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Research Paper

# Modest enhancement of sensory axon regeneration in the sciatic nerve with conditional co-deletion of PTEN and SOCS3 in the dorsal root ganglia of adult mice

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ABSTRACT

Axons within the peripheral nervous system are capable of regeneration, but full functional recovery is rare. Recent work has shown that conditional deletion of two key signaling inhibitors of the PI3K and Jak/Stat pathways—phosphatase and tensin homolog (PTEN) and suppressor of cytokine signaling-3 (SOCS3), respectively—promotes regeneration of normally non-regenerative central nervous system axons. Moreover, in studies of optic nerve regeneration, co-deletion of both PTEN and SOCS3 has an even greater effect. Here, we test the hypotheses (1) that PTEN deletion enhances axon regeneration following sciatic nerve crush and (2) that PTEN/SOCS3 co-deletion further promotes regeneration. PTEN$^{fl/fl}$ and PTEN/SOCS3$^{fl/fl}$ mice received direct injections of AAV-Cre into the fourth and fifth lumbar dorsal root ganglia (DRG) two weeks prior to sciatic nerve crush. Western blot analysis of whole cell lysates from DRG using phospho-specific antibodies revealed that PTEN deletion did not enhance or prolong PI3K signaling following sciatic nerve crush. However, PTEN/SOCS3 co-deletion activated PI3K for at least 7 days post-injury in contrast to controls, where activation peaked at 3 days. Quantification of SCG10-expressing regenerating sensory axons in the sciatic nerve after crush injury revealed longer distance regeneration at 3 days post-injury with both PTEN and PTEN/SOCS3 co-deletion. Additionally, analysis of noxious thermosensation and mechanosensation with PTEN/SOCS3 co-deletion revealed enhanced sensation at 14 and 21 days after crush, respectively, after which all treatment groups reached the same functional plateau. These findings indicate that co-deletion of PTEN and SOCS3 results in modest but measureable enhancement of early regeneration of DRG axons following crush injury.

## 1. Introduction

Peripheral nerve damage occurs in approximately 5% of all trauma cases (Noble et al., 1998). Given that there are roughly 40 million trauma cases in the United States each year (National Hospital Ambulatory Medical Care Survey: 2011, n.d.), this equates to approximately 2 million peripheral nerve injuries annually. Unfortunately, while axons in the peripheral nervous system are capable of regeneration, full functional recovery is rarely achieved. Together, the alarming incidence of peripheral nerve damage and the lack of consistent recovery highlight the need for new therapeutic interventions aimed at improving axon regeneration in the periphery.

The environment of the damaged peripheral nerve is conducive to axonal regeneration. Schwann cells de-differentiate and clear debris along with macrophages (reviewed in Navarro et al., 2007). These de-differentiated Schwann cells provide neurotrophic and physical support for regenerating axons. Additionally, neurons of the peripheral nervous system have an intrinsic capacity for regeneration. Even so, regeneration in the periphery may be limited by a number of factors including severity of the injury, nerve gaps that require bridging, distance to the peripheral target, age, and a lack of specific innervation (de Ruiter et al., 2008; Fu and Gordon, 1995).

Recent studies have been aimed at improving intrinsic regenerative capacity by deleting the signaling inhibitors phosphatase and tensin homolog (PTEN) and suppressor of cytokine signaling 3 (SOCS3). Conditional genetic deletion of PTEN promotes regeneration of optic nerve axons (Park et al., 2008). Similarly, conditional deletion or shRNA-mediated suppression of PTEN promotes regeneration of corticospinal tract axons (Danilov and Steward, 2015; Lewandowski and Steward, 2014; Liu et al., 2010). These findings stand out due to the

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**Table 1**
Animal use.

| Analysis (n) | Genotype (n) | DRG injection (n) | Injury (n) |
|---|---|---|---|
| Immunofluorescence within the DRG (3) | PTEN$^{fl/fl}$ (3) | None | None (3) |
| Western blot of whole DRG lysates for signaling after nerve crush (72) | PTEN$^{fl/fl}$ (30) | None | None (5)<br>SNC (25) |
| | PTEN/ SOCS3$^{fl/fl}$ (42) | None | None (7)<br>SNC (35) |
| Western blot of whole DRG lysates for signaling after deletion and subsequent nerve crush (96) | PTEN$^{fl/fl}$ (43) | None<br>AAV-GFP<br>AAV-Cre (36) | None (4)<br>SNC (3)<br>None (12)<br>SNC (24) |
| | PTEN/ SOCS3$^{fl/fl}$ (53) | None<br>AAV-GFP<br>AAV-Cre (36) | None (8)<br>SNC (9)<br>None (12)<br>SNC (24) |
| Immunofluorescence within the sciatic nerve (12) | PTEN$^{fl/fl}$ (9) | Sham<br>AAV-GFP<br>AAV-Cre | SNC (3)<br>SNC (3)<br>SNC (3) |
| | PTEN/ SOCS3$^{fl/fl}$ (3) | AAV-Cre | SNC (3) |
| Behavioral recovery (43) | PTEN$^{fl/fl}$ (23) | Sham<br>AAV-GFP<br>AAV-Cre | SNC (5)<br>SNC (7)<br>SNC (11) |
| | PTEN/ SOCS3$^{fl/fl}$ (20) | Sham<br>AAV-GFP<br>AAV-Cre | SNC (6)<br>SNC (4)<br>SNC (10) |
| Total N = 226 | | | |

SNC = sciatic nerve crush.

complete lack of axonal regeneration of these CNS axons under normal conditions. Additionally, co-deletion of PTEN and SOCS3 further increases axon regeneration in the optic nerve (Sun et al., 2011). While there is evidence to suggest that manipulation of the PI3K pathway may enhance regeneration in the periphery (Abe et al., 2010; Christie et al., 2010), conditional deletion of PTEN in dorsal root ganglia sensory neurons has not been investigated. Furthermore, to the best of our knowledge, there have been no studies involving co-suppression of PTEN and SOCS3 on axon regeneration in peripheral nerves. Thus, the goal here is to assess whether PTEN deletion alone or PTEN/SOCS3 co-deletion will enhance regeneration of peripheral sensory axons that already have a capacity for regeneration.

## 2. Materials and methods

### 2.1. Animals

A total of 244 adult male mice (3- to 5-months-old at first surgery) were used in this study. A complete breakdown of the number of animals from each genetic background used in the experiments performed here is presented in Table 1. Animals were obtained from two local colonies of transgenic mouse lines bred in pairs as homozygous mutants. PTEN-floxed mice (PTEN$^{fl/fl}$; n = 123) on a 129S background contain flox sequences around exon 5 of the PTEN gene. PTEN/SOCS3-floxed mice (PTEN/SOCS3$^{fl/fl}$; n = 118) on a B6/129S background were generated from breeding pairs donated by Dr. Zhigang He, Children's Hospital, Boston. Cre-reporter ROSA$^{tdT}$ mice (n = 3) contain a floxed stop cassette upstream of the coding sequence for tdT.

Animals were group housed with littermates in a temperature controlled vivarium on a 12 h light-dark cycle. All procedures were approved by the University of California Irvine Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition, 2011).

### 2.2. Surgical procedures

Sciatic nerve crushes were performed under ketamine/xylazine anesthesia (10 and 1 mg/kg, respectively) administered intraperitoneally (i.p.). A dorsal incision was made on the left side from hip to knee so the biceps femoris and gluteus superficialis could be split by blunt dissection, exposing the left sciatic nerve. The sciatic nerve was separated from the surrounding muscle up to the medial junction of the biceps femoris and gluteus superficialis, where the sciatic nerve turns away from midline. The nerve was crushed twice at this location for 15 s each at 90 degree angles relative to one another to reduce the possibility of sparing. Following sciatic nerve crush, the overlying muscles were closed with a single cat gut suture and the overlying skin was closed with staples.

Mice used to determine the effect of PTEN or PTEN/SOCS3 co-deletion received direct injections of AAV vectors into the fourth and fifth lumbar dorsal root ganglia (L4/5 DRG) two weeks prior to sciatic nerve crush. Following a midline incision over the L2 to S2 vertebrae, the left L4/5 DRG were exposed by hemi-laminectomy. A volume of 1 μL of AAV-Cre ($1 \times 10^9$ infectious units/μL) was injected into each of the DRG using a Hamilton syringe attached to a 35 ga. beveled needle. Vector controls received the same volume of AAV-GFP and sham controls underwent hemi-laminectomy alone. Fast Green dye was added to both AAV-Cre and AAV-GFP solutions (final concentration of 0.1%) to allow confirmation of successful intraganglionic injection. Following injections, the surrounding erector spinae muscles were closed with two cat gut sutures and the overlying skin was closed with staples.

### 2.3. Behavioral testing

Four groups of mice were used for behavioral experiments (n = 10–11 per group, Table 1). Sham surgery and AAV-GFP vector control groups included both PTEN$^{fl/fl}$ and PTEN/SOCS3$^{fl/fl}$ mice. Mice were handled daily for one week and trained for two weeks prior to data collection. Baseline measurements were recorded two weeks prior to DRG injections or sham surgeries. An additional baseline was recorded 4 h prior to sciatic nerve crush to determine if DRG injections affected behavior. Following sciatic nerve crush, measurements were recorded weekly for eight weeks by a blinded observer.

Noxious thermosensation was measured using a Hargreave's apparatus (#390G, IITC). Mice were placed into individual plexiglass compartments with opaque dividers over a glass base plate. The base plate was heated to 33 °C to prevent the base from acting as a heat sink. Mice were acclimated for 20 min prior to testing. The active intensity of the light source was set at 53% to achieve a baseline withdrawal latency near 5 s. The maximum stimulation time was set at 15 s (3× baseline) to avoid damaging the hind paw. The light source was focused on the lateral surface of the hind paw behind the hypothenar foot pad to avoid stimulation of the medial regions of the hind paw innervated by the saphenous nerve. Reported measurements are the average of three trials separated by at least 5 min.

Mechanosensation was measured using graded von Frey filaments. Mice were tested in the same plexiglass compartments used for testing with the Hargreave's apparatus on top of an anodized metal mesh platform with 1/4″ waffle holes that allow access to the plantar surface of the paw (#410, IITC Life Science, Woodland Hills, CA). Mice were allowed to acclimate for 20 min prior to testing. Filaments of 3.22, 3.61, 3.84, 4.08, 4.31, and 4.56 log units (mean distance = 0.238) were applied to the center of the left hind paw between the foot pads beginning with the 3.84 filament. Fifty percent paw withdrawal threshold was calculated using the up-down method (Chaplan et al., 1994). Mice that did not respond to any filaments following sciatic nerve crush were assigned a paw withdrawal threshold of 4.5 g.

Motor function was assessed using an automated catwalk system equipped with CatWalk XT 8.1 software (Noldus Information Technology, Wageningen, Netherlands). Mice were placed in a dark box