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Heparin-Binding EGF-like Growth Factor (HB-EGF) stimulates the proliferation of Müller glia-derived progenitor cells in avian and murine retinas

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Abstract

Müller glia can be stimulated to de-differentiate, proliferate and form Müller glia-derived progenitor cells (MGPCs) that regenerate retinal neurons. In the zebrafish retina, Heparin-Binding EGF-like Growth Factor (HB-EGF) may be one of the key factors that stimulate the formation of proliferating MGPCs. Currently nothing is known about the influence of HB-EGF on the proliferative potential of Müller glia in retinas of birds and rodents. In the chick retina, we found that levels of both *hb-egf* and *egf-receptor* are rapidly and transiently up-regulated following NMDA-induced damage. Although intraocular injections of HB-EGF failed to stimulate cell-signaling or proliferation of Müller glia in normal retinas, HB-EGF stimulated proliferation of MGPCs in damaged retinas. By comparison, inhibition of the EGF-receptor (EGFR) decreased the proliferation of MGPCs in damaged retinas. HB-EGF failed to act synergistically with FGF2 to stimulate the formation of MGPCs in the undamaged retina and inhibition of EGF-receptor did not suppress FGF2-mediated formation of MGPCs. In the mouse retina, HB-EGF stimulated the proliferation of Müller glia following NMDA-induced damage. Furthermore, HB-EGF stimulated not only MAPK-signaling in Müller glia/MGPCs, but also activated mTor- and Jak/Stat-signaling. We propose that levels of expression of EGFR are rate-limiting to the responses of Müller glia to HB-EGF and the expression of EGFR can be induced by retinal damage, but not by FGF2-treatment. We conclude that HB-EGF is mitogenic to Müller glia in both chick and mouse retinas, and HB-EGF is an important player in the formation of MGPCs in damaged retinas.

Keywords

retina; Müller glia; proliferation; progenitor

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Introduction

Müller glia in the retina can be stimulated to become proliferating progenitor cells. The reprogramming of Müller glia into Müller glia-derived progenitor cells (MGPCs) involves de-differentiation, up-regulation of transcription factors expressed by retinal stem cells, and proliferation as MGPCs (Gallina et al. 2014a; Goldman 2014). MGPCs have the capacity to produce progeny that differentiate into retinal neurons. The regenerative capacity of Müller glia varies substantially across vertebrate classes. The teleost fish retina has a robust capacity to regenerate functional neurons after injury and this regeneration is mediated by MGPCs (Lenkowski and Raymond 2014). By comparison, the avian retina has a limited regenerative response; numerous proliferating MGPCs are generated but their neurogenic capacity is not sufficient to restore visual function (Fischer 2005; Gallina et al. 2014a). Müller glia in the mammalian retina typically respond to damage by undergoing reactive gliosis (Dyer and Cepko 2000). However, damage coupled with growth factor treatment can stimulate the formation of MGPCs with limited neurogenic potential (Karl and Reh 2010; Ooto et al. 2004). One of the major hurdles to harnessing the regenerative potential of the retina in warm-blooded vertebrates is the identification of the factors and cell-signaling pathways that drive the transition of Müller glia into proliferating MGPCs.

Understanding the cell-signaling events that influence the formation of MGPCs in different vertebrate classes is expected to unlock the regenerative potential of MGPCs in humans. Recent studies have indicated that a network of cell-signaling pathways is initiated during the reprogramming of Müller glia into MGPCs in the zebrafish model system (Lenkowski and Raymond 2014; Wan et al. 2014). This network can be initiated in Müller glia in the absence of damage and is known to involve MAPK, Notch, PI3K/Akt, Jak/Stat, Wnt/ β -catenin (reviewed by Goldman 2014). In the chick model system, we have reported FGF2/ MAPK-signaling initiates a network of signaling pathways that includes Notch (Ghai et al. 2010; Hayes et al. 2007), glucocorticoid (Gallina et al. 2014c), Wnt/ β -catenin (unpublished observations), and Hedgehog-pathways (Todd & Fischer, 2015).

In the zebrafish retina, HB-EGF has been shown to be necessary and sufficient to drive Müller glia-mediated retinal regeneration through MAPK-signaling (Wan et al. 2012; Wan et al. 2014). However, this result remains controversial due to a conflicting report indicating that HB-EGF does not stimulate MGPCs in the fish retina (Nelson et al. 2013). *In vivo*, EGF or FGF stimulate the proliferation of MGPCs in damaged mouse retinas ostensibly via MAPK activation (Karl et al. 2008).

HB-EGF is first synthesized as a membrane-anchored glycoprotein precursor and is cleaved to a soluble form that potently activates EGFR family receptors and MAPK-signaling (Prenzel et al. 1999). It remains unknown whether HB-EGF influences Müller glia proliferation in bird and rodent model systems. Accordingly, the purpose of this study was to determine whether HB-EGF influences the Müller glia in chick and mouse retinas *in vivo*, and identify the different cell-signaling pathways that are activated by HB-EGF.

Methods and Materials

Animals

The use of animals in these experiments was in accordance with the guidelines established by the National Institutes of Health and the Ohio State University. Newly hatched leghorn chickens (*Gallus gallusdomesticus*) were obtained from Meyer Hatchery (Polk, Ohio). Chicks were kept on a cycle of 12 hours light, 12 hours dark (lights on at 8:00 AM). Chicks were housed in a stainless steel brooder at about 25°C and received water and Purinatm chick starter *ad libitum*. All experiments utilized chicks between the ages of P7–P21.

C57/bl6 mice were used for all experiments. All animal breeding and experimental procedures were approved by the Ohio State University Animal Care and Use Committee (protocol number: 2009A0139). All experiments utilized mice between P30–P40.

Intraocular injections

Chickens were anesthetized via inhalation of 2.5% isoflurane in oxygen and intraocular injections were performed as described previously (Fischer et al. 1999; Fischer et al. 1998). Injections were made using a 25- μ l Hamilton syringe and a 26-gauge needle with a beveled, curved tip. The injection was consistently made into the dorsal quadrant of the vitreous chamber. Mice were anesthetized via inhalation of 2.5% isoflurane in oxygen, and intravitreal injections were made using a custom 31G needle and a 20 μ l Hamilton Syringe. For all experiments, the left eyes were injected with the “test” compound and the contralateral right eyes were injected with vehicle as a control. Compounds were injected in 20 μ l sterile saline for chicken retinas and 3 μ l for mouse retinas with 0.05 mg/ml bovine serum albumin added as a carrier. Compounds used in these studies included NMDA (28.3 or 0.9 μ g/dose – chick; 4.2 μ g/dose mouse; Sigma-Aldrich), HB-EGF (500 ng/dose – chick; 750ng ng/dose - mouse; R&D System), EGF-receptor inhibitor, PD153 (2 μ g dose; Tocris), FGF2 (250 ng/dose; Sigma-Aldrich), IGF1 (400ng/dose; R&D Systems). 2 μ g of BrdU was included with the intraocular injections to label proliferating cells. Mice received injections of 2mg BrdU intraperitoneally to label proliferating cells. Injection paradigms are included in each figure.

Quantitative Reverse transcriptase PCR

Individual retinas were placed in 1 ml of Trizol Reagent (Invitrogen) and total RNA was isolated according to the Trizol protocol and resuspended in 50 μ l RNase free water. Genomic DNA was removed by using the *DNA FREE* kit provided by Ambion. cDNA was synthesized from mRNA by using Superscripttm III First Strand Synthesis System (Invitrogen) and oligodT primers according to the manufacturer’s protocol. Control reactions were performed using all components with the exception of the reverse transcriptase to exclude the possibility that primers were amplifying genomic DNA.

PCR primers were designed by using the Primer-BLAST primer design tool at NCBI (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primers used in this study included: *hb-egf* forward 5’ TGGTGTGGTCATACGTGCTC 3’; reverse 5’ GCCTGCGGAAGTACAAGGAT 3’, product size 159. *egfr* forward 5’

CAGCCGCGATAGATGGAG 3'; reverse 5' TACACGAGGGTGCAAAGGAC 3', product size 71. PCR reactions were performed by using standard protocols, Platinum[™]Taq (Invitrogen) and an Eppendorf thermal cycler. PCR products were run on an agarose gel to verify the predicted product sizes. Significance of difference (*p<0.05; **p<0.01) was determined by using a Mann-Whitney U test.

Fixation, sectioning and immunocytochemistry

Tissues were fixed, sectioned and immunolabeled as described previously (Fischer et al. 2008; Fischer et al. 2009b). Chicks were euthanized via CO₂-inhalation. Eyes were enucleated, hemi-sectioned equatorially and the gel vitreous was removed. Eye cups were fixed for 30 minutes in 4% paraformaldehyde plus 2% sucrose in 0.1 M phosphate buffer, pH 7.4. Working dilutions and sources of antibodies used in this study are included in Table 1.

None of the observed labeling was due to non-specific labeling of secondary antibodies or autofluorescence because sections labeled with secondary antibodies alone were devoid of fluorescence. Secondary antibodies included donkey-anti-goat-Alexa488/568, goat-anti-rabbit-Alexa488/568/647, goat-anti-mouse-Alexa488/568/647, goat anti-rat-Alexa488 and goat-anti-mouse-IgM-Alexa568 (Invitrogen) diluted to 1:1000 in PBS plus 0.2% Triton X-100.

Terminal deoxy-nucleotidyltransferaseUTP nick end labeling (TUNEL)

To identify dying cells that contained fragmented DNA the TUNEL method was used. We used an *in situ* Cell Death Kit (TMR red; Roche Applied Science), as per the manufacturer's instructions. Slides were washed twice for 15 minutes in PBS plus 0.2% Triton X-100. Slides were then washed twice for 10 minutes in PBS. 5 µL of TUNEL enzyme, 55 µL buffer, and 60 µL of distilled water were added per slide and incubated in a dark humid chamber at 37°C for one hour. Slides were then washed 3 times in PBS for 5 minutes each. Slides were then mounted in a 4:1 mix of glycerol and water.

In situ hybridization

Standard procedures were used for *in situ* hybridization, as described elsewhere (Fischer et al. 2004b; Fischer and Reh 2002). PCR products were cloned into Topo TA vector (pCR-II; Invitrogen). RNA polymerase (T7) was used to generate digoxigenin-labeled riboprobes by using a kit provided by Roche and stored at -80°C until use. The riboprobe covered nucleotides 900 to 2.4kb of *hb-egf*. Eyes were dissected in RNase-free Hanks Balanced Salt Solution (HBSS), fixed overnight at 4°C in 4% paraformaldehyde (PFA) buffered in 0.1 M dibasic sodium phosphate (pH 7.4), and embedded in OCT-compound. Cryosections were processed for *in situ* hybridization as described previously (Fischer et al. 2002a; Fischer et al. 2004a). In short, slides were warmed to room temperature, sections ringed using rubber cement, and endogenous phosphatases inactivated by washing in 0.2 M HCl for 15 min. Sections were then treated with 0.5 ug/ml of Proteinase K for 10 min at 37°C, washed in PBS in DEPC-treated H₂O, and fixed in 4% PFA for 15 min. Sections were soaked in 0.25% acetic acid for 15 min, washed in DEPC-treated H₂O, and then incubated at 60°C with a hybridization solution (200–500ng of riboprobe, 50% formamide, 4X SSC, 10X Denhardt's solution, 500 µg of salmon sperm DNA and torula RNA). Post-hybridization treatment

included washes in standard sodium citrate (SSC) at 60°C followed by RNase A digestion for 20 minutes at 37°C. Sections were washed at room temperature in a MABT solution (0.05M maleic acid buffer, 0.1% Tween-20) and incubated overnight at 4°C with Fab fragments raised to dioxygenin (DIG) that were conjugated to alkaline phosphatase (anti-DIG-AP; Roche) plus 10% normal goat serum, 10 mM levamisole, and 10 mM glycine in MABT. NBT/BCIP (BioChemika) in 0.1 M NaCl, 0.1 M tris-HCl pH 9.5, 0.05 M MgCl₂ and 0.01% Tween-20 was used to precipitate chromophore from the anti-DIG-AP.

Measurements of eye size and intraocular pressure

Similar to previous reports (Fischer et al. 2008; Ritchey et al. 2012), we obtained measurements of corneal circumference, corneal arc, axial length and equatorial circumference. Digital photographs of enucleated eyes were taken using a 6.1 megapixel Nikon D100 SLR camera. Images of enucleated eyes were measured by using Image Pro Plus 6.2 (Mediacybernetics). Measurements obtained using Image Pro Plus 6.2 were highly reproducible and had very low levels of sampling error ($\pm 0.22\%$) at a resolution of 50 pixels/mm or greater. Eye size can vary greatly between individual chicks, similar to overall body size, even for animals that hatch at the same time. Thus, to account for inter-individual variability, the data is presented as treated minus control.

ATonoLabtm tonometer was used to measure IOP. The device was used with the factory-set calibration for rat eyes, similar to our previous report (Ritchey et al. 2012). *In vivo* measurements of IOP were made daily through the course of each experiment and at the termination of each experiment. Measurements of IOP were made consistently at the same time of day.

Photography, measurements, cell counts and statistics

Photomicrographs were obtained using a Leica DM5000B microscope equipped with epifluorescence and Leica DC500 digital camera. Confocal images were obtained using a Zeiss LSM 510 imaging system at the Hunt-Curtis Imaging Facility at the Ohio State University. Images were optimized for color, brightness and contrast, multiple channels overlaid and figures constructed by using Adobe Photoshop. Cell counts were performed on representative images. To avoid the possibility of region-specific differences within the retina, cell counts were consistently made from the same region of retina for each data set. Central retina was defined as the region within a 3mm radius of the posterior pole of the eye, and peripheral retina was defined as an annular region between 3mm and 0.5mm from the CMZ. Cell counts for proliferation were taken from the peripheral retina.

The identity of BrdU-labeled cells was determined based on previous findings that 100% of the proliferating cells in the chick retina are comprised of Sox2/9⁺ Müller glia in the INL/ONL, Sox2/9/Nkx2.2⁺ NIRC cells in the IPL, GCL and NFL (the NIRC cells do not migrate distally into the retina), and CD45⁺ (Sox2/9⁻) microglia (Fischer et al. 2010; Zelinka et al. 2012). Non-astrocytic Inner Retinal Glial (NIRC) cells are a distinct type of glial cell found in the inner layers of chick retinas (Fischer et al. 2010) and possibly the retinas of reptiles (Todd et al. 2015). Sox2⁺ nuclei in the INL were identified as Müller glia based on their large size and fusiform shape which are distinctly different from the Sox2⁺ nuclei of

cholinergic amacrine cells which are small and round (Fischer et al. 2010). In whole-mount preparation of the mouse retina, the BrdU⁺/Sox2⁺ nuclei of astrocytes were distinguished from the BrdU⁺/Sox2⁺ nuclei of Müller glia based on the size, shape and location within the retina. Astrocyte nuclei were found in the nerve fiber layer, IPL, and GCL, and their nuclei were relatively large, ovoid and widely spaced. Proliferating, Müller glia nuclei were observed in the INL and ONL, and were relatively narrow, fusiform, and narrowly spaced. Confocal images across retinal layers were obtained to distinguish between the nuclei of astrocytes and the nuclei of Müller glia.

Where significance of difference was determined between two treatment groups accounting for inter-individual variability (means of treated-control values) we performed a two-tailed, paired t-test. Where significance of difference was determined between two treatment groups we performed a two-tailed, unpaired t-test.

Results

Levels of *hb-egf* and *egf-receptor* in NMDA-damaged chick retinas

We used qRT-PCR to quantify mRNA levels of *hb-egf* and *egf-receptor* in retinas damaged by NMDA, where proliferating MGPCs are known to form (Fischer and Reh 2001). We found that levels of *hb-egf* were increased more than 7-fold within 4 hrs of NMDA-treatment, and levels of *hb-egf* gradually decreased over the next 3 days (Fig. 1a). However, levels of *hb-egf* remained elevated at nearly 1-fold above levels of control retinas up to 3 days after damage (Fig. 1a). This sustained up-regulation of *hb-egf* could influence the formation of MGPCs that is seen after NMDA-induced retinal damage. Similarly, levels of *egf-receptor* were increased nearly 8-fold within 4 hrs of NMDA-treatment (Fig. 1b). However, levels of *egf-receptor* rapidly returned to control levels at 1 and 2 days after NMDA-treatment, and were modestly increased at 3 days after treatment (Fig. 1b). To identify the cells that up-regulated *hb-egf*, we performed *in situ* hybridization (ISH). In control retinas, we failed to detect ISH-signal for *hb-egf* (Fig. 1c), where there were few PCNA-positive cells (Fig. 1e). By comparison, we detected ISH-signal scattered across the INL at 3 days after NMDA-treatment (Fig. 1d), where many PCNA-positive cells were present in the INL (Fig. 1f). Many of the *hb-egf*-positive cells were likely to be Müller glia with vertically oriented processes spanning the INL (Fig. 1d), and nuclei labeled for PCNA (Figs. 1g–i). The PCNA-positive nuclei in the INL (Müller glia) appeared to overlap with the ISH-signal for *hb-egf*, but signal also appeared in PCNA-negative cells (presumptive amacrine cells) in the inner half of the INL (Figs. 1g–i). By comparison, the PCNA-positive cells in the IPL (presumptive NIRC cells) appeared to be negative for *hb-egf* (Figs. 1g–i).

HB-EGF stimulates Müller glia proliferation in NMDA-damaged chick retinas

HB-EGF is known to activate cell-signaling in Müller glia through the MAPK-pathway (Wan et al. 2012). However, intraocular injections of HB-EGF failed to stimulate cell-signaling; we did not detect increases in readouts of MAPK-signaling including pERK, p38 MAPK, pCREB, cFos and Egr1 (not shown). Consistent with these findings, 4 consecutive daily intraocular injections of HB-EGF failed to stimulate the formation of MGPCs (not shown). Since NMDA-treatment induced the expression of *egf-receptor*, we tested whether

HB-EGF stimulated the formation of MGPCs following a diminished level of damage. After intraocular application of a relatively low dose of NMDA, we found that HB-EGF more than doubled the number of proliferating BrdU+/Sox9+ MGPCs (Figs. 2a–e). By comparison, HB-EGF had no significant effect upon the proliferation of NRG cells (4.1 ± 1.4 vs 4.8 ± 2.1 BrdU+ NRG cells; $n = 5$; NMDA vs NMDA+HB-EGF). Levels of cell death are known to influence the formation of MGPCs (Fischer and Reh 2001). Thus, we used the TUNEL assay to examine whether HB-EGF influenced numbers of dying cells. We found no difference in numbers of dying cells in retinas treated with NMDA+HB-EGF compared to numbers seen in retinas treated with NMDA+vehicle (Fig. 2f). We failed to detect a significant increase in readouts of cell-signaling in retinas treated with NMDA and HB-EGF (not shown). Different branches of the MAPK-signaling pathway are highly active in NMDA-treated Müller glia (Fischer et al. 2009b), and our methods of detection may not have been sufficiently sensitive to detect further increases.

NMDA-induced retinal damage resulted in the up-regulation of the *egfr* in addition to *hb-egf*. Therefore, we tested whether inhibition of EGFR reduced the number of proliferating MGPCs. To test this we injected a relatively large dose (500 nmol) of NMDA, followed by two doses of an EGFR inhibitor. We found a decrease in the number of Sox9+/BrdU+ proliferating MGPCs in retinas treated with NMDA+EGFRi compared to numbers of proliferating MGPCs seen in retinas treated with NMDA+vehicle (Figs. 2g–k). The effects of EGFR-inhibition were not due to cell death; there was no significant difference in numbers of TUNEL-positive cells between treated and control retinas (Fig. 2l).

HB-EGF fails to synergize with FGF2 to stimulate the formation of MGPCs in the chick retina

In the zebrafish model system, HB-EGF is sufficient to stimulate the formation of proliferating MGPCs in the absence of retinal damage (Wan et al. 2012). We have found that 4 consecutive daily doses of FGF2 are sufficient to stimulate the formation of proliferating MGPCs in the absence of retinal damage (Fischer et al. 2014b), and that activation of Hedgehog-signaling potentiates FGF2-mediated formation of MGPCs (Todd and Fischer 2015). Thus, we investigated whether HB-EGF influenced the formation of MGPCs in the absence of retinal damage in the chick retina. We applied 3 consecutive daily injections of FGF2±HB-EGF and tested whether the formation of MGPCs was potentiated. There were relatively few proliferating MGPCs in retinas treated with 3 doses of FGF2, and HB-EGF did not further stimulate the formation of MGPCs (Figs. 3a, b). By comparison, there were many proliferating MGPCs in retinas treated with 4 doses of FGF2, and inhibition of EGFR failed to influence the formation of MGPCs (Fig. 3c). HB-EGF and EGFR-inhibitor likely had no effect upon the formation of MGPCs because FGF2-treatment does not up-regulate the *egf-receptor* (Fig. 3d).

The combination of IGF1 and HB-EGF in the avian retina

We have reported previously that the combination of IGF1 and FGF2 stimulates ocular enlargement, angle closure associated with transient increases in intraocular pressure (IOP), damage to inner retinal neurons, and the proliferation of MGPCs (Fischer et al. 2002b; Ritchey et al. 2012). Thus, we tested whether the combination of IGF1 and HB-EGF

influenced ocular growth and the formation of MGPCs. We found that 5 consecutive daily intraocular injections of IGF1+HB-EGF resulted in significantly larger eyes with increases in corneal circumference, corneal arc, axial length and equatorial circumference (Figs. 4a–c). Unlike the combination of IGF1 and FGF2, we found no increases in IOP, no evidence of angle closure, no evidence of proliferating MGPCs, and no evidence of cell death during or after treatment with IGF1 and HB-EGF (not shown). Compared to treatment with IGF1 alone, the combination of IGF1 and HB-EGF resulted in no further increase in microglial or Müller glial reactivity. Levels of CD45 and GFAP, respectively, did not appear increased in retinas treated with IGF1+HB-EGF compared to levels seen in retinas treated with IGF1 alone (Fig. 4d).

The combination of insulin and EGF is known to stimulate the proliferation of cells in the CMZ and the non-pigmented epithelium (NPE) of the ciliary body (Fischer and Reh 2003). Accordingly, we tested whether the combination of IGF1 and HB-EGF stimulated the formation of MGPCs or the proliferation of cells in the CMZ and NPE of the ciliary body. We found that the combination of IGF1 and HB-EGF failed to stimulate the formation of proliferating MGPCs (not shown). The combination of HB-EGF with IGF1 had no additive effect upon MAPK-signaling; we observed no significant changes in retinal levels of pERK, pCREB or cFos in retinas treated with HB-EGF and IGF1 compared to those treated with IGF1 alone (not shown). Furthermore, the application HB-EGF and IGF1 had no additive effect upon dying cells (TUNEL-positive cells), intraocular pressure (not shown), or the proliferation progenitors in the CMZ (Fig. 5a, b). HB-EGF alone did not stimulate the proliferation of progenitors in the CMZ (Fig. 5a, b); in contrast to the effects of EGF alone which is known to stimulate the proliferation of CMZ progenitors (Fischer and Reh 2000). Compared to numbers of proliferating NPE cells in eyes treated with IGF1 or HB-EGF alone, we found that the combination of HB-EGF and IGF1 significantly increased proliferation (Figs. 5a, b). The proliferation of cells in the NPE treated with IGF1 and HB-EGF was large enough to result in an obvious increase in the thickness of the NPE (Fig. 5a).

HB-EGF and proliferation in the mouse retina

EGF has been shown to stimulate the proliferation of Müller glia in damaged rodent retinas *in vivo and in vitro* (Close et al. 2006; Karl et al. 2008; Ueki and Reh 2013). Thus, we tested whether HB-EGF stimulated the proliferation of Müller glia in damaged mouse retinas. We found that intraocular injections of NMDA followed by four consecutive daily doses of HB-EGF significantly increased numbers of BrdU-labeled Sox2⁺ nuclei (Müller glia) in the INL (Fig. 6a, b). By comparison, HB-EGF did not significantly increase numbers of proliferating astrocytes (Sox2⁺/BrdU⁺) in the NFL/IPL (Fig. 6a, c).

We next tested whether HB-EGF influenced cell-signaling in Müller glia in the mouse retina. In normal retinas HB-EGF had no effect upon Müller glia (not shown). In NMDA-damaged retinas, low levels of pERK were observed in glutamine synthetase (GS) -positive Müller glia (Fig. 7a). By comparison, a single injection of HB-EGF into NMDA-treated eyes resulted in a significant induction of pERK in the Müller glia (Fig. 7a). pS6, a readout of mTor-signaling, was apparent in presumptive amacrine cells, but not in GS-positive Müller glia in damaged retinas (Fig. 7b). By comparison, pS6 was up-regulated in Müller

glia, but was no longer apparent in amacrine cells in retinas treated with NMDA and HB-EGF (Fig. 7b). In NMDA-damaged retinas, pCREB was apparent in the nuclei of GS-positive Müller glia and numerous neurons in the INL (Fig. 7c). In retinas treated with NMDA and HB-EGF, pCREB was present in Müller glia, but seemingly reduced in neurons in the INL (Fig. 7c). In NMDA-damaged retinas, pStat3 was observed in the nuclei of GS-positive Müller glia in the INL (Fig. 7d). By comparison, pStat3 appeared up-regulated in Müller glia treated with NMDA and HB-EGF (Fig. 7d). p38 MAPK was prominent in GS-positive Müller glia in NMDA-damaged retinas, and levels of p38 MAPK were not influenced by HB-EGF (Fig. 7e).

Discussion

We find that HB-EGF can stimulate the proliferation of MGPCs, but only in damaged retinas. We propose that the responses of Müller glia to HB-EGF are limited by levels of expression of EGF-receptor. In normal, undamaged retinas, HB-EGF had no apparent effect upon the Müller glia in chick or mouse retinas. Our evidence suggests that retinal damage increases levels of EGF-receptor. Similarly, in the rat retina, Müller glia are unresponsive to exogenous EGF (Close et al. 2006). However, following light-induced damage EGF-receptor is up-regulated in Müller glia and this renders these cells responsive to EGF (Close et al. 2006).

There is a growing volume of evidence to indicate that a network of cell-signaling participates in the formation of proliferating MGPCs. This network of signaling pathways includes MAPK/ERK (Fischer et al. 2009a; Fischer et al. 2009b; Wan et al. 2012), Notch (Conner et al. 2014; Ghai et al. 2010; Hayes et al. 2007), Jak/Stat (Kassen et al. 2009; Wan et al. 2014; Zhao et al. 2014), glucocorticoid (Gallina et al. 2014c), Wnt (Meyers et al. 2012; Osakada et al. 2007; Ramachandran et al. 2011), Hedgehog (Todd and Fischer 2015), and PI3K (Wan et al. 2014). Recently, Stat3 and β -catenin have been shown to be key bottlenecks to the signaling cascades activated in the formation of MGPCs in the zebrafish (Wan et al. 2014).

The ability of HB-EGF to stimulate MGPC formation in the zebrafish is controversial (Nelson et al. 2013; Wan et al. 2012). Our data suggests that HB-EGF can stimulate MGPC formation in chicken and mouse models, but only in damaged retinas. Our data indicate that HB-EGF and EGFR-signaling is not involved in the formation of MGPCs in undamaged FGF-treated chick retinas. This is distinctly different from the effects of HB-EGF in undamaged zebrafish retinas, where HB-EGF alone is sufficient to stimulate the formation of proliferating MGPCs (Wan et al., 2012). In the mouse retina, HB-EGF seems to predominantly activate MAPK signaling via activation of ERK. In addition, we find that application of HB-EGF to damaged retinas activates effectors of Jak/Stat- and mTor-signaling. This suggests that, similar to the effects observed in the zebrafish retina, HB-EGF activates a network of signaling pathways that ultimately leads to MGPC formation in the avian and mouse retina. In the human retina, HB-EGF expression was detected in patients with proliferative vitreoretinopathy but not in healthy controls (Hollborn et al. 2005). HB-EGF also stimulated proliferation of the human Müller cell line MIO-M1 and this effect was dependent on MAPK signaling (Hollborn et al. 2005). Collectively, these studies support the

notion that HB-EGF is a mitogen for retinal glia and can promote the transition of Müller glia into proliferating MGPCs.

Although HB-EGF is able to stimulate proliferation in the damaged retina, we saw no effect of exogenous HB-EGF in FGF-mediated formation of MGPCs. HB-EGF is known to predominantly activate EGFR-MAPK-signaling (Lemmon and Schlessinger 2010), (Schneider and Wolf 2009). Since FGF2 potently activates MAPK-signaling in Müller glia (Fischer et al. 2009a), HB-EGF may not further activate these pathways that are already saturated by FGF2. Additionally, we failed to detect an increase in expression of the EGF-receptor in the FGF2-treated retina. This most likely explains why the Müller glia were unresponsive to HB-EGF when combined with FGF2. In damaged retinas, we failed to detect significant increases in cell-signaling in NMDA-damaged chick retinas treated with HB-EGF, whereas different signaling pathways were dramatically up-regulated in Müller glia in NMDA-damaged mouse retinas treated with HB-EGF. Previous studies have shown large and selective activation of cell-signaling in Müller glia in NMDA-damaged chick retina (Fischer et al. 2009b; Gallina et al. 2014b; Ghai et al. 2010). By comparison, Jak-Stat, mTor and MAPK-signaling pathways are modestly elevated in NMDA-damaged rodent retinas. This may represent a fundamental difference in the response of Müller glia to excitotoxic damage in chick and mouse retinas.

Rates of ocular growth can be influenced by exogenous growth factors. For example, application of FGF2 inhibits the excessive ocular growth that results from form-deprivation, and TGF β 1 overrides the growth-slowing effects of FGF2 (Rohrer and Stell 1994). Intraocular injections of insulin, in opposition to the growth-slowing effects of glucagon, have been shown to exacerbate the excessive ocular growth that results from form-deprivation and lens-induced myopia (Feldkaemper et al. 2009; Zhu and Wallman 2009). We found that the combination of IGF1 and FGF2, in the absence of vision-restricting manipulations, induced excessive ocular growth (Ritchey et al. 2012). However, it remains unclear whether the excessive ocular growth resulting from IGF1+FGF2 was secondary to angle closure and increased IOP, damage to retinal neurons that slow rates of ocular growth, direct activation of scleral growth, or a combination of these possibilities (Ritchey et al. 2012). Few factors that leave the retina undamaged have been shown to influence rates of ocular growth in eyes with unrestricted vision. For example, in eyes with unrestricted vision and no retinal damage, glucagon peptide has been shown to slow equatorial, but not axial, ocular growth and a small molecule inhibitor of the glucagon receptor increases both axial and equatorial ocular growth (Fischer et al. 2008). By comparison, our current findings have shown that the combination of IGF1 and HB-EGF stimulates excessive ocular growth in eyes with unrestricted vision without damaging the retina or closing the angle and increasing IOP. Ocular treatment with IGF1 and HB-EGF may serve as a model system by which to better understand the factors that regulate rates of ocular growth and the pathogenesis of myopia. Further studies are required to identify the sites of action and cell-signaling pathways activated by IGF1 and HB-EGF that accelerate rate of ocular growth.

Many different factors have been shown to regulate the addition of new cells to the peripheral edge of the retina (reviewed by Fischer et al. 2014a). The CMZ represents a zone of neural progenitors that continuously adds cells to the retinal margin throughout the life of

the zebrafish (Hitchcock and Raymond 2004). The chicken maintains a zone of proliferating cells at the retinal margin after hatching, although these cells have a restricted potency (Fischer and Reh 2000). The proliferation of the chicken CMZ can be elicited by growth factors such as Insulin, IGF, FGF, Shh, EGF (Fischer and Reh 2003; Moshiri et al. 2005; Todd and Fischer 2015). We report here that HB-EGF alone, or in combination with IGF1, fails to stimulate the proliferation of progenitors in the CMZ. However, the combination of HB-EGF and IGF acts synergistically to stimulate the proliferation of cells in the NPE. This is reminiscent of previous findings where the combination of insulin and EGF stimulated proliferation cells and resulted in a significant thickening of the NPE of the pars plana directly anterior to the CMZ (Fischer and Reh 2003).

Conclusions

We conclude that HB-EGF and EGF-receptor can influence proliferation of Müller glia/MGPCs in damaged retinas of birds and rodents. In untreated retinas, levels of EGF-receptor may be too low for HB-EGF to activate cell-signaling in Müller glia. In retinas treated with NMDA, levels of *egf-receptor* are increased and, thereafter, exogenous HB-EGF or EGFR-inhibitor are capable of influencing the proliferation of Müller glia. By comparison, in the absence of damage where Müller glia have been primed by FGF2 to become MGPCs, HB-EGF has no impact upon proliferation, likely because levels of *egf-receptor* is not increased and/or signaling pathways are already saturated. In conclusion, HB-EGF mediated signaling substantially impacts MGPC formation in the damaged avian and mammalian retina.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- In contrast to the zebrafish, Muller glia in the chick and mouse retina do not reprogram in response to HB-EGF stimulation.
- HB-EGF signaling promotes the proliferation of Muller glia in both avian and murine NMDA-damaged retina.
- In mouse, HB-EGF stimulated not only MAPK-signaling in Müller glia, but also activated mTor- and Jak/Stat-signaling.

Figure 1.

Levels of *hb-egf* and *egf-receptor* are rapidly up-regulated in the NMDA-damaged chick retinas. **a, b**; cDNA was generated from retinas (n = 4) that were treated with saline (control) or NMDA (treated). Retinas were harvested at 4hrs, 1 day, 2 days or 3 days after treatment. qRT-PCR was used to measure relative levels of expression. Significance of difference (* $p < 0.05$; ** $p < 0.01$) was determined by using a Mann-Whitney U test. **c-i**; *In situ* hybridization (ISH) was performed on control (**c, e**) and NMDA-treated (**d, f-i**) retinas at 3 days after treatment. This time-point was selected because levels of *hb-egf* remained significantly elevated and proliferating MGPCs are expected to be positive for PCNA to permit double-labeling. Immunofluorescence labeling for PCNA (red; **e, f, h**) was performed after the ISH-procedure. Arrows indicate the nuclei of MGPCs, and small double-arrows indicate the nuclei of presumptive NIRC cells. The scale bar (50 μ m) in panel **f** applies to **c-f**. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer.

Figure 2.

HB-EGF and EGF-receptor inhibitor (EGFRi) influence the proliferation of MGPCs in NMDA-damaged chick retinas. The injection paradigms are schematized above each data-set. **a–f**; Eyes were injected with 60 nmol NMDA at P6, HB-EGF at 4 and 24 hrs later, BrdU at P8, and retinas harvested at P9. Sections of the retina were immunolabeled for BrdU (green) and Sox9 (red; **a–d** and **g–j**). Arrows indicate the nuclei of BrdU/Sox9-positive MGPCs in the INL or ONL. The histograms in **e** and **k** illustrate the mean ($\pm$ SD; $n=8$) number of proliferating MGPCs in control and HB-EGF-treated retinas. The histograms in **f** and **l** represent the mean ($\pm$ SD; $n=5$) number of TUNEL⁺ cells. Significance of difference (* $p<0.05$; ** $p<0.01$) was determined by using an unpaired, two-tailed t-test. The scale bar (50 μ m) in panel applies to **a–d** and **f–i**. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer.

Figure 3.

HB-EGF and EGF-receptor inhibitor (EGFRi) have no influence upon the proliferation of MGPCs in FGF2-treated chick retinas in the absence of damage. The injection paradigms are outlined above each data-set. **(a, b)** Eyes were injected with FGF2 $\pm$ HB-EGF at P7–P9, BrdU at P10, and retinas harvested at P11. **(c)** Eyes were injected with BrdU + FGF2 $\pm$ EGFRi at P7–P10, and retinas harvested at P11. **(d)** Eyes were injected with saline + BrdU $\pm$ FGF2 at P7–P10, and retinas harvested at P11. Sections of the retina were immunolabeled for BrdU (green) and Sox9 (red) **(a)**. Arrows indicate the nuclei of BrdU/Sox9-positive MGPCs in the INL or ONL. The histograms in **b** and **c** illustrate the mean ($\pm$ SD; $n=8$) number of proliferating MGPCs in control and HB-EGF or EGFRi-treated retinas. Significance of difference was determined by using an unpaired, two-tailed t-test. **(d)** cDNA was generated from retinas ($n=4$) that were treated with saline (control) or FGF for three consecutive days (treated). Retinas were harvested at 1 day after the last injection of saline $\pm$ FGF2. qRT-PCR was used to measure relative levels of expression of *egf-receptor*. Significance of difference ($*p<0.05$) was determined by using a Mann-Whitney U test.

Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer.

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Figure 4.

The combination of HB-EGF and IGF1 in the chick retina, stimulates ocular growth and nuclear migration of Müller glia, but not reactive gliosis. Eyes received 5 consecutive daily injections of IGF1 ± HB-EGF and retinas were harvested 48 hours after the last injection. Measurements were made by using ImagePro6.2 on digital images of enucleated eyes; measurements included corneal circumference, corneal arc, axial length and equatorial circumference (**a**, **b**). Histograms represent the mean (n=8) and standard deviation for data-sets (**c**). Significance of difference was determined by using an unpaired, two-tailed t-test. Retinal sections were labeled with antibodies to Sox9 (red) or CD45 (red) and GFAP (green) (**d**). The scale bar (5mm) in panel **a** applies to **a** and **b**, and the bar (50 μm) in **d** applies to **d** alone. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer.

Figure 5.

The IGF1 and HB-EGF stimulate the proliferation of CMZ progenitors and cells in the NPE of the ciliary body in the chick. Eyes received 5 consecutive daily injections of saline+BrdU (vehicle), IGF1+BrdU, HB-EGF+BrdU, or IGF1+HB-EGF+BrdU. An additional injection of BrdU was applied 1 day after the last injection of factors and retinas were harvested 1 day later. (a) Sections were labeled with antibodies to BrdU (green) and Sox2 (red). The histograms in **b** illustrate the mean ($\pm$ SD; $n = 4$) number of BrdU cells in the retina+CMZ or NPE of ciliary body directly anterior to the retina. Significance of difference ($p < 0.001$) among the treatment groups was determined using one-way ANOVA. Significance of difference ($*P < 0.05$) between treatment groups was determined using a two-tailed t -test with Bonferroni's correction. The scale bar in **a** represents 50 μ m. Abbreviations: CMZ – circumferential marginal zone, NPE – non-pigmented epithelium.

Figure 6.

HB-EGF stimulates the formation of proliferating MGPCs in NMDA-damaged rodent retina. The injection paradigm is outlined at the top of the figure. NMDA was injected into eyes at P33 followed by four daily consecutive injections of HB-EGF or saline, and eyes were harvested one day later. Intraocular and intraperitoneal injections of BrdU were applied with each dose of HB-EGF. Whole-mounts of the retina were labeled with antibodies to BrdU (green) and Sox2 (red). Confocal microscopy was used to obtain Z-series stacks, and 5–9 optical sections were projected to obtain composite images through the depth of the INL or through the NFL to IPL. Arrows indicate the nuclei of BrdU/Sox2-positive astrocytes in the NFL-IPL and MGPCs in the INL. The histograms in **b** and **c** illustrate the mean ($\pm$ SD; $n=4$) number of proliferating MGPCs in control and HB-EGF treated retinas. Significance of difference ($*p<0.05$) was determined by using an unpaired, two-tailed t-test. The scale bar (50 μ m) in the bottom right applies to all panels. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, NFL – nerve fiber layer.

Figure 7.

HB-EGF activates different signaling pathways in Müller glia in damaged rodent retinas. NMDA-treated eyes were injected with saline or HB-EGF and retinas were harvested 24 hrs after injection. Sections of the retina were labeled with antibodies to GS (red) to label the cytoplasm of Müller glia and pERK, pS6, pCREB, pStat3 or p38MAPK (green). Arrows indicate the nuclei or cell bodies of Müller glia, and hollow arrow-heads indicate retinal neurons. The lower panels in **d** represents the orthogonal projection to show co-labeling in the z-plane. The scale bar (50 μ m) in **c** applies to **b** and **c**, and the bar in **e** applies to **a**, **d** and

e. Abbreviations: ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer.

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Table 1

Antibodies, working dilutions, host and source.

Antigen	Working dilution	Host	Clone or catalog number	Source
BrdU	1:200	rat	OBT00030S	AbD Serotec
BrdU	1:100	mouse	G3G4	Developmental Studies Hybridoma Bank (DSHB)
CD45	1:200	mouse	HIS-C7	Cedi Diagnostic
cFos	1:400	rabbit	K-25	Santa Cruz Immunochemicals
Egr1	1:1000	goat	AF2818	R&D Systems
GFAP	1:2000	rabbit	N1506	Dako
GS	1:2000	mouse	Ab125724	Abcam
Nkx2.2	1:50	mouse	74.5A5	Developmental Studies Hybridoma Bank (DSHB)
p38 MAPK	1:400	rabbit	12F8	Cell Signaling Technologies
PCNA	1:1000	mouse	M0879	Dako
pCREB	1:500	rabbit	87G3	Cell Signaling Technologies
pERK1/2	1:200	rabbit	137F5	Cell Signaling Technologies
pS6	1:750	rabbit	2211	Cell Signaling Technologies
pSTAT3	1:300	rabbit	9131	Cell Signaling Technologies
Sox2	1:1000	goat	Y-17	Santa Cruz Immunochemicals
Sox9	1:2000	mouse	AB5535	Chemicon