MST2 Acts via AKT Activity to Promote Neuronal Axon Regeneration and Functional Recovery after Spinal Cord Injury in Mice

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Research Article

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Abstract

Background

Mammalian sterile 20-like kinase 2 (MST2), a core component of the Hippo signaling pathway, plays an crucial role in apoptosis and cell growth. However, its role in axon regeneration after spinal cord injury (SCI) was first elucidated in this study.

Methods

We first screened the proteins involved in spinal cord contusion injury using a mouse model utilizing 4D label-free proteomic analysis. We selected the neuronal axon regeneration-associated protein MST2 as our target protein. In vitro and in vivo experiments were applied to explore the function of MST2. We then performed additional experiments, such as co-immunoprecipitation and so on to confirm the downstream molecular mechanisms of MST2.

Results

We confirmed that MST2, mainly expressed in neurons, promoted axon regeneration by positively regulating AKT activity in neuronal cell models and primary cortical neurons. Mechanistically, our studies found that MST2 protein could interact both with AKT and p-AKT in vitro. In vivo, MST2 knockdown inhibits axon regeneration and motor functional recovery, while lentiviral-mediated overexpression of MST2 promoted axon regeneration and motor functional recovery after SCI. However, MST2 induced axon regeneration and motor functional recovery were reversed by AKT inhibitors.

Conclusion

This study provided evidence that how MST2 acts as a new regulator that controls axon regeneration after SCI, MST2 promotes neuronal axon regeneration by positively regulating AKT activity, indicating MST2 may be a target with great therapeutic potential for SCI.

Background

Spinal cord injury (SCI) is a devastating neurological condition that causes physical dependency, morbidity, psychological stress, and financial strain [1]. Many factors lead to the central nervous system (CNS) axon regeneration failure, possibly due to diminished intrinsic capacity and an external inhibitory environment [2, 3]. External factors have been intensively studied, such as glial scarring, inflammation, and myelin-associated protein, aiming to develop axon regeneration strategies by eliminating inhibitory influences, but their efficacy is limited. Thus, only modifying the inhibitory environment is not sufficient to obtain robust axon regeneration [3, 4]. Several cell autonomous molecules, including Nogo [5], Krüppel-like factors (KLFs) [6], mammalian rapamycin target (mTOR) [2], phosphatase and tensin homolog (PTEN) [2, 7, 8], play a significant role in axon regeneration. Intrinsic mechanisms also play essential roles in regulating axonal regeneration, especially in the first two weeks of the glial scar maturation [9]. Therefore, it is important to find novel and necessary regulators that effectively promote the regeneration of neuronal axons.
The Hippo pathway is a conserved evolutionary signaling pathway. The phosphorylation of MST1/2 activates its downstream LATS1/2, then LATS1/2 phosphorylates downstream YAP or TAZ, which could regulate cell proliferation, differentiation, and survival in the neuronal system [10–13]. In the brain, highly expressed MST1/2, SAV1, LATS1/2, and TAZ are in major cell types, however, highly expressed YAP is only in astrocytes and endothelial cells [14]. Subsequently, these diverse expression patterns of components of the Hippo signaling pathway suggest their diverse roles among the different cell types in the brain. However, few components of the Hippo pathway regulate neuronal axon regeneration. For example, NDR1/2 knockdown or mutants promoted dendrite length and proximal branching of mammalian pyramidal neurons in vitro and in vivo [15]. In a zebrafish SCI model, ctgfa, a target of YAP, promoted glial bridging and axonal regeneration in the injured area [16]. Thus, it is important to clarify that the role and regulatory mechanism of Hippo signaling in neuronal axon regeneration.

Mammalian sterile 20-like kinase 2 (MST2) is a multifunctional serine/threonine kinase (also called STK3) that plays a central role in the Hippo pathway. MST2 regulates the proliferation, differentiation, and apoptosis of tumor growth and the immune system [17–20]. MST2 has important roles in the neuronal system, such as neuronal apoptotic death [21], the aging process [22], huntingtin disease [23], and retinal detachment (RD) [24]. MST2 regulates microtubule stability/dynamics in a noncanonical way. For example, the deficiency of MST2 or NDR1 destabilizes microtubule attachment during mitosis [25]. In mammalian cells, the basal body and the centrosome share the centriole as a microtubule organizing center, which is mainly for generating cilia. Moreover, the MST2-SAV1 complex promotes ciliogenesis and establishes a polarized cell structure [26]. These results suggest that MST2 was involved in microtubule stability. Additionally, MST2 was activated in mitosis, catalyzing the mitotic phosphorylation of MOB 1 A/B, which is required for mitotic nuclear Dbf2-related 1 (NDR1) kinase activation and chromosome alignment [25]. Our previous study confirmed that MOB1, a downstream substrate for MST2, promoted axon regeneration and functional recovery after SCI [27]. Given these results and the fact that MOB1 is a physiological substrate of MST2, we sought to determine whether MST2 regulates axon regeneration. Our previous proteomic analysis indicated that MST2 can positively modulate neuronal axon regeneration and the AKT (protein kinase B) signal after SCI. Herein, we elucidate that the roles and mechanisms of MST2 in promoting axon regeneration and functional recovery after SCI.

Phosphatidylinositol 3-kinase (PI3K)/AKT is the most representative signaling pathways that promotes axon regeneration [28], and AKT is the main effector [29]. The classical activation pathway of AKT is PI3K phosphorylation to activate phosphoinositol (3,4,5)-triphosphate (PIP3), while PTEN antagonizes the response by dephosphorylation. AKT relies on PIP3 localization to the cytoplasmic membrane and is activated to act on classical downstream substrates such as mTOR and GSK3β to promote axon regeneration. Theoretically, axonal regeneration could be promoted as long as it activates AKT activity. Therefore, we speculate that MST2 may promote axonal regeneration after SCI by positively regulating AKT activity.

Utilizing 4D label-free proteomic-based analysis, the study aimed to screen the proteins that differentially express in the lesion center of injured spinal cord compared to the normal at day seven post-SCI. Through screening, we identified MST2 as a new molecule that promotes neuronal axon regeneration and function.
recovery after SCI in mice. Additionally, the experiments have investigated the underlying molecular mechanism in vitro and in vivo.

Materials And Methods

Animals

C57BL/6 female mice were provided by Soochow University Animal Experimental Center. All mice maintained in SPF level room with temperature 21 ± 2 °C, humidity 30%–35%, 12/12-h dark/light cycle, and free access to food and water. All experimental procedures followed the guidelines of animal research of the Science and technology department of China and were approved by the ethics committee of animal research Soochow University.

Model of SCI and tissue preparation

Surgical procedures for SCI model were performed as previous report [30]. C57BL/6 female mice were anesthetized by intraperitoneal injection of 3% pentobarbital. A laminectomy was performed at T9-T11 levels to expose the T10 thoracic spinal cord. The spinal cord was contused with a Multicenter Animal Spinal Cord Injury Study (MASCIS) Impactor weight-drop device. A 5-g weight impact rod dropped from 25 mm in height to produce a reliable contused SCI model (impacting force: 125 kdyn). In contrast, mice in the sham group were only performed laminectomy. After the surgery, all mice were individually housed. An intramuscular injection of antibiotics (20,000 units penicillin) was provided for three consecutive days. Massage the bladder of mice in the SCI group to help void twice a day until spontaneous voiding resumed.

Mice were sacrificed at different time points for the specific experiments. Mice were anesthetized and transcardially perfused with cold phosphate-buffered saline (PBS). For proteomic analysis (n = 3/time point) and Western blot assays (n = 3/time point), the spinal cord tissues (a 10 mm length of spinal cord tissue, including the center of SCI) were removed and stored at −80 °C for processing. To perform H&E and fluorescent staining (n = 5/time point), the animals were perfused with PBS followed by 4% ice-cold paraformaldehyde. The spinal cord tissue samples were dissected and post-fixed with paraformaldehyde overnight for subsequent experiments.

Mass spectrometry (MS) analysis

Briefly, total protein was extracted from spinal cord tissue, followed by filter-aided sample preparation (FASP Digestion) [31]. Then, the above sample was loaded onto the C18-reversed phase analytical column (Thermo Fisher Scientific, Acclaim PepMap RSLC 50um X 15cm, nano viper, P/N164943) at a flow rate of 300 nL/min. LC-MS/MS analysis was performed using an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific) coupled with Easy nLC (Thermo Scientific). Finally, MS data were analyzed using MaxQuant software v1.6.17.0.

Cell culture
HT22 and Neuro2A cell lines were purchased from the type culture collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (Hyclone, Beijing, China), containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Thermo Scientific) at 37 °C with 5% CO2.

Primary cortical neurons were isolated from embryonic stage 15 (E15) mice and cultured. Briefly, the cerebral cortex was separated and then digested with type I collagenase (Gibco) and Accutase (Sigma, St. Louis, MO, USA) for 30 min at 37 °C in sequence, and gently agitated by every 5 min. After digestion inactivated with 10% FBS, and filtrated through a 70 µm pore-sized mesh, centrifuged at 1,000 rpm for 5 min at 4 °C, and cell precipitate was collected. Neurons were then plated onto Poly-D-Lysine (Gibco) coated dishes in Neurobasal medium (Gibco) supplemented with 2% B27 supplements (Gibco), 0.5 mM L-glutamine (Sigma) and 1% penicillin/streptomycin (Thermo Scientific) at a density of 300 neurons/mm². Neuronal cultures were incubated at 37 °C with 5% CO2. The neurons were used for subsequent experiments after 5–7 days.

**Lentivirus infection and production of stable cell lines**

The lentivirus infection was manipulated by referring to instructions. All cells were in good condition. HT22 and Neuro2A were seeded in six-well plates for 12–16 h to be 20%–30% confluence. Neurons cultured for 3–5 days in vitro were infected with lentivirus. Then, the shRNA lentivirus, the control lentivirus, and the overexpression lentivirus (GeneChem) were added to the plates based on cell MOI and virus titer. Fresh medium was replaced after 16 h, and culture was continued. After 48–72 h of viral infection, the cells, except primary cortical neurons, were treated with a medium containing puromycin (1 µg/ml, Sigma-Aldrich) to remove noninfected cells. Subsequently, the medium was replaced with fresh medium, and cells were evaluated under a microscope within 3–5 days. The stable cells were used for further experiments.

**Real-time PCR**

*Total RNA was first extracted with Trizol* (Thermo Scientific) and then converted to cDNA using a RevertAid cDNA Synthesis Kit (Thermo Scientific). Real-time PCR was performed three times in 20 µL reactions using iQ SYBR Premix Ex Taq Perfect Real Time (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 50 ng of first-strand cDNA, and 0.2 mg of each primer. The cycle threshold (CT) method was used to calculate relative expression levels. Relative mRNA levels were determined by the $2^{-\Delta\Delta CT}$ method using GAPDH as endogenous control. For RT-PCR, the primers were as follows:

**Mus-MST2**
forward primer: 5'-CCAGGCCCTATGTCCAACAG-3', reverse primer: 5'-GGTCTAGTGCTTTTAGCCGC-3';

**Mus-NF200**
forward primer: 5'-GCAGCCAAAGTGAACACAGAT-3', reverse primer: 5'-TCCCACTTGGTGTTTCTCAGC-3';

**Mus-β-tubulin**
forward primer: 5'-CACCTCCCCATTCCCTG-3', reverse primer: 5'-CATCGAACATCTGCTGCGT-3'.

For internal control, we used GAPDH primers as follows:
Mus-GAPDH forward primer: 5'-ATCACTGCCACCCAGAAGAC-3', reverse primer: 5'-ATCCACGACGGACACATTGG-3' (Sangon Biotech, Shanghai, China).

**Immunoprecipitation and western blot analysis**

Western blot analysis was performed as previously reported[32]. Briefly, protein from spinal cord tissues and cells was extracted using RIPA lysis buffer containing protease inhibitor cocktail at 4 °C for 10 min and centrifuged at 4 °C and 12,000 rpm for 15 min. After that, equivalent protein samples were separated using SDS-PAGE and transferred to PVDF membrane (Millipore, Burlington, MA, USA). Afterward, the membrane was blocked with 5% BSA for 1 h and then incubated overnight at 4 °C with primary antibodies, including β3-tubulin (#5568; CST, Danvers, MA, USA), GAPDH (#2118; CST), p-AKT (S473) (#4060; CST), AKT (#4691; CST), MST2 (#ab52641; Abcam, Cambridge, UK), Flag (#AE005, ABclonal, Wuhan, China), Flag (#AE092; ABclonal), MST2 (#A9036; ABclonal), NF200 (#DF6060; Affinity Biosciences, Cincinnati, OH, USA). On the next day, PVDF membranes were incubated with the enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific) to visualize protein bands using Tanon-5500 (Tanon Science & Technology, Shanghai, China), which were then quantified using densitometric analysis of ImageJ v1.57[33].

For immunoprecipitation, cells were lysed in IP buffer (Thermo Scientific) and centrifuged at 14,000 rpm for 15 min at 4 °C. Only 5% of the lysate was saved as input control. The remaining lysate was mixed with antibodies at 4 °C overnight and then incubated with protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, USA) at 4 °C for an additional 4 h. After being washed with IP buffer, the precipitates were analyzed using western blotting. Light chain-specific secondary antibody was used to eliminate heavy chain interference.

**Immunofluorescence(IF) on neurons**

The neurons were seeded in the 24-well plate and washed with cold PBS 72 h after transfection. Neurons were fixed in −20 °C methanol for 15 min, washed with PBS supplemented with Tween 80 (PBST), and permeabilized with 0.3% Triton X-100 for 10 min. The neurons were then incubated overnight with primary antibodies at 4 °C. The next day, cells were incubated with secondary antibodies at room temperature for 1 h, and the nuclei were stained with DAPI for 5–10 min. The images were sequentially photographed using a fluorescent microscope (Nikon, Tokyo, Japan). For quantitative morphological evaluation, the Image J software v1.57 was used.

**Lentiviral vector injection after SCI**

Immediately following SCI, lentiviral vectors were injected into two points at a distance of 1.5 mm rostral and caudal to the center of the lesion site with a depth of 1.0 mm. 5 μL of lentiviral solution (1×10⁸TU/mL) were injected at a flow rate of 50 μL/min for each site through a microinjection needle, which connected to a Hamilton syringe driven by a microinjection pump. After injection, the microinjection needle was left for 2 min and then withdrawn slowly from the injection site. Muscles and skin were then sutured.

**Hematoxylin and eosin(HE) staining**
After deparaffinization and rehydration, spinal cord tissue sections were stained in hematoxylin solution (beyotime, China) for 5 min and eosin solution (beyotime, China) for 2 min, followed by dehydration with graded alcohol and clearing in xylene. The slides were mounted, examined, and photographed using a microscope (Nikon, Tokyo, Japan).

**Immunofluorescence on tissue**

IF staining was conducted as previously described [27]. Briefly, the spinal cord was collected, and 20-µm-thick frozen sections were prepared. The samples were then incubated overnight at 4 °C with primary antibodies. Subsequently, sections were incubated at 37 °C for 1 h with secondary antibodies labeled with Cy3/FITC. The slides were then washed and stained with DAPI, and coverslipped using Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). All tissue samples were processed under the same conditions and observed using a fluorescent microscope (Nikon, Tokyo, Japan). Morphological evaluation was quantified using the ImageJ software v1.57 [33].

**Basso mouse scale (BMS) and footprint test**

Recovery of motor function was assessed using the BMS for locomotion [34]. All mice were observed for 5 min by two observers in a blinded manner. Scoring was based on different parameters such as ankle movements, paw placement, stepping pattern, coordination, trunk instability, and tail position. Mice were tested before injury (day -1) and on days 1, 3, 7, 14, 28, and 42 after injury. Mice with a BMS score of > 3 (some stepping) at one day after injury were excluded from the analysis. For the footprint test, the hind paws with non-toxic dyes were recorded during continuous locomotion on a 50-cm runway. Stride length width between two sides of the prints were measured and calculated from multiple steps.

**Radiographic assessment**

Magnetic resonance imaging (MRI) scans of mice were performed 7 days and 42 days after SCI to assess the integrity of spinal cord. Briefly, mice were anesthetized as described above and placed in an MR scanner (Supernova 1.5T, QuantumTec Medical Devices Limited, Beijing, China) with a whole-body coil to obtain MR images of the spinal cord.

**Statistical analysis**

All results are expressed as mean value ± SD of three independent experiments. Differences between experimental groups were evaluated using two-tailed unpaired Student’s t-test or one-way ANOVA followed by post hoc test analysis. Two-way repeated-measures ANOVA was used to compare time course data of groups, and post hoc Bonferroni test was used to compare means at each time point unless otherwise stated. Statistical analyses were performed using GraphPad Prism software, and P < 0.05 was considered statistically significant.

**Results**
Proteomic analysis for the identification of MST2 (STK3) and expression of MST2 in the spinal cord tissues of mice

To find a new molecule for axon regeneration, spinal cord tissue samples were collected from uninjured and injured mice seven days after surgery. Then, LC-MS/MS analysis was used via a 4D label-free strategy to obtain their proteome profiles. Fig. 1a shows the experimental flowchart. Proteins that met the following screening criteria were considered differently expressed: expression fold change > 1.5 (up or downregulation) and P-value < 0.05. A total of 609 proteins were successfully identified as differentially expressed proteins (DEPs); of them, 337 were up-regulated, and 272 were down-regulated in the SCI versus NC group and were identified as DEPs. Fig. 1b shows the heatmap, and Fig. 1c illustrates the volcano plot.

Subsequently, the data were further analyzed based on the gene ontology (GO) term to ascertain which proteins affect axon regeneration. Four DEPs were found in the term of biological process: positive regulation of axon regeneration (GO-ID: 0048680), including Q91ZX7 (Lrp1), Q9JI10 (MST2), Q8K0T0 (Rtn1), Q9ES97 (Rtn3) (Fig. 1d). Among them, two proteins (Q9ES97 (Rtn3) and Q8K0T0 (Rtn1)) were downregulated, while (Q9JI10 (MST2) and Q91ZX7 (Lrp1)) were up-regulated with a fold change of 2.00 and 1.66, respectively (Fig. 1e depicts the scatter plot). Lrp1 was excluded as it can promote CNS axon regeneration [35, 36]. However, the effect of MST2 on axon regeneration has been unreported in the literature. The results of the annotation of the GO term indicated that MST2 had multiple functional roles in the CNS (Fig. 1f). Therefore, MST2 was finally selected for further study.

In the mammalian CNS, MST2 plays an essential role in early embryonic development [37, 38]. To confirm whether MST2 is involved after SCI, a Western blot was used to test its expression in SCI mice. We found that the expression level of MST2 gradually increased after injury and that its expression at one week was consistent with the result of proteomics (Figs. 1g–h).

To further determine whether MST2 is expressed in the nervous system, double immunofluorescence staining was performed to examine the co-localization between MST2 and GFAP/IBA-1/NeuN in the uninjured and injured spinal cord, respectively. The results showed that MST2 is especially expressed in Neun+ cells (Figs. 1i–j), both in normal and injured mice spinal cord. These results indicated that MST2 was especially expressed in spinal neurons, and expression was elevated after SCI.

MST2 knockdown inhibits axon regeneration in vitro

To demonstrate whether MST2 affects axon regeneration, we first designed three siRNA sequences to reduce MST2 expression (Figs. 2a–b). The optimal knockdown effect was observed using the sh-MST2-3 sequence (Figs. 2c–f). Subsequently, we established stable MST2 knockdown cells using lentiviral shRNAs in Neuro2A and HT22 cell lines for subsequent studies.

Then, MST2 was knockdown in Neuro2A, HT22 cells, and primary mouse cortical neurons, respectively. The results of Western blot indicated that MST2 knockdown significantly decreased protein levels of NF200 and β3-tubulin (Figs. 2g–j). Similar results showed that mRNA levels of NF200 and β3-tubulin were decreased using PCR (Figs. 2k–m). Finally, to examine the effect of MST2 knockdown on the neuronal axon, the morphological changes of the primary mouse cortical neurons after lentiviral transfection were detected.
using fluorescence microscopy. The results revealed that the axonal length of sh-MST2 group was significantly shorter half than that of the sh-control group (Figs. 2n–o). Together, these results demonstrated that MST2 knockdown inhibited axon regeneration in vitro.

**MST2 overexpression promotes axon regeneration in vitro**

To further confirm the observations described above, the cells above were infected with MST2 overexpression lentivirus. The western blot and PCR results showed that both protein (Figs. 3a–d) and mRNA (Figs. 3e–g) levels of NF200, β3-tubulin protein, and MST2 were elevated in these cell lines. Neuronal axons were examined after primary cortical neurons were infected with the MST2 overexpression lentivirus using fluorescence microscopy. The axon length of MST2 overexpression group was more than double the length of control group (Figs. 3h–i). These results suggested that increased MST2 expression promoted axon regeneration in vitro.

**MST2 positively regulates AKT activity in vitro**

The mechanism of MST2 in promoting neuronal axon regeneration was discovered. The previous proteomics results were positively correlated with MST2 and AKT (Fig. 1f). AKT positively influences the axon regeneration of primary neurons, and AKT pathway activation regulates neuronal axon regeneration [29, 39-42]. First, western blots were performed to confirm whether MST2 could regulate AKT expression. We found that MST2 knockdown significantly decreased the expression of p-AKT (S473) while total AKT expression was unchanged (Fig. 4a–c).

Then, specific inhibitors of PI3K pathways and AKT were used to explore how MST2 affected the protein expression of AKT activity. Stable MST2 overexpression cells were treated with LY294002 (PI3K inhibitor) [43], perifosine (AKT inhibitor) [44]. We observed that both inhibitors could reverse the elevated protein expression of NF200, β3-tubulin, and p-AKT (S473) induced by MST2 overexpression (Figs. 4d–h).

To more clearly observe the length changes of the axons, primary mouse cortical neurons were selected. IF results revealed that both inhibitors could reverse axon elongation induced by MST2 overexpression (Figs. 4i–j). These data showed that MST2 positively regulates AKT activity and promotes axon regeneration in vitro, consistent with previous proteomic results.

**MST2 interacts with AKT in a neuronal cell model**

Previous report shows that AKT interacts with MST2 in MCF7 and Hela cells [45]. IP assays were performed to evaluate whether MST2 interacts with AKT or p-AKT (S473) in the neuronal cell model (Figs. 5a–d). The result showed that MST2 interacted with AKT and p-AKT (S473), either endogenous or exogenous. Double IF staining of MST2 and AKT in normal and injured spinal cords indicated that MST2 co-localized with AKT in neurons (Figs. 5e–f), supporting an interaction between MST2 and AKT.

**MST2 knockdown inhibits axon regeneration in vivo and motor function recovery**

The effects of MST2 expression on neuron axon regeneration in vitro suggested the physiological significance of MST2. Therefore, the impact of MST2 on neuron axon regeneration and function recovery
after SCI were investigated in vivo by local injection of lentiviral vector into the lesion area. After SCI in mice, LV-sh-MST2 was locally injected into the lesion area. At one- and two-week time points, the protein expression of MST2, NF200, and β3-tubulin was detected using western blot. We found that the LV-sh-MST2 group showed significantly decreased expressions (Figs. 6a–c). Four weeks after SCI, Hematoxylin and eosin (H&E) staining of spinal cord sections showed similar lesion sizes in both groups. However, more tissue voids were observed in the LV-sh-MST2 group (Figs. 6d–e). To investigate the role of MST2 inactivation in neurons in vivo, β3-tubulin was used to sign neurons. The results of IF revealed that, compared to the other groups, the area of β3-tubulin positive staining in the LV-sh-MST2 group was the smallest in the central parts of lesion at four weeks after injury(Figs. 6f–g).

Subsequently, radiographic assessment and functional recovery were evaluated. MRI scans were used to assess tissue damage one week and six weeks after SCI. Compared to the sh-control group, a more severe tissue injury (loss of spinal cord continuity) that T2-weighted images showed was found in the LV-sh-MST2 group (Fig. 6h). Moreover, hindlimb function was evaluated using the Basso mouse scale for six weeks post-SCI. The BMS scores of all mice were 9 points before the injury and the scores were reduced to 0 immediately after SCI. Consequently, the BMS score in all mice gradually increased, then reached 2.50 ± 0.55 in the LV-sh-MST2 group and 4.33 ± 0.71 in the sh-control group six weeks after injury (Fig. 6i). Footprints were then evaluated six weeks after SCI mice. Due to the animals dragged their legs, the footprint distance could not been measured consistently (Fig. 6j).

**MST2 overexpression promotes neuronal axon regeneration in vivo and motor functional recovery after SCI**

In contrast, following MST2 overexpression lentivirus local injection, the same experimental approaches described above were used. As expected, opposite results were obtained, demonstrating that MST2 played a functional role in vivo. In detail, the western blot showed that the expression of MST2, NF200, and β3-tubulin in the LV-MST2 group was significantly increased at one-week and two-week time points (Figs. 7a–c). Results of HE staining showed that lesion size in the LV-MST2 group was smaller than in the vector group (Figs. 7d–e). IF showed that the area of β3-tubulin positive staining in the central lesion parts in the LV-MST2 group was the largest at four weeks after injury (Figs. 7f–g).

Furthermore, to determine whether MST2 overexpression would be reversed by AKT inhibitor in vivo, MST2 overexpression lentivirus injection after SCI was used, and one group of mice was treated with perifosine at 250 mg/kg/wk by oral gavage for four weeks. The injured tissue sections were collected on days 7 and 28 after SCI and then subjected to IF for β3-tubulin. The results revealed that the area of β3-tubulin positive staining in the central lesion parts in the LV-MST2 group was significantly reversed by perifosine (Figs.7h–j), which was consistent with the in vitro results.

Moreover, compared to the vector group, MRI images of spinal cords in the LV-MST2 group showed better spinal cord continuity, which was also reversed by perifosine (Fig. 7k). Compared to the other groups, the BMS scores increased significantly in the LV-MST2 group at two weeks post-injury. In comparison, after four weeks post-injury, the BMS scores in the LV-MST2 + perifosine group were significantly reversed (Fig. 7l). The footprint distance also could not be unmeasured due to the mice dragged their legs, but the motor function
of the hind limbs in the LV-MST2 group was significantly improved compared to the other two SCI groups (Fig. 7m).

Discussion

Herein, we identified MST2 as a potential intrinsic facilitator of axon regeneration using the 4D label-free proteomic technique. Furthermore, we found that MST2 promotes axon regeneration by increasing AKT activity in vivo and in vitro. At last, we revealed that the MST2 promotes motor function recovery after SCI. This is the first report that MST2 promotes axon regeneration and functional recovery after SCI in mice.

The components of Hippo signaling pathway have different functions in the nervous system. For example, YAP is mainly expressed in astrocytes. The Hippo/YAP signaling pathway is actively involved in the self-renewal of neural stem cells (NSCs), proliferation of neural progenitor cells (NPCs), differentiation and activation of glial cells and astrocyte, myelination of glial cells, and development of neurological diseases [46]. In contrast, some of these components regulate axon regeneration. MOB1, a downstream substrate for MST2, promotes neurite outgrowth and functional recovery after SCI [27]. Ctgfa, a target of YAP, promotes glial bridging and axonal regeneration in the injured area in a zebrafish SCI model [16]. Down-regulated expression of NDR1/2 increases length of dendrite and proximal branching of mammalian pyramidal neurons in vitro and in vivo [15]. Accordingly, it is worth exploring the potential function of axon regeneration of other components.

Previous studies on the functional role of MST2 in mammals have been largely confined to cancers, and other roles of MST2 are still needed to be clarified. MST2 kinase plays an important role in nervous system development and neurological disorders [47]. As mentioned in the introduction, MST2 is involved in microtubule stability [25] and contributes to establishing a polarized cell structure [26]. Moreover, MST2 is required for mitotic activation of the NDR1 kinase and alignment of chromosomes [25]. In the polarization process of hippocampal neurons, NDR1/2 double knockdown inhibits the formation of supernumerary axons [48]. These results prove that MST2 contributes to axon regeneration in mammalian CNS. Herein, we found that the expression level of MST2 was elevated after SCI and MST2 promoted axon regeneration in vivo and in vitro.

Two main factors contribute to the failure of adult CNS neurons to regenerate, including the low intrinsic capacity of CNS neurons and the presence of myelin and scar-associated inhibitory molecules in the lesion microenvironment [3, 49, 50]. Therefore, intrinsic mechanisms have important roles in controlling the axon regeneration [2]. However, we reported that MST2 overexpression significantly promoted axon regeneration in neuronal cell models and primary cortical neurons in vitro, and its overexpression by local injection of lentiviral vectors enhanced axon regeneration in vivo, leading to significant improvements in functional recovery after SCI. Conversely, MST2 knockdown greatly eliminated axon regeneration in vitro and in vivo. These results proved that MST2 contributes to axon regeneration in the mammalian CNS. Surprisingly, H&E staining showed a similar lesion size in both sh-control and sh-MST2 groups, while the lesion size in the LV-MST2 group was smaller than in the sham and vector groups. This implied that exogenous MST2 overexpression has a stronger role in promoting axon regeneration, which can be a new target for treating SCI.
Regulation of axon regeneration is an essential growth factor during the development of the nervous system. Promoting axon regeneration is crucial in treating CNS injuries and neurodegenerative diseases. Numerous kinase signaling pathways promote axon regeneration. The PTEN/PI3K/AKT pathway is an important signaling pathway in the regulation of axon regeneration. The inactivation of PTEN[7, 51, 52], upregulation of the PI3K/AKT signaling pathway, and upregulation of its downstream effectors, including mTOR and GSK3β [8, 53], promote axon regeneration in CNS injuries. Our previous study also obtained results consistent with previous reports [27, 30]. AKT plays a major role in the PI3K/AKT signaling pathway and is an important positive regulator of axon regeneration. Activation of the AKT pathway can promote the sprouting and regeneration of axons [39–42]. AKT positively impacts the axon regeneration of many primary cell types, such as hippocampal neurons, superior cervical ganglion (SCG), dorsal root ganglion (DRG), hypoglossal motoneurons, and PC12 cells. AKT phosphorylates or interacts with many other proteins, such as GSK3β and mTOR, which may positively affect neuronal morphology and development [27, 53–55]. Herein, we found that MST2 interacted with AKT and p-AKT (S473) in IP experiments, either exogenous or endogenous, which is consistent with previous studies in tumor cells [56].

We also found that MST2 positively regulates the AKT activity, which is consistent with the result of proteomics. Furthermore, MST2 overexpression-induced axon regeneration could be reversed by an AKT inhibitor, suggesting that MST2 is a regulator of AKT activity in axon regeneration in the injured CNS. However, the regulatory mechanism of AKT is complex and involves many factors and pathways; the interaction between AKT and MST2 still needs further exploration.

Here are the results that confirm the importance of MST2. However, axon regeneration is a complex process that likely involves multiple mechanisms, and further studies are still needed to determine the detailed mechanisms between MST2 and AKT. As a key component of the Hippo pathway, MST2 may be a target with great therapeutic potential for SCI. Therefore, more studies are required that focus on the role of MST2 in axon regeneration to discover drugs for treating SCI.

**Conclusion**

In summary, current study shows the role of MST2 in axon regeneration and reveals a novel molecular mechanism where MST2 regulates the AKT activity to promote axon regeneration. These results offer a new candidate for treatment of CNS injuries.

**Abbreviations**

SCI  Spinal cord injury
CNS  Central nervous system
KLFs  Krüppel-like factors
mTOR  mammalian rapamycin target
PTEN  phosphatase and tensin homolog
MST2  Mammalian sterile 20-like kinase 2
PI3K  Phosphatidylinositol 3-kinase
PIP3  Phosphoinositol (3,4,5) -triphosphate
PBS  phosphate-buffered saline
MS  Mass spectrometry
HE  Hematoxylin and eosin
IF  Immunofluorescence
BMS  Basso mouse scale
MRI  Magnetic resonance imaging
DEPs  Differentially expressed proteins
NSCs  Neural stem cells
NPCs  Neural progenitor cells
SCG  Superior cervical ganglion
DRG  Dorsal root ganglion

Declarations

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Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Soochow University. Consent to participate was not applicable.

**Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

**Consent for publication**

Not applicable.

**Author Contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by HZ. The first draft of the manuscript was written by HZ, HW, ZS and JL and all authors commented on previous versions of the manuscript. Visualization, resource purchase were performed by HZ, YX, XX, ZZ, JF. All authors read and approved the final manuscript.

**References**


Figures
Figure 1

Proteomic analysis for identifying MST2 (STK3) and expression in the spinal cord tissues of mice. (a) Flowchart of the 4D label-free proteomics procedure; (b) heatmap of DEPs; where yellow represents upregulated DEPs and blue represents downregulated DEPs; (c) volcano plot of DEPs; the X axis is the fold change (log2), and the Y axis represents the \( P \) value (-log10); blue points (fold change > 1.5) indicate upregulated proteins; and green points (fold change < -1.5) indicate downregulated proteins; (d) proteins of GO-ID: 0048680 (positive regulation of axon regeneration); (e) scatter plots of Q9JI10 (MST2) levels observed in...
the SCI group and NC group (*P < 0.05 vs. NC group); (f) part of GO terms for MST2 (P; biological process); (g–h) western blot analysis showing expression profiles of MST2 after SCI and GAPDH serve as a control (**)P < 0.01 sham vs. seven days after SCI; (i–j) representative co-localization of MST2 with GFAP/Iba1/Neun in normal and injured mouse spinal cords; Scale bar: left = 500 μm; right = 20 μm; arrows indicate co-localized IF; DEPs, differentially expressed proteins; SCI, spinal cord injury; NC; negative control.

Figure 2
MST2 is efficiently knockdown, and MST2 knockdown inhibits axon regeneration in vitro. (a) Three independent MST2 shRNA sequences (322, 323, and 324) are designed to construct lentiviral-shRNA vectors; (b) Lentiviral vector backbones; (c–f) the changes in MST2 levels detected using western blot analysis in Neuro 2A and HT22 cells transfected with three independent MST2-shRNAs: sh-MST2-1, sh-MST2-2, and sh-MST2-3, or sh-control; GAPDH was used as an internal control; (g–j) MST2, β3-tubulin, and NF200 protein expression levels measured using western blotting, and (k–m) mRNA expression levels measured using real-time PCR in Neuro2A and HT22 cells, and primary cortical neurons with MST2 knockdown, and GAPDH serve as an internal control; (n) representative images of primary cortical neurons are photographed with MST2 knockdown; magnification: 40×; (o) quantification of relative axon length in primary cortical neurons (**P < 0.01 vs. sh-control).
Figure 3

**MST2 overexpression promotes axon regeneration in vitro.** (a–d) MST2, β3-tubulin; and NF200 expression levels measured using western blotting; (e–g) and real-time PCR in stable Neuro2A and HT22 cells and primary cortical neurons with MST2 overexpression, and GAPDH serve as an internal control; (h) representative images of primary cortical neurons photographed with MST2 overexpression; magnification: 40×; (i) quantification of relative axon length in primary cortical neurons (**P < 0.01 vs. vector).
**Figure 4**

**MST2 positively regulates AKT activity in vitro.** (a–c) AKT, p-AKT(S473), and MST2 protein levels are measured using western blotting in stable HT22 and Neuro2A cells with MST2 knockdown; and GAPDH serves as an internal control (**P < 0.01 vs. sh-control); (d–h) stable MST2 overexpression Neuro2A and HT22 cells treated with LY294002 (PI3K inhibitor) and perifosine (AKT inhibitor); GAPDH serves as an internal control (**P < 0.01 vs. Flag-MST2); (i) representative images of primary cortical neurons are
photographed with MST2 overexpression and treated with perifosine; magnification: 40×; (j) quantification of relative axon length in primary cortical neurons (**P < 0.01 vs. LV-MST2).

**Figure 5**

**MST2 interacts with AKT and p-AKT.** (a–b) Endogenous and exogenous MST2 and AKT interact in Neuro2A and HT22 cells; (c–d) endogenous and exogenous MST2 and p-AKT(S473) interact in Neuro2A and HT22 cells; input and IP analyzed using western blotting; (e–f) representative co-localization of MST2 with AKT in the normal and injured mouse spinal cords; scale bar: left = 500 μm; right = 20 μm; arrows indicate co-localized IF.
**Figure 6**

**MST2 knockdown inhibits axon regeneration in vivo and functional recovery after mouse SCI.** (a–c) MST2, β3-tubulin, and NF200 protein levels of spinal cord tissue are measured using western blotting at one and two weeks after SCI; and GAPDH serve as an internal control (\(**P < 0.01; *P < 0.05\) vs. sh-control); (d–e) HE staining results for sections and quantification of relative lesion area of the injured spinal cord at four weeks after SCI; (f) representative IF images of the longitudinal sections of spinal cord show β3-tubulin labeled neurites (red) and DAPI (blue) in the various groups at four weeks after SCI (scale bar: left = 500 μm; right =
20 μm); (g) quantitative analysis of the β3-tubulin* cells in the central parts of spinal cord longitudinal sections (**P < 0.01 vs. sh-control); (h) representative T2-weighted MR images of spines one and six weeks after SCI or sham surgery; (i) time course of BMS scores following SCI (*P < 0.05 vs. sh-control); (j) footprint data in false-color mode; four or more consecutive steps used to determine the mean values of each measurement; scale bar: 1.5 cm.

Figure 7
MST2 overexpression promotes axon regeneration and functional recovery, whereas can be reversed by AKT inhibitors. (a–c) MST2, β3-tubulin, and NF200 protein levels of spinal cord tissue measured using western blotting at one and two weeks after SCI, and GAPDH serve as an internal control (**P < 0.01; *P < 0.05 vs. vector); (d–e) HE staining results for sections and quantification of relative lesion area of the injured spinal cord at four weeks after SCI; (f) representative IF images of the longitudinal sections of spinal cord show β3-tubulin labeled neurites (red) and DAPI (blue) in the various groups at four weeks after SCI (scale bar: left = 500 μm; right = 20 μm); (g) quantitative analysis of the β3-tubulin+ cells in the central parts of spinal cord longitudinal sections; (**P < 0.01 vs. vector); (h) representative IF images of the longitudinal sections of spinal cord show β3-tubulin-labeled neurites (red) and DAPI (blue) in the various groups at four weeks after SCI (scale bar: left = 500 μm; right = 20 μm); (i) quantitative analysis of the β3-tubulin+ cells in the central parts of spinal cord longitudinal sections, respectively (**P < 0.01; *P < 0.05 vs. LV-MST2 group; ##P < 0.01, #P < 0.05 vector vs. LV-MST2 + perifosine group); (j) representative T2-weighted MR images of spines one and six weeks after SCI or sham surgery; (k) time course of BMS scores following SCI (*P < 0.05 vs. LV-MST2 group, #P < 0.05 LV-MST2 group vs. LV-MST2 + perifosine group); (l) footprint data in false-color mode; four or more consecutive steps used to determine the mean values of each measurement; scale bar: 1.5 cm.