

Neogenesis of cerebellar Purkinje neurons from gene-marked bone marrow cells in vivo

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The versatility of stem cells has only recently been fully recognized. There is evidence that upon adoptive bone marrow (BM) transplantation (BMT), donor-derived cells can give rise to neuronal phenotypes in the brains of recipient mice. Yet only few cells with the characteristic shape of neurons were detected 1–6 mo post-BMT using transgenic or newborn mutant mice. To evaluate the potential of BM to generate mature neurons in adult C57BL/6 mice, we transferred the enhanced green fluorescent protein (GFP) gene into BM cells using a murine stem cell virus-based retroviral vector. Stable and high level long-

term GFP expression was observed in mice transplanted with the transduced BM. Engraftment of GFP-expressing cells in the brain was monitored by intravital microscopy. In a long-term follow up of 15 mo post-BMT, fully developed Purkinje neurons were found to express GFP in both cerebellar hemispheres and in all chimeric mice. GFP-positive Purkinje cells were also detected in BM chimeras from transgenic mice that ubiquitously express GFP. Based on morphologic criteria and the expression of glutamic acid decarboxylase, the newly generated Purkinje cells were functional.

Introduction

Several recent studies suggest that adult cell types may cross the boundaries determined by embryologic trilaminar origin. Bone marrow (BM)*-derived cells were found to differentiate into skeletal muscle (Ferrari et al., 1998; Gussoni et al., 1999), cardiomyocytes (Orlic et al., 2001), and epithelial cells of liver, lung, intestinal tract, and skin (Petersen et al., 1999; Lagasse et al., 2000; Krause et al., 2001). Stem cells isolated from skeletal muscle or brain adopted a hematopoietic fate in vivo (Jackson et al., 1999; Bjornson et al., 1999). In the central nervous system (CNS), which has traditionally been considered a secluded site, BM-derived cells gave rise to astrocytes and cells expressing neuronal antigens (Eglitis and Mezey, 1997; Kopen et al., 1999; Brazelton et al., 2000; Mezey et al., 2000). At least 0.2% of all cells expressing the

neuronal marker, NeuN, were BM-derived 4 mo after BM transplantation (BMT). It is at present unclear whether bone marrow stem cells overcome their intrinsic restrictions upon exposure to nervous system environment (“transdifferentiation”), or are truly pluripotent stem cells capable of supplying the adult CNS with neurons and glia. It is also unclear whether the bone marrow-derived neuronal phenotypes observed in previous studies persist in the brain after BMT and develop into functional neurons. Recently, single hematopoietic stem cells were found to long-term repopulate irradiated mice and differentiate into epithelia of various organs (Krause et al., 2001). In this study, engraftment of gene-marked BM cells was investigated in the brain up to 15 mo posttransplantation. Single, fully developed cerebellar Purkinje neurons were found to derive from the donor BM, underscoring the tremendous differentiative capacity of adult BM cells.

Results and discussion

BM cells were transduced with a retroviral vector encoding green fluorescent protein (GFP) and an antifolate-resistant dihydrofolate reductase gene. This system permits selection at the stem cell level to increase the number of gene-modi-

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*Abbreviations used in this paper: BM, bone marrow; BMT, BM transplantation; CNS, central nervous system; GABA, γ -aminobutyric acid; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein.

Key words: bone marrow transplantation; gene transfer; green fluorescent protein; nervous system; Purkinje cells

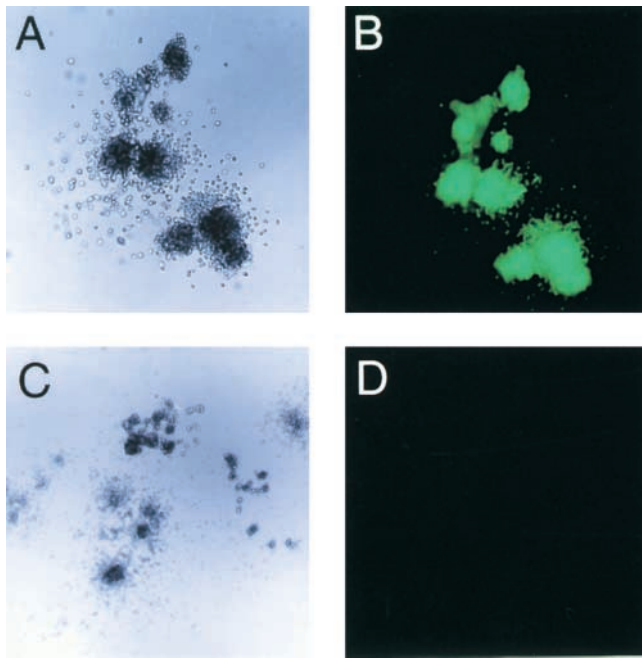


Figure 1. Retroviral-mediated transfer of the GFP gene into hematopoietic cells. Adult BM cells were transduced with a murine stem cell virus-based retroviral vector containing the GFP marker gene. Cells were subsequently plated in methylcellulose supplemented with hematopoietic growth factors, and analyzed for GFP expression after 7 d in culture. The top two panels show identical GFP-transduced hematopoietic colonies, visualized separately under phase-contrast (A) and fluorescence (B) microscopy. The bottom two panels demonstrate nontransduced hematopoietic colonies under phase-contrast (C) and fluorescence (D) microscopy. Note the lack of background fluorescence in (D).

fied cells after BMT (Allay et al., 1998), which is of particular relevance for human hematopoietic stem cell transplantation. GFP is a naturally occurring fluorophore that requires no substrates or additional gene products for its fluorescence activity and can thus be scored directly by fluorescence microscopy (Chalfie, 1995). Transduction efficiency into murine myeloid clonogenic progenitors averaged 80–90% ($n = 7$). Retrovirally transduced hematopoietic colonies expressed high levels of GFP as determined by direct visualization with phase contrast and fluorescence microscopy (Fig. 1, A and B). Nontransduced clonogenic progenitors showed no detectable fluorescence (Fig. 1, C and D), indicating the specificity of GFP as a marker for genetically modified hematopoietic cells.

Transplantation of GFP-expressing BM cells into lethally irradiated mice led to long-term reconstitution of hematopoiesis with GFP-marked peripheral blood cell progeny (Fig. 2). All myeloid and lymphocytic lineages displayed a 50–80% range of GFP labeling, and GFP expression was stable during the entire observation period of 15 mo posttransplantation. The sera of BM chimeras were free of replication-competent retrovirus, which was demonstrated by the failure to transduce hematopoietic cells. Mice reconstituted with BM cells from transgenic mice that ubiquitously express GFP displayed ~80% GFP marking in peripheral blood mononuclear cells. Multilineage and long-term repopulation of irradiated hosts suggests engraftment at the stem cell level.

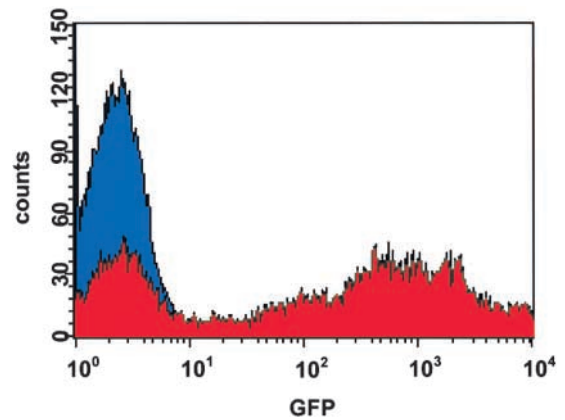


Figure 2. Reconstitution of hematopoiesis with GFP-marked peripheral blood cell progeny in myeloablated mice. Flow cytometric analysis of GFP expression was performed in peripheral blood leukocytes of lethally irradiated mice 8 mo after transplantation of GFP-expressing BM cells (red) and in leukocytes of control mice that did not receive GFP-labeled BM (blue). Approximately 70% of white blood cells were stably expressing the fluorophore in GFP-BM chimeras. All myeloid and lymphocytic populations were labeled, but the levels of GFP expression varied within lineages. Highest expression was observed in monocytes/macrophages and granulocytes (unpublished data).

GFP-positive cells were detected in the brain as early as 2 wk post-BMT. By virtue of their GFP expression, BM-derived cells could be tracked in the brains of living animals (Fig. 3 A). Donor-derived cells were located primarily in perivascular sites, between the basal laminas of endothelia and astrocytes (Fig. 3, A and B). These cells expressed Iba1, a marker of monocytes and macrophages (Imai et al., 1996). Some GFP-expressing cells were found to have left the perivascular space for the brain parenchyma at 1 mo post-BMT (Fig. 3 C). By 4 mo after transplantation, the number of BM-derived cells increased significantly in the brain parenchyma and highest engraftment was observed in the cerebellum. All donor-derived cells in cerebellum expressed monocyte/macrophage-specific antigens, such as F4/80 and Iba1, up to 4 mo post-BMT ($n = 15$; unpublished data). No GFP-marked cells in the cerebellum expressed the astroglial marker, glial fibrillary acidic protein (GFAP), or displayed a morphology characteristic of neurons and expressed the neuronal antigen, NeuN. On very rare occasions were cells detected in the olfactory bulb that coexpressed GFP and NeuN (Fig. 3, D–F), but none of these cells showed a morphology characteristic of neurons. However, 12–15 mo after transplantation, up to 0.1% of fully developed cerebellar neurons were found to express GFP in the perikaryon, axon, and dendritic tree (Figs. 4, A and B, 5, A, D, and F, and Table I). No other brain region contained GFP-marked neurons at this time after transplantation. The GFP-positive cerebellar neurons were identified as Purkinje cells by their characteristic morphology, and the expression of calbindin-D28K (Figs. 4, B–D and 5, A–C), which in the mouse cerebellum is uniquely expressed in Purkinje neurons (Nordquist et al., 1988). The cells also contained the γ -aminobutyric acid (GABA)-synthesizing enzyme, glutamic acid decarboxylase, indicating neurotransmitter synthesis in Purkinje cells (Fig. 5, E–H; Ottersen and Storm-Mathisen,

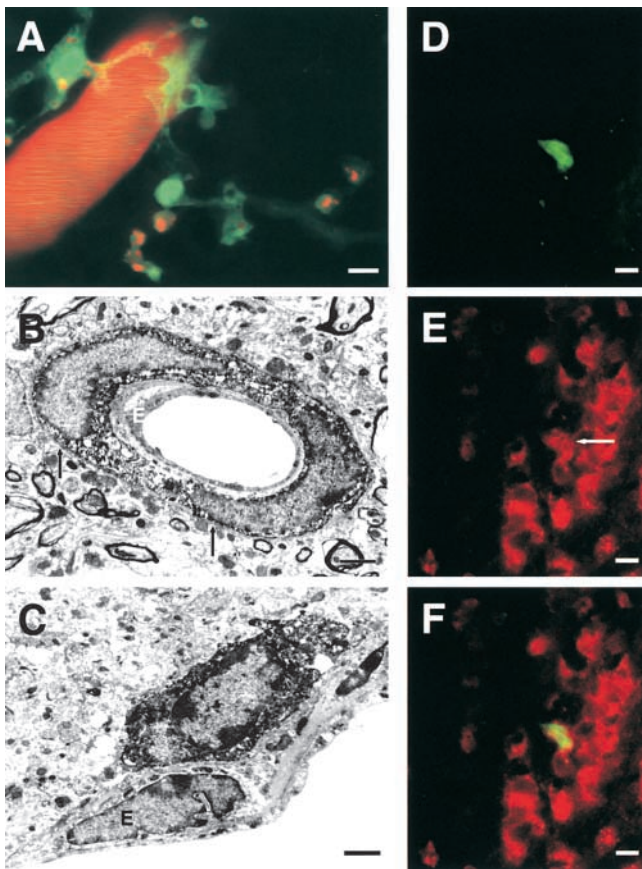


Figure 3. Detection of GFP-expressing cells in the brains of BM chimeras. 4 wk after transplantation of GFP-expressing BM cells, a closed cranial window was implanted in anesthetized mice. After intravenous injection of the dye rhodamine B dextran to contrast the lumen of cerebral vessels, GFP-expressing cells were detected in vivo using laser-scanning microscopy through the cranial window (A). Some extraluminal GFP-positive cells extended short cellular processes and showed a punctate rhodamine staining from dye uptake. These cells were located in the perivascular space (the basement membrane is indicated by arrows), as demonstrated by immunoelectron microscopy for GFP (B). Note that endothelia (E) did not express GFP. In C, a donor-derived cell is visible that has crossed the blood brain barrier and is located in the brain parenchyma. 13 wk post-BMT, a GFP-expressing cell in the olfactory bulb (D) demonstrates NeuN-immunoreactivity (arrow in E, immunohistochemistry using Texas red). The overlay of images D and E is shown in F. Bars: (A) 5 μm ; (B and C) 1 μm ; (D and F) 10 μm .

1984). They were negative for glial markers, such as Iba1, GFAP, and S100 β . Multiple synaptic contacts (Fig. 4 G) suggest that the engrafted Purkinje cells are functional. The differentiation of BM-derived cells into Purkinje cells occurred in both cerebellar hemispheres and in all animals examined ($n = 7$). The cell fluorescence resulted from true expression of the marker gene, as determined by immunohistochemistry using an anti-GFP antibody (Fig. 4 E). Immunoelectron microscopic localization of GFP revealed a homogenous cytoplasmic distribution in dendrites (Fig. 4, F and G), excluding the possibility of GFP uptake by host Purkinje cells. Although infection of Purkinje cells by recombinant retrovirus was unlikely given the postmitotic character of CNS neurons, and virtually excluded by

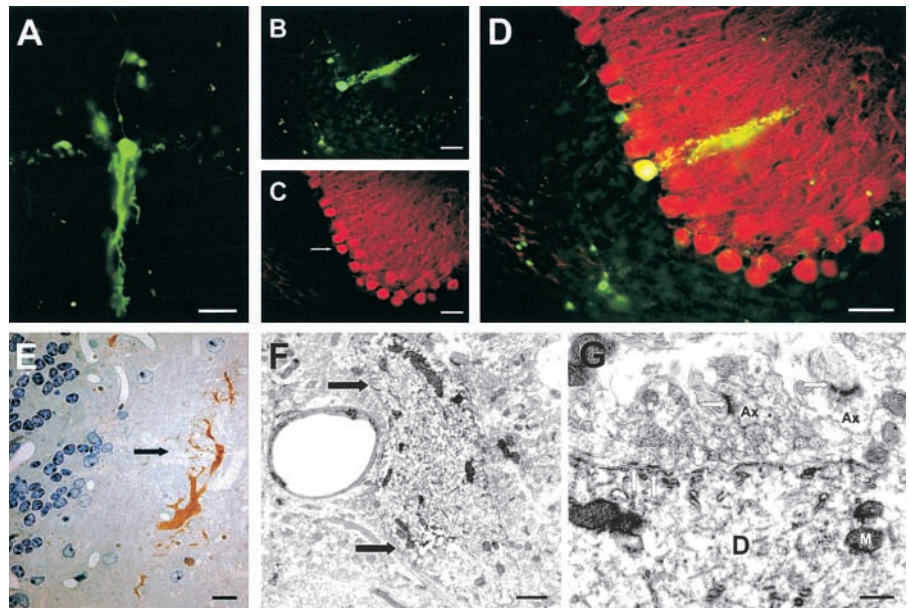
the absence of recombinant retrovirus in the blood of chimeras, we transplanted BM from transgenic mice that ubiquitously express GFP into lethally irradiated adult mice ($n = 2$). Thus avoiding retroviral gene transfer, the same differentiation of BM-derived cells into Purkinje cells was observed at 10–12 mo post-BMT (Fig. 5, A–C and Table I).

This study demonstrates engraftment of Purkinje cells in adult mice after BM transplantation for the first time. The data are in line with recent observations suggesting that BM cells can give rise to cells expressing neuronal antigens in vitro and in vivo (Kopen et al., 1999; Brazelton et al., 2000; Mezey et al., 2000; Sanchez-Ramos et al., 2000; Woodbury et al., 2000). Although the detection of neuronal protein markers alone could be misleading, the differentiated morphology of the GFP-expressing Purkinje cells observed in our study substantiates the capacity of BM-derived cells to generate mature neurons in the adult CNS. Functional activity of the engrafted Purkinje neurons is suggested by the presence of neurotransmitter synthesizing enzyme and synaptic junctions. Moreover, denervated Purkinje cells would show marked changes of dendritic arborization (Anderson and Flumerfeldt, 1984).

In contrast to previous findings, our data suggest that neuronal differentiation of BM cells is a rare biological event and occurs late, after BMT. The observation period in the present study almost covered the entire life span of a laboratory mouse. The discrepancy may relate to the fact that we scored only those GFP-expressing cells as neurons that showed the characteristic morphology with axon/dendrites and expression of neuronal antigens. In the regions of adult neurogenesis, such as the olfactory bulb, engrafted cells underwent apoptosis before full neuronal differentiation (unpublished data). Interestingly, a recent BMT study in mice using GFP-marked BM cells revealed that no donor-derived neurons were detectable in the brain at 6 mo posttransplantation, which is in perfect agreement with our findings (Nakano et al., 2001). In terms of macrophage/microglial engraftment and lack of astroglial differentiation of BM-derived cells, our data are also in line with recently published results (Brazelton et al., 2000; Nakano et al., 2001). With regard to the cerebellum, it remains enigmatic why engraftment of Purkinje cells was seen at 12 mo, but not at 4 mo post-BMT. Moreover, it is unclear why BM cells did not contribute to the population of granule cells, which are the most numerous neurons in the brain. It is tempting to speculate that late neogenesis of Purkinje cells represents a physiological response to ageing when almost 30% of the Purkinje cell population, but none of the cerebellar granule cells, are lost (Sturrock, 1990). The decrease in Purkinje cell number is noticeable in mice at 15 mo, but not at 6 mo of age. Importantly, recent experimental evidence suggests that BM stem cells have regenerative capacity (Ferrari et al., 1998; Lagasse et al., 2000; Orlic et al., 2001).

Purkinje cells are large, GABAergic neurons that serve as sole output of the cerebellar cortex (Voogd and Glickstein, 1998). Chick–quail chimera work revealed that they arise from clonally related embryonic founder cells whose progeny populate each side of the cerebellum, respecting the cerebellar midline (Alvarez Otero et al., 1993). In this context, it is interesting to note that we found GFP-expressing Purkinje cells in both cerebellar hemispheres, suggesting that the factors governing neogenesis of Purkinje cells in the adult may be dif-

Figure 4. Characterization of GFP-expressing Purkinje cells in the brains of BM chimeras. 12–15 mo after transplantation of GFP-expressing BM cells, CNS engraftment of marrow-derived cells was evaluated in brain sections using direct fluorescence microscopy. In the cerebellum, single neurons located at the border of the granular and the molecular layer were found to express GFP in their perikaryon, axon, and dendrites (A). These cells were identified as Purkinje neurons by their expression of the calcium-binding protein, calbindin-D28K: section through a GFP-marked neuron (B, direct fluorescence microscopy) that shows calbindin immunoreactivity (arrow in C, immunohistochemistry using Texas red). The overlay (D) demonstrates that the two fluorophores are in the same cell. Immunohistochemical staining of semithin brain sections using an anti-GFP antibody visualized by diaminobenzidine revealed GFP immunoreactivity in Purkinje cell dendrites (E, arrow). Nuclei of adjacent granule cells and molecular layer interneurons were counterstained with methylene blue. Note that these cells did not show GFP immunoreactivity (E). Cytoplasmic GFP expression in Purkinje cell dendrites was demonstrated by immunoelectron microscopy for GFP (F and G). In F, a large GFP-labeled dendrite (indicated by arrows) is located in proximity to a blood vessel. Part of this dendrite is shown at higher magnification in G. The cytoplasm of the dendrite (D) contains mitochondria (M). Basket fibers terminate on the surface of the dendrite, and axons (Ax) of parallel fibers form asymmetric synaptic junctions with Purkinje cell dendritic thorns (arrows). Bars: (A–D) 50 μ m; (E) 10 μ m; (F) 1 μ m; (G) 0.25 μ m.



ferent from those during cerebellar development. It remains to be determined which types of BM stem cells give rise to Purkinje neurons in the adult brain. In the absence of a consensus regarding which markers are consistently expressed on BM stem cell populations, an analysis of the genomic insertion sites of the retrovirus may in the future help to define the clonal precursors of BM-derived Purkinje cells. Mesenchymal stem cells have been shown to generate neurons in vitro and after intraventricular injection into neonatal mouse brains (Kopen et al., 1999; Sanchez-Ramos et al., 2000; Woodbury et al., 2000), but failed to engraft in the brain parenchyma following in utero transplantation (Liechty et al., 2000).

Several technical points need to be addressed. The DNA content of certain large neurons, notably Purkinje cells, has been suggested to exceed diploid levels, but a subsequent critical appraisal of techniques contradicted the findings (Mann et al., 1978). Hyperdiploid DNA values may represent changes in chromatin compaction and the presence of single-stranded DNA related to the metabolic state of Purkinje cells (Bernocchi et al., 1986). Thus, fusion of host Purkinje cells with GFP-marked blood-borne cells is an unlikely scenario. No

temporal or spatial correlation between the appearance of donor-derived Purkinje cells and the engraftment of macrophages in the cerebellum was observed, and GFP-marked Purkinje cells did not express Iba1 (unpublished data). Although GFP may have promoted the differentiation of BM cells into neurons, an overwhelming body of evidence suggests that the fluorophore is biologically inert (Goldman and Roy, 2000). Finally, using transgenic mice that ubiquitously express GFP, we could exclude the possibility that GFP labeling of Purkinje cells was a mere result of recombinant retrovirus taken up by host neurons. The bicistronic vector used in this study provided high and stable gene expression in hematopoietic cells in the absence of replication-competent retrovirus.

The data suggest that BM cells can, upon adoptive transplantation, generate Purkinje cells in the adult brain. These findings may have important implications for the therapy of CNS disorders that are associated with Purkinje cell loss, as engraftment of BM cells might be enhanced during pathology. Interestingly, reduced ataxia and increased life span was observed in mouse models of Niemann-Pick disease after transplantation of normal BM, and Purkinje cells were

Table I. Semiquantitative analysis of Purkinje cell engraftment

| Mouse | GFP labelling | Time post-BMT | GFP ⁺ microglia | GFP ⁺ cells Perivascular/leptomeningeal | GFP ⁺ Purkinje cells | Total Purkinje cells |
|-------|---------------|---------------|----------------------------|--|---------------------------------|----------------------|
| | | <i>mo</i> | | | | |
| 1 | Retroviral | 12 | 1,107 | 2,515 | 10 | 13,519 |
| 2 | Retroviral | 12 | 2,064 | 2,989 | 10 | 13,691 |
| 3 | Transgenic | 10 | 747 | 1,331 | 2 | 13,110 |

10–12 mo after transplantation of BM cells expressing GFP under the control of a retroviral or β -actin promoter, every seventh cerebellar section of the chimeric mice was analyzed for GFP-expressing cells. Purkinje cells and microglia were identified by their morphology and immunoreactivity for calbindin/GAD and Iba1, respectively. GFP-positive cells located in perivascular and leptomeningeal sites were also evaluated.

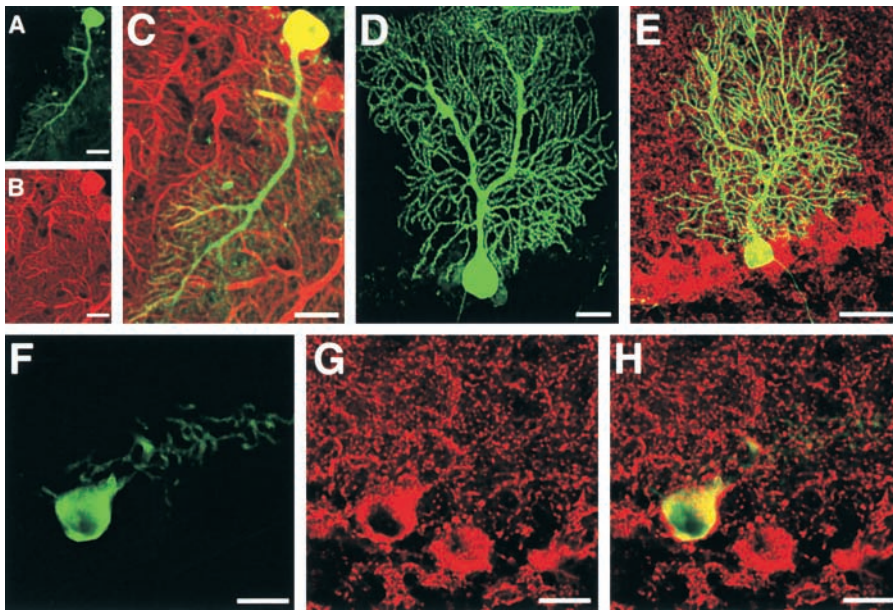


Figure 5. Confocal microscopic analysis of BM-derived Purkinje cells.

12 mo after transplantation of BM cells from transgenic mice that ubiquitously express GFP, donor-derived Purkinje cells were visualized by confocal laser-scanning microscopy. The GFP fluorescence of an engrafted neuron (A) colocalized with calbindin immunoreactivity (B, immunohistochemistry using Texas red), characterizing the neuron as a Purkinje cell (C, overlay of images A and B). GFP-expressing Purkinje cells were also detected in chimeric mice 12 mo after transplantation of BM cells transduced with a GFP retroviral vector (D, maximum intensity projection of 16 consecutive scans). Another GFP-marked neuron is visible in the layer of GAD-expressing Purkinje cells (E, overlay of the maximum intensity projections of GFP and Texas red fluorescence visualizing GAD immunoreactivity). The single optical section through a GFP-

positive Purkinje cell (F) revealed expression of GAD (G, immunohistochemistry using Texas red) in the Purkinje cell soma as well as in GABAergic nerve terminals (F, overlay of images F and G). Bars: (A–D) 25 μ m; (E) 50 μ m; (F–H) 25 μ m.

present in the cerebellum despite the lack of enzyme activity in the brain (Miranda et al., 1998).

Materials and methods

Retroviral transduction of BM cells and transplantation

The MGirL22Y murine stem cell virus–based vector was generated by replacing the neomycin coding cassette in MSCVNEO2.1 (Hawley et al., 1994) with an internal ribosomal entry site from the encephalomyocarditis virus linked to a gene encoding a mutant dihydrofolate reductase (L22Y). The enhanced GFP gene from EGFP-1 (CLONTECH Laboratories, Inc.) was cloned into the resulting plasmid upstream of the internal ribosomal entry site. High-titer, ecotropic MGirL22Y virus–producer cells were generated by transduction of GP plus E86 cells with MGirL22Y vector particles derived by cotransfection of 293T cells with the plasmid containing the MGirL22Y sequences and a helper plasmid encoding the required gag, pol, and env retroviral genes (Persons et al., 1997). The presence of replication-competent retrovirus from the MGirL22Y virus–producer cells was ruled out by testing the ability of conditioned medium from the virus producer cells to mobilize, in a *mus dunni* test cell line, a recombinant, integrated Moloney leukemia virus (G1Na) which encodes for neomycin phosphotransferase (McLachlin et al., 1993). Briefly, conditioned media from the MGirL22Y virus–producer cells was used to infect *mus dunni* cells harboring two integrated copies of G1Na (provided by Dr. Vanin, St. Jude's Children's Research Hospital, Memphis, TN). The transduced *mus dunni* cells were cultured for 3 wk, and conditioned media was taken from these cells and tested on naive *mus dunni* cells for the ability to confer neomycin resistance. No neomycin-resistant colonies were ever detected over multiple independent assays.

BM was harvested from 8–12-wk-old male C57BL/6 mice 2 d after treatment with 150 mg/kg 5-fluorouracil (Sigma-Aldrich). After prestimulation for 48 h in DME supplemented with 15% heat-inactivated fetal calf serum (Biochrom), 20 ng/ml murine interleukin-3, 50 ng/ml human interleukin-6 (PromoCell), and 50 ng/ml rat stem cell factor (provided by Amgen). BM cells were cocultured with irradiated (1,300 cGy) MGirL22Y virus–producer cells in the presence of 6 μ g/ml polybrene (Sigma-Aldrich). After 48 h, nonadherent BM cells were rinsed off the producer cell monolayer and transplanted into lethally irradiated (two doses of 550 cGy separated by 3 h) adult male C57BL/6 mice. Alternatively, BM was harvested from “green mice” (Okabe et al., 1997) and transplanted directly into lethally irradiated adult male C57BL/6 mice. Recipient mice received 5×10^6 cells by tail vein injection. For in vitro clonogenic progenitor assays, transduced BM cells were suspended in 2 ml of methylcellulose supplemented with interleukin-3, interleukin-6, erythropoietin, and stem cell factor (StemCell Technologies), supporting the growth of myeloid colonies.

Flow cytometric analysis of GFP expression in peripheral blood cells

1, 4, 8, and 12 mo after BMT, peripheral blood obtained by tail vein puncture was examined by fluorescent-activated cell sorting (Becton Dickinson). After erylisis, cells labeled with Mac-1-Pe, B220-Pe, Gr-1-Pe (PharMingen), and CD4-Pe (Becton Dickinson) were analyzed for GFP expression. Dead cells (propidium iodide–positive) were excluded. Data were evaluated using the Cellquest™ software.

Immunohistochemical analysis of donor cell engraftment in the cerebellum

BM chimeric mice ($n = 24$) were killed between 2 wk and 15 mo post-transplantation, and perfused transcardially with 4% paraformaldehyde in PBS. 30 μ m vibratome or 20 μ m cryosections were obtained from the brains. For immunohistochemistry, IgG block was performed and sections were incubated in PBS supplemented with 5–10% normal goat serum and 0.5% Triton X-100. Primary antibodies were added overnight at dilutions of 1:200 for anti-calbindin-D28K (mouse monoclonal; provided by Dr. Veh, Charité, Berlin, Germany), 1:250 for anti-glutamic acid decarboxylase (GAD) (rabbit polyclonal; Chemicon), 1:100 for anti-NeuN (mouse monoclonal; Chemicon), 1:100 for anti-Iba1 (rabbit polyclonal; provided by Dr. Imai, National Institute of Neuroscience, Tokyo, Japan), 1:50 for anti-F4/80 (rat monoclonal; Serotec), 1:2,500 for anti-GFAP (rabbit polyclonal; Dako), and 1:100 for anti-S100 β (rabbit polyclonal; provided by Dr. Veh, Charité, Berlin, Germany). Texas red–conjugated secondary antibodies (goat anti–mouse and goat anti–rabbit; Molecular Probes) were added at 1:200 for 1 h. Omission of primary antibodies served as negative control and resulted in no detectable staining. Digital micrographs of GFP and Texas red fluorescence were obtained on a conventional fluorescence microscope equipped with a color scanner (Leica) or on a confocal laser scanning microscope (ZEISS).

Quantification of engraftment

Semiquantitative analysis of Purkinje cell engraftment was performed in three representative animals. Every seventh cerebellar section was analyzed, and the number of GFP-expressing Purkinje cells was determined in relation to the total number of calbindin- or GAD-stained Purkinje cells in these sections. Moreover, the remaining GFP-expressing microglia and macrophages, demonstrating Iba1 immunoreactivity, were counted. In two representative chimeras (retroviral and transgenic GFP expression), every single section from the olfactory bulb to the cerebellum was analyzed for GFP-marked neurons.

Ultrastructural analysis of engrafted cells

Preembedding immunohistochemistry was performed on 80- μ m vibratome sections as described above. A preabsorbed polyclonal anti-GFP

antibody (CLONTECH Laboratories, Inc.) was used at a dilution of 1:250. Omission of primary antibody served as negative control. Addition of the secondary biotinylated goat anti-rabbit antibody (Vector Laboratories) was followed by incubation with the avidin-biotin peroxidase complex (ABC; Vector Laboratories) and 3,3'-diaminobenzidine/H₂O₂ (Sigma-Aldrich) according to the manufacturer's instructions. Sections were then fixed in 2% glutaraldehyde/PBS and immersed in 2% Dalton's osmium. After dehydration, the tissue was embedded in araldite. For high resolution light microscopy, 1- μ m semithin araldite sections were scanned with a Leica color scanner. Each cell immunoreactive for GFP was sought in adjacent ultrathin sections cut on a LKB-ultramicrotome. The sections were mounted on mesh copper grids and processed for electron microscopy.

Intravital microscopy

In vivo confocal laser imaging of GFP-expressing cells was performed according to a modified protocol of Villringer et al. (1991). Anesthesia was induced in BM chimeric mice by inhalation of 2% halothane in a mixture of 30% O₂/70% N₂O. Anesthesia was maintained throughout the experiment by intraperitoneal administration of 750 mg/kg urethane and 150 mg/kg α -chloralose. The femoral artery and vein were cannulated and mean arterial blood pressure was monitored. Systemic arterial pressure was 82 ± 8 mm Hg (mean \pm SD; $n = 3$). Body temperature was maintained between 36.5°C and 37.5°C using a heating pad. Mice were intubated and ventilated with a mixture of O₂ and air. End tidal CO₂ partial pressure was monitored and kept between 2.5–3.5% by adjusting the respiratory rate, tidal volume, and inspiration time. After fixation to a stereotaxic frame, a craniotomy was performed over the parietal cortex. The dura mater was removed and a closed cranial window was implanted. Mice received 250 mg/kg rhodamine B dextran (Sigma-Aldrich) by intravenous injection. Subsequently, the brains were examined for GFP-expressing cells through the coverglass of the cranial window for 3 h using a confocal laser scanning microscope (Leica).

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