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Neurons and Tumor Suppressors

Douglas W. Zochodne*

Hotchkiss Brain Institute and Department of Clinical Neurosciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 4N1, Canada

ABSTRACT: Neurons choose growth pathways with half hearted reluctance, behavior that may be appropriate to maintain fixed long lasting connections but not to regenerate them. We now recognize that intrinsic brakes on regrowth are widely expressed in these hesitant neurons and include classical tumor suppressor molecules. Here, we review how two brakes, PTEN (phosphatase and tensin homolog deleted on chromosome 10) and retinoblastoma emerge as new and exciting knockdown targets to enhance neuron plasticity and improve outcome from damage or disease.

KEYWORDS: *peripheral nerve, regeneration, retinoblastoma protein, PTEN*

Unrestrained growth and sprouting of axons in the mature adult nervous system might disrupt stable and critical connections established during neurodevelopment. However, after injury, robust plasticity would favor regrowth of injured axons to their target organs. While neurons display greater axon regrowth following peripheral nervous system (PNS) injury than central nervous system (CNS) injury, severe limitations to peripheral axon regrowth are nonetheless common. For example, a major human sciatic lesion at the level of the thigh is unlikely to be associated with regrowth of axons into the foot; prolonged distal stump denervation, reluctant initial regrowth, and impaired directionally competent regeneration all contribute to failure to recover. Thus, like the CNS, PNS lesions pose major regenerative challenges.

Adult peripheral neurons express a growing list of regenerative "brakes", inhibition or knockdown of which encourages greater plasticity. These are intrinsic pathways that appear to operate downstream of growth factor receptors but may suppress growth in response to a wide array of signals. The RHOA-ROK (RHO kinase) GTPase pathway mediates growth cone retraction in response to extracellular cues that include myelin associated glycoprotein (MAG), Nogo-66, oligodendrocyte myelin glycoprotein, semaphorins, and chondroitin sulfate proteoglycans. RHOA-ROK inhibition appears to operate on distal growth cone pathways including LIM kinase and cofilin (ADF, actin depolymerising factor) that alter actin dynamics and myosin phosphatase leading to increased myosin ATPase activity and growth cone retraction. Several inhibitors of RHOA-ROK are identified. Fasudil (HA-1077) increases neurite outgrowth of adult sensory neurons *in vitro* and increases the outgrowth of axons distal to a nerve transection.[1]

Tumor suppressors include a range of molecules that inhibit key growth pathways. These have operated in a variety of contexts including cell cycle inhibition, interruption of growth factor receptor pathways, facilitation of cell death, and other mechanisms. We hypothesized that among this pool of molecules, several might be expressed in and offer their restraining properties to neurons. Our first choice was PTEN (phosphatase and tensin homolog deleted on chromosome ten), a lipid and protein phosphatase that specifically dephosphorylates phosphatidylinositol 3,4,5 triphosphate (PIP3), a intermediary in the growth factor receptor-PI3K p85-pAkt growth pathway. pAkt, in turn, is an important integrator of growth signals that act on a number of targets including inhibiting GSK-3β through phosphorylation; GSK-3β, in turn, is an inhibitor of growth cone microtubule dynamics. Thus, PTEN actively suppresses signals upstream of this critical growth pathway. Mutations in PTEN are associated with Cowden's disease, a disorder associated with carcinogenesis, and PTEN mutations are associated with glioblastoma development. PTEN was expressed widely in peripheral sensory neurons, but prominent expression in small caliber IB-4 nonpeptidergic neurons was intriguing, given the more restrained regrowth these neurons exhibit.[2] Both inhibition of PTEN pharmacologically, using bpV(pic), and PTEN knockdown, using siRNA, increased the neurite outgrowth of adult sensory neurons (Figure 1). Moreover, the impact of this strategy in preinjured neurons with an accelerated growth

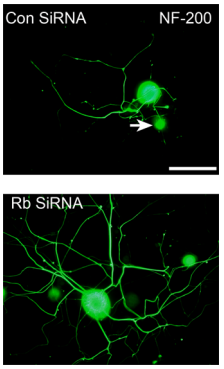

Figure 1. Adult rat sensory neurons, stained with a neurofilament marker exposed to either scrambled control siRNA or Rb1 siRNA. Knockdown of Rb1 was associated with increased neurite outgrowth *in vivo* and enhanced regeneration *in vivo*. Bar = 100 μm. The image was created by Dr. Bhagat Singh, Zochodne Laboratory, University of Calgary.

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phenotype was yet greater. "Preconditioning" of neurons with a previous axotomy injury ramps up regenerative programs and by itself dramatically increases neurite outgrowth. It is remarkable that the impact of PTEN was substantially greater in preconditioned neurons, indicating that the persistent expression of this roadblock to regrowth restrained growth patterns previously considered optimal for neurons. During our ongoing work investigating PTEN in peripheral neuron outgrowth, Park, He, and colleagues published evidence that viral mediated PTEN knockdown increased adult retinal axon regrowth in the CNS, an outcome involving signaling through the mTOR pathway and inhibited by rapamycin.[3] However, in peripheral neurons, accelerated outgrowth was independent of this pathway without inhibition by rapamycin. PTEN inhibition with bp(V)pic or local PTEN siRNA, applied locally within the milieu of axon outgrowth from a nerve transection, increased the outgrowth of emerging axons.

PTEN was also colocalized in sensory neurons with the ubiquitin proteosome system (UPS) E3 ligase Nedd4 (neural precursor cell-expressed developmentally down-regulated protein 4) that degrades it. Knockdown of Nedd4 was associated with rises in PTEN levels and attenuated neurite outgrowth.

The potential significance of PTEN manipulation in clinical peripheral nerve disorders is well tested in diabetes mellitus, a metabolic disorder that not only leads to neurodegeneration or polyneuropathy but also attenuates regeneration. This "double hit" on peripheral neurons is a challenging patient problem. Of interest is the finding that PTEN protein and mRNA levels were elevated in DRG sensory neurons of mice with chronic diabetes.[4] Moreover, knockdown of PTEN in diabetic neurons *in vitro* similarly enhanced neuite outgrowth. Taken further, PTEN siRNA, administered at the site of an injured diabetic nerve underwent retrograde transport, where it knocked down PTEN expression and improved indices of subsequent regeneration: compound muscle action potentials, reflecting numbers of reconnected motor axons to end plates, conduction velocities of regenerating motor and sensory axons, numbers and caliber of regrowing myelinated axons distal to the injury site, reinnervation of the epidermis by unmyelinated axons, and restoration of mechanical sensation. Taken together, all of the findings concerning neuronal actions of PTEN indicate a robust impact of manipulating this tumor suppressor on regenerative capacity.

The retinoblastoma protein (Rb1) inhibits cell cycle progression and other forms of plasticity by binding to and suppressing E2F transcriptional activity, a pathway with divergent outputs. Phosphorylation of Rb inhibits its binding and unfetters E2F mediated plasticity. Unlike PTEN, Rb1 is not clearly linked to recognized growth pathways such as pAkt and MEK-ERK-Myc but instead may act downstream of them. At least one of its mediators may be the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) since its levels rise in neurons with Rb1 knockdown. Furthermore, PPARγ agonists and antagonists influence the growth properties of neurons. Beyond this insight, the Rb1 pathway appears to operate through unclarified mechanisms, very much worth exploring in the search for palates of neuronal regeneration molecules. In peripheral neurons, Rb1 was widely expressed in sensory neuronal cytoplasm, axons, and nuclei, unlike the preferential expression of PTEN in IB4 non-peptidergic neurons. Knockdown of Rb1 with siRNA or knockout of Rb1 using neurons from Rb1 floxed mice exposed to Cre recombinase expressing adenovirus were both associated with robust increases in neurite outgrowth, including previously preconditioned neurons.[5] Like PTEN suppression, Rb1 inhibition added to the enhanced outgrowth properties of neurons beyond that offered by preconditioning alone. For both molecules, this is a remarkable finding since a number of previous investigations have sought to exploit the preconditioning effect alone to further growth.

Most small molecules designed to interact with Rb1 emphasize inhibiting its deactivation (blocking phosphorylation) to limit tumor growth. In neurons, however, its deactivation or inhibition is the goal, and for this, we relied on specific knockdown using siRNA, examining two separate siRNAs in rats and knockdown in adult mouse neurons. Like PTEN, local Rb1 siRNA enhanced the outgrowth of axons beyond a nerve trunk transection. Taking the *in vivo* analysis one step further, however, we noted that Rb1 siRNA, applied locally to injured nerves in mice, improved several facets of regeneration: mechanical and thermal sensitivity, hindpaw grip strength, and sensory conduction velocity of regenerating axons.

These findings validate arguments that the growth behavior of adult sensory neurons *in vitro* regularly predicts how new strategies might impact growth *in vivo*. This has been the case with RHOA-ROK, insulin, PTEN in diabetic mice, and Rb1. Finally, it may be that molecular manipulation of peripheral neurons may be more easily achieved than surmised. In the case of Rb1, PTEN, and previously Unc5H, we have achieved ipsilateral knockdown of molecules of interest without a viral vector, instead employing local lipotransfection of axons, with evidence that the siRNAs are taken up and retrogradely transported to their perikarya. Thus, in addition to the new repertoire of molecules that may be exploited to coax reluctant growth of neurons, including tumor suppressor members, nonviral delivery of siRNA may offer an exciting new approach toward influencing them. The critical piece of the story will be that delivery be designed as spatially and temporally constrained to avoid the risk of oncogenesis that could occur with long-term unleashing of tumor suppressors.

AUTHOR INFORMATION

Corresponding Author

*168 Heritage Medical Research Bldg, 3330 Hospital Dr. NW, Calgary, Alberta, Canada, T2N 4N1. Phone: 403-220-8759. Fax: 403-283-8731. E-mail: dzochodn@ucalgary.ca.

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Notes

The authors declare the following competing financial interest(s): The author has filed for patent protection for the use of Rb1 siRNA in regeneration.

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