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Authors

Yang, Peng
Seiler, Magdalene J
Aramant, Robert B
et al.

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Differential Lineage Restriction of Rat Retinal Progenitor Cells in Vitro and in Vivo

Peng Yang,¹ Magdalene J. Seiler,^{1,2*} Robert B. Aramant,^{1,2} and Scott R. Whittemore^{2–4}

¹Department of Ophthalmology and Visual Sciences, University of Louisville, Louisville, Kentucky

²Department of Anatomical Sciences and Neurobiology, University of Louisville, Louisville, Kentucky

³Department of Neurological Surgery, University of Louisville, Louisville, Kentucky

⁴Kentucky Spinal Cord Injury Research Center, University of Louisville, Louisville, Kentucky

To identify and characterize the lineage potential of rat neural retina progenitor cells (NRPCs) in vitro and engrafted into rats with retinal degeneration, NRPCs were isolated from neural retinas of embryonic day 17 Long Evans rats and cultured in serum-free or serum-containing media with fibroblast growth factor 2 and neurotrophin 3. After expansion, cellular differentiation was initiated by the withdrawal of these growth factors. Despite forming primary neurospheres, NRPCs cultured in serum-free medium survived poorly after passage. In contrast, NRPCs cultured in serum-containing medium could be expanded for up to 12 passages and differentiated into glial fibrillary acidic protein-positive glial cells and retina-specific neurons expressing rhodopsin, S-antigen, calbindin, recoverin, and calretinin. For in vivo analysis, passage 1 (P1) undifferentiated NRPCs were labeled with bromodeoxyuridine (BrdU), implanted into the subretinal space of Royal College of Surgeons (RCS) rats, and analyzed immunohistochemically 4 weeks postgrafting. The grafted NRPCs showed extensive glial differentiation, irrespective of their topographic localization. A few BrdU-labeled grafted NRPCs expressed protein kinase C, a marker for bipolar and amacrine interneuron-specific differentiation. Other retina-specific or oligodendrocytic differentiation was not detected in the grafted cells. Although NRPCs are capable of self-renewal and multilineage differentiation in vitro, they developed mostly into glial cells following engraftment into the adult retina. These data suggest that the adult retina retains epigenetic signals that are either restrictive for neuronal differentiation or instructive for glial differentiation. Induction of lineage-specific cell differentiation of engrafted NRPCs to facilitate retinal repair will likely require initiation of specific differentiation in vitro prior to grafting and/or modification of the host environment concomitantly with NRPC grafting. © 2002 Wiley-Liss, Inc.

Key words: retinal progenitor cell; photoreceptor; bipolar cell; transplantation

The mammalian neural retina is composed of six classes of distinct neurons: cone and rod photoreceptors;

horizontal, bipolar, amacrine, and ganglion cells; and Müller glial cells and astrocytes. Retinal neuronal differentiation occurs in late embryonic development and early postnatal life in the rat (Cepko et al., 1996). Similarly to neurons in other CNS and PNS locations, retinal neurons retain no intrinsic capability of self-regeneration in response to inherited or acquired damage. Therefore, extrinsic cells and tissues with varying degrees of differentiation, such as retinal pieces (Turner and Blair, 1986), photoreceptor cells (Silverman and Hughes, 1989; Kaplan et al., 1997), and tissue sheets (Seiler and Aramant, 1998; Aramant et al., 1999; Woch et al., 2001), introduced through surgical transplantation are the necessary sources for replacing damaged retinal neurons.

Another approach to replacing degenerated neurons is to use neural stem and progenitor cells, which are intrinsic to the hippocampus (Altman and Das, 1965; Kaplan and Hinds, 1977; Bayer et al., 1982; Kuhn et al., 1996), subventricular zone (Lewis, 1968; Reynolds and Weiss, 1992; Lois and Alvarez-Buylla, 1993; Morshead et al., 1994), spinal cord (Weiss et al., 1996; Kalyani et al., 1997), retinal pigmented ciliary margin (Ahmad et al., 2000; Tropepe et al., 2000), and neural retina (Ahmad et al., 1999) of the CNS. CNS neural stem and progenitor cells are undifferentiated, expressing the neural precursor intermediate filament nestin (Hockfield and McKay, 1985; Cattaneo and McKay, 1990). After differentiation in vitro or in vivo, neural stem and progenitor cells can develop along neuronal, astrocytic, and oligodendrocytic pathways (Whittemore and Snyder, 1996; McKay, 1997; Gage, 2000). Moreover, stem cells can transdifferentiate when grafted to ectopic sites (Bjornson et al., 1999; Clarke et al., 2000).

*Correspondence to: Magdalene J. Seiler, PhD, Department of Ophthalmology and Visual Sciences, University of Louisville School of Medicine, 301 East Muhammad Ali Boulevard, Louisville, KY 40202.

E-mail: magdalene.seiler@louisville.edu

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Differentiation of in vitro expanded neural stem and progenitor cells into cells expressing retinal cell phenotypes either has not been investigated or has been found only in cells isolated from the eye (Ahmad et al., 1999; Tropepe et al., 2000; Yang et al., 2002), suggesting that there are intrinsic and/or extrinsic limitations affecting retinal cell differential fate. The present data show that rat neural retina progenitor cells (NRPCs) develop into retinal neurons in vitro, expressing the retinal cell-specific antigens rhodopsin and S-antigen, whereas the undifferentiated NRPCs transplanted into the subretinal space of rats with retinal degeneration did not show corresponding differentiation. Engrafted NRPCs adopted mostly a glial fate and occasionally a bipolar/amacrine cell phenotype.

MATERIALS AND METHODS

NRPC Isolation

Embryonic day (E) 17 fetuses ($n = 17$) were harvested from timed-pregnant, outbred Long Evans rats (Harlan Sprague Dawley, Indianapolis, IN). All animals were treated according to the *Guidelines for the Use of Animals in Neuroscience Research* and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, with the approval of the University of Louisville Institutional Animal Care and Use Committee. The fetal eyes were collected in Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) and transferred to fresh HBSS in a 35-mm petri dish on ice. Neural retinas were separated free of vitreous, lens, retinal pigmented epithelium (RPE), and cornea. Special care was taken to exclude the optic disk and the ciliary margin by cutting off a circular 1.5-mm zone from the center of the optic nerve and the margin of the ciliary bodies. Neural retinas were digested in 0.05% trypsin (Invitrogen) in artificial cerebral spinal fluid [ACSF; 124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose, pH 7.35, aerated with 95% O_2 /5% CO_2 (Reynolds and Weiss, 1992)] for 10 min at room temperature and triturated gently with a fire-polished, siliconized Pasteur glass pipette to release single cells. The cell suspension was centrifuged at 150g for 5 min, and the resulting pellets were resuspended in fresh culture medium. Cell viability was evaluated with trypan blue, and the cell concentration was adjusted for plating.

Expansion and Passage

Isolated cells were cultured at 5×10^4 cells/cm² under two conditions: as a cell suspension in uncoated flasks (Ahmad et al., 1999) or as a monolayer on dishes precoated with 10 μ g/ml poly-L-ornithine overnight and 5 μ g/ml fibronectin (PO/FN; Invitrogen) for 5 hr at 37°C. For the suspension cultures, serum-free medium containing DMEM/F-12, N2 supplement, 10 ng/ml neurotrophin 3 (NT3; Regeneron Pharmaceuticals, Tarrytown, NY), and 10 ng/ml fibroblast growth factor 2 (FGF-2; Invitrogen) was used and cultured at 37°C in a 5% CO_2 incubator. FGF-2 was added every 2 days, but the medium was not changed. After 6–8 days in culture, primary neurospheres were collected and digested with or without 0.05% trypsin in ACSF for 5 min at room temperature. The spheres were then gently triturated to release single cells, which were plated at the same density and identically cultured. To establish the extent

and efficiency with which the sphere-forming cells retained the ability to form secondary (P1) spheres, the numbers of P0 and P1 spheres at day 6 after plating were counted.

For the adherent cultures, the media included 1% fetal bovine serum (FBS; Invitrogen) in addition to the components in the serum-free medium. In pilot studies (data not shown), many different variations of serum-free medium were tried. However, the cells did not grow well and could not be passaged. Therefore, 1% FBS was included in all experiments described in this paper. In addition, FGF-2 was tested in combination with platelet-derived growth factor (PDGF; 10 ng/ml; Caldwell et al., 2001). No difference in cell proliferation and cell phenotypes after differentiation was observed (data not shown). Therefore, PDGF was not included in the experiments in this study. Half the culture medium was changed every 2 days and FGF-2 added every other day. Cells grew to 70–80% confluence in 7–14 days and were harvested with a cell lifter (Fisher Scientific, Fair Lawn, NJ). P1 and later passages of cells were plated at the same density as the P0 cells in the PO/FN-coated dishes.

For immunocytochemical identification of dividing cells from proliferating cultures, 10 μ M bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) was added either overnight or 5 days immediately before the day when induction of differentiation began or termination of the cultures. The cells used for transplantation were expanded in expansion cultures and pulsed with 10 μ M BrdU for 5 days immediately before the day of transplantation. Before each transplantation experiment, the percentages of nestin-positive (undifferentiated) and GFAP-positive (differentiated) immunophenotypes of the cells to be used were examined to ensure that the cells were mostly undifferentiated.

In Vitro Differentiation

Expanded cells were harvested and replated on PO/FN-coated 12-well dishes (Costar Corning Inc, Corning, NY) at a density of 10^5 cells/cm². Cells were induced to differentiate by replacing the FGF-2 and NT3 with 10% FBS for 5–21 days, with one or two medium changes. No attempt was made to differentiate the cells under serum-free conditions. To test the effect of Matrigel on the cells, cells were grown on Matrigel-coated dishes (growth factor-reduced and laminin- and collagen IV-based Matrigel matrix; 1:20; Becton Dickinson Labware, Bedford, MA). After differentiation, no difference in phenotypes was observed in P1 and P2 cells (data not shown).

Transplantation

Five inbred albino Royal College of Surgeons (RCS) rats aged 29–57 days, derived from our in-house colony (originally obtained from D. Organicziak, Wayne State University, Dayton, OH), were used as recipients of transplants; rats at these ages show advanced neural retina degeneration (LaVail, 1981). Because the donor cells were derived from outbred Long Evans rats, the transplanted cells were allografts, mismatched in MHC antigens. Implantation was performed unilaterally in the subretinal space through an incision behind the pars plana. A volume of 0.3–0.5 μ l cell suspension containing 10^4 – 10^5 BrdU-prelabeled, undifferentiated P1 cells was embedded in growth factor-reduced Matrigel matrix (Becton Dickinson Labware) prior to grafting for a better attachment to the host retina. Expanded cells were transplanted, using a custom-made implan-

tation tool (Seiler and Aramant, 1998; Aramant et al., 1999), into the subretinal space in the superior quadrant close to the optic disc. The engrafted rats were sacrificed by injection with sodium pentobarbital (300 mg/kg; Abbott Laboratories, North Chicago, IL) 4 weeks after surgery and perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. After washing with 0.1 M sodium phosphate buffer, grafted eye cups were immersed in 30% sucrose in 0.1 N phosphate buffer overnight and embedded in OCT compound on dry ice for cryostat sectioning.

Immunocytochemistry

Undifferentiated and differentiated P1 cells ($n = 6$ independent experiments), cells from expansion cultures (Table I), and retinas with grafts ($n = 5$ experiments) were immunocytochemically analyzed. Primary antibodies included anti-BrdU (1:400; sheep; Biotest International, Saco, ME), nestin (Rat-401; 1:50; mouse; DSHB, Iowa City, IA), β III tubulin (1:200; mouse; ICN Pharmaceuticals, Costa Mesa, CA), glial fibrillary acidic protein (GFAP; 1:10,000; mouse; Sigma), galactocerebroside (GalC; 1:50; rabbit; Advanced Immunochemical, Long Beach, CA), neuron-specific enolase (NSE; 1:10,000; rabbit; Dako, Burlingame, CA), synaptophysin (1:500; mouse; Sigma), calbindin (1:1,000; rabbit; Calbiochem, San Diego, CA), recoverin [1:200; rabbit; a gift from T. Müller (Hisatomi et al., 1999) or from J.F. McGinnis (McGinnis et al., 1999)], calretinin (1:500; rabbit; Chemicon, Temecula, CA), S-antigen [arrestin; A9C6; 1:10,000; mouse; a gift from Larry A. Donoso (Donoso et al., 1987)], rhodopsin [rho 1D4; 1:50; mouse; a gift from Robert S. Molday (Hicks and Molday, 1986)], and protein kinase C (PKC) α (1:50; mouse; Amersham Pharmacia Biotech, Piscataway, NJ). Briefly, cultured cells and tissue sections were incubated in 20% appropriate normal serum in phosphate-buffered saline (PBS) containing 0.25% Triton X-100 (PBST) for 30 minutes at room temperature, followed by incubation in primary antibodies for 20 hours at 4°C. Cells and sections were then washed with PBST and incubated with secondary antibodies conjugated with the fluorescent dyes Alexa Fluor-350 or -488 or rhodamine X (Molecular Probes, Eugene, OR) for 1–2 hr at room temperature. Diamidinophenylindole dihydrochloride (DAPI; Molecular Probes) was used to counterstain nuclei. For BrdU detection, cells and sections were treated at room temperature before immunostaining with 2 N HCl for 10 and 20 minutes, respectively. For double and triple staining, normal horse serum was used to block nonspecific background binding. Cells/tissues were incubated in a mixture of primary antibodies, washed, and incubated sequentially with secondary antibodies. Tissues known to express or lack a specific antigen were used as positive and negative controls.

Data Quantification

Images from the immunostained cultured and grafted cells as well as host tissues were captured on a Nikon Eclipse TE300 inverted microscope and an Optronix three-chip cooled CCD camera connected to a Power Macintosh 9600 computer equipped with a Scion CG7 frame grabber and NIH Image software. Quantification was achieved by recording the numbers of positive cells vs. all cells in a field. At least 200 cells for each condition were counted. For the *in vivo* specimens, BrdU-

positive cells were identified and recorded in terms of their topographic localization and antigenic expression. Selected grafted tissues were examined further with a laser scanning confocal microscope (Zeiss 510; Carl Zeiss) to confirm that BrdU-labeled cells were simultaneously immunopositive for different markers. Original images were edited with Adobe Photoshop and final figures assembled with Adobe Illustrator (Adobe, San Jose, CA).

RESULTS

NRPCs in Primary Neurospheres

NRPCs began to divide 1 day after plating and formed small neurospheres (Fig. 1A) in serum-free media and uncoated flasks. After 8–10 days in culture, the number of spheres had increased, and most had become larger (Fig. 1B). When spheres grew for longer than 10 days, they began to die. Overnight incubation with BrdU revealed that small numbers of cells (fewer than 20%) in the spheres were dividing (Fig. 1C, inset), indicating that the spheres were formed by slowly dividing cells and not simply an aggregation of previously dissociated cells. By day 6, more than 90% of cells in the spheres expressed nestin (Fig. 1C), an intermediate filament found exclusively in undifferentiated neural precursor cells (Hockfield and McKay, 1985). Other markers for more mature phenotypes, including β III tubulin, NSE, GFAP, GalC, PKC, rhodopsin, S-antigen, calbindin, and calretinin, were not detected (data not shown).

Poor Survival of Secondary Neurospheres

If P1 cells were cultured under the same conditions as primary (P0) cells, most cells died shortly after plating, and few survived to form secondary spheres. Repeated trials (five independent experiments) demonstrated that, at day 6 after passage, the number of P1 neurospheres was on average only 3 (range 0–6) per 10 embryonic eyes (that were used to start the initial culture experiment), significantly fewer than the average of 18 spheres (range 14–25) per 10 embryonic eyes of the P0 cells (Fig. 1D), indicating that the NRPCs were incapable of division under these culture conditions. Furthermore, the P1 spheres appeared smaller and looser, which was unlike the healthy, densely packed appearance of the primary spheres (Fig. 1A,B). After 5 days in culture, most secondary spheres died, which is consistent with one previous result (Ahmad et al., 1999). Attempts to culture P2 cells from P1 spheres were unsuccessful. Because of the rare formation of P1 neurospheres, it was impossible to obtain sufficient numbers of cells for *in vivo* analysis.

NRPC Expansion in Adherent Cultures

Table I summarizes the properties of NRPCs grown as adherent cultures. Because of the difficulty in passaging P0 neurospheres under the initial culture conditions, we attempted to culture cells in media containing FGF-2 and 1% FBS on PO/FN-coated dishes. FBS is known to be important for cell survival and differentiation (Ye and Sontheimer, 1998). The NRPCs cultured under this con-

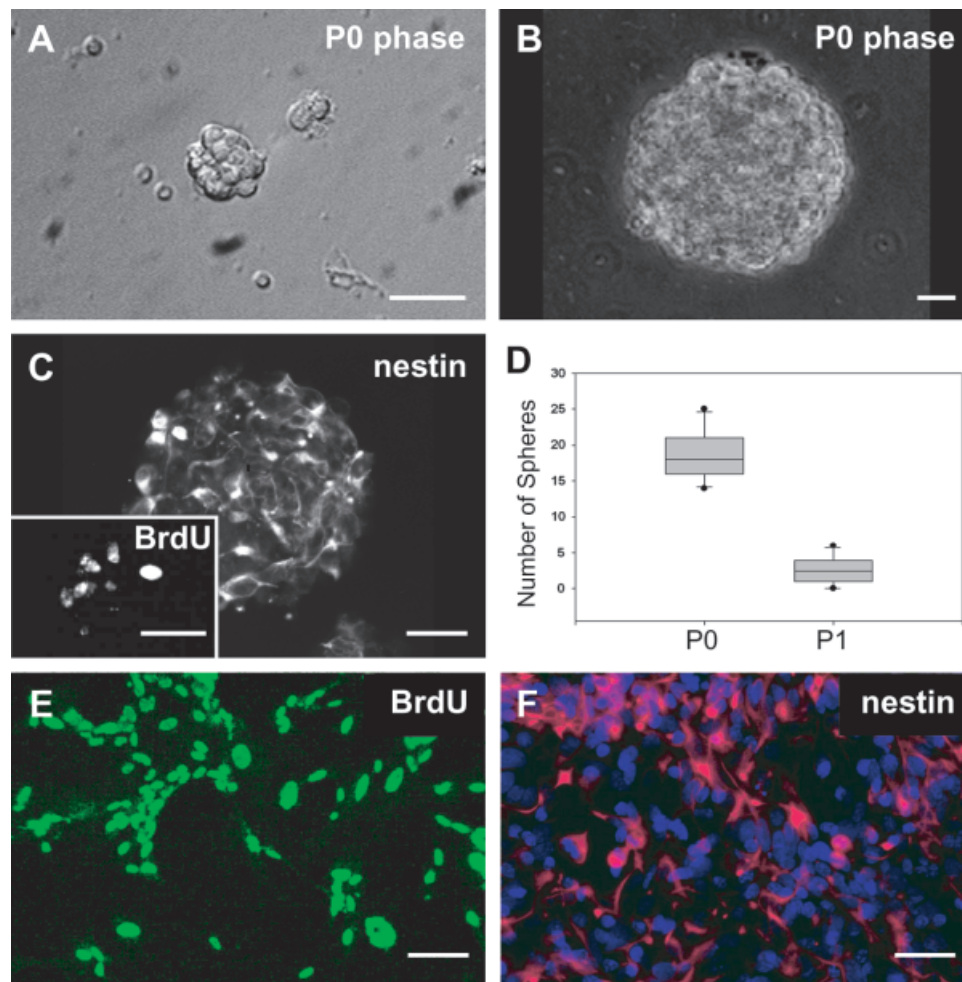


Fig. 1. Neurospheres cultured with serum-free medium containing FGF-2 in uncoated flasks. **A:** A small P0 sphere and a doublet of dividing cells at day 2 after plating. **B:** A larger P0 sphere at day 6 after plating. **C:** The majority of cells in the proliferating neurospheres expressed nestin, an intermediate filament characteristically present in undifferentiated neural precursor cells. **Inset:** After incubation with 10 μ M BrdU overnight, small numbers of dividing cells in the proliferating P0 sphere were positive for BrdU. **D:** Expansion of proliferating NRPCs. The number of primary (P0) spheres at day 6 after initial

passage was 14–25 per 10 embryonic eyes, whereas only 0–6 P1 spheres were formed at the same time point after passaging. Data represent the mean $\pm$ SEM of six independent experiments. **E,F:** Proliferating NRPCs cultured in medium containing FGF-2 and 1% FBS on PO/FN-coated dishes. After a 5-day exposure to 10 μ M BrdU, most P1 cells were shown to have divided over that time frame, being immunopositive for BrdU (**E**), and expressed nestin (**F**). In **E**, the nuclei are counterstained blue with DAPI. Scale bars = 100 μ m in A–C; 50 μ m in E,F.

dition continued to divide for as long as P12 (the latest passage tested). The time that these cells took to grow to near confluence (days to passage) varied greatly. Early cells tended to proliferate more quickly than cells from later passages. Although a small proportion of the cells from each passage expressed the differentiated cell marker GFAP, most were undifferentiated and immunopositive for nestin. Although the percentage of nestin-positive cells in the expansion cultures remained relatively constant over the first six passages (73–90%), by P12 this had dropped to <70% (Table I). However, the numbers of late-passage preparations were small, limiting our ability to draw statistically meaningful conclusions. Nevertheless, these data

indicated that the expanded cells we used in transplantation and in vitro differentiation analyses were mostly undifferentiated. Approximately 90% of the P1 cells at day 6 had undergone cell division (Fig. 1E) over the previous 5 days when BrdU was pulsed in the culture media and remained undifferentiated as suggested by nestin immunoreactivity (82%; Fig. 1F). The proliferating P1 cells typically had limited cytoplasm, with no or few cell processes. It was not uncommon to observe aggregates of small, phase-bright cells, with no cell processes. In contrast, the few differentiated cells displayed a larger cytoplasm, with more cell processes, and an irregular cell shape (data not shown).

TABLE I. Properties of Proliferating NRPCs In Vitro*

Passage (n)	Days to passage	-Fold increase ^b	Viability	Percentage nestin (n)	Percentage GFAP (n)
P0 (17)			82.7 ± 11.4	83.9 ± 11.8(6)	9.2 ± 6.3(6)
P1 (17)	7.2 ± 0.6	1.1 ± 0.7	85.2 ± 6.8	82.2 ± 4.7 (6)	10.2 ± 4.5(6)
P2 (12) ^a	7.6 ± 1.3	2.0 ± 0.3	84.9 ± 9.8	86.2 ± 8.1 (6)	9.6 ± 8.1(3)
P3 (9) ^a	9.1 ± 2.9	1.8 ± 0.5	81.3 ± 7.0	75.1 ± 11.6(3)	NT
P4 (9)	8.5 ± 1.7	2.2 ± 1.2	84.5 ± 3.2	NT	NT
P5 (8) ^a	14.2 ± 5.0	0.7 ± 0.2	87.4 ± 6.3	NT	NT
P6 (6) ^a	13.5 ± 2.8	3.2 ± 0.6	82.7 ± 11.2	72.5 ± 9.1 (3)	NT
P7 (5) ^a	14.7 ± 5.9	0.9 ± 0.2	78.3 ± 6.2	NT	NT
P8 (4) ^a	9.8 ± 2.1	2.3 ± 1.2	85.9 ± 4.7	NT	NT
P9 (3)	12.7 ± 2.6	3.1 ± 0.7	83.5 ± 9.6	NT	NT
P10 (3)	22.2 ± 6.8	1.5 ± 0.4	74.6 ± 1.8	NT	NT
P11 (3)	28.6 ± 9.4	1.5 ± 0.9	76.9 ± 12.3	68.2 ± 3.8 (2)	NT
P12 (1) ^a	32	1.5	64.1	62.5 (1)	47.3 (1)

*NRPCs were grown in the presence of FGF-2 and NT3 and passaged at about 80% confluence. The numeric data represent the mean ± SEM. The number of independent preparations (n) for a passage or nestin or GFAP staining is indicated in the parentheses. NT, not tested.

^aNumber of successfully passaged preparations after excluding the preparations used in immunocytochemical staining and/or grafting studies (some preparations from P5, P7, and P8 were used for immunostaining for markers other than nestin and GFAP; data not shown).

^bIncrease compared to previous passage.

NRPC Differentiation In Vitro

NRPC differentiation was induced in vitro by withdrawal of FGF-2 and NT3 and addition of 10% FBS. The differentiated cells from the P1 passage appeared larger, with more cell processes. Many cells had a small soma and several long, thin cell processes, often connected to each other. Small numbers of cells appeared polygonal and were very large. Nearly 90% of cells differentiated into neurons, expressing the early neuronal marker β III tubulin (Figs. 2A, 3) and 75% and 30% expressed the more mature neuronal markers NSE (Figs. 2B, 3) and synaptophysin (Figs. 2C, 3), respectively. Triple immunostaining demonstrated that most cells were positive for BrdU (red), about 10% for GFAP (green), and 0% for PKC (blue; Figs. 2D, 3). As the passage number increased, more cells tended to have a GFAP-positive phenotype (Table I). Oligodendrocytic differentiation was not detected. Interestingly, many differentiated neurons also expressed photoreceptor-specific markers; 60% expressed rhodopsin (Figs. 2E, 3) and 40% S-antigen (Figs. 2F, 3). A few percent of the cells were recoverin positive (Figs. 2G, 3) and calretinin positive (Fig. 3). Forty-three percent of the cells expressed calbindin (Figs. 2H, 3), a marker expressed on retinal horizontal cells as well as other restricted populations of CNS neurons.

NRPC Differentiation In Vivo

Many grafted cells survived in the host retinas but showed no cellular morphology similar to that of the surrounding endogenous retinal cells. Small numbers of NRPCs implanted into the subretinal space migrated into various layers of the host neural retina (Fig. 4A). These BrdU-positive cells expressed GFAP, both in the outer neural retina (Fig. 4B) and in the subretinal space (Fig. 4C). Small numbers of BrdU-positive NRPCs also ex-

pressed PKC in the subretinal space (Fig. 4D), the outer neural retina (Fig. 4E), and the inner neural retina (Fig. 4F). The topographic localization of these cells did not correspond to the normal cellular layer of endogenous PKC expression, the inner nuclear layer of the host retina, where the bipolar cells reside. No nestin-positive, graft-derived cells were found, indicating that the cells did not differentiate from NRPCs. Immunostaining with other antibodies (β III tubulin, NSE, synaptophysin, rhodopsin, S-antigen, calbindin, recoverin, and calretinin) did not reveal immunopositive engrafted cells in the neural retina and subretinal space (data not shown). The overall differentiated phenotype of the engrafted NRPCs did not correlate with that of NRPC differentiation in vitro, with respect to the expression of proteins reflective of more mature retinal cell lineages. Moreover, no specific organization of the grafted cells was observed. The morphologies of the recipient retinas were maintained after transplantation, except that mild infiltration of inflammatory cells, mainly plasma cells, was seen occasionally (in two of five experiments) in the subretinal space (data not shown).

DISCUSSION

Proliferation and Passaging of NRPCs

In vitro, the E17 neural retina-derived NRPCs proliferated for at least 12 passages and differentiated into neurons (87%) and glial cells (7%). Later passages differentiated into a decreased number of neurons (data not shown) and an increased number of glial cells (Table I). Before differentiation, most of the NRPCs expressed only the neural precursor cell marker nestin. However, a minor proportion of cells in the proliferating culture did express the more differentiated astrocytic marker GFAP, likely because of the presence of FBS. On the other hand, the earliest stem cell located in the CNS has been postulated to

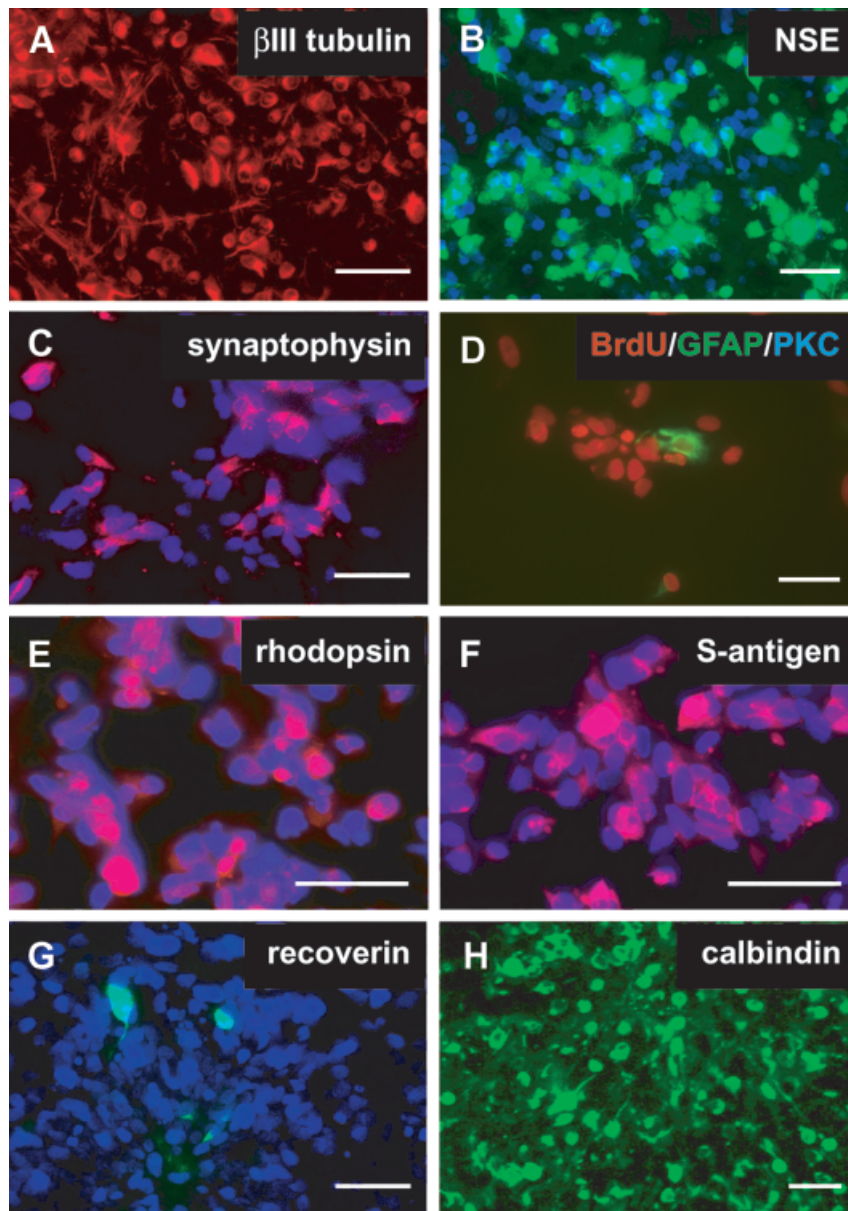


Fig. 2. Immunophenotypic profile of NRPCs differentiated in medium containing 10% FBS. Most cells differentiated into neurons immunopositive for β III tubulin (A), NSE (B), and/or synaptophysin (C). Triple immunostaining demonstrated that most of the cells were divided cells immunopositive for BrdU (red; D), about 10% for GFAP (green; D), and 0% for PKC (blue; D). Oligodendrocytic differentiation

was not detected. A photoreceptor phenotype was suggested by rhodopsin (E), S-antigen (F), and recoverin (G) expression. Many cells expressed calbindin (H), a marker for retinal horizontal cells as well as other restricted populations of CNS neurons. Except for D, blue nuclear stains in B,C,E–G represent DAPI counterstain. Scale bars = 50 μ m.

be GFAP-positive (Doetsch et al., 1999), raising the possibility that these cells are also precursors. Most of the NRPCs (90%) were dividing precursors, incorporated BrdU, and did not express markers for differentiated cells. The possibility cannot be completely excluded that at least some of these proliferating BrdU-labeled cells were reprogrammed from a differentiated to an undifferentiated dividing state in the presence of FGF-2. Oligodendrocyte precursor cells differentiate to type 2 astrocytes in the

presence of 15% FBS, but they can be completely reprogrammed to undifferentiated cells in the presence of FGF-2 and are capable of differentiating later into neurons, type 1 astrocytes, and oligodendrocytes (Kondo and Raff, 2000).

Addition of serum to the suspension culture did not increase the quantity or the passaging efficiency of the spheres (data not shown), indicating that serum is not essential for the NRPCs growing in suspension culture. In

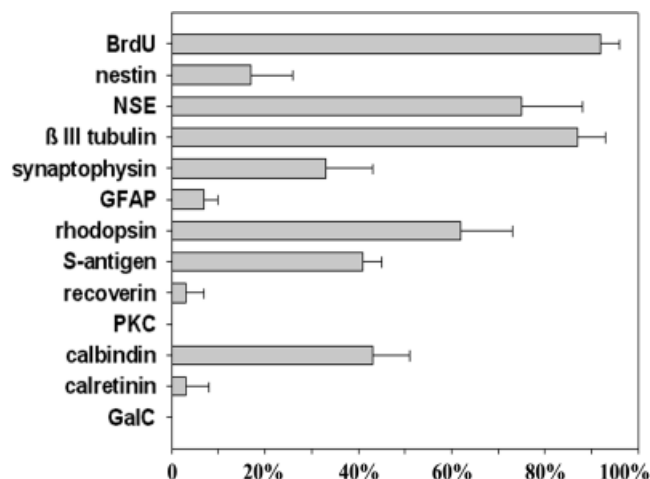


Fig. 3. Quantification of NRPC differentiation in vitro. After withdrawal of mitogens and differentiation with 10% serum for 5–21 days, the NRPCs developed mostly into neurons, expressing not only general neuronal markers, such as NSE ($75\% \pm 13\%$), synaptophysin ($33\% \pm 10\%$), and β III tubulin ($87\% \pm 6\%$), but also retinal cell-specific markers, such as the photoreceptor markers rhodopsin ($62\% \pm 11\%$), S-antigen ($41\% \pm 4\%$), and recoverin ($3\% \pm 4\%$); the retinal horizontal cell marker calbindin ($43\% \pm 8\%$); and the amacrine cell marker calretinin ($3\% \pm 5\%$). Positive immunoreactivity for the bipolar cell marker PKC and the oligodendrocytic marker GalC was not observed. Only a small proportion of NRPCs developed into glial cells, immunopositive for GFAP ($7\% \pm 3\%$). Moreover, reduced expression for nestin ($17\% \pm 9\%$) was seen. The number of dividing cells as assessed by BrdU incorporation was consistently high ($92\% \pm 4\%$). Data represent the mean $\pm$ SEM of six independent experiments.

contrast, addition of a low percentage of serum to the adherent cultures increased the survival and the passaging efficiency of the cells so that the NRPCs could be maintained for at least 6 months (P12, the latest passage tested). Thus, an adherent substrate is apparently important for the NRPCs to survive after passage, and serum enhances that activity. The mechanism for the improved efficiency of passaging NRPCs is not clear. It will be important to clarify this mechanism, because different combinations of substrate and/or mitogen had different effects on the growth (Adler et al., 1985) and lineage restriction (Whittemore et al., 1999) of neural stem cells.

NRPCs Do Not Differentiate Into Oligodendrocytes In Vitro

During dissection, a circular zone around the optic disk of the E17 neural retina was removed, to avoid contamination by oligodendrocyte precursors from the optic nerve (Watanabe and Raff, 1988). At this stage of development (rat E17), there are no differentiated oligodendrocytes in the optic nerve (Miller et al., 1985; Watanabe and Raff, 1988), and oligodendrocyte precursor cells are kept out of the retina by a barrier at the optic disk (Ffrench-Constant et al., 1988). Although our culture conditions did not favor oligodendrocyte differentiation (10% serum and absence of PDGF), it is likely that the

absence of the oligodendrocyte lineage reflects an intrinsic predetermination of the NRPCs at the time of isolation (E17). Oligodendrocytes and type 2 astrocytes arise from a common glial-restricted precursor, distinct from the neuronal lineage (Raff and Lillien, 1988; Rao and Mayer-Proschel, 1997). Primary cultures of mouse and rat retinal precursor cells have shown that these cells can develop both into neurons and into glia, but development of oligodendrocytes has not been reported (Jensen and Raff, 1997; Watanabe et al., 1997). Previous studies have shown that neural stem and precursor cells isolated from the subventricular zone (SVZ) and spinal cord developed into oligodendrocytes as well as neurons and astrocytes (Lois and Alvarez-Buylla, 1993; Gritti et al., 1996; Weiss et al., 1996). Consistently with present data, oligodendrocytes could not be obtained from populations of proliferating fetal human spinal cord precursor cells (Quinn et al., 1999) or neural differentiating human ES cell-derived cultures (Carpenter et al., 2002). The neural rat retina consists of neurons and Müller glial cells, with astrocytes migrating from the optic nerve after birth (Bussow, 1980; Watanabe and Raff, 1988; Huxlin et al., 1992), and no oligodendrocytes. Thus, the GFAP-expressing cells in differentiated cultures likely represented Müller cells. In contrast, a previous study (Ahmad et al., 1999) detected a minor population of oligodendrocytes (10%) among differentiated primary cultures of (P0) NRPCs derived from E17 rat retina. However, it was impossible to determine whether the oligodendrocytes arose from stem cells or were already committed precursors found in those unpassaged cultures. Thus, this discrepancy could be the result of the difference in the mitogens in the previous (epidermal growth factor; EGF) and present (FGF-2) studies or in the methodologies used to culture the cells. In other CNS stem/precursor cell populations, FGF-2 and EGF have different effects: FGF-2 tended to increase the number of neurons, whereas EGF promoted glial development (Craig et al., 1996; Kuhn et al., 1997; Whittemore et al., 1999). Consistently with the present data, Tropepe et al. (2000) reported that the neural stem cells isolated from the pigmented ciliary margin of the eye were incapable of oligodendrocytic differentiation and suggested that these cells are restricted to generating retinal cells.

Retinal-Specific Differentiation of Passaged NRPCs In Vitro

Many previous studies indicated that fetal retinal cells in primary culture sustained their proliferative state and developed later a retinal-specific fate in the presence of exogenous growth factors (Anchan et al., 1991; Ahmad et al., 1999). Retinal cell-specific differentiation in vitro from passaged neural stem or progenitor cells has been reported only from studies of stem cells isolated from pigmented ciliary margin of the eye (Ahmad et al., 2000; Tropepe et al., 2000) and not from the brain or spinal cord. In our study, the NRPCs, different from those fetal cells in previous primary culture studies, were self-renewing, passaged cells (Table I) and differentiated in vitro mostly with a rod photoreceptor phenotype, express-

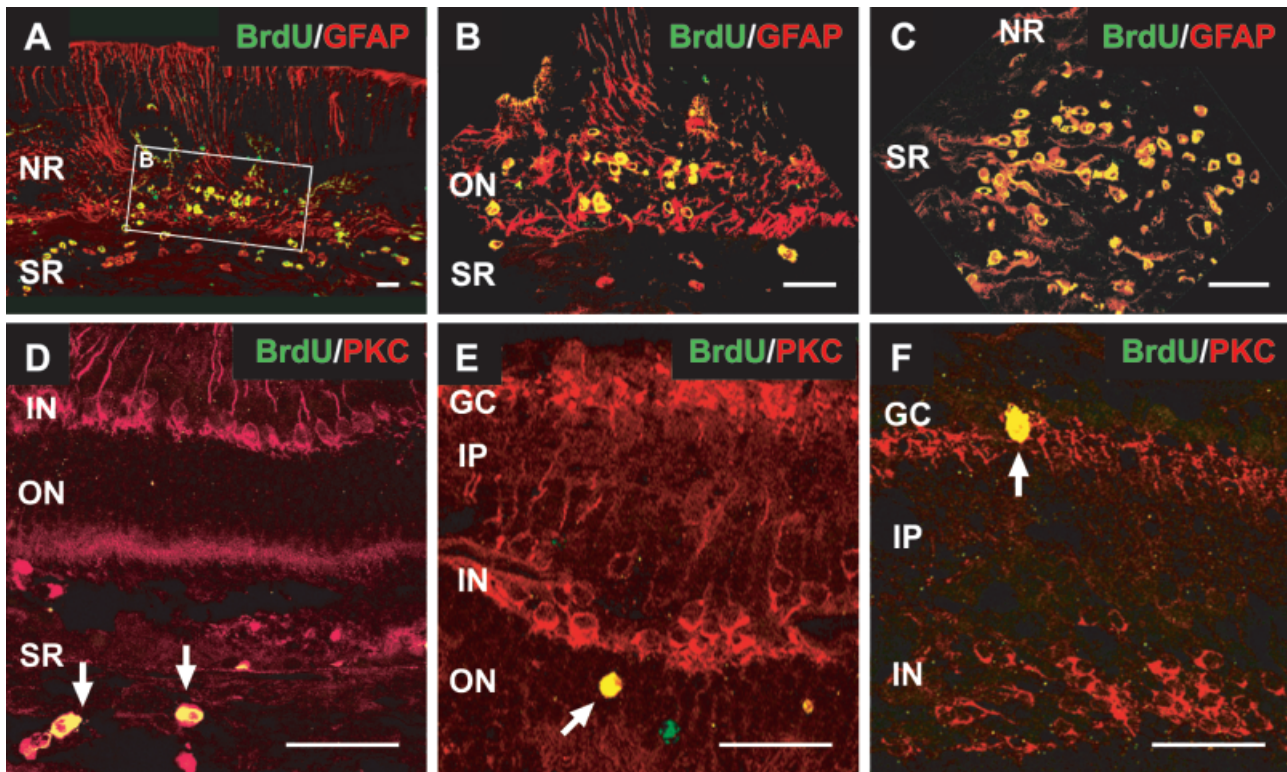


Fig. 4. Confocal images of undifferentiated NRPCs 4 weeks after grafting into the subretinal space. The grafted NRPCs survived, and a small group of individual cells migrated into the neural retina. Many BrdU-labeled cells expressed GFAP (yellow; **A**) in the outer neural retina (yellow; **B**; higher magnification of the highlighted area in **A**) and in the subretinal space (yellow; **C**). A few BrdU⁺ cells also

expressed PKC in the subretinal space (yellow; arrow; **D**), outer neural retina (yellow; arrow; **E**), and inner neural retina (yellow; arrow; **F**). NR, neural retina; SR, subretinal space; GC, ganglion cell layer; IP, inner plexiform layer; IN, inner nuclear layer; ON, outer nuclear layer. Scale bars = 20 μ m.

ing rhodopsin. Expression of recoverin, a marker for rods, cones, and cone bipolar cells, was relatively low, suggesting that the differentiation toward photoreceptor cells was not complete. In addition, the expression of S-antigen, a marker for rods and blue cones, was unusually high (40%), indicating that the current culture condition was different from the normal in situ environment. Photoreceptor cell differentiation was also found in previous studies of the pigmented ciliary margin and retina (Ahmad et al., 1999; Tropepe et al., 2000). Differentiation toward other retinal cells, such as horizontal cells, bipolar cells, and amacrine cells, was not found uniformly in any of these studies. In the present study, a minor population of the NRPCs in vitro differentiated into amacrine or horizontal cell phenotypes but not into rod bipolar cells (PKC⁺). This could have resulted from the culture conditions, suggesting the need for soluble factors present in the media (Jasoni and Reh, 1996; Levine et al., 1997). More likely, it could be caused by lack of the appropriate temporal progression of retinal cell types and their cell surface cues. During retinal development, bipolar cells are born significantly later than cone photoreceptors, horizontal cells, amacrine cells, ganglion cells, and rod photoreceptors (Cepko et al., 1996).

The development of all retinal cell lineages depends on specific extracellular signals in a precise sequence.

It is interesting to note that, in vitro, the NRPCs developed a high percentage of β III tubulin- and NSE- and few recoverin-immunoreactive neurons. Most neuronal retinal cell types of mouse (Rich et al., 1997) and rat (Fujieda et al., 1994) express NSE from late development to adult life. Rat rod photoreceptors transitionally express this marker during the first 2 postnatal weeks (Fujieda et al., 1994), whereas mouse rods do not (Rich et al., 1997). Insofar as rods account for the vast majority of retinal neuronal populations (Jeon et al., 1998), the high NSE expression (75%) in the differentiated NRPCs may indicate a rod-like differentiation, as is reflected in rhodopsin immunoreactivity (60%). β III tubulin is a marker expressed in the early development of neurons, in contrast to NSE and other mature retinal neuron-specific markers. It is normally not expressed in the adult retina. A high expression level (43%) was also found for calbindin, a marker for horizontal cells and, to a lesser extent, amacrine cells (Hamano et al., 1990). Both types of cells account for only a small portion of the retinal cell population. Therefore, the present findings suggest that the in vitro micro-

environment induces the cells to mature, whereas some retina-specific markers remain unchanged. This may explain the differences seen between the *in vitro* and the *in vivo* conditions. In addition, differential cell death, as suggested from previous studies (Kirsch et al., 1998; Soderpalm et al., 2000), may have a role in the *in vitro* and *in vivo* differentiation of NRPCs and should be characterized systematically in future studies.

NRPC Differentiation In Vivo

The use of neural stem and progenitor cells in transplantation has a great potential to advance toward the replacement of degenerated neurons in the CNS and the retina. Vision loss caused by diseases such as retinitis pigmentosa is directly associated with retinal neuronal degeneration (Stone et al., 1999). In the present study, the grafted NRPCs differentiated preferentially into glial cells *in vivo*. The pluripotentiality observed *in vitro* was not confirmed in the *in vivo* grafting results, except that a very few grafted cells expressed PKC, a marker for retinal bipolar and amacrine cells. This indicates that the lineage potential of NRPCs is restricted by the microenvironment. The immunophenotypic differentiation of NRPCs into glial cells *in vivo* was not influenced by the presence of a mild inflammatory reaction (in two of five experiments). *In vivo*, NRPCs seem to adopt a reactive or protective pathway, mimicking the reaction of retinas in response to injury, when Müller cells and astrocytes proliferate (Erickson et al., 1987; Seiler and Turner, 1988). After intraventricular infusion of mitogens, Craig et al. (1996) found a significant astrocytic (28%) and a limited neuronal (3%) differentiation of BrdU-labeled cells in the SVZ. Glial restriction of CNS stem cells that were pluripotent *in vitro* was also reported after engraftment into the injured spinal cord (Cao et al., 2001). Therefore, the use of neuronally restricted progenitor cells (Mayer-Proschel et al., 1997) should be considered in future retinal transplantation studies in which neuronal replacement is the goal. Alternatively, short-term differentiation of the neural progenitor cells may be helpful in obtaining multilineaged cell populations *in vivo* (Vescovi et al., 1999).

Photoreceptor differentiation was not detected in the present study after transplantation *in vivo*. A previous study using unpassaged NRPCs showed photoreceptor cell differentiation after transplantation (Chacko et al., 2000), although it is unknown whether these cells differentiated from stem cells or were already committed to the photoreceptor lineage at the time of grafting. Other retinal transplantation studies using hippocampus-derived (Nishida et al., 2000; Young et al., 2000) and human brain-derived (Mizumoto et al., 2001) neural precursor cells showed a predominant neuronal differentiation in the vitreous cavity and neural retina, but retinal cell-specific differentiation was not detected. When engrafted into the brain, these cells differentiated into neurons only in the locations where neurogenesis occurred (Gage et al., 1995; Suhonen et al., 1996; Svendsen et al., 1996). Limited numbers of cells immunopositive for PKC were observed after NRPC retinal grafting *in vivo*. *In vitro*, NRPCs did

not show expression of PKC. It is possible that the degenerated retinas of RCS rats retain cues to promote bipolar/amacrine cell development. Alternatively, this may suggest that the *in vitro* differentiation conditions used in the present study do not encourage bipolar cell differentiation.

Taken together, the studies with brain-derived neural stem and precursor cells implanted into the brain (Gage et al., 1995; Suhonen et al., 1996; Svendsen et al., 1996), spinal cord (Cao et al., 2001), and retina (Nishida et al., 2000; Young et al., 2000; Mizumoto et al., 2001; Warfvinge et al., 2001) and the present study with NRPCs grafted into the neural retina indicate that further long-term *in vitro* and *in vivo* studies under more complex, defined conditions are needed to elucidate the mechanism involved in the specific differentiation after implantation *in vivo*. It is likely that induction of NRPCs along specific lineages *in vitro* prior to engraftment and the modification of epigenetic signals in the host retina together with NRPC grafting will be needed to allow the appropriate cell-specific differentiation.

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