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 regrow 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 normally regenerate in the mature CNS.

Other pathways known to have a role in RGC axon regeneration include those activated by inflammatory stimulation, such as oncomodulin and activation of the JAK/STAT3 pathway. Transcriptional repressors, such as Kruppel-like factors (KLFs), Sox11, and c-Myc. However, the regenerative effects remain limited, since for example, activation of mTOR or overexpression of osteopontin and insulin-like growth factor 1 (IGF1) promotes selective regeneration of a small proportion of α-RGC that comprise only 6% of RGC in an intact retina. Therefore, other pathways must be identified to promote regeneration of a greater population of RGC.

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 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 by 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...
BMP4/Smad1-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.52 Retinal cells were dissociated using a P2000 pipette tip and 12X 1 ml tissue culture 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% CO2.16

For LCM, dissociated retinal cells were grown on polyethylenyl 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) were used.16 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)31.34 Retinal cultures were incubated for 3 days with the appropriate treatments before fixing in 4% paraformaldehyde in PBS (TAAB, Peterborough, United Kingdom) and viewed under a Zeiss Axioplan 2 fluorescent microscope equipped with an Axiocam HRc and Axiovision software (all from Zeiss, Hertfordshire, United Kingdom). Negative controls had primary antibody omitted and were used to set the background threshold levels for nontarget staining before image capture.

RGC Neurite Outgrowth and Survival. The mean number of surviving βIII-tubulin+ RGC, those with neurites and the mean neurite lengths, were quantified at day 4 after plating and treatment as described by us previously.29 Briefly, the identity of chamber slides was masked by a second investigator and wells split into nine quadrants. Images were captured by the masked investigator from each quadrant using a Zeiss Axioplan 2 fluorescent microscope equipped with an Axiocam HRc and Axiovision software (all from Zeiss). The number of βIII-tubulin+ RGC and neurite lengths were quantified using Axiovision software (Version 4.8; Zeiss) and ImagePro (Version 6.3; Media Cybernetics, Bethesda, MD, USA).

RGC survival was determined by counting the number of βIII-tubulin+ RGC in each quadrant and multiplying by the surface 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 FMI-43 [N-(3- triethylammoniumpropyl)-4-[4-(4-dibutylamino)styryl]pyridinium dibromide] (Invitrogen) for 20 minutes,42 fixed with 4% parafomaldehyde 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 FMI-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/group) 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), 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 1010/63/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 3R’s principle. Adult, female 6- to 8-week-old Sprague-Dawley or Fischer rats (according to experiment) were studied in three rats/group (repeated on three independent occasions, total n = 18 eyes/ON/group) 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), 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.

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**RESULTS**

**LCM of RGC With Neurites Correlated With Increased Expression of Regeneration Related Genes**

LCM was efficient at targeted removal of cell bodies from RGC with and without neurites in naive retinal cultures treated with combined NTF (Fig. 2A). When RGC with and without neurites were compared, regeneration-related gap43, Sprt1a, e/ebp-epsilon, atf3, and galanin mRNA were 8-, 34-, 30-, 5.4-, and 10-fold upregulated in RGC with neurites, respectively, while SOCS3 mRNA levels remained unchanged (Fig. 2C). Analysis of components of the BMP4/Smad1 pathway in RGC treated with combined NTF in culture also showed that bmp4, smad1, smad4, smad5, smad8, smif, and msg1 mRNA were upregulated 3.2-, 4.5-, 5.5-, 2-, 3-, and 2-fold, respectively (P < 0.05–0.0001), in RGC with neurites compared to RGC without neurites (Fig. 2D). These results suggested that RGC that grow neurites in culture have a regenerative phenotype and upregulate genes in the BMP4/Smad1 pathway.

**LCM of RGC With Neurites 21 days After ONC+PN Correlates With Changes in Regeneration-Related mRNA**

After ONC+PN and subsequent culture of RGC, LRD injection into the distal segment of the optic nerve, beyond the ONC site, identified RGC that had regenerated axons through and beyond (Fig. 2E), whereas FMI-43 labeled all cells in culture, including RGC without neurites which were LRD− (Fig. 2F). There was a 15-, 40-, 35-, 12.4-, and 12-fold increase in regeneration-related gap43, Sprt1a, e/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+ RGC that also were BMP4+ (Fig. 2I). However, after ONC+PN treatment, many βIII-tubulin+ RGC were BMP4+ (Fig. 2I). Few βIII-tubulin+ RGC were phosphorylated (p) Smad1+ in intact controls (Fig. 2I), but after ONC, some βIII-tubulin+ RGC were pSmad1+, while after ONC+PN treatment, most βIII-tubulin+ RGC were pSmad1+ (Fig. 2I), 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+ RGC that were also LRD+ (Fig. 2J), indicating that the BMP4/Smad1 pathway was highly active in RGC regenerating their axons in vivo.

**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 siBMPR1α (Fig. 4A), siBMPR1b (Fig. 4B), siSmad1 (Fig. 4C), and siACVR1 (Fig. 4D). Knockdown of BMPR1α, 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 blocked the survival and neurite outgrowth properties of BMP4 peptide.
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.\(^4^6\) 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 RBPMS\(^+\) RGC/mm at 24 days after ONC and treatment reduced from 61 \(\pm\) 6 in intact controls to 5 \(\pm\) 1 after ONC and vehicle treatment (Figs. 5D, 5E). BMP4 treatment promoted the survival of 16 \(\pm\) 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...

**FIGURE 2.** Regenerating RGC upregulate BMP4/Smad1 signaling. LCM of (A) before and (B) after collection of RGC somata from FMI-43\(^+\) RGC with (red arrows) and without (white arrowbeads) neurites, stimulated by combined NTF (FGF2/BDNF/NGF). (C) Changes in regeneration-related mRNA in RGC (normalized to freshly isolated intact controls) with and without neurites and (D) in BMP4/Smad1 pathway. LCM of ONC+PN-stimulated regenerating (E) LRD\(^+\)/(F) FMI-43\(^+\) (red arrows) and nonregenerating (LRD\(^+\)/FMI-43\(^+\); white arrowbeads) RGC showed similar changes in (G) regeneration-related mRNA in RGC with/without neurites compared to naïve retinal cultures. (H) Changes in mRNA levels of BMP4/Smad1 pathway molecules in regenerating and nonregenerating RGC prepared at 21 days after ONC+PN implantation mirrored that in intact control cultures except that levels of all mRNA were significantly higher. Immunohistochemistry to demonstrate (I) absence of BMP4 (red) in βIII-tubulin\(^+\) RGC (green) in intact or after ONC, but most RGC in the GCL were positive for BMP4 after ONC+PN treatment. (J) No pSmad1\(^+\) (red) immunoreactivity was localized in βIII-tubulin\(^-\) RGC (green) in intact eyes. However, some pSmad1\(^+\) RGC were present after ONC, but nearly all RGC in the GCL were positive for pSmad1 after ONC+PN treatment. (A, B, E, F) Scale bars: 50 μm, (I, J) Scale bars: 25 μm. **\(P < 0.01\); ***\(P < 0.0001\).
increase in GAP43+ axons present at 100 (P < 0.05), 200 (P < 0.005), and 400 (P < 0.05) μm beyond the lesion site compared to vehicle-treated groups in vivo (Figs. 5E, 5G). These data showed that BMP4 significantly enhanced RGC survival and axon regeneration in vivo.

The number of GFAP+ fibers crossing the IPL increased significantly to 16 ± 6 in vehicle-treated compared to intact eyes (Figs. 5H, 5I; P < 0.009). However, BMP4 treatment further increased the number of GFAP+ fibers crossing the IPL to 35 ± 2 compared to vehicle-treated eyes (Figs. 5H, 5I; P < 0.004) amounting to a 46% increase compared to vehicle-treated eyes. These results demonstrated that BMP4 treatment also activates retinal glia.

**BMP4-Stimulated RGC Neurite Outgrowth and Axon Regeneration was Rapamycin-Insensitive**

The addition of neither vehicle nor rapamycin (Fig. 6A) to optimal BMP4 peptide-treated retinal cultures (200 ng/mL) did not affect the levels of RGC survival (Fig. 6B), the percentage RGC with neurites (Fig. 6C), and the mean neurite length (Fig. 6D) in the presence of CME, suggesting that PI3K/mTOR/pS6 signaling was not involved in BMP4-stimulated RGC survival and neurite outgrowth (compare Figs. 6A–D with Figs. 3A–D). In addition, neither vehicle nor rapamycin affected BMP4-stimulated RGC axon regeneration in vivo (Figs. 5E, 5F). These results demonstrated that BMP4 treatment also activates retinal glia.

**DISCUSSION**

The results of this study demonstrated that the BMP4/Smad1 pathway is highly active and correlates positively with RGC survival and neurite outgrowth/axon regeneration. BMP4 peptide addition in vitro and intravitreal delivery in vivo promoted significant disinhibited RGC neurite outgrowth and RGC survival and axon regeneration after ONC. In addition, delivery of BMP4 peptide in vivo promoted glial activity. Moreover, BMP4-stimulated RGC survival and neurite outgrowth and axon regeneration were insensitive to rapamycin and, hence, were mTORC1-independent.

**,***P < 0.0001. (D) Scale bars: 100 μm.

**FIGURE 3.** BMP4 peptide promotes RGC survival and disinhibited neurite outgrowth, effects that were insensitive to rapamycin. (A) Dose-dependent increase in the percentage of surviving RGC, (B) percentage of RGC with neurites, and (C) the mean neurite length. (D) Examples of RGC neurite outgrowth in treated cultures. ** ***P < 0.0001. (D) Scale bars: 100 μm.
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.

**FIGURE 5.** BMP4 promotes RGC survival, axon regeneration and activates glia in vivo after ONC. (A) Representative images. (B) quantification, and (C) double immunolabelling for pSmad1 and bIII-tubulin to show that endogenous BMP4 upregulates Smad1 signaling such that nearly all RGC in the GCL at 7 days after ONC were pSmad1+. (D) RBPMS+ RGC immunohistochemistry and (E) quantification show reduced numbers of RBPMS+ RGC in vehicle-treated ONC eyes compared to intact eyes; note that BMP4 treatment neuroprotected RGC. (F) GAP43 immunohistochemistry and (G) quantification show significantly greater numbers of GAP43+ RGC axons regenerated 100, 200, and 400 μm beyond the ONC lesion site (*) after BMP4 treatment compared to vehicle-treated groups. (H) GFAP reactive astrocyte somata and processes in the inner retinal layers and (I) quantification show significantly increased numbers of reactive astrocyte processes traverse the inner plexiform layer (IPL) after treatment of ONC eyes with BMP4 compared to vehicle treated. (A, C, E, G) Scale bars: 100 μm. **P < 0.001; ***P < 0.0001, ANOVA; #P < 0.05 and ##P < 0.003, GLMM.
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