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Journal

Experimental neurology, 177(1)

ISSN

0014-4886

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Publication Date

2002-09-01

DOI

10.1006/exnr.2002.7955

Peer reviewed

BRIEF COMMUNICATION

In Vitro Isolation and Expansion of Human Retinal Progenitor Cells

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Received October 15, 2001; accepted April 19, 2002

Human retinal development proceeds with temporal and spatial precision. Although differentiation starts around the beginning of the third month of gestation, the majority of cells in the outer neuroblastic layer of human neural retina are still proliferating, as evidenced by their Ki-67 immunoreactivity. In the present study, the proliferating human retinal progenitor cells (HRPCs) were isolated and expanded in culture. They were capable of dividing for multiple generations (with passage 8, the latest tested) and differentiating to several retinal cell phenotypes. These findings indicate that human retina at the 10th–13th week of gestation harbors progenitor cells that can be maintained and expanded *in vitro* for multiple generations. The availability of such cells may have important implications with respect to human degenerative retinal diseases, as these HRPCs have the potential to be used therapeutically to replace damaged retinal neurons. © 2002 Elsevier Science (USA)

Key Words: neural stem cell; progenitor cell; neuron; retina; human.

The mammalian CNS develops from neural stem and progenitor cells in temporal and spatial precision during organogenesis. In human, the embryogenesis and organogenesis of the eye and its adnexa occur before the third month of gestation when differentiation initiates. Mounting evidence indicates that neural stem or progenitor cells isolated from rodent brain, spinal cord, and the ciliary margin of the eye can divide and differentiate into neurons and glial cells in culture (see, 13, 21, 34). Thus, these cells may be used for *in vitro* and *in vivo* modeling of some human retinal diseases. More importantly, replacement of damaged CNS or PNS neurons might be achieved by transplantation of these precursors. In human, neural stem and progenitor cells which can be maintained for extended passages *in vitro* have been identified from the brain (4, 6, 8, 10, 17, 19, 20, 23, 24, 26) and spinal cord (25). These cells, similar to their *in situ* counterparts in the

normal brain and spinal cord, retain the capabilities of multilineage differentiation after engraftment into the rodent brain and spinal cord (2, 3, 11, 12, 22, 27, 29, 30, 33). Human fetal retinal cells have been found previously proliferative *in vitro* in the presence of fetal bovine serum (FBS) and absence of mitogens (18). Multilineage differentiation from passaged retinal cells has not been established. The human retinal progenitor cells (HRPCs) described in this report are isolated from fetal (10–13 weeks of gestation) eyes, divide for multiple generations, and develop along multiple neural lineages as demonstrated by BrdU-labeling and retinal cell-specific immunocytochemistry.

Human fetal eyes ($n = 18$) at the 10th–13th week of gestation were obtained from legal routine therapeutic abortions, with patients' consent and University of Louisville Institutional Review Board (IRB) approval. To identify proliferating cells in the neural retina *in situ*, two eyes at the 13th week of gestation were frozen and embedded for cryosectioning. Other isolated eyes were kept in ice-cold Hank's balanced salt solution (HBSS, Invitrogen, Carlsbad, CA) and dissected under a Leica Wild M8 microscope. The neural retina was separated from the retinal pigmented epithelium, vitreous, lens, and cornea. A band (1.5 mm in width) of retinal tissue that surrounds the optic nerve and the ciliary bodies was trimmed away to eliminate the possible contamination of astrocytic and oligodendrocytic precursors in the optic nerve and neural stem cells in the ciliary body. The remaining retina was incubated in 0.05% trypsin (Invitrogen) in HBSS for 20 min at 37°C and triturated gently with a fire-polished, siliconized Pasteur glass pipette to release single cells. The single cell suspension was centrifuged at 2500 rpm for 5 min. The resulting pellet was resuspended in expansion medium: DMEM/F-12, N2 supplement (Invitrogen), 1% FBS, 10 ng/ml neurotrophin 3 (NT3, a gift of Regeneron Pharmaceuticals, Tarrytown, NY), and 10 ng/ml fibroblast growth factor 2 (FGF2, Invitrogen). The cells were plated at 5×10^4 cells/cm² in uncoated flasks (initial experiments) or dishes pre-

coated with 10 $\mu\text{g/ml}$ poly-L-ornithine and 5 $\mu\text{g/ml}$ laminin (PO/LM) and cultured at 37°C in a humidified 5% CO₂ incubator. FGF2 (10 ng/ml) was added daily. Media in the coated dishes was changed every other day. Media in the uncoated flasks was not changed because medium change would disrupt the growth of neurospheres.

The HRPCs in the uncoated flasks and coated dishes grew to confluence in 8–12 days. For passaging, primary neurospheres at day 8 were collected, trypsinized, and triturated to release single cells. The resulting single cells were plated at the same density and cultured under the same condition as the previous generation (Passage 0, P0) of cells. Since the P1 cells derived from the P0 spheres in the non-adherent culture survived poorly, further passaging of the P1 spheres was not attempted. The primary HRPCs in the adherent culture were passaged similarly, except that they were replated onto PO/LM-coated dishes. The secondary HRPCs kept dividing for at least 3 months (P8, the latest tested) in the presence of mitogens and were passaged every 8–12 days when they grew near confluence. The HRPCs from early passages (P1–P3) were differentiated for 10–20 days in media containing 5% FBS and no FGF2/NT3. To identify dividing cells in the culture, 10 μM bromodeoxyuridine (BrdU, Sigma Chemical, St. Louis, MO) was added into the media either overnight or for 5 days immediately before differentiation or fixation (in expansion culture). The expanded and differentiated cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS, and kept at 4°C in PBS.

Immunophenotypic analysis was carried out in frozen sections for the proliferating cell analysis and undifferentiated and differentiated P1–P3 cells for lineage analysis, using primary antibodies against Ki-67 (antigen retrieval, 1:50, mouse, Zymed, South San Francisco, CA), BrdU (1:400, sheep, Biodesign International, Saco, ME), nestin (Rat-401, 1:50, mouse, DSHB, Iowa City, IA), neuron-specific enolase (NSE, 1:10,000, rabbit, DAKO, Burlingame, CA), β III tubulin (1:200, mouse, ICN Pharmaceuticals, Costa Mesa, CA), synaptophysin (1:500, mouse, Sigma Chemical), rhodopsin [rho 1D4, 1:50, mouse, gift from Robert S. Molday (14)], S-antigen [arrestin, A9C6, 1:10,000, mouse, gift from Larry A. Donoso (9)], recoverin [1:200, rabbit, gift from T. Müller (15)], protein kinase C (α isoform, 1:50, mouse, Amersham Pharmacia Biotech, Piscataway, NJ), calbindin (1:1,000, rabbit, Calbiochem, San Diego, CA), calretinin (1:500, rabbit, Chemicon, Temecula, CA), glial fibrillary acidic protein (GFAP, 1:10,000, mouse, Sigma Chemical), and galactocerebroside (GalC, 1:50, rabbit, Advanced Immunochemical, Long Beach, CA). Briefly, the cultured cells and tissue sections were incubated in 20% goat normal serum in PBS containing 0.25% Triton X-100 (PBST) for 30 min at room temperature followed by incubation with primary

antibodies for 20 h at 4°C. Cells and sections were then washed with PBST and incubated with secondary antibodies conjugated with the fluorescent dyes Alexa Fluor-488, or 546 (Molecular Probes, Eugene, OR) overnight at 4°C. To enhance BrdU detection, the fixed cells were incubated first with 2 N HCl for 10 min at room temperature followed by PBST washing. Normal human and rat retinas were used as positive and negative controls for each immunostaining procedure. The immunostained cells were analyzed on a Nikon Eclipse TE300 inverted microscope with an Optronix 3-chip cooled CCD camera connected to a Power Macintosh 9600 computer that was equipped with a Scion CG7 frame grabber and NIH Image software. The numbers of positive cells versus all cells in a respective field were recorded. At least 200 cells from each slide were counted.

The proliferating cells in the human fetal retinas at the 13th week of gestation were localized in the outer neuroblastic layer and immunoreactive for the proliferating cell marker Ki-67 (Fig. 1A). A central area of neural retina exhibited no immunoreactivity (Fig. 1A). This finding indicates that the majority of neuroblastic cells in the fetal retina are dividing cells and the remaining population, notably in the area around the posterior pole, represents differentiated cells. Therefore, HRPCs are proliferating before or around the 13th week of gestation. In addition, diffuse, Ki-67-positive cells were found in the ciliary margin, the optic nerve, the choroid tissue, and the hyaloid artery (Fig. 1A), suggesting there are precursor cells proliferating in these areas.

The P0 cells in the nonadherent culture divided 1–2 days after initial plating and formed neurospheres (Fig. 1B). The HRPCs in these neurospheres were immunopositive for BrdU (Fig. 1B, insert), a marker for proliferating cells, indicating that the increase in the size of the spheres is associated with cell division. However, the cells from the P0 spheres survived poorly after initial passage. Therefore, further experimentation was not attempted in the nonadherent culture. As an alternative culturing method, growth as adherent cells was examined. Under these conditions, the cells kept dividing after plating. They appeared phase bright, with or without a few short processes (Fig. 1C). A few cells were polygonal in shape, with a large flat body of cytoplasm, consistent with that of glial cells (Fig. 1C, arrows). Many of these large cells attached to the bottom of the plates and the small, phase bright cells often grew on top of these cells. They kept proliferating for at least eight passages in 3 months (the latest tested). The early passages (P1–P3) were stable with respect to their morphologies and immunophenotypes (see below), while cells from later passages tended to grow more slowly, with a greater variation in the proportion of cells expressing GFAP (data not shown). As revealed by immunostaining, the vast ma-

FIG. 1. (A) Low power photograph of the eye cup immunostained for Ki-67 at the 13th week of gestation. The main population of Ki-67-positive cells is located in the outer neuroblastic layer (NB), with others found in the optic nerve (ON), the hyaloid artery (HA), and the ciliary margin (CM). At this stage of development, the central area of the neural retina (CR) has started to differentiate and no longer contains a population of dividing cells. (B) A P0 neurosphere at day 4 after the first plating. The insert shows that few cells in the neurosphere are immunopositive for BrdU after overnight exposure, suggesting that the constituent cells in the sphere are slowly dividing. (C) Phase photomicrograph showing P1 HRPCs at day 2–3 after plating. Many HRPCs grow on top of glial cells (arrows). (D) More than 90% of the P1 HRPCs are BrdU-positive ($92.3 \pm 4.6\%$, mean $\pm$ SEM of nine independent experiments) after 5-day exposure. (E) The majority of HRPCs in the proliferating culture are undifferentiated, expressing the neural precursor marker nestin ($84.0 \pm 12.5\%$). (F–I) After 10–20 days of differentiation, many HRPCs differentiate into neurons, immunopositive for β III tubulin (F, $74.7 \pm 6.2\%$), NSE (G, $56.9 \pm 11.2\%$), and synaptophysin (H and insert, $41.1 \pm 3.6\%$), and GFAP-positive Müller cells (I, $11.8 \pm 7.2\%$). Many of the neurons have cell processes (F, H and insert). Magnification bars: A, 1 mm; B–I, 100 μ m.

majority of HRPCs in the adherent culture were BrdU-positive ($92.3 \pm 4.6\%$, Fig. 1D), indicating that over the 5-day period when BrdU was included in the media, the majority of the cells had divided at least once. Most of the BrdU-positive HRPCs expressed nestin ($84.0 \pm$

12.5% , Fig. 1E), an early marker for neural precursors, but not more mature neuronal or glial markers such as synaptophysin, NSE, or GFAP (data not shown), suggesting that they were indeed undifferentiated cells. Some of the undifferentiated cells did show a spindle-

FIG. 2. P1 HRPCs express the retinal cell-specific markers: rhodopsin (A, $6.7 \pm 4.6\%$) and recoverin (B, $23.1 \pm 11.7\%$) for retinal photoreceptor cells, PKC (C, $2.6 \pm 1.5\%$) for retinal bipolar and amacrine cells, and calbindin (D, $33.6 \pm 9.8\%$) for retinal horizontal interneurons and other CNS neurons. Magnification bars, 100 μm .

shaped morphology, but were distinct from the differentiated GFAP-positive glial cells, which were usually much larger in size. These results indicate that the HRPCs cultured under the present condition are self-renewing, a critical property that all neural stem and progenitor cells share.

After differentiation for 10–20 days in the absence of mitogens, the majority of cells stopped dividing and grew larger in size, with several long cellular processes. The differentiated cells from independent experiments showed consistent morphological features. A few cells in the differentiation culture remained undifferentiated, proliferating slowly and forming cellular aggregates. Most of the differentiated cells did not express the early neural precursor marker nestin (data not shown). Instead, they were immunoreactive for the early neuronal marker β III tubulin ($74.7 \pm 6.2\%$, Fig. 1F) and the more mature neuronal markers NSE ($56.9 \pm 11.2\%$, Fig. 1G) and synaptophysin ($41.1 \pm 3.6\%$, Fig. 1H). A small number of cells developed into GFAP-positive glial cells ($11.8 \pm 7.2\%$, Fig. 1I). In view of the fact that neurons and Müller glial cells share a common progenitor cell in the retina (32), it is likely the GFAP-positive cells are Müller cells, but not astrocytes. The latter migrate into the retina only after birth in rodents (5, 16) and in later gestational stages (20 wks) in humans (28). No cells immunoreactive with anti-GalC were detected, suggesting an inability of these HRPCs to differentiate along the oligodendrocytic lineage. Taken together with the facts that oligo-

dendrocytes are not an intrinsic component of the neural retina and that *in vitro* neural stem cells from the ciliary margin of rodent eyes also do not differentiate into oligodendrocytes (31), we propose that human retinal progenitor cells are intrinsically programmed, at least at the time point of isolation (10th–13th weeks of gestation), to develop exclusively into retinal neurons and Müller cells.

To confirm whether these HRPCs are restricted to a retinal cell lineage, the differentiated HRPCs were stained with a panel of retinal cell-specific antibodies. A subset of the cells expressed rhodopsin ($6.7 \pm 4.6\%$, Fig. 2A), a marker for rod photoreceptors, S-antigen ($3.2 \pm 2.0\%$), a marker for rod and blue cone photoreceptors, and recoverin ($23.1 \pm 11.7\%$, Fig. 2B), a marker for photoreceptors and cone bipolar cells. A few cells were immunopositive for the bipolar and amacrine cell marker PKC ($2.6 \pm 1.5\%$, Fig. 2C) and about one third of HRPCs were positive for calbindin ($33.6 \pm 9.8\%$, Fig. 2D), a marker for retinal horizontal interneurons, ganglion cells and other CNS neurons. Immunoreactivity for calretinin, a marker for retinal amacrine interneurons, was found in only a few cells ($0.7 \pm 0.5\%$). It is of interest to note that, although varying populations of the HRPCs that were differentiated in culture for 10–20 days were immunoreactive with markers of mature retinal cells, these HRPCs did not present the retinal cell morphologies that develop *in situ*. For example, we did not observe the outer segment formation in the cells expressing photoreceptor

markers. Some retinal interneurons developed *in vitro* and did show multiple processes, but varied greatly in size.

The various neural differentiations observed in the present study suggest that the passaged HRPCs have potential to develop *in vitro* along multiple retinal cell lineages. In the rodent eye, neural stem and progenitor cells have been found in the ciliary margin (31) and the neural retina (1, 35). Like the human retinal progenitors described here, the rodent retinal progenitor cells from others' (1, 31) and our data (35) show varying degrees of differentiation *in vitro* into retina-specific cell types. These disparate results likely reflect differences in culture conditions and passage number. Importantly, all data indicate that cell populations differentiated *in vitro* differ markedly from the phenotypes and ratios of retinal cells observed *in vivo*. Rat retinal cells are derived clonally from a common progenitor cell and develop in a sequentially restricted fashion (7), likely involving selective cell death and responses to specific epigenetic signals that appear in a precise temporal and spatial sequence. Therefore, not surprisingly, the precise temporal and spatial differentiation seen *in situ* was not observed *in vitro*.

The HRPCs cultured under the present condition were responsive to FGF2. The absence of FGF2 and presence of FBS in the medium lead to HRPC differentiation (data not shown). The latter finding was in contrast to a previous study that found continuous proliferation of fetal retinal cells in the absence of FGF2 and presence of FBS (18). FGF2 has been used extensively as an exogenous mitogen to promote *in vitro* propagation of neural stem or progenitor cells. The FGF2-independent proliferation might be related to the earlier developmental stages of embryos used in the previous study. It is not clear when the fate of these passaged HRPCs is restricted and to what extent they retain their lineage potential. Unlike the retinal precursors of the rat (35), the HRPCs display a much lower tendency to differentiate *in vitro* with retinal cell-specific phenotypes in the time frame of 10–20 days tested. Most likely, the culture conditions lack the appropriate soluble and cell surface signals to direct that differentiation. As demonstrated previously, retinoid acid and thyroid hormone have roles in promoting rod and cone photoreceptor development *in vitro* (18). The extensive neuronal phenotypes observed in the differentiated HRPC populations indicate the potential of these cells to develop in response to exogenous stimuli. As we better understand the nature of the intrinsic signals that regulate retinal development *in vivo*, it is likely that the phenotypic fate of these cells can be specifically controlled after engraftment back into the injured retina. The availability of expanded populations of HRPCs may have important clinical significance in using these cells to save or even restore function in many human retina-related diseases.

ACKNOWLEDGMENTS

This work was supported by an Anonymous Sponsor, Kentucky Lions Eye Foundation, Research to Prevent Blindness (M.J.S. and R.B.A.), and Norton Healthcare, the Commonwealth of Kentucky Research Challenge Trust Fund, RR1557, and NS38665 (S.R.W.). The authors thank Russell Howard, Marija Sasek, Miranda Messer, and Betty Flood for their technical assistance.

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