

# Ameliorative Effects of p75NTR-ED-Fc on Axonal Regeneration and Functional Recovery in Spinal Cord-Injured Rats

Yong-Tang Wang · Xiu-Min Lu · Feng Zhu ·  
Peng Huang · Ying Yu · Zai-Yun Long · Ya-Min Wu

Received: 10 August 2014 / Accepted: 29 October 2014 / Published online: 15 November 2014  
© Springer Science+Business Media New York 2014

**Abstract** As a co-receptor of Nogo-66 receptor (NgR) and a critical receptor for paired immunoglobulin-like receptor (PirB), p75 neurotrophin receptor (p75NTR) mediates the inhibitory effects of myelin-associated inhibitors on axonal regeneration after spinal cord injury. Therefore, the p75NTR antagonist, such as recombinant p75NTR protein or its homogenates may block the inhibitory effects of myelin and promote the axonal regeneration and functional recovery. The purposes of this study are to subclone and express the extracellular domain gene of human p75NTR with IgG-Fc (hp75NTR-ED-Fc) in prokaryotic expression system and investigate the effects of the recombinant protein on axonal regeneration and functional recovery in spinal cord-injured rats. The hp75NTR-ED-Fc coding sequence was amplified from pcDNA-hp75NTR-ED-Fc by polymerase chain reaction (PCR) and subcloned into vector pET32a (+), then the effects of the purified recombinant protein on neurite outgrowth of dorsal root ganglion (DRG) neurons cultured with myelin-associated glycoprotein (MAG) were determined, and the effects of the fusion protein on axonal regeneration, functional recovery, and its possible mechanisms in spinal cord-injured rats were further investigated. The results indicated that the purified infusion protein could promote neurite outgrowth of DRG neurons, promote axonal regeneration and functional recovery, and decrease RhoA activation in spinal cord-injured

rats. Taken together, the findings revealed that p75NTR still may be a potential and novel target for therapeutic intervention for spinal cord injury and that the hp75NTR-ED-Fc fusion protein treatment enhances functional recovery by limiting tissue loss and stimulating axonal growth in spinal cord-injured rats, which may result from decreasing the activation of RhoA.

**Keywords** p75NTR-ED-Fc · Neurite outgrowth · Axonal regeneration · Functional recovery · Spinal cord injury

## Introduction

Traumatic spinal cord injury (SCI) is one of the leading causes of disabilities in young adults. Growing evidence indicates that the regeneration failure following central nervous system (CNS) injury in adult is not due to intrinsic properties of CNS neurons but to the neurite outgrowth inhibitors associated with the CNS myelin, such as Nogo-A, the myelin-associated glycoprotein (MAG), and the oligodendrocyte-myelin glycoprotein (OMgp) [1–3]. Interestingly, these three structurally different inhibitors share two common receptors, Nogo-66 receptor (NgR) and paired immunoglobulin-like receptor B (PirB) [4–8]. Each of the three inhibitors can interact in high affinity with NgR and transduce the inhibitory signal into neurons via a complex composed of NgR and its co-receptors [9–11]. Co-immunoprecipitation, structural analysis, and deletion mutation have demonstrated that the p75 neurotrophin receptor (p75NTR) could conjunct with the C-terminal of NgR via the extracellular domain (ED), and mediates the inhibitory effects of myelin without being blocked by neurotrophic factors [9, 12]. As a result, p75NTR expressed in neurons can specifically participate in regulating the inhibitory effects of myelin-associated inhibitors on axonal regeneration after central nervous system injury, while not

Y.-T. Wang (✉) · Z.-Y. Long · Y.-M. Wu  
State Key Laboratory of Trauma, Burns and Combined Injury,  
Institute of Surgery Research, Daping Hospital, Third Military  
Medical University, Chongqing 400042, People's Republic of China  
e-mail: wangytlu@163.com

X.-M. Lu (✉) · F. Zhu · P. Huang · Y. Yu  
College of Pharmacy and Bioengineering, Chongqing University of  
Technology, Chongqing 400054, People's Republic of China  
e-mail: luxm@cqut.edu.cn

neurotrophic factors or their high-affinity TrkA receptor. The reason may be the lack of expression of p75NTR in oligodendrocytes where neurotrophic factors and TrkA receptor play an important role in axonal regeneration following CNS injury [13]. Recently, it also has been indicated that p75NTR mediates axon growth inhibition through an association with PirB, another common receptor of three different inhibitors [14]. Moreover, p75NTR can stimulate the downstream signal through the interaction with ganglioside GT1b and induces the MAG-dependent inhibition to axon outgrowth [15–17]. The final result of myelin signal transduction shows that p75NTR appears to enhance the activation of RhoA, a small GTPase known to affect the regulation cytoskeleton, and achieves the inhibitory effect [18]. These research studies indicate that p75NTR, as a co-receptor of NgR and PirB, could mediate the inhibitory effects of myelin or through some mechanisms unknown. Therefore, recombinant p75NTR protein or its homogenates may block the inhibitory effects of myelin and promote the axonal regeneration and functional recovery in spinal cord-injured rats.

The purposes of this study are to subclone and express the extracellular domain gene of human p75NTR with IgG-Fc (hp75NTR-ED-Fc) in prokaryotic expression system and further investigate the effect of the recombinant protein on neurite outgrowth of dorsal root ganglion (DRG) neurons, axonal regeneration, and functional recovery in spinal cord-injured rats, which may pave a way to the further research of central nervous system (CNS) regeneration on the target of p75NTR.

## Materials and Methods

### Animals

Adult female Sprague–Dawley (SD) rats ( $n=31$ ) were supplied by the Animal Breeding Center of Institute of Surgery Research, Daping Hospital, Third Military Medical University, and housed in a light- and temperature-controlled room. All surgical interventions and postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA) and approved by the Animal Use and Care Committee of School of Medicine, Third Military Medical University.

### Recombinant hp75NTR-ED-Fc Fusion Protein

The DNA fragment of human p75NTR extracellular domain and Fc fragment of human IgG (hp75NTR-ED-Fc) were amplified from eukaryotic expression vector pcDNA-hp75NTR-ED-Fc by using polymerase chain reaction (PCR) and subcloned into prokaryotic expression vector pET32a (+),

and then we constructed pET-hp75NTR-ED-Fc expressing Trx-hp75NTR-ED-Fc fusion protein in *Escherichia coli*. The recombinant vectors were amplified in *E. coli* DH5 $\alpha$  and identified by using PCR, enzyme digestion, and sequencing and then transformed into *E. coli* BL21 (DE3). After being identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, the recombinant Trx-hp75NTR-ED-Fc protein was purified with His-tag affinity chromatograph and digested with restriction enzymes, and then the hp75NTR-ED-Fc protein was monitored by SDS-PAGE analysis.

### Effects of hp75NTR-ED-Fc on the Neurite Outgrowth of Dorsal Root Ganglia Neurons

Neurite outgrowth assay was performed with dorsal root ganglia (DRG) neurons isolated from newly born SD rats as previously described [19, 20]. Glass coverslips in 24-well plates were coated with 100  $\mu\text{g/ml}$  poly-L-lysine (PLL), washed, and dried. The DRG neurons were dissected, dissociated, and resuspended with Neurobasal (NB) medium in presence of 1  $\mu\text{l}$  phosphate-buffered saline (PBS) with 100 ng MAG-Fc (R&D systems), 100 ng MAG-Fc and hp75NTR-ED-Fc, or 100 ng bovine serum albumin (BSA) used as a positive control, and then plated separately at a density of  $1 \times 10^6$  cells/ml onto corresponding coverslips pre-coated with immobilized substrates (PLL). Cells were cultured 24 h before fixation with 4 % paraformaldehyde and immunostained with a mouse anti- $\beta$  III tubulin monoclonal antibody (Millipore, USA). Neurite length was quantified by measuring the lengths of individual neurites using the Image-Pro Plus 5.0 image analysis software from Media Cybernetics, from at least 10 areas under light microscopy per condition, and from three independent experiments in duplicate wells. Average neurite length was used for statistic analysis.

### Spinal Cord Dorsal Hemisection Model

Spinal cord dorsal hemisection model was performed as previously described [21]. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg), and body temperature was maintained at 37 °C during the period of anesthesia with a temperature-controlled heating pad. In the dorsal hemisection model, rat spinal cords were exposed by laminectomy at the level of T9–T11. The dorsal hemisection was performed at a depth of 1.6 mm from the dorsal surface of the cord, using a pair of microscissors, to sever the left dorsal corticospinal tracts (CSTs). A total of 19 rats received spinal cord hemisection, 9 rats for hp75NTR-ED-Fc protein administered group (SCI+hp75NTR-ED-Fc group) injected multipointly and slowly with 25  $\mu\text{l}$  hp75NTR-ED-Fc protein (0.4 mg/ml) at the points 2 mm to the site of injury, and 10 rats for SCI group injected the same

as hp75NTR-ED-Fc protein with BSA as a negative control. Among them, three hp75NTR-ED-Fc protein administered and four SCI rats died during the first week after SCI. The remaining six hp75NTR-ED-Fc protein administered and six SCI rats underwent the entire experiment procedures, with other six rats whose spinal cords were only exposed by laminectomy without dorsal hemisection as the sham group ( $n=6$ ), and the other six rats untouched as normal control ( $n=6$ ).

#### Assessments of Motor Function

In order to assess the motor function, long-term behavioral experiments were then carried out using the same injury model. Rat locomotor recovery after SCI was assessed according to Basso, Beattie, and Bresnahan (BBB) locomotor scale [22]. This scale provides a measure of hindlimb function ranging scale from 0 (complete paralysis) to 21 (normal locomotion) by assessing hindlimb joint movements, stepping, trunk position and stability, forelimb-hindlimb coordination, paw placement, toe clearance, and tail position. The rats were observed in an open field and evaluated within a period of 4 min. BBB was performed at 24 h and 3 days post-injury, and once weekly thereafter up to 6 weeks post-injury. Blind scoring ensured that observers were not aware of the treatment received by individual rats.

Footprint assay was modified from the protocol reported previously [23]. The fore paws and hind paws of the animals were inked in red and blue, respectively, and then the rats were made to walk down a narrow runway of 100 cm long and 7 cm wide with a white paper placed on the floor, leaving the footprints on the paper. The base of support was determined by measuring the core to core distance of the central pads of the hind paws. Each rat was tested for its footprints before and 6 weeks after spinal cord injury, respectively. Blind scoring ensured that observers were not aware of the treatments received by each rat.

#### Electrophysiological Evaluation

To further evaluate the recovery of the sensory and motor systems as total nervous systems after SCI, both somatosensory evoked potentials (SEPs) and motor evoked potentials (MEPs) were recorded. Animals were anesthetized and placed in a stereotaxic frame adjusted so that the surface of the skull was horizontal between the lambda and bregma. The cranium was exposed and a small diameter hole drilled above the primary somatosensory cortex and motor cortex of right hemisphere. Stereotaxic coordinates were measured from bregma and calculated using a rat atlas (SEP: P,  $-0.42$ – $-2.20$  mm; L,  $1.50$ – $3.00$  mm; MEP: P,  $-0.48$ – $3.14$  mm; L,  $1.00$ – $3.00$  mm). A metal microelectrode was positioned 0.5 mm below the cortical surface. Signals were band-pass filtered (2–2000 Hz)

and recorded digitally on a personal computer (PowerLab system, AD Instruments). SEPs were recorded from the skull over the right somatosensory cortex following electrical stimulation (10–40 mA, 0.2-ms duration, 4 Hz) of the left sciatic nerve, and MEPs were recorded from the left sciatic nerve following stimulation (10–40 mA, 0.2-ms duration, 4 Hz) of the right motor cortex. The electrical stimulations were produced by a constant current stimulator and isolation unit (AD Instruments, Australia). Measurements were taken at the same stimulation and recording sites and in the same posture to obtain reproducible results. SEP and MEP recordings were evaluated from latency measured from the first prominent peak by a custom-made software.

#### Corticospinal Tract Retrograde Tracing

Sixteen to 18 h before perfusion 6 weeks after the dorsal hemisection, an retrograde axonal tracer nuclear yellow (NY, 376.37 MW; 0.5 % in PBS; Sigma) was slowly injected into the left lateral sciatic nerve of the hind limb, as previously described [21, 24]. For each injection, 6  $\mu$ l NY was delivered for a period of 15 min via a 10–15-nm inner diameter glass pipette attached to a Picopump, and stuck needles for 10 min. Sixteen to 18 h later, the animals were perfused transcardially with PBS followed by 4 % paraformaldehyde. The spinal cord 3 mm rostral to the lesion site was sagittally sectioned at a thickness of 20  $\mu$ m using a cryostat (Leica CM1900, Bannockburn, IL) and thaw-mounted on gelatin-coated slides. Transverse sections were collected from the spinal cord 4–6 mm rostral to the injury site. The sections were directly examined, and all pictures were captured at the same area of tissue sections using a ZEISS LSM510 Meta confocal microscope.

#### Histological Analysis

After the last functional tests at the 6 weeks after injury, rats were deeply anesthetized by intraperitoneal injections of sodium pentobarbital (80 mg/kg) and killed by transcardial perfusion with 100 ml of cold 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 400 ml of 4 % paraformaldehyde in cold 0.1 M PBS (pH 7.4). After perfusion, a 12-mm block of the spinal cord containing the injury site in the middle was carefully removed, and 6 mm containing the entire injury site in the middle for longitudinal sections (20  $\mu$ m) and 3–6 mm rostral and 3–6 mm caudal to the injury site for transverse sections (20  $\mu$ m) were postfixed in the same fixation solution, dehydrated overnight in ethanol, and embedded in a paraffin block. Serial longitudinal and transverse sections were made. For further morphological and morphometric analyses, two sets of slides each containing serial sections were respectively stained with hematoxylin and eosin (HE) and toluidine blue (Nissl), and all images were collected at the

same area of tissue sections by an Olympus BX60 microscope. Morphometric analysis of spared spinal cord (SC) parenchyma was carried out in the most central portion of the SC judging from the presence of ependyma in the longitudinal sections of tissue by HE stain. Area measurements were performed using the Image-Pro Plus 5.0 image analysis software (Media Cybernetics Inc., Atlanta, GA, USA). The sections 3–6 mm rostral to the injury site were analyzed for residual ventral horn motoneurons, and the number of surviving ventral horn neurons were confirmed by the exhibition of Nissl substance, euchromatic nucleus, and nucleolus [25]. From each spinal cord, approximately 50 sections were inspected, and all slides were assessed blindly with respect to treatment. For frozen section preparation, the specimen was transferred to a solution containing 30 % sucrose in 0.1 M PBS (pH 7.4) at 4 °C, until it was stayed at the bottom of the container, and 6-mm spinal cord segment containing the entire injury site for longitudinal sections and 3–6 mm rostral and 3–6 mm caudal to the injury site for transverse sections were embedded in tissue freezing medium (Tissue-Tek, Miles, Elkhart, IN), serial 20- $\mu$ m-thick cross sections were obtained using a cryostat (Leica CM1900, Bannockburn, IL) and thaw-mounted on gelatin-coated slides. The frozen sections were stored at 4 °C and would be further used for immunofluorescence staining.

#### Immunofluorescence Staining

The immunofluorescence staining was used to identify the extent of axons regeneration through the expression of growth-associated protein-43 (GAP-43) in normal and injured spinal cord. In brief, spinal cord frozen sections were mounted on gelatin-coated slides. After incubation with a blocking buffer (10 % normal goat serum, 0.3 % Triton X-100 in 0.01 M PBS) for 1 h at room temperature, sections reacted with the primary antibody, a mouse anti-GAP-43 monoclonal antibody (1:4000, Millipore) at 4 °C overnight, and then with Cy3 conjugated goat anti-mouse antibody (1:100, Sigma) for 1 h at 37 °C. After incubation with Cy3 conjugated goat anti-rabbit antibody, slides were incubated with DAPI for 5 min, cover slipped, and all pictures were captured at the same area of tissue sections using a ZEISS LSM510 Meta confocal microscope.

#### RhoA Activity Assay

RhoA activity was assessed by a pull-down assay according to the manufacturer's instructions for a RhoA Activation Assay Biochem Kit (Cytoskeleton, Denver, CO). As previously reported [26], injured spinal cords were rapidly removed 3 days after transection and 5-mm blocks of tissue taken each side of the epicenter of the lesion. The same blocks of spinal cord

tissues were also taken from the normal and sham rats. All the tissues were lysed in cell lysis buffer with protease inhibitors and processed as described previously [27]. Active GTP-RhoA was isolated from tissue homogenates of injured spinal cord 3 days after transection by pull-down assay and detected by Western blot analysis with RhoA-specific monoclonal antibody (Santa Cruz). Total RhoA in the tissue homogenates from the same animals was also detected and used as a control for the cross-comparison of Rho activity. Total RhoA and GTP-RhoA levels were quantified by densitometry, and the Rho-GTP level was normalized to total RhoA bands.

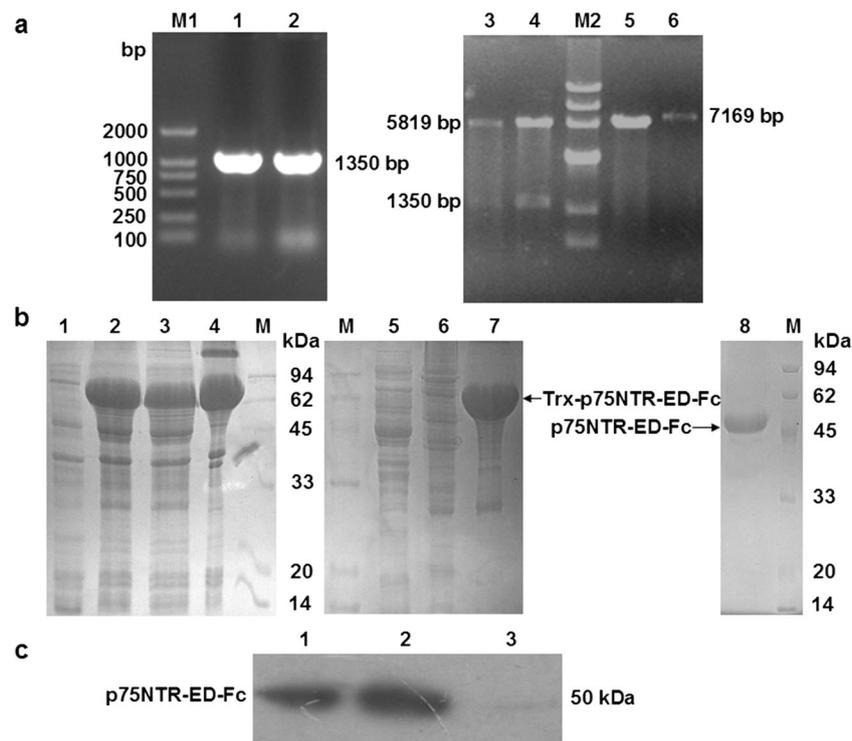
#### Statistical Analysis

All the data were presented as mean $\pm$ SEM and were analyzed with SPSS10.0 application software. Quantification of the fluorescence intensity of GAP-43 immunohistochemical staining, NY-labeled neurons around the lesion site, the histological assay, and RhoA activity assay were statistically analyzed using the two-tailed Student's *t* test. The length of neurite outgrowth was analyzed by one-way analysis of variance (ANOVA) followed by a post hoc Tukey test. BBB behavioral scores and footprint analysis were analyzed by repeated measures ANOVA followed by the post hoc Tukey test. Differences were considered to be statistically significant when  $P < 0.05$ .

## Results

#### Preparation of Recombinant hp75NTR-ED-Fc Fusion Protein

The choice of polypeptide sequence is important for the therapeutic efficacy of protein antagonist. In the present study, the extracellular domain gene of human p75NTR and the Fc fragment of human IgG (hp75NTR-ED-Fc) were subcloned into prokaryotic expression vector pET32a (+). Besides targeting effects of the extracellular domain of p75NTR, the Fc fragment could improve the efficiency of antigen presentation, extend the half-life in the body, and provide a tag for affinity chromatograph of fusion protein purification. After identified by PCR, restriction endonuclease digestion, and DNA sequencing analysis (Fig. 1a), the recombinant plasmid was induced by IPTG. The recombinant fusion protein hp75NTR-ED-Fc was monitored by SDS-PAGE (Fig. 1b) and Western blot with an anti-IgG-Fc monoclonal antibody (Fig. 1c) and then was purified with high-performance liquid chromatography (HPLC). The above results showed that the recombinant prokaryotic expression plasmid pET32a-hp75NTR-ED-Fc was constructed and expressed



**Fig. 1** Preparation of recombinant hp75NTR-ED-Fc fusion protein. **a** Colony PCR and restriction enzyme analysis of recombinant vector of pET32a-hp75NTR-ED-Fc. Lanes 1–2, positive PCR products of pET32a-hp75NTR-ED-Fc; lane 3, pET32a(+) digested with Kpn I and Xho I; lane 4, pET32a-hp75NTR-ED-Fc digested with Kpn I and Xho I; lane 5, pET32a(+) digested with Xho I; lane 6, pET32a-hp75NTR-ED-Fc digested with Xho I; M1, M2, DNA marker. **b** SDS-PAGE analysis of the recombinant hp75NTR-ED-Fc protein. Lane 1, full bacterial proteins from transformed *E. coli* without induction of IPTG; lane 2, full bacterial proteins from transformed *E. coli* with induction of IPTG; lane 3, supernatant bacterial proteins from transformed *E. coli* with induction of IPTG;

lane 4, precipitation bacterial proteins from transformed *E. coli* with induction of IPTG; lanes 5–6, supernatant bacterial proteins without conjugation with His-tag column; lane 7, elution of supernatant bacterial proteins (Trx-p75NTR-ED-Fc) from His-tag column; lane 8, Purified recombinant hp75NTR-ED-Fc protein; M, protein molecular weight standard. **c** Identification of the recombinant hp75NTR-ED-Fc protein using Western blot by an anti-IgG-Fc monoclonal antibody. Lanes 1–2, supernatant bacterial proteins from transformed *E. coli* with induction of IPTG; lane 3, supernatant bacterial proteins from transformed *E. coli* without induction of IPTG

successfully and that abundant purified hp75NTR-ED-Fc protein was obtained for antagonist.

#### Promotional Effects of hp75NTR-ED-Fc on Nuerite Outgrowth

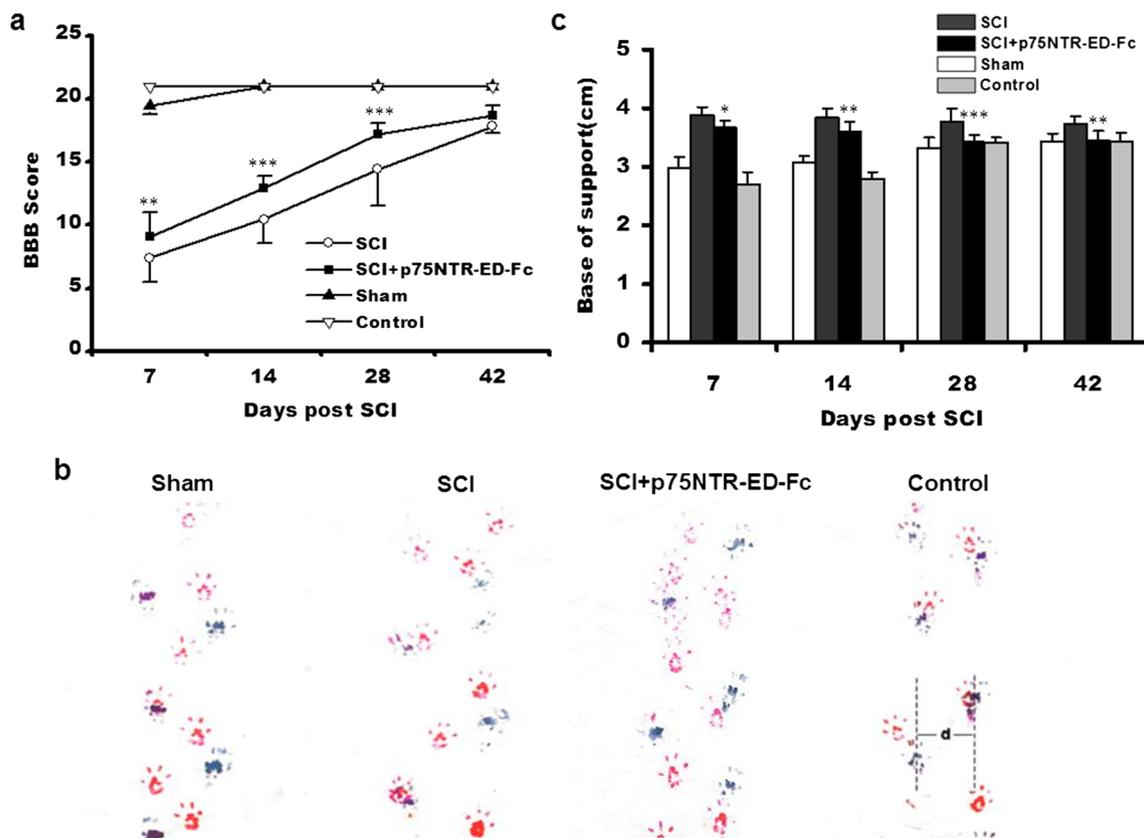
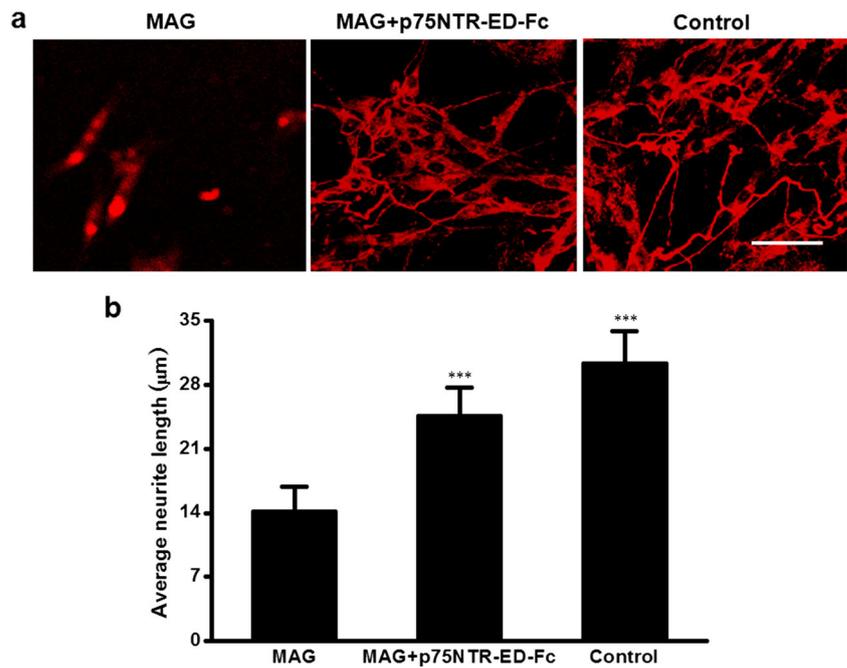
To determine if hp75NTR-ED-Fc fusion protein could neutralize myelin-associated inhibitors and promote neurite outgrowth in vitro, the hp75NTR-ED-Fc fusion protein was added into the cultures of rat dorsal root ganglia neurons in presence of MAG, one of the three known myelin-associated inhibitors, with MAG used as a negative control and BSA as a positive control, respectively. After being cultured for 24 h, neurite lengths were measured according to the neurons immunostained with a mouse anti- $\beta$  III tubulin monoclonal antibody. The results showed that hp75NTR-ED-Fc and the positive control significantly enhanced neurite outgrowth in DRG neurons as compared with MAG (Fig. 2a, c) and that the average length of neurites treated with hp75NTR-ED-Fc and

BSA significantly increased than that of MAG (Fig. 2b; one-way ANOVA,  $F=52.212$ ,  $P<0.0001$ ). These results strongly indicated that hp75NTR-ED-Fc protein could reverse the inhibition of myelin-associated inhibitors.

#### hp75NTR-ED-Fc Improved Functional Recovery After SCI

To determine the effect of hp75NTR-ED-Fc fusion protein on functional recovery, besides BBB locomotor, another functional measure, footprint analysis was performed. The results of BBB locomotor showed significant improvements in locomotor recovery in hp75NTR-ED-Fc-treated rats starting as early as 7 days, and lasted up to 6 weeks after SCI as compared to the SCI group (Fig. 3a; repeated measures ANOVA and post hoc Tukey test,  $F=109.329$ ,  $P<0.0001$ ). In the footprint analysis at the sixth week after SCI, the prints of all hind toes in the hp75NTR-ED-Fc-treated rats were very visible, while in the SCI group, they were not clearly separated which indicated the signs of toe dragging (Fig. 3b). Furthermore, the base of

**Fig. 2** The inhibitory effect of MAG on neurite outgrowth was reversed by hp75NTR-ED-Fc fusion protein. **a** Immunostaining with a mouse anti- $\beta$  III tubulin monoclonal antibody for the neurite of DRG neurons cultured with MAG-Fc, MAG-Fc in presence of hp75NTR-ED-Fc, and BSA. Scale bar represents 50  $\mu$ m and is valid for all pictures. **b** Quantifications of the average neurite lengths of DRG neurons. Recombinant hp75NTR-ED-Fc fusion protein significantly reversed the inhibition of MAG and promoted neurite outgrowth compared with MAG. \*\*\* $P < 0.0001$ , compared with MAG group



**Fig. 3** Treated with hp75NTR-ED-Fc fusion protein promoted functional recovery after SCI. **a** The BBB scores in hp75NTR-ED-Fc-treated rats were consistently higher than those in SCI group. **b** Footprint analysis showed that the prints of all hind toes in hp75NTR-ED-Fc-treated rats were very visible, while in SCI rats, they were not clearly separated which indicated the signs of toe dragging. Fore paw footprints (red) and hind

paw footprints (blue) from rats tested. “d” shows the vertical distance between the hind paws. **c** The distance of the base of support between the hind paws in hp75NTR-ED-Fc-treated rats was significantly reduced compared with SCI rats. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ , compared with SCI group (Color figure online)

support was significantly reduced in both the hp75NTR-ED-Fc-treated and the sham rats compared with the SCI rats (Fig. 3c; repeated measures ANOVA and post hoc Tukey test,  $F=41.146$ ,  $P<0.0001$ ). The above results suggested that hp75NTR-ED-Fc fusion protein noticeably promoted recovery of motor function after SCI.

#### Electrophysiological Evaluation

To further evaluate the recovery of the sensory and motor systems as total nervous systems after SCI, both SEP and MEP were recorded, and the latency was analyzed from the first prominent peak by a custom-made software. When recorded following electrical stimulation of the left sciatic nerve, SEPs in each group were all in stable wave form, as well as MEPs recorded following electrical stimulation of the right motor cortex (Fig. 4a, b). However, both latencies of MEP and SEP in SCI rats were obviously longer than those in sham and control rats, and when treated with p75NTR-ED-Fc fusion protein, both were decreased significantly (Tables 1 and 2 and Fig. 4; two-tailed Student's *t* test,  $P<0.05$ ,  $P<0.01$ , compared with SCI).

#### Treatment with hp75NTR-ED-Fc Promoted Axon Regeneration After Spinal Cord Hemisection

To evaluate whether hp75NTR-ED-Fc fusion protein-mediated functional recovery was the consequence of axon regeneration, axonal transport was detected by NY-labeled neurons of spinal cord corticospinal tract in sham, SCI, hp75NTR-ED-Fc-treated, and normal control rats. In transverse and longitudinal sections rostral to the lesion site, the number of labeled positive neurons and corresponding fluorescence intensity in hp75NTR-ED-Fc-treated rats was much more and higher than that in SCI rats, and the same change was found in sham and control groups when compared with SCI group. (Fig. 5a–d; two-tailed Student's *t* test,  $P<0.001$ ,  $P<0.0001$ , compared with SCI). These results of NY retrograde tracing suggested that the hp75NTR-ED-Fc fusion protein could efficiently promote axonal outgrowth and repair the fiber contact of injured spinal cord.

#### hp75NTR-ED-Fc Improved Tissue Repair and Neuroprotection After SCI

To determine whether hp75NTR-ED-Fc antagonist-mediated tissue repair resulted from the survival of motoneurons in the ventral horn, and the neuroprotective efficacy of hp75NTR-ED-Fc protein following SCI, the sections at the sixth week after SCI were stained with HE and Nissl, respectively. HE stain showed cysts of variable size and shape in the left portion of the cord, often trabeculated and containing foamy macrophages. Although showing microcysts, white and gray

substances surrounding the larger cysts were considered spared tissue. In contrast to spinal cord-injured animals, in hp75NTR-ED-Fc-treated rats, the glial scar surrounding the connective tissue matrix appeared to be less extensive, and there were many longitudinally oriented blood vessels growing into the lesion site several millimeters distant from the crush site (Fig. 6a). The percentage of spared spinal cord (SC) tissue in hp75NTR-ED-Fc treated group was significantly smaller than that in SCI group ( $P=0.019$ , compared with SCI), as well as in sham and normal control group ( $P=0.001$ , compared with SCI;  $P=0.001$ , compared with SCI) (Fig. 6c; two-tailed Student's *t* test). As shown in Fig. 6b by Nissl stain, in hp75NTR-ED-Fc-treated group, more residual motoneurons were found in the rostral to the lesion sites. Quantitative assessment of numbers of neuronal survival indicated that hp75NTR-ED-Fc antagonist resulted in a significant enhancement of survival of motoneurons in the ventral horn following SCI, compared to the SCI group ( $P=0.024$ , compared with SCI), as well as the sham and normal control group compared with the SCI group ( $P<0.0001$ , compared with SCI;  $P<0.0001$ , compared with SCI) (Fig. 6d; two-tailed Student's *t* test). The results indicated that antagonism of hp75NTR-ED-Fc resulted in significantly better tissue repair and neuroprotection after SCI.

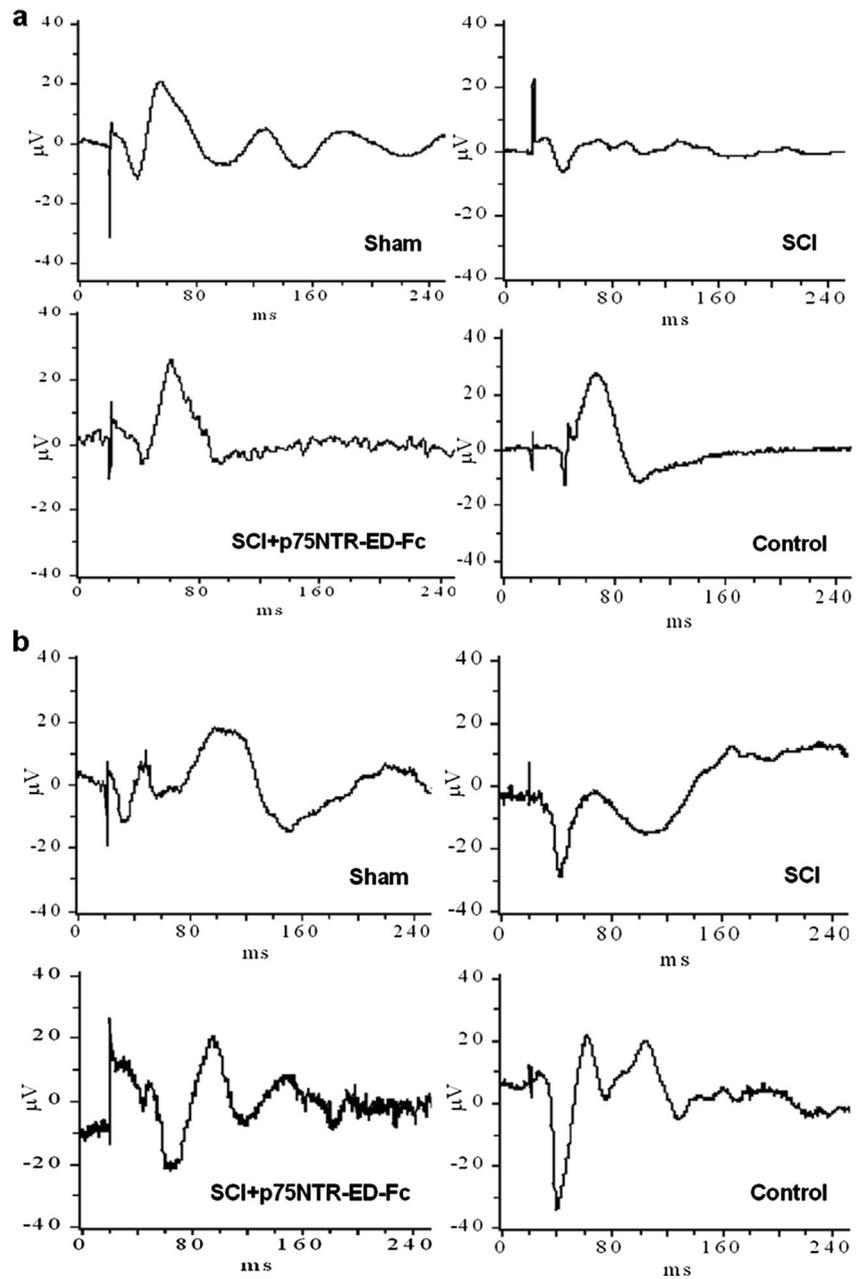
#### The Expression of GAP-43

To further elucidate the mechanism of action of p75NTR-ED-Fc on functional recovery and axonal regeneration, its effects on the expression of GAP-43, a crucial marker of neurite growth, was investigated by immunofluorescence stained with a mouse anti-GAP-43 monoclonal antibody. The results showed that the fluorescence intensity of anti-GAP-43 staining in hp75NTR-ED-Fc-treated rats was much weaker than that in SCI rats (Fig. 7a, b; two-tailed Student's *t* test,  $P<0.0001$ ). However, the same expression was found in the sham and normal control rats compared with the SCI rats (Fig. 7a, b). These results suggest that the hp75NTR-ED-Fc fusion protein as an antagonist could improve the functional recovery significantly and promote axon regeneration effectively through upregulation of the expression of GAP-43 in spinal cord injured-rats.

#### RhoA Activity Assay

It has been confirmed that GTP-Rho levels are low in uninjured spinal cord homogenates and increase in tissue surrounding the site of transection after SCI, and the activation of RhoA inhibits axon regeneration after SCI [28]. Interestingly, they found that RhoA was active as early as 1.5 h after injury, the significant increase in activation observed by 24 h, peaked at 3 days, and was sustained for at least 7 days. In the present study, to determine whether p75NTR-ED-Fc inhibits

**Fig. 4** Electrophysiological recordings of motor and cortical somatosensory evoked potential



**Table 1** The latencies of SEPs (ms) after SCI

Group	Time after SCI			
	1 week	2 weeks	4 weeks	6 weeks
Sham	13.58±0.80	12.25±0.67	11.72±0.86	11.78±0.67
SCI	31.5±0.75	29.33±0.52	22.87±0.53	18.62±0.17
SCI+p75NTR-ED-Fc	30.47±0.95**	27.85±0.71**	20.99±0.56**	15.16±0.79**
Control	11.75±0.5	12.16±0.38	12.21±0.96	12.48±0.82

The data given are the mean±SEM of six separate experiments

SEP somatosensory evoked potential, SCI spinal cord injury

\*\* $P < 0.01$ , compared with SCI

**Table 2** The latencies of MEPs (ms) after SCI

Group	Time after SCI			
	1 week	2 weeks	4 weeks	6 weeks
Sham	13.25±0.5	13±0.5	12.89±0.64	12.92±0.90
SCI	32.5±0.87	32.4±0.62	31.37±0.88	26.25±0.7
SCI+p75NTR-ED-Fc	31.77±0.89*	30.97±0.85**	27.45±0.76**	21.31±0.68**
Control	12.66±0.52	12.33±0.38	12.70±0.77	13.11±0.84

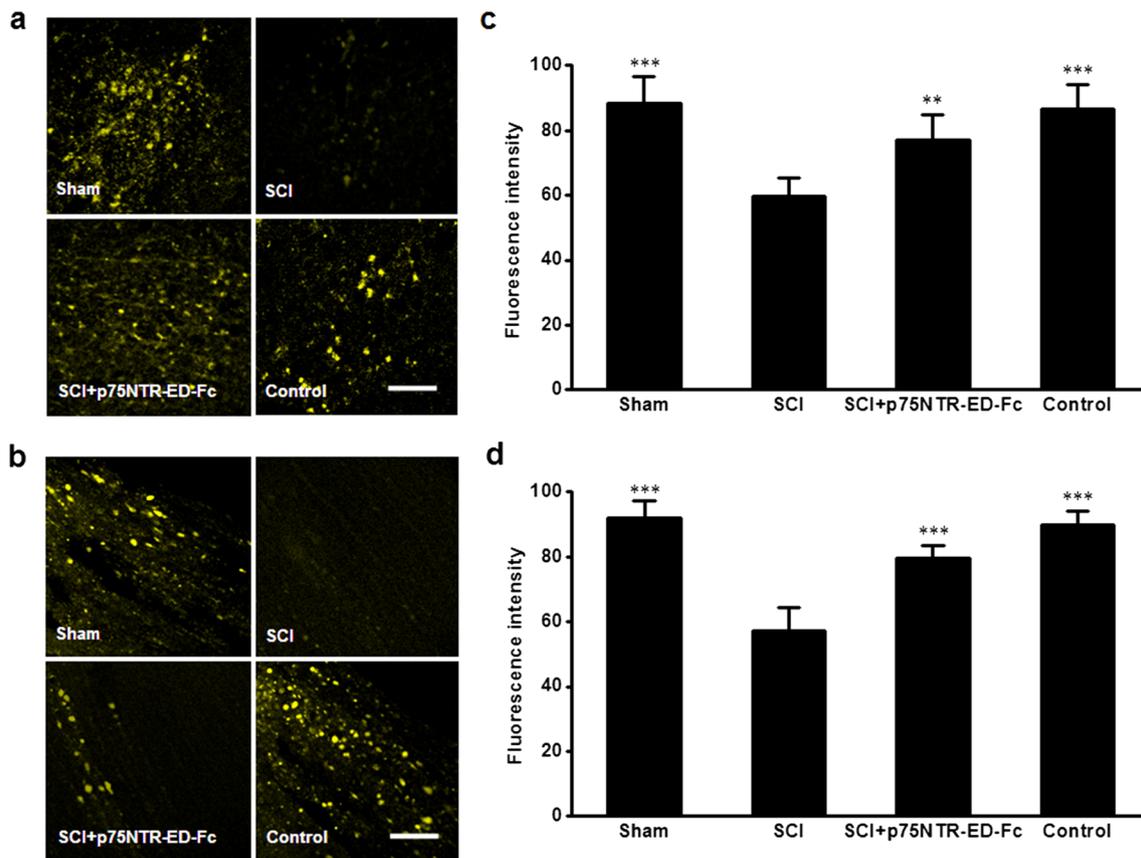
The data given are the mean±SEM of six separate experiments

MEP motor evoked potential, SCI spinal cord injury

\* $P<0.05$ , \*\* $P<0.01$ , compared with SCI

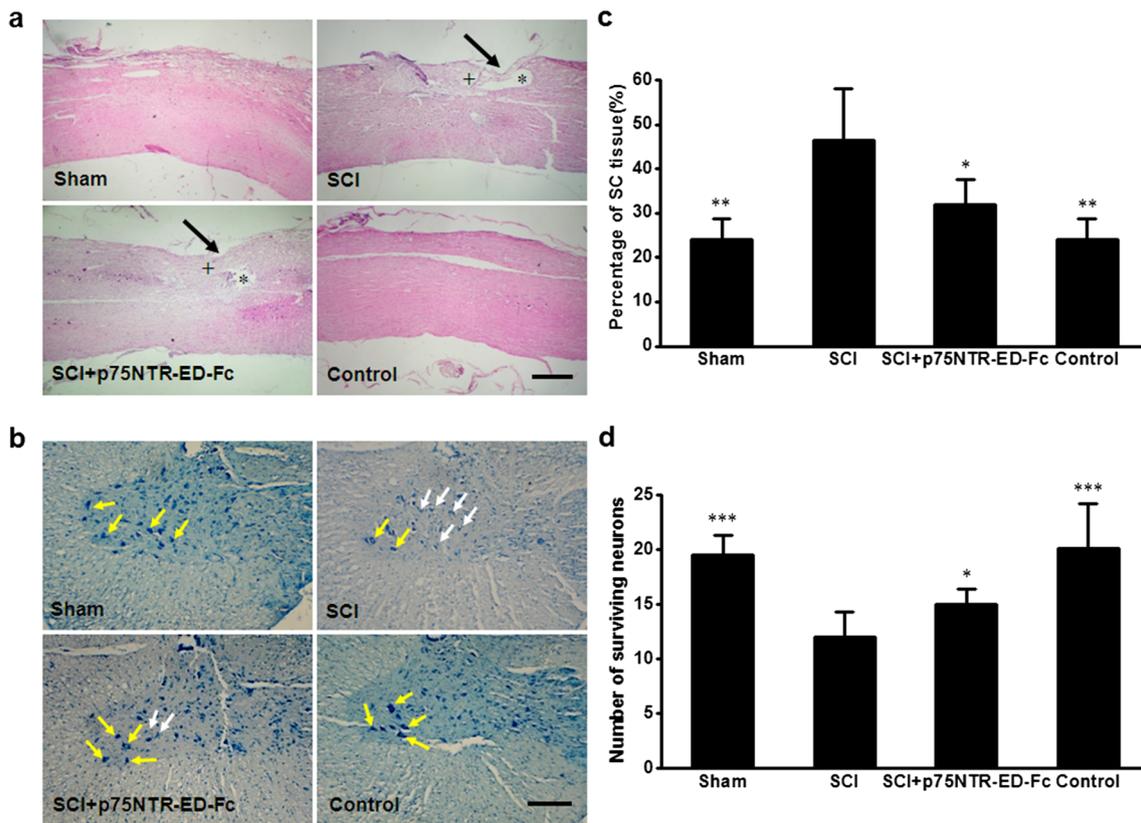
RhoA activation, and further elucidate the possible mechanism of action of p75NTR-ED-Fc on functional recovery and axonal regeneration after SCI, RhoA activity assay was detected in spinal cord tissue homogenates from sham, injured, p75NTR-ED-Fc-treated, and normal rats after injured 3 days. The results indicated that spinal cord tissue homogenates from p75NTR-ED-Fc-treated rats, as well as the normal and sham

rats, had significantly lower GTP-RhoA levels than those from spinal cord-injured rats measured 3 days after injury in dorsal hemisection models (Fig. 8a, b; two-tailed Student's *t* test,  $P<0.0001$ ). These results suggest that most of the RhoA activation was blocked by the antagonism of hp75NTR-ED-Fc protein in the spinal cord following injury, which resulted in upregulation of the expression of GAP-43, and



**Fig. 5** hp75NTR-ED-Fc promoted axon regeneration following SCI. **a** NY-labeled neurons of transverse sections of the spinal cords from the sham, normal control, SCI, and hp75NTR-ED-Fc-antagonistic rats at levels rostral to the lesion site. Scale bar represents 80  $\mu\text{m}$  and is valid for all pictures. **b** NY-labeled neurons of sagittal sections of the spinal cord from the sham, normal control, SCI, and hp75NTR-ED-Fc-antagonistic rats at levels rostral to the lesion site. Scale bar represents 150  $\mu\text{m}$

and is valid for all pictures. **c** Quantifications of fluorescence intensity in neurons labeled with NY in the sham, normal control, SCI, and hp75NTR-ED-Fc-antagonistic rats at transverse sections rostral to the lesion site. **d** Quantifications of fluorescence intensity in neurons labeled with NY in the sham, normal control, SCI, and hp75NTR-ED-Fc-antagonistic rats at sagittal sections rostral to the lesion site. \*\* $P<0.01$ , \*\*\* $P<0.0001$ , compared with the SCI



**Fig. 6** Histological analyses of spinal cord sections after SCI. **a** HE staining of the sections of spinal cord segment containing the rostrocaudal extension of the lesion cavity (*black arrows*). Trabeculated cysts (*asterisk*) in the left portion of the cord containing foamy macrophages; spared nervous tissue with microcysts (*plus sign*). *Scale bar* represents 1 mm and is valid for all pictures. **b** The transverse sections 3–6 mm rostral to the injury site were analyzed for residual ventral horn motoneurons by Nissl stain. In the hp75NTR-ED-Fc-treated group, the majority of neurons presented undisturbed morphology (*yellow arrows*). However,

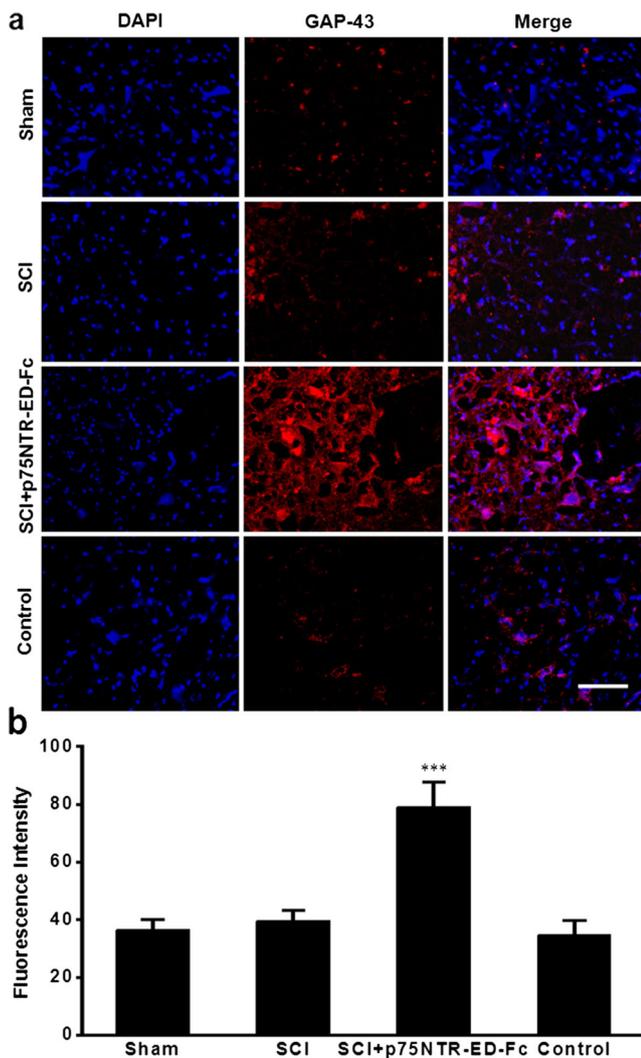
transverse sections taken from the SCI group were associated with the presence of ischemic neurons (*white arrows*) in ventral horn rostral to the lesion site. *Scale bar* represents 100  $\mu$ m and is valid for all pictures. **c** Quantifications for the percentage of spared spinal cord (SC) tissue in the sham, normal control, SCI, and hp75NTR-ED-Fc-treated groups by HE stain. **d** Quantifications for the number of surviving ventral horn neurons in spinal cord tissue of the sham, normal control, SCI, and hp75NTR-ED-Fc-treated groups by Nissl stain. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ , compared with the SCI (Color figure online)

improvement of the functional recovery significantly and promotion of axon regeneration effectively in spinal cord-injured rats.

## Discussion

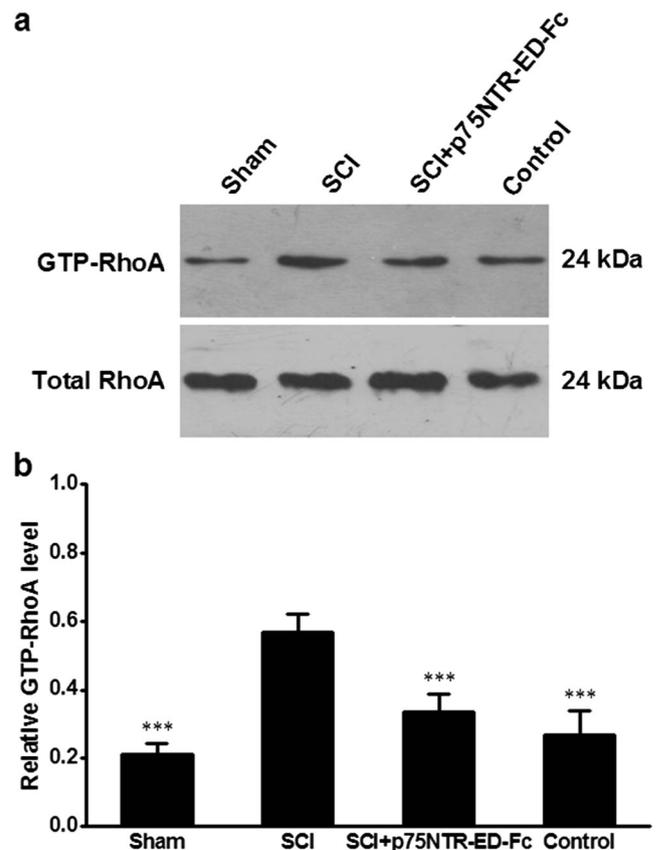
The function of p75NTR is extremely complex and difficult to dissect [13, 29]. As the common neurotrophin receptor, it has been shown to promote nerve regeneration or cell survival either in association with Trk receptors or by itself [30]. Paradoxically, its activation has also been shown to promote apoptotic cell death [31]. Besides these, p75NTR has also been shown to be a co-receptor to the NgR and PirB, thereby possibly playing a role in inhibition of axonal regeneration after spinal cord injury [9, 14]. Growing evidence has demonstrated that p75NTR could conjunct with the C-terminal of NgR via the extracellular domain (ED) and mediates the

inhibitory effects of myelin without being blocked by neurotrophic factors after CNS injury [9, 12], which indicate that p75NTR plays a more important role in the inhibitory effects of myelin in CNS compared with neurotrophic factors and their high-affinity TrkA receptor. The reason may be that the lack of expression of p75NTR in oligodendrocytes, where neurotrophic factors and TrkA receptor play a pivotal role in axonal regeneration following CNS injury, results in the diffusion of neurotrophins and the reduction of the neurotrophin gradient developed around oligodendrocyte, which could not attract and guide incoming axons to regenerate into injured distal central nerves, and decrease the axonal regeneration after CNS injury [13]. In other words, the p75NTR expressed in neurons mainly inhibits axonal regeneration as a co-receptor for NgR and PirB mediating the inhibitory signal of myelin-associated inhibitors on axonal regeneration after spinal cord injury. Therefore, antagonism of the function of p75NTR and the interaction between p75NTR and NgR or PirB may efficiently block the inhibitory signals from multiple



**Fig. 7** The expression of GAP-43 in the spinal cord injured-rats. **a** Sagittal sections of the spinal cord showed that the expression of GAP-43 in hp75NTR-ED-Fc-treated rats was much higher than that in the SCI rats killed at 6 weeks after SCI. The same expression was found in the sham and normal control compared with the SCI group. The cell nucleus was stained *blue* with DAPI, and immunoreactivity of GAP-43 was labeled with *red*. Scale bar represents 80  $\mu$ m and is valid for all pictures. **b** Quantifications of GAP-43 immunoreactivity in sham, normal control, SCI, and hp75NTR-ED-Fc-treated rats. \*\*\* $P < 0.0001$ , compared with the SCI (Color figure online)

myelin inhibitors without influencing the function and expression of neurotrophic factors and TrkA receptor on the surface of oligodendrocytes. It had been indicated that p75NTR antibody could block the inhibition of myelin through neutralizing the interaction between p75NTR and NgR [12]. Moreover, the interaction of p75NTR and Rho-GDI had been blocked with a peptide ligand associated with the fifth alpha helix of p75NTR (Pep5). Pep5 could promote neurite outgrowth through interfering with the interaction between p75NTR and Rho-GDI and preventing the activation of Rho [18]. It was also reported that the small interfering RNA (siRNA) had been used to suppress endogenous p75NTR protein levels by triggering



**Fig. 8** p75NTR-ED-Fc reduces RhoA activation in injured spinal cord tissue. **a** Active GTP-RhoA was isolated from tissue homogenates of injured spinal cord 3 days after transection by pull-down assay and detected by Western blot analysis with RhoA-specific monoclonal antibody. Total RhoA in the tissue homogenates from the same animals was also detected and used as a control for the cross-comparison of Rho activity. **b** Total RhoA and GTP-RhoA levels were quantified by densitometry and the Rho-GTP level was normalized to total RhoA bands. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with the SCI

posttranscriptional gene silencing [15]. RNA interference (RNAi) was achieved by using siRNAs to silence gene expression in a sequence-specific manner. Therefore, p75NTR interfered by siRNA may provide a more accurate method to study p75NTR function than traditional studies of knockout mice where there are likely differences in gene expression that modify the cellular context [32]. However, it is shown that gene knockouts often result in inferior regeneration compared to treatment with neutralizing agents [19, 20, 33–37].

The aim of this study was to design an hp75NTR-ED-Fc fusion protein used as an antagonist through combining with the C-terminal of NgR and PirB via the extracellular domain (ED) and neutralize the inhibitory effects of endogenous myelin and its receptors. For this purpose, the present study first designed and prepared the hp75NTR-ED-Fc fusion protein, and then the fusion protein was monitored by using SDS-PAGE. Moreover, the result of Western blot also demonstrates that the fusion protein can be specifically recognized by rabbit anti-NGFRp75 polyclonal antibody, which further indicates

that the fusion protein is the interest protein hp75NTR-ED-Fc indeed. The conjunction of the fusion protein is between the hinge region of p75NTR extracellular domain and IgG-Fc. As a result, it could enhance the stability and extend the half-life in the body without influencing its activity and the correct folding of either part of the fusion protein. In order to investigate the function of hp75NTR-ED-Fc infusion protein, after the recombinant protein purified with His-tag affinity chromatograph, the effect of the recombinant protein on DRG neuron neuritis was further investigated. The results showed that the infusion protein could markedly promote neurite outgrowth of DRG neurons when cultured with MAG, one of main myelin inhibitors.

To evaluate the efficacy of hp75NTR-ED-Fc fusion protein on functional recovery after SCI, a dorsal hemisection model was used for studying axon regeneration, tissue sparing, and functional recovery *in vivo*. GAP-43 immunohistochemical staining and NY tracing in the dorsal hemisection model showed that, in most of the hp75NTR-ED-Fc-treated rats after SCI, injured CST axons could grow beyond the lesion site into the rostral spinal cord as compared to the lack of such growth in the SCI rats. This was likely a result of the antagonism target on p75NTR that reversed some inhibitory activities of the myelin inhibitors such as NgR and PirB and consequently inhibited the activation of RhoA in the following signal transduction pathway after SCI, which resulted in promotion of axonal regeneration of the CST. The following experiments of the present study well confirmed the above supposition, and the activation of RhoA was significantly decreased in p75NTR-ED-Fc-treated rats compared with spinal cord-injured rats. In addition to inducing axonal regeneration, the fusion protein may also have an effect on tissue protection and functional recovery through some multifarious mechanisms [14]. In the present study, antagonism of hp75NTR-ED-Fc fusion protein after SCI induced significantly better morphological and functional recovery compared with spinal cord-injured rats. These findings suggest that, in addition to stimulating axon regeneration, the fusion protein may also have some neuroprotective effect which may contribute to the overall functional recovery after SCI.

Besides inhibition of axonal regeneration as common signal pathway of myelin inhibitors, RhoA also can regulate apoptotic cell death pathways [28, 38, 39]. Overexpression of RhoA can induce apoptosis *in vitro* after serum deprivation [38, 39]. Caspase-3 is required and activation of the JNK pathway can also occur in response to Rho activation for this process [38, 40, 41]. Inactivation of RhoA *in vivo* by treatment with the RhoA antagonist C3-05 protects neurons and oligodendrocytes from apoptotic cell death after SCI and results in improvement of functional recovery that was attributed in part to the neuroprotective effect of the RhoA antagonist [28]. It also have been demonstrated that LINGO-1-Fc, another co-receptor of NgR in signal pathway of myelin

inhibitors, may protect neurons from apoptotic death possibly through reducing the activation of RhoA, caspase-3, and JNK phosphorylation in LINGO-1-Fc-treated spinal cord [26]. Based on the above research and present study, the neuroprotective effect of p75NTR-ED-Fc fusion protein in spinal cord-injured rats may also result from inactivation of RhoA, may be related to caspase-3 activation and JNK phosphorylation, and the mechanism underlying such neuroprotection remains to be further investigated.

Recently, a p75NTR homolog, TAJ/TROY, has been implicated in the same role as p75NTR in the Nogo/myelin inhibition of axonal regeneration [42, 43]. It is possible that this protein may play a similar role as p75NTR in functional recovery after SCI. However, little is known regarding Troy's specific role in axon regeneration relative to p75NTR. As we all know, p75NTR can regulate neuronal survival and axonal regeneration upon binding to neurotrophins, while Troy does not and its ligands remain undiscovered [43]. It is not known if Troy, like p75NTR, inhibits axon regeneration through PirB or RhoA-GDI binding [14, 18], nor if its activation requires proteolysis by  $\alpha$ - and  $\gamma$ -secretases. Much remains to be done to understand Troy's specific role in axon regeneration, and the potentially diverse CNS biology that it may contribute to. Troy's potential to interact with diverse proteins and its varied cell type expression pattern indicates a context-dependent role that has yet to be elaborated [44]. As this result, it is significance to treat the SCI on the target of p75NTR.

These results seem to conflict with the previous study of Song et al. [45], which had concluded that suppression of p75NTR may not promote regeneration of injured spinal cord in mice 2 weeks after SCI by using p75NTR-deficient mice and local administration of p75NTR-Fc fusion molecule. However, because of some unknown mechanisms, it is shown that gene knockouts often result in inferior regeneration compared to treatment with neutralizing agents [19, 20, 33–37], and there are also some crucial differences between their study and ours. In the present study, we used a hemisection spinal cord injury model of rats and observed the kinetics of the effects of the hp75NTR-ED-Fc on the spinal cord-injured rats until 6 weeks after SCI. The results of our study indicated that hp75NTR-ED-Fc, as an antagonist for p75NTR, might block the interaction of exogenous p75NTR and NgR or PirB, result in inhibiting the activation of RhoA during early injury of spinal cord, promote nerve regeneration, and improve functional recovery at least until 6 weeks after SCI; the therapeutic efficacy might be long-termed, and set on about 2 weeks after SCI. On the other hand, short-term efficacy of p75NTR-Fc was observed in the study of Song et al. only 2 weeks after SCI, and the therapeutic efficacy might not be showed at this time [45]. The results of our study are in concordance with those previously reported by Ji et al. [26], which demonstrate that treatment with LINGO-1-Fc, another co-receptor of NgR,

promotes the recovery of function at 3 and 4 weeks after spinal cord injury.

## Conclusions

The results of the current study indicated that antagonist on the target of p75NTR can be used therapeutically to treat SCI in adult rats. The recombinant hp75NTR-ED-Fc fusion protein treatment enhances functional recovery by limiting tissue loss and stimulating axonal growth in spinal cord-injured rats, which may result from decreasing the activation of RhoA. Therefore, our present study may further confirm a strong rationale that p75NTR is still a potential and novel target for therapeutic intervention after SCI, and provide a new therapeutic strategy or pharmacal design of neurodegenerative disease and neurotrauma on the target of p75NTR.

**Acknowledgments** This study was supported by the National Natural Science Foundation of China (81372040 and 81101464), the Independence Project Foundation of State Key Laboratory of Trauma, Burns and Combined Injury (SKLZZ201215), and the Natural Science Foundation Project of CQ CSTC (CSTC, 2008BB5107). We appreciate technical assistance and helpful comments provided by Prof. X.-F. Zhou and Dr. H.-Y.Li.

## References

- Oertle T, Schwab ME (2003) Nogo and its partners. *Trends Cell Biol* 13:187–194
- Spencer T, Domeniconi M, Cao Z, Filbin MT (2003) New roles for old proteins in adult CNS axonal regeneration. *Curr Opin Neurobiol* 13:133–139
- Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* 417:941–944
- Fournier AE, GrandPre T, Strittmatter SM (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* 409:341–346
- Gonzenbach RR, Schwab ME (2008) Disinhibition of neurite growth to repair the injured adult CNS: Focusing on Nogo. *Cell Mol Life Sci* 65:161–176
- Atwal JK, Pinkston-Gosse J, Syken J, Stawicki S, Wu Y, Shatz C, Tessier-Lavigne M (2008) PirB is a functional receptor for myelin inhibitors of axonal regeneration. *Science* 322:967–970
- Filbin MT (2008) PirB, a second receptor for the myelin inhibitors of axonal regeneration Nogo66, MAG, and OMgp: Implications for regeneration in vivo. *Neuron* 60:740–742
- Matsushita H, Endo S, Kobayashi E, Sakamoto Y, Kobayashi K, Kitaguchi K, Kuroki K, Söderhäll A, Maenaka K, Nakamura A, Strittmatter SM, Takai T (2011) Differential but competitive binding of Nogo protein and class I major histocompatibility complex (MHC) to the PIR-B ectodomain provides an inhibition of cells. *J Biol Chem* 286:25739–25747
- Wang KC, Kim JA, Sivasankaran R, Segal R, He Z (2002) p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* 420:74–78
- McGee AW, Strittmatter SM (2003) The Nogo-66 receptor: focusing myelin inhibition of axon regeneration. *Trends Neurosci* 26:193–198
- Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB (2004) LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat Neurosci* 7:221–228
- Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, Poo MM (2002) A p75NTR and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. *Nat Neurosci* 5:1302–1308
- Zhou XF, Li HY (2007) Roles of glial p75NTR in axonal regeneration. *J Neurosci Res* 85:1601–1605
- Fujita Y, Takashima R, Endo S, Takai T, Yamashita T (2011) The p75 receptor mediates axon growth inhibition through an association with PIR-B. *Cell Death Dis* 2:e198–e204
- Yamashita T, Higuchi H, Tohyama M (2002) The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. *J Cell Biol* 157:565–570
- Higuchi H, Yamashita T, Yoshikawa H, Tohyama M (2003) Functional inhibition of the p75 receptor using a small interfering RNA. *Biochem Biophys Res Commun* 301:804–809
- Blöchl A, Blöchl R (2007) A cell-biological model of p75NTR signaling. *J Neurochem* 102:289–305
- Yamashita T, Tohyama M (2003) The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat Neurosci* 6:461–467
- Ji B, Case LC, Liu K, Shao Z, Lee X, Yang Z, Wang J, Tian T, Shulga-Morskaya S, Scott M, He Z, Relton JK, Mi S (2008) Assessment of functional recovery and axonal sprouting in oligodendrocyte-myelin glycoprotein (OMgp) null mice after spinal cord injury. *Mol Cell Neurosci* 39:258–267
- Nakamura Y, Fujita Y, Ueno M, Takai T, Yamashita T (2011) Paired immunoglobulin-like receptor B knockout does not enhance axonal regeneration or locomotor recovery after spinal cord injury. *J Biol Chem* 286:1876–1883
- Wang YT, Lu XM, Zhu F, Huang P, Yu Y, Zeng L, Long ZY, Wu YM (2011) The use of a gold nanoparticle-based adjuvant to improve the therapeutic efficacy of hNgR-Fc protein immunization in spinal cord-injured rats. *Biomaterials* 32:7988–7998
- Basso DM, Beattie MS, Bresnahan JC (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 12:1–21
- Stirling DP, Khodarahmi K, Liu J, McPhail LT, McBride CB, Steeves JD, Ramer MS, Tetzlaff W (2004) Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. *J Neurosci* 24:2182–2190
- Decherchi P, Gauthier P (2000) Regrowth of acute and chronic injured spinal pathways within supra-lesional post-traumatic nerve grafts. *Neuroscience* 101:197–210
- Teng YD, Mocchetti I, Wrathall JR (1998) Basic and acidic fibroblast growth factors protect spinal motor neurons in vivo after experimental spinal cord injury. *Eur J Neurosci* 10:798–802
- Ji B, Li M, Wu WT, Yick LW, Lee X, Shao Z, Wang J, So KF, McCoy JM, Pepinsky RB, Mi S, Relton JK (2006) LINGO-1 antagonist promotes functional recovery and axonal sprouting after spinal cord injury. *Mol Cell Neurosci* 33:311–320
- Xie X, Peng J, Chang X, Huang K, Huang J, Wang S, Shen X, Liu P, Huang H (2013) Activation of RhoA/ROCK regulates NF- $\kappa$ B signaling pathway in experimental diabetic nephropathy. *Mol Cell Endocrinol* 369:86–97
- Dubreuil CI, Winton MJ, McKerracher L (2003) Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. *J Cell Biol* 162:233–243

29. Chu GKT, Yu W, Fehlings MG (2007) The p75 neurotrophin receptor is essential for neuronal cell survival and improvement of functional recovery after spinal cord injury. *Neuroscience* 148:668–682
30. Barker PA (1998) p75NTR: a study in contrasts. *Cell Death Differ* 5: 346–356
31. Roux PP, Barker PA (2002) Neurotrophin signaling through the p75 neurotrophin receptor. *Prog Neurobiol* 67:203–233
32. Gino BF, Yazan ZA, Alyson EF (2004) Molecular targets to promote central nervous system regeneration. *Curr Neurovasc Res* 1:61–75
33. Zheng B, Ho C, Li S, Keirstead H, Steward O, Tessier-Lavigne M (2003) Lack of enhanced spinal regeneration in Nogo-deficient mice. *Neuron* 38:213–224
34. Teng FYH, Tang BL (2005) Why do Nogo/Nogo-66 receptor gene knockouts result in inferior regeneration compared to treatment with neutralizing agents? *J Neurochem* 94:865–874
35. Zheng B, Atwal J, Ho C, Case L, He XL, Garcia KC, Steward O, Tessier-Lavigne N (2005) Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. *Proc Natl Acad Sci U S A* 102:1205–1210
36. Lee JK, Geoffroy CG, Chan AF, Tolentino KE, Crawford MJ, Leal MA, Kang B, Zheng B (2010) Assessing spinal axon regeneration and sprouting in Nogo, MAG and OMgp deficient mice. *Neuron* 66: 663–670
37. Omoto S, Ueno M, Mochio S, Takai T, Yamashita T (2010) Genetic deletion of Paired immunoglobulin-like receptor B does not promote axonal plasticity or functional recovery after traumatic brain injury. *J Neurol Sci* 30:13045–13052
38. Jimenez B, Arends M, Esteve P, Perona R, Sanchez R, Ramony Cajal S, Wyllie A, Lacal JC (1995) Induction of apoptosis in NIH3T3 cells after serum deprivation by over expression of Rho-p21, a GTPase protein of the ras superfamily. *Oncogene* 10:811–816
39. Aznar S, Lacal JC (2001) Rho signals to cell growth and apoptosis. *Cancer Lett* 165:1–10
40. Marinissen MJ, Chiariello M, Tanos T, Bernard O, Narumiya S, Gutkind JS (2004) The small GTP-binding protein RhoA regulates c-jun by a ROCK-JNK signaling axis. *Mol Cell* 14: 29–41
41. Zhan H, Sun SJ, Cai J, Li YQ, Hu CL, Lee DH, So KF, Li X (2013) The effect of an NgR1 antagonist on the neuroprotection of cortical axons after cortical infarction in rats. *Neurochem Res* 38:1333–1340
42. Park JB, Yiu G, Kaneko S, Wang J, Chang J, He XL, Garcia KC, He Z (2005) ATNF receptor family member, TROY, is a coreceptor with Nogo receptor in mediating the inhibitory activity of myelin inhibitors. *Neuron* 45:345–351
43. Shao Z, Browning JL, Lee X, Scott ML, Shulga-Morskaya S, Allaire N, Thill G, Levesque M, Sah D, McCoy JM, Murray B, Jung V, Pepinsky RB, Mi S (2005) TAJ/TROY, an orphan TNF receptor family member, binds Nogo-66 receptor 1 and regulates axonal regeneration. *Neuron* 45:353–359
44. Mi S (2008) Troy/Taj and its role in CNS axon regeneration. *Cytokine Growth Factor Rev* 19:245–251
45. Song XY, Zhong JH, Wang X, Zhou XF (2004) Suppression of p75NTR does not promote regeneration of injured spinal cord in mice. *J Neurol Sci* 24:542–546