Published in final edited form as:
Exp Eye Res. 2008 November ; 87(5): 433–444. doi:10.1016/j.exer.2008.07.009.

Characterization of Müller glia and neuronal progenitors during adult zebrafish retinal regeneration

Ryan Thummel[‡], **Sean C. Kassen**[‡], **Jennifer M. Enright**, **Craig M. Nelson**, **Jacob E. Montgomery**, and **David R. Hyde**[*]

Department of Biological Sciences and the Center for Zebrafish Research University of Notre Dame, Notre Dame, Indiana, 46556 USA

Abstract

The adult zebrafish retina exhibits a robust regenerative response following light-induced photoreceptor cell death. This response is initiated by the Müller glia proliferating in the inner nuclear layer (INL), which gives rise to neuronal progenitor cells that continue to divide and migrate to the outer nuclear layer (ONL), where they differentiate into rod and cone photoreceptors. We previously conducted a microarray analysis of retinal gene expression at 16, 31, 51, 68, and 96 hours of constant intense-light treatment to identify genes and their corresponding proteins that may be involved in the generation and proliferation of the neuronal progenitor cells. We examined the expression of two candidate transcription factors, Pax6 and Ngn1, and one candidate transgene, *olig2:EGFP*, in the regenerating light-damaged retina. We compared the temporal and spatial expression patterns of these markers relative to PCNA (proliferating cell nuclear antigen), an established marker for proliferating cells in the zebrafish retina, and the *Tg(gfap:EGFP)*^{nt11} transgenic line that specifically labels Müller glial cells. We found that Müller glial cells dedifferentiate during regeneration, based on the loss of cell-specific markers such as GFAP (glial fibrillary acidic protein) and glutamine synthetase following their reentry into the cell cycle to produce neuronal progenitors. Pax6 expression was first detected in the proliferating neuronal progenitors by 51 hours of constant light treatment, which is significantly after the Müller glia first reenter the cell cycle after 31 hours of light. This suggests that Pax6 expression increases in neuronal progenitors, rather than in the proliferating Müller glia. EGFP expression from the *olig2* promoter was first detected by 68 hours of constant light treatment in the dedifferentiated Müller glia, with Pax6 expressed in the closely-associated proliferating neuronal progenitors migrating to the ONL. Both Pax6 and *olig2* expression persisted until three days post light-treatment, when the neuronal progenitors begin differentiating into new rod and cone photoreceptors. Ngn1 protein expression was initially detected in proliferating neuronal progenitors at 68 hours of light treatment. However, Ngn1 expression persisted in a subset of the INL nuclei until 17 days post-light treatment. Using the *Tg(gfap:EGFP)*^{nt11} transgenic line, Ngn1 was localized to the Müller glial nuclei that were reestablished following the regenerative response. These markers, therefore, can be used to identify different cell types at particular stages of retinal regeneration: neuronal progenitor formation, proliferation, and the reestablishment of the Müller glia cells. These markers will be important to further characterize the regeneration response in other

*Correspondence sent to: David R. Hyde, Ph.D. Department of Biological Sciences Galvin Life Science Building University of Notre Dame Notre Dame, Indiana, 46556 Email: dhyde@nd.edu Phone: 574-631-8054 Fax 574-631-7413.
‡These authors contributed equally to the manuscript
Email addresses: Ryan Thummel rthumme1@nd.edu Sean C. Kassen skassen@nd.edu Jennifer M. Enright jenright@nd.edu Craig M. Nelson nelson.124@nd.edu Jacob E. Montgomery jmontgo2@nd.edu David R. Hyde dhyde@nd.edu

retinal damage models and to elucidate the defects associated with mutants and morphants that disrupt the regeneration response.

Keywords

Pax6; Ngn1; Olig2; retinal regeneration; Müller glia; neuronal progenitor; stem cells; photoreceptor degeneration

Introduction

Unlike mammals, there are two areas of persistent neurogenesis in the adult zebrafish retina, the circumferential marginal zone (CMZ) and the Müller glial cells in the INL (Fausett & Goldman, 2006; Hitchcock et al., 2004; Otteson et al., 2001; Raymond et al., 2006; Raymond & Hitchcock, 1997). Neurons that differentiate from the CMZ stem cell niche, which include all the retinal cell types except rod photoreceptors, are added to the periphery of the growing retina. The proliferative neuronal progenitors in the CMZ are characterized by diffuse expression of N-cadherin, activation of Notch-Delta signaling, and expression of *rx1, vsx2/ Chx10*, and *pax6a* (Raymond et al., 2006). The rod photoreceptor lineage begins with proliferating Müller glia, which generate neuronal progenitor cells that continue to proliferate and migrate to the outer nuclear layer (ONL), where they become rod precursor cells that are committed to differentiating into rod photoreceptors (Otteson et al., 2001). The proliferation of Müller glia to generate neuronal progenitors is analogous to the well-established role of the radial glia in mammalian brain neurogenesis (Anthony et al., 2004; Bignami & Dahl, 1979; Gotz & Barde, 2005).

Upon damage to the zebrafish retina, increased numbers of Müller glia reenter the cell cycle and produce multipotent neuronal progenitor cells that can replenish any of the retinal cell types. Many different damage paradigms have been established to induce a regenerative response including, laser injury (Wu et al., 2001), injection of neurotoxins (Fimbel et al., 2007; Sherpa et al., 2008), surgical removal or puncture of the retina (Cameron, 2000; Fausett & Goldman, 2006), localized heat (Raymond et al., 2006), and constant intense-light damage (Bernardos et al., 2007; Vihtelic & Hyde, 2000). While all cell types in the zebrafish retina can be regenerated, extensive research has been aimed at a light-damage model that specifically targets all photoreceptor cell types (Vihtelic & Hyde, 2000; Vihtelic et al., 2006). Constant intense-light exposure of dark-adapted *albino* zebrafish causes widespread photoreceptor cell death in the dorsal and central retina within 24 hours, which initiates the photoreceptor regeneration response (Vihtelic & Hyde, 2000; Vihtelic et al., 2006). Proliferating cells in the INL are detected by PCNA expression after 31 hours of light exposure and many clusters of dividing fusiform-shaped neuronal progenitor cells are observed 37 hours later (Kassen et al., 2007). The proliferating INL cells generated during this phase of massive cell division, migrate to the ONL within 96 hours after starting the constant light exposure and begin to differentiate into rod and cone photoreceptors (Kassen et al., 2007; Vihtelic & Hyde, 2000; Wu et al., 2001).

Multiple lines of evidence suggest that the source of the INL neuronal progenitor cells in the damaged zebrafish retina is Müller glia (Bernardos et al., 2007; Fausett & Goldman, 2006; Fischer, 2005; Thummel et al., 2008). First, Müller glial cells colabel with multiple proliferation cell markers, including BrdU (Bernardos et al., 2007), PCNA (Thummel et al., 2008; Vihtelic & Hyde, 2000), and the *Tg(ccnb1:EGFP)* transgenic line that expresses EGFP from the *cyclin B1* promoter (Kassen et al., 2008). Second, *Tg(alpha-1 tubulin:EGFP)* transgenic zebrafish expressed EGFP in Müller glia, indicating that the Müller glia adopted a neuronal progenitor cell identity after a puncture wound (Fausett & Goldman, 2006; Goldman

et al., 2001). In addition, the *alpha-1 tubulin:EGFP* transgene was coexpressed with proliferating cell markers in the Müller glia of regenerating retinas (Fausett & Goldman, 2006). Finally, knockdown of PCNA expression in the light-damaged zebrafish retina inhibited INL proliferation and initiated Müller glia apoptosis (Thummel et al., 2008). Together, these data indicate that the Müller glia are the source of neuronal progenitor cells in the regenerating zebrafish retina.

Thus, the different cellular phases of the light-lesion-induced retinal response, such as cell death, Müller glial cell changes, and neuronal progenitor cell proliferation and migration are histologically and immunohistochemically definable and largely occur during the first 4 days of the constant light treatment (Kassen et al., 2007; Vihtelic & Hyde, 2000; Vihtelic et al., 2006). Recently, the subset of Müller glia that reenter the cell cycle following damage were shown to express several transcription factors that typically are restricted to the developing neural retina and CMZ, such as *rx1, vsx2* (*Chx10*), and *pax6a* (Raymond et al., 2006). However, little is known about the dynamic temporal gene and protein expression patterns through the regeneration process. Here we show that as Müller glia reenter the cell cycle, they dedifferentiate and terminate expression of characteristic Müller glial cell markers, such as GFAP (glial fibrillary acidic protein) and glutamine synthetase. Following the initiation of Müller glial proliferation, Pax6 is detected in the neuronal progenitor cells. The expression of the *olig2:EGFP* transgene then follows the start of Pax6 expression, however, the *olig2* expression occurs in the dedifferentiated Müller glia. By three days after terminating the 96 hours of constant light treatment, Pax6 expression returned to the pre-light treatment expression pattern, while *olig2* expression persisted in a subset of Müller glia. The transcription factor Neurogenin 1 (Ngn1) is first detected in either the dedifferentiated Müller glia or the progenitor cells that will differentiate into the reestablished Müller glia following 68 hours of light treatment. Ngn1 expression persists in these new Müller glia through 17 days after terminating the constant intense-light treatment. These antibodies and the *olig2:EGFP* transgene define specific cell types and events through regeneration: Müller glial proliferation, dedifferentiation, neuronal progenitor formation and amplification, and photoreceptor differentiation. These markers will be extremely valuable in characterizing these events and allow us to identify when and where specific mutants and morphants disrupt regeneration of the light-damaged retina.

Materials and Methods

Fish maintenance

The *albino, albino Tg(olig2:EGFP)*^{vu12} (Shin et al., 2003b), and *albino Tg*(*gfap:EGFP*)^{nt11} (Kassen et al., 2007) stocks were maintained in the Center for Zebrafish Research at the University of Notre Dame Freimann Life Science Center. Fish were raised on a diet of brine shrimp and flake food three times daily under a daily light cycle of 14 hr light (250 lux):10 hr dark at 28.5°C (Detrich et al., 1999; Westerfield, 1995). All protocols used in this study were approved by the animal use committee at the University of Notre Dame and are in compliance with the ARVO statement for the use of animals in vision research.

Constant intense light treatment protocol

To induce rod and cone photoreceptor damage, constant intense-light treatment was used as previously described (Kassen et al., 2007; Thummel et al., 2008; Vihtelic & Hyde, 2000; Vihtelic et al., 2006). The *albino* and *albino* transgenic lines were dark adapted for 14 days, then transferred to clear polycarbonate tanks located between four 150W halogen bulbs. This constant bright light (2,800 lux) was maintained for up to 4 days, after which the fish were returned to standard light conditions (14 hr light: 10 hr dark).

Quantitative real-time PCR

Quantitative real-time PCR was performed and analyzed as previously described (Kassen et al., 2007). Gene-specific primers for the three target genes were designed using the ABI Primer Express program (Applied Biosystems; Foster City, CA). The PCR primers used were: *olig2* forward, 5′-GGAGGTCATGCCCTACGCT-3′; *olig2* reverse, 5′-GCAGCAGAGTGGCTATTTTAG-3′; *pax6a* forward, 5′-CACATACACACCCCCGCAC-3′, *pax6a* reverse, 5′-CCGAGGCGGCCATTG-3′; *pax6b* forward, 5′-ACACCGTACGGGATTCAAAC-3′, *pax6b* reverse, 5′-CAGCGTCCCTCTTATCTGGA-3′. Total RNA was isolated from six to ten pooled *albino* retinas that were treated in constant intense light for designated lengths of time (Kassen et al., 2007). This biological sample was independently performed three times. The cDNA from each time point in each biological replicate was reverse transcribed from total RNA using a mixture of random hexamers and oligo-dT and quantified using a NanoDrop (Thermo Scientific; Wilmington, DE). Sample preparation and run conditions were performed as described in the ABI 7500 (Applied Biosystems) user manual. Briefly, a SYBR Green master mix (Applied Biosystems) was combined with the various primer pairs (~3 μl of each primer pair at 10 μM each) and cDNA (~1 μl) in a total volume of 20 μl. The reactions were heated for 2 min at 50° C, 10 min at 95°C, and then 40 cycles of 15 sec at 95°C and 1 min at 60°C. Agarose gel electrophoresis and dissociation curve analysis verified that single DNA products were amplified with each of the primer pairs used in these experiments.

The ΔΔCT method was used for data analysis (Johnson et al., 2000; Kassen et al., 2007; Vong et al., 2003). Two types of preliminary validation experiments were performed to ensure the applicability of this analysis method for each of the genes examined. We previously determined that the 18S rRNA gene is an appropriate internal reference for data normalization in the light-damaged and regenerating zebrafish retinal tissue (Kassen et al., 2007). cDNA corresponding to each time point was quantified using a NanoDrop and equal amounts of cDNA template from each time point were used for real-time PCR amplification. Consistent CT values for each sample were obtained, which verified the suitability of 18S rRNA gene expression during the experimental time course. Second, we determined that the amplification efficiency for each target gene was approximately equal to the 18S rRNA gene (Johnson et al., 2000; Kassen et al., 2007; Vong et al., 2003). To determine the amplification efficiencies, the differences in the CT values between each target gene and the 18S rRNA reference gene (ΔCT) were obtained for at least three template dilutions. The slope of the line obtained when the input template amount was plotted against ΔCT was less than or equal to 0.1 in all cases (Johnson et al., 2000; Kassen et al., 2007; Vong et al., 2003).

For each gene examined by qRT-PCR during the light treatment time course, serial dilutions of cDNA from each time point were run in triplicate and the mean CT value was normalized against the 18S rRNA CT, giving the ΔCT value. The ΔCT value for each time point was then averaged across the three biological replicates. The normalized ΔCT values for each gene from each light treatment time point were then normalized against the dark-treated control groups, yielding the ΔΔCT. After obtaining the ΔΔCT value for each gene, the $\log_2$-fold change in gene expression levels was calculated for the time course. Statistically significant differences between *pax6a* and *pax6b* transcript amounts were determined with a Student's t-test using the normalized ΔCT values for each gene as input values.

Immunohistochemistry and confocal microscopy

Fish were euthanized at various time points by anesthetic overdose in 2-phenoxyethanol, eyes were collected, and fixed in either 4% paraformaldehyde in 5% sucrose/1X PBS or 9:1 ethanolic formaldehyde (100% ethanol:37% formaldehyde). After overnight incubation at 4° C, eyes were washed in 5% sucrose/1X PBS at room temperature and cryoprotected in 30%

sucrose/1X PBS overnight at 4°C. Eyes were subsequently washed in 1:1 (Tissue Freezing Medium (Triangle Biomedical Sciences; Durham, NC):30% sucrose/1X PBS) for four hours at room temperature and with 2:1 (TFM:30% sucrose/1X PBS) at 4°C overnight. The retinas were embedded in TFM and maintained at −80°C until sectioning at a thickness of 14 microns. Frozen sections were dried at 50°C for two hours and subsequently stored at −80°C. Prior to immunolabeling, slides were thawed for 20 minutes at 50°C.

Retinal sections were rehydrated in 1X PBS and incubated in blocking solution 1X PBS/2% NGS (normal goat serum)/0.2% Triton X-100/1% DMSO. A stronger blocking solution consisting of 1X PBS/2% NGS/0.4% Triton X-100/2% DMSO was used with mouse anti-green fluorescent protein monoclonal antibody. The sections were incubated overnight at 4°C with the primary antibody diluted in blocking buffer. Primary antibodies used for these studies include the mouse monoclonal anti-PCNA antibody (1:1000, clone PC10, Sigma Chemical, St. Louis, MO), rabbit polyclonal anti-PCNA antiserum (1:1500 Abcam, Cambridge, MA), mouse monoclonal anti-green fluorescent protein antibody (1:750 Molecular Probes, Eugene, OR), rabbit polyclonal anti-rhodopsin antiserum (1:5000, Vihtelic et al. 1999), rabbit polyclonal anti-blue opsin antiserum (1:250, Vihtelic et al. 1999), rabbit polyclonal anti-Pax6 antiserum (1:100, Covance, Berkeley, CA), mouse monoclonal anti-glutamine synthetase antibody (1:500, Chemicon International, Temecula, CA), and mouse polyclonal anti-Ngn1 antibody (1:500, Abcam). After overnight incubation, sections were washed in 1X PBS/0.05% Tween-20. The sections were then incubated for one hour at room temperature with the secondary antibody diluted 1:500 in 1X PBS/0.05% Tween-20. Secondary antibodies included Alexa Fluor goat anti-primary 488, 568, and 594 (Molecular Probes). Nuclei were labeled with TO-PRO-3 (Molecular Probes) at a 1:750 dilution in 1X PBS/0.05% Tween-20. Sections were again washed in 1X PBS/0.05% Tween-20 and 1X PBS, before coverslips were mounted using ProLong Gold (Molecular Probes).

Antigen retrieval was utilized for Pax6 immunostaining. Immediately following tissue rehydration, retinal sections were incubated at 95°C in preheated 1X Antigen Plus pH 10 buffer (Novagen, Madison, WI) for 25 minutes. Sections were cooled for 40 minutes and washed in 1X PBS. Subsequently, they were incubated for one hour in blocking solution and immunolabeled as previously described.

Confocal microscopy was performed with either a 1024 BioRad or Leica TCS SP5 confocal microscope on retinal sections that either contained or were immediately adjacent to the optic nerve. A minimum of eight retinas were analyzed per experimental time point.

Results

Müller glia proliferate and dedifferentiate during light-induced zebrafish retinal regeneration

We examined the temporal and spatial pattern of cell proliferation in the light-damaged retina by immunolocalizing PCNA in the *Tg(gfap:EGFP)*^{nt11} transgenic zebrafish line, which expresses enhanced green fluorescent protein in zebrafish Müller glia (Kassen et al., 2007). At 0 and 16 hours of constant light treatment, the majority of PCNA labeling in the central and dorsal retina was observed in the ONL (Fig. 1, Panels I-J and Q-R), which likely represented proliferating rod precursor cells (Hitchcock & Kakuk-Atkins, 2004; Otteson et al., 2001). By 31 hours of constant light exposure, there was a reduction in the number of PCNA-positive cells in the ONL and an increased number of PCNA-expressing cells in the INL (Fig. 1, Panels K and S). The PCNA-positive INL cells colocalized with the EGFP-positive Müller glia (Fig. 1, Panels C, K, and S), which demonstrated that the Müller glia had reentered the cell cycle. At 51 hours of constant light damage, PCNA-positive neuronal progenitor cells, which arose from Müller glial cell division, were first observed (Fig. 1, Panels D, L, and T).

By 68 hours of constant light treatment, there were two apparent groups of Muller glia. One group maintained normal levels of EGFP expression and normal Müller glial cell morphology. Because this group was not associated with any PCNA-positive cells (Fig. 1, Panels E, M, and U, arrowheads), these Müller glia most likely had not reentered the cell cycle. The second group of Müller glia exhibited a reduced amount of EGFP expression that colabeled with columns of migrating PCNA-positive neuronal progenitor cells (Fig. 1, Panels E, M, and U, arrow). This group likely represented the subset of Müller glial cells that reduced their EGFP expression from the *gfap* promoter and reentered the cell cycle. Using confocal microscopy to generate a single optical section of the immunolabeled retina confirmed the presence of two groups of Müller glia (Fig. 2, Panels A-C). The morphologically normal Müller glia expressed relatively high amounts of EGFP and did not coexpress PCNA (Fig. 2, Panels A-C, arrowheads), while the morphologically hypertrophied Müller glial cells expressed low levels of EGFP and were associated with multiple PCNA-positive nuclei (Fig. 2, Panels A-C, arrow). The merged confocal z-stack of this retinal section showed the morphologically normal Müller glia expressed high levels of EGFP and were not within the clusters of EGFP-positive nuclei (Fig. 2D, arrowheads), while the morphologically hypertrophied Müller glia expressed reduced levels of EGFP and contained clusters of PCNA-positive nuclei (Fig. 2D, arrow). Therefore, only the Müller glia that had reentered the cell cycle and produced clusters of PCNA-positive neuronal progenitor cells decreased their expression of Müller glial cell markers. We termed this group dedifferentiated Müller glia, because of their decreased expression of Müller glial-specific markers.

At 96 hours of light damage, it was difficult to detect EGFP expression in the dedifferentiated Müller glia (Fig. 1, Panels F and V), even though the PCNA-positive neuronal progenitor cells retained their columnar organization and were present in regions devoid of EGFP-positive Müller glia (Fig. 1, Panels N and V). By 3 days post-light treatment, EGFP expression had not returned to this group of Müller glia (Fig. 1, Panels G and W), even though INL proliferation was largely absent (Fig. 1, Panels O and W) and rod and cone photoreceptor differentiation had already initiated in the ONL (Fig. 3, Panels C and F). By 17 days post-light treatment, however, EGFP expression had returned to near the levels observed in the 0 hr control retinas (Fig. 1, Panels H and X). These data suggested that a subset of Müller glia in the light-damaged zebrafish retina proliferated to generate neuronal progenitor cells and then dedifferentiated and hypertrophied, possibly to provide a scaffold for the migrating neuronal progenitor cells to the ONL. By 17 days post-light treatment, the dedifferentiated Müller glia had redifferentiated into normal-shaped Müller glial cells.

To further examine the dedifferentiated Müller glia, we immunolocalized glutamine synthetase, a Müller glial-specific protein, and PCNA in light-treated *albino* retinas (Fig. 4). In control retinas, glutamine synthetase was localized throughout all the Müller glia and PCNA was not detected (Fig. 4, Panels A, F, and K). At 31 hours of constant light treatment, some Müller glia coexpressed PCNA (Fig. 4, Panels B, G, and L, arrow), while the remaining Müller glia did not express PCNA (Fig. 4, Panels B, G, and L, arrowhead). At 51 hours, the dedifferentiated Müller glia exhibited a diffuse pattern of glutamine synthetase and were associated with PCNA-positive neuronal progenitor nuclei (Fig. 4, Panels C, H, and M, arrow) relative to the PCNA-negative Müller glia (Fig. 4, Panels C, H, and M, arrowhead). The dedifferentiated Müller glia continued to exhibit the hypertrophied pattern of glutamine synthetase expression and were associated with PCNA-positive neuronal progenitor nuclei at 68 hours (Fig. 4, Panels D, I, and N, outlined in dashed lines) and 96 hours of constant light treatment (Fig. 4, Panels E, J, and O, arrow). Thus, the dedifferentiated Müller glia hypertrophied, reduced their expression of both *gfap* and glutamine synthetase, and were associated with the proliferating neuronal progenitors.

Pax6 is expressed in newly generated and proliferating neuronal progenitor cells during regeneration of the light-damaged zebrafish retina

The *pax6* gene is expressed in neuronal progenitor cells during the persistent neurogenesis of rod photoreceptors in the adult zebrafish retina and during regeneration of the damaged zebrafish retina (Otteson et al., 2001; Raymond et al., 2006). We examined Pax6 expression throughout regeneration in the light-damaged retina. Pax6 was constitutively expressed in the amacrine cells in the INL and ganglion cells in the GCL of the undamaged adult zebrafish retina (Fig. 5A, double and single arrowheads, respectively). This is consistent with the Pax6 expression pattern in the adult chicken, rodent, and human retinas (Lakowski et al., 2007; Shin et al., 2003a; Stanescu et al., 2007). Additionally, Pax6 was weakly expressed in cone cell ellipsoids, which may represent non-specific immunoreactivity (Fig. 5A). The regeneration response was initiated by proliferating (PCNA-positive) rod precursor cells in the ONL after 16 hours of constant light treatment (Fig. 5B, arrowhead), followed by PCNA-positive Müller glia in the INL at 31 hours (Fig. 5C, arrowheads). There was a reduction in the number of PCNA-positive ONL rod precursors at 31 hours, when the Müller glia began proliferating. Although Pax6 was recently reported to be very weakly expressed in Müller glia prior to proliferation (Bernardos et al., 2007), we did not detect Pax6 expression in the Müller glia at these earlier time points. We first observed the induced Pax6 expression in columns of PCNA-positive INL neuronal progenitors at 51 hours (Fig. 5D). This colocalization of Pax6 and PCNA persisted through 96 hours (Fig. 5, Panels E and F), as the neuronal progenitors continued to proliferate and migrate towards the ONL. Coincident with the observed Pax6 expression in the neuronal progenitors, we also detected weak Pax6 expression in the Müller glial cell processes in the inner plexiform layer (Fig. 5, Panels D-F, double arrows). This suggested that Pax6 was also expressed in the Müller glia, but could not be detected until nearly 20 hours after the Müller glia reentered the cell cycle. Pax6 expression in the neuronal progenitors was largely absent by 3 days post-light treatment (Fig. 5G), with only the few remaining clusters of INL neuronal progenitors retaining weak Pax6 expression (Fig. 5G, arrow). These data suggested that Pax6 played an active role in the proliferation and migration of the neuronal progenitor cells, and that Pax6 expression terminated shortly after the neuronal progenitors reached the ONL and photoreceptor differentiation began.

Expression of*olig2:EGFP* increases following Pax6 expression in the dedifferentiated Müller glia

One potential transcriptional target of Pax6 is the *oligodendrocyte lineage transcription factor2* (*olig2*) gene, which functions downstream of Pax6 to regulate motor neuron and oligodendrocyte development (Park et al., 2002; Buffo, 2007; Sugimori et al., 2007). Because an antibody to the Olig2 protein does not exist, we studied regeneration in the *Tg (olig2:EGFP)*^{vu12} transgenic line, which faithfully expresses EGFP from the *olig2* promoter (Shin et al., 2003b).

In the regenerating *albino Tg(olig2:EGFP)*^{vu12} zebrafish line, EGFP expression was not detected in the INL through 51 hours of constant light treatment, even though PCNA-positive Müller glia and neuronal progenitors were present at 31 and 51 hours of light treatment, respectively (Fig. 6, Panels A-D). EGFP expression was first observed at 68 hours of constant light treatment in cells with Müller glial cell morphology (Fig. 6E). By 96 hours, all of the EGFP-positive Müller glia were associated with multiple migrating PCNA-positive neuronal progenitor cells (Fig. 6F). Thus, *olig2:EGFP* was only expressed in the dedifferentiated Müller glia during zebrafish retinal regeneration.

To examine the relationship between Pax6 and *olig2* expression during the constant light treatment, adult *albino* and *albino Tg(olig2:EGFP)*^{vu12} transgenic zebrafish were simultaneously light treated and collected for Pax6 and EGFP immunolocalization. Pax6

expression was first detected at 51 hours of light treatment in the neuronal progenitors in the INL and the Müller glial cell processes in the IPL (Fig. 7A, arrows and double arrows, respectively), whereas EGFP from the *olig2:EGFP* transgene was not observed in the dedifferentiated Müller glia until 68 hours of light treatment (Fig. 7G). Pax6 and EGFP, however, colabeled proliferating cell clusters at 68 and 96 hrs, with Pax6 primarily localized in the neuronal progenitor nuclei and EGFP filling the surrounding dedifferentiated Müller glial cells (Fig. 8, Panels A and B, arrows and arrowheads, respectively).

Zebrafish possess two *pax6* genes, *pax6a* and *pax6b*, which are functionally redundant during retinal development (Blader et al., 2004). The anti-rabbit Pax6 polyclonal antiserum recognizes both zebrafish Pax6a and Pax6b (data not shown). Therefore, to determine what *pax6* gene increased in expression during the light treatment, quantitative real-time PCR was performed on RNA collected at each time point (Fig. 9). Neither *pax6a* nor *pax6b* transcript levels changed significantly through 31 hours of light treatment (Fig. 9). At 51 hours of light treatment, however, *pax6a* levels decreased, and *pax6b* levels increased (Fig. 9). This suggested that Pax6b was immunolocalized in the newly formed INL neuronal progenitors at 51 hours. To further support the observation that *pax6* expression increased prior to *olig2*, quantitative real-time PCR was performed and revealed that *olig2* transcript levels decreased from 0 - 51 hours of light treatment, but increased at 68 hours (Fig. 9). This increased *olig2* expression at 68 hours was consistent with the increased Pax6 expression at 51 hours preceding the EGFP expression in the *albino Tg(olig2:EGFP)*^{vu12} transgenic fish at 68 hours.

Ngn1 labels proliferating neuronal progenitors and post-mitotic Müller glia during regeneration of the light-damaged zebrafish retina

The Ngn1 protein is involved in cell fate decisions in the developing nervous system (Blader et al., 2004; Sugimori et al., 2007). During zebrafish retinal development, Ngn1 colabeled with the Müller glial cell marker, glutamine synthetase, at 72 hours post-fertilization (Fig. 10, Panels A-C). However, the Ngn1 expression did not persist into the adult retina (Fig. 11A). During regeneration of the light-damaged zebrafish retina, Ngn1 was first weakly detected in the INL after 51 hours of light treatment (Fig. 11B). Ngn1 expression increased in the INL through 68 hours (Fig. 11C). At this time, all of the Ngn1-expressing cells colabeled with PCNA-positive neuronal progenitor cells as they migrated to the ONL (Fig. 11D). At 96 hours of constant light, some Ngn1-positive cells remained basally in the INL columns of neuronal progenitors (Fig. 11E, arrowheads). These basally-located Ngn1-positive cells no longer colabeled with PCNA (Fig. 11F, arrowhead). These Ngn1-positive cells persisted through 17 days post-light treatment (Fig. 11, Panels G and H) and remained PCNA-negative (data not shown).

To determine whether the Ngn1-labeled cells will ultimately reestablish the Müller glia following retinal regeneration, Ngn1 expression was examined in the light-treated *Tg (gfap:EGFP)*^{nt11} retina, which expressed EGFP in Müller glia (Kassen et al., 2007). At 0 hours, there were no Ngn1-positive cells, although the EGFP expression in the Müller glia remained strong (Fig. 12, Panels A, D, and G). At 3 days post-light treatment, Ngn1 expression was detected in the INL, where it colabeled with the diffuse EGFP-positive dedifferentiated Müller glia (Fig. 12, Panels B, E, and H, arrowhead). At 17 days post-light treatment, Ngn1-positive cells colabeled with a subset of the EGFP-positive Müller glia (Fig. 12, Panels C, F, and I, arrow). These data suggest that Ngn1 labeled a distinct population of dedifferentiated Müller glia late in the regeneration response that will eventually reestablish the Müller glia population.

Discussion

Characterizing the cellular response in the light-damaged zebrafish retina, through changes in gene and protein expression, provides a framework to examine the processes underlying the initiation and maintenance of the regeneration response. The time points selected in this study

corresponded to equivalent points used in a gene microarray study (Kassen et al., 2007), which included a time point during maximal photoreceptor cell death (16 hours), a time point when a subset of Müller glia reentered the cell cycle (31 hours), a time point when neuronal progenitors were generated (51 hours), and two relatively evenly-spaced time points during neuronal progenitor cell proliferation and migration (68 and 96 hours). Following 96 hours of constant light treatment, the fish were returned to a normal light:dark cycle. We also selected one time point that corresponded to the start of photoreceptor differentiation from the neuronal progenitors (3 days post-light treatment) and one time point that corresponded to the reexpression of GFAP and glutamine synthetase in the regenerated Müller glia (17 days post light treatment).

Pax6 was examined because of its known expression in neuronal progenitor cells during retinal development and during the persistent neurogenesis in the rod photoreceptor lineage in the adult retina (Hitchcock et al., 1996; Otteson et al., 2001). In the regenerating chick retina, the pigmented epithelia can transdifferentiate into retinal neurons (Coulombre & Coulombre, 1965). Overexpression of only *pax6* is sufficient to promote this transdifferentiation event (Azuma et al., 2005). Regeneration of the postnatal chick retina can also occur through the dedifferentiation and proliferation of the Müller glia (Fischer, 2005; Fischer & Reh, 2001). These proliferating Müller glia express several transcription factors, including *pax6* (Fischer et al., 2002). In the regenerating newt retina, Pax6 is expressed in all the retinal precursor cells (Kaneko et al., 1999). In the undamaged adult zebrafish retina, Pax6 labels INL neuronal progenitor cells that arise from proliferating Müller glia, which migrate to the ONL and become rod precursors that are committed to differentiate into only rod photoreceptors (Hitchcock et al., 1996; Otteson et al., 2001; Raymond et al., 2006). Recently, very weak Pax6 expression was described in the undamaged adult zebrafish Müller glia (Bernardos et al., 2007). In contrast, we did not observe Pax6 expression until 51 hours of light treatment (Fig. 5D), following Müller glial cell division at 31 hours. Mutation and/or morphant analysis will be required to elucidate the roles of Pax6a and Pax6b in the neuronal progenitor cells and Müller glia during retinal regeneration.

Olig2 is a basic helix-loop-helix (bHLH) transcription factor that belongs to the Oligodendrocyte lineage transcription factor (Olig) family (Ligon et al., 2006). Olig2 was originally characterized as a key regulator for the differentiation of progenitor motor neurons (pMN) into either motor neurons or oligodendrocytes (Lee et al., 2005; Lu et al., 2002; Park et al., 2002; Zhou & Anderson, 2002). During development, a variety of homeodomain transcription factors, including Pax6, promote the specification of motor neurons (Ericson et al., 1997; Osumi et al., 1997). Morpholino knockdown studies demonstrated that loss of Olig2 expression prevented both motor neuron and oligodendrocyte differentiation in zebrafish (Park et al., 2002). Thus, Olig2 maintains the pMN in an undifferentiated state. In the central nervous system, Olig2 has been implicated in regulating the multipotent neural progenitors that lie in the subventricular zone of the adult rat brain (Buffo et al., 2005; Takemura, 2005). Under stressed conditions, these neural progenitor cells begin proliferating and expressing both Olig2 and Pax6; however, Olig2 is expressed in the neuronal precursors and not the proliferative precursors in higher mammals (Hack et al., 2004; Tonchev et al., 2006). In mouse, Olig2 expression was strongly upregulated in glial cells in response to a stab wound to the brain and inhibition of Olig2 expression forced these cells to prematurely differentiate (Buffo, 2007; Buffo et al., 2005).

Using the *Tg(olig2:EGFP)*^{vu12} transgenic zebrafish line, we demonstrated that *olig2:EGFP* expression increased, not during neuronal progenitor formation at 51 hours of light treatment, but at 68 hours, when neuronal progenitors were actively proliferating (Fig. 6). Thus, *olig2* expression follows Pax6 expression. Further, *olig2:EGFP* expression appears to be in the dedifferentiated Müller glia, while Pax6 is expressed in both the Müller glia and the

proliferating neuronal progenitors (Fig. 8). Our current resolution cannot definitively differentiate if *olig2* expression is restricted to the Müller glia or may also be expressed in the neuronal progenitors. Olig2 may be required to maintain the Müller glia in a dedifferentiated state to allow the neuronal progenitors to migrate to the ONL. By 3 days post-light treatment, Olig2 is significantly down-regulated in the INL, as neuronal progenitors have migrated to the ONL and are beginning to differentiate into rod and cone photoreceptors (Figs. 7 and 3). These data are consistent with the role of Olig2 maintaining the Müller glia in a dedifferentiated state during retinal regeneration.

Neurogenin1 (Ngn1), a transcription factor that regulates neuronal differentiation in the central nervous system (Blader et al., 2004; Lee et al., 2005), was upregulated in the gene microarray analysis of the light-damaged retina (Kassen et al., 2007). Because zebrafish do not appear to possess a Ngn2 ortholog, Ngn1 may be the functional equivalent of the mouse Ngn2 (Lee et al., 2005). In rodents, Olig2 activates the expression of *ngn2* in the pMNs (Lee et al., 2005), which suggests that Olig2 might regulate the expression of Ngn1 in the regenerating retina. While both *olig2* and Ngn1 expression were first observed in the dedifferentiated Müller glia at 68 hours of light treatment (Figs. 6E and 11C), it remains possible that *olig2* expression preceded Ngn1 between 51 and 68 hours. Ngn1 expression also persisted in the regenerating retina through 17 days post-light treatment, beyond when *olig2* expression was no longer detected. While the potential regulatory relationship between *olig2* and Ngn1 remains unclear, it is obvious that Ngn1 labeled relatively new post-mitotic Müller glia in both the developing retina and the regenerated retina following light damage (Figs. 10-12).

The goal of this work was to identify markers that differentiated between neuronal progenitor cells and Müller glia and elucidate changes that both cell types underwent during retinal regeneration. We found that Müller glial cells loose cell-specific markers such as GFAP (glial fibrillary acidic protein) and glutamine synthetase following their reentry into the cell cycle, suggesting that the Müller glia dedifferentiated during regeneration. Pax6 expression was first detected in the proliferating neuronal progenitors by 51 hours of constant light treatment, which is significantly after the Müller glia first reentered the cell cycle after 31 hours of light. This indicated that Pax6 expression increased in neuronal progenitors, rather than in the proliferating Müller glia. Real-time PCR performed on RNA collected at each time point confirmed that neither *pax6a* nor *pax6b* transcript levels changed significantly through 31 hours of light treatment. However, *pax6b*, but not *pax6a*, transcript levels increased at 51 hours of light treatment (Fig. 9), which suggested that Pax6b was immunolocalized in the INL neuronal progenitors after 51 hours of constant light treatment. EGFP expression from the *olig2:EGFP* transgene was first detected at 68 hours of constant light treatment in the dedifferentiated Müller glia. It was not possible to determine if *olig2* was also expressed in the proliferating neuronal progenitors. The expression of olig2 in the dedifferentiated Müller glia is consistent with Olig2 functioning to prevent premature differentiation of stem/progenitor cells. While Pax6 expression was largely lost by 3 days post-light treatment, *olig2* expression persisted through this time, which corresponded to when the progenitors began differentiating into new rod and cone photoreceptors. Similar to *olig2*, Ngn1 protein expression was initially detected in the dedifferentiated Müller glia at 68 hours of light treatment. In contrast, however, Ngn1 expression persisted in a subset of inner nuclear layer nuclei until 17 days post-light treatment. These Ngn1-positive cells ultimately differentiated back into Müller glia. This suggests that light damage induces a subset of Müller glia to proliferate and dedifferentiate, and then differentiate back into Müller glia at the completion of regeneration. It then remains unclear if the neuronal progenitors possess the ability to differentiate into Müller glia. These markers, therefore, can be used to identify different cell types at particular stages of retinal regeneration, which will aid in the further characterization of the regeneration response in the light-damaged retina.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Suzyanne Guzicki, Debbie Bang, and the staff of Freimann Life Science Center for providing zebrafish husbandry and care. We appreciate Bruce Appel (Vanderbilt University) for generously providing the Tg (*olig2:EGFP*)^{vu12} transgenic line. We thank members of the Hyde lab for thoughtful discussions and comments through the course of this project. This work was funded by NIH grant R21EY017134 to D.R.H., funds from the Center for Zebrafish Research at the University of Notre Dame, and a Fight for Sight Postdoctoral Research Fellowship to R. T.

References

Anthony TE, Klein C, Fishell G, Heintz N. Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 2004;41:881–890. [PubMed: 15046721]

Azuma N, Tadokoro K, Asaka A, Yamada M, Yamaguchi Y, Handa H, Matsushima S, Watanabe T, Kida Y, Ogura T, Torii M, Shimamura K, Nakafuku M. Transdifferentiation of the retinal pigment epithelia to the neural retina by transfer of the Pax6 transcriptional factor. Hum. Mol. Genet 2005;14:1059–1068. [PubMed: 15757974]

Bernardos RL, Barthel LK, Meyers JR, Raymond PA. Late-stage neuronal progenitors in the retina are radial Muller glia that function as retinal stem cells. J. Neurosci 2007;27:7028–7040. [PubMed: 17596452]

Bignami A, Dahl D. The radial glia of Muller in the rat retina and their response to injury. An immunofluorescence study with antibodies to the glial fibrillary acidic (GFA) protein. Exp. Eye Res 1979;28:63–69. [PubMed: 376324]

Blader P, Lam CS, Rastegar S, Scardigli R, Nicod JC, Simplicio N, Plessy C, Fischer N, Schuurmans C, Guillemot F, Strahle U. Conserved and acquired features of neurogenin1 regulation. Development 2004;131:5627–5637. [PubMed: 15496438]

Buffo A. Fate determinant expression in the lesioned brain: Olig2 induction and its implications for neuronal repair. Neurodegener Dis 2007;4:328–332. [PubMed: 17627137]

Buffo A, Vosko MR, Erturk D, Hamann GF, Jucker M, Rowitch D, Gotz M. Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. Proc. Natl. Acad. Sci. U. S. A 2005;102:18183–18188. [PubMed: 16330768]

Cameron DA. Cellular proliferation and neurogenesis in the injured retina of adult zebrafish. Vis. Neurosci 2000;17:789–797. [PubMed: 11153658]

Coulombre JL, Coulombre AJ. Regeneration of neural retina from the pigmented epithelium in the chick embryo. Dev. Biol 1965;12:79–92. [PubMed: 5833111]

Detrich HW 3rd, Westerfield M, Zon LI. Overview of the Zebrafish system. Methods Cell Biol 1999;59:3–10. [PubMed: 9891351]

Ericson J, Rashbass P, Schedl A, Brenner-Morton S, Kawakami A, van Heyningen V, Jessell TM, Briscoe J. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell 1997;90:169–180. [PubMed: 9230312]

Fausett BV, Goldman D. A role for alpha1 tubulin-expressing Muller glia in regeneration of the injured zebrafish retina. J. Neurosci 2006;26:6303–6313. [PubMed: 16763038]

Fimbel SM, Montgomery JE, Burket CT, Hyde DR. Regeneration of inner retinal neurons after intravitreal injection of ouabain in zebrafish. J. Neurosci 2007;27:1712–1724. [PubMed: 17301179]

Fischer AJ. Neural regeneration in the chick retina. Prog. Retin. Eye Res 2005;24:161–182. [PubMed: 15610972]

Fischer AJ, McGuire CR, Dierks BD, Reh TA. Insulin and fibroblast growth factor 2 activate a neurogenic program in Muller glia of the chicken retina. J. Neurosci 2002;22:9387–9398. [PubMed: 12417664]

Fischer AJ, Reh TA. Muller glia are a potential source of neural regeneration in the postnatal chicken retina. Nat. Neurosci 2001;4:247–252. [PubMed: 11224540]

Goldman D, Hankin M, Li Z, Dai X, Ding J. Transgenic zebrafish for studying nervous system development and regeneration. Transgenic Res 2001;10:21–33. [PubMed: 11252380]

Gotz M, Barde YA. Radial glial cells defined and major intermediates between embryonic stem cells and CNS neurons. Neuron 2005;46:369–372. [PubMed: 15882633]

Hack MA, Sugimori M, Lundberg C, Nakafuku M, Gotz M. Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. Mol. Cell Neurosci 2004;25:664–678. [PubMed: 15080895]

Hitchcock P, Kakuk-Atkins L. The basic helix-loop-helix transcription factor neuroD is expressed in the rod lineage of the teleost retina. J. Comp. Neurol 2004;477:108–117. [PubMed: 15281083]

Hitchcock P, Ochocinska M, Sieh A, Otteson D. Persistent and injury-induced neurogenesis in the vertebrate retina. Prog. Retin. Eye Res 2004;23:183–194. [PubMed: 15094130]

Hitchcock PF, Macdonald RE, VanDeRyt JT, Wilson SW. Antibodies against Pax6 immunostain amacrine and ganglion cells and neuronal progenitors, but not rod precursors, in the normal and regenerating retina of the goldfish. J. Neurobiol 1996;29:399–413. [PubMed: 8907167]

Johnson MR, Wang K, Smith JB, Heslin MJ, Diasio RB. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. Anal. Biochem 2000;278:175–184. [PubMed: 10660460]

Kaneko Y, Matsumoto G, Hanyu Y. Pax-6 expression during retinal regeneration in the adult newt. Dev. Growth Differ 1999;41:723–729. [PubMed: 10646802]

Kassen SC, Ramanan V, Montgomery JE, C TB, Liu CG, Vihtelic TS, Hyde DR. Time course analysis of gene expression during light-induced photoreceptor cell death and regeneration in albino zebrafish. Dev. Neurobiol 2007;67:1009–1031. [PubMed: 17565703]

Kassen, SC.; Thummel, R.; Burket, CT.; Campochiaro, LA.; Harding, MJ.; Hyde, DR. Mol. Vis. 2008. The Tg(cyclinB1:EGFP) transgenic zebrafish line is a marker for proliferation in retinal development and regeneration. in press

Lakowski J, Majumder A, Lauderdale JD. Mechanisms controlling Pax6 isoform expression in the retina have been conserved between teleosts and mammals. Dev. Biol 2007;307:498–520. [PubMed: 17509554]

Lee SK, Lee B, Ruiz EC, Pfaff SL. Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. Genes Dev 2005;19:282–294. [PubMed: 15655114]

Ligon KL, Fancy SP, Franklin RJ, Rowitch DH. Olig gene function in CNS development and disease. Glia 2006;54:1–10. [PubMed: 16652341]

Lu QR, Sun T, Zhu Z, Ma N, Garcia M, Stiles CD, Rowitch DH. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell 2002;109:75–86. [PubMed: 11955448]

Osumi N, Hirota A, Ohuchi H, Nakafuku M, Iimura T, Kuratani S, Fujiwara M, Noji S, Eto K. Pax-6 is involved in the specification of hindbrain motor neuron subtype. Development 1997;124:2961–2972. [PubMed: 9247338]

Otteson DC, D'Costa AR, Hitchcock PF. Putative stem cells and the lineage of rod photoreceptors in the mature retina of the goldfish. Dev. Biol 2001;232:62–76. [PubMed: 11254348]

Park HC, Mehta A, Richardson JS, Appel B. olig2 is required for zebrafish primary motor neuron and oligodendrocyte development. Dev. Biol 2002;248:356–368. [PubMed: 12167410]

Raymond PA, Barthel LK, Bernardos RL, Perkowski JJ. Molecular characterization of retinal stem cells and their niches in adult zebrafish. BMC Dev. Biol 2006;6:36. [PubMed: 16872490]

Raymond PA, Hitchcock PF. Retinal regeneration: common principles but a diversity of mechanisms. Adv. Neurol 1997;72:171–184. [PubMed: 8993697]

Sherpa T, Fimbel SM, Mallory DE, Maaswinkel H, Spritzer SD, Sand JA, Li L, Hyde DR, Stenkamp DL. Ganglion cell regeneration following whole-retina destruction in zebrafish. Dev. Neurobiol 2008;68:166–181. [PubMed: 18000816]

Shin DH, Kwon BS, Chang YP, Bae SR, Kim J, Kim JW. Ultramicroscopical immunolocalization of PAX6 in the adult chicken retina. Acta Histochem 2003a;105:267–272. [PubMed: 13677621]

Shin J, Park HC, Topczewska JM, Mawdsley DJ, Appel B. Neural cell fate analysis in zebrafish using olig2 BAC transgenics. Methods. Cell Sci 2003b;25:7–14.

Stanescu D, Iseli HP, Schwerdtfeger K, Ittner LM, Reme CE, Hafezi F. Continuous expression of the homeobox gene Pax6 in the ageing human retina. Eye 2007;21:90–93. [PubMed: 16254589]

Sugimori M, Nagao M, Bertrand N, Parras CM, Guillemot F, Nakafuku M. Combinatorial actions of patterning and HLH transcription factors in the spatiotemporal control of neurogenesis and gliogenesis in the developing spinal cord. Development 2007;134:1617–1629. [PubMed: 17344230]

Takemura NU. Evidence for neurogenesis within the white matter beneath the temporal neocortex of the adult rat brain. Neuroscience 2005;134:121–132. [PubMed: 15964698]

Thummel R, Kassen SC, Montgomery JE, Enright JM, Hyde DR. Inhibition of Muller glial cell division blocks regeneration of the light-damaged zebrafish retina. Dev. Neurobiol 2008;68:392–408. [PubMed: 18161852]

Tonchev AB, Yamashima T, Sawamoto K, Okano H. Transcription factor protein expression patterns by neural or neuronal progenitor cells of adult monkey subventricular zone. Neuroscience 2006;139:1355–1367. [PubMed: 16580139]

Vihtelic TS, Doro CJ, Hyde DR. Cloning and characterization of six zebrafish photoreceptor opsin cDNAs and immunolocalization of their corresponding proteins. Vis. Neurosci 1999;16:571–585. [PubMed: 10349976]

Vihtelic TS, Hyde DR. Light-induced rod and cone cell death and regeneration in the adult albino zebrafish (Danio rerio) retina. J. Neurobiol 2000;44:289–307. [PubMed: 10942883]

Vihtelic TS, Soverly JE, Kassen SC, Hyde DR. Retinal regional differences in photoreceptor cell death and regeneration in light-lesioned albino zebrafish. Exp. Eye Res 2006;82:558–575. [PubMed: 16199033]

Vong QP, Chan KM, Cheng CH. Quantification of common carp (Cyprinus carpio) IGF-I and IGF-II mRNA by real-time PCR: differential regulation of expression by GH. J. Endocrinol 2003;178:513–521. [PubMed: 12967342]

Westerfield, M. The Zebrafish Book: A guide for the laboratory use of zebrafish (Danio rerio). Univ. of Oregon Press; Eugene, OR.: 1995.

Wu DM, Schneiderman T, Burgett J, Gokhale P, Barthel L, Raymond PA. Cones regenerate from retinal stem cells sequestered in the inner nuclear layer of adult goldfish retina. Invest. Ophthalmol. Vis. Sci 2001;42:2115–2124. [PubMed: 11481280]

Zhou Q, Anderson DJ. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 2002;109:61–73. [PubMed: 11955447]

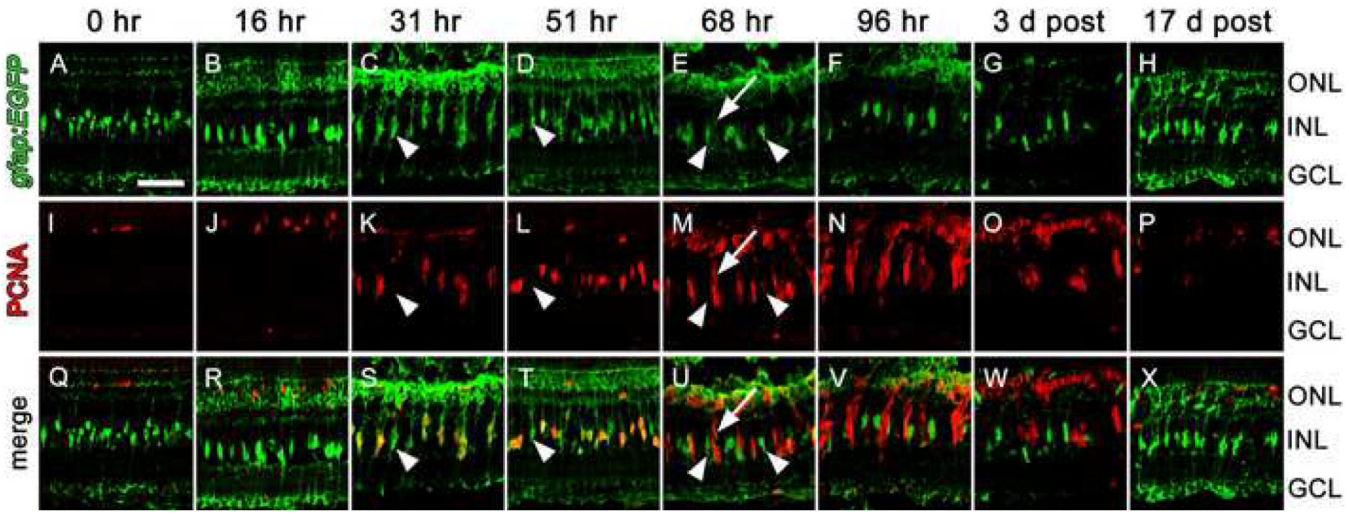

Figure 1. Müller glia express PCNA during light-induced zebrafish retinal regeneration.

Timecourse of EGFP expression (A-H), PCNA expression (I-P), and merged images (Q-X) in the *albino Tg(gfap:EGFP)*^{nt11} zebrafish line. PCNA was expressed in the ONL at 0 and 16 hours of light damage (I and J) and in the INL from 31 hours of light treatment through 3 days post-light treatment (K-O). Multiple PCNA-positive neuronal progenitors take on a fusiform shape and migrate from the INL to the ONL from 51-96 hours of constant light treatment (L-N). At 3 days post light, a single layer of PCNA-positive ONL nuclei and only a few PCNA-positive INL cells persist (O). At 17 days post light, only a few PCNA-positive nuclei are observed in the ONL (P). The *Tg*(*gfap:EGFP*)^{nt11} transgenic line expressed EGFP in Müller glia (A-H and Q-X). Throughout the timecourse, many Muller glia remained PCNA-negative (arrowheads). However, at 31 hours of light treatment, a subset of Müller glia nuclei expressed PCNA (S), which persisted until 51 hours (T). At 68 hours of constant light, EGFP expression decreased in the hypertrophied Müller glia associated with PCNA-positive neuronal progenitors (arrow; Panels E, M and U). EGFP expression remained high in the Müller glial cells that did not reenter the cell cycle (arrowheads; Panels E, M, and U). At 96 hours of light treatment, EGFP did not appear to colabel with migrating PCNA-positive neuronal progenitor cells in the INL (V). Scale bar in Panel A is 25 microns. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PCNA, proliferating cell nuclear antigen; *gfap:EGFP, glial fibrillary associated protein promoter:enhanced green fluorescent protein transgene*.

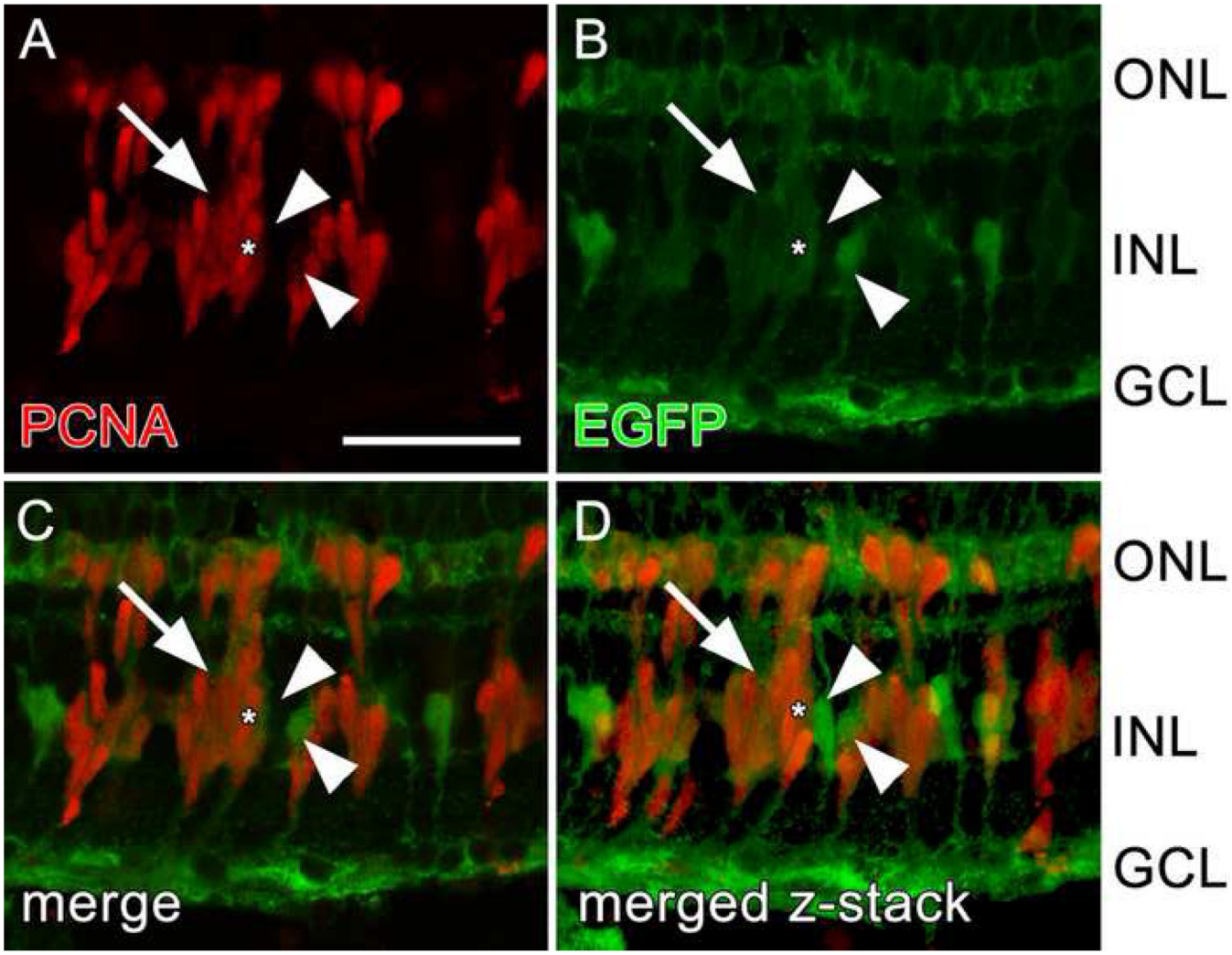

Figure 2. Expression of EGFP and PCNA in the *albino Tg(gfap:EGFP)*^{nt11} retina after 68 hours of constant light treatment.

Dark-adapted adult *albino Tg(gfap:EGFP)*^{nt11} zebrafish were placed in constant intense light for 68 hours, immunolabeled for PCNA and then examined by confocal microscopy at either a single optical plane (A-C) or a merged z-stack. PCNA was detected in clusters of neuronal progenitor nuclei (A, C, D). EGFP was present at either high levels in morphologically normal Müller glial cells (B, C, D, arrowheads) or at low levels in hypertrophied Müller glia (B, C, D, arrows). Either merging the single optical plane or the z-stack revealed that the hypertrophied Müller glia, but not the morphologically normal Müller glia, were always associated around a cluster of PCNA-positive neuronal progenitors. In the single optical plane, each PCNA-positive neuronal progenitor appeared to be surrounded by slightly elevated levels of EGFP-positive Müller glial cell (A, B, and C, asterisks). Scale bar in Panel A is 25 microns. PCNA, proliferating cell nuclear antigen; EGFP, enhanced green fluorescent protein; ONL, outer nuclear layer; INL, inner nuclear layer; GCL ganglion cell layer.

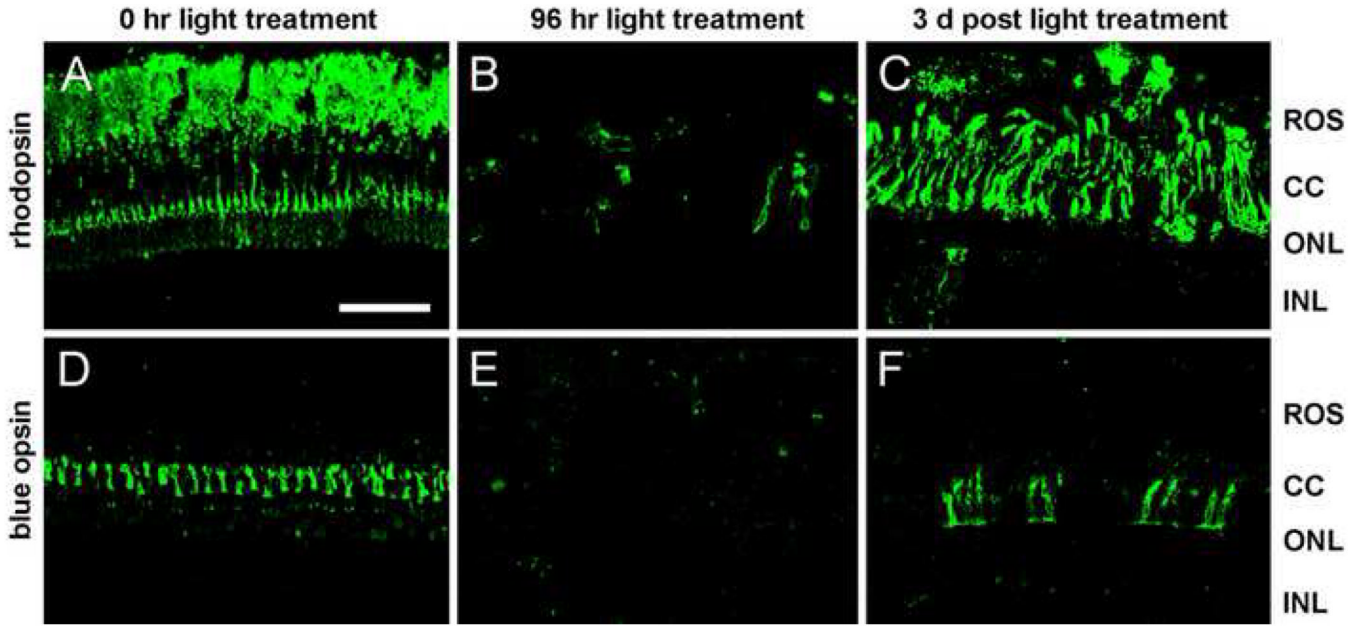

Figure 3. Regeneration of rod and cones at 3 days post light treatment.

Dark-adapted adult *albino* zebrafish were placed in constant intense light for either 0 (A, D) or 96 hours and then examined either immediately after (B, E) or 3 days after the 96-hour light treatment (C, F). The retinas were sectioned and immunolabeled with either anti-rhodopsin (A-C) or anti-blue cone opsin (D-F). At 0 hours of light treatment, rhodopsin labels rod outer segments (ROS) and blue opsin labels long single cones in the cone cell layer (CC) as previously reported (Vihtelic et al., 1999). Following 96 hours of constant intense light treatment, rhodopsin (B) and blue opsin (E) immunolabeling was largely undetected. However, as early as 3 days post-light treatment, both rhodopsin (C) and blue opsin (F) immunolabeling was beginning to return, suggesting that the rod and cone photoreceptors were starting to differentiate. Scale bar in Panel A is 25 microns. ROS, rod outer segments; CC, cone cells; ONL, outer nuclear layer; INL, inner nuclear layer.

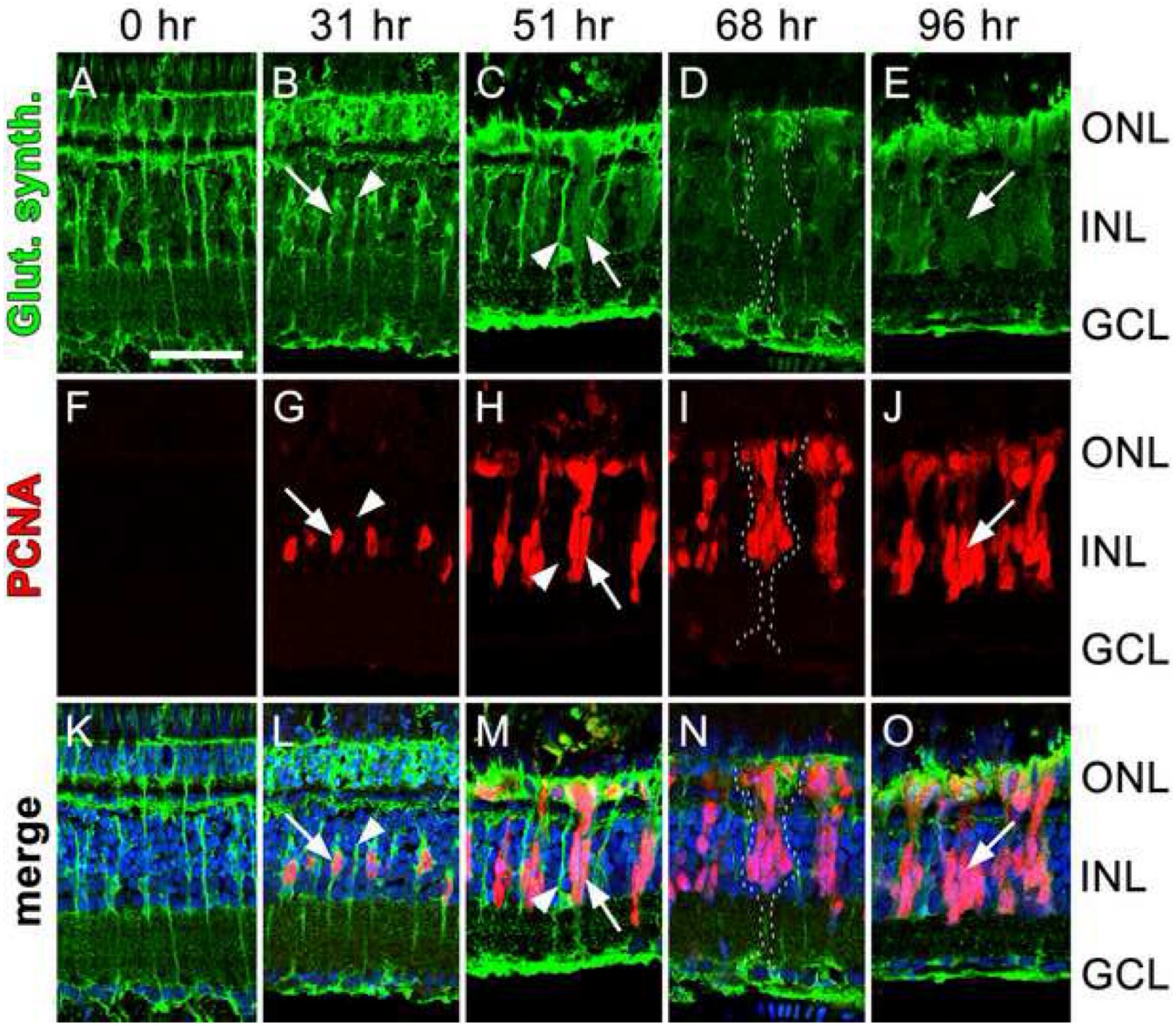

Figure 4. PCNA and glutamine synthetase expression during constant-light treatment.

Dark-adapted *albino* zebrafish were exposed to constant intense light for 0 (A, F, K), 31 (B, G, L), 51 (C, H, M), 68 (D, I, N) and 96 hours (E, J, O) and eyes were collected and processed for immunolabeling of glutamine synthetase (A-E, green), PCNA (F-J, red), or the two labels merged (K-O). At 0 hours, the glutamine synthetase evenly labeled the Müller glia and PCNA was not present. At 31 hours, the single PCNA-positive cells in the INL colocalized with a subset of glutamine synthetase-positive Müller glia (arrow), while the remaining glutamine synthetase-positive Müller glia remained PCNA-negative (arrowhead). By 51 hours, the Müller glia that were associated with clusters of PCNA-positive cells had hypertrophied and were expressing reduced levels of glutamine synthetase relative to the remaining Müller glia (arrow and arrowhead, respectively). At 68 and 96 hours, the Müller glia that were associated with clusters of PCNA-positive cells reached a maximal level of hypertrophy (dashed outline and arrow, respectively). Scale bar in Panel A is 25 microns. PCNA, proliferating cell nuclear antigen; GS, glutamine synthetase; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

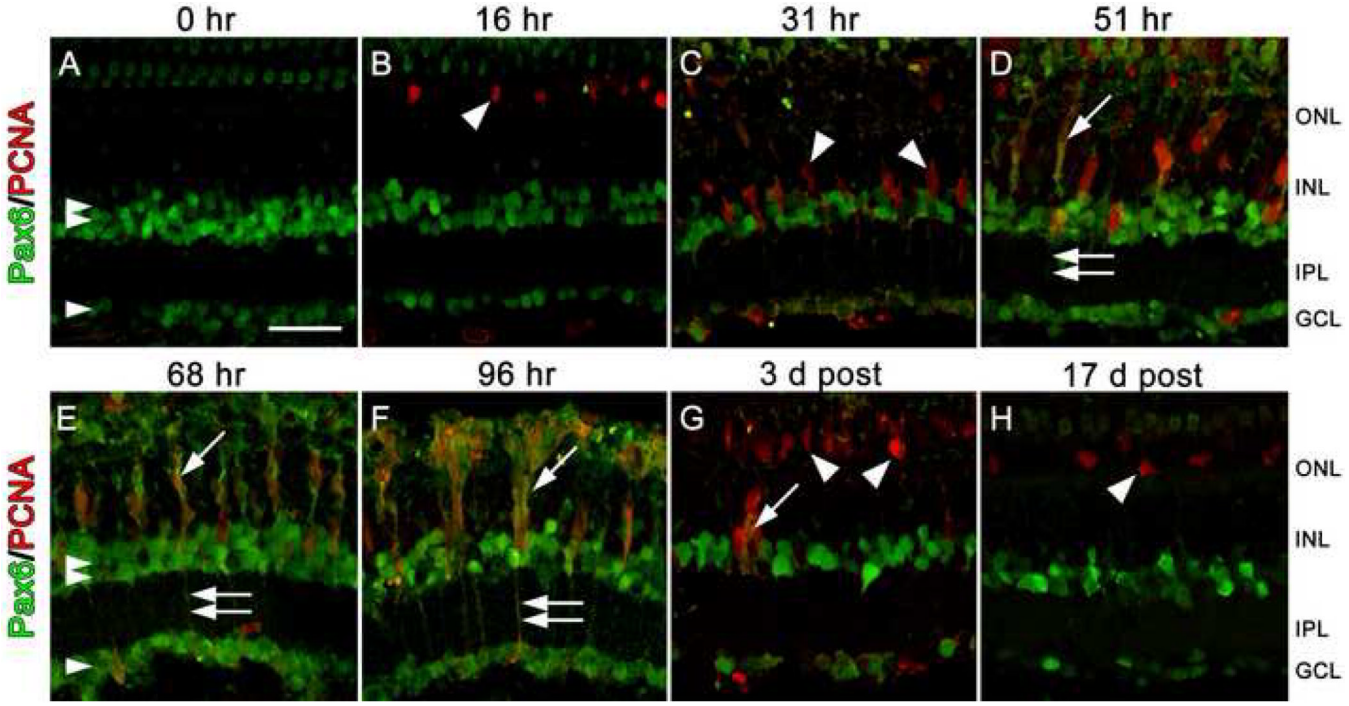

Figure 5. Pax6 expression in proliferating neuronal progenitor cells during regeneration of the light-damaged retina.

Dark-adapted adult *albino* zebrafish were placed in constant intense light for 0, 16, 31, 51, 68, and 96 hours. Two additional time points following 4 days of light exposure, 3 days post and 17 days post light treatment, were also analyzed. The retinas were sectioned and immunolabeled with anti-Pax6 (green) and anti-PCNA (red). At all time points, Pax6 expression was detected in the amacrine (A and E; double arrowheads) and ganglion cells (A and E; arrowhead). The PCNA-positive rod precursors (arrowhead in Panel B) were proliferating after 16 hours of light, but did not colabel with Pax6. At 31 hours of light treatment, Pax6 did not colabel with PCNA-positive Müller glia in the INL (C; arrowheads). At 51 hours, migrating PCNA-positive progenitor cells were expressing Pax6 (D; arrow). This is significantly later than when the Müller glia reentered the cell cycle and started to express PCNA (arrowheads in Panel C). At 68 and 96 hours of light treatment, Pax6 was expressed in the proliferating neuronal progenitors that were present in the INL and ONL (E and F; arrows). Pax6 expression was also detected in cellular processes in the IPL from 51 through 96 hours (D-F; double arrows). By 3 days post light, however, Pax6 did not colabel with PCNA-positive progenitors in the ONL (G; arrowheads). Pax6 expression returned to 0 hour control levels by 17 days post light treatment. Scale bar in Panel A is 25 microns. PCNA, proliferating cell nuclear antigen; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

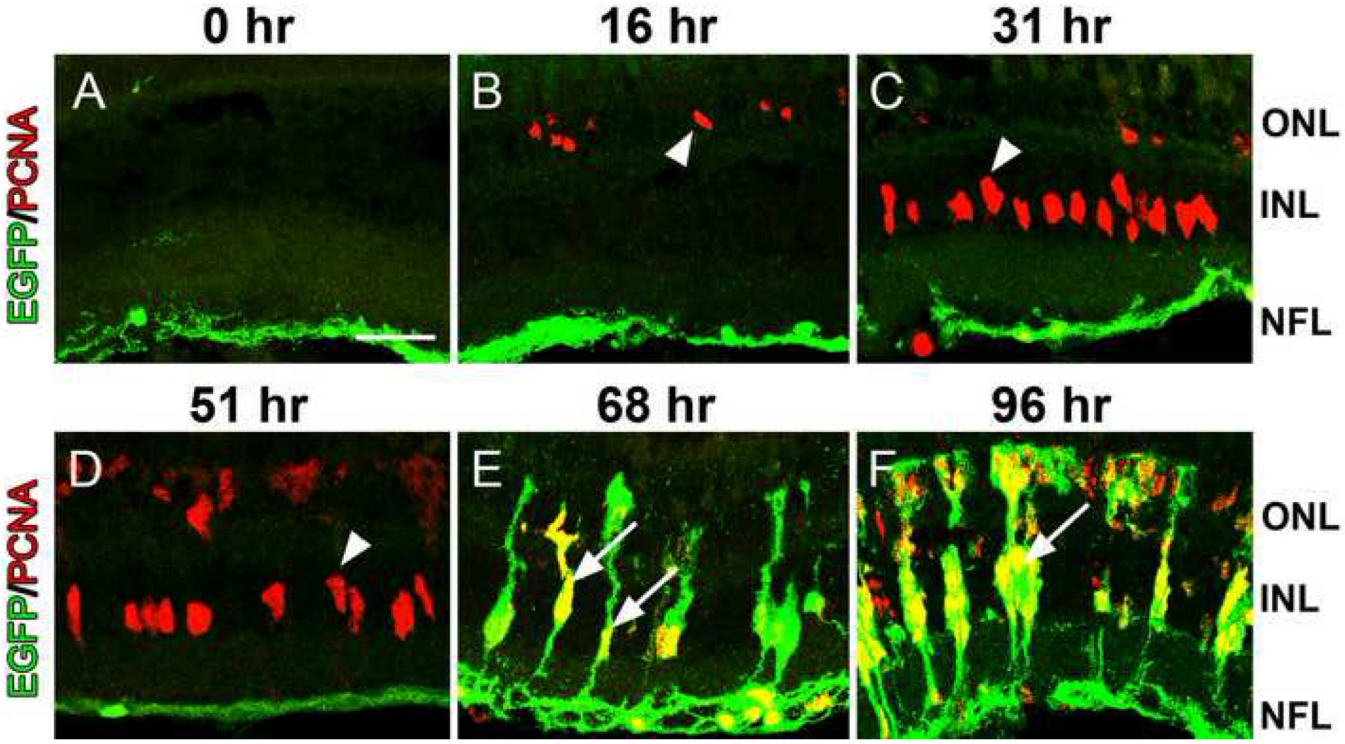

Figure 6. EGFP and PCNA expression during light-induced zebrafish retinal regeneration of the *Tg(olig2:EGFP)*^{vu12} transgenic fish line.

EGFP in the *albino Tg(olig2:EGFP)*^{vu12} adult zebrafish retina was expressed in the nerve fiber layer throughout the light treatment timecourse. At 16 hours of constant light, PCNA (red) was expressed in the rod precursor cells in the ONL (B; arrowhead). At 31 and 51 hours of constant light, PCNA-expressing Müller glia in the INL were observed (C and D; arrowhead). At 68 and 96 hours of constant light treatment, EGFP filled cells with a Müller glial morphology and colabeled with PCNA-positive nuclei in the ONL and INL (E and F; arrows). Scale bar in Panel A is 25 microns. PCNA, proliferating cell nuclear antigen; EGFP, enhanced green fluorescent protein; ONL, outer nuclear layer; INL, inner nuclear layer; NFL, nerve fiber layer.

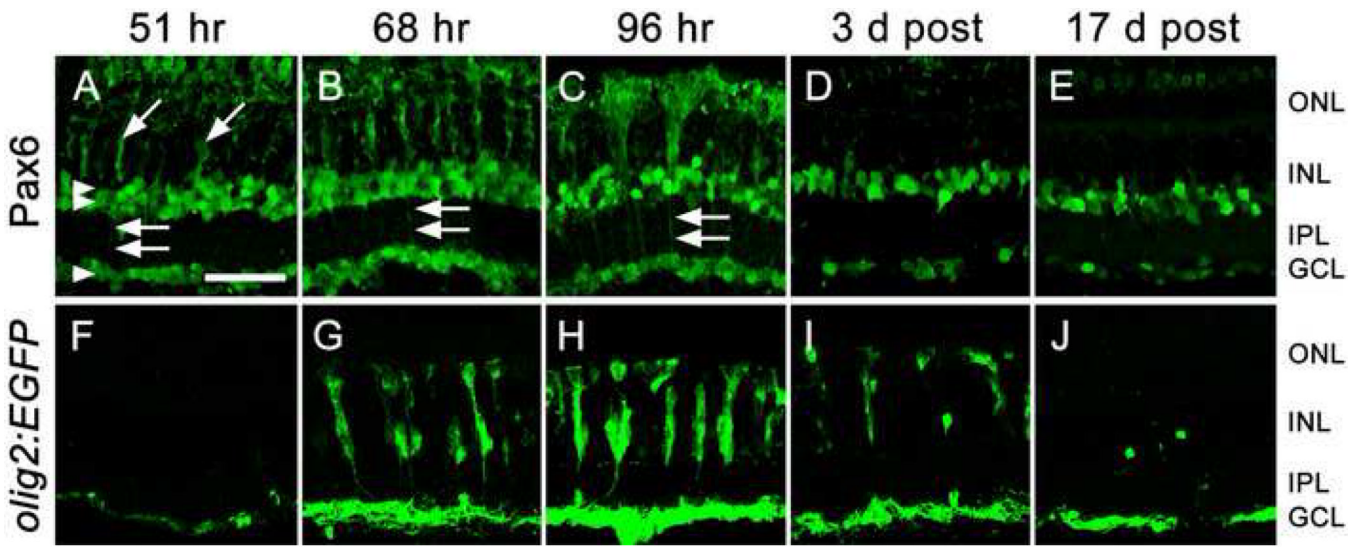

Figure 7. Pax6 expression precedes*olig2*expression in the regenerating retina.

To determine if expression of the *olig2:EGFP* transgene increased following the formation of Pax6-positive neuronal progenitors, dark-adapted adult *albino* (A-E) and *albino Tg (olig2:EGFP)*^{vu12} zebrafish (F-J) were simultaneous light treated for various lengths of time. Pax6 was observed in amacrine and ganglion cells at all timepoints (A; double and single arrowheads, respectively). Increased Pax6 expression was first detected in the columns of INL neuronal progenitors at 51 hours (A; arrows), and continued to be expressed in the progenitors until 3 days post light (B-D). Pax6 expression was also detected in cellular processes in the IPL from 51 through 96 hours (A-C; double arrows). EGFP expression from the *olig2:EGFP* transgene was not observed until 68 hours (G) and labeled dedifferentiated Müller glia until 3 days post light (H and I). Both Pax6 and *olig2:EGFP* were dramatically reduced in expression by 17 days post light (panels E and J, respectively). Scale bar in Panel A is 25 microns. *olig2:EGFP*, *olig2 promoter:enhanced green fluorescent protein transgene*; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

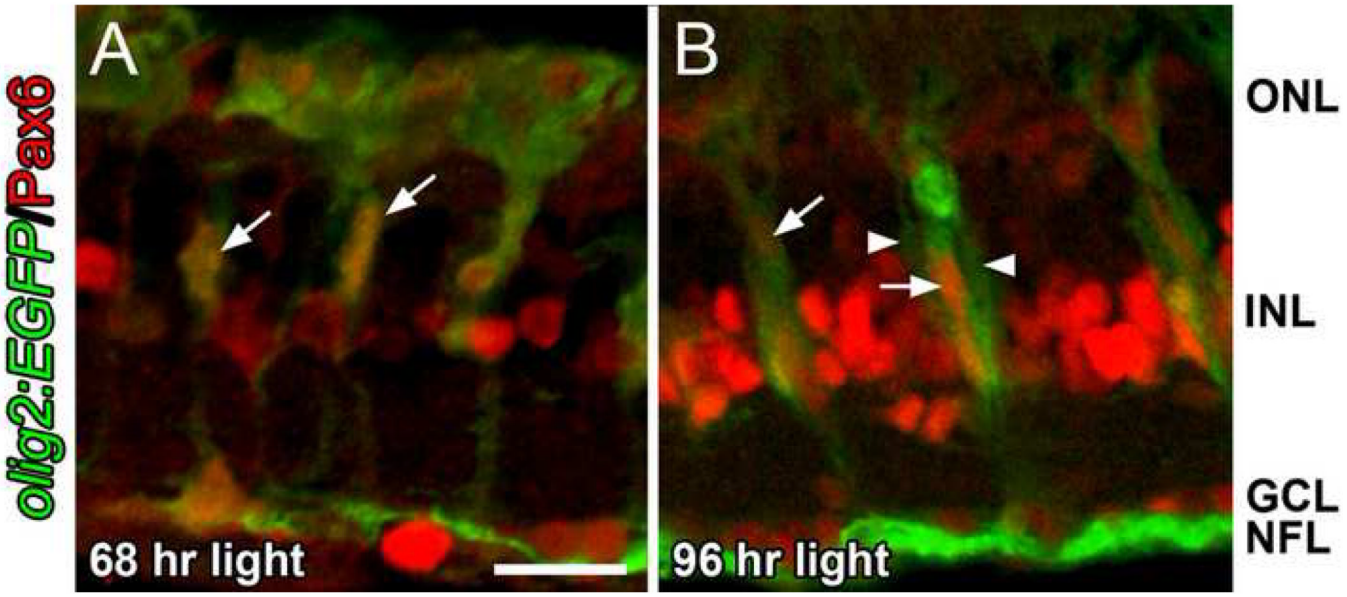

Figure 8. Colocalization of Pax6 and*olig2*in the regenerating retina.

Dark-treated *albino Tg(olig2:EGFP)*^{vu12} zebrafish were exposed to either 68 or 96 hours of constant intense light and immunolabeled for Pax6 (red) and EGFP (green), which is expressed form the *olig2* promoter. Amacrine and ganglion cells (small, spherical red cells in the INL and GCL, respectively) constitutively express Pax6 at both timepoints, while EGFP is constitutively expressed in the nerve fiber layer of the ganglion cells. Pax6-positive neuronal progenitor nuclei appear at both 68 and 96 hours (arrows) and are surrounded by or adjacent to EGFP-positive dedifferentiated Müller glia (arrowheads). While it is clear that the dedifferentiated Müller glia are *olig2*-positive and Pax6-negative, it is difficult to discern if the Pax6-positive neuronal progenitors are *olig2*-positive or negative. Scale bar in Panel A is 25 microns. *olig2:EGFP*, *olig2 promoter:enhanced green fluorescent protein transgene*; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; NFL, nerve fiber layer.

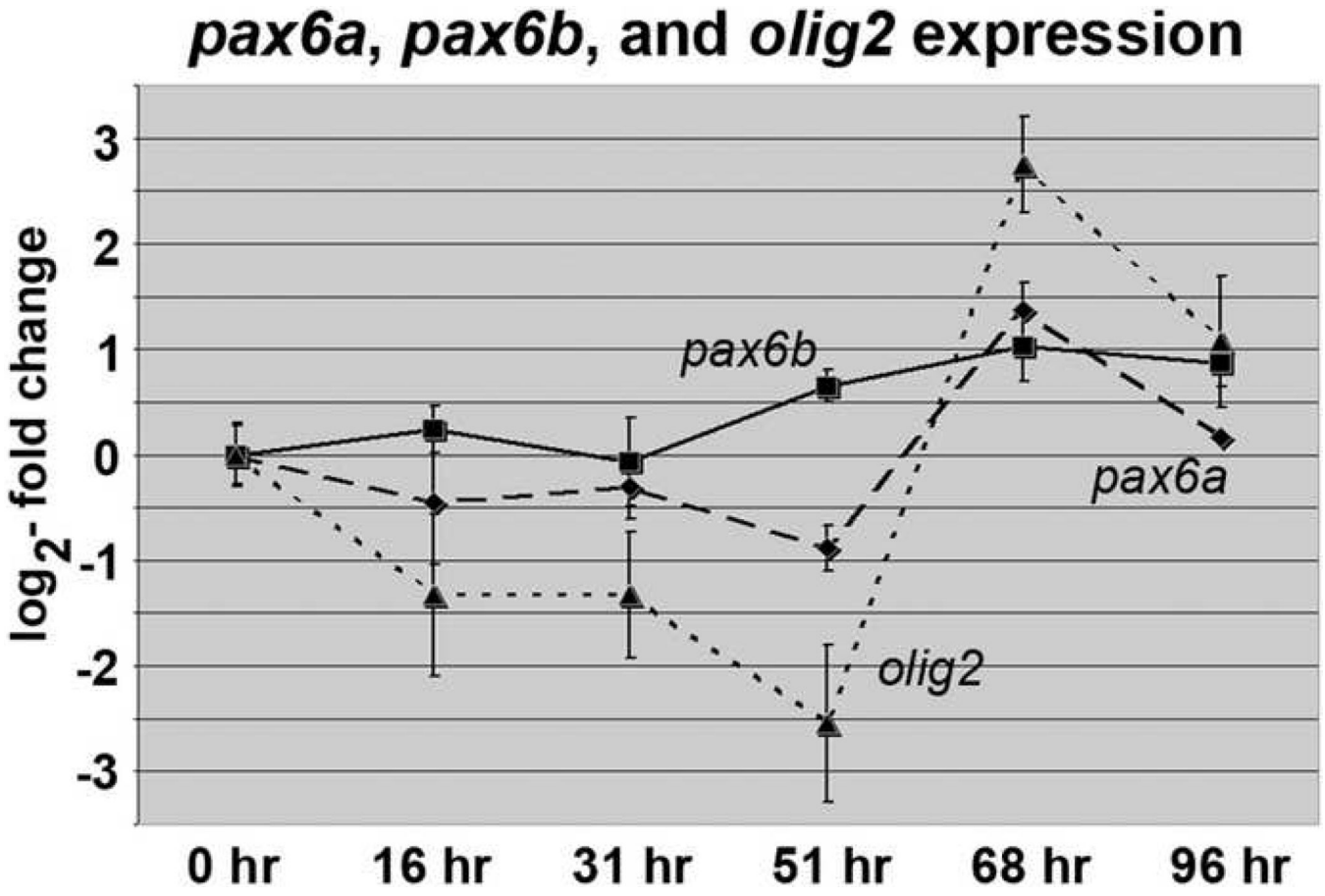

Figure 9. Expression patterns of*pax6a, pax6b*, and *olig2* by quantitative real-time PCR.

Dark-treated *albino* zebrafish were exposed to 0, 16, 31, 51, 68, and 96 hours of constant light treatment. RNA was isolated from pooled retinas at each time point for quantitative real-time PCR analysis. The $\log_2$-fold changes in the expression of *pax6a*, *pax6b*, and *olig2* were analyzed at each timepoint in biological and technical triplicates using gene-specific primers. Consistent with the immunohistochemical data, transcript levels for each gene remained near or below the 0 hour control levels through 31 hours of light treatment. Starting at 51 hours of light treatment, *pax6b* transcript levels increased (solid line with squares), while *pax6a* (long dashed line with diamonds) and *olig2* (short dashed line with triangles) levels decreased. At 68 hours of light, at which time the neuronal progenitors were rapidly proliferating, all three transcripts increased dramatically. These data support the immunohistochemical data that showed an increase in Pax6 expression prior to *olig2*. Using the Student's *t*-test, the differences between the *pax6a* and *pax6b* values at 16, 31, and 68 hours were not statistically significant, while they were statistically significant at 51 ($p = 0.004$) and 96 hours ($p = 0.003$). Vertical lines at each time point represent
the standard deviation of the means.

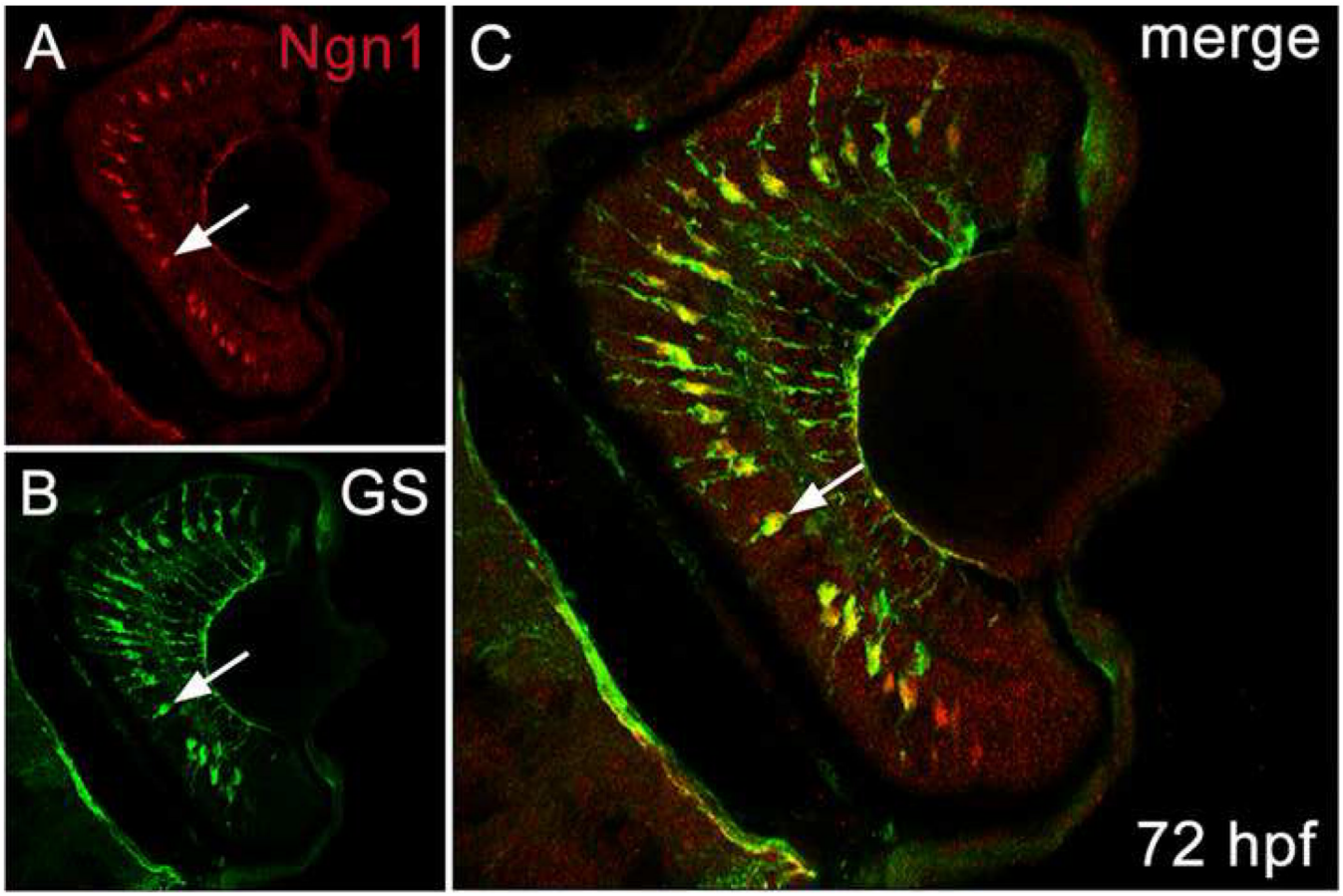

Figure 10. Polyclonal Ngn1 antiserum labels Müller glia in the developing retina.

Wild-type embryos were collected at 72 hours post-fertilization (hpf), processed and sectioned. Retinal sections were immunolabled with a polyclonal anti-Ngn1 antiserum (red; A and C) and a polyclonal anti-glutamine synthetase antiserum (green; B and C), which specifically labels Müller glia. The merged image (C) revealed that Ngn1 localization is restricted to the Müller glia nuclei (arrow) at 72 hpf. Ngn1, Neurogenin 1; GS, glutamine synthetase; hpf, hours post-fertilization.

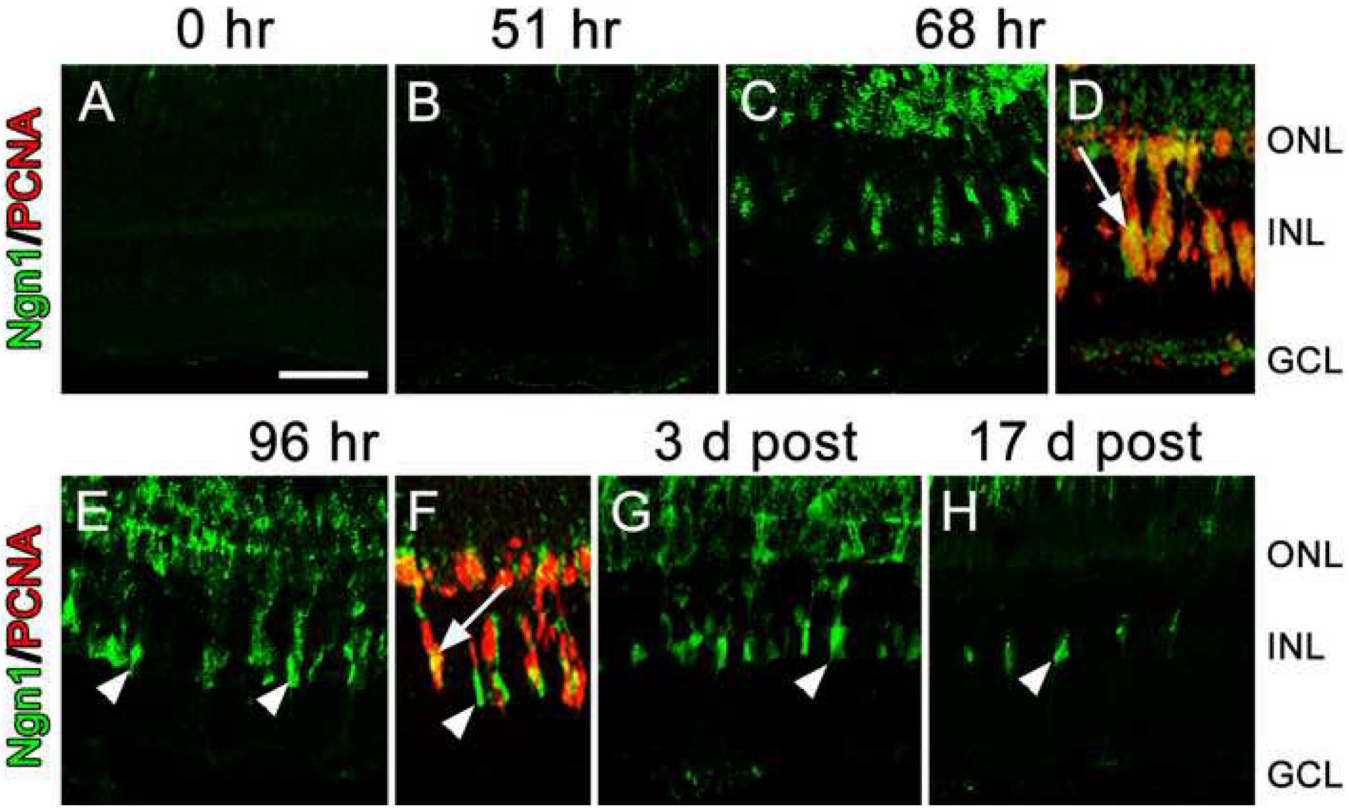

Figure 11. Ngn1 expression during regeneration of the light-damaged zebrafish retina.

Dark-treated *albino* zebrafish were exposed to constant intense light for 0, 51, 68, and 96 hours. Retinas were also examined at 3 and 17 days after the 96 hours of constant intense light treatment. The retinas were sectioned and immunolabeled for Ngn1 (A-H, green) and PCNA (D and F, red). Ngn1 expression was not observed until 68 hours (C and D, green) and colabeled with columns of PCNA-positive neuronal progenitors (D, arrow). At 96 hours of constant light treatment (E and F), some Ngn1-positive cells in the INL and ONL were also PCNA-positive (F, arrow); however, there were also Ngn1-positive cells in the INL that were PCNA-negative (F, arrowhead). By 3 days post-light treatment (G), Ngn1 expression was restricted to a small number of INL nuclei (arrowhead). These Ngn1-positive nuclei, which had the morphology of Müller glia, were still observed at 17 days post-light treatment (H, arrowhead). Scale bar in Panel A is 25 microns. PCNA, proliferating cell nuclear antigen; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

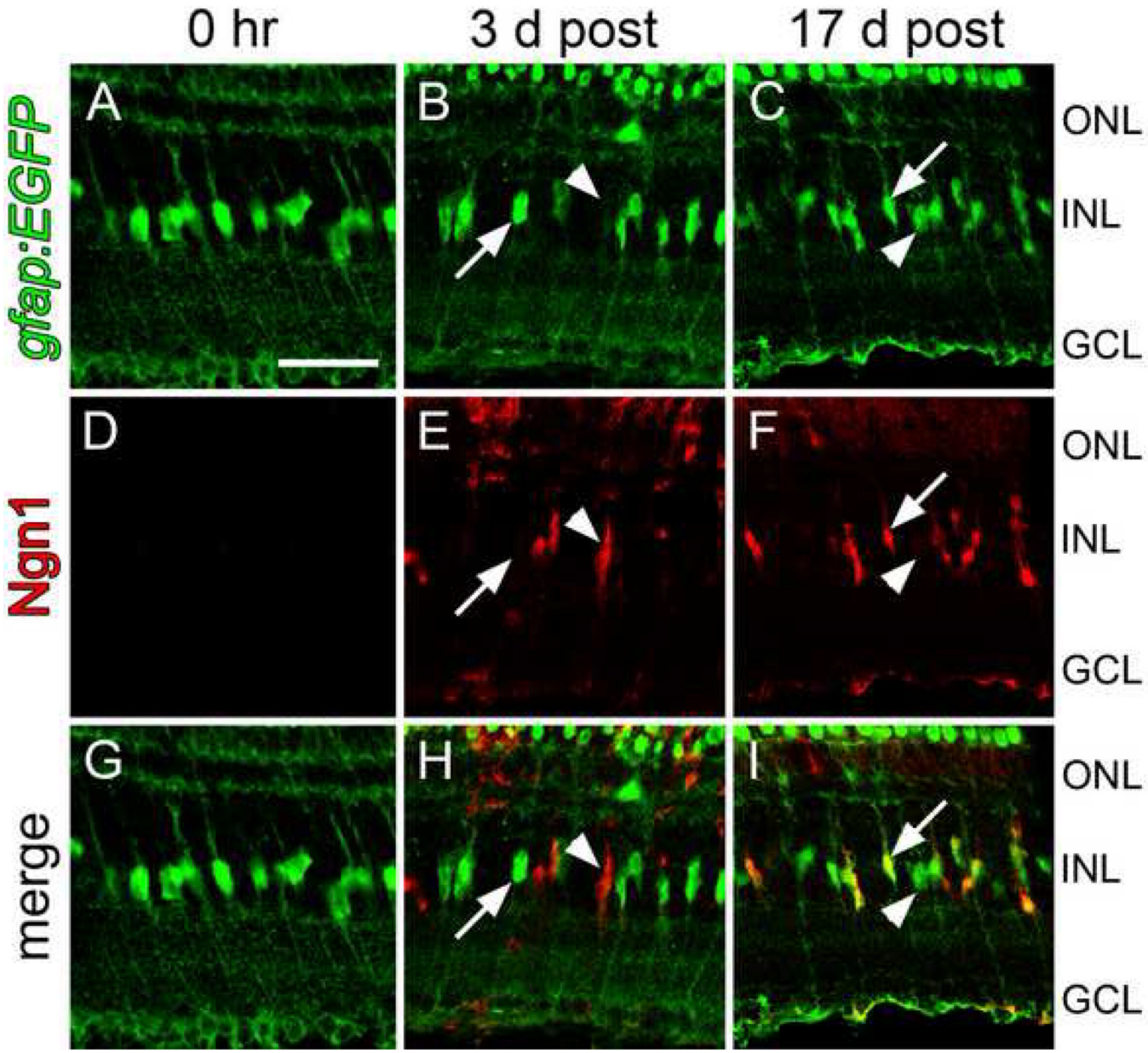

Figure 12. Anti-Ngn1 polyclonal antiserum labels post-mitotic Müller glia during light-induced zebrafish retinal regeneration.

In order to determine the nature of the Ngn1-positive nuclei and 3 and 17 days post light treatment, we analyzed Ngn1 expression in *albino Tg*(*gfap:EGFP*)^{nt11} zebrafish. Ngn1 (red) was not expressed in Müller glia, labeled by the *gfap:EGFP* transgene (green), at 0 hours of constant light treatment (A, D, and G). At 3 days post light treatment, a subset of Ngn1-positive cells did not colabel with Müller glia (arrowhead; B, E, and H). In addition, many EGFP-positive Müller glia were Ngn1-negative (arrow; B, E, and H). At 17 days post light treatment, a subset of Müller glial cells did not colabel with Ngn1 (arrowhead; C, F, and I), while a subset of Müller glia colabeled with Ngn1 (arrow; C, F, and I). This was consistent with the expression data from 72 hours post-fertilization embryos (Figure 10), in which Ngn1 labeled post-mitotic, immature Müller glia. These data indicate that Ngn1 labels immature Müller glial cells that are reestablished following the light treatment. Scale bar in Panel A is 25 microns.

gfap:EGFP, *glial fibrillary acidic protein promoter:enhanced green fluorescent protein transgene*; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.