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Responses of Müller glia to retinal injury in adult zebrafish

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Abstract

In an effort to identify the cellular events that enable neuronal regeneration in the vertebrate retina, the identity and characteristics of mitotic and apoptotic cells were examined in lesioned retinas of adult zebrafish. Following lesion a complex spatiotemporal pattern of mitosis was observed, including a delayed entry of Müller glia into the cell cycle. Characteristics of these proliferative Müller glia indicated they might serve as a stem/precursor cell of regenerated retina. The results suggested a model of retinal regeneration in which lesions are filled, in part, by a localized *en place* cytogenesis within intact retina surrounding the lesion site.

Keywords: Regeneration; Retina; Proliferation; Apoptosis

1. Introduction

The central nervous system (CNS) of most adult vertebrates, including humans, has a limited capacity for cellular repair. Whether a lesion involves damage to axonal pathways or a loss of entire neurons, few axons are re-grown and few neurons are regenerated to replace those that have been lost. This overall dearth of regeneration contributes to the significant, and often chronic, loss of neurological function that can accompany disease- or injury-induced damage to the cortex, spinal cord, retina, and other neural structures.

In some adult vertebrates, however, the CNS can regenerate. Pioneering studies of Matthey (1926), Sperry (1949), and others demonstrated that the neural retina of adult anamniotes (fish and amphibians) possess an ability to regenerate cells. The retinas of anamniotes have since proven to be valuable model systems for the investigation of cellular regeneration in the vertebrate CNS (reviews: Otteson & Hitchcock, 2003; Raymond & Hitchcock, 2000; Reh & Levine, 1998; Stenkamp & Cameron, 2002). Key advantages of the retina as a model system are that its cellular composition permits precise characterization of the cellular outcome of regeneration, and targeted examination of the specific cell type(s) that might serve as regenerative stem/precursor cells. Little is known, however, regarding the cellular and molecular mechanisms that enable regeneration in the adult vertebrate retina. By extension our understanding of why the retinas of most adult vertebrates, including humans, do not or cannot regenerate is correspondingly thin.

The aim of the current investigation was to identify cellular events that enable retinal regeneration in the zebrafish, a popular model system for the study of retinal development (Brockerhoff, Dowling, & Hurley, 1998; Easter & Malicki, 2002; Malicki, Pujic, Thisse, Thisse, & Wei, 2002). Experiments were performed to identify the spatiotemporal profiles of mitotic and apoptotic cells in the lesioned zebrafish retina, a structure known to regenerate (Cameron, 2000; Cameron & Carney, 2000; Vihtelic & Hyde, 2000). Surgical removal of a

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small portion of retina induced complex spatiotemporal profiles of mitosis and apoptosis, the former including a proliferative "activation" of Müller glia, a type of radial glia that is present in all vertebrate retinas (Dowling, 1987) and which has previously been identified as a potential stem/precursor cell (e.g., Fischer & Reh, 2001; Wu et al., 2001). Apoptotic cells were observed in the lesioned retina but were limited primarily to rod photoreceptors, suggesting that proliferative Müller glia do not necessarily progress to cellular death. Proliferative Müller glia also displayed morphological features suggestive of other stem/precursor cells within the CNS (Kakita & Goldman, 1999; Morest & Silver, 2003). Lastly, proliferative cells displayed evidence of radial migration, with Müller glia perhaps providing a conduit for this dispersal.

The results indicate that the retina of adult zebrafish responds to lesion with complex patterns of apoptosis, mitosis, and cellular migration. Because they participate in the latter two phenomena, Müller glia are hypothesized to contribute mechanistically to the retina's ability to regenerate. The results further motivated the development of a cellular model of retinal regeneration in which lesions sites are resolved, at least in part, by a localized *en place* cytogenesis within intact retina surrounding the lesion.

2. Methods

Zebrafish (*Danio rerio*) ranging in standard length from 2.5–3.2 cm were used for all experiments, and appropriate animal care guidelines were followed. Fish were maintained in plastic tanks using standard procedures (Westerfield, 2000). The methodology for inducing retinal injury was similar to that reported previously (Cameron & Easter, 1995; Hitchcock, Myrh, Easter, Mangione-Smith, & Jones, 1992). Anesthetized fish received an excision of dorsal retina from the right eye (approximately 0.25 mm^2 in area), approximately halfway between the optic nerve head and the retinal margin, and were returned to their tanks for various survival periods ranging from 1 to 21 days. At least three animals were analyzed for each experimental condition and time point. Two hours prior to euthanasia some fish were transferred to tank water that contained 5 mM 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO), a thymidine analogue that is incorporated into the DNA of cells within S phase of the cell cycle. The timing of these experiments was controlled so that the BrdU exposure began exactly 2 h prior to euthanasia.

The methodology for preparing and processing retinas for cryosectioning and indirect immunohistochemistry (IHC) was as previously described (Cameron & Carney, 2000; Cameron, 2000). Mounted sections were stored at −80 °C until processed for IHC, in situ hybridization, or apoptosis analysis. The primary antibodies used in the IHC analyses recognized proliferating cell nuclear antigen (PCNA; Chemicon; Temecula, CA), BrdU (Amersham; Piscataway, NJ), zebrafish carbonic anhydrase (zCAH), or glutamine synthetase (GS; the latter two antibodies provided by Paul Linser, Florida State University). For anti-BrdU labeling the sections were briefly exposed to low pH (15 min, 2N HCl/PBS/0.3% Triton-X) to disrupt the structure of genomic DNA; this procedure had no discernable adverse affect upon the labeling efficacy of the other antibodies. The labeling patterns of the primary antibodies were detected with Cy2- or Cy3-conjugated antibodies raised against the immunoglobulins of the primary antibodies' source animal (mouse or rabbit; Jackson Immuno Research; West Grove, PA). Sections screened with the antibodies were visualized with standard epifluorescence microscopy, and digital images were captured (MetaMorph; Universal Imaging; Downingtown, PA). Great care was taken to ensure the accurate identification and digital capturing of immuno-positive cells, particularly double-labeled cells. Quantification of the mitogenic activity in control and lesioned retinas was achieved by counting from reacted sections, for all retinal layers, the number of BrdU- or PCNA-positive cells within 100 μm-wide bins. The counts derived from all retinal layers were combined to derive the "Total" count function (see Fig. 3). In lesioned retinas these bins originated at the ventral edge of lesion sites, and in control retinas the bins originated at corresponding retinal location. For each experimental time point at least three sections were analyzed from each of three different fish (control and lesioned). Reacted slides were stored at −20 °C.

Non-isotopic in situ hybridization was performed using a digoxigenin-labeled cRNA probe that recognized the mRNA encoding for zebrafish rhodopsin. Briefly, mRNA was isolated from 4–8 dark-adapted zebrafish retinas (QuickPrep Micro mRNA Purification kit; Amersham), from which first-strand cDNA was synthesized (First Strand cDNA Synthesis kit; Amersham). Samples of mRNA were tested with PCR for genomic DNA contamination and discarded if such contamination was detected. Sequence-specific primers for zebrafish rhodopsin (Genbank accession #AF331797; Vihtelic, Doro, & Hyde, 1999) were prepared (forward: 5′-TCTACAACCCCTGCATCTAC-3′; reverse: 5′-CCTCTACAATCACAACAAAGCC-3′; Integrated DNA Technologies; Coralville, IA) and using the first-strand cDNA as a template, PCR was used to amplify a partial-length fragment, approximately 600 base pairs in length, encoding for rhodopsin. The amplified product was purified by gel electrophoresis (QiaQuick; Qiagen; Valencia, CA) and cloned into pCR4-TOPO (Invitrogen; Carlsbad, CA); the identity of the cloned inserts

was confirmed by sequencing (Biotechnology Resource Center, Cornell University; Ithaca, NY). Plasmid DNA from expanded, transformed colonies (TOP10; Invitrogen) was purified and used as a template for an additional round of PCR using M13 forward and reverse primers (Integrated DNA Technologies). The linear, amplified product was purified as above, and 10 μg of material was used as a template for the synthesis of complementary digoxigenin (DIG)-UTP-labeled RNA, using T3 or T7 DNA-dependent RNA polymerase (Roche; Indianapolis, IN). Dot blot analysis with an alkaline phosphatase-conjugated anti-DIG antibody (anti-DIG-AP; Roche) was used to confirm the presence of DIG-labeled product. Working aliquots of the RNA probes (1500 ng) were stored at −20 °C, and used for non-isotopic in situ hybridization analysis of retinal cryosections, using an NBT/BCIP chromogen visualization procedure similar to that described previously (Barthel & Raymond, 1993; also Manglapus and Schwob, personal communication). Digital images of reacted slides were collected.

Apoptotic cells were detected in cryosections with the terminal UTP nick-end labeling (TUNEL) technique, using a commercial kit (ApopTag; Serologicals, Norcross, GA). The kit's procedure was modified so that labeled cells were visualized with the same anti-DIG-AP and NBT/BCIP visualization procedure as above. In double label experiments apoptotic cells were labeled first, using the procedure above with the Vector Red chromogen (Vector Laboratories; Burlingame, CA) replacing NBT/BCIP, followed by indirect IHC detection of zCAH- or GS-positive cells with a Cy2-conjugated secondary antibody.

3. Results

3.1. Spatiotemporal profile of proliferative cells in lesioned retina

The spatiotemporal distribution of retinal cells that were induced by lesion to progress through the cell cycle was determined. Progression through the cell cycle was independently assessed by either cellular incorporation of the thymidine analogue BrdU, or positive immunoreactivity for proliferating cell nuclear antigen (PCNA). In control, unlesioned retinas there were few mitotically active cells evident in the retina (data not shown), consistent with earlier reports utilizing the adult zebrafish model system (Marcus, Delaney, & Easter, 1999; Cameron, 2000), but unlike other adult fish species (Julian, Ennis, & Korenbrot, 1998; Otteson, D'Costa, & Hitchcock, 2001; Raymond & Rivlin, 1987).

At 1 d post-lesion there was little evidence for proliferative cells in extant retina, including regions proximal to the lesion site. At this time point the few observed proliferative cells were typically located within the axonal fiber layer or within the lesion site itself (Fig. 1A), and were assumed, based upon earlier work, to include microglia and cellular components of the immune system, respectively (Lillo et al., 2001). At 2 d post-lesion proliferative cells were evident within the extant retina (Fig. 1B). These proliferative cells were located primarily within 100 μm of the lesion site, in the outer and inner nuclear layers (ONL and INL). Based upon their laminar location the cells in the ONL are likely to include rod precursor cells (Johns & Fernald, 1981; Raymond & Rivlin, 1987). Identification of proliferative cells in the INL is considered below. These results indicated that retinal lesion induces a delayed, proliferative activation

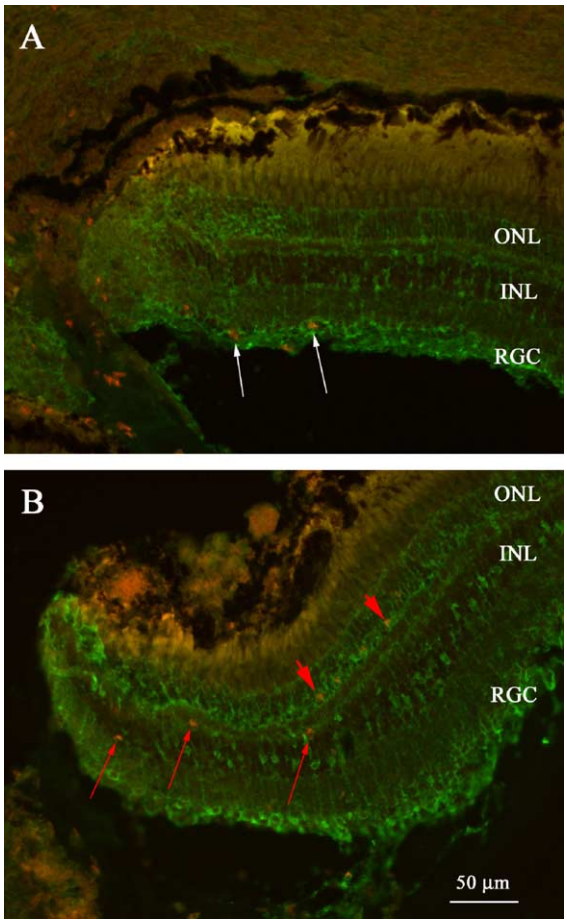

Fig. 1. Short-term cellular proliferation in lesioned zebrafish retina. Indirect IHC techniques were used to screen retinas for cellular incorporation of BrdU (red) and carbonic anhydrase immunoreactivity (zCAH, green), a marker for Müller glia (see Section 2). (A) at 1 d post-lesion there are few proliferative cells in the retina, with the few cells being located at the axonal fiber layer (white arrows) or at the edge of the lesion site (white arrowheads, left side of panel). *B*, At 2 d post-lesion proliferative cells are evident in the extant retina, located within the inner nuclear (INL, long red arrows) and outer nuclear layers (ONL, short red arrows). Note the distance of proliferative cells from the lesion edge (left side of panel). The former include rod precursor cells (Raymond and Rivlin, 1987). RGC, retinal ganglion cell layer.

of cells that are intrinsic to the ONL and INL of retina surrounding a lesion site.

At 7 d post-lesion a large number of proliferative cells was evident within both the ONL and INL, where they were typically observed as clusters (Fig. 2; cf. all panels of Fig. 1). The spatial distribution of the proliferative cells indicated that the elevated numbers were present contiguously up to 800 μm from the lesion site (Fig. 3A), suggesting either (a) that proliferative cells are originating, and migrating laterally away, from sites proximal to the lesion, and/or (b) the operation of a long-range, proliferation-inducing mechanism(s) that is spatially centered upon the lesion site. The long-range triggering mechanism is judged to be more likely because of the lack of evidence for tangentially oriented cells with migrational morphology (i.e., nuclei with fusiform profiles that are parallel to the retinal sheet; Fig. 6), and the lack of evidence for a "wave" of proliferative cells originating from the lesion site and moving into surrounding retina as a function of time. Between 600–800 μm from the lesion edge an abrupt diminution in the number of proliferative cells was consistently observed, particularly within the INL, with considerably fewer, but non-zero, numbers of proliferative cells evident beyond 800 μm from the lesion site (Figs. 2B and 3A). The overall amplitude of cellular proliferation was estimated by integrating under the "Total" function (Fig. 3A, see Section 2) up to 800 μm, and multiplying by the total estimated number of 15 μm-thick sections, perpendicular to the tangent of the lesion edge, that could be accommodated within the annulus of retina surrounding the lesion site (assuming an approximate annular circumference of 5.3 mm). This estimate revealed that at 7 d post-lesion >33,000 cells are progressing through the cell cycle within the retina surrounding the lesion site (which represents between 5% and 10% of the total retinal area), approximately 67% of which are located within the INL (Fig. 3A). This observation indicated a degree of cellular proliferation that is four orders of magnitude greater than that observed in control retina.

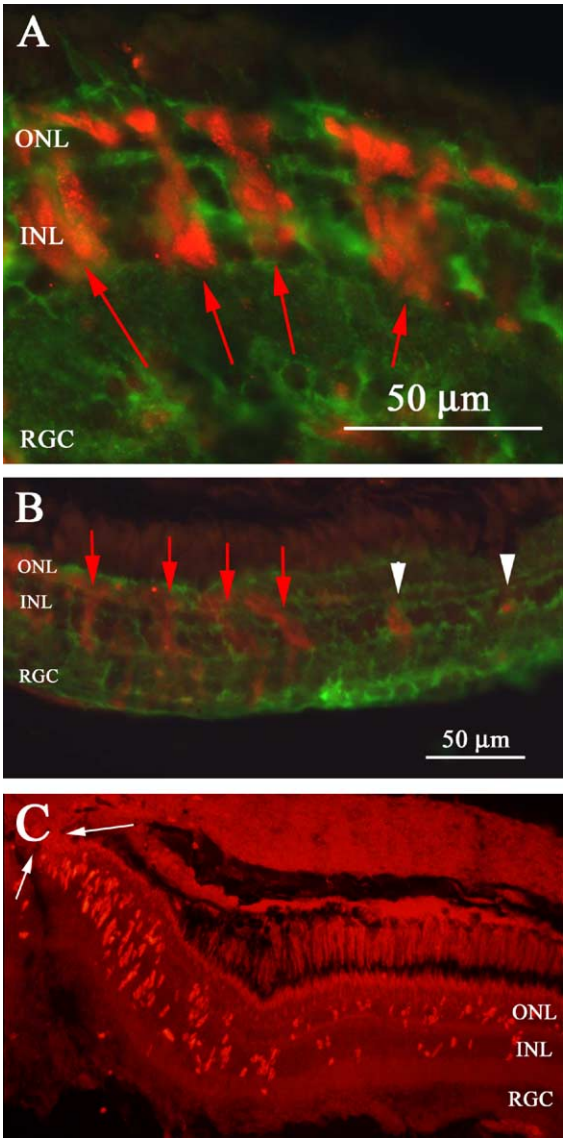

Fig. 2. Clustering of proliferative cells in lesioned zebrafish retina. (A) at 7 d post-lesion proliferative cells in the extant retina, particularly within the INL, are often observed as clusters (red arrows). The imaged region is approximately 250 μm from the lesion site. (B) with distance away from a lesion site (off panel to the left, approximately 500 μm away), there is a relatively homogeneous distribution of proliferative cells (red arrows) up to approximately 600–800 μm from the lesion site, after which there is a sudden diminution of proliferative cells (white arrowheads). Quantification of this effect is presented in Fig. 3 (see Section 2). (C) low magnification view of the distribution of BrdU-positive cells relative to the edge of a lesion site (between the two arrows), at 7 d post-lesion. Scale bar as in panel B.

A similar spatial profile of cellular proliferation was observed at 14 d post-lesion, although with a smaller amplitude than at 7 d post-lesion (Fig. 3B). Cellular proliferation diminished further at 21 d post-lesion (Fig. 3C), beyond which time the proliferation profile returned to the control condition. The overall amplitude of cellular proliferation was estimated as above, and revealed that >12,000 and >4000 cells in extant retina are progressing through the cell cycle at 14 and 21 d post-lesion, respectively. The amplitude of cellular proliferation suggested a mechanism for substantial neurogenesis within intact retina surrounding a lesion site (see Section 4).

These analyses indicated a complex spatiotemporal profile of cellular proliferation in lesioned retina of adult zebrafish. This profile was characterized by a delayed cellular proliferation within a large but restricted area of retina surrounding the lesion site, particularly within the INL, followed by a time-dependent diminution in the number of proliferative cells within the same spatial area. Mechanistically, the data suggest that retinal lesion induces, following a temporal delay of 1–2 d, a mito-

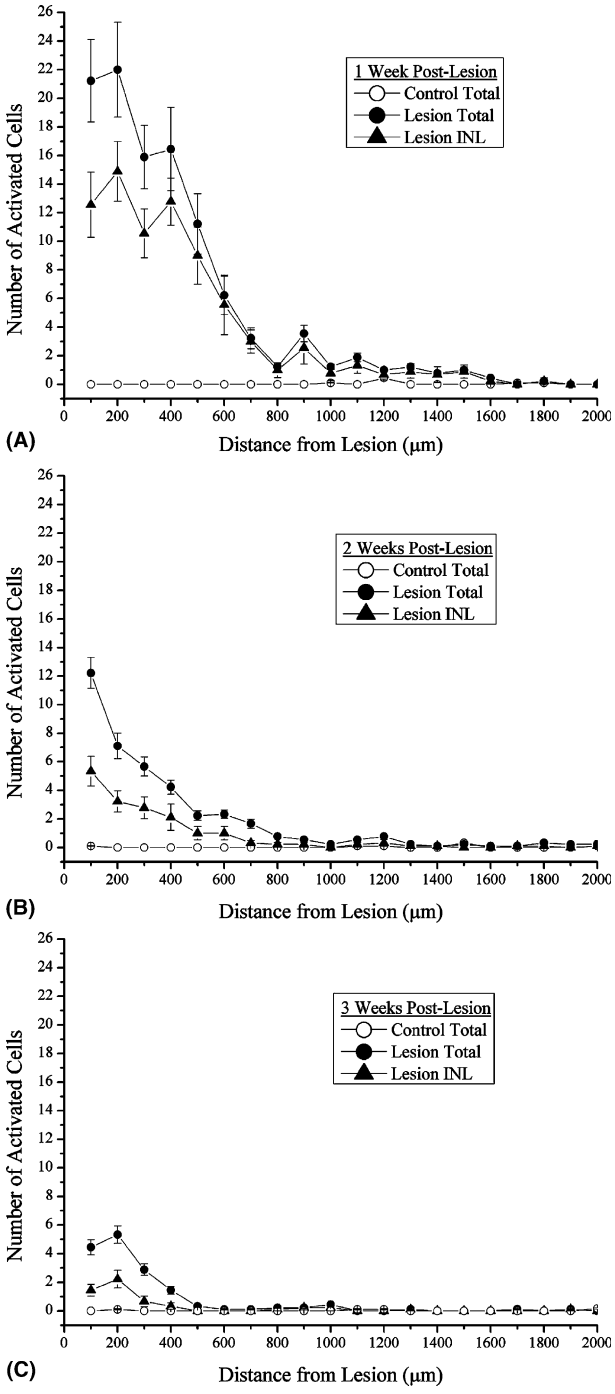

Fig. 3. Quantitative analysis of the spatiotemporal distribution of proliferative cells in lesioned zebrafish retina. The number of proliferative cells within defined retinal lamina (ONL/OPL, INL, IPL, and RGC) was determined within 100 μm-wide bins, with the lesion edge serving as the origin (see Section 2 for additional details). The data are displayed as mean ± standard error. (A) At 7 d post-lesion there is a significant elevation in the total number of proliferative cells within extant retina (solid circles, "Lesion Total" function), with the majority of proliferative cells located within the INL (triangles). This elevation extends to approximately 800 μm from the lesion edge. Thereafter, occasional, punctate loci of proliferative cells are observed, to approximately 1700 μm from the lesion edge. In control retinas there is a near-zero number of proliferative cells throughout central retina (open circles). (B) At 14 d post-lesion there remains a significant elevation in the number of proliferative cells compared to control (symbols as in panel A), although the amplitude of proliferation is diminished compared to 7 d post-lesion. (C) At 21 d post-lesion the cellular proliferation remains evident, although the amplitude and spatial extent of proliferation are diminished compared to earlier time points. Beyond this time point the profile of cellular proliferation returns to near-control levels.

genic triggering mechanism(s) that originates at or proximal to the lesion site, operates over relatively long temporal and spatial extents, and induces responsive cells in the surrounding retina to progress through the cell cycle.

3.2. Müller glia contribute to the population of proliferative cells in lesioned retina

Previous studies have suggested that glial cells can function as neuronal precursors within the CNS (review: Goldman, 2003). To investigate if glial cells contribute to the population of proliferative cells in lesioned zebrafish retina (i.e., if they are candidate stem/precursor cells), double labeling IHC experiments were performed using independent markers for cell cycle passage and Müller glia. By 5 d post-lesion direct evidence for the presence of proliferative Müller glia was observed: cells double-labeled with markers for cell cycle progression (e.g., BrdU) and carbonic anhydrase (zCAH, a marker for Müller glia; Linser, Smith, & Angelides, 1985; Peterson, Fadool, McClintock, & Linser, 2001) were present in extant retina (Fig. 4). Selectivity of the antibody markers was supported by the simultaneous presence of zCAH+/BrdU+, zCAH−/BrdU+, and zCAH+/BrdU− cells within local fields of the retina (cf. arrowheads and arrows, Fig. 4). Similar results were obtained in double labeling experiments that utilized anti-GS, an independent marker for Müller glia (Fig. 5). Proliferative Müller glia were observed with anti-BrdU (Figs. 4 and 5) and anti-PCNA labeling (Fig. 6B), indicating that (a) the double-labeled profiles were not due to non-specific labeling of the markers for cell cycle progression, and (b) both the BrdU-incorporation and PCNA-labeling techniques were valid indicators of cell cycle progression. As above, selectivity of the combined zCAH and PCNA markers was confirmed (arrows, Fig. 6B). These results indicate that Müller glia, which in control retina

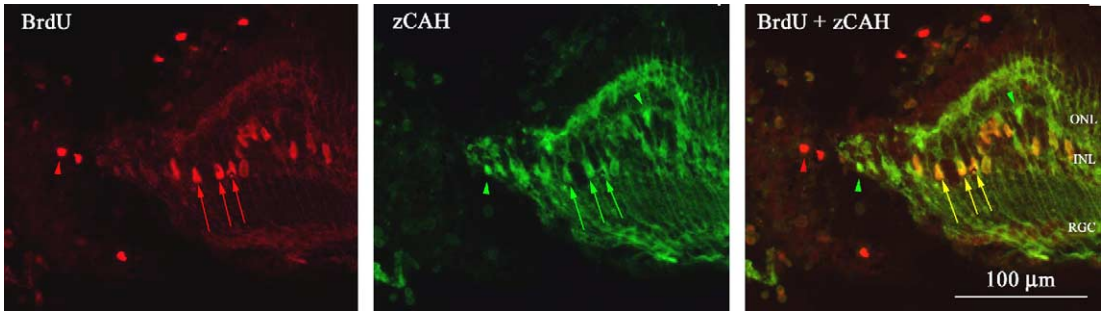

Fig. 4. Evidence that Müller glia progress through the cell cycle subsequent to retinal lesion. All panels present the same sample at equivalent magnification, which was screened for both BrdU incorporation (left panel, Cy3; red) and zCAH immunoreactivity (center panel, Cy2; green). In each panel the lesion site is at the left, best observed as the termination of the zCAH label in the center and right panels. At 5 d post-lesion proliferative cells (BrdU-positive) are observed in extant retina, including cells located within the INL (e.g., red arrows); BrdU-positive cells are also located outside the retina, including within the lesion site (e.g., red arrowhead). In the same section zCAH-positive cells (Müller glia) are located within central retina, with their somata located within the INL (e.g., green arrows and arrowheads). Combining the two images (right panel) reveals the presence of BrdU-positive Müller glia (e.g., yellow arrows). Cells that are labeled with one of the antibody markers, but not the other are also evident in the combined image (arrowheads; note that not all Müller glia are BrdU-positive), indicating selectivity of the antibody markers, as well as optical resolution of the fluorescence signals.

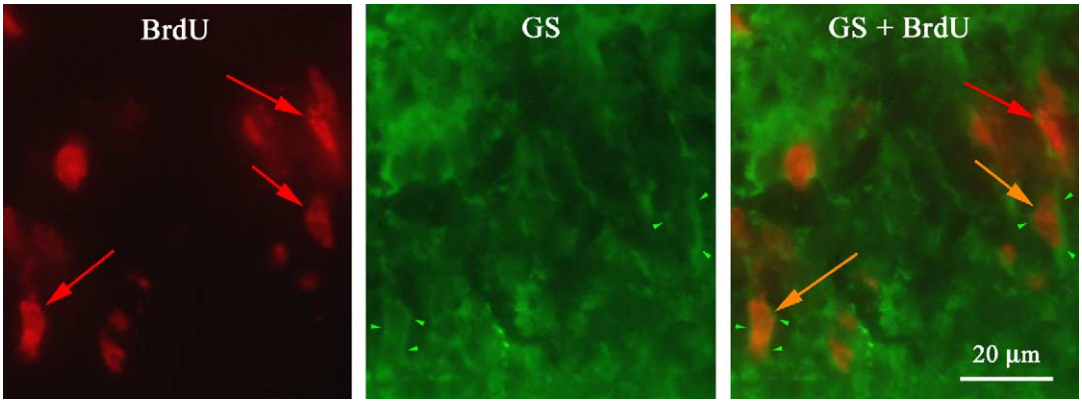

Fig. 5. Evidence that Müller glia progress through the cell cycle subsequent to retinal lesion. As in Fig. 4, all panels present the same sample, at 5 d post-lesion. In this case the section was screened for both BrdU incorporation (left panel, Cy3; red marker and arrows) and GS immunoreactivity (center panel, Cy2; green marker and arrowheads). Combining the two images (right panel) reveals the presence of BrdU-positive Müller glia (e.g., orange arrows). Note the BrdU-positive nuclei indicated by the orange arrows are enveloped by a thin cytoplasmic "shell" of GS-positive material (green arrowheads). As in Fig. 4, cells that are labeled with one of the antibody markers, but not the other, are evident in the combined image, indicating both selectivity of the antibody markers and optical resolution of the fluorescence signals.

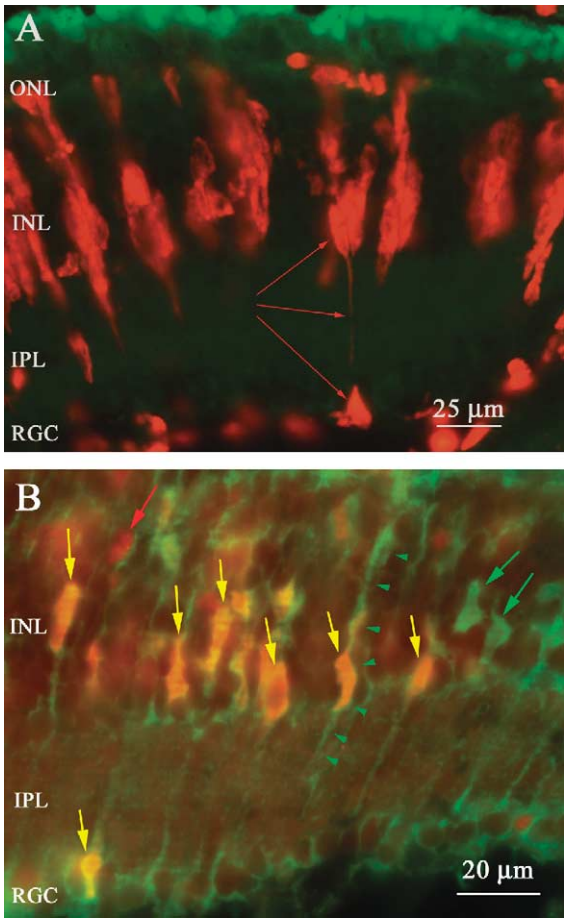

Fig. 6. Spatial dispersal and identity of proliferative cells in lesioned zebrafish retina. The panels are from two different retinas, and the views are approximately 300 µm from a lesion site, off panel to the right. (A) during the second week post-lesion proliferative (12 d post-lesion) BrdU-positive cells (red) that are located primarily within the INL display evidence of nuclei that extend into the inner plexiform layer (IPL; red arrows). Labeled nuclei are also present that extend between the ONL and INL. These labeling patterns are consistent with cellular migration between the retinal lamina, within the radial dimension of the retina. Proliferative cells are also present within the RGC layer. The apparent "green" signal at the outer retina is actually auto-fluorescence of cone ellipsoids. (B) evidence that Müller glia contribute to the radial dispersal of proliferative cells, and that proliferative Müller glia could potentially function as stem/precursor cells. Firstly, proliferative cells with a fusiform profile, that are not labeled with the Müller glia marker, are observed in close association with radial-extending processes of Müller glia (e.g., red arrow), suggesting that proliferative cells migrate along the Müller glia through the radial depth of the retina. As in panel A, no corresponding evidence for cellular migration within the tangential plane of the retina is observed. Secondly, proliferative Müller glia (PCNA+/zCAH+; yellow profiles and arrows) display a physical profile suggestive of intracellular nuclear kinetic mechanisms: nuclei are present throughout the depth of the INL, some nuclei appear to be extending toward and into the IPL, and some zCAH+ nuclei are observed proximal to the RGC layer (note double-labeled profile at the left side of the panel). The spatial profile of Müller glia processes, including those of proliferative cells (*e.g.*, green arrow heads) is consistent with a physical substrate for intracellular nuclear kinesis within this plane and to the observed extent. Specificity of antibody labeling is indicated by the simultaneous presence of PCNA+/zCAH− (red arrow), PCNA+/zCAH+ (yellow arrows), and PCNA−/zCAH+ cells (green arrows).

of adult zebrafish rarely, if ever, progress through the cell cycle (control data, Fig. 3) do so in response to retinal lesion. Because regenerated retina arises, at least in part, from mitotic stem/precursor cells (e.g., Hitchcock et al., 1992; Wu et al., 2001; Cameron, 2000), these data further suggest that Müller glia could be a source of regenerated retinal cells.

Additional evidence was observed that proliferative Müller glia could potentially function as an induced stem/precursor cell in the lesioned zebrafish retina. The nuclei of Müller glia are normally restricted to the INL (Figs. 4 and 6B). By the end of the first week post-lesion, nuclei of some proliferative (PCNA-positive) Müller glia retina were observed extending into the inner plexiform layer (Fig. 6B). Additionally, some PCNA-positive profiles that were co-localized with a Müller glia marker were also observed in the area of the "end foot", a specialized structure that is located proximal to ganglion cell somata and axons (Fig. 6B). Because such "activated" nuclei resided within the spatial extent of immunoreactivity appropriate for Müller glia (but also microglia; see Section 4), and inasmuch as they were located at atypical positions within the physical dimensions of Müller glia cells, the operation

of an intracellular, nuclear kinetic mechanism was suggested (Das, Payer, Cayouette, & Harris, 2003). Intracellular nuclear kinesis has been identified as a characteristic of precursor cells in the CNS (Kakita & Goldman, 1999; Morest & Silver, 2003), and its hypothesized presence in this system indirectly suggests that proliferative Müller glia perform a stem/precursor cell function in lesioned retina.

3.3. Müller glia are spared from lesion-induced apoptotic cell death

Using the TUNEL method cells exhibiting evidence of DNA cleavage consistent with apoptotic cell death were observed, principally during the first week post-injury. Few TUNEL-positive cells were observed in control retina (data not shown). The apoptotic cells in lesioned retina were primarily located within the ONL, proximal to the lesion site, in spatial correspondence with a region in which rhodopsin expression is absent (cf. Fig. 7A and B). This data was consistent with the hypothesis that the population of apoptotic cells within lesioned retina was composed primarily of rod photoreceptors. The number of cells exhibiting evidence of apoptosis decreased with time after lesion, such that few apoptotic cells were observed in the retina by 14 d post-lesion (data not shown).

In lesioned retina there was little evidence for apoptosis within the population of Müller glia. Qualitative inspection revealed that the number of proliferative Müller glia greatly exceeded the number of TUNEL-positive cells observed in the INL during the first week following lesion (cf. Figs. 4–6B and 7B). To directly determine if Müller glia exhibit evidence of apoptosis, double label experiments were performed using the anti-zCAH antibody combined with the TUNEL technique (see Section 2). Consistent with the data presented above, in which relatively few cells in the INL exhibited evidence of apoptosis (Fig. 7B), no TUNEL-positive Müller glia were observed (Fig. 7C). This data further suggested that subsequent to retinal lesion, Müller glia that progress through the cell cycle do not necessarily progress to apoptotic cell death.

3.4. Müller glia contribute to the radial dispersal of proliferative cells

If a spatially restricted precursor of regenerated retinal cells exists, such as Müller glia, a mechanism should also exist to disperse the precursor cells (or their prog-

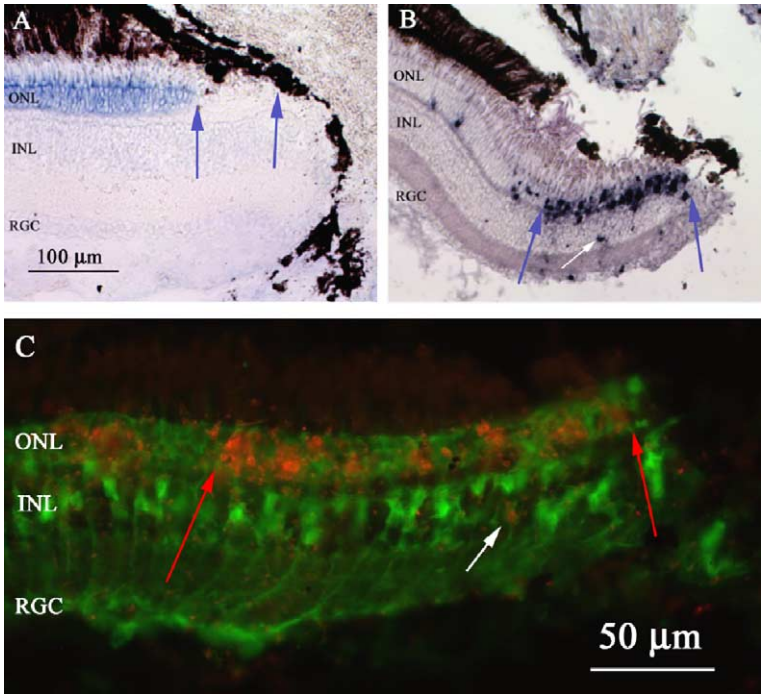

Fig. 7. Spatial profile and identity of apoptotic cells in lesioned zebrafish retina. All panels represent retinas at 6 d post-lesion, with lesion sites to the right. (A) rhodopsin expression in lesioned retinas is restricted to the ONL, as expected (blue reaction product). Proximal to the lesion edge there is no evidence of rhodopsin expression, even though nuclei are evident within the ONL (region between the two blue arrows). (B) the TUNEL technique of apoptosis detection revealed labeled cells primarily within the ONL, proximal to the lesion edge. The spatial location and extent of this region corresponded well with the region in panel A within which there was no rhodopsin expression, suggesting that rod photoreceptors are a principal contributor to the population of cells undergoing apoptosis. Relatively few TUNEL-positive cells were observed in the INL (white arrow). *C*, Double labeling for apoptosis (red; see Section 2) and Müller glia (anti-zCAH, green) indicated the presence of apoptotic cells within the ONL proximal to the lesion site (region between the two red arrows). As above, evidence of apoptosis was observed in the INL (white arrow) but few, if any, zCAH-positive cells were TUNEL-positive.

eny) to the other nuclear layers of the retina. Evidence for dispersion mechanisms was observed in lesioned retina. At post-lesion time points prior to 5 d, proliferative cells were restricted to the retina's nuclear layers, and were absent from the plexiform layers. After 5 d post-lesion, however, and especially during the second week following lesion, individual BrdU-positive cells were observed at the INL extending, as relatively thin processes, into the inner or outer plexiform layers (Fig. 6A). Because the BrdU label is restricted to nuclei, and BrdU-positive nuclei were not observed in the retina's plexiform layers at earlier time points, the data indicated that such BrdU-positive cells were dispersing out from (or moving into) the INL. Furthermore, dispersal of proliferative cells was observed no earlier than 5 d post-lesion (cf. all panels of Figs. 1, 4, and 6A), suggesting that as a function of time, cellular dispersal is secondary to the lesion-induced triggering of cell cycle progression.

Double label experiments suggested that Müller glia provide a spatial conduit for the dispersal of proliferative cells across the retina's radial dimension. Specifically, proliferative, non-Müller glia cells were observed in close association with the zCAH-positive processes of Müller glia (e.g., red arrow of Fig. 6B). Along with their spatial association with Müller glia these proliferative cells displayed a fusiform profile reminiscent of that associated with migrating cells, and the long dimension of these profiles was parallel to the radially oriented Müller glia processes. These cytoarchitectural features are consistent with the operation of cellular migration mechanisms, and suggest that proliferative cells can disperse radially to all retinal layers by migrating along the processes of Müller glia (Noctor, Flint, Weissman, Dammerman, & Kriegstein, 2001). The presence of cellular migration parallel to the retinal tangent (i.e., parallel to the retinal nuclear and plexiform layers) could not be ruled out, although the expected morphological profile of such migrating cells—a fusiform or pyriform nuclear profile with the long axis parallel to the retinal tangent—was not observed.

4. Discussion

4.1. Cellular mechanisms of retinal regeneration—Müller glia as a potential stem/precursor cell

Regenerated cells in the adult fish retina are produced by mitotic stem/precursors. Although the identity of these stem/precursor cells remains unclear, the list of candidate cell types is quite long and includes rod precursor cells, cells within the circumferential germinal zone, and other retinal cell types (Otteson & Hitchcock, 2003). It is known, however, that in order to regenerate a retina with the proper cellular components and laminar organization a candidate stem/precursor cell must meet certain requirements. First-order requirements of a stem/precursor cell in this model system are progression through the cell cycle, survival subsequent to cell cycle passage, and that the stem/precursor cells, or their progeny, disperse to other retinal layers.

Because previous studies have implicated glial cells as neuronal precursors in the CNS (review: Goldman, 2003), we performed experiments to determine if Müller glia are a candidate stem/precursor cell of regenerated retina in the adult zebrafish. Recent investigations have explicitly identified Müller glia as a potential, regenerative stem/precursor cell in the vertebrate retina (Fischer & Reh, 2001; Wu et al., 2001; reviews: Fischer & Reh, 2003; Garcia & Vecino, 2003), and the current study supports the hypothesis that Müller glia in zebrafish retina display requisite characteristics of stem/precursor cells. Firstly, Müller glia proximal to a lesion site progress through the cell cycle. Secondly, Müller glia survive passage through the cell cycle (that is, Müller glia induced to proliferate do not invariably exhibit subsequent evidence of cell death). Thirdly, Müller glia may exhibit cellular phenomena associated with stem/precursor cells in other parts of the CNS (Kakita & Goldman, 1999; Morest & Silver, 2003). Microglia, however, are also labeled with anti-zCAH (Peterson et al., 2001) and so it remains possible that some double-labeled cells in zebrafish retina are not Müller glia but rather microglia that, in response to lesion, have entered the cell cycle and migrated into the neural retina proper, perhaps as far as the INL. Injury-induced proliferation of microglia at the inner surface of fish retina has been reported previously (Cameron & Easter, 1995; Lillo et al., 2001).

Because they can survive entry into the cell cycle it follows that proliferative Müller glia could potentially engage in subsequent stem cell functions, such as de-differentiation into transitional cell types (e.g., neuroblasts, neuronal precursors) from whence regenerated retinal neurons are produced. Furthermore, evidence was observed that Müller glia contribute to the dispersal of proliferative cells by providing a radially oriented conduit for cellular migration (see Mack, Papanikolaou, & Lillo, 2003). Ongoing experiments are aimed at determining if progression of Müller glia through the cell cycle is a necessary and/or sufficient component of cellular regeneration in the adult zebrafish retina, if the proliferative activation of Müller glia can be uncoupled from retinal damage *per se*, and if there is a direct lineage from proliferative Müller glia to regenerated retinal neurons.

The signaling mechanism(s) that triggers Müller glia to progress through the cell cycle is unknown. Recent evidence from chicken retina suggests that FGF2 and insulin-like growth factors (IGF) could perform this triggering function (Fischer, McGuire, Dierks, & Reh, 2002). Furthermore, a mitogenic role for IGF signaling has been demonstrated in the retina of adult goldfish

(Boucher & Hitchcock, 1998a, 1998b) and a similar, mitogenic effect of IGF, and FGF2, has also been observed in the retina of adult zebrafish (Shears and Cameron, unpublished observations). The current study does provide some insight into the operational characteristics of the mitogenic triggering mechanism. Firstly, the spatial profile of cellular proliferation suggests the operation of a signal that originates at, or proximal to, the lesion site itself, and is operational for hundreds of microns away from the lesion edge. This spatial profile is suggestive, although not formally definitive, of a diffusible/paracrine signaling mechanism, and we note that both FGF2 and the IGFs are diffusible agents. Secondly, the triggering mechanism functions during a restricted temporal window subsequent to retinal lesion. These spatiotemporal characteristics provide a clear, specific set of parameters that must be matched by any hypothesized triggering mechanism.

In lesioned mammalian retinas Müller glia provide a cellular basis for sub-retinal fibrosis (a "glial scar") and proliferative vitreoretinopathy (Fisher & Lewis, 2003), phenomena that might have non-, and perhaps anti-, regenerative effects upon the neural retina. That Müller glia might, in some model systems, function as an inducible stem/precursor cell of regenerated retinal neurons raises the possibility of molecularly dissecting the attendant mechanisms with precise cellular resolution. A recently described mutant strain of zebrafish, termed *lazy eye*, manifests a degeneration of Müller glial cells (Kainz, Adolph, Wong, & Dowling, 2003), making it a potentially interesting system for investigating such mechanisms. Furthermore, by implicating Müller glia as a candidate stem/precursor cell, a cellular and molecular basis is established for directly determining why a corresponding cellular function is apparently not displayed in the retinas of other adult vertebrates, including humans.

4.2. A mechanistic model of lesion resolution in the zebrafish retina

The results of the current study motivated the development of a mechanistic model of retinal regeneration (Fig. 8). This model incorporates cellular phenomena known to occur subsequent to retinal injury, including the progression of Müller glia through the cell cycle and the atypical production of new retinal cells outside lesion sites (Cameron & Easter, 1995; Cameron, 2000). The main feature of the model is the prediction that the resolution of a retinal lesion is achieved, at least in part, by cytogenic events that occur within the extant, nominally intact retina surrounding a lesion site. This mechanism of lesion resolution, termed *en place* regeneration, is distinct from those previously described in the adult fish retina, including the scattered neurogenic foci mechanism, which operates in experimental conditions in which virtually all retina has been destroyed (Raymond, Reifler, & Rivlin, 1988; Stenkamp, Powers, Carney, & Cameron, 2001), and the wound blastema mechanism (Hitchcock et al., 1992), which involves the production and appositional addition of regenerated cells directly at the lesion edge. A combination of all three mechanisms may contribute to retinal regeneration in this and other systems.

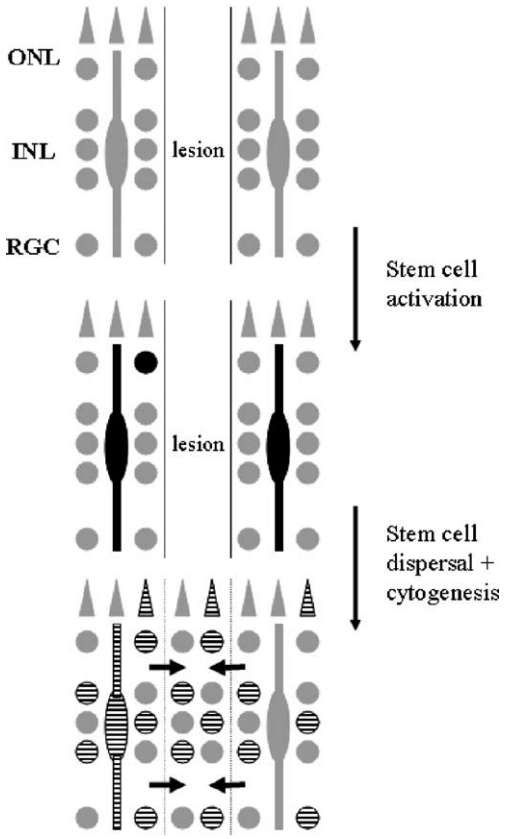

Fig. 8. A cellular model of *en place* regeneration in the lesioned zebrafish retina. Based upon the results of this and earlier investigations, the following cellular mechanism of lesion resolution in the adult zebrafish retina is suggested. Subsequent to the loss of a patch of retina (top panel), there is a delayed proliferation of cells within the ONL and INL (dark profiles, middle panel). The population of proliferative cells includes Müller glia (elongated profiles extending throughout the retina's radial depth). Secondarily to the proliferation event, there is a radial dispersal of proliferative cells via cell migration, with Müller glia functioning as a conduit (see Section 3; Fig. 6). The proliferative cells are hypothesized to be stem/precursor cells, and in that role produce new retinal cells within the extant retina (Cameron and Easter, 1995; Cameron, 2000), a process that is here termed *en place* regeneration. These new cells, because they occupy three-dimensional space, cause the extant retina to expand. It is hypothesized that a free vector of expansion is toward, and into, the lesion site (arrows, bottom panel). The lesion site is thus ultimately filled, in part, by pre-existing (grey profiles) and new retinal cells (hatched profiles); the latter include cells produced through the proposed, *en place* regeneration mechanism. This cellular model of retinal regeneration does not rule out the operation of other regenerative mechanisms, and it is hypothesized to function in parallel with the wound blastema mechanism of lesion closure (Hitchcock et al., 1992).

The model begins with a retina within which a localized lesion has been introduced (Fig. 8, top). Following

a delay of approximately 2–3 d, two distinct cellular events occur within the surrounding retina: an activation (cell cycle progression) of cells located in the ONL and INL (Fig. 8, middle), followed by a radial dispersal of proliferative cells and/or their progeny. The results of the current study indicate that Müller glia contribute to both phenomena, but other potential stem/precursor cell-types might be involved, including the microglia discussed above, and the rod precursors located within the ONL (Raymond et al., 1988). In the model the proliferative stem/precursor cells begin to produce new retinal cells, including neurons that are interstitially added into the extant retina (Fig. 8, bottom). A conservative estimate of the amplitude of lesion-induced cellular proliferation reveals that $>10^5$ retinal cells progress through the cell cycle following lesion, implying that a comparable number of new, differentiated cells might be produced within the extant retina (note the spatial profile of apoptosis in Fig. 7). A proposed effect of this substantial, *en place* cytogenesis is the physical expansion of extant retina. That is, the new cells, as they are added into the retina, physically push aside the pre-existing cells. We postulate that a permissive vector of expansion is toward, and into, the lesion site (Cameron & Easter, 1995). Thus, the lesion site becomes occupied by a combination of newly generated cells (hatched profiles, Fig. 8) and pre-existing cells, the latter having formerly resided outside the lesion site proper (grey profiles, Fig. 8). Evidence for this type of *en place* cytogenesis in lesioned fish retina has previously been reported (Cameron & Easter, 1995), and it is also known that some of these new cells arise from mitotic precursors (Cameron, 2000). The new model suggests that *en place* regeneration contributes directly to wound closure, and the current report suggests that Müller glia could be the underlying stem/precursor cells of this regenerative mechanism.

Within this model regenerative neurogenesis occurs in such a way that the proper number, types, and positions of retinal cells are restored within the lesion site. Because regenerated retina in the zebrafish contains few differentiated cells that are located within inappropriate lamina (Cameron & Carney, 2000), we further speculate that at each retinal lamina the proliferative, dispersed stem/precursor cells access position-dependent signals that help to determine the identity of regenerated cells at that location. In this way, for example, position-dependent cues within the ONL might help to favor the local production of photoreceptors, as opposed to ganglion cells. Alternatively, position dependent cues may serve as attractant/repellent factors that direct the radial dispersal of differentiated cells to their proper retinal lamina. We further hypothesize that homotypic, lateral inhibitory mechanisms that affect cell fate decisions—which have been implicated during normal zebrafish retinal growth (Cameron & Carney, 2004)—also contribute to the identity and spatial patterning of cells within regenerated retina.

Lastly, the ability of an otherwise intact retina to engage in *en place* regeneration is of potential significance to retinal lesions that are restricted to distinct cell types. Several diseases of the human retina, such as glaucoma, macular degeneration, and retinitis pigmentosa, result in a semi-selective loss of retinal cells, at least early in the disease process. For example, macular degeneration primarily involves a loss of photoreceptors, whereas glaucoma involves a loss of ganglion cells (Chader, 2002; Harwerth et al., 2002). Because the current investigation suggests that a proliferative activation of Müller glia could result in an *en place* regeneration of retinal cells, it raises the possibility of identifying, and ultimately harnessing, a similar cellular mechanism for the targeted restoration of specific cell types in the damaged, but otherwise cellularly intact, human retina.

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