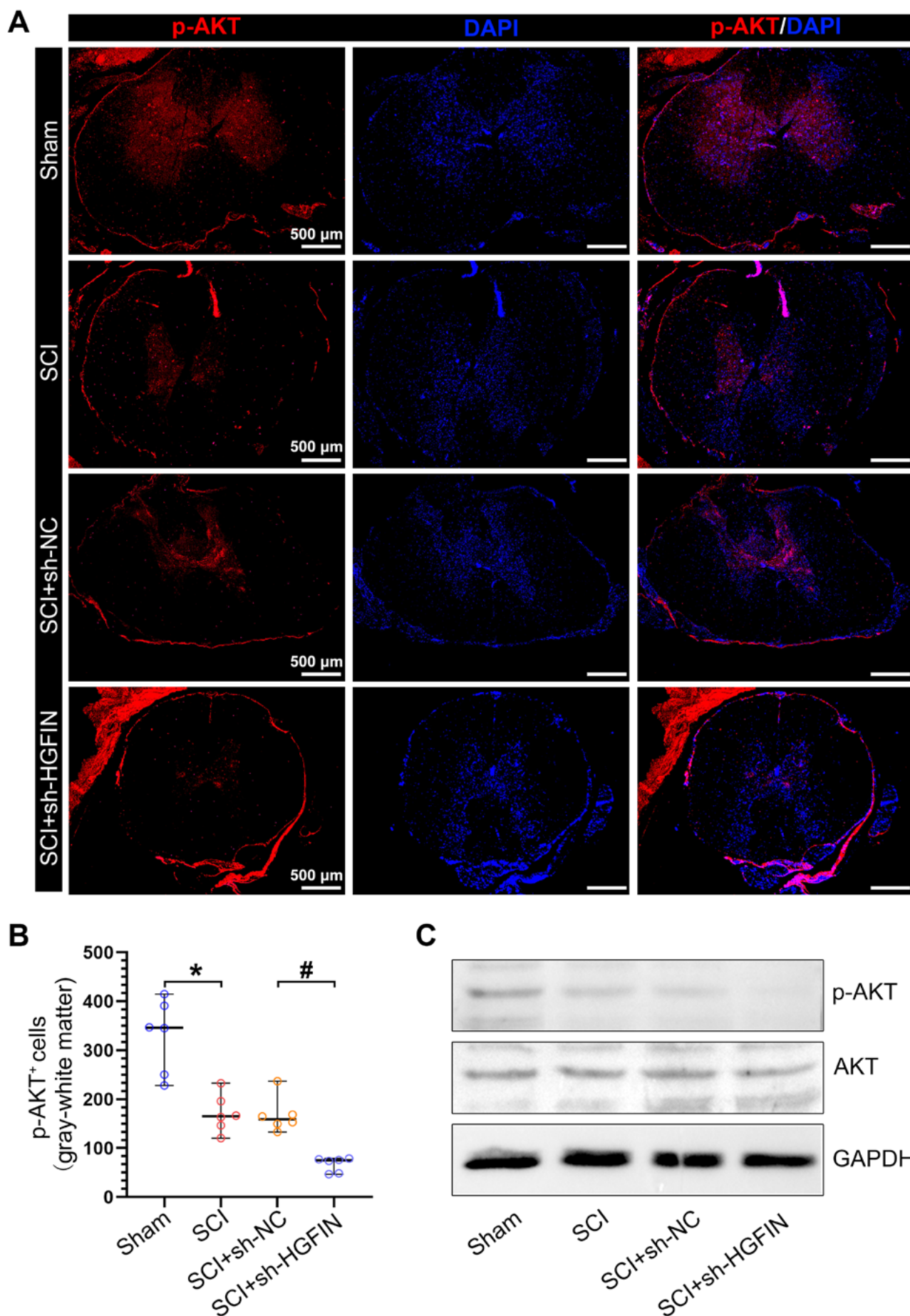
Spinal cord injury is a complex and multifaceted disease process, and numerous therapeutic approaches against SCI have generated successful results,<sup>2,22,23</sup> among which neuroprotection and improving the immune environment are promising strategies.<sup>24</sup> Accumulating evidence demonstrates that HGFIN has neuroprotective roles and is implicated in inflammatory responses.<sup>18,19</sup> Thus, we explored whether HGFIN exerts a regulatory function in SCI progression and can be a potential marker for SCI. In the present study, a rat model of SCI was successfully established, and the expression of HGFIN in the spinal cord tissues of the SCI rats was detected. It has been indicated that HGFIN was aggregated in the grey and white matter of spinal cord tissue in amyotrophic lateral sclerosis patients.<sup>25</sup> Consistently, in the present study, immunofluorescent staining for HGFIN revealed that HGFIN was highly expressed in the grey matter of spinal cord tissue after SCI. Hematopoietic growth factor inducible neurokinin-1 type was found to be highly expressed in the spinal cord tissue of SCI rats. Emerging evidence has indicated that HGFIN is recognized as a potential neurodegenerative disease-related marker and is found to be upregulated in numerous neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis.<sup>26–29</sup> Therefore, lentiviruses carrying HGFIN shRNA were injected into SCI rats to knock down HGFIN, and the effect of HGFIN deficiency on inflammation and neuronal apoptosis was explored.

The destructive effect of neuronal apoptosis in neuronal diseases, including SCI, has been well-documented.<sup>30,31</sup> It is generally accepted that apoptosis is important in the pathophysiology of SCI, and apoptotic-related signaling and mediators, such as caspase cascades, Bax/Bcl-2, and TNF- $\alpha$ , in apoptosis, are shown to modulate the SCI progression.<sup>32,33</sup> Previous studies have indicated that the expression of pro-apoptotic proteins was increased after neuronal injury, and anti-apoptotic protein expression was commonly decreased.<sup>34,35</sup> Following SCI, extensive activation of PARP and activated caspase-3 occurred, and Bcl-2 expression was decreased in spinal cord tissues.<sup>36</sup> These findings demonstrated that modifying neuronal apoptosis and improving neuron survival might



**Fig. 3.** Effect of hematopoietic growth factor inducible neurokinin-1 type (HGFN)-deficiency on neuronal apoptosis after spinal cord injury (SCI). **A.** Representative images of TUNEL/NeuN double staining after SCI by immunofluorescence in all groups; **B.** Quantitative analysis of the results in panel A ( $n = 6$ ). The Kruskal–Wallis test was used to calculate the significant differences; **C.** Western blot results of apoptosis-related factors, including cleaved caspase-3, cleaved PARP and Bcl-2. Data are presented using the median (range)

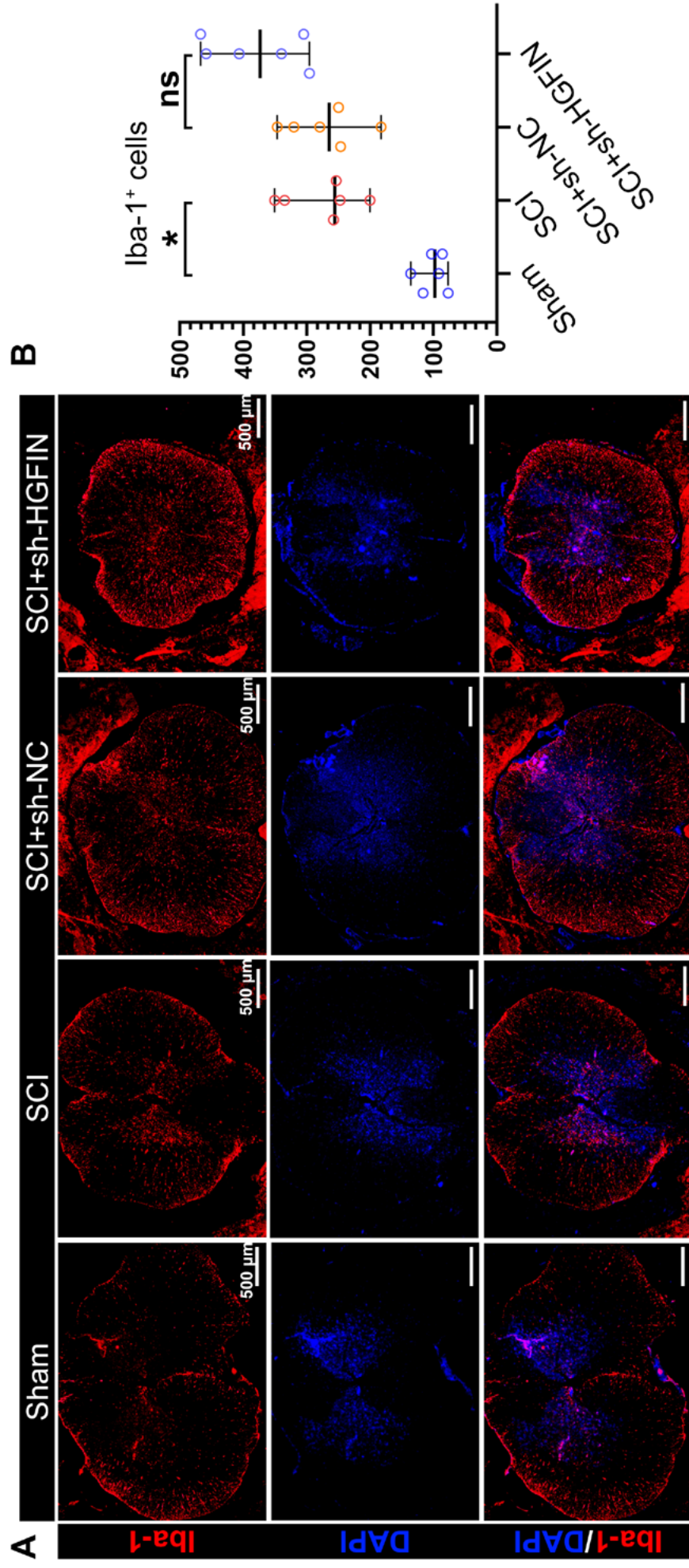
\* $p < 0.05$  compared to the sham group; ns – not significant, compared to the SCI+sh-NC group.



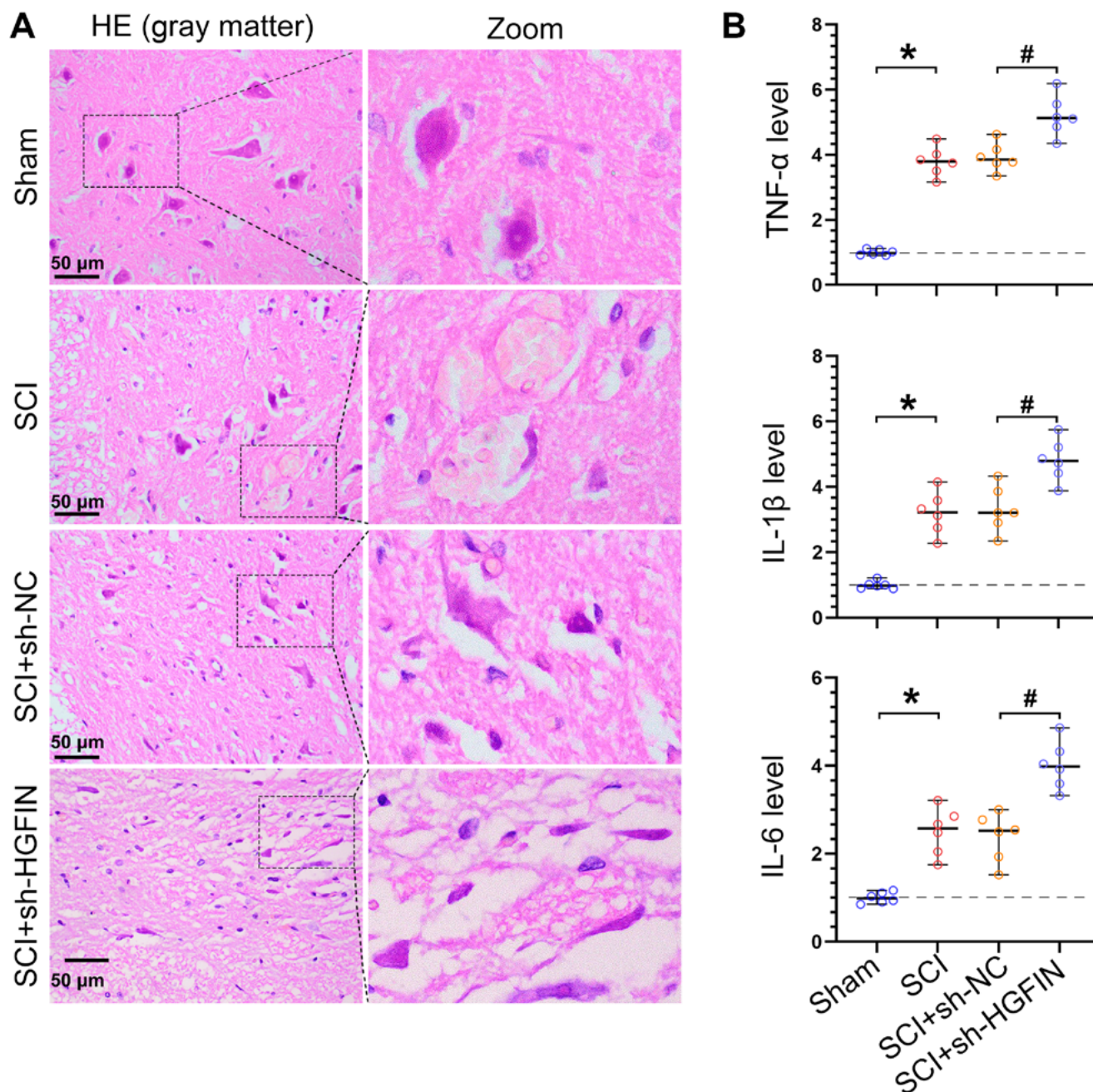
**Fig. 4.** Downregulation of hematopoietic growth factor inducible neurokinin-1 type (HGFIN) represses activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway after spinal cord injury (SCI). A. Immunofluorescence staining for p-AKT in the grey-white matter after injection of sh-HGFIN lentivirus in SCI models; B. Quantitative analysis of the number of p-AKT positive cells ( $n = 6$ ). The Kruskal–Wallis test was used to calculate the significant differences; C. Western blot results of AKT and p-AKT protein levels. Data are presented using the median (range)

\* $p < 0.05$  compared to the sham group; # $p < 0.05$  compared to the SCI+sh-NC group.





**Fig. 5.** Effect of hematopoietic growth factor inducible neurokinin-1 type (HGFIN) on Iba-1 expression in the spinal cord after spinal cord injury (SCI). A. Representative images of Iba-1 immunofluorescence staining; B. Quantitative analysis of the number of Iba-1 positive cells ( $n = 6$ ). The Kruskal–Wallis test was used to calculate the significant differences. Data are presented using medians (range). \* $p < 0.05$  compared to the sham group; ns – not significant, compared to the SCI+sh-NC group.



**Fig. 6.** Downregulation of hematopoietic growth factor inducible neurokinin-1 type (HGFIN) promotes the inflammation response after spinal cord injury (SCI). A. Hematoxylin and eosin staining of spinal cord sections in different groups; B. Relative mRNA expression levels of pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$  and IL-6, were detected by real-time polymerase chain reaction (PCR). The Kruskal–Wallis test was used to calculate the significant differences. Data are presented using medians (range)

\* $p < 0.05$  compared to the sham group; # $p < 0.05$  compared to the SCI+sh-NC group.

be an important strategy for the improvement of SCI. In the present study, the apoptotic mechanism was confirmed by evaluating the extent of apoptosis by TUNEL staining. Consistently, SCI surgery promoted neuronal apoptosis, and the apoptosis-related proteins were significantly changed in the spinal cord tissues of SCI rats. The impact of HGFIN on cell apoptosis was investigated, and SCI rats subjected to LV-shHGFIN aggravated the occurrence of apoptosis, accompanied by increased

pro-apoptotic marker expression and downregulated anti-apoptotic marker Bcl-2. These data suggested that HGFIN might exert an anti-apoptotic effect on SCI progression.

The activation of the PI3K/AKT signaling pathway is crucial for neuron development, which exerts anti-neuroinflammation and anti-apoptotic properties in neurons.<sup>6,37</sup> Recent studies have focused on the PI3K/AKT pathway in spinal cord neuron progressions, and targeting PI3K/AKT may be an innovative therapeutic approach for

SCI.<sup>3,38</sup> The PI3K/AKT is a well-known survival signaling pathway that has been shown to suppress neuronal apoptosis, thus improving neural function.<sup>39,40</sup> In the present research, we have focused on the PI3K/AKT pathway and explored whether HGFIN exerted function through the regulation of PI3K/AKT signaling. It has been proposed that recombinant HGFIN increased the expression of phosphorylated ERK1/2 and AKT in amyotrophic lateral sclerosis patients.<sup>25</sup> Consistent with this finding, results of the present study revealed that HGFIN knockdown decreased p-AKT expression after SCI, suggesting that HGFIN exerted its function partly through the regulation of the PI3K/AKT pathway. However, the specific mechanism of HGFIN associated with PI3K/AKT signaling in SCI was not investigated in the current study.

Given the involvement of HGFIN in the inflammatory responses that negatively regulate inflammation,<sup>14</sup> we speculated that HGFIN deficiency promoted the inflammation process in SCI. The Iba-1 is a key glial cell activation marker that is generally used to assess the inflammatory response.<sup>41,42</sup> Here, we found that HGFIN knockdown increased the number of Iba-1 positive cells following SCI. In addition, research on the progression of SCI found that inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, were significantly increased in spinal cord tissues of the SCI model.<sup>4</sup> Consistently, the results of the present study demonstrated that SCI rats exhibited inflammatory responses, as indicated by the upregulation of these cytokines in the present study. Moreover, HGFIN expression repressed the production of pro-inflammatory cytokines in macrophages.<sup>16</sup> Here, we demonstrated that HGFIN depletion aggravated inflammatory responses, indicated by the increased expression of pro-inflammatory cytokines and a series of histological alterations.

Spinal cord tissue was collected on days 7, 14 and 28 from SCI rats, which were chosen to represent acute, followed by a subacute phase involving inflammatory processes, and intermediate phases of regeneration, respectively.<sup>43</sup> The response to injury starts at 12 h after injury and is peaking on the 7<sup>th</sup> day. In addition, after 7 days, the spontaneous functional recovery reached a plateau.<sup>44</sup> Therefore, we investigated the functional role of HGFIN on SCI at 7 days post-SCI. In the present study, the increased apoptosis-related factors and pro-inflammatory cytokines were detected on day 7 post-injury.

## Limitations

Our current study was mainly focused on the potential effect of HGFIN on SCI. Nevertheless, there were several limitations. Our data indicated that HGFIN knockdown promoted apoptosis and inflammatory responses, but the role of HGFIN overexpression in these functions is lacking in the present study. This may potentially assist in exploring mechanisms for neuron functional recovery after SCI. Hematopoietic growth

factor inducible neurokinin-1 type plays a dual function in the inflammation process, thus, the underlying mechanisms of the neuroprotective effects of HGFIN against SCI need further research. In addition, we speculated that HGFIN exerted its anti-inflammatory and anti-apoptotic properties by regulating the PI3K/AKT pathway. The specific mechanism associated with the HGFIN-mediated PI3K/AKT pathway should be more deeply evaluated. In addition, the sample size of each group was small and may have limited the generalizability of our results. The nonparametric tests do not indicate significant differences in the quantification of TUNEL/NeuN and Iba-1 positive cells.

## Conclusions

Our findings provide evidence that HGFIN might attenuate neuronal apoptosis and inflammation response via regulation of the PI3K/AKT signaling pathway, indicating that targeting HGFIN in the spinal cord tissue might be a promising therapeutic strategy for the treatment of SCI.

## Supplementary data

The Supplementary materials are available at <https://doi.org/10.5281/zenodo.8420072>. The package includes the following file: Supplementary Table 1 and information on statistical analysis.

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