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EGF Stimulates Müller Glial Proliferation via a BMP Dependent Mechanism

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Abstract

Müller glia, the major type of glia in the retina, are mitotically quiescent under normal condition, though they can be stimulated to proliferate in some pathological states. Among these stimuli, EGF is known to be a potent mitogen for Müller glia. However, the signaling pathways required for EGF-mediated proliferation of Müller glia are not clearly understood. In this study, postnatal day 12 (P12) or adult *trp53*^{-/-} mouse retinas were explanted and cultured in the presence of EGF to stimulate Müller glial proliferation. Treatment with signaling inhibitors showed that activation of both MEK/ERK1/2 and PI3K/AKT pathways is required for EGF-induced proliferation of Müller glia. Interestingly, BMP/Smad1/5/8 activation downstream of PI3K/AKT signaling was also necessary for robust Müller glial proliferation, though activation of BMP/Smad1/5/8 signaling alone failed to stimulate their proliferation. In dissociated Müller glial culture, treatment with EGF induced the upregulation of *Bmp7*, and this upregulation was blocked significantly by co-treatment with the BMP inhibitor dorsomorphin, suggesting that BMP/Smad1/5/8 activation is mediated at least in part by an autocrine mechanism in Müller glia. A better understanding of how BMP/Smad1/5/8 signaling is involved in glial proliferation may have important implications for proliferative disorders, as well as for retinal regeneration in mammalian retinas.

INTRODUCTION

The factors that control the proliferation of Müller glia in the retina are important to understand for both proliferative disease states and potentially for regenerative medicine approaches to treat retinal degenerations. Although Müller glia do not proliferate under normal conditions, they can enter the mitotic cell cycle in some pathological states. For example, retinal detachment induces Müller glial proliferation in many species and these cells contribute to the accumulation of periretinal cellular membranes that characterize proliferative vitreoretinopathy (PVR) (Fisher and Lewis 2003; Sethi et al. 2005). In addition, retinal injury in fish and birds triggers extensive proliferation in the Müller glia (Bernardos et al. 2007; Fausett and Goldman 2006; Fischer and Reh 2001; Fischer and Reh 2002), which in fish can lead to nearly complete restoration of cells lost by the injury (Fimbel et al. 2007; Vihtelic and Hyde 2000; Wu et al. 2001).

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Müller glial proliferation has been studied in a variety of species, both *in vitro* and *in vivo*, and some of the mitogenic factors for these cells are known. For example, insulin-like growth factor (IGF) and insulin are potent mitogens for Müller glia (Fischer et al. 2002; Ikeda et al. 1995). In the posthatch chick, a combination of insulin and fibroblast growth factor (FGF) causes a large percentage of the Müller glia to enter the mitotic cell cycle (Fischer et al. 2002; Fischer and Reh 2002). P2Y-receptor activation stimulates Müller glial proliferation in guinea pig, as does the activation of the platelet-derived growth factor (PDGF) receptor (Milenkovic et al. 2003). Perhaps the most well-studied mitogenic factor for Müller glia is epidermal growth factor (EGF). EGF has been shown to stimulate Müller glial proliferation in mice (Close et al. 2006; Karl et al. 2008; Ueki et al. 2012), rats (Close et al. 2005), rabbits (Scherer and Schnitzer 1994), guinea pig (Milenkovic et al. 2003), and human (Hollborn et al. 2005).

There is evidence that these various signals converge on the EGF receptor (EGFR) (Milenkovic et al. 2003). In this model, ATP stimulates the P2Y receptor, which then causes an increase in intracellular calcium and an autocrine release of PDGF. The activation of the PDGF receptor then leads to an increase in matrix metalloproteinase activity, which allows the heparin-binding EGF (HB-EGF) to stimulate the EGFR. There is support for this model in analysis of PVR retinas, since HB-EGF is increased in the retinas of PVR patients (Hollborn et al. 2005). In addition, in zebrafish, HB-EGF is increased after damage and is necessary for the proliferation of Müller glia and their regeneration of new neurons (Wan et al. 2012); however, at this time the signaling events that occur downstream of EGFR activation are not well described.

In addition to the increase in HB-EGF observed in PVR retinas, two components of the bone morphogenetic protein (BMP) pathway, Smad1 and BMP4, were also among the most highly up-regulated genes (Hollborn et al. 2005). However, little is known about the role of BMPs in Müller glial proliferation. In this report we describe a novel role for BMP in the control of Müller glial proliferation. We have found that treatment of the retina with EGF in mice leads to stimulation of the BMP signaling pathway in the Müller glia. EGF treatment of the retina causes a release of BMP7, at least in part from the Müller glia. This autocrine BMP release goes on to activate the BMP receptors on Müller glia and leads to Smad1/5/8 phosphorylation and an increase in Id1 in these cells. However, while activation of the BMP pathway is required for EGF stimulated proliferation in the Müller glia, it is not sufficient, since direct activation of this pathway with BMP did not elicit a mitogenic effect. These results demonstrate a role for BMP in the mitogenic pathway regulating Müller glial proliferation and have implications for both PVR and regenerative approaches for retinal disease.

MATERIALS AND METHODS

Animals

All procedures involving animals were performed according to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the IACUC at the University of Washington. Wild type mice of the C57BL/6 strain were used for most of the experiments in this study; however, in some experiments, Hes5-GFP mice (Basak and

Taylor 2007) were used to mark Müller glia (Nelson et al. 2011). In addition, we used 6-week old *trp53*^{-/-} mice (Donehower et al. 1992) for the adult retinal explant and dissociated Müller glial cultures.

Retinal Explant and Müller Glial Cultures

Retinal explant and dissociated Müller glial cultures were prepared as described previously (Ueki et al. 2012). Briefly, retinas were isolated and cultured as explants on tissue culture filter inserts (Millipore, Billerica, MA) (Supporting Figure S1). For dissociated Müller glia cultures, adult *trp53*^{-/-} retinas were dissociated using a papain dissociation kit (Worthington, Lakewood, NJ) and then plated with EGF to promote initial growth. Subsequently, Müller glia were plated at equal density without EGF 2 days prior to experiments. Indicated factors and inhibitors were added to the culture media; those include 100 ng/mL recombinant mouse (rm) EGF, 100 ng/mL rmBMP4, 100 ng/mL rmBMP7, 100 ng/mL Noggin (R&D systems, Minneapolis, MN), 25 μ M PD98059, 10 μ M LY294002 (Cell Signaling, Danvers, MA), and 4 μ M Dorsomorphin (Tocris, Bristol, United Kingdom). 10 μ g/mL EdU (Invitrogen, Grand Island, NY) was added to the culture media for the duration of the culture period.

Immunohistochemistry

Retinas or cells were fixed with 2% paraformaldehyde in PBS for 30–40 min at room temperature. For cross-sections, retinas were cryoprotected in increasing concentrations of sucrose (10–30%) in PBS at room temperature, followed by overnight incubation at 4°C in PBS containing 30% sucrose. Retinas were placed in Tissue-Tek OCT Compound (Sakura Fintek, Torrance, CA), frozen with dry ice, and then sectioned on a cryostat at a thickness of 12 μ m. Slides were dried at room temperature, and stored at –20°C until use. Slides or explants were washed once with PBS containing 1% Triton X-100 prior to immunofluorescence labeling. Nonspecific binding was blocked by incubating sections with 10% horse serum in PBS for 1 h at room temperature, and the primary antibody was applied overnight at 4°C. The Primary antibodies used were chicken anti-GFP (Abcam, Cambridge, MA), rabbit anti-Pax6 (Covance, Princeton, NJ), rabbit anti-pSmad1/5/8 (Cell Signaling, Danvers, MA), and rabbit anti-Id1 (BioCheck, Foster City, CA). Sections were washed, and incubated with Alexa Fluor 488 goat anti-rabbit IgG (#A11008), Alexa Fluor 488 goat anti-chicken IgG, or Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, Grand Island, NY) for 1 h at room temperature. Nuclei were counterstained with DAPI (Sigma, St. Louis, MO). EdU staining was performed following immunohistochemistry using Click-iT EdU Alexa Fluor 555 imaging kit (Invitrogen, Grand Island, NY), and sections or whole retinas were coverslipped with PBS containing 50% glycerol. Imaging was performed using an Olympus FluoView confocal laser scanning microscope (Olympus, Center Valley, PA). To ensure quantitative image quality, laser power, pinhole settings, PMT settings, and intensity thresholds were kept constant for a given antibody.

Western Blot

Retinas were harvested at indicated time points and homogenized in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 5% (v/v) glycerol,

phosphatase inhibitor cocktail (Roche, Indianapolis, IN), and protease inhibitor cocktail (Sigma, St. Louis, MO)]. Equal amounts of the total protein lysate were separated by electrophoresis on 4–20% gradient SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% bovine serum albumin (BSA) in TBS-T (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween-20) for 1 hr at room temperature, and then incubated overnight at 4°C with the indicated primary antibodies. Primary antibodies used were rabbit anti-pAKT (S473), rabbit anti-AKT, rabbit anti-pERK1/2, rabbit anti-ERK1/2 (Cell Signaling, Danvers, MA), rabbit anti-Id1 (BioCheck, Foster City, CA), and mouse anti-beta actin (Abcam, Cambridge, MA) antibodies. Membranes were then incubated for 1 hr at room temperature with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Bio-Rad, Hercules, CA). Signals were exposed to x-ray films using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA), and quantified using ImageJ. The phosphorylated protein signal was normalized to the corresponding total protein signal. β -actin was used as a loading control.

Real-Time qPCR

Total RNA was isolated from each retina using Trizol (Invitrogen, Grand Island, NY). RNA was treated with RQ1 DNase (Promega, Madison, WI), and then cleaned with RNeasy kit (Qiagen, Germantown, MD). 1 μ g of RNA from each sample was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), and real-time qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). *Gapdh* was used as a normalization control. Primer sequences were as follows: *Id1* F 5'-TACGACATGAACGGCTGCTACTCA-3', *Id1* R 5'-TTACATGCTGCAGGATCTCCACCT-3', *Id3* F 5'-CTGTCGGAACGTAGCCTGG-3', *Id3* R 5'-GTGGTTCATGTCGTCCAAGAG-3', *Bmp7* F 5'-ACGGACAGGGCTTCTCCTAC-3', *Bmp7* R 5'-ATGGTGGTATCGAGGGTGGAA-3', *Gapdh* F 5'-GGCATTGCTCTCAATGACAA-3', and *Gapdh* R 5'-CTTGCTCAGTGTCTTGCTG-3'.

RESULTS

Proliferation of Müller Glia is Dependent on MEK/ERK1/2 and PI3K/AKT Pathways

Previously we have shown that EGF stimulation of retinal explants from postnatal day 12 (P12) mice for 5 days in vitro (5DIV) induced robust proliferation of Müller glia (Ueki et al. 2012). In this study, we investigated the signaling pathways mediating Müller cell proliferation in the mouse retina using this explant culture system (Supporting Figure S1). For immunohistochemical analysis, explants were either sectioned or analyzed as flatmounts (Supporting Figure S1B and C). Retinas from P12 Hes5-GFP mice were cultured as explants for 5 days (P12+5DIV) in the presence of EGF, and the thymidine analog EdU to label the proliferating cells (Figure 1A). In the first series of experiments, we fixed the explants at the end of the culture period and analyzed the numbers of S-phase cells. As reported previously (Close et al. 2005; Ueki et al. 2012), EGF induced proliferation of the cells in the retina. Figure 2B and C show examples of explant sections that were double-labeled for EdU and Hes5-GFP, a marker of Müller glia (Nelson et al. 2011). After 5 days of EGF treatment, the

majority of the EdU⁺ cells in the inner nuclear layer (INL) and outer nuclear layer (ONL) were Hes5-GFP⁺ Müller glia (Figure 1B), and these proliferating glia migrated toward ONL (Figure 1C). EdU⁺ cells in the ganglion cell layer (GCL) were not Hes5-GFP⁺, and therefore excluded from further analysis (arrows in Figure 1B). In a second series of experiments, we imaged fields in flatmounted retinas (Figure 1D and E). Imaging the full depth of the INL and ONL for EdU⁺ cells in projected confocal stacks shows the extensive proliferation that occurred in the explants following 5 days of EGF treatment (Figure 1D). Previous reports have shown that Müller glia express Pax6, and that they move to the ONL after entering the mitotic cycle (Ueki et al. 2012; Vazquez-Chona et al. 2011). In explants co-labeled with Pax6 and EdU, the majority of the cells that proliferate are Pax6⁺ (Figure 1E), and the proliferating Müller glia migrate from the INL to the ONL. Müller glial proliferation in the explants was EGF dependent, since untreated explants did not have a substantial number of EdU⁺ cells in either the INL or the ONL (NT in Figure 1D and E).

EGF is known to activate both the MEK/ERK1/2 and the PI3K/AKT pathways (Citri and Yarden 2006). Therefore, we investigated if EGF-induced proliferation of Müller cells depended on activation of these pathways using inhibitors for MEK or PI3K, PD98059 (PD) or LY294002 (LY), respectively. Unlike normal retinas, where phosphorylated ERK1/2 and AKT are undetectable or present at only a low level (data not shown), retinal explants after 5 DIV (NT) showed some activation of ERK1/2 and AKT (Figure 1F) even when cultured in the absence of EGF. Nevertheless, increased activation of ERK1/2 (pERK1/2) was observed with EGF treatment (Figure 1F), and this effect could be blocked by the addition of the MEK inhibitor PD. Addition of LY along with EGF effectively blocked activation of the AKT pathway in the explants (Figure 1F). These effects are quantified in Figure 1G (AKT) and Figure 1H (ERK1/2). Interestingly, treatment with either PD or LY almost completely inhibited EGF-induced proliferation of Müller glia in the retinal explants (Figure 1D and E), suggesting that activation of both MEK/ERK1/2 and PI3K/AKT pathways are essential for Müller glia to proliferate through an EGF-dependent pathway.

Robust Müller Glial Proliferation Requires BMP/Smad1/5/8 Signaling

As noted in the Introduction, although there is a great deal known about the molecular pathways that lead to EGFR activation in Müller glia, there is less known about the downstream consequences of EGFR activity in these cells. In a screen for other signaling pathways that were active in the EGF treated Müller glia, we were surprised to discover that treatment of the explants with EGF activates the BMP/Smad1/5/8 pathway. Figure 2A shows an example of an explant labeled with an antibody against the activated form of Smad1/5/8 (pSmad1/5/8; red), a key downstream protein in the BMP pathway. In untreated explants, pSmad1/5/8 is undetectable in the Hes5-GFP⁺ Müller glia, while there is robust labeling in these cells in EGF treated explants (Figure 2A). Additionally, the BMP responsive genes, Inhibitor of differentiation 1 (*Id1*) and 3 (*Id3*), are significantly up-regulated in the EGF treated explants. This up-regulation can be blocked by the addition of dorsomorphin (DM), a BMP receptor type I inhibitor, or noggin, which blocks BMP binding to the receptor, (Figure 2B and C). Moreover, real-time qPCR data showed that EGF treatment induces expression of *Bmp7* that can be blocked by DM or noggin in the explants

(Figure 2C) (*Bmps* 2, 4, and 5 were undetectable; data not shown), suggesting that EGF may activate BMP/Smad1/5/8 signaling through a positive feedback loop with BMP7.

To determine whether the activation of the BMP pathway in response to EGF was important for the proliferation of Müller glia, we assayed the number of EdU⁺ cells when EGF stimulation was combined with inhibitors of the BMP pathway. Representative flatmount images taken in the INL and ONL show that treatment with either DM or noggin largely blocked EGF-induced Müller cell proliferation (Figure 2D). Together, our data demonstrate that EGF activates BMP/Smad1/5/8 signaling in Müller glia, and this activation is necessary for the robust activation of Müller cell proliferation induced by EGF.

PI3K/AKT Signaling is Necessary for EGF-dependent Activation of BMP/Smad1/5/8 in Retinal Explants

To determine whether the activation of the BMP pathway in Müller glia requires ERK1/2 and AKT activation, we treated explants with PD or LY along with EGF, and analyzed activation of pSmad1/5/8 (Figure 3). We found that EGF-induced activation of Smad1/5/8 in Müller glia (Hes5-GFP; green) was blocked by LY treatment but not by PD (Figure 3A). This result indicates that the AKT, but not the ERK1/2 pathway, is required for the activation of the BMP pathway after EGF treatment. By contrast, treating explants with EGF and DM or noggin had no effect on ERK1/2 or AKT phosphorylation (Supporting Figure S2), further implying that BMP/Smad1/5/8 is likely downstream of PI3K/AKT activation. However, activation of PI3K/AKT pathway does not seem to be sufficient to induce BMP/Smad1/5/8 signaling since non-treated explants show activation of AKT (NT in Figure 1F) but not Smad1/5/8 (Figure 3A) or Id1 (Figure 3C).

The conclusion that BMP pathway activation in Müller glia is downstream of the AKT pathway is further supported by experiments measuring levels of the BMP signaling components, BMP7 and Id1. PD treatment of retinal explants did not significantly inhibit EGF-induced *Bmp7* expression, while LY treatment had a profound effect on *Bmp7* expression, leading to levels even lower than the no-treatment control explants (Figure 3B). LY also blocked EGF-induced Id1 expression significantly (Figure 3C and D). Together, these data strongly suggest that the activation of BMP/Smad1/5/8 signaling in Müller glia is one of the downstream events of PI3K/AKT activation.

BMP/Smad1/5/8 Signaling is Required, but Not Sufficient for Robust Müller Glial Proliferation

Our results demonstrate that activation of BMP/Smad1/5/8 signaling is necessary for robust Müller glial proliferation in retinal explants (Figure 2). In order to test if BMP/Smad1/5/8 signaling is sufficient to stimulate Müller glial proliferation, we treated retinal explants with recombinant mouse BMP4 or BMP7 for 5DIV, and quantified Müller glial proliferation (Figure 4). Treatment of explants with BMP4 or BMP7 increased Id1 expression significantly (Figure 4A and B), showing that the treatment effectively activated BMP/Smad1/5/8 signaling. However, this activation did not stimulate Müller glial proliferation. Immunohistochemistry of flatmounted explants showed that there were only a few EdU⁺/Hes5-GFP⁺ cells (proliferating Müller glia) in the INL of BMP4- or BMP7-treated explants,

while many of these double positive cells were present after EGF treatment (Figure 4C and D). Quantification of EdU⁺ cells in the INL and ONL of the retinal explants demonstrated that treatment of BMP alone did not promote robust Müller glial proliferation (Figure 4D). Thus, BMP/Smad1/5/8 signaling is necessary for EGF stimulated Müller glial proliferation, but it is not sufficient for Müller glial proliferation on its own.

Activation of BMP/Smad1/5/8 Signaling Promotes Proliferation of Adult *trp53*^{-/-} Müller Glia

Though mature Müller glia do not divide *in vivo* under normal conditions, we previously reported that dissociated Müller glia from adult *trp53*^{-/-} retinas proliferated with EGF stimulation *in vitro* (Ueki et al. 2012). In this study, we investigated if the proliferation of dissociated Müller glia depended on MEK/ERK1/2, PI3K/AKT and BMP/Smad1/5/8 signaling activation as seen in Müller glia in P12 retinal explants. The same number of adult *trp53*^{-/-} Müller glia were plated in each well, and cells were grown for 2 days with the indicated factors. The number of nuclei in each random field was counted after nuclear staining with DAPI (Figure 5A). EGF induced rapid proliferation of dissociated Müller glia, while co-treatment with inhibitors, PD, LY, or DM blocked robust proliferation induced by EGF (Figure 5B). Interestingly, inhibition of BMP/Smad1/5/8 signaling with DM only partially decreased Müller glial growth, which was also observed in Müller glial proliferation in retinal explants (Figure 2D). Treatment of dissociated Müller glia with EGF induced robust *Bmp7* mRNA expression, and the expression was attenuated when co-treated with DM (Figure 5C), suggesting that the activation of BMP/Smad1/5/8 signaling was autocrine in Müller glia.

We recently reported that Müller glia in adult *trp53*^{-/-} retinal explants proliferated in response to EGF treatment (Ueki et al. 2012). Here we investigated if proliferation of mature Müller glia from adult retinal explants was also mediated through activation of the same signaling pathways as in P12 retinal explants. Retinas from adult *trp53*^{-/-} mice were cultured for 5DIV as explants with the same factors as used in the P12 cultures. EdU incorporation was used to assess the degree of Müller glial proliferation (Figure 5D and E). As expected from our previous study, EGF stimulated EdU incorporation in the Müller glia in the *trp53*^{-/-} adult mouse retina; however, treatment of the adult explants with PD, LY, or DM significantly reduced the number of EdU⁺ cells (Figure 5D and E), similar to the effects we observed on Müller glial of P12 explants (Figures 1D and 2D). Together, these results demonstrate that activation of MEK/ERK1/2, PI3K/AKT and BMP/Smad1/5/8 pathways in adult Müller glia is essential for their robust proliferation. Müller glial proliferation is mediated by the same signaling pathways regardless of their age (P12 or adult).

DISCUSSION

In this report we have found that BMP/Smad1/5/8 signaling, activated in response to EGF downstream of PI3K/AKT signaling, is essential for robust Müller glial proliferation (Figure 6). EGF-stimulated Müller glia induce BMP7 expression, activate Smad1/5/8, and increase expression of a known BMP signaling target Id1. While BMP/Smad1/5/8 signaling is necessary for robust Müller glial proliferation, it is not sufficient. This is the first study

demonstrating a requirement for BMP/Smad1/5/8 signaling for Müller glial proliferation. Our data show that ERK1/2 signaling is also required for Müller glia to proliferate.

MEK/ERK1/2 and PI3K/AKT pathways are well-characterized downstream signaling pathways activated in response to EGF (Citri and Yarden 2006). Indeed, we detected activation of ERK1/2 and PI3K/AKT in EGF-treated retinal explants (Figure 1F–G). Inhibition of either pathway significantly reduced Müller glial proliferation in P12 and adult retinal explants as well as dissociated Müller glial cultures (Figures 1D, 1E and 5), demonstrating both MEK/ERK1/2 and PI3K/AKT pathways are required for Müller glia to proliferate. This observation is supported by previous studies showing a requirement for ERK1/2 and PI3K/AKT signaling activation in growth factor-induced proliferation of the human Müller cell line MIO-M1 (Hollborn et al. 2004; Hollborn et al. 2005) and guinea pig primary Müller glial cultures (Milenkovic et al. 2003).

Although the role of MEK/ERK1/2 and PI3K/AKT signaling in growth factor-induced proliferation of Müller glia have been demonstrated previously in Müller glial cultures (Hollborn et al. 2004; Hollborn et al. 2005; Milenkovic et al. 2003), the mechanisms by which these pathways stimulate proliferation were not clearly characterized. Here, we demonstrate that EGF-induced Müller glial proliferation is mediated at least in part by autocrine activation of BMP/Smad1/5/8 signaling in Müller glial (Figure 6). Expression of inhibitor of differentiation (Id) 1 and 3, known downstream targets for BMP/Smad1/5/8 signaling (Lopez-Rovira et al. 2002; Samanta and Kessler 2004), is also induced in the retina with EGF treatment (Figure 2B and C). The expression of Id proteins antagonizes proneural bHLH transcription factors during development, and overexpression of Id1-4 promotes cell proliferation and glial differentiation of progenitors in the CNS (Cai et al. 2000; Hirai et al. 2012; Jung et al. 2010). Moreover, treatment of telencephalic neural progenitors with BMP2 induces Id1 and Id3 expression and promotes astrocytogenesis instead of neurogenesis (Nakashima et al. 2001). Therefore, it is possible that the expression of Id proteins through BMP/Smad1/5/8 signaling in Müller glia promotes their proliferation, as well as maintains their glial state in the mouse retina. Consistent with this idea, we recently reported that BMP/Smad1/5/8 signaling is activated and Id1/3 expression is induced in Müller glia in the damaged mouse retina (Ueki and Reh 2012), which has a limited ability to regenerate lost neurons from Müller glia after damage (Karl et al. 2008). On the other hand, damage induces zebrafish Müller glia to undergo robust proliferation, dedifferentiation and neural differentiation to completely restore lost neurons (Bernardos et al. 2007; Fausett and Goldman 2006; Fimbel et al. 2007). Interestingly, a recent study shows that in damaged zebrafish retina, Id3 expression declines as Müller glia proliferate and differentiate into new neurons (Qin et al. 2009), further suggesting a role of BMP/Smad1/5/8 signaling in glial fate maintenance.

It is also possible that BMP/Smad1/5/8 signaling regulates cell cycle progression. For example, in other systems, Id1 inhibits cyclin dependent kinase inhibitors p21 (Prabhu et al. 1997) and p16 (Ohtani et al. 2001) to promote cell proliferation. Alternatively, BMP/Smad1/5/8 signaling may upregulate D-type cyclins in Müller glia. Cyclin D is a cell cycle regulator that mediates the G1 to S-phase transition. Cyclin D1 and D3 are up-regulated in Müller glia after light damage in mouse (Joly et al. 2011) and laser injury in rat (Kohn et

al. 2006), and both light damage and neurotoxin damage rapidly induce BMP/Smad1/5/8 signaling in mouse Müller glia (Ueki and Reh 2012). In addition, cyclin D1 is essential for cell proliferation during retinal development, and mice lacking cyclin D1 show significant reduction in cell number (Sicinski et al. 1995). Therefore, EGF-induced activation of BMP/Smad1/5/8 signaling in the retina may induce expression of D-type cyclins in Müller glia to promote their proliferation.

Our results show that EGF treatment induces *Bmp7* up-regulation in P12 retinal explants or adult *trp53*^{-/-} dissociated Müller glial cultures (Figures 2C and 5C). Hollborn *et al.* reported that expression of *Bmp4* was upregulated along with HB-EGF in human proliferative vitreoretinopathy (PVR) patient retinas (Hollborn et al. 2004), in which Müller glial proliferation is observed. Although in mouse retinas we did not detect upregulation of *Bmp2*, -4 and -5 with EGF treatment (data not shown), treatment of Müller glial cultures with EGF induces expression of *Bmp7* (Figure 5C). In addition, co-treatment of retinas or Müller glial cultures with EGF and BMP inhibitors DM and/or noggin significantly attenuates expression of *Bmp7* (Figures 2C and 5C). These data suggest that BMP/Smad1/5/8 signaling is at least in part activated in an autocrine manner in Müller glia, and that there may be a positive feedback loop to activate BMP/Smad1/5/8 signaling.

Our results demonstrate that activation of BMP/Smad1/5/8 signaling in mouse Müller glia is essential for their proliferation induced by EGF. We propose BMP/Smad1/5/8 signaling is a key proliferative signal for Müller glia. Gene expression studies in human PVR, in which Müller glia and other retinal cells proliferate to form fibroproliferative tissue, show increased expression of heparin binding EGF-like growth factor (HB-EGF), as well as BMP/Smad1/5/8 signaling components, BMP4 and Smad1 in PVR patient retinas (Hollborn et al. 2004). This is consistent with our observations in mouse Müller glia, where EGF activates BMP/Smad1/5/8 signaling to promote their proliferation. Moreover, an increase in BMP signaling has been associated with damage in other regions of the central nervous system (CNS), and therefore the mechanism we propose for Müller glia may also apply to other CNS glia.

We recently reported that BMP/Smad1/5/8 signaling is activated in the cells of the inner retina, including Müller glia, after retinal damage in mouse, and provides a neuroprotective effect on retinal ganglion cells possibly through the activation of Müller glia (Ueki and Reh 2012). At the site of rat spinal cord demyelinating lesions, expression of BMP4 and -7 and Smad1/5/8 phosphorylation are rapidly induced; these factors may promote gliosis and glial scar formation (Fuller et al. 2007). However, an analysis of BMP receptor-null mice found that the effects of injury on gliotic scar formation depended on which receptor was deleted, and the role of BMP signaling in gliosis and glial scar formation following spinal cord injury is likely to be complex (Sahni et al. 2010). In addition to its role in injury, the results of our study may have implications for CNS tumours. Overexpression or unregulated activation of EGFR is commonly associated with gliomas and contributes to uncontrolled proliferation of glia (Halatsch et al. 2006; Huang et al. 1997). It is known that EGFR mainly transmit signals through the MEK/ERK1/2 and PI3K/AKT pathways in gliomas (McLendon et al. 2007), but it is not known whether BMP signaling is downstream of the EGF receptor in astrocytes or

gliomas. Our results may therefore provide new therapeutic targets for regulating glial growth in pathological conditions in the retina in particular and the CNS more generally.

Understanding how BMP/Smad1/5/8 signaling promotes Müller glial proliferation may also have important implications for mammalian retinal regeneration. As noted in the Introduction, retinal damage in non-mammalian vertebrates triggers proliferation of the Müller glia (Bernardos et al. 2007; Fausett and Goldman 2006; Fischer and Reh 2001; Fischer and Reh 2002). In fish, the progeny of the Müller glia differentiate into various types of retinal neurons to repair the injury (Fimbel et al. 2007; Vihtelic and Hyde 2000; Wu et al. 2001). One of the key signals important in regeneration of the zebrafish retina is HB-EGF (Wan et al, 2012). Goldman and colleagues have shown that damage to zebrafish retina causes an increase in the expression of this Müller glial mitogen, like that observed in the mammalian retina. In addition, the subsequent EGFR activation in zebrafish also requires MAPK signaling (Wan et al, 2012). Moreover, the Cntf/LIF-Stat pathway is also known to be activated by retinal damage in both zebrafish (Kassen et al, 2009) and mammalian Müller glia (Ueki et al, 2008). Thus, it appears that some of the early events in the Müller glial response to injury may be conserved between these species. However, a key difference in these animals is that in the fish, injury causes at least some of the Müller glia to develop characteristics of retinal progenitors (eg. Ascl1 expression), while in the mouse, Müller glia do not express Ascl1 after damage. One possibility for this differential response to injury might be that the BMP pathway is activated in mammalian Müller glia, but not zebrafish Müller glia after injury; BMP is known to promote gliogenesis, particularly when co-activated with Cntf/LIF-Stat3 signaling. Future comparative studies of Müller glia from zebrafish and mammals will likely provide insight into the molecular pathways underlying the differences between their regenerative responses to injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. EGF stimulates Müller glial proliferation through ERK1/2 and PI3K/AKT pathways in explant cultures

A. Retinas from P12 mice were cultured onto a tissue culture insert for 5 days (5DIV) with daily addition of EGF, EdU (to mark proliferating cells), and inhibitors as indicated. Explants were collected after 5DIV and processed for IHC, protein, or RNA analyses. **B.** EGF stimulated cell proliferation (EdU⁺) in the retina. All the EdU⁺ cells in ONL and INL were also Hes5-GFP⁺ suggesting that those proliferating cells were Müller glia. EdU⁺ cells in GCL are not Hes5-GFP⁺ (arrows), and therefore excluded from the further analyses. Scale bar: 100 μ m. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. **C.** Many proliferating Müller glia (EdU⁺/Hes5-GFP⁺) migrated to ONL. Scale bar: 20 μ m. **D.** Representative stack images (only ONL+INL) of EdU staining on retinal flatmounts are shown. EGF stimulated Müller glia to proliferate. This proliferation was blocked by the addition of the MEK inhibitor (PD98059 or PD) or the PI3K inhibitor (LY294002 or LY), demonstrating that ERK1/2 and PI3K/AKT pathways were both essential for Müller glia to proliferate. Scale bar: 100 μ m. **E.** Representative single slice (2 μ m) flatmount images of ONL are shown. Proliferating Müller glia (EdU⁺/Pax6⁺) migrate to ONL with EGF stimulation, but the proliferation and migration were blocked by MEK or PI3K inhibitors. Scale bar: 100 μ m. **F.** Representative Western Blot of retinal explants showing that inhibitors effectively blocked phosphorylation of their intended targets. **G.** Quantification of Western blots showing that LY blocked AKT phosphorylation. The pAKT/AKT ratio of non-treated (NT) explants is normalized to 1 for each set of experiment (n = 3). **H.** Quantification of Western blots showing that PD blocked EGF induced phosphorylation of ERK. The pERK/ERK ratio of non-treated (NT) explants is normalized to 1 for each set of experiment (n = 3). In **G** and **H**: *p < 0.05 with t-test. Error bars: SEM.

Figure 2. EGF-induced Müller glial proliferation is partially dependent on activation of BMP/Smad1/5/8 signaling

A. EGF induced phosphorylation of Smad1/5/8 (pSmad1/5/8; red) in Müller glia (Hes5-GFP; green), demonstrating that BMP/Smad1/5/8 signaling was activated by EGF in retinal explants. **B.** One μm single slice flatmount images of the INL are shown. EGF induced Id1 (white) expression, a known target of BMP/Smad1/5/8 signaling, and the expression was partially blocked by inhibition of BMP/Smad1/5/8 signaling by a BMP signaling inhibitor dorsomorphin (DM). **C.** Real-time PCR of BMP/Smad1/5/8 signaling components. EGF induced expression of a BMP/Smad1/5/8 ligand (*Bmp7*) and targets (*Id1* and *Id3*). The expression is completely blocked by BMP inhibitors DM or noggin. Expression was normalized to NT value for each set of experiment ($n = 5$). * $p < 0.05$ with ANOVA. Error bars: SEM. **D.** Representative stack images of the EdU staining (ONL+INL in flatmounts) are shown. Both DM and noggin largely inhibited EGF-induced Müller glial proliferation. Scale bars (in **A**, **B**, **D**): 100 μm .

Figure 3. Activation of BMP/Smad1/5/8 signaling is downstream of PI3K/AKT pathway

A. Smad1/5/8 phosphorylation (pSmad1/5/8; red) induced by EGF in Müller glia (Hes5-GFP; green) is blocked by inhibition of the PI3K/AKT (EGF+LY) or the BMP/Smad1/5/8 (EGF+DM) pathways, whereas inhibition of the ERK1/2 pathway (EGF+PD) did not affect Smad1/5/8 phosphorylation. **B.** Real-time PCR showing that EGF-induced expression of *Bmp7* is significantly attenuated by PI3K/AKT inhibition. Expression was normalized to NT value for each set of experiment (n = 4). *p < 0.05 with t-test. **C.** A representative Western Blot for Id1, a BMP/Smad1/5/8 signaling target. **D.** Quantification of Western Blots showing that EGF-induced Id1 expression was blocked significantly by PI3K/AKT inhibition. The Id1/actin ratio of non-treated (NT) explants is normalized to 1 for each set of experiment (n = 3). *p < 0.05 with t-test. Error bars: SEM.

Figure 4. Activation of BMP/Smad1/5/8 signaling is not sufficient to induce Müller glial proliferation

A–B. Representative Western Blot (**A**) and quantification (**B**) showing that treatment of explants with EGF, BMP4 or BMP7 for 5DIV effectively induced expression of a BMP signaling target, Id1. The Id1/actin ratio of non-treated (NT) explants is normalized to 1 for each set of experiment (n = 3). *p < 0.05 with t-test. Error bars: SEM. **C.** One μm single optical section of confocal images taken in the INL of retinal explants. Hes5-GFP⁺/EdU⁺ cells indicate proliferating Müller glia. Scale bar: 50 μm . **D.** Treatment of explants with BMP4 or BMP7 did not induce significant Müller glial proliferation, while EGF stimulated many Müller glia to proliferate. *p < 0.05 with t-test (n = 3). Error bars: SEM.

Figure 5. Activation of ERK1/2, PI3K/AKT, and BMP/Smad1/5/8 pathways is essential for EGF-induced proliferation of mature Müller glia from *trp53*^{-/-} retinas

Müller glia from adult *trp53*^{-/-} retinas were dissociated and cultured as previously described (Ueki et al. 2012). Equal numbers of Müller glia were plated, and then treated with the indicated factors for 2 days. **A.** Representative images of DAPI staining of the Müller glial cultures after 2 days. Scale bar: 100 μ m. **B.** Number of nuclei was counted in random fields after DAPI staining.

While EGF promoted significant Müller glial proliferation, inhibition of ERK1/2, PI3K/AKT, or BMP/Smad1/5/8 pathways with PD, LY, or DM, respectively, blocked this proliferation, demonstrating that activation of these pathways was essential for dissociated Müller glia to proliferate. * $p < 0.05$ with t-test ($n = 3$). **C.** Müller glia were collected after the indicated treatment for 2 days and the level of *Bmp7* was measured by qPCR. EGF induces robust upregulation of *Bmp7*, which can be repressed by treatment with a BMP inhibitor DM. Expression was normalized to NT value for each set of experiment ($n = 4$). * $p < 0.05$ with t-test.

D. Adult *trp53*^{-/-} retinas were explanted and cultured for 5DIV. Representative confocal images taken in ONL+INL of retinal explants are shown (flatmounts). Scale bar: 100 μ m. **E.** As reported previously (Ueki et al. 2012), many Müller glia of adult *trp53*^{-/-} explants proliferated in response to EGF. Treatment of explants with PD, LY, or DM significantly reduced EGF-induced proliferation of Müller glia in adult *trp53*^{-/-} explants, suggesting that activation of these pathways is essential for adult Müller glia to proliferate. * $p < 0.05$ with t-test ($n = 3$). Error bars in **B**, **C** and **E**: SEM.

Figure 6. Model for EGF-induced Müller glial proliferation

Activation of BMP/Smad1/5/8 signaling downstream of PI3K/AKT signaling is essential for inducing robust Müller glial proliferation. Our data suggest autocrine activation of BMP/Smad1/5/8 signaling in Müller glia. ERK1/2 signaling is also necessary for proliferation.