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The effect of systemic PTEN antagonist peptides on axon growth and functional recovery after spinal cord injury

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Abstract

Knockout studies suggest that PTEN limits the regenerative capacities of CNS axons as a dominant antagonist of PI3 kinase, but the transgenic approach is not feasible for treating patients. Although application of bisperoxovanadium may block PTEN function, it is a general inhibitor of phosphotyrosine phosphatases and may target enzymes other than PTEN, causing side effects and preventing firm conclusions about PTEN inhibition on regulating neuronal growth. A pharmacological method to selectively suppress PTEN post-injury could be a valuable strategy for promoting CNS axon regeneration. We identified PTEN antagonist peptides (PAPs) by targeting PTEN critical functional domains and evaluated their efficacy for promoting axon growth. Four PAPs (PAP1-4) bound to PTEN protein expressed in COS7 cells and blocked PTEN signaling *in vivo*. Subcutaneous administration of PAPs initiated two days after dorsal over-hemisection injury significantly stimulated growth of descending serotonergic fibers in the caudal spinal cord of adult mice. Systemic PAPs induce significant sprouting of corticospinal fibers in the rostral spinal cord and limited growth of corticospinal axons in the caudal spinal cord. More importantly, PAP treatment enhanced recovery of locomotor function in adult rodents with spinal cord injury. This study may facilitate development of effective therapeutic agents for CNS injuries.

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Keywords

PTEN antagonist peptide; spinal cord injury; intrinsic growth capacity; axon regeneration; functional recovery

1. Introduction

Axonal disconnection in the CNS results in persistent deficits in many neurological disorders, such as spinal cord injury (SCI), because axons in the mammalian CNS do not regenerate. Failure of axon regeneration in the CNS is believed to be due to both the non-permissive environment and the reduced intrinsic growth capacity of mature neurons [1]. Among extrinsic factors, myelin-associated growth inhibitors [2, 3], scar-generating chondroitin sulfate proteoglycans [4, 5], repulsive axon guidance cues [6, 7] and lack of neurotrophic factors [8–10] appear to contribute to growth failure. Among neuronal intrinsic factors, several cell-autonomous molecules, including cAMP, RhoA, Krüppel-like factors, mammalian target of rapamycin (mTOR) and phosphatase and tensin homolog (PTEN), have been reported to play roles in determining neuronal growth ability [11–14].

PTEN appears to be particularly important for controlling the regenerative capacity of injured axons [15]. Conditional deletion of PTEN, a negative regulator of mTOR, remarkably enhanced axon growth after SCI or optic nerve injury and protected retinal ganglion cells (RGCs) from death following axotomy [12, 14, 16]. The axon growth-promoting action of PTEN deletion is reduced by the mTOR inhibitor rapamycin. Deletion of tuberous sclerosis complex 1 (TSC1), a negative regulator of mTOR, also activated mTOR and enhanced axon regeneration principally by mimicking the effect of PTEN deficiency [12]. PTEN deletion or inhibition has been reported to stimulate axon growth of several neuronal populations, including RGCs, dorsal root ganglion (DRG) sensory neurons, corticospinal tract (CST) and other motor neurons [12, 14, 15, 17, 18]. In addition, PTEN blockade with the general phosphatase inhibitor bisperoxovanadium (bpV) protected spinal cord tissues after SCI [19, 20]. While suppressing PTEN activity is very promising for promoting CNS axon regeneration, the transgenic approach used in previous studies is not feasible for treating patients. Application of bpV may block PTEN function [17], but bpV compounds also target many other enzymes [21] and may cause clinical side effects, such as lowering blood glucose by activating insulin signaling [22]. Therefore, it is important to develop a pharmacological method to suppress PTEN activity efficiently and selectively. In the present study, we designed small peptides to block the critical domains of PTEN, and tested their ability to inhibit PTEN signaling, stimulate axon growth *in vitro* and *in vivo*, and promote recovery of locomotor function in adult rodents with SCI.

2. Materials and Methods

2.1. Design and preparation of PTEN antagonist peptides

Peptides derived from the active regions of a protein may selectively interact with the same molecule and alter its activity. PTEN protein consists of 403 amino acid residues and multiple activity domains, including the phosphatidylinositol-4,5-bisphosphate (PIP2)

binding motif and the protein tyrosine phosphatase, C2, and C-terminal tail regions [23]. The PIP2 binding motif at the N-terminus serves a dual role of localizing PTEN to the membrane and regulating its activity [24]. The ATP-binding motifs associate PTEN subcellular localization and its tumor-suppressor activity [25, 26]. The C-terminal tail contains the PDZ domain-binding sequence and multiple phosphorylation sites and contributes to PTEN stabilization and targeting for phosphorylation by microtubule-associated serine/threonine kinases [27, 28]. To selectively block PTEN function with a pharmacological approach, we designed five PTEN antagonist peptides (PAP1-5) by targeting the PIP2, ATP-type A or B, PDZ, and C-terminal tail regions of PTEN (Fig. 1A, Table 1). To facilitate peptide access into cells, we included the transactivator of transcription (TAT) sequence (GRKKRRQRRRC) at the C-terminus of all the PAPs. TAT-fusion proteins or peptides have been shown to retain their biological activity and to be able to rapidly access the intracellular space of cultured cells and intact tissues following extracellular applications *in vitro* and *in vivo* [29]. All the PTEN antagonist peptides and a random sequence peptide (RP, control peptide) were designed in our lab and synthesized by CHI Scientific Inc (Maynard, MA). The peptide purity was analyzed via HPLC. Peptides were dissolved in DMSO (20 µg/ml), stored at -20°C before starting experiments and then diluted to required concentrations with culture medium or sterile PBS just before application.

2.2. Sources of compounds

Antibodies against the following proteins were used: PTEN (138G6) rabbit mAb, phospho-Akt (Ser473, 587F11, 1:1000) mouse mAb, Akt (pan, 11E7, 1:000) rabbit mAb, phospho-glycogen synthase kinase 3β (GSK-3β Ser9, 1:1000), GSK-3β (27C10, 1:1000) rabbit mAb, phospho-S6 ribosomal protein (Ser235/236, 1:2000 for Western blots) Rabbit mAb, phospho-Erk1/2 (p44/42, 1:2000, Cell Signaling Technology), 5-HT serotonin rabbit (1:4000, ImmunoStar), monoclonal anti-gial fibrillary acidic protein (GFAP, 1:400, Sigma), neuron-specific class III β-tubulin (Tuj1, 1:400, Covance), mouse myelin basic protein (MBP, SMI 99, 1:400, Convence) and goat anti-biotin agarose (Vector Lab). The major proteins employed include purified myelin from bovine brain white matter (Pel-Freez Biologicals, Cat#57104-1), laminin (10 µg/ml, Sigma) and avidin/biotin complex kit (ABC, PK-6100, Vector Lab). CNS myelin was purified in our lab with two concentrations of sucrose as reported previously [30]. The PTEN plasmid was purchased from Addgene (Cat# 10750) and amplified in our lab. The biotinylated peptides were made with EZ-Link Sulfo-NHS-LC-LC-Biotin (Cat# PI-21338) and dialyzed with Tube-O-Dialyzer™, Micro (1K MWCO, Cat# 786-620, G-Bioscience) following protocols from the manufacturer. As a control, a plasmid containing DNA that does not express a gene product was used (provided by Dr. Robert Bachoo at UT Southwestern Medical Center).

2.3. Neurite outgrowth assay in primary neuronal cultures

Neurite outgrowth in DRG and cerebellar granular neuron (CGN) cultures was performed 1–5 days after growth. Before cell plating, culture coverslips were coated with poly-L-lysine (100 µg/ml, Sigma). For DRG assay on myelin spots, the center of each culture coverslip was spotted with 2 µl of purified CNS myelin (200 µg/ml, plus 10 µg/ml of laminin), which contains a number of axon growth inhibitors [10]. DRGs were dissected out from C57BL/6

mice aged 7–10 weeks. After incubation with collagenase (120 unit/ml, Worthington, Freehold, NJ) for 90 min and then with a mixture of 0.25% trypsin/EDTA (Sigma) and collagenase, dissociated DRG neurons were plated onto plastic coverslips in 24-well plate after washing and grown in culture medium (DMEM/F12 in 10% fetal bovine serum, 2 mM glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin, Sigma) for 5 days at 37°C [31, 32]. Culture medium was changed 2 days after plating. Primary CGN cultures were prepared from cerebelli of postnatal 7–9 day (P7-9) C57/BL6 mice [31, 33]. Following dura mater removal, dissected cerebelli were chopped into small pieces (~1×1×1 mm) and digested with 0.25% trypsin for 15 min. CGNs were dissociated, filtered with a strainer (mesh size: 70 µm, Falcon) and grown in culture medium (DMEM/F12 containing 10% fetal bovine serum, 2 mM glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin, 25 mM KCl and 25 mM glucose) for 24 hrs at 37°C. Before DRG and CGN plating, the above prepared coverslips were incubated with laminin (10 µg/ml) for 2 hrs at 37°C.

After plating, dissociated neurons were treated with vehicle, RP (10 µg/ml), PAP1 (2.5 µg/ml), PAP2 (10 µg/ml), PAP3 (6.7 µg/ml), PAP4 (6.7 µg/ml) and PAP5 (5 µg/ml) for 5 days (DRGs) or 24 hrs (CGNs). The doses of PAPs were chosen based on the dose-response curve obtained from neurite outgrowth assays of DRGs on CNS myelin. Neurons were fixed 5 days (DRGs) or 24 hrs (CGNs) after plating and stained for Tuj1. Images of each culture were captured with a Nikon image-collecting system and neurite outgrowth was quantified with Photoshop and NIH image software [31]. DRG neurites per myelin spot were traced manually and the total length of neurites per spot was determined from 5–8 representative experiments. The neurites of CGNs were also traced manually from multiple areas of each coverslip selected randomly and the neurite length per neuron was measured from 120–185 CGNs per group in 4 separated experiments [31, 33]. To determine cellular distribution of PTEN, we stained DRGs with anti-PTEN and anti-Tuj1 antibodies in selected cultures.

2.4. PTEN peptide binding assay in COS-7 cells

COS-7 cells were cultured as we reported [33]. Forty-eight hrs after transfection with PTEN DNA or a control plasmid DNA that does not express a gene product, cells were incubated with purified biotinylated RP or PAPs 1-4 (15 µM) for 1 hour. Following 3 washes with PBS and fixation with 4% formaldehyde, COS-7 cells were incubated in ABC and PAP binding signals were visualized with ABC-diaminobenzidine (DAB)-based color reactions. The intensity of reaction color in multiple wells was read with a 96-well plate reader at 405 nm. The binding experiments were carried out in 6–12 replicates.

2.5. Spinal cord and brain tissue preparations for biochemical assays in mice with spinal cord injury

To detect the potential changes of PTEN signaling pathway *in vivo*, we performed a dorsal over-hemitranssection injury at T7 (see below) in two sets of mouse experiments and examined several signaling proteins in the spinal cord or in the brain with Western blots. To examine expression changes of PTEN, p-S6 and p-Erk at different time points, we performed SCI in 5 groups of adult C57BL/6 mice (3 mice per group, Fig. 3) and collected fresh spinal cord 1, 3, 7, 14 and 21 days after the injury. These animals did not receive any peptide treatment. At the indicated time points, mice were transcardially perfused with cold

PBS and 3 fresh spinal cord blocks were harvested from each animal immediately after perfusion: lesion (2 mm rostral to and 2 mm caudal to injury, containing the injury site), rostral (2–6 mm rostral to lesion) and caudal (2–6 mm caudal to lesion) areas. In addition, 3 age-matched adult female mice received sham surgery and were used as no-injury controls. In this experiment (Fig. 3), a total of 18 mice were used in the 6 groups.

To evaluate potential role of PAPs in blocking PTEN signaling pathway *in vivo*, we made a dorsal transection injury at T7 in 5 separate groups of adult C57BL/6 mice (Fig. 4). To evaluate the role of PAPs in blocking the PTEN signaling pathway *in vivo*, beginning one day after injury, mice were injected subcutaneously with RP, PAP1, PAP2, PAP3 or PAP4 (71 µg/mouse/day, 2 times per day) for two days. Three days after SCI, mice were perfused through heart with cold PBS and the following fresh tissue samples were harvested immediately from 4 mice per group: the spinal cord containing the injury site (2 mm rostral to and 2 mm caudal to the injury), the cortex around the sensorimotor area at a 2-mm depth (see Table 2 for coordinates), and the brainstem including midbrain, pons and medulla. In addition, the above-mentioned fresh tissue samples were also collected from 4 age-matched no-SCI adult mice (no-injury controls), which received 2-day RP treatment. The fresh tissue collection was limited to 5 min per animal after perfusion. Collected fresh tissue samples were harvested on dry ice immediately and then stored at -80°C. A total of 24 mice (6 groups of 4) were employed in this section.

2.6. PTEN, Akt, GSK-3β, S6 kinase and Erk assays

The tissue samples of the spinal cord, sensorimotor cortex and brainstem were prepared in lysis buffer supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM orthovanadate, 10 µg/ml leupeptin and 10 µg/ml aprotinin). Following total protein quantification in supernatants, samples containing the same amount of proteins were prepared for Western blots using antibodies against phosphorylated S6 (p-S6), Erk1/2 (p-Erk) and Akt (p-Akt), and total proteins of PTEN, Akt (t-Akt), GSK-3β (t-GSK) or actin. The levels of phosphorylated Akt, S6 and Erk1/2 represent active form of these proteins, but p-GSK indicates the inactive form of this kinase. For band densitometry, the images of protein bands were captured with a Bio-Rad Gel Doc XR documentation system and band density was determined using Quantity One software [31]. The values of band density measured from different time points after SCI were compared to those in no-injury controls (Fig. 3). The signaling proteins measured from PAP treated mice were compared to those in RP-treated SCI controls (Fig. 4).

2.7. Dorsal over-hemisection of the spinal cord, peptide treatments, peptide penetration, histology, and behavioral tests in mice

Adult female C57BL/6 mice (9–10 weeks old) were deeply anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (15 mg/kg). A complete laminectomy was performed and the dorsal part of the spinal cord was fully exposed at T6 and T7 levels [34]. A dorsal over-hemisection at 1.0 mm was performed at T7 (~1.5 mm in dorsoventral diameter) with a 30-gauge needle and microscissors to completely sever the dorsal part of the spinal cord as reported previously [33, 34]. The lesion depth of 1 mm was ensured by passing a marked 30-gauge of needle 4 times across the dorsal spinal cord. To study axon growth (Fig. 5–10),

mice received subcutaneous injections (two times per day) of RP, PAP2 or PAP4 at 71 μ g/mouse/day beginning two days after SCI for 14 successive days. Three weeks after SCI, mice received biotin dextran amine (BDA) tracer injections into the sensorimotor cortex with 5 injection sites and the coordinates summarized in Table 2 (0.9 mm depth, 1 μ l/spot) [34].

Mice were perfused 5 weeks after SCI and spinal cord around the lesion was processed for 5-HT and CST labeling as we reported previously [33, 34]. The spinal cord extending from 0–4 mm rostral to and caudal to the lesion (8 mm long, containing the injury site) was cut parasagittally (30 μ m). The spinal cord 5–7 mm rostral to and caudal to the injury was transversely sectioned (30 μ m). All the parasagittal sections and 20 transverse sections (10 rostral to injury, 10 caudal to injury) were processed for BDA tracer via DAB-based color reaction [34, 35]. For raphe axon labeling, all the parasagittal sections and 20 transverse sections rostral to and caudal to the lesion (10 sections per location) were immunostained with a 5-HT antibody and an Alexa594-conjugated secondary antibody. To visualize the lesion area, all the parasagittal sections were triply stained for GFAP with an Alexa488-conjugated secondary antibody.

To determine serotonergic fiber density in the spinal cord 5–7 mm caudal to the lesion, individual fibers stained by 5-HT antibody in the ventral and dorsal half of the spinal cord were traced manually from images captured through a 10x objective. The mean density of traced 5-HT fibers was measured from 4 randomly-selected transverse sections in each mouse using NIH and Photoshop software. To further compare axon numbers in the caudal spinal cord among different groups, we counted 5-HT axons in all the parasagittal sections of the spinal cord from 0–3.2 mm caudal to the lesion epicenter in each animal (~25 sections/mouse), using Nikon 80i microscope, digital DXM1200 color camera and ACT-1 software. The injury center was determined as the midpoint of histological abnormalities produced by the lesion cavitations, reactive astrocytes, and morphological changes of injured axons. The 5-HT-positive axons that crossed lines perpendicular to the parasagittal plane at the lesion center and at 0.8, 1.6, 2.4 and 3.2 mm caudal to the lesion were counted from live images (10x lens) on a computer screen. The numbers of 5-HT axons at a given distance were summed from all the sections of each mouse and compared among different groups. To quantify CST axons in the caudal spinal cord, we counted the total numbers of BDA-labeled individual axons and CST axon arbors (i.e., axons with branching structures) from 0–3.5 mm caudal to the lesion in all the parasagittal sections of each animal. To measure transection depth in each animal, all the parasagittal sections greater than 1 mm in dorsoventral diameter were photographed for GFAP staining around the lesion. The lesion depths were measured based on the characteristic lesion cavities, scar formation and other morphologic changes at the injury site and calibrated with the dorsoventral diameter of the same section.

To determine functional recovery, we evaluated locomotion recovery during 5 weeks of survival by measuring the Basso mouse scale for locomotion (BMS) two days and once per week after SCI, performance on a grid walk and footprint analysis 5 weeks after SCI. The BMS scores were evaluated while animal was walking in an open field and re-evaluated from digital video documents. The grid walk errors were counted from videotapes played at

a slow speed (4 separate trials per test) and averaged from different trials. For footprint analysis, walking patterns of mouse hindpaws were recorded with ink during continuous locomotion across a 50-cm runway, and the stride length on each side and stride width between two sides of the prints were measured and calculated from multiple steps [34]. All of the behavioral tests were performed by two persons. For axon quantification and behavioral analysis, 7, 10, and 8 mice were used in the RP, PAP2 and PAP4 groups, respectively.

For peptide penetration experiments, mice received vehicle DMSO, PAP2, or biotinylated RP (BioRP), BioPAP2 or BioPAP4 (S.C., 71 µg/mouse) starting 2 or 28 hours (Fig. 11A) after a dorsal over-hemisection lesion at T7. Mice received the 2nd treatment 12 hrs after the first injection and were perfused for histology or pull-down assay 12 hrs after the 2nd peptide injection. To detect biotin signal along the spinal cord, parasagittal sections of the fixed spinal cord 2 mm rostral to and caudal to the lesion (4 mm long in total) were cut and processed for biotin-labeled signaling with ABC-DAB-based reaction (Fig. 11A, 4 mice/treatment group). In Fig. 11A, a total of 24 mice were used, including 20 SCI mice and 4 uninjured controls. In separate groups (Fig. 11B, 3 mice/group), blocks of fresh spinal cord 2 mm rostral to and 2 mm caudal to the lesion were harvested immediately after perfusion with cold PBS. BioRP and BioPAPs in the supernatants of spinal cord lysates were precipitated with antibiotin beads (SP-3030, Vector lab) and the levels of biotinylated peptides were detected with ABCDAB- based reactions and measured with a 96-well plate reader at a wavelength of 405 nm. In Fig. 11B, a total of 15 mice (12 SCI mice and 3 uninjured controls) were employed.

2.8. Statistical analysis

SigmaPlot software was used for statistical analysis. Data in graphs are shown as means $\pm$ SEM. The comparisons between multiple groups including the BMS data were analyzed with a repeated measures ANOVA followed by Bonferroni post-hoc tests. The experiments involving a single determination of means between two independent groups were analyzed with Student's *t*-test. Differences between groups with $p < 0.05$ were considered significant. During the experimental procedures, including neurite growth assay, surgery, histology, axon quantification and behavioral evaluations, researchers were blind to the drug treatments.

3. Results

3.1. Effects of PTEN antagonist peptides 1–4 on neurite outgrowth on inhibitory substrates

To test whether PTEN blockade with PAPs promotes neurite outgrowth *in vitro*, we evaluated axon growth of adult DRG neurons cultured in the presence of axon growth inhibitors. Use of mature neurons should be relevant to repair of CNS axonal injuries. We assessed neurite elongation of dissociated DRGs using a spot assay of purified CNS myelin, which contains a number of axon growth inhibitors [10]. Following 5 days of neuronal growth, we measured the length of neurites present on myelin spots. The neurites originated from neuronal perikarya either outside or inside of myelin spots. Although a very high density of neurites was detected on all the coverslips, a limited number of neurites were

present on myelin spots under control conditions (Fig. 1B). In contrast, treatments with PAP1-4 (2.5-10 $\mu\text{g/ml}$), but not with PAP5 (5 $\mu\text{g/ml}$), significantly enhanced the number of neurites on myelin spots (Fig. 1B, D). PAP1-4 showed a similar growth-promoting effect on neurite extension on highly concentrated CNS myelin (200 $\mu\text{g/ml}$). Double immunostaining confirmed the expression of PTEN in the cell bodies and neurites of DRGs (Fig. 1C). To confirm the PTEN-blocking activity of PAPs, we assessed neurite growth of cultured CGNs, a type of central neuron, derived from postnatal 7–9 day mice [31]. Like DRG neurons, CGNs are sensitive to myelin inhibitors [31, 36]. While PAPs 1-4 target different domains of PTEN, they induced a similar degree of neurite extension of CGNs on coverslips evenly coated with purified CNS myelin (50 $\mu\text{g/ml}$, Fig. 1E). Thus, out of the 5 PTEN-targeting peptides, PAPs1-4 at low μM doses significantly promoted axon elongation on inhibitory substrates *in vitro*.

3.2. Binding of PAPs 1-4 to PTEN protein expressed in COS-7 cells

To confirm the interactions between PAPs and PTEN protein, we performed a peptide binding assay for PTEN expressed in non-neuronal COS-7 cells two days after transfection with PTEN DNA or a control plasmid. After incubation with purified biotinylated RP (BioRP) or PAPs (BioPAP, 15 μM), we evaluated peptide binding by measuring biotin signals with ABC-DAB-based color reactions. We detected a weak baseline staining in control DNA-transfected cells probably due to low level of endogenous PTEN expression in COS-7 cells. However, PAPs1-4 exhibited enhanced binding to cells transfected with PTEN (Fig. 2A, B). PAPs1, 3 and 4 revealed higher binding affinity than PAP2 at 15 μM . The increased binding of peptides was due to PTEN expression (Fig. 2C, D) because COS-7 cells transfected with a control plasmid did not display enhanced binding to BioPAP2.

3.3. PTEN expression and S6 and Erk activity in the injured spinal cord

To determine whether spinal cord trauma caused a change in expression of PTEN protein and its downstream signaling pathway, we examined the levels of PTEN, p-S6 and p-Erk in the lesioned spinal cord 1–21 days after injury. We detected enhanced PTEN expression in the rostral spinal cord 3–7 days after SCI although PTEN in the lesion center area was moderately reduced (Fig. 3A, D, G). As the downstream pathway of Akt/PTEN, mTOR kinase is critical for regulating neuronal growth [12]. One of the well-studied mTOR targets is ribosomal S6 kinase 1, which phosphorylates ribosomal protein S6. The cellular levels of p-S6 properly represent mTOR activity [12]. Also, PTEN appears to prevent Erk activation in neurons [37]. Thus, we monitored alterations of p-S6 and p-Erk in the lesioned spinal cord in addition to PTEN. Although we detected p-Erk in uninjured spinal cord (Fig. 3C, F, I), the basic level of p-S6 in uninjured spinal cord was very low (Fig. 3B, E, H). SCI significantly increased the levels of both p-S6 and p-Erk, especially 1–7 days after trauma. These findings suggest inactivation of PTEN and activation of mTOR several days after SCI. The enhanced p-S6 and p-Erk signals detected around the lesion are likely to mainly occur in reactive glial cells.

3.4. Effects of PAPs 1-4 on Akt, S6 and GSK-3 β activities in the spinal cord, sensorimotor cortex and brainstem of mice with SCI

To validate biological activity of PAPs1-4 *in vivo*, we examined the downstream signaling changes of PTEN in injured spinal cord following systemic PAP treatments. Akt, GSK-3 β and S6 are the best-characterized downstream signals of PTEN and measurement of phosphorylated Akt, GSK-3 β and S6 is widely used to determine PTEN and mTOR activities [12, 38, 39]. Akt phosphorylation at residue Ser473 (p-Akt) in the C-terminal hydrophobic motif is necessary for full activation of this signaling and the cellular levels of p-Akt at Ser473 represent its activity. GSK-3 β is inactivated by extracellular signals through phosphorylation of an N-terminal serine residue at Ser-9. The levels of phosphorylated GSK-3 β at Ser9 (p-GSK) are inversely correlated with GSK-3 β activity in neurons [39]. Phosphorylation of S6 ribosomal protein highly correlates with increased translation of mRNA transcripts and cell growth. Thus, we examined the levels of p-Akt, p-GSK and p-S6 in lysates of the spinal cord in adult SCI mice treated with our peptides. The p-Akt and p-GSK levels in the injured spinal cord of RP-treated animals were not significantly altered 48 hrs after lesioning compared to uninjured controls (Fig. 4A, B). In contrast, the spinal cord in SCI mice systemically treated with PAPs1-4 (71 μ g/mouse/day) exhibited higher levels of p-Akt, p-GSK and p-S6 than RP-treated SCI controls (Fig. 4A-C).

PTEN deletion by local injections of adeno-associated virus (AAV)-cre into the sensorimotor cortex induced CST axon growth in PTEN^{flox/flox} mice [14]. Given the penetration properties of TAT-conjugated peptides into the CNS following systemic application, and the breakdown of the blood-brain barrier after SCI [40–42], systemic application of PAPs is also likely to block PTEN activity in neurons at supraspinal levels. We tested this hypothesis by measuring PTEN-related signaling proteins in the sensorimotor cortical area and in the brainstem of SCI mice treated with PAPs or a RP systemically. Compared to no-SCI controls (Fig. 4D, E, G, H), spinal cord trauma did not alter the levels of p-Akt and p-GSK in the cortex and brainstem, but remarkably reduced the levels of p-S6, especially in the cortex (Fig. 4F). Similarly, crush injury of optic nerve axons in mice reduced p-S6 level in their neuronal cell bodies (retina ganglion cells) [12]. Importantly, systemic treatments with PAPs enhanced the levels of p-Akt, p-GSK and p-S6 in the sensorimotor cortex and in the brainstem (Fig. 4D-I). Therefore, treatments with PAPs1-4 activate Akt and mTOR, but inactivate GSK-3 β in the lesioned spinal cord and supraspinal structures. These results support that systemic treatments with PAPs efficiently block PTEN activity in the brain, as well as in the spinal cord.

3.5. Effect of systemic PAP2 and PAP4 treatments on growth of raphespinal and CST fiber tracts

We next studied whether PTEN suppression with PAPs would stimulate axon growth and functional recovery following dorsal over-hemisection SCI at T7, a model that typically transects ~67% of the spinal cord, including the dorsal, dorsolateral and lateral CST fibers, but spares some ventral spinal cord [33]. Because PAPs1-4 exhibited similar growth-promoting actions *in vitro* (Fig. 1), we chose two peptides, PAPs2 and 4 (71 μ g/mouse/day), and evaluated their axon growth-promoting potential following daily subcutaneous injections for 14 consecutive days initiated 2 days after SCI. This dose is anticipated to

achieve ~3.6 µg/ml of peptide in the animal's body (based on 20g body weight per mouse and 24 hr period of stability after injection), which is close to the optimal doses of these peptides (6.7-10 µg/ml) for promoting neurite outgrowth *in vitro* (Table 1). We have confirmed the biological activity of this dose of PAPs 2 and 4 by measuring several signaling proteins downstream of PTEN (Fig. 4). Based on the time windows of primary alterations of PTEN-related pathways (Fig. 3), we delivered the PAPs 2–16 days after SCI.

We characterized descending serotonergic fibers from transverse sections of the spinal cord by immunostaining 5-HT. The 5-HT tracts contribute to behavioral recovery after SCI [43–45]. Five weeks after dorsal over-hemisection, the density of 5-HT fibers was reduced in the caudal spinal cord compared to sections rostral to the lesion [34]. However, in SCI mice treated with PAPs2 and 4, the density of 5-HT fibers in the spinal cord 5–7 mm caudal to the lesion was significantly increased compared to RP-treated SCI controls (Fig. 5A, C), although the density of 5-HT fiber 5–7 mm rostral to the lesion was similar among different groups. Parasagittal sections containing the lesion site also displayed projection of a greater number of 5-HT axons into the caudal spinal cord in the PAP-treated mice (Fig. 5B, D and Fig. 6). In particular, we detected fasciculated 5-HT fibers in the spinal cord caudal to the injury in PAP-treated mice (Fig. 6E and I), but not in RP-treated SCI controls. Because dorsal over-hemisection spared a small portion of 5-HT axons in the ventral spinal cord [33, 34], the increased 5-HT fibers detected in the caudal spinal cord of PAP-treated mice could be derived from regenerating fibers of lesioned tracts and/or sprouting of spared fibers.

PTEN deletion has been reported to promote compensatory sprouting and impressive regeneration of CST axons in SCI mice [14]. We evaluated the integrity of CST tracts, which appear to contribute to fine voluntary movements of the limbs [46]. In RP-treated animals, we observed the prominent dorsal CST axons and a number of ipsilateral dorsolateral CST fibers in transverse sections rostral to the lesion (Fig. 7A). The BDA-labeled dorsal CST axons sent many collateral sprouts into the gray matter, particularly into the ventral region. The sprouting was primarily confined to the side of the spinal cord contralateral to tracer injection. By contrast, in PAP-treated SCI animals, transverse sections rostral to the lesion exhibited a different pattern of CST axon distribution, with a higher density of BDA-labeled fibers outside of the dorsal CST area. The ectopic CST fibers extended throughout the gray matter in most PAP-treated mice and some fibers reached the lateral and dorsolateral white matter. Moreover, a greater number of CST sprouts extended into the gray matter of the opposite side of the spinal cord, i.e., ipsilateral to the side of tracer injection. Quantification of the collateral CST sprouts from transverse sections indicated a 2-fold increase in the PAP-treated animals (Fig. 7B).

A critical issue is whether pharmacological PTEN blockade stimulates growth of CST axons into and beyond the lesion area after SCI. We examined BDA-traced CST fibers in parasagittal sections around the lesions, which were visualized by GFAP staining. CST fibers terminated a few mm rostral to the lesions and did not regenerate into the caudal spinal cord in RP-treated mice (Fig. 8A). We did not observe any ventral CST axons in these mice. However, sections from PAP-treated mice displayed a higher density of CST sprouts immediately rostral to the injury and some sprouts approached to lesion epicenter (Fig. 8B, C). A small number of CST axons bypassed the lesion epicenter and projected into the

caudal spinal cord (Fig. 8B). These fibers exhibited highly branching patterns in the gray matter areas and were only observed in PAP-treated mice. Counts of BDA-labeled individual axons and axonal arbors from parasagittal sections indicate the increased numbers of CST axons in the caudal spinal cord of the mice treated with either PAP2 or PAP4 (Fig. 8D). GFAP staining around the lesion indicates similar transection injury in these animals (bottom images in Fig. 8A, B and C).

Quantification of the lesion depth from multiple sections of each mouse demonstrates a similar degree of injury in the 3 groups of SCI mice (Fig. 9). Thus, only a few CST axons were observed in the caudal spinal cord, although PAP treatments stimulated CST sprouting rostral to the lesion.

3.6. Effect of PAP treatments on recovery of locomotor function

We evaluated functional recovery in these SCI mice by measuring the standardized BMS locomotor scores, grid walk and footprints of the hindpaws [33, 34, 47]. Four hours and two days after dorsal over-hemisection, mice showed no observable or slight-extensive hindlimb movement (BMS: 0–3) and there are no differences among the 3 groups (Fig. 10A). For example, RP, PAP2 and PAP4 groups have the BMS scores of 0.44 ± 0.24 , 0.6 ± 0.08 and 0.5 ± 0.4 four hours after injury, respectively, indicating a similar degree of injury among the 3 groups of mice. The RP-treated controls exhibited partial recovery several weeks after SCI, probably due to short-range sprouting of spared fibers and reorganization of segmental circuitry, including propriospinal re-connections [48]. The recovery reached a stable level by ~3 weeks after lesioning and mice generally had some coordination and hindpaw rotation when making initial contact with the testing surface and on lift off. In contrast, the PAP-treated mice exhibited increased BMS scores 2–5 weeks after SCI and most had consistent coordination and parallel paw position when contacting the testing surface (Fig. 10A). We also performed a grid walking test by evaluating the incidence of the hindlimbs slipping below the grid plane from videotapes watched at a slow speed 5 weeks after SCI. The RP-treated SCI mice made numerous errors by misplacing their hindpaws and falling into grid holes, but the PAP-treated mice made significantly fewer errors by correctly placing their hindpaws on the grid (Fig. 10B). Moreover, PAP4-treated mice had enhanced stride length of the hindpaws measured from footprints 5 weeks after injury (Fig. 10C, D). Together, PAP2 and PAP4 treatments significantly improve growth of descending fiber tracts and recovery of locomotor function in adult rodents with SCI.

3.7. Penetration of systemically administered PAPs into lesioned spinal cord

Following SCI, there is breakdown of the blood-brain barrier around the lesion for weeks [41, 42], which could permit penetration of peptides into the CNS following systemic administration. Conjugation of the TAT sequence to PAPs should further facilitate their penetration into the CNS and cells [40]. To confirm access of PAPs into injured spinal cord, we evaluated distribution of BioPAP2 and BioPAP4 in mice with T7 dorsal transection by initiating the treatments either 2 or 28 hours after injury and examining biotin-labeled signals one day after subcutaneous peptide delivery. We found stronger biotin signals in both gray matter and white matter of the spinal cord in SCI mice treated with BioPAP2 and BioPAP4 (Fig. 11A), especially in the areas of cell bodies and axon cylinders. In contrast,

we detected low levels of biotin signals in no-SCI mice treated with BioPAP2 and in SCI mice treated with bioRP compared to no-injury and injury controls, suggesting moderate access of TAT-conjugated PAP2 into un-injured CNS and partial washout of biotinylated RP in SCI mice during animal perfusion and tissue processing. Moreover, we confirmed the considerable diffusion of BioPAP2 and BioPAP4 into the lesioned spinal cord by precipitating biotin-conjugated peptides in the lysates of fresh spinal cord tissue samples in one set of SCI mice (Fig. 11B). Because mice were perfused with cold PBS for about 5 min before spinal cord tissue collection, the detected peptide signals should mainly come from the intracellular compartments. Thus, PTEN peptides penetrated into the lesioned spinal cord following systemic application.

4. Discussion

Recent work using conditional knockout mice suggested that PTEN is potentially important for controlling the regenerative capacity of injured axons [12, 14, 16]. Transgenic deletion of PTEN is scientifically very powerful, but it only targets a single neuronal population via local injections of AAV-cre, and not the diffusely-dispersed neurons, such as 5-HT neurons or propriospinal interneurons known to contribute to functional recovery after SCI [43–45, 48]. PTEN deletion has not been shown to contribute to functional return after CNS injury and it is not feasible for treating patients. In this study, we identified selective antagonist peptides for PTEN by targeting its PIP2, ATP-type, PDZ and C-terminal tail domains. We demonstrated their binding to PTEN over-expressed in COS7 cells, promotion on neurite outgrowth and blockade of PTEN downstream signals. Systemic PAP treatment stimulated growth of descending serotonergic fibers in the caudal spinal cord of SCI rodents. We found enhanced sprouting of CST axons rostral to the lesion and a limited degree of growth of CST axons in the caudal spinal cord. Importantly, PAP treatments enhanced recovery of locomotor function after SCI. Thus, we developed systemically deliverable small peptides to block PTEN selectively, which were able to promote regrowth of injured CNS axons following post-injury administration. Our peptide approach shows benefits in several areas where the transgenic approach is highly deficient.

4.1. Axon growth in adult SCI rodents following systemic PTEN peptide treatments

Like most other reports on axonal growth, the present study employed a partial injury model because of the experimental difficulties posed by complete spinal transection. In these circumstances, the existence of spared axons make it very difficult to be certain that an increase in density of anterogradely labeled fibers in the caudal spinal cord represents regeneration of severed axons, sprouts of spared fibers, or both. Thus, even though we have attempted to characterize the nature of the growth as regenerative or sprouted, some caution must be maintained regarding interpretation. This caveat also would apply to many lesions in the brain, such as stroke and brain injury.

The intracellular PI3K/Akt/mTOR pathway appears to be essential for regulating axon growth by various cytokines generated by glial cells [16]. Deletion of PTEN or TSC1 stimulated growth in injured RGCs and descending CST neurons by activating mTOR [15, 16], a key signal to regulate protein synthesis during cell growth. Thus, PTEN and mTOR

are critical signals for controlling growth of mature neurons. PTEN blockade with small peptides significantly stimulated growth of descending serotonergic tracts (Fig. 5, 6), which contribute to locomotor recovery after SCI [43–45].

PTEN deletion in the motor cortex promoted CST regeneration in conditional KO mice with SCI [14]. Given that systemic PAP treatments increased the levels of phosphorylated Akt, GSK and S6 in multiple regions of the CNS after SCI, including the sensorimotor cortical area, brainstem and lesioned spinal cord (Fig. 4), PTEN inhibition at different neuronal levels might contribute to the enhanced axonal growth in axotomized neurons. In particular, both PTEN inhibition in the neuronal bodies of the brain and blockade of locally-enriched PTEN at growth cones of the spinal cord are likely to regulate axon elongation by altering the activity of the PI3K/Akt/GSK3 signaling pathway [49, 50]. PTEN accumulated rapidly at the growth cone membrane following Sema3A application and contributed to Sema3A-induced growth cone collapse in neurons [49].

Many groups have reported a limited degree of sprouting or regenerative growth of injured CST axons by inhibiting myelin-associated inhibitors and scar-rich proteoglycans [5, 34], applying neurotrophic factors [51], suppressing intracellular RhoA and GSK-3 β signals [31, 52], transplanting grafts, or combining different strategies [53–55]. In contrast, the He group demonstrated greater regeneration of injured CST axons in addition to compensatory sprouting in conditional PTEN knockout mice [14]. Thus, we evaluated the integrity of CST axons in mice with a dorsal transection, a model that was used in that study [14]. Although we detected sprouting of transected CST axons rostral to the lesion, almost all the sprouts terminated before reaching the lesion epicenter and only a limited number of BDA-labeled axons projected into the caudal spinal cord (Figs. 7 and 8). Thus, inhibitory factors associated with scar around the lesion appear to contribute to the growth failure of CST axons in PAPtreated mice. It has been reported that mTor activation increases reactive astrogliosis in rodents with ischemic SCI [56]. Although we did not observe obvious changes in the scar tissues 5 weeks after injury (Fig. 6, 8 and 9), it is also possible that activation of the Akt/mTor pathway following PAP treatment stimulates reactive gliosis and alters the inhibitory properties of scar tissues around the lesion. It is less clear how regenerating CST axons crossed scar tissues following PTEN deletion in conditional knockout mice [14].

The reasons for different CST growth between this study and the previous report using transgenic mice are not clear [14]. By using several approaches, we have confirmed high efficiency of our sequence-targeting peptides for blocking PTEN signaling pathway. PAPs1–4 bound to PTEN protein, blocked its downstream signals and promoted significant neurite extension in adult and postnatal neurons cultured on inhibitory substrates (Fig. 12 and 4). The injury models used in two studies were unlikely to cause notable differences because both groups employed mid-thoracic dorsal transection. It is possible that we transected the spinal cord to a greater degree than the He group (1.0 vs. 0.8 mm), but Liu et al. also reported robust CST axon regeneration in a more severe crush injury model. In both studies, axonal injuries were induced in adult mice (> 2 months) and animal ages were unlikely to cause discrepancy for CST growth. Nevertheless, the time periods for suppressing PTEN activity were very distinct between the two studies. To develop a feasible

therapy for patients, we initiated PAP treatments 2 days after axon lesion. In contrast, Liu et al. began deletion of PTEN 4–8 weeks before injury by injecting AAV-Cre into the sensorimotor cortex of neonatal or 4-week old mice. Thus, mTOR activation in immature animals and/or several weeks before axotomy is probably a major determinant of robust CST axon regeneration. Notably, we cannot exclude the possible higher efficiency of transgenic method by locally injecting AAV-Cre into PTEN^{flox/flox} mice, which affected ~25% of the cortical area determined by measuring Cre-dependent expression of placental alkaline phosphatase [14].

4.2. Improved functional recovery in SCI mice treated with PAPs

PTEN inhibition following PAP treatment is probably the molecular basis for the increased axon growth and improved behavioral recovery in this study. Our peptides selectively bind PTEN expressed in transfected mammalian cells and block PTEN activity (Fig. 2 and 4) due to the sequence overlap with certain domains of PTEN. We have also confirmed the biological activity of our PAPs by detecting enhanced DRG neurite growth, diffusion into injured spinal cord, enhanced PTEN-related signaling proteins in the CNS, and increased axonal growth in the caudal spinal cord following PAP treatments (Fig. 1, 4–8, 11). In contrast, the previously reported protective and beneficial effects of bpV in SCI rodents [19, 20] or in a stroke model [57] might be attributed to inhibition of PTEN as well as other tyrosine phosphatases due to less selectivity of bpV [21]. bpV compounds are able to block many phosphatases, including Cdc25A, Yersinia outer protein H, T cell protein tyrosine phosphatase and leukocyte common antigen-related phosphatase (LAR) [21]. Among them, LAR has been shown to mediate axonal growth as a functional receptor of scar-sourced inhibitors [33].

The enhanced supraspinal sprouting and/or regeneration into the caudal spinal cord probably contributed to the functional improvement in PAP-treated mice although segmental mechanisms appeared to play a major role in the locomotor recovery of control SCI mice due to lack of obvious axon regeneration. Systemic PAPs induced axonal growth in the spinal cord below the lesion and significantly increased the number of raphespinal fibers. We only detected a very small number of CST axons in the caudal spinal cord, but the actual regenerating CST fibers might be much greater than that observed because anterograde tracing with BDA only labels a small fraction of total CSTs [58]. Given that propriospinal relay connections have been shown to bypass injury sites and mediate spontaneous functional recovery in adult rodents with a partial spinal cord transection [48], it is also possible that the increased CST sprouts rostral to the lesion contribute to functional recovery through establishment of propriospinal neuronal relays. It is likely that sprouting, regeneration and reorganization of other descending fibers, such as rubrospinal axons, also contribute to enhanced functional recovery due to widespread expression of PTEN in the CNS [59].

PTEN peptides might promote recovery also through other mechanisms, such as protecting injured spinal cord tissues, enhancing myelination and stimulating proliferation and differentiation of endogenous stem cells. PAP treatments increased the BMS scores 2–5 weeks after SCI (Fig. 10A). The early better recovery might be attributed to neuroprotection

and short-range axon sprouting following PTEN suppression. In fact, PTEN deletion protected RGCs from death after axotomy [12]. Intrathecal bpV treatment protected long-projecting axons, myelin and blood vessels and improved functional recovery in SCI rats [19]. Systemic bpV, which elevated the levels of phosphorylated Akt and S6 in the lesioned spinal cord, reduced neuronal death and cavity formation, increased tissue sparing, and improved functional recovery in SCI rats [20]. PTEN deficiency resulted in increased myelin thickness and profound and progressive enlargement of most white matter tracts in mice [60]. Moreover, PTEN has been shown to regulate development and proliferation of CNS stem cells or progenitors [61].

Activation of mTOR several weeks before injury stimulated extensive CST regeneration [14], suggesting that we may achieve a greater extent of axon regeneration and functional recovery by initiating peptide treatment at an earlier time point, such as 2–3 hours after lesioning, a time frame also feasible for treating many acutely-injured patients. Moreover, we can easily combine our peptides with other effective strategies, such as small peptides to block scar-mediated suppression [33], to target multiple mechanisms for neuronal growth failure. Of note, PTEN is expressed in different types of cells and regulates many fundamental cellular functions in the body, including apoptosis, migration, proliferation and growth. Dysregulation of PTEN has been linked to several disorders, such as tumorigenesis and epilepsy [62–64]. In particular, loss of PTEN function has been shown to contribute to tumor formation in prostate, skin and intestine. The risk of tumor formation may be slight when PTEN inhibition with PAP treatment is limited to 2–3 weeks [64]. Indeed, we did not observe tumors or other obvious signs of side effects in our animal studies. Nevertheless, it would be important to perform a safety assessment in a clinically-relevant model before moving PAPs to clinical trial.

5. Conclusion

We have designed selective PTEN-blocking peptides and demonstrated that they significantly promote axonal growth and behavioral recovery after CNS injuries in adult mammals. Subcutaneous injections of our PAPs initiated two days after lesions may provide a basis for improving axon growth and locomotor recovery in patients, given the wide applications of FDA-approved peptide drugs in humans. Thus, systemically deliverable PAPs might have therapeutic advantages over more invasive therapies and facilitate development of a successful therapy for CNS axonal injuries.

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Fig. 1.

PAP1-4 increased neurite outgrowth of DRG neurons and CGNs cultured on purified CNS myelin. A, Schematic indicates the targeting sequence of PAP1-5 and the targeted functional domains of PTEN. B, Adult DRG neurons were cultured for 5 days on coverslips spotted with CNS myelin in the center area and stained with Tuj1. Very few neurites were detected on myelin spots in cultures treated with DMSO or RP. However, a greater number of neurites were detected on myelin spots in cultures treated with PAPs1- 4, but not PAP5. At high magnification (right images), a number of clustered neurites (red) were seen on myelin

spots in PAPs1-4-treated neurons. Scale bars: 200 μm in left images, 50 μm in right images. C, High level of PTEN protein was detected in the bodies and neurites of cultured DRGs and was colocalized with neuronal marker Tuj1. D, Quantification indicates longer axon length of DRG neurons treated with PAPs1-4, but not PAP5, on myelin spots. The numbers indicate means $\pm$ SEM from 6–10 myelin spots per group. The differences indicated were compared to RP-treated controls. E, Dissociated CGNs cultured from postnatal day 7 mice were treated with vehicle DMSO or PAPs1-5 in the absence or presence of CNS myelin (Mye, 50 $\mu\text{g}/\text{ml}$). Neurite outgrowth per CGN was manually traced and quantified 24 hrs after cell plating using NIH and Photoshop software. The numbers in graph indicate means $\pm$ SEM from 3-4 experiments. The differences indicated are compared to vehicle controls. Treatment with PAPs1-4, but not with PAP5, significantly increased neurite length of CGNs cultured on CNS myelin. In D and E, * $p < 0.05$, ** $p < 0.01$, *Student's t-test*.

Fig. 2.

PAPs1-4 significantly bind PTEN protein in transfected COS-7 cells. A, COS-7 cells were transfected with control or PTEN plasmid. The control plasmid contains DNA that does not express a gene product. Forty-eight hrs after transfection, cells were incubated with purified PAP2, BioRP or BioPAPs1-4. Peptide-binding signals were detected by staining biotin with ABC-DAB-based color reactions. Scale: 50 μ m. B, Bar graph indicates the quantified peptide binding signals in each group with a 96-well plate reader at 405 nm. The differences indicated are compared to BioRP-treated control (**<0.01, *Student's t* test, n = 6 wells in

each group). C, Western blot confirmed the expression of PTEN in transfected COS-7 cells. D, The representative images indicated expression of PTEN in COS-7 cells. Scale: 25 μ m.

Fig. 3.

Traumatic injury altered PTEN expression and increased S6 and Erk activity in the lesioned spinal cord. The levels of PTEN (A, D, G), p-S6(active form, B, E, H) and p-Erk (active form, C, F, I) were analyzed with Western blots from tissue lysates of fresh spinal cord blocks 2–6 mm rostral to lesion (A, B, C), at lesion site (2 mm rostral to and caudal to injury center, containing the lesion, D, E, F), 2–6 mm caudal to the lesion (G, H, I), or from the same level of spinal cord in no-SCI controls. Actin was also detected in the same blots (bottom bands). Bar graphs below the bands indicate densitometric analysis of PTEN, p-S6

and p-Erk from multiple animals. Trauma significantly increased PTEN levels rostral to the lesion although there was a slight reduction at the lesion site. SCI also significantly enhanced levels of p-S6 and p-Erk several days after lesion. Means $\pm$ SEM were shown. n=3 from 3 mice in each group, *p < 0.05 compared to no-SCI controls.

Fig. 4.

PAPs1-4 enhance Akt and S6 activity and suppress GSK-3 β activity in the spinal cord, sensorimotor cortex and brainstem in mice with SCI. Western blots indicate the levels of phosphorylated Akt (p-Akt, active form, A, D, G), GSK-3 β (p-GSK, inactive form, B, E, H) and S-6 (p-S6, C, F, I) in the supernatants of fresh spinal cord tissues (2 mm rostral to and 2 mm caudal to dorsal transection), sensorimotor cortex and brainstem 3 days after a lesion at T7 (or the same level of spinal cord in no-SCI controls). Although the total protein levels (t-Akt, t-GSK or actin) were similar in the supernatants, treatments with PAPs1-4 significantly

increased the levels of p-Akt, p-GSK and p-S6, especially in the spinal cord and cortical area. Bar graphs below the bands indicate densitometric analysis of p-Akt, p-GSK and p-S6 levels in the lesioned spinal cord, sensorimotor cortex and brainstem (n= 4 mice per group). P1: PAP1; P2: PAP2; P3: PAP3; P4: PAP4. Total Akt, GSK-3 β or actin were detected from the same tissue samples via immunoblotting (bottom bands). Treatment with PAPs1-4 enhanced the levels of p-Akt, p- GSK and p-S6. Means $\pm$ SEM were shown and the differences indicated were compared to RP-treated SCI controls. * p < 0.05, ** p < 0.01.

Fig. 5.

Systemic treatments with PAP2 and PAP4 enhance serotonergic fibers in the caudal spinal cord of SCI mice. **A**, Transverse sections of the spinal cord 5–7 mm caudal to the lesion at upper lumbar levels displayed reduced 5-HT fibers 5 weeks after dorsal transection at T7, but treatments with PAP2 or PAP4 increased the density of serotonergic fibers in central and ventral part of the spinal cord compared to RP-treated mice. Scale: 20 μ m. **B**, Camera lucida drawings of 5-HT fibers from all parasagittal sections of each representative mouse are shown from the RP, PAP2 and PAP4-treated animals (2 example mice per group). Scale:

200 μ m. C, Serotonergic fiber length was measured from the gray and white matter in dorso-central areas and from the gray matter in the ventral horn of the spinal cord 5–7 mm caudal to the lesion in transverse sections. The animals from RP group show a number of 5-HT axons in the distal spinal cord, indicating the spared serotonin axons following dorsal over-hemisection. In contrast, subcutaneous injections of PAP2 or PAP4 started 2 days after SCI resulted in a greater number of 5-HT axons in the distal spinal cord, particularly in the ventral areas. The dorsal is up in all these sections. D, Serotonergic fiber number was counted from all the parasagittal sections of the spinal cord 0–3.2 mm caudal to the lesion epicenter in each group. In C and D, means $\pm$ SEM were shown and the differences were compared to RP-treated SCI controls. * $p < 0.05$, ** $p < 0.01$.

Fig. 6.

PAP2 and PAP4 treatments enhance the number of 5-HT axons in the caudal spinal cord of SCI mice. Immunostaining images for 5-HT (red) from parasagittal sections around the lesion (arrows) show serotonin axons in the control, PAP2 and PAP4 animals. The mouse from RP group shows termination of 5-HT axons around the lesion and low density of 5-HT fibers in the spinal cord caudal to the lesion (A, C, D). In contrast, systemic treatments with PAP2 (E, G, H) and PAP4 (I, K, L) started 2 days after SCI induce higher density of 5-HT axons in the caudal spinal cord. The dorsal is up and caudal is on the right in all these

sections. Immunostaining images for GFAP (green) in B, F, and J show the lesion areas (arrows) in sections A, E and I. Scales: 200 μ m in A, B, E, F, I and J; 25 μ m in C, D, G, H, K and L.

Fig. 7. PAP2 and PAP4 treatments induce CST sprouting rostral to the lesion in SCI mice. A, Transverse sections 5–7 mm rostral to the lesion display a similar degree of dorsal CST labeling in SCI mice. The midline is to the left and dorsal is up in all the sections. Increased density of ectopic sprouts lateral to the dorsal CST was detected in the PAP2 and PAP4-treated animals. Scale: 50 μ m. B, Ectopic CST sprouts outside of the dorsal CST axons were quantified from transverse sections 5–7 mm rostral to injury (means $\pm$ SEM).

Fig. 8.

PAP2 and PAP4 treatments induce CST sprouting and limited CST growth caudal to the lesion in SCI mice. A, Parasagittal sections around the lesion in SCI controls indicated termination of dorsal CST axons and a number of CST sprouts immediately rostral to the lesion. B, Similar sections around the lesion in PAP2-treated mice indicated higher density of CST sprouts immediately rostral to the lesion. A small number of CST sprouts grew into the distal spinal cord. C, Parasagittal sections from a PAP4- treated mouse near the lesion indicated a very high density of CST sprouts immediately rostral to the lesion, but they

terminated rostral to the lesion epicenter. The bottom images in A, B and C indicated GFAP staining and lesion areas in these sections. Scales in A, B and C: 200 μm for the top and bottom images, and 20 μm for the middle images. D, BDA-labeled individual CST axons and CST arbors were counted from all the parasagittal sections of the spinal cord 0–3.5 mm caudal to the lesion in each animal (means $\pm$ SEM).

Fig. 9.

Parasagittal sections immunostained for GFAP around the lesion indicate similar degree of transection lesions among 3 groups of SCI mice. A, Multiple representative images of GFAP staining from 3 animals display similar transection injury in RP, PAP2 and PAP4 groups. Scales: 250 μ m. B, Quantification of the transection depth from multiple parasagittal sections greater than 1 mm in dorsoventral diameter indicates similar degree of transection injury in 3 groups of SCI mice.

Fig. 10.

Systemic treatments with PAP2 and PAP4 improve locomotor recovery in SCI mice. A, Graph indicates the locomotor BMS scores in RP- (black color), PAP2- (red) or PAP4-treated (green) mice following T7 dorsal transection SCI. B, Graph indicates grid walk errors in RP (black), PAP2 (red) or PAP4-treated (green) mice 5 weeks after SCI. C, Graph indicates stride length of the hindlimbs 5 weeks after injury in 3 groups. D, The representative footprints of the hindlimbs are shown from 3 groups of mice. Means $\pm$ SEM were shown and the differences indicated were compared to RP-treated SCI controls. * $p < 0.05$, ** $p < 0.01$.

Fig. 11.

Biotinylated PAP2 and PAP4 penetrate into the spinal cord in mice with T7 dorsal transection. Two or 28 hours after a dorsal over-hemisection injury, mice started to receive vehicle or peptide injections. One day after the treatments, SCI mice were perfused and biotin labeling signals were examined from parasagittal sections of fixed spinal cord around the lesion (2 mm rostral and 2 mm caudal to injury, 4 mm long). Treatments with biotinylated PAP2 and PAP4 induced stronger staining signals for biotin in the gray matter and white matter of the spinal cord than control animals (A). Scale: 25 μ m. Biotin-conjugated peptides were pulled down from the lysates of fresh spinal cord tissue with anti-biotin beads in SCI mice receiving 2-hr delayed treatments (B). Biotin signals were measured with ABC-DAB color reactions. Higher biotin signals were detected in mice treated with BioRP, BioPAP2 (BioP2) or BioPAP4 (BioP4), but not with non-biotin-labeled PAP2 (P2), suggesting penetration of peptides into the lesioned spinal cord. Means $\pm$ SEM

were shown and the differences indicated were compared to PAP2- treated SCI controls. * p < 0.05, n=3 mice per group.

Table 1

Summary of the sequence-targeting peptides for PTEN

Name	Targeting domain	Sequence	AA	MW	Optimal dose (µg/ml)
PAP1	PIP2	Mtaiikeivsmkrr + TAT	26	3297	2.5
PAP2	ATP-type B	khknhykiynlcae + TAT	25	3243	10
PAP3	ATP-type A	lhckagkgrtgvmic + TAT	26	3056	6.7
PAP4	C-terminal tail	tveepspnpeassstsvpd + TAT	30	3416	6.7
PAP5	PDZ	penepfdedqhsqitkv + TAT	28	3824	5.0

AA: Amino acid number. TAT Sequence: GRKKRRQRRRPQ

Table 2

Coordinates for BDA injections for CST tracing (unit: mm; 0.9 mm depth; 1 μ l/spot).

Injection sites	From Bregma	From midline
Spot 1	0	1
Spot 2	−0.5	0.5
Spot 3	−0.5	1
Spot 4	−0.5	1.5
Spot 5	−1	1