UNIVERSITY OF BIRMINGHAM

University of Birmingham

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

Thompson, Adam; Berry, Martin; Logan, Ann; Ahmed, Zubair

[Link to publication on Research at Birmingham portal](#)

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

Adam Thompson, Martin Berry, Ann Logan, and Zubair Ahmed

Neuroscience and Ophthalmology, Institute of Inflammation and Ageing, University of Birmingham, Edgbaston, Birmingham, United Kingdom

Correspondence: Zubair Ahmed, Neuroscience and Ophthalmology, Institute of Inflammation and Ageing, College of Medical and Dental Sciences, University of Birmingham, Room 386, Robert Aitken Institute of Clinical Research, Edgbaston, Birmingham B15 2TT, UK; z.ahmed.1@bham.ac.uk.

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 disinhibited 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.[1-5] 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.[6-9] 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.[10,11]

Other pathways known to have a role in RGC axon regeneration include those activated by inflammatory stimulation, such as oncomodulin[12-14] and activation of the JAK/STAT3 pathway,[15-17] transcriptional repressors, such as Kruppel-like-factors (KLFs),[18,19] Sox11,[20] and c-Myc.[21] 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.[22] 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.[23-28] In addition, exogenous addition of BMP4 promotes the survival of RGC after NMDA-mediated damage,[28] 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.[29] 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.[30] 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
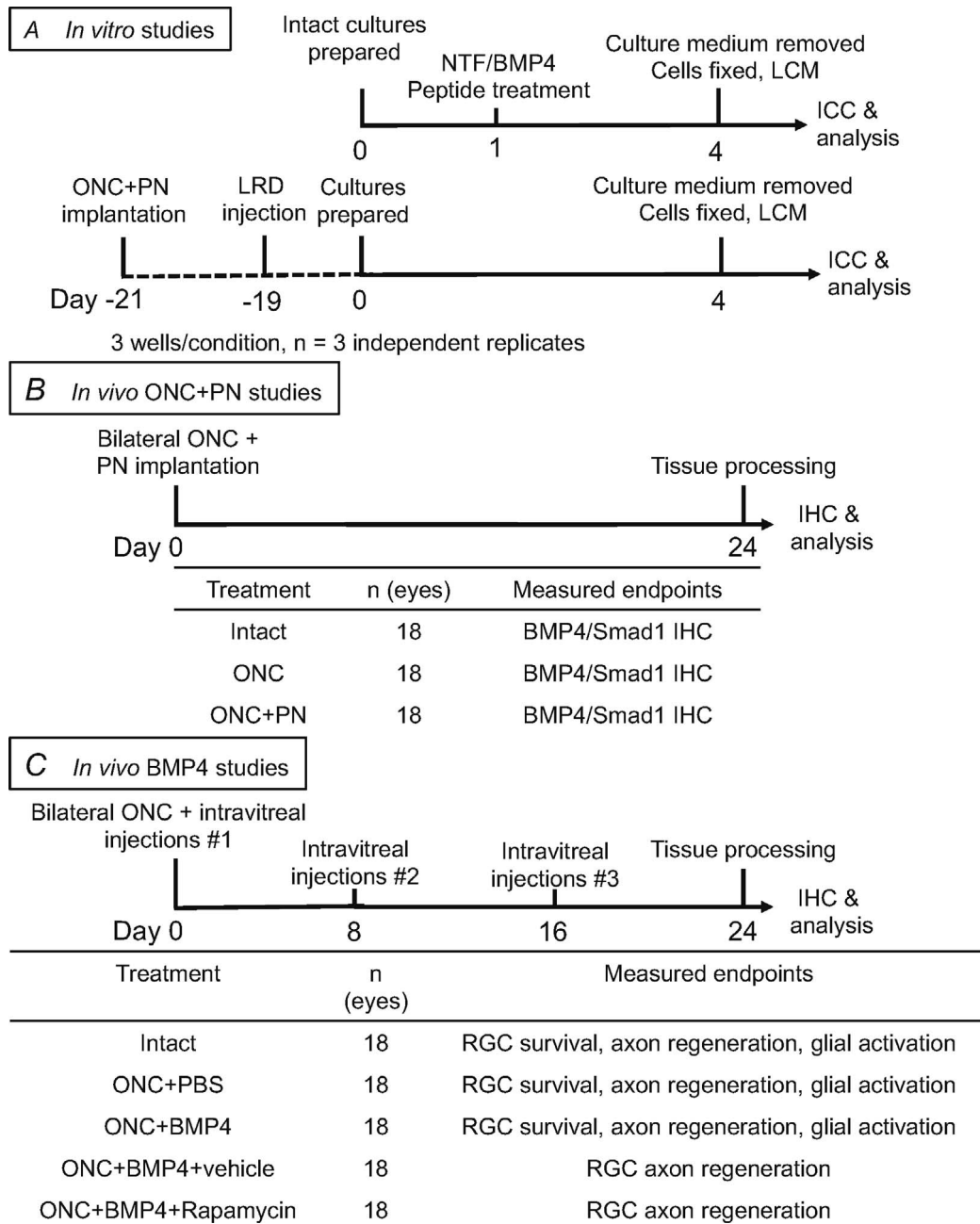
FIGURE 1. Experimental design for in vitro and in vivo experiments. (A) Timelines for the in vitro studies including retinal cultures prepared from naïve rats and 21 days after ONC+PN implantation. (B) Timeline of in vivo ONC+PN implantation study, treatment groups, and measured endpoints. (C) Timeline of in vivo BMP4 study, treatment groups, and measured endpoints.

(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[31] 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)[32] 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.[32] Retinal cells were dissociated using a Papain dissociation kit and 125×10^3 cells cultured in 8-well 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% CO_2.[16]

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.[33]

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 0–300 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) for 10 minutes at room temperature (RT) as described previously[35] and subjected to βIII-tubulin immunocytochemistry and RGC neurite outgrowth analysis, as described above (Fig. 1A).

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.[36]

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 TGFβ-superfamily that binds BMP4, namely type 1A BMP receptor (BMPR-1A), type 1B BMP receptor (BMPR-1B), and type 1A activin receptor (ACVR1).[37] We purchased a set of four siRNAs of each, pooled together to guarantee knockdown and targeted against rat BMPR1a (SMARTpool siRNA, M-088678-01-005; siBMPR1A), BMPR1b (SMARTpool siRNA, M089942-01-0005; siBMPR1b), ACVR1 (SMARTpool siRNA, M-099048-01-0005; siACVR1), and Smad1 (SMARTpool, M094977-01-0005; siSmad1; all from Dharmacon, Lafayette, CO, USA). A negative control, comprised of four nontargeting siRNAs (siControl) also was included in each run as well as transfection reagent alone controls. In a preliminary experiment, we determined 5 nM of each siRNA to optimally knock down the relevant mRNA in retinal cultures. Retinal cultures then were cultured in the presence of optimal concentrations of BMP4, allowed to incubate for 3 days before immunocytochemistry for βIII-tubulin to determine RGC survival and neurite outgrowth, as described below.

Mixed retinal cells were transfected with siRNA using Lipofectamine 2000 reagent (Invitrogen) as described previously.[38] Breifly, siRNA and transfection reagent were each diluted in supplemented neomycin anaerobic blood agar (NBA) culture medium (without antibiotics) and incubated for 5 minutes at room temperature. The two solutions were combined, gently mixed, and incubated for a further 25 minutes at room temperature to form siRNA-reagent complexes. Complexes were diluted to the desired concentrations in NBA, added to the cells, and transfected for 5 hours before addition of supplemented NBA to a final volume of 500 μL per

well, and incubated at 37°C and 5% CO_2 for a further 3 days. Culture medium alone, lipofectamine reagent without siRNA, and control siRNA were used as controls. A dose-response assay was done initially, for all siRNAs and tested at 2.5, 5, 10, 20, and 50 nM concentrations, confirming that a concentration of 5 nM caused optimal mRNA knockdown in retinal cultures.

Immunocytochemistry of Retinal Cultures. Retinal cells were permeabilized and blocked in PBS containing 3% BSA and 0.1% Triton X-100 for 30 minutes at RT before incubation with monoclonal anti-βIII-tubulin antibodies (1:200 dilution, Sigma-Aldrich, Poole, United Kingdom) for 1 hour at RT. Cells then were washed in PBS and incubated with Alexa Fluor 488 anti-mouse IgG (1:400 dilution, Invitrogen) for 1 hour at RT. After final washes in PBS, cells were coverslip-mounted in Vectamount containing 4′,6-diamidino-2-phenyl-endole (DAPI; Vector Laboratories, 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 nonspecific 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.[39] 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 decribed 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,[40] fixed with 4% paraformaldehyde and 4% sucrose in PBS for 10 minutes, dehydrated through a graded series of ethanols 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), $n = 3$ 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.[10] 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 anesthetized using isofluorane inhalation and bilateral ONC performed 2 mm from the lamina cribrosa as described by us previously.[32] 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 portion of the optic nerve 5 mm beyond the ONC site. LRD+ RGC was used to identify cells that grew axons 5 mm beyond the lesion site in vivo.

Immunohistochemistry for RBPMS and βIII-tubulin in radial sections of the eye were used to assess RGC survival. Phosphorylated (p) Smad1 immunoreactivity was used to detect Smad1 pathway activation. GAP43 immunoreactivity in longitudinal sections of the optic nerve was used to assess RGC axon regeneration.[38] All data were captured and analyzed with the investigator masked to the treatment conditions.

RNA Extraction and Amplification. RNA was extracted from LCM captured RGC using RNAqueous Microkit (Ambion, Inc., Foster City, CA, USA; typically a yield of 1 ng from 1000 RGC) following the protocol of Zivraj et al.[40] The extracted RNA was linearly amplified using the two-cycle TargetAmp kit

TABLE 1. List of Primers Used for the BMP4/Smad1 and PTEN/mTOR Pathways

Gene	Primer Sequence Cat No/Reaction ID	Source
gap43	4331182, Rn00567901_m1	ThermoFisher
sprr1a	4331182, Rn02061965_s1	ThermoFisher
cebp-epsilon	4331182, Rn00567306_g1	ThermoFisher
atf3	4331182, Rn00563784_m1	ThermoFisher
galanin	4331182, Rn00583681_m1	ThermoFisher
socs-3	4331182, Rn00585674_s1	ThermoFisher
bmp-4	4331182, Rn0043287_m1	ThermoFisher
smad1	4331182, Rn00565555_m1	ThermoFisher
smad5	4331182, Rn00572484_m1	ThermoFisher
smad8	4331182, Rn01494797_m1	ThermoFisher
smurf1	4331182, Rn01412801_m1	ThermoFisher
erk1/2	4331182, Rn00820922_g1	ThermoFisher
smif	4351372, Rn01214158_m1	ThermoFisher
msg1	4351372, Rn06396495_g1	ThermoFisher
tgif	4331182, Rn01418715_m1	ThermoFisher
sip1	4331182, Rn00584534_m1	ThermoFisher
pten	4331182, Rn00477208_m1	ThermoFisher
rheb	4331182, Rn00566263_m1	ThermoFisher
mtor	4331182, Rn00693900_m1	ThermoFisher
s6k1	4331182, Rn01760380_m1	ThermoFisher
4ebp1	4331182, Rn00587524_m1	ThermoFisher
tsc1	4331182, Rn00573107_m1	ThermoFisher
tsc2	4331182, Rn00562086_m1	ThermoFisher
redd1	4331182, Rn01433735_g1	ThermoFisher
akt	4331182, Rn00583646_m1	ThermoFisher
gapdh	4331182, Rn01775763_g1	ThermoFisher

(Epicentre, Madison, WI, USA) with a final yield of 36 μg amplified (a)RNA. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies) before qRT-PCR.

qRT-PCR Analysis. Selected mRNA (Table 1) were validated by qRT-PCR by preparing complementary DNA from extracted RNA and qRT-PCR was performed using a Light-Cycler real time qRT-PCR machine (Roche, Burgess Hill, United Kingdom) according to previously published methods.[41] Fold-changes in mRNA were calculated using the ΔΔCt method.[41]

Immunohistochemistry. Eyes from perfusion-fixed animals were removed, cryoprotected through a graded series of sucrose solution and blocked in OCT (TAAB Laboratories, Berks, United Kingdom). Radial cryosections of eyes were adhered onto charged glass slides and immunohistochemistry (IHC) performed on sections thawed and washed in PBS. Nonspecific binding was blocked before incubation with the relevant primary antibody (Table 2) overnight at 4°C. Negative controls, including ommision 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).[34,42] Sections were examined using a Zeiss epi-fluorescent microscope attached to an Axiocam HRc and run using Axiovision software (all from Zeiss), with an experimenter masked to the treatment conditions.

Quantification of RGC Survival. RGC survival was quantified as described previously.[43] Briefly, sections contining the optic disc were selected from each eye and the number of RBPMS+ RGC were quantified along 250 μm lengths either side of the optic nerve head in four consecutive sections/retina.

Quantification of Müller Cell Activation. Retinal Müller cell activation was quantified in glial fibrillary acidic protein

TABLE 2. List of Primary and Secondary Antibodies Used in This Study

Antibody	Source	Dilution
Primary antibodies		
Mouse anti-βIII-tubulin	Sigma-Aldrich	1:400
Rabbit anti-pSmad1	Cell Signaling Technology	1:200
Rabbit anti-BMP-4	Abcam	1:400
Mouse anti-GAP-43	Invitrogen	1:200
Rabbit anti-GFAP	Sigma-Aldrich	1:400
Rabbit anti-RBPMS	Abcam	1:400
Secondary antibodies		
Alexa-488 anti-rabbit	Invitrogen	1:400
Alexa-488 anti-mouse	Invitrogen	1:400
Alexa-594 anti-mouse	Invitrogen	1:400
Alexa-594 anti-rabbit	Invitrogen	1:400

(GFAP)–stained radial eye sections by counting the number of GFAP$^+$ Müller cell processes along a 250 μm long sampling line set orthogonal to the radial plane through the middle of the inner plexiform layer (IPL), with the experimenter masked to the treatment conditions, as described by us previously.[44]

Statistical Analysis

Significant differences were calculated between sample means by 1-way ANOVA with post hoc test using Tukey's method in SPSS Version 24 (IMB Corporation, Armonk, NY, USA).

To determine statistical differences between treated and vehicle groups for GAP43$^+$ RGC axon regeneration, a generalized linear mixed model was used in R statistics package (available in the public domain at www.r-project. org). Binomial GLMMs were fitted in R using package *lme4* with the *glmer* function. *P* values then were calculated using parametric bootstrap.[45]

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 naïve retinal cultures treated with combined NTF (Fig. 2A). When RGC with and without neurites were compared, regeneration-related *gap43*, *Sprr1a*, c/*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-, 3-, and 2-fold, respectively ($P < 0.05$–$P < 0.0001$), 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*, *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 naïve 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. 2J), but after ONC, some βIII-tubulin$^+$ RGC were pSmad1$^+$, while after ONC+PN treatment, most βIII-tubulin$^+$ RGC were pSmad1$^+$ (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$^+$ 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% ($P < 0.0001$) with 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 stimultaion 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.
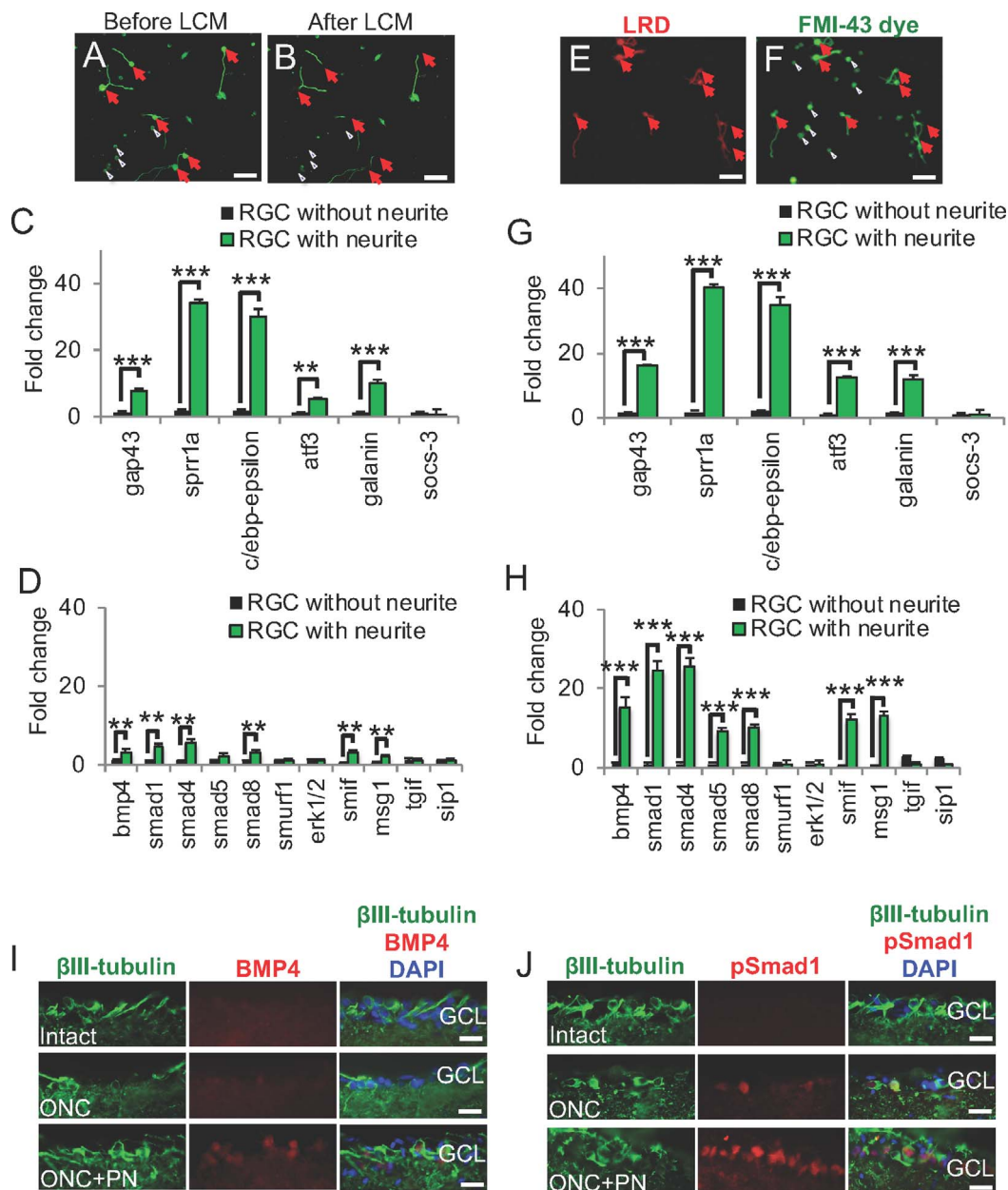
FIGURE 2. Regenerating RGC upregulate BMP4/Smad1 signaling. LCM of (**A**) before and (**B**) after collection of RGC somata from FMI-43$^+$ RGC with (*red arrows*) and without (*white arrowheads*) neurites, stimulated by combined NTF (FGF2/BDNF/NGF). (**C**) Changes in regeneration-related mRNA in RGC (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 arrowheads*) 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. **$P < 0.01$; ***$P < 0.0001$.

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 RBPMS$^+$ RGC/mm at 24 days after ONC and treatment reduced from 61 ± 3 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
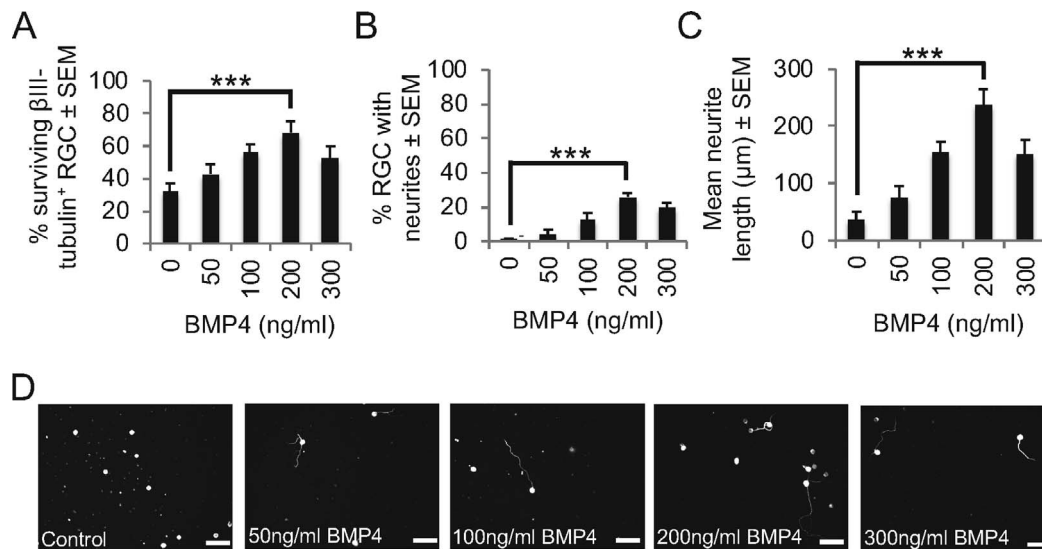
FIGURE 3. BMP4 peptide promotes RGC survival and disinhibited neurite outgrowth, effects that were insensitive to rapamycin. **(A)** Dose-dependent increase in the percentage of surviving RGC, **(B)** percentage of RGC with neurites, and **(C)** the mean neurite length. **(D)** Examples of RGC neurite outgrowth in treated cultures. ***$P < 0.0001$. **(D)** *Scale bars*: 100 μm.

increase in GAP43$^+$ axons present at 100 ($P < 0.05$), 200 ($P < 0.003$), and 400 ($P < 0.05$) μm beyond the lesion site compared to vehicle-treated groups in vivo (Figs. 5F, 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 suggested that BMP4-stimulated RGC neurite outgrowth and axon regeneration are insensitive to rapamycin and, hence, mTORC1-independent.

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 activation. Moreover, BMP4-stimulated RGC survival and neurite outgrowth and axon regeneration were insensitive to rapamycin

and, hence, were mTORC1-independent. These results implied that activation of the BMP4/Smad1 pathway may be an additional target for therapeutic manipulation in the search for pan RGC axon regeneration.

Several studies have demonstrated that activation of the PTEN/mTOR pathway, usually by genetic deletion of PTEN or shRNA against PTEN[10,22,47,48] and codeletion of PTEN/SOCS3,[5] promotes the survival and regeneration of approximately 10% of all RGC in the murine retina.[22] 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).[49,50] This is despite the multiplicity of neuroprotective/axogenic factors that includes mTOR activation (either deletion of PTEN or tuberous sclerosis complex 1 (TSC-1),[10,11] trophic factors derived from Schwann cells,[39,51] macrophages,[12,13] retinal glia,[52,53] onco-modulin,[12–14] and ciliary neurotrophic factor/leukemia inhibitory factor.[17,54–56]

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 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.[57,58] 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.[58–60] Moreover, overexpression of BMP4 in adult DRGN in vitro and in vivo activated Smad1 and enhanced the axon growth potential of DRGN in culture and after SCI in a mouse model.[30]

BMPs have essential roles in retinal development[23–25,61] and BMP-Smad1/5/8 is neuroprotective for RGC since inhibiting BMP signaling reduces NMDA-induced RGC cytotoxicity.[28] In addition, treatment with exogenous BMP4 promoted RGC survival after NMDA-induced damage.[28] We also showed that exogenous BMP4 not only promoted significant RGC survival, but also promoted significant RGC neurite outgrowth. RGC growing neurites correlated with
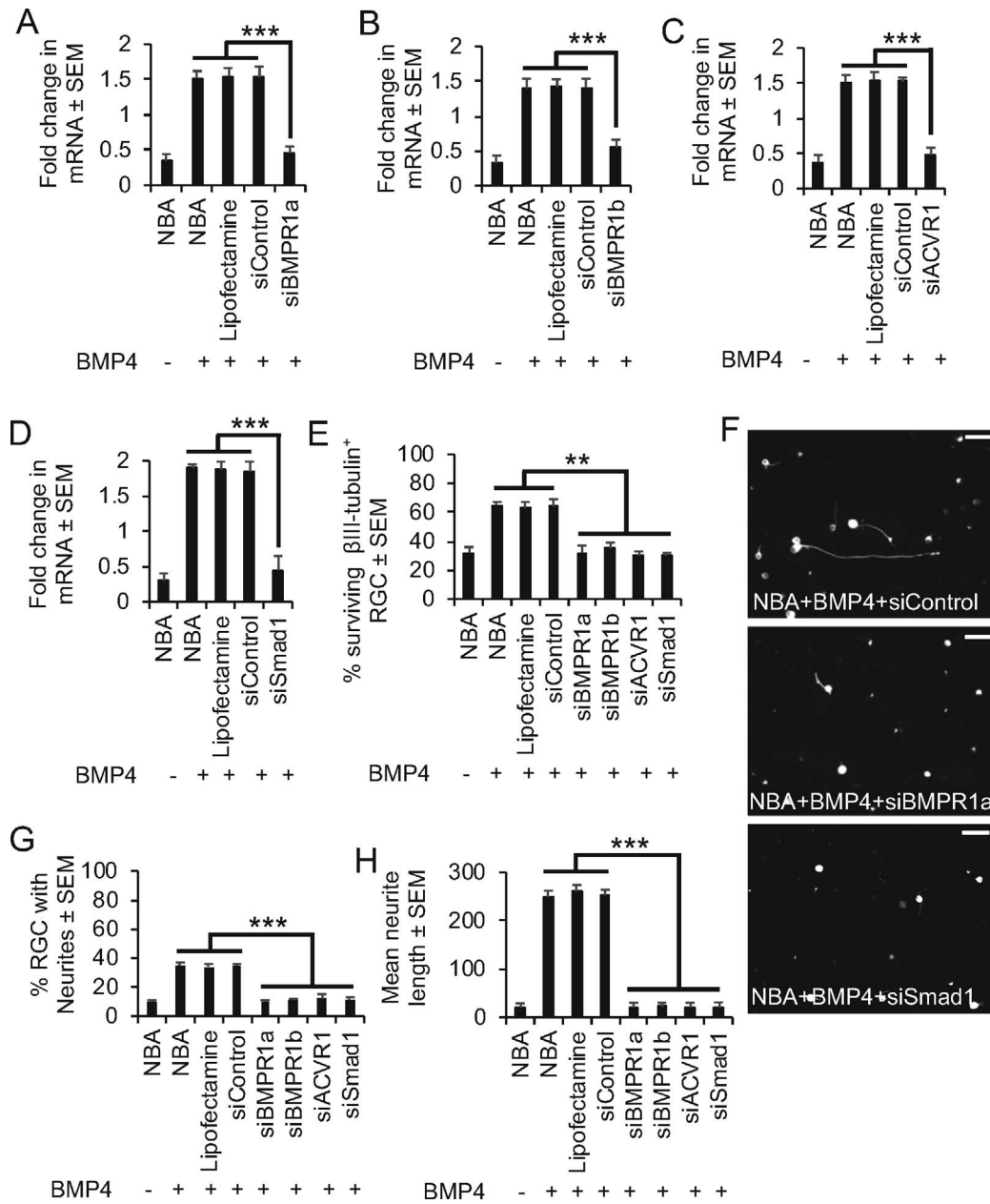
FIGURE 4. Knockdown of BMPR and Smad1 attenuates BMP4-stimulated RGC survival and neurite outgrowth. Confirmation of approximately 70% knockdown in (**A**) BMPR1a, (**B**) BMPR1b, (**C**) ACVR1, and (**D**) Smad1 mRNA in retinal cells by appropriate siRNAs. (**E**) Knockdown of BMPR and Smad1 significantly reduced RGC survival. (**F**) Representative images from siControl and siBMPR1a and siSmad1-treated wells. Knockdown of BMPR and Smad1 reduces (**G**) % RGC with neurites and (**H**) the mean neurite length. **$P < 0.01$; ***$P < 0.0001$, ANOVA. (**F**) *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/
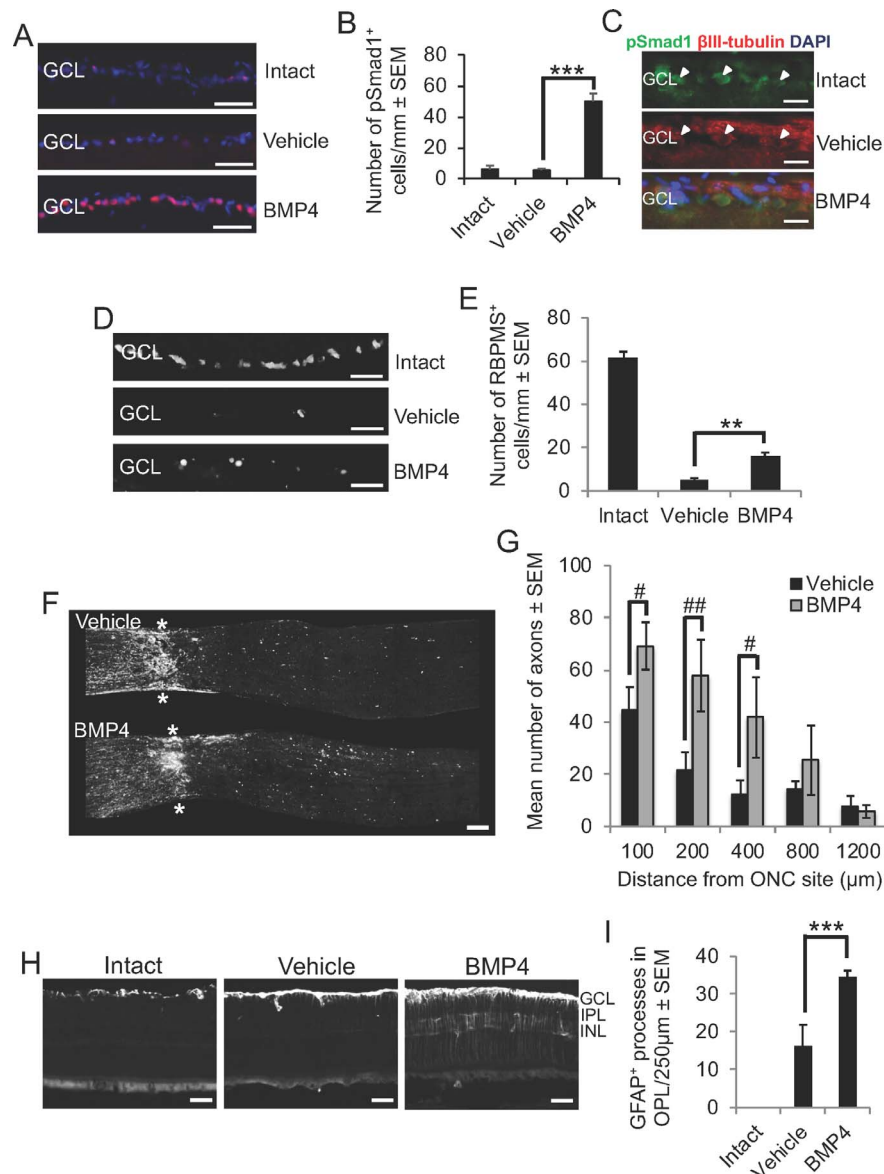
FIGURE 5. BMP4 promotes RGC survival, axon regeneration and activates glia in vivo after ONC. (**A**) Representative images, (**B**) quantification, and (**C**) double immunolabelling for pSmad1 and βIII-tubulin to show that exogenous BMP4 upregulates Smad1 signaling such that nearly all RGC in the GCL 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 treatment. (**A, C, E, G**) *Scale bars*: 100 μm. **$P < 0.001$; ***$P < 0.0001$, ANOVA; #$P < 0.05$ and ##$P < 0.003$, GLMM.

Smad1 activation, future experiments could address whether these constitute W3-RGC, M2-RGC, or ooDSGCs.[22] 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.[46,62,63] However, despite the survival of >95% of RGC, siCASP2 treatment did not cause RGC axon regeneration.[62]

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.
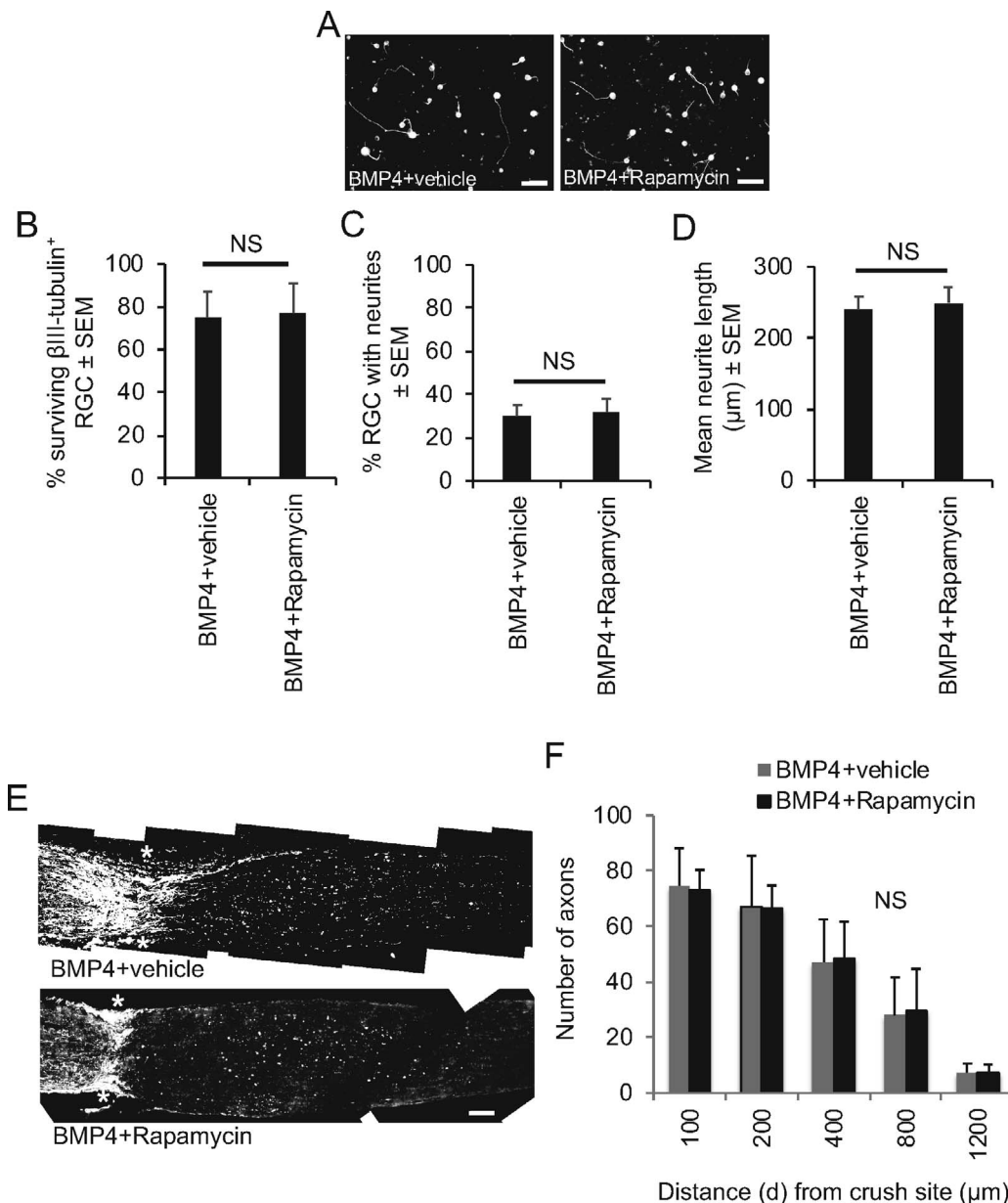
FIGURE 6. BMP4-stimulated RGC survival and neurite outgrowth/axon regeneration is rapamycin insensitive. (**A**) Representative BMP4+vehicle and BMP4+rapamycin-treated cultures to demonstrate rapamycin insensitivity. Quantification of BMP4-stimulated (**B**) RGC survival, (**C**) % RGC with neurites and (**D**) mean neurite length showed no attenuation in rapamycin-treated cultures. (**E**) BMP4-stimulated GAP43⁺ RGC axon regeneration in vivo and its (**F**) quantification showed no sensitivity to rapamycin. (**A**, **E**) *Scale bars*: 100 μm. NS, not significant. *Lesion site.

References

1. Sandvig A, Berry M, Barrett LB, Butt A, Logan A. Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. *Glia.* 2004;46:225–251.

2. Goldberg JL. Intrinsic neuronal regulation of axon and dendrite growth. *Curr Opin Neurobiol.* 2004;14:551–557.

3. Tang F, Dent EW, Kalil K. Spontaneous calcium transients in developing cortical neurons regulate axon outgrowth. *J Neurosci.* 2003;23:927–936.

4. Cafferty WB, McGee AW, Strittmatter SM. Axonal growth therapeutics: regeneration or sprouting or plasticity? *Trends Neurosci.* 2008;31:215–220.

5. Sun F, Park KK, Belin S, et al. Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. *Nature.* 2011; 480:372–375.

6. Verma P, Chierzi S, Codd AM, et al. Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *J Neurosci.* 2005;25:331–342.

7. Smith PD, Sun F, Park KK, et al. SOCS3 deletion promotes optic nerve regeneration in vivo. *Neuron.* 2009;64:617–623.

8. Hellstrom M, Muhling J, Ehlert EM, et al. Negative impact of rAAV2 mediated expression of SOCS3 on the regeneration of

adult retinal ganglion cell axons. *Mol Cell Neurosci*. 2011;46:507–515.

9. Hellstrom M, Harvey AR. Cyclic AMP and the regeneration of retinal ganglion cell axons. *Int J Biochem Cell Biol*. 2014;56:66–73.

10. Park KK, Liu K, Hu Y, et al. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science*. 2008;322:963–966.

11. Park KK, Liu K, Hu Y, Kanter JL, He Z. PTEN/mTOR and axon regeneration. *Exp Neurol*. 2010;223:45–50.

12. Yin Y, Cui Q, Li Y, et al. Macrophage-derived factors stimulate optic nerve regeneration. *J Neurosci*. 2003;23:2284–2293.

13. Yin Y, Cui Q, Gilbert HY, et al. Oncomodulin links inflammation to optic nerve regeneration. *Proc Natl Acad Sci U S A*. 2009;106:19587–19592.

14. Leon S, Yin Y, Nguyen J, Irwin N, Benowitz LI. Lens injury stimulates axon regeneration in the mature rat optic nerve. *J Neurosci*. 2000;20:4615–4626.

15. Leibinger M, Andreadaki A, Fischer D. Role of mTOR in neuroprotection and axon regeneration after inflammatory stimulation. *Neurobiol Dis*. 2012;46:314–324.

16. Leibinger M, Andreadaki A, Diekmann H, Fischer D. Neuronal STAT3 activation is essential for CNTF- and inflammatory stimulation-induced CNS axon regeneration. *Cell Death Dis*. 2013;4:e805.

17. Muller A, Hauk TG, Leibinger M, Marienfeld R, Fischer D. Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF. *Mol Cell Neurosci*. 2009;41:233–246.

18. Moore DL, Blackmore MG, Hu Y, et al. KLF family members regulate intrinsic axon regeneration ability. *Science*. 2009;326:298–301.

19. Moore DL, Apara A, Goldberg JL. Kruppel-like transcription factors in the nervous system: novel players in neurite outgrowth and axon regeneration. *Mol Cell Neurosci*. 2011;47:233–243.

20. Norsworthy MW, Bei F, Kawaguchi R, et al. Sox11 expression promotes regeneration of some retinal ganglion cell types but kills others. *Neuron*. 2017;94:1112–1120.

21. Belin S, Nawabi H, Wang C, et al. Injury-induced decline of intrinsic regenerative ability revealed by quantitative proteomics. *Neuron*. 2015;86:1000–1014.

22. Duan X, Qiao M, Bei F, Kim IJ, He Z, Sanes JR. Subtype-specific regeneration of retinal ganglion cells following axotomy: effects of osteopontin and mTOR signaling. *Neuron*. 2015;85:1244–1256.

23. Franke AG, Gubbe C, Beier M, Duenker N. Transforming growth factor-beta and bone morphogenetic proteins: cooperative players in chick and murine programmed retinal cell death. *J Comp Neurol*. 2006;495:263–278.

24. Furuta Y, Hogan BL. BMP4 is essential for lens induction in the mouse embryo. *Genes Dev*. 1998;12:3764–3775.

25. Liu J, Wilson S, Reh T. BMP receptor 1b is required for axon guidance and cell survival in the developing retina. *Dev Biol*. 2003;256:34–48.

26. Murali D, Kawaguchi-Niida M, Deng CX, Furuta Y. Smad4 is required predominantly in the developmental processes dependent on the BMP branch of the TGF-beta signaling system in the embryonic mouse retina. *Invest Ophthalmol Vis Sci*. 2011;52:2930–2937.

27. Murali D, Yoshikawa S, Corrigan RR, et al. Distinct developmental programs require different levels of Bmp signaling during mouse retinal development. *Development*. 2005;132:913–923.

28. Ueki Y, Reh TA. Activation of BMP-Smad1/5/8 signaling promotes survival of retinal ganglion cells after damage in vivo. *PLoS One*. 2012;7:e38690.

29. Zou H, Ho C, Wong K, Tessier-Lavigne M. Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. *J Neurosci*. 2009;29:7116–7123.

30. Parikh P, Hao Y, Hosseinkhani M, et al. Regeneration of axons in injured spinal cord by activation of bone morphogenetic protein/Smad1 signaling pathway in adult neurons. *Proc Natl Acad Sci U S A*. 2011;108:E99–EE107.

31. Ahmed Z, Dent RG, Suggate EL, et al. Disinhibition of neurotrophin-induced dorsal root ganglion cell neurite outgrowth on CNS myelin by siRNA-mediated knockdown of NgR, p75NTR and Rho-A. *Mol Cell Neurosci*. 2005;28:509–523.

32. Berry M, Carlile J, Hunter A. Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. *J Neurocytol*. 1996;25:147–170.

33. Logan A, Ahmed Z, Baird A, Gonzalez AM, Berry M. Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. *Brain*. 2006;129:490–502.

34. Ahmed Z, Mazibrada G, Seabright RJ, Dent RG, Berry M, Logan A. TACE-induced cleavage of NgR and p75NTR in dorsal root ganglion cultures disinhibits outgrowth and promotes branching of neurites in the presence of inhibitory CNS myelin. *FASEB J*. 2006;20:1939–1941.

35. Ahmed Z, Aslam M, Lorber B, Suggate EL, Berry M, Logan A. Optic nerve and vitreal inflammation are both RGC neuroprotective but only the latter is RGC axogenic. *Neurobiol Dis*. 2010;37:441–454.

36. Gobrecht P, Leibinger M, Andreadaki A, Fischer D. Sustained GSK3 activity markedly facilitates nerve regeneration. *Nat Commun*. 2014;5:4561.

37. Wang RN, Green J, Wang Z, et al. Bone morphogenetic protein (BMP) signaling in development and human diseases. *Genes Dis*. 2014;1:87–105.

38. Morgan-Warren PJ, O'Neill J, de Cogan F, et al. siRNA-mediated knockdown of the mTOR inhibitor RTP801 promotes retinal ganglion cell survival and axon elongation by direct and indirect mechanisms. *Invest Ophthalmol Vis Sci*. 2016;57:429–443.

39. Ahmed Z, Suggate EL, Brown ER, et al. Schwann cell-derived factor-induced modulation of the NgR/p75NTR/EGFR axis disinhibits axon growth through CNS myelin in vivo and in vitro. *Brain*. 2006;129:1517–1533.

40. Zivraj KH, Tung YC, Piper M, et al. Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. *J Neurosci*. 2010;30:15464–15478.

41. Read ML, Mir S, Spice R, et al. Profiling RNA interference (RNAi)-mediated toxicity in neural cultures for effective short interfering RNA design. *J Gene Med*. 2009;11:523–534.

42. Douglas MR, Morrison KC, Jacques SJ, et al. Off-target effects of epidermal growth factor receptor antagonists mediate retinal ganglion cell disinhibited axon growth. *Brain*. 2009;132:3102–3121.

43. Mead B, Thompson A, Scheven BA, Logan A, Berry M, Leadbeater W. Comparative evaluation of methods for estimating retinal ganglion cell loss in retinal sections and wholemounts. *PLoS One*. 2014;9:e110612.

44. Ahmed Z, Read ML, Berry M, Logan A. Satellite glia not DRG neurons constitutively activate EGFR but EGFR inactivation is not correlated with axon regeneration. *Neurobiol Dis*. 2010;39:292–300.

45. Fagoe ND, Attwell CL, Eggers R, et al. Evaluation of five tests for sensitivity to functional deficits following cervical or thoracic dorsal column transection in the rat. *PLoS One*. 2016;11:e0150141.

46. Ahmed Z, Kalinski H, Berry M, et al. Ocular neuroprotection by siRNA targeting caspase-2. *Cell Death Dis*. 2011;2:e173.

Investigative Ophthalmology & Visual Science

47. Huang Z, Hu Z, Xie P, Liu Q. Tyrosine-mutated AAV2-mediated shRNA silencing of PTEN promotes axon regeneration of adult optic nerve. *PLoS One*. 2017;12:e0174096.

48. Huang ZR, Chen HY, Hu ZZ, Xie P, Liu QH. PTEN knockdown with the Y444F mutant AAV2 vector promotes axonal regeneration in the adult optic nerve. *Neural Regen Res*. 2018;13:135–144.

49. Berry M, Ahmed Z, Lorber B, Douglas M, Logan A. Regeneration of axons in the visual system. *Restor Neurol Neurosci*. 2008;26:147–174.

50. Berry M, Ahmed Z, Morgan-Warren P, Fulton D, Logan A. Prospects for mTOR-mediated functional repair after central nervous system trauma. *Neurobiol Dis*. 2016;85:99–110.

51. Berry M, Carlile J, Hunter A, Tsang W, Rosenstiel P, Sievers J. Optic nerve regeneration after intravitreal peripheral nerve implants: trajectories of axons regrowing through the optic chiasm into the optic tracts. *J Neurocytol*. 1999;28:721–741.

52. Garcia M, Forster V, Hicks D, Vecino E. Effects of muller glia on cell survival and neuritogenesis in adult porcine retina in vitro. *Invest Ophthalmol Vis Sci*. 2002;43:3735–3743.

53. Lorber B, Berry M, Douglas MR, Nakazawa T, Logan A. Activated retinal glia promote neurite outgrowth of retinal ganglion cells via apolipoprotein E. *J Neurosci Res*. 2009;87:2645–2652.

54. Diekmann H, Leibinger M, Fischer D. Do growth-stimulated retinal ganglion cell axons find their central targets after optic nerve injury? New insights by three-dimensional imaging of the visual pathway. *Exp Neurol*. 2013;248:254–257.

55. Leibinger M, Muller A, Andreadaki A, Hauk TG, Kirsch M, Fischer D. Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on ciliary neurotrophic factor and leukemia inhibitory factor. *J Neurosci*. 2009;29:14334–14341.

56. Muller A, Hauk TG, Fischer D. Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation. *Brain*. 2007;130:3308–3320.

57. Miyazono K, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. *J Biochem*. 2010;147:35–51.

58. Mehler MF, Mabie PC, Zhang D, Kessler JA. Bone morphogenetic proteins in the nervous system. *Trends Neurosci*. 1997;20:309–317.

59. Fuller ML, DeChant AK, Rothstein B, et al. Bone morphogenetic proteins promote gliosis in demyelinating spinal cord lesions. *Ann Neurol*. 2007;62:288–300.

60. Setoguchi T, Yone K, Matsuoka E, et al. Traumatic injury-induced BMP7 expression in the adult rat spinal cord. *Brain Res*. 2001;921:219–225.

61. Dudley AT, Lyons KM, Robertson EJ. A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev*. 1995;9:2795–2807.

62. Vigneswara V, Akpan N, Berry M, Logan A, Troy CM, Ahmed Z. Combined suppression of CASP2 and CASP6 protects retinal ganglion cells from apoptosis and promotes axon regeneration through CNTF-mediated JAK/STAT signalling. *Brain*. 2014;137:1656–1675.

63. Vigneswara V, Ahmed Z. Long-term neuroprotection of retinal ganglion cells by inhibiting caspase-2. *Cell Death Discov*. 2016;2:16044.

Investigative Ophthalmology & Visual Science