

Available online at www.sciencedirect.com

ScienceDirect

Developmental Biology 299 (2006) 283–302

**DEVELOPMENTAL
BIOLOGY**

www.elsevier.com/locate/ydbio

Neural stem cell properties of Müller glia in the mammalian retina: Regulation by Notch and Wnt signaling

Ani V. Das, Kavita B. Mallya, Xing Zhao, Faraz Ahmad, Sumitra Bhattacharya,
Wallace B. Thoreson, Ganapati V. Hegde, Iqbal Ahmad*

*Department of Ophthalmology and Visual Sciences, DRC 4034, 98-5840 Nebraska Medical Center, University of Nebraska Medical Center,
Omaha, NE 68198-5840, USA*

Received for publication 31 May 2006; revised 16 July 2006; accepted 25 July 2006
Available online 29 July 2006

Abstract

The retina in adult mammals, unlike those in lower vertebrates such as fish and amphibians, is not known to support neurogenesis. However, when injured, the adult mammalian retina displays neurogenic changes, raising the possibility that neurogenic potential may be evolutionarily conserved and could be exploited for regenerative therapy. Here, we show that Müller cells, when retrospectively enriched from the normal retina, like their radial glial counterparts in the central nervous system (CNS), display cardinal features of neural stem cells (NSCs), i.e., they self-renew and generate all three basic cell types of the CNS. In addition, they possess the potential to generate retinal neurons, both in vitro and in vivo. We also provide direct evidence, by transplanting prospectively enriched injury-activated Müller cells into normal eye, that Müller cells have neurogenic potential and can generate retinal neurons, confirming a hypothesis, first proposed in lower vertebrates. This potential is likely due to the NSC nature of Müller cells that remains dormant under the constraint of non-neurogenic environment of the adult normal retina. Additionally, we demonstrate that the mechanism of activating the dormant stem cell properties in Müller cells involves Wnt and Notch pathways. Together, these results identify Müller cells as latent NSCs in the mammalian retina and hence, may serve as a potential target for cellular manipulation for treating retinal degeneration.

© 2006 Elsevier Inc. All rights reserved.

Keywords: Müller cells; Stem cells; Retina; Progenitors; Notch signaling; Wnt signaling; Chemical injury

Introduction

Two recent developments have major impact on our understanding of brain development and the potential of treating neurodegenerative changes. The first is the reaffirmation of long-standing observations that the adult brain harbors proliferating populations of cells and that neurons are born throughout life, particularly in the subventricular zone (SVZ) of the lateral ventricle and subgranular layer (SGL) of the dentate gyrus of the hippocampus (Alvarez-Buylla and Lim, 2004). The second is that glia perform dual functions of providing homeostatic support and participating in neurogene-

sis (Alvarez-Buylla et al., 2001; Doetsch, 2003; Gotz and Barde, 2005). A variety of approaches demonstrated that SVZ and SGL astrocytes possess NSC properties, and are the primary source of neurogenesis in the lateral ventricle-rostral migratory zone and hippocampus (Doetsch et al., 1999; Seri et al., 2001). Radial glia, popularly known for providing scaffolds for migrating neuroblasts, were demonstrated to serve as NSCs in the embryonic brain and a prevalent source of cortical projection neurons (Malatesta et al., 2003; Noctor et al., 2001). Recently, SVZ radial glia in the adult brain were observed to be the source of SVZ astrocytic stem cells (Merkle et al., 2004).

Unlike the SVZ and SGL, active neurogenesis has not been detected in the normal adult mammalian retina. However, neurogenic changes have been observed in injured retina, and one of the sources of injury-induced neurogenesis is thought to

* Corresponding author. Fax: +1 402 559 3251.

E-mail address: iahmad@unmc.edu (I. Ahmad).

be the major glial cell type of the retina, the Müller cells (Braisted et al., 1994; Ooto et al., 2004; Reh and Fischer, 2001). Are Müller cells latent NSCs in retina, that can be activated by extrinsic cues, including those that are injury-induced, to proliferate and generate new neurons? To answer this question, we purified Müller cells from the rodent retina and demonstrated that they display cardinal features of NSCs *in vitro*, i.e., they self-renew, and generate both neurons and glia, in addition to generating retinal neurons in conducive culture conditions. Müller cells, identified and enriched prospectively as side population (SP) cells, when transplanted into retina generated lamina-specific retinal neurons, thus providing a direct proof of their neurogenic potential. In addition, our results suggested that Notch and Wnt pathways acted in concert to regulate the stem cell properties of Müller cells. This raises the possibility of treating degenerating retinas from within, by targeted manipulation of these cells.

Materials and methods

Müller cell culture

Enrichment of Müller cells was done according to a previously described method (Hicks and Courtois, 1990). Briefly, eyes from postnatal (PN) days 10–21 rats were enucleated and incubated overnight in DMEM. Eyecups were transferred to dissociation solution (DMEM containing 0.1% Trypsin and 70 U/ml collagenase) and incubated at 37°C in CO₂ incubator for 1 h. Eyecups were washed with DMEM and the retina was dissected out with care to avoid contamination from RPE and ciliary epithelium. The retina was mechanically dissociated into small aggregates and cultured in DMEM containing 10% FBS for 8–10 days. The floating retinal aggregates and debris were removed leaving purified flat cell population of Müller cells attached to the bottom of the dish. Cells were trypsinized and cultured in DMEM containing 10% FBS for another 5 days to get a further purified population. Cells were dissociated using Trypsin–EDTA and cultured in DMEM/F12 (containing 1× N2 supplement (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin) supplemented with FGF2 (10 ng/ml; Collaborative Research) and EGF (20 ng/ml; Collaborative Research) at a density of $\sim 5 \times 10^4$ cells/cm² for 5–7 days to generate neurospheres. To remove microglia, cell dissociates were subjected to immunopanning using Mac-1 antibody (Barres et al., 1988; Meucci et al., 1998). To examine their neurosphere-forming ability, microglia was purified from retina according to a previously described method (Roque and Caldwell, 1993) and cultured in the same medium mentioned above for 4–6 days. To ascertain the clonal generation of neurospheres by co-culturing green (GFP-expressing) and non-green cells (Zhao et al., 2002), Müller cells were enriched from the retina of the green [constitutively expressing the green fluorescent protein (GFP)] and wild-type mice as described above. LDA analysis was carried out as previously described (Ahmad et al., 2004; Das et al., 2004). Cells were diluted to give an initial concentration of 5000 cells/ml from which serial dilutions in 200 µl aliquots were plated in individual wells of a 96-well plate. Culture was carried out for 7 days, after which the fraction of wells not containing neurospheres for each cell-plating density was calculated. The negative logarithm of fraction of negative wells was plotted against the number of cells plated/well to provide a straight line in a semi logarithm plot. The zero term of Poisson equation ($F_0 = e^{-x}$, where F_0 is the fraction of well without neurospheres and x is the mean number of cell/well) predicts that when 37% of test wells are negative, there is an average of one stem cell/well. To examine the differentiation potential of Müller cell-derived stem cells/progenitors, neurospheres were exposed to 10 µM BrdU (Sigma) for the final 48 h to tag the dividing cells and plated on poly-D-lysine (500 µg/ml) and laminin (5 µg/ml) coated 12-mm glass coverslips. In order to promote differentiation, mitogens were substituted with brain-derived neurotrophic factor (BDNF; 1 ng/ml), retinoic acid (RA; 1 µM) and 1% FBS and culture was continued for 5–7 days. Cells were fixed using cold 4% paraformaldehyde for immunocytochemical analysis.

Table 1
List of antibodies used

Name	Species	Dilution	Company
Vimentin	Mouse	1:10	Hybridoma
GS	Rabbit	1:5000	Sigma
Nestin	Mouse	1:4	Hybridoma
Sox2	Rabbit	1:500	Chemicon
Notch1	Rat	1:1	Gift
β-Tubulin	Rabbit	1:1000	Babco
GFAP	Rabbit	1:100	Sigma
O4	Mouse	1:30	Chemicon
Map2	Mouse	1:200	Chemicon
Calbindin	Rabbit	1:10,000	Gift
Pax6	Rabbit	1:1000	Babco
Chx10	Rabbit	1:250	Gift
Rx	Mouse	1:250	Gift
CD31	Mouse	1:50	Chemicon
Mac-1	Rabbit	1:100	Accurate Chemical and Corp.
Bm3b	Rabbit	1:300	Babco
Opsin	Mouse	1:1000	Gift
PKC	Rabbit	1:1000	Sigma
Syntaxin	Mouse	1:100	Gift
BrdU	Rat	1:100	Accurate Chemical and Corp.

FACS analysis

In order to find out the purity of enriched Müller cells, we carried out FACS analysis using antibodies specific to Müller glia such as glutamine synthetase (GS) and vimentin. Briefly, cells in the monolayer culture were dissociated into single cells and fixed using ice-cold ethanol. After washing with 1× PBS containing 1% BSA, cells (2×10^6) were incubated with appropriate dilution of GS/vimentin (Table 1) in 1× PBS containing 1% BSA for 1 h at 4°C. Prior to antibody incubation, cells were blocked for non-specific binding in 1× PBS containing 0.1% Triton-X100 for 30 min at 4°C. After antibody incubation, cells were washed and incubated in PBS-BSA solution containing appropriate secondary antibodies linked to FITC for 1 h at 4°C. Cells were washed with PBS-BSA and resuspended in PBS-BSA for FACS analysis.

Immunocytochemical analysis

Immunocytochemical analysis was carried out for the detection of BrdU and/or cell-specific markers as previously described (Zhao et al., 2002). Briefly, paraformaldehyde-fixed cells were incubated in PBS containing 5% NGS and 0, 0.2 or 0.4% Triton-X100 followed by an overnight incubation in antibodies at 4°C. The list of antibodies used is given in Table 1. Cells were examined for epifluorescence following incubation in IgG conjugated to Cy3/FITC. Images were captured using cooled CCD-camera (Princeton Instruments) and Openlab software (Improvision).

RT-PCR analysis

Isolation of total RNA and cDNA synthesis were carried out as previously described (James et al., 2003). Briefly, 4 µg of RNA was transcribed into cDNA in total volume of 50 µl. Specific transcripts were amplified with gene-specific forward and reverse primers using a step cycle program on a Robocycler (Stratagene). Amplifications were carried out for 25 cycles so that they remained within the range of linearity to yield a semi-quantitative estimation of changes in the relative levels of transcripts. Products were visualized by ethidium bromide staining after electrophoresis on 2% agarose gel. Gene-specific primers used for RT-PCR analyses are given in Table 2.

Co-culture experiments

BrdU-labeled neurospheres derived from enriched Müller cells were collected, washed extensively to remove excess BrdU and co-cultured on

Table 2
List of primers used for the RT-PCR analysis

Name	Sequence	Annealing temp (°C)	Size (bp)	Acc. No
Vimentin	Forward: 5'AAGGCACTAATGAGTCCCTGGAG3' Reverse: 5'GTTTGAAGAGGCAGAGAAATCC3'	56	251	NM031140
Glutamine synthetase (GS)	Forward: 5'TCACAGGGACAAATGCCGAG3' Reverse: 5'GTTGATGTTGGAGGTTTCGTGG3'	58	362	M96152
CRALBP	Forward: 5'CTGAGTTTGGAGGAATCTTGC3' Reverse: 5'TGGATTGGGGGAGAGTTC3'	54	150	XM217702
Opsin	Forward: 5'CATGCAGTGTTTCATGTGGGA 3' Reverse: 5'AGCAGAGGCTGGTGAGCATG 3'	64	422	U22180
mGluR6	Forward: 5'CACAGCGTGATTGACTACGAG3' Reverse: 5'CTCAGGCTCAGTGACACAGTTAG3'	56	317	D13963
HPC1	Forward: 5'AAGAGCATCGAGCAGCAGAGCATC3' Reverse: 5'CATGGCCATGTCCATGAACAT3'	60	342	NM016801
Brn3b	Forward: 5'GGCTGGAGGAAGCAGAGAAATC 3' Reverse: 5'TTGGCTGGATGGCGAAGTAG 3'	60	141	AF390076
β-Tubulin	Forward: 5'TGCGTGTGTACAGGTGAATGC3' Reverse: 5'AGGCTGCATAGTCATTCCAAG3'	54	250	NM139254
GFAP	Forward: 5'ATCTGGAGAGGAAGGTTGAGTCG3' Reverse: 5'TGGCGGCGATAGTCATTAGA3'	58	310	NM017009
PLP	Forward: 5'CGGGTGTGTCATTGTTGGG3' Reverse: 5'ACAGGTGGAAGGTCATTGGAAC3'	58	310	NM030990
Notch1	Forward: 5'TCTGGACAAGATTGATGGCTACG3' Reverse: 5'CGTTGACACAAGGGTTGGACTC3'	56	329	NM008714
Nestin	Forward: 5'TGGAGCAGGAGAAGCAAGGTCTAC3' Reverse: 5'TCAAGGGTATTAGGCAAGGGGG3'	56	295	NM012987
Pax6	Forward: 5'CCATCTTTGCTTGGGAAATCC3' Reverse: 5'TCATCCGAGTCTTCTCCATTGG3'	56	310	NM_013001
Musashi1	Forward: 5'TGAAAGAGTGTCTGGTGTATGCG3' Reverse: 5'GCCTGTTGGTGGTTTTGTTCG3'	52	309	NM148890
Sox2	Forward: 5'AGGGCTGGGAGAAAGAAGAG3' Reverse: 5'GGAGAATAGTTGGGGGAAG3'	56	179	NM_011443
ABCG2	Forward: 5'GATGGCTACTACTCACAAGG3' Reverse: 5'ACTGAATCTAACCAAGGAAGG3'	54	325	NM_011920
Clusterin	Forward: 5'CCTCCAGTCCAAGATGCTCAAC Reverse: 5'TTCTGCGGTATTCTGTAGC	58	292	NM_053021
Carbonic anhydrase	Forward: 5'TTGCCAATGGAGACCCAG Reverse: 5'TGAGCCCCAGTGAAGTGAAAC	58	233	NM_019291
CD31	Forward: 5'AAGAGCAACTTCCAGACCGTCC Reverse: 5'AAGCACATTTCATCTCCAGACTG	58	222	NM_031591
Tyrosinase	Forward: 5'TCAGTCTATGTCATCCCCACAGG Reverse: 5'GTTCTATCCCCAGTTAGTTCTCG	56	252	NM_011661
Ath5	Forward: 5'TGGGG(I)CA(GA)GA(CT)AA(GA)AA(GA)3' Reverse: 5'CAT(I)GG(GA)AA(I)GG(CT)TC(I)GG(CT)TG3'	52	231	AF071223
Ath3	Forward: 5'CGACTGGCAAGGAACTACATCTG3' Reverse: 5'ACTAATGCTCAGGGGTGGTGTG3'	50	629	NM007501
Otx2	Forward: 5'GAGAGGAGGTGGCACTGAAAATC3' Reverse: 5'CCCCAAAGTAGGAAGTTGAGC3'	56	391	U96488
NeuroD	Forward: 5'AAGAGGAGGAGGAAGAGGAGGAG3' Reverse: 5'TTGGTAGTGGGCTGGGACAAAAC3'	58	337	AF107728
CyclinA	Forward: 5'TACACACACGAGGTAGTGACGCTG3' Reverse: 5'CCAAGCCGTTTTTCATCCAGG3'	56	307	X60767
CyclinD1	Forward: 5'ACACCAATCTCCTCAACCGACC3' Reverse: 5'GTTCAACAGAAGCAGTTCCATTG3'	56	383	NM171992
CyclinD3	Forward: 5'CGAAACCACACCCCTGACTATTG3' Reverse: 5'TGCTTTTTGACCAGTGCCTGC3'	60	201	NM012766
Delta1	Forward: 5'CGACCTCGAACAGAAAAC3' Reverse: 5'CGACCTCGAACAGAAAAC3'	56	397	NM032063
Hes1	Forward: 5'GCTTTCCTCATCCCCAATG3' Reverse: 5'CGTATTAGTGTCCGTCAGAAGAG3'	56	224	NM024360
Crx	Forward: 5'CCTCACTATTCCGGTCAATGCC3' Reverse: 5'ATGTGCCTGCCTTCTCTTC3'	58	346	NM_021855
Lef1	Forward: 5'CACACAACGGCATCCCTCATC3' Reverse: 5'TACTCCTGGCTACGACATTCCG3'	56	205	NM130429
Fzd1	Forward: 5'TGGTTTCGTGTCGCTCTTCC 3'	60	223	XM216082

(continued on next page)

Table 2 (continued)

Name	Sequence	Annealing temp (°C)	Size (bp)	Acc. No
Fzd2	Reverse: 5'AGGGCAAGGGATGCATAAC3' Forward: 5'CGCTTCTACTTTCTTCACGGTCAC3'	58	211	NM172035
Fzd4	Reverse: 5'GCCTTCTTTCTTAGTGCCCTGC3' Forward: 5'TGCTTCATCTCTACCACCTTCACTG3'	60	282	NM022623
Ki67	Reverse: 5'CAGAATAACCCACCAAATGGAGC3' Forward: 5'GAGCAGTTACAGGGAACCGAAG3'	58	262	X82786
p27Kip1	Reverse: 5'CCTACTTTGGGTGAAGAGGCTG3' Forward: 5'GACTTTCAGAATCATAAGCCCTG3'	58	261	NM031762
β-Actin	Reverse: 5'TGGACACTGCTCCGCTAAC33' Forward: 5'GTGGGGCGCCCCAGGCACCA 3'	50	548	XM_037235

poly-D-lysine, laminin coated 12-mm glass coverslips with E3 chick/PN1 rat retinal cells in 1% FBS for 5–7 days, across 0.4 μm membrane (Millipore). At the end of culture, cells were fixed with 4% paraformaldehyde for 15 min at 4°C followed by immunocytochemical analysis for various retinal neuronal markers. Media were changed every other day and neurospheres were allowed to differentiate for 5–7 days. To obtain E3 chick retinal cells, fertilized hens eggs (SPAFAS, Wilmington, MA) were incubated in a humidified chamber at 37°C for 3 days. Embryos were staged according to [Hamburger and Hamilton \(1951\)](#). Retina were dissected out from eyes and dissociated into single cells as previously described ([James et al., 2003](#)).

Hoechst dye efflux assay

Hoechst dye efflux assay was carried as previously reported ([Ahmad et al., 2004](#); [Bhattacharya et al., 2003](#)). Briefly, dissociated retinal cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) at a concentration of 10⁶ cells/ml and incubated at 4°C overnight followed by staining with Hoechst 33342 (4 μg/ml) at 37°C for 60 min and sorted on a FACStar Plus (BD Biosciences, Lincoln Park, NJ) cell sorter. Hoechst dye was excited at 350 nm, and fluorescence was measured at two wavelengths using a 485 BP22 (485-nm bandpass filter) and a 675 EFLP (675-nm long-pass edge filter) optical filter (Omega Optical, Brattleboro, VT). The SP region was defined on the flow cytometer on the basis of its fluorescence emission in both blue and red wavelengths. Dead cells and debris were excluded by establishing a live gate on the flow cytometer using forward versus side scatter. The sorted SP cells were processed for immunocytochemical and RT-PCR analyses. The specificity of the assay was ascertained by incubating cells with 150 μM of verapamil during the staining stage.

In vivo activation and enrichment of Müller cells

PN21 rats were used for this experiment. The rats were anesthetized with ketamine and xylazine and right eyes were injected with 0.5 μl–10 μl of a mixed solution of neurotoxins [kainate (40 mM) and NMDA (4 mM)], growth factors (FGF2, 20 ng/eye; insulin, 1 μg/eye) and BrdU (1 μg/eye) by intravitreal injection using a glass micropipette attached to a 10-μl Hamilton syringe as previously described ([Zhao et al., 2005](#); [Chacko et al., 2003](#)). The left eyes were injected with the vehicle and were used as controls. Rats were given intraperitoneal injections of BrdU (0.12 mg/g body weight) everyday until the end of the experiment on 4th day. Eyes were enucleated. Some eyes were processed for immunohistochemical analysis and retina from the rest was subjected to Hoechst dye efflux assay as described above. To examine the role of Notch signaling on the in vivo activated Müller cells, DAPT (25 μM), a γ-secretase inhibitor ([James et al., 2004](#)), was injected along with the growth factors. To examine the involvement of Wnt signaling in the in vivo activation of Müller cells, Wnt2b and FzdCRD conditioned medium was injected intravitreally. Wnt2b and FzdCRD conditioned medium was obtained by transiently transfecting pEF-Wnt2b vector ([Kubo et al., 2003](#)) and Mfz8CRD-IgG ([Hsieh et al., 1999](#)) in 293 cells by using Effectene transfection kit (Qiagen). One day after transfection, cells were transferred to serum-free DMEM/F-12, and the medium was collected after an additional 24 h. Control conditioned medium was obtained from untransfected 293T cells. To

compensate for the artifactual differences in levels of cell-specific transcripts in the injured retina due to cell loss, examination of levels of transcripts by PCR was carried out after normalization of levels of beta actin transcripts in injured and uninjured retina.

Brain and retinal transplantation

Transplantation of GFP⁺ cells from Müller cell-derived neurospheres was carried out as previously described ([Vicario-Abejon et al., 1995](#); [Zhao et al., 2002](#)). Briefly, to implant cells in the hippocampus of the PN2 rat pups ($n=4$) animals were anesthetized by hypothermia. Each animal was placed on a miniaturized hypothermic stereotaxic instrument (Stoelting Co.). A hole was drilled in the skull using a dental burr at 1.7 mm lateral to midline and 1.2 mm posterior to bregma. A pulled glass pipette with an external diameter of ~70 μm was placed ~1.4 mm below dura to deliver 0.5 μl of cell suspension over a period of 2 min. The surgical area was cleaned and the incision was closed with Superglue. The animal was revived on a 37°C heating pad and returned to its mother. Intravitreal transplantation of GFP⁺ cells from Müller cell-derived neurospheres cells or SP cells was performed in PN1 rats ($n=3$) as previously described ([Chacko et al., 2003](#)). Briefly, animals were anesthetized with 20–25 μl of a 1:1 dilution of ketamine (60 mg/ml) and xylazine (8 ng/ml), injected intraperitoneally. A 30-gauge needle was inserted into the eye near the equator. The needle was retracted, and a glass micropipette connected to a 10 μl Hamilton syringe was inserted through the wound to deliver 50,000–100,000 cells in a 1–2 μl volume. Animals were sacrificed 1 week after transplantation. Eyes were enucleated and processed for immunohistochemical analysis. The SP cells were labeled with CFDA-SE as per manufacturer's instructions (Molecular Probes) for tracking purpose. In order to determine the lamina-specific incorporation of grafted cells, retinal sections (75 sections) from different transplantation experiments were examined and total cells integrated in a particular section were counted. Next, the number of cells incorporated in each lamina was counted to determine the lamina-specific proportion of cells with respect to total cell integrated.

Brain overlay culture

To analyze whether Müller cells differentiate into physiologically active neuronal cells in vivo, we carried out brain slice overlay culture according to a previously described method ([Benninger et al., 2003](#); [Polleux et al., 1998](#)). Briefly, PN1 pups were sacrificed by decapitation, brains removed in ice-cold HBSS, and placed on a clean Whatman filter paper. A solution of low-melting agarose was poured on top of the brain and was allowed to solidify by placing the filter on a cooled surface. 300-μm-thick coronal sections were cut using a mechanical tissue chopper (Stoelting Co.). Slices were collected using a flat brush and cultured in a 6-well plate on millicell filter (Millipore) covered with a film of slice culture medium (DMEM containing 25% HBSS and 10% FBS). Müller cell-derived neurospheres from GFP-transgenic mice were dissociated and resuspended in slice culture medium. Approximately 1 × 10⁵ cells were added onto and around the slices and cultured for 3–4 days. At the end of culture, the slices were analyzed for the presence of GFP⁺ cells and used for electrophysiological and immunohistochemical analyses.

Electrophysiological analysis

For electrophysiological studies, brain slices with GFP⁺ cells from Müller cell-derived neurospheres were placed in a chamber, and perfused on the stage of an upright, fixed-stage microscope (for electrophysiology, model BHWI; Olympus, Lake Success, NY; for imaging experiments, model E600FN; Nikon, Melville, NY) with an oxygenated solution containing NaCl, 140 mM; KCl, 5 mM; CaCl₂, 2 mM; MgCl₂, 1 mM; HEPES, 10 mM; glucose, 10 mM (pH 7.4). Experiments were performed at room temperature. For whole-cell recording, patch pipettes were pulled on a vertical puller (model PB-7; Narishige, Tokyo, Japan) from borosilicate glass pipettes (1.2-mm outer diameter, 0.95-mm inner diameter; with internal filament (World Precision Instruments) and had tips of 1- to 2- μ m outer diameter with tip resistances of 6 to 12 M Ω). Pipettes were filled with a bathing solution containing KCH₃SO₄, 98 mM; KCl, 44 mM; NaCl, 3 mM; HEPES, 5 mM; EGTA, 3 mM; MgCl₂, 3 mM; CaCl₂, 1 mM; glucose, 2 mM; Mg-adenosine triphosphate (ATP), 1 mM; guanosine triphosphate (GTP), 1 mM; and reduced glutathione, 1 mM (pH 7.2) (Das et al., 2005b; James et al., 2003). GFP-positive cells were identified under epifluorescence and patched for whole-cell recording.

Adenovirus infection

To eliminate the immigrant astrocytes from the Müller cell culture, we infected the purified Müller cells with *Adgfap2Tk* vector for 24 h and exposed them to Ganciclovir (GCV; 150 μ g/ml) (Das et al., 2006; Vandier et al., 2000) for another 24 h. GCV specifically eliminate Tk-expressing cells. Cells were collected, washed and then cultured in the presence of FGF2 to generate neurospheres.

Results

Enriched Müller cells display properties of NSCs

To examine whether or not Müller cells possess NSC properties we carried out neurosphere assay (Doetsch et al., 1999; Laywell et al., 2000; Merkle et al., 2004) on Müller cells, enriched from rodent retina, ranging from PN10 to PN21. We adopted a monolayer culture protocol that has been demonstrated to enrich Müller cells to the range of greater than 95%, free from neuronal, fibroblastic or astrocytic contaminations (Hicks and Courtois, 1990). In our hands, approximately 95% of cells in the monolayer culture were Müller cells as revealed by the proportion of cells expressing vimentin and glutamine synthetase (GS) immunoreactivities (Figs. 1A–N). To further ascertain the enrichment and purity of monolayer Müller cell culture, we examined the expression of cell-specific transcripts. RT-PCR analysis revealed that cells in culture expressed a battery of transcripts characteristic of Müller cells such as *GS*, *CRALBP*, *Vimentin*, *Clusterin* and *Carbonic anhydrase* (Blackshaw et al., 2004) (Fig. 1O). In contrast, transcripts corresponding to rod photoreceptors (*opsin*), bipolar cells (*mGluR6*), amacrine cells (*Syntaxin 1*), retinal ganglion cells or RCGs (*Brn3b*), endothelial cells (*CD31*) and retinal pigmented epithelium (RPE)/pigmented ciliary epithelium (*tyrosinase*) were not detected suggesting that the monolayer culture was enriched for Müller cells and not contaminated with the abovementioned cells (Fig. 1P). However, low levels of transcripts characteristic of microglia (*Iba1*) were detected, suggesting the presence of microglia in the enriched Müller cell culture. Subsequent immunocytochemical analysis revealed the presence of microglia in the monolayer culture in the range of

2–4%, which could be removed by immunopanning (see below).

Having determined the purity of the culture, we first examined the proliferating and self-renewing abilities of enriched Müller cells. When these cells were cultured in the presence of FGF2 for 4–5 days, a small subset (0.5%–1%) of cells proliferated to generate spheres of an average size of 200 μ m. The size of neurospheres was dependent on culture time [Fig. 2A (5 days in culture) versus Fig. 2P (2 days in culture)]. The spheres displayed classic features of neurospheres; the majority (72.4 \pm 2.6%) of cells in spheres incorporated BrdU, attesting to their proliferative nature and expressed general (Notch1; 83.0 \pm 3.0) and neural (Sox2; 74.0 \pm 3.4; Nestin; 86.0 \pm 1.8, Musashi; 73.0 \pm 4.3) stem cell markers (Figs. 2A–F and G). The majority (88.0 \pm 1.5%) of proliferating cells co-expressed Nestin and Notch, suggesting that these cells did not represent distinct sub-populations of cells but rather a single population of neural progenitors, expressing multiple neural progenitor markers (Figs. 2H–L). Although the absence of cells expressing CD31, retinal neuron-specific markers and tyrosinase ensured that endothelial cells, retinal neurons, RPE/pigmented ciliary epithelium, respectively, were not the source of neurospheres, a possibility remained that the source could be the contaminating microglia. To examine this possibility, we purified microglia from retina (Roque and Caldwell, 1993) and cultured them identically like enriched Müller cells in the presence of FGF2. At the end of 7 days in culture, neurospheres were not detected (Fig. 2M). In addition, we observed that enriched Müller cells, following the removal of microglia by immunopanning, still formed neurospheres, when exposed to FGF2 for 2 days (Fig. 2N). Together, these observations suggested that enriched Müller cells and not contaminating microglia were the source of neurospheres. It could be argued that immigrant astrocytes, as a possible minor contaminant in the monolayer culture, could be the source of neurospheres. To address this issue, we adapted an approach of selectively ablating GFAP-expressing astrocytes in the monolayer culture, followed by examining the generation of neurospheres (Das et al., 2006; Morshead et al., 2003). Cells in the monolayer culture, before their exposure to FGF2, were infected with adenoviral vector *Adgfap2Tk* in which the HSV-Tk gene is driven by GFAP promoter. Following infection and exposure to ganciclovir (GCV) for 24 h, cells were cultured in the presence of FGF2 for 1–2 days. GCV is converted into a toxic triphosphate form in the presence of HSV-Tk, killing dividing cells by incorporating in replicating DNA (Vandier et al., 2000). We observed the generation of neurospheres in culture of *Adgfap2Tk* infected cells and those that were infected with the control virus, providing evidence against immigrant astrocytes as the source of neurospheres (Fig. 2O).

To address the issue that neurospheres generated in bulk culture may not have clonal origin but arise due to cell aggregation, we co-cultured green [obtained from green fluorescent protein (GFP)-expressing mice] and non-green (obtained from wild-type mice) enriched Müller cells in the presence of FGF2. The culture was carried out for 2 days since

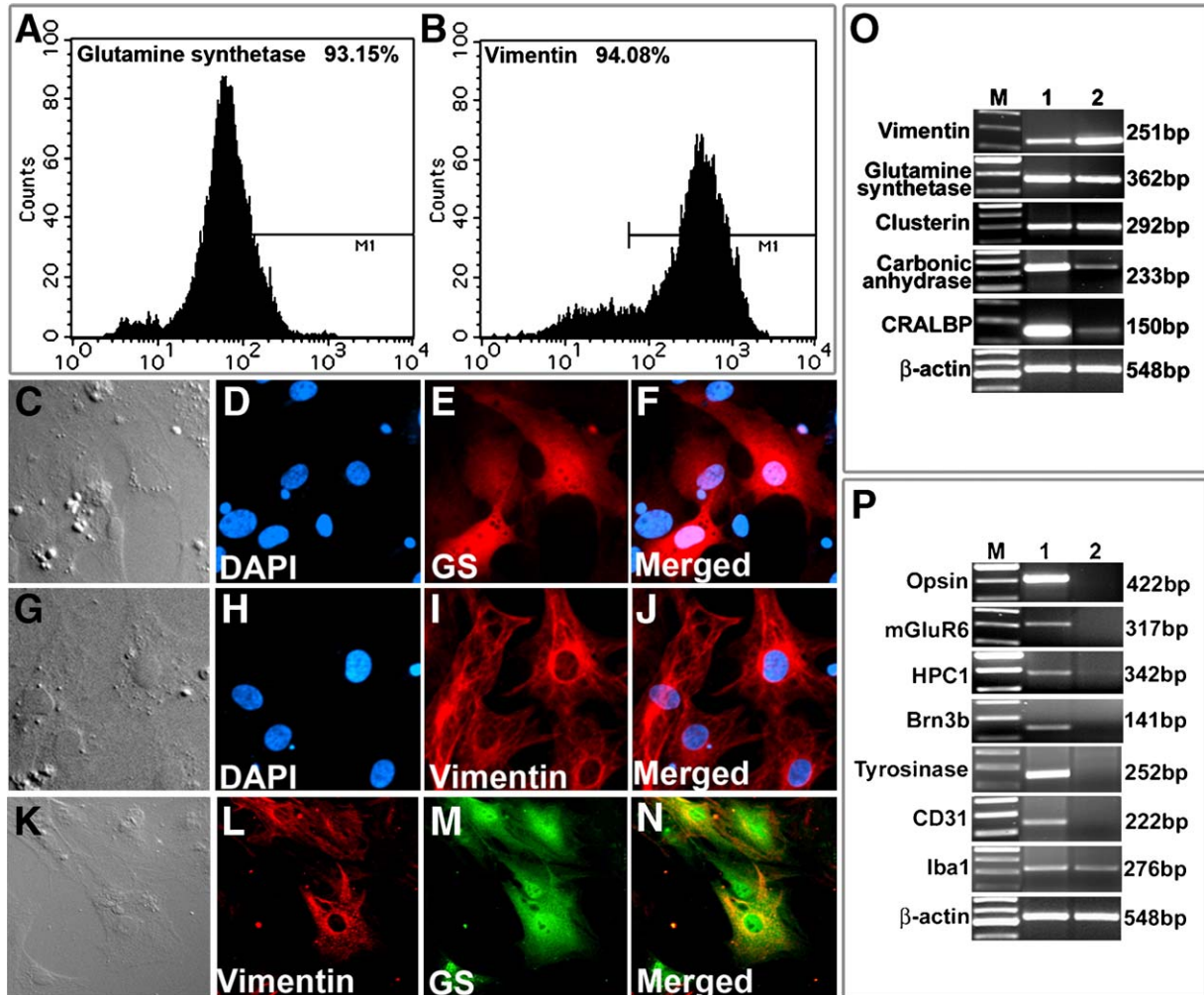


Fig. 1. Purity of enriched Müller cells: FACS and immunocytochemical analysis showed that ~95% of purified cells expressed immunoreactivities corresponding to Müller cell markers, GS (A, C–F) and vimentin (B, G–J). Double immunocytochemical analysis showed that ~99% of the cells co-expressed both vimentin and GS further attesting their purity (K–N). RT-PCR analysis was carried out to detect the expression of Müller cell (O) and other cell type (P)-specific transcripts to determine the purity of enriched cells. Lane M=DNA marker; lane 1=PN1 retina, lane 2=purified Müller cells, $\times 400$.

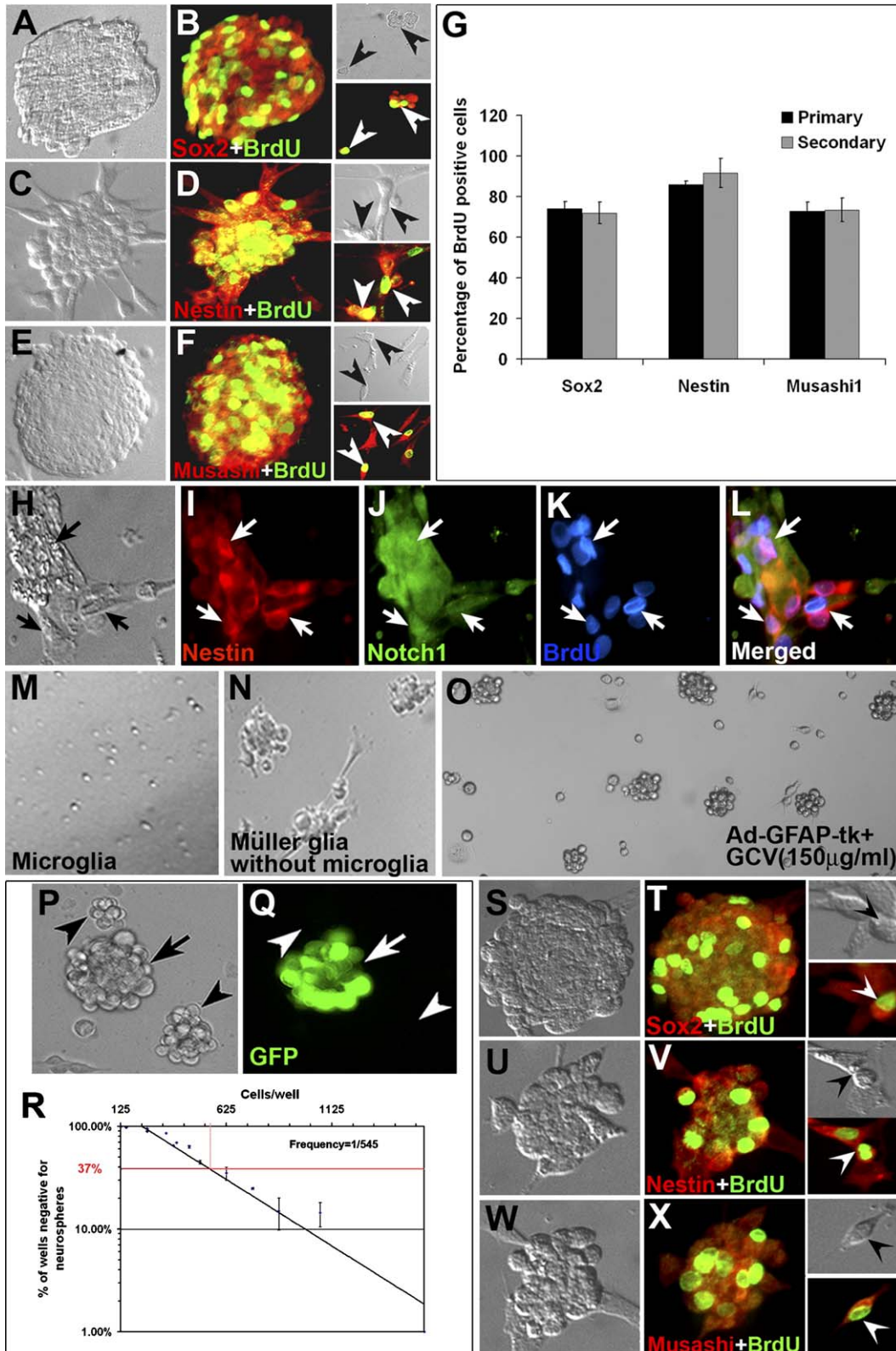
determination of cell composition in smaller neurospheres is more reliable than in big neurospheres in which cells are tightly packed. Neurospheres were generated that consisted of solely green cells or non-green cells, ruling out cell aggregation as the cause of neurosphere formation (Figs. 2P and Q). The bulk neurosphere assay has the tendency to overestimate the frequency of cells that generate neurospheres (Reynolds and

Rietze, 2005). Therefore, to obtain a better estimate of the frequency of cells in the monolayer cells culture that generate neurospheres, we carried out limiting dilution analysis (LDA). The assay demonstrated that the frequency of the single limiting cell type approximated to 0.18% in the culture and that, based the single hit kinetic of the assay, the generation of neurospheres was a cell-autonomous process (Fig. 2R). Finally, to test

Fig. 2. Stem cell properties of enriched Müller cells. Enriched Müller cells when exposed to mitogens formed neurospheres, which consisted of proliferating cells as they incorporated BrdU, and expressed neural progenitor markers, Sox2 (A, B and inset), Nestin (C, D and inset) and Musashi1 (E, F and inset). Graph (G) represents the percentage of BrdU⁺ cells expressed neural progenitor markers. Triple immunocytochemical analysis showed BrdU⁺ cells co-expressing Notch and Nestin immunoreactivities, thus demonstrating that enriched Müller cells expressed multiple progenitor markers (H–L). To ensure that neurospheres were generated from Müller cells and not from contaminating microglia, enriched microglia (M) and enriched Müller cells minus microglia (N) were exposed to mitogens. No neurospheres were generated by microglia (M) whereas Müller cells still generated neurospheres even after the removal of microglia (N). To further prove that the neurospheres are generated by Müller cells and not by migrating astrocytes, we selectively ablated astrocytes by infecting cells with Ad-GFP-Tk virus and then exposing them to ganciclovir (GCV: 150 μ g/ml) for 24 h. The remaining cells were cultured in the presence of mitogens for another 24 h and still generated neurospheres (O), thus eliminating the possibility of migrating astrocytes as the source of neurospheres. When enriched green and non-green cells were mixed and plated, neurospheres generated were either green (GFP⁺) or non-green, thus demonstrating their clonal generation (P, Q). LDA carried out on enriched Müller cells showed that there was a single limited cell type for the generation of neurospheres, at the frequency of 1 cell/550 cells in the monolayer Müller cell culture (R). Cells in the primary neurospheres when dissociated and re-plated, formed secondary neurospheres, which expressed neural stem cell/progenitor markers, Sox2 (S, T and inset), Nestin (U, V and inset) and Musashi1 (W, X and inset). Data are expressed as mean \pm SEM from triplicate culture of three different experiments. $\times 200$ except for panels M–O ($\times 100$).

whether or not enriched Müller cells could generate secondary neurospheres with parental properties, the true test of self-renewal potential, primary neurospheres were dissociated and cultured in the presence of FGF2 for 5 days. Secondary neurospheres were observed with a frequency (0.5%–1%, in

bulk culture) and progenitor properties (BrdU⁺ and Sox2⁺ cells=72.0±5.3%; BrdU⁺ and Nestin⁺ cells=92.0±7.2%; BrdU⁺ and Musashi⁺ cells=73.0±5.8) similar to that of primary neurospheres (Figs. 2S–X and G). In addition, cells in the secondary neurospheres displayed potential similar to



those in primary neurospheres (see below), thus affirming their ability to self-renew. That these cells could self-renew for extended period of time was apparent from their ability to generate neurospheres through five passages.

Next, we wanted to know whether or not enriched Müller cells were multipotential, another key feature of neural stem cells. BrdU-tagged neurospheres were shifted to differentiating conditions, characterized by the absence of FGF2 and presence of serum in the culture medium. With the addition of serum in the culture medium, cells in neurospheres changed their morphology, depicting bipolar and multipolar shapes. Immunocytochemical analysis identified BrdU-tagged cells expressing neuronal marker, β -tubulin ($35.0 \pm 2.3\%$; Figs. 3A, B and J) and astrocytic marker, GFAP ($48.0 \pm 3.6\%$; Figs. 3C, D and J). To confirm the ability of glial cells to generate neurons, we further examined the neuronal differentiation of enriched Müller cells. Double immunocytochemical analysis of neurospheres

cultured in differentiating conditions revealed cells that co-expressed multiple neuronal markers, such as Map2 and β -tubulin, attesting to the specificity of neuronal differentiation (Figs. 3E, F and J). Although, ocular progenitors are not known to differentiate along oligodendrocytic lineage in vivo, those exposed to mitogens in vitro have been observed to express markers corresponding to oligodendrocytes (Ahmad et al., 1999, 2000). We examined whether or not enriched Müller cells possessed this potential and observed that $28.0 \pm 3.0\%$ of BrdU-positive cells expressed oligodendrocyte-specific marker, O4 (Figs. 3G, H and J). However, as observed previously with retinal and ciliary epithelial progenitors (Ahmad et al., 1999, 2000), the O4⁺ cells did not display the morphological features of typical oligodendrocytes, suggesting their immaturity or incomplete differentiation. Together, these observations suggested that, at least in vitro, enriched Müller cells have the potential to differentiate along the three basic CNS lineages. To

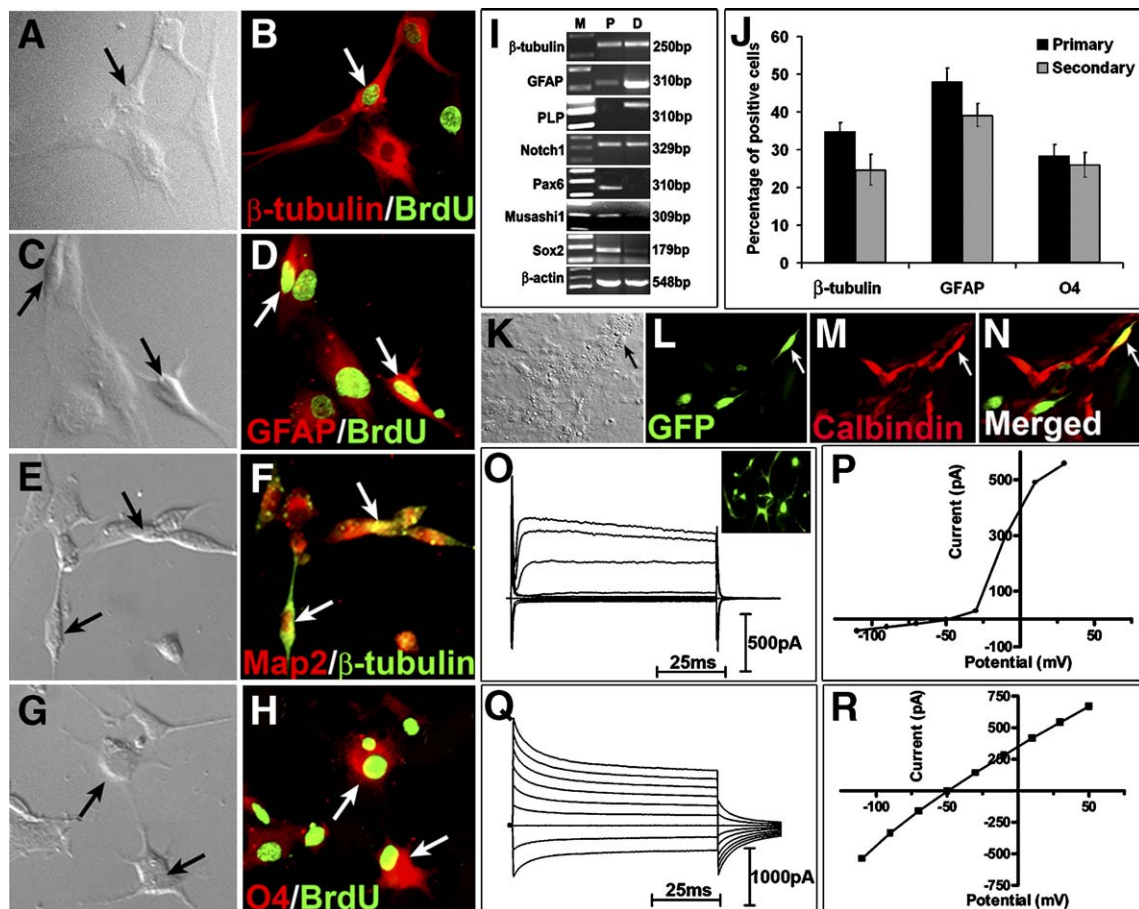


Fig. 3. Multipotent nature of enriched Müller cells: BrdU-tagged cells from Müller cell-derived neurospheres (arrows), when shifted to differentiating conditions, expressed markers corresponding to neurons [β -tubulin (A, B)] and astrocytes [GFAP (C, D)]. Double immunocytochemical analysis showed that cells co-expressed both β -tubulin and Map2 (E, F), confirming their differentiation along neuronal lineage. Even though ocular progenitors are not known to generate oligodendrocytes, we observed that Müller cell-derived progenitors expressed O4, an oligodendrocytic marker (G, H). RT-PCR analysis showed that levels of transcripts corresponding to cell-specific markers (β -tubulin, GFAP and PLP) increased in differentiating condition (lane D) as compared to those in proliferating conditions (lane P) (I). In contrast, those corresponding to stem cell/progenitor markers (Pax6, Musashi1 and Sox2) decreased in differentiating conditions. Lane M=DNA marker. The frequency of cells expressing each of the differentiated markers is presented in the graph (J). GFP⁺ cells from Müller cell-derived neurospheres, transplanted into the hippocampus of PN2 rats, expressed calbindin, a site-specific neuronal marker, demonstrating that Müller cells can generate neurons at heterotopic sites (K–N). GFP⁺ cells from Müller cell-derived neurospheres, cultured as an overlay on PN1 hippocampal explants acquired neuronal morphology (inset) and whole-cell recording such GFP⁺ cells showed sustained outward currents (I_k), characteristic of emerging physiological characteristics of a neuron (O, P). In contrast some GFP⁺ cells retained their Müller cell characteristics in differentiating conditions as displayed by current profiles (Q, R). Data are expressed as mean \pm SEM from triplicate culture of three different experiments, $\times 200$.

further corroborate multipotentiality of Müller cells, we examined the change in the expression of transcripts corresponding to progenitor, neuronal and glial markers when neurospheres were shifted from proliferating to differentiating conditions. RT-PCR analysis revealed an increase in levels of transcripts corresponding to β -tubulin (neuron), *GFAP* (astrocytes) and *PLP* (oligodendrocytes) in neurospheres in differentiating conditions, compared to those in proliferating conditions (Fig. 3I). In contrast, levels of transcripts corresponding to neural progenitor markers such as *Pax6*, *Musashi* and *Sox2* decreased in differentiating conditions. The inverse relationship between the expression of progenitor- and the cell-type-specific transcripts suggested that cells in Müller cell-derived neurospheres down-regulated the expression of progenitor markers as they differentiate into neurons and glia. To determine the potential of self-renewing cells, secondary neurospheres were cultured in differentiating conditions, followed by the examination of the expression of cell-type-specific markers. Just as observed in primary neurospheres, cells in secondary neurospheres, following the exposure to differentiating conditions, expressed neuronal- and glial-specific markers. The proportion of cells expressing cell-type-specific markers was similar to those in the primary neurospheres (secondary neurospheres versus primary neurospheres; β -tubulin: 25.0 ± 4.0 versus 35.0 ± 2.3 ; *GFAP*: 39.0 ± 3.0 versus 48.0 ± 3.6 ; *O4*: 26.0 ± 3.3 versus 28 ± 3.0), suggesting that self-renewing cells possess the multipotentiality of their parents (Fig. 3J).

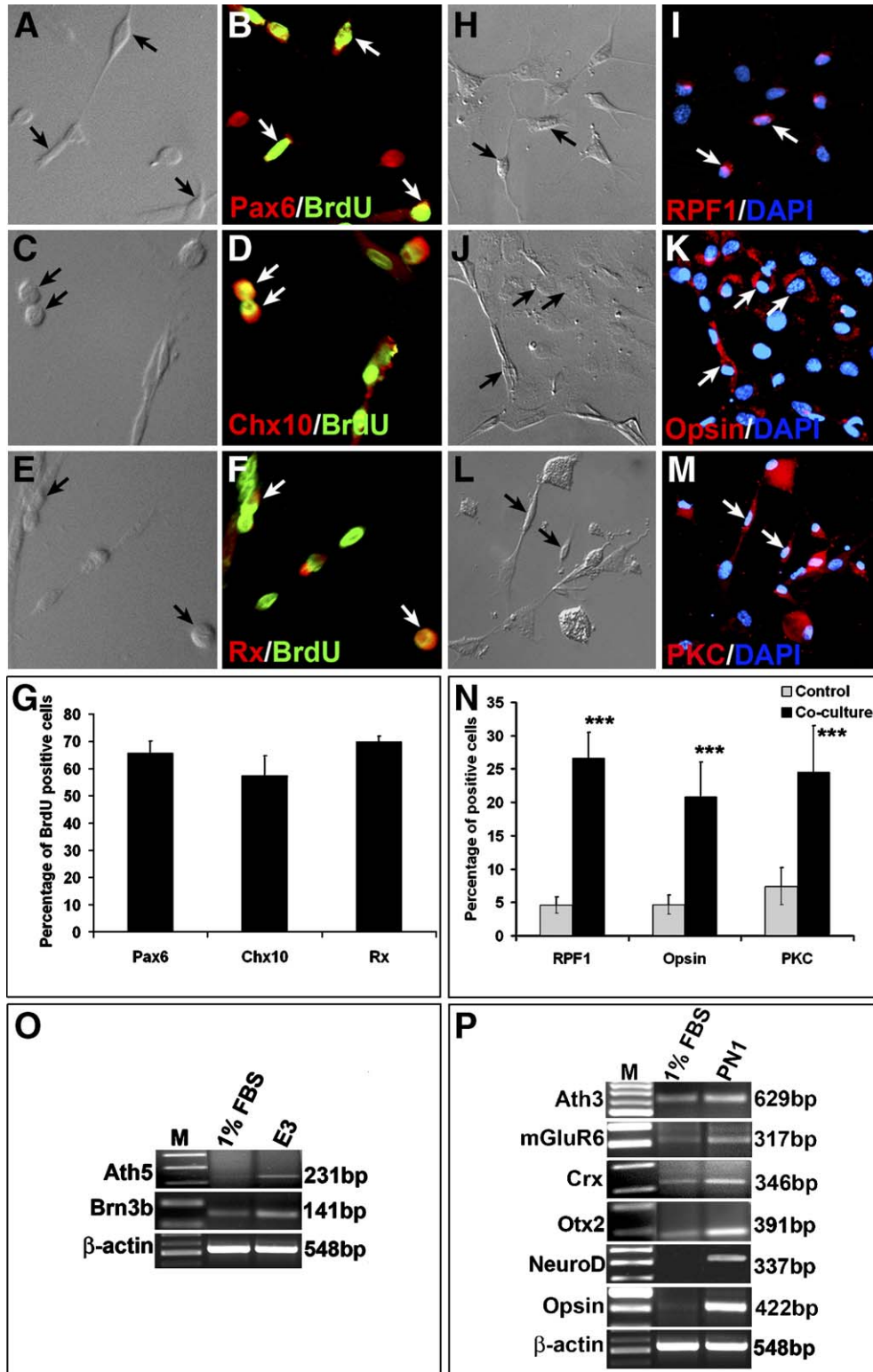
To investigate the neurogenic potential of Müller cells in the context of the in vivo microenvironment, we transplanted GFP-expressing cells from Müller cell-derived neurospheres in the hippocampus of the PN2 rats. One week following transplantation, immunohistochemical analysis of the brain sections revealed GFP⁺ cells expressing calbindin, a marker expressed by hippocampal neurons, demonstrating their capacity for neuronal differentiation at heterologous sites (Figs. 3K–N). It must be mentioned that calbindin expression is not exclusive to hippocampus. However, its expression in cells, grafted in hippocampus, suggests that they may have acquired hippocampal phenotype. To ascertain that cells that appeared neuronal by biochemical and molecular criteria possessed properties of a functional neuron, we carried out electrophysiological analysis of GFP⁺ cells from Müller cell-derived neurospheres in a brain slice overlay culture. Within 2 days in culture, GFP⁺ cells were observed within and on brain slices that had elaborated multipolar structure resembling differentiated neurons (Fig. 3O, inset). GFP⁺ cells in the slice culture were identified under epifluorescence and patched for electrophysiological analysis. Whole-cell recording of GFP⁺ cells (70%) revealed prominent outward currents activated by potentials above -30 mV, similar to delayed rectifying currents found in retinal neurons (Figs. 3O and P). In contrast, few GFP⁺ cells exhibited large inward rectifying currents as observed in matured Müller cells in vivo (Figs. 3Q and R) (Newman, 1993). Together, these observations underscored the multipotential nature of enriched Müller cells and their capacity to generate functional neurons.

Enriched Müller cells can generate retinal neurons

Our observation that proliferating cells in neurosphere culture, besides expressing pan neural progenitor markers, expressed multiple retinal progenitor markers such as *Pax6* (Marquardt et al., 2001), *Chx10* (Belecky-Adams et al., 1997) and *Rx* (Mathers et al., 1997) (Figs. 4A–G) raised the possibility that Müller cells, retrospectively enriched from the normal retina, can generate retinal neurons. To test this premise, we examined their ability to generate both early (e.g., RGCs) and late (e.g., photoreceptors and bipolar cells) born retinal neurons, when exposed to environment conducive for cell-type-specific differentiation. First we co-cultured BrdU-tagged Müller cell-derived neurospheres with chick embryonic day 3 (E3) retinal cells across a membrane. We have previously demonstrated that E3 chick retinal cells elaborate diffusible and evolutionarily conserved RGC-promoting activities and therefore, such activities may influence the differentiation of enriched Müller cells (James et al., 2003). Co-culture was carried out across a membrane to ensure that the result was not due to cell fusion (James et al., 2003). BrdU-tagged cells in neurospheres were observed expressing immunoreactivities corresponding to RPF1, a pou-domain factor expressed in developing RGCs (Yang et al., 2003; Zhou et al., 1996), suggesting their differentiation along RGC lineage. The proportion of BrdU⁺ and RPF-1⁺ cells was ~ 5 -fold higher in the presence E3 chick retinal cells than in the FBS control ($26.6 \pm 3.9\%$ versus $4.6 \pm 1.2\%$ and $p < 0.0001$) (Figs. 4H–I and N). To further ascertain the differentiation of Müller cells along RGC lineage, we examined the expression of transcripts corresponding to known regulators of RGC differentiation, *Ath5* and *Brn3b* (Mu and Klein, 2004). *Ath5* encodes a bHLH transcription factors that regulate the competence of retinal progenitors for RGC differentiation and *Brn3b* encodes a pou-domain transcription factor that is the downstream target of *Ath5*, regulating the differentiation and survival of committed RGC precursors (Mu and Klein, 2004; Yang et al., 2003). There was an increase in levels of both *Ath5* and *Brn3b* transcripts when Müller cell-derived neurospheres were co-cultured with E3 chick retinal cells as compared to those in FBS control, suggesting that the differentiation of Müller cells along RGC lineage involved normal regulatory mechanism (Fig. 4O). Second, we used a similar paradigm to test the ability of Müller cells to generate late born retinal neurons, rod photoreceptors and bipolar cells. BrdU-tagged Müller cell-derived neurospheres were co-cultured with PN1 rat retinal cells, known to induce the differentiation of late born retinal neurons (James et al., 2003). Immunocytochemical examination of cells after 5 days in co-culture revealed BrdU⁺ cells expressing a rod photoreceptor-specific marker, opsin (Figs. 4J, K and N) or a bipolar cell-specific marker, PKC (Figs. 4L, M and N). The proportions of cells expressing these markers were significantly higher in co-culture conditions than those in FBS controls (opsin: $21.0 \pm 5.2\%$ versus $4.7 \pm 1.4\%$; $p < 0.0001$, PKC: $25.0 \pm 7\%$ versus $7.0 \pm 3\%$; $p < 0.0001$), suggesting their differentiation along rod photoreceptor and bipolar cell lineages, which could be enhanced in conducive environment. PKC is expressed in neurons other than bipolar cells. Since cells are

grafted in the retina where only bipolar cells express PKC and grafted cells expressing PKC are always localized in the inner nuclear layer where bipolar cells are found, it could be surmised that PKC immunoreactivities belonged to those grafted cells that differentiated along bipolar cell lineage. That the differentiation of Müller cells along the lineages of late born neurons involved normal regulatory mechanism was demonstrated by a con-

comitant increase in levels of transcripts corresponding to rod photoreceptor regulators, *NeuroD* (Ahmad et al., 1998; Morrow et al., 1999) and *Otx2* (Nishida et al., 2003) and a regulator of bipolar cells, *Ath3* (Bhattacharya et al., 2004; Hatakeyama and Kageyama, 2004), when neurospheres were co-cultured with PN1 retinal cells, compared to those in FBS controls (Fig. 4P). Together, these observations suggested that, given a conducive



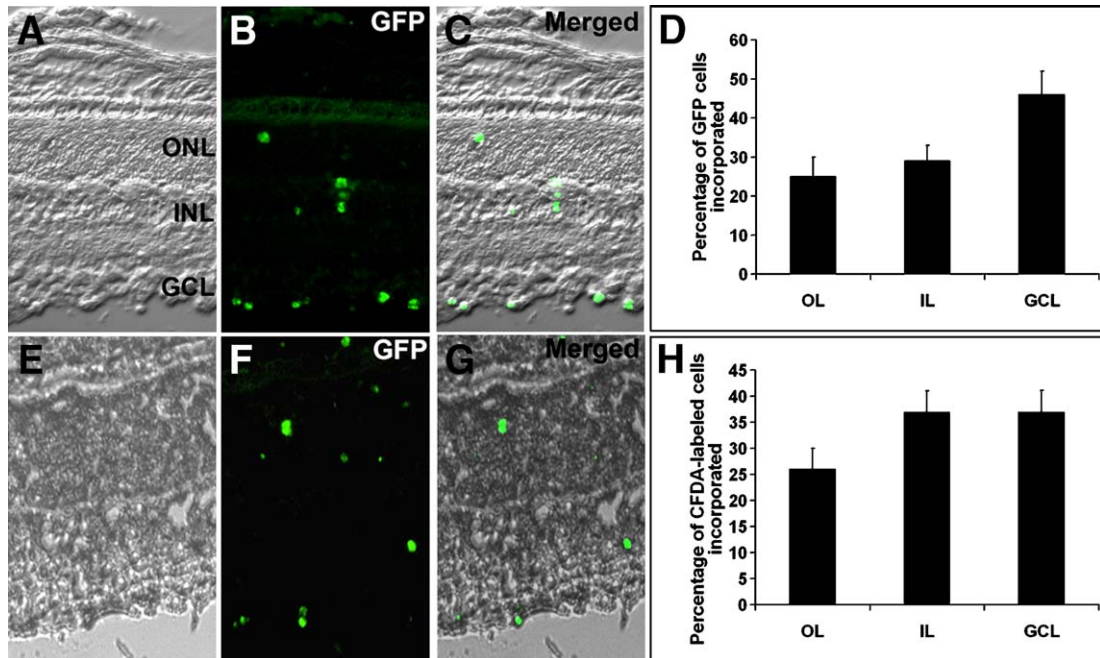


Fig. 5. Incorporation of transplanted Müller cells in the host retina. GFP⁺ cells from Müller cell-derived neurospheres when transplanted in PN1 rats incorporated differentially in different layers of the host retina (A–D). CFDA-labeled and prospectively enriched Müller SP cells when transplanted intravitreally, incorporated differentially into different layers of the host retina (E–H), $\times 200$. ONL=outer nuclear layer; INL=inner nuclear layer; GCL=ganglion cell layer.

microenvironment, enriched Müller cells differentiated into retinal neurons using normal regulatory mechanisms.

To know whether or not these cells can generate retinal neurons *in vivo*, GFP-expressing Müller cell-derived neurospheres were dissociated into single cells, and transplanted intravitreally in postnatal day 7 (PN7) rat eyes. The host retina contained focal mechanical damage, which is known to facilitate incorporation of grafted cells (Chacko et al., 2003). The outcomes of transplantation were examined a week later. Since the grafted cells were obtained from neurospheres in proliferating conditions that did not express retinal neuron-specific genes (Fig. 1L), the possibility that the outcome could be due to transplantation of already differentiated cells was eliminated. We observed that labeled Müller cells migrated into all layers of retina (Figs. 5A–D) in different proportions (OL: $25.0 \pm 5.0\%$; IL: $29.0 \pm 4.0\%$; GCL: $46.0 \pm 6\%$, total of 71 sections counted) and some of these cells expressed retinal cell-type-specific markers (Fig. 6). For example, labeled cells were detected expressing rod photoreceptor-specific marker (i.e., opsin) near the outer nuclear layer, bipolar cell-specific marker (i.e., PKC) in the inner nuclear layer and RGC marker (i.e.,

Brn3b) in RGC layer, demonstrating the potential of retro-spectively enriched Müller cells to differentiate along layer-specific retinal neuron lineages in the host retina. Of all the labeled cells, those expressing opsin immunoreactivities were rare and did not display typical photoreceptor morphology. This may be due to short post-transplantation time (1 week), lack of cues for maturation of nascent photoreceptor or lack of the potential of grafted cells to give rise to mature photoreceptor. However, differentiation of photoreceptors, characterized by both biochemical and distinct morphological features, has not been achieved until date even with late retinal progenitors as the source of transplanted cells.

Müller cells display neurogenic potential in vivo

Next, we wanted to know if Müller cells possess stem cell properties and neurogenic potential *in vivo*. Towards this end, we tested a hypothesis that the injury-induced proliferative and neurogenic potential entailed activation of dormant stem cell properties in a subset of Müller cells. We argued that test of this hypothesis would lead to prospective isolation of

Fig. 4. Retinal potential of enriched Müller cells *in vitro*: BrdU-tagged cells in Müller cell-derived neurospheres expressed immunoreactivities corresponding to retinal progenitor markers, Pax6 (A, B), Chx10 (C, D) and Rx (E, F) in proliferating conditions. The frequency of these markers in neurospheres in proliferating conditions is represented in the graph (G). Cells in Müller cell-derived neurospheres, when co-cultured with E3 chick retinal cells across a membrane, expressed RGC-specific marker, RPF1 (H, I) and the proportion of such cells was significantly higher as compared to those in controls, suggesting their differentiation along RGC lineage (N, $***p < 0.0001$). Levels of transcripts corresponding to RGC regulators, *Ath5* and *Brn3b* increased in co-culture conditions as compared to controls, as revealed by RT-PCR analysis thus corroborating immunocytochemical results (O). Similarly, cells in Müller cell-derived neurospheres, when co-cultured with PN1 rat retinal cells across a membrane, expressed rod photoreceptor-specific markers, rhodopsin (J, K) and bipolar cell-specific markers, PKC (L, M) and proportion of such cells was significantly higher as compared to those in controls suggesting their differentiation along rod photoreceptor and bipolar lineages (N, $***p < 0.0001$). Levels of transcripts corresponding rod photoreceptor regulators and markers, *Crx*, *NeuroD*, *Otx2*, and *Opsin* and that of bipolar cells, *Ath3* and *mGluR6* increased in co-culture condition as compared to controls, as revealed by RT-PCR analysis corroborating immunocytochemical results (P). Lane M in all RT-PCR data = DNA marker. Data are expressed as mean \pm SEM from triplicate culture of three different experiments, $\times 200$.

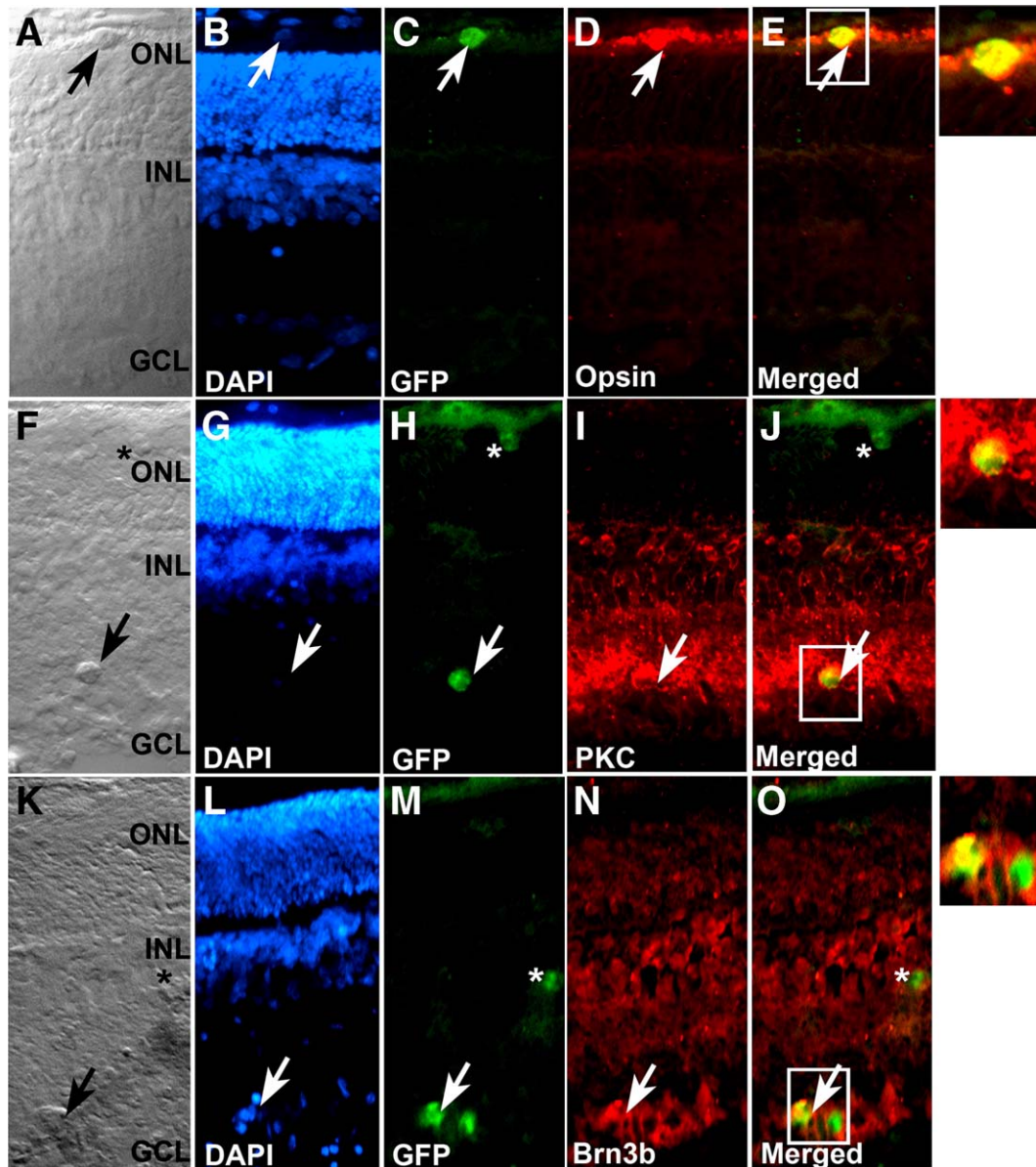


Fig. 6. Retinal potential of enriched Müller cells in vivo: GFP⁺ cells from Müller cell-derived neurospheres (arrows) were transplanted into the vitreous cavity of PN1 rat eyes and the outcomes of transplantation were examined 1 week later by immunohistochemical analysis. A subset of incorporated cells expressed lamina-specific retinal markers. For example: (1) a GFP⁺ cell, migrated near the inner segments of photoreceptors and outer nuclear layer (ONL) expressed rod photoreceptor-specific marker, opsin (A–E). (2) A GFP⁺ cell in the inner nuclear layer (INL) expressed bipolar cell-specific marker, PKC (F–J). (3) GFP⁺ cells in the ganglion cell layer (GCL) expressed RGC-specific marker, Brn3b (K–O). Inset shows magnified views of incorporated GFP⁺ cells expressing specific markers, $\times 200$.

activated Müller cells for the examination of their stem cell properties. Therefore, we subjected adult retina to neurotoxin treatment, an experimental paradigm used to study the injury-induced activation of resident Müller cells (Fischer and Reh, 2001; Ooto et al., 2004) and examined the expression of progenitor markers. Neurotoxins (NMDA+KA) in culture medium, supplemented with growth factors (FGF2+insulin) to increase the number of activated Müller cells, were injected intravitreally in eyes of PN21 rats. The rats received daily intraperitoneal injection of BrdU to label dividing cells for 4 days. Immunohistochemical analysis revealed the presence of BrdU⁺ cells in neurotoxin-treated retina that were predominantly localized in the inner retina. Such cells were not

detected in control retina in which PBS was injected and rarely in those that received growth factors only (Figs. 7A and B). The BrdU⁺ cells in neurotoxin-damaged retina expressed immunoreactivities corresponding to GS (Figs. 7C and D) and vimentin (Figs. 7E and F), demonstrating the injury-induced activation of Müller cells. The characteristic radial staining pattern of Müller cell marker was compromised in neurotoxin-treated retina because of the remarkable thinning of the inner retina (compare with staining pattern in normal retina in Figs. 7A, B and Figs. 9H–K).

Next, we were interested in knowing the properties and potential of activated Müller cells. First, we examined the expression patterns of stem cell/progenitor markers in injured

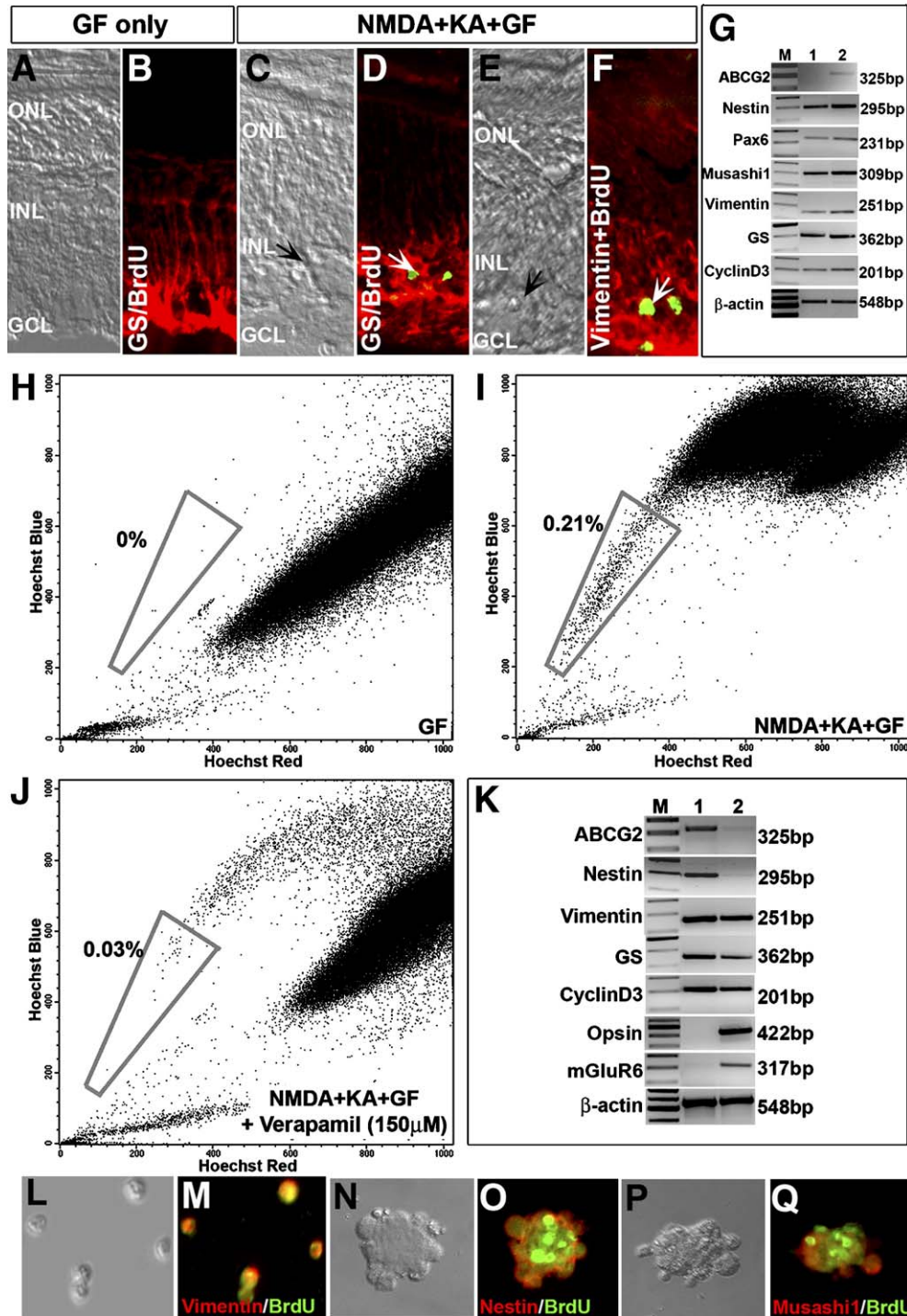


Fig. 7. Prospective identification and characterization of activated Müller cells: the stem cell potential of Müller cells in vivo was examined in response to injury to the retina. The intravitreal injection of neurotoxin included BrdU to tag proliferating cells and FGF2+insulin (GF=growth factors) to enhance injury-induced cell proliferation. Immunocytochemical analysis revealed BrdU+ cells in neurotoxin-treated (D and F) retina, but not in controls (A, B). The BrdU+ cells were positive for GS (C, D) and vimentin (E, F) immunoreactivities suggesting their Müller cell nature. Analysis of transcripts corresponding to different progenitor-specific markers (*abcg2*, *Pax6*, *Nestin* and *Musashi1*) revealed that their levels were increased in injured retina (lane 2) than in controls (lane 1), suggesting that in response to injury Müller cells acquired progenitor properties (G). Cell dissociates obtained from retina, exposed to GF/NMDA+KA+GF, were subjected to Hoechst dye efflux assay demonstrated the presence of side population (SP) cells, as revealed by FACS analysis. While SP cells could not be detected in the control retina (H), ~0.2% of total cells in neurotoxin-treated retina were enriched as SP cells (I) and these cells were sensitive to verapamil, an inhibitor of ABCG2 (J). RT-PCR analysis of SP (lane 1) and non-SP cells (lane 2) revealed that both SP and Non-SP cells expressed transcripts corresponding to the Müller cell markers (*Vimentin*, *GS* and *CyclinD3*) but only SP cells expressed stem cell-/progenitor-specific *abcg2* and *Nestin* mRNA (K). Transcripts corresponding to *opsin* and *mGluR6*, expressed in differentiated rod photoreceptors and bipolar cells, respectively, were present in non-SP cells (lane 2) and absent in SP cells (lane 1) (K). Immunocytochemical analysis revealed that SP cells were BrdU-positive and expressed Müller cell marker, vimentin (L, M). When SP cells were cultured in the presence of FGF2, they gave rise to clonal neurospheres consisting of BrdU⁺ cells expressing Nestin (N, O) and Musashi (P, Q) immunoreactivities, indicating their stem cell nature. Lane M in all RT-PCR data=DNA marker, $\times 200$.

and control retina, which led to the second approach, i.e., prospective enrichment and characterization of activated Müller cells. An examination of the expression of stem cell-/progenitor-specific transcripts (*abcg2*, *Pax6*, *Nestin* and *Musashi1*) revealed that their levels were higher in injured retina than in controls (Fig. 7G). Interestingly, one of the genes whose transcripts appeared to be injury induced was *abcg2* that encodes a protein belonging to the ABC membrane transporter family, which includes drug efflux pumps (Krishnamurthy and Schuetz, 2006). Expression of ABCG2 enables cells to preferentially exclude a fluorescent DNA intercalating dye called Hoechst 33342, thus allowing their enrichment as side population (SP) cells, using fluorescent activated cell sorting (FACS) (Bhattacharya et al., 2003; Goodell et al., 1996; Krishnamurthy and Schuetz, 2005). The SP cell phenotype has emerged as a universal feature of stem cells including those from blood (Goodell et al., 1996), heart (Martin et al., 2004), gonads (Lassalle et al., 2004) and brain (Ahmad et al., 2004; Hulspas and Quesenberry, 2000). We sought to prospectively enrich activated Müller cells as SP cells using the Hoechst dye efflux assay. The assay showed that SP cells were not detected in the control retina (Fig. 7H). In contrast, when the assay was performed on cell dissociates obtained from neurotoxin-injured retina, ~0.2% of the total cells were enriched as SP cells (Fig. 7I). Treatment of cell dissociates with verapamil, an inhibitor of ABCG2 transporter, led to a decrease in the proportion of SP cells, demonstrating the specificity of SP cell phenotype (Fig. 7J). Together, these observations demonstrated that the injury-induced activation of *abcg2* was accompanied by an increase in the proportion SP cells in the injured retina.

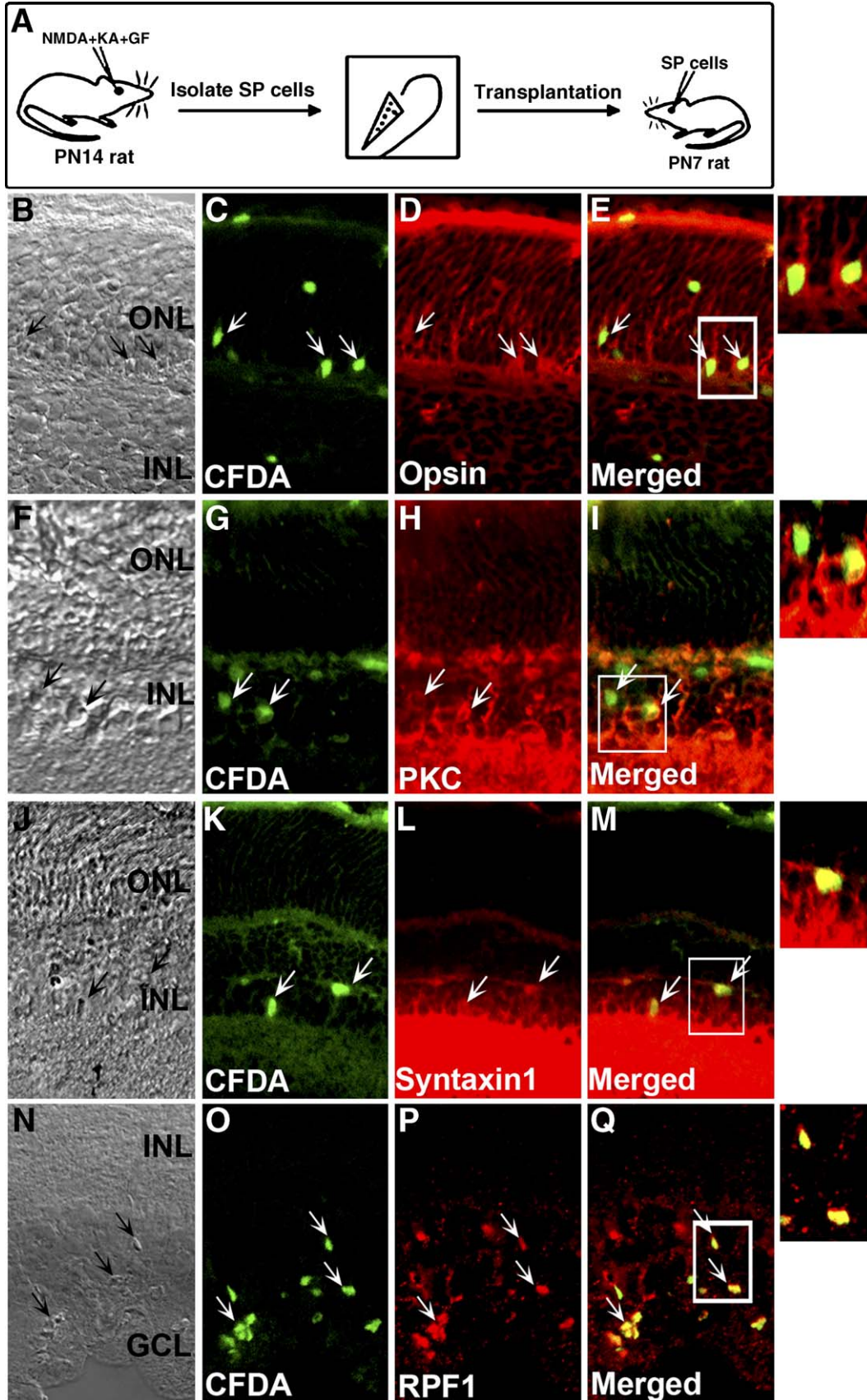
The next question was whether or not the SP cells represented activated Müller cells and if they had the ability to generate neurospheres. We addressed this question, first, by examining the expression of transcripts corresponding to Müller cell- and stem cell-specific markers in SP and non-SP (NSP) cells. We observed that SP cells expressed both Müller cell (*Vimentin*, *GS* and *CyclinD3*)- and stem cell (i.e., *Nestin* and *abcg2*)-specific markers, attesting to their Müller cell identity and yet revealing their progenitor nature, a proof of injury-induced activation (Fig. 7J). In contrast, NSP cells expressed Müller cell (*Vimentin*, *GS* and *CyclinD3*)-, rod photoreceptor (*Opsin*)- and bipolar cell (*mGluR6*)-specific transcripts suggesting their post-mitotic and terminally differentiated nature (Fig. 7K). Second, we carried out immunocytochemical analysis on SP cells, which revealed that they were BrdU-positive and expressed vimentin, thus corroborating their proliferative nature and Müller cell identity (Figs. 7L and M). When SP cells were cultured in the presence FGF2, neurospheres were generated which, like those generated by enriched Müller cells, consisted of proliferating cells expressing stem cell markers, *Nestin* (Figs. 7N and O) and *Musashi1* (Figs. 7P and Q).

Having determined that SP cells represented activated Müller cells, we were interested in knowing if they possessed neurogenic potential. To test this premise, we enriched Müller SP cells from freshly dissociated neurotoxin-injured retina, labeled them with CFDA-SE for tracking purpose, and transplanted intravitreally in postnatal day 7 (PN7) rat eyes as described above. One week after transplantation, we observed labeled Müller cells migrated into all layers of retina (Figs. 5E–H) (OL: 26.0±4.0%; IL: 37.0±4.0; GCL: 37.0±4.1, total of 52 sections counted) and could be detected expressing retinal cell-type-specific markers. For example, labeled cells were detected expressing rod photoreceptor-specific marker (i.e., Rhodopsin) in the outer nuclear layer, amacrine cell-specific marker (i.e., Syntaxin 1) in the inner nuclear layer and RGC marker (i.e., Brn3b) in RGC layer. These cells were unlikely to represent differentiated cells from the donor retina because SP cells did not express transcripts corresponding to retinal neuron-specific gene (Fig. 7K). These cells are unlikely to be host cells that got labeled by the dye from dying donor cells, as transplantation of freeze-thawed CFDA-labeled donor cells did not label cells in the host retina. Together, our results demonstrated the potential of prospectively enriched Müller cells to differentiate along layer-specific retinal neuron lineages in the host retina, thus providing a direct proof of their neurogenic potential (Fig. 8).

Notch and Wnt pathways influence NSC properties of Müller cells

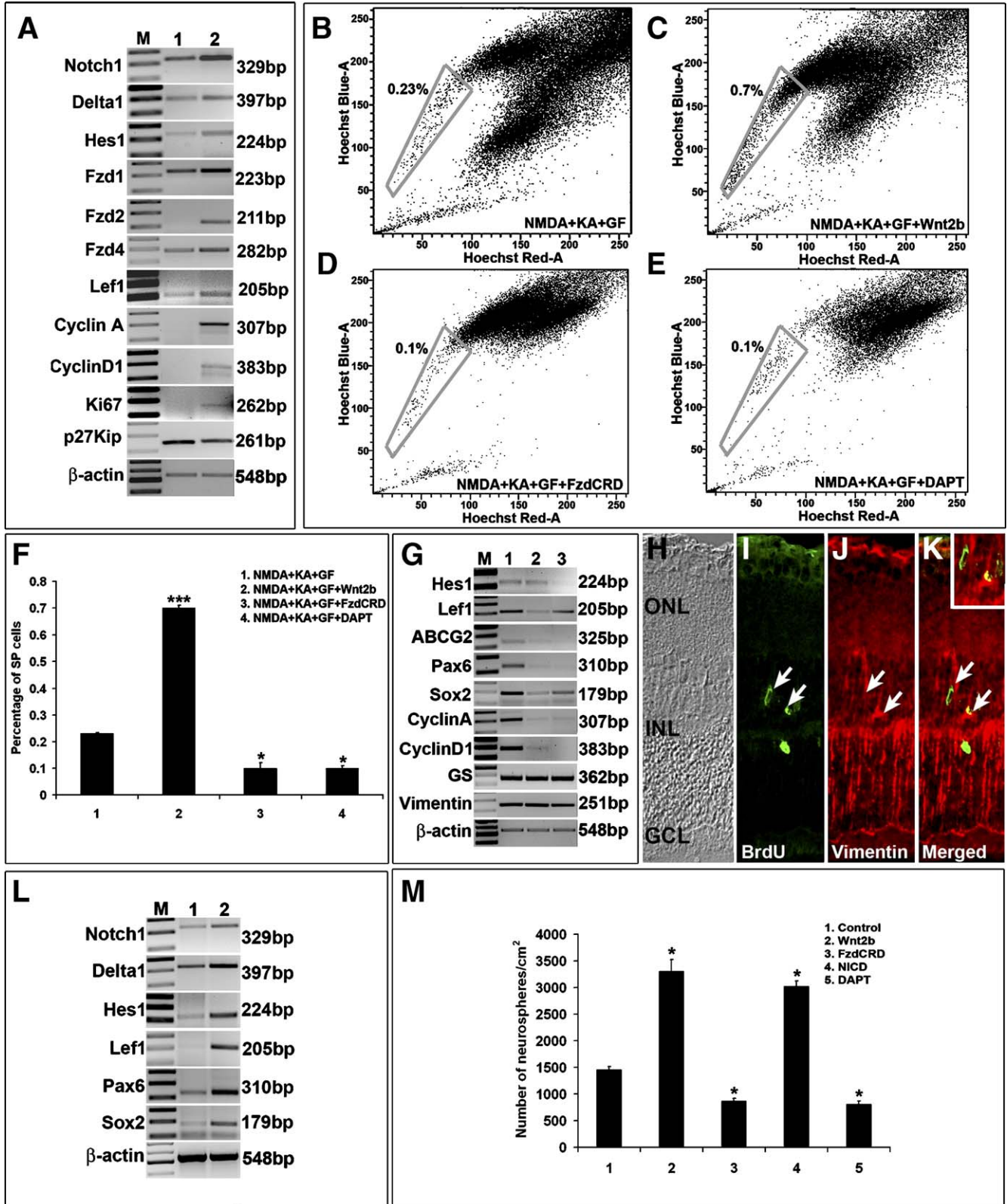
To get an insight into the mechanism underlying the activation of the latent NSC properties of Müller cells, we examined the involvement of Notch and Wnt pathways, both known to regulate stem cells in general, and retinal stem cells/progenitors in particular (Ahmad et al., 2004). We observed that the expression of Notch (*Notch1*, *Delta1* and *Hes1*) and Wnt (*Fzd1*, *Fzd2*, *Fzd4* and *Lef1*) pathway genes were increased in neurotoxin-damaged retina as compared to controls, suggesting the engagement of these pathways in response to injury (Fig. 9A). Additionally, the expression of *cyclinA* and *cyclinD1*, positive regulators of cell cycle, were increased, while that of *p27kip1*, a negative regulator of cell cycle, and was decreased. This suggested that one of the consequences of the engagement of Wnt and Notch pathways was to positively influence the cell cycle. As a functional test of this premise, we examined the effects of perturbing Wnt and Notch pathways on the number of prospectively identified Müller SP cells in neurotoxin-injured retina. When neurotoxins were injected along with Wnt2b conditioned medium to accentuate Wnt signaling, there was ~3-fold increase in the number of the Müller SP cells as compared to those obtained from the eyes injected with neurotoxins only (0.7±0.01% versus 0.23±0.003%, $p < 0.001$) (Figs. 9B, C and F). The vehicle for neurotoxins was culture

Fig. 8. Neurogenic potential of Müller cells in vivo: Müller cells were prospectively enriched as SP cells from the retina of rats with neurotoxin injury, labeled with CFDA-SE and transplanted intravitreally in eyes of PN1 rats with mechanical damage to retina (A). The outcomes of transplantation were analyzed immunohistochemically, 1 week later. CFDA-labeled Müller cells were detected in the outer nuclear layer, expressing rod photoreceptor-specific marker, opsin (B–E), in the inner nuclear layer, expressing bipolar cell-specific marker, PKC (F–I) and amacrine cell-specific marker, Syntaxin 1 (J–M), and in the ganglion cell layer, expressing RGC-specific marker, Brn3b (Q–U). Inset shows magnified views of transplanted CFDA-labeled cells, expressing retinal cell-specific markers, ×600.



medium, therefore, the effect was attributed to the presence of Wnt2b in the condition medium. In contrast, injection of Wnt pathway antagonist, Fzd-CRD led to a ~2-fold decrease in the number of Müller SP cells ($0.1 \pm 0.02\%$ versus $0.23 \pm 0.003\%$,

$p < 0.05$) (Figs. 9D and F). When Notch signaling was inhibited by injecting DAPT, a gamma secretase inhibitor (James et al., 2004), the number of Müller SP cells was likewise decreased ~2-fold ($0.1 \pm 0.01\%$ versus $0.23 \pm 0.003\%$, $p < 0.05$) (Figs. 9E



and F). Taken together, these observations suggested that both Wnt and Notch pathways were involved in the emergence of Müller SP cell phenotype in injured retina. The observations that the number of Müller SP cells in the presence of antagonists of Wnt (Fzd-CRD) and Notch (DAPT) signaling was lower than that in controls suggested the involvement of endogenous Wnt and Notch signaling in the maintenance of stem cell properties of Müller cells in response to injury (Figs. 9B, D–F). To test the specificity and intracellular response to perturbation in Wnt and Notch signaling, cells from neurotoxin-injured retina (controls) and similarly injured retina in which Wnt and Notch signaling pathways were attenuated by Fzd-CRD and DAPT injection, respectively, were collected and subjected to RT-PCR analysis (Fig. 9G). In all groups, constitutive expression of *vimentin* and *GS* was observed, attesting to the fact that the Müller cell phenotype remained unchanged in response to injury. Transcripts corresponding to effectors of Notch (*Hes1*) and Wnt (*Lef1*) and those encoding progenitor regulators and markers (*abcg2*, *Pax6* and *Sox2*) could be detected in cells in control groups, but their levels decreased significantly in groups in which Wnt and Notch signaling had been attenuated. Similarly, levels of *cyclinA* and *cyclinD1* transcripts, present in the controls groups, were decreased after the attenuation of Wnt and Notch signaling. Together, these observations suggested that both Wnt and Notch signaling were involved in injury-induced activation of stem cell properties of Müller cells in vivo. Since the exposure of injured retina to Wnt2b conditioned medium caused a remarkable increase in Müller SP cells, we wanted to know if accentuation of Wnt signaling could activate Müller cells in uninjured retina. The identification of BrdU⁺ cells expressing vimentin in the retina injected with Wnt2b (Figs. 9H–K) demonstrated that Wnt signaling might regulate Müller cells in vivo in the normal retina.

Do Wnt and Notch pathways regulate stem cell properties of Müller cells enriched from normal adult retina? Levels of *Notch1*, *Delta1*, *Hes1* and *Lef1* transcripts were increased in enriched Müller cells in proliferating conditions relative to those not exposed to FGF2, suggesting that Wnt and Notch pathway are operational in vitro in sustaining stem cell pro-

erties in enriched Müller cells (Fig. 9L). There was a significant increase in the number of neurospheres generated when enriched Müller cells were cultured in the presence of Wnt2b to accentuate Wnt signaling and a significant decrease when cultured in the presence of Fzd-CRD to attenuate it as compared to controls (Wnt2b; 3302±221 versus 1460±55, $p<0.05$; Fzd-CRD; 869±50 versus 1460±55, $p<0.05$) (Fig. 9M). Similarly, there was a significant increase in the number of neurospheres when enriched Müller cells were transfected with NICD to constitutively activate Notch signaling and a decrease in the presence of DAPT to attenuate it as compared to controls (NICD, 3025±101 versus 1460±55, $p<0.05$; 811±58 versus 1460±55, $p<0.05$) (Fig. 9M). These observations that perturbation of Wnt and Notch pathways affected generation of neurospheres suggest their roles in regulating the stem cell properties of the Müller cells enriched from the normal retina. The observations that the number of neurospheres generated in the presence of antagonists of Wnt (Fzd-CRD) and Notch (DAPT) signaling was lower than that in controls suggested the involvement of endogenous Wnt and Notch signaling in the maintenance of stem cell properties of enriched Müller cells (Fig. 9M).

Discussion

Müller cells morphologically and biochemically resemble radial glia. For example, Müller cells elaborate processes radiating towards the outer and inner surfaces of the retina and express markers such as Vimentin (Dahl et al., 1981; Schnitzer, 1988), GLAST (Hartfuss et al., 2001; Lehre et al., 1997) and Nestin (Lendahl et al., 1990; Walcott and Provis, 2003), which are also expressed by radial glia. We have tested the hypothesis that, like radial glia, Müller cells possess NSC potential, which remains dormant under the constraints imposed by the non-neurogenic nature of the niche in the adult retina. Their NSC properties become apparent only when the Müller cell environment is altered, either by removing them from their niche or perturbing the niche. This hypothesis was confirmed by the following observations.

Fig. 9. Involvement of Notch and Wnt pathways in stem cell potential of Müller cells: analysis of transcripts corresponding to components of Notch (*Notch1*, *Delta1*, and *Hes1*) and Wnt (*Fzd1*, *Fzd2*, *Fzd4* and *Lef1*) pathways by RT-PCR revealed that their levels increased in the retina with neurotoxin injury (A, lane 1) as compared to controls (A, lane 2), suggesting a role for Notch and Wnt signaling in Müller cell activation in vivo. There was an increase in the cell cycle regulators (*cyclinA* and *cyclinD1*) and an indicator (*Ki67*), and a decrease in cell cycle inhibitor (*p27Kip1*) in the neurotoxin-treated retina (A, lane 2) when compared to uninjured retina, suggesting a shift in cell cycle stages in response to neurotoxin injury (A, lane 1). To determine whether or not the emergence of the Müller SP cells in injured retinas was influenced by Wnt and Notch signaling, neurotoxin injury was done in the presence of Wnt pathway agonist (Wnt2b) and antagonist (Fzd-CRD), and Notch pathway antagonist, DAPT. The number of Müller SP cells increased in the presence of Wnt2b (C and F) as compared to controls (B and F). In contrast, Müller SP cell number decreased in the presence of Fzd-CRD (D and F) and DAPT (E and F) as compared to controls (B and F). These observations suggested that the SP cell phenotype of Müller cells was sensitive to Notch and Wnt signaling. RT-PCR analysis of neurotoxin-injured retina in which Wnt (lane 2) and Notch (lane 3) pathways were inhibited by Fzd-CRD and DAPT, respectively, revealed that transcripts corresponding to intercellular regulators of these pathways, *Lef1* and *Hes1* including that of cell cycle regulators (*cyclinA* and *cyclinD1*), neuronal regulators (*Sox2* and *Pax6*) and stem cell regulator/marker (*abcg2*) were decreased, as compared to controls (lane 1) (G). Levels of *vimentin* and *GS* transcripts remained unchanged, suggesting that stem cell properties such as cell proliferation and SP cell phenotype and not Müller cell phenotype were affected through these pathways during injury. There was an increase in the vimentin-expressing BrdU⁺ cells in the Wnt2b treated normal retina (H–K and inset) suggesting that Wnt signaling regulates proliferation of Müller cells in vivo. Levels of *Notch1*, *Delta1*, *Hes1* and *Lef1* transcripts along with that of neural stem cell regulators, *Sox2* and *Pax6* were increased in enriched Müller cells in proliferating conditions (L, lane 2) as compared to those that were not exposed to mitogens (L, lane 1), suggesting that Notch and Wnt pathways influenced the proliferative and neurogenic state of enriched Müller cells in vitro. The generation of clonal neurospheres by enriched Müller cells was enhanced and attenuated in the presence of Wnt2b and FzdCRD, respectively, compared to controls (M). A similar increase and decrease in the number of neurospheres was observed when NICD was over-expressed or in the presence of DAPT, respectively (M). These observations suggested that both Wnt and Notch signaling regulated stem cell properties of enriched Müller cells. Data are expressed as mean±SEM from triplicate culture of three different experiments. Lane M in all RT-PCR data=DNA marker. *** $p<0.001$, * $p<0.05$, ×200.

First, Müller cells, enriched from the normal retina, generate clonal neurospheres. These neurospheres consist of self-renewing and multipotent cells, thus demonstrating the cardinal features of stem cells in vitro. A similar approach has been previously used to confirm the stem cell nature of radial glia and SVZ astrocytes, enriched from the adult brain (Doetsch et al., 1999; Laywell et al., 2000; Merkle et al., 2004). Furthermore, cells in the clonal neurospheres demonstrate the capability of differentiating into functional neurons in vitro, and ability to generate site-specific neurons upon homotopic and heterotopic transplantation. Second, changes in the milieu of the adult retina, due to neurotoxin injury, confer SP cell phenotype on Müller cells. The SP cell phenotype has emerged as a universal feature of stem cells (Das et al., 2005a; Pevny and Rao, 2003). Evidence suggests that the molecular determinant of SP cell phenotype in a wide variety of stem cells, including those from blood (Goodell et al., 1996), heart (Martin et al., 2004), gonads (Lassalle et al., 2004) and brain (Ahmad et al., 2004; Hulsbas and Quesenberry, 2000), is the expression of ABCG2, a protein belonging to the ABC membrane transporter family which include drug efflux pumps (Krishnamurthy and Schuetz, 2006). It is thought that ABCG2 may constitute a core group of proteins expressed in stem cells to maintain their homeostasis and protect their genome from damage by excluding toxins and harmful chemicals (Das et al., 2005a) and maintain their uncommitted state by excluding differentiation promoting factor(s) (Krishnamurthy and Schuetz, 2006). The expression of *abcg2* and the associated SP cell phenotypes is a prominent feature of retinal stem cells/progenitors throughout retinal histogenesis (Das et al., 2005a). The expression of *abcg2* declines precipitously in the adult retina (data not shown) and thus few cells emerge as SP cells when the Hoechst dye efflux assay was carried out on uninjured retina. The emergence of SP cells in neurotoxin-injured retina, expressing neural progenitor regulators/markers, *Pax6*, *Sox2* and *Musashi1*, is an indication of the activation of NSC potential. This notion is further supported by the observation that the Müller SP cells can generate clonal neurospheres, consisting of self-renewing cells with neural properties and potential, demonstrated both in vitro and in vivo.

Several studies have suggested that Müller cells can acquire neurogenic potential in response to injury to the retina. For example, it is thought that proliferating and apically migrating Müller cells in goldfish retina, injured by laser lesion, may be the source of observed photoreceptor regeneration (Braisted et al., 1994). Studies in rats suggested mitogenic effects in the retina in response excitatory amino acids which was mediated through action on glial cationic channels (Sahel et al., 1991). Müller cells have been observed to proliferate and express progenitor properties in neurotoxin-injured retina of postnatal chicken (Fischer and Reh, 2001) and adult mouse (Ooto et al., 2004). In both cases, the evidence of neurogenic potential was indirect and based on the observation that BrdU-positive cells in injured retina expressed markers corresponding to differentiated neurons or specific retinal cell types. Due to the lack of direct evidence, it remained possible that neurons formed after injury were derived from a dormant population of non-

Müller stem cells within the postnatal retina, similar to those found in fish (Fischer and Reh, 2001). To test the neurogenic potential of Müller cells, we demonstrated that enriched Müller cells generated cells with biochemical, molecular and physiological characteristics of a generic neuron in vitro and also had the capacity to differentiate into specific retinal neurons both in vitro and in vivo. As a direct evidence of neurogenic potential in vivo, we demonstrated that Müller cells, prospectively enriched as SP cells, integrated and generated lamina-specific retinal neurons after transplantation into mechanically injured retina. Our observations, therefore, suggest that the neurogenic potential of Müller cells is an inherent, but dormant, feature. These cells express multiple stem cell/progenitor-specific genes whose expression is environment-sensitive. The dual phenotype of Müller cells is reminiscent of radial glia and suggestive of an ambivalent nature that allows them to carry out dual roles of supporting neuronal functions and participating in neurogenesis, when the microenvironment allows.

The mechanism of activation of the dormant NSC properties and potential of Müller cells are likely to involve signal(s) that integrate cell-intrinsic and cell-extrinsic information. Our observations suggest that Notch and Wnt pathway may mediate the integration of such signal(s). For example, the activities of these pathways are enhanced in Müller cells in response to injury and that the stem cell properties of Müller cells are sensitive to perturbation of these pathways. Both Notch and Wnt pathways, like elsewhere in the CNS, regulate the maintenance of stem cells/progenitors in the developing retina (Ahmad et al., 2004; Kubo et al., 2003; Liu et al., 2003). Similar to its context-dependent involvement in radial glia development, the Notch pathway is utilized for the differentiation of Müller cells (Furukawa et al., 2000; James et al., 2004). Although the involvement of Wnt signaling in Müller cell differentiation has not been previously reported, its role in progenitor properties of radial glia has recently emerged (Zhou et al., 2004). Therefore, it is quite likely that both radial glia and Müller cells retain their ability to respond to Notch and Wnt signaling long after their differentiation is completed as a part of their malleable nature. Therefore, injury-induced extrinsic cues, such as cytokines and growth factors may lead to the activation of Notch (Faux et al., 2001; Liao et al., 2004) and Wnt (Viti et al., 2003; Zhou et al., 2004) pathways in Müller cells, prompting them to shift from a maintenance to a stem cell mode. These pathways act cooperatively towards the activation of Müller cells (to be published elsewhere). Based on previous observations that *p27kip1* keeps Müller cells from entering cell cycle (Dyer and Cepko, 2000), and the recent evidence that Notch and Wnt signaling adversely affect the activities of *p27kip1* (Castelo-Branco et al., 2003; Murata et al., 2005; Sarmiento et al., 2005), it is reasonable to postulate that Notch and Wnt signaling-dependent inhibition of *p27kip1* may constitute one of the first steps towards the shift of Müller cells to the stem cell mode. The process is furthered by accentuating the expression of cell cycle regulators such as cyclins, neurogenic genes such as *Sox2* (Graham et al., 2003) and *Pax6* (Heins et al., 2002; Kohwi et al., 2005) and homeostasis-promoting genes such as *abcg2*. Taken together, our study suggests that the Müller cells are the NSCs of

the adult retina. These cells keep their stem cell potential dormant under the constraints of non-neurogenic niche of the adult retina. When the environment changes demanding stem cell activity, they are capable of functioning as NSCs and as such can serve as a target of therapy for regenerative medicine of the retina.

Acknowledgments

We would like to thank Dr. Spyros Artavanis-Tsakonas for the Notch1 antibody, Dr. Shinichi Nakagawa for the Wnt2b construct, Dr. Jeremy Nathans for the FzdCRD constructs and Dr. Anuja Ghorpade for the Iba-1 antibody. Thanks are due to Dr. Jim Rogers for his valuable advice and guidance on LDA, Drs. Graham Sharp and Gregory Bennett for critiquing the manuscript and helpful suggestions, Dr. Kenneth Cowan for the adenovirus vectors and Gaurav Sahay for the technical assistance. This work was supported by the Nebraska Tobacco Settlement Biomedical Research Development, NIH and Research to Prevent Blindness.

References

- Ahmad, I., Acharya, H.R., Rogers, J.A., Shibata, A., Smithgall, T.E., Dooley, C.M., 1998. The role of NeuroD as a differentiation factor in the mammalian retina. *J. Mol. Neurosci.* 11, 165–178.
- Ahmad, I., Dooley, C.M., Thoreson, W.B., Rogers, J.A., Afiat, S., 1999. In vitro analysis of a mammalian retinal progenitor that gives rise to neurons and glia. *Brain Res.* 831, 1–10.
- Ahmad, I., Tang, L., Pham, H., 2000. Identification of neural progenitors in the adult mammalian eye. *Biochem. Biophys. Res. Commun.* 270, 517–521.
- Ahmad, I., Das, A.V., James, J., Bhattacharya, S., Zhao, X., 2004. Neural stem cells in the mammalian eye: types and regulation. *Semin. Cell Biol.* 15, 53–62.
- Alvarez-Buylla, A., Lim, D.A., 2004. For the long run: maintaining germinal niches in the adult brain. *Neuron* 41, 683–686.
- Alvarez-Buylla, A., Garcia-Verdugo, J.M., Tramontin, A.D., 2001. A unified hypothesis on the lineage of neural stem cells. *Nat. Rev., Neurosci.* 2, 287–293.
- Barres, B.A., Silverstein, B.E., Corey, D.P., Chun, L.L., 1988. Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron* 1, 791–803.
- Belecky-Adams, T., Tomarev, S., Li, H.S., Ploder, L., McInnes, R.R., Sundin, O., Adler, R., 1997. Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. *Invest. Ophthalmol. Visual Sci.* 38, 1293–1303.
- Benninger, F., Beck, H., Wernig, M., Tucker, K.L., Brustle, O., Scheffler, B., 2003. Functional integration of embryonic stem cell-derived neurons in hippocampal slice cultures. *J. Neurosci.* 23, 7075–7083.
- Bhattacharya, S., Jackson, J.D., Das, A.V., Thoreson, W.B., Kuszynski, C., James, J., Joshi, S., Ahmad, I., 2003. Direct identification and enrichment of retinal stem cells/progenitors using Hoechst dye efflux assay. *IOVS* 44, 2764–2773.
- Bhattacharya, S., Dooley, C., Soto, F., Madson, J., Das, A.V., Ahmad, I., 2004. Involvement of Ath3 in CNTF-mediated differentiation of the late retinal progenitors. *Mol. Cell. Neurosci.* 27, 32–43.
- Blackshaw, S., Harpavat, S., Trimarchi, J., Cai, L., Huang, H., Kuo, W.P., Weber, G., Lee, K., Fraioli, R.E., Cho, S.H., Yung, R., Asch, E., Ohno-Machado, L., Wong, W.H., Cepko, C.L., 2004. Genomic analysis of mouse retinal development. *PLoS Biol.* 2, E247.
- Braisted, J.E., Essman, T.F., Raymond, P.A., 1994. Selective regeneration of photoreceptors in goldfish retina. *Development* 120, 2409–2419.
- Castelo-Branco, G., Wagner, J., Rodriguez, F.J., Kele, J., Sousa, K., Rawal, N., Pasolli, H.A., Fuchs, E., Kitajewski, J., Arenas, E., 2003. Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. *Proc. Natl. Acad. Sci. U. S. A.* 100, 12747–12752.
- Chacko, D.M., Das, A.V., Zhao, X., James, J., Bhattacharya, S., Ahmad, I., 2003. Transplantation of ocular stem cells: the role of injury in incorporation and differentiation of grafted cells in the retina. *Vision Res.* 43, 937–946.
- Dahl, D., Rueger, D.C., Bignami, A., Weber, K., Osborn, M., 1981. Vimentin, the 57,000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia. *Eur. J. Cell Biol.* 24, 191–196.
- Das, A., James, J., Zhao, X., Bhattacharya, S., Ahmad, I., 2004. Involvement of c-Kit receptor tyrosine kinase in the maintenance of ciliary epithelial neural stem cells; interaction with notch signaling. *Dev. Biol.* 273, 87–105.
- Das, A.V., Edakkot, S., Thoreson, W.B., James, J., Bhattacharya, S., Ahmad, I., 2005a. Membrane properties of retinal stem cells/progenitors. *Prog. Retinal Eye Res.* 24, 663–681.
- Das, A., James, J., Rahnenführer, J., Thoreson, W.B., Bhattacharya, S., Zhao, X., Ahmad, I., 2005b. Retinal properties and potential of the adult mammalian ciliary epithelium stem cells. *Vision Res.* 45, 1653–1666.
- Das, A.V., Zhao, X., James, J., Kim, M., Cowan, K.H., Ahmad, I., 2006. Neural stem cells in the adult ciliary epithelium express GFAP and are regulated by Wnt signaling. *Biochem. Biophys. Res. Commun.* 339, 708–716.
- Doetsch, F., 2003. The glial identity of neural stem cells. *Nat. Neurosci.* 6, 1127–1134.
- Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M., Alvarez-Buylla, A., 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97, 703–716.
- Dyer, M.A., Cepko, C.L., 2000. Control of Muller glial cell proliferation and activation following retinal injury. *Nat. Neurosci.* 3, 873–880.
- Faux, C.H., Turnley, A.M., Epa, R., Cappai, R., Bartlett, P.F., 2001. Interactions between fibroblast growth factors and Notch regulate neuronal differentiation. *J. Neurosci.* 21, 5587–5596.
- Fischer, A.J., Reh, T.A., 2001. Muller glia are a potential source of neural regeneration in the postnatal chicken retina. *Nat. Neurosci.* 4, 247–252.
- Furukawa, T., Mukherjee, S., Bao, Z.Z., Morrow, E.M., Cepko, C.L., 2000. *rax*, *Hes1*, and *notch1* promote the formation of Muller glia by postnatal retinal progenitor cells. *Neuron* 26, 383–394.
- Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., Mulligan, R.C., 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* 183, 1797–1806.
- Gotz, M., Barde, Y.A., 2005. Radial glial cells defined and major intermediates between embryonic stem cells and CNS neurons. *Neuron* 46, 369–372.
- Graham, V., Khudyakov, J., Ellis, P., Pevny, L., 2003. SOX2 functions to maintain neural progenitor identity. *Neuron* 39, 749–765.
- Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88, 49–92.
- Hartfuss, E., Galli, R., Heins, N., Gotz, M., 2001. Characterization of CNS precursor subtypes and radial glia. *Dev. Biol.* 229, 15–30.
- Hatakeyama, J., Kageyama, R., 2004. Retinal cell fate determination and bHLH factors. *Semin. Cell Biol.* 15, 83–89.
- Heins, N., Malatesta, P., Cecconi, F., Nakafuku, M., Tucker, K.L., Hack, M.A., Chapouton, P., Barde, Y.A., Gotz, M., 2002. Glial cells generate neurons: the role of the transcription factor Pax6. *Nat. Neurosci.* 5, 308–315.
- Hicks, D., Courtois, Y., 1990. The growth and behaviour of rat retinal Muller cells in vitro: I. An improved method for isolation and culture. *Exp. Eye Res.* 51, 119–129.
- Hsieh, J.C., Rattner, A., Smallwood, P.M., Nathans, J., 1999. Biochemical characterization of Wnt-frizzled interactions using a soluble, biologically active vertebrate Wnt protein. *Proc. Natl. Acad. Sci. U. S. A.* 96, 3546–3551.
- Hulspas, R., Quesenberry, P.J., 2000. Characterization of neurosphere cell phenotypes by flow cytometry. *Cytometry* 40, 245–250.
- James, J., Das, A.V., Bhattacharya, S., Chacko, D.M., Zhao, X., Ahmad, I., 2003. In vitro generation of early-born neurons from late retinal progenitors. *J. Neurosci.* 23, 8193–8203.
- James, J., Das, A.V., Rahnenführer, J., Ahmad, I., 2004. Cellular and molecular characterization of early and late retinal stem cells/progenitors: differential regulation of proliferation and context dependent role of Notch signaling. *J. Neurobiol.* 61, 359–376.

- Kohwi, M., Osumi, N., Rubenstein, J.L., Alvarez-Buylla, A., 2005. Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. *J. Neurosci.* 25, 6997–7003.
- Krishnamurthy, P., Schuetz, J.D., 2006. Role of ABCG2/BCRP in biology and medicine. *Annu. Rev. Pharmacol. Toxicol.* 46, 381–410.
- Kubo, F., Takeichi, M., Nakagawa, S., 2003. Wnt2b controls retinal cell differentiation at the ciliary marginal zone. *Development* 130, 587–598.
- Lassalle, B., Bastos, H., Louis, J.P., Riou, L., Testart, J., Dutrillaux, B., Fouchet, P., Allemand, I., 2004. 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. *Development* 131, 479–487.
- Laywell, E.D., Rakic, P., Kukekov, V.G., Holland, E.C., Steindler, D.A., 2000. Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proc. Natl. Acad. Sci. U. S. A.* 97, 13883–13888.
- Lehre, K.P., Davanger, S., Danbolt, N.C., 1997. Localization of the glutamate transporter protein GLAST in rat retina. *Brain Res.* 744, 129–137.
- Lendahl, U., Zimmerman, L.B., McKay, R.D., 1990. CNS stem cells express a new class of intermediate filament protein. *Cell* 60, 585–595.
- Liao, Y.F., Wang, B.J., Cheng, H.T., Kuo, L.H., Wolfe, M.S., 2004. Tumor necrosis factor- α , interleukin-1 β , and interferon- γ stimulate gamma-secretase-mediated cleavage of amyloid precursor protein through a JNK-dependent MAPK pathway. *J. Biol. Chem.* 279, 49523–49532.
- Liu, H., Mohamed, O., Dufort, D., Wallace, V.A., 2003. Characterization of Wnt signaling components and activation of the Wnt canonical pathway in the murine retina. *Dev. Dyn.* 227, 323–334.
- Malatesta, P., Hack, M.A., Hartfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F., Gotz, M., 2003. Neuronal or glial progeny: regional differences in radial glia fate. *Neuron* 37, 751–764.
- Marquardt, T., Ashery-Padan, R., Andrejewski, N., Scardigli, R., Guillemot, F., Gruss, P., 2001. Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* 105, 43–55.
- Martin, C.M., Meeson, A.P., Robertson, S.M., Hawke, T.J., Richardson, J.A., Bates, S., Goetsch, S.C., Gallardo, T.D., Garry, D.J., 2004. Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. *Dev. Biol.* 265, 262–275.
- Mathers, P.H., Grinberg, A., Mahon, K.A., Jamrich, M., 1997. The Rx homeobox gene is essential for vertebrate eye development. *Nature* 387, 603–607.
- Merkle, F.T., Tramontin, A.D., Garcia-Verdugo, J.M., Alvarez-Buylla, A., 2004. Radial glia give rise to adult neural stem cells in the subventricular zone. *Proc. Natl. Acad. Sci. U. S. A.* 101, 17528–17532.
- Meucci, O., Fatatis, A., Simen, A.A., Bushell, T.J., Gray, P.W., Miller, R.J., 1998. Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* 95, 14500–14505.
- Morrow, E.M., Furukawa, T., Lee, J.E., Cepko, C.L., 1999. NeuroD regulates multiple functions in the developing neural retina in rodent. *Development* 126, 23–36.
- Morshead, C.M., Garcia, A.D., Sofroniew, M.V., van Der Kooy, D., 2003. 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. *Eur. J. Neurosci.* 18, 76–84.
- Mu, X., Klein, W.H., 2004. A gene regulatory hierarchy for retinal ganglion cell specification and differentiation. *Semin. Cell Dev. Biol.* 15, 115–123.
- Murata, K., Hattori, M., Hirai, N., Shinozuka, Y., Hirata, H., Kageyama, R., Sakai, T., Minato, N., 2005. Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1. *Mol. Cell. Biol.* 25, 4262–4271.
- Newman, E.A., 1993. Inward-rectifying potassium channels in retinal glial (Muller) cells. *J. Neurosci.* 13, 3333–3345.
- Nishida, A., Furukawa, A., Koike, C., Tano, Y., Aizawa, S., Matsuo, I., Furukawa, T., 2003. Otx2 homeobox gene controls retinal photoreceptor cell fate and pineal gland development. *Nat. Neurosci.* 6, 1255–1263.
- Noctor, S.C., Flint, A.C., Weissman, T.A., Dammerman, R.S., Kriegstein, A.R., 2001. Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409, 714–720.
- 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.
- Pevny, L., Rao, M.S., 2003. The stem-cell menagerie. *Trends Neurosci.* 26, 351–359.
- Polleux, F., Giger, R.J., Ginty, D.D., Kolodkin, A.L., Ghosh, A., 1998. Patterning of cortical efferent projections by semaphorin–neuropilin interactions. *Science* 282, 1904–1906.
- Reh, T.A., Fischer, A.J., 2001. Stem cells in the vertebrate retina. *Brain Behav. Evol.* 58, 296–305.
- Reynolds, B.A., Rietze, R.L., 2005. Neural stem cells and neurospheres—re-evaluating the relationship. *Nat. Methods* 2, 333–336.
- Roque, R.S., Caldwell, R.B., 1993. Isolation and culture of retinal microglia. *Curr. Eye Res.* 12, 285–290.
- Sahel, J.A., Albert, D.M., Lessell, S., Adler, H., McGee, T.L., Konrad-Rastegar, J., 1991. Mitogenic effects of excitatory amino acids in the adult rat retina. *Exp. Eye Res.* 53, 657–664.
- Sarmiento, L.M., Huang, H., Limon, A., Gordon, W., Fernandes, J., Tavares, M.J., Miele, L., Cardoso, A.A., Classon, M., Carlesso, N., 2005. Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. *J. Exp. Med.* 202, 157–168.
- Schnitzer, J., 1988. Immunocytochemical studies on the development of astrocytes, Muller (glial) cells, and oligodendrocytes in the rabbit retina. *Brain Res. Dev. Brain Res.* 44, 59–72.
- Seri, B., Garcia-Verdugo, J.M., McEwen, B.S., Alvarez-Buylla, A., 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J. Neurosci.* 21, 7153–7160.
- Vandier, D., Rixe, O., Besnard, F., Kim, M., Rikiyama, T., Goldsmith, M., Brenner, M., Gouyette, A., Cowan, K.H., 2000. Inhibition of glioma cells in vitro and in vivo using a recombinant adenoviral vector containing an astrocyte-specific promoter. *Cancer Gene Ther.* 7, 1120–1126.
- Vicario-Abejon, C., Cunningham, M.G., McKay, R.D., 1995. Cerebellar precursors transplanted to the neonatal dentate gyrus express features characteristic of hippocampal neurons. *J. Neurosci.* 15, 6351–6363.
- Viti, J., Gulacsi, A., Lillien, L., 2003. Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. *J. Neurosci.* 23, 5919–5927.
- Walcott, J.C., Provis, J.M., 2003. Muller cells express the neuronal progenitor cell marker nestin in both differentiated and undifferentiated human foetal retina. *Clin. Exp. Ophthalmol.* 31, 246–249.
- Yang, Z., Ding, K., Pan, L., Deng, M., Gan, L., 2003. Math5 determines the competence state of retinal ganglion cell progenitors. *Dev. Biol.* 264, 240–254.
- Zhao, X., Das, A., Thoreson, W.B., Rodriguez-Sierra, J.F., Ahmad, I., 2002. Adult corneal limbal epithelium; a model for studying neural potential of non-neural stem cells. *Dev. Biol.* 250, 317–331.
- Zhao, X., Das, A.V., Soto-Leon, F., Ahmad, I., 2005. Growth factor-responsive progenitors in the postnatal mammalian retina. *Dev. Dyn.* 232, 349–358.
- Zhou, H., Yoshioka, T., Nathans, J., 1996. Retina-derived POU-domain factor-1: a complex POU-domain gene implicated in the development of retinal ganglion and amacrine cells. *J. Neurosci.* 16, 2261–2274.
- Zhou, C.J., Zhao, C., Pleasure, S.J., 2004. Wnt signaling mutants have decreased dentate granule cell production and radial glial scaffolding abnormalities. *J. Neurosci.* 24, 121–126.