Proliferation and Expression of Progenitor and Mature Retinal Phenotypes in the Adult Mammalian Ciliary Body after Retinal Ganglion Cell Injury

Philip E. B. Nickerson,¹ Jason G. Emsley,² Tanya Myers,¹ and David B. Clarke¹,³

PURPOSE. Despite the identification of a small population of cells residing in the ciliary body (CB) of the adult mammalian eye that have the capacity to generate retina-like cells in vitro, their activity in vivo remains quiescent. The authors sought to identify whether the predictable and time-dependent death of retinal ganglion cells (RGCs) results in activation of progenitor-like cells within the CB.

METHODS. RGC injury was induced by optic nerve axotomy in adult mice. Thymidine-analogue lineage tracing and immunocytochemistry were used to identify dividing cells and the phenotype of newly generated progeny.

RESULTS. Two populations of nestin-expressing cells are present in the CB of the uninjured eye. One population resides in periendothelial cells of blood vessels, and a second resides in the ciliary epithelium. Axotomy increases proliferation in the CB, a response that begins before the onset of RGC death and continues during a time that corresponds with the peak in RGC death. In addition, a subpopulation of nestin-positive cells in the CB upregulates the homeodomain protein Chx10. Finally, recoverin, the expression of which is normally restricted to like cells within the CB.

CONCLUSIONS. Together, these results suggest that progenitor-like cells of the CB respond to cues associated with the loss of a single retinal cell type and that a subpopulation of those cells may differentiate into a cell that bears phenotypic resemblance to those seen in the retina. (Invest Ophthalmol Vis Sci. 2007; 48:5266–5275) DOI:10.1167/iovs.07-0167

The vertebrate retina originates from a population of retinal progenitor cells (RPCs) in the embryonic primordium of the diencephalon. During retinogenesis, a temporally choreographed sequence of progenitor proliferation and subsequent induction of retinal cell phenotypes is initiated. These distinct cellular phenotypes, which constitute all seven neural cell types of the adult retina, emerge in a well-characterized hierarchical order.¹,² Throughout adulthood, the neurogenic capacity of the vertebrate eye is attenuated; in the mammalian eye, it is virtually abolished. Continually active postnatal and adult RPCs have been described in the lower vertebrate eye, including cells located at the border of the retina and adjacent epithelium, termed the ciliary margin (CM) of the amphibian eye³ and retinal pigmented epithelium (RPE), rods, and Müller glia in fish.⁴ Cells originating from the CM of the lower vertebrate eye continuously generate a population of retinal neurons throughout metamorphosis and adulthood.⁵ These progenitors thus contribute to the prodigious capacity for neural regeneration in many lower vertebrates. Some higher nonmammalian vertebrates, such as the postnatal chicken, also retain the capacity to repopulate the adult retina throughout adulthood and under pathologic conditions.⁶,⁷

In mammalian systems, the existence of adult retinal neurogenic potential was demonstrated using an in vitro colony-forming (neurosphere) assay.⁸,⁹ This research identified a small population of nestin-positive cells derived from the pigmented ciliary epithelium (CE), a bilayer of cells that cover the ciliary body (CB), a structure involved in mediating lens shape. These nestin-expressing cells retain the ability to clonally proliferate, generate sphere colonies, and self-renew for many passages. When exposed to differentiating media conditions, they generate progeny with phenotypes reminiscent of retinal neurons and glia. These CE cells, termed retinal stem cells (RSCs), have been identified in rodent and human eyes.⁸,⁹ RSCs differ from many other neural precursors in that they can proliferate in the absence of mitogenic factors in vitro and are not abolished after the genetic deletion of glial fibrillary acidic protein (GFAP) during development.⁸,¹⁰ In vitro, these cells exhibit multipotentiality and express transcription factors (Pax6, Six3, Chx10, Rx, Lhx2) and phenotypes associated with other general precursors in the central nervous system (Nestin, Musashi1, SSEA-1).¹¹ In vivo, mammalian RSCs reside in a quiescent state and show no proliferative activity under control conditions. This quiescence, coupled with the absence of a putative marker, challenge our ability to characterize and determine a role for endogenous RSCs.

In vivo responses of cells in the adult mammalian CB and CM to changes in their intrinsic gene expression have also been investigated. Mutant mice undergoing constitutive activation of the canonical sonic hedgehog signaling pathway display enriched populations of proliferating cells in the adult CM.¹² Cross-breeding of these mutants with a model of retinal degeneration, a pro23his rhodopsin mutant, generated CM-derived divided cells with phenotypes consistent with neurons and photoreceptors. This evidence suggests that adult CM progenitors can be stimulated to contribute to the repopulation of the adult mammalian retina.

Responses of adult mammalian CM and CE cells to injury have not been fully characterized. Injury to CNS tissue has been shown to generate changes in the local microenvironment through the release of diffusible factors and proteins mediating cell-cell interactions.¹³–¹⁵ Neurogenic regions in

From the Departments of ¹Anatomy and Neurobiology and ³Sur- gery (Neurosurgery), Dalhousie University, Halifax, Nova Scotia, Can- ada, and the ²Departments of Neurosurgery and Neurology, MGH-HMS Center for Nervous System Repair, Harvard Medical School, and Pro- gram in Neuroscience, Harvard Stem Cell Institute, Harvard University, Boston, Massachusetts.

Supported by the Capital Health Research Fund, Halifax, Nova Scotia, and the Department of Surgery, Dalhousie University. JGE was partially supported by fellowships from the Nova Scotia Health Re- search Foundation and the Heart and Stroke Foundation of Canada.

Submitted for publication February 9, 2007; revised April 19 and May 26, 2007; accepted August 22, 2007.

Disclosure: P.E.B. Nickerson, None; J.G. Emsley, None; T. Myers, None; D.B. Clarke, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: David B. Clarke, Department of Anatomy and Neurobiology, Dalhousie University, 5850 College Street, Halifax, Nova Scotia, Canada, B3H 1X5; d.clarke@dal.ca.
brain respond aggressively to pathologic conditions brought on by a wide range of damaging stimuli. In lower vertebrates, a robust neurogenic response in the CM is elicited after injury to teleost and chicken retinas. To a lesser extent, adult mammalian Müller glial cells re-enter the cell cycle and undergo reactive gliosis in response to excitotoxic lesions of the retina, upregulate appropriate progenitor machinery when exposed to exogenous growth factors, and, in some instances, generate neuron-like progeny in vivo and in vitro. The influence of specific classes of retinal cells on the activity of RPCs during development, including aspects of proliferation and the fate of cells generated by RPCs, has also been described. Of specific interest is the well-characterized regulation of fate determination during early retinogenesis by accumulating numbers of RGCs. RGCs are a source of diffusible factors that act to regulate progenitor proliferation and the further production of RGCs. Recent evidence suggests that the proliferative capacity of RPCs is also partially dependent on the presence of newly generated RGCs. The influence of RGCs on the activity of quiescent neural precursors in the adult mammalian CB, however, has not been described.

The goals of this study were to examine the following in the adult rodent: levels of proliferation within the CB, CM, and adjacent retina; phenotypes of proliferating cells and their progeny; and response alterations of these cells after RGC injury. We describe a novel proliferative and phenotypic response of cells of the CB to optic nerve (ON) transection, an effect that is initiated before and increases during the period of RGC death. Taken together, our results demonstrate that quiescent progenitors can be activated after selective injury of at least one class of retinal neuron.

**Materials and Methods**

**Balb-c Mice**

The use of Balb-c mice (Charles River, St. Constant, Quebec), which are albino, eliminates pigment autofluorescence and allows for fluorescent immunocytochemical phenotyping in the CB. Thirty-day-old female mice were housed two per cage in a colony vivarium maintained on a 12-hour light/12-hour dark cycle at constant temperature (21°C) and humidity (40%-50%). Food and water were available ad libitum. All animals were cared for by Dalhousie University animal care, following standards described by the Canadian Council for Animal Care and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**ON Transection**

The following methods for ON transection were adapted from those described in rats. Briefly, the left eye was transected with microscissors approximately 0.5 mm from the posterior aspect of the sclera. Sham animals underwent the same surgical procedures without transection of the ON. Preservation of the blood supply was confirmed by microscopic examination of the retina through the dilated pupil. The right eye of each animal served as an internal, uninjured control.

**BrdU Labeling of Proliferating Cells and Their Progeny**

Proliferating cells were labeled during the S-phase of mitosis by administration of 5-bromo-2'-deoxy-uridine (BrdU). To detect proliferation during early and later time periods after injury, two labeling paradigms were used (Fig. 1). In a “pulse” group, three daily intraperitoneal injections of BrdU (0.5 mL, 50 mg/kg, and a dosage shown to consistently label proliferating cells in the CNS) were performed for 5 days beginning on the day of surgery to detect early postinjury cell division. A second “chase” group received BrdU in its drinking water (1.5 mg/mL) from the time of injury until killing. Water consumption was recorded for individual animals to control for differences in BrdU availability. Analysis of water consumption showed no significant differences between individuals and groups (not shown).

**Kill and Retinal Tissue Processing**

Animals were killed 4, 14, and 28 days after surgery (n = 4–5 per group). Animals were anesthetized by intraperitoneal injection of a lethal dose of sodium pentobarbital (100 mg/kg) and underwent transcardial perfusion with chilled solutions of 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Before the eyes were removed, sutures were placed in the conjunctiva as a reference point for retinal orientation. Eyes and brain were removed, postfixed for 3 hours in 4% paraformaldehyde, and cryoprotected in 30% sucrose. Eyes were embedded in gelatin, and the same postfix and cryoprotection procedure was repeated before sectioning (35 μm) on a freezing microtome. Brains, which were used to confirm CNS bioavailability of BrdU, were removed, postfixed in 4% paraformaldehyde, and cryoprotected in 30% sucrose before sectioning at 40 μm. Sections were stored in Milonig solution (0.1 M PBS/0.075% sodium azide) until staining.

**Immunocytochemistry**

Cellular proliferation and phenotype were determined by fluorescent double-and triple-label immunocytochemistry. Tissue was washed in
0.1 M PB for 5 minutes, briefly dipped in ddH2O, and placed in 2 NHCl for 2 hours at room temperature. After washing, sections were placed in blocking solution (8% serum, 0.3% BSA in 0.1 M PBS/3% Triton X-100) for 1 hour at 4°C. Sections were placed in diluent (3% serum in 0.1 M PBS/3% Triton X-100) containing primary antibody against BrdU alone, or they were multilabeled using two or more primary antibodies (Table 1). Sections were then rinsed and incubated in either cyanine-conjugated or fluorescent (Alexa; Invitrogen, Carlsbad, CA) second-ary antibodies for 1 hour at room temperature. Sections were mounted onto gelatin-coated glass slides and dried. Fluorescent mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) was used for fluorescent dye-stained (Alexa 488; Invitrogen) sections, and another (Citifluor; Marivac Limited, Halifax, NS, Canada) was used for sections stained using Cy2. All antibodies were tested using positive control tissue and primary antibody omission. Nuclei within sections were counterstained (Topro-3 Iodide; Molecular Probes, Eugene, OR).

Confocal Microscopy

Labeled sections were imaged using a confocal microscope (LSM 510 or 510 META; Carl Zeiss, Oberkochen, Germany). Objective lenses (1.4 oil/DIC; Plan-Apochromat; Carl Zeiss) ranging from 40× to 100× magnification were used. Pinhole diameters were maintained at 1.0 to 2.0 Airy units for all wavelengths when quantifying double- and triple-labeled cells. Laser outputs were set at 5% (488 nm), 80% (543 nm), and 9% (633 nm). Emission filters were 505 to 530 nm (Cy2), 560 to 615 nm (Cy3), and more than 650 nm (Cy5). Orthogonal analysis was used to ensure colocalization of all multilabeled sections.

Cell Counting and Statistics

We used at least four animals per group for our experiments. For BrdU-labeling experiments, three sagittal sections at the level of the ON per animal were visualized on a fluorescence microscope (DM400; Leica, Wetzlar, Germany) equipped with an electronic stepper stage (Ludl, Hawthorne, NY). BrdU-positive nuclei within the CE, CM, and adjacent neural retina were counted and plotted using scientific soft-ware (Neurolucida and Stereo Investigator; MBF Bioscience, Williston, VT). All counting was performed with investigators masked to the experimental conditions. Unused sections at the level of and adjacent to the ON were used for further phenotypic analysis.

BrdU data are expressed as the mean number of BrdU-positive nuclei per retinal section, at the level of the ON ± 1.0 SEM. Repeated-

---

### Table 1. Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cellular Phenotype</th>
<th>Source and Immunizing Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRALBP</td>
<td>Adult Müller glia</td>
<td>1:1000, Abcam, Cambridge, MA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. ab15051</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monoclonal clone B2, IgG2a</td>
</tr>
<tr>
<td>GFAP</td>
<td>Activated Müller glia and nonretinal astrocytes</td>
<td>1:100, Novo Castra, Newcastle, UK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. NCL_GFAP_GA5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clone GA5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Species and isotype mouse anti-GFAP IgG1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1000, Chemicon, Temecula, CA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. MAB302</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Species and isotype mouse anti-GS IgG2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glutamine synthetase whole protein (1–375 bp) used as the immunizing antigen</td>
</tr>
<tr>
<td>Nestin</td>
<td>Neuroectodermal, stem/progenitors, RPCs, and activated Müller glia</td>
<td>1:500, BD PharMingen, San Diego, CA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. 556309</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clone 401</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Species and isotype mouse anti-nestin IgG1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1000, Chemicon, Temecula, CA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. AB5977</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Species is rabbit anti-musashi-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:500, Covance Research, Berkeley, CA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. PRB-278P</td>
</tr>
<tr>
<td>Pax6</td>
<td>RPCs, amacrine, horizontal, and RGCs</td>
<td>1:500, Chemicon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. AB9014 and AB9016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Species sheep anti-recombinant human Chx10</td>
</tr>
<tr>
<td>Chx10</td>
<td>RPCs, bipolar, and a small subpopulation of Müller glia</td>
<td>1:500, Oncogene Research Products, San</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diego, CA</td>
</tr>
<tr>
<td>Factor 8</td>
<td>Vascular endothelium</td>
<td>1:500, Oncogene Research Products, San</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diego, CA</td>
</tr>
<tr>
<td>DCX</td>
<td>Immature, postmitotic neurons</td>
<td>1:500, Chemicon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. AB9010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Species guinea pig anti-DCX</td>
</tr>
<tr>
<td>β-III tubulin (Tuj1)</td>
<td>Immature and mature neurons</td>
<td>1:1000, Chemicon</td>
</tr>
<tr>
<td>NeuN</td>
<td>Postmitotic CNS neurons</td>
<td>1:1000, Chemicon, Temecula, CA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. MAB377</td>
</tr>
<tr>
<td>MAP-2</td>
<td>Mature CNS neurons</td>
<td>1:500, Chemicon, Temecula, CA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. AB5622</td>
</tr>
<tr>
<td>Recoverin</td>
<td>Photoreceptors and bipolar cells</td>
<td>1:4000, Chemicon</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Catalog no. AB5585</td>
</tr>
<tr>
<td>BrdU</td>
<td>Anti-5-bromo 2’-deoxy-uridine</td>
<td>1:1000, Research Diagnostics Inc., Flanders, NJ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sheep anti-BrdU</td>
</tr>
</tbody>
</table>
measures analysis of variance was performed, using the independent variable of surgical group (three levels: transected ON, non-transected ON [right eyes], and sham operated). Repeated-measures analysis allowed us to use the right eye as an internal control for each subject. When faced with a significant main effect, Scheffé and *t*-test post hoc analyses were performed to test differences among individual groups.

To analyze changes in the number of Chx10-positive nuclei in the CE of animals across all groups, we used analysis of covariance, with the total number of cells in the CE (counts from CE at either end of the retinal section are pooled) of each section as the covariate. This analysis was used to control for differences in the total number of nuclei within the CE of different sections. Chx10 data are expressed as the mean number of Chx10-positive cells ± SEM, per retinal section. Post hoc *t*-test comparisons were used to compare differences among groups after a significant main effect.

**RESULTS**

**CB and Epithelium Contain Two Distinct Populations of Nestin-Positive Cells in the Uninjured Eye**

Nestin is an intermediate filament protein previously identified in adult neural stem/progenitor cells, embryonic neuroectodermal stem cells, reactive adult astrocytes and radial glia after injury. RSCs and progenitors, neurogenic Müller glial cells and in floating spheres generated from the CE of adult mice. Consistent with these in vitro reports, immunocytochemical staining of retinas in our in vivo study revealed a population of nestin-positive cells in the CB (Figs. 2B–D). Sparingly distributed single nestin-immunoreactive cells were present in the CE adjacent to the vitreous (Fig. 2B), consistent with the nonpigmented layer of the CE. These cells radially one or two thin processes deep to the inner CE, nestin-immunoreactive cell population. Nestin-positive cells are also present in epithelium from the peripheral edge of the retina and RPE to the CB (Fig. 2C). Double-label immunocytochemistry revealed that nestin-positive cells of the CB do not express GFAP, cellular retinaldehyde-binding protein, or glutamine synthetase (data not shown), lending further evidence in support of previous work reporting that CB stem/progenitor cells do not have a glial phenotype.

A previous report indicates that periendothelial cells and pericytes of CNS vasculature express nestin. To determine whether nestin-positive cells in the CB are associated with vasculature, we double-labeled for nestin and endothelial-specific anti–factor 8 (Figs. 2C, 2D). Double labeling with anti–factor 8 revealed two distinct populations of nestin-positive cells. In the deep layers of the CB, nestin colocalized with factor 8–positive blood vessels (Figs. 2C, 2D), corresponding...
to pericytes and/or periendothelial cells (high magnification in Fig. 2D, arrowhead) and not luminal endothelia (Fig. 2D, arrows). Nestin-positive cells in the CE (including individual "displaced" cells and those that form a chain continuous with RPE), however, are distinct from those nestin/anti-factor 8-positive cells of the CB vasculature because of their location within the superficial epithelium of the CB and the absence of factor 8 expression.

**Nestin-Positive Cells of the CE Undergo Low-Level Proliferation in Response to RGC Injury**

Examination of retinas from sham-operated and right eye, non-injured controls revealed extremely low-level proliferation in the CB and CM, as seen by BrdU incorporation (typically 1 or 2 nuclei per section). Cellular proliferation followed a temporal progression in injured and noninjured retinas. However, proliferation was significantly greater in injured than in uninjured eyes at 4 and 14 days, when eyes were examined with a BrdU chase paradigm. Differences in proliferation over 14 to 28 days were not detectable after a pulse of BrdU, likely because of dilution of BrdU by subsequent cell division or death. Furthermore, BrdU administration restricted to the first few days after axotomy would not be seen because of a delay of several days in CB proliferative response to axotomy. Given these observations, data from animals exposed to chronic (chase paradigm; Fig. 1) BrdU exposure are presented for the remainder of the study when assessing proliferation.

Interestingly, proliferation increases in the CB of noninjured eyes over time, suggesting that cells of the CB undergo a constitutive, slow rate of cell division or that cells recently generated outside the eye migrate to the CB. However, the proliferative response after RGC injury is increased and is temporally restricted: a significant increase in the number of BrdU-positive nuclei in the CB is seen at 4 days ($\text{MEAN}_{\text{INJURED}} = 1.67$, $\text{MEAN}_{\text{UNINJURED}} = 0.17$; $P < 0.003$), peaks at 14 days ($\text{MEAN}_{\text{INJURED}} = 10.9$, $\text{MEAN}_{\text{UNINJURED}} = 2.0$; $P < 0.004$), and returns to levels that are not significantly different from those of controls by 28 days ($\text{MEAN}_{\text{INJURED}} = 13.7$, $\text{MEAN}_{\text{UNINJURED}} = 9.9$; $P > 0.05$) after injury.

To determine whether cells of the CB and CE that proliferate in response to RGC injury express nestin, double-label immunocytochemistry was performed. Staining revealed a population of recently divided cells in the CE that expressed nestin after ON transection (Fig. 3A). Orthogonal confocal analysis confirmed the presence of BrdU-positive nuclei within chains of nestin-expressing cells, which themselves extended to the RPE (Fig. 3B). These dividing cells, however, were restricted to the pars plicata and did not extend to the pars plana, suggesting that dividing progeny did not migrate to the CM or adjacent neural retina.

Orthogonal analysis was used to quantify the number of nestin-, BrdU-, and nestin/BrdU-positive cells present in the CE within injured eyes (Fig. 3D). Twenty-eight days after injury, when the number of BrdU-labeled nuclei was the highest, $58 \pm 9$ cells per section were nestin positive; of those, $27\% (16 \pm 6)$ were BrdU positive. Conversely, approximately $80\%$ of the BrdU-positive cells in the CB at this time expressed nestin,
indicating that most recently dividing cells exhibited this progenitor-like phenotype.

**CE Cells Upregulate Nestin and Chx10 in Response to Injury**

To determine whether progenitor-like cells in the CB respond to retinal injury, we analyzed expression of the transcriptional regulators musashi-1, Pax6, and Chx10, all of which are phenotypes expressed in retinal stem cells and RPCs.11 No cells in the CM or CB expressed musashi-1 or Pax6, with or without retinal injury (data not shown). Under control conditions, Chx10 expression was restricted to inner nuclear layer cells of the retina (arrow for example; nuclei are blue). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Outline of CB in fluorescent dye-stained images. No Chx10 staining is detected in the CB. At 4, 14, and 28 days after injury, Chx10-positive cells can be seen in the CE (arrowheads). Chx10-positive nuclei appear close to nestin immunoreactivity (green). (B) Cell counts reveal a significant increase in the number (mean per retinal section) of Chx10-positive nuclei in the CE after injury. (C) High-magnification, orthogonal analysis confirmed colocalization of Chx10-positive nuclei within nestin-positive cells. (D) After injury, the proportion of Chx10-positive cells that also expressed nestin increased relative to control. (B, D) *P < 0.05; significant relative to control. Scale bar: (A) 50 μm; (C) 20 μm.

**Figure 4.** Chx10 is upregulated within nestin-positive cells in the CE after injury. (A) In control and sham-operated retinas, Chx10 (red) immunoreactivity is restricted to inner nuclear layer cells of the retina (arrow for example; nuclei are blue). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Outline of CB in fluorescent dye-stained images. No Chx10 staining is detected in the CB. At 4, 14, and 28 days after injury, Chx10-positive cells can be seen in the CE (arrowheads). Chx10-positive nuclei appear close to nestin immunoreactivity (green). (B) Cell counts reveal a significant increase in the number (mean per retinal section) of Chx10-positive nuclei in the CE after injury. (C) High-magnification, orthogonal analysis confirmed colocalization of Chx10-positive nuclei within nestin-positive cells. (D) After injury, the proportion of Chx10-positive cells that also expressed nestin increased relative to control. (B, D) *P < 0.05; significant relative to control. Scale bar: (A) 50 μm; (C) 20 μm.
4; \( P < 0.02 \), 14 days (55 ± 16; \( P < 0.02 \)), and 28 days (74 ± 32; \( P < 0.05 \)) after injury compared with controls (2.3 ± 0.9; Fig. 4B).

Low-magnification observations indicated that Chx10-positive nuclei resided close to nestin-positive cells. Orthogonal confocal analysis confirmed the coexpression of nestin and Chx10 in CE cells (Fig. 4C). Quantification revealed that most nestin-positive cells expressed Chx10 in their nuclei 4 (85.4%), 14 (94.3%), and 28 (90.6%) days after injury (\( P \leq 0.01 \)) relative to controls (16%; Fig. 4D). These data indicate that two previously identified RSC phenotypes (nestin and Chx10) are upregulated and are expressed by cells in the CE after RGC injury.

Recoverin Is Upregulated in Nondividing Cells of the CE after RGC Axotomy

The observation that 20% of BrdU-positive cells in the CB were not nestin positive (Fig. 3D) prompted us to investigate whether newly generated, terminally differentiated neuronal phenotypes were present. To examine this possibility, double-label immunocytochemistry for BrdU and either βIII-tubulin or doublecortin was performed. Despite the presence of βIII-tubulin–positive cells in the CB (consistent with parasympathetic innervation), none of these cells was BrdU positive. The absence of doublecortin labeling in all conditions suggested that no new neurons were generated. In support of these data, generic postmitotic, mature neuronal phenotypes and RGCs (MAP-2, NF-200, and NeuN) were also negative (data not shown).

To determine whether BrdU-positive cells generated after injury resembled any remaining specific retinal cells, immunocytochemistry against a select panel of retina-specific antibodies was performed (Pax6 for amacrine cells, PKCa for bipolar cells, Gs for Müller glia, calbindin for horizontal cells, and recoverin for photoreceptors and bipolar cells). Under control and uninjured conditions, no cells, including newborn (BrdU-positive) cells, had these phenotypes (data not shown). In contrast, a small population of recoverin-expressing cells (e.g., Fig. 5A, arrow) was seen at 14 and 28 days after injury, suggesting that cells resembling photoreceptors or bipolar cells were present. However, none of these cells was BrdU positive (Fig. 5), suggesting that nonproliferative cells in the CE upregulate recoverin after injury.

DISCUSSION

Injury-Induced Activation of Progenitor-Like Cells of the CB

Taken together, our results show that the selective injury of at least one retinal cell type, the RGC, induces the activation of a small population of cells in the CB that resemble RPCs. Nestin-expressing cells of the CB proliferate and upregulate Chx10, a transcription factor expressed in active RPCs and RSCs. Finally,
nonproliferative cells present in the CB upregulate recoverin, a calcium sensor protein expressed in photoreceptors and bipolar cells, after injury, suggesting an attempt at retinal cell production by endogenous precursors.

In this report, we describe a population of cells in the CB of adult mice that express proteins similar to those seen in retinal stem and progenitor cells previously described by in vitro methods. The influence of the adult retina on the activity of retinal stem/progenitor cells in the CB has been investigated. Previous reports in lower vertebrates show a salient influence on adult marginal progenitors located in the peripheral retina by glucagon-expressing neurons. Similarly, the capacity for murine RPCs and Müller glia to enter the cell cycle is influenced by retinal tissue. These results, supported by those reported here, clearly show that even into adulthood, neural progenitors in the peripheral aspect of the adult eye retain the capacity to respond to cues provided by the injury or death of retinal neurons.

Previous studies have identified a number of secreted factors that are released in the retinal microenvironment in response to RGC injury and death. Possible mechanisms of release include recently characterized indirect pathways by which Müller glia release a variety of neurotrophic factors in response to ON transection. In addition, the release of bFGF and glial-derived neurotrophic factor are induced by signaling from infiltrating microglia, cells that are present and active during RGC death.

The specific mechanism by which CE cells respond to RGC injury is still unclear. Recent evidence suggests that an increase in proliferation (as measured by Ki67 and Cyclin D1 expression) within nestin-positive cells of the CB follows exogenous application of insulin and FGF-2, observations that are consistent with those reported in chick and that support a role for growth factors in the regulation of RSC activity. An unexpected finding evident in our data is the moderate proliferative response detected in retinas 4 days after ON transection. As discussed, factors released as a function of cell death can elicit a mitogenic response by CNS precursors. This proliferative response 4 days after axotomy, however, precedes the onset of RGC death after axotomy by 1 day, indicating that although the proliferative response in the CE may be augmented by RGC death, it is initiated by injury.

Of particular interest in our study is the activation of transcriptional machinery appropriate for progenitor activity after RGC injury. Chx10 has been shown to play a pivotal role in the development of the neural retina. Although not necessary for the genesis of all retinal cell types, mutations in Chx10 result in abnormal eye growth, including microphthalmia, cataracts, and iris malformations. Furthermore, the absence of Chx10 expression in Chx10 or/ or mice results in small eyes but a several-fold increase in the number of adult retinal stem cells, an effect thought to be attributable to the loss in negative regulators elicited by the diminished RPC cell population. The initial in vitro phenotypic screening during the discovery of RSCs demonstrated that these cells express nestin and Chx10, two keystone phenotypes of RPCs. Consistent with these findings, we report in vivo evidence of axotomy-induced activation of a population of cells expressing both nestin and Chx10 in the same anatomic location. Although this observation is not sufficient to conclude that RSCs are being activated, it is consistent with previous findings.

One well-characterized role of Chx10 is the maintenance of proliferation within pools of RPCs. Chx10 mutations result in premature depletion of RPCs and subsequent reduction in retinal volume. In our study, we observed a robust increase in Chx10 expression with a relatively modest increase in proliferation. Although we would hypothesize that Chx10 upregulation should be coincident with proliferation within the CE, it is possible that its expression is insufficient to elicit a robust response in the absence of other mitogenic genes such as Pax6, which is not upregulated after RGC injury.

**Recoverin Expression in the CB after Injury**

We identified a population of cells in the CE that up-regulate the calcium-binding protein recoverin after retinal injury. In the neural retina, recoverin is expressed at relatively early stages of photoreceptor differentiation and in a subpopulation of bipolar cells. The emergence of recoverin-positive cells in the CE after injury suggests an attempt for the differentiation of retinal cell types. CM progenitors in mice can be induced to generate photoreceptor-like cells in vivo after the combined overactivation of sonic hedgehog signaling and the presence of retinal degeneration. Given that most recoverin-positive cells in the retina are photoreceptors, one initial speculation might be that an attempt is made toward their genesis. However, previous findings also suggest that cells of the mature bovine CB express components of phototransduction and retain the capacity to contract in response to light in the absence of neural pathways. In addition, CE and iris tissues can be induced to generate photoreceptor-like cells in vitro in the absence of stem cell culture conditions. In light of evidence supporting a phototransductive ability by CB tissue, it remains possible that RSCs in the ciliary epithelium may represent a residual pool from which young, photoreceptive CE cells may be generated. Specific events leading to the production of recoverin-positive cells after axotomy appear to involve its upregulation in the absence of proliferation. This observation supports the idea that CE progenitors may themselves differentiate into a postmitotic cell type independent of proliferation, an effect reported in subventricular zone neural precursors after growth factor treatment. The attempted genesis of an alternative cell type, a bipolar neuron for example, is also possible because recoverin is present in bipolar cells.

**Conclusions**

Our results show that, in vivo, cells of the CB respond to axotomy with a relatively low level of proliferation that is initiated before, and increases during, the period of RGC death. In addition, RGC injury increases the number of cells expressing Chx10 within the CB and the proportion of those Chx10-positive cells that express nestin. Finally, it is evident that cells of the CE have the capacity to express a phototypic marker (recoverin) seen in retinal photoreceptors and bipolar neurons, a response that is not coincident with cell division. From these data, we conclude that CB cells express phototypes reminiscent of RPCs and RSCs in response to RGC injury in a time-dependent manner, in accordance with the known temporal progression of RGC death. In addition, cells of the CE can be induced in vivo to express phototypes normally seen in retinal neurons. Further understanding of the mechanisms underlying the activation of RSCs, and their responses to different pathologic conditions, may provide important insight into the future development of cell replacement strategies for treating various retinal abnormalities.

**References**


18. Szele FG, Chesselet MF. Cortical lesions induce an increase in cell proliferation but not cell-fate determination or signaling. 

17. Weinstein DE, Burrola P, Kilpatrick TJ. Increased proliferation of retinal progenitor cells due to retinal injury. 

12. Moshiri A, Reh TA. Persistent progenitors at the retinal margin of the mammalian retina. 


20. Morshead CM, Garcia AD, Sofroniew MV, van der Kooy D. The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells. 

21. Mohsiri A, Reh TA. Persistent progenitors at the retinal margin of the mammalian retina. 

22. Garcia M, Vecino E. Role of Muller glia in neuroprotection and regeneration in the retina. 


24. Das AV, Mallya KB, Zhao X, et al. Neural stem cell properties of Muller glia in the mammalian retina: regulation by Notch and Wnt signaling. 


27. Mu X, Fu X, Sun H, et al. Ganglion cells are required for normal progenitor cell proliferation but not cell-fate determination or patterning in the developing mouse retina. 


29. Clarke DB, Bray GM, Aguayo AJ. Prolonged administration of NT-4/5 fails to rescue most axotomized retinal ganglion cells in adult rats. 


31. Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. 

32. Hayes NL, Nowakowski RS. Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. 

33. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intercellular filament protein. 

34. Clarke SR, Shetty AK, Bradley JL, Turner DA. Reactive astrocytes express the embryonic intermediate neurofilament nestin. 

35. Sheedlo HJ, Turner JE. Influence of a retinal pigment epithelial cell factor on rat retinal progenitor cells. 


40. Close JL, Gumsuco B, Reh TA. Retinal neurons regulate proliferation of postnatal progenitors and Muller glia in the retina via TGF beta signaling. 


42. Thanos S, Richter W. The migratory potential of vitally labelled microglial cells within the retina of rats with hereditary photoreceptor dystrophy. 

43. Abduh M, Bernier G. In vivo reactivation of a quiescent cell population located in the ocular ciliary body of adult mammals. 

44. Fischer AJ, Diersk DB, Reh TA. Exogenous growth factors induce the production of ganglion cells at the retinal margin. 

45. Murphy JA, Franklin TB, Rufase VF, Clarke DB. The neuron cell adhesion molecule is necessary for normal adult retinal ganglion cell number and survival. 


48. Coles BL, Horsford DJ, McInnes RR, van der KD. Loss of retinal progenitor cells leads to an increase in the retinal stem cell population in vivo. 


