Subtype-Specific Regeneration of Retinal Ganglion Cells following Axotomy: Effects of Osteopontin and mTOR Signaling

Highlights
- RGC subtypes differ dramatically in ability to survive axotomy
- Axotomized αRGCs regenerate preferentially when mTOR signaling is stimulated
- αRGCs selectively express osteopontin and IGF1 receptors
- Administration of osteopontin plus IGF1 promotes αRGC axon regeneration

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In Brief
Duan et al. show that limited regeneration of retinal ganglion cell (RGC) axons following transection reflects preferential survival and selective regeneration of one subset, αRGCs. αRGCs selectively express osteopontin and IGF1 receptor; administered together, osteopontin and IGF1 promote αRGC regeneration.
Subtype-Specific Regeneration of Retinal Ganglion Cells following Axotomy: Effects of Osteopontin and mTOR Signaling

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SUMMARY

In mammals, few retinal ganglion cells (RGCs) survive following axotomy, and even fewer regenerate axons. This could reflect differential extrinsic influences or the existence of subpopulations that vary in their responses to injury. We tested these alternatives by comparing responses of molecularly distinct subsets of mouse RGCs to axotomy. Survival rates varied dramatically among subtypes, with alpha-RGCs (αRGCs) surviving preferentially. Among survivors, αRGCs accounted for nearly all regeneration following downregulation of PTEN, which activates the mTOR pathway. αRGCs have uniquely high mTOR signaling levels among RGCs and also selectively express osteopontin (OPN) and receptors for the insulin-like growth factor 1 (IGF-1). Administration of OPN plus IGF-1 promotes regeneration as effectively as downregulation of PTEN; however, regeneration is still confined to αRGCs. Our results reveal dramatic subtype-specific differences in the ability of RGCs to survive and regenerate following injury, and they identify promising agents for promoting axonal regeneration.

INTRODUCTION

Regeneration following injury to the mammalian brain or spinal cord is notoriously poor: few survivors extend axons beyond the injury site (Ramon y Cajal, 1928), and in some cases, many of the axotomized neurons die (Mansour-Robaey et al., 1994; Conta Steencken et al., 2011). Limited regeneration can be explained in at least two different ways. First, non-genetic differences among neurons could account for differences in outcome—for example, stochastic variation, history of activity, or proximity to environmental cues that modulate growth. Alternatively or in addition, distinct subpopulations within a seemingly homogeneous population could regenerate preferentially, owing to preexisting qualities that improve their lot. Distinguishing among these and other alternatives is important both in guiding searches for protective factors and in assessing interventions designed to enhance regeneration.

Here, we analyzed retinal ganglion cells (RGCs) to address this issue. All visual information is conveyed from the eye to the brain by RGC axons, which run through the optic nerve to retinorecipient areas such as the superior colliculus and lateral geniculate nucleus. Although all RGCs share numerous attributes, they can be divided into ~30 distinct subpopulations, on the basis of morphological, physiological, and molecular criteria (Masland, 2012; Sanes and Masland, 2015). After damage to the mouse optic nerve, >80% of RGCs die, and <1% of the survivors extend axons past the site of damage (Mansour-Robaey et al., 1994; Park et al., 2008). Regeneration of a substantial number of RGC axons can be elicited, however, by manipulations of the neurons themselves or the environment through which they grow (Aguayo et al., 1991; Benowitz and Popovich, 2011; Liu et al., 2011; Maier and Schwab, 2006; Park et al., 2008; Smith et al., 2009; Sun et al., 2011). Thus, one can ask whether specific subsets of RGCs differ in their abilities to survive following nerve crush and/or regenerate axons following treatment.

In the first part of this study, we assessed the survival of 11 RGC subtypes after transection of the optic nerve in mice. Subtypes differed dramatically in susceptibility to damage, with the largest RGC types, alpha-RGCs (αRGCs), surviving preferentially but not exclusively. We then promoted regeneration by suppressing expression of PTEN, which acts, at least in part, by enhancing mTOR activity (Jaworski et al., 2005; Kim et al., 2009; Park et al., 2008; Zukor et al., 2013). We found that αRGCs accounted for nearly all of the regenerating axons in this paradigm.

On the basis of these results, we sought features of αRGCs that might account for their regenerative ability and found three: they have high endogenous levels of mTOR activity, they selectively express a secreted phosphoprotein, osteopontin (OPN) (Bellahcène et al., 2008; Wang and Denhardt, 2008), which is capable of stimulating mTOR activity (Ahmed and Kundu, 2012).
2010), and they selectively express receptors for insulin-like growth factor 1 (IGF-1), which promotes the regeneration of some neuronal types (Dupraz et al., 2013). Ectopic expression of OPN in combination with IGF-1 promotes regeneration of αRGCs as effectively as PTEN suppression. Together, our work identifies a neuronal-intrinsic factor that can promote regeneration and provides a strategy to identify additional regeneration-promoting factors.

RESULTS

Differential Survival of RGC Subtypes

We used immunohistochemical and transgenic approaches to mark molecularly distinct subsets of RGCs in mice: (1) There are four groups of ON-OFF direction-selective RGCs (ooDSGCs), each tuned to motion in a single direction: ventral, dorsal, nasal, and temporal. Antibodies to the neuropeptide cocaine- and amphetamine-regulated transcript (CART) label all four groups (Kay et al., 2011), while a transgenic line, HB9-GFP, labels the subset tuned to ventral motion (Trenholme et al., 2011). (2) W3-RGCs are labeled with yellow fluorescent protein (YFP) in the TYW3 mouse line (Kim et al., 2010). W3-RGCs are among the smallest RGCs in terms of soma size and dendritic diameter and are among the most numerous RGCs. They comprise at least two populations: W3B, which are motion sensitive but not direction selective, and W3D, which remain physiologically uncharacterized (Zhang et al., 2012). (3) Antibodies to melanopsin label M1- and M2-RGCs, two subsets of intrinsically photosensitive RGCs that can be distinguished by the sublaminae of the inner plexiform layer within which their dendrites arborize (Berson et al., 2010; Ecker et al., 2010). (4) The RGCs with the largest somata are αRGCs. In mice, they comprise at least three subtypes, which differ in physiological properties as well as dendritic stratification (Estevez et al., 2014; Pang et al., 2003; Schubert et al., 2005; van Wyk et al., 2009; Vögel et al., 2005). We recently generated and characterized a mouse line in which Cre recombinase is inserted into the locus encoding a potassium channel modifier, kcng4. When crossed to a reporter line (Buffelli et al., 2003), subsets of bipolar cells and RGCs were YFP positive in double-transgenic offspring (Kcng4-cre;Thy1-stop-YFP line 1; called Kcng4-YFP here) (Duan et al., 2012; Buffelli et al., 2003), subsets of bipolar cells and RGCs were YFP positive in double-transgenic offspring (Kcng4-cre;Thy1-stop-YFP line 1; called Kcng4-YFP here) (Duan et al., 2012). Further analysis revealed that the labeled RGCs had large somata and dendrites and were rich in a neurofilament-associated epitope, SMII32 (Figure S1). These features identified them as αRGCs (Benson, 2008; Peichl, 1991). Morphological and physiological analysis revealed that labeled RGCs included all three types of αRGCs, and no other RGCs (Figure 1B; M.Q., X.D., J.R.S., B. Krieger, and M. Meister, unpublished data). Thus, the Kcng4-cre line provides selective genetic access to αRGCs. Together, these markers allowed us to assay the survival of 11 RGC subtypes (4 ooDSGCs, 2 W3-RGCs, 3 αRGCs, and 2 melanopsin-positive RGCs).

We crushed the optic nerves of wild-type or transgenic mice and then assessed RGC survival 14 days post crush (dpc). Consistent with previous reports (Park et al., 2008), ~20% of RGCs survived, as assessed by staining for class III beta-tubulin (TuJ1), a pan-RGC marker (Figures 1A and 1C). The survival rate varied greatly among RGC subtypes. More than 80% of the αRGCs (Kcng4-YFP RGCs) and more than 70% of the M1-RGCs survived, whereas few if any M2-RGCs or ooDSGCs (HB9-GFP and CART⁺RGCs) survived (Figures 1A–1C). Survival of W3-RGCs was intermediate (~10%). Examination of Kcng4-YFP retina cross-sections, in which the three αRGC subtypes can be distinguished by dendritic lamination, indicated that all three subtypes survived (Figure 1B). As a consequence, αRGCs and M1-RGCs, which comprise ~6% and ~3% of all RGCs in the normal retina, respectively, accounted for 23% and 11% of surviving RGCs by 14 dpc (Figure 1E).

To assess whether the apparently preferential survival of αRGCs represented a delayed cell loss, we examined animals after 2 additional weeks (28 dpc). Although ~10% of the RGCs were lost between 14 and 28 dpc, αRGCs and M1-RGCs were still preferentially spared and constituted ~25% and ~15% of all surviving RGCs, respectively (Figures 1D and 1E). Thus, αRGCs and M1-RGCs survive preferentially but not exclusively following nerve crush.

Selective Regeneration of αRGCs

Next, we compared the ability of RGC subtypes to extend axons following injury. To promote regeneration, we injected an adeno-associated virus (AAV) encoding a previously validated short hairpin RNA directed against PTEN (shPTEN) (Zukor et al., 2013), a negative regulator of mTOR signaling. We used immunostaining for a phosphorylated form (Ser235/236) of ribosome protein S6 (pS6) to assay the efficacy of shPTEN, on the basis of previous studies showing that pS6 provides a reliable estimate of mTOR activity (Laplante and Sabatini, 2012; Park et al., 2008). Levels of pS6 were increased in ~60% of RGCs in optimally infected areas by 2 weeks after infection with AAV-shPTEN (Figure 2A). Thus, AAV infects, and PTEN restricts mTOR signaling in, most if not all RGC subtypes. A control AAV (AAV-GFP) infected a similar fraction of RGCs but had no effects on pS6. AAV-shPTEN had no detectable effect on retinal structure or integrity (data not shown).

To assess regeneration, we crushed the optic nerve in Kcng4-YFP, HB9-GFP, and TYW3 mice 2 weeks after AAV-shPTEN administration, then analyzed retina and optic nerve 14 dpc. Selective survival of αRGCs following depletion of PTEN was similar to that observed in control retinas after crush (Figure S2A compared with Figure 1A). Regeneration was detected by counting YFP- or GFP-positive axons in longitudinal sections through the optic nerves. We observed substantial regeneration of αRGC axons but no detectable regeneration of ooDSGC or W3-RGC axons (Figure 2B).

To visualize all regenerating axons, we injected the anterograde tracer cholera toxin subunit B (CTB) into the retina 2 to 3 days before sacrifice. In all lines, approximately 150 axons per retina regenerated at least 0.5 mm past the crush site, accounting for ~2.5% of surviving RGCs (Figures 2C and 2D). Importantly, >90% of the CTB-positive axons in the Kcng4-YFP retina were also YFP positive (Figure 2D). No markers were available to label M1-RGC axons, but the near complete overlap of CTB- and YFP-positive axons in the Kcng4-YFP line suggests that few if any M1-RGCs or other RGC subtypes regenerate. Thus, αRGC account for the vast majority of the regenerating axons after downregulation of PTEN.
**αRGCs Have High mTOR Activity and Are Rich in OPN**

In the adult retina, ~10% of RGCs are stained intensely with anti-pS6 and therefore have high mTOR activity (Park et al., 2008). We asked whether the pS6-rich cells are αRGCs. In initial studies, we found variable pS6 levels in RGCs of adult mice (data not shown). We reasoned that neuronal activity, which is known to stimulate phosphorylation of pS6 (Knight et al., 2012), contributed to this variability. We therefore dark-adapted mice overnight to decrease activity of RGCs, then stained retinas of Kcng4-YFP mice for pS6. In dark-adapted retinas, 6% of RGCs were clearly pS6 rich, and the others were not detectably pS6 positive (Figure 3A). The pS6-rich RGCs were αRGCs: >90% of Kcng4-YFP+ RGCs neurons were rich in pS6, and >85% of pS6-rich neurons were YFP+ (Figure 3B).

We then asked whether αRGCs express other genes that could play roles in their selective survival and regeneration. In initial studies, we detected expression of OPN (gene symbol Spp1, for secreted phosphoprotein-1) in large RGCs in adult retina (M. Yamagata and J.R.S, unpublished data). We selected this candidate for further analysis because OPN is expressed by a subset of RGCs in rats (Ju et al., 2000), can enhance mTOR activity (Ahmed and Kundu, 2010), and has been implicated in injury responses of other neuronal types (see Discussion). Immunostaining in the Kcng4-YFP line revealed that >90% of αRGCs were OPN+, and 84% of OPN+ RGCs were αRGCs (Figures 3E and 3F). Likewise, OPN and neurofilament SMI32 staining overlapped by >90% (Figure S1B).
To assess whether OPN regulates mTOR activity in the retina, we used AAV-mediated gene transfer to express OPN in multiple RGC subtypes. More than 60% of RGCs were strongly OPN⁺ in optimally infected areas 2 weeks after infection, and levels of pS6 were high in ~60% of OPN-rich RGCs (Figures 3G and 3H). Because αRGCs constitute only 6% of RGCs, OPN is clearly able to stimulate mTOR signaling in non-αRGCs. We also used OPN null mutant mice (OPN⁻/⁻) to ask whether the high mTOR levels of αRGCs require expression of OPN. Levels of pS6 in αRGCs did not differ detectably between controls and OPN⁻/⁻ mice (Figures 3I and 3J). Thus, OPN stimulates mTOR activity but is presumably not the only factor that maintains high levels of mTOR activity in αRGCs.

**OPN Promotes RGC Growth of Non-αRGCs**

Before assessing the ability of OPN to affect regeneration, we investigated its role in the normal development of αRGCs. We found that the difference in size between αRGC and non-αRGC somata arose during the first postnatal week, whereas OPN was not detectable in RGCs until the second postnatal week (Figures 4A and 4B). These results suggested that OPN is dispensable for the initial growth of RGCs. Consistent with this idea, we found no significant difference in size between control and OPN⁻/⁻ αRGCs during the period of peak growth or in adulthood (Figure S3A and data not shown). Moreover, the majority of RGCs were rich in pS6 during the first postnatal week, and pS6 immunoreactivity did not become restricted to αRGCs until the third postnatal week (Park et al., 2008). Thus, neither selective mTOR signaling nor selective expression of OPN is required for αRGCs to reach their normal size.

We also asked whether OPN can promote growth of RGCs in adults using AAV-mediated gene transfer of OPN, as described above. RGC soma size (measured by area in sections) increased by ~30% over controls 4 weeks after the introduction of OPN to a broad range of RGC types using Thy1-cre mice (Figures 4C and 4D). Analysis with subtype-specific markers indicated that small W3-RGCs were affected disproportionately (80% increase in area), whereas the size of αRGCs did not increase in the presence of supernormal levels of OPN (Figures 4E and S3B); this is consistent with the presence of additional growth-promoting factors in these neurons. Together, these results indicate that OPN does not play an essential role in the development of αRGCs but can elicit RGC growth and mTOR signaling in adult retina.

**OPN plus IGF-1 Promotes Axonal Regeneration**

To determine whether OPN could promote axonal regeneration following nerve injury, we used the optic nerve crush protocol described above but injured retinal cells with AAV expressing OPN instead of shPTEN. Immunostaining showed expression of OPN in ~80% of RGCs in injured retina (data not shown), but regeneration was not significantly more effective in its presence than in control retinas (Figures 5A and 5B). We therefore combined AAV-OPN administration with intravitreal injection of a growth factor. We chose IGF-1 because it has neuroprotective and regeneration-promoting abilities in other contexts (Dupraz et al., 2013; Hollis et al., 2009) and because its receptor, IGF1R, is expressed by RGCs (Bu et al., 2013; Tagami et al., 2009; and see below). IGF-1 alone had no detectable effect on regeneration, but the combination promoted regeneration as effectively as shPTEN (Figures 5A and 5B). In some cases, axons regenerated and extended more than 2 mm (Figure S4A). OPN and IGF-1 promoted RGC survival to a modest extent (Figures S5A and S5B), but the effect on survival was insufficient to account for the effect on axonal regeneration.
We then tested the relationship of OPN, IGF-1, and mTOR activation as promoters of regeneration. IGF-1 on its own had no detectable effect on mTOR signaling in RGCs following axotomy, but expression of OPN, with or without IGF-1 enhanced mTOR signaling, after axotomy, as it did in normal retinas (Figures S6A and S6B). Moreover, rapamycin, a potent and specific inhibitor of mTOR (Laplante and Sabatini, 2012), blocked axonal regeneration promoted by OPN plus IGF-1 (Figures 5C and 5D) as well as the OPN-induced increase in pS6 levels (Figure S6C) with minimal effects on neuronal survival (Figure S5C). Conversely, regeneration promoted by shPTEN is affected little if at all in OPN−/− mice (data not shown). These data place mTOR signaling downstream of OPN in a molecular pathway that promotes regeneration.

We also tested the combination of OPN and brain-derived neurotrophic factor (BDNF), because BDNF promotes RGC branching, and its receptor, TrkB, is expressed by RGCs (Cui et al., 2002; Sawai et al., 1996). Like IGF-1, BDNF was ineffective on its own at promoting regeneration and had a modest effect on neuronal survival (Figure S5C). Conversely, regeneration promoted by shPTEN is affected little if at all in OPN−/− mice (data not shown). These data place mTOR signaling downstream of OPN in a molecular pathway that promotes regeneration.

Figure 3. Selective mTOR Activity and OPN Expression in αRGCs
(A and B) Section of Kcng4-YFP retina labeled with antibodies to pS6 plus YFP (A) and quantification of their overlap (B).
(C and D) Sections of Kcng4-YFP retina labeled with antibodies to pRaptor (C) or pRictor (D) plus YFP.
(E and F) Section of Kcng4-YFP retina labeled with antibodies to OPN and YFP (E) and quantification of their overlap (F).
(G and H) Section of control retina (top) and retina infected with AAV-OPN (bottom) 2 weeks previously, labeled with antibodies to OPN and pS6 (G). (H) Fraction of OPN+ and pS6+ cells in both conditions.
(I and J) Section of Kcng4-YFP;OPN−/− retina labeled with antibodies to pS6 and YFP (I) and fraction of YFP+ cells that were pS6+ (J).

n = 3 retinas per condition. The scale bars represent 50 μm.
survival. However, OPN plus BDNF stimulated regeneration to a similar extent as OPN plus IGF-1 (Figures 5E, 5F, and S5B). Thus the stimulatory role of IGF-1 was not unique, and OPN can promote substantial axon regeneration when paired with growth factors.

**OPN plus IGF-1 Promotes Selective Regeneration of αRGCs**

We expected that administration of OPN plus IGF-1 to most or all RGCs would promote regeneration in multiple RGC types. To test this idea, we introduced AAV-OPN and IGF-1 intravitreally...
in Kcng4-YFP, HB9-GFP, and TYW3 mice to label αRGCs, o0DSGCs, and W3-RGCs, respectively. Surprisingly, using protocols and criteria described above (Figure 2), we found that nearly all regenerating axons arose from αRGCs (Figures 6A and 6B).

The ability to selectively target αRGCs provides an opportunity to ask whether the delivery of OPN directly to αRGCs promotes regeneration. To that end, we used an AAV in which expression of OPN was Cre dependent and limited overexpression to αRGCs by infecting retinas of Kcng4-YFP mice. αRGC-specific expression of OPN promoted αRGC regeneration after nerve crush (Figures 6C and 6D).

Selective IGF1R Expression and mTOR Signaling in Axotomized αRGCs
The result that AAV-OPN and IGF-1 are insufficient to promote regeneration of other RGC subtypes to which they are delivered implies that αRGCs differ from non-α-RGCs in some quality that enhances their regenerative responses after injury. The restriction of shPTEN-induced regeneration to αRGCs (Figure 2) leads to the same conclusion. The difference could be in the ability of RGC subtypes to respond to IGF-1, to upregulate mTOR signaling, or to respond appropriately to IGF-1-and mTOR-initiated signals. As a first step in distinguishing these alternatives, we compared levels of the IGF-1 receptor (IGF1R) in αRGCs and non-α-RGCs. IGF1R was selectively expressed in αRGCs in both control adult retinas and in retinas 3 and 7 dpc, although some expression in non-α-RGCs and Müller glial cells was observed after axotomy (Figure 7A). TrkB showed a similar expression pattern, although the expression was less selective and the neuropil was also intensely stained (Figure S7). Thus, one factor contributing to selective responsiveness of αRGCs to OPN plus growth factors is selective expression of the growth factor receptors.

We also compared levels of mTOR signaling in axotomized αRGCs and non-α-RGCs, using pS6 as a marker. Levels of pS6 fell dramatically in RGCs following axotomy, as shown previously (Park et al., 2008), and introduction of shPTEN or OPN plus IGF-1 restored its levels. These treatments were 9-fold and 3-fold more effective, respectively, in increasing pS6 levels in axotomized αRGCs than in neighboring non-α-RGCs (Figures 7B and 7E). Moreover, even though αRGCs constituted only 20% of surviving RGCs at 14 dpc, they accounted for >90% of pS6-rich RGCs following shPTEN treatment and ~60% of pS6-RGCs after OPN plus IGF-1 treatment. As in control retinas (Figure 3A), levels of pRaptor paralleled those of pS6: αRGCs accounted for >90% of pRaptor-rich RGCs after either shPTEN treatment or OPN plus IGF-1 administration (Figure 7C and data not shown). In contrast, the levels of pRictor were low, with <5% of total αRGCs being pRictor-positive. Moreover, the pRictor level did not differ detectably between αRGCs and non-α-RGCs and did not change detectably following shPTEN treatment or
OPN plus IGF-1 administration (Figure 7D). (The ability of the pRictor antibody to detect mTORC2 signaling is demonstrated by staining of non-neuronal cells in injured but not intact retina; compare Figures 3D and 7D.) Thus, a second property of aRGCs that can help account for their ability to regenerate is their ability to maintain or restore mTORC1 levels after axotomy.

DISCUSSION

After damage to the optic nerve, few RGCs survive, and even fewer can be coaxed to extend new axons (Aguayo et al., 1991; Liu et al., 2011). Using markers of 11 RGC subtypes, we found that limited survival and regeneration do not reflect uniformly low vigor of many subtypes; instead, they result from selective survival and regeneration of specific subtypes. We then analyzed factors that promote regeneration of those RGCs that survive and identified OPN as a promoter of RGC growth and regeneration. Together, our results provide new insights into both the cellular and molecular bases of axon regeneration in the mammalian CNS.

Selective Survival and Regeneration of aRGCs

We found dramatic differences among RGC subtypes in their ability to survive axotomy. Some populations, such as ooDSGCs, are almost completely eliminated within 2 weeks, whereas most aRGCs and M1-RGCs survive. These results are consistent with previous reports that M1-RGCs preferentially survive axotomy in rats and that alpha-like RGCs selectively regenerate in cats (Pérez de Sevilla Müller et al., 2014; Robinson and Madison, 2004; Watanabe et al., 1993; Watanabe and Fukuda, 2002; Watanabe et al., 1995). Differential susceptibility to injury has also been reported in a mouse model of glaucoma, although the subtypes affected were not molecularly identified (Della Santina et al., 2013). As a consequence of selective survival, the repertoire of visual features that the retina could potentially report to the brain is fundamentally altered (Figure 1D), a change that will need to be taken into account if efforts to promote regeneration of RGC survivors succeed.

Among surviving RGCs, the ability to regenerate is specific to aRGCs, an evolutionarily conserved RGC type characterized by large somata, smooth dendrites, high levels of neurofilaments, and large receptive fields (Berson, 2008; Peichl, 1991). aRGCs comprise ~6% of all RGCs in intact retina and ~25% of the RGCs that survive axotomy but give rise to >90% of the axons that extend >0.5 mm beyond the site of nerve crush following downregulation of PTEN. In that aRGCs and non-aRGCs are intermingled in the retina, and their axons are intermingled in the optic nerve, it is almost certain that the differences in their regenerative abilities reflect intrinsic differences rather than differences in their environments.

One caveat to the conclusion that aRGCs regenerate selectively is that we tested only two regeneration-promoting treatments, PTEN knockdown and OPN plus a growth factor. It will be important to ask whether other interventions, such as deletion of suppressor of cytokine signaling 3 or provoking an inflammatory response (Benowitz and Popovich, 2011; Morgan-Warren et al., 2013; Park et al., 2008; Smith et al., 2009; Sun et al., 2011; Watkins et al., 2013), can promote regeneration of additional RGC subtypes.

OPN as a Promoter of Axon Regeneration

OPN promotes RGC regeneration when introduced in combination with either IGF-1 or BDNF, neither of which promotes significant regeneration on its own. OPN is a secreted, glycosylated phosphoprotein; it was discovered as a component of bone matrix but has since been shown to be synthesized by many cell types and to affect multiple cellular processes, including

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Figure 7. Selective Expression of IGF1R and Activation of mTOR Signaling in Axotomized αRGCs

(A) IGF1R expression in αRGCs (labeled with OPN) in control retinas, 3 and 7 dpc.

(B–D) Sections from mice injected with control vector, AAV-shPTEN, or AAV-OPN plus IGF-1. Optic nerves were crushed 14 days later and retinas analyzed 14 dpc with anti-pS6 (B), anti-pRaptor (C), or anti-pRictor (D). The scale bars represent 50 μm.

(E) Fraction of αRGC and other RGCs (YFP + and YFP −, respectively, in Kcng4-YFP) that are pS6+, from sections such as those in (B), n = 4 to 6 retinas per treatment.

(F) Model showing pathways by which PTEN knockdown or exogenous OPN expression could promote regeneration. a, b, and c indicate steps at which differences between αRGC and non-αRGCs could affect their regenerative abilities. Results in (A–E) implicate steps a and b as critical differences (see Discussion).

See also Figure S7.
adhesion, proliferation, and survival (Kahles et al., 2014; Kazanecki et al., 2007; Wang and Denhardt, 2008). It is expressed by subsets of neurons as well as several classes of glial cells, including Schwann cells, Müller glia, and microglia. Levels are affected by neural injury in several systems, and OPN is reported to exert both pro- and anti-inflammatory roles that can promote neuronal survival and regeneration (Careccio and Comi, 2011; Chidlow et al., 2008; Del Rio et al., 2011; Hashimoto et al., 2007; Misawa et al., 2012; Wright et al., 2014). In most cases, these effects have been ascribed to depots in glial cells; for OPN-stimulated regeneration of injured motor axons, elegant transplantation experiments have demonstrated that this is the case (Wright et al., 2014). In the retina, in contrast, directed delivery reveals that neuron-derived OPN promotes regeneration.

OPN may be useful for promoting regeneration for several reasons. First, PTEN is a tumor suppressor. Whereas OPN, like PTEN knockdown, acts in part by elevating mTOR, PTEN inhibition also activates many additional pathways that likely contribute to its tumor suppressor activity. OPN may circumvent this potentially dangerous activity. Second, soluble protein therapeutics are clearly promising for treatment of a variety of neural injuries and neurological diseases (Thoenen and Sendtner, 2002). In other systems, OPN acts as an extracellular cytokine for identifying these and other factors that promote survival and regeneration.

**Growth-Promoting Capabilities of αRGCs**

Regeneration promoted by OPN plus IGF-1 or shPTEN is restricted to αRGCs. This surprising result raises two questions. First, why are endogenous OPN and mTOR, both of which are enriched in adult αRGCs, insufficient to promote their regeneration? Second, why is regeneration restricted to αRGCs even when OPN plus IGF-1 or shPTEN is supplied to most RGCs?

One answer to the first question is that mTOR signaling decreases dramatically following axon damage (Park et al., 2008). We hypothesized that OPN might also decrease following axotomy, but we detected no striking changes in OPN levels by immunohistochemistry or in OPN mRNA levels by RT-PCR (data not shown). However, our immunohistochemical methods are non-quantitative, and decreases in OPN mRNA abundance in RGCs would have been masked by known increases in microglia (Chidlow et al., 2008). Moreover, OPN is heterogeneous in several respects: there are several alternatively spliced isoforms (at least in humans), many forms of post-translational modification, and an alternative translation product that remains intracellular and mediates activities distinct from those of the secreted isoform (Gimba and Tilli, 2013; Inoue and Shinohara, 2011; Kazanecki et al., 2007). It remains to be determined which forms promote regeneration and whether the active forms, which may be a small fraction of the total, are affected by axotomy.

In considering the second question, we note that at least two signaling pathways are activated by the interventions we have assayed here (Figure 7F). The first is mTOR signaling, as indicated by the sensitivity of PTEN small hairpin RNA and OPN-induced regeneration to the mTOR-specific blocker rapamycin (Park et al., 2008) (“a” in Figure 7F). For reasons we do not yet understand, OPN upregulates mTOR signaling selectively in αRGCs following axotomy, even though it can upregulate mTOR signaling in most RGCs in uninjured retina.

mTOR signaling alone is insufficient for robust regeneration, however. PTEN regulates several pathways other than mTOR (Hill and Wu, 2009; Manning and Cantley, 2007; Morgan-Warren et al., 2013) and interventions that more selectively upregulate mTOR signaling, such as deletion of TSC1, are significantly less effective in eliciting regeneration than downregulation of PTEN (Park et al., 2008). Likewise, OPN can upregulate mTOR signaling in the presence or absence of a growth factor but does not induce regeneration unless accompanied by a growth factor. Thus, mTOR-dependent and mTOR-independent pathways must be co-activated for optimal regeneration. We suggest that IGF-1, which does not detectably enhance mTOR signaling, activates the mTOR-independent pathway (“b” in Figure 7F). Selective activation of this pathway in αRGCs is likely based on the selective expression of IGF1R by αRGCs. Other salient differences between αRGCs and non-αRGCs may well exist, for example at later steps in the signaling pathway (“c” in Figure 7F). Molecular comparison of subtypes refractory and susceptible to the effects of injury will be a promising approach for identifying these and other factors that promote survival and regeneration.

**EXPERIMENTAL PROCEDURES**

**Animals**

OPN mutant mice were produced by inserting CreER into the translation start codon of the spp1 gene using lambda phage-mediated recombineering (Chan et al., 2007), followed by homologous recombination in embryonic stem cells. Chimeras were produced by the Harvard University Genome Modification Facility. High-percentage chimeras transmitting the knockin allele were bred to animals expressing FLP recombinase from the β-actin promoter (Rodriguez et al., 2003) to remove the PGK-NEO cassette. Primers used for genotyping OPNCreER are OPN Common Forward Primer (TGGTGTGTAAGATCGTTGCT), CreER Reverse Primer (CATCGACCGGTAATGCAGGCAAAT), and OPN wild-type Reverse Primer (CAGGAAATGCGTGTGCT). The primers amplify fragments of 450 bp for the knockin allele and 225 bp for the wild-type allele. Insertion of CreER led to generation of a null allele (Figure 4A and data not shown). However, we detected very low levels of CreER expression and activity in this line, so it was not useful for marking OPN-expressing cells.

hB9-GFP transgenic mice (Trenholm et al., 2011) were obtained from K. Eggan (Stem Cell and Regenerative Biology Department, Harvard University), and Thy1-cre transgenic mice (Dewachter et al., 2002) were obtained from Jackson Laboratories. Other lines were generated and characterized in our laboratory as described previously: Kcng4-Cre (Duan et al., 2014), TW3-YFP (Kim et al., 2010; Zhang et al., 2012), TW7-YFP (Kim et al., 2010), and Thy1-STOP-YFP (Buffelli et al., 2003). Mice were maintained on a C57BL/6 background and experiments were done according to protocols approved by both the Harvard University Standing Committee on the Use of Animals in Research and Teaching and IACUC at Boston Children’s Hospital.

**Gene Transfer and Surgical Methods**

A cDNA encoding OPN was cloned from a mouse retina cDNA library (Kay et al., 2011) and inserted into AAV plasmids for ubiquitous or Cre-dependent expression (Cardin et al., 2009). The coding sequence corresponds to that in accession number BC020355 (NCBI-Nucleotide). AAV-U6-shPTEN-CMV-mCherry was modified from a previously characterized vector AAV-U6-shPTEN-CMV-GFP (Zukor et al., 2013) by changing the fluorescent protein. The targeting sequence was AGGTGAAGATATATTCCTCCAA. AAV serotype 2/2 was produced at Boston Children’s Hospital Viral Core. AAV2 was titered (Wright et al., 2014) by changing the fluorescent protein. ShPTEN-CMV-GFP (Zukor et al., 2013) by changing the fluorescent protein.
Detailed surgical methods were described by Park et al. (2008). For injection, adult animals were anesthetized with ketamine/xylazine (100/10 mg/kg). AAV (−3 μl) was injected intravitreally with a fine glass pipette. Optic nerves were crushed with a pair of Dumont #5 forceps (Roboz) 2 weeks after injection. IGF-1 or BDNF (1 μl, 1 μg/μl, Peprotech) was injected into the vitreal space of the eye at 0 and 7 dpc. One microliter of Alexa-conjugated CTB568 or 647 (Invitrogen) was injected intravitreally 2 to 3 days before euthanasia to label all regenerating axons. Rapamycin (6 mg/kg, LC Laboratories) was delivered intraperitoneally every 2 days from the time of AAV injection.

Histology

Anesthetized mice were transcardially perfused with 4% paraformaldehyde (PFA). Eyes and optic nerves were dissected out and post-fixed in 4% PFA at 4°C overnight. For frozen sections, tissues were immersed in 30% sucrose for 2 days before sectioning in a cryostat (20 μm for retina, 10 μm for optic nerve). For some experiments, eyes were fixed in 4% PFA at 4°C by immersion for ~30 to 60 min, immediately after the mice were sacrificed by a lethal overdose of anesthesia.

For immunohistochemistry, sections were incubated in PBS with 3% donkey serum and 0.3% Triton X-100 for blocking, followed by primary antibodies overnight at 4°C and secondary antibodies for ~2 hr at room temperature. Whole mounts were incubated in PBS with 5% donkey serum and 0.5% Triton X-100 for blocking, followed by primary antibodies for ≥48 hr at 4°C and secondary antibodies for ≥16 hr at 4°C. Finally, sections were washed with PBS and mounted in Vectashield (Vectorlabs).

Primary antibodies used were rabbit anti-GFP (1:1,000, Millipore), chicken anti-α-TUBulin (1:500, Abcam), rabbit anti-red fluorescent protein (1:500, Abcam), rabbit anti-CART (1:2,500, Phoenix Peptide), rabbit anti-melanopin (1:1,000, gift from I. Provencio, University of Virginia), rabbit anti-phosphorylated S6 Ser235/236 (1:200, Cell Signaling Technology), mouse anti-neurofilament (SMI32, 1:1,000, Covance), rabbit anti-IGF1R (1:1,000, Sigma), goat anti-mouse TryK (1:500, R&D Systems), rabbit anti-phosphorylated-Raptor (Ser792) (1:200, Cell Signaling Technology), rabbit anti-phosphorylated-Rictor (Thr1135) (1:200, Cell Signaling Technology), and goat anti-OPN (1:1,000, R&D Systems). Nuclei were labeled with NeuroTrace Nissl 435/455 (1:1,000, Invitrogen). Secondary antibodies were conjugated to DyLight 649 (Jackson ImmunoResearch), Alexa Fluor 568, or Alexa Fluor 488 (Invitrogen) and used at 1:500.

Imaging and Quantification

For whole mounts of retinas, at least eight areas (~0.5 × 0.5 mm) across the whole retinas were imaged with a standard epi-fluorescence microscope (Nikon) focusing on the retinal ganglion layer. Cells were counted, and the counts obtained from all areas were averaged to generate a single value for each retina.

For retina sections, images were taken with a confocal microscope (Olympus FV1000 or Zeiss LSM-710) using 440/488–515/568 and 647 lasers with a step size of 0.5 μm and a 40× (NA 1.3) lens. Images were analyzed using ImageJ software (NIH). One field from at least eight sections per sample were imaged and analyzed. The numbers from all sections were averaged to generate a single value for each retina.

For soma size measurement, Z stacks were projected onto a single plane, and the largest area was measured with ImageJ. To measure level of immunoreactivity, slides were stained, mounted, and imaged in parallel, and the signals were imaged within a linear range. Fluorescent intensity was measured in ImageJ to indicate the relative expression level.

For nerve sections, regenerating axons were identified and counted as described previously (Park et al., 2008). In analyzing YFP+ axons, signals from fluorescent or autofluorescent tissue debris was excluded.

Only contrast and brightness were adjusted for all images. Caution was taken not to oversaturate the images, and only brightly stained cells were counted when positive staining was to be identified.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.02.017.

AUTHOR CONTRIBUTIONS

X.D., M.Q., and F.B. planned and performed all experiments and analyzed data. I.-J.K. performed initial studies showing that OPN is expressed in sRGCs. Z.H. and J.R.S. planned the study and analyzed data. X.D. and J.R.S. wrote the paper with input from all authors.

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