Original Article

SENP5 deteriorates traumatic brain injury via SUMO2-dependent suppression of E2F1 SUMOylation

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Abstract

Traumatic brain injury (TBI) represents a main public health concern during the past decade, attracting considerable interest because of its rising prevalence, wide-ranging risk factors and lifelong familial and societal influence. SUMO2 can conjugate to substrates upon various cellular stresses. Nevertheless, whether and how SUMO2-specific proteases partake in TBI is less understood. The aim of this study is to dissect the effects of SUMO-specific peptidase 5 (SENP5) on accentuating TBI in rats in an effort to unveil its underlying mechanism. SENP5 is overexpressed in hippocampal tissues of TBI rats, and inhibition of SENP5 reduces neurological function scores, decreases brain water content, inhibits apoptosis in hippocampal tissues, and attenuates brain injury caused in rats. Moreover, SENP5 inhibits the SUMOylation level of E2F transcription factor 1 (E2F1) and increases the protein expression of E2F1. Silencing of E2F1 blocks the p53 signaling pathway. Overexpression of E2F1 partially reverses the protective effect of sh-SENP5 on TBI in rats. These findings reveal an essential role of SENP5 and the SUMOylation status of E2F1 in the TBI development.

Key words Traumatic brain injury, SENP5, E2F1, SUMOylation, p53 signaling pathway

Introduction

Traumatic brain injury (TBI), a structural and/or physiological impairment of brain function due to an external force, poses a considerable threat to the public health and has strong economic repercussions [1]. China has more patients with TBI than the majority of other countries worldwide, and population-based mortality of TBI is projected to be nearly 13 cases per 100,000 people in China [2]. Most civilian TBI is initiated by falls, motor vehicle collisions, being struck by an object, or physical assault, and this diverse etiology is reflected by the highly heterogeneous pathology (focal or diffuse) [3]. Moreover, TBI induces secondary biochemical changes that resulted in neuroinflammation, neuronal death, and neurological dysfunction [4]. Therefore, targeting neuronal apoptosis is of great importance for improving prognosis of patients with TBI.

Post-translational protein modifications are recognized to play essential roles in regulating protein function and turnover [5]. SUMOs are polypeptides resembling ubiquitin (Ub) which are covalently attached to lysine residues of targets via a specific enzymatic pathway, and SUMOylation is regarded as one of the major posttranslational modifications in various biological processes [6]. In neurons, SUMOylation orchestrates cellular processes from neuronal differentiation and regulation of synapse development to the control of synaptic transmission and cell survival [7]. Aberrant SUMOylation hampers the Ub-proteasome degradation pathway, which causes the accumulation of toxic proteins [8]. Suppression of the proteasome leads to the accumulation of proteins that are conjugated with SUMOylation [9]. Conjugated SUMOs are removed from substrates via SUMO-specific proteases (SENP5s), and there are six SENPs: SENP1-3 and SENP5-7 [10]. In the present study, data mining using GEO website (https://www.ncbi.nlm.nih.gov/geo/) revealed that SENP5 is the most significantly upregulated gene in TBI rats versus sham-operated rats in the GSE59645 dataset. More compelling, SUMO deconjugation by SENP5s is paralogue-specific, and SENP5 has a preference for SUMO2/3 relative to SUMO1 [11]. However, the role of SENP5 in TBI has rarely been studied. Therefore, we postulated that SENP5 regulates the SUMOylation of its target protein via SUMO2/3. Many SUMOylation
targets, including transcription factors, ion channels, signaling kinases, and diverse synaptic proteins, can modulate neuronal activity [12]. Intriguingly, SUMO2 is significantly conjugated to E2F1 mainly at lysine 266 residue following oxidative insult, which specifically modulates E2F1 transcription to enhance cell cycle arrest for cell survival [13]. We, therefore, investigated whether E2F1 is the physiological substrate for SENP5 upon TBI and whether the deSUMO2 is linked to its role in controlling neuronal apoptosis in TBI.

Materials and Methods

Animals and treatments
Male Sprague-Dawley rats (12 weeks, weight 360-400 g) were obtained from Vital River (Beijing, China). Before the experiment, the rats were acclimatized for 7 days under a 12 h light/dark cycle at 23–25°C with 50%–60% humidity with free access to food and water. All procedures were implemented in strict accordance with the NIH guidelines for the Care and Use of Laboratory Animals (1996). This study was approved by the Institutional Animal Care and Use Committee of the Zhejiang University of Technology (Hangzhou, China).

Rats were arbitrarily divided into 6 groups (n = 6 in each): the sham group, the TBI group, the sh-NC group (sh-NC lentivirus was injected intracerebroventricularrly in rats 72 h before TBI establishment), the sh-SENP5 group (sh-SENP5 lentivirus was injected intracerebroventricularly in rats 72 h before TBI establishment), the sh-SENP5 + overexpression (oe)-NC group (sh-SENP5 lentivirus and oe-NC lentivirus were injected intracerebroventricularrly in rats 72 h before TBI establishment), and the sh-SENP5 + oe-E2F1 group (sh-SENP5 lentivirus and oe-E2F1 lentivirus were injected intracerebroventricularly in rats 72 h before TBI establishment). Mammalian shRNA interference lentiviral vector targeting the coding sequences locus of SENP5 pLV[shRNA]-EGFP-U6 > rSenp5[shRNA 1#] (5′-GCTGGTAAACCCAGAGGCTATAA-3′), pLV[shRNA]-EGFP-U6 > rSenp5[shRNA 2#] (5′-GGAAGAAAGGATTCACCTAG-3′), and the negative control (NC) sh-NC (5′-TCCGTTGCGGAGAGGAAG-3′) as well as mammalian gene expression lentiviral vector pLV[Exp]-EGFP-CMV (> rE2f1[NM_001100778.1] and its control oe-NC were acquired from VectorBuilder (Guangzhou, Guangdong, China) with a virus titer of 1×10^9 TU/mL. Briefly, customized viral packaging plasmids were transduced into 293T cells. The cells were subsequently placed in a 37°C incubator with 5% CO₂ for further incubation. For proteasome inhibition, the PC12 cells infected with lentivirus of sh-SENP5 and screened with puromycin were treated with the proteasome inhibitor MG132 (MedChemExpress, Monmouth Junction, USA) at a concentration of 25 μM or its control DMSO for 4 h. In the protein stability assay, the PC12 cells were treated with 20 μM Cycloheximide (CHX; MedChemExpress) for 0, 3, 6, 12 and 24 h. The protein expression of E2F1 in the cells was measured to assess its protein degradation.

Modified neurological severity scores (mNSS)
The neurological function of each animal was assessed by researchers who were unaware of the experimental groups. The test was performed 24 h postoperatively. The scale was scored from 0 to 18 (normal, 0; maximal deficit, 18). The mNSS system includes motor (muscle status and abnormal movement), sensory (visual, tactile and proprioceptive) and reflex responses, and balance tests. Higher score indicates severer injury.

Brain water content
The rats were euthanized by sodium pentobarbital (200 mg/kg) i.p., and the brains of the rats were removed. The wet weights of the impacted hemispheres were weighed using an electronic analytical balance. After that, the tissues were heated at 110°C for 24 h and weighed for the dry weight. The brain water content [(wet weight - dry weight)/wet weight] × 100%.

RT-qPCR
TRizol (Invitrogen, Carlsbad, USA) was used for total RNA extraction from cells and tissues, followed by reverse transcription using PrimeScript® RT Master Mix (Takara, Dalian, China). ‘TB Green’ Fast qPCR Mix (TaKaRa) and ABI7500 real-time qPCR instrument (Applied Biosystems, Inc., Foster City, USA) were used for RT-qPCR. The primers sequences were as follows: SENP1, 5′-CGGCTAGGACT-GAAAGAGAGA-3′ (forward) and 5′-GAGGCTTTCGGGTTTCC-
GAG-3′ (reverse); SENP2, 5′-CTCTAGCTGTGACCCGAAGG-3′ (forward) and 5′-CTCCGTGACCCGAAGG-3′ (reverse); SENP3, 5′-TCGTTTCTCATTGGGACCC-3′ (forward) and 5′-TCGTTTCTCATTGGGACCC-3′ (reverse); SENP5, 5′-ACTGGTTTTAGCCCTGTAGAAGC-3′ (forward) and 5′-CCAGCTGTGATCATCTCCAGC-3′ (reverse); SENP6, 5′-ACACACAAGCTACGCTTA-3′ (forward) and 5′-TCGACTGACTACGCTTA-3′ (reverse); SENP7, 5′-GAATGCTGTCGACAAAGC-3′ (forward) and 5′-CCAGGTCGTCCATATCCAGC-3′ (reverse); SENP8, 5′-GATGTTGATGGGTCCGT-3′ (reverse). For visualization, Alexa Fluor 488-coupled goat anti-mouse (1:200; Abcam) or Alexa Fluor 647-coupled goat anti-rabbit (1:200; Abcam) was used. Finally, an enhanced chemiluminescence reagent (Thermo Fisher Scientific) was used for visualization.

**Western blot analysis**

Total protein in the cells and tissues was isolated using RIPA buffer (Beyotime, Shanghai, China) and quantified using bicinchoninic acid protein assay kit. Total protein (30 μg) was separated by 10% SDS-PAGE (Beyotime), and the protein (30 μg/lane) was transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, USA). The membranes were blocked with 5% BSA (Gibco) for 1 h at room temperature, and then incubated with specific antibodies to SENP5 (1:1000; GeneTex, Inc., Irvine, USA), E2F1 (1:1000; GeneTex), p53 (1:1000; Abcam, Cambridge, UK), p21 (1:1000; Abcam), HA (1:500; GeneTex), Myc (1:500; Abcam), Flag (1:200; GeneTex), or GAPDH (1:10,000; Abcam), followed by incubation with the appropriate secondary antibody goat anti-rabbit IgG H&L (HRP) (1:10,000; Abcam). Finally, an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Waltham, USA) was used for visualization.

**HE staining**

The rat hippocampal tissues were fixed with 4% paraformaldehyde for 1 d, paraffin-embedded, and sectioned. The sections (4 μm) were dehydrated with xylene, rehydrated, and stained with hematoxylin for 8 min and with eosin for 2 min. Finally, the sections were dehydrated, sealed with resin, and observed under a high-powered microscope (Olympus, Tokyo, Japan).

**TUNEL analysis**

TUNEL Assay Apoptosis Detection Kit (Beyotime) was used to assess apoptosis. Briefly, the hippocampal tissues were made into 4-μm paraffin sections. After routine dewaxing, hydration, and permeabilization, 20 mg/L proteinase K was added and incubated for a 15-min culture at room temperature. The sections were reacted with 500 μL TUNEL reaction mix (50 μL reaction solution TdT + 450 μL fluorescein-labeled dUTP solution) for 60 min in the dark at room temperature. The sections were washed with phosphate-buffered saline, sealed with anti-fluorescence quenching blocking solution, and observed under a fluorescence microscope (Nikon Instruments Inc., Melville, USA).

**Dual-labeled immunofluorescence**

Rat hippocampal tissues and cerebral cortex tissues were separated, made into paraffin-embedded sections, dehydrated with xylene, rehydrated with ethanol, and then heated in a microwave oven with citrate buffer for antigen retrieval. After washing, the sections were permeabilized with Triton X-100 for 15 min and blocked with goat serum for 1 h. The sections were then incubated with rabbit antibody to SENP5 (1:200; Thermo Fisher Scientific) or mouse antibody to NeuN (1:100; GeneTex) overnight at 4°C, and then with Alexa Fluor® 647-coupled goat anti-rabbit (1:200; Abcam) or Alexa Fluor® 488-coupled goat anti-mouse (1:200; Abcam) for 1 h at room temperature. DAPI was used to stain the nuclei, and the sections were dehydrated and permeabilized using ethanol and xylene, respectively. The intensity of SENP5 staining in the hippocampus and cerebral cortex was observed under a fluorescence microscope.

**Co-immunoprecipitation (Co-IP)**

The Pierce® Co-IP kit (Thermo Fisher Scientific) was used for the Co-IP assay. Briefly, the cells were lysed in RIPA buffer (Beyotime) containing protease and phosphatase inhibitors. Following centrifugation at 12,000 g for 10 min at 4°C, the supernatant was immunoprecipitated with primary antibody for 4 h at 4°C. The mixture was treated with 50 μL magnetic protein A/G beads for 120 min at 4°C. The immunoprecipitation was resuspended in SDS-PAGE buffer and evaluated by western blot analysis. The fusion protein vector HA-E2F1, Myc-SUMO1, Myc-SUMO2, Myc-SUMO3 and Flag-SENP5 used in Co-IP for cell transfection were designed and synthesized by LMAI (Shanghai, China). After immunoprecipitation using E2F1 (1:100; Thermo Fisher Scientific), ubiquitination and SUMO2 modification levels of E2F1 in the pulled-down immune complexes were analyzed by western blot analysis using anti-Ub antibody (1:1000; Abcam) or anti-SUMO2 antibody (1:1000; Thermo Fisher Scientific).

**Dual-luciferase assay**

For the assessment of E2F1 transcription, the E2F luciferase reporter plasmid (Yeasen Biotechnology Co., Ltd., Shanghai, China) overexpressing SENP5 was transfected into the cells using the lipofectamine 2000 transfection kit (Thermo Fisher Scientific). At 48 h after transfection, a dual-luciferase reporter assay system (Promega, Madison, USA) was used to assess the transcription of E2F1.

**Statistical analyses**

In vitro experiments were repeated three times. Results were expressed as the mean ± SD. Data were analyzed by unpaired t-test or one-way/two-way ANOVA, followed by Tukey’s post-hoc test. The criterion for statistical significance was set at P < 0.05 assessed by GraphPad Prism 8.02 (GraphPad, San Diego, USA).

**Results**

SENP5 is overexpressed in hippocampal tissues of TBI rats

The GEO dataset GSE59645 which comprises of rats subjected to fluid percussion TBI or sham injury was analyzed for differentially expressed genes in TBI. GSM1441405, GSM1441406, GSM1441407 are samples from the hippocampal region of rats with TBI, whereas GSM1441403, GSM1441404 are samples from the hippocampal region of rats in the sham group. The gene with the largest elevation foldchange in TBI, A_64_p076530, was screened with adj P < 0.05 and log2FoldChange > 2. It was annotated by the GPL14746 platform as SENP5 (Figure 1A). We developed a rat model of TBI. The neurological severity score was assessed to determine the neurological damage of the rats 24 h after TBI. Significant increases in neurological function scores and severe nerve damage were observed in the TBI rats (Figure 1B).
Figure 1. SENP5 is highly expressed in hippocampal tissues of TBI rats
(A) Screening for differentially expressed genes in the hippocampal region of TBI rats from GSE59645. (B) Neurological severity score of rats. (C) Water content in the brain tissues of rats. (D) The apoptosis in hippocampal tissues examined using TUNEL assay. (E) Evaluation of pathological changes in rat hippocampal tissues by HE staining. (F) Detection of SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7 mRNA expression in hippocampal tissues of rats by RT-qPCR. (G) SENP5 protein expression in hippocampal tissues of rats detected by western blot analysis. (H) SENP5 protein expression in the cerebral cortex of rats detected by western blot analysis. (I) SENP5 expression in neurons of cerebral cortex and hippocampus of rats revealed by dual-label immunofluorescence. Data are presented as the mean ± SD (n = 6). Comparisons between two groups were performed by unpaired t-tests. **P < 0.01.
SEN5 promotes TBI via inhibiting E2F1 SUMOylation

content, and an elevation in brain water content was found in the TBI group (Figure 1C). The number of apoptotic cells was increased in hippocampal tissues in the TBI rats, as revealed by TUNEL assay (Figure 1D).

The pathological changes in the hippocampal tissues of rats were assessed by HE staining. Neurons in the left hippocampal tissues of the sham-operated rats were found to be pyramidal or round, with three to four layers of cells, dense and orderly, with centered and completely transparent nuclei. The nuclei of cells in the hippocampal region of the rats with TBI were concentrated, and the cytosol was contracted and deformed (Figure 1E). The results of RT-qPCR and western blot analysis confirmed that sh-SENP5 1# and sh-SENP5 2# were significantly overexpressed in hippocampal tissues of TBI rats, and the upregulation of SENP5 was more pronounced (Figure 1F).

Therefore, we choose SENP5 as the study subject. The overexpression of SENP5 at the protein level in hippocampal and cerebral cortex tissues of TBI rats was also confirmed by western blot analysis (Figure 1G,H). In addition, dual-labeled immunofluorescence using NeuN and SENP5 showed significantly elevated expression of SENP5 in neurons in the cerebral cortex and hippocampus of TBI rats (Figure 1I).

Inhibition of SENP5 attenuates TBI in rats

To investigate whether SENP5 affects TBI in rats, we designed three rat-specific inhibitory sh-SENP5 lentiviruses. To avoid off-target effects, we performed knockdown efficiency experiments in PC12 cells, and sh-SENP5 1# and sh-SENP5 2# with better efficiency were selected for the subsequent functional assays (Figure 2A). The rats were subjected to intracerebroventricular injection of sh-SENP5 1# and sh-SENP5 2#, and successful infection of lentiviral vectors in the rat cerebral cortex and hippocampus was observed by fluorescence microscopy (Figure 2B). RT-qPCR and western blot analysis confirmed that sh-SENP5 1# and sh-SENP5 2# were effective in reducing SENP5 in hippocampal tissues of rats (Figure 2C,D). Inhibition of SENP5 expression was found to lower neurological function scores and to attenuate neurological damage in rats (Figure 2E). Silencing of SENP5 reduced water content in injured brain tissues (Figure 2F) and inhibited apoptosis in hippocampal tissues (Figure 2G). The HE staining results demonstrated that the cells in the hippocampal tissues of the sh-SENP5 group were more neatly arranged, and the nuclei were less shrunken and deformed (Figure 2H).

SUMO2-mediated modification of E2F1 is suppressed by SENP5

The SUMOpilot™ Analysis Program database (https://www.abcepta.com/sumopilot) was used to predict the SUMOylated modification sites of E2F1 protein based on the protein sequence of Rattus norvegicus E2F1, and the results showed that there were multiple motifs with high probability of SUMOylation sites (Figure 3A). To check whether SUMO2 can modify E2F1, we co-transfected PC12 cells with HA-labeled E2F1 and Myc-labeled SUMO1, 2 or 3. When E2F1 was co-expressed with SUMO2, the HA antibody detected a band with a much higher molecular weight than E2F1, indicating that E2F1 can bind to SUMO2 (Figure 3B). Next, a significant decrease in SUMO2 levels was found in hippocampal tissues of TBI rats (Figure 3C), indicating that TBI resulted in a significant decrease in the level of SUMOylated modifications on E2F1 in rats. Co-IP analysis in 293T and PC12 cells co-transfected with HA-tagged E2F1 and Myc-tagged SENP5 confirmed that E2F1 and SENP5 can interact with each other since anti-HA significantly pulled-down SENP5-tagged protein and anti-Myc significantly pulled-down E2F1 protein (Figure 3D).

To further investigate the role of SENP5 in SUMO2-mediated modification of E2F1, we transfected oe-NC or oe-SENP5 into PC12 cells. We tagged E2F1 with HA and SUMO2 with Myc. SENP5 significantly inhibited the modification level of SUMO2 on E2F1 protein, thereby increasing its protein expression, as revealed by co-IP analysis (Figure 3E). In addition, the dual-luciferase reporter system showed that the transcription of E2F1 was elevated, and luciferase activity was enhanced after overexpression of SENP5 (Figure 3F). We treated PC12 cells with the proteasome inhibitor MG132 in combination with SENP5 knockdown. Co-IP analysis revealed that the SUMO2 modification of E2F1 was increased, and the expression of E2F1 was reduced in cells with SENP5 loss (Figure 3G). After the combined treatment with MG132, the results of western blot analysis showed that the protein expression of E2F1 was elevated (Figure 3H). PC12 cells overexpressing SENP5 were treated with CHX. We found that overexpression of SENP5 was able to inhibit the degradation of E2F1, and the protein stability of E2F1 was increased (Figure 3I). Subsequently, we evaluated the level of ubiquitination on E2F1 in PC12 cells overexpressing SENP5. The level of ubiquitination on E2F1 was significantly reduced after overexpression of SENP5, indicating that the de-SUMOylation of E2F1 by SENP5 inhibited its degradation by the Ub-proteasome system (Figure 3J). The above results indicate that SENP5 can inhibit the SUMOylation of E2F1 and its degradation by the Ub-proteasome pathway, thus increasing the protein expression of E2F1.

Silencing of SENP5 inhibits E2F1 expression and blocks the p53 signaling activation

E2F1-related genes were analyzed by KEGG enrichment and visualized using bubble mapping. E2F1-related genes were enriched in the p53 signaling (Figure 4A,B). Whether E2F1 acts on the p53 signaling in TBI rats remains unclear. Our western blot analysis results demonstrated that the E2F1 protein expression was elevated in the TBI rats, along with augmented protein expressions of p53 and p21. The protein expressions of E2F1, p53 and p21 were significantly reduced after inhibition of SENP5 expression (Figure 4C). Thus, silencing of SENP5 impaired the activation of the p53 signaling pathway via E2F1.

Overexpression of E2F1 partially reverses the effect of sh-SENP5 on TBI in rats

To minimize animal sacrifice, we only administered the overexpression lentivirus oe-E2F1 to rats pre-injected with sh-SENP5 1# to further confirm the effect of E2F1 on TBI in rats. Western blot analysis results showed that the protein expression of E2F1 was much higher in the hippocampal tissues of sh-SENP5 + oe-E2F1-treated rats than in the hippocampal tissues of sh-SENP5 + oe-NC-treated rats, and there was no substantial difference in the protein expression of SENP5 relative to the sh-SENP5 + oe-NC-treated rats (Figure 5A). The mNSS assessment revealed increased neurological damage in rats after overexpression of E2F1 (Figure 5B). After further overexpression of E2F1, we also found a significant increase in water content in injured brain tissues of rats (Figure 5C) and elevated apoptosis in hippocampal tissues (Figure 5D). The
Pyramidal layer of hippocampal tissues in the sh-SENP5+oe-E2F1 group was thinned, with disordered cell arrangement and concentrated nucleus. In addition, capillaries showed edema, and contact between adjacent neurons was lost (Figure 5E). Western blot analysis results demonstrated that p53 and p21 expressions were also significantly augmented in the hippocampal tissues after overexpression of E2F1 with SENP5 silencing, and the p53 signaling pathway was activated (Figure 5F).

Figure 2. Inhibition of SENP5 attenuates TBI in rats  (A) SENP5 mRNA expression in lentiviral vector-infected PC12 cells was assessed by RT-qPCR. (B) Infection of lentiviral vectors in rat cerebral cortex and hippocampal tissues observed by fluorescence microscopy. (C) SENP5 mRNA expression in hippocampal tissues of TBI rats in response to sh-SENP5 1# and sh-SENP5 2# was detected by RT-qPCR. (D) SENP5 protein expression in hippocampal tissues of TBI rats in response to sh-SENP5 1# and sh-SENP5 2# detected by western blot analysis. (E) Neurological severity score of TBI rats in response to sh-SENP5 1# and sh-SENP5 2#. (F) Water content in the brain tissues of TBI rats in response to sh-SENP5 1# and sh-SENP5 2#. (G) The apoptosis in hippocampal tissues of TBI rats in response to sh-SENP5 1# and sh-SENP5 2# examined by TUNEL assay. (H) Evaluation of pathological changes in rat hippocampal tissues in response to sh-SENP5 1# and sh-SENP5 2# by HE staining. Data are presented as the mean ± SD (n=6). Comparisons between two groups were performed by one-way ANOVA and Tukey’s post hoc analysis. **P < 0.01.
Figure 3. E2F1 transcriptional activity is altered by SENP5, which is dependent on SUMO2

(A) Prediction of SUMOylation sites based on the protein sequence of *Rattus norvegicus* E2F1 using the SUMOplot® Analysis Program database. (B) PC12 cells were co-transfected with HA-tagged E2F1 and Myc-tagged SUMO1, 2 or 3, followed by western blot analysis with anti-HA antibody. (C) The changes of SUMO2 modification levels on E2F1 in hippocampal tissues of TBI rats detected by co-IP. (D) Co-IP analysis of the interaction between E2F1 and SENP5 in HEK293T and PC12 cells. (E) Effect of overexpression of SENP5 on the SUMO2 modification and protein expression of E2F1 using HA-tagged E2F1 and Myc-tagged SUMO2 detected by co-IP analysis. (F) The transcriptional activity of E2F1 measured using a dual-luciferase reporter assay system. (G) Co-IP analysis of SUMO2 modification and protein expression of E2F1 after knockdown of SENP5 using HA-tagged E2F1 and Myc-tagged SUMO2. (H) The protein expression of E2F1 after treatment with the proteasome inhibitor MG132 and knockdown of SENP5 detected by western blot analysis. (I) The protein degradation of E2F1 after treatment with CHX and overexpression of SENP5. (J) The ubiquitination level of E2F1 in PC12 cells after overexpression of SENP5 detected by co-IP assay. Error bars represent mean ± SD (n = 3). Comparisons were performed by unpaired t-tests or two-way ANOVA and Tukey’s post hoc analysis. *P < 0.05, **P < 0.01.
Discussion

Since its discovery in the mid-1990s, posttranslational modification with SUMO has been revealed to modulate proteins in a vast network of pathways [14]. For instance, SUMOylation of proteins plays a major role in mediating neuronal function; therefore, the balance between protein SUMOylation and deSUMOylation requires fine-tuning to maintain the homeostasis of neural tissues [15]. Our results specified that intracerebroventricular administration of sh-SENP5 remarkably reduced neuronal apoptosis and attenuated histopathological alterations after TBI. The protective effects of sh-SENP5 against TBI contribute to the molecular mechanisms of SUMO2-mediated SUMOylation of E2F1.

In the present study, we found that SENP3 and SENP5 were both upregulated in hippocampal tissues of rats with TBI relative to sham-operated rats, and the alteration of SENP5 was more pronounced. SENP3 has been reported to be gradually augmented from 3 h after TBI and peaked at 24 h in the brain of adult mice [16]. Therefore, the role and mechanism of SENP5 in TBI were further examined in the present study. According to Shimizu et al. [17], the expression of SENP5 was enhanced in the heart following the myocardial ischemia-reperfusion injury in mice. Kim et al. [18] revealed that overexpression of SENP5 contributed to cardiac dysfunction, accompanied by declined cardiomyocyte proliferation and raised apoptosis. Our loss-of-function assays using intracerebroventricular injection of suppressive lentivirus with SENP5 showed that depletion of SENP5 successfully lowered mNSS and ameliorated pathological changes in rats, while reducing neuronal apoptosis. Benzothiophene-2-carboxamide derivatives have been reported so far as SENPs inhibitors with selectivity within the SENPs family [19]. Therefore, pharmacologic targeting SENP5 is a promising option for the treatment of TBI from a therapeutic perspective.

Mechanistically, Saikosaponin-d, the main bioactive component of Radix bupleuri, could reverse the hypoxia-induced effects by specifically activating SENP5 in a time- and dose-dependent manner while downregulating SUMO1 [20]. Meanwhile, SENP3-controlled de-conjugation of SUMO2 was related to augmented leukemia cell proliferation under oxidative stress [21]. Therefore, we linked the neuroprotective effects of sh-SENP5 with SUMO2 in TBI. SUMO1 regulation of E2F1 binding to the EZH2 promoter has been demonstrated as an imperative post-translational mechanism for governing E2F1 function in cancer cells [22]. Consistently, we substantiated the direct binding interaction between E2F1 and SENP5 here and corroborated that overexpression of SENP5 decreased the SUMO2-mediated SUMOylation of E2F1, thereby increasing E2F1 expression in PC12 cells using co-IP analysis.

According to a recent study by Dzreyan et al. [23], the earliest proapoptotic event in the injured dorsal root ganglia was the overexpression of E2F1 at 4 h following sciatic nerve transection, which preceded the induction of p53 at 24-h post-axotomy.

Figure 4. Silencing of SENP5 inhibits E2F1 expression and blocks the p53 signaling pathway. (A) KEGG enrichment analysis of genes associated with E2F1. (B) Visualization by bubble mapping. (C) Detection of protein expression of p53, p21 and E2F1 in rats underwent different treatments by western blot analysis. n = 6/group for all groups. Data are presented as the mean ± SD of two-way ANOVA and Tukey’s post hoc analysis. **P < 0.01.
Aubrecht et al. [24] revealed that the neuroprotective effects of CR8, a cyclin-dependent kinase inhibitor, may be explained by inhibition of major upstream injury responses, including inhibition of p53 transactivation. We identified the p53 signaling pathway as the main pathway enriched by E2F1-relevant genes in this study. For validation, we performed western blot analysis to assess the protein expressions of p53 and p21. Their expressions were enhanced in the brain tissues of TBI rats and reduced following sh-SENP5 administration. Similar to our findings, p53 was found to be activated in the ischemic areas of brain, which resulted in neuronal apoptosis [25], and p53 loss or applications of p53 inhibitors significantly attenuated brain damage in several stroke models [26]. Furthermore, 17β-estradiol administration rescued p53-stimulated apoptotic cell death in the cortical stab wound injury model by modulating the expressions of Bax and Bcl-2 and caspase-3 activation [27]. However, the specific role of p53 in SENP5-mediated TBI progression needs to be further verified. E2F1, a major cell cycle driver, is correlated with apoptotic cell death in a TBI model [28]. Ablation of E2F1 has been revealed to limit neuroinflammation and neurological deficits in contusive spinal cord injury [29]. Here, the observation derived from our rescue experiments showed that overexpression of E2F1 perturbed the protective effects of sh-SENP5 against TBI, as evidenced by higher mNSS, brain water content and apoptosis than TBI rats injected with sh-SENP5 + oe-NC.

In summary, our results showed that sh-SENP5 protects the neurons against apoptosis in a rat TBI model (Figure 6). The study also provides evidence that the mechanism of this protection is related to impaired p53 pathway, which is mediated by the

Figure 5. Overexpression of E2F1 partially reverses the protective effect of sh-SENP5 on TBI in rats (A) The protein expression of SENP5 and E2F1 after further overexpression of E2F1 in rats with sh-SENP5 detected by western blot analysis. (B) Neurological severity score of rats treated with sh-SENP5 + oe-E2F1. (C) Water content in the brain tissues of rats treated with sh-SENP5 + oe-E2F1. (D) The apoptosis in hippocampal tissues of rats treated with sh-SENP5 + oe-E2F1 examined by TUNEL assay. (E) Evaluation of pathological changes in hippocampal tissues of rats treated with sh-SENP5 + oe-E2F1 by HE staining. (F) Detection of protein expression of p53 and p21 in rats treated with sh-SENP5 + oe-E2F1 by western blot analysis. Data are presented as the mean ± SD (n = 6). Comparisons between two groups were performed by unpaired t-tests. Two-way ANOVA was utilized for the comparisons of data among multiple groups, accompanied by Tukey’s post hoc tests. *P < 0.05, **P < 0.01.
repression of E2F1 via SUMO2. The precise mechanism of E2F1 on the p53 pathway remains unclear, and we also need to further study this. To support our hypothesis, we plan to carry out an in vitro experiment in the future.

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Conflict of Interest
The authors declare that they have no conflict of interest.

References


