Development/Plasticity/Repair

Aberrant Growth and Differentiation of Oligodendrocyte Progenitors in Neurofibromatosis Type 1 Mutants

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Neurofibromatosis type 1 (NF1) patients are predisposed to learning disabilities, macrocephaly, and brain tumors as well as abnormalities on magnetic resonance imaging that are postulated to result from abnormal myelination. Here we show that *Nf1*+/− spinal cords in adult mice have more than twofold-increased numbers of NG2+ progenitor cells. *Nf1*−/− embryonic spinal cords have increased numbers of Olig2+ progenitors. Also, cultures from *Nf1* mutant embryos with hemizygous and biallelic *Nf1* mutations have dramatically increased numbers of CNS oligodendrocyte progenitor cells. In medium that allows growth of neuroepithelial cells and glial progenitors, mutant cells hyper-respond to FGF2, have increased basal and FGF-stimulated Ras-GTP, and fail to accumulate when treated with a farnesyltransferase inhibitor. Cell accumulation results in part from increased proliferation and decreased cell death. In contrast to wild-type cells, *Nf1*−/− progenitors express the glial differentiation marker O4 while retaining expression of the progenitor marker nestin. *Nf1* mutant progenitors also abnormally coexpress the glial differentiation markers O4 and GFAP. Importantly, *Nf1*−/− spinal cord-derived oligodendrocyte progenitors, which are amplified 12-fold, retain the ability to form oligodendrocytes after *in vivo* transplantation. The data reveal a key role for neurofibromin and Ras signaling in the maintenance of CNS progenitor cell pools and also suggest a potential role for progenitor cell defects in the CNS abnormalities of NF1 patients.

Key words: **NF1; progenitor; oligodendrocyte; FGF; brain; Ras**

Introduction

Neuroepithelial (NEP) cells give rise to neurons, astrocytes, and oligodendrocytes in the CNS. The lineage relationships among CNS progenitor cells and their differentiated progeny are under intense investigation (Temple and Alvarez-Buylla, 1999; Sauvageot and Stiles, 2002). Little is known about signaling cascades that regulate CNS progenitor cells in general and oligodendrocyte progenitors in specific, *in vivo*, or cell culture. Markers such as NG2 and Olig2 that identify oligodendrocyte progenitors in embryos can also be expressed by neuronal progenitors and in postnatal rodent NG2+ cells that are multipotent (Miller et al., 1999; Rao, 1999; Rowitch et al., 2002; Belachew et al., 2003). Adult NG2+ cells are present in uninjured CNS and multiple sclerosis lesions and can remyelinate after injury (Redwine and Armstrong, 1998; Chang et al., 2000; Watanabe et al., 2002). PDGF and FGF2 stimulate proliferation and inhibit differentiation in NG2+ cells *in vivo* that develop into oligodendrocytes (Armstrong et al., 2002; Frost et al., 2003; Oh et al., 2003) and drive oligodendrocyte progenitor growth *in vitro* (McKinnon et al., 1990; Mujtaba et al., 1999).

Neurofibromin is a tumor suppressor that is a GTPase-activating protein (GAP) for Ras (for review, see Donovan et al., 2002) and acts as a negative regulator of Ras signaling. In many cell types, loss of neurofibromin is correlated with increased GTP-bound active Ras. Neurofibromin is widely expressed in the developing brain (Daston and Ratner, 1992). In the adult brain, neurofibromin is present in some neurons and oligodendrocytes (Daston et al., 1992; Nordlund et al., 1993). After brain injury, astrocytes upregulate neurofibromin (Giordano et al., 1996). Neurofibromin is of particular interest to brain function. Humans with *NF1* gene mutations are predisposed to learning disabilities, macrocephaly, and abnormalities on T2-weighted magnetic resonance (MR) imaging postulated to be focally severe patches of a widespread myelin disorder (DiPaolo et al., 1995; Ozonoff, 1999; Wang et al., 2000; Eastwood et al., 2001; North and Ratner, 2002). Macrocephaly results from enlarged white matter tracts (Steen et al., 2001). Pilocytic astrocytomas occur in ~25% of children with NF1, and cells in these benign tumors express the oligodendroglial lineage marker PEN5 (Listernick et al., 1997; Li et al., 2001). Astrogliosis is a feature of NF1 patient brains (Nordlund et al., 1995). Thus, NF1 patients may have

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altered CNS glia, but glial lineages have not been evaluated in NF1 disease models. Neurofibromin may be important in progenitor cells, because hematopoietic progenitors from *Nf1* mutant mice are hypersensitive to cytokines resulting from defective Ras and phosphoinositide-3 kinase signaling (Bollag et al., 1996; Largaespada et al., 1996; Zhang et al., 1998; Birnbaum et al., 2000).

Mice that completely lack *Nf1* die *in utero* at or before embryonic day (E) 13 (Brannan et al., 1994; Jacks et al., 1994). Mice with hemizygous *Nf1* mutations show profound brain dysfunction including astrogliosis (Gutmann et al., 1999; Rizvi et al., 1999; Bajenaru et al., 2001; Zhu et al., 2001) and learning defects (Silva et al., 1997). Here we show that the development of the oligodendrocyte lineage is abnormal in cells from mice with one or two mutant *Nf1* alleles. We suggest that brain dysfunction in NF1 results from effects on progenitor cell pools and demonstrate a key role for Ras signaling in oligodendrocyte progenitors.

Materials and Methods

Animals and genotyping

C57BL/6 wild-type female mice (breeders) were obtained from Harlan Bioproducts for Science (Indianapolis, IN). The *Nf1* gene was targeted in mice (Brannan et al., 1994). For genotyping, DNA was isolated, and the targeted allele was determined by PCR as described previously (Brannan et al., 1994). *Nf1*+/− mice had been back-crossed at least 10 generations onto the C57BL/6 background at the time of these experiments. Wild-type, *Nf1*+/−, and *Nf1*−/− embryos were obtained 12.5 d after mating *Nf1*+/− C57BL/6 males with *Nf1*+/− C57BL/6 females.

Immunohistochemistry for NG2

Wild-type and *Nf1* animals (three per genotype) were perfused with 0.9% saline followed by 2% paraformaldehyde, 0.01 M sodium meta-periodate, and 0.1 M lysine fixative (Wu et al., 2000). The spinal cord was dissected out, postfixed in the same fixative overnight, and then cryoprotected overnight in 20% sucrose. Forty micrometer thick free-floating coronal serial sections, at the level of the cervical enlargement, were processed for immunohistochemistry using a biotinylated goat anti-rabbit secondary and the ABC-DAB method for visualization (Vector Laboratories, Burlingame, CA). Rabbit anti-NG2 (Chemicon, Temecula, CA) antibody was used at a dilution of 1:1000. NG2+ cells were counted in gray and white matter at 200× under bright-field optics.

Immunohistochemistry for Olig2

Wild-type, *Nf1*+/−, and *Nf1*−/− E12.5 embryos (three per genotype) were removed and fixed overnight at 4°C in 4% paraformaldehyde in PBS. Embryos were then cryoprotected overnight in 20% sucrose. Ten micrometer thick cryostat coronal serial sections, at the level of the cervical enlargement, were processed for immunohistochemistry using a biotinylated goat anti-rabbit secondary (Jackson ImmunoResearch, West Grove, PA) and the ABC-DAB method for visualization (Vector Laboratories). Rabbit anti-Olig2 (a kind gift from D. Rowitch and C. Stiles, Dana-Farber Cancer Institute, Boston, MA) was used at a dilution of 1:2000. Olig2+ cells were counted at 400× under bright-field optics.

Cell cultures

Glial restricted precursor cells. Twelve-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) were double coated with fibronectin (Becton Dickinson) and laminin (Becton Dickinson). Fibronectin (250 μg/ml) was applied to the wells and immediately withdrawn, and the plates were allowed to dry for 1 hr before coating with laminin (20 μg/ml) overnight at 4°C (Rao and Mayer-Proschel, 1997). Spinal cords were dissected from E12.5 mouse embryos using sharpened number 5 forceps in Leibovitz's L-15 (Invitrogen, Rockville, MD) medium. Spinal cords, with dorsal root ganglia removed, were incubated at 37°C in 0.25% trypsin (Invitrogen) for 30 min with agitation. Cords were subsequently washed by placing them in DMEM (Invitrogen) with 10% fetal bovine serum (FBS) and penicillin–streptomycin and centrifuging at 800 rpm for 5 min at room temperature (RT). The supernatant was then aspirated and the pellet was resuspended in 0.5 ml of NEP cell media (Kalyani et al., 1997) (see below) by gently triturating with a glass Pasteur pipette. Laminin was aspirated from the wells and replaced with 1.5 ml of NEP medium just before the addition of resuspended cells. Cells were cultured at a density of one cord per five wells and maintained at 37°C in 7.5% CO_2 with growth factors added or omitted as described in this study.

NEP medium. Based on the Bottenstein and Sato N2 medium, NEP medium contains DMEM–F12 (Invitrogen), N2 additives (progesterone, putresine, selenium, insulin, transferrin) plus complex B27 supplements (Invitrogen), 1% BSA, FGF2 (25 ng/ml, or at doses specified below; R & D Systems, Minneapolis, MN), and 10% chick embryo extract (Invitrogen). Growth factors and medium were replaced every 3 d. In some experiments, PDGFAA (10 ng/ml; R & D Systems) and farnesyl transferase inhibitor (1 μM; L744,832; Merck, Darmstadt, Germany) were added as specified in this study.

Colony counts. Four random fields (1.9 mm) were selected from each well and marked 1 d after plating. Colonies of progenitor-like cells were identified by morphology (small, phase-dark cells), counted, and grouped by colony size. Colony sizes were as follows: <40 cells (small), 40–150 (medium), and >150 (large). Counts were performed daily at days 1–5 after plating.

Ras activation assay. Cells from wild-type, *Nf1*+/−, and *Nf1*−/− embryos were cultured in 60 mm culture dishes, as described above, in the presence of FGF2 (25 ng/ml) until generally confluent (7 d). FGF2 was then removed from the media for 24 hr. One-half of the dishes were then stimulated with FGF2 (25 ng/ml) for 10 min. The cells were immediately processed using the Ras activation assay kit (catalog #17–218; Upstate Biotechnology, Lake Placid, NY) according to the instructions of the manufacturer.

Immunocytochemistry

For nonexpanded marker analysis, E12.5 mouse spinal cords were dissociated and prepared as described above and plated directly onto eight-well RS-treated Labtek II chamber slides (Nalge Nunc International, Naperville, IL) at a density of 5000 cells per well, fixed with 4% paraformaldehyde in PBS at 24 hr, and then labeled as described below. For marker analysis of expanded spinal cord cultures 5 d after plating, cultures were treated with a nonenzymatic cell dissociation medium (Sigma, St. Louis, MO) for 15 min at 37°C. With a glass Pasteur pipette, the medium was gently washed over the cells to dislodge them from the dish. The cells were washed in DMEM plus 10% FBS by centrifugation at 800 rpm for 5 min. The medium was aspirated, and the pellet was resuspended in 0.5 ml of NEP media with FGF2 plus PDGFAA. Cell number was determined on a hemacytometer, and the cells were diluted to a density of 10,000 cells per milliliter. Fibronectin–laminin-coated eight-well RS-treated Labtek II chamber slides were prepared as described above. Five thousand cells were placed in each well and maintained in 7.5% CO_2 at 37°C for 3 d. On day 3, the media was aspirated, and the cells were gently rinsed once with PBS and fixed in 4% paraformaldehyde for 15 min at RT. For nestin, NG2, and GFAP, cells were treated with 0.05% Triton X-100 in PBS for 15 min before incubation with the primary antibody. For lipid antigens (O4, A2B5), cells were not exposed to detergent. Cells were incubated with primary antibody for 1 hr at RT, washed three times (5 min each) in PBS, incubated in the dark with the appropriate secondary antibody for 45 min at RT, and washed three times in PBS. Cells were then stained with 5 μg/ml bis-Benzimide (Sigma) for 15 min, washed once in PBS, and coverslipped using Fluoromount G (Electron Microscopy Sciences, Ft. Washington, PA). In cases of double labeling, cell-surface antigens were stained first and then cells were refixed in 4% paraformaldehyde before permeabilization and staining internal antigens. The following antibodies and dilutions were used: Rat-401 (nestin) mouse IgG conditioned medium (CM) (Developmental Studies Hybridoma Bank, Iowa City, IA), A2B5 mouse IgM CM (Eisenbarth et al., 1979), O4 mouse IgM CM (cells from T. Gard, University of South Alabama College of Medicine, Mobile, AL) (Sommer and Schachner, 1981; Gard and Pfeiffer, 1990), GFAP rabbit IgG 1:1000 (Dako, Carpenteria, CA), 5A5 [embryonic neural cell adhesion molecule (E-NCAM)] mouse IgM CM (Developmental Studies Hybridoma Bank), PDGF receptor (PDGFR)-α rabbit IgG 1:500 (Upstate Biotechnology), and NG2 rabbit IgG 1:150 (Chemicon). Rhodamine [tetramethylrhodamine iso-

thiocyanate (TRITC)]-conjugated secondary antibodies (Jackson ImmunoResearch) were used in single-labeling experiments. For double labeling, internal antigens were stained with TRITC-conjugated secondary antibodies, and cell-surface antigens with FITC-conjugated secondary antibodies (Jackson ImmunoResearch). This minimized overlap of bis-Benzimide nuclear staining with the internal antigen staining. Quantitation was performed on a Zeiss (Thornwood, NY) Axiophot fluorescent microscope by counting randomly selected fields of bis-Benzimide-stained nuclei and then determining the percentage of cells positive for a specific antigen. At least 100 cells were counted per condition.

DNA fragmentation assay. Cells from E12.5 wild-type and *Nf1*+/− mouse spinal cords were cultured as described above in the presence of FGF2 (25 ng/ml) until nearly confluent. The cells were then plated at a density of 20,000 cells per well onto fibronectin–laminin-coated eight-well RS-treated Labtek II chamber slides, as described previously, in the presence or absence of FGF2 (25 ng/ml) and maintained for 3 d at 37°C. The cells were fixed in 4% paraformaldehyde for 15 min at RT and processed using the fluorescein-FragEL DNA fragmentation detection kit (Oncogene Research Products, Boston, MA) according to the instructions of the manufacturer. The kit uses terminal deoxynucleotidyl transferase (TdT) to attach fluorescein-conjugated deoxynucleotides to free 3′-OH groups at the end of DNA fragments resulting from endogenous endonucleases cleaving cellular DNA as the cell undergoes apoptosis. In brief, the slides were immersed in PBS for 15 min at RT and then permeabilized with 20 ng/ml of proteinase K in 10 mM Tris, pH 8.0, for 5 min. The slides were rinsed three times in PBS and then incubated at RT in TdT equilibration buffer for 30 min. The TdT-labeling reaction mix was applied to the specimens, and slides were then incubated at 37°C in a humidified chamber for 90 min. The slides were rinsed three times for 1 min each in fresh PBS and coverslipped using the supplied fluorescein-FragEL mounting media. Cells were visualized as described above. The number of fragmented nuclei was compared with the total number of nuclei for each condition and genotype.

Bromodeoxyuridine labeling. Cells were cultured until nearly confluent, as described above, and then plated at a density of 5000 cells per well on fibronectin–laminin-coated eight-well RS-treated Labtek II chamber slides in the absence of FGF2 for 48 hr. FGF2 (10 ng/ml) was added for 24 hr, and the cells were pulsed with 10 μM bromodeoxyuridine (BrdU; Sigma) for the final 4 hr. The cells were fixed in 3.7% formaldehyde in PBS for 15 min at RT and rinsed with PBS. The cells were permeabilized in 0.3% Triton X-100 (Fisher Scientific, Pittsburgh, PA) in PBS for 15 min at RT. Rat anti-BrdU (Accurate Chemicals, Westbury, NY) was applied at 1:500 for 45 min at 37°C in the following mixture: immunofluorescence (IF) buffer (0.5% NP-40, 5 mg/ml BSA in PBS), 20 mM $MgCl_2$, and 200 U/ml DNase 1 (Calbiochem, San Diego, CA). The slides were washed three times for 5 min in PBS and then incubated with TRITC-conjugated donkey anti-rat (Jackson ImmunoResearch) at 1:100 in IF buffer with 5 μg/ml bis-Benzimide (Sigma) for 45 min at 37°C. The slides were washed three times for 5 min in PBS, coverslipped, and visualized as described above. The number of BrdU-positive nuclei were counted and compared with the total number of nuclei in each condition and genotype. A minimum of 100 cells was counted for each sample.

Oligodendrocyte progenitor cells. A2B5-immunoreactive oligodendrocyte progenitor cells were isolated from spinal cords by immunoselection as described previously (McKinnon et al., 1990). Purified cells were plated on Falcon culture dishes (Becton Dickinson) precoated by incubating with a solution of 100 μg/ml poly-L-ornithine (Sigma) in 15 mM boric acid, pH 8.4, and then extensively washing in sterile water. The cells were cultured in DMEM containing 4.5 gm/l D-glucose (Invitrogen), penicillin (50 U/ml), streptomycin and transferrin (50 μg/ml each), sodium selenite and triiodothyronine (30 nM each), 50 ng/ml bovine insulin, and 0.5% FBS (Invitrogen). The cells were expanded as secondary cultures (McKinnon and Zazanis, 1996) by supplementing with B104 neuroblastoma-conditioned medium (B104 cm; 20% v/v) (Schubert, 1974; Louis et al., 1992). The cells were subcultured as described for the oligodendrocyte line CG-4 (Louis et al., 1992) using ATV trypsin solution (Irvine Scientific, Irvine, CA) to dislodge the cells from the culture flasks. Transplantations were done with these secondary oligodendrocyte progenitor cultures maintained *in vitro* for <15 passages (at a 1:3 split ratio).

Grafting of oligodendrocyte progenitor cells into postnatal day 2 mouse brain

When oligodendrocyte progenitor cells (OPCs) became confluent, they were labeled with the fluorescent dye PKH26 (Sigma), and 10,000–20,000 pooled cells were injected into postnatal day (P) 2 shiverer mutant mouse brains lacking myelin basic protein (MBP). Cell grafts were placed into the mid-thalamus by injection through the cortex and right lateral ventricle (Osterhout et al., 1997). *Shi/+* and *shi/shi* mouse were used as positive and negative controls for MBP staining, respectively. Pups were killed at P14 except one Shi/Shi host, which was killed at P21. Brains were removed, fixed in 4% paraformaldehyde, and cryoprotected in 20% sucrose. Free-floating coronal serial sections (40 μm) were cut on a freezing microtome and processed for immunofluorescence using a polyclonal rabbit anti-MBP (1:400) overnight (gift from Dr. D. Colman, Montreal Neurologic Institute, Montreal, Canada). Sections were then rinsed, incubated in goat anti-rabbit FITC for 90 min, mounted on gelatin-coated slides, air-dried overnight, and coverslipped with Fluoromount G. Sections were viewed under the fluorescent microscope using a TRITC filter to visualize PKH26 and a FITC filter to visualize MBP staining.

Results

We tested whether increased numbers of progenitors might reside in the *Nf1*+/− adult nervous system. NG2 has been used as a marker of oligodendrocyte progenitors in uninjured adult mouse spinal cord (Wu et al., 2000). Cross sections of adult wild-type and *Nf1*+/− cervical spinal cords were cut and labeled with anti-NG2. Figure 1, *A* and *B*, shows examples of stained cells. Labeled cells in both genotypes had characteristic small cell bodies and multiple branched processes characteristic of oligodendrocyte progenitors (Watanabe et al., 2002). Counts of NG2+ cells revealed a significant increase ($p = 0.001$) in NG2+ cells in both gray and white matter in *Nf1*+/− spinal cord (Fig. 1*C*).

Increased *Nf1* mutant progenitors exist *in vivo* and after acute dissociation of E12.5 spinal cords

We tested whether increased numbers of glial progenitors present *in vivo* in the adult are also present in the developing CNS in E12.5 spinal cord. The earliest known markers for oligodendrocyte progenitors are the basic helix-loop-helix transcription factors Olig1 and Olig2 (Lu et al., 2002; Zhou and Anderson, 2002). We used the spinal cord as a model system because, at E12.5, glial progenitors developed in significant numbers, whereas in brain and optic nerve, these cells arise after embryonic lethality. Spinal cords were sectioned and labeled with anti-Olig2 (kindly provided by D. Rowitch and C. Stiles). Sections were not labeled for Olig1 because the available antibody is not as reliable (J. Alberta, personal communication). Figure 1, *D*, *E*, and *F*, shows examples of stained cells. Counts of Olig2+ cells revealed a significant increase ($p = 0.01$) in *Nf1*−/− spinal cords compared with wild-type spinal cords (Fig. 1*G*).

It is now clear that Olig2 and NG2 can mark cells capable of becoming neurons as well as oligodendrocytes at certain times during development (Sun et al., 2001; Belachew et al., 2003). To define progenitors amplified in *Nf1*mutants, we performed marker analysis on cells from E12.5 mutant spinal cord after acute dissociation. Cells were dissociated, plated, fixed within 24 hr, and then stained for markers of CNS progenitors. As shown in Table 1, similar percentages of E-NCAM-positive putative neuronal progenitors were present across genotypes. In contrast, the percentage of cells expressing nestin, A2B5 or NG2 (progenitor and glial lineage markers *in vitro*, respectively), was increased

threefold to fourfold in *Nf1*−/− spinal cords. Again, *Nf1*+/− spinal cord cells had intermediate levels of precursor accumulation, supporting the idea that there is an expanded pool of glial progenitors in *Nf1* mutant spinal cords.

In NEP medium, *Nf1* mutant cells give rise to glia but not neurons

To determine how *Nf1* mutation results in increased numbers of oligodendrocyte progenitors, we turned to some well studied *in vitro* systems. *In vitro*, it is possible to grow spinal cord cells under conditions that maintain them as relatively undifferentiated neuroepithelial cells. This medium also allows the expansion of oligodendrocyte progenitors (Kalyani et al., 1997; Rao and Mayer-Proschel, 1997; Rao et al., 1998; Rao, 1999; Herrera et al., 2001; Gregori et al., 2002).

We tested whether cell populations are altered when cells derived from *Nf1* mutant spinal cords are cultured in this medium, as described for mouse cells (Mujtaba et al., 1999). Small dark putative progenitors overtook other cell types in *Nf1*−/− cultures, forming a monolayer by day 6. In contrast, wild-type and *Nf1*+/− cultures failed to reach this level of growth even by day 14 (data not shown). For all three genotypes, the cells expanding under these culture conditions had an antigenic phenotype (A2B5+, NG2−, PDGFRα−) characteristic of glial lineage cells (Table 2).

Early oligodendrocyte progenitors lack expression of the PDGF receptor and require FGF2 for growth (Rao and Mayer-Proschel, 1997; Rao, 1999). We first determined whether FGF2 was necessary for the appearance of mutant progenitors. Cultures were generated in NEP medium either without growth factors, with FGF2 (5 ng/ml) alone, with PDGFAA (10 ng/ml) alone, or with FGF2 and PDGFAA in combination, and colony number and size were counted daily for 5 d (Fig. 2). Colonies did not appear in large numbers when cells were maintained in the absence of growth factors or with PDGFAA alone. In contrast, large colonies appeared when FGF2 was present, and the combination of FGF2 and PDGFAA was not significantly different from FGF2 alone.

Because some *Nf1* mutant cell types are hypersensitive to certain growth factors, we varied the dose of FGF2 and assayed colony number. If *Nf1*-deficient cells are hypersensitive to FGF2, the low dose used in the above study might have masked an *Nf1*+/− phenotype. We therefore varied the concentration of FGF2 between 1 and 25 ng/ml, and colony counts were again performed daily for 5 d (Fig. 3*A*). At the lowest doses tested (1–2.5 ng/ml), there was little or no colony formation with cells from any genotype. At 5 ng/ml, in keeping with the above study, abundant cell accumulation was observed in *Nf1*−/− cultures, and significantly less growth was observed in *Nf1*+/− and wild-type cultures. At 10 ng/ml of FGF2, *Nf1*+/− cultures developed significantly more colonies than did wild-type

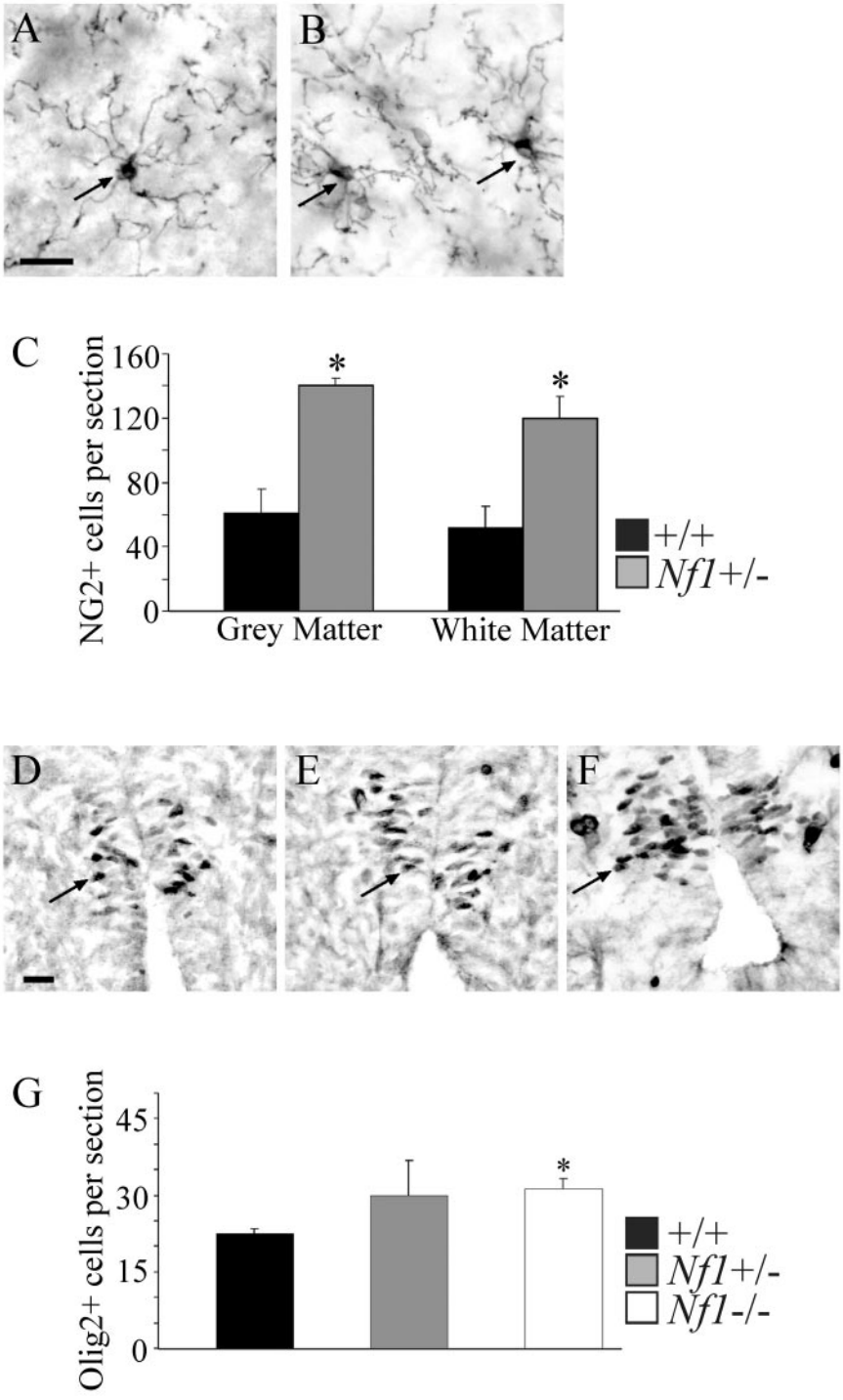

Figure 1. NG2 labeling of adult mouse spinal cords and Olig2 labeling of E12.5 spinal cords. *A*, *B*, Photomicrographs of transverse sections of adult wild-type (*A*) and *Nf1*+/− (*B*) spinal cords showing immunostained NG2+ cells (arrows). Scale bar, 20 μm. *C*, Significantly more NG2+ cells were found in the *Nf1*+/− ($n = 3$) versus wild-type ($n = 3$) spinal cords in both gray matter (*$p = 0.01$; paired Student's *t* test) and white matter (*$p = 0.001$; paired Student's *t* test). *D*–*F*, Photomicrographs of transverse sections of E12.5 wild-type (*D*), Nf1+/− (*E*), and Nf1−/− (*F*) spinal cords showing Olig2+ cells (arrows). Scale bar, 20 μm. *G*, Significantly more Olig2+ cells were found in the *Nf1*−/− ($n = 3$) versus wild-type ($n = 3$) spinal cords (*$p = 0.01$; paired Student's *t* test). *Nf1*+/− ($n = 3$) spinal cords were not significantly different from either wild-type or *Nf1*−/− spinal cords.

Table 1. Marker analysis of E12.5 embryonic spinal cord cells

	+/+	*Nf1*+/−	*Nf1*−/−
Antigen	Percentage of cells expressing antigen		
Nestin	17 ± 1	30 ± 1	54 ± 2
A2B5	17 ± 5	37 ± 2	65 ± 1
ENCAM	12 ± 1	17 ± 1	15 ± 1
NG2	8 ± 1	16 ± 2	35 ± 2

Cells of designated genotype were plated and immunostained 24 hr after dissociation. Cells from three embryos of each genotype were analyzed. Data are ±SE.

cells (Fig. 3*B*). At 25 ng/ml, colonies formed in cells of all three genotypes, although they appeared earlier in *Nf1* mutant cultures; *Nf1*−/− cultures became confluent at day 3, *Nf1*+/− cells at day 4, and wild-type cultures by day 5.

The hypersensitivity of *Nf1*+/− and *Nf1*−/− cells to FGF2 suggested a negative role for *Nf1* in regulation of FGF signaling. The best-defined neurofibromin-signaling pathway is control of Ras-GTP levels. NF1 returns active Ras-GTP to its inactive (GDP) state, and in some cell types (likely when Ha-Ras is important; see Discussion), Ras activation can be blocked by farnesylprotein transferase inhibitors (FTI) (Yan et al., 1995; Kim et al., 1997; Costa et al., 2002). To begin to examine whether Ras-GTP was required for the accumulation of progenitors, cells were maintained in the presence of 5 ng/ml FGF2 (and PDGFAA), either with or without 1 μM of the FTI, L744,832 (a gift from Merck Research Labs) (Kohl et al., 1995). The presence of FTI completely inhibited the aberrant accumulation of cells in the *Nf1*−/− cultures (Fig. 4*A*,*B*). In contrast, the drug did not affect the appearance of the wild-type cultures (Fig. 4*A*,*B*).

Together, results from these experiments suggest that the accumulation of cells in both *Nf1*−/− and *Nf1*+/− cultures depends on FGF2 and may depend on activation of the Ras-signaling pathway. To test directly whether there is increased Ras activation in the *Nf1*+/− cells, a Ras-activation assay was performed. We chose to use *Nf1*+/− cells rather than *Nf1*−/− cells as a stringent test of possible alterations in Ras-GTP levels. We compared *Nf1*+/+ with *Nf1*+/− cells. Wild-type and *Nf1*+/− cells were FGF2-starved for 24 hr and then stimulated with FGF2 for 10 min. Cell extracts were incubated with a bead-conjugated fragment of Raf1 with high affinity for Ras-GTP but not Ras-GDP, and bound protein eluted from beads and levels of GTP-bound Ras proteins were determined by immune blotting with a Ras10 antibody. Wild-type cells had low levels of Ras-GTP in uninduced conditions and readily detectable levels after FGF stimulation (Fig. 4*C*). In contrast, *Nf1*+/− cells had elevated levels of Ras-GTP in uninduced conditions and showed super maximal stimulation during FGF stimulation (Fig. 4*C*). These results are consistent with NF1 acting as a Ras-GAP in the FGF2 signaling pathway and with the interpretation that a decrease in neurofibromin function leads to a hypersensitivity to FGF2.

Marker analysis of expanded embryonic spinal cord cultures

Cells from *Nf1*+/−, *Nf1*−/−, and wild-type cultures were stained with markers that distinguish among CNS progenitor types defined *in vitro* (Rao, 1999) (Table 2). In general, the *Nf1* mutant cells were similar to wild-type cells cultured under the same conditions. Neither cell type expressed E-NCAM, a marker of neuronal precursors (Mujtaba et al., 1999), PDGFRα, or NG2 chondroitin sulfate, which is expressed in concert with PDGFR. Absence of these markers is characteristic of early oligodendrocyte precursors (Rao, 1999) and consistent with the lack of response of mutant cells to PDGFAA (see above). Both *Nf1* mutant and wild-type cells also expressed antigenic determinants of early oligodendrocyte lineage phenotype, including nestin and A2B5 immunoreactivity, and the ability to generate O4-immunoreactive cells (Table 2).

Several important differences were observed between cells in *Nf1* mutant and wild-type cultures. Almost all *Nf1*−/− cultures were positive for the progenitor marker nestin (87%) and A2B5 (74%), whereas only approximately one-half the cells expressed these markers in wild-type cultures. Wild-type cells generally lose nestin expression before acquiring O4 immunoreactivity, a marker for the oligodendrocyte lineage (Rao, 1999). In contrast, 72% of *Nf1*−/− cells were positive for O4, most of which (87%) retained nestin expression (Table 2). *Nf1*+/− cells were intermediate between wild-type and *Nf1*−/− cells in marker expression.

In *Nf1* mutant cultures, 16% of the cells coexpressed O4 and GFAP immunoreactivity (Fig. 5*A*; Table 2). Wild-type cultures did not contain cells with this phenotype; *Nf1*+/− cells were intermediate. This mixed glial phenotype was observed under culture conditions (FGF2) predicted to prevent cells from acquiring astrocytic characteristics (Rao, 1999). Similar phenotypes have been reported previously in pathologic white matter (Dyer et al., 2000). Thus, immunolabeling studies indicate that abnormal progenitor cells arise in large numbers in *Nf1* mutant cultures.

Cell proliferation and death

The accumulation of large numbers of cells in *Nf1* mutant cultures must result from an increase in cell proliferation or a decrease in cell death. To begin to test whether numbers of dying cells were different in wild-type and mutant cells, Hoechst-labeled nuclei were visualized. Overall, there were three times fewer pyknotic nuclei in *Nf1*−/− cultures versus wild-type cul-

Table 2. Immunocytochemical marker analysis of expanded embryonic spinal cord cells

	NEP	GRP	O2A	Oligo	Type 1 astrocyte	Type 2 astrocyte	+/+	*Nf1*+/−	*Nf1*−/−
Antigen							Percentage of cells expressing antigen		
Nestin	+	+	+	−	+	+	44 ± 3	78 ± 2	87 ± 3
A2B5	−	+	+	−	−	+	56 ± 2	55 ± 2	74 ± 2
E-NCAM	−	−	−	−	+	−	0	0	0
NG2	−	−*	+	+			7 ± 2	7 ± 1	3 ± 1
PDGFR-α	−	−*	+	+			N.D.		0
O4	−	−	+*	+	−	−	49 ± 3	56 ± 2	72 ± 2
GFAP	−	−	−	−	+	+	33 ± 2	36 ± 1	48 ± 1
O4/GFAP	−	−	−	−	−	−	<1	6 ± 1	17 ± 1
Pyknotic							17 ± 2	10 ± 1	5 ± 1
O4/GFAP plus Pyknotic							0	1 ± 1	20 ± 2

Eight days after dissociation from E12.5 spinal cords, cells were stained with designated markers. For comparison, antigenic phenotype of cells defined in the literature is shown. Antigenic characterization of NEP cells, GRPs, O2A, type 1 astrocytes, and type 2 astrocytes is reviewed by Rao (1999). *Do not initially express, but can gain expression maintaining multipotentiality (Gregori et al., 2002). Data is ±SE. N.D., Not determined.

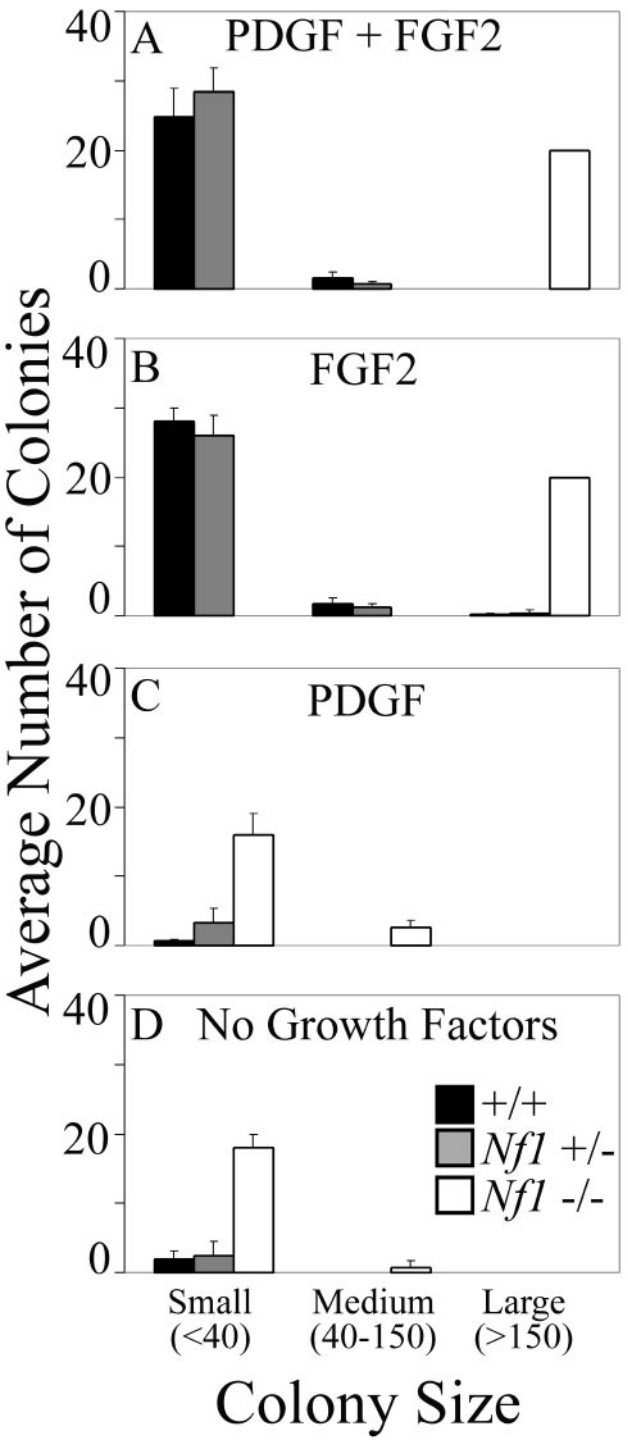

Figure 2. Growth factor requirements of *Nf1* mutant glial progenitors. *A--D*, Colony number and size from wild-type ($n = 9$), *Nf1*+/− ($n = 6$), and *Nf1*−/− ($n = 2$) E12.5 spinal cord cultures at day 5 in the presence of FGF2 plus PDGF (*A*), FGF2 (*B*), PDGF (*C*), and no growth factors (*D*).

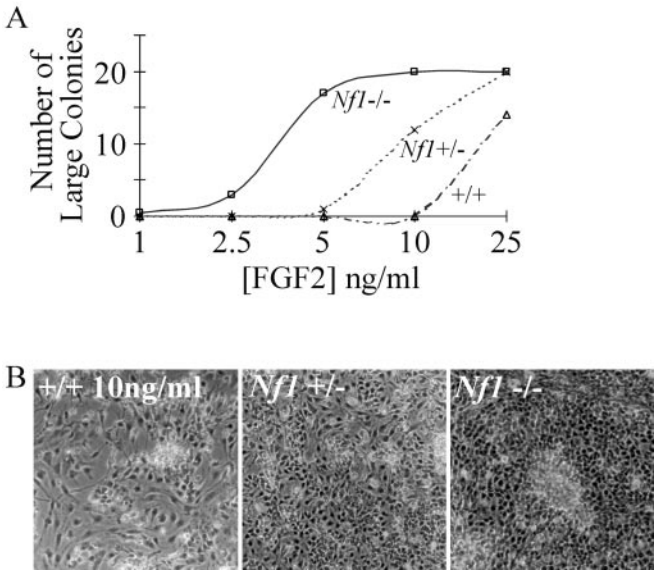

Figure 3. FGF2 dose–response curves. *A*, Number of large (<150 cells) colonies observed at day 5 of wild-type ($n = 3$), *Nf1*+/− ($n = 3$), and *Nf1*−/− ($n = 3$) E12.5 spinal cord cultures grown in the presence of 1, 2.5, 5, 10, and 25 ng/ml FGF2. *B*, Day 5 photographs of wild-type, *Nf1*+/−, and *Nf1*−/− E12.5 spinal cord cultures grown in the presence of 10 ng/ml of FGF2.

tures (Table 2). Strikingly, O4–GFAP double-labeled *Nf1*−/− cells exhibited a high level of pyknotic nuclei (20%).

To confirm these results, we performed a fluorescent DNA fragmentation assay on cultures of wild-type, *Nf1*+/−, and *Nf1*−/− cells. Primary cells grown to 90% confluence were replated and allowed to grow for 3 d in the presence or absence of FGF2 and then examined for cell death (Fig. 5*B*). In the unstimulated condition, there were significantly fewer dying *Nf1*+/− mutant cells compared with wild-type cells ($p = 0.03$), suggesting that activated Ras-GTP promoted increased survival. In FGF2-stimulated conditions, both wild-type and *Nf1*+/− cultures had fewer TUNEL-labeled cells, although the results were not significantly different from the untreated *Nf1* mutant cultures. *Nf1*−/− cells showed an additional decrease in TUNEL-labeled cells. Thus, *Nf1* mutation is sufficient to promote increased survival.

To test whether proliferation might also contribute to the accumulation of *Nf1* mutant cells, we performed a BrdU incorporation assay on wild-type, *Nf1*+/−, and *Nf1*−/− cells. Primary cells grown to 90% confluence were subcultured for 3 d in the absence of FGF2 and then pulsed with FGF2 (10 ng/ml) for 24 hr with 10 nM BrdU present for the final 4 hr. The number of nuclei that incorporated BrdU was then counted (Fig. 5*C*). FGF stimulation resulted in increased numbers of BrdU-positive nuclei in *Nf1*−/− cells ($p = 0.02$; one-tailed Student's t test). Therefore, both increased proliferation and increased cell survival likely contribute to increases in oligodendrocyte progenitors in *Nf1* mutant cells.

OPC colony formation

To confirm that *Nf1* mutant oligodendrocyte lineage cell expansion was not dependent on NEP medium, we tested a second well studied OPC culture system, in which OPCs proliferate and do not differentiate in medium containing FGF2 (McKinnon et al., 1990; Miller et al., 1999) or PDGF and FGF2 (Bogler et al., 1990). In media containing uncharacterized mitogens from neuroblastoma B104-conditioned media, OPCs expand as focal colonies (Louis et al., 1992). Equal numbers of cells from E12.5 wild-type and *Nf1* mutant spinal cords were plated in the presence of B104 mitogens, and colonies were observed in all cultures after several weeks of continuous culture (Fig. 6*A*). The frequency of colony formation varied from 2.5×10^{-3} (*Nf1*+/+) to 14.1×10^{-3} (*Nf1*+/−) and 28.5×10^{-3} (*Nf1*−/−). Consistent with the cells being primarily oligodendroglial lineage, colonies contained ei-

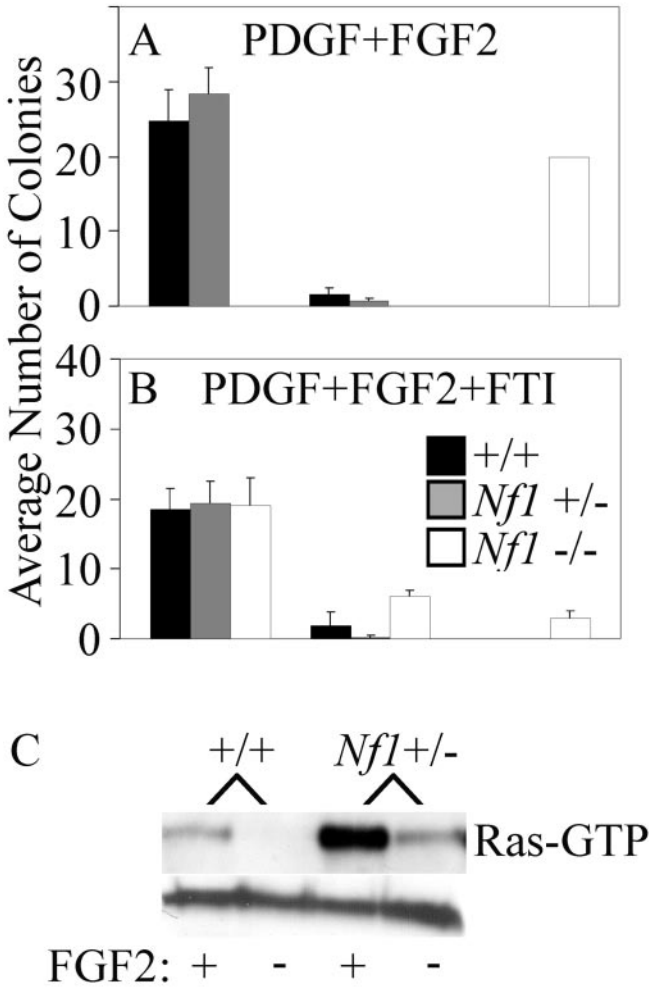

Figure 4. Inhibition of growth by FTI and Ras activation assay. *A*, *B*, Colony number and size from wild-type ($n = 9$), *Nf1*+/− ($n = 6$), and *Nf1*−/− ($n = 2$) E12.5 spinal cord cultures at day 5 in the presence of FGF2 plus PDGF (*A*) and FGF2, PDGF, and FTI (*B*). *C*, Immunoblot of a Ras-GTP pull-down assay showing increased basal and FGF2-stimulated Ras-GTP in *Nf1*+/− cells compared with wild-type cells from E12.5 mouse spinal cord cultures.

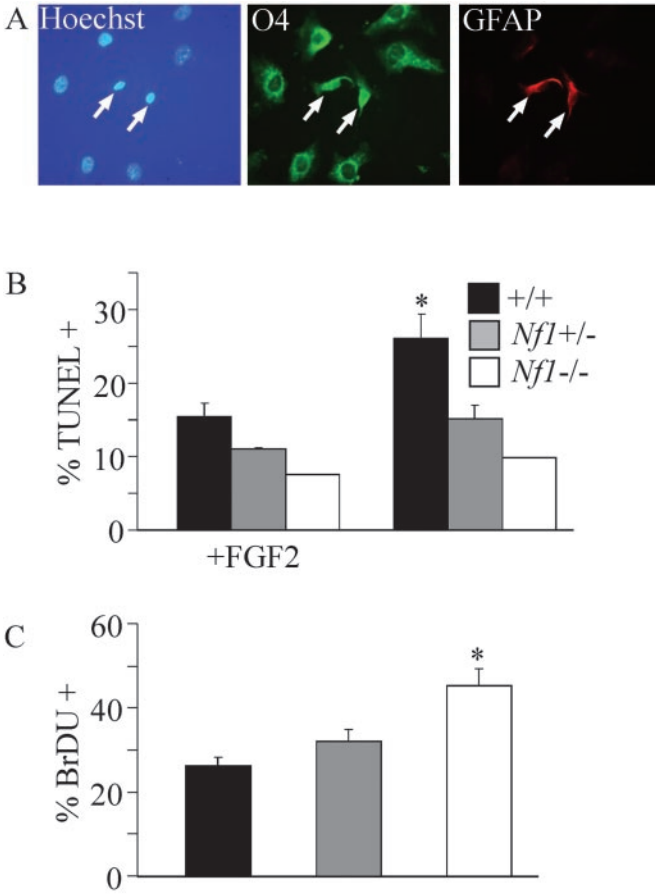

Figure 5. *Nf1*−/− mixed glial cells, DNA fragmentation, and cell proliferation assays. *A*, Triple labeling of *Nf1*−/− spinal cord cultures showing O4+/GFAP+ cells (arrows). Expanded cultures of E12.5 mouse spinal cord cells were assayed for DNA fragmentation and BrdU incorporation. *B*, Significantly fewer (*$p = 0.03$; paired Student's *t* test) *Nf1*+/− ($n = 3$) cells were undergoing cell death in the unstimulated condition versus wild-type cells ($n = 3$). A significant difference was not found when stimulated with 25 ng/ml FGF2. Fewer *Nf1*−/− ($n = 1$) cells were dying in each condition. *C*, A 4 hr pulse revealed a significant increase (*$p = 0.02$; paired Student's *t* test) in BrdU incorporation in the *Nf1*−/− ($n = 3$) cultures versus both *Nf1*+/− ($n = 3$) and wild-type ($n = 3$) cultures after 24 hr of FGF2 stimulation (10 ng/ml). There was a slight increase in BrdU incorporation in the *Nf1*+/− cultures versus wild-type cultures, but it failed to reach significance.

ther A2B5+ OPCs or mixtures of OPCs and A2B5+, GFAP+ cells (type 2 astrocyte; a cell type only found in culture); only a few GFAP+/A2B5− type 1 astrocytes were found (Fig. 6*B*).

To determine whether cells isolated from *Nf1* mutant mice were capable of differentiating into oligodendrocytes, *Nf1*−/− OPCs were dye-labeled and then implanted into P2 myelin basic protein (*MBP*−/−) (shiverer) mouse brains. If MBP-expressing cells are detected, they must arise from the grafted cells. Numerous grafted cells became MBP positive and showed myelin sheath formation 12 d after grafting (Figs. 6*C*,*D*,*E*). Thus, cells expanded in *Nf1*−/− cultures can give rise to oligodendrocytes, and the absence of neurofibromin in these cells did not affect their ability to form myelin *in vivo*.

Discussion

The findings presented here demonstrate that loss of neurofibromin results in an abnormal accumulation of CNS progenitor cells. Oligodendrocyte progenitors are increased in number in the adult and embryonic *Nf1*mutant spinal cord. Marker analysis suggests that glial progenitors are enriched *in vivo* and immediately after dissociation from embryonic spinal cords and expand in culture. Amplified cells express markers of the oligodendrocyte lineage, including NG2, Olig2, and O4, but *in vitro* lack expression of the neuronal precursor marker E-NCAM. Although the full potential of the *Nf1* mutant cells studied here remains to be determined, the abnormal progenitor cell population is dependent on FGF2 for expansion and forms oligodendrocytes on transplantation *in vivo*. Together, the data support a role for *Nf1* in oligodendrocyte development.

The antigenic profile of the *Nf1* mutant cells characterized shows some similarities to a cell type identified *in vitro* as a GRP (Rao and Mayer-Proschel, 1997; Mujtaba et al., 1999). GRPs can give rise to astrocytes and OPC cells, but not neurons, *in vitro* and to astrocytes and oligodendrocytes during transplantation *in vivo* (Herrera et al., 2001). We did not address the ability of *Nf1* mutant cells grown in NEP medium to differentiate into neurons or astrocytes. Indeed, cell fate studies suggest that astrocytes and oligodendrocytes may be derived from separate progenitor cell pools *in vivo* (Lu et al., 2002; Zhou and Anderson, 2002), and thus the full range of phenotypic choices available to progenitors may not be fully exercised under normal developmental conditions. When grown under conditions that promote the expansion of

PDGF-responsive OPCs (Louis et al., 1992; McKinnon and Zazanis, 1996), *Nf1* mutant cultures generated A2B5+ progenitors and A2B5+/GFAP+ type 2 astrocytes. Because *in vivo* analyses have not detected type 2 astrocytes, we focused on oligodendrocyte differentiation of the mutant cells. Strikingly, during transplantation into MBP-deficient mice, *Nf1* mutant progenitors differentiated into myelin-forming oligodendrocytes.

A significant fraction of cells in *Nf1* mutant cultures simultaneously expressed precursor markers (nestin, A2B5) and later emerging differentiation markers (e.g., O4). In contrast, wild-type oligodendrocytes stop expressing nestin as they begin to express differentiation markers (Rao, 1999). Defects in CNS progenitors may also arise in nestin+ NEPs earlier in development, accounting for high nestin expression. Many *Nf1* mutant cells also concomitantly expressed GFAP and O4, exhibiting an aberrant mixed glial phenotype (Godfraind et al., 1989; Dyer et al., 2000). Thus, *Nf1* mutation uncouples the normal cessation of proliferation that occurs under *in vitro* differentiation conditions. However, the ability of *Nf1* mutant cultures to generate myelin-forming oligodendrocytes during transplantation into MBP-deficient mice indicates that such alterations do not uniformly interfere with normal differentiation.

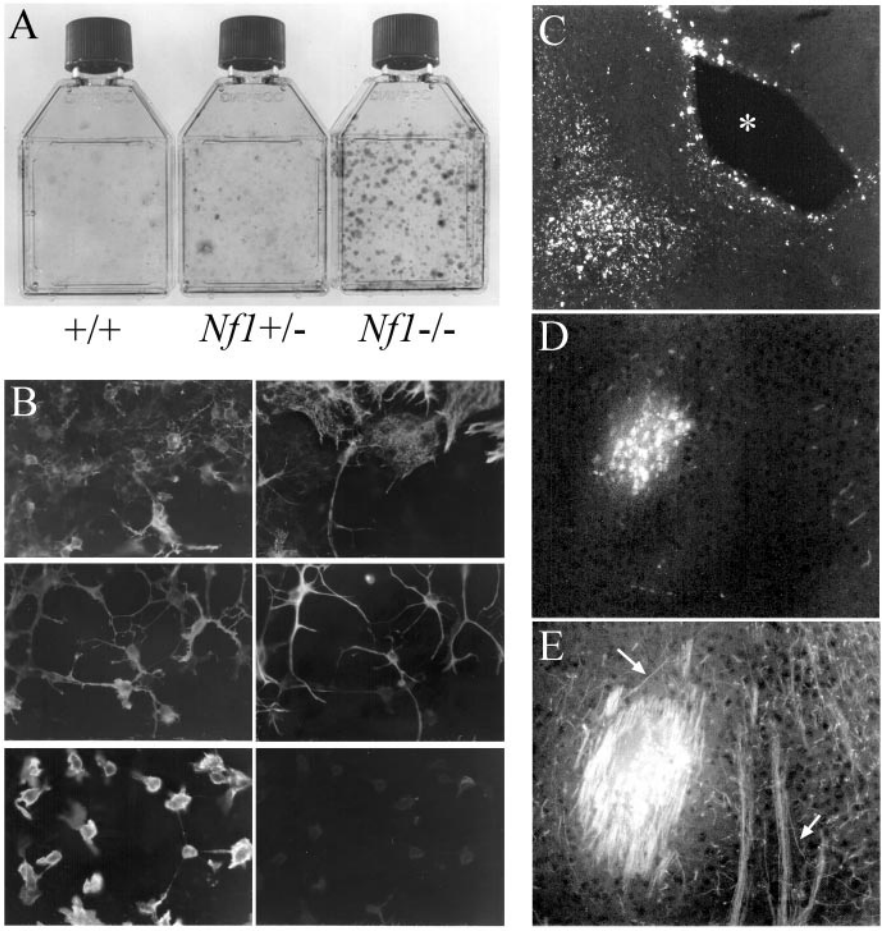

Figure 6. E12.5 spinal cord cultures and grafting of dye-labeled *Nf1*−/− OPCs (O2A cells) into the forebrain of MBP−/− mice. *A*, OPC cultures from wild-type, *Nf1*+/−, and *Nf1*−/− E12.5 spinal cords stained with cresyl violet. *B*, Cells expanded from *Nf1*−/− cultures were identified by double-label immunocytochemistry using A2B5 (left) and anti-GFAP antibodies (right). Left and right images represent the same field of cells in each pair. Top, Colony containing both type 1 astrocytes (A2B5−/GFAP+) and oligodendrocyte progenitor cells (A2B5+/GFAP−). Center, Colony with type 2 (A2B5+/GFAP+) astrocytes. Bottom, Colony with only A2B5+/GFAP− oligodendrocyte progenitor cells. *C*, A low-magnification micrograph shows the relationship to the third ventricle (*) of dye-labeled grafted OPCs in MBP− mouse brain. A group of dye-labeled cells is shown at higher magnification in *D* with associated MBP+ myelin sheaths in *E*. Arrows point to a small group of longitudinally cut myelin sheaths.

Cell defects driven by increased response to growth factors may be a common feature of loss of *Nf1*. *Nf1* null embryonic peripheral neurons show that Ras-mediated hyperresponsiveness to neurotrophic factors (Vogel et al., 1995, 2000; Zhu et al., 2001) and astrocytoma cells from *Nf1* patients, as well as *Nf1* mutant fibroblasts, are hyper-responsive to epidermal growth factor (Atit et al., 1999; Gutmann et al., 1999). Hematopoietic progenitors from *Nf1*−/− murine fetal liver form abnormally high numbers of progenitor colonies in response to low levels of cytokines *in vitro* (Largaespada et al., 1996; Zhang et al., 1998). Our observation that FGF2-stimulated Ras-GTP is elevated in *Nf1* mutant glial progenitors is the first direct demonstration of abnormal Ras activation by FGF2 in an *Nf1*-deficient background. Previous evidence implicated neurofibromin in modulation of response to FGF2 signaling (Kitano et al., 1992; Griesser et al., 1997; Kaufmann et al., 1999). Ras-GTP was elevated in CNS progenitors under basal conditions, as in *Nf1* mutant Schwann cells (Kim et al., 1995; Sherman et al., 2000). In contrast, *Nf1* mutant hematopoietic progenitors show increased Ras-GTP only after cytokine stimulation (Zhang et al., 1998). Thus, the effects of loss of *Nf1* on Ras regulation among progenitor populations appear to be cell-type specific.

Although neurofibromin has poorly defined non-Ras functions (Johnson et al., 1993, 1994; Guo et al., 1997, 2000; Kim et al., 1997; Tong et al., 2002), these appear not to be relevant to phenotypes defined here, because abnormal accumulation of *Nf1* mutant glial progenitor cells was abolished by the addition of FTIs. This finding suggests the involvement of aberrant Ras signaling in the mutant phenotype. Blocking farnesylation inhibits Ras processing that is required for Ras membrane localization and activation. The data are consistent with the ability of FTIs to rescue *Nf1*-dependent phenotypes in Schwann cells (Yan et al., 1995; Kim et al., 1997) and learning deficits in *Nf1* heterozygous mice (Costa et al., 2002). We cannot presently exclude effects of FTIs on non-Ras farnesylated proteins, such as RhoB (Prendergast, 2000), in this system. However, consistent with effects of FTIs on progenitor cells being mediated through Ras, Ras-GTP was elevated in *Nf1* mutant CNS progenitor cells. Together, these data support a key role for farnesylation-sensitive form(s) of Ras, such as H-Ras (Sebti and Hamilton, 1997), upstream of neurofibromin in glial progenitors.

Nf1 mutant cells in our experiments accumulated, at least in part, through increased cell survival. There were significantly fewer dying (pyknotic and TUNEL+) cells in *Nf1*+/− mutant cultures compared with wild-type cells, and a trend toward increased survival in the *Nf1* mutants was stimulated with FGF2. Differences were also detected in cell proliferation rates. We conclude that survival and proliferation are both affected. Previous

studies identified FGF2 as a survival factor for early glial progenitors (Yasuda et al., 1995). Increased survival of *Nf1* mutant progenitors in low doses of FGF implicates Ras signaling downstream of FGF-mediated survival. In later OPCs, activation of phosphotidyl inositol 3-kinase is essential for survival (Vemuri and McMorris, 1996), and this survival pathway is activated by PDGF but not FGF (Ebner et al., 2000). The data suggest that signaling pathways required for glial progenitor survival differ as the cells undergo maturation.

It is especially relevant to note that we observed abnormalities in *Nf1* hemizygous glial lineage cells, in both embryos and adult mice. Mutant human *NF1*+/− cells could account for some nonfocal effects in the NF1 patient nervous system. Magnetic resonance spectroscopy of NF1 patients shows an increase in choline, suggestive of focal edema and vacuolization of myelin (Wang et al., 2000). Eastwood et al. (2001) showed increases in water diffusibility by MR imaging and proposed that this also reflected widespread myelin disorder in the NF1 brain. On pathologic examination, T2-hyperintense lesions in NF1 patients appeared to contain vacuolated myelin (DiPaolo et al., 1995); these lesions have even higher water diffusibility than the surrounding NF1 brain (Eastwood et al., 2001). Although nests of demyelinated cells (T2 hyperintensities) in NF1 patients may represent either heterozygous or null *NF1* mutations, the global changes in brain metabolism likely result from *NF1* haplo-insufficiency. It is also plausible that with increased numbers of *NF1* glial progenitors, some lose the second *NF1* allele, causing astrocyte tumor formation; loss of heterozygosity at *NF1* is frequent in NF1 patient pilocytic astrocytomas (Gutmann et al., 2000; Kluwe et al., 2001; for review, see Cichowski and Jacks, 2001). In this context, it is of interest that astrocytes arise from cells that would normally make oligodendrocytes or motor neurons when signaling is perturbed (Zhou et al., 2002).

A striking finding in this study is the marked increase *in vivo* of NG2+ cells in the adult *Nf1*+/− mouse spinal cord. Previous studies identified proliferating NG2+ cells in the adult human and rodent CNS that are distinct from astrocytes, oligodendrocytes, and microglia (Chang et al., 2000; Wu et al., 2000; McTigue et al., 2001). Cells with these characteristics can give rise to mature oligodendrocytes *in vitro* (Wolswijk and Noble, 1989) and *in vivo* (Watanabe et al., 2002). Abnormalities in the embryonic glial progenitor pool that we defined *in vitro* are likely to exist *in vivo* and persist into the adult, accounting for the increase in adult NG2+ cells. Indeed, FGF2 maintains NG2+ progenitors in a proliferative state in adult CNS (Armstrong et al., 2002).

Our data suggest the hypothesis that abnormalities in the glial lineage contribute to brain dysfunction in NF1 patients. Small increases in brain progenitor pools result in a magnified increase in brain volume (Rakic, 1995). For example, increased progenitor numbers could contribute to the enlarged white matter tracts that underlie macrocephaly in NF1 patients.

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