

Diabetic Complications Consortium

Application Title: PTEN KNOCKDOWN: A NOVEL STRATEGY TO REVERSE DIABETIC NEUROPATHY

Principal Investigator: Douglas W. Zochodne.

1. Project Accomplishments:

- The role of PTEN contributing to impaired diabetic nerve regeneration was explored. A major paper from our laboratory addressing this topic was published (Singh et al., 2014).
- The support also allowed our laboratory to explore new connections between diabetes, impaired nerve regeneration and a novel strategy for repair-ES (electrical stimulation). This work is in preparation for submission.
- Our laboratory has published three recent reviews on the role of PTEN in peripheral nervous system regeneration (Zochodne, 2014a; Zochodne, 2014b; Krishnan and Zochodne, 2014).

2. Specific Aims:

1. To evaluate expression of PTEN and downstream molecules in type 1 and 2 experimental diabetic neuropathy models in mice;

These data are now reported in our publication (attached)(Singh et al., 2014).

2: To evaluate the impact of PTEN pharmacological or siRNA knockdown on experimental type 1 and 2 diabetic neuropathy in mice.

These data are now reported in our publication (attached)(Singh et al., 2014).

3. Publications:

Krishnan A, Zochodne DW (2014) Is Cytoplasmic PTEN a Specific Target for Neuronal Survival? Mol Neurobiol (in press)

Singh B, Singh V, Krishnan A, Koshy K, Martinez JA, Cheng C, Almquist C, Zochodne DW (2014) Regeneration of diabetic axons is enhanced by selective knockdown of the PTEN gene. *Brain* 137:1051-1067.

Zochodne DW (2014a) Diabetes and the plasticity of sensory neurons. *Neurosci Lett* (in press).

Zochodne DW (2014b) Neurons and tumor suppressors. *ACS Chem Neurosci* 5:618-620.

Regeneration of diabetic axons is enhanced by selective knockdown of the *PTEN* gene

Bhagat Singh, Vandana Singh,* Anand Krishnan,* Kurien Koshy, Jose A. Martinez, Chu Cheng, Chris Almqvist and Douglas W. Zochodne

Department of Clinical Neurosciences and the Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada

*These authors contributed equally to this work.

Correspondence to: Douglas W. Zochodne,
168 Heritage Medical Research Bldg;
3330 Hospital Dr. NW,
Department of Clinical Neurosciences and the Hotchkiss Brain Institute,
University of Calgary,
Calgary,
Alberta,
Canada T2N 4N1
E-mail: dzochodn@ucalgary.ca

Diabetes mellitus renders both widespread and localized irreversible damage to peripheral axons while imposing critical limitations on their ability to regenerate. A major failure of regenerative capacity thereby imposes a 'double hit' in diabetic patients who frequently develop focal neuropathies such as carpal tunnel syndrome in addition to generalized diffuse polyneuropathy. The mechanisms of diabetic neuron regenerative failure have been speculative and few approaches have offered therapeutic opportunities. In this work we identify an unexpected but major role for *PTEN* upregulation in diabetic peripheral neurons in attenuating axon regrowth. In chronic diabetic neuropathy models in mice, we identified significant *PTEN* upregulation in peripheral sensory neurons of messenger RNA and protein compared to littermate controls. *In vitro*, sensory neurons from these mice responded to *PTEN* knockdown with substantial rises in neurite outgrowth and branching. To test regenerative plasticity in a chronic diabetic model with established neuropathy, we superimposed an additional focal sciatic nerve crush injury and assessed morphological, electrophysiological and behavioural recovery. Knockdown of *PTEN* in dorsal root ganglia ipsilateral to the side of injury was achieved using a unique form of non-viral short interfering RNA delivery to the ipsilateral nerve injury site and paw. In comparison with scrambled sequence control short interfering RNA, *PTEN* short interfering RNA improved several facets of regeneration: recovery of compound muscle action potentials, reflecting numbers of reconnected motor axons to endplates, conduction velocities of both motor and sensory axons, reflecting their maturation during regrowth, numbers and calibre of regenerating myelinated axons distal to the injury site, reinnervation of the skin by unmyelinated epidermal axons and recovery of mechanical sensation. Collectively, these findings identify a novel therapeutic approach, potentially applicable to other neurological conditions requiring specific forms of molecular knockdown, and also identify a unique target, *PTEN*, to treat diabetic neuroregenerative failure.

Keywords: diabetic polyneuropathy; diabetes; peripheral nervous system; peripheral nerve; axonal injury

Abbreviations: CMAP = compound muscle action potential; siRNA = short interfering RNA

Introduction

Diabetes mellitus, a health burden of enormous and growing prevalence, is the most common cause of damage to the PNS. Diabetes renders diffuse nerve damage, or polyneuropathy, as well as focal damage known as mononeuropathy (Dyck *et al.*, 1993; Zochodne, 1999). Polyneuropathy is a progressive neurodegenerative disorder that particularly targets sensory neurons and their axons, starting in their distal terminals (Zochodne *et al.*, 2008). In addition to polyneuropathy and mononeuropathy, however, diabetes substantially attenuates axon regeneration and recovery, imposing a 'double hit' on the nervous system (Longo *et al.*, 1986; Ishii and Lupien, 1995; Terada *et al.*, 1998; Kennedy and Zochodne, 2000). Although several mechanisms of neuroregenerative failure in diabetes have been considered, few have offered robust avenues for repair (Triban *et al.*, 1989; Ishii and Lupien, 1995; Terada *et al.*, 1998; Yasuda *et al.*, 1999, 2003; Xu and Sima, 2001; Kennedy and Zochodne, 2002; Xu *et al.*, 2002).

Diminished intrinsic regenerative capacity of mature neurons is a major aspect of regeneration failure. Given this limitation, removal of extracellular inhibitory cues and addition of growth factors may not be sufficient to accomplish the desired regeneration outcomes (Brewster *et al.*, 1994; Mukhopadhyay *et al.*, 1994; Schnell *et al.*, 1994; GrandPre *et al.*, 2002). Most growth factors offer selective support for only the neuron subclasses that express appropriate receptors. In diabetes, growth factor receptors are downregulated (Zochodne *et al.*, 2001) and neuropathic damage involves a wide spectrum of axons, especially sensory (Malik *et al.*, 2001). PTEN is a key and widely expressed intrinsic neuronal mechanism that regulates axonal regrowth through inhibition of PI3K, a central pathway involved in survival, growth, proliferation and regeneration of axons (Soltoff *et al.*, 1992; Gallo and Letourneau, 1998; Jones *et al.*, 2003). PI3K activation through growth factor tyrosine kinase receptors phosphorylates the serine/threonine kinase Akt (Carter and Downes, 1992; Raffioni and Bradshaw, 1992). Specifically PTEN inhibits the PI3K signalling pathway by dephosphorylating PIP3 (Leslie and Downes, 2002). Several key downstream consequences of attenuated pAkt signalling are: increased levels of GSK-3 β , a molecule that promotes growth cone collapse (Eickholt *et al.*, 2002); enhanced nuclear export of pFOXO1, a member of Forkhead family of transcription factors (Nakae *et al.*, 2002); reduced mTOR signalling, a molecule that regulates protein translation (Park *et al.*, 2010). Deletion of PTEN enhanced regeneration of corticospinal tract and retinal ganglionic cells in the CNS (Park *et al.*, 2008; Liu *et al.*, 2010) and axons of peripheral sensory neurons (Christie *et al.*, 2010). After axon injury, PTEN expression in peripheral neurons persists, causing an ongoing regenerative block despite the need for plasticity in regrowing neurons. However, dismantling the roadblock by PTEN inhibition or short interfering RNA (siRNA) knockdown substantially increases neurite outgrowth from adult sensory neurons *in vitro* and accelerates the outgrowth of peripheral axons beyond a nerve transection (Christie *et al.*, 2010). As PTEN attenuates common transduction pathways activated by a number of growth factors, its inhibition or knockdown encourages a wide spectrum of neurons to grow.

In this work, we explored whether augmenting intrinsic neuron growth through PTEN knockdown in diabetic neurons might allow them to overcome their inherent growth limitations. Unexpectedly, we found that expression of the PTEN blockade was elevated in diabetes. Adult diabetic sensory neurons had robust responsiveness to its knockdown *in vitro* whereas *in vivo* its knockdown accelerated recovery of several facets of nerve regrowth in a chronic model of diabetic polyneuropathy. PTEN knockdown was accomplished by a unique and selective form of delivery to injured neurons through ipsilateral hindlimb uptake of an siRNA.

Materials and methods

Injury and induction of diabetes

Outbred adult male, 22–25 g CD-1 mice (Charles River), and male db/db (BKS.Cg-Dock7m +/+ Leprdb/J) mice (Jackson Labs) were used in the study. The procedures were reviewed and approved by the University of Calgary Animal Care Committee in conjunction with guidelines from the Canadian Council of Animal Care (CCAC). Mice (8 wks old) were made diabetic by 3-day consecutive injections of intraperitoneal streptozotocin (Sigma) at a dose of 85, 70 and 55 mg/kg dissolved in citrate buffer (pH 4.5). Control animals received intraperitoneal citrate buffer alone. Diabetes status was confirmed as ≥ 16 mmol/l fasting using a glucometer (Ultra one). Under isoflurane anaesthesia, and using aseptic techniques the left sciatic nerve of diabetic mice ($n = 7$ –8/group) was exposed and crushed just distal to the sciatic notch with a sterile forceps for 15 s.

PTEN expression

For immunohistochemistry, published methods were followed (Kan *et al.*, 2012). In brief, dorsal root ganglia were harvested and fixed in modified Zamboni's fixative (2% paraformaldehyde, 0.5% picric acid and 0.1% PBS) overnight at 4°C and then cryoprotected in 20% sucrose/PBS. After embedding in optimum cutting temperature (O.C.T.) compound (Miles), 14- μ m thick sections were placed onto poly-L-lysine coated glass slides. Primary antibodies applied were monoclonal anti-NF200 (heavy subunit of neurofilament, axons, 1:800, Sigma cat.#N0142), rabbit polyclonal PTEN (1:50; Santa Cruz cat.#6817), rabbit monoclonal ps6K (1:50; Cell Signaling cat.#4858), and rabbit polyclonal FOXO1 (1:50; Cell Signaling Cat. #9461), and incubated at 4°C for 24 h. The day after, slides were washed with PBS and incubated with secondary antibodies, anti-mouse IgG CY3 conjugate (1:100, Sigma) or Alexa Fluor[®] 488 goat anti-rabbit IgG (H + L) conjugate (1:400, Cedarlane) for 1 h at room temperature. Finally, cover slips were mounted on the slides with bicarbonate buffered glycerol (pH 8.6) and the slides were viewed with a fluorescent microscope (Zeiss Axioskope). Negative controls included omission of primary antibodies on parallel sections (not shown). Pixel intensity measurements and analysis was performed using Adobe Photoshop CS4 (Adobe Systems). For semi-quantitative analysis of PTEN expression in neurons, they were divided into four categories of arbitrary luminosity intensity levels: >100 (arbitrary units) were considered positive, medium (100–150), strong (150–200) and very strong (>200). Measurements included the total number of neurons in each category. A total of three sections per mouse were analysed (~250–500 neurons/mouse). For neuronal size analysis, luminosity and size of each neuron from $n = 3$ dorsal root ganglia sections/group

(~400–500 neurons from each of control and diabetic group) were measured provided they were approximately centrally sectioned, as indicated by DAPI nuclear staining. Luminosity and size was measured using ImageJ (NIH software). Neuronal size and their respective luminosities were plotted on a graph by subdividing neurons into a pool of >15 μm , 15–20 μm , 20–30 μm , 30–40 μm and >40 μm based on their size. To confirm the retrograde transport of PTEN siRNA, we ligated the sciatic nerve proximal to the injury site. Fluorescent-labelled PTEN siRNA (Cat. # S102734494, Mm_PTEN_6) was applied for 6 days, at the injury site with additional intraplantar injections each day (see below). On Day 7, mice were perfused and tissue samples were processed as above. Fourteen-micrometre thick longitudinal sections of sciatic nerves and 10- μm thick transverse sections of dorsal root ganglia were dried and washed with PBS buffer three times. Sciatic samples were mounted and covers slipped and were visualized with an Olympus laser scanning confocal microscope equipped with epifluorescence ($\times 60$ magnification and scanning step size 1 μm). The dorsal root ganglia sections were immunostained with PTEN and visualized with Zeiss Axioskope fluorescent microscope.

Analysis of regenerating axons

Ipsilateral, tibial nerves distal (~10 mm) to the crush site and footpads were harvested at 28 days after sciatic injury. Diabetic (3 month) and nondiabetic mice underwent sciatic crush then daily application, for 6 days, of PTEN or scrambled siRNA (1.56 μl or 0.5 μg siRNA with 20 μl HiPerfect transfection reagent (Qiagen) plus 3.44 μl saline [25 μl for footpad] or 28.44 μl saline [50 μl for injury site]). Tibial nerves were fixed in glutaraldehyde (2.5%) buffered in cacodylate (0.025 M) overnight, washed and stored in cacodylate buffer (0.15 M), then fixed in osmium tetroxide (2%), washed in graded alcohols then embedded in Epon® (Ohnishi *et al.*, 1976). Transverse sections of 1- μm thickness were made through the approximate centre of the tibial nerve and stained with toluidine blue. The whole nerve sections were photographed under oil immersion microscopy ($\times 100$) in non-overlapping fields. In each field, the numbers and calibre of unequivocal myelinated axons were then measured using an image analysis program (Scion image) by an observer blinded to treatment groups. Final measurements included total number and mean axonal diameter of regenerating myelinated axons. Footpad skin samples were harvested using skin punch and processed as described previously (Kan *et al.*, 2012). Briefly, samples were fixed in 2% PLP (2% paraformaldehyde, L-lysine and sodium periodate) for 18 h at 4°C and cryoprotected overnight in 20% glycerol/0.1 M Sorenson phosphate buffer at 4°C. Skin sections of 25- μm thickness were blocked in 10% goat serum for 1 h at room temperature. Primary antibody PGP9.5 (rabbit polyclonal; 1:1000, Encore biotechnology) was applied overnight at 4°C followed by goat anti-rabbit Cy3; 1:100 (Jackson ImmunoResearch) secondary for 1 h at room temperature. Images were captured using an Olympus laser scanning confocal microscope ($\times 100$ magnification and step size of 1 μm). Epidermal fibres labelled with PGP9.5 were counted in five adjacent fields of six sections for a total of 30 fields per mouse at each time point. Both vertical (trajectory ~90° to the surface of the skin) and total (all axon profiles) were analysed. All analyses were conducted with the examiner blinded to the identity of the samples being studied.

Electrophysiology

Multifibre motor and sensory conduction recordings were carried out in left sciatic-tibial fibres in mice anaesthetized with isoflurane at a near-nerve subcutaneous temperature of 37°C, maintained by a thermosensitive heat lamp as previously described (Kan *et al.*, 2012). In

brief, supramaximal stimuli were delivered to the sciatic nerve at the notch and knee and compound muscle action potentials (CMAPs) recorded from tibial innervated interosseous muscles in the hindpaw. Sensory nerve action potentials were recorded from the sciatic nerve at the knee following stimulation of plantar digital nerves.

Functional recovery of sensation

Mice underwent mechanical and thermal testing at Day 0 (before crush injury), 14 and 28 days following sciatic nerve crush with and without PTEN siRNA, as described above. There were 5-min intervals provided between a total of three trials performed during the same day. Mechanical sensitivity was measured using an automated Von Frey apparatus (dynamic plantar anaesthesiometer, UGO). A filament with a progressively-increasing force (2 g/s) was applied to the plantar surface of the mice through the wired mesh until a withdrawal reflex. Three separate trials were conducted and the mean latency (time to withdraw) and amount of threshold force were calculated. For testing the recovery of thermal sensation, we used the Hargreaves apparatus (Hargreaves *et al.*, 1988). In brief, a radiant heat source was applied individually to the middle of the hindpaw and the latency (in seconds) to withdrawal was measured. Three separate trials were performed for the withdrawal response. Mechanical and thermal testing was performed on identical days with an interval of at least 1 h between the two tests.

Western immunoblots and phosphatase assay

Dorsal root ganglia (L4–L6) and sciatic nerve samples were added to RIPA lysis buffer (Fisher Scientific) containing protease and phosphatase inhibitors (Roche). As previously described (Singh *et al.*, 2012), 20 μg of total protein was electrophoresed on 10% SDS-PAGE and then transferred on PVDF membrane in Tris-glycine-methanol buffer for 2 h at 4°C. After blocking with 5% non-fat dry milk, membranes were incubated overnight with a polyclonal antibody to PTEN, 1:1000 (Mouse monoclonal; Cat. #9556, or rabbit monoclonal, Cat. #9559, Cell Signaling), pS6k, 1:1000 (rabbit monoclonal; Cat. #4858, Cell Signaling), s6k, 1:1000 (mouse monoclonal; Cat. #2317, Cell Signaling), pGSK-3 β 1:1000 (rabbit monoclonal; Cell Signaling), GSK-3 β 1:1000 (Mouse monoclonal; Cat. #9832, Cell Signaling), pFOXO1, 1:1000 (rabbit polyclonal; Cat. #9461, Cell Signaling), phospho-p38, 1:500 (mouse monoclonal; Cell Signaling), p38, 1:500 (Rabbit polyclonal monoclonal; Cat. #9212, Cell Signaling) and tubulin or actin, 1:2000 (mouse monoclonal; Sigma) as a loading control in 2% bovine serum albumin in Tris-buffered saline (TBS). To confirm the specificity of the anti-PTEN antibody, a PTEN blocking peptide (1:500, Cat. #1250, Cell Signaling) was incubated with the PTEN antibody for 30 min at room temperature before use. The next day, after TBST [TBS with Tween 20 (0.1%)] rinses, horseradish peroxidase-labelled (HRP) secondary antibodies, anti-rabbit IgG HRP and anti-mouse IgG HRP (Santa Cruz), were incubated with the immunoblot at 1:5000 dilutions for 1 h at room temperature. The signals were developed by exposing the blot to enhanced chemiluminescent reagents (Amersham) and subsequently exposure on Hyperfilm (GE Healthcare). The film images were digitized and the difference between the treatments was measured by analysing pixel intensity and the size of the band using Photoshop (Adobe).

To determine the PTEN activity, PTEN protein from either wild-type or diabetic (3-month-old) mice dorsal root ganglia was immunoprecipitated using the PTEN antibody. In brief, dorsal root ganglia were harvested in a phosphate-free tris lysis buffer (25 mM Tris, 150 mM

NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol) including protease inhibitor cocktail (Roche). The protein lysate was incubated with the anti-PTEN antibody (1:50, Cell Signaling) overnight at 4°C with slight agitation. Protein A-sepharose (50% aqueous slurry, Thermo Scientific) was added to each sample and incubated for 4 h at 4°C with brief shaking. The bead complex was washed three times with Tris buffer and finally suspended in PTEN reaction buffer (25 mM Tris, 140 mM NaCl, 2.7 mM KCl, 10 mM dithiothreitol). The recombinant human PTEN (150 ng/reaction; Echelon) and the dorsal root ganglia lysate were incubated with dipotassium bisperoxo(pyridine-2-carboxyl) ox-ovanadate [bpV(pic)], 1 μ M, (EMD Chemicals), for 30 min. at 37°C in a 96-well plate. The substrate (PIP3, Echelon) was added at 3 nmol concentration to each well and incubated for 40 min at 37°C. Finally, the malachite green (Echelon) was added and the absorbance was read at 620 nm using Spectramax (Molecular devices). The standard curve was plotted and the amount of free phosphate in each well was calculated by interpolation from the standard curve (linear regression analysis). The graph was represented as a release of free phosphate compared to wild-type control samples.

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR was performed according to previous descriptions (Christie *et al.*, 2010). Briefly, total RNA was extracted using TRIzol[®] reagent (Invitrogen) and first strand DNA was synthesized using SuperScript[®] II First-strand Kit (Invitrogen). Real-time quantitative PCR was performed on the StepOnePlus[™] sequence detection system (Applied Biosystems). Primers of interest were designed with Primer Express 2.0 (Applied Biosystems) and synthesized by the University of Calgary DNA Lab. The purity of each amplicon was determined using melting curve analysis. Quantification of amplified products was done using the SYBR[®] Green I fluorophore (Invitrogen). The cycle number at which the fluorescence signal crossed a fixed threshold (threshold cycle, CT) with an exponential growth of PCR product during the linear phase was recorded. Relative expression values were generated using the comparative CT method ($2^{-\Delta\Delta CT}$) where all genes of interest were standardized to expression of RPLP0. Primer sequences were as follows: PTEN: F, 5'-GATAGC CCTAACCCCAAGAACG-3'; R, 5'-TGAAACCTCCCATGTGCTGAT-3'; RPLP0: F, 5'-AAGAACCACCATGATGCGCAAG-3'; R, 5'-TTGGTGAAC ACGAAGCCCA-3'; p38: MRF5'-ATCACAGCAGCCCAAGCTCTT-3'; MRR5'-CCTGCTTCAAAGGACTGGTCA-3'.

In vitro studies of adult sensory neurons

The procedure for adult sensory neuronal cell culture was similar to previous work (Lindsay, 1988; Andersen *et al.*, 2000; Singh *et al.*, 2012). L4–L6 dorsal root ganglia were dissected from adult wild-type and diabetic mice washed with Hank's balanced salt solution (HBSS) and dissociated by incubating in L15 medium containing 0.1% collagenase (90 min, 37°C) followed by trituration and then passage through a 70- μ m mesh. To partially remove Schwann cells, the cell suspension was loaded onto 15% bovine serum albumin (Sigma) in L15 and spun at 1000 rpm for 5 min. One ml of growth medium [Dulbecco's modified Eagle medium/F12 (Invitrogen) containing N2 nutrients supplement (Invitrogen), 10 μ M cytosine β -D-arabino-furanoside hydrochloride (Sigma), 5 U/ml penicillin-streptomycin (Invitrogen)] was added to each dish. Scrambled and fluorescent PTEN siRNA was prepared in BSA-free media with 1:10 ratio of transfection buffer (Invitrogen) and was applied at a 20 nM dose. Sensory

neurons were viewed by phase contrast using an inverted microscope (Zeiss Axiovert 40 CFL). Forty-eight hours later, neurons were fixed with 2% paraformaldehyde for 2 h at 4°C followed by PBS and then blocked with 10% goat serum (0.3% Triton[™] X-100/PBS) for 30 min at room temperature. Neurons and neurites were stained with 1 h incubation of mouse monoclonal Nf-200 antibody (1:800, Sigma) at room temperature. Secondary antibody, sheep anti-mouse IgG (whole molecule), F(ab)2 fragment-Cy3 (1:200; Sigma) was applied following PBS wash at room temperature for 1 h. The samples were then washed in PBS and mounted using DAPI vectashield (Vector laboratories) to label the nuclei and imaged using a Zeiss Axioskope fluorescent microscope. The total neurite outgrowth, number of primary neurites (defined as processes extending from the soma), length of the longest neurite and number of branches of a primary neurite were analysed and quantified by MetaXpress software and an observer blinded to the treatment condition (Molecular Devices).

Statistical analysis

Results were represented as mean \pm standard error of the mean (SEM) and compared with a one-way ANOVA with *post hoc* Tukey's comparisons, or Student's *t*-tests (one-tailed in an expected direction of change) as appropriate.

Results

PTEN expression is elevated in diabetic peripheral sensory neurons

Previous work has suggested that neurons with elevated expression of PTEN, particularly the small calibre IB4 population, have attenuated intrinsic growth characteristics (Leclere *et al.*, 2007; Christie *et al.*, 2010). We examined messenger RNA and protein expression of PTEN in experimental diabetes, known to exhibit marked evidence of regenerative failure (Kennedy and Zochodne, 2000). Diabetic dorsal root ganglia had elevated levels of PTEN compared to littermate controls, verified using several approaches. Firstly, we identified rises in PTEN messenger RNA in dorsal root ganglia of a mouse model of type 1 diabetes mellitus induced by streptozotocin, with a duration of diabetes of 3 months (Fig. 1A). Next we confirmed elevated expression of PTEN protein levels by western immunoblots in both diabetic dorsal root ganglia and sciatic nerves [Fig. 1B and C (Day 0, basal) and Supplementary Fig. 1]. To confirm that upregulated PTEN in dorsal root ganglia specifically involved neurons, we examined two separate diabetes models, streptozotocin-induced diabetes of 3 months in outbred CD-1 mice, a model of type 1 diabetes and db/db mice at 12 weeks of age, a model of type 2 diabetes mellitus (Fig. 1D). We noted prominent neuronal expression, particularly involving small calibre sensory neurons, as previously reported (Christie *et al.*, 2010). Using a neuronal size expression analysis however, we noted that in diabetes, PTEN expression was also upregulated in medium to large calibre neurons, unlike controls (Fig. 1E). Using a semi-quantitative luminosity analysis, under identical conditions of staining and imaging, we also confirmed that higher numbers overall, of sensory neurons, expressed PTEN (significant for type 1 diabetic mice; a similar but

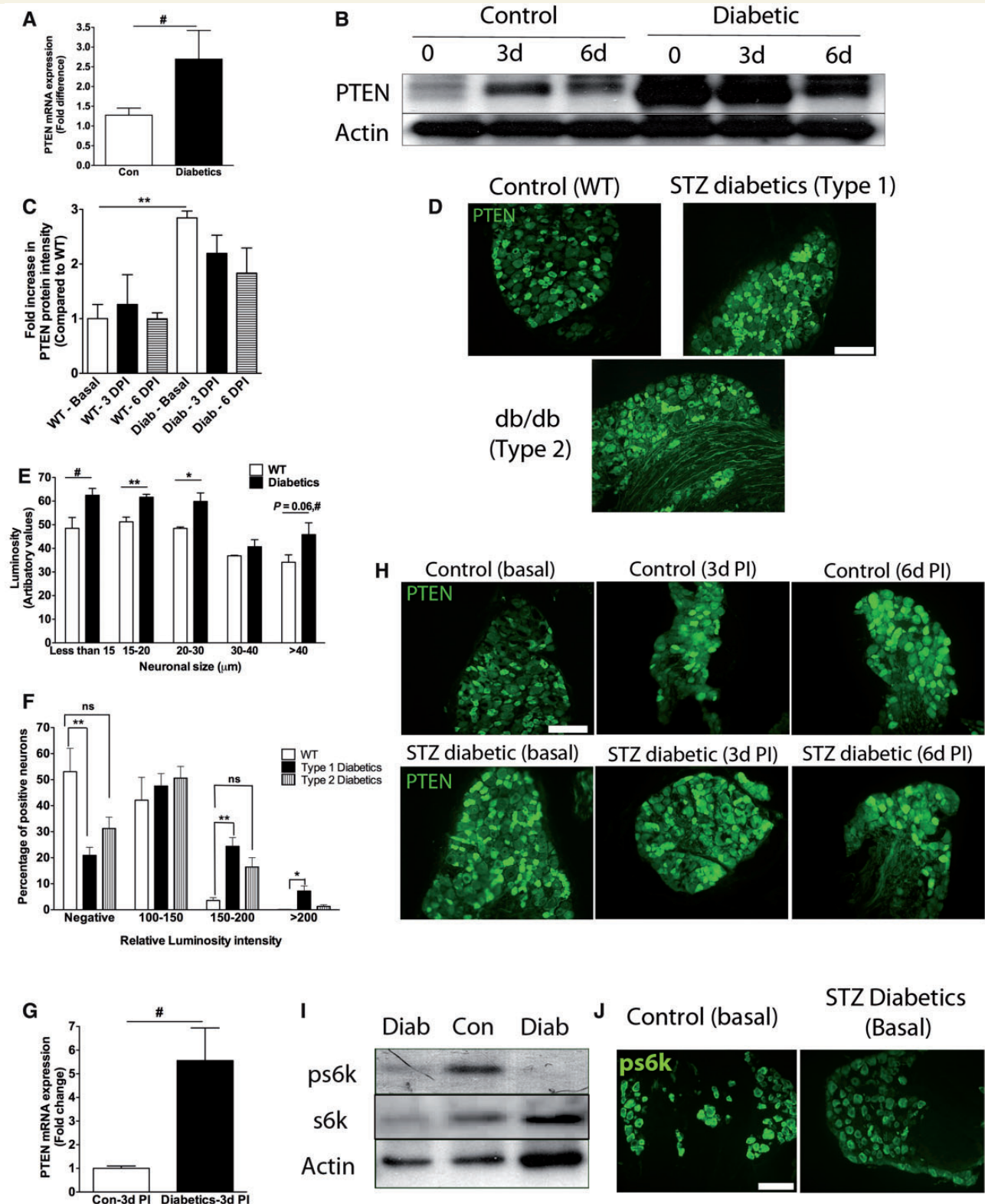


Figure 1 PTEN expression is robustly elevated and extensive in adult diabetic sensory neurons. Expression is analysed in 3-month type 1 diabetic mice with or without nerve injury and 12-week-old type 2 diabetic (db/db) mice. (A) PTEN messenger RNA transcripts in wild-type (WT) and diabetic dorsal root ganglia indicating higher expression of PTEN in diabetics. (B) Western immunoblots labelled with PTEN in wild-type and diabetic dorsal root ganglia before (basal, Day 0) and 3 and 6 days after sciatic nerve injury. Beta-actin is used as a loading control. (C) Quantification of protein data from B. A robust rise in basal expression of PTEN protein in diabetic dorsal root ganglia compared to non-diabetics. (D) Examples of adult sensory neurons from wild-type, streptozotocin (STZ) induced type 1 diabetic, and db/db, type 2 diabetic dorsal root ganglia labelled with PTEN (green). Note that PTEN is expressed in a larger number of neurons with brighter

(continued)

non-significant trend in type 2 diabetic mice) (Fig. 1F). As a possible upstream signal accounting for enhanced PTEN expression we also observed rises in p38 messenger RNA and phospho-p38 protein compared to parent p38 protein levels (Supplementary Fig. 2) (Koistinen et al., 2003; Wang et al., 2006). PTEN is normally associated with downregulation of downstream ps6k ribosomal protein, a molecule essential for protein synthesis and growth cone formation (Park et al., 2010). Although we were unable to confirm overall changes in pAkt, a promiscuous and central signalling molecule that might not be subject to chronic declines in diabetes (data not shown), levels of ps6k in diabetic dorsal root ganglia were substantially lower than non-diabetic controls and its expression involved dorsal root ganglia sensory neurons (Fig. 1I and J). We further explored downstream of PI3-K signalling and noted a decrease in protein expression of pGSK-3 β (an inactive form of GSK-3 β) and pFOXO1 in adult diabetic sensory neurons, indicating an enhancement of PTEN activity (Supplementary Fig. 3). Unphosphorylated FOXO1 was identified in the nucleus, where it resides in the absence of pAkt phosphorylation, in diabetic dorsal root ganglia. We further tested PTEN activation using a PTEN lipid phosphatase assay. Diabetic dorsal root ganglia lysate produced a trend, although not statistically significant, towards higher free phosphate through conversion of PIP3 substrate compared with wild-type controls. The rise in free phosphate with PTEN was inhibited by bpV(pic) (1 μ M), a pharmacological inhibitor of PTEN (Supplementary Fig. 3). Taken together, these findings identified a substantial upregulation of PTEN in larger numbers of expressing neurons and a shift in the populations that express it. Finally, we asked whether PTEN upregulation in diabetes persists following injury, where it may dampen regenerative activity. In previous work, although PTEN levels have been unchanged or even declined following injury, persistent expression continues to inhibit axon regrowth (Christie et al., 2010). Thus persistent expression of its messenger RNA and protein expression in diabetic dorsal root ganglia after injury indicated a potential to play an ongoing role in influencing regeneration (Fig. 1B, G and H). There was also a decrease in pGSK-3 β activity after injury (Supplementary Fig. 3).

Diabetic neurons are responsive to PTEN inhibition

We next tested whether adult diabetic sensory neurons retained their ability to respond to PTEN inhibition *in vitro*. Previously, we showed that non-diabetic neurons increased neurite outgrowth

and branching in response to PTEN inhibition or knockdown, a rise in plasticity that translated into enhanced *in vivo* outgrowth (Christie et al., 2010). At the 2 days *in vitro* time point studied, baseline neurite outgrowth of adult diabetic dorsal root ganglia neurons was not impaired, a change identified in other models of longer duration (Fig. 2) (Zherebitskaya et al., 2009). Despite this caveat, fluorescent PTEN siRNA was incorporated in sensory neurons (Fig. 2A), knocked down PTEN messenger RNA (Fig. 2B) and generated a robust increase in neurite outgrowth and branching (Fig. 2C–G).

Knockdown of PTEN by non-viral retrograde short interfering RNA uptake

To provide selective knock down of PTEN expression strictly in regenerating peripheral neurons, we exploited non-viral siRNA retrograde axon uptake from the injury site, an approach we have previously identified as capable of knocking down ipsilateral parent neuron gene expression (Webber et al., 2011). An important advantage of this approach, for potential translation into human work, is that viral vectors are not required for delivery. We applied Gelfoam[®] soaked in PTEN or scrambled siRNA at a nerve crush injury site in non-diabetic control mice immediately for 20 min, then by percutaneous injection at the sciatic notch, supplemented with intraplantar injections daily for 5 days. The approach was empirical. Critical factors seem to be repeated exposure, application at the injury site and the capability of retrograde transfer of the nucleotide. It is possible that other local forms of delivery, not explored here might also provide sufficient retrograde delivery. PTEN messenger RNA and protein were reduced and ps6k protein seemed to be upregulated in ipsilateral dorsal root ganglia at Day 7 and PTEN protein decreased in the sciatic nerve (Fig. 3A, C and D and Supplementary Fig. 4). PTEN messenger RNA was similarly reduced in ipsilateral samples of the lumbar spinal cord where spinal motor neurons reside (Fig. 3B). By immunohistochemistry, there was a qualitative reduction in the intensity of PTEN expression in ipsilateral dorsal root ganglia neuronal cell bodies in wild-type and diabetic mice (Fig. 3E). To provide evidence for retrograde transport of the siRNA, we ligated the sciatic nerves proximal to the injury, injected a 3'-Alexa Fluor[®] 488 labelled siRNA into the crush zone and plantar footpads and noted accumulation of labelled nucleotide distal to the ligation site (Fig. 3F–H). PTEN siRNA had no impact on blood glucose and body weights (data not shown).

Figure 1 Continued

luminosity in diabetics. (E) Neuronal size analysis from wild-type and type 1 diabetic sensory neurons indicating a wider expression of PTEN. PTEN expression is significantly higher in smaller neurons with concurrent expression in large calibre sensory neurons in diabetics. (F) Quantification of neuronal luminosity (described in methods section). Note a shift in percentage of neurons with higher luminosity intensity specifically in type 1 diabetes compared to controls. A similar but non-significant trend is observed in type 2 diabetic dorsal root ganglia. (G) PTEN messenger RNA levels in wild-type and diabetic dorsal root ganglia 3 days after sciatic nerve injury. (H) Wild-type and type 1 diabetic dorsal root ganglia immunostained with PTEN (green), before (basal, Day 0) and 3 and 6 days after sciatic nerve injury. (I and J) Marked decline in ps6k protein in adult 3-month diabetic dorsal root ganglia and littermate controls analysed using western blot (I) and immunostaining (J). ps6k is prominently expressed in sensory neurons. For each of immunohistochemistry, western blotting and quantitative real-time-PCR experiment, $n = 3$ –5 animals per group/time point was used. [Values are mean \pm SEM; A, C, G and E, * $P \leq 0.05$ Student's t -test (#one-tailed in A, G and E), two-tailed otherwise; F, * $P \leq 0.05$, ** $P < 0.01$, one way ANOVA; *post hoc* Tukey's multiple comparison test. Scale bar = 100 μ m.]

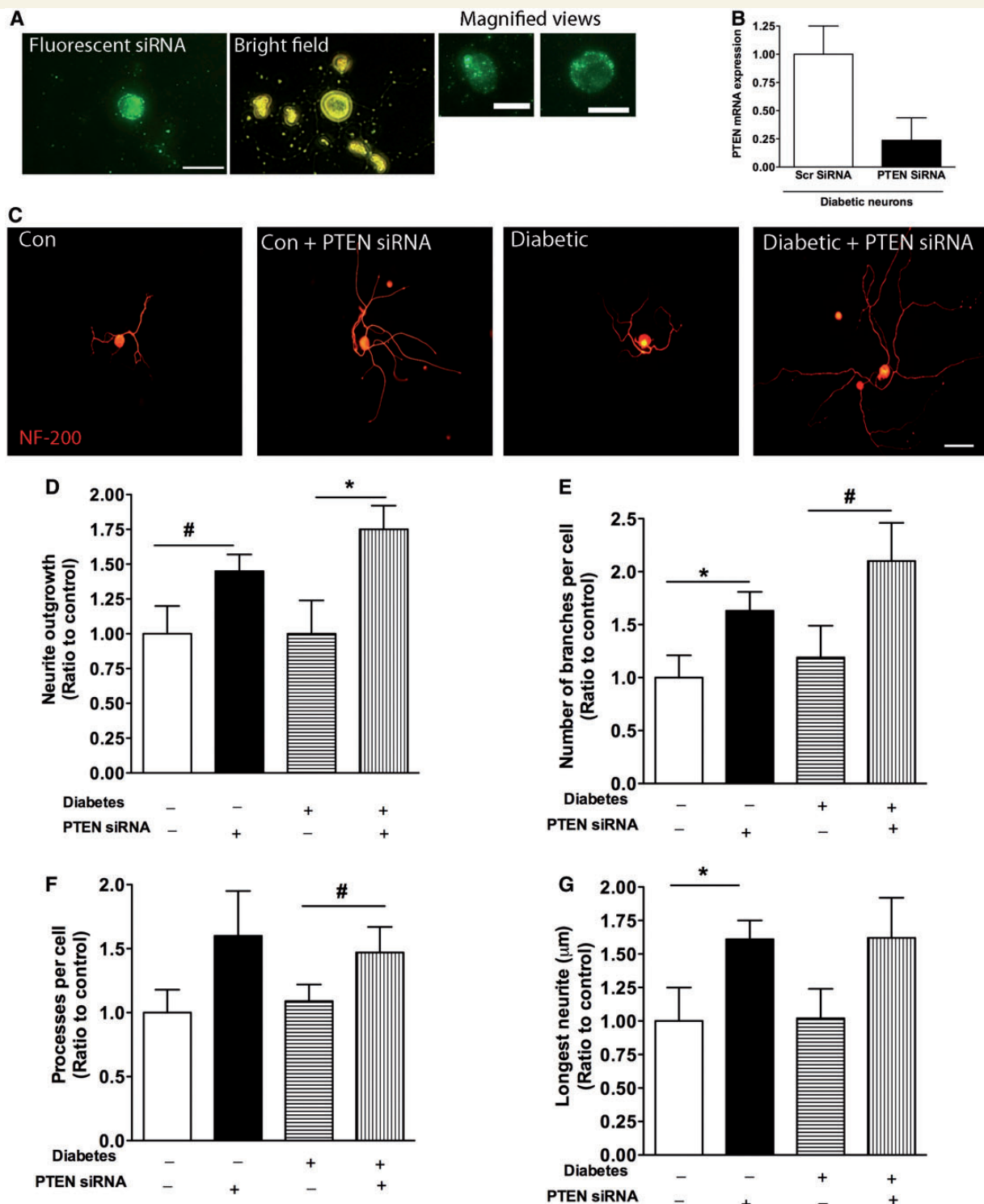


Figure 2 PTEN inhibition increased neurite outgrowth of adult diabetic sensory neurons. Neurons are harvested for *in vitro* studies from adult non-diabetic and diabetic (3 months of diabetes) mice. (A) Fluorescent-labelled PTEN siRNA was integrated into adult diabetic sensory neurons *in vitro*. The right panel represents a bright field image showing the neuronal cell body and neurites (Scale bar = 100 μm). Also illustrated are high power views of single neurons (Scale bar = 50 μm). (B) *In vitro*, PTEN messenger RNA transcripts in isolated sensory neurons with the siRNA ($P = 0.07$ one-tailed Student's *t*-test). (C) Representative images of adult wild-type and type 1 diabetic sensory neurons *in vitro* were labelled with an antibody to neurofilament (NF-200, red, scale bar = 100 μm). Sensory neurons were exposed to culture media containing either scrambled or PTEN siRNA (20 nM). (D–G) Quantification of the total neurite outgrowth (D), mean number of branches per primary branch (E), mean number of processes per cell (processes extending from the soma) (F) and longest neurite (G). Note that PTEN siRNA increased outgrowth under both diabetic and non-diabetic conditions. Values are represented as mean \pm SEM; D–G, # $P < 0.05$, one-tailed; * $P < 0.05$, two-tailed Student's *t*-test.

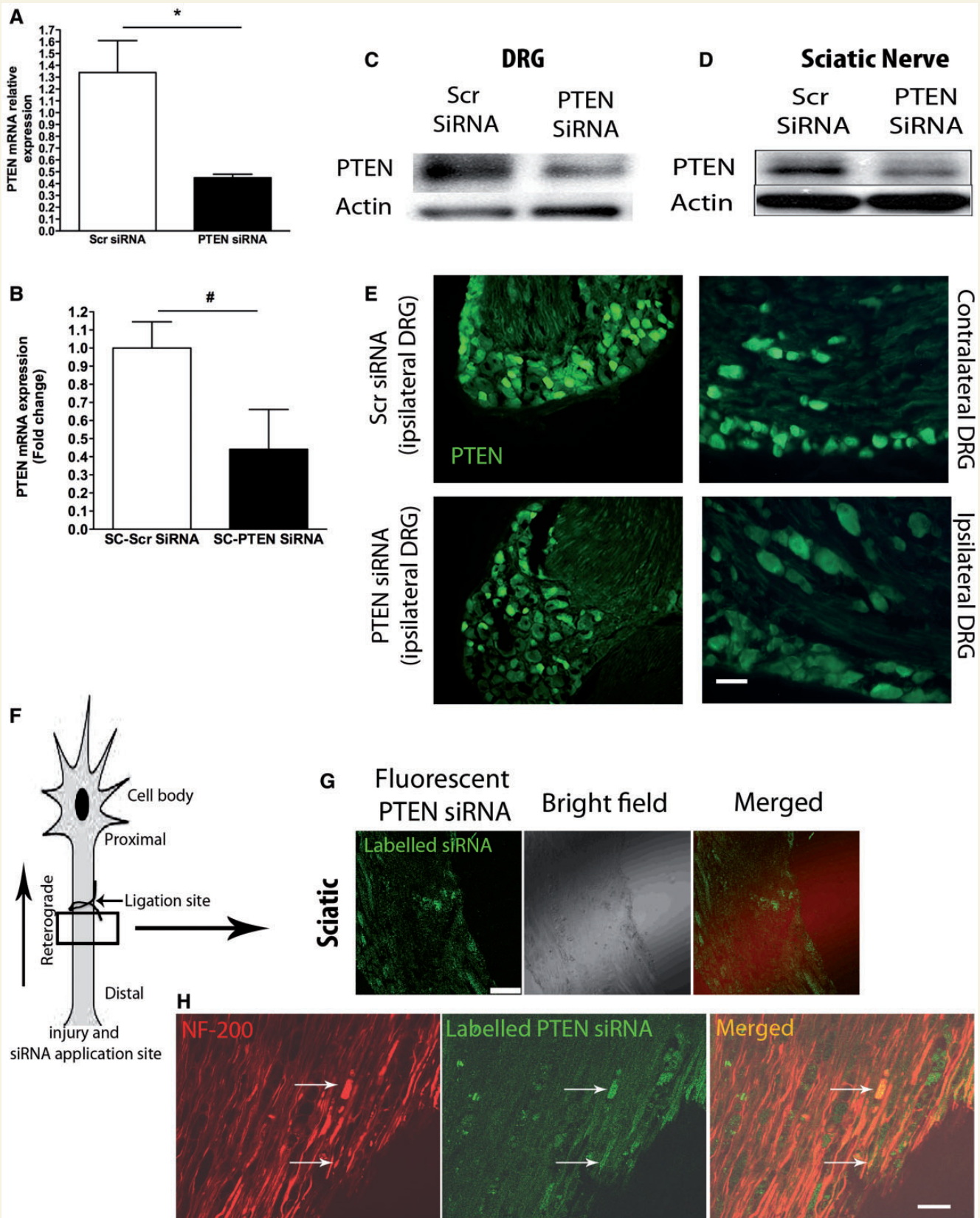


Figure 3 Local PTEN siRNA is retrogradely transported to alter gene expression in ipsilateral parent dorsal root ganglia (DRG) sensory neurons. (A) Administration of PTEN siRNA locally at the sciatic nerve and additional intraplantar injections successfully knocked down the PTEN messenger RNA expression in ipsilateral non-diabetic dorsal root ganglia ($n = 3$ separate experiments). (B) Knockdown of PTEN messenger RNA in the ipsilateral non-diabetic lumbar spinal cord where motor neurons reside ($n = 3$ mice/treatment). (C and D) Western

(continued)

PTEN inhibition accelerates electrophysiological recovery after injury in mice with chronic diabetes

We next addressed whether PTEN inhibition might impact neuroregenerative failure in a chronic model of experimental diabetes. Neuropathy relevant models replicate several findings in humans that include electrophysiological abnormalities, axon atrophy, loss of sensation and retraction of epidermal axons. When a focal nerve injury or mononeuropathy is then superimposed, epidermal reinnervation, myelinated fibre regrowth and electrophysiological maturation are all significantly impaired in diabetes (Sima *et al.*, 1988; Kennedy and Zochodne, 2000). Our 2.5-month-old diabetic mice developed expected and robust features of the model before injury: severe hyperglycaemia, less weight gain (Fig. 4A and B), slowing of motor and sensory conduction velocities (Fig. 4C and D) and loss of thermal and mechanical sensitivity (Fig. 4G and H). In particular, conduction slowing is a hallmark of significant neuropathy; the model is associated with preserved amplitudes of CMAPs and sensory nerve action potentials despite the presence of neuropathy as observed here. However following injury, CMAPs and sensory nerve action potentials provide a robust index of axon regrowth. We then superimposed focal sciatic crush injury to address regeneration.

After crush injury, CMAPs and sensory nerve action potentials disappear, then gradually reappear at lower amplitudes as reconnection to targets is established (Fig. 5A, B, G and H). Similarly, motor and sensory conduction velocities of regenerating axons at 14 and 28 days post-injury trended below pre-injury levels, consistent with their immaturity (Fig. 5C–F) and at most time points were lower in diabetic mice. Ipsilateral PTEN retrograde siRNA knockdown (identical amounts applied to diabetic and non-diabetic mice) enhanced repair of three of four electrophysiological indices of recovery in diabetic mice: CMAP amplitudes and conduction velocities of motor and sensory axons. In non-diabetic mice, the impact was less robust: a trend toward improved CMAPs and improved sensory nerve action potentials and sensory conduction velocity. Interestingly, the magnitude of improvement in diabetics was large, rendering some values, such as motor conduction velocity, that exceeded control values (Fig. 5 C–F).

PTEN inhibition accelerates myelinated axon regrowth and skin reinnervation

We next examined whether PTEN retrograde siRNA knockdown influenced the repopulation of newly regenerated myelinated axons distal to the crush zone 28 days after injury. In keeping with previous observations of neuroregenerative failure, the numbers and mean calibre of myelinated axons in regenerating tibial nerves were substantially lower in diabetic mice (Fig. 6A–C). In diabetic, but not non-diabetic nerves, ipsilateral to PTEN knock-down there was an increase in both the number and mean axon calibre (mean axon diameter of all sampled myelinated axons) of regenerating myelinating axons (Fig. 6A–C).

Reinnervation of the skin target organ involves fine unmyelinated afferent fibres that repopulate the epidermis. As PTEN expression was especially high in parent neurons of small calibre axons in the dorsal root ganglia, it was important to establish their responsiveness to knockdown. Similarly, loss of epidermal afferents is a key feature of diabetic neuropathies (Polydefkis *et al.*, 2001). The density of axons (the intraepidermal nerve fibre density) reinnervating the epidermis 3 weeks after sciatic nerve injury was substantially reduced compared with uninjured values (not shown), most marked in diabetics. As in previous work in our laboratory, we measured both vertically oriented axon profiles and total profiles so that all branching behaviour could be included (Cheng *et al.*, 2010). PTEN retrograde siRNA knockdown was associated with a significant increase in total and vertically directed epidermal axons in diabetic mice. In controls, there were only non-significant trends toward improvement (Fig. 6D–F).

Behavioural sensory changes after sciatic nerve injury proved a less robust marker of injury and recovery, likely because the sciatic nerve is not the sole contributor to hindpaw innervation. In this study we evaluated mechanical and thermal sensory recovery following injury in wild-type and diabetic mice. In both non-diabetic and diabetic mice there were non-significant trends toward heightened mechanical sensitivity after injury. In diabetic mice, PTEN knockdown was associated with greater mechanical sensitivity at 28 days after injury. No significant changes in thermal sensitivity following injury were demonstrable beyond trends toward lesser sensitivity and there was no clear impact of PTEN knock-down (Fig. 7).

Figure 3 Continued

immunoblots of dorsal root ganglia and sciatic nerve lysates labelled with PTEN. Partial knockdown of PTEN protein in the ipsilateral non-diabetic dorsal root ganglia (C) and locally in the sciatic nerve (D) ($n = 3$ mice/treatment). (E) Labelling of wild-type ipsilateral dorsal root ganglia sensory neurons with PTEN antibody (green) after application of scrambled and PTEN siRNA injections (*left*, non-diabetic). *Right* side panels shows contralateral and ipsilateral dorsal root ganglia labelled with PTEN (green) in 3 month diabetics after PTEN siRNA treatment. (Scale bar = 100 μm for *left* panels and 50 μm for the *right* panels). PTEN luminosity decreased after siRNA application in ipsilateral dorsal root ganglia in both wild-type and diabetics similar to changes demonstrated with western immunoblots (C) and with quantitative real-time PCR (A). (D) Schematic representation of the injury and ligation sites and retrograde transport of PTEN siRNA. (F–H) Accumulation of fluorescent PTEN siRNA distal to the ligation site (box, F), and proximal to the injury and siRNA delivery site, indicating possible retrograde transport. (G) Bright field and merged views are also shown. (H) Longitudinal sections of ipsilateral sciatic nerves proximal to the ligation site labelled with NF-200 (axons) indicating close localization with the fluorescent siRNA in swollen axon profiles, sometimes double labelled with neurofilament (arrows). Values are represented as mean \pm SEM; A, * $P < 0.05$, two-tailed Student's *t*-test; B, # $P < 0.05$, one-tailed Student's *t*-test; $n = 3$ –4 mice/treatment.

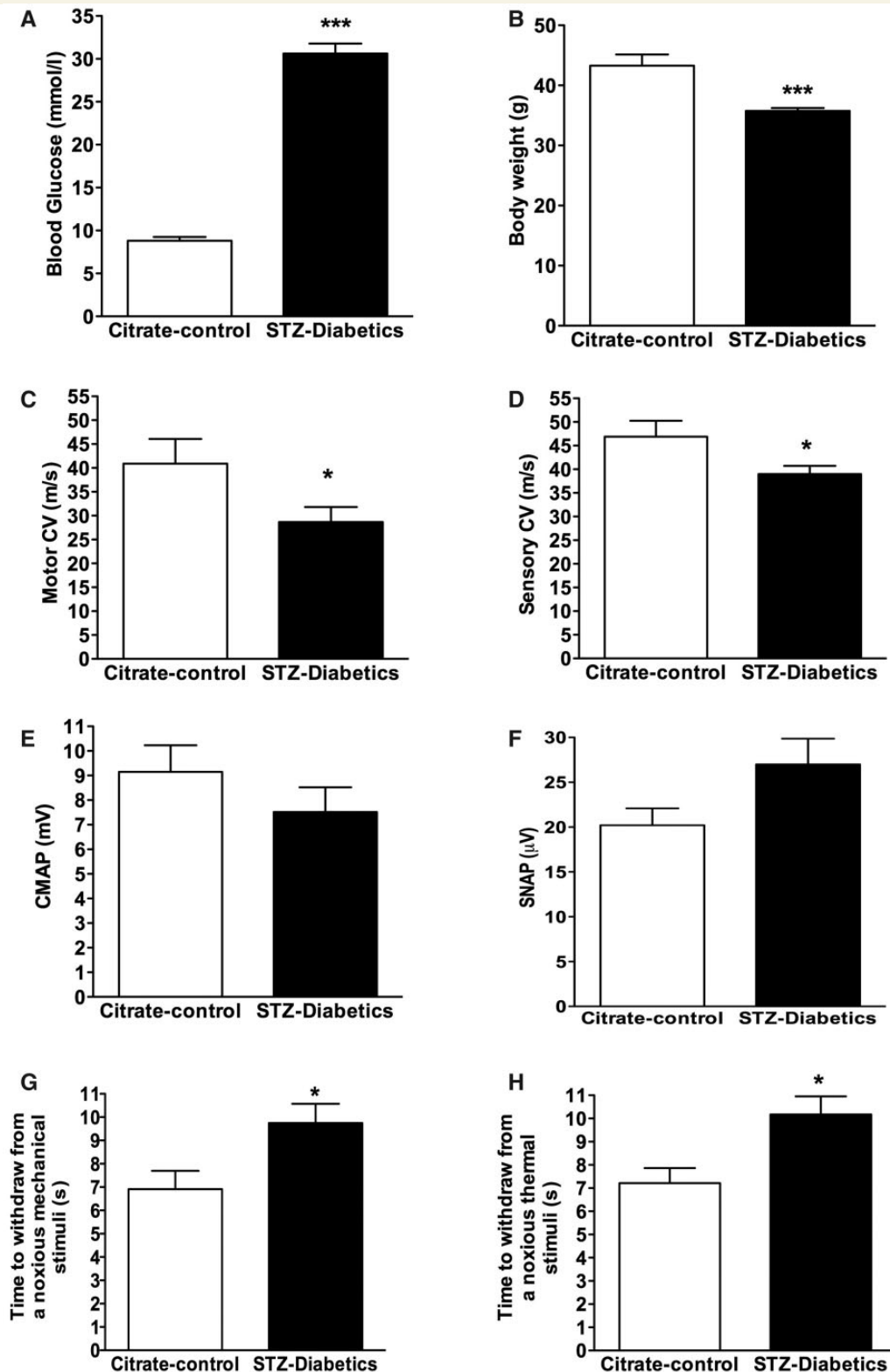


Figure 4 Chronic diabetes is associated with electrophysiological and behavioural features of polyneuropathy. Analysis is carried out in non-diabetic and mice with type 1 diabetes (2.5 months). (A and B) Blood glucose levels 2.5 months after streptozotocin (STZ) injection were significantly higher (A) and gain in body weight was reduced in outbred diabetic mice compared to wild-type non-diabetic controls (B). (C–E) Electrophysiological recordings of control and diabetic nerves. Motor and sensory conduction velocities (CV) were significantly reduced in 2-month-old diabetics (C and D) with no change in CMAP and sensory nerve action potential amplitudes (E and F). (G and H) Diabetic animals were hyposensitive to mechanical (G) and thermal stimuli (H). Ten wild-type and 26 type 1 diabetic animals were analysed. Values are represented as mean \pm SEM; A–H, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-tailed Student's *t*-test.

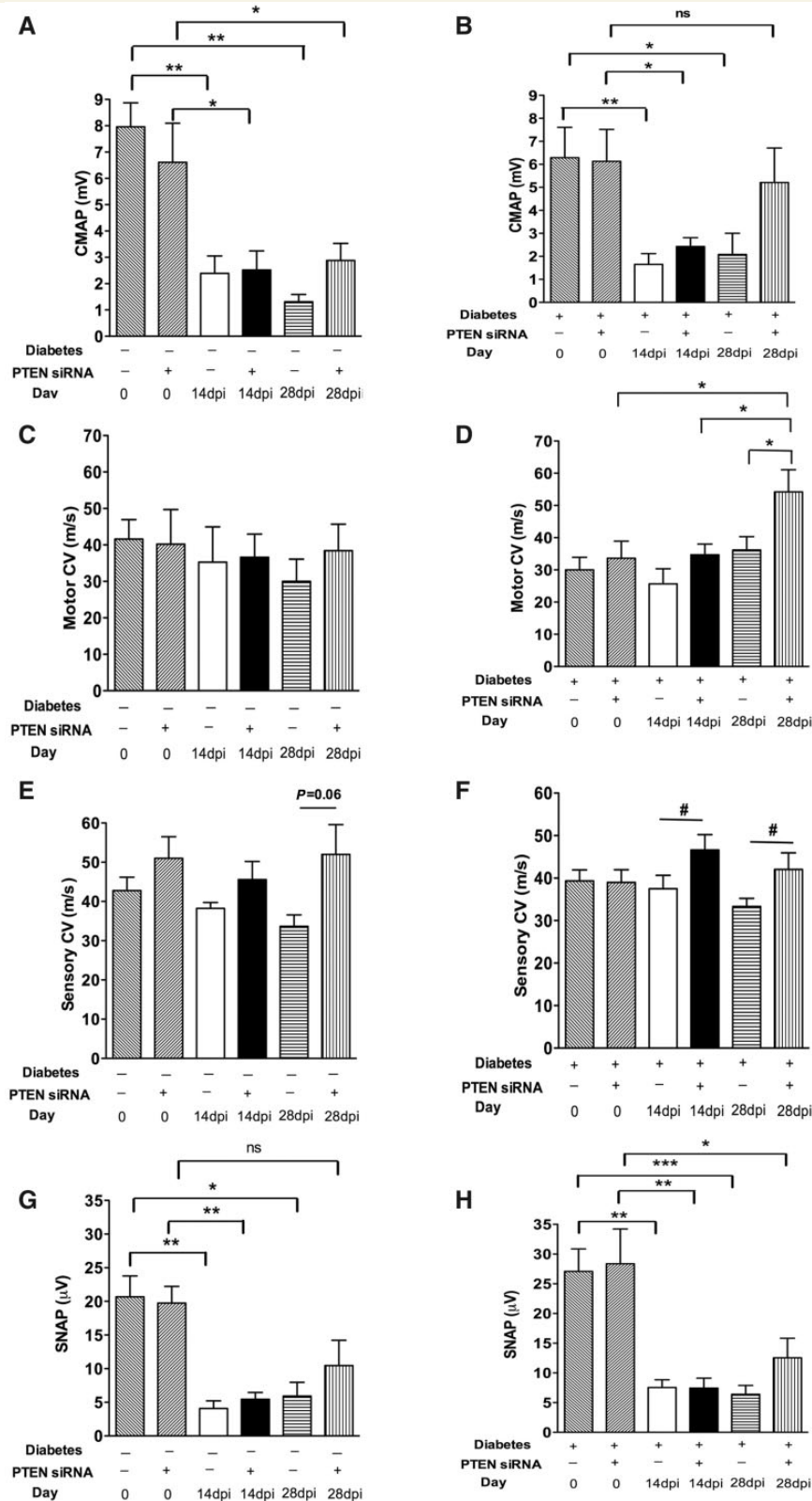


Figure 5 Knockdown of PTEN at the neuronal dorsal root ganglia cell body is associated with electrophysiological recovery in diabetics. Analysis is carried out in non-diabetic mice and mice with type 1 diabetes (3 months) treated for an additional 1 month (28 d). (A and B) CMAPs in wild-type (A) and diabetics (B) before (Day 0) and 14 and 28 days after injury (dpi). CMAPs were reduced after sciatic nerve injury in both wild-type and diabetic mice (14 dpi in A and B). PTEN inhibition was associated with significant improvement of CMAP amplitudes in both diabetics and non-diabetics but amplitudes were almost double in diabetics compared to controls at Day 28 post-injury. (C and D) Motor conduction velocities were lower in diabetics (D) than wild-type mice (C) before injury (Day 0) and similarly at 14 days

(continued)

Discussion

The major findings of this work were: (i) peripheral sensory neurons substantially upregulated PTEN expression at the messenger RNA and protein level during chronic diabetes; (ii) peripheral sensory neurons from diabetic mice were capable of responding to PTEN knockdown with evidence of increased plasticity; (iii) local siRNA, delivered to the injured nerve and hindpaw knocked down PTEN messenger RNA in the ipsilateral dorsal root ganglia and spinal cord; and (iv) selective PTEN knockdown in injured diabetic neurons and axons accelerated regrowth: motor axon reconnection to endplates, electrophysiological maturation of motor and sensory axons, regrowth and maturation of myelinated axons, reinnervation of the epidermis and improved mechanical sensitivity. These benefits were more robust and consistent than those conferred by a similar strategy in injured non-diabetic nerves.

Several aspects of this study were specifically designed to mimic clinical scenarios. Over 50% of diabetic subjects have evidence of polyneuropathy (Dyck *et al.*, 1993) identified by clinical loss of sensation, loss of epidermal innervation of the skin and electrophysiological abnormalities, especially slowing of motor and sensory conduction velocity (Bril, 1994). We have previously provided evidence that the outbred diabetic mouse model, generated by streptozotocin injection to model type 1 diabetes mellitus, as used in this work, provides robust modelling of these features (Kennedy and Zochodne, 2000, 2005). Moreover, this diabetic model demonstrates severe regenerative failure when a focal injury is superimposed (Kennedy and Zochodne, 2000). In the present study, our mice had evidence of polyneuropathy after 2 months of diabetes with slowing of motor and sensory conduction velocity and loss of sensation to thermal and mechanical stimuli before injury.

Regeneration in diabetic peripheral nerves is a significant clinical consideration. For example, focal nerve injuries, mononeuropathies, are commonly identified in patients with pre-existing polyneuropathy. Examples include entrapment neuropathies at the carpal tunnel and cubital fossa or ischaemic neuropathies. In addition, established neuropathy is difficult to reverse even in the setting of tight glycaemic control or pancreatic transplantation (Kennedy *et al.*, 1990; Mehra *et al.*, 2007; Kan *et al.*, 2012). Targeted approaches to reverse abnormalities by exploiting axon regrowth are therefore a compelling therapeutic priority.

In this work, we addressed specific and clinically relevant regenerative endpoints. The CMAP amplitude correlates with the numbers of motor axons that regrow and reconnect to tibial motor endplates. Its recovery after crush injury is a functional measure of

axon reconnection. Conduction velocities in regenerating motor and sensory axons are indices of maturation: regrowth of axon calibre, remyelination and reinsertion of nodal and paranodal proteins. These electrophysiological endpoints were improved after PTEN knockdown. We examined numbers and calibre of myelinated axons distal to the crush zone and identified improvements in nerves after PTEN knockdown. Finally we identified enhanced reinnervation of the skin by fine unmyelinated axons. Although we did demonstrate that mice with PTEN knockdown exhibited greater mechanical sensitivity after injury, behavioural sensory data in mice after selective nerve injury can be difficult to interpret, a feature also observed in the present study. Improved mechanical sensitivity could be related to the rises in levels of PTEN in larger dorsal root ganglia neurons that we observed. As hindlimbs are also innervated by the saphenous nerve, mice (or rats) undergoing sciatic injury do not demonstrate complete analgesia or anaesthesia despite complete nerve interruption. Testing modalities can also pose challenges, as testing for thermal paw sensation involves heating up a large superficial and deep portion of the hindpaw that is innervated by both the sciatic and saphenous nerve. No difference in thermal sensitivity was demonstrated in this study. Finally, nerve injury is associated with the development of both peripheral and central features of neuropathic pain. In the setting of injury to only one sensory territory supplying the hindlimb, neuropathic pain can paradoxically heighten behavioural responses to sensory stimuli. Despite these caveats, by 28 days post-injury, when early hindpaw reinnervation had occurred, diabetic mice with PTEN knockdown were more sensitive to mechanical stimuli.

Mechanisms that might account for regenerative failure in diabetic peripheral nerve have been widely considered. Reviewed elsewhere in detail (Yasuda *et al.*, 2003; Kennedy and Zochodne, 2000), these have included abnormal polyol flux (Love *et al.*, 1995; Yasuda *et al.*, 1999), microangiopathy (Yamamoto *et al.*, 1998; Kennedy and Zochodne, 2002), attenuated expression of neurotrophic receptors (Zochodne *et al.*, 2001), delayed upregulation of regeneration associated genes (Xu and Sima, 2001; Xu *et al.*, 2002), failed IGF signalling (Glazner *et al.*, 1993; Ishii and Lupien, 1995), 'effete' Schwann cells that fail to support axons, defective inflammatory clearance of the products of Wallerian degeneration (Conti *et al.*, 1997; Terada *et al.*, 1998) and abnormalities of structural axon proteins (Williams *et al.*, 1982; Terada *et al.*, 1998). The linkage of impaired regeneration in diabetes to PTEN expression is novel and was unexpected. In a recent clinical study, PTEN haploinsufficiency was found to be associated with a profound increase in insulin sensitivity in diabetic patients (Pal *et al.*, 2012); indicating its

Figure 5 Continued

post-injury. Recovery in motor conduction velocities was significant only in diabetics exposed to PTEN siRNA (D). (E and F) Sensory conduction velocities in wild-type mice (E) and diabetics (F) before and 14 and 28 days post-injury. Pre-injured levels were lower in diabetics (Day 0) (F) than controls (E). A significant benefit on sensory conduction velocity using PTEN siRNA was noted only in diabetics (F) but there was a similar trend in non-diabetics (E). (G and H) SNAP amplitudes before and after injury in wild-type mice (G) and diabetics (H). Sensory nerve action potential amplitudes improved with the PTEN inhibition in non-diabetics and tended toward higher values in diabetics (NS) (G and H). Values are represented as mean \pm SEM; A–H, $^{\#}P < 0.05$, one-tailed Student's *t*-test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA; *post hoc* Tukey's multiple comparison test, $n = 6$ –8 animals/group/time point).

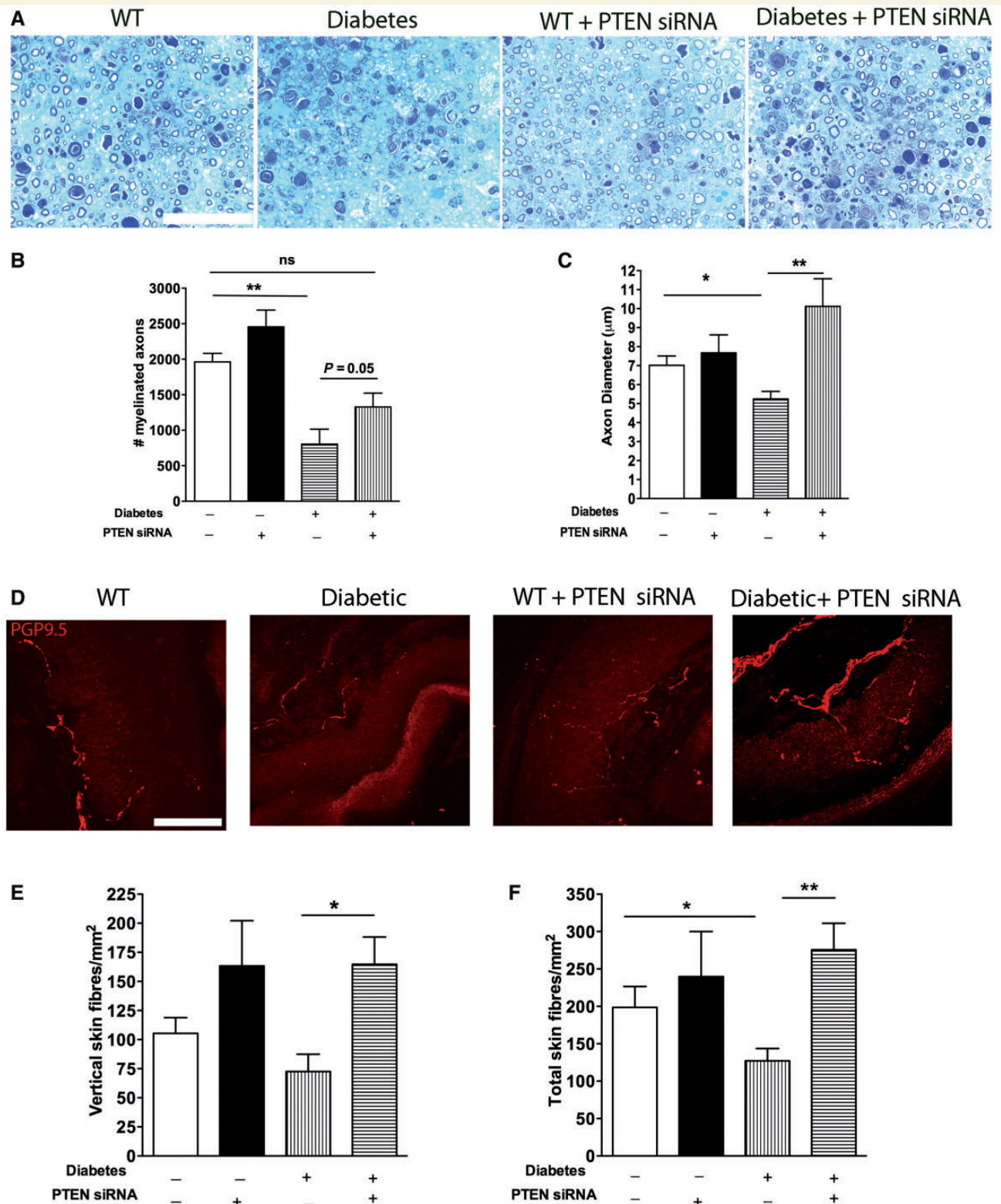
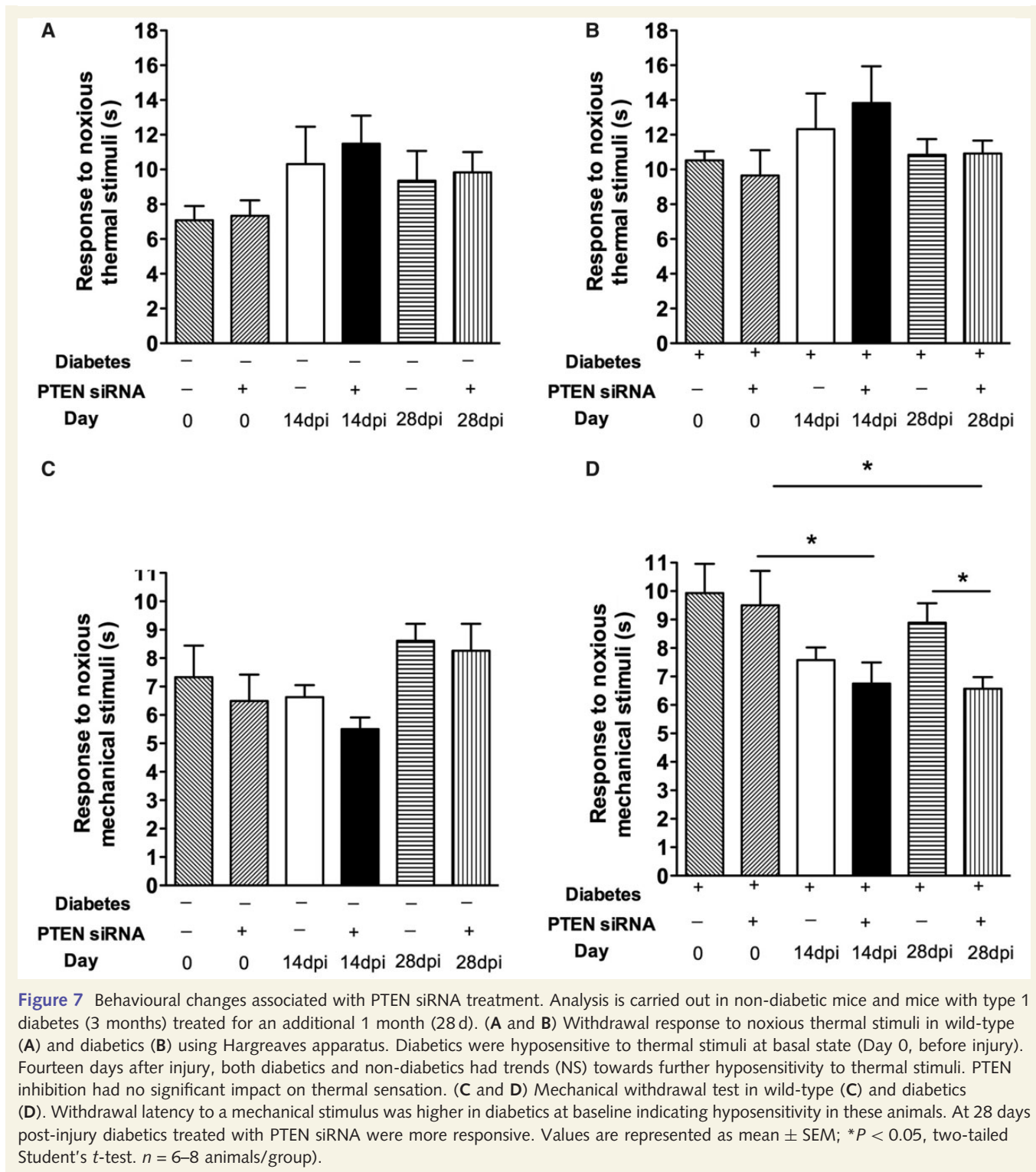


Figure 6 Repopulation of myelinated axons and skin target reinnervation with PTEN knockdown. Analysis is carried out in non-diabetic mice and mice with type 1 diabetes (3 months) treated for an additional 1 month (28 d). (A) Representative images of semi-thin sections of regenerating distal tibial nerve (10 mm from the injury site) from wild-type and diabetics 21 days after sciatic nerve injury with and without PTEN siRNA. (B and C) Quantification of total myelinated axons (B) and axon calibre (C) measured as mean axonal diameter. Diabetic animals displayed regeneration deficits with significantly fewer and smaller calibre axons regenerating 3 weeks after injury. PTEN inhibition partially rescued the deficit. (D) Representative images of footpads from wild-type and diabetics with and without PTEN siRNA indicating newly regenerating sensory afferents in the epidermis. Reinnervation was reduced in diabetics with few reinnervating axons crossing into

(continued)

**Figure 6** Continued

the epidermis. (E and F) Quantification of vertical skin fibre density (E) and total skin fibre density (F) identifying lower numbers in diabetics. These deficits were rescued by PTEN inhibition in diabetics, but not in non-diabetics. Values are represented as mean \pm SEM; B and C, * P < 0.05, ** P < 0.01, one-way ANOVA; *post hoc* Tukey's multiple comparison tests and two-tailed Student's t -test, #one-tailed Student's t -test, n = 5–7 animals/group; E and F * P < 0.05, ** P < 0.01, one-way ANOVA; *post hoc* Tukey's multiple comparison test and two-tailed Student's t -test; * P < 0.05). Scale bars; A = 50 μ m; D = 100 μ m.

potential role in the development of diabetic neuropathy given the identification of insulin resistance in adult sensory neurons (Singh *et al.*, 2012). The mechanisms that upregulate PTEN are of significant importance in understanding why diabetic axons regenerate less well. In muscle and endothelial cells, upregulation of PTEN associated with diabetes has been linked to palmitic acid acting through p38 MAPK and ATF2 (Koistinen *et al.*, 2003; Wang *et al.*, 2006; Hu *et al.*, 2007). Similarly, in this study, we observed a rise in p38 messenger RNA and phospho-p38 protein in diabetic sensory neurons. How PTEN might be influenced by mechanisms described above including rises in p38 expression is uncertain and requires further investigation. Although it is likely that PTEN is expressed in lower levels in glial and other non-neuronal cells, its major expression in this and previous work was neuronal, suggesting a direct interaction between diabetes and neuronal PTEN synthesis. In non-diabetic nerves, PTEN expression is downregulated after injury but residual expression nonetheless attenuated regenerative outgrowth (Christie *et al.*, 2010). In the setting of heightened expression, its importance may be correspondingly greater, a possibility suggested in our comparative studies of knockdown in littermate diabetic and non-diabetic mice. Similarly, upregulation of PTEN in small calibre IB-4 neurons has been linked to their limited growth capacity (Leclere *et al.*, 2007; Christie *et al.*, 2010).

We believe our approach towards the delivery of siRNA at the site of a nerve injury offers interesting possibilities for the future treatment of other forms of neurological damage. The mechanisms of siRNA retrograde transport to parent neurons, as demonstrated in this work, are unknown but may be similar to those involving viral vectors (Kaspar *et al.*, 2002). Recent findings also indicate that small RNA species may undergo significant intercellular exchange (Chitwood and Timmermans, 2010). That siRNA lends itself to axonal capture and selective retrograde uptake by injured neurons is of exceptional importance. The present work confirms similar observations in previous published (Webber *et al.*, 2011) and unpublished work from our laboratory using a variety of targeted siRNAs. Although the efficiency of transport and retrograde gene knockdown may be less than with viral vectors, this approach nonetheless offers a pathogen-free solution to targeted neuronal delivery. As PTEN is a tumour suppressor molecule (Ali *et al.*, 1999; Waite and Eng, 2002), it is of translational interest to strictly circumscribe its potential role in both time and tissue. PTEN mutations are associated with Cowden syndrome, a disorder of cutaneous hamartomas with enhanced susceptibility to carcinoma, and with glioblastoma development (Mellinghoff *et al.*, 2005). To avoid potential oncogenesis, direct and selective neuronal delivery during critical periods of early axon outgrowth are required.

Taken together, our findings demonstrate that non-viral strategies for selective targeting of neurons may offer a new approach toward treating localized injuries. This approach may be of value in other neurological conditions. More importantly, we show that PTEN targeting offers interesting and significant benefits to diabetic regenerating axons, over and above the lesser impacts discovered in non-diabetic axons. The importance of PTEN in understanding diabetic complications comes as a surprise, as a variety of intrinsic and extrinsic mechanisms of neuroregenerative failure have long been considered. Our results do not exclude

these mechanisms, but may be superimposed, adding a novel but apparently detrimental response to diabetic nerve injury.

Acknowledgements

We would like to thank Dr. Kimberly Christie for her technical help with the tissue harvesting.

Funding

Operating funds from National Institutes of Health Diabetes Complications Consortium Pilot and Feasibility 12GHSU172 (NIH DCC P+F), Canadian Institutes of Health Research (CIHR 184584) and the Canadian Diabetes Association (CDA) OG-3-12-3669 supported the work. BS held a studentship from Alberta Innovates-Health Solutions (AI-HS). The work was supported by the RUN (Regeneration Unit in Neurobiology) facility, Hotchkiss Brain Institute at the University of Calgary.

Supplementary material

Supplementary material is available at *Brain* online.

References

- Ali IU, Schriml LM, Dean M. Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. *J Natl Cancer Inst* 1999; 91: 1922–32.
- Andersen PL, Webber CA, Kimura KA, Schreyer DJ. Cyclic AMP prevents an increase in GAP-43 but promotes neurite growth in cultured adult rat dorsal root ganglion neurons. *Exp Neurol* 2000; 166: 153–65.
- Brewster WJ, Fernyhough P, Diemel LT, Mohiuddin L, Tomlinson DR. Diabetic neuropathy, nerve growth factor and other neurotrophic factors. *Trends Neurosci* 1994; 17: 321–5.
- Bril V. Role of electrophysiological studies in diabetic neuropathy. *Can J Neurol Sci* 1994; 21 (Suppl 4): S8–12.
- Carter AN, Downes CP. Phosphatidylinositol 3-kinase is activated by nerve growth factor and epidermal growth factor in PC12 cells. *J Biol Chem* 1992; 267: 14563–7.
- Cheng C, Guo GF, Martinez JA, Singh V, Zochodne DW. Dynamic plasticity of axons within a cutaneous milieu. *J Neurosci* 2010; 30: 14735–44.
- Chitwood DH, Timmermans MC. Small RNAs are on the move. *Nature* 2010; 467: 415–19.
- Christie KJ, Webber CA, Martinez JA, Singh B, Zochodne DW. PTEN inhibition to facilitate intrinsic regenerative outgrowth of adult peripheral axons. *J Neurosci* 2010; 30: 9306–15.
- Conti G, Stoll G, Scarpini E, Baron PL, Bianchi R, Livraghi S, et al. p75 neurotrophin receptor induction and macrophage infiltration in peripheral nerve during experimental diabetic neuropathy: possible relevance on regeneration. *Exp Neurol* 1997; 146: 206–11.
- Dyck PJ, Kratz KM, Karnes JL, Litchy WJ, Klein R, Pach JM, et al. The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester diabetic neuropathy study. *Neurology* 1993; 43: 817–24.
- Eickholt BJ, Walsh FS, Doherty P. An inactive pool of GSK-3 at the leading edge of growth cones is implicated in Semaphorin 3A signaling. *J Cell Biol* 2002; 157: 211–17.
- Gallo G, Letourneau PC. Localized sources of neurotrophins initiate axon collateral sprouting. *J Neurosci* 1998; 18: 5403–14.

- Glazner GW, Lupien S, Miller JA, Ishii DN. Insulin-like growth factor II increases the rate of sciatic nerve regeneration in rats. *Neuroscience* 1993; 54: 791–7.
- GrandPre T, Li S, Strittmatter SM. Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature* 2002; 417: 547–51.
- Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988; 32: 77–88.
- Hu Z, Lee IH, Wang X, Sheng H, Zhang L, Du J, et al. PTEN expression contributes to the regulation of muscle protein degradation in diabetes. *Diabetes* 2007; 56: 2449–56.
- Ishii DN, Lupien SB. Insulin-like growth factors protect against diabetic neuropathy: effects on sensory nerve regeneration in rats. *J Neurosci Res* 1995; 40: 138–44.
- Jones DM, Tucker BA, Rahimtula M, Mearow KM. The synergistic effects of NGF and IGF-1 on neurite growth in adult sensory neurons: convergence on the PI 3-kinase signaling pathway. *J Neurochem* 2003; 86: 1116–28.
- Kan M, Guo G, Singh B, Singh V, Zochodne DW. Glucagon-like peptide 1, insulin, sensory neurons, and diabetic neuropathy. *J Neuropathol Exp Neurol* 2012; 71: 494–510.
- Kaspar BK, Erickson D, Schaffer D, Hinh L, Gage FH, Peterson DA. Targeted retrograde gene delivery for neuronal protection. *Mol Ther* 2002; 5: 50–6.
- Kennedy JM, Zochodne DW. The regenerative deficit of peripheral nerves in experimental diabetes: its extent, timing and possible mechanisms. *Brain* 2000; 123: 2118–29.
- Kennedy JM, Zochodne DW. Influence of experimental diabetes on the microcirculation of injured peripheral nerve: Functional and morphological aspects. *Diabetes* 2002; 51: 2233–40.
- Kennedy JM, Zochodne DW. Experimental diabetic neuropathy and spontaneous recovery: Is there irreparable damage? *Diabetes* 2005; 54: 830–7.
- Kennedy WR, Navarro X, Goetz FC, Sutherland DE, Najarian JS. Effects of pancreatic transplantation on diabetic neuropathy. *N Engl J Med* 1990; 322: 1031–7.
- Koistinen HA, Chibalin AV, Zierath JR. Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from Type 2 diabetic patients. *Diabetologia* 2003; 46: 1324–8.
- Leclere PG, Norman E, Groutsi F, et al. Impaired axonal regeneration by isolectin B4-binding dorsal root ganglion neurons *in vitro*. *J Neurosci* 2007; 27: 1190–9.
- Leslie NR, Downes CP. PTEN: The down side of PI 3-kinase signalling. *Cell Signal* 2002; 14: 285–95.
- Lindsay RM. Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J Neurosci* 1988; 8: 2394–405.
- Liu K, Lu Y, Lee JK, Samara R, Willenberg R, Sears-Kraxberger I, et al. PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nat Neurosci* 2010; 13: 1075–81.
- Longo FM, Powell HC, Lebeau J, Gerrero MR, Heckman H, Myers RR. Delayed nerve regeneration in streptozotocin diabetic rats. *Muscle Nerve* 1986; 9: 385–93.
- Love A, Cotter MA, Cameron NE. Impaired myelinated fiber regeneration following freeze-injury in rats with streptozotocin-induced diabetes: involvement of the polyol pathway. *Brain Res* 1995; 703: 105–10.
- Malik RA, Veves A, Walker D, Siddique I, Lye RH, Schady W, et al. Sural nerve fibre pathology in diabetic patients with mild neuropathy: relationship to pain, quantitative sensory testing and peripheral nerve electrophysiology. *Acta Neuropathol* 2001; 101: 367–74.
- Mehra S, Tavakoli M, Kallinikos PA, Efron N, Boulton AJ, Augustine T, et al. Corneal confocal microscopy detects early nerve regeneration after pancreas transplantation in patients with type 1 diabetes. *Diabetes Care* 2007; 30: 2608–12.
- Mellinghoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med* 2005; 353: 2012–24.
- Mukhopadhyay G, Koherty P, Walsh FS, Crocker PR, Filbin MT. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* 1994; 13: 757–67.
- Nakae J, Biggs WH III, Kitamura T, Cavenee WK, Wright CV, Arden KC, et al. Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat Genet* 2002; 32: 245–53.
- Ohnishi A, O'Brien PC, Dyck PJ. Studies to improve fixation of human nerves. Part 3. Effect of osmolality of glutaraldehyde solutions on relationship of axonal area to number of myelin lamellae. *J Neurol Sci* 1976; 27: 193–9.
- Pal A, Barber TM, Van de Bunt M, Rudge SA, Zhang Q, Lachlan KL, et al. PTEN mutations as a cause of constitutive insulin sensitivity and obesity. *N Engl J Med* 2012; 367: 1002–11.
- Park KK, Liu K, Hu Y, Kanter JL, He Z. PTEN/mTOR and axon regeneration. *Exp Neurol* 2010; 223: 45–50.
- Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, et al. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science* 2008; 322: 963–6.
- Polydefkis M, Hauer P, Griffin JW, McArthur JC. Skin biopsy as a tool to assess distal small fiber innervation in diabetic neuropathy. *Diabetes Technol Ther* 2001; 3: 23–8.
- Raffin S, Bradshaw RA. Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. *Proc Natl Acad Sci USA* 1992; 89: 9121–5.
- Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME. Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature* 1994; 367: 170–3.
- Sima AA, Bril V, Nathaniel V, McEwen TA, Brown MB, Lattimer SA, et al. Regeneration and repair of myelinated fibers in sural-nerve biopsy specimens from patients with diabetic neuropathy treated with sorbinil. *N Engl J Med* 1988; 319: 548–55.
- Singh B, Xu Y, McLaughlin T, Singh V, Martinez JA, Krishnan A, et al. Resistance to trophic neurite outgrowth of sensory neurons exposed to insulin. *J Neurochem* 2012; 121: 263–76.
- Soltoff SP, Rabin SL, Cantley LC, Kaplan DR. Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with the trk tyrosine kinase. *J Biol Chem* 1992; 267: 17472–7.
- Terada M, Yasuda H, Kikkawa R. Delayed Wallerian degeneration and increased neurofilament phosphorylation in sciatic nerves of rats with streptozotocin-induced diabetes. *J Neurol Sci* 1998; 155: 23–30.
- Triban C, Guidolin D, Fabris M, Marini P, Schiavinato A, Donà M, et al. Ganglioside treatment and improved axonal regeneration capacity in experimental diabetic neuropathy. *Diabetes* 1989; 38: 1012–22.
- Waite KA, Eng C. Protean PTEN: form and function. *Am J Hum Genet* 2002; 70: 829–44.
- Wang XL, Zhang L, Youker K, Zhang MX, Wang J, LeMaire SA, et al. Free fatty acids inhibit insulin signaling-stimulated endothelial nitric oxide synthase activation through upregulating PTEN or inhibiting Akt kinase. *Diabetes* 2006; 55: 2301–10.
- Webber CA, Christie KJ, Cheng C, Martinez JA, Singh B, Singh V, et al. Schwann cells direct peripheral nerve regeneration through the netrin-1 receptors, DCC and Unc5H2. *Glia* 2011; 59: 1503–17.
- Williams SK, Howarth NL, Devenny JJ, Bitensky MW. Structural and functional consequences of increased tubulin glycosylation in diabetes mellitus. *Proc Natl Acad Sci USA* 1982; 79: 6546–50.
- Xu G, Pierson CR, Murakawa Y, Sima AA. Altered tubulin and neurofilament expression and impaired axonal growth in diabetic nerve regeneration. *J Neuropathol Exp Neurol* 2002; 61: 164–75.
- Xu G, Sima AA. Altered immediate early gene expression in injured diabetic nerve: implications in regeneration. *J Neuropathol Exp Neurol* 2001; 60: 972–83.
- Yamamoto Y, Yasuda Y, Kimura Y, Komiya Y. Effects of cilostazol, an antiplatelet agent, on axonal regeneration following nerve injury in diabetic rats. *Eur J Pharmacol* 1998; 352: 171–8.

- Yasuda H, Terada M, Maeda K, Kogawa S, Sanada M, Haneda M, et al. Diabetic neuropathy and nerve regeneration. *Prog Neurobiol* 2003; 69: 229–85.
- Yasuda H, Terada M, Taniguchi Y, Sasaki T, Maeda K, Haneda M, et al. Impaired regeneration and no amelioration with aldose reductase inhibitor in crushed unmyelinated nerve fibers of diabetic rats. *NeuroReport* 1999; 10: 2405–9.
- Zherebitskaya E, Akude E, Smith DR, Fernyhough P. Development of selective axonopathy in adult sensory neurons isolated from diabetic rats: role of glucose-induced oxidative stress. *Diabetes* 2009; 58: 1356–64.
- Zochodne DW. Diabetic neuropathies: features and mechanisms. *Brain Pathol* 1999; 9: 369–91.
- Zochodne DW, Ramji N, Toth C. Neuronal targeting in diabetes mellitus: a story of sensory neurons and motor neurons. *Neuroscientist* 2008; 14: 311–18.
- Zochodne DW, Verge VMK, Cheng C, Sun H, Johnston J. Does diabetes target ganglion neurons? Progressive sensory neuron involvement in long term experimental diabetes. *Brain* 2001; 124: 2319–34.