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Green Fluorescent Protein Bone Marrow Cells Express Hematopoietic and Neural Antigens in Culture and Migrate Within the Neonatal Rat Brain

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Finding a reliable source of alternative neural stem cells for treatment of various diseases and injuries affecting the central nervous system is a challenge. Numerous studies have shown that hematopoietic and nonhematopoietic progenitors derived from bone marrow (BM) under specific conditions are able to differentiate into cells of all three germ layers. Recently, it was reported that cultured, unfractionated (whole) adult BM cells form nestin-positive spheres that can later initiate neural differentiation (Kabos et al., 2002). The identity of the subpopulation of BM cells that contributes to neural differentiation remains unknown. We therefore analyzed the hematopoietic and neural features of cultured, unfractionated BM cells derived from a transgenic mouse that expresses green fluorescent protein (GFP) in all tissues. We also transplanted the BM cells into the subventricular zone (SVZ), a region known to support postnatal neurogenesis. After injection of BM cells into the neurogenic SVZ in neonatal rats, we found surviving GFP^+ BM cells close to the injection site and in various brain regions, including corpus callosum and subcortical white matter. Many of the grafted cells were detected within the rostral migratory stream (RMS), moving toward the olfactory bulb (OB), and some cells reached the subependymal zone of the OB. Our in vitro experiments revealed that murine GFP^+ BM cells retained their proliferation and differentiation potential and predominantly preserved their hematopoietic identity (CD45, CD90, CD133), although a few expressed neural antigens (nestin, glial fibrillary acdiic protein, TuJ1). © 2004 Wiley-Liss, Inc.

Key words: bone marrow; green mouse; grafting; subventricular zone, developing rat brain

The identification of nonfetal cells capable of neuronal differentiation has great potential for numerous cellular therapies. Bone marrow (BM) contains therapeutically useful stem/progenitor cells and may be considered a possible alternative source of cells for neural grafting in the treatment of neurological diseases.

Several investigators have published reports on hematopoietic and nonhematopoietic stem cells derived from adult BM. Under certain, specific conditions, the nonhematopoietic BM cells differentiated into cells expressing neuronal and glial antigens (Azizi et al., 1998; Sanchez-Ramos et al., 2000; Woodbury et al., 2000, 2002) and also into myogenic progenitors (Ferrari et al., 1998). Multipotentiality was also noticed in unfractionated BM-derived cells. In transplantation studies, these cells were shown to express neural markers in the brain (Eglitis and Mezey, 1997; Mezey et al., 2000, 2003; Brazelton et al., 2000; Priller et al., 2001, Corti et al., 2002a; Hess et al., 2002) and spinal cord (Corti et al., 2002b) and also to differentiate into heart (Orlic et al., 2001) and liver (Petersen et al., 1999) cells.

In in vitro experiments under conditions commonly used for differentiating neural stem cells, whole BM was induced to form cellular spheres indistinguishable from neural stem cell neurospheres. These BM-derived spheres expressed neurogenin 1, a transcription factor found during specific stages of neural development (Kabos et al., 2002). After grafting into the neurogenically active hippocampus of adult rat, some of the transplanted BM cells integrated and tested positive for the neuronal marker NeuN. Thus, these whole BM-derived stem/progenitor cells can be differentiated in vitro by chemicals and growth factors or in vivo, in a suitable microenvironment.

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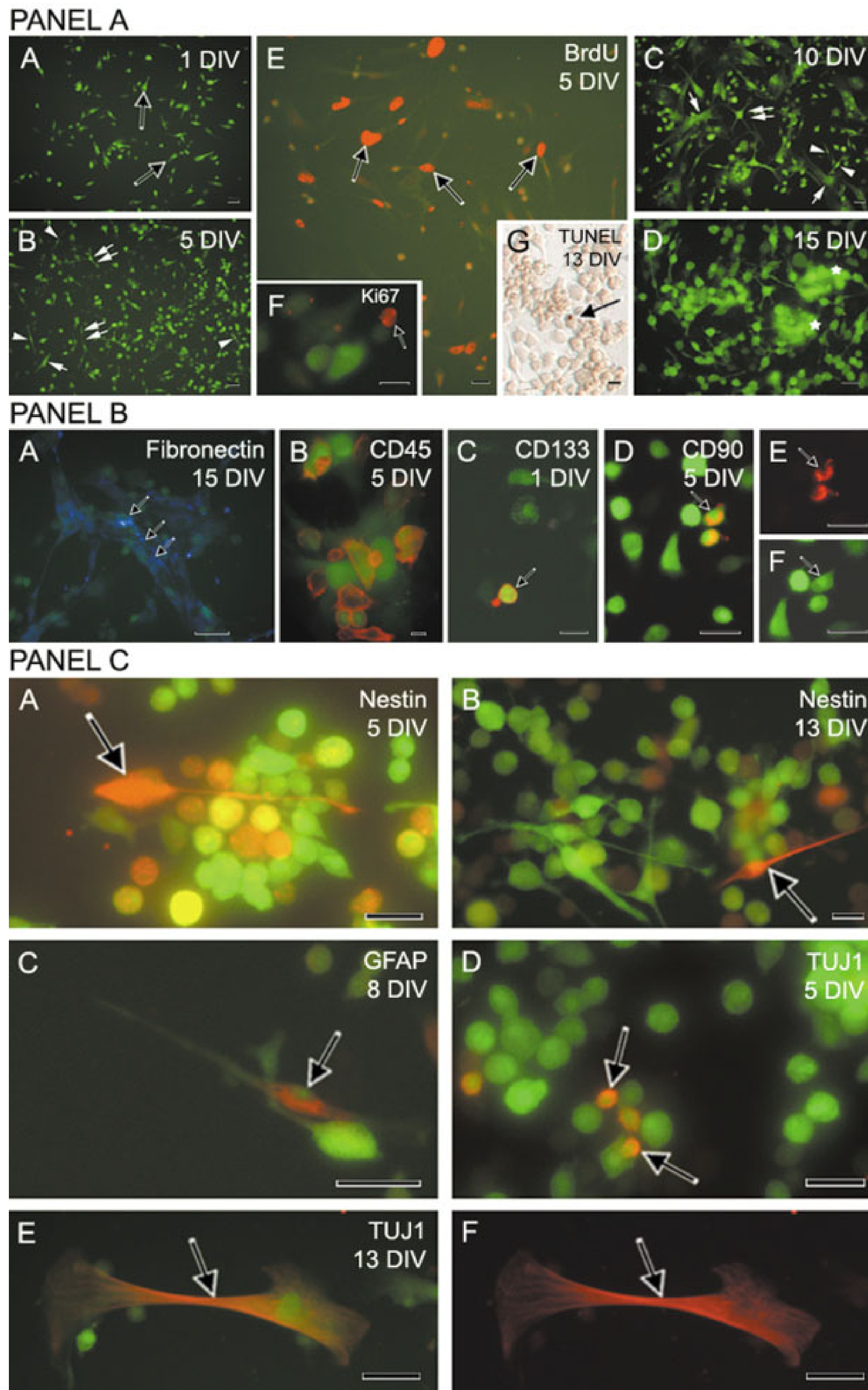


Figure 1.

In this study, we focused on the subventricular zone (SVZ), a life-long neurogenic region that provides developmentally important cues, such as epidermal growth factor (EGF), fibroblast growth factor-2 (FGF2), sonic hedgehog, cytokines, neurotrophic factors, bone morphogenic proteins (BMPs), and noggin (Reynolds and Weiss, 1992; Morshead et al., 1994; Palmer et al., 1995; Seroogy et al., 1995; Gross et al., 1996; Michaelson et al., 1996; Gritti et al., 1999; Lim et al., 2000; Sobeih and Corfas, 2002; Marshall et al., 2003). These signals are able to determine the cell's phenotypic and positional fate and to maintain a migratory state by providing guidance cues to motile cells. Our own previous studies demonstrated that the SVZ and its natural extension, the RMS, can support the survival and migration of various grafted cell types, from neural (Zigova et al., 1996, 2000; Yang et al., 2000) and nonneural (Zigova et al., 2002) sources. We used neonatal (0–2 days old) rats, because we expected these cues to be stronger in the younger, developing brain.

In the current study, we injected unfractionated BM cells that express green fluorescent protein (GFP) (Okabe et al., 1997) into the anterior part of the SVZ to determine whether progenitor cells from a different dermal origin would be able to survive, take distinct migratory pathways, and eventually adopt neural phenotypes after exposure to this young, highly neurogenic environment. At the same time, we plated GFP^{+} BM cells into media used for neuronal cultures to examine the fate of these cells by immunocytochemistry and to compare morphological and phenotypic features with the grafted cells.

MATERIALS AND METHODS

Preparation of BM- and SVZ-Derived Progenitors

BM cells were harvested from transgenic "green mice" that constitutively express GFP (C57BL/6-TgN; Jackson Laboratory, Bar Harbor, ME) according to the procedure described previously by Song and Sanchez-Ramos (2002). Briefly, after sacrifice of the animal, the femur and tibia were removed and flushed out with phosphate-buffered saline with 0.5% bovine serum albumin (pH 7.2). The marrow was collected and spun down, the supernatant discarded, and the resulting cell pellet suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, La Jolla, CA) with 10% fetal bovine serum (Invitrogen) and gentamicin (50 μl/ml; Sigma, St. Louis, MO) in a 15-ml conical tube (Falcon, Oxnard, CA) and centrifuged (400 g/7 min). The supernatant was removed, and the cells were resuspended in 1 ml of the above-described fresh medium. Their viability was assessed (85–92%) by using a rapid staining procedure with 0.4% trypan blue (Invitrogen). For culture, cells were plated at a density of 100,000 cells/cm^2 either on poly-L-lysine-coated (10 μg/ml; Sigma) eight-well chamber slides (Lab-Tek) or in 75-ml flasks (Corning, Corning, NY) and incubated at 37°C in 5% CO_2. The slides and flasks were incubated for up to 20 days with media changes every 3 days. For transplantation, BM-GFP cells were adjusted to 30,000 cells/μl.

SVZ progenitors were also prepared from the transgenic GFP mice. The SVZ was microdissected from the brain (Zigova et al., 1996; Zigova and Newman, 2002; Laywell et al., 2002) and collected into incubation medium [10 ml of Hank's balanced salt solution (HBSS; Invitrogen) containing 0.1% trypsin (Sigma) and 0.01 DNase (Sigma)] for 20 min at 37°C. After washing, the tissue was mechanically dissociated by gentle trituration, transferred into DMEM containing 10% fetal bovine serum, and spun down. The supernatant was removed and the resulting cell pellet resuspended into 1 ml of fresh medium for estimation of cell viability (~90%) and for transplantation (30,000 cells/μl).

Cell cultures from murine BM were fixed with 4% paraformaldehyde after 1–20 days in vitro (DIV) for 10 min at room temperature and stored in 0.1 M phosphate buffer (pH 7.4) at

Fig. 1. Morphology and proliferative capacity of cultured murine green-fluorescing BM cells. **A:** Parts A–D: Fluorescent photomicrograph showing the unfractionated population of green-fluorescing BM cells at 1 (part A), 5 (part B), and 10 (part C) days in vitro (DIV). Shortly after plating, round green-fluorescing cells attached to the dish and started to send out processes (part A). On days 5 (part B) and 10 (part C), we noticed several morphologically distinct cell types: very large, flat stromal cells (single arrows); spindle-shaped or elongated cells (arrowheads); and cells with two or more thin processes (double arrows). Around day 15, we found numerous clusters of cells (stars) throughout the entire culture dish (part D), suggesting that, under this culture condition, BM cells continue to proliferate (see part E). At this time point, the nuclearly localized fluorescing signal (green) was very strong, and in many instances we observed it in cellular bodies and processes. Part E: In cultures pulsed with BrdU 2 hr before fixation, we detected immunopositivity (red) in numerous cells regardless of their morphology, but mainly in small round cells without processes (5 DIV). Another proliferation marker, Ki67 (5 DIV), confirmed the proliferative capacity of BM-derived cells (part F). Occasional tdt-positive BM cells (arrow) were found in 13-day-old cultures (part G). **B:** Bone marrow cells express fibronectin and hematopoietic (CD) markers. Part A: Fluorescent photomicrograph showing AMCA-conjugated fibronectin positivity (blue, arrowheads) in processes and somas of BM cells (green) cultured for 15 days. Part B: At 5 DIV, approximately 80% of cultured BM cells expressed CD45, a surface antigen typical for hematopoietic lineages. Part C: Few green BM-derived cells revealed positivity for CD133, an early marker of hematopoietic progenitor cells (red, arrow). Part D: The presence of CD90 antigen indicated the presence of murine T cells (red, arrow). Parts E,F: Same image viewed through a dual filter (D). **C:** Cultured BM cells express neural antigens. Part A: Fluorescent photomicrographs demonstrating nestin positivity (red) in BM cells (green) at 5 (arrow) and 13 (part B) DIV (arrow). Part C: Fluorescent images of cultured (8 DIV) BM-derived cells (green) expressing glial fibrillary acidic protein (red) antigen, a marker of mature astrocytes. Parts D–F: Occasional positivity for early neuronal markers, IIIβ-tubulin (red), in 5 (part D)- and 13 (parts E,F)-day-old cultures taken under dual (part E) and single (part F) rhodamine filters. Immunopositivity was observed in small, round (part D) or stromal-like cells (parts E,F). The morphology of glial-positive cells varied substantially, and their overall number was higher than the number of cells revealing TuJ1 positivity. Scale bars = 20 μm in A(A–C,E); 25 μm in A(D), B(A,C–F), C(A–F); 16 μm in B(B); 10 μm in A(F,G); 10 μm in C (B).

4°C until immunocytochemistry for hematopoietic and neural markers was performed. To assess the proliferative capacity of BM cells, the cultures were exposed to the S-phase marker 5-bromo-2′-deoxyuridine (BrdU; Sigma; 10 μM) for 2 hr before fixation.

Transplantation of Cell Suspension Into the Neonatal SVZ

Timed-pregnant rats (Sprague-Dawley) were purchased from Harlan, Inc. (Indianapolis, IN). After birth, neonatal 1-day-old rats from separate litters were anesthetized by hypothermia according to the procedure described previously (Zigova et al., 1996, 2002b). Briefly, the head of the neonate pup was placed in a contoured Styrofoam mold, and an incision was made through the skin overlying the sagittal suture. A small hole was created in the skull on the right hemisphere (1 mm right from the midline, 2 mm anterior to Bregma), and a Hamilton syringe was lowered (2 mm deep to the pial surface) through the opening in the skull and 2 μl of cell suspension slowly delivered into the SVZ at a rate of 1 μl/min for 2–3 min (BM-GFP, n = 16; SVZ-GFP, n = 5). After transplantation, the skin was repositioned and sealed with surgical glue (Nexaband Liquid; Vet Prod Labs). The pups were placed under a heat lamp for recovery before returning them to their mother. This entire study followed the USF IACUC guidelines and the Principles of Laboratory Animal Care.

Cell and Tissue Processing and Immunocytochemistry

The purpose of our tissue culture assay was twofold: to establish the basic characteristics (phenotype and proliferative potential) of unfractionated, freshly prepared, and cultured BM-GFP cells and to work out double-labeling procedures for cell identification and further phenotypic characterization. These results were crucial for troubleshooting double- and triple-labeling procedures related to identification of transplanted cells, particularly in those cases when the green signal in the brain sections was not strong enough and the signal enhancement was followed by additional immunoprocedures.

At various times after implantation (2–70 days), the animals were anesthetized by ether inhalation and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were postfixed for 24 hr, immersed in cryopreservative (20% sucrose in 0.1 M PBS) overnight at 4°C, embedded in tissue freezing compound (TBS), and cryosectioned in the sagittal plane at 20 μm thickness.

For immunohistochemistry, the sections were thawed, rinsed thrice with PBS, and incubated in blocking solution (0.1 M PBS containing 10% goat serum and 0.01% Triton X-100) for 1 hr at room temperature, followed by incubation at 4°C overnight with the primary antibody. To enhance the GFP signal, a rabbit polyclonal anti-GFP antibody (Molecular Probes, Eugene, OR; 1:500) was employed. Rabbit polyclonal antifibronectin (Sigma; 1:400) to detect the presence of extracellular matrix molecules in BM cells and the rat monoclonal panleukocyte marker CD45 (Serotec; 1:100) to detect hematopoietic cells were utilized. The following mouse monoclonal antibodies were used: CD90.2 (Thy-1.2; Pharmingen, San Diego, CA; 1:20) for T lymphocytes and CD133 (Miltenyi Biotec; 1:35) to recognize hematopoietic stem/progenitor cells. The microglia were identified by the rat monoclonal OX42 (1:100; Serotec, Bicester, United Kingdom), whereas mouse anti-nestin (Pharmingen; 1:100), mouse anti-TuJ1 (Covance; 1:1,000), 1:500), and rabbit anti-glial fibrillary acidic protein (GFAP; Dako, Carpenteria, CA; 1:500) were used to recognize neural antigens. Controls in which the primary antibody was eliminated resulted in no staining.

For BrdU staining, cells were pretreated with 2 N HCl at 60°C and washed with borate buffer (pH 8.5) at room temperature, blocked for 1 hr, then incubated with rat anti-BrdU (Accurate, Westbury, NY; 1:500). To estimate the number of fluorescently labeled BrdU cells, the number of BrdU-positive and the total number of cells were counted after randomly choosing 20 fields. In addition, some slides were immunolabeled with sheep polyclonal Ki67 antibody (Chemicon, Temecula, CA; 1:100). Apoptosis was detected by using the terminal deoxynucleotidyl transferase (tdt) labeling technique (TACS apoptosis detection kit; R&D Systems, Minneapolis, MN). Positive and negative controls were carried out in parallel.

After being rinsed with PBS three times, the sections or cultured cells were incubated with species-appropriate fluorescent secondary antibodies conjugated to Alexa Fluor 594 (Molecular Probes; 1:700), Alexa Fluor 488 (Molecular Probes; 1:600), or Alexa Fluor 350 (Molecular Probes; 1:450) for 2 hr at room temperature. After being rinsed with PBS, the slides were coverslipped with either 95% glycerol or Vectashield mounting medium. Cell nuclei were visualized with DAPI (2 mg/ml; Roche). Cresyl violet-stained brain sections were used for identification of the injection site and for morphological evaluation of the brain. All cultures and brain sections were examined using the Olympus BX60 and IX71 microscopes. Selected slides were analyzed by confocal microscope (Nikon Inverted Diaphot), and images were processed using the Oncor Image software.

RESULTS

Morphology, Proliferative Capacity, and Phenotype of Green BM Cells

Shortly after plating (1–5 DIV), BM GFP cells began to develop into a heterogeneous population of cells with very distinct cellular morphologies (Fig. 1A, parts A, B). The diversified morphology became even more evident with longer culture periods (Fig. 1A, parts C, D). We frequently observed, in addition to small, round, spindle-shaped, and elongated cells with two or more processes, large, flat, stromal-like cells (Fig. 1A, parts B–D). When we pulsed BM cultures with the cell proliferation marker BrdU, we found nuclear labeling in all studied intervals and in all cell types (Fig. 1A, part E). Cell proliferation was also confirmed by the second proliferation marker, Ki67 (Fig. 1A, part E, inset F). At the same time, the low incidence of apoptotic cells (Fig. 1A, part E, inset G) suggested that BrdU incorporation reflects the proliferative capacity of cultured BM cells (for at least the 3 weeks for which they had been in culture; Table I) and not their near-death stage.

The expression of fibronectin, a marker typical for BM cells (Bentley and Tralka, 1983), was found mainly in round and stromal-like cells (Fig. 1B, part 1) at 5, 10, and

TABLE I. Proliferative Capacity of Cultured BM Cells Expressing GFP

	DIV			
	1 (%)	5 (%)	10 (%)	15 (%)
BrdU$^+$/total[a]	94/383 (24.5)	179/462 (38.7)	462/1,035 (44.6)	330/805 (41.0)
Prevalent morphology[b]	1	1, 2	1	1

[a]Ratio between the total number of cells and those incorporating the proliferation marker BrdU. The counts were collected from 20–25 fields/slide/time point.
[b]Prevalent morphology of plated BM-GFP$^+$ cells incorporating BrdU: 1, round, small cells; 2, other cell types (including spindle-shaped, elongated, and large, flat cells).

TABLE II. Spatiotemporal Distribution of Grafted Murine GFP$^+$ Cells Following Their Transplantation Into the Neonatal SVZ*

	Rat No.	Age of host	Survival (days)	Final position of GFP$^+$ cells						
				SVZ	RMS	OB	CC	CT	SWM	LV
Homotopic SVZa grafts	1	P1	14	+	+	+	+	−	−	−
	2	P1	21	+	+	+	+	−	−	−
	3	P1	21	−	+	+	−	+	−	−
	4	P1	31	−	+	+	−	+	−	+
	5	P1	56	+	+	+	−	+	−	−
Bone marrow grafts	1	P2	2	+	+	−	+	+	−	−
	2	P2	8	−	−	+	+	+	−	−
	3	P1	14	+	−	+	+	−	+	−
	4	P2	21	+	−	−	+	+	−	−
	5	P1	30	−	−	−	+	−	−	−
	6	P2	35	−	+	−	+	+	−	−
	7	P2	43	+	−	−	+	+	−	−
	8	P1	56	−	+	−	+	−	−	+
	9	P1	70	+	+	+	+	−	−	−
	10	P1	70	+	+	+	+	−	−	+

*Summary of the distribution of SVZ-GFP$^+$ and BM-GFP$^+$ cells within the brain regions at various postinjection survival times. Among 22 animals that received the transplant of GFP$^+$ cells, 15 were used for analysis; in 4 animals the processing was not satisfactory, and in 3 animals the transplant was not found, so they were excluded from total counts and final analysis. Representative images were taken from sections prepared from rats 1 and 2 for SVZ and rats 2 and 3 for BM transplants. CC, corpus callosum; CT, cortex; LV, lateral ventricle; OB, olfactory bulb; RMS, rostral migratory stream; SWM, subcortical white matter; SVZ, subventricular zone; +, presence of cells; −, absence of cells.

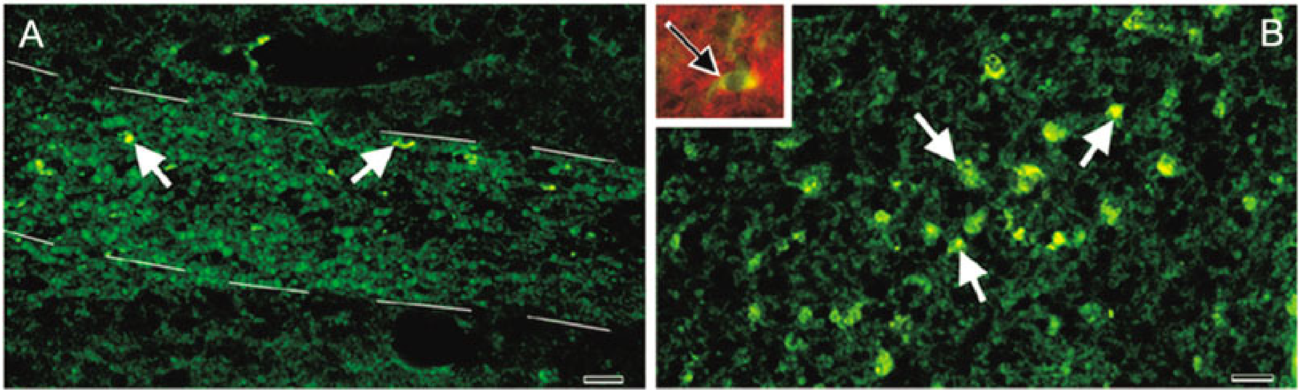
15 DIV. At 5 DIV, 8.1% of the population showed fibronectin positivity that increased to 26.4% at 10 DIV and remained at this level at 15 DIV (28.0%).

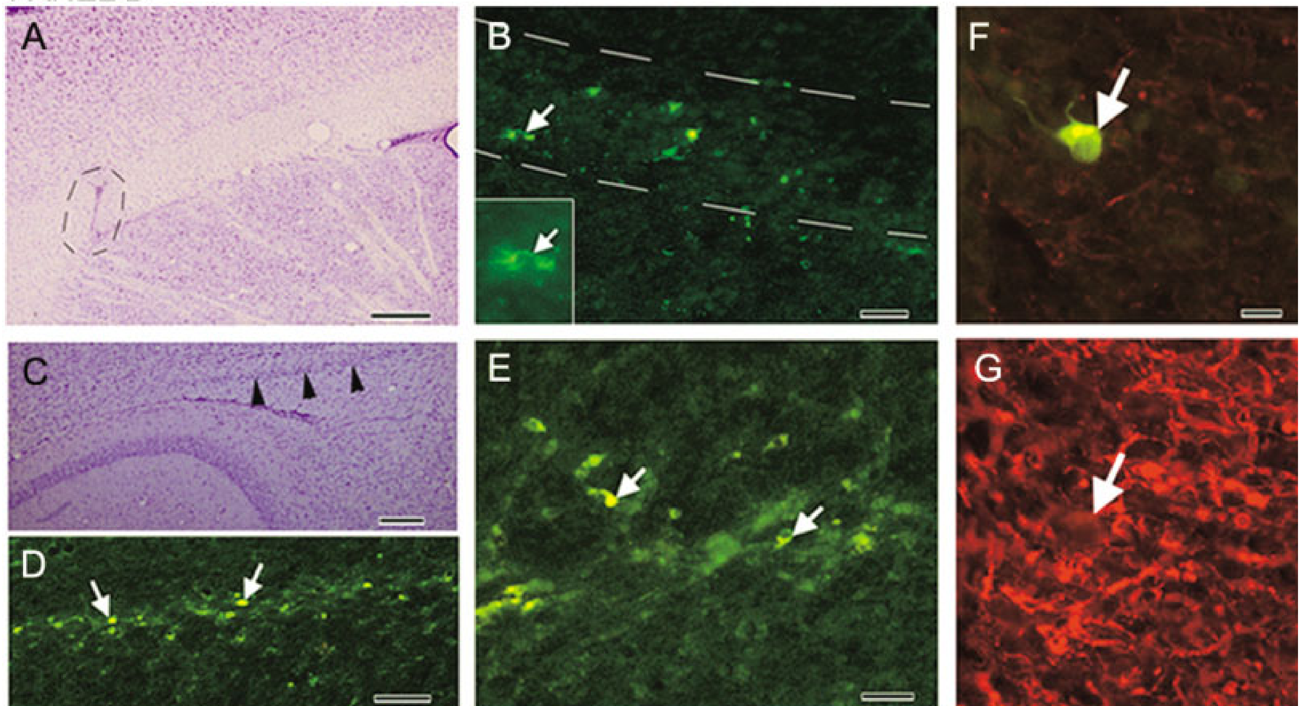
The pan-leukocyte marker CD45 was observed in all cell types (85%) except for the large stromal cells (Fig. 1B, part B). The majority of BM cells retained the expression of this marker throughout the culture time points studied (5–20 DIV). The rare occurrence of the T-cell marker CD90 (1.2–1.8%) and CD133 (>1%), presumable markers of stem/progenitor cells, was also observed (Fig. 1B, parts C–F). The cells expressing these antigens were usually round and small.

We also wanted to determine whether the culture environment could induce the BM cells to express neural phenotypes and whether these phenotypes could be confirmed by distinct neuronal and glial morphologies. We noticed, in 5- and 13-day-old cultures, immunopositivity for nestin, a marker for neural progenitor cells (Lendahl et al., 1990). The immunolabeling for this antigen was strictly related to round, small cells or to cells with few processes (Fig. 1C, parts A, B). The presence of cells expressing glial fibrillary acidic protein (GFAP), an antigen typical for mature glial cells (Dahl et al., 1985), was very rare, and, interestingly, morphology of GFAP-positive BM cells did not match the classical description or appearance of glial cells (Fig. 1C, part C). The early neuronal marker TuJ1 was detected in some GFP$^+$ cells (Fig. 1C, parts D–F). Morphologically, the GFP/TuJ1-colabeled cells were either small, round cells or cells with stromal-like appearance. In general, the number of cells expressing GFAP (~4.2%) was higher than the number revealing immunopositivity for IIIβ-tubulin (~2.6%) at days 5, 8, and 13.

Transplantation of Murine SVZ-GFP$^+$ Progenitors Into the SVZ of the Neonatal Rat

This experiment was performed as a control experiment for the BM transplantation. Knowing from our previous studies that homotopically grafted progenitors (rat SVZ progenitors into the rat SVZ) behave like endogenous SVZ-derived cells (Zigova et al., 1996), we based our current control experiments on those findings. In this experiment, we transplanted SVZ cells from transgenic GFP mice into rat SVZ. This provided the advan-





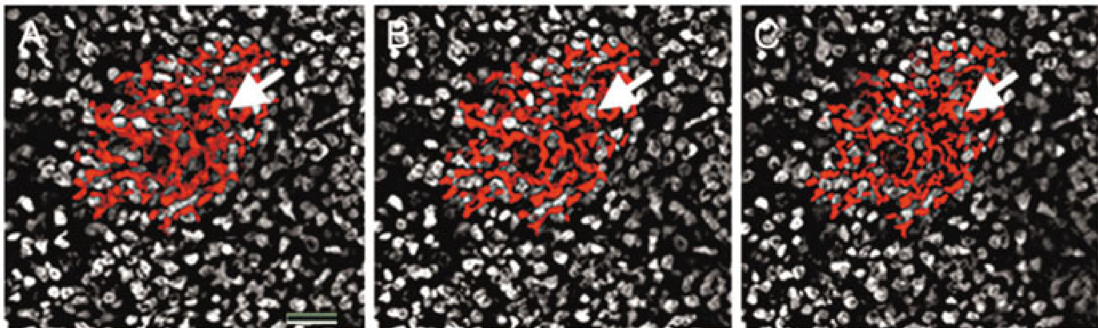


Figure 2.

tage of easy identification of the grafted cells by their green fluorescence, which remained detectable by enhanced imunolabeling even throughout the longest postgrafting period.

Depending on the survival time (Table II), transplanted murine SVZ progenitors were found around the injection site in the anterior part of the SVZ and along the full extent of the RMS (Fig. 2A, part A). Within 1 week, many of these cells reached the subependymal layer of the olfactory bulb (OB) and mingled with endogenous interneurons (Fig. 2A, part B). Two weeks after grafting, many of the green cells began to send out processes and morphologically resemble OB cells (Fig. 2A, part B, and inset). At the same time, these cells acquired a neuron-specific class IIIβ-tubulin immunoreactivity. These findings confirm the ability of murine SVZ-derived progenitors to follow the same migratory route as endogenous analogs and to settle in the OB while expressing neuronal cell type-specific antigen commonly found in this region (Zigova et al., 1998).

Distribution and Phenotype of GFP$^+$ BM Cells After Transplantation Into the SVZ of the Neonatal Rat

To test the potential of the whole BM for neural differentiation in vivo, we transplanted freshly prepared cells into the anterior part of the SVZ of neonatal (1-day-old) rat pups. It is believed that this neurogenically active region, rich in neurotrophic factors, may support neural differentiation of those cells in the BM that are not fully committed to hematopoietic fate.

After transplantation, animals survived from 2 days up to 10 weeks (Table II). In those animals with shorter survival (2 days), the GFP$^+$ BM cells were located only close to the injection site (Fig. 2B, part A). With increased survival time after grafting (1–3 weeks), the cells were found in corpus callosum and cortex and posteriorly in the subcortical white matter (Fig. 2B, parts C–E). At the same time, many BM cells were observed within the vertical or horizontal limb of the RMS, and some of them resembled the morphology of migrating neuroblasts (Fig. 2B, part B). Few single cells reached the subependymal zone of the OB. Morphologically, these cells were small, usually with one or two relatively short processes, similar, in some cases, to the OB granule cells (Fig. 2B, parts F, G). In one instance, we found a clump of BM cells with very short and thick processes, in the core of the OB (Fig. 2C, parts A–C). Phenotypically, these BM cells either as single cells or in clump formation displayed very low positivity for hematopoietic (CD45) antigens in vivo and did not reveal immunoreactivity to any of the neural (nestin, TuJ1, GFAP) antigens.

DISCUSSION

In this study, we demonstrated that GFP$^+$ murine BM cells transplanted into the neonatal SVZ were able to migrate from the injection site in the anterior part of the SVZ along the RMS and reach the OB. Some green donor cells were found dorsally by the SVZ–white matter interface. In almost every case, we noted cells confined to the injection site. The green fluorescence signal allowed easy detection of donor cells even in the longest survival periods after grafting (2–70 days). In some instances, the signal was converted with an antibody against GFP, confirming that the cell fluorescence resulted from the expression of the marker gene. Phenotypically, these BM cells in the most remote locations from the injection site (OB and subcortical white matter) did not show glial or neuronal immunoreactivity, and CD45 expression was very weak. In cultures (1–15 days), which served as comparisons of cellular morphology and phenotype for grafted BM cells, we found positivity for CD markers (CD45, CD90,

Fig. 2. **A:** After implantation into the neonatal SVZ, precursors isolated from green mouse SVZ followed the same migratory route, resided in the OB, and expressed neuronal antigens as their endogenous counterparts. The animals (n = 5) survived 14–56 days after surgery (see Table II). Part A: After injection, green-fluorescing SVZ cells were found in the RMS (marked by dashed lines) leading to the OB. Part B: Note many green SVZ-derived cells within the subependyma of the OB (arrows). **Inset** shows one of the numerous GFP-SVZ cells (green) reaching the OB, colabeled with antibody against IIIβ-tubulin (red, 14 days after grafting). **B:** BM cells isolated from green mouse were found in the SVZ, the RMS, and the OB. These cells did not express neural markers after reaching the OB. The animals (n = 10) survived for 2–70 days after grafting (see Table II). Part A: Cresyl violet-stained parasagittal section through the host brain showing the injection site (encircled area) targeting the SVZ. The confined, darkly stained BM cells were easily recognized within the brain parenchyma (70 days survival after grafting). Part B: Several GFP-positive BM-derived cells were found within the delineated (dashed lines) migratory route. The arrow points to the cell, which is magnified in the **inset**. Part C: Brightfield image of a cresyl violet-stained section through the neonatal rat brain showing the area in the posterior subcortical white matter overlying the hippocampus (arrowheads). We detected within this region several BM-derived green-fluorescing cells (arrows), shown in part D. Part E: Higher magnification allows detailed observation of several cells with green fluorescent signal (arrows). Part F: Fluorescent photomicrograph of the single GFP-positive BM-derived cell (arrow) within the OB subependyma (14 days after grafting) observed under the dual (rhodamine and FITC) filter. Part G: The same section was stained for glial fibrillary acidic protein (GFAP), a marker of astrocytic glia. The cell indicated by the arrow is GFAP negative (rhodamine filter). **C:** Confocal images of the transplanted BM cells isolated from GFP mouse. These cells were implanted into the anterior part of the SVZ of a 1-day-old rat pup. Eight days later, the cells reached the subependymal layer of the OB. On this particular section, the green signal was converted through the rhodamine-conjugated anti-GFP antibody to obtain stronger red signal. DAPI counterstain confirmed the singleness of BM-derived nuclei surrounded by GFP immunopositivity. Parts A–C: Fluorescent photomicrographs showing the cluster of BM-derived cells (red) in the OB subependyma taken through a 20-μm-thick section. The arrow points to a single cell that was followed in three consecutive images, 2 μm apart. Scale bars = 20 μm in A(A), B(B), in C(A) for C(A–C); 10 μm in A(B), in B(F) for B(F,G); 200 μm in B(A,C,D); 50 μm in B(E).

CD133) and occasionally for common neural antigens (nestin, GFAP, TuJ1); however, none of these cells displayed morphology characteristic of neurons or mature glia.

Early Postnatal SVZ Supports Migration of Endogenous Progenitors Originating From SVZ

Previous studies suggested two distinct migratory patterns of SVZ-derived progenitor cells: radial (coronal) in the posterior part and tangential (rostrocaudal) in the anterior part of the forebrain. These migration pathways were also closely associated with either neuronal (neurogenic pathways) or glial (gliogenic pathways) phenotypes. However, the long-distance migration of progenitor cells from the SVZ toward the OB differs in neonatal and adult rodent brain. Luskin (1993) described the tangential migration of dividing neuronal progenitors originating from the anterior aspect of the early postnatal SVZ, giving rise to OB interneurons. Doetsch and Alvarez-Buylla (1996) noted in juvenile and adult animals a network of chains of neuronal progenitors throughout the lateral wall of the lateral ventricle rising from the full rostrocaudal extent of the SVZ. Retroviral injection studies examining the migratory patterns of neural progenitors within and out of the SVZ (Suzuki and Goldman, 2003) confirmed that, depending on the injection placement, neonatal SVZ progenitors migrated along the entire rostrocaudal extent of the SVZ, including the caudal tip of the SVZ at the hippocampal border and out of the RMS toward the OB or along the SVZ adjacent to the striatum.

Neonatal Brain Supports Migration of Neural and Nonneural Cell Types

Transplantation studies designed to examine the migratory properties of various cell types within the SVZ showed that cells derived from the anterior SVZ are able to resume migratory behavior and spatiotemporal distribution within the RMS and OB similar to that of their endogenous analogs (Zigova et al., 1996). In the current study, we used GFP^+ mouse cells to trace their migratory fate after implantation into the anterior part of the SVZ in neonatal rats. Regardless of the different species and age of the host and donor (adult mouse SVZ progenitors were grafted into the neonatal rat SVZ), the GFP^+ SVZ-derived cells were found in the subependymal OB of the rat within 3 weeks after transplantation. Easy identification of green-fluorescing GFP^+ cells allowed for their detection in distant locations from the injection placement. In one case, we found cells in the subcortical white matter (Fig. 2B, parts C–E) posterior to the injection site. One explanation would be that injection of cells was placed more posterior than in the rest of animals, so some injected cells first migrated to the posterior SVZ and then commenced the radial migratory movement toward the overlying subcortical white matter. Another possible explanation would be that these cells migrated along myelinated (Fulton et al., 1992) and unmyelinated (Golden et al., 1997) axons in the developing subcortical white matter pathways, such as the superior longitudinal or the arcuate fasciculi. This, however, has to be elucidated.

Previously we have also tested the migratory properties of other cells, known as hNT neurons (Zigova et al., 2000). These human, neuronally committed cells were also able to move within the RMS, and a few of them even reached the granule cell layer of the OB, similarly to postnatally generated OB interneurons. Most of them, however, stayed close to the injection site in the SVZ. This reduction in number of cells reaching the OB after tangential movement along the RMS and radial migration through the OB granule cell layer was attributed to the human origin of these cells as well as to different stages of their neuronal commitment at the time of grafting.

Transplantation of predifferentiated human umbilical cord blood cells into the anterior part of the SVZ revealed that these originally nonneural cells had migrated only through the very initial part of the RMS and never reached the OB. Many of them were trapped in the cortical parenchyma, in the corpus callosum, or around cortical blood vessels (Zigova et al., 2002b). Here, the freshly prepared whole bone marrow-derived cells were found within the entire extent of the RMS and also in the OB, indicating that these two cell sources, both derived from a mesodermal germ layer, have different abilities to read the cues within the RMS and OB. It is possible that dissimilarities in the migratory behavior of human and mouse donor cells reflect the species incompatibility and the differences in the development of progenitor cells. Extensive migratory behavior of BM cells derived from marrow stromal cells was demonstrated by Kopen at al. (1999). These investigators engrafted murine marrow stromal cells into the lateral ventricle of the neonatal mice and followed their migratory patterns and phenotypic fate over the 12-day period. Engrafted cells were detected in many brain regions, including the islands of Calleja, OB, cerebellum, and brainstem.

Migratory Properties, Phenotype, and Proliferation of BM-Derived Cells

There is now evidence that BM cells can engraft into the brain parenchyma regardless of the route by which they were administered (systemic vs. intraparenchymal). However, differences, such as final phenotype of transplanted cells, distribution, and proliferative capacity, were noted in various experimental models. Systemic BM transplantation with GFP-expressing cells from transgenic mice resulted in migration of cells into peripheral tissues (Ono et al., 2003) and brain parenchyma (Ono et al., 1999, 2003; Corti et al., 2002a; Hess et al., 2002). In our study, the BM cells were delivered into a small target area within the neurogenic SVZ, the region that is known to be a part of the natural migratory pathway of progenitor glial and neuronal cells. After placing BM cells into this region, we found them in two distinct places (RMS/OB and subcortical white matter), which were relatively remote from the injection site, so this could not be explained as a passive distribution or artifact caused by a needle or volume or pressure developed during the cell delivery. This observa-

tion also argues for two possible phenotypes of transplanted cells, neuronal within the subependymal zone of the OB and glial in the subcortical white matter. In our study, we did not confirm the colocalization of the GFP signal either with GFAP or with TuJ1, even though some cultured BM cells revealed it (Fig. 1C). The most probable explanation for the absence of neural antigens in grafted cells may be the short survival time (2 weeks), insufficient for the cells to start any differentiation. Other authors usually report immunopositivity for neural antigens in grafted BM progenitors after extended periods (Mezey et al., 2000, 2003; Brazelton et al., 2000; Priller et al., 2001).

Ono et al. (2003) investigated the cytochemical differences in cells that had migrated into the brain and other tissues after 7 days with antibodies against CD34 (hematopoietic stem cell marker), Mac-1 (C3bi receptor marker), and ER-MP12 (murine early-stage myeloid precursor marker). They found that clusters of BM-derived cells in the liver and other organs were mainly $CD34^{-}$, $Mac\text{-}1^{+/-}$, $ER\text{-}MP12^{-}$, whereas those in the brain were $CD34^{-}$, $Mac\text{-}1^{+}$, $ER\text{-}MP12^{+}$, suggesting that clusters in the brain were in a different state of differentiation from those in other tissues. In our studies, however, the cluster of BM cells within the OB (14 days postgrafting) did not show colocalized OX42 (marker of microglia) antibody reactivity. This may be explained again by the shortness of survival, or perhaps other microglia-recognizing antibodies such as F4/80 and ER-MP12 have to be employed.

Another interesting finding reported by other authors was the presence of clusters of BM cells in the brain parenchyma, suggesting that these cells are actively proliferating while reaching their final destination (Ono et al., 2003). In our study, we noted the cluster of BM-derived cells within the OB, probably also indicating the proliferative capacity of specific subpopulation of BM cells. Incorporation of BrdU in cultured (1–15 DIV) BM cells supports this finding. Here we can speculate that migratory behavior associated with proliferative capacity of progenitor cells can reflect two scenarios. The first possibility is that some of these cells were delivered to the ventricle and were moved with the cerebrospinal fluid toward the OB ventricular system. This would explain the facts that some early hematopoietic progenitors started to proliferate and that the clump was trapped in the subventricular zone of the OB. Ono et al. (2003) reported the presence of similar clumps of cells within the brain parenchyma after systemic administration of the same cell source. The second explanation would be based on the parallel observation of endogenous neural progenitors from SVZ/RMS/OB region, for which migration of proliferating neuroblasts has been previously described (Menezes et al., 1995). The second possibility would also suggest (indirectly) that hematopoietic and neural cells might have common characteristics or that immature cells within those two cell sources have an overlapping set of cytochemical properties (receptors, proteins, etc.) that allow them to behave similarly in the specific brain environment.

Further studies are warranted to understand better the migratory and phenotypic potential of progenitors originating from hematopoietic sources such as BM and human umbilical cord blood and also to explain the differences between theses cell sources with respect to recipient age. This will allow us to determine effective therapeutic strategies for treatment of neurodegenerative diseases occurring predominantly in older individuals.

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REFERENCES

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ. 1998. Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats—similarities to astrocyte grafts. Proc Natl Acad Sci USA 95:3908–3913.

Bentley SA, Tralka TS. 1983. Fibronectin-mediated attachment of hematopoietic cells to stromal elements in continuous bone marrow culture. Exp Hematol 11:129–138.

Brazelton TR, Rossi FM, Keshet GI, Blau HM. 2000. From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290: 1775–1779.

Corti S, Locatelli F, Strazzer S, Salani S, Del Bo R, Soligo D, Bossolasco P, Bresolin N, Scarlato G, Comi GP. 2002a. Modulated generation of neuronal cells from bone marrow by expansion and mobilization of circulating stem cells with in vivo cytokine treatment. Exp Neurol 177:443–452.

Corti S, Locatelli F, Donadoni C, Strazzer S, Salani S, Del Bo R, Caccialanza M, Bresolin N, Scarlato G, Comi GP. 2002b. Neuroectodermal and microglial differentiation of bone marrow cells in the mouse spinal cord and sensory ganglia. J Neurosci Res 70:721–733.

Dahl D, Crosby CJ, Sethi JS, Bignami A. 1985. Glial fibrillary acidic (GFA) protein in vertebrates: immunofluorescence and immunoblotting study with monoclonal and polyclonal antibodies. J Comp Neurol 239:75–88.

Doetsch F, Alvarez-Buylla A. 1996. Network of tangential pathways for neuronal migration in adult mammalian brain. Proc Natl Acad Sci USA 93:14895–14900.

Eglitis MA, Mezey E. 1997. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. Proc Natl Acad Sci USA 94:4080–4085.

Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528–1530.

Fulton BP, Burne JF, Raff MC. 1992. Visualization of O-2A progenitor cells in developing and adult rat optic nerve by quisqualate-stimulated cobalt uptake. J Neurosci 12:4816–4833.

Golden JA, Zitz JC, McFadden K, Cepko CL. 1997. Cell migration in the developing chick diencephalon. Development 124:3525–3533.

Gritti A, Frolichsthal-Schoeller P, Galli R, Parati EA, Cova L, Pagano SF, Bjornson CR, Vescovi AL. 1999. Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. J Neurosci 19:3287–3297.

Gross RE, Mehler MF, Mabie PC, Zang Z, Santschi L, Kessler JA. 1996. Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron 17:595–606.

Hess DC, Hill WD, Martin-Studdard A, Carroll J, Brailer J, Carothers J. 2002. Bone marrow as a source of endothelial cells and NeuN-expressing cells after stroke. Stroke 33:1362–1368.

Kabos P, Ehtesham M, Kabosova A, Black KL, Yu JS. 2002. Generation of neural progenitor cells from whole adult bone marrow. Exp Neurol 178:288–293.

Kopen GC, Prockop DJ, Phinney DG. 1999. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 96:10711–10716.

Laywell ED, Kukekov VG, Suslov O, Zheng T, Steindler DA. 2002. Production and analysis of neurospheres from acutely dissociated and postmortem CNS specimens. Methods Mol Biol 198:15–27.

Lendahl U, Zimmerman LB, McKay RD. 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595.

Lim DA, Tramontin AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A. 2000. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron 28:713–726.

Luskin MB. 1993. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron 11:173–189.

Marshall CA, Suzuki SO, Goldman JE. 2003. Gliogenic and neurogenic progenitors of the subventricular zone: who are they, where did they come from, and where are they going? Glia 43:52–61.

Menezes JR, Smith CM, Nelson KC, Luskin MB. 1995. The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. Mol Cell Neurosci 6:496–508.

Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. 2000. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 290:1779–1782.

Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. 2003. Transplanted bone marrow generates new neurons in human brains. Proc Natl Acad Sci USA 100:1364–1369.

Michaelson MD, Mehler MF, Xu H, Gross RE, Kessler JA. 1996. Interleukin-7 is trophic for embryonic neurons and is expressed in developing brain. Dev Biol 179:251–263.

Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D. 1994. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 13:1071–1082.

Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 1997. "Green mice" as a source of ubiquitous green cells. FEBS Lett 407:313–319.

Ono K, Takii T, Onozaki K, Ikawa M, Okabe M, Sawada M. 1999. Migration of exogenous immature hematopoietic cells into adult mouse brain parenchyma under GFP-expressing bone marrow chimera. Biochem Biophys Res Commun 262:610–614.

Ono K, Yoshihara K, Suzuki H, Tanaka KF, Takii T, Onozaki K, Sawada M. 2003. Preservation of hematopoietic properties in transplanted bone marrow cells in the brain. J Neurosci Res 72:503–507.

Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. 2001. Bone marrow cells regenerate infarcted myocardium. Nature 410:701–705.

Palmer TD, Ray J, Gage FH. 1995. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci 6:474–486.

Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. 1999. Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170.

Priller J, Persons DA, Klett FF, Kempermann G, Kreutzberg GW, Dirnagl U. 2001. Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo. J Cell Biol 155:733–738.

Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256.

Seroogy KB, Gall CM, Lee DC, Kornblum HI. 1995. Proliferative zones of postnatal rat brain express epidermal growth factor receptor mRNA. Brain Res 670:157–164.

Sobeih MM, Corfas G. 2002. Extracellular factors that regulate neuronal migration in the central nervous system. Int J Dev Neurosci 20:349–357.

Song S, Sanchez-Ramos J. 2002. Preparation of neural progenitors from bone marrow and umbilical cord blood. Methods Mol Biol 198:79–88.

Suzuki SO, Goldman JE. 2003. Multiple cell populations in the early postnatal subventricular zone take distinct migratory pathways: a dynamic study of glial and neuronal progenitor migration. J Neurosci 23:4240–4250.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. 2000. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370.

Woodbury D, Reynolds K, Black IB. 2002. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J Neurosci Res 69:908–917.

Yang H, Mujtaba T, Venkatraman G, Wu YY, Rao MS, Luskin MB. 2000. Region-specific differentiation of neural tube-derived neuronal restricted progenitor cells after heterotopic transplantation. Proc Natl Acad Sci USA 97:13366–13371.

Zigova T, Betarbet R, Soteres BJ, Brock S, Bakay RA, Luskin MB. 1996. A comparison of the patterns of migration and the destinations of homotopically transplanted neonatal subventricular zone cells and heterotopically transplanted telencephalic ventricular zone cells. Dev Biol 173:459–474.

Zigova T, Pencea V, Wiegand SJ, Luskin MB. 1998. Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. Mol Cell Neurosci 11:234–245.

Zigova T, Pencea V, Sanberg PR, Luskin MB. 2000. The properties of hNT cells following transplantation into the subventricular zone of the neonatal forebrain. Exp Neurol 163:31–38.

Zigova T, Newman MB. 2002a. Transplantation into neonatal rat brain as a tool to study properties of stem cells. Methods Mol Biol 198:341–356.

Zigova T, Song S, Willing AE, Hudson JE, Newman MB, Saporta S, Sanchez-Ramos J, Sanberg PR. 2002b. Human umbilical cord blood cells express neural antigens after transplantation into the developing rat brain. Cell Transplant 11:265–274.