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Incorporation of Murine Brain Progenitor Cells into the Developing Mammalian Retina

Samantha J. Van Hoffelen,^{1,2} Michael J. Young,³ Marie A. Shatos,³ and Donald S. Sakaguchi^{1,2}

PURPOSE. To investigate the influence of a developing host environment on the survival, differentiation, and morphologic integration of murine brain progenitor cells (mBPCs) transplanted into the mammalian retina.

METHODS. Enhanced green fluorescent protein (GFP)-expressing murine brain progenitor cells were transplanted into developing and mature Brazilian opossums (*Monodelphis domestica*). Animals were allowed to survive for up to 4 weeks after transplantation, at which time the eyes were prepared for immunohistochemical analysis.

RESULTS. Transplanted mBPCs survived and differentiated *in vivo*, and extensive morphologic integration was observed within the host retinas. GFP-expressing cells often displayed morphologies characteristic of retinal neurons. GFP somata were located in nuclear layers, and their processes ramified throughout the inner (IPL) and outer (OPL) plexiform layers. Furthermore, in some cases, GFP-expressing neurites were confined to specific sublamina within the IPL. The greatest morphologic integration and differentiation were observed after transplantation into the youngest-aged host eyes. Some transplanted mBPCs incorporated within the inner retina expressed the neuronal markers microtubule associated protein (MAP)-2 or calretinin. Transplanted cells coexpressed GFP and recoverin only in the ONL.

CONCLUSIONS. mBPCs survived and morphologically integrated after xenotransplantation without immunosuppression. mBPCs were capable of incorporating into specific layers of the retina and expressing neuronal and retinal markers. The age of the host appeared to play a key role in determining cell fate *in vivo*. (*Invest Ophthalmol Vis Sci.* 2003;44:426-434) DOI:10.1167/iovs.02-0269

A greater understanding into the biology of neural stem cells will provide important information relevant to studies of central nervous system (CNS) regeneration and repair. Neural

stem cells have been isolated from adult, developing,¹⁻³ and embryonic⁴ brain. *In vitro* studies have revealed that neural stem cells possess the ability to adopt a variety of cellular fates. Furthermore, neural stem cell transplantation has been proposed as a method of repairing the damaged and diseased nervous system, including the retina. It is important to note that in studies examining the transplantation of "neural stem cells," investigators are in fact grafting mixed populations of cells, some of which may be "true" neural stem cells, but also contain cells that are more differentiated. These cells are best termed neural progenitor cells or precursor cells. This is true for both single adherent cells, and neurosphere cultures.

The work of Gage et al.⁵ revealed that isolated progenitor cells from the adult rat hippocampus retain the capacity to generate mature neurons when transplanted into the adult brain. Furthermore, these cells are capable of functionally integrating into the host hippocampal circuitry.⁶ When transplanted into neonatal or adult eyes, adult rat hippocampal progenitor cells survive, migrate, integrate, and in some cases differentiate into what appear to be mature neurons, but fail to differentiate into cells of retina-specific lineages.⁷⁻⁹ Although the normal immature retina is receptive to the integration of transplanted progenitor cells, the studies just cited show that a damaged retinal environment, induced mechanically⁸ or by transient ischemia,¹⁰ is needed to induce incorporation and differentiation of transplanted progenitor cells in mature hosts. Similarly, differentiation and morphologic integration of adult rat hippocampal progenitor cells has been observed in mature dystrophic rat retinas,⁹ but not in the congenic control. These results show that damaged or diseased environments are capable of supporting the differentiation of transplanted neural stem cells. Taken together, the findings in these studies suggest that incorporation of transplanted neural stem cells into the mature host neural retina cannot occur unless the tissue is damaged. Furthermore, it appears that the age of the recipient may affect the degree of integration after transplantation. This hypothesis, however, has remained relatively untested due to the limitations of performing transplants at embryonic or fetal stages.

In this report, we present a novel system for investigating such developmental questions. The Brazilian opossum, *Monodelphis domestica*, is a pouchless marsupial born in an extremely immature, fetal-like state. Brazilian opossum pups undergo an extensive period of postnatal development.¹¹ Access to an embryonic-like environment provides an excellent model system with which to investigate the fate of progenitor cells *in vivo*. Using enhanced green fluorescent protein (GFP)-expressing neural progenitor cells isolated from neonatal mice,² we investigated the cells' survival, integration, and differentiation after transplantation into the developing and mature opossum eye. We present the first systematic study showing the fate of murine brain progenitor cells (mBPCs) after transplantation into the developing environment of the eye.

MATERIALS AND METHODS

Animals

Brazilian gray short-tailed opossums (*Monodelphis domestica*) were obtained from a colony maintained at Iowa State University (Ames, IA).

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The animals were maintained in a constant environment (temperature: 26°C; humidity ~80%) and kept on a 14/10-hour light-dark cycle. Animals were provided with food and water ad libitum (Reproduction Fox Chow, Milk Specialties Products, Madison, WI) and fresh fruit. Gestation was approximately 13.5 days and litters of 3 to 13 pups were obtained. The day of birth was designated as postnatal day 1 (1 PN). Eye opening occurred at approximately 35 PN, and pups were weaned from their mothers at 60 PN. Pups of ages 5 to 35 PN ($n = 28$) and mature animals of 79 PN and 2.5 years ($n = 8$) of age were used in this study. All animal procedures for this study adhered to the provisions of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and had the approval of the Iowa State University Committee on Animal Care, and were performed in accordance with committee guidelines.

Murine Brain Progenitor Cell Culture

The murine brain progenitor cells (mBPCs) used in this study were isolated from brains of newborn enhanced green fluorescent protein (GFP)-expressing transgenic mice (TgN(β -act-eGFP)04Obs)¹² as reported by Shatos et al.² Murine brain progenitor cells were maintained as neurospheres in plastic tissue culture flasks (T-25 Falcon; Fisher Scientific, Pittsburgh, PA) in complete culture medium containing DMEM/Ham's F12 1:1 (Omega Scientific, Tarzana, CA) supplemented with N2 (Life Technologies, Rockville, MD), nystatin suspension (Life Technologies), penicillin-streptomycin (Sigma, St. Louis, MO), epidermal growth factor 20 ng/mL (recombinant human EGF; Life Technologies), and basic fibroblast growth factor 20 ng/mL (human recombinant bFGF; Promega Corp., Madison, WI). For in vitro analysis, mBPCs were collected by centrifugation at 800g for 3 minutes and the pellets resuspended in concentrated culture medium. The cells were then plated on 12-mm poly-L-ornithine-laminin or poly-L-lysine-coated glass coverslips. To prepare the substrates, the coverslips were washed with detergent (2% RBS; Pierce Chemical Co, Rockford, IL), coated with 50 μ L/mL poly-L-ornithine (Sigma) in sterile water, incubated overnight, washed, and coated with 5 μ g/mL mouse-derived laminin (Mouse; BD Biosciences, Bedford, MA) in phosphate-buffered saline (PBS, 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ per liter ddH₂O [pH 7.4]) for 6 to 8 hours. Laminin-coated coverslip substrates were used immediately after preparation. Coverslips were also coated with 1 mg/mL poly-L-lysine prepared in borate buffer (310 mg H₃BO₃, 475 mg Na₂B₄O₇, 10 H₂O in 100 mL ddH₂O [pH 8.4]) and incubated for 3 hours. Coated coverslips were rinsed in culture water, air dried, and used as needed. No clear difference in the number of attached cells was observed with either substrate. To begin the differentiation process, the neurospheres were harvested, dissociated, and plated onto substrate-coated glass coverslips in medium without bFGF and EGF (referred to as differentiation medium).

Transplantation of mBPCs into the Developing Eye

Cultured mBPCs were collected as spheres within the culture medium and spun at 800g for 3 minutes, after which the pelleted mBPCs were resuspended in Dulbecco's PBS (Life Technologies). Adult animals were anesthetized in an induction chamber. Anesthesia was induced with 3% halothane combined with 30% NO and 70% O₂ and was maintained with 1.5% halothane in NO and O₂ for the duration of the experimental procedure. Opossum pups remain attached securely to the mother's nipples until 20 PN. When performing transplantations on 5, 8, and 10 PN pups, the mother was anesthetized in the halothane induction chamber (as described above) with the litter still attached. Older pups were anesthetized individually.

The cell injection apparatus consisted of a 20- μ L syringe (Hamilton, Reno, NV) connected through a 0.9% saline-filled polyethylene tube to a beveled glass micropipette. Animals received intraocular injections of mBPCs through the dorsolateral aspect of the eye. Two microliters of cell suspension (~50,000 cells/ μ L) was slowly injected into the vitreal chamber of adult eyes and 1.0 to 1.5 μ L into the eyes of younger hosts.

An aliquot of cells used for each transplantation session was plated into a sterile culture dish and visualized with fluorescence microscopy to verify the viability and GFP expression of the transplanted cells. Animals were monitored daily, and those receiving transplants were allowed to survive for 1, 2, and 4 weeks. After appropriate survival periods the opossum pups and adults were deeply anesthetized with halothane and perfused transcardially with 4% paraformaldehyde in 0.1 M PO₄ buffer. The heads were removed and postfixed for 48 hours in 4% paraformaldehyde. Eyes were removed, immersion fixed for an additional 2 to 6 hours, and cryoprotected in 30% sucrose in 0.1 M PO₄ buffer. Tissue was embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; VWR International, West Chester, PA) frozen and sectioned coronally at 20 μ m using a cryostat (American Optical, Buffalo, NY). Sections were thaw mounted onto microscope slides (Superfrost; Fisher Scientific) and stored at -20°C until processed.

Analysis of mBPCs In Vitro: Immunocytochemistry

Cells cultured on coverslips were processed for immunocytochemistry according to standard protocols. Briefly, cells were first rinsed on 0.1 M PO₄ buffer, fixed in 4% paraformaldehyde in 0.1 M PO₄, rinsed, and processed as described for tissue sections. Specific primary antibodies (see Antibodies section) were used to identify differentiated neurons and glia. Cultured cells were incubated in primary antibodies for 18 hours, rinsed, and subsequently incubated in the dark for 2 hours in fluorescent conjugated secondary antibody. They were then rinsed and mounted on microscope slides with antifade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). Antibody experiments were repeated three times with cells plated from separate culturing sessions.

Preparations were examined on a photomicroscope (Microphot FXA; Nikon Corp., Melville, NY). A 20 \times objective was used to examine 6 to 10 microscope fields, each field representing 0.1 mm² (360 \times 280 μ m). In each microscope field the following counts were made: total number of cells (using light microscopy), number of cells expressing GFP (using an FITC filter cube), and number of cells labeled with the primary antibody of interest (using a rhodamine isothiocyanate [RITC] filter cube). These data were used to calculate the percentage of GFP-expressing cells labeled with one of the antibody markers on each coverslip. The data collected after 1 and 3 days in culture, in both complete and differentiation media for each antibody was compared and analyzed using a two-sample, student *t*-test. All data analyses were performed blind, to eliminate experimental bias.

Analysis of Tissue Sections: Immunohistochemistry

Sections were washed in potassium PBS (KPBS; 0.15 M NaCl, 0.034 M K₂HPO₄, 0.017 M KH₂PO₄ [pH 7.4]) and incubated in blocking solution (1.5% blocking serum, 1% bovine serum albumin [BSA; Sigma], and 0.4% Triton X-100, Fisher Scientific) for 2 hours. Sections were incubated in primary antibody overnight at room temperature in a humid chamber, washed in KPBS with Triton X-100, incubated in appropriate biotinylated secondary antibodies for 2 hours, rinsed, and incubated with streptavidin Cy3 (Jackson ImmunoResearch, West Grove, PA) in the dark for 30 minutes. Slides were then rinsed and coverslipped with antifade mounting medium (Vectashield; Vector Laboratories).

Bromodeoxyuridine Injection and Analysis

Opossum pups were injected with bromodeoxyuridine (BrdU) at 5, 10, and 20 PN. Individual pups were injected without separation from the mother. Each pup ($n = 3$ at each time point) was given a subcutaneous injection of 1.5 μ L of 20 mg/mL BrdU solution (in sterile saline) along the dorsal midline. Pups were allowed to survive for 2 hours, at which time the tissue was prepared for immunohistologic analysis, as previously described.¹³ Briefly, tissue sections were rinsed with KPBS and pretreated with 0.06% trypsin (bovine type III; Sigma) and 5.4×10^{-3} M CaCl₂ in KPBS for 30 minutes at 37°C. After washing for 10 minutes with KPBS, tissue was treated with 0.1 N HCl for 10 minutes, followed

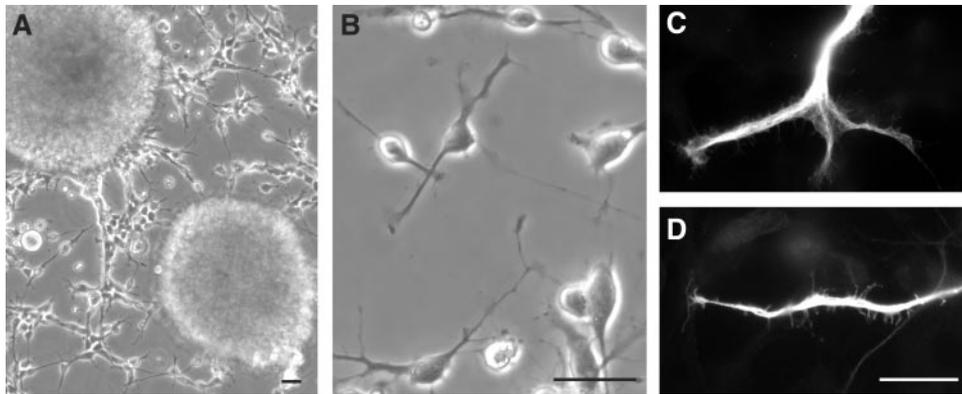


FIGURE 1. mBPCs in vitro as neurospheres and dissociated cells. (A, B) Phase-contrast images of mBPCs. (A) mBPCs were maintained as neurospheres when cultured in complete media. (B) mBPCs adhered and extended processes when cultured in differentiation media on adhesive substrates. (C, D) Fluorescent images illustrating the pattern of TUJ1 IR in processes of cultured mBPCs. Scale bars, 20 μm .

by incubation in 2 N HCl at 37°C for 30 minutes. The tissue sections were neutralized in basic 0.5 M KPBS (pH 8.5) and subsequently processed for routine immunohistochemistry with an anti-BrdU antibody.

Antibodies

Anti-microtubule associated protein (MAP)-2 (mouse IgG; Roche Molecular Biochemicals, Indianapolis, IN) was diluted at 1:1000 in blocking solution and used as a marker of ganglion cells and the inner plexiform layer (IPL).¹⁴ An antibody against class III β -tubulin (TUJ1) was used as a neuronal marker and was diluted at 1:200 (mouse IgG; Chemicon International, Temecula, CA). Anti-gial fibrillary acidic protein (GFAP; GA5, mouse IgG; ICN Immunobiologicals, Costa Mesa, CA) was diluted at 1:1000 and used as a marker of astrocytes and reactive Müller glia of the retina.¹⁵ Anti-calretinin was used to identify this calcium-binding protein (rabbit IgG; Chemicon), which has been used as a marker of a subclass of horizontal cells, amacrine cells, and ganglion cells,^{16,17} and was diluted at 1:3000. Anti-synaptogamin I (P65, mouse IgG obtained from Reinhard Jahn, Howard Hughes Medical Institute [HHMI], Yale University, New Haven, CT) was diluted at 1:2000. Anti-recoverin (rabbit IgG, obtained from Alexander Dizhoor, Wayne State University, Detroit, MI) was diluted at 1:2000 and used as a marker of photoreceptors.¹⁸ The O4 antibody (1:400, mouse IgM, Chemicon) was used as a marker for oligodendrocytes. BrdU was detected using anti-BrdU (rat 1:2000; Dako Corp., Carpinteria, CA). All primary and secondary antibodies were diluted in KPBS with 1% BSA, 0.4% Triton X-100, and 1% normal blocking serum, corresponding to the species in which the secondary antibody was produced. All biotinylated secondary antibodies (horse anti-mouse and goat anti-rabbit; Jackson ImmunoResearch) were diluted to 1:500. Streptavidin Cy3 was diluted in KPBS to 1:10,000. Negative controls were used in parallel during all immunohistologic processing by the omission of the primary or secondary antibody. No antibody labeling was observed in the control experiments.

Analysis of Tissue Sections

Tissue sections containing transplanted GFP-expressing mBPCs and labeled with the antibodies were examined with a photomicroscope (Microphot FXA; Nikon Corp.). Retinas that received transplanted mBPCs were compared with control, noninjected, age-matched retinas to determine whether normal morphologic development had occurred during the survival period after transplantation. Murine brain progenitor cells were analyzed for location, morphology, and colocalization of antibody markers. Images were captured with a charge-coupled device camera (Megaplus; Model 1.4; Kodak Corp., San Diego, CA) connected to a framemaker (MegaGrabber; Perceptics, Knoxville, TN, in a Macintosh 8100/80AV computer; Apple Computer, Cupertino, CA) using NIH Image 1.58VDM software (available by ftp from zippy.nimh.nih.gov; Wayne Rasband, National Institutes of Health, Bethesda, MD). Some sections were visualized and images captured using a confocal scanning laser microscope (TCS-NT; Leica Microsystems Inc., Exton,

PA). Figures were prepared on computer (iMac, Power PC G3; Apple Computer, using Photoshop, ver. 4.0, Adobe, San Jose, CA, and Freehand, ver. 9.0, for the Macintosh; Macromedia, San Francisco, CA). Outputs were generated on a continuous-tone color printer (Phaser; Tectronix, Beaverton, OR).

Reconstruction of GFP-Expressing mBPCs Integrated into the Retina

Reconstructions were drawn from a series of six to eight confocal images of the same cell. Confocal image series were obtained from eight retinas. Reconstructed images were digitized with a flatbed scanner (Astra 2400S; UMAX Corp., Fremont, CA, final image constructed in Freehand ver. 9.0; Macromedia).

RESULTS

Murine Brain Progenitor Cells In Vitro

Murine brain progenitor cells were maintained in medium supplemented with bFGF and EGF as a suspension of neurospheres (Fig. 1). To begin differentiation, the neurospheres were harvested, dissociated, and plated onto adhesive substrates in medium without bFGF and EGF. A small proportion of cells remained free floating in the culture medium as single cells or small neurospheres. These nonadherent cells were removed during processing and therefore were not analyzed. Cells in differentiation medium appeared to cease proliferation, whereas those cells in complete medium appeared to continue proliferation, in that the number and density of cells increased. Murine brain progenitor cells plated in complete medium for 3 days (Fig. 1) had an average cell density of 200 ± 56 (mean \pm SD) cells per 0.1 mm^2 . Cells grown in the differentiation medium had an average density of 28 ± 16 cells per 0.1 mm^2 .

Cells were evenly distributed across the coverslip; however, when large clumps were observed, they were not included in the analysis because of the difficulty of quantifying them. Cells remained healthy after plating, as verified by their continued strong expression of GFP. Cells adopted a variety of morphologies when cultured on adhesive substrata in the differentiation medium. Many mBPCs were unipolar, bipolar, or multipolar, possessing neuritelike processes of various lengths. As illustrated in Figure 1, neurites displayed complex growth cone-line structures at their tips. Cultured mBPCs also displayed flattened morphologies reminiscent of astrocytes, and some mBPCs retained their simple spherical morphology.

Specific antibodies were used to assess the phenotypes of the mBPCs after culturing 1 and 3 days, in the presence or absence of the growth factors. Figure 2 illustrates the expression of MAP2, TUJ1, and GFAP in populations of GFP-expressing mBPCs. Many mBPCs expressed MAP2 and TUJ1, proteins

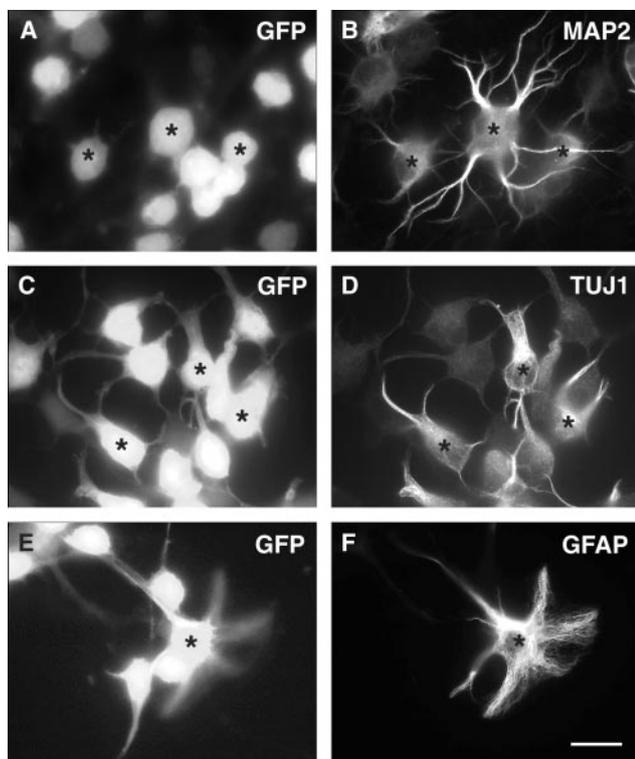


FIGURE 2. Expression of neuronal and glial markers by mBPCs in vitro. (A, C, E) Fluorescent images illustrating GFP-expressing mBPCs and immunoreactivity for (B) MAP2 (D) TUJ1, and (F) GFAP. (*) Examples of GFP-expressing mBPCs that also expressed neural markers. Scale bar, 20 μ m.

characteristic of neurons. These cells often displayed neuronal morphologies but could also be round cells with small somata. MAP2-immunoreactive (IR) cells often displayed extensive processes as well as more simple bipolar morphologies. The TUJ1 antibody revealed extensive detail of the neurites (Figs. 1, 2). In contrast, cells were rarely labeled with the anti-GFAP antibody. GFAP IR was detected in cells displaying a mature glial morphology with star-shaped, short, broad processes (Fig. 2).

The percentage of MAP2- and TUJ1-expressing mBPCs observed in differentiation medium was significantly greater than in mBPCs cultured in complete medium after 3 days (MAP2 $P < 0.001$, TUJ1 $P < 0.001$, GFAP $P < 0.4$, two-sample Student's t -test, Table 1). These in vitro results revealed that the differ-

TABLE 1. mBPCs Differentiate after Withdrawal of Growth Factors

	1 Day		3 Days	
	Complete	Differentiation	Complete	Differentiation
MAP2	5 \pm 3.7	26 \pm 6.2*	22 \pm 18	55 \pm 11.9*
TUJ1	30 \pm 8	35 \pm 12.9	24 \pm 3.8	86 \pm 8.3*
GFAP	1 \pm 1.2	2 \pm 3.1	2 \pm 3	5 \pm 6.5

Complete: mBPCs cultured in medium containing bFGF and EGF. Differentiation: mBPCs cultured in medium without bFGF and EGF. Data are the percentage of immunoreactive cells mean \pm SD and represent pooled data from three separate culture sessions. The expression of MAP2 was significantly different between mBPCs maintained in differentiation medium and those in complete medium after both 1 and 3 days ($P < 0.001$ and 0.004, respectively), and the expression of TUJ1 was significantly different after 3 days in differentiation medium ($P < 0.001$).

* Significantly from complete cell cultures.

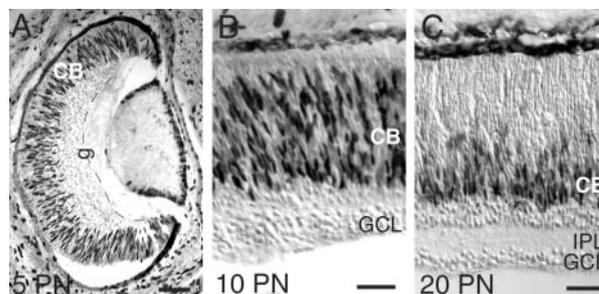


FIGURE 3. Neurogenesis within the developing opossum retina. Images showing BrdU-IR within the postnatal retina. Pups received subcutaneous injections of BrdU at (A) 5, (B) 10, and (C) 20 PN and were killed 2 hours later. Extensive BrdU incorporation was observed during early postnatal development. CB, cytotblast layer; GCL and g, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer. Scale bars: (A) 500 μ m; (B, C) 300 μ m.

entiation conditions used in this analysis facilitated the morphologic and phenotypic differentiation of mBPCs and confirmed their ability to generate cells expressing neuronal and glial markers.

Murine Brain Progenitor Cells In Vivo: Survival, Differentiation, and Morphologic Integration after Transplantation into the Developing Retina

To investigate the influence of the age of the host environment on neural progenitor cell survival, differentiation, and integration, we transplanted mBPCs into the developing and mature eyes of Brazilian opossums (*M. domestica*; Figs. 3, 4). At birth, the opossum retina is relatively undifferentiated.^{13,19,20} Most retinal cytotgenesis occurs postnatally between 1 and 25 PN (Fig. 3; Sakaguchi DS, unpublished results, 1997). The 12 to 15 PN opossum retina is developmentally comparable to a 1-PN rat retina based on cellular differentiation and lamination patterns.²⁰ In the present study, we used a developmental series of hosts, including 5, 8, 10, 30, and 34 PN and mature animals of 79 PN and more than 2 years. By using fetal-like hosts of 10 PN or younger, maturing hosts (30–34 PN), and mature hosts (older than 79 PN), we were able to investigate the influence of the cellular environment on the mBPCs in vivo. Figure 3 shows the retinal environment at the time of transplantation into the young hosts. Incorporation of BrdU was used as an indicator of cell division to determine the amount and location of cytotgenesis within the developing retina. At 5 PN, BrdU IR was located throughout the outer half of the retina (Fig. 3A). Extensive BrdU IR was present in the 10 PN eye and was still localized in the outer half of the retina (Fig. 3B). Cytogenesis continued throughout early postnatal development, and many BrdU IR cells were still observed at 20 PN (Fig. 3C).

One Week after Transplantation. To determine whether mBPCs were capable of survival after xenotransplantation into the opossum eye, tissue sections were examined for the presence of GFP-expressing cells 1 week after transplantation. The transplanted mBPCs were reliably identified based on GFP fluorescence. Transplanted GFP-expressing cells were observed throughout the posterior segment of the eye. Cells were within the vitreous, adjacent to the lens, and juxtaposed to the inner limiting membrane (ILM). The GFP-expressing cells were present, both as large aggregates and as dispersed cells within all ages of recipient. After 1 week's survival, no cells were fully integrated within the neural retina in hosts of any age. On occasion, isolated mBPCs were observed within the ILM; however, these cells appeared relatively simple in morphology. One week after transplantation, GFP-expressing mBPCs survived in hosts of all ages. The age of the host environment did

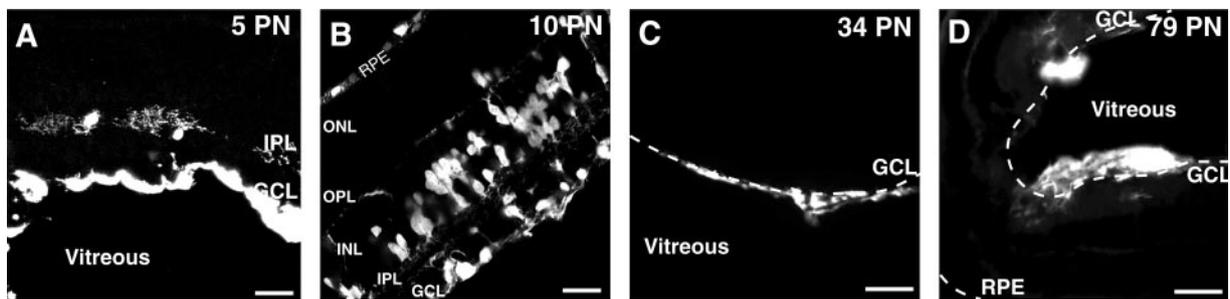


FIGURE 4. Fluorescent images of mBPCs illustrating their survival and integration into the developing neural retina 4 weeks after transplantation. GFP-expressing cells were observed within the eye at all ages. (A) Within the 5-PN host, GFP-expressing cells were integrated throughout the host retina. (B) At 10 PN, mBPCs were observed within all layers of the retina; GFP-expressing cells were observed in the RPE, ONL, INL, IPL, and GCL. (C) In an older host (34 PN), mBPCs were within the ILM, and processes extended into the inner retina, but somata did not. (D) At 79 PN, isolated GFP-expressing processes had integrated within the inner retina; however, GFP-expressing somata were mainly within the vitreous or adjacent to the ILM. Scale bars: (A, B) 100 μm ; (C, D) 250 μm .

not appear to affect survival or morphologic differentiation at 1 week after grafting. We observed no cases of complete morphologic integration of transplanted cells within the host retina. GFP-expressing processes were observed occasionally within and along the ILM.

Two Weeks after Transplantation. GFP-expressing mBPCs were again found throughout the posterior segment of the eye 2 weeks after transplantation. In contrast to tissue analyzed after 1 week, more cells were adjacent to the ILM. Although mBPC somata were seldom observed within the nuclear layers of the retina, GFP-expressing processes were present within the ganglion cell layer (GCL) and IPL. At 2 weeks after transplantation most mBPCs were observed bordering the inner retina or abutting the lens within the eyes of the youngest hosts (5, 8, and 10 PN). Transplanted cells within older hosts (30, 34, and 79 PN) were generally dispersed within the vitreous of the posterior segment; however, these cells displayed more differentiated morphologies. Cells in these older hosts rarely integrated or associated with the ILM. We observed GFP-expressing mBPCs throughout the eye at 2 weeks after

transplantation, suggesting the transplanted cells may be capable of longer-term survival. After 2 weeks' survival, it became apparent that the host's age influenced the fate of cells *in vivo*. Although still very limited, the younger the host environment, the more incorporation we observed within the host inner retina.

Four Weeks after Transplantation. At this time point, GFP-expressing mBPCs were incorporated within all layers of the retina after transplantation into 5, 8, and 10 PN hosts (Figs. 4A, 4B, 5). Many GFP-expressing cells integrated into the neural retina of 5 and 10 PN hosts (Figs. 5A–C, 5D–G). Transplanted mBPCs were observed within the GCL, inner nuclear layer (INL), and outer nuclear layer (ONL). Processes were observed extending throughout the ILM, GCL, IPL and ONL (Figs. 4A, 4B, 5A, 5D, 5F). The lens and the ciliary margin were also attractive environments for mBPC differentiation. Transplanted mBPCs were observed adjacent to and extending into the outer layers of the lens. GFP-expressing cells were throughout the posterior segment of the eye. In striking contrast, mBPCs survived and differentiated morphologically but in general remained within

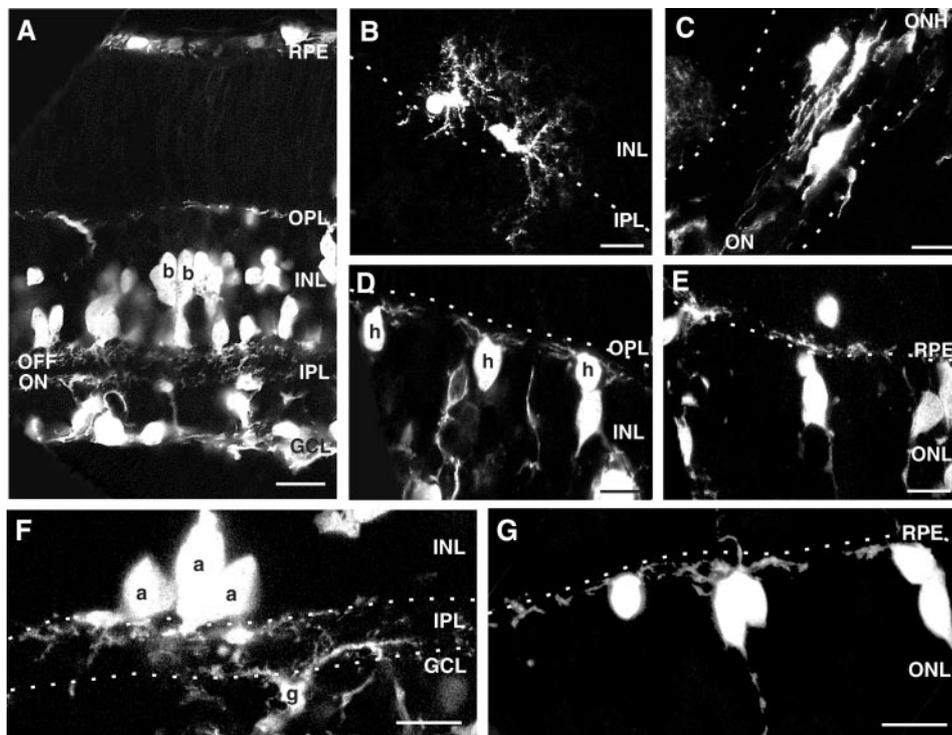
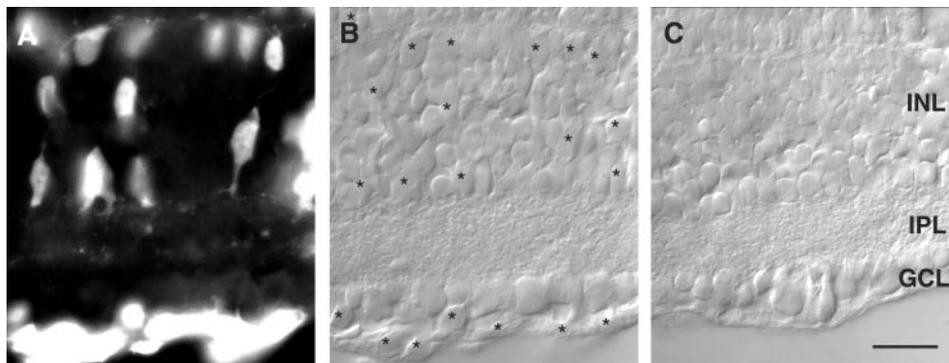


FIGURE 5. Morphologic differentiation and integration of mBPCs into the 5 and 10 PN retinas 4 weeks after transplantation. GFP-expressing cells appeared intertwined between host cells in an organized, extensive network in both ages of host (images captured with confocal microscopy). (A) GFP-expressing cells were throughout the host retina, with somata located within nuclear layers and processes extending throughout the plexiform layers. At 5 PN cells incorporated into the INL extended elaborate processes. (B) GFP-expressing cells extended processes within the INL. (C) GFP-expressing cells with long processes were located in the optic nerve at 5 PN. *Dotted line*: boundary of the optic nerve. Cells appeared morphologically analogous to bipolar cells (A), horizontal cells with lateral-extending processes (D), amacrine cells with large soma and extensive dendritic arborizations (F) and ganglion cells (A, F). Cells incorporated within the ONL did not adopt morphologies comparable to photoreceptors (G). ONH, optic nerve head; ON, optic nerve; h, horizontal-like cell; b, bipolar-like cell; a, amacrine-like cell; g, ganglion-like cell. Scale bars, 20 μm .

FIGURE 6. The architectural integrity of the retina was maintained after incorporation of mBPCs. (A) GFP-expressing cells integrated throughout the 10 PN host retina 4 weeks after transplantation. (B) Differential interference contrast (DIC) microscopic image of the retina illustrated in (A) and an age-matched control retina (C). (B, ★) GFP-expressing transplanted somata from (A). All tissue sections were obtained from 38 PN animals (i.e., 10 PN host plus 4 weeks' [28 days'] survival after transplantation). Scale bar, 20 μ m.



the vitreous or in close contact with the ILM after transplantation into the older and adult hosts (30, 34, and 79 PN; Figs. 4C, 4D). Isolated cells appeared to extend into the inner retina at 34 and 79 PN; however, no somata invaded the host retina at the older ages. At 4 weeks after transplantation, many mBPCs integrated within all nuclear layers of the retina of 5 and 10 PN hosts, but few cells were observed integrated into the more mature host retinas.

Morphologic Differentiation of Murine Brain Progenitor Cells after Transplantation

After transplantation, recipient retinas appeared to develop normally. Tissue taken after the various survival periods was indistinguishable from age-matched control tissue. As shown in Figure 6, lamination of the IPL and organization within the INL appear normal within retinas hosting integrated cells.

Extensive morphologic differentiation and integration were observed after transplantation of the mBPCs into 5 to 10 PN host eyes after 4 weeks' survival. A striking observation was that transplanted mBPCs appeared to respect the host tissue's architecture. The transplanted mBPCs appeared to obey the nuclear boundaries of the retina. As shown in Figures 4A, 4B, and 5A, mBPCs were within the GCL and INL; few somata were within the plexiform layers. We observed extensive outgrowth of processes within the plexiform layers (Figs. 5A, 5D, 5F, 5G, 8D). The observation that GFP-expressing processes extending into the IPL were often organized within specific sublamina was most intriguing (Figs. 5A, 7, 8D). As shown in Figure 5A, processes from GFP-expressing somata located within both the

INL and GCL extended specifically into what appeared to be OFF and ON sublamina. Transplanted cells often possessed morphologies similar to specific retinal cell types. Figures 5 and 7 illustrate cells with a bipolar cell morphology (Fig. 5A), with apical and basal processes extending to the OPL and IPL. Some transplanted GFP-expressing cells located in the outer portion of the INL, adjacent to the OPL, adopted morphologies similar to horizontal cells with neurites extending along the inner portion of the OPL (Figs. 5D, 7). We observed mBPCs within the INL that displayed different morphologies, depending on location. Cells with morphologies similar to horizontal cells were observed in the outer INL (Figs. 5D, 7), bipolar-like cells were throughout the INL (Fig. 5A) and amacrine-like cells were present within the inner INL (Figs. 5A, 5F, 7). GFP-expressing cells residing in the GCL often displayed ganglion cell-like morphologies (or displaced amacrine cell morphologies; Fig. 5F, 7). Transplanted cells were also observed along the ILM, extending processes along the inner retinal surface. GFP-expressing processes within the optic nerve head region were observed only at 4 weeks after transplantation in the youngest recipient (5 PN; Fig. 5C). Cells within this region of the optic nerve possessed small somata but extended extensive processes into proximal regions of the optic nerve.

In contrast to observations in the younger hosts, we rarely observed GFP-expressing cells that were morphologically integrated into the neural retina in the older transplant recipients. However, cells with a variety of morphologies were observed within the vitreous of older hosts. Transplanted mBPCs comparable in structural complexity to those seen *in vitro* were found in the vitreous and in proximity to the neural retina. Murine brain progenitor cells were also observed to form a monolayer along the inner retinal surface in older hosts (30–79 PN). These cells lining the retina were simple and uniform in shape. They often elaborated processes along the ILM, forming a continuous monolayer of GFP-expressing somata and processes (Figs. 4C, 4D).

Evaluation of the Phenotypes Adopted by mBPCs In Vivo

A panel of specific antibodies was used to evaluate whether the transplanted cells adopted mature neural phenotypes *in vivo*. Within the mammalian retina, MAP2 IR localizes within neurons of the inner retina.^{21,22} Some GFP-expressing cells expressed MAP2 when located in the inner retina (Fig. 8A). Integrated cells expressed MAP2 within the GCL and INL. Figure 8A, shows several MAP2-IR cells located within both these nuclear layers. In addition, some GFP-expressing cells located within the vitreous were also MAP2 IR (data not shown). Cells incorporated within the outer layers of the retina were not MAP2 IR.

A subpopulation of GFP-expressing cells were observed to coexpress calretinin (Fig. 8B). In the mature retina, antibodies

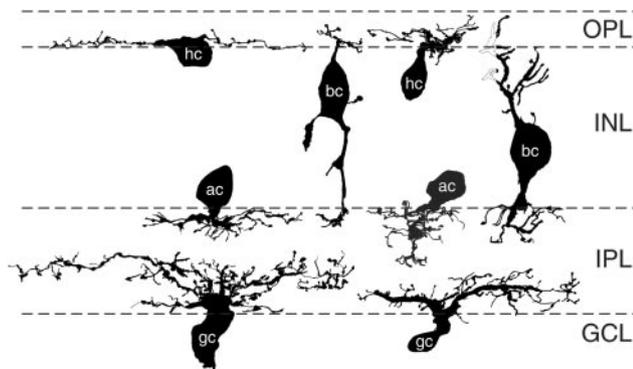


FIGURE 7. Transplanted GFP-expressing mBPCs adapted morphologies similar to retinal cell types. Reconstructions of GFP-expressing cells morphologically integrated into the developing retina. Each cell was reconstructed from a series of confocal images. Cells exhibiting morphologies similar to retinal neurons are displayed together in a single field. *Dashed lines:* approximate boundaries of the plexiform layers. hc, horizontal-like cell; bc, bipolar-like cell; ac, amacrine-like cell; gc, retinal ganglion-like cells (or displaced amacrine-like cell).

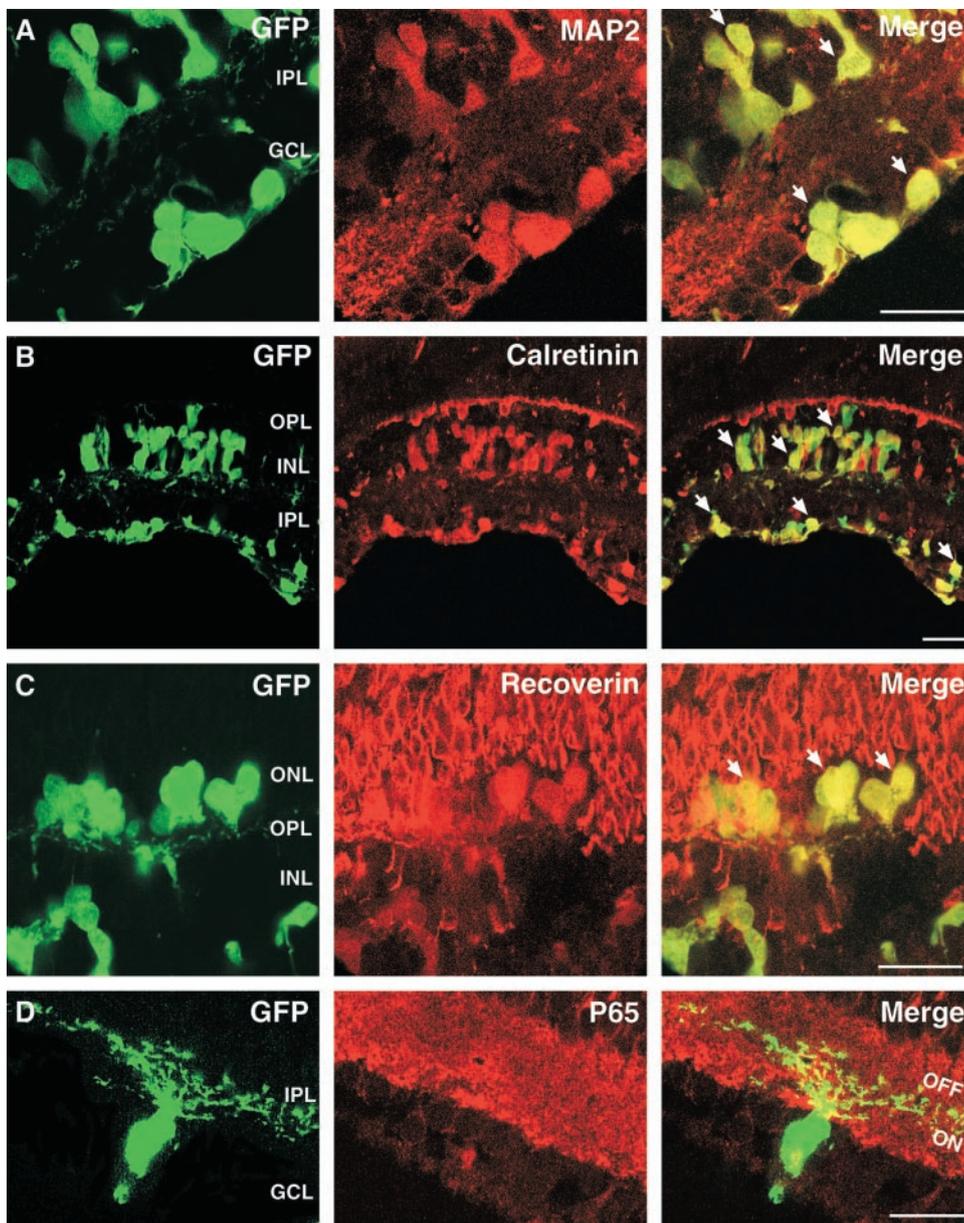


FIGURE 8. mBPCs expressed neuronal markers after transplantation into the 10 PN host retina. Images captured with confocal microscopy. (A–D) *Left column:* GFP-expressing cells within the host retina; *middle column:* antibody IR within the host retina and among transplanted cells; *right column:* merged image, illustrating colocalization of GFP and antibody. Merged images were created by merging confocal images of GFP fluorescence (*green*) with antibody (*red*) labeling patterns. (A) mBPCs incorporated within the INL were MAP2 IR. Cells incorporated into the outer layers of the retina were not MAP2 IR. (B) Calretinin IR was observed among GFP-expressing cells in the inner retina. Cells were located among calretinin-IR amacrine and ganglion cells. No calretinin-IR mBPCs were in the vitreous of the outer retina at this age. (C) Recoverin IR was observed among GFP-expressing cells located within the ONL. mBPCs expressed recoverin only when located within the outer retina. (D) GFP-expressing processes ramified throughout the IPL. These processes were not P65 IR but were intertwined among the processes of the IPL. GFP (*green*); MAP2, calretinin, recoverin-IR, and P65 (*red*); colocalization (*yellow*). *Arrows* in merged images: examples of colocalization. Scale bars, 20 μ m.

against calretinin labels some amacrine and horizontal cells, as well as the IPL.^{23,24} The merged image in Figure 8B illustrates examples of GFP-expressing cells that coexpressed calretinin (arrows). In some cases the calretinin-IR mBPCs were relatively evenly distributed similar to host cells in the surrounding retina. We observed no calretinin expression within the outer retina.

mBPCs integrated within the ONL did not adopt morphologies characteristic of mature photoreceptors. However, a relatively small subpopulation of GFP-expressing cells coexpressed recoverin, a marker for photoreceptors and cone bipolar cells (Fig. 8C). The merged image in Figure 8C illustrates a cluster of GFP-expressing somata that coexpressed recoverin. The GFP-expressing cells were located along the inner border of the ONL, adjacent to the OPL.

An antibody against the presynaptic terminal protein P65 was used to determine whether GFP-expressing processes express a synapse-associated protein. In this analysis, very little P65 IR was observed within GFP-expressing processes. However, GFP-expressing processes within the IPL were intertwined among host processes (Fig. 8D).

We used an antibody against GFAP to determine whether the transplanted cells expressed this glial marker. In this analysis, GFAP IR was observed only among host astrocytes. mBPCs adjacent to and along the inner retina were not GFAP IR. We observed GFAP-IR (at all ages examined) only among mBPCs situated in the vitreous, generally among clusters of GFP-expressing cells (data not shown).

DISCUSSION

Neural progenitor and neural stem cells have been successfully transplanted into the injured and diseased mammalian retina.^{8–10} Incorporation within the intact mammalian retina has been observed in young hosts, yet no study has systematically analyzed the effect of the developing environment on neural progenitor cell fate *in vivo*. In the present study, we have demonstrated for the first time that transplanted neural progenitor cells can survive, differentiate, and morphologically integrate into the developing mammalian retina of the Brazilian opossum. Using a marsupial as an experimental model system

we have demonstrated that the age of the host environment strongly influences the ability of mBPCs to differentiate and integrate into the host tissue. This is the first study to demonstrate extensive morphologic integration of mBPCs into the intact mammalian retina. The finding that neural progenitor cells not only migrate into a highly organized tissue such as the retina, but also adopt characteristic morphologies without disrupting the host retina strongly supports their use in further transplantation studies.

In Vitro Differentiation of mBPCs

Capable of self-renewing in culture, mBPCs were maintained as neurospheres in growth factor-supplemented culture medium. After plating onto adhesive substrates, mBPCs cultured in the absence of bFGF and epithelial growth factor (EGF) adopted mature cellular phenotypes. mBPCs often adopted elaborate morphologies *in vitro* and often appeared to interconnect through complex neuritic processes. Using markers against mature neuronal and glial proteins, we observed a significant increase in the number of mBPCs immunoreactive for both MAP2 and TUJ1. Because the culture environment was not manipulated, except for the withdrawal of the mitogenic factors, the potential to express neuronal proteins must be intrinsic to the cell after exiting the cell cycle. Few mBPCs were labeled with the GFAP antibody in either culture condition suggesting more complex regulation of the astrocytic phenotype from these neural progenitor cells. Furthermore, no mBPCs were labeled with the O4 antibody directed against oligodendrocytes,^{25,26} (data not shown), although the antibody clearly labeled oligodendrocytes in sections of brain tissue.

Survival and Integration of Transplanted mBPCs

After transplantation, the GFP-expressing cells dispersed and migrated throughout the posterior segment of the eye and were easily identified with fluorescence microscopy. As a standard procedure, an aliquot of cells used for transplants was always cultured for 24 hours to verify the condition of the mBPCs at the time of transplantation. These mBPCs always strongly expressed GFP, and thus we are confident that at the time of transplantation the cells were in a healthy condition.

Our results clearly demonstrate that mBPCs were capable of survival after xenotransplantation, even in the absence of immunosuppression. This may be due to the relative purity of cultured mBPCs, which lack antigen-presenting cells and passenger leukocytes that would be present in conventional grafts of neural tissue. A large number of GFP-expressing cells survived after transplantation in all ages of recipient. After 1 week, transplanted cells were observed within the vitreous or adjacent to the lens. After 2 weeks, cells began to integrate within the retina but were not incorporated within the nuclear layers. At 4 weeks after transplantation, mBPCs incorporated within all layers of the younger host retinas (5–10 PN). The physical barrier of the ILM could prevent invasion of cells into the older retinas.

The incidence of incorporation of GFP-expressing cells decreased with increasing age of the host eye. At 5 and 10 PN, GFP-expressing cells were within all layers of the retina. In general, cells transplanted into older hosts remained in the posterior segment of the eye or aligned themselves along the ILM and were seldom observed integrated into the host tissue. Survival and differentiation cues, such as growth or neurotrophic factors and cell–extracellular adhesion molecules are especially abundant early in retinal development.^{27–30} It is likely that factors such as these facilitate the survival, differentiation, and migration of the transplanted cells at younger ages. Furthermore, it is possible that immature retinal tissue acts as a

weaker physical barrier to the emigration of the mBPCs. The vitreal surface of the developing retina of the youngest hosts (5–10 PN) is composed principally of the processes of the neuroepithelial progenitor cells, the nascent axons of the retinal ganglion cells (RGCs) and the retinal basal lamina. With continued development, the vitreal surface becomes thicker and more complex as additional RGC axons accumulate on the optic fiber layer. In addition, the ILM is formed by end feet of the Müller glial (retinal gliogenesis begins at approximately 15 PN) and the astrocytes begin migrating into the retina through the optic nerve and line the vitreal surface beginning at approximately 20 to 25 PN. Thus, the increase in physical complexity of the vitreal surface may inhibit or slow the migration of transplanted cells from the posterior chamber into the more mature retinas. It is interesting to note that we observed extensive morphologic integration of the mBPCs 4 weeks after transplantation into the youngest hosts (5–10 PN). The extensive morphologic integration occurred between 2 and 4 weeks after the transplantation of the mBPCs. In the case of the 10 PN hosts this would correspond to a developmental window between 24 and 38 PN. Transplants in comparable-aged hosts (24–38 PN) produced no incorporation. Additional studies are necessary to identify and determine what factors regulate the migration and integration of transplanted neural progenitor cells into the CNS.

Morphologically integrated mBPCs respected the architectural organization of the retina. Transplanted cells were principally localized to the nuclear layers, and their processes organized into appropriate layers based on retinal laminar position. Transplanted mBPCs located within the GCL and inner regions of the INL extended processes into the IPL. In many cases, the processes were restricted to specific sublamina within the IPL, either the ON or OFF sublamina. These results suggest that the migrating mBPCs were capable of detecting molecular cues present within the host retina. It is possible that the cues are intimately involved in regulating the incorporation of the transplanted cells. Of course, we cannot rule out the possibility that the grafted cells are simply restricted mechanically by the host microenvironment and adopt their morphologic attributes due to this mechanism.

Differentiation of mBPCs In Vivo

mBPCs can differentiate *in vivo*, producing characteristic retinal morphologies. Transplanted mBPCs adopted a variety of morphologies both within the vitreal chamber (all ages) and within the retina of young hosts. Transplanted cells with morphologies reminiscent of amacrine, bipolar, and horizontal cells were in the INL, and cells with morphologies similar to RGCs and displaced amacrine cells were in the GCL. No GFP-expressing cells adopted morphologies characteristic of mature photoreceptors. Our results also suggest mBPCs are capable *in vivo* of extending long processes after transplantation into 5-PN hosts, as GFP-expressing processes were observed extending within the optic nerve.

Many transplanted cells expressed mature neuronal phenotypes. MAP2 was coexpressed by many GFP-expressing cells positioned within the inner retina and vitreous. Because we observed no MAP2 expression in the mBPCs that were incorporated into the outer layers of the retina, this suggests an endogenous inhibitory signal present in the outer retina that may be involved in the regulation of MAP2. In the host retina, calretinin-IR amacrine cells are located in the inner INL and are equally spaced around the curvature of the retina. GFP-expressing calretinin-IR mBPCs were in locations appropriate for native amacrine cells. GFP-expressing cells located in the ONL were recoverin IR. These cells did not have typical photoreceptor morphologies but were nestled among inner segments

of surrounding receptors. No previous study has reported retina-specific differentiation of brain-derived cells. The regionalization of expression of specific neural phenotypic markers by transplanted cells strongly suggests that the mBPCs are capable of responding to local environmental cues within the retina.

Our results are consistent with previous work in rats with transplanted hippocampal progenitor cells.⁶⁻⁸ These studies demonstrated that although the mature diseased microenvironment is supportive of progenitor cell integration, only the immature normal retina possesses this property. In the current study we showed that by systematically evaluating recipient age and transplant outcome, we could determine the contribution of the host's developmental stage to the differentiation capacity of grafted progenitor cells. Future studies are needed to elucidate the molecular mechanism for this effect.

CONCLUSION

Neural stem-progenitor cells clearly possess a remarkable degree of plasticity. The data presented in this report demonstrate that mBPCs can survive intravitreal transplantation and incorporate within the intact, developing retina. We have shown for the first time that the developing environment may be critical in determining the fate of transplanted progenitor cells. Four weeks after transplantation into a developing environment, murine-derived neural progenitors integrated with the neural retina, respected the architectural organization, and adopted retinal-like morphologies and phenotypes.

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