Journal of Neuroscience Research

Journal of Neuroscience Research 90:407–421 (2012)

Müller Glial Cells Induce Stem Cell Properties in Retinal Progenitors In Vitro and Promote Their Further Differentiation Into Photoreceptors

María V. Simón,[1,2] Pablo De Genaro,[1,2] Carolina E. Abrahan,[1,2] Beatriz de los Santos,[1] Nora P. Rotstein,[1,2] and Luis E. Politi[1,2]*

[1]Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB), Buenos Aires, Argentina
[2]Universidad Nacional del Sur, Buenos Aires, Argentina

Using stem cells to replace lost neurons is a promising strategy for treating retinal neurodegenerative diseases. Among their multiple functions, Müller glial cells are retina stem cells, with a robust regenerative potential in lower vertebrates, which is much more restricted in mammals. In rodents, most retina progenitors exit the cell cycle immediately after birth, differentiate as neurons, and then cannot reenter the cell cycle. Here we demonstrate that, in mixed cultures with Müller glial cells, rat retina progenitor cells expressed stem cell properties, maintained their proliferative potential, and were able to preserve these properties and remain mitotically active after several consecutive passages. Notably, these progenitors retained the capacity to differentiate as photoreceptors, even after successive reseedings. Müller glial cells markedly stimulated differentiation of retina progenitors; these cells initially expressed Crx and then developed as mature photoreceptors that expressed characteristic markers, such as opsin and peripherin. Moreover, they were light responsive, insofar as they decreased their cGMP levels when exposed to light, and they also showed high-affinity glutamate uptake, a characteristic of mature photoreceptors. Our present findings indicate that, in addition to giving rise to new photoreceptors, Müller glial cells might instruct a pool of undifferentiated cells to develop and preserve stem cell characteristics, even after successive reseedings, and then stimulate their differentiation as functional photoreceptors. This complementary mechanism might contribute to enlarge the limited regenerative capacity of mammalian Müller cells. © 2011 Wiley Periodicals, Inc.

Key words: regeneration; retina stem cells; progenitors; photoreceptor differentiation

The use of stem cells to replace lost neurons is a promising strategy to treat neurodegenerative diseases, including those affecting the human retina, such as retinitis pigmentosa and macular degeneration. In the eye, stem cells were initially found in the ciliary marginal zone (van der Kooy and Weiss, 2000). Later, Müller glial cells were shown to display stem cell properties in the vertebrate retina. Müller glial cells are the major glial cell type in the retina and play multiple roles in this tissue, such as recycling of neurotransmitters and provision of trophic and metabolic support (Poitry-Yamate et al., 1995; Derouiche, 1996; Poitry et al., 2000). Increasing evidence demonstrates that after retinal injury these cells dedifferentiate, proliferate, and then differentiate into several neuronal types, among them photoreceptors, in fish, birds, and mammals, including humans (Kirn and Nottebohm, 1993; van Praag et al., 2002; Fischer and Reh, 2003; Haynes and Del Rio-Tsonis, 2004; Bernardos et al., 2007; Karl et al., 2008). The potential usefulness of Müller glial cells for replacing lost photoreceptors in retina neurodegeneration is widely accepted; however, their utilization still requires unraveling how to control their proliferation and induce their differentiation as photoreceptors.

Answering these questions will require improving our understanding of the cross-talk between Müller glial cells and their neighboring cells. Retina Müller glial cells remain mitotically active for several days in culture and express many stem cell markers, along with Pax6, a transcription factor indispensable for retina development and regeneration (Hill et al., 1991; Insua et al., 2008). In contrast, many retina neuronal progenitors retain their mitotic potential for only 2–3 days in vitro and then irreversibly exit the cell cycle and differentiate mostly as

Contract grant sponsor: FONCyT; Contract grant number: PICT 2006-711; Contract grant sponsor: Argentinean National Research Council (CONICET); Contract grant sponsor: Universidad Nacional del Sur, Bahía Blanca, Argentina.

*Correspondence to: Luis E. Politi, Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB), Camino La Carrindanga Km 7, 8000 Bahía Blanca, Buenos Aires, Argentina. E-mail: inpoliti@criba.edu.ar

Received 22 December 2010; Revised 18 June 2011; Accepted 21 June 2011

Published online 4 October 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.22747

© 2011 Wiley Periodicals, Inc.

photoreceptors (Garelli et al., 2006). We have shown that, in mixed neuron–glia cultures from rat retina, both Müller glial cells and neuronal progenitors retain their proliferative potential for long periods, implying that their interaction mutually regulates their mitogenic capacity (Insua et al., 2008). Different trophic factors regulate proliferation; e.g., glial-derived neurotrophic factor (GDNF) allows neuronal progenitors to stay longer in their cell cycle (Insua et al., 2003) and restores the proliferative potential and Pax6 expression in Müller glial cells (Insua et al., 2008). Hence, local environmental cues might be crucial to create a favorable environment for regeneration.

Being able to reseed and obtain consecutive passages of photoreceptor progenitors in vitro is necessary, though not sufficient, to establish these cells as stem cells. Photoreceptors can be successfully maintained in vitro in purified cultures of retina neurons (Rotstein et al., 1996, 1997), but, as occurs with other neuronal cells, they cannot be reseeded, so attempts to obtain secondary cultures of these neurons lead to a generalized cell death. Our recent findings suggest that Müller glial cells enhance the mitotic capacity of progenitor cells (Insua et al., 2008), releasing them from cell cycle arrest. We have exploited the advantages of diverse cell culture conditions to investigate the contribution of different cell types, particularly Müller glial cells, to the generation, maintenance, and later differentiation of retina stem cells. Our results show that, in mixed cultures with neurons, Müller glial cells generated and maintained a pool of retina progenitors with stem cell characteristics, and these progenitors successfully survived consecutive reseedings. These progenitors preserved the ability to differentiate as photoreceptors, even after successive reseeding, and Müller glial cells in mixed cultures advanced this differentiation, turning them into functional photoreceptors. The present study shows that one of the effects of Müller glial cells might be to induce retina progenitor cells (RPCs) to acquire stem cell properties, thus favoring the ability to replace lost neurons in the retina.

MATERIALS AND METHODS

Materials

Two-day-old albino Wistar rats bred in our own colony were used in all experiments. All proceedings concerning animal use were in accordance with the guidelines published in the NIH *Guide for the care and use of laboratory animals*. Plastic 35-mm-diameter culture dishes were purchased from Inter Med (Naperville, IL). Fetal calf serum (FCS) was from Bioser (Buenos Aires, Argentina). Dulbecco's modified Eagle's medium (DME) was purchased from Life Technologies (Grand Island, NY).

Trypsin, trypsin inhibitor, transferrin, hydrocortisone, putrescine, insulin, polyornithine, selenium, gentamycin, 4,6-diamidino-2-phenylindole (DAPI), fluorescein-conjugated secondary antibodies, poly-ornithine, paraformaldehyde (PF), mouse monoclonal anti-α-acetylated tubulin antibody, and monoclonal antisyntaxin clone HPC-1 were from Sigma (St. Louis, MO). Type 2 collagenase was purchased from Invitrogen. Monoclonal antibodies against BrdU (clone G3G4), vimentin (clone 40E-C), and nestin (clone Rat-401) were from DSHB (developed under the auspices of the NICHD and maintained by the University of Iowa Department of Biological Sciences). Mouse monoclonal neuron-specific beta III tubulin (Tuj1) was purchased from Abcam (Cambridge, MA). Polyclonal rabbit anti-Crx was a generous gift from Cheryl M. Craft (University of Southern California). Monoclonal Rho4D2 and anti-peripherin (clone Per3B6) were gently donated by Dr. R. Molday (University of British Columbia); polyclonal antibodies against cellular retinaldehyde binding protein (CRALBP) and Pax6 were kindly donated by Dr. J. Saari (University of Washington, Seattle, WA) and Dr. G.S. Mastick (University of Nevada, Reno, NV) respectively. 5-Bromo-2-deoxyuridine (BrdU) and secondary antibody Alexa 488-conjugated goat anti-mouse were from Molecular Probes, Invitrogen (Carlsbad, CA). Tyramide was from NEN Life Science Products (Boston, MA), and avidin-peroxidase was from Vector Laboratories (Burlingame, CA). CellTrace CFSE Cell Proliferation Kit (C34554) was from Molecular Probes, Invitrogen. All other reagents used were of analytical grade.

Retina Cultures

Pure Müller glial cell cultures. Purified cultures of Müller glial cells were prepared by using protocols previously described (Hicks and Courtois, 1990). Briefly, newborn rat eyes were excised and incubated overnight in DME at room temperature and then treated with trypsin (1 mg/ml) and type 2 collagenase (2 mg/ml). Retinas were then dissected, chopped into small pieces, and seeded in culture medium supplemented with 10% FCS. The medium was routinely replaced every 3–4 days. After 8–10 days, pure Müller glial cells became confluent and were used for different experiments (Fig. 1A).

Pure neuronal cultures. Neuronal cultures from 2-day rat retinas were obtained following previously described procedures, with slight modifications (Politi et al., 1996; Rotstein et al., 1996, 1997). In brief, after dissection and thorough dissociation of the retinas (to avoid the formation of clumps, where Müller glial cells usually develop), cells were resuspended at low density in 10% serum-containing medium. The resulting cell suspension was seeded onto 35-mm-diameter dishes, pretreated with poly-ornithine and schwannoma-conditioned medium (Adler, 1982). Cultures were incubated at 36°C in a humidified atmosphere of 5% CO_2. Two hours after seeding, most cells were undifferentiated RPCs, displaying an irregular or round morphology. Many of them underwent their last mitotic division during the first 2 days in vitro, and soon after they began their differentiation as photoreceptors (Fig. 1C).

Mixed neuron–glia cultures. To obtain long-term mixed cultures of Müller glial cells and retina neurons, we followed previously described protocols (Politi et al., 1996), with slight modifications. Briefly, retinas from 2-day-old rats were chemically and mechanically dissociated with trypsin (350 μl of 0.25% trypsin in 6 ml of calcium- and magnesium-free Hanks

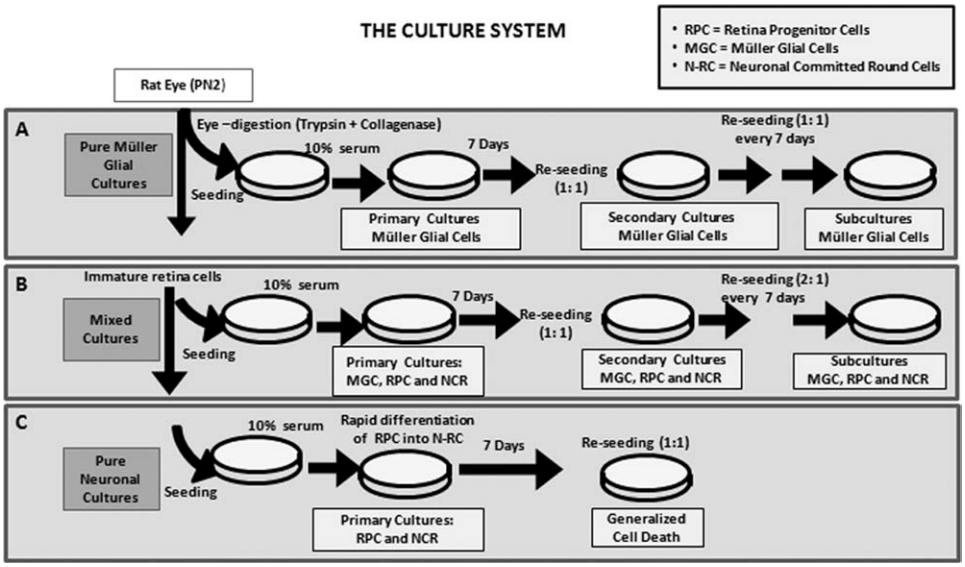

Fig. 1. The culture systems. Two-day-old rat retinas were dissected, dissociated, and seeded under different culture conditions. To obtain pure Müller glial cell cultures (**A**), eyes were dissected, kept overnight in DME, and then treated with a trypsin-collagenase solution (see Materials and Methods). Dissociated cells were seeded and incubated in 10% serum-containing medium. The cultures were reseeded every 7 days in a 1:1 dilution. To obtain mixed cultures of retinal neurons and Müller glial cells (**B**), eyes were dissected, retinas were digested with trypsin and then dissociated, and cells were resuspended in 10% serum-containing medium and seeded. Müller glial cells grew and differentiated along with a significant amount of retina progenitor cells (RPCs). These RPCs either progressed in the cell cycle, generating more RPCs, or exited the cell cycle as neuronal committed round cells (N-RC), which differentiated mainly into photoreceptors. Cells were harvested and reseeded after every 7 days in culture, to obtain secondary cultures or subcultures. To obtain purified neuronal cultures of photoreceptors and amacrine neurons (**C**), eyes were dissected, retinas dissociated, and cells seeded at low density in 10% serum-containing medium. During the first 1–2 days in vitro, a population of RPCs was present in the cultures, but they soon differentiated, mostly as photoreceptors. After 7 days, cells were harvested and reseeded to obtain secondary cultures, but in these cultures reseeding led to a generalized cell death.

[CMF]) for 13 min. Cells were then resuspended in DME medium with 10% FCS and seeded at a density of 1.0×10^6 cells per dish on 35-mm-diameter plastic dishes with no previous treatment (Fig. 1B).

Subcultures of pure neuronal, Müller glial cell, and mixed neuron–glia cultures. To obtain subcultures, pure neuronal cultures and mixed neuron–glia cultures were grown for 7 days, while primary pure glial cultures were grown until confluence. Cells from these three different culture conditions were then separately harvested with 0.25% trypsin and 5 mM EDTA in CMF, pooled, and rapidly reseeded at 1:1 dilution in new culture dishes. Secondary pure Müller glial cell cultures were grown in DME and reseeded every 7 days. Secondary mixed cultures were incubated in DME with 10% FCS for 1 day to promote cell reentry into the cell cycle, then washed twice with DME and incubated with a serum-free, chemically defined differentiation medium (Politi et al., 1996), supplemented with 100 nM insulin to induce cell differentiation. After 7 days, cells were harvested and reseeded to obtain tertiary mixed cultures. This procedure was repeated several times to obtain at least the fifth subcultures; by the fourth passage, cells had to be concentrated at a 2:1 dilution in order to increase the number of cells/dish (Fig. 1A–C). Reseeding primary neuronal cultures led to widespread cell death (Fig. 1C).

Cell Tracing Experiments

To determine whether RPCs had originated from Müller glial cells, we performed experiments using CellTrace, a fluorescent dye that labels cells and is retained throughout development and mitosis. Pure Müller glial cultures were incubated with this dye, following the manufacturer's specifications. They were then harvested and reseeded with mixed neuron–glia cultures, which were not labeled. Similar reciprocal experiments were performed, labeling mixed neuron–glia cultures with CellTrace, resuspending these cells, mixing them with pure Müller glial cells, and reseeding these cells. After 4 days, the resulting cultures were fixed and then labeled with nestin and Pax6, and the presence of RPC showing CellTrace labeling was analyzed.

Cell Identification

Cells were fixed for at least 1 hr with 2% paraformaldehyde in phosphate-buffered saline (PBS), followed by permeation with Triton X-100 (0.1%) for 15 min. Müller glial cells were identified by their morphology and by immunocytochemistry, with polyclonal antibodies against cellular retinaldehyde binding protein (CRALBP), glial fibrillary acidic protein (GFAP), and vimentin (Table I). To exclude the possibility of astrocyte contamination in Müller glial cell cultures, Pax2 immunostaining was performed in pure Müller glial cultures.

TABLE I. Characteristics of Different Cell Types in Culture*

Cell type	Abbreviation	Approximate size (μm)	Morphology	Cell markers
Müller glial cells	MGC	20–200	Flat, irregular	CRALBP, GFAP, Vimentin, Pax-2(–)
Retina progenitor cells	RPC	5–20	Round	Nestin, Sox2, Pax-6, BrdU uptake, mitotic figures
Neuronal commited- round cells	N-RC	5–20	Round, with axons and/or neurites	Any neuronal or PHR marker (Tuj I, NeuN, HPC-1, Crx, opsin, peripherin, etc)
Round cells (in general), include RPC and N-RC	RC	5–20	Round	Markers of either RPC or N-RC
Photoreceptors	PHR	5–10	Round cell body, short axon, cilium	Crx, opsin, peripherin, high-affinity uptake of glutamate, light response

*Description of the different cell types that developed and differentiated under the different culture conditions used. The description contains the main characteristics of Müller glial cells; retina progenitor cells; round cells, and photoreceptors, including their specific markers, approximate sizes, and abbreviations used through the text.

None of the glial cells in the cultures was Pax2 positive, indicating that Müller glial cells and not astrocytes were present in these cultures (not shown).

Photoreceptors and amacrine cells were the two main retina neurons in neuron–glia mixed cultures and were identified by their morphology by using phase-contrast microscopy and by immunocytochemistry with monoclonal antibodies. Alexa 488-conjugated goat anti-mouse was used as the secondary antibody. Tyramide signal amplification was occasionally used to improve visualization, following the procedure described by the manufacturer. Controls for immunocytochemistry were done by omitting either the primary or the secondary antibody. Photoreceptors were identified using Rho4D2 (Barnstable, 1980; Hicks and Barnstable, 1987), Crx, and peripherin antibodies (Table I), as previously described (Rotstein et al., 1998). In addition to these characteristics, photoreceptors have a small round cell body (3–5 μm) with a single neurite at one end, which usually ends in a conspicuous synaptic "spherule"; sometimes they display a connecting cilium at the opposite end (Table I), but they fail to develop their characteristic outer segments. Opsin is diffusely distributed over their cell body, which is usually darker than that of amacrine neurons. To be identified as photoreceptors, cells had to display at least three of the criteria described above (Table I). Amacrine cells are larger than photoreceptors (7–20 μm) and have multiple neurites. All of them show anti-syntaxin (HPC-1) immunoreactivity (Barnstable, 1980), starting at early stages of development, and this immunoreactivity is retained even after undergoing degenerative changes that alter the morphological appearance (Politi et al., 2001). Amacrine cells also express Pax6. Although this expression occurs in all proliferating retina progenitors, photoreceptors stop expressing Pax6 after their last mitotic division, whereas differentiated amacrine neurons retain Pax6 expression (Garelli et al., 2006).

One of the main objects of our study was the heterogeneous population of round cells, comprising two subpopulations: RPCs and neuronal committed round cells (N-RC). RPC were defined as small (5–20 μm), round cells that maintained their mitotic potential and expressed nestin, Sox-2,and Pax6 (Table I). In addition, they showed no expression of Crx, opsin, peripherin, HPC-1, or Tuj-1, the markers of differentiated neurons or photoreceptors used in the present study. Nestin is a good marker for RPC, because its expression disappears as soon as cells exit the cell cycle and start to express markers of differentiated neurons (Garelli et al., 2006). On the other hand, N-RC represented the subpopulation of round cells that had already exited the cell cycle and initiated their differentiation. These cells still had a round morphology but had lost nestin expression and already expressed at least one of the retina neuronal/photoreceptor markers, such as neuron-specific Tuj1, acetylated alpha tubulin, opsin, or Crx (Table I).

Evaluation of Cell Proliferation

To identify cells that were replicating their DNA, pure neuron and pure Müller glial cultures and neuron–glial mixed cultures were incubated with BrdU for the last 24 hr. Cultures were then fixed, and BrdU uptake was analyzed by immunocytochemistry. To determine whether proliferating RPC in secondary mixed cultures were later differentiating into photoreceptors, BrdU was added at day 2, and coexpression of Crx and BrdU uptake was analyzed at day 7. Proliferation was also evaluated by quantification of mitotic figures with DAPI, a fluorescent probe that binds to DNA.

Flow Cytometry

For quantitative examination of RPCs, 2-day secondary neuron–glial mixed cultures were analized by flow cytometry. Single cell suspensions were fixed in 4% paraformaldehyde (PF) for 12 hr, washed twice with PBS, and then permeated for 15 min with 0.1% Triton X-100. Cells were then successively stained with primary (nestin 1/100) and secondary (Cy2 anti-mouse 1/200) antibodies, then collected and filtered using a 32-μm-pore-size nylon mesh. Flow cytometry analyses were done with a FACSCalibur flow cytometer (BD Biosciences). Quantification of dissociated retina cells by FACS was performed using FACSComp. A minimum of 10,000 events was analyzed for each sample.

Microscopy

Cultures were then analyzed by phase-contrast and fluorescence microscopy, using a Nikon Eclipse E600 microscope,

and by a laser scanning confocal microscope (Leica DMIRE2) with a ×63 water objective. x-y (Top to bottom) section images were collected and processed with LCS software (Leica) and Photoshop 8.0 (Adobe Systems, San Jose, CA). In some cases, phase pictures were contrasted as pseudo-Nomarsky, using XN-View software.

Effect of Light on cGMP Hydrolysis

To evaluate the functional maturation of cells displaying photoreceptor characteristics, we assessed whether phototransduction pathways were already active by measuring the extent of cGMP hydrolysis after light exposure. Secondary, 7-day mixed neuron–glia cultures were kept in the dark for 24 hr and then exposed or not to light, as reported elsewhere (Jomary and Jones, 2008). cGMP levels were quantified using an enzyme immunoassay kit (Biotrack [EIA] System; GE Healthcare, Buckinghamshire, United Kingdom). Similar experiments with neuronal and pure Müller glial cultures were done as controls.

To investigate whether Müller glial cells released soluble factors that induced photoreceptor differentiation, pure neuronal cultures were incubated with glia- conditioned media. Pure Müller glial cell cultures were grown until confluent in 10% FCS. Incubation medium was then replaced, and cells were incubated for another 48 hr in DME. This medium was collected, and 250 μl of it was added to pure neuronal cultures (2.25 ml final volume), immediately after seeding the cells.

High-Affinity Glutamate Uptake

In the retina, photoreceptors take up glutamate by high-affinity mechanisms (Politi and Adler, 1986; Schmitt et al., 2002; Harada et al., 2007). In vitro, uptake experiments were done by incubating secondary, 7-day mixed neuron–glia cultures with [^{3}H]glutamate (1 μCi/μl, 0.4 μl) in Tris-Hepes-buffered medium (40 mM Tris-Hepes, 1 nM $MgCl_2$, 5 mM KCl, and 140 mM NaCl at pH 7.4) during 15 min at 36.5°C. Cells were then rinsed twice in buffer and lysed in water to determine total intracellular radioactivity by liquid scintillation counting. Replicate dishes were fixed in glutaraldehyde, rinsed in buffer, dehydrated in increasing concentrations of ethanol, and coated for autoradiography using a Kodak nuclear track emulsion NTB2. After 7 days of dark exposure at 4°C, the autoradiograms were developed in Dektol, fixed in Kodak fixer, and examined under the microscope. Since high-affinity glutamate uptake is Na^+ and temperature dependent, the uptake of [^{3}H]glutamate in secondary mixed cultures kept in an Na^+-free medium and at low temperature was evaluated and used as control.

Western Blot

To investigate neuronal differentiation further, changes in the levels of alpha acetylated tubulin, an axon-specific protein, during the time in culture were analyzed by Western blot. Cells were rinsed in PBS and then treated with lysis buffer (with protease and phosphatase inhibitors) on ice for 20 min. Proteins were separated by one-dimensional SDS-PAGE (Laemmli et al., 1970) and then transferred to Immobilon-P (PVDF) membranes. They were then immersed in PBS containing 5% skim milk for 30 min at room temperature to block nonspecific binding. Anti-acetylated tubulin and anti-actin monoclonal primary antibodies were allowed to react with the proteins overnight at 4°C. Membranes were then washed twice for 15 min and once for 10 min with PBS-T (0.1% Tween 20) and incubated with peroxidase-labeled goat anti-mouse IgG antibody. Finally, proteins were visualized using an enhanced chemiluminiscent technique (ECL Plus Western Blotting Detection Reagents RPN 2132; GE Healthcare), according to the manufacturer's instructions.

Statistical Analysis

For cytochemical studies, 10 fields per sample, randomly chosen, were analyzed in each case. Each value represents the average of at least three experiments, with three or four dishes for each condition ± SD, unless otherwise indicated. Statistical significance was determined by Student's two-tailed *t*-test, with $P < 0.05$ considered significant.

RESULTS

Persistence of RPCs In Vitro

We previously demonstrated that, in mixed neuron–glia cultures, neuronal progenitors maintain their proliferative potential for several days in vitro (Insua et al., 2008). To explore the possibility that Müller glial cells might release progenitor cells from their cell cycle arrest, primary mixed cultures of Müller glial cells, retina progenitors, and neurons were reseeded after 7 days in vitro (Fig. 1B). In the resulting secondary mixed cultures, Müller cells were found to coexist with a pool of round cells, which took up BrdU and expressed nestin, Pax6, and Sox-2 (Fig. 2D–K). These round cells were defined as RPCs and were observed in every consecutive passage of the mixed cultures, even after their fifth reseeding (Fig. 2L–Q). An additional finding in these fifth reseedings was the presence of Crx-positive cells (Fig. 2R,S). Hence, regardless of their time in vitro, under suitable conditions, round cells initially retained their mitotic potential and later the capacity to adopt a photoreceptor fate. On the other hand, when pure neuronal cultures were reseeded, no proliferative cells were seen (Fig. 2A–C). After reseeding, most neurons had pycnotic or fragmented nuclei (arrowheads in Fig. 2C), indicating that the procedure led to a generalized cell death.

The persistence of proliferative progenitors after successive reseedings of mixed cultures was unexpected. Retina progenitors have a limited capacity for remaining in the cell cycle (Insua et al., 2003). Hence, an important question was whether neighbor cells had a role in preserving mitotically active progenitors in the successive passages of mixed neuron–glia cultures. We initially evaluated the effects of Müller glial cells on cell cycle progression and on the maintenance of stem cell markers in RPC. Dissociation of cells from primary mixed neuron–glia cultures gave rise to small clumps of cells, most of which had attached to the substrata by day 1. Soon after,

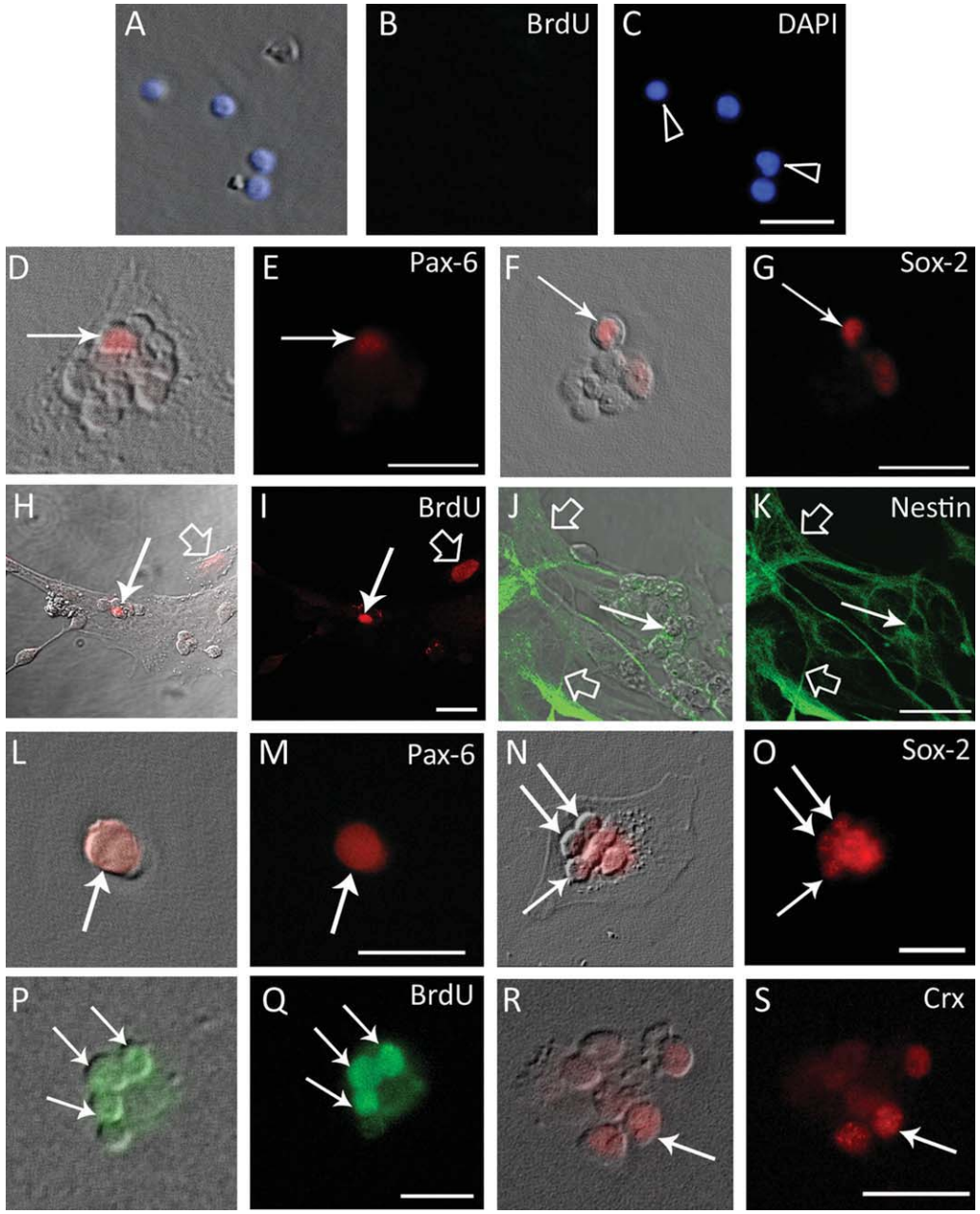

Fig. 2. Retinal progenitors were preserved after successive reseedings of mixed neuron–glia cultures. Nomarsky (A,D,F,H,J,L,N,P,R) and fluorescence (B,C,E,G,I,K,M,O,Q,S) micrographs of RPCs (thin arrows) in secondary mixed cultures (D–K) and after four passages (L–S). Pure neuronal cultures did not survive passaging (A–C); no proliferative RPC were found in this condition (B), and most neuronal nuclei were fragmented or pycnotic (arrowheads in C). On the contrary, BrdU-positive RPC (open arrows) were observed in secondary mixed neuron–glia cultures (H,I) and after four consecutive passages (P,Q). RPCs (thin arrows) in these subcultures displayed several stem cell markers, such as Pax6 (D,E,L,M), Sox-2 (F,G,N,O), and nestin (J,K) expression. Note that, after four passages, although a population of PRCs retained stem cell markers, many of them differentiated as photoreceptor-like cells, which expressed Crx (arrows in R,S). Nuclei were stained with DAPI (A,C). Scale bars = 20 μm.

Müller glial cells began to extend beneath the clusters. Nearly all these Müller glial cells incorporated BrdU and expressed nestin (open arrows in Fig. 2H–K). In addition to Müller glial cells, secondary mixed cultures had a significant amount of undifferentiated RPCs that remained in the cell cycle, as evidenced by their BrdU uptake and nestin, Pax6, and Sox-2 expression (thin arrows in Fig. 2D–K). The expression of these markers and of BrdU-labeled cells persisted even after 7 days in vitro (Fig. 3A). About 4.5% of round cells expressed nestin by day 1; this percentage progressively decreased to 3.7%, 2.5%, and 1.7% by days 3, 5, and 7, respectively (Fig. 3A).

Flow cytometric analysis confirmed the presence of nestin-positive round cells in these secondary mixed cultures: by day 2, over 68% of the cells were round cells, and about 5% of them were nestin-positive round cells (Fig. 3B). As a whole, these results provide evidence that a small pool of round cells consisted of RPCs, which remained cycling and retained stem cell characteristics at every time studied.

To evaluate further the total amount of RPCs, we quantified Pax6-positive cells. We have previously shown that all RPCs express Pax6 before their differentiation as retinal neurons (Garelli et al., 2006). Amacrine cells also express Pax6, so, in order to evaluate only RPCs, we excluded cells double-labeled with the amacrine marker HPC1 and Pax6. About 10% of total round cells in secondary mixed neuron–glia cultures expressed Pax6 (and did not coexpress HPC1; Fig. 3A). This amount remained almost constant between days 1 and 7, implying that a pool of progenitors with the potential to give rise to retinal neurons was preserved in mixed neuron–glia cultures after reseeding.

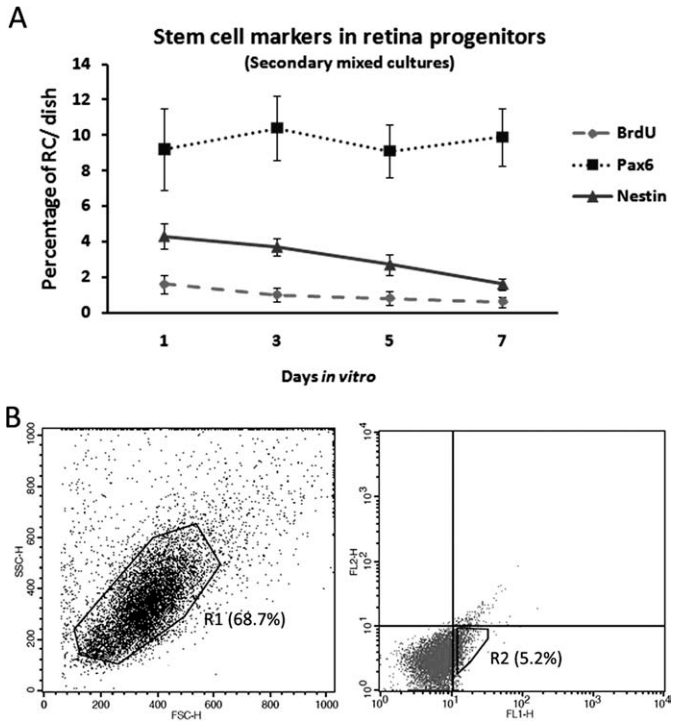

Fig. 3. Progression of stem cell marker expression during development in vitro in secondary mixed neuron–glia cultures. Percentage of round cells showing BrdU uptake and Pax6 and nestin expression is shown in A. Flow cytometry analysis indicating that almost 70% of total cells in secondary mixed neuron–glia cultures were round cells, and among them about 5% were nestin-positive RPCs (B). Each value in A represents the mean of three experiments (±SD).

Possible Sources of RPCs in Secondary Mixed Neuron–Glia Cultures

We then addressed the question of which was the source of RPCs in secondary mixed neuron–glia cultures. Among their multiple functions, Müller glial cells are stem cells in the retina and have been shown to generate diverse neuronal types (Reh and Fischer, 2001; Fischer and Reh, 2003; Bernardos et al., 2007). To establish whether their dedifferentiation originated RPCs, we evaluated the amount of RPCs in primary and secondary cultures of pure Müller glial cells that took up BrdU or expressed nestin. Primary purified Müller glial cell cultures consisted of 99.98% Müller glial cells (Table II), which expressed the glial markers vimentin and CRALBP (Fig. 4A,B, respectively), and showed a negligible amount of round cells (white arrow in Fig. 4D), amounting to less than 0.02%, which might represent RPCs (Table II). After reseeding these pure Müller glial cells, nearly all (99.9%) BrdU-positive cells in 7-day secondary cultures were Müller glial cells (Fig. 4F); round cells were virtually absent in both primary and secondary purified glial cultures (72 cells/35-mm dish [±175 cells/dish], n = 4; see also Table II). Hence, under these conditions, purified Müller glial cells were ineffective to preserve or generate viable and proliferative RPCs.

An alternative source for the RPCs observed in the consecutive passages might be progenitors that were initially present in the newborn retina and were preserved, maintaining their mitotic activity in successive mixed cultures, because of their interactions with Müller cells. To investigate whether RPCs preserved their stem cell characteristics after reseeding the mixed cultures, we evaluated the amount of RPCs expressing nestin before and after reseeding. At day 7, nestin-positive RPCs in primary mixed cultures amounted to 49,000 ± 5,280 cells/dish (Table III), whereas, in secondary-mixed cultures, 10,535 ± 1,715 nestin-positive cells/dish were still present at day 1, implying that RPCs were highly conserved after reseeding (Table III). As stated above, RPCs retained their stem cell properties not only in secondary mixed cultures but also after successive passages. These

TABLE II. Quantitative Analysis of Different Cell Types in Primary Cultures*

Cell types	Pure neuronal cultures	Pure Müller glial cultures	Mixed cultures
Müller glial cells/dish	2,131 ± 1,735 (0.9)	361,320 ± 45,611	112,883 ± 24,693
Round cells/dish	234,616 ± 45,861	72 ± 175 (0.019)	239,878 ± 17,638
Total cells/dish	236,747 ± 62,728	361,393 ± 26,931	352,762 ± 28,997

*The cells were seeded under different culture conditions, as explained in Materials and Methods. The data represent the numbers (±SD; n = 3) of the different cell type populations obtained in primary cultures. The numbers in brackets represent the percentage of given cell types.

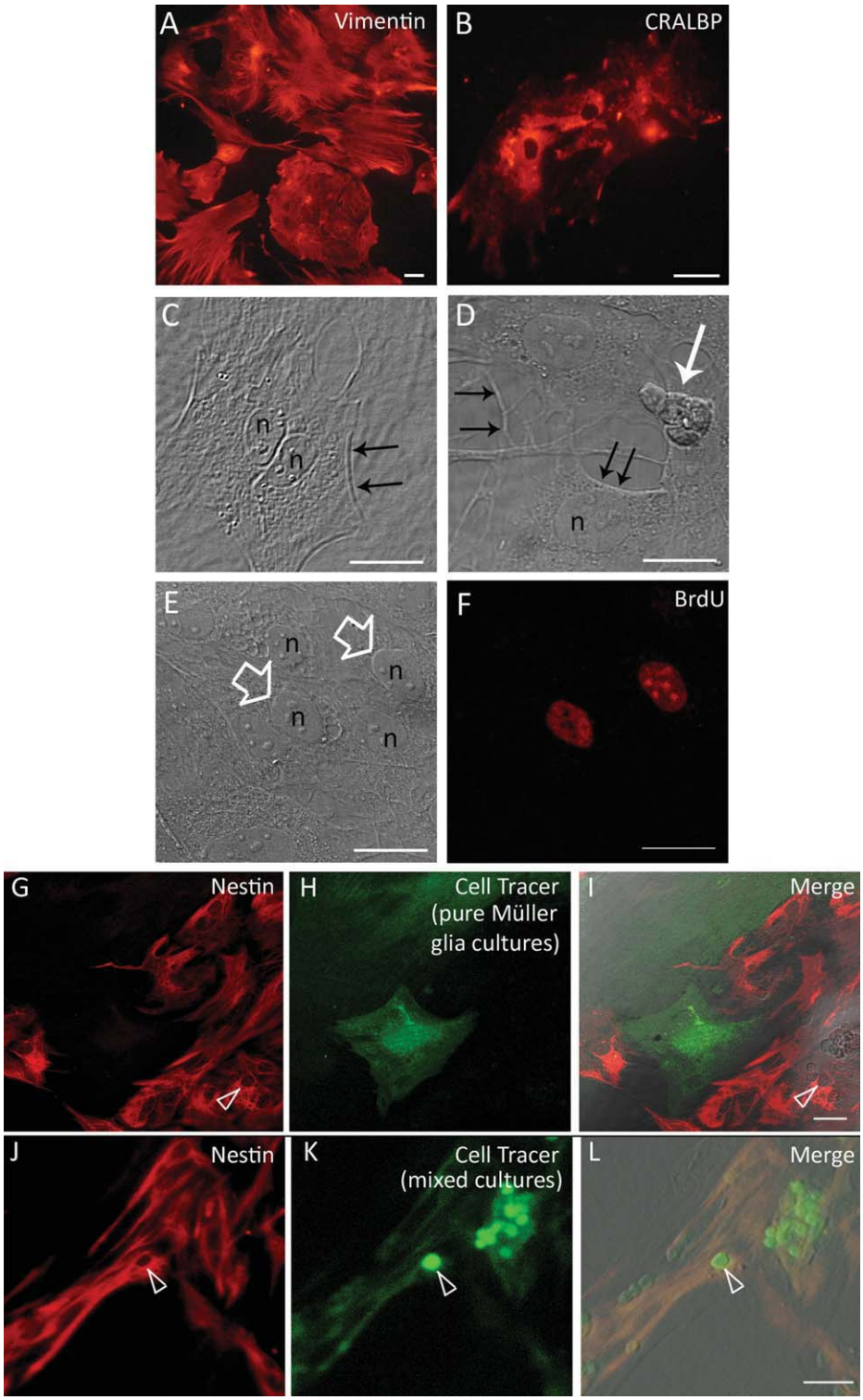

Fig. 4. Purified cultures of Müller glial cells failed to generate RPCs after reseeding. Phase (C–E) and fluorescence (A,B,F) photomicrographs of purified Müller glial cell cultures. Müller glial cells expressed vimentin (A) and CRALBP (B). Primary cultures of these cells (A–D) showed few RPCs, which tended to form clusters (white arrow in D) on top of glial cells (black arrows in C,D). After reseeding, many Müller cells took up BrdU (F, and open arrows in E) but almost no proliferative RPCs were seen in this condition. Nuclei (n) stained with BrdU are shown in F. Nomarsky (I,L) and fluorescence (G,H,J,K) photomicrographs obtained from CellTrace-labeled pure Müller glial cells seeded with (unlabeled) mixed neuron–glial cultures (G–I) or from the reciprocal culture condition, CellTrace-labeled cells from mixed neuron–glial cultures (J–L) pooled with (unlabeled) pure Müller glial cells. The cells were initially labeled with CellTrace (green fluorescence in H,K), cultured for 4 days and after being fixed, labeled with nestin (red fluorescence in G,J). Nestin-labeled Müller glial cells and RPCs were observed in both culture conditions (G,J). However, when pure Müller glial cells were labeled with CellTrace, none of the RPCs (arrowheads) was CellTrace positive (H). On the contrary, when mixed neuron–glia cultures were labeled with the dye, all RPCs showed both CellTrace and nestin staining (K). Scale bars = 20 μm.

TABLE III. Development of Round Cells in Both Primary and Secondary Mixed Cultures

	Days in vitro	Round cells/dish	Crx-positive round cells/dish	Nestin-positive round cells/dish
Primary mixed cultures	1	323,555 ± 15,440	174,720 ± 19,530	76,800 ± 4,800
	7	349,863 ± 28,997	277,441 ± 31,488	49,000 ± 5,280
Secondary mixed cultures	1	230,681 ± 57,070	177,624 ± 11,534	10,535 ± 1,715
	7	178,714 ± 15,062	142,971 ± 16,084	3,040 ± 570

*Mixed neuron–glial cultures were obtained as described in Materials and Methods. The data depict the numbers (±SD; n = 3) of round cells expressing markers of stem cells (nestin) or differentiated photoreceptors (Crx) in both primary cultures and after reseeding the cells to obtain secondary cultures.

results imply that interaction with Müller glial cells maintained a pool of RPCs that preserved their proliferative potential and stem cell characteristics and suggest that at least part of them derived from mitotic divisions of pre-existing RPCs.

To investigate further whether RPCs came from Müller glial cells or from pre-existing RPCs, we labeled cells in different culture conditions with CellTrace, a green fluorescent dye that stained cells and was retained in them even after passaging. In green-labeled pure Müller glial cells incubated with (unlabeled) mixed neuron–glia cultures, many nestin-positive RPCs were present, but none of them showed green fluorescent labeling (Fig. 4G–I). On the contrary, when green-labeled mixed neuron–glia cultures were incubated with (unlabeled) pure Müller glial cultures, every nestin-positive RPC also showed the green fluorescent dye (Fig. 4J–L). Consistently, by day 4, the percentage of Pax6-positive RPCs in both culture conditions was 9.05% (±1.85%, n = 3; not shown), and this value was in close concordance with that shown in Figure 3A at the same time point. Altogether, these data suggest that RPCs in secondary mixed cultures originated from RPCs already present in primary cultures and not from Müller glial cells. Moreover, their interaction with glial cells in mixed cultures allowed these RPCs to be preserved in successive passages.

Differentiation of RPCs as Photoreceptors

Although the differentiation as photoreceptors of an initial pool of round, undifferentiated cells was predictable in primary mixed neuron–glia cultures, the finding of photoreceptors after consecutive reseedings was unexpected. As occurs with other neurons, differentiated photoreceptors cannot be reseeded; hence, their occurrence after successive passages raised the question of whether they were generated from progenitor cells that were present in the donor retinas and preserved in mixed neuron–glia cultures because of their interaction with Müller glial cells. We explored whether these progenitors eventually differentiated as retina neurons and whether Müller cells stimulated this differentiation. In primary mixed cultures, the total amount of round cells remained fairly stable during time in vitro, being about 325,000 cells/dish and 350,000 cells/dish at days 1 and 7, respectively (Table III). However, nestin-positive RPCs decreased (Table III), while the amount of round cells expressing the photoreceptor transcription factor Crx increased during development (Table III). Round cells in secondary mixed cultures showed a similar decrease in nestin-positive RPCs and a concomitant increase in the percentage of Crx-expressing round cells, i.e., neuronal committed round cells, with time in vitro (Table III). This suggests that RPCs in both primary and secondary mixed cultures were gradually exiting the cell cycle to adopt a photoreceptor fate.

To confirm that at least some of these photoreceptors derived from RPCs generated in secondary mixed cultures, 2 days after seeding the cells, we incubated them with BrdU and at day 7 searched for cells doubly labeled cells with BrdU and Crx. The percentage of BrdU-positive round cells that also showed Crx expression (Fig. 5A–D) was 50% (±27%; not shown), suggesting that RPCs in secondary mixed cultures initially proliferated and then exited the cell cycle to adopt a photoreceptor fate.

To confirm the differentiation of RPC along a neuronal, and photoreceptor, pathway, we analyzed whether they developed neurites and expressed neuronal markers, such as Tuj1, an early marker of neuronal differentiation. Many neuronal-like cells in secondary mixed cultures showed Tuj1-labeled processes by day 7 (Fig. 6A). Western blot analysis and immunocytochemistry indicated that these cells already expressed acetylated alpha tubulin, an axonal marker, after 2 days in secondary mixed cultures. Its levels (Fig. 6B) and the length of the acetylated alpha tubulin-labeled processes had markedly increased at day 7 (Fig. 6C–F), suggesting that many round cells were already committed to a neuronal fate.

Differentiation toward a photoreceptor phenotype increased with time in culture. After 7 days in secondary mixed cultures, many cells with photoreceptor morphology showed an extensive network of Tuj1-stained axon-like processes (Fig. 7C, wide arrows). About 80% and 10% of total round cells expressed Crx and opsin, respectively (Fig. 5E), and coexpression of Tuj1 with Crx (Fig. 7G) was observed in 16% (±3.8%) of them (not shown). To corroborate differentiation of round cells as photoreceptors, we investigated the expression of specific photoreceptor markers, such as the visual protein opsin, and peripherin (a structural glycoprotein found in the disk rims in photoreceptor outer segments). Many round cells in secondary mixed cultures showed opsin and peripherin expression, which was localized in apical

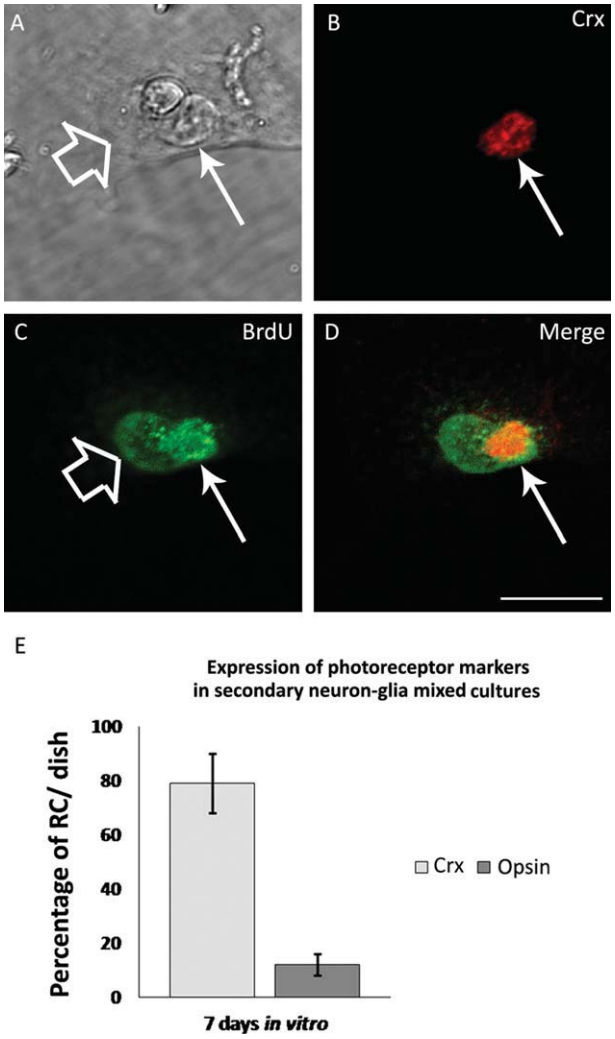

Fig. 5. Proliferative RPCs adopt a photoreceptor fate. Confocal Nomarsky (A) and fluorescence (B–D) photomicrographs of 7-day secondary mixed neuron–glia cultures, incubated at day 2 with BrdU. Photoreceptor-like round cells (thin white arrows) growing on top of Müller cells (open arrows) showed simultaneous labeling with Crx (B) expression and BrdU uptake (C); merged image is shown in D. B–D are projection images on the x and y axes. Note that both Müller cells and RPC took up BrdU (A,C), but only round cells showed its coexpression with Crx (D). The percentages of total round cells (RCs) expressing Crx and opsin are shown in E. Scale bar = 20 μm.

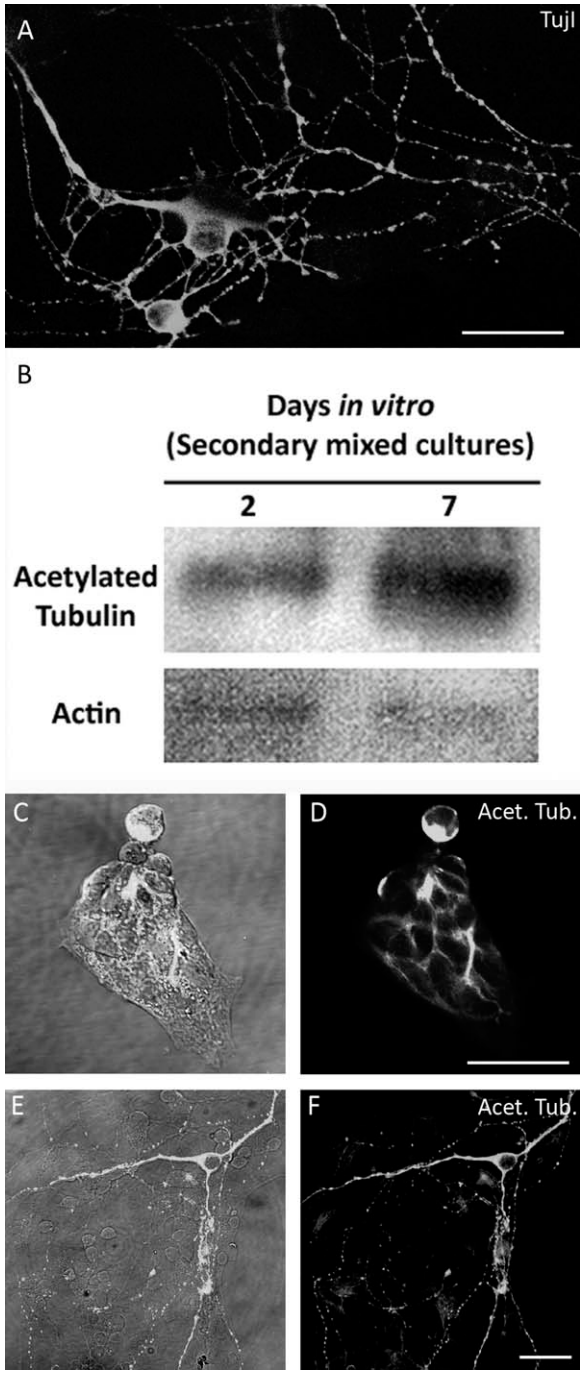

Fig. 6. Round cells were induced to differentiate as neurons in secondary mixed cultures. Confocal fluorescence photomicrographs (A,C–F) show both the expression of Tuj1 (an early neuronal marker) in the extensive neurite network (A) after 7 days in secondary mixed culture and the increase in acetylated alpha tubulin (an axonal neuronal marker) between days 2 (C,D) and 7 (E,F) in secondary mixed cultures. This increase in acetylated alpha tubulin levels between days 2 and 7 is shown by Western blot (B). Scale bars = 20 μm.

processes resembling rudimentary outer segments (arrowheads in Fig. 7B,D,F,H). Over 10% of round cells already expressed opsin (Fig. 5E), indicating that they had gone farther along the photoreceptor differentiation pathway.

Newly Generated Photoreceptor-Like Cells Show Functional Characteristics

Next we investigated whether these cells expressing photoreceptor markers also had characteristics of functional photoreceptors. The expression of opsin in many photoreceptor-like cells in secondary mixed cultures suggested that exposure to light might activate the visual

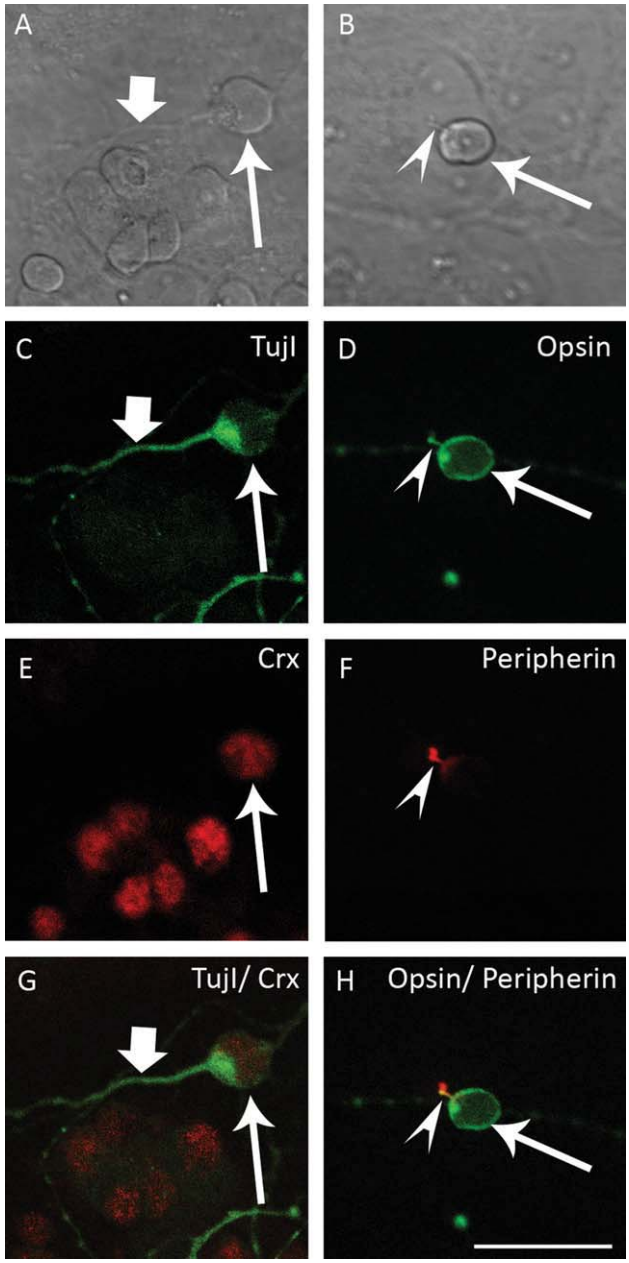

Fig. 7. Round cells developed photoreceptor characteristics in secondary mixed cultures. Confocal Nomarsky (A,B) and fluorescence (C–H) photomicrographs of 7-day secondary mixed neuron–glia cultures showing expression of Tuj1 (C), opsin (D), Crx (E), and peripherin (F) in round cells (thin arrows). Merged images (G,H) show coexpression of Tuj-1 and Crx (G) and of peripherin and opsin (H). Note that most round cells expressed Crx (E), and many developed conspicuous Tuj1-positive axons (wide arrows in C,G) and also showed apical processes, which localized opsin and peripherin labeling (arrrowheads in D,F,H). Scale bar = 20 μm.

transduction cascade in these cells. If so, this would turn on complex biochemical pathways leading to a reduction in cGMP levels. To test this possibility, we evaluated cGMP levels in cultures exposed to ambient light or kept in the dark. When secondary mixed cultures were exposed to light, cGMP levels decreased by over 40% compared with cGMP levels in cultures kept in the dark (Fig. 8A). In contrast, neither pure neuronal cultures nor pure Müller glial cell cultures showed significant changes in cGMP levels after light exposure (Fig. 8A). However, when pure neuronal cultures were supplemented with glial conditioned medium, a light response similar to that observed in secondary mixed cultures was obtained (Fig. 8A). These results suggest that Müller glial cells might release soluble factors that advanced photoreceptor differentiation.

To evaluate further the functionality of photoreceptor-like cells in secondary mixed cultures, we investigated [^{3}H]glutamate uptake in the secondary mixed cultures. In the retina in vivo, both neurons and Müller glial cells have glutamate transporters that control extracellular glutamate levels in extracellular fluid, thus keeping nontoxic levels of this metabolite (Danbolt, 2001). Glutamate is one of the main neurotransmitters in photoreceptors, and high-affinity uptake of synaptically released glutamate is one of the functional properties of mature photoreceptors (Politi and Adler, 1986). The uptake of [^{3}H]glutamate in secondary mixed cultures was over 25,000 dpm/dish (Fig. 8B). For controls, we evaluated the uptake of [^{3}H]glutamate in mixed cultures kept in an Na^+-free medium and at low temperature; under both conditions, there was almost no [^{3}H]glutamate uptake. Next we investigated by autoradiographic analysis which cellular type was responsible for [^{3}H]glutamate uptake. Nearly all ^{3}H-labeled cells were photoreceptor-like cells, with round cell bodies (Fig. 8C,D, arrowhead), some of them with axon-like processes. The uptake of [^{3}H]glutamate in Müller glial cells (Fig. 8C,D, arrows) was negligible. These results imply that photoreceptor neurons were mainly responsible for high-affinity glutamate uptake. As a whole, this evidence suggests that, in mixed cultures, Müller glial cells instructed RPCs to differentiate into functional photoreceptors.

DISCUSSION

In response to injuries, Müller glial cells are able to replace lost neurons in fish and chick retina and have a similar, though more restricted, behavior in rat and mouse retina, generating progenitors that eventually express neuronal markers (Karl and Reh, 2010). Our present findings suggest that, at least in vitro, Müller glial cells might also have other effects. First, Müller glial cells in culture with retina neurons induced and/or preserved stem cell characteristics in RPCs that allowed these cells to survive and proliferate, even after successive passages. Second, Müller glial cells influenced the fate of the generated progenitors: with time in culture, many RPCs left the cell cycle and exhibited morphological, molecular, and functional characteristics that suggested their differentiation as mature photoreceptors.

Understanding the mechanisms that activate neurogenesis in the retina is a prerequisite for developing

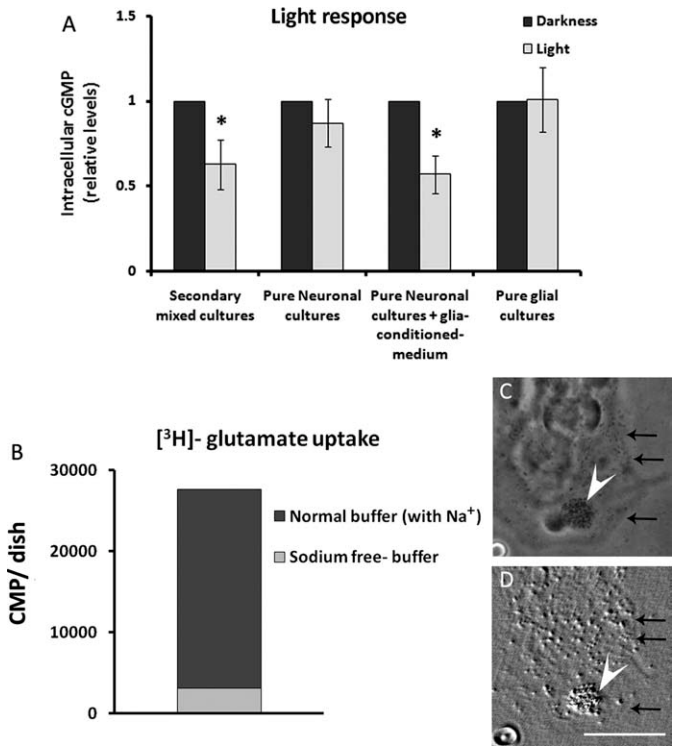

Fig. 8. Photoreceptors in secondary mixed neuron–glia cultures displayed functional characteristics. Light response was assessed in secondary mixed neuron–glia cultures by measuring cGMP hydrolysis (A). Day-6 mixed cultures were kept in the dark for 24 hr and then exposed (or not) to light for 10 min. The decrease in cGMP levels after light exposure, compared with cultures left in the dark, suggests the presence of functional phototransduction pathways in secondary mixed cultures. On the contrary, pure neuronal and pure Müller glial cell cultures showed no significant changes in cGMP levels after light exposure (A). A significant cGMP reduction was observed after light exposure of pure neuronal cultures supplemented with glial-conditioned medium (A). [^{3}H]glutamate uptake (B–D) was evaluated in day-7 secondary mixed neuron–glia cultures, incubated with 1 μCi/μl for 15 min; cells were then lysed to determine uptake (B) or fixed to perform autoradiographic studies (C,D). Photoreceptor-like round cells (arrowheads) took up [^{3}H]glutamate, as shown in phase (C) and Nomarsky (D) photomicrographs, whereas Müller glial cells (arrows in C,D) showed no high-affinity glutamate uptake. The amount of cpm of [^{3}H]glutamate per dish taken up by cells in control and sodium-free buffer is shown in B. Note that this uptake occurred mostly in a sodium-dependent manner, as occurs in vivo. $\star P < 0.05$ compared with controls left in the darkness. Scale bar = 20 μm.

effective replacement strategies to treat retinal degenerative diseases. Retinas of lower vertebrates display robust regenerative capacity either by activation of retinal pigment epithelium transdifferentiation (Sakaguchi et al., 1997; Ikegami et al., 2002; Liang et al., 2006; Vergara and Del Rio-Tsonis, 2009) or through proliferation and differentiation of stem cells such as Müller glial cells (Raymond et al., 1988; Cameron, 2000; Stenkamp and Cameron, 2002). The later are among the main stem cells proposed to generate replacement neurons in the retina. Diverse injuries induce their proliferation and dedifferentiation, both in vivo (Das et al., 2006) and in vitro (Abrahan et al., 2009). The role of Müller glial cells in the generation of new photoreceptors during neurodegenerative diseases of the retina is now widely accepted. Müller glial cells have been shown to proliferate in response to insults, generating progenitors that then express neuronal markers of different neuronal types (Reh and Fischer, 2001; Ooto et al., 2004). Unfortunately, this response is very limited in birds and mammals (Hitchcock et al., 2004; Karl et al., 2008; Karl and Reh, 2010). We have previously demonstrated that RPC from rat retina exit the cell cycle soon after birth; Müller glial cells allow them to preserve their proliferative potential in primary mixed cultures, and stimulation with GDNF maintains them in the cell cycle for one or two mitotic cycles at the most (Insua et al., 2003, 2008). Our present findings show that Müller cells in mixed cultures instructed a pool of undifferentiated RPCs to remain active in the cell cycle for extended periods and preserved them with stem cell characteristics in successive passages. These RPCs took up BrdU and expressed nestin, Sox-2, and Pax6, required for the proliferation and expansion of retina progenitors; the perpetuation of these attributes after successive reseedings is a characteristic of stem cells. Interestingly, the percentage of RPCs is similar to that obtained after chronically passaging Müller glial cells from adult rat and mouse retinas (Nickerson et al., 2008). Preservation of proliferative RPCs occurred only in mixed neuron–glia cultures; RPC in neuronal cultures rapidly exited the cell cycle, and pure Müller glial cell cultures showed very few RPCs, which were unable to self-renew. This suggests that the cross-talk between Müller glial cells and a significant pool of RPCs initially present might be responsible for the maintenance of proliferation and/or induction of stem-like characteristics in these cells. Müller glial cells produce and release many molecular cues, such as bFGF, GDNF, and DHA, which have been shown to promote neuronal survival and promote cell cycle progression (Politi et al., 2001; Harada et al., 2002; Insua et al., 2003). Similarly, the release of specific cues by Müller glial cells might maintain the mitotic potential and induce or preserve stem cell characteristics in RPC in mixed cultures. Taking into account the low rate of regeneration achieved by the transdifferentiation of Müller cells in mammals (Tackenberg et al., 2009), this novel role of Müller glial cells in the generation and perpetuation of retina progenitors might be critical to increase the pool of progenitors during regenerative processes of the retina.

We have previously shown that, in pure neuronal cultures lacking trophic factors for photoreceptors, differentiation of these cells seems to be arrested. Although most of these cells express Crx, very few express opsin, and most of them lack their characteristic outer segments (Rotstein et al., 1998; Garelli et al., 2006). In secondary mixed cultures, Müller glial cells instructed a subpopulation of RPC to exit the cell cycle, adopt a photoreceptor

fate, and then proceed with their differentiation to achieve a high degree of maturation. The occurrence of RPCs showing BrdU uptake and expression of Crx, the earliest photoreceptor-specific transcription factor (Chen et al., 1997; Furukawa et al., 1997) in secondary mixed cultures, indicates that these progenitors had their last mitotic division in these cultures and soon after initiated their differentiation as neurons, as evidenced by the presence of Tuj1, an early neuronal marker, and in particular as photoreceptors. Crx-expressing cells were still found after the fifth successive reseeding. It is well known that reseeding of neurons leads to extensive death; hence, those Crx-positive cells had to originate from RPCs that differentiated in that passage, implying that the capacity to give rise to photoreceptors was preserved in these progenitors.

After they had left the cell cycle, these RPC rapidly developed many morphological and molecular features characteristic of mature photoreceptors, such as the expression of opsin and peripherin and the formation of conspicuous apical processes in which both proteins were localized. These cells also developed extensive axon-like processes, labeled with Tuj1 and acetylated alpha tubulin, both of which stabilize microtubule structures in neuronal axons. Moreover, photoreceptor-like cells in secondary mixed cultures showed a high degree of functional maturation, evidenced by the activation of the phototransduction cascade after light exposure and by their high-affinity glutamate uptake. Exposure to light induced a significant cGMP hydrolysis in secondary mixed cultures, which was absent in pure neuronal cultures. Light absorption by photoreceptors in the retina in vivo activates a cascade of reactions, the phototransduction cascade, which leads to cGMP hydrolysis by phosphodiesterases (Lolley et al., 1977, 1992; Capovilla et al., 1982). Our results indicate that such light-activated phosphodiesterases were present in photoreceptors in secondary mixed cultures; notably, photoreceptors also became functional when incubated with glial-conditioned media. This suggests that Müller glial cells not only induced the differentiation of progenitors as photoreceptors but also remarkably stimulated their maturation, giving rise to light-sensitive photoreceptor cells. Our results imply that the cross-talk with Müller glial cells was indispensable in further advancing photoreceptor differentiation; Müller glial cells might release soluble factors that permit photoreceptor differentiation to progress until the molecular pathways involved in light responses are activated.

Retina neurons have high-affinity uptake mechanisms for their neurotransmitters, with nonphotoreceptor cells taking up γ-aminobutyric acid and photoreceptors taking up glutamate by high-affinity mechanisms via glutamate transporter 1 (GLT1; Politi and Adler, 1986, 1988; Schmitt et al., 2002; Harada et al., 2007). Glutamate transporters located in the plasma membrane transport glutamate in an Na^+- and voltage-dependent manner (Brew and Attwell, 1987; Barbour et al., 1988; Kanai et al., 1994). In contrast, the vesicular transporter selectively concentrates glutamate into vesicles in an Na^+-independent, ATP-dependent manner (Naito and Ueda, 1983; Tabb and Ueda, 1991; Fykse and Fonnum, 1996). Our results indicated a marked high-affinity [^{3}H]glutamate uptake in secondary mixed cultures, and autoradiographic studies revealed that photoreceptor-like cells were mainly responsible for this uptake. Together with the ability of these cells to respond to light, these results reveal that photoreceptor cells in mixed cultures show a remarkable progress in their differentiation, developing distinctive features of mature photoreceptors. In a seminal work, Adler and Hatlee (1989) showed that differentiation as photoreceptors represents a default pathway followed by progenitor cells in the absence of neuron-inducing signals. Our results indicate that RPCs behave like multipotent cells that preferentially follow a rod photoreceptor differentiation pathway. Müller glial cells are well known for their relevant role in photoreceptor morphogenesis, promoting extensive neurite outgrowth in rod photoreceptors (Kljavin and Reh, 1991). Our findings are consistent with this role of Müller glial cells and show that they effectively promote photoreceptor differentiation.

As a whole, our results suggest that the dialog with Müller cells is crucial for RPCs to persist and maintain their stem cell characteristics and eventually to adopt a photoreceptor fate and develop as functional photoreceptors. The delicate balance between self-renewal and differentiation of stem cells depends on myriad intracellular and extracellular factors. Together, stem cells and elements of the microenvironment constitute a niche, in which the later play both anatomical and functional roles in the survival and differentiation of stem cells (Moore and Lemischka, 2006; Scadden, 2006). Niche cells nurture stem cells and regulate their number and fate, integrating environmental signs to mediate the balanced response of stem cells to the requirement of the organism. Our findings suggest that Müller glial cells might have roles that resemble those proposed for niche cells. Müller glial cells might act as niche cells in the retina, making possible the self-renewal of progenitor cells and, upon adequate environmental signals, advancing their differentiation as photoreceptors.

In summary, we propose that Müller cells contribute in different ways to photoreceptor renewal during retina regeneration. In addition to activating their own dedifferentiation and further differentiation into photoreceptors, they might instruct a subpopulation of RPCs to acquire characteristics of neural stem cells and then induce their differentiation to become functional photoreceptors. This might increase the pool of ptoliferating progenitors, enhancing the limited regenerative capacity of mammalian Müller cells.

ACKNOWLEDGMENTS

The authors are deeply indebted to Dr. Grant S. Mastick (University of Nevada, Reno), Dr. Cheryl M. Craft (University of Southern California), and Dr. Robert S. Molday, (University of British Columbia) for

generously supplying polyclonal rabbit anti-Pax6, rabbit anti-Crx, and monoclonal antiopsin (Rho4D2) and anti-peripherin (clone Per3B6) antibodies, respectively.

REFERENCES

Abrahan CE, Insua MF, Politi LE, German OL, Rotstein NP. 2009. Oxidative stress promotes proliferation and dedifferentiation of retina glial cells in vitro. J Neurosci Res 87:964–977.

Adler R. 1982. Regulation of neurite growth in purified retina neuronal cultures: effects of PNPF, a substratum-bound, neurite-promoting factor. J Neurosci Res 8:165–177.

Adler R, Hatlee M. 1989. Plasticity and differentiation of embryonic retinal cells after terminal mitosis. Science 243:391–393.

Barbour B, Brew H, Attwell D. 1988. Electrogenic glutamate uptake in glial cells is activated by intracellular potassium. Nature 335:433–435.

Barnstable CJ. 1980. Monoclonal antibodies which recognize different cell types in the rat retina. Nature 286:231–235.

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

Brew H, Attwell D. 1987. Electrogenic glutamate uptake is a major current carrier in the membrane of axolotl retinal glial cells. Nature 327:707–709.

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

Capovilla M, Cervetto L, Torre V. 1982. Antagonism between steady light and phosphodiesterase inhibitors on the kinetics of rod photoresponses. Proc Natl Acad Sci U S A 79:6698–6702.

Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ. 1997. Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron 19:1017–1030.

Danbolt NC. 2001. Glutamate uptake. Prog Neurobiol 65:1–105.

Das AV, Mallya KB, Zhao X, Ahmad F, Bhattacharya S, Thoreson WB, Hegde GV, Ahmad I. 2006. Neural stem cell properties of Müller glia in the mammalian retina: regulation by Notch and Wnt signaling. Dev Biol 299:283–302.

Derouiche A. 1996. Possible role of the Müller cell in uptake and metabolism of glutamate in the mammalian outer retina. Vis Res 36:3875–3878.

Fischer AJ, Reh TA. 2003. Potential of Müller glia to become neurogenic retinal progenitor cells. Glia 43:70–76.

Fontaine V, Kinkl N, Sahel J, Dreyfus H, Hicks D. 1998. Survival of purified rat photoreceptors in vitro is stimulated directly by fibroblast growth factor-2. J Neurosci 18:9662–9672.

Furukawa T, Morrow EM, Cepko CL. 1997. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91:531–541.

Fykse EM, Fonnum F. 1996. Amino acid neurotransmission: dynamics of vesicular uptake. Neurochem Res 21:1053–1060.

Garelli A, Rotstein NP, Politi LE. 2006. Docosahexaenoic acid promotes photoreceptor differentiation without altering Crx expression. Invest Ophthalmol Vis Sci 47:3017–3027.

Harada T, Harada C, Kohsaka S, Wada E, Yoshida K, Ohno S, Mamada H, Tanaka K, Parada LF, Wada K. 2002. Microglia–Müller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. J Neurosci 22:9228–9236.

Harada T, Harada C, Nakamura K, Quah HM, Okumura A, Namekata K, Saeki T, Aihara M, Yoshida H, Mitani A, Tanaka K. 2007. The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma. J Clin Invest 117:1763–1770.

Haynes T, Del Rio-Tsonis K. 2004. Retina repair, stem cells and beyond. Curr Neurovasc Res 1:231–239.

Hicks D, Barnstable CJ. 1987. Different rhodopsin monoclonal antibodies reveal different binding patterns on developing and adult rat retina. J Histochem Cytochem 35:1317–1328.

Hicks D, Courtois Y. 1990. The growth and behaviour of rat retinal Müller cells in vitro. 1. An improved method for isolation and culture. Exp Eye Res 51:119–129.

Hill RE, Favor J, Hogan BL, Ton CC, Saunders GF, Hanson IM, Prosser J, Jordan T, Hastie ND. 1991. Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature 354:522–525.

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

Ikegami Y, Mitsuda S, Araki M. 2002. Neural cell differentiation from retinal pigment epithelial cells of the newt: an organ culture model for the urodele retinal regeneration. J Neurobiol 50:209–220.

Insua MF, Garelli A, Rotstein NP, German OL, Arias A, Politi LE. 2003. Cell cycle regulation in retinal progenitors by glia-derived neurotrophic factor and docosahexaenoic acid. Invest Ophthalmol Vis Sci 44:2235–2244.

Insua MF, Simon MV, Garelli A, de Los SB, Rotstein NP, Politi LE. 2008. Trophic factors and neuronal interactions regulate the cell cycle and Pax6 expression in Müller stem cells. J Neurosci Res 86:1459–1471.

Jomary C, Jones SE. 2008. Induction of functional photoreceptor phenotype by exogenous Crx expression in mouse retinal stem cells. Invest Ophthalmol Vis Sci 49:429–437.

Kanai Y, Stelzner M, Nussberger S, Khawaja S, Hebert SC, Smith CP, Hediger MA. 1994. The neuronal and epithelial human high affinity glutamate transporter. Insights into structure and mechanism of transport. J Biol Chem 269:20599–20606.

Karl MO, Reh TA. 2010. Regenerative medicine for retinal diseases: activating endogenous repair mechanisms. Trends Mol Med 16:193–202.

Karl MO, Hayes S, Nelson BR, Tan K, Buckingham B, Reh TA. 2008. Stimulation of neural regeneration in the mouse retina. Proc Natl Acad Sci U S A 105:19508–19513.

Kirn JR, Nottebohm F. 1993. Direct evidence for loss and replacement of projection neurons in adult canary brain. J Neurosci 13:1654–1663.

Kljavin IJ, Reh TA. 1991. Müller cells are a preferred substrate for in vitro neurite extension by rod photoreceptor cells. J Neurosci 11:2985–2994.

Laemmli UK, Beguin F, Gujer-Kellenberger G. 1970. A factor preventing the major head protein of bacteriophage T4 from random aggregation. J Mol Biol 47:69–85.

Liang L, Yan RT, Ma W, Zhang H, Wang SZ. 2006. Exploring RPE as a source of photoreceptors: differentiation and integration of transdifferentiating cells grafted into embryonic chick eyes. Invest Ophthalmol Vis Sci 47:5066–5074.

Lolley RN, Farber DB, Rayborn ME, Hollyfield JG. 1977. Cyclic GMP accumulation causes degeneration of photoreceptor cells: simulation of an inherited disease. Science 196:664–666.

Lolley RN, Craft CM, Lee RH. 1992. Photoreceptors of the retina and pinealocytes of the pineal gland share common components of signal transduction. Neurochem Res 17:81–89.

Moore KA, Lemischka IR. 2006. Stem cells and their niches. Science 311:1880–1885.

Naito S, Ueda T. 1983. Adenosine triphosphate-dependent uptake of glutamate into protein I-associated synaptic vesicles. J Biol Chem 258:696–699.

Nickerson PE, Da SN, Myers T, Stevens K, Clarke DB. 2008. Neural progenitor potential in cultured Müller glia: effects of passaging and exogenous growth factor exposure. Brain Res 1230:1–12.

Ooto S, Akagi T, Kageyama R, Akita J, Mandai M, Honda Y, Takahashi M. 2004. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. Proc Natl Acad Sci U S A 101:13654–13659.

Poitry S, Poitry-Yamate C, Ueberfeld J, MacLeish PR, Tsacopoulos M. 2000. Mechanisms of glutamate metabolic signaling in retinal glial (Müller) cells. J Neurosci 20:1809–1821.

Poitry-Yamate CL, Poitry S, Tsacopoulos M. 1995. Lactate released by Müller glial cells is metabolized by photoreceptors from mammalian retina. J Neurosci 15:5179–5191.

Politi LE, Adler R. 1986. Generation of enriched populations of cultured photoreceptor cells. Invest Ophthalmol Vis Sci 27:656–665.

Politi L, Adler R. 1988. Selective failure of long-term survival of isolated photoreceptors from both homozygous and heterozygous rd (retinal degeneration) mice. Exp Eye Res 47:269–282.

Politi LE, Bouzat C, los Santos EB, Barrantes FJ. 1996. Heterologous retinal cultured neurons and cell adhesion molecules induce clustering of acetylcholine receptors and polynucleation in mouse muscle BC3H-1 clonal cell line. J Neurosci Res 43:639–651.

Politi LE, Rotstein NP, Carri NG. 2001. Effect of GDNF on neuroblast proliferation and photoreceptor survival: additive protection with docosahexaenoic acid. Invest Ophthalmol Vis Sci 42:3008–3015.

Raymond PA, Reifler MJ, Rivlin PK. 1988. Regeneration of goldfish retina: rod precursors are a likely source of regenerated cells. J Neurobiol 19:431–463.

Reh TA, Fischer AJ. 2001. Stem cells in the vertebrate retina. Brain Behav Evol 58:296–305.

Rotstein NP, Aveldano MI, Barrantes FJ, Politi LE. 1996. Docosahexaenoic acid is required for the survival of rat retinal photoreceptors in vitro. J Neurochem 66:1851–1859.

Rotstein NP, Aveldano MI, Barrantes FJ, Roccamo AM, Politi LE. 1997. Apoptosis of retinal photoreceptors during development in vitro: protective effect of docosahexaenoic acid. J Neurochem 69:504–513.

Rotstein NP, Politi LE, Aveldano MI. 1998. Docosahexaenoic acid promotes differentiation of developing photoreceptors in culture. Invest Ophthalmol Vis Sci 39:2750–2758.

Sakaguchi DS, Janick LM, Reh TA. 1997. Basic fibroblast growth factor (FGF-2) induced transdifferentiation of retinal pigment epithelium: generation of retinal neurons and glia. Dev Dyn 209:387–398.

Scadden DT. 2006. The stem-cell niche as an entity of action. Nature 441:1075–1079.

Schmitt A, Asan E, Lesch KP, Kugler P. 2002. A splice variant of glutamate transporter GLT1/EAAT2 expressed in neurons: cloning and localization in rat nervous system. Neuroscience 109:45–61.

Stenkamp DL, Cameron DA. 2002. Cellular pattern formation in the retina: retinal regeneration as a model system. Mol Vis 8:280–293.

Tabb JS, Ueda T. 1991. Phylogenetic studies on the synaptic vesicle glutamate transport system. J Neurosci 11:1822–1828.

Tackenberg MA, Tucker BA, Swift JS, Jiang C, Redenti S, Greenberg KP, Flannery JG, Reichenbach A, Young MJ. 2009. Müller cell activation, proliferation and migration following laser injury. Mol Vis 15:1886–1896.

van der Kooy D, Weiss S. 2000. Why stem cells? Science 287:1439–1441.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. 2002. Functional neurogenesis in the adult hippocampus. Nature 415:1030–1034.

Vergara MN, Del Rio-Tsonis K. 2009. Retinal regeneration in the *Xenopus laevis* tadpole: a new model system. Mol Vis 15:1000–1013.