RESEARCH PAPER

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Ubiquitin-specific peptidase 53 promotes chronic constriction injury-induced neuropathic pain through the RhoA/ROCK pathway

Qi Li[1#], Rui Li[2#], Xiaolei Chu[1], Xiaoqiong An[3], Ming Chen[3], Yanlong Yu[4], Lei Zhang[5], Long Chen[5]*, Xiaoxi Zhu[6]*

[1] Department of Rehabilitation Medicine, Tianjin Hospital, Tianjin University, Tianjin City, China,
[2] Department of Traditional Chinese Medicine, Guizhou Provincial People's Hospital, Guiyang, Guizhou Province, China,
[3] School of Basic Medical Science, Guizhou Medical University, Guiyang, Guizhou Province, China,
[4] Laboratory of Medical Molecular Biology, Guizhou Medical University, Guiyang, Guizhou Province, China,
[5] Academy of Medical Engineering and Translational Medicine, Tianjin University, Tianjin City, China,
[6] Key Laboratory of Molecular Biology, Guizhou Medical University, Guiyang, Guizhou Province, China,
[#] These authors contributed equally to the work and should be considered co-first authors,
** Email: cagor@tju.edu.cn, formpt@hotmail.com*

Neuropathic pain is associated with nervous system injury and the production of pro-inflammatory factors. Critical functions for ubiquitin-specific peptidase 53 (USP53) have been demonstrated in various diseases. However, the role and mechanism of USP53 in chronic constriction injury (CCI)-induced neuropathic remains unclear. In our current study, a model of neuropathic pain was induced by CCI in rats. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blotting results demonstrated that USP53 was significantly up-regulated in CCI rats. In addition, silencing of USP53 alleviated neuropathic pain and reduced the production of pro-inflammatory factors in CCI rats according to paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) tests and enzyme-linked immunosorbent assay (ELISA), respectively. Moreover, knockdown of USP53 inhibited the activation of FK506-binding protein 51 (FKBP51)/RhoA/ROCK signaling in CCI rats. In summary, this study revealed that USP53 exacerbated CCI-induced neuropathic pain, potentially *via* regulation of the FKBP51/RhoA/ROCK pathway.

Key words: USP53, chronic constriction injury, neuropathic pain, FKBP51, RhoA/ROCK pathway

INTRODUCTION

Neuropathic pain is identified as hypersensitivity to diverse stimuli induced *via* primary injury and malfunction of the somatic nervous system (Tibbs et al., 2016), and as the leading subtype of pathologic pain, it is characterized by hyperpathia, allodynia, secondary algesia and spontaneous pain (Liu et al., 2019). It has been reported that neuropathic pain affects no less than 7% of the worldwide population, and its morbidity is likely to increase due to factors such as aging and improved cancer prognoses (Zilliox, 2017). Based on its major pathological aspects, neuropathic pain includes peripheral, pathological, myelopathic, and cerebral types (Cohen et al., 2014). Chronic constriction injury (CCI) is an established model for studying neuropathic pain (Liu et al., 2012). Recently, progress has been made in the detection and treatment of neuropathic pain. However, effective therapeutic measures are still limited because of the complicated pathogenesis (Cao et al., 2015). Therefore, it is essential to explore valid target genes and the detailed molecular mechanisms involved in neuropathic pain caused by CCI.

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Ubiquitination is a reversible posttranslational modification capable of modulating diverse vital activities such as DNA repair, cell apoptosis, and glycolysis (Hershko and Ciechanover, 1998; Zhao et al., 2020). Deubiquitinating enzymes (DUBs) play crucial roles in reversing this modification and counteracting the effects of ubiquitination (Hariri et al., 2021; Komander et al., 2009). Previous studies revealed that the DUB family includes approximately 97 members, of which the ubiquitin-specific peptidase (USP) subfamily is the largest (Nijman et al., 2005). USPs such as USP18, USP38, and USP14 have been found to play critical roles in a variety of biological conditions including cancer and inflammatory response (Tan et al., 2018; Li et al., 2019; Zhan et al., 2020). USP53, a member of the USP subfamily, contains a catalytically inactive ubiquitin-specific protease domain. Recent reports suggest that USP53 is involved in several types of cancer as a tumor suppressor. For example, USP53 suppresses proliferation and glycolysis and facilitates apoptosis in lung adenocarcinoma cells (Zhao et al., 2020). Gui et al. (2021) found that overexpression of USP53 slowed the development of clear cell renal cell carcinoma. In addition, an anticancer effect of USP53 has been reported in hepatocellular carcinoma (Yao et al., 2022) and esophageal carcinoma (Cheng et al., 2022). However, the role and molecular mechanism of USP53 in neuropathic pain remains unclear.

The Rho GTPase family is classified into 7 subtypes and plays important roles in regulating the formation of focal contact and fibers. Stress fibers are able to modulate cell contraction and affect cell adhesion, migration, and morphological changes (Stankiewicz et al., 2014; Wu et al., 2016). RhoA, as one of the most widely studied members of the Rho GTPases, is closely associated with multiple biological actions (Nakamura et al., 2017). RhoA, combined with its effector protein Rho-associated 5 coiled-coil containing protein kinase (ROCK), have been shown to be involved in cytoskeletal dynamics (Yamamoto et al., 2018). Recent data demonstrates that neuropathic injury is also capable of activating the RhoA/ROCK signaling pathway (Kishima et al., 2021).

FK506 binding protein FKBP51 (FKBP51) belongs to the immunophilin family (Yamamoto et al., 2018) and plays vital roles in a variety of cancers such as colitis-associated colorectal cancer (Xia et al., 2021) and prostate cancer (Maeda et al., 2021). FKBP51 has been found to generate and maintain various chronic pain states (Bortsov et al., 2013; Linnstaedt et al., 2018). Additionally, FKBP51 knockdown significantly improved neuropathic pain in a rat CCI model by suppressing the nuclear factor-kappa B (NF-κB) pathway and inflammatory reaction (Yu et al., 2017). FKBP51 overexpression was also found to up-regulate the expression of RhoA, thereby activating the RhoA-ROCK signaling pathway, and USP53 was capable of deubiquitinating FKBP51 (Takaoka et al., 2017; Zhao et al., 2020). However, genes upstream of the FKBP51/RhoA/ROCK pathway associated with neuropathic pain have received little attention. Therefore, our study intended to explore whether USP53 affects neuropathic pain in rats by serving as a mediator of the FKBP51/RhoA/ROCK pathway.

This study aimed to reveal whether USP53 exerts a crucial role in CCI-induced neuropathic pain and provide a preliminary exploration of molecular mechanisms regulated by USP53 underlying CCI in rats.

METHODS

Establishment of CCI model

A total of 60 Sprague-Dawley (SD) rats (male, 200-250 g) were purchased from Guizhou Medical University (the Experimental Animals Center of, Guiyang, China) and groups of 20 rats were raised in separate rooms for 7 days with food and water. The food was bought from Jiangsu Syony Pharmaceutical Biological Engineering Co. LTD (China) and the water was sterilized before being used. Lentivirus containing short hairpin RNA USP53 (sh-USP53) or scramble sequences (sh-NC) were purchased from HANBIO (China). Then, the rats were randomly divided into the following groups: sham, CCI, CCI+sh-NC, and CCI+sh-USP53 (5 rats in each group were fed in a cage). The CCI rat model was established as described in a previous study (Zhang et al., 2019). In brief, pentobarbital sodium was used to anesthetize rats by intraperitoneal injection at 40 mg/kg. In each rat, the sciatic nerves of both thighs were exposed using blunt dissection. Next, the sciatic nerves of rats were ligated near the trijunction of the sciatic nerve. The same operation was performed on rats in the sham group, except for no ligation. Additionally, recombinant adenovirus sh-USP53 or sh-NC (Linmei Biotechnology, China) at a multiplicity of infection (MOI) of 20 was injected into rats three days before surgery through intrathecal catheter. All protocols related to rats in this investigation were approved by the Animal Care Welfare Committee of Guizhou Medical University (Approval no. 2101406). The relevant operations followed the Guidelines of the International Association for the Study of Pain (Zimmermann, 1983). The sequences of shRNAs used in this study were presented here:
sh-NC: sense-5'-CCCATAAGAGTAATAATAT-3',
antisense-5'-ATATTATTACTCTTATGGG-3' (antisense);
sh-USP53: sense-5'-CCTGAAATGTTTGCAGAAT-3',
antisense-5'-ATTCTGCAAACATTTCAGG-3'.

Mechanical hypersensitivity

Mechanical hypersensitivity was determined by measuring the paw withdrawal threshold (PWT) of hind paws in the same and opposite directions (Wan et al., 2020). Briefly, rats were quickly placed on a wire and allowed to stand for 20 min to acclimatize to the equipment. An electronic von Frey aesthesiometer (2393, IITC Life Science, USA) was employed to detect the PWT of hind paws of rats according to the manufacturer's instructions. PWT was measured every 5 min for three trials in total, and the average was taken as the final PWT value.

Thermal hyperalgesia

Thermal hyperalgesia was determined by measuring the paw withdrawal latency (PWL) of hind paws of rats with the Plantar Test (Hargreave's Method) Plantar Analgesia Meter (mouse claw pressure pain meter, IITC, USA), as described in previous report (Jia et al., 2020; Huang et al., 2022). The rats were kept in a thick plexiglass chamber and tested with thermal radiation stimulation. PWL refers to the time from the beginning of stimuli to the retraction of the hind paw. The time of PWL was recorded every 10 minutes for three trials in total and the average was taken as the final PWL value.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent was used to extract the total RNA from spinal cord tissues according to the manufacturer protocol. Then, RNA was reverse transcribed into cDNA by using a reverse transcription kit (K1671, Thermo Scientific, USA). Next, a MonAmp™ ChemoHS qPCR Mix (MQ00401S, Monad, China) was employed to conduct q-PCR. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene (Sato et al., 2021). The sequences of all primers were listed:
USP53-forward-5'-GAAGTGTCCTAGTAACTGTGGCC-3',
USP53-reverse-5'-GAATGAAAGCAACTGTGATACCCC-3';
tumor necrosis factor alpha (TNF-α)-forward-
-5'-TCAGTTCCATGGCCCAGAC-3',
TNF-α-reverse-5'-GTTGTCTTTGAGATCCATGCCT-3';
interleukin (IL)-6-forward-5'-
-GATTGTATGAACAGCGATGATGC-3',
IL-6-reverse-5'-AGAAACGGAACTCCAGAAGACC-3';
IL-1 beta (IL-1β)-forward-5'-
-CCCAACTGGTACATCAGCACCTCTC-3',
IL-1β-reverse-5'-CTATGTCCCGACCATTGCTG-3';
GAPDH-forward-5'-
-CACGAATTCGGTCATCATCTCTGCCCCCTCTGC-3',
GAPDH-reverse-5'-
-GCTGGATCCGACGCCTGCTTCACCACCTTCTT-3'.

Enzyme-linked immunosorbent assay (ELISA)

Spinal cord tissues were collected and homogenized using radioimmunoprecipitation assay (RIPA) lysis buffer (20-188, Millipore, USA) containing Cocktail (HY-K0011, MedChemExpress, USA) on ice. After 5 min, the protein solution was sonicated and centrifuged at 12000 rpm for 15 min, followed by harvesting of the supernatants into another centrifuge tube. The protein expression level of TNF-α, IL-6, and IL-1β in spinal cord tissues was determined by utilizing a specific ELISA kit (Invitrogen, USA) according to the manufacturer's protocol. The concentration of TNF-α, IL-6, and IL-1β was presented in pg/mL.

Western blotting

The spinal cord tissues were isolated and homogenized using RIPA lysis buffer (20-188, Millipore, USA) containing Cocktail (HY-K0011, MedChemExpress, USA) on ice. After 5 min, the protein solution was sonicated and centrifuged at 12000 rpm for 15 min, followed by harvesting of the supernatants into another centrifuge tube. A BCA Protein Assay Kit (23235, Thermo Scientific) was introduced to measure the protein concentration. 20 μg protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (88585, Thermo Scientific, USA). Then the membranes were blocked with 3% skim milk (LP0031B, Thermo Scientific, USA) for 1 h. Next, the membranes were incubated with primary antibodies including USP53 (HPA035844, MilliporeSigma, USA, 1:1000), FKBP51 (ab2901, Abcam, UK, 1:500), ROCK1 (sc-17794, Santa Cruz Biotechnology, USA, 1:1000), active RhoA-GTP (26904, NewEast Biosciences, USA, 1:1000), RhoA (sc-418, Santa Cruz Biotechnology, USA, 1:1000) or GAPDH (60004-1-Ig, Proteintech, USA, 1:1000) overnight at 4°C. Finally, the membranes were incubated with Mouse Anti-Rabbit IgG Antibody (M205) [HRP], mAb (A01827-200, Genscript, China, 1:10000), or MonoRab™ Anti-Mouse IgG (H&L) (76F10), mAb, Rabbit (V90301, Genscript, China, 1:10000) for 2 h at room temperature. The protein band was observed using High sensitivity ECL luminescent solution (PW30601S, Monad, China) and a Hesper chemiluminescence imaging system (GD50401, Monad, China). Finally,

ImageJ 1.8.0 was employed to calculate the relative protein expression.

Statistical analysis

The data in this study were presented as mean ± standard deviation (SD). The statistical analyses were performed using SPSS 21.0 (IBM Co., Armonk, NY, USA). The statistical analysis between 2 groups was conducted by employing Student's *t*-test, while the statistical analysis of 3 or more groups was performed utilizing One-way analysis of variance (ANOVA) with *post hoc* Tukey test. All experiments were repeated at minimum 3 times.

RESULTS

USP53 was up-regulated in CCI rats

To explore the role of USP53 in CCI-induced neuropathic pain, we established a CCI model in SD rats and estimated USP53 expression in spinal cord tissues. As shown in Fig. 1A, B, both mRNA ($F_{(4,4)}$=20.06, P<0.001) and protein levels ($F_{(4,4)}$=71.38, P<0.001) of USP53 were remarkably increased in CCI rats compared with sham rats. These findings suggested that USP35 might be associated with neuropathic pain.

USP53 knockdown ameliorates neuropathic pain in CCI rats

We hypothesized that USP53 might influence CCI-induced neuropathic pain. To evaluate the assumption, we introduced a construct to reduce USP53 expression in spinal cord tissues ($F_{(4,4)}$=3.772, P<0.001) (Fig. 2A). PWT was postoperatively measured at 0, 3, 7, and 14 days. CCI induction notably decreased the PWT, and silencing USP53 significantly restored the postoperative mechanical hypersensitivity from the seventh day. PWL was also tested at 0, 3, 7, and 14 days postoperation. Reduced PWL was observed in the CCI group, whereas knockdown of USP53 dramatically improved thermal hyperalgesia from the third day (PWT, respectively: $F_{(4,4)}$=3.6, P<0.001, $F_{(4,4)}$=1.625, P<0.001; PWL, respectively: $F_{(4,4)}$=2.304, P<0.001, $F_{(4,4)}$=1.769, P<0.001) (Fig. 2B). Collectively, these results illustrated that USP53 ameliorated neuropathic pain in CCI rats.

USP53 knockdown inhibited the inflammatory response in CCI rats

Pro-inflammatory factors, including TNF-α, IL-6, and IL-1β, are involved in stimulating neuropathic pain. An increased load of pro-inflammatory factors in spinal cord tissues indicates an aggravation of neuropathic pain. In this study, qRT-PCR and ELISA were employed to determine the expression of TNF-α, IL-6, and IL-1β in spinal cord tissues. The results showed that TNF-α, IL-6 and IL-1β were distinctly elevated after inducing the CCI model, while silencing USP53 remarkably decreased TNF-α, IL-6 and IL-1β at the mRNA (TNF-α, respectively: $F_{(4,4)}$=9.707, P<0.001, $F_{(4,4)}$=6.817, P<0.001; IL-6, respectively: $F_{(4,4)}$=263.9, P<0.001, $F_{(4,4)}$=3.929, P<0.001; IL-1β, respectively: $F_{(4,4)}$=19.86, P<0.001, $F_{(4,4)}$=6.123, P<0.001) (Fig. 3A) and protein level (TNF-α, respectively: $F_{(4,4)}$=45.21, P<0.001, $F_{(4,4)}$=1.262, P<0.001; IL-6, respectively: $F_{(4,4)}$=9.947, P<0.001, $F_{(4,4)}$=7.574, P<0.001; IL-1β, respectively: $F_{(4,4)}$=49.57, P<0.001, $F_{(4,4)}$ =3.335, P<0.001) (Fig. 3B). Thus, the knockdown of USP53 clearly suppressed the pro-inflammatory reaction in CCI rats.

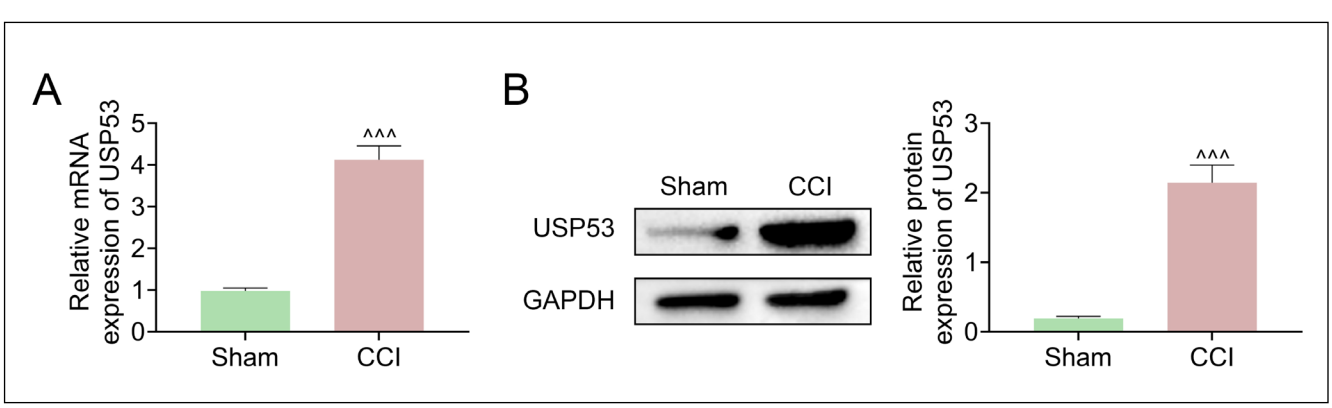

Fig. 1. USP53 was up-regulated in CCI rats. (A) USP53 mRNA levels detected with qRT-PCR. (B) USP53 protein levels detected using western blotting. ^^^P<0.001 CCI group *vs.* sham group.

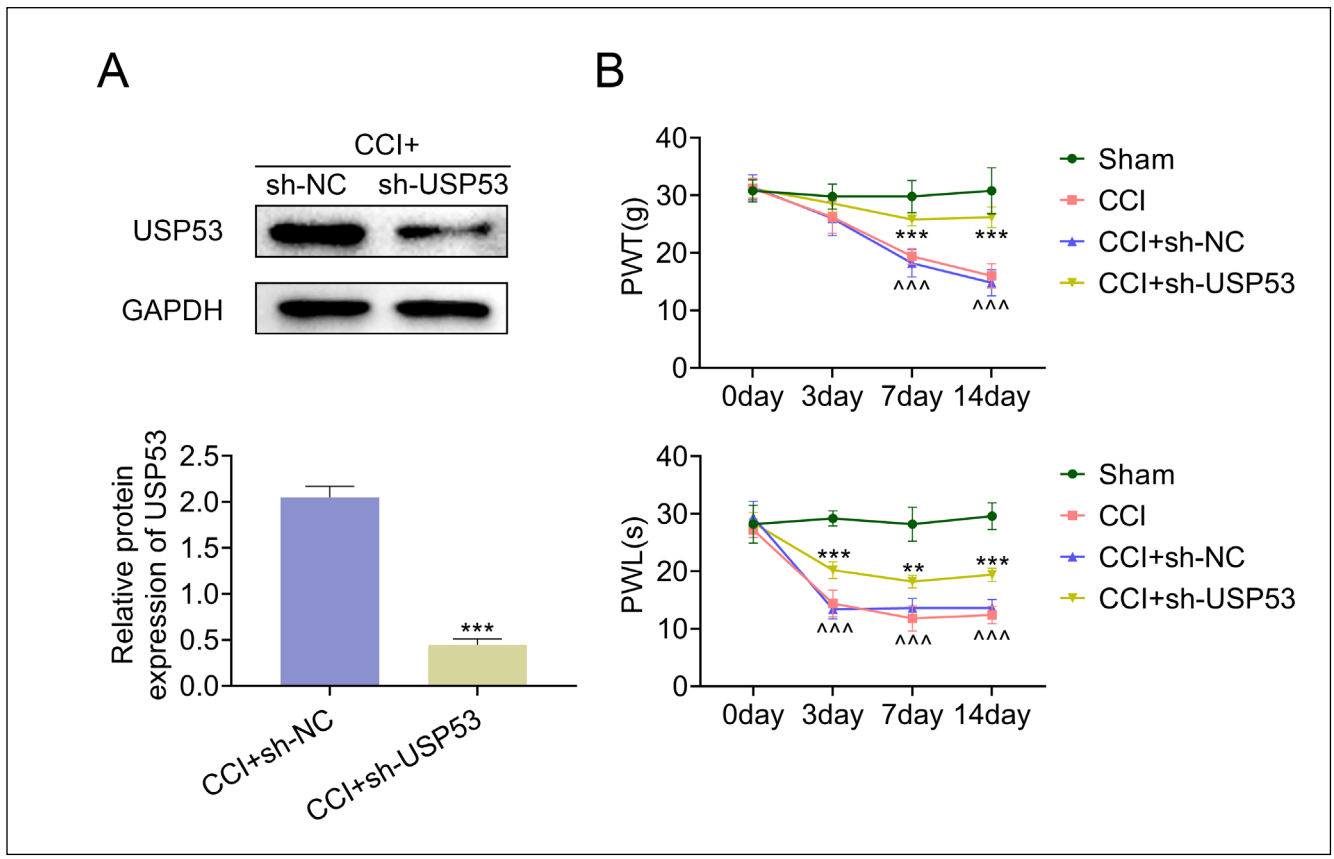

Fig. 2. USP53 knockdown ameliorates neuropathic pain in CCI rats. (A) USP53 protein levels detected using western blotting. (B) The effects of USP53 knockdown on PWT and PWL. ^^^*P*<0.001 CCI group *vs.* sham group, ***P<0.001 and **P<0.01 CCI + sh-USP53 group *vs.* CCI + sh-NC group.

USP53 regulates the FKBP51/RhoA-ROCK pathway

Given that FKBP51 and RhoA/ROCK play a vital role in neuropathic pain, and FKBP51 is able to activate the RhoA-ROCK pathway, we explored whether USP53 affects CCI-induced neuropathic pain *via* the FKBP51/RhoA/ROCK pathway. As shown in Fig. 4A–D, the expression levels of FKBP51 ($F_{(4,4)}$=2.204, P<0.001, $F_{(4,4)}$=8.789, P<0.001), ROCK1 ($F_{(4,4)}$=2.734, P<0.001, $F_{(4,4)}$=4.084, P<0.001) and active RhoA(GTP-RhoA) ($F_{(4,4)}$=4.249, P<0.001, $F_{(4,4)}$=1.191, P<0.001) were significantly up-regulated in CCI rats compared with sham group. However, a reduction in FKBP51, GTP-RhoA, and ROCK levels was observed after silencing USP53 (Fig. 4A–C). Taken together, these results revealed that USP53 might inactivate FKBP51/RhoA/ROCK signaling in CCI rats.

DISCUSSION

Chronic neuropathic pain is a common disease, but a lack in the understanding of its molecular mechanisms has severely restricted the development of effective treatment strategies (Tian et al., 2020). Recent studies showed that USP53, a member of the DUB family, has critical roles in various cellular functions such as apoptosis, glycolysis, and osteogenic differentiation (Zhao et al., 2020; Baek et al., 2021). Additionally, USP53 was found to be closely associated with a variety of cancer progressions. For example, USP53 suppressed the growth and metastasis of renal cell carcinoma by mediating the NF-κB pathway (Gui et al., 2021). However, whether USP53 is involved in CCI-induced neuropathic pain remains unclear.

As a well-established tool for studying neuropathic pain in rats, we utilized a CCI model in this study to confirm the specific role of USP53 in neuropathic pain. The results demonstrated that USP53 expression increased significantly in CCI rats and suggested that USP53 might be related to CCI-induced neuropathic pain. Thus, adenovirus carrying USP53 short hairpin RNAs was injected into rats to reduce the expression level of USP53. In addition to PWL measurements, PWT measurements showed that silencing USP53 effectively alleviated neuropathic pain in the CCI rats.

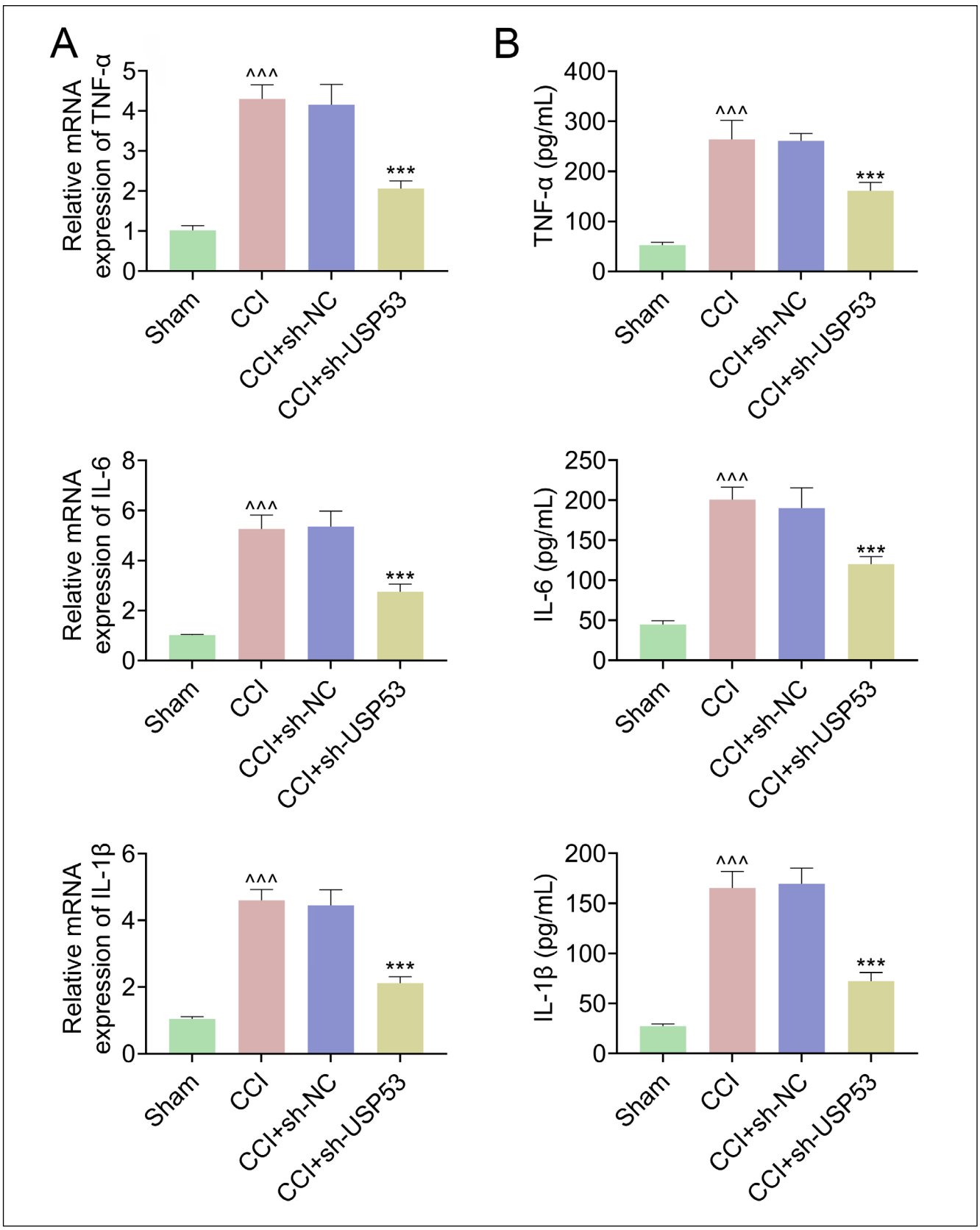

Fig. 3. USP53 knockdown alleviates the inflammatory response in CCI rats. (A) mRNA levels of TNF-α, IL-6 and IL-1β measured by qRT-PCR. (B) Protein levels of TNF-α, IL-6 and IL-1β measured by ELISA. ^^^*P*<0.001 CCI group *vs.* sham group; ***P<0.001 CCI + sh-USP53 group *vs.* CCI + sh-NC group.

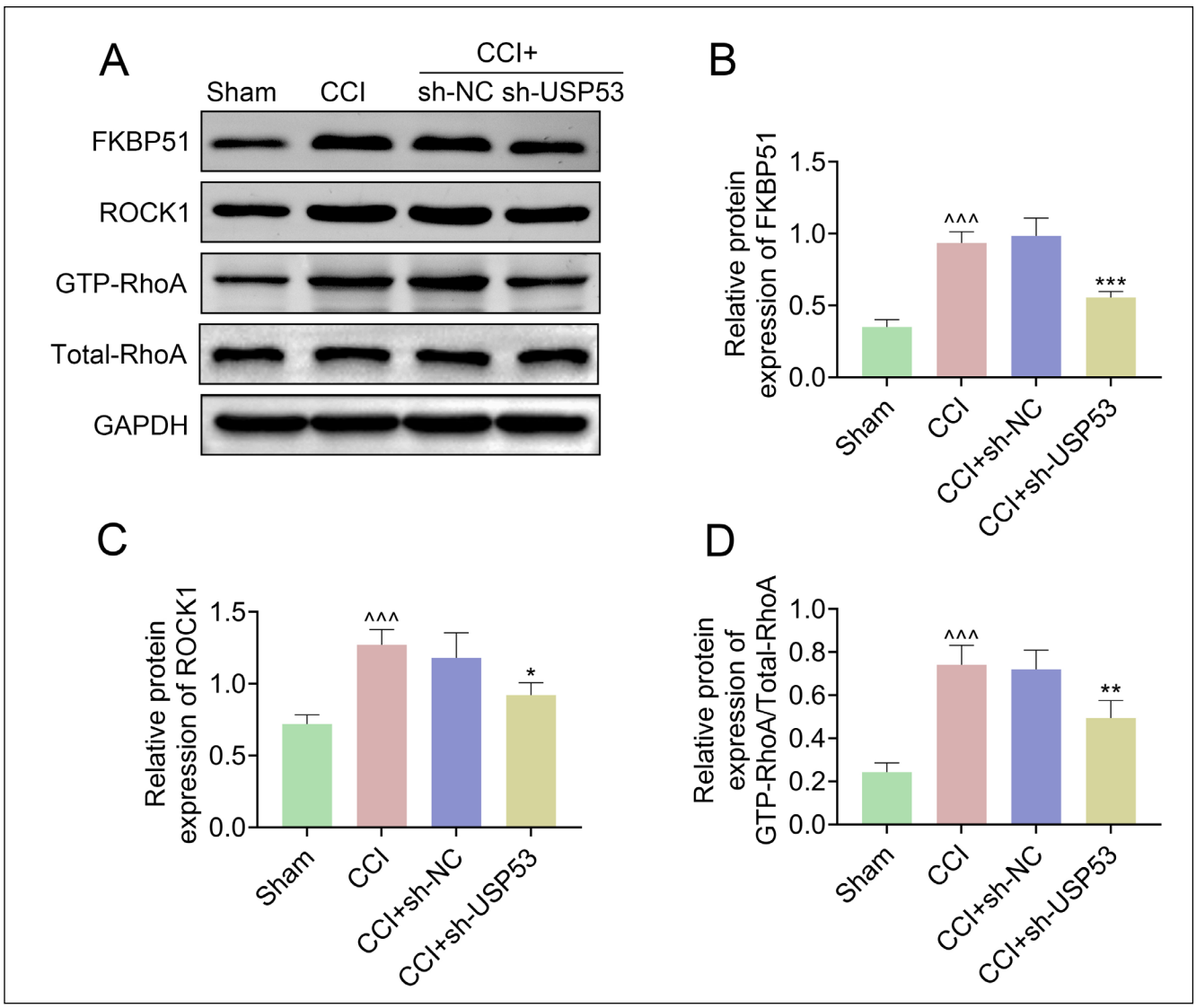

Fig. 4. USP53 regulates the FKBP51/RhoA-ROCK pathway. (A) The protein level of FKBP51, GTP-RhoA, RhoA and ROCK. (B) The quantitative analysis of FKBP51 protein level. (C) The quantitative analysis of ROCK1 protein level. (D) The quantitative analysis of GTP-RhoA protein level. ^^^P<0.001 CCI group *vs.* sham group, ***P<0.001, **P<0.01, *P<0.05 CCI + sh-USP53 group *vs.* CCI + sh-NC group.

These results support that USP53 knockdown might be a potent therapeutic strategy for targeting neuropathic pain.

Additionally, emerging evidence has revealed that proinflammatory mediators are closely correlated to neuropathic pain and that the production of pro-inflammatory factors, such as TNF-α, IL-6, and IL-1β, is an important marker of neuropathic pain (Zhou et al., 2019; Liu et al., 2020; Wang et al., 2020). However, no previous studies have reported on how USP53 affects the production of inflammatory factors. This study is the first to show that CCI clearly increased the load of TNF-α, IL-6, and IL-1β, while USP53 inhibition effectively counteracted these effects, suggesting that the silencing of USP53 improved neuropathic pain, at least in part, by inhibiting inflammatory response.

Previous studies have reported that FKBP51 is involved in diverse biological processes, including various malignancies and neurodegenerative disorders (Maiarù et al., 2016; Yu et al., 2017). Recently, Yu et al. (2017) verified that FKBP1 knockdown significantly ameliorated neuropathic pain in CCI rats. Moreover, it was reported that the RhoA/ROCK pathway plays an essential role in neuropathic pain and FKBP51 was able to promote the activation of the RhoA/ROCK cascade (Tatsumi et al., 2015; Ohsawa et al., 2016). However, the genetic regulation of the effects of FKBP51 and RhoA/ROCK pathway on neuropathic pain remains to

be explored. Interestingly, Zhao et al. (2020) found that USP53 is able to deubiquitinate and stabilize FKBP51 expression. Therefore, we considered the possibility that silencing USP53 could mediate the FKBP51/RhoA/ROCK pathway in CCI rats. As predicted, FKBP51, ROCK1, and active RhoA were increased in the CCI model, indicating that FKBP51/RhoA/ROCK signaling was activated in CCI rats. Meanwhile, USP53 knockdown dramatically inhibited the activation of the FKBP51/RhoA/ROCK pathway induced in the CCI model. These findings demonstrated that USP53 silencing improved neuropathic pain in CCI rats by modulating the FKBP51/RhoA/ROCK pathway.

This investigation prompts further questions that would lead to a better understanding of the specific molecular mechanisms related to USP53 in CCI rates. For example, we only verified that USP53 regulated RhoA/ROCK signaling in the CCI rats, but whether USP53 affects neuropathic pain induced by other factors through this pathway requires further study. Moreover, additional experiments should be conducted to explore whether USP53 modulates the activation of RhoA/ROCK pathway through direct deubiquitylation of FKBP51 in CCI rats.

CONCLUSION

In summary, this study provides the first report that USP53 aggravated CCI-induced neuropathic pain, at least in part, by modulating FKBP51/RhoA/ROCK signaling, suggesting that USP53 might be a promising target gene for neuropathic pain treatment.

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