Activation of the BMP4/Smad1 Pathway Promotes Retinal Ganglion Cell Survival and Axon Regeneration

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PURPOSE. We investigate if the BMP4/Smad1 intracellular signaling pathway is neuroprotective and axogenic in adult rodent retinal ganglion cells (RGC) in vivo and in vitro.

METHODS. Adult retinal cultures were prepared from intact and after optic nerve crush (ONC) injured rats that have been stimulated to survive and regenerate using an intravitreal peripheral nerve (PN) graft. Laser capture microdissection (LCM) then was used to isolate RGC with and without neurites. Quantitative RT-PCR determined changes in BMP4/Smad1 signaling pathway mRNA. Immunohistochemistry confirmed localization of BMP4 and activation of Smad1 in ONC+PN-stimulated RGC in vivo. BMP4 peptide was used to stimulate RGC survival and neurite/axon regeneration in vitro and in vivo. Finally, the rapamycin sensitivity of the effects was determined in BMP4-stimulated RGC in vitro and in vivo.

RESULTS. In retinal cultures prepared from intact and ONC+PN-stimulated rats, RGC with neurites had upregulated regeneration-related and BMP4/Smad1 signaling pathway mRNA levels, while low levels of these mRNAs were present in RGC isolated without neurites. An optimal dose of 200 ng/mL BMP4 peptide in vitro promoted approximately 30% RGC survival and inhibited RGC neurite outgrowth, despite the presence of inhibitory CNS myelin extracts. BMP4 also promoted approximately 30% RGC survival in vivo and stimulated significant RGC axon regeneration at 100, 200, and 400 μm beyond the lesion site. Finally, the response of RGC to BMP4 treatment in vitro and in vivo was rapamycin-insensitive.

CONCLUSIONS. Activation of the BMP4/Smad1 pathway promotes survival and axon regeneration independent of mTOR and, therefore, may be of therapeutic interest.

Keywords: optic nerve crush, retinal ganglion cells, neuroprotection, laser capture microdissection, BMP4, Smad1

Axon regeneration in the adult mammalian central nervous system (CNS) is limited after injury by a multiplicity of factors, including a low intrinsic capacity of adult neurons to reprogram their axons and the presence of myelin- and scar-derived axon inhibitory factors. However, intrinsic factors, such as cyclic adenosine monophosphate (cAMP), mammalian target of rapamycin (mTOR), and the repressors phosphatase and tensin homolog (PTEN) and suppressor of cytokine signaling 3 (SOCS3) promote CNS axon regeneration. The observation that mTOR activity is developmentally downregulated and new protein synthesis is suppressed after mTOR inactivation probably explains why some axons do not regenerate in the mature CNS.

One possible novel candidate therapeutic target is bone morphogenetic protein 4 (BMP4) and the downstream small mothers against decapentaplegic 1 (Smad1) signaling pathway that it activates. BMPs are essential in retinal development, upregulated after retinal injury, and activated downstream Smad1 signaling is neuroprotective for RGC. In addition, exogenous addition of BMP4 promotes the survival of RGC after NMDA-mediated damage, but RGC axon regeneration has not been studied after BMP4/Smad1 activation. However, in a conditioning lesion, where the peripheral branch of the dorsal root ganglia (DRG) is axotomized, Smad1 signaling is induced and is required for the enhanced axon growth potential, while a central axotomy failed to activate Smad1. Intrathecal injection of AAV-BMP4 to overexpress BMP4 in DRG neurons activated Smad1 signaling and promoted sensory axon regeneration after spinal cord injury in the mouse. Therefore, we reasoned that BMP4/Smad1 signaling might also be activated in RGC regenerating their axons and that intravitreal delivery of a BMP4 peptide may promote RGC survival and axon regeneration.

We isolated RGC somata by laser capture microdissection (LCM) from two RGC populations: those that did and did not grow neurites. LCM was undertaken in the same culture dish in intact and optic nerve crush plus intravitreal peripheral nerve

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(ONC+PN)-treated retinal cultures and activity of the BMP4/Smad1 pathway investigated in RGC that grew neurites versus those that did not. In both RGC populations, signaling components of the BMP4/Smad1 pathway were highly active in RGC with neurites. BMP4 peptides disinhibited RGC neurite outgrowth in the presence of inhibitory CNS myelin extracts (CME) in vitro and promoted significant RGC survival and axon regeneration in vivo. Taken together, these results showed that BMP4/Smad1 could be a constituent of a neurotrophic factor cocktail required to promote RGC axon regeneration, thereby achieving greater RGC axon reconnection with denervated targets after visual pathway trauma and the reinstatement of visual function.

**METHODS**

**In Vitro Experiments**

**Experimental Design.** For the LCM studies, dissociated retinal cultures from \( n = 12 \) intact (uninjured) adult female Sprague-Dawley rats (170–220 g; Charles River, Margate, United Kingdom) were grown in triplicates (3 independent repeats, \( n = 9 \) wells/treatment) or adult Fischer rats (Charles River) prestimulated to grow by intravitreal sciatic nerve graft implantation (ONC+PN) for 21 days before harvesting retinae and dissociating retinal cells (\( n = 3 \) rats/experiment, three independent repeats, \( n = 9 \) wells/treatment) as described above (Figs. 1A, 1B).
**Mixed Adult Rat Retinal Cultures.** Retinal cultures were prepared from either intact 6- to 8-week-old Sprague-Dawley rats (180-220 g; Charles River) or 6- to 8-week-old Fischer rats (Charles River; to avoid SN graft rejection) at 21 days after ONC-PN implantation. Retinal cells were dissociated using a P500 dissociation kit and 12% 105 ml of 0.1% trypsin for 5 hours before chamber slides and grown in Neurobasal-A medium supplemented with B27 supplement and L-glutamine (all from Invitrogen, Paisley, United Kingdom), with appropriate treatments for 3 days at 37°C and 5% CO2.

For LCM, dissociated retinal cells were grown on polyethylene terephthalate (PET) membranes (Leica Microsystems, Milton Keynes, United Kingdom) coated with 10 μg/mL poly-L-lysine for 3 days in a humidified chamber. To stimulate neurite outgrowth, preoptimized NTF consisting of neurotrophin (NT)-3 (50 ng/mL), fibroblast growth factor 2 (FGF2; 10 ng/mL) and brain-derived neurotrophic factor (BDNF; 50 ng/mL; referred to as “combined NTF” from herein; all purchased from Peprotech, London, United Kingdom) were used.

To assess BMP4-mediated disinhibition of RGC neurite outgrowth, retinal cells were prepared from intact 6- to 8-week-old Sprague-Dawley rats (Charles River) as above and treated with increasing concentrations of BMP4 peptide (Peprotech) from 0–500 ng/mL in the presence of preoptimized CNS myelin extracts (CME).

Retinal cultures were incubated for 3 days with the appropriate treatments before fixing in 4% paraformaldehyde in PBS (TAAB, Peterborough, United Kingdom) for 10 minutes at room temperature (RT) as described previously and subjected to βIII-tubulin immunocytochemistry and RGC neurite outgrowth analysis, as described above.

To test if BMP4-mediated RGC neurite outgrowth is sensitive to Rapamycin, retinal cells also were treated with 10 nM Rapamycin (LC Laboratories, Woburn, Boston, USA) for 3 days.

**siRNA Experiments.** To confirm a role for BMP4, we knocked down BMP receptors and downstream Smad1 in BMP4 stimulated and assessed RGC survival and neurite outgrowth. There are three type I receptors of the TGF-β superfamily that binds BMP4, namely type I BMP receptor (BMPR-1A), type 1B BMP receptor (BMPR-1B), and type 1A activin receptor (ACVR1). We purchased a set of four siRNAs of each, pooled together to guarantee knockdown and targeted to βIII-tubulin.

LCM of RGC With and Without Neurites. LCM microscope (Arcturus Pixcell II; Applied Biosystems, Rugby, United Kingdom) was used to isolate 1000 FMI-43+ RGC from each quadrant using a Leica PixCell. The identity of chamber slides was masked by a second investigator and wells split into nine quadrants. Images were captured by the investigator from each quadrant using a Zeiss Axioscop 2 microscope equipped with an Axioscam HRc and Axiovision software (all from Zeiss, Hertfordshire, United Kingdom).

**In Vivo Experiments**

**Experimental Design.** To assess the effects of intravitreal PN grafts on BMP4/Smad1 activation, three Fischer rats/group (repeated on three independent occasions; total n = 18 eyes/optic nerves/group) were used (Fig. 1B) to avoid graft rejection. Animals were assigned randomly to treatment groups and masked to the investigator. Groups included: (1) intact, (2) ONC, and (3) ONC-PN.

To assess activation of Smad1 after intravitreal BMP4 injection, three rats/group (three independent repeats; total number of surviving βIII-tubulin+ RGC in each quadrant and multiplying by the area of each well (n = 3 wells/condition; three independent repeats; total n = 9 well/condition). The proportion of RGC survival at 4 days after plating and appropriate treatments was determined by counting the number of βIII-tubulin+ RGC immediately after plating, as described above. All data were analyzed by the investigator masked to the treatment conditions.

**LCM of RGC With and Without Neurites.** Retinal cells were stained with fluorescent lipophilic dye FM1-43 (N-[3-(triethylammonium)propyl]-4-(4-(4-dihydro-6-methyl-1H-pyrindinum dibromide) (Invitrogen) for 20 minutes, fixed with 4% paraformaldehyde and 4% sucrose in PBS for 10 minutes, dehydrated through a graded series of ethanol and air-dried. An LCM microscope (Arcturus Pixcell II; Applied Biosystems, Rugby, United Kingdom) was used to isolate 1000 FM1-43+ RGC with and without neurites in each experiment and the RNA extracted, amplified, and quantitative (q)RT-PCR performed as described below. Experiments were repeated on three independent occasions.

**In Vivo Experiments**

**Experimental Design.** To assess the effects of intravitreal PN grafts on BMP4/Smad1 activation, three Fischer rats/group (repeated on three independent occasions; total n = 18 eyes/optic nerves/group) were used (Fig. 1B) to avoid graft rejection. Animals were assigned randomly to treatment groups and masked to the investigator. Groups included: (1) intact, (2) ONC, and (3) ONC-PN.

To assess activation of Smad1 after intravitreal BMP4 injection, three rats/group (three independent repeats; total
18 eyes/group) were used and included: (1) intact controls, (2) ONC+PBS (vehicle), and (3) ONC+BMP4 (5 μg BMP4 dissolved in a final volume of 5 μL PBS; dosage preoptimized to cause maximal RGC survival in vivo [not shown]). Animals received intravitreal injections of PBS or BMP4 immediately after injury and were killed at 7 days and prepared for immunohistochemistry for phosphorylated (p) Smad1 (pSmad1).

The effects of BMP4 on RGC survival and axon regeneration were studied in three rats/group (repeated on three independent occasions, total n = 18 eyes/ON/treatment) at 24 days after ONC and treatment; the groups included: (1) intact controls, (2) ONC+PBS (vehicle; to control for the effects of multiple intravitreal injections), and (3) ONC+BMP4 (5 μg BMP4; Fig. 1C). To assess if RGC axon regeneration promoted by BMP4 was rapamycin-sensitive (i.e., mTORC1-dependent), n = 3 rats/group (n = 18 eyes/ON/treatment); the groups included: (1) BMP4+vehicle and (2) BMP4+rapamycin. Rapamycin was prepared and administered as described previously. Briefly, rapamycin stock solutions were dissolved at 20 mg/mL in ethanol and, before each administration, diluted in 5% Tween 80, 5% polyethylene glycol 400 in PBS. Either rapamycin or vehicle was injected intraperitoneally immediately after ONC and every 2 days thereafter at a final concentration of 6 mg/mL.

**ONC Group**

All animal procedures were approved by the University of Birmingham local animal welfare and ethical review board and licensed by the UK Home Office. Surgery was done in strict accordance with the UK Animals Scientific Procedures Act, 1986 and the Revised European Directive 1010/65/EU according to the guidelines and recommendations for the use of animals by the Federation of the European Laboratory Animal Science Associations. Experiments also conformed to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research except that bilateral ONC was enforced by the UK Home Office as a means of reducing animal numbers, in keeping with the 3Rs principle. Adult, female 6- to 8-week-old Sprague-Dawley or Fischer rats (according to experiment) were anesthetized using isoflurane inhalation and bilateral ONC performed 2 mm from the lamina cribrosa as described by us previously. Animals were monitored throughout the study using an ophthalmoscope to detect damage to the lens or cataracts. No animal showed lens damage or suffered cataracts confirming that the lens had not been injured during surgery and, thus, all eyes were included for analysis. Animals were killed 5 days after ONC and retinal cells cultured as described below. For implantation of a PN graft, a 0.5 cm length of donor sciatic nerve was excised, teased between watchmaker’s forceps and grafted intravitreally and held in place using Spongostan. Two days before killing animals at 24 days, lysinated rhodamine dextran (LRD) was injected into the distal end of the PN. Animals were studied in three rats/group (repeated on three independent occasions, total n = 9 rats/group, n = 27 eyes/ON/treatment).

**Quantification of RGC Survival.** RGC survival was quantified as described previously. Briefly, sections containing the optic disc were selected from each eye and the number of mRNA (Table 1) were validated by qRT-PCR by preparing complementary DNA from extracted RNA and qRT-PCR was performed using a LightCycler real time qRT-PCR machine (Roche, Burgess Hill, United Kingdom) according to previously published methods.

**Immunohistochemistry.** Eyes from perfusion-fixed animals were removed, cryoprotected through a graded series of sucrose solution and blocked in OCT (TAAB Laboratories, Berkshire, United Kingdom). Radial cross-sections of eyes were adhered onto charged glass slides and immunohistochemistry (IHC) performed on sections thawed and washed in PBS. Non-specific binding was blocked before incubation with the relevant primary antibody (Table 2) overnight at 4°C. Negative controls, including omission of primary antibodies were included in each run to set the background threshold before image capture. Sections then were washed in PBS, incubated with relevant secondary antibodies conjugated to either Alexa488 or Texas Red for 1 hour at RT, washed in further changes of PBS and mounted using Vectashield containing DAPI (Vector Laboratories).

**Quantification of Mül1er Cell Activation.** Retinal Müller cell activation was quantified in glial fibrillary acidic protein (GFAP) immunoreactive cells by us previously. Animals were monitored throughout the study using an ophthalmoscope to detect damage to the lens or cataracts. No animal showed lens damage or suffered cataracts confirming that the lens had not been injured during surgery and, thus, all eyes were included for analysis. Animals were killed 5 days after ONC and retinal cells cultured as described below. For implantation of a PN graft, a 0.5 cm length of donor sciatic nerve was excised, teased between watchmaker’s forceps and grafted intravitreally and held in place using Spongostan. Two days before killing animals at 24 days, lysinated rhodamine dextran (LRD) was injected into the distal end of the PN. Animals were studied in three rats/group (repeated on three independent occasions, total n = 9 rats/group, n = 27 eyes/ON/treatment).

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including RGC without neurites which were LRD<sup>−</sup> (Fig. 2F). There was a 15-, 40-, 35-, 12-, and 12-fold increase in regeneration-related gap43, Sprr1a, c/ebp-epsilon, atf3, and galanin mRNA, respectively, with no change in SOCS3 mRNA levels in RGC with and without neurites (Fig. 2G). These results are comparable with those seen in RGC treated with combined NTF (compare Figs. 2C and 2G), suggesting that a consistent profile of regeneration-related genes was switched on in RGC with neurites compared to RGC without neurites. The results also showed that some RGC survived for 21 days in vivo after ONC+PN, but did not grow their neurites when placed in culture.

Levels of mRNA for the BMP4/Smad1 pathway were significantly higher in cultured RGC that had been harvested 21d after ONC+PN treatment when compared to RGC in cultures prepared from naive eyes treated with combined NTF (Fig. 2H). For example, bmp4, smad1, smad4, smad5, smad8, smif, and msg1 mRNA levels were 15-, 24.5-, 25.5-, 9-, 10-, 12-, and 13-fold upregulated, respectively (P < 0.0001), in RGC with neurites compared to RGC without neurites (Fig. 2H). These results suggested that intact and ONC+PN-treated cultured RGC that grow neurites activate the BMP4/Smad1 signaling pathway.

### ONC+PN Activated the BMP4/Smad1 Signaling Pathway in RGC In Vivo

Immunohistochemistry in intact control and ONC eye sections contained few βIII-tubulin<sup>+</sup> RGC that also were BMP4<sup>+</sup> (Fig. 2I). However, after ONC+PN treatment, many βIII-tubulin<sup>+</sup> RGC were BMP4<sup>+</sup> (Fig. 2I). Few βIII-tubulin<sup>+</sup> RGC were phosphorylated (p) Smad1<sup>+</sup> in intact controls (Fig. 2I), but after ONC, some βIII-tubulin<sup>+</sup> RGC were pSmad1<sup>+</sup>, while after ONC+PN treatment, most βIII-tubulin<sup>+</sup> RGC were pSmad4<sup>+</sup> (Fig. 2J), indicating that the BMP4/Smad1 pathway was highly active in RGC regenerating their axons in vivo.

### BMP4 Peptide Promoted RGC Survival and Neurite Outgrowth on a CME Substrate

Increasing concentrations of BMP4 peptide from 0 to 300 ng/mL increased the % βIII-tubulin<sup>+</sup> RGC in retinal cultures to a maximum of 68% ± 7% (P < 0.0001) at 200 ng/mL, significantly greater than that observed in cultures without added BMP4 (32 ± 9%; Figs. 3A, 3D). The mean percentage of RGC with neurites and mean neurite length also increased in a dose-dependent manner to a maximum of 26 ± 3% (P < 0.0001) at 200 ng/mL, and 238 ± 27 μm (P < 0.0001), respectively (Figs. 3B–D), despite the presence of inhibitory CME. These results suggested that BMP4 is a potent RGC survival and neuritogenic factor.

### Knockdown of BMP4 Receptors or Downstream Smad1 Abrogates BMP4-Induced RGC Survival and Neurite Outgrowth

Approximately 70% knockdown of appropriate mRNA was achieved in RGC cultures treated with siBMPR1a (Fig. 4A), siBMPR1b (Fig. 4B), siSmad1 (Fig. 4C), and siACVR1 (Fig. 4D). Knockdown of BMPR1a, BMPR1b, ACVR1, or Smad1 in the presence of BMP4 peptide stimulation not only suppressed RGC survival to levels achieved with NBA alone (Fig. 4E), but also significantly inhibited RGC neurite outgrowth (Fig. 4F) in terms of percentage RGC with neurites (Fig. 4G) and the mean neurite length (Fig. 4H). These results suggested that inhibition of BMPR or Smad1 by siRNA block the survival and neurite outgrowth properties of BMP4 peptide.
Few, if any cells in the ganglion cell layer (GCL) were pSmad1⁺ in either intact retina or in retinae at 7 days after ONC+vehicle treatment (Figs. 5A, 5B), a time-point where there is some 20% to 40% RGC loss.⁴⁶ However, intravitreal injection of BMP4 after ONC led to a significant increase in the number of ganglion cells with pSmad1⁺ immunoreactivity (Figs. 5A, 5B). Immunolabeling for pSmad1 (green) colocalized to βIII-tubulin⁺ (red) RGC in intact or after ONC, but most RGC in the GCL were positive for BMP4 after ONC+PN treatment. (A–J) Scale bars: 50 µm. **P < 0.01; ***P < 0.0001.

**BMP4 Enhanced RGC Survival, Axon Regeneration and Müller Glial Activation In Vivo**

Few, if any cells in the ganglion cell layer (GCL) were pSmad1⁺ in either intact retina or in retinae at 7 days after ONC+vehicle treatment (Figs. 5A, 5B), a time-point where there is some 20% to 40% RGC loss.⁴⁶ However, intravitreal injection of BMP4 after ONC led to a significant increase in the number of ganglion cells with pSmad1⁺ immunoreactivity (Figs. 5A, 5B). Immunolabeling for pSmad1 (green) colocalized to βIII-tubulin⁺ (red) cells in the GCL (Fig. 5C; arrowheads), suggesting activation of the BMP4/Smad1 signaling pathway in RGC. The number of RBPM5⁺ RGC/mm at 24 days after ONC and treatment reduced from 61 ± 6 in intact controls to 5 ± 1 after ONC and vehicle treatment (Figs. 5D, 5E). BMP4 treatment promoted the survival of 16 ± 2 RGC/mm, equating to a significant increase in RGC of 31% compared to vehicle treated groups (P < 0.001). BMP4 also promoted a significant increase in the number of RBPMS⁺ RGC/mm at 24 days after ONC and treatment reduced from 61 ± 6 in intact controls to 15 ± 1 after ONC and vehicle treatment (Figs. 5D, 5E).
BMPs have essential roles in retinal development and BMP-Smad1 promotes RGC survival and axon regeneration in vivo, suggesting that this pathway is an additional determinant of RGC neurite outgrowth/axon regeneration. BMPs constitute a large family of proteins and BMP signaling leads to phosphorylation of Smad1/5/8, which then forms a complex with Smad4, translocates to the nucleus, and regulates target gene expression. BMP has important functions during development of the nervous system, but BMP4 and BMP7 increase rapidly after spinal cord injury (SCI) and BMP signaling reduces NMDA-induced RGC cytotoxicity. In this study, we showed that the BMP4/Smad1 pathway was highly upregulated in RGC with neurites and also during RGC axon regeneration in vivo, suggesting that this pathway is an additional determinant of RGC neurite outgrowth/axon regeneration. BMPs promote the survival and regeneration of approximately 10% of all RGC in the murine retina. It seems apparent that no matter what the regenerative strategy in the optic nerve, only the ipRGC survive and regenerate their axons (i.e., <10% RGC). This is despite the multiplicity of neuroprotective/axogenic factors that include mTOR activation (either deletion of PTEN or tuberous sclerosis complex 1 (TSC-1), trophic factors derived from Schwann cells, macrophages, retinal glia, oncomodulin, and ciliary neurotrophic factor/leukemia inhibitory factor. BMP has important functions during development of the nervous system, but BMP4 and BMP7 increase rapidly after spinal cord injury (SCI) in the rat, with phosphorylation of Smad1/5/8 at the lesion site. Moreover, overexpression of BMP4 in adult DRG in vitro and in vivo activated Smad1 and enhanced the axon growth potential of DRGN in culture and after SCI in a mouse model. BMPs have essential roles in retinal development and BMP-Smad1/5/8 is neuroprotective for RGC since inhibiting BMP signaling reduces NMDA-induced RGC cytotoxicity. In addition, treatment with exogenous BMP4 promoted RGC survival after NMDA-induced damage. We also showed that exogenous BMP4 not only promoted significant RGC survival, but also promoted significant RGC neurite outgrowth. RGC growing neurites correlated with...
significant activation of components in the BMP4/Smad1 pathway. In regenerating ONC+PN-treated eyes, we observed activation of BMP4 and pSmad1 in almost all RGC in the ganglion cell layer suggesting that BMP4/Smad1 signaling correlates with a regenerative RGC phenotype. Furthermore, BMP4 treatment in vivo promoted RGC survival, axon regeneration, and retinal glial activation. Moreover, BMP4-stimulated RGC neurite outgrowth/axon regeneration was insensitive to rapamycin, suggesting that the BMP4/Smad1 pathway is mTORC1-independent. Together these results suggested that BMP4/Smad1 is an additional signaling pathway, important in regulating non-ipRGC survival and axon regeneration. Our results agree with the observations made in adult DRGN in the spinal cord and suggested that reactivation of the BMP4/Smad1 pathway in adult CNS neurons can restore their axon growth potential.

It is clear that activation of the BMP4/Smad1 pathway stimulated RGC survival and axon regeneration (initiation and elongation). However, the pro-survival effect of BMP4 was small in scale and the morphology of surviving RBPMS+ RGC in retinal cross-sections appear smaller and rounded compared to untreated control retinas. Therefore, it is possible that BMP4 delays the death of RGC and future experiments will need to address this question before BMP4 can be used therapeutically as a neuroprotective agent. Nonetheless, the regenerative response to BMP4 was rapamycin-insensitive and, hence, mTOR-independent, suggesting that the regeneration observed was probably in non-ipRGC. Although we did not determine the subtype of RGC responding to BMP4/
Smad1 activation, future experiments could address whether these constitute W3-RGC, M2-RGC, or ooDSGCs. It remains to be investigated if any of the other 30 subtypes of RGC are affected by BMP4 activation and, hence, the BMP4/Smad1 pathway presents itself as a valuable tool in determining the differential survival and regenerative responses of different RGC subtypes in the retina. Approximately 30% RGC survival was achieved after BMP4 treatment in vitro and in vivo with only 30% of those RGC growing neurites in culture and probably a similar small number also regenerating their axons in vivo. This suggested that RGC survival and axon regeneration are likely to be signaled differently and, hence, may require different combinations of neuroprotective/trophic factors. Indeed, we have shown that RGC exclusively activate cleaved caspase-2 after ONC injury and suppression of caspase-2 with a siRNA (siCASP2) protects >95% RGC in the rat retina. However, despite the survival of >95% of RGC, siCASP2 treatment did not cause RGC axon regeneration.

In conclusion, our results showed that the BMP4/Smad1 pathway is activated in surviving and regenerating RGC. Addition of exogenous BMP4 promoted non-ipRGC RGC survival and disinhibited neurite outgrowth/axon regeneration in vitro and in vivo, effects that were independent of mTOR signaling. Our results suggested that the BMP4/Smad1 represents an additional therapeutic target to achieve pan RGC survival and axon regeneration and, hence, restoration of lost function.
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