Research article

# Reactive gliosis in the adult zebrafish retina

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ABSTRACT

In contrast to mammals, zebrafish posses the remarkable ability to regenerate retinal neurons. Damage to the zebrafish retina induces Müller glia to act as stem cells, generating retinal progenitors for regeneration. In contrast, injury in the mammalian retina results in Müller glial reactive gliosis, a characteristic gliotic response that is normally detrimental to vision. Understanding the signaling pathways that determine how Müller glia respond to injury is a critical step toward promoting regeneration in the mammalian retina. Here we report that zebrafish Müller glia exhibit signs of reactive gliosis even under normal regenerative conditions and that cell cycle inhibition increases this response. Persistently reactive Müller glia increase their neuroprotective functions, temporarily saving photoreceptors from a cytotoxic light lesion. However, the absence of a sustained proliferation response results in a significant inhibition of retinal regeneration. Interestingly, when cell cycle inhibition is released, a partial recovery of regeneration is observed. Together, these data demonstrate that zebrafish Müller glia possess both gliotic and regenerative potential.

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## 1. Introduction

Müller glial cells are the principal glial cell of the retina, and under normal conditions provide a variety of critical roles in retinal neuron support and maintenance. In addition to providing structural support for the retina lamina and vasculature (Bringmann et al., 2006; Hollander et al., 1991; Newman and Reichenbach, 1996), Müller glia maintain retinal neurons by regulating retinal glucose metabolism (Poitry-Yamate et al., 1995; Tsacopoulos and Magistretti, 1996), and recycling the neurotransmitter Glutamate (Masai et al., 2005). In addition, Müller glia regulate retinal blood flow and water homeostasis (Bringmann et al., 2004; Newman and Reichenbach, 1996; Newman, 1993; Paulson and Newman, 1987).

Upon damage to the mammalian retina, Müller glial cells respond with reactive gliosis. Cell hypertrophy and upregulation of GFAP (Glial Fibrillary Acidic Protein) following retinal damage serves as the earliest and most-widely accepted indicators of retinal stress and Müller glial cell reactivity (Bignami and Dahl, 1979; Grosche et al., 1995; Lewis and Fisher, 2003; Xue et al., 2010). Reactive Müller glia also upregulate and release neurotrophic factors, such as FGF-2, and phagocytose of blood-derived proteins (Bringmann and Wiedemann, 2012). Although reactive gliosis is initially neuroprotective, slowing neuronal cell death, persistent gliosis results in loss of normal Müller glial cell function and subsequent loss of retinal neurons (Bringmann et al., 2006; Ganesh and Chintala, 2011).

Persistent reactive gliosis has two forms, non-proliferative and proliferative. Non-proliferative reactive gliosis is associated with mild retinal damage and is characterized by persistent Müller glial cell hypertrophy, upregulation of GFAP, and down-regulation of Glutamine Synthetase, which is necessary for glutamate recycling (Bringmann and Wiedemann, 2012). Down-regulation of Glutamine Synthetase leads to retinal neuron excitotoxicity, ultimately resulting in neuronal degeneration in diseases such as glaucoma or following a minor retinal detachment (Bringmann and Wiedemann, 2012; Inman and Horner, 2007; Lewis et al., 1989). In the case of severe damage, such as ischemia-reperfusion, light injury, severe retinal detachment, proliferative retinopathy, and age-dependant retinopathies, Müller glial cells undergo proliferative gliosis (Bringmann and Wiedemann, 2012; McGillem and Dacheux, 1999; Sethi et al., 2005; Ulbricht et al., 2008). In addition to losing normal homeostatic function, proliferative reactive

gliosis is characterized by unregulated Müller glial cell proliferation, which appears to be regulated by inactivation of the cyclin-dependent kinase inhibitor *Cdkn1b* (Dyer and Cepko, 2000; Vazquez-Chona et al., 2011). Within the context of the inflammation associated with retinal damage, this proliferative response can lead to mislocalized retinal neurons or progenitor cells that contribute to the formation of a glial scar, which severely compromises normal vision (Bringmann and Wiedemann, 2009; Lewis et al., 2010).

In contrast to the mammalian response, adult zebrafish possess the ability to regenerate their neural retina in response to a variety of damage paradigms (Bernardos and Raymond, 2006; Fausett et al., 2008; Fimbel et al., 2007; Vihtelic and Hyde, 2000). Regardless of the method used to damage the zebrafish retina, the mechanism of regeneration remains the same. Following damage, a subset of Müller glial cells re-enter the cell cycle in a controlled proliferative response. Each participating Müller cell creates a small cluster of retinal progenitors, which migrate to the area of damage and differentiate into the appropriate retinal cell type (Bernardos and Raymond, 2006; Fausett et al., 2008; Fimbel et al., 2007; Thummel et al., 2008b). While multiple studies have revealed signaling pathways that are required for Müller glial cell proliferation and progenitor amplification in zebrafish, it is currently unclear what triggers a Müller cell act as stem cells or remain persistently reactive following damage.

Using photolesioned or Ouabain-damaged zebrafish retinas, here we show that Müller glial cells exhibit signs of reactive gliosis prior to acting as stem cells and that the gliotic response is localized to the area of damage. Next we tested whether inhibiting Müller glial cell proliferation would result in persistent reactive gliosis. We used two distinct methods to inhibit cell cycle re-entry: 1) 5-Fluorouracil (5-FU), a pharmacological compound previously shown to inhibit Müller glial cell proliferation *in vivo* (Negishi, 1994), and 2) the *in vivo* electroporation of anti-sense *pcna* morpholinos, which was previously shown to inhibit Müller glial cell proliferation during adult zebrafish regeneration (Thummel et al., 2008b). We found that inhibition of proliferation using 5-FU or PCNA knockdown resulted in a persistent reactive gliosis response which included persistent cell hypertrophy, an upregulation of GFAP, increased *fgf2* expression, and increased retinal cell neuroprotection. These data characterize reactive gliosis in adult zebrafish and provide evidence that zebrafish Müller glia have the capacity to be both persistently reactive and a source of retinal progenitors.

## 2. Materials and methods

### 2.1. Fish maintenance

Adult *albino*, Tg(*gfap:egfp*)/*alb* and Tg(*nrd:egfp*)/*alb* zebrafish were used for this study. Fish were maintained under a daily light cycle of 14 h light (250 lux):10 h dark at 28.5 °C (Westerfield, 1995) and fed a combination of flake food and brine shrimp. All protocols used in this study were approved by the Institutional Animal Care and Use Committee at Wayne State University School of Medicine and are in compliance with the ARVO statement for the use of animals in vision research.

### 2.2. Light treatment protocol

Extensive rod and cone photoreceptor loss was achieved using a previously-described photolesion protocol (Thomas et al., 2012). Fish were dark-adapted for 10 days followed by 30 min of UV light, and then transferred to a clear 1.8 L tank positioned between 4 halogen lamps (250 W each). Fish were subjected to constant light

exposure for up to four days, and then transferred back to standard light:dark conditions. During the light treatment, water temperature remained between 30 and 33 °C.

### 2.3. Intravitreal injections

Intravitreal injections were performed as previously described (Qin et al., 2011). Injections of saline (sterile 1X PBS) or 5-fluorouracil (5-FU; 5 mg/mL; Sigma—Aldrich) began two days prior to light onset (hpL) and continued daily. Eyes collected at 18 and 24 hpL received 3 injections, 36 hLt received 4 injections, and all subsequent time points received 5 injections. Retinas specifically labeled in Fig. 8 received 12 injections. Fish were anesthetized and a small incision was made in the cornea using a Safety Sideport Straight Knife (15°; Beaver-Vistec International). A 33 gauge Hamilton Syringe was used to inject 0.5 μL of 5-FU or saline.

### 2.4. Immunohistochemistry and microscopy

Immunohistochemistry was performed as previously described (Thomas et al., 2012). Untreated control and light damaged fish were euthanized at 18, 24, 36 or 72 h or 7 or 11 days after light onset. Tissue was fixed in 9:1 ethanolic formaldehyde (100% ethanol: 37% formaldehyde) overnight at 4 °C. Eyes were cryopreserved, sectioned and immunohistochemistry was preformed as previously described. Primary antibodies used for this study included rabbit polyclonal anti-GFP antisera (1:1500; Abcam, Cambridge, MA), mouse monoclonal anti-PCNA antibody (1:1000; Sigma—Aldrich, St. Louis, MO), mouse monoclonal anti-Zpr-1 antibody (1:200; Zebrafish International Resource Center, Eugene, OR). Secondary antibodies used included AlexaFluor conjugated 488, 594 and 647 goat anti-primary (1:500, Life Technologies, Grand Island, NY). Nuclei were stained using TO-PRO-3 (TP3; 1:750; Life Technologies, Grand Island, NY). Coverslips were mounted using ProLong Gold (Molecular Probes, Eugene, OR) and confocal microscopy was performed with a Leica TCS SP2 or SP8 confocal microscope.

### 2.5. BrdU and EdU incorporation

For BrdU incorporation, adult zebrafish were placed in a 5 mM BrdU solution 1000 mL De-ionized water, 0.66 g NaCl, 0.1 g Neutral Regulator ((SeaChem Laboratories, Inc., Stone Mountain, GA), and 1.5 g BrdU (B5002-5G/Sigma—Aldrich Co.)) for 24 h starting at 24 hpL. Following tissue processing, retinal sections were incubated in a Sodium Citrate Buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) at 100 °C for 30 min and then cooled to room temperature for 30 min. Retinal sections were washed in PBS +0.5% Triton-X 100 (PBS-Tx) and then blocked with 20% sheep serum in PBS-Tx for 1 h at room temperature. Rat anti-BrdU primary antibody (Clone: BU1/75, ICR1; Accurate Chemical and Scientific Corporation, Westbury, NY) was diluted (1:200) into 2% sheep serum in PBS-Tx and incubated on retinal sections overnight at 4 °C. AlexaFlour conjugated anti-rat antibodies were diluted (1:500) in 2% sheep serum in PBS-Tx incubated on retinal sections at room temperature for 1 h.

For EdU incorporation, 59-ethynyl-29-deoxyuridine (EdU; Invitrogen, Carlsbad, CA) was diluted in 1X PBS to 1 mg/mL and injected intraperitoneally (IP; 50 μL) into adult zebrafish as previously described (Bailey et al., 2010). Daily EdU injections began at 24 hpL and continued through 72 hpL (for 96 hpL time point). Immunolocalization of EdU was performed using Click-iT EdU AlexaFluor 594 Imaging Kit per the manufacturer's instructions (Invitrogen), followed by PCNA co-localization as described above.