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Ablation of IGFBP5 expression alleviates neurogenic erectile dysfunction by inducing neurovascular regeneration

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Purpose: To investigate the therapeutic potential of eliminating insulin-like growth factor-binding protein 5 (IGFBP5) expression in improving erectile function in mice with cavernous nerve injury (CNI)-induced erectile dysfunction (ED).

Materials and Methods: Eight-week-old male C57BL/6 mice were divided into four groups: a sham-operated group and three CNI-induced ED groups. The CNI-induced ED groups were treated with intracavernous injections 3 days before the CNI procedure. These injections included phosphate-buffered saline, scrambled control short hairpin RNA (shRNA), or shRNA targeting mouse IGFBP5 lentiviral particles. One week after CNI, erectile function was evaluated and the penile tissue was then harvested for histological examination and western blot analysis. Additionally, the major pelvic ganglia (MPG) and dorsal root ganglia (DRG) were cultured for *ex vivo* neurite outgrowth assays.

Results: Following CNI, IGFBP5 expression in the cavernous tissues significantly increased, reaching its peak at day 7. First, ablation of IGFBP5 expression promotes neurite sprouting in MPG and DRG when exposed to lipopolysaccharide. Second, ablating IGFBP5 expression in CNI-induced ED mice improved erectile function, likely owing to increased neurovascular contents, including endothelial cells, pericytes, and neuronal processes. Third, ablating IGFBP5 expression in CNI-induced ED mice promoted neurovascular regeneration by increasing cell proliferation, reducing apoptosis, and decreasing Reactive oxygen species production. Finally, western blot analysis demonstrated that IGFBP5 ablation attenuated the JNK/c-Jun signaling pathway, activated the PI3K/AKT signaling pathway, and increased vascular endothelial growth factor and neurotrophic factor expression.

Conclusions: Ablating IGFBP5 expression enhanced neurovascular regeneration and ultimately improved erectile function in CNI-induced ED mice.

Keywords: Apoptosis; Erectile dysfunction; Insulin-like growth factor binding protein 5; Nerve regeneration; Reactive oxygen species

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INTRODUCTION

Surgical techniques for radical prostatectomy (RP) for prostate cancer have improved, but the risk of cavernous nerve injury (CNI) remains high [1]. Long-term denervation following RP leads to cavernous hypoxia and structural changes in erectile tissue, reducing the effectiveness of oral phosphodiesterase 5 inhibitors (PDE5Is) [2]. This highlights the need for novel therapeutic strategies and targets to protect and regenerate the penile neurovascular structure after CNI.

Insulin-like growth factor-binding protein 5 (IGFBP5) is a secreted protein that plays a key role in regulating IGF-1 bioavailability [3]. IGFBP5 is the most highly conserved across species and has multiple biological functions. Studies increasingly show that IGFBP5 is frequently dysregulated in various diseases, depending on the cellular context [4,5]. Its numerous biological activities make it a potential marker for disease progression, and changes in its expression may have pathophysiological significance. Ock et al. [6] reported that IGFBP5 knockdown improves erectile dysfunction (ED) by promoting cavernosal angiogenesis in streptozotocin-induced type-1 diabetic mice. However, the effects of IGFBP5 and its underlying mechanisms in peripheral nerve disorder, particularly CNI-induced ED, remain unclear. Therefore, this study aims to investigate the role of IGFBP5 in a CNI-induced ED mouse model using *in vivo* and *ex vivo* systems.

In this study, IGFBP5 expression in the penis of CNI-induced ED mice was found to increase over time after injury, peaking at 7 days. Furthermore, under neuroinflammatory conditions, IGFBP5 ablation promoted neuronal regeneration. Immunohistochemistry and western blot analysis revealed that IGFBP5 ablation inhibited the JNK/c-Jun signaling pathway, activated the PI3K/AKT signaling pathway, and increased the expression of vascular endothelial growth factor (VEGF) and neurotrophic factors, ultimately improving the erectile function in CNI-induced ED mice.

MATERIALS AND METHODS

1. Ethics statement and animal treatment

The Institutional Animal Care and Use Committee (IACUC) at Inha University approved the procedures and animal care used in this study (approval number: 230209-858) [6]. The C57BL/6 male mice were divided into four groups (n=10 per group): a control sham operation group and three groups of bilateral CNI-induced ED mice receiving different treatments. The CNI-induced ED mice model was created following a previously described method [7]. These treatments included intracavernous injections of phosphate-buffered saline (PBS) (20 μL/mouse), scrambled control short hairpin RNA (shRNA) (shCon, 5×10^4 infection units [IFU] in 20 μL), and shRNA targeting mouse IGFBP5 (shIGFBP5) lentiviral particles (shIGFBP5, 5×10^4 IFU in 20 μL; Santa Cruz Biotechnology). The shRNA sequence targeting mouse IGFBP5 was ACGGCTTATGGGTCATTTA.

2. *Ex vivo* neurite sprouting assay

The mouse major pelvic ganglion (MPG) or dorsal root ganglion (DRG) tissues were harvested and processed as previously described [8].

3. Erectile function examination

Penile erectile function was measured as previously described [9]. The stimulation settings were as follows: a 5 V amplitude, frequency of 12 Hz, pulse duration of one millisecond, and total stimulation time of 1 minute. Systemic blood pressure was measured using a noninvasive tail-cuff system (Visitech Systems). To account for changes in systemic blood pressure, the ratios of maximal or total intracavernous pressure (ICP) to mean systolic blood pressure (MSBP) were calculated.

4. TUNEL assay

Cavernous endothelial cell apoptosis was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Chemicon) following the instruction of the manufacturer.

5. Reactive oxygen species detection

Reactive oxygen species (ROS) production in cavernous tissues was assessed using hydroethidine (an oxidative fluorescent dye; Molecular Probes), which detects intracellular superoxide anions, and nitrotyrosine (a peroxynitrite marker, 1:50; Millipore) staining as previously described [10]. Additionally, the expression of inducible nitric oxide synthase (iNOS) and $p47^{phox}$ (a subunit of neutrophil NADPH oxidase) was evaluated using western blot analysis.

6. Histological examination

Mouse corpus cavernosum (CC), MPG, or DRG tissues for immunofluorescence staining were fixed in 4% paraformaldehyde for 24 hours at 4℃. Frozen tissue sections (12 μm) or *ex vivo* MPG and DRG samples were then incubated overnight at 4℃ with the following antibodies: IGFBP5 (Santa Cruz Biotechnology), CD31 (Millipore), neuron-glial antigen 2 (NG2; Millipore), neurofilament (NF; Sigma-Aldrich), NOS1 antibody (nNOS; Santa Cruz Biotechnology), phospho-his-

tone 3 (pH3; Millipore), nitrotyrosine (Millipore), or species-appropriate secondary antibodies conjugated to tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC). The samples were then mounted with a DAPI-containing solution (4′,6-diamidino-2-phenylindole, a nuclear stain) (Vector Laboratories Inc.). All fluorescent images were captured using a confocal microscope (K1-Fluo; Nanoscope Systems Inc.), and immunopositive areas were quantified using an image analyzer (National Institutes of Health 1.34; https://imagej.net/ij/nih-image/).

7. Western blotting

Western blot was performed against the following antibodies: IGFBP5 (antibodies-online Inc.), $p47^{phox}$ (Santa Cruz Biotechnology), iNOS (BD Biosciences), p-c-Jun (Cell Signaling), c-Jun (Cell Signaling), p-JNK (Cell Signaling), JNK (Cell Signaling), p-AKT (Cell Signaling), AKT (Cell Signaling), p-PI3K (Cell Signaling), PI3K (Cell Signaling), BDNF (Santa Cruz Biotechnology), NT-3 (Santa Cruz Biotechnology), NGF (Thermo Fisher Scientific), p-IGF1R (Novus Biologicals), IGF1R (Abcam), Ang-1 (Abcam), VEGF (Santa Cruz Biotechnol-

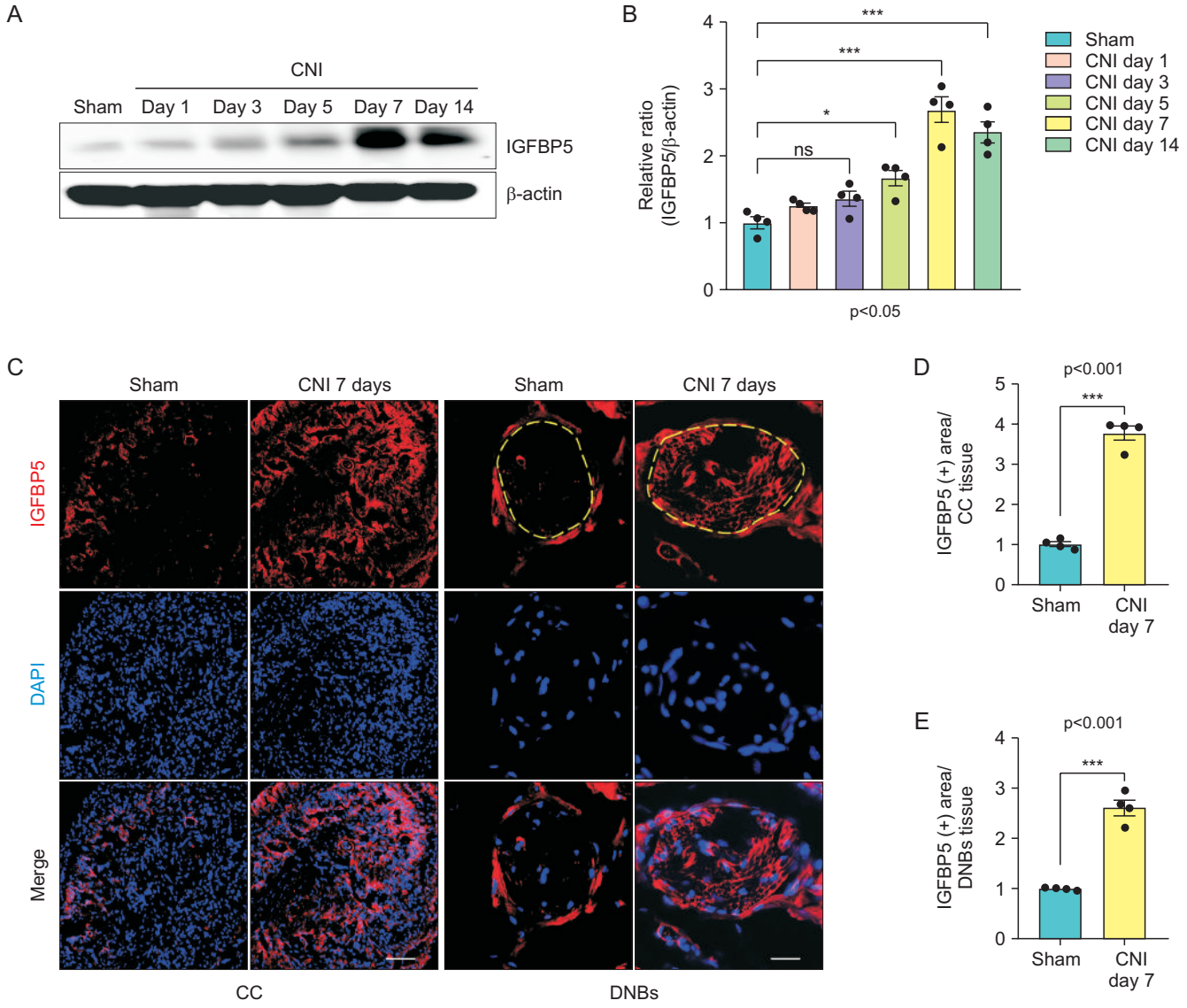

Fig. 1. Increased IGFBP5 expression in the penis of CNI-induced ED mice. (A) Representative western blot showing IGFBP5 expression in the cavernous tissue from sham operation and CNI-induced ED mice. (B) Normalized band intensity values were analyzed using an image analyzer (n=4, *p<0.05; ***p<0.001). (C) IGFBP5 staining (red) in the corpus cavernosum (CC) and dorsal nerve bundles (DNBs) from sham operation and CNI-induced ED mice (7 days). Nuclei were labeled with DAPI (blue). Scale bars=100 μm (CC) and 25 μm (DNBs). (D, E) Quantitative analysis of IGFBP5 immunopositive area in the CC (D) and DNBs (E, measured within the dotted lines) was conducted using an image analyzer system (n=4, ***p<0.001). Data are presented as mean±standard error of the mean, with the sham operation group set to a relative ratio of 1. IGFBP5, insulin-like growth factor-binding protein 5; CNI, cavernous nerve injury; ED, erectile dysfunction; DAPI, 4′,6-diamidino-2-phenylindole; ns, not significant.

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ogy), or β-actin (ABclonal Inc.). The membranes were washed and then incubated with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 2 hours at room temperature.

8. Statistical analysis

The data are expressed as mean±standard error of the mean of values from three or more independent experiments. Statistical analyses were performed using one-way ANOVA followed by Tukey's post-hoc tests, with data analyzed using GraphPad Prism version 8 (GraphPad Software Inc.). A p-value of <0.05 was considered statistically significant.

RESULTS

1. IGFBP5 expression increases in the penis of CNI-induced ED mice

A previous study demonstrates that IGFBP5 expression is significantly elevated in diabetic ED mice and that knocking it down promotes the regeneration of dorsal nerve bundles (DNBs) in this ED animal model [6]. Based on these findings, we hypothesized that IGFBP5 knockdown may have therapeutic effects on CNI-induced ED in mice. To test this hypothesis, we first examined the time-dependent expression of IGFBP5 in cavernous tissue from sham-operated and CNI-induced ED mice. The western blot analysis revealed that IGFBP5 expression increased progressively from 1 to 14 days after CNI, with peak levels observed on day 7 (Fig. 1A, B). Immunofluorescence staining with anti-IGFBP5 antibodies revealed an increase in IGFBP5 expression in the CC and DNBs (indicated by dashed lines) of CNI-induced ED mice on day 7 (Fig. 1C-E). These findings suggest that IGFBP5 may play a role in cavernous nerve regeneration and cavernous angiogenesis.

2. Ablation of IGFBP5 expression promotes neurite sprouting

To assess the effect of IGFBP5 ablation on neural regeneration in *ex vivo* studies, we first cultured MPG or DRG. IGFBP5 expression was inhibited by infecting these tissues with shIGFBP5 lentiviral particles for 2 days. The tissues were then exposed to lipopolysaccharide (LPS) (10 μg/mL). After 5 days, neurofilament immunofluorescence staining

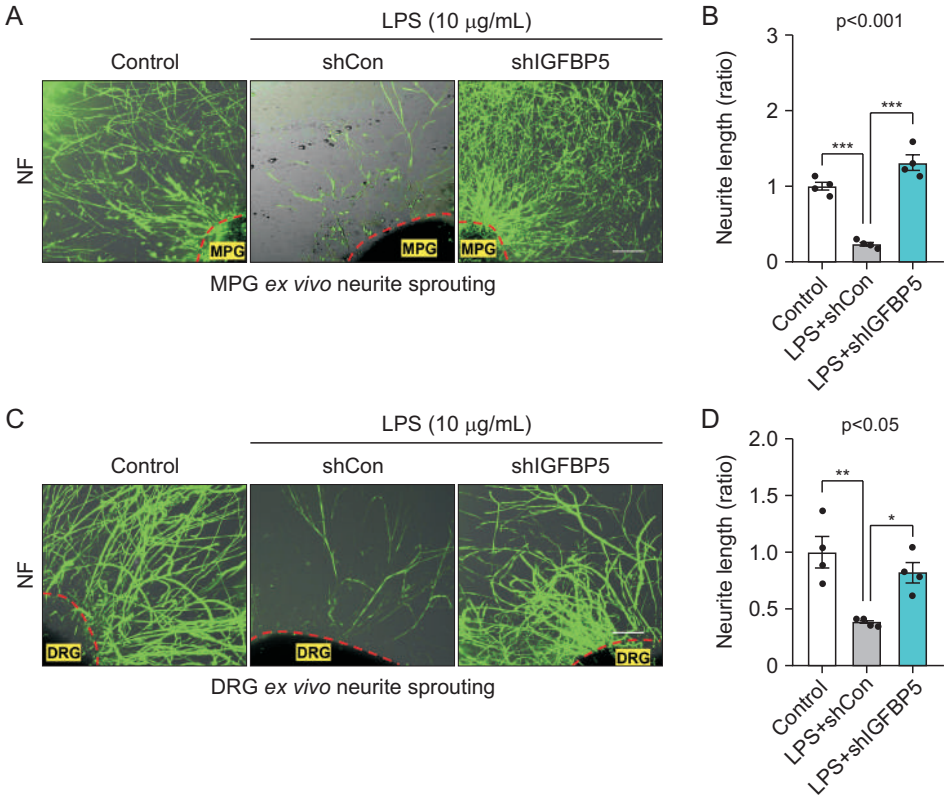

Fig. 2. Ablation of IGFBP5 expression promotes neurite sprouting. (A, C) Neurofilament (NF, green) staining in mouse MPG or DRG tissues from the indicated conditions. Scale bars=100 μm. (B, D) Quantitative analysis of NF-immunopositive neurite length was performed using an image analyzer system (n=4, *p<0.05; **p<0.01; ***p<0.001). Data are presented as mean±standard error of the mean, with the control group set to a relative ratio of 1. IGFBP5, insulin-like growth factor-binding protein 5; LPS, lipopolysaccharide; shCon, scrambled short hairpin RNA control; shIGFBP5, short hairpin RNA targeting mouse IGFBP5; MPG, mouse major pelvic ganglion; DRG, dorsal root ganglion.

revealed that neurite sprouting from MPG or DRG tissues in the LPS+shCon group was significantly reduced compared to those in the control group. However, IGFBP5 ablation alleviated the LPS-induced reduction in neurite sprouting in MPG or DRG tissues (Fig. 2). These findings suggest that IGFBP5 ablation may have a neuroprotective effect in CNI-induced ED mice.

3. Ablation of IGFBP5 expression improves erectile function in CNI-induced ED mice

To determine whether IGFBP5 ablation improves erectile function in CNI-induced ED mice, we inhibited IGFBP5 expression by injecting shRNA lentiviruses targeting mouse IGFBP5 (shIGFBP5) intracavernosally (5×10^4 IFU/mouse, based on a previous study [6]) into the mouse penis 3 days before inducing CNI. Fig. 3A shows a schematic of the experimental procedure. One week after CNI, erectile function was assessed during electrical stimulation. The ratios of

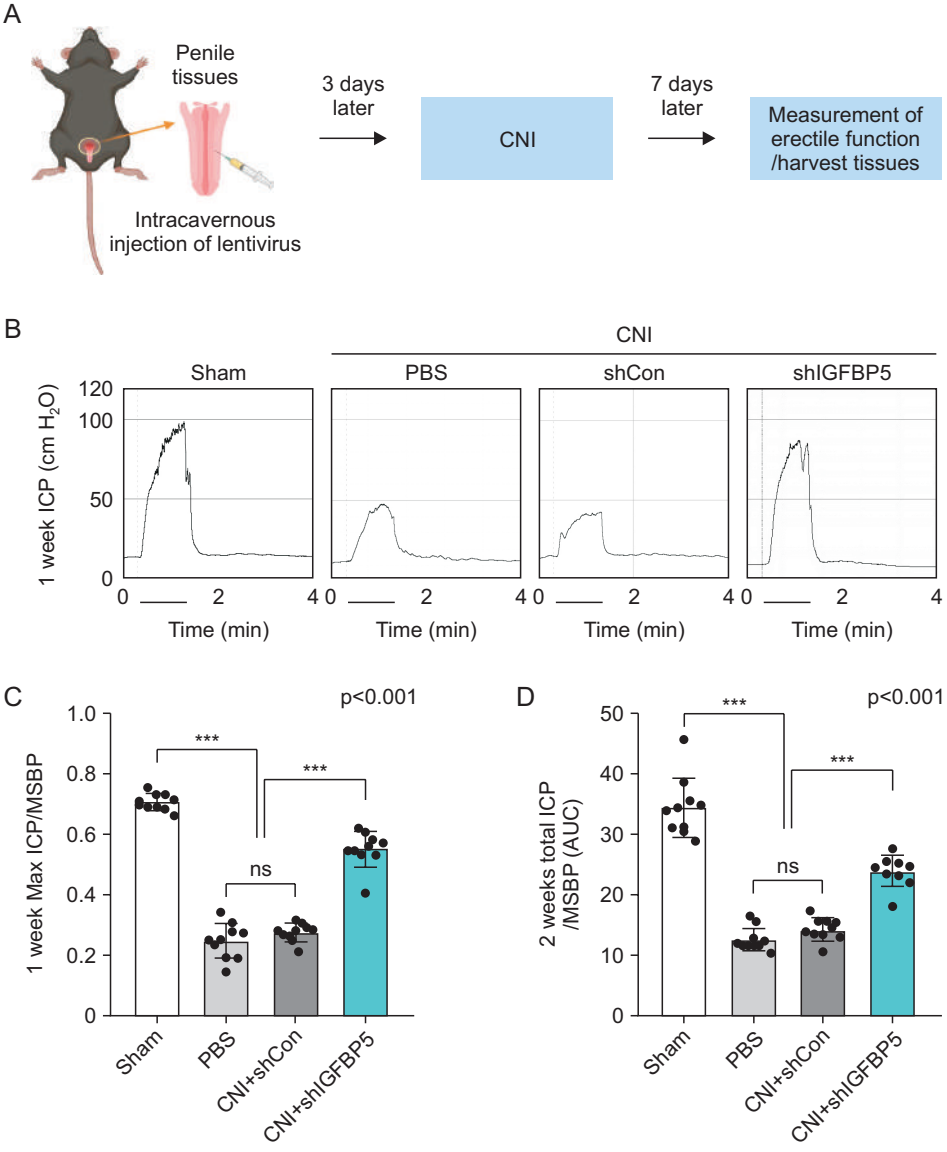

Fig. 3. Ablation of IGFBP5 expression improves erectile function in CNI-induced ED mice. (A) Schematic diagram of the mouse CNI model and experimental procedures. CNI was performed 3 days after intracavernous injection of PBS (20 μL), shCon-lentivirus, or shIGFBP5-lentivirus (5×10^4 infection units [IFU]/mouse for shRNA lentiviral particles). Erectile function measurements and additional studies were conducted 7 days later. (B) Representative ICP responses for sham operation and CNI-induced ED mice. Electrical stimulation of the cavernous nerve was performed 1 week after CNI. The stimulus interval is indicated by the solid bar. (C, D) The ratio of the mean maximal and total ICP (area under the curve) to MSBP was evaluated for each group (n=10, ***p<0.001). IGFBP5, insulin-like growth factor-binding protein 5; CNI, cavernous nerve injury; ED, erectile dysfunction; PBS, phosphate-buffered saline; shCon, scrambled short hairpin RNA control; shIGFBP5, short hairpin RNA targeting mouse IGFBP5; ICP, intracavernous pressure; MSBP, mean systolic blood pressure; ns, not significant; AUC, area under the curve.

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maximum and total ICP to MSBP was significantly reduced in PBS-treated or shCon lentivirus-injected CNI mice compared to those in the sham operation group. However, these erectile function parameters improved in the shIGFBP5 injection group, reaching 75% of the levels observed in the sham group (Fig. 3B-D). No significant differences in body weight or MSBP were observed between the sham operation control group and other experimental groups. Immunofluorescence staining for CD31, NG2, nNOS, and neurofilament in cavernosal and DNBs tissue revealed that IGFBP5 inhibition significantly restored endothelial cell and pericyte content in the CC (Fig. 4A, C, D), alongside neuronal content in the DNBs (Fig. 4B, E, F) in CNI-induced ED mice. These findings indicate that IGFBP5 ablation may promote the restoration of cavernous endothelial cells, pericytes, and neuronal cell contents, thereby improving erectile function in CNI-induced ED mice.

4. Ablation of IGFBP5 expression reduces apoptosis and induces proliferation by promoting survival of cavernous endothelial cells in CNI-induced ED mice

To assess whether IGFBP5 ablation promotes the survival of cavernous endothelial cells, we analyzed cell prolifera-

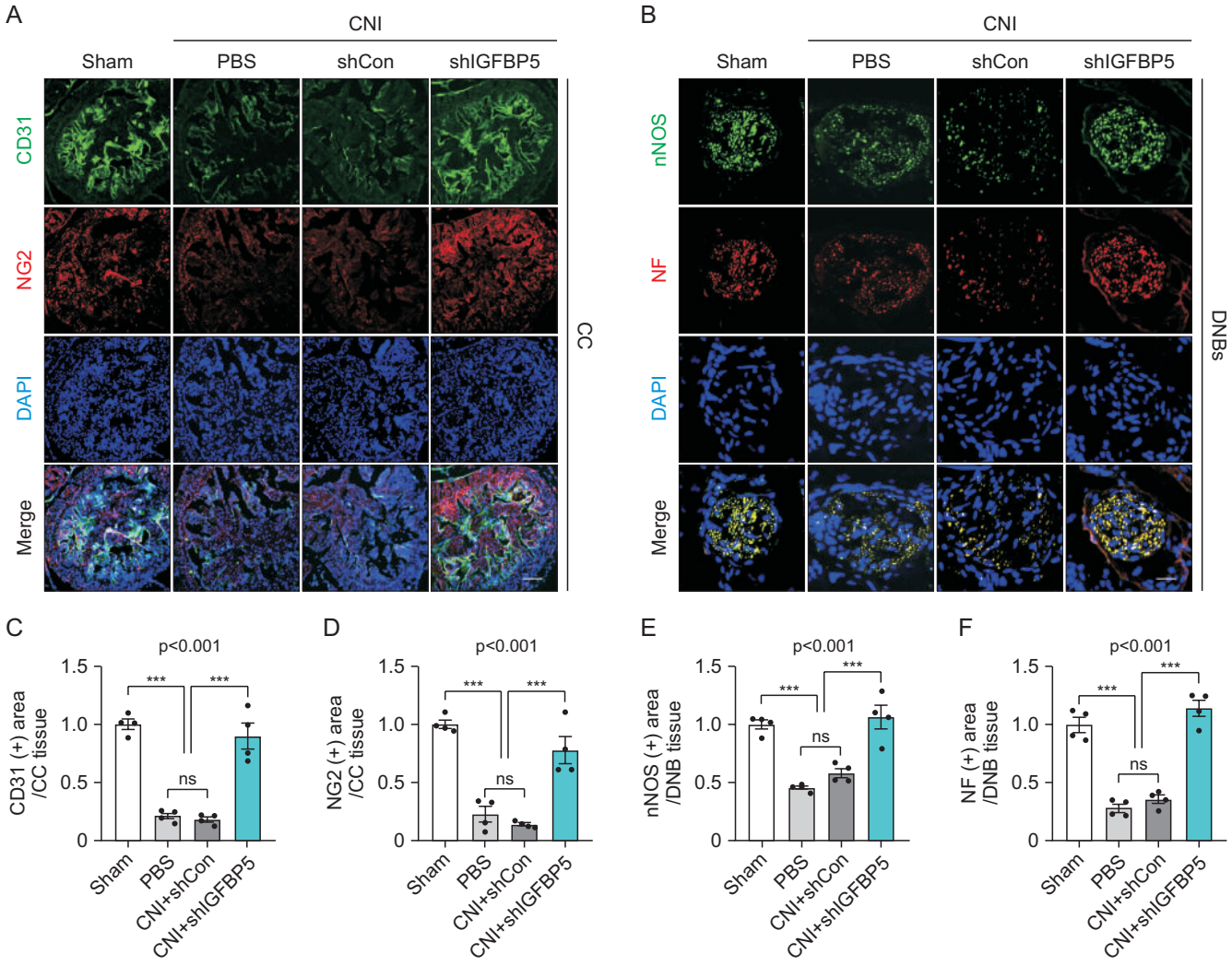

Fig. 4. Ablation of IGFBP5 expression increases cavernous endothelial-mural cell and neuronal cell contents in CNI-induced ED mice. (A, B) Immunofluorescence staining of CD31 (green), NG2 (red), nNOS (green), and neurofilament (NF, red) in the corpus cavernosum (CC) and dorsal nerve bundle (DNB) tissues from sham operation and CNI-induced ED mice, which received intracavernous injection of PBS (20 μL), shCon-lentivirus, or shIGFBP5-lentivirus (5×10^4 infection units [IFU]/mouse for shRNA lentiviral particles). Nuclei are labeled with DAPI (blue). Scale bars=100 μm (CC) and 25 μm (DNBs). (C-F) Quantitative analysis of CD31-, NG2-, nNOS-, and NF-immunopositive areas in CC and DNB tissues was performed using an image analyzer system (n=4, ***p<0.001). Data are presented as mean±standard error of the mean, with the sham operation group set to a relative ratio of 1. IGFBP5, insulin-like growth factor-binding protein 5; CNI, cavernous nerve injury; ED, erectile dysfunction; PBS, phosphate-buffered saline; shCon, scrambled short hairpin RNA control; shIGFBP5, short hairpin RNA targeting mouse IGFBP5; DAPI, 4′,6-diamidino-2-phenylindole; ns, not significant.

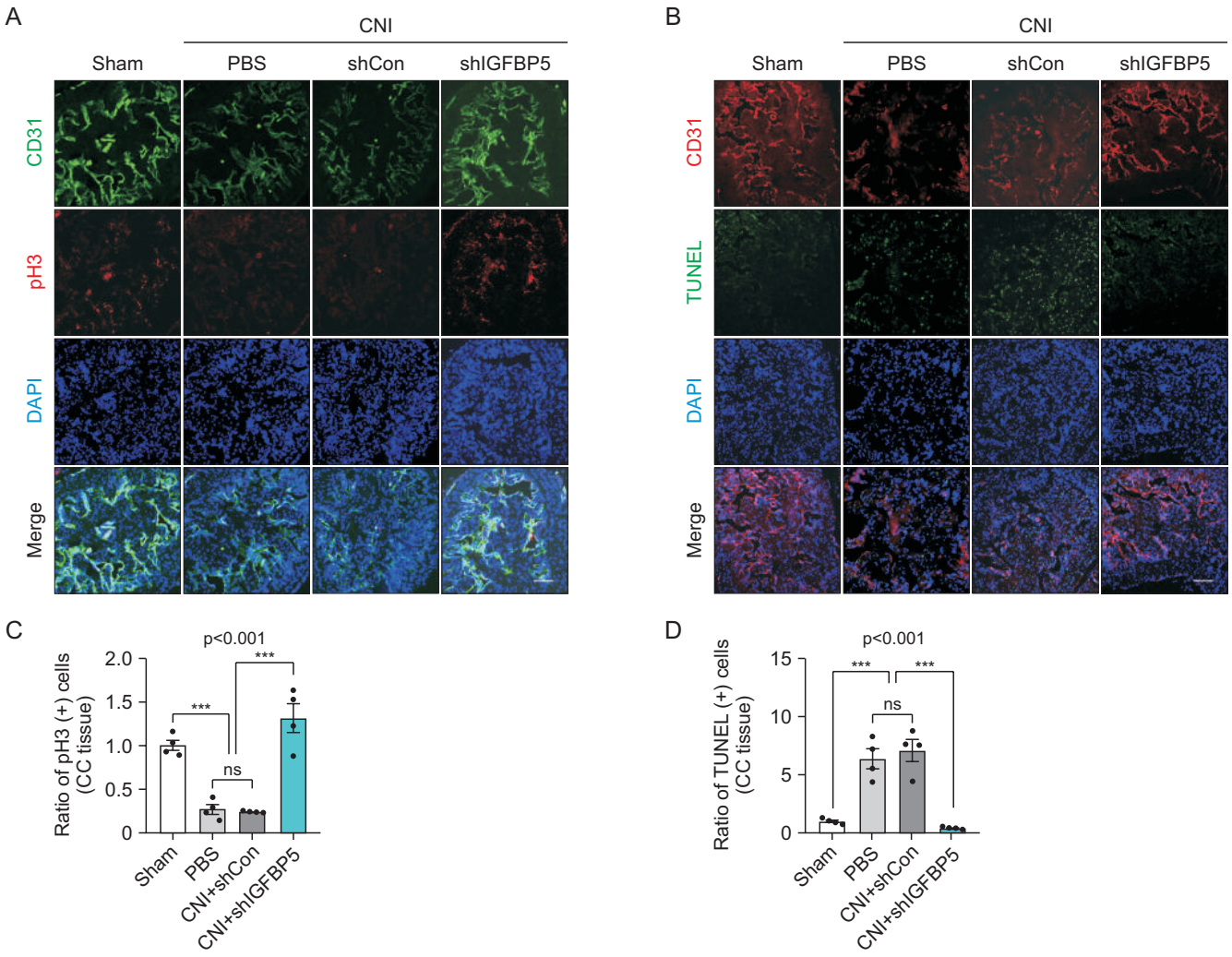

Fig. 5. Ablation of IGFBP5 expression induces endothelial proliferation and reduced apoptosis in CNI-induced ED mice. (A) Representative immunofluorescence images staining of corpus cavernosum (CC) tissue showing CD31 (green) and phospho-histone H3 (pH3; red) after ICP studies. Scale bar=100 μm. (B) Immunofluorescence staining of CC tissue for CD31 (red) and TUNEL assay (green) after ICP studies. Scale bar=100 μm. Nuclei are labeled with DAPI (blue). (C, D) The number of pH3-positive or TUNEL-positive endothelial cells was quantified using an image analyzer system (n=4, ***p<0.001). Data are presented as mean±standard error of the mean values, with the sham operation group set to a relative ratio of 1. IGFBP5, insulin-like growth factor-binding protein 5; CNI, cavernous nerve injury; ED, erectile dysfunction; PBS, phosphate-buffered saline; shCon, scrambled short hairpin RNA control; shIGFBP5, short hairpin RNA targeting mouse IGFBP5; ICP, intracavernous pressure; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; DAPI, 4′,6-diamidino-2-phenylindole; ns, not significant.

tion using pH3 staining and apoptosis using the TUNEL assay in CNI-induced ED mice. Compared to the sham-operated group, cavernous endothelial cell proliferation was markedly reduced (Fig. 5A, C), and apoptosis significantly increased (Fig. 5B, D) in PBS- or shCon lentivirus-treated CNI mice. However, these effects were normalized in the shIGFBP5 injection group (Fig. 5). In conclusion, IGFBP5 ablation may promote the survival of cavernous endothelial cells in CNI-induced ED mice by enhancing cell proliferation and reducing apoptosis.

5. Ablation of IGFBP5 expression reduces ROS production in CNI-induced ED mice

Increased cavernous ROS is a known pathophysiological factor in RP-induced ED [11]. Therefore, we performed immunofluorescence experiments using nitrotyrosine and hydroethidine to detect peroxynitrite and superoxide anion production in the cavernous tissue of CNI-induced ED mice. We found that the expression levels of nitrotyrosine and hydroethidine were significantly higher in PBS-treated or shCon lentivirus-injected CNI-induced ED mice than those in the sham operation group. However, these elevated levels were significantly reduced in shIGFBP5 lentivirus-infected CNI-induced ED mice groups (Fig. 6A-C). Additionally, $p47^{phox}$,

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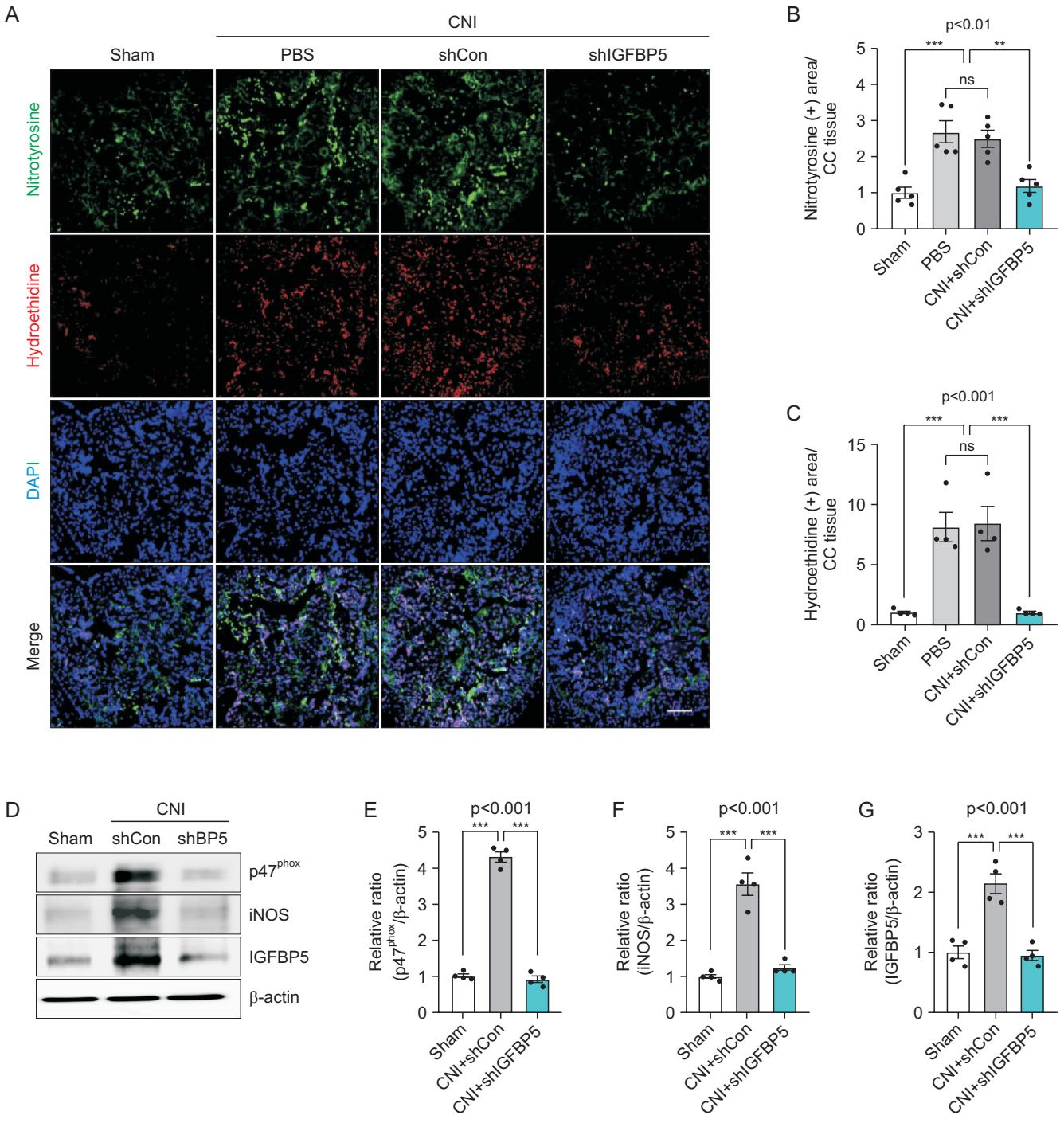

Fig. 6. Ablation of IGFBP5 expression reduces ROS production in CNI-induced ED mice. (A) Representative images of immunofluorescence staining of corpus cavernosum (CC) tissue for nitrotyrosine (a marker for peroxynitrite generation, green) and hydroethidine (an oxidative fluorescent dye detecting superoxide anions, red) after ICP studies. Scale bar=100 μm. Nuclei are labeled with DAPI (blue). (B, C) The nitrotyrosine- and hydroethidine-immunopositive areas were quantified using an image analyzer system (n=4, **p<0.01; ***p<0.001). (D) Representative western blot showing $p47^{phox}$, iNOS, and IGFBP5 levels in the mouse cavernous tissue after ICP studies. (E-G) Normalized band intensity values for $p47^{phox}$, iNOS, and IGFBP5 (n=4, ***p<0.001). Data are presented as mean±standard error of the mean, with the sham operation group set to a relative ratio of 1. IGFBP5, insulin-like growth factor-binding protein 5; ROS, reactive oxygen species; CNI, cavernous nerve injury; ED, erectile dysfunction; PBS, phosphate-buffered saline; shCon, scrambled short hairpin RNA control; shIGFBP5, short hairpin RNA targeting mouse IGFBP5; ICP, intracavernous pressure; DAPI, 4′,6-diamidino-2-phenylindole; ns, not significant; iNOS, inducible nitric oxide synthase.

a regulatory subunit of NADPH oxidases, is known to produce superoxide in endothelial cells [12]. Excessive NADPH oxidase activity promotes iNOS expression and iNOS-mediated NO, with its metabolite peroxynitrite potentially causing endothelial dysfunction [13]. To investigate this, we assessed the expression of $p47^{phox}$ and iNOS in the cavernous tissue of CNI-induced ED mice. The results showed that $p47^{phox}$ and iNOS levels were significantly higher in shCon lentivirus-injected CNI-induced ED mice, whereas these levels returned to normal levels in the shIGFBP5 injection group (Fig. 6D-G).

6. Alterations in neurovascular regeneration signaling following IGFBP5 ablation in CNI-induced ED mice

To investigate the mechanism by which IGFBP5 ablation affects neurovascular regeneration, we analyzed ROS-mediated cell death signaling pathways (JNK and c-Jun), cell survival signaling pathways (PI3K and AKT), the expression of neurotrophic factors (BDNF, NGF, and NT-3), IGF1 signaling pathways (p-IGF1R and IGF1R), and angiogenesis factor (Ang-1 and VEGF) in CNI-induced ED mice using western blot analysis. Our results showed that in shCon lentivirus-injected CNI-induced ED mice, the JNK signaling pathway (Fig. 7A-C) was activated, while the PI3K-AKT signaling pathway (Fig. 7A, D, E), neurotrophic factor expressions (Fig. 7A, F-H), and VEGF expression (Fig. 7A, K) were downregulated. In contrast, IGFBP5 ablation via shIGFBP5 injection restored these pathways to baseline levels in CNI mice when compared to those in the sham-operated group (Fig. 7). However, IGF1 signaling pathways and Ang-1 expression was not modulated by shIGFBP5 injection (Fig. 7I, J). These findings indicate that IGFBP5 influences the pathogenesis of CNI-induced ED by modulating the JNK, c-Jun, PI3K-Akt, and VEGF signaling pathways.

DISCUSSION

IGFBP5 was identified approximately 30 years ago [14]. Since then, changes in IGFBP5 levels have been observed in various cancers, leading to extensive research on its role in malignancies such as retinoblastoma, glioblastoma, breast cancer, prostate cancer, and non-functioning pituitary adenoma [4,5,15-20]. Additionally, alterations in IGFBP5 expression in different cellular contexts have been related to the development of numerous diseases [21,22]. For example, IGFBP5 can enhance endothelial cell inflammation, contributing to the progression of diabetic nephropathy [21], and it also plays a crucial role in inducing neuronal apoptosis [23]. Furthermore, as of August 2024, approximately 63 studies have investigated IGFBP5 in the context of the central nervous system (search keywords "IGFBP5" and "central nervous system"), while only five studies have explored its role in the peripheral nervous system (search keywords "IGFBP5" and "peripheral nervous system"). Recent research demonstrates that IGFBP5 expression is significantly elevated in type 1 diabetic mice and that suppressing IGFBP5 expression can enhance cavernous neurovascular regeneration, leading to improved erectile function [6]. Despite these findings, the specific role of IGFBP5 in peripheral nerve disorders, particularly in CNI-induced ED, and its underlying mechanisms remain unclear. Therefore, this study aims to evaluate the role of IGFBP5 in a CNI-induced ED mouse model using *in vivo* and *ex vivo* systems.

We first confirmed the expression of IGFBP5 in the CNI-induced ED mouse model over 1 to 14 days following nerve injury, using western blotting and immunofluorescence staining. IGFBP5 expression in the penile tissue of CNI-induced ED mice peaked on day 7 and then began to decline. The erectile function in the CNI-induced ED mice model is typically at its lowest until 2 weeks after nerve injury, after which it begins to recover spontaneously [24]. Therefore, we speculate that the elevated expression of endogenous IGFBP5 following nerve injury may be a key factor impeding neurovascular regeneration and exacerbating neurovascular injury. To test our hypothesis, we first infected MPG and DRG cultures with lentiviral particles carrying shRNA targeting mouse IGFBP5 (shIGFBP5) under inflammatory conditions to inhibit the IGFBP5 expression. We then assessed changes in neurite sprouting. *Ex vivo* experiments demonstrated that ablation of IGFBP5 expression in MPG or DRG cells cultured under LPS-treatment conditions significantly promoted neurite sprouting, supporting our hypothesis (Fig. 2). Additionally, *in vivo* experiments confirmed that reducing IGFBP5 expression in CNI-induced ED mice (1 week after CNI) significantly restored erectile function (Fig. 3). However, this study only evaluated the recovery of erectile function within 1 week after nerve injury and did not assess recovery for ≥2 weeks. Because a previous study showed that in a mouse CNI model, injury effects such as apoptosis peaked at 1 week after injury and then gradually recovered over time [24]. In further studies, we will evaluate the long-term recovery of IGFBP5 ablation using the neurectomy model, which induces ED and typically persists for over 12 weeks [24].

Oxidative stress, caused by an imbalance between antioxidant levels and the accumulation of ROS [25], is a well-known contributor to cell death and tissue dysfunction [25]. Studies show that oxidative stress impairs nerve regenera-

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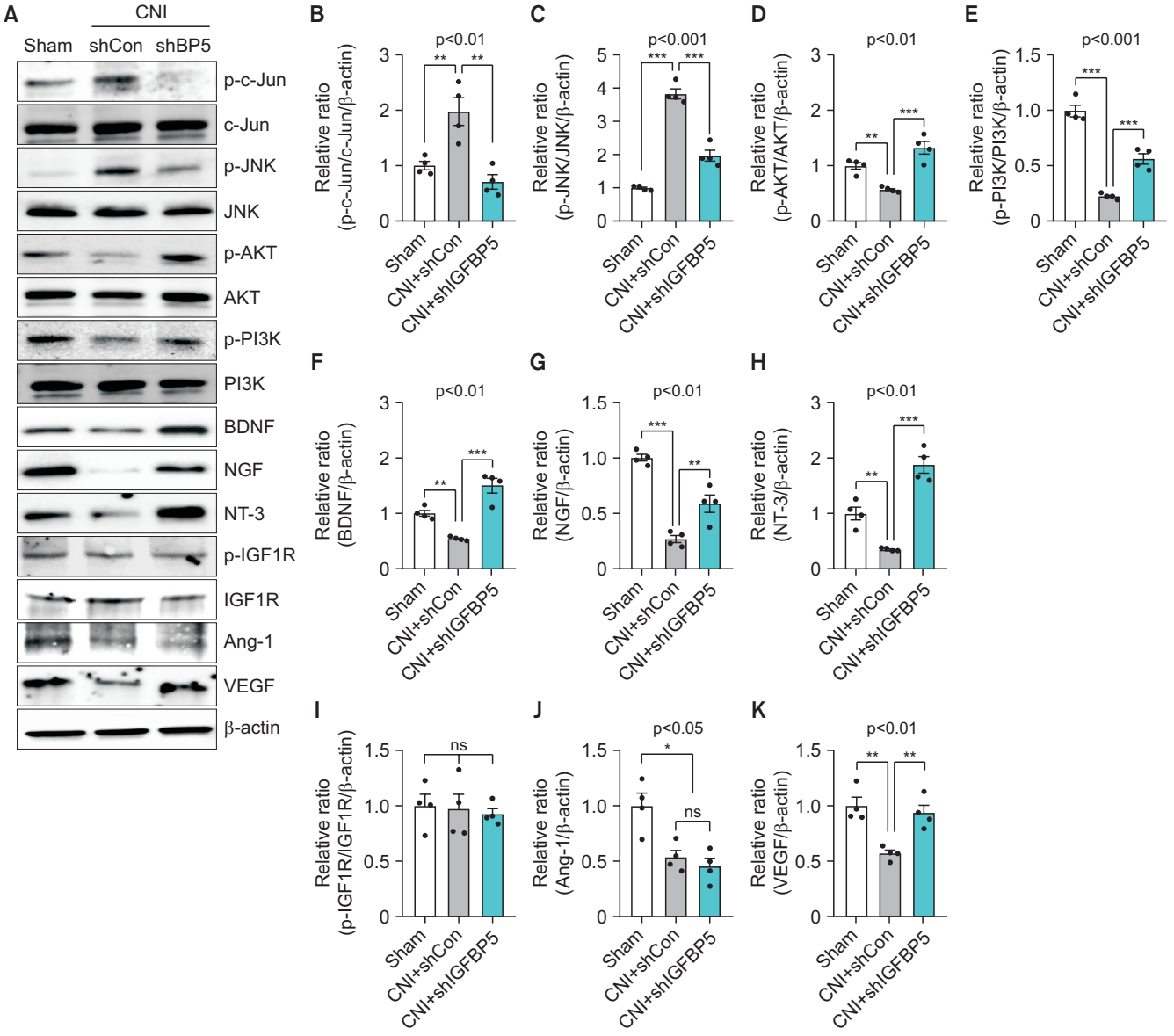

Fig. 7. Ablation of IGFBP5 expression modulates PI3K/AKT and JNK/c-Jun signaling and enhances the expression of neurotrophic factors in CNI-induced ED mice. (A) Representative western blot showing levels of p-c-Jun, c-Jun, p-JNK, JNK, p-AKT, AKT, p-PI3K, PI3K, neurotrophic factors (BDNF, NGF, and NT-3), p-IGF1R, IGF1R, Ang-1, and VEGF in the mouse cavernous tissue after ICP studies. (B-K) Normalized band intensity values for the indicated targets (n=4, *p<0.05; **p<0.01; ***p<0.001). Data are presented as mean±standard error of the mean, with the sham operation group set to a relative ratio of 1. IGFBP5, insulin-like growth factor-binding protein 5; CNI, cavernous nerve injury; ED, erectile dysfunction; shCon, scrambled short hairpin RNA control; shIGFBP5, short hairpin RNA targeting mouse IGFBP5; ICP, intracavernous pressure; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3; IGF1R, insulin like growth factor 1 receptor; VEGF, vascular endothelial growth factor; ns, not significant.

tion following CNI [26,27]. In a rat model of CN injury, Wang et al. [28] found that elevated oxidative stress in serum and cavernous tissue contributes to ED. IGFBP5 has been implicated in ROS production in primary human lung fibroblasts. Silencing IGFBP5 significantly reduces ROS levels in fibroblasts in patients with systemic sclerosis and idiopathic pulmonary fibrosis [29]. Furthermore, Yasuoka et al. [29] demonstrated that IGFBP5-induced ROS production is mediated by the activation of the JNK signaling pathway. This present study demonstrated that the ablation of IGFBP5 expression mitigated CNI-mediated ROS production by reducing the levels of superoxide anions, peroxynitrite, $p47^{phox}$, and iNOS. This protective effect on nerves ultimately led to improved erectile function. However, the precise mechanism linking IGFBP5 and ROS was not elucidated. Based on existing literature [29], we hypothesize that NADPH oxidase plays a pivotal role in IGFBP5-induced ROS generation. Further research is necessary to determine whether IGFBP5 directly

or indirectly influences NADPH oxidase expression in CC cells.

Insulin-like growth factor (IGF) is known to bind to the IGF-1 receptor (IGF1R) and regulates a variety of behaviors such as cell survival, differentiation, migration and proliferation [30]. Many studies have shown that IGFBP5 can inhibit IGF1-IGF1R binding and thus inhibit its signaling pathway [30]. However, recent studies have shown that IGFBP5 can act independently of its IGF-interfering activity. This IGF-independent action may be executed intracellularly (intrinsic action) or may involve IGFBP5 binding to a putative plasma membrane receptor [21]. In the present study, we found that the phosphorylated IGF1R, IGF1R was not significantly correlated with the presence or absence of IGFBP5. In addition, we also found that angiogenesis-related signaling pathways (VEGF) were activated after ablation of IGFBP5. However, overexpression of VEGF may have corresponding negative effects. Therefore, while targeting IGFBP5 to treat ED, it is necessary to focus on controlling the expression of VEGF to improve the safety of targeted IGFBP5 therapy. In addition, we also conducted relevant toxicity tests. One week after IGFBP5 ablation, we extracted some important tissues, such as liver, kidney, and heart for relevant toxicity tests. Although the short-term evaluation results showed no relevant toxicity, in the future subsequent clinical transformation process, we need to conduct long-term monitoring to determine its safety.

Our findings confirm that the ablation of IGFBP5 expression has protective and restorative effects on erectile function in CNI-induced ED mice. Nevertheless, it has several limitations. First, we only evaluated the effect of IGFBP5 ablation in mouse models, in order to better apply the strategy of targeting IGFBP5 to clinical practice. We need to collect penile tissues from normal and neurogenic ED patients, or use human IGFBP5 lentivirus in human-derived cells to reduce the expression of IGFBP5, and these studies will enhance the translational relevance of our study. Second, when considering translation to the clinical settings by using lentivirus, thoroughly investigating the potential side effects, long-term consequences, and safety of targeting IGFBP5, including nonclinical toxicity testing, through GLP preclinical trials is crucial. Additionally, the precise mechanism by which IGFBP5 ablation influences neurotrophic and angiogenic factors remains unclear and warrants further investigation. Despite these limitations, this study may provide valuable insights in addressing the current shortcomings of oral PDE5Is in treating RP-induced ED in men and may even have implications for other neurovascular diseases.

CONCLUSIONS

The effect of IGFBP5 expression ablation on erectile function in mice with CNI-induced ED was assessed in this study. Our results showed that ablating IGFBP5 expression restored the contents of cavernous endothelial cells, pericytes, and neurons, regulated ROS levels, and PI3K/Akt and JNK/c-Jun signaling activities, ultimately improving erectile function in mice with CNI-induced ED.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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