Direct Identification and Enrichment of Retinal Stem Cells/Progenitors by Hoechst Dye Efflux Assay

Sumitra Bhattacharya,1 John D. Jackson,2 Ani V. Das,1 Wallace B. Thoreson,1 Charles Kuszynski,2 Jackson James,1 Shantanam Joshi,5 and Iqbal Ahmad1

PURPOSE. The present study describes a method for isolating neural stem cells/progenitors directly from the freshly dissociated embryonic retina (prospective identification) and compares their characteristics with those enriched from mitogen-exposed embryonic retinal cell culture.

METHODS. Cell dissociates from embryonic rat retina and mitogen-exposed embryonic retinal cultures were stained with Hoechst 33342 fluorescent dye. The emission patterns of cells were analyzed in both blue and red wavelength using flow cytometry to enrich cells that retained or excluded the dye. The phenotype characteristics and differentiation potential of enriched cells were analyzed by immunocytochemical, RT-PCR, and electrophysiological analyses.

RESULTS. The Hoechst dye efflux assay identified a minor population of cells, called side population (SP) cells, in fresh retinal dissociates. These cells that preferentially excluded the Hoechst 33342 fluorescent dye were proliferative and expressed both neural and retinal progenitor markers. The retinal SP cells generated functional neurons and glias and possessed the ability to differentiate along lineages of different late-born retinal cell types. Cells of similar phenotypes and potential were observed in the SP obtained from mitogen-exposed retinal cultures.

CONCLUSIONS. The Hoechst dye efflux assay represents an effective method for direct identification of retinal stem cells/progenitors. These results demonstrate that the prospectively isolated retinal stem cells/progenitors and those enriched as SP cells from mitogen-exposed retinal cell culture may be similar in their properties and potential. (Invest Ophthalmol Vis Sci. 2003;44:2764–2773) DOI:10.1167/iovs.02-0899

Neural stem cells/progenitors that give rise to neurons and glia have been identified in different regions of the brain, including the retina.1 These cells can be maintained in a proliferative state in culture, usually in the presence of mitogens, epidermal growth factor (EGF), and/or fibroblast growth factor (FGF2). Withdrawal of mitogens from the culture and addition of serum or growth factors induce their differentiation into neurons and glias.2 The fact that cultured stem cells/progenitors can acquire site-specific phenotypes on transplantation suggests their potential usefulness in treating neurodegenerative changes.3–6 However, in most cases, neural stem cells/progenitors have not been enriched directly from freshly dissociated neural tissue (prospective identification) and the question therefore remains of whether the potential that they display is inherent or acquired from prolonged exposure to mitogens used to maintain them in culture. This issue is a significant one for both the biology of stem cells and their use as therapeutic reagents. To investigate the inherent potential of neural stem cells/progenitors, characterization of properties is necessary that allows direct identification of neural stem cells/progenitors and study of their primitive characteristics over generations. With this objective in mind we have begun the identification and characterization of retinal stem cells/progenitors based on strategies developed for the isolation of hematopoietic stem cells (HSCs).

There are currently two different approaches for prospective identification of HSCs. The first approach involves fluorescence-activated cell sorting (FACS), in which monoclonal antibodies to specific cell surface markers is used. This approach has been used successfully to isolate self-renewing, multipotent cells from the peripheral nervous system with the low-affinity neurotrophin receptor p75 used as a surface marker.7 More recently, specific monoclonal antibodies that recognize the surface markers, CD133 and 5E12, have been used for prospective identification of self-renewing and multipotent neural stem cells from human fetal brain.8 Another approach to identify hematopoietic stem cells/progenitors is based on the ability of these cells to exclude selectively Hoechst 33342 dye, which can be enriched as a part of a minor population, called the side population (SP), by FACS.9 This approach has been used to enrich neural progenitors from neurospherically cultured.10 We used the Hoechst dye exclusion assay for direct identification of neural stem cells/progenitors from freshly dissociated embryonic day (E)18 rat retina and demonstrate that their properties and potential are similar to those enriched from in vitro expanded E18 retinal cells.

METHODS

Dissociation of Embryonic Retina

Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Timed-pregnant E18 Sprague-Dawley rats were obtained from the supplier (Sasco, Wilmington, MA), and embryos were harvested in Hanks’ balanced salt solution (HBSS). Eyes were enucleated with minimum extraneous tissue and placed in HBSS in a separate Petri dish. The optic nerve and remaining mesenchymal tissues were carefully removed before the retina was isolated, to prevent the possible contamination of retina with brain tissues. The retina was carefully teased away from the retinal pigmented epithelium (RPE), and the central portion of the retina surrounding the optic nerve was removed and discarded. The isolated retina was collected in a sterile 15-ml polypropylene tube and dissociated into single cells, as previously described.2 Briefly, the retina was digested with 0.25% trypsin at 37°C for 10 minutes. Cells were washed with fresh retinal culture medium (RCM: DMEM-F12, N2 sup-

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rospheres were collected by centrifugation, dissociated into single
retinal cells were resuspended in Hoechst Iscove’s modified Dulbecco’s
medium (10% cells/mL; IMDM) containing 2% fetal calf serum
(FCS) at 4°C overnight, followed by staining with Hoechst 33342 (2.0
μg/mL). Total RNA was isolated with a kit (Qiagen, Valencia, CA). cDNA (2
μL) was synthesized using random hexamers. Real-time (RT) PCR was
performed with gene-specific primers (Table 2) by using the fol-
lowing step-cycle program (Robocycler; Stratagene, La Jolla, CA): de-
naturation at 94°C for 30 seconds, annealing at specific temperature
wavelengths (Omega Optical, Inc.). First, a live gate was defined on the
flow cytometer using Hoechst red and blue axes to exclude dead cells
that are Hoechst red (very bright), red cells (no Hoechst stain), and
debris. After 10^6 events were collected within the live gates, the SP
and non-SP (NSP) cells were defined as Hoechst-low and -bright,
respectively. The region between SP and NSP cells was defined as
extra-SP or upper SP. SP, NSP, and extra-SP cells were sorted into
tubes (Eppendorf, Fremont, CA) containing 100% FBS. The sorted cells
were cytocentrifuged for immunofluorescence analysis or cultured as
described earlier and then subjected to immunofluorescence or
electrophysiological analyses.

**Immunofluorescence Analysis**

Immunofluorescence analysis on undifferentiated and differentiated SP
and NSP cells was performed as previously described. Briefly, cells were
fixed in ice-cold 4% paraformaldehyde and incubated with cell-
specific marker antibodies (Table 1) in blocking serum at 4°C over-
night. After the incubation in species-specific IgG conjugated with CY3
and/or 7-amino-4-methyl-coumarin-3-acetic acid (AMCA) or FITC, cells
were washed with PBS and examined by microscope.

**RT-PCR Analysis**

Total RNA was isolated with a kit (Qiagen, Valencia, CA). cDNA (2 μL)
was amplified with gene-specific primers (Table 2) by using the fol-
lowing step-cycle program (Robocycler; Stratagene, La Jolla, CA): de-
naturation at 94°C for 30 seconds, annealing at specific temperature

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### Table 1. List of Antibodies Used for Immunofluorescence Analyses

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Cell Types</th>
<th>Dilution</th>
<th>Source</th>
<th>References</th>
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<tr>
<td>CD4</td>
<td>Mature lymphocytes</td>
<td>1:50</td>
<td>Pharmingen</td>
<td>12</td>
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<td>CD8a</td>
<td>Mature lymphocytes</td>
<td>1:50</td>
<td>Pharmingen</td>
<td>13</td>
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<td>CD11b</td>
<td>Neutrophils</td>
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<td>Pharmingen</td>
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<td>RP-1</td>
<td>Granulocytes</td>
<td>1:50</td>
<td>Pharmingen</td>
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<td>Pharmingen</td>
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<td>Thy1 Antigen</td>
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<td>Pharmingen</td>
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<td>Neural progenitors</td>
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<td>Progenitors</td>
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<tr>
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<td>Proliferating cells</td>
<td>1:500</td>
<td>Pharmingen</td>
<td>20</td>
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<td>Chx10</td>
<td>Retinal progenitors</td>
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<td>Ref. 21</td>
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<td>Map2</td>
<td>Neurons</td>
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<td>Chemicon†</td>
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<td>β-tubulin</td>
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<td>Covance‡</td>
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<td>GFAP</td>
<td>Astrocytes</td>
<td>1:100</td>
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<td>Syntaxin1</td>
<td>Aminacrine cells</td>
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<td>Ref. 28</td>
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<tr>
<td>Rhodopsin</td>
<td>Photo receptors</td>
<td>1:5000</td>
<td>Ref. 28</td>
<td>28</td>
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</table>

* Division of BD Biosciences, San Diego, CA.
† Developmental Studies Hybridoma Bank, University of Iowa, Iowa City.
‡ Temecula, CA.
§ St. Louis, MO.

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### Table 2. List of Primers and Their Respective Sequences Used for RT-PCR Analyses

<table>
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<tr>
<th>Genes</th>
<th>Product Accession</th>
<th>Genbank Accession Number</th>
<th>Temperatures (°C)</th>
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<td>β-Actin</td>
<td>Forward: 5’ GTGGGGCCGGCCACGGACCA 3’</td>
<td>543</td>
<td>XMO37235</td>
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<td>Nestin</td>
<td>Forward: 5’TGGAGGAGGAGAAGGAGGTCTAC 3’</td>
<td>295</td>
<td>NM012987</td>
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<td>Opsin</td>
<td>Reverse: 5’TGAAGGTTTATGGAAGGGG 3’</td>
<td>382</td>
<td>U22180</td>
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<tr>
<td>Syntaxin1</td>
<td>Reverse: 5’AGCAGAGGCTGTGAGGACTG 3’</td>
<td>342</td>
<td>NM016801</td>
</tr>
<tr>
<td>Map2</td>
<td>Forward: 5’CGGACACAAAGAAAATCCCCCACG 3’</td>
<td>329</td>
<td>X54100</td>
</tr>
<tr>
<td>GFAP</td>
<td>Reverse: 5’GTCAAACTTATCCTTCCATC 3’</td>
<td>310</td>
<td>NM017809</td>
</tr>
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(Table 2) for 35 seconds, and extension at 72°C for 40 seconds for 30 cycles, followed by a final extension at 72°C for 5 minutes. PCR products were resolved on 2.0% agarose gel against 100-bp DNA marker (MBI Fermentas, USA).

**Electrophysiological Analysis and Ca^{2+} Imaging**

For electrophysiological and Ca^{2+} imaging studies, cells were plated on coverslips, 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; Omega Dot; Stoelting Co., Chicago, IL) and had tips of 1 to 2 μm outer diameter with tip resistances of 6 to 12 MΩ. Pipettes were filled with a bathing solution containing 140 mM NaCl; 5 mM KCl; 2 mM CaCl₂; 1 mM MgCl₂; 10 mM HEPES; and 10 mM glucose (pH 7.4).

**FIGURE 1.** A subpopulation of freshly dissociated E18 retinal cells excluded Hoechst dye. Freshly isolated rat bone marrow (A), E18 hippocampal (B), and retinal (C) cells were stained with Hoechst 33342 fluorescent dye, and emission patterns of respective cell types were analyzed in both blue and red wavelength by flow cytometry. Boxed region: SP cells that selectively excluded the Hoechst 33342 dye.

**FIGURE 2.** The exclusion of Hoechst dye by E18 retinal cells was verapamil sensitive. Freshly isolated E18 retinal cells were stained with Hoechst 33342 dye in the presence or absence of verapamil. The Hoechst dye staining and emission patterns of retinal cells revealed that the efflux of Hoechst dye by SP cells took place in the absence (A) and in the presence (B) of verapamil. The effect of verapamil on the Hoechst dye efflux was concentration dependent (C).
was concentration dependent (Fig. 2). To investigate the possibility that SP cells may be present due to contamination with blood during the isolation of retinal cells, expression of different hematopoietic lineage antigens by retinal cells from either the fresh tissue or neurospheres obtained from an EGF-exposed culture of E18 retinal cells was analyzed (Table 3). The proportion of retinal cells expressing Thy1 antigen (CD 90) because mature retinal ganglion cells are Thy1 positive. In contrast, nestin, a neuroectodermal stem cell marker, was expressed only by retinal dissociates and not by bone marrow cells (Table 3).

**Expression of Retinal Progenitor Markers by Retinal SP Cells**

To determine the nature of cells in the retinal SP, we investigated the proliferative potential of cells and the expression of different markers that characterize neural and retinal progenitors (Fig. 3). The majority (88.2% ± 5.9%) of SP cells were proliferative, as they expressed Ki67 antigen, a cell proliferation marker. These cells expressed the neural progenitor marker Notch1 because cells were cytocentrifuged, resulting in the loss of cytoplasmic antigens could not be distinguished, because stem cells/progenitors generally have a high nuclear-to-cytoplasmic ratio and because cells were cytocentrifuged, resulting in the loss of cytoplasmic processes. Magnification, ×200.
ers nestin (91.1% ± 6.1%) and Notch1 (92.6% ± 6.4%) and the retinal progenitor marker Chx10 (86.4% ± 2.8%). There was no discrimination between nuclear and cytoplasmic distributions of specific antigens because stem cells/progenitors generally have a high nuclear-to-cytoplasmic ratio and because cells were cytocentrifuged for the purpose of immunocytochemical analysis, resulting in the loss of cytoplasmic processes. To determine whether SP cells represent distinct subpopulations, the expression of pan-neural and retinal progenitor markers was performed by double immunocytochemical analysis. Cells that expressed pan-neural markers, nestin, and/or Notch1, also expressed the retinal progenitor marker Chx10 (data not shown).

RT-PCR analysis was used to compare the expression of cell-type–specific markers between SP and NSP cells (Fig. 4). Transcripts corresponding to the general neural markers Map2 and glial fibrillary acidic protein (GFAP) and those encoding the retinal cell–specific markers opsin and syntaxin 1 were expressed in NSP cells, but not in SP cells, suggesting that the latter largely consists of undifferentiated cells. Results of RT-PCR analyses were corroborated by immunocytochemical analysis; while nestin (91.1% ± 6.1%) was expressed exclusively in SP, opsin (15.38% ± 5.5%) and syntaxin 1 (3.15% ± 0.9%) immunoreactivities were detected only in NSP cells. In addition, Ki67 positive cells (88.2% ± 5.9%) were found only in SP cells.

**Enrichment of Retinal SP Cells from Primary Neurospheres**

To analyze the characteristics of progenitors exposed to mitogens, dissociates from E18 retina were cultured in the presence of EGF. In this culture condition, subsets of proliferating cells generated individual colonies called neurospheres (Figs. 5A–C). Neurospheres were collected and dissociated, and cells were subjected to a Hoechst dye efflux assay. A robust SP was obtained that contained 1.0% of total retinal cells analyzed compared with 0.1% obtained from freshly dissociated retina (Fig. 5D). These cells were not likely to be present because of contamination of neurospheric cells with blood cells, because the proportion of cells expressing hematopoietic lineage markers was insignificant (Table 3). RT-PCR analysis showed transcript differences.
scripts corresponding to Map2, GFAP, opsin, and syntaxin in the NSP cells only, suggesting that the SP consists of undifferentiated cells (Fig. 5E). Immunocytochemical analysis of these SP cells showed that similar to those directly isolated from E18 retina (Fig. 3), they were Ki67 positive (88.3% ± 3.7%) and expressed both neural and retinal progenitor markers in similar proportions (nestin-positive cells: 92.1% ± 4.8%, Notch1-positive cells: 95.0% ± 5.5%, and Chx10-positive cells: 89.1% ± 5.5%) as those that were prospectively identified.

To understand the proliferative nature and differentiation potential of retinal stem cells/progenitors, SP cells obtained from the primary neurospheres were cultured to high density (1.5–2.0 × 10⁴ cells/well) in a 96-well culture plate in the presence of EGF. A subset of the SP cells generated clonal secondary spheres (Fig. 6A). Most of the cells in the NSP (Fig. 6B), plated at the same density died, and few that survived did not generate neurospheres. Cells in the secondary neurospheres were proliferative and expressed neural progenitor markers (Figs. 6C–J). When EGF was withdrawn from the culture medium and supplemented with 1% serum, SP cells enriched from either freshly dissociated retina or mitogen-exposed neurospheres expressed the pan-neural markers, β-tubulin and GFAP, suggesting that a subset of SP cells is multipotent (Figs. 7 and 8). The multipotent nature of SP cells was corroborated by RT-PCR analyses that showed that SP cells in differentiation conditions express transcripts corresponding to neuronal (Map2) and glial (GFAP) markers (Figs. 7F, 8F).

**Electrophysiological Analysis of Retinal SP Cells**

Analyses of cell-type-specific antigenic markers and transcripts suggested that SP cells are multipotent and differentiate along neuronal and glial lineage. We wanted to know whether differentiated SP cells possess distinct electrophysiological properties in addition to expressing neural markers. This possibility was explored by electrophysiological analysis using...
whole-cell recording from SP cells in differentiation conditions (Fig. 9). Voltage-dependent currents were evoked by a series of −20-mV voltage steps (150 ms) from −110 to +70 mV. The following phenotypes were observed based on current-voltage profiles: (1) cells that displayed neuronal features, such as a rapidly activating inward current evoked above −30 mV, attributed to sodium current (I\text{Na}) and a sustained outward current attributed to potassium current (I\text{K}) (Figs. 9A, 9B); (2) cells that displayed glial features such as a prominent inward rectifying current at hyperpolarizing potential as observed in the Müller glia of primates\textsuperscript{29} (Figs. 9C, 9D); and (3) cells that displayed a delayed, outward rectifying current uncharacteristic of either neurons or glia (data not shown). It is likely that these cells represent precursors in the process of differentiation, and the current-voltage profile displayed by them may be indicative of their immature nature. We further ascertained the functional differentiation of SP cells by analyzing the expression of N-methyl-D-aspartate (NMDA) and non-NMDA ionotropic glutamate, using Ca\textsuperscript{2+} imaging with Fura-2 (Fig. 10). Application of kainic acid (KA, 30 μM) evoked an increase in the 340:380 ratio indicating an increase in intracellular [Ca\textsuperscript{2+}] in three cells in the field (Fig. 10A2). The changed ratio, within the circular region of interest, is plotted as a function of time (Fig. 10B). After recovery from kainic acid, an Mg\textsuperscript{2+}-free solution containing glycine (0.1 mM) was applied for 2 minutes, followed by application of the same solution with NMDA (0.1 mM) for 1 minute. Similar to kainic acid, NMDA evoked a Ca\textsuperscript{2+} increase in the cell, denoted by the circular region of interest (Figs. 10A3, 10B). Application of a high K\textsuperscript{+} solution to strongly depolarize the cell also evoked a Ca\textsuperscript{2+} increase in this cell as well as in two others in the field, suggesting the presence of voltage-gated Ca\textsuperscript{2+} channels. Thus, consistent with previous studies on retinal progenitor cells, differentiating conditions promote the expression of various physiological properties, including those commonly associated with neurons (e.g., voltage-dependent Na\textsuperscript{+} currents and ionotropic glutamate receptors) and glia (e.g., prominent inward rectifying currents).

**Generation of Retinal Neurons**

The foregoing observations demonstrated that SP cells have neural potential. To determine whether they retain their ability to differentiate into retinal neurons, SP cells enriched from freshly dissociated E18 retina or neurospheres were cocultured with an excess of cells isolated from PN1 retina. We have shown that PN1 retinal cells facilitate the differentiation of cultured retinal progenitors into retinal neurons.\textsuperscript{2} Because the SP cells represented the late stage of retinal histogenesis, we examined their potential to generate late-born retinal neurons (rod photoreceptors, amacrine cells, and bipolar cells) and the Müller glia. We observed that retinal SP cells obtained from either source could express rod photoreceptor-specific (rhodopsin), bipolar cell-specific (PKC), amacrine cell-specific (synaptin), and glia-specific (vimentin) markers when cocultured with PN1 cells for 5 to 7 days (Fig. 11). This observation suggests that, besides possessing the potential to give rise to neurons and glia, SP cells enriched from either a freshly dissociated retina or neurospheres demonstrate a similar capability to differentiate along lineages of different late-born retinal cell types.

**DISCUSSION**

One of the most significant barriers in the study of the biology of neural stem cells/progenitors and in their therapeutic use is the lack of information about their inherent proliferative and differentiation potential.\textsuperscript{1} A significant proportion of information regarding the properties of neural stem cells/progenitors has emerged from in vitro studies. It is likely that these cells, exposed to high concentrations of mitogens to maintain them in culture, undergo changes and thereby acquire potential differences from their parents. Therefore, information regarding the inherent and acquired potential of these cells becomes important to shed light on mechanisms and the extent of their plasticity. This information is particularly important for therapeutic use of neural stem cells/progenitors in highly ordered and laminated sensory structures such as the retina, where the presence of undesirable neurons or glia due to heterologous differentiation may exacerbate rather than solve problems.

In this study we have demonstrated that the Hoechst dye efflux assay can be used effectively for direct identification of retinal stem cells/progenitors and for cross-comparison of properties between populations of progenitors enriched from
different sources. Because the number of dividing cells is greater in the developing retina at the stage of late neurogenesis (E18) than at early neurogenesis (E14), we performed the assay on the former to maximize the enrichment of proliferating progenitors. We observed that the dual-wavelength flow cytometric analysis of Hoechst-dye–stained E18 retinal cells yielded two basic populations of cells: the NSP and SP. The differentiated cells were largely distributed in the NSP. The stem cells/progenitors, in contrast, reside in SP that, like HSC SP, is verapamil sensitive. The notion that retinal SP cells represent stem cells/progenitors is supported by the fact that these cells are proliferative and express a number of markers that are characteristic of both neural (i.e., nestin) and retinal (i.e., Chx10) progenitors. However, SP cells account for a small proportion of nestin-positive cells present in either E18 retina or neurospheres (Table 3). The rest of the proliferating nestin-positive cells found in an extra-SP region between the SP and upper NSP (data not shown) are likely to represent committed precursors in different stages of differentiation. In addition to dividing and expressing progenitor markers, retinal SP cells are undifferentiated, as ascertained by the absence of expression of neural cell- and retinal cell-type–specific markers. However, retinal SP cells are not homogeneous in the expression of progenitor markers. For example, although most cells expressed both nestin and Chx10, a subset of cells was observed that was nestin positive and Chx10 negative. Cellular heterogeneity has also been observed in HSCs and human cord blood SP cells.

The prospectively identified retinal progenitors appeared to be similar in characteristics and potential to progenitor cells

![Figure 9](image-url)  
**Figure 9.** SP cells displayed electrophysiological properties of differentiated cells. SP cells obtained from E18 retina were cultured in the presence of serum, and current–voltage relationships were obtained from a subset of cells by whole-cell recording. (A, C) Currents evoked by a series of 150-ms voltage steps applied from a holding potential of −70 mV. In the current–voltage plot in (B), both the maximal inward (I) and outward (O) currents are plotted. The data show that both the sustained outward rectifying currents and the presumptive sodium current activated above −50 mV. The current–voltage plot in (D) shows that the currents were inwardly rectifying below −90 mV and outwardly rectifying above −50 mV. The cell in (A) and (B) exhibited an electrophysiological phenotype similar to glial Müller cells. The cell in (C) and (D) exhibited a neuronal phenotype.

![Figure 10](image-url)  
**Figure 10.** SP cells express ionotropic glutamate receptors. SP cells obtained E18 retina were cultured in the presence of serum and effects of kainic acid, NMDA, and high K⁺ solutions on intracellular Ca²⁺ levels were measured with Fura-2 in differentiated cells. (A) Bright-field image (top left) and a series of pseudocolor images illustrating 340:380 ratio levels in the control (A1), 30 μM kainic acid (A2), 100 μM NMDA (A3), and 140 mM K⁺ (A4). (B) Graph of ratio changes obtained from the circular region of interest indicated in (A). The numbers 1 to 4 in (B) indicate the time points at which the corresponding images in (A) were obtained.
enriched from EGF-exposed retinal neurospheres. This notion is supported by the following observations. First, both were distributed in the verapamil-sensitive SP. Second, the range and proportion of progenitor markers expressed by cells in both SPs were similar, suggesting that they represent similar progenitor populations and third, both are multipotent, capable of giving rise to neurons and glia in differentiation conditions. Cells from both SPs possessed the capacity to differentiate into late-born retinal neurons. The differentiated progeny, in addition to expressing cell-type–specific markers, were distinguishable on the basis of voltage-gated current profiles and the presence of ionotropic glutamate receptors, as demonstrated previously for cultured retinal stem cells/progenitors. These observations suggest that the differentiation of retinal SP cells involves several steps that are characterized by distinct molecular as well as electrophysiological properties.

Retinal progenitors are selectively expanded when exposed to EGF, as suggested by a 10-fold increase in the proportion of the SP cells obtained from the neurospheres compared with those obtained from the fresh retina. Both progenitor populations failed to generate neurospheres when SP cells were seeded at a clonal density. Generation of secondary neurospheres was observed when SP cells enriched from the EGF-exposed neurospheres were cultured at high density. Such a culture condition was not possible with prospectively identified cells, because of their low number (0.1% vs. 1.0% of total cells). This suggests two possibilities: The proliferating cells isolated from retina are not stem cells but rather neural progenitors with a limited self-renewal property, or these cells are indeed stem cells, but conditions have not been identified that promote their self-renewal in vitro. The fact that clonal generation of neurospheres can be observed in high-density culture suggests that the self-renewal property of retinal stem cells/progenitors is a non–cell-autonomous process and therefore requires contributions from other cells.

Taken together, our results suggest that the Hoechst dye exclusion assay represents an efficient method for the direct identification and enrichment of retinal progenitors and that progenitors enriched from mitogen-exposed retinal culture appear to be similar in characteristics and differentiation potential to those that are prospectively identified and therefore may be equally suitable for therapeutic purposes.

**FIGURE 11.** SP cells, isolated directly from E18 retina or mitogen-exposed retinal neurospheres differentiated into retinal neurons. SP cells from either source were cocultured in the presence of PN1 retinal cells across a membrane for 5 to 7 days followed by immunocytochemical analyses to detect retinal cell-type-specific markers. SP cells, enriched from either source, expressed marker for rod photoreceptor (Rhodopsin; A, B, I, J), amacrine cells (Syntaxin; C, D, K, L), bipolar cells (PKC; E, F, M, N), and Müller glia (Vimentin; G, H, O, P). Arrows: retinal cells of the cell type of interest. (Q) Relative proportion of specific retinal cell types in SP cells after co-culture. Magnification, ×200.
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References