MOLECULAR AND CELLULAR BIOLOGY, Feb. 1997, p. 862–872 Vol. 17, No. 2
0270-7306/97/$04.00+0

Nf1-Deficient Mouse Schwann Cells Are Angiogenic and Invasive and Can Be Induced To Hyperproliferate: Reversion of Some Phenotypes by an Inhibitor of Farnesyl Protein Transferase

HAESUN A. KIM,† BO LING, AND NANCY RATNER*

Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0521

Received 23 August 1996/Returned for modification 21 October 1996/Accepted 5 November 1996

We have developed a potential model of Schwann cell tumor formation in neurofibromatosis type 1 (NF1). We show that mouse Schwann cells heterozygous or null at *Nf1* display angiogenic and invasive properties, mimicking the behavior of Schwann cells from human neurofibromas. Mutations at *Nf1* are insufficient to promote Schwann cell hyperplasia. Here we show that Schwann cell hyperplasia can be induced by protein kinase A activation in mutant cells. Removal of serum from the culture medium also stimulates hyperplasia, but only in some mutant cells. After serum removal, clones of hyperproliferating Schwann cells lose contact with axons in vitro, develop growth factor-independent proliferation, and exhibit decreased expression of the cell differentiation marker P0 protein; hyperproliferating cells develop after a 1-week lag in Schwann cells heterozygous at *Nf1*. The experiments suggest that events subsequent to *Nf1* mutations are required for development of Schwann cell hyperplasia. Finally, an anti-Ras farnesyl protein transferase inhibitor greatly diminished both clone formation and hyperproliferation of null mutant cells, but not invasion; farnesyl transferase inhibitors could be useful in treating benign manifestations of NF1.

Neurofibromatosis type 1 (NF1) is one of the most common inherited human autosomal dominant diseases, with a worldwide incidence of 1 in 3,500 individuals (56). Benign manifestations frequently associated with NF1 include pigmented lesions of the skin (café au lait spots), hamartomas of the iris (Lisch nodules), learning disabilities, formation of optic pathway gliomas, and neurofibromas (22). Neurofibromas are one of the major defining features of NF1. Cutaneous neurofibromas (benign peripheral nerve sheath tumors) are associated with small nerve branches, plexiform neurofibromas develop along major peripheral nerves, and malignant peripheral nerve sheath tumors (MPNST) develop in about 4% of NF1 patients (56). Genetic studies show that malignant tumors in NF1, including MPNSTs, contain cells with mutations in both the constitutionally inactivated and previously normal somatic allele, consistent with *NF1* acting as a tumor suppressor (19, 36, 64). The mechanism of neurofibroma formation is less clear. While recent studies demonstrated loss of heterozygosity (LOH) at the *NF1* locus in some neurofibromas (12, 60), LOH could be associated with either of the major cell types in the benign tumors, fibroblasts or Schwann cells.

Schwann cells may be the primary pathogenic cells in neurofibromas. The majority (40 to 85%) of cells in neurofibromas are Schwann cells (18, 49), and in contrast to Schwann cells in normal nerve, neurofibroma Schwann cells are found without association with axons (28, 50). Furthermore, in vitro, Schwann cells, but not fibroblasts, from neurofibromas show angiogenic and invasive properties (63). Because the genetic status of neurofibroma Schwann cells is unknown, it remains unclear if a single *NF1* mutation is sufficient for manifestation of some neurofibroma Schwann cell phenotypes or if mutations (or epigenetic changes) subsequent to the inherited, predisposing mutation are required.

Alteration in cell signaling pathways are likely to contribute to abnormalities in *NF1*-deficient Schwann cells. The *NF1* gene product, neurofibromin, contains a region homologous to yeast IRA proteins and mammalian Ras-GTPase-activating proteins (4, 72, 73) that function as negative regulators of Ras by accelerating the conversion of Ras-GTP to Ras-GDP. The Ras-GTPase-activating protein-related domain (GRD) of neurofibromin complements *ira* mutations in the yeast *Saccharomyces cerevisiae* and functions as a negative regulator of Ras in vitro (4, 21, 41, 72). Cell lines derived from MPNSTs have little neurofibromin and high levels of Ras activation (7, 16). Lowering Ras-GTP in these cells inhibits cell growth. In contrast, expression of an oncogenic Ras in cultured rat Schwann cells is correlated with decreased cell proliferation (29, 57), and transformation of Ras-expressing Schwann cells requires introduction of a second oncogene (29, 57). It is not known whether phenotypes of neurofibroma Schwann cells result from loss of *NF1* alone or require additional gain-of-function mutation in some other oncogene(s), loss-of-function mutation in tumor suppressor genes other than *NF1*, or epigenetic (environmental) alterations.

To address these issues, we have taken advantage of mice with targeted mutations in the *Nf1* gene (9, 25). Neurons from the mice show trophic factor-independent survival (70), and myeloid cells display hypersensitivity to growth factors (35). While mice heterozygous at *Nf1* appear normal and do not develop neurofibromas, it was speculated that homozygous null cells might be required to develop tumors. Since *Nf1*-null embryos die in utero by 14.5 days of gestation, we developed

* Corresponding author. Mailing address: Department of Cell Biology, Neurobiology and Anatomy, University of Cincinnati College of Medicine, P.O. Box 670521, Cincinnati, OH 45267-0521. Phone: (513) 558-6079. Fax: (513) 558-4454. E-mail: nancy.ratner@uc.edu.

† Present address: Division of Cellular and Molecular Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115.

methods to isolate Schwann cells from embryonic day 12.5 *Nf1*-null mouse embryos prior to death and showed that *Nf1*-null Schwann cells do not hyperproliferate or lose contact with axons in vitro (29, 59). Heterozygous and null Schwann cells derived from *Nf1*-deficient mice show increased ratios of Ras-GTP to Ras-GDP (29) but, like Schwann cells expressing v-Ras, exhibit decreased proliferation in response to axonal signals and increased expression of protein zero (P0), a marker of Schwann cell differentiation (58). Thus, additional genetic or epigenetic events might be required for *Nf1*-deficient cells to develop tumor phenotypes.

Here we report that several characteristics identified in human neurofibroma Schwann cells are mimicked by *Nf1*-deficient mouse Schwann cells. Loss of *Nf1* by itself is sufficient to induce angiogenic and invasive properties in Schwann cells. We have identified two epigenetic signals that trigger hyperproliferation in *Nf1*-null Schwann cells: (i) *Nf1*-null mutant Schwann cells undergo hyperplasia when exposed to forskolin, an agent that increases cyclic AMP (cAMP) in cells; and (ii) hyperplasia and dissociation from axons are induced in a subpopulation of null mutant cells upon withdrawal of serum from the culture medium. Because loss of *Nf1* activates Ras in Schwann cells, drugs designed to reverse the oncogenic effects of activated Ras might be useful to reverse phenotypes of *Nf1*-deficient Schwann cells in benign neurofibromas. Ras proteins are farnesylated posttranslationally for membrane targeting and activation by farnesyl protein transferase. Farnesyl protein transferase inhibitors (FPTIs) reverse Ras-transformed phenotypes in cultured cells (26, 52) and growth of malignant tumors in vivo (31, 45). FPTIs inhibited growth of MPNST cell lines derived from an NF1 patient (74). We now show, using the FPTI L-739,749 (52), that hyperplasia but not invasion of *Nf1*-deficient mouse Schwann cells can be reversed in culture. These data are consistent with a model in which loss-of-function mutations at *Nf1* act in concert with epigenetic signals to trigger Schwann cell growth. Further, the data suggest that anti-Ras drugs might reverse at least some phenotypes of *Nf1*-deficient Schwann cells.

MATERIALS AND METHODS

Mouse Schwann cell culture. Mouse Schwann cells were isolated from wild-type, heterozygous, and *Nf1*-null mutant mouse embryo dorsal root ganglia at embryonic day 12.5 essentially as described previously (29). Dorsal root ganglia of the embryos were enzymatically dissociated and cells from single embryos plated onto two wells of six-well culture plates in 10% human placental serum containing Dulbecco modified Eagle medium (DMEM) supplemented with nerve growth factor (NGF). The next day, the medium was switched to serum-free defined N2 medium (54) containing NGF and gentamicin (5 μg/ml). After 5 to 6 days, Schwann cells and neurons were separated from fibroblasts by lifting up the Schwann cell-neuron layers from the dish, leaving most of the fibroblasts behind. Cells from the same genotype were pooled, and Schwann cells were enzymatically dissociated from the neurons in 0.01% collagenase. Cells were centrifuged, resuspended in DMEM with 10% fetal bovine serum (FBS), and plated on poly-L-lysine-coated 100-mm-diameter cell culture plates at a density of approximately 10^6 cells/plate. These cells were considered passage 0. The next day, cells were switched to Schwann cell growth medium containing recombinant human glial growth factor2 (rhGGF2; 10 ng/ml; Cambridge Neuroscience) and 10% FBS, with 2 μM forskolin (Calbiochem) added to suppress fibroblast growth. Contaminating neurons were removed from the cultures by omitting NGF from the medium. After 1 week, cells were trypsinized and replated at the plating density (passage 1). When cultures were examined for expression of a Schwann cell antigen, the low-affinity NGF receptor (NGFR), by using a rat anti-mouse NGFR antibody (obtained from California Institute of Technology), David Anderson, 99.5 to 99.9% of the cells were positive for NGFR expression. NGFR-expressing cells were also positive for the Schwann cell marker S100. In all experiments, cells prepared between passage 1 and 3 were used. In cultures where morphologically transformed *Nf1* mutant Schwann cells were obtained, cells were kept in serum-free N2 medium supplemented with rhGGF2 and forskolin from passage 0 throughout the culture period. For experiments where L-739,749 (Merck Pharmaceutical) was used, cells were preincubated with 10 μM L-739,749 for 3 to 5 days in Schwann cell growth medium prior to experiments unless otherwise indicated.

CAM assay for angiogenesis. Chorioallantoic membranes (CAM) of day 6 postfertilization standard chicken eggs (SPAFAS, Inc., Roanoke, Ill.) were dropped and exposed by cutting a window (1 cm^2) on one side of the egg, using the false air sac technique (2, 63). After exposure of the membrane, windows were sealed with Transpore tape (3M) and the egg was put into a humid incubator at 35 to 37°C. Three days later, cultured cells were trypsinized, washed three times in DMEM, and seeded onto the CAM at a density of 1×10^6 to 1.5×10^6 cells/10 μl/egg. Windows were sealed again, and eggs were incubated for another 48 h. Forty-eight hours later, eggs were removed from incubator for observation with 6× stereoscopic dissecting microscope and assessed for angiogenesis; more than five loops of host blood vessels delineating the added cells was scored as a positive angiogenic response.

In vitro Matrigel invasion assay. The ability of cells to migrate through Matrigel was evaluated by a vital dye method (8). The 8-μm-pore-size polycarbonate filter of a 2-cm^2 transwell unit (Costar) was coated with 30 μg of Matrigel (Collaborative Biomedical Products). Frozen Matrigel was thawed overnight at 4°C and reconstituted in cold phosphate-buffered saline to obtain a concentration of 0.3 μg/ml. To each chamber, 100 μl of diluted Matrigel was added and air dried overnight. The next day, the membranes were reconstituted with 200 μl of serum-free DMEM; after removal of the excess medium, 200 μl of cell suspension in 10% FBS (400,000 cells/ml) was loaded on the membrane (in triplicate). The transwells were then placed in 24-well dishes containing 800 μl of culture medium per well. After 3 or 5 days incubation at 37°C, 20 and 80 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; 5 mg/ml; Sigma) in phosphate-buffered saline were added to the transwells and the bottom wells, respectively. Metabolic conversion of MTT to formazan was allowed to proceed for at least 2 h at 37°C. Cells and formazan crystals were scraped from the bottom of the membrane with precut Whatman 3M filter papers and placed in 150 μl of *n*-butanol. After removal of cells and formazan from the bottom of the filter, the filter was cut from the well and placed in a separate tube that contained 150 μl of *n*-butanol. Subsequently, the formazan was allowed to dissolve at 4°C for 24 h, and 100 μl of *n*-butanol was added. Then 150 μl from each tube was transferred to each well of a 96-multiwell dish, and the optical density (OD) of the formazan product was measured at 540 nm. The invasion rate (Ir) was determined by the ratio of the OD from the bottom of the filter to those from the bottom and top of the filter combined.

Ras processing assay in *Nf1*-null mutant mouse Schwann cells. Schwann cells isolated from *Nf1*-null mutant mouse embryos were plated onto T75 flasks in DMEM plus 10% FBS supplemented with rhGGF2 (10 ng/ml) and grown to near confluence. Cells were then treated with or without 10 μM L739,439 for 2 days in the absence of growth factor. At the end of the incubation period, cells were switched to 5 ml of labeling medium containing 200 μCi of [^{35}S]methionine per ml and 10% dialyzed FBS with or without L-739,749. After 16 to 18 h, cell lysates were prepared by adding 1 ml of lysis buffer (20 mM Tris HCl [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1% Nonidet P-40 [NP-40], 0.1% sodium dodecyl sulfate [SDS], 16 mg of aprotinin per ml, 1 mM dithiothreitol, 1 μM leupeptin, 0.7 μg of pepstatin per ml, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 0.8 mM *N*-ethylmaleimide) to each flask. Cells were scrapped from the flasks, incubated on ice for 30 min, and then centrifuged for 10 min at 14,000 rpm. Equal amounts of trichloroacetic acid-precipitable counts of each extract were precipitated with 10 μg of anti-Ras antibody Y13-259 (Oncogene Science) conjugated to 10 μg of rabbit anti-rat immunoglobulin G complexed with protein G-agarose. For the peptide competition control, Y13-259 was preincubated with 100 μg of Ras peptide for 1 h at 4°C prior to use. After 3 h of incubation at 4°C, beads were washed twice in wash buffer I (0.1 M NaCl, 1 mM EDTA, 0.1 M Tris HCl [pH 8.0], 1% NP-40, 0.3% SDS), twice in wash buffer II (1 M NaCl, 0.1 M Tris HCl [pH 8.0], 0.1% NP-40), and three times in wash buffer III (10 mM Tris HCl [pH 8.0], 0.1% NP-40). Beads were then boiled for 5 min, samples were run on an SDS–15% acrylamide gel, and the gel was processed for autoradiography.

Schwann cell proliferation assay. Schwann cells (25,000 cells/well) obtained from embryonal day 12.5 mouse embryos as described above were plated on poly-L-lysine-coated eight-chamber well Lab-Tek glass slides in DMEM plus 10% FBS. Two days after plating, the medium was switched to serum-free medium (N2) supplemented with appropriate growth factors. Cells were incubated in the presence or absence of growth factors for another 48 h, and [^{3}H]thymidine (1 μCi/ml) was added during the last 24-h incubation period. Cells were fixed in 4% paraformaldehyde and immunostained with a Schwann cell-specific antigen, NGFR, and processed for autoradiography (29). The Schwann cell labeling index was determined by the percentage of NGFR-positive cells that had incorporated [^{3}H]thymidine in the nucleus. In some experiments, Schwann cell proliferation was tested by using a conventional 96-well plate assay as described previously (47).

P0 extraction and Western blotting. Schwann cells isolated from wild-type or *Nf1*-null mouse embryos were plated (1×10^6 to 1.5×10^6 cells/dish) onto poly-L-lysine-coated 60-mm-diameter culture dishes in DMEM with 10% fetal calf serum. After 24 h, dishes were treated with rhGGF2 (10 ng/ml) or with 5 μM forskolin, a condition which induces P0 expression in cultured Schwann cells (43), both in serum-free medium (N2). Cells were cultured for another 4 days; the medium was changed each day. At the end of the culture period, cells were lysed in 50 μl of buffer (2% SDS, 2 mM EGTA, 2 mM EDTA, 5 mM Tris HCl [pH 6.8], 2 mM phenylmethylsulfonyl fluoride), boiled for 5 min, and centrifuged

TABLE 1. Angiogenic response of the chicken CAM to mouse Schwann cells isolated from +/+, +/−, or −/− mutant mouse embryos[a]

Cell type	Genotype	No. of eggs tested	No. positive for angiogenesis
Fibroblasts	+/+	3	0
	+/−	5	0
	−/−	6	0
Schwann cells	+/+	8	0
	+/−	7	4
	−/−	9	6

[a] Dissociated fibroblasts or Schwann cells (10^6) prepared from +/+, +/−, and homozygous −/− mutant mice were placed onto the 9-day postfertilization chick CAM. After 2 to 3 days, angiogenic responses on CAM were assessed; more than five loops of host blood vessels delineating the added cells was scored as a positive response.

for 15 min at 14,000 rpm (43). The amount of proteins in each sample was determined from the resulting supernatant by Lowry assay (40); equal amounts of protein per sample were loaded and separated in SDS–10% polyacrylamide gels. Proteins were then transferred onto nitrocellulose, and P0 was detected by Western blot analysis using a polyclonal antibody (1:500) raised against rat P0 protein from D. Colman.

RESULTS

***Nf1* mutant mouse Schwann cells are both angiogenic and invasive in vitro.** Schwann cells derived from neurofibromas of NF1 patients are abnormal, for unlike normal Schwann cells or neurofibroma-derived fibroblasts, they promote angiogenesis and invade basement membranes in the chick CAM model system (63). To investigate directly the effect of *Nf1* mutation on these properties of Schwann cells, we isolated Schwann cells from wild-type (+/+) or *Nf1* mutant mice at embryonic day 12.5 (29). After growth in culture in serum containing medium supplemented with rhGGF2, both wild-type and *Nf1* mutant Schwann cells were tested in the CAM assay. Schwann cells derived from heterozygous (+/−) or homozygous (−/−) *Nf1* mutant embryos induced angiogenic responses within 2 to 3 days after the cells were seeded on the CAM, while +/+ Schwann cells did not promote angiogenic responses (Table 1). Fifty-seven percent (four of seven) of eggs tested with +/− Schwann cells were positive for angiogenesis, as were 66% (six of nine) of eggs seeded with −/− Schwann cells. This result was obtained in two separate experiments with cells isolated from different embryos. In contrast, fibroblasts derived from wild-type or *Nf1* mutant mouse embryos did not induce an angiogenic response on the CAM.

To determine if Schwann cells lacking *Nf1* are invasive, they were tested in an in vitro Matrigel invasion assay. Cells were plated on Matrigel-coated polycarbonate membranes, and the Ir was calculated 3 or 5 days later by determining the percentage of cells that had passed through the Matrigel and were present on the other side of the membrane. After 3 days (Fig. 1A), while +/+ mouse Schwann cells showed an Ir of 1 to 2%, −/− Schwann cells showed a sevenfold increase in invasiveness compared to +/+ cells. +/− cells had an intermediate Ir of 5 to 7%. By 5 days (Fig. 1B), 10% of +/+ cells invaded the Matrigel, while 23% of +/− cells and 35% −/− cells had crossed the Matrigel-coated filters. This result, which was consistent in four separate experiments using Schwann cells from different embryos, suggests that *Nf1*-deficient Schwann cells can degrade Matrigel and that loss of neurofibromin is sufficient to generate an invasive phenotype.

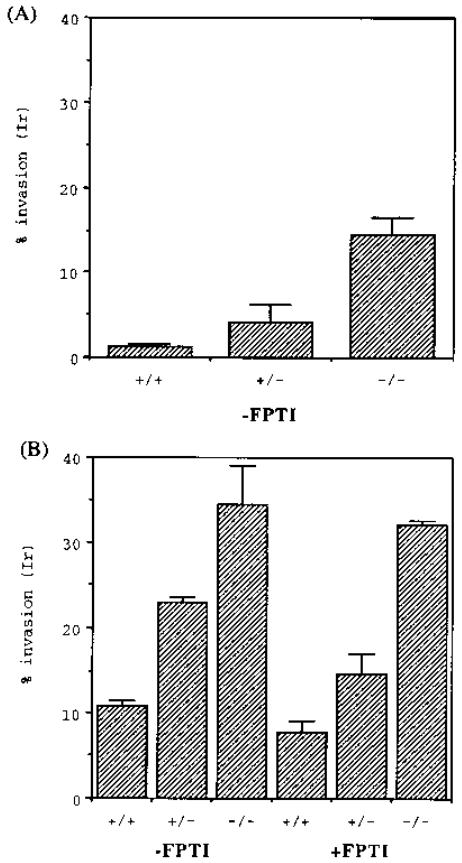


FIG. 1. In vitro Matrigel invasion assay using wild-type or *Nf1*-deficient mouse Schwann cells with or without FPTI (L-739,749) treatment. (A) Three-day invasion assay without FPTI treatment. (B) Five-day invasion assay with or without FPTI. Wild-type (+/+) or *Nf1*-deficient (+/− or −/−) Schwann cells grown in DMEM–10% FBS supplemented with rhGGF2 and forskolin were preincubated with or without FPTI (10 μM) for 2 days. Cells (80,000 cells/chamber) were then plated on an Matrigel-coated invasion chamber membrane (polycarbonate filter, 8-μm pore size) in DMEM–10% FBS without growth factors and incubated for another 3 or 5 days. Irs of cells were determined by the ratio of OD from the bottom of the filter to those from the bottom and top of the filter combined. Results are representative of four separate experiments with each value run in triplicate.

Invasive properties of *Nf1*-deficient mouse Schwann cells are unaffected by treatment with an inhibitor of farnesyl protein transferase. We showed previously that loss of neurofibromin in Schwann cells correlates with elevated levels of activated Ras, suggesting that neurofibromin acts as a negative regulator of Ras in this cell type. To begin to establish whether the invasive properties of Schwann cells lacking neurofibromin might be directly related to the activation of Ras pathway in these cells, we treated cells with an inhibitor of farnesyl protein transferase (L-739,749) to block cellular Ras activity and tested their invasive capacity.

First, to evaluate the activity of L-739,749 in whole cells, a Ras processing assay similar to that described previously (32) was carried out on −/− Schwann cells. Cells were pretreated with 10 μM L-739,749 for 2 days and metabolically labeled with [^{35}S]methionine for an additional 16 to 18 h in the presence or absence of the drug. The amount of processed Ras was monitored by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). Cellular Ras protein present in untreated cells (lane 2) exhibited a gel mobility shift following treatment of cells with L-739,749 (lane 3), indicative of unprocessed forms of Ras in drug-treated cells. No obvious cell death was observed with the drug treatment. This experiment

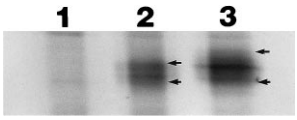


FIG. 2. Inhibition of Ras processing in *Nf1*-deficient mouse Schwann cells by L-739,749. Cells were preincubated with 10 μM L-739,749, metabolically labeled Ras proteins were immunoprecipitated, and Ras processing was analyzed by SDS-PAGE. Lane 1 shows the specificity of the antibody; Schwann cell extract was incubated with antibody-bead complexes that had been treated with Ras peptides that block antigen-antibody interaction prior to immunoprecipitation. Normal processing of cellular Ras proteins (arrows) is shown in lane 2 (no drug treatment). In lane 3, multiple bands of slower mobility than those in lane 2 indicate the presence of unprocessed forms of Ras.

does not rule out additional effects of L-739,749 on non-Ras farnesylated proteins.

After confirming the drug's capacity to alter the levels of processed Ras in mutant Schwann cells, cells were pretreated with L-739,749 for 2 days and tested in the 5-day in vitro Matrigel invasion assay with replenishment of drug every 2 days. As shown in Fig. 1B, pretreatment with the drug failed to inhibit the invasion of +/− or −/− Schwann cells through Matrigel-coated basement membrane. Similar results were obtained even when cells were pretreated with L-739,749 for 5 days and analyzed in a 3-day invasion assay. This result suggests that the acquired invasive phenotype in *Nf1*-deficient Schwann cells could be independent of the elevated level of activated Ras in these cells. However, it is possible that inhibition of Ras processing by FPTI is only partial and insufficient to alter the invasive phenotype of *Nf1* mutant Schwann cells. Another possibility is that Ras activation causes long-term changes in Schwann cells that cannot be reversed by FPTI exposure. To test if FPTI treatment can reverse any *Nf1* mutant phenotype, we tested the drug in a Schwann cell proliferation assay.

We showed previously that *Nf1* mutant mouse Schwann cells proliferate less than +/+ cells in response to contact with axons or exposure to a soluble growth factor (rhGGF2) (29). When rhGGF2-treated −/− Schwann cells were exposed to L-739,749, there was a dose-dependent increase in cell proliferation up to a level comparable to that of +/+ cells (Fig. 3A). Thus, FPTI can reverse some effects of *Nf1* loss in Schwann cells. These results are consistent with the idea that growth inhibition in *Nf1*-deficient Schwann cells is due to the increased Ras activity and consistent with the demonstration that normal rat Schwann cells that express v-Ras also show decreased proliferation (29, 57).

Forskolin induces hyperplasia in mouse Schwann cells deficient for neurofibromin: inhibition of the forskolin effect by FPTIs. Decreased proliferation of mutant Schwann cells cannot explain Schwann cell hyperplasia in NF1 patients. Since loss of neurofibromin might result in an increased response to growth factors other than axons or rhGGF2, we tested −/− Schwann cell responses to two growth factors present in neurofibromas, basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF). Cells were exposed to growth factor, incubated with [^{3}H]thymidine, and subjected to autoradiography, and the percentage of cells labeled cells was determined. Neurofibromin-deficient cells showed decreased proliferation, a result similar to that obtained after rhGGF2 stimulation (data not shown).

A second messenger known to play an important role during Schwann cell development (proliferation and differentiation) is cAMP. To test whether mutations in *Nf1* alter the Schwann cell response to intracellular cAMP elevation, both +/+ and −/− mouse Schwann cells were treated with forskolin, an activator of adenylate cyclase (62), under serum-free conditions. In rat Schwann cells, cAMP elevation has no effect on Schwann cell proliferation unless it is accompanied by the presence of serum or soluble growth factors (67). Normal mouse Schwann cells also did not show any proliferative response to forskolin (Fig. 3B). In contrast, proliferation of −/− Schwann cells was about 40-fold greater than that of the untreated control even at the lowest concentration (2 μM) of forskolin tested. Like +/+ Schwann cells, +/− Schwann cells did not show any response to forskolin (data not shown). Treatment of cells with dideoxyforskolin, a forskolin analog which lacks the function to activate adenylate cyclase (62), did not stimulate −/− cell proliferation (data not shown). These results suggests that activation of cAMP pathway alone can induce hyperplasia in *Nf1*-deficient Schwann cells and that loss of both *Nf1* alleles is required for this phenotype.

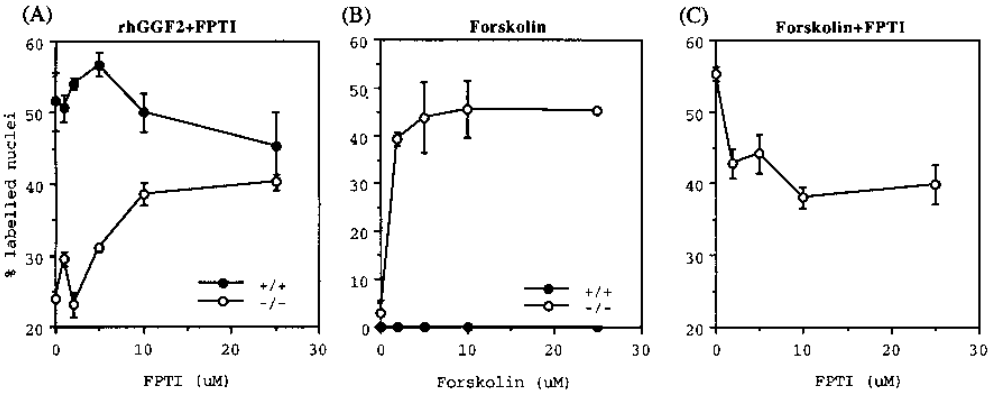
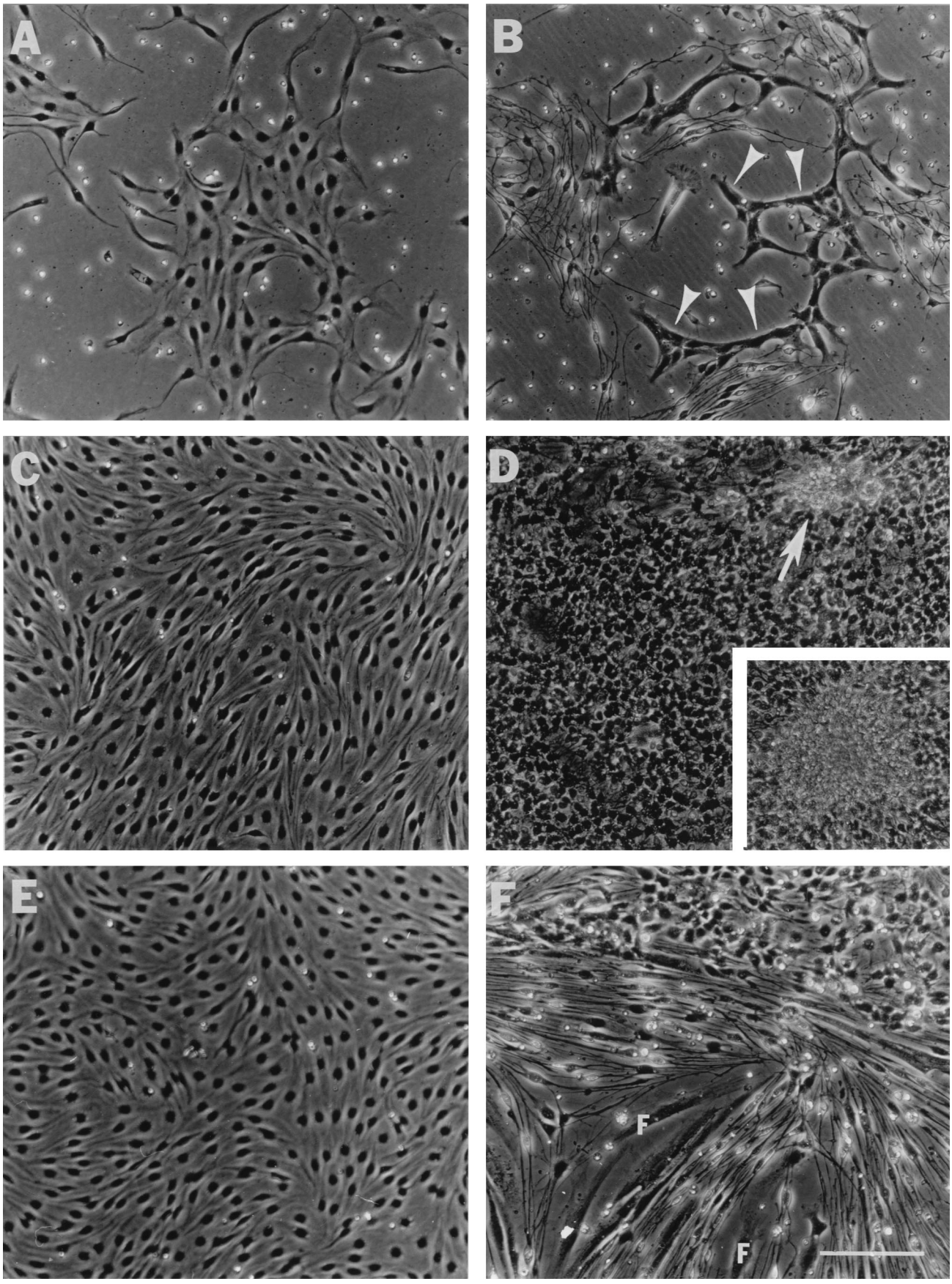


FIG. 3. Induction by forskolin of hyperplasia in *Nf1*-null mutant Schwann cells and reversion of growth responses of mutant Schwann cells to rhGGF2 and forskolin by L-739,749. (A) Effects L-739,749 on rhGGF2-stimulated growth of +/+ and −/− mouse Schwann cells. (B) Forskolin treatment of −/− mouse Schwann cells results in hyperproliferation but has no effect on +/+ Schwann cells. (C) Partial inhibition of forskolin-induced −/− mouse Schwann cell growth by L-739,749. Cells were plated on poly-L-lysine-coated eight-chamber glass Lab-Tek slides (25,000 cells/chamber) and preincubated with (A and C) or without (B) different doses (0 to 25 μM) of L-739,749 in DMEM–10% FBS for 2 days. Medium was switched to serum-free N2 medium containing either rhGGF2 (10 ng/ml) plus L-739,749 (0 to 25 μM) (A), different doses of forskolin (0 to 25 μM) (B), or 2 μM forskolin plus L-739,749 (0 to 25 μM) (C); after 16 to 18 hours, cells were labeled with [^{3}H]thymidine (0.5 μCi/ml) for another 24 h. Proliferation of Schwann cells was determined by the percentage of NGFR-positive cells with labeled nuclei; 800 to 1,000 cells were counted in each condition, and each point is an average of duplicate samples. Shown are representative results of three to four separate experiments. Note that the assays in panels B and C were carried out at different times, and so maximal percent labeled nuclei cannot be directly compared.


A
B
C
D
E
F
F
F

When forskolin was tested on −/− Schwann cells preincubated with different doses of L-739,749, there was a partial (30%), dose-dependent inhibition of forskolin (2 μM)-mediated proliferation (Fig. 3C). Drug treatment had no effect on +/+ Schwann cells (not shown). Thus, hyperplasia of −/− Schwann cells in response to forskolin may be due only partially to increased Ras activity in these cells.

Populations of *Nf1* mutant mouse Schwann cells exhibit morphological transformation accompanied by growth factor independence of growth and hyperplasia when cultured in the absence of serum. While culturing isolated −/− mouse Schwann cells in serum-free medium, we noticed the appearance of clones of cells with a morphology distinct from that of the surrounding cells (Fig. 4B). Unlike typical −/− Schwann cell morphology, distinguished from that of +/+ cells (Fig. 4A) by narrowed and bright cell bodies with elongated processes (29), these cells were flattened, round, and shorter. All of these cells were immunoreactive with antibodies recognizing S100 and NGFR (NGFR^{p75}), confirming that they were Schwann cells (not shown). These cells proliferated faster than surrounding −/− cells. As the culture became confluent (1 week after initial plating), most of the growth area in a plate became occupied by these morphologically transformed cells (Fig. 4D). In confluent cultures, these cells formed foci (Fig. 4D, inset), whereas +/+ cells (Fig. 4C) or −/− cells maintained in serum containing media showed contact inhibition of growth.

To quantitate the effect of serum on morphological transformation, we counted the number of morphologically transformed cell clones that arose from wild type +/− and −/− Schwann cell cultures kept in either 10% FBS or serum-free medium (N2) for 1 week after the first passage of the cells (Table 2). While no clones of transformed cells arose in cultures maintained in 10% FBS-containing medium, clones were observed in −/− Schwann cell culture kept in N2 medium. Transformed cell clones also developed in +/− cultures in N2 medium after a 1-week lag compared to the time of appearance of clones in −/− cultures.

To test the proliferative properties of morphologically transformed −/− (TXF−/−) Schwann cells, we pooled clones of these cells from a confluent TXF−/− culture and performed in vitro proliferation assays with and without added growth factor (rhGGF2) in the presence or absence of serum. Wild-type mouse Schwann cells showed a proliferative response similar to that of rat Schwann cells, in that they are quiescent unless growth factor is present (29) (Fig. 5A). In contrast, TXF−/− Schwann cells showed high basal-level proliferation under serum-free conditions in the absence of rhGGF2, indicating growth factor independence (Fig. 5B). The proliferation rate was higher under serum-free conditions than in the presence of serum. Thus, serum both inhibited initial clone development and slowed growth of cells once they developed morphological transformation. When TXF−/− cells were tested for invasion, they retained their invasive properties (not shown).

L-739,749 blocks the appearance of transformed cell clones in serum-free cultures and inhibits the growth of transformed −/− Schwann cells. To determine if the transformation of *Nf1* mutant Schwann cells was related to increased Ras activity, −/− Schwann cells were kept in either N2 medium or N2 medium with 10 μM L-739,749 for 10 or 16 days, and the TXF−/− cell clones that arose in cultures were counted (Table 3). In the continuous presence of the drug, at day 10, the appearance of clones of transformed cells in −/− Schwann cell culture was dramatically reduced. In addition, the clones that were present were smaller than those in untreated cultures. By day 16, while the number and the size of clones increased in untreated −/− cell cultures, in the presence of L-739,749, no additional clones developed and the ones observed at day 10 disappeared. Focus formation was also inhibited in confluent cultures of −/− Schwann cells maintained in the presence of L-739,749 (Fig. 4F).

To determine whether inhibition of appearance of clones in the −/− cell culture by L-739,749 is due to inhibition of TXF−/− cell growth, TXF−/− cells were pretreated with 10 μM L-739,749 for 4 days and growth in N2 medium was compared to that of untreated TXF−/− cells by counting cells every 2 days for 6 days (Fig. 5C). Increase in the cell number of TXF−/− cells was completely abolished by L-739,749 treatment; untreated cells continued to increase in number. Thus, the growth of TXF−/− is likely due to elevated level of Ras-GTP in these cells, and L-739,749-mediated inhibition of clone formation in −/− Schwann cell cultures is probably due to inhibition of transformed cell growth. Whether L-739,749 can actually inhibit the critical transformation event in −/− Schwann cell culture is unknown.

Transformed *Nf1*-null mutant Schwann cells grow independent of axonal contact. Isolated −/− Schwann cells are prepared from mouse embryonic dorsal root ganglion cultures that contain neurons as well as Schwann cells. Transformed Schwann cell clones appeared 5 to 6 days after dissociation of Schwann cells from the neurons present in these cultures. However, when we carefully examined Schwann cells in original dorsal root ganglion cultures 4 days after removal from −/− embryos, we detected clones of both S100- and NGFR^{p75}-positive cells with transformed morphology. Strikingly, these cells had lost contact with neurons (Fig. 6). This result indicates that during the process of morphological transformation, Schwann cells acquire growth factor independence, hyperproliferate, and show one of the characteristic features of human neurofibromas, loss of contact with axons.

P0 expression is suppressed in morphologically transformed *Nf1* mutant Schwann cells. We have shown previously that loss of neurofibromin in Schwann cells correlates with constitutive expression of P0 protein, normally a marker for Schwann cells stimulated to differentiate (58). To determine if the differentiated phenotype is maintained in TXF−/− cells, confluent cultures were grown in N2 medium supplemented with either rhGGF2 or 5 μM forskolin for 5 days, conditions under which Schwann cells lacking neurofibromin express P0 protein. Figure 7 shows Western blots probed for P0 protein from protein extracts prepared from −/− or TXF−/− Schwann cells. As shown before, −/− cells express P0 in both rhGGF2 and forskolin conditions. In TXF−/− Schwann cells, basal levels of P0 were completely lost (lane G) and cells did not express P0 even in the presence of forskolin, conditions that induce P0 expression in wild-type cells. This result sug-

FIG. 4. Growth of *Nf1*-deficient mouse Schwann cells in a continuous serum-free medium results in morphological transformation and focus formation. Phase-contrast micrographs were taken from wild-type (A, C, and E) and *Nf1* −/− (B, D, and F) Schwann cell cultures grown in serum-free medium. Cells with morphology distinct from that of surrounding cells start to appear in *Nf1* −/− cell cultures (B, arrowheads), whereas wild-type cells remain unchanged (A). As cultures become confluent, morphologically transformed *Nf1* −/− cells take over the whole growth area (D) and foci are formed (arrow and inset), whereas wild-type cells show contact inhibition of growth (C). In the presence of 10 μM L-739,749, growth of transformed *Nf1* −/− cells is inhibited and no focus formation is observed (F). L-739,749 has no effect on wild-type cell growth (E). Contaminating fibroblasts (F) are also indicated. Bar = 135 μm.

TABLE 2. Colony formation in *Nf1*-deficient mouse Schwann cell cultures in serum-free defined medium and inhibition of colony formation by serum

Genotype	No. of colonies/cm^2		
	7 days[a]		14 days N2[b]
	N2	10% FBS	
+/+	0	0	0
+/−	0	0	47.75
−/−	115.7	0	577.5

[a] Schwann cells from +/+, +/−, or −/− mouse embryos were expanded in DMEM plus 10% FBS supplemented with rhGGF2 (10 ng/ml) and forskolin (2 μM). After 3 to 4 days, cells were replated on poly-L-lysine-coated 60-mm-diameter culture plates at a density of 0.5×10^6 cells/plate in the presence of growth factors in serum-free N2 or DMEM–10% FBS. Seven days later, the cell colonies (>4 cells) were counted.

[b] In a separate experiment, 7 days after initial plating on 60-mm-diameter plates in N2, cultures became confluent and no morphological transformation was observed in +/− cultures. Cells were trypsinized and replated at a density of 0.5×10^6 cells/plate. A week later, the colonies were counted.

TABLE 3. Colony formation in *Nf1*-null mutant mouse Schwann cell cultures in serum-free defined medium and inhibition of colony formation by FPTI (10 μM)[a]

Size of colonies (no. of cells)	No. of colonies/60-mm-diam plate			
	10 days		16 days	
	−FPTI	+FPTI	−FPTI	+FPTI
<10	10	2	15	0
<40	8	0	8	0
>40	14	1	14	0

[a] Schwann cells isolated from *Nf1*-null mutant mouse embryos were expanded in DMEM–10% FBS supplemented with rhGGF2 (10 ng/ml) and forskolin (2 μM). At the second passage, cells were plated onto poly-L-lysine-coated 60-mm-diameter plates (0.5×10^6/plate), and the medium was switched to serum-free N2 medium containing rhGGF2 and forskolin, with or without FPTI (10 μM). The medium was changed every 5 days, and the colonies were counted on days 10 and 16.

gests that the TXF−/− cells not only increase proliferation but also have lost the ability to differentiate in vitro.

DISCUSSION

The basis of tumor formation in NF1 is not understood, because the phenotype of Schwann cells with mutant *Nf1* has not been explored. In this report, we demonstrate significant behavioral differences between mutant Schwann cells and their wild-type counterparts: *Nf1*-deficient Schwann cells are invasive, are angiogenic, lose contact with axons, form foci in the absence of serum components, and hyperproliferate after forskolin treatment. These phenotypes might be sufficient to give rise to abnormal growth in vivo and account for the multiple neurofibromas that arise in NF1 patients.

We showed that loss of *Nf1* in mouse Schwann cell is by itself sufficient to induce angiogenesis and invasion, features of Schwann cells from human neurofibromas (63). We have also shown that when loss of *Nf1* is accompanied by epigenetic changes, such as removal of serum components, or activation of protein kinase A (PKA), Schwann cell hyperplasia is induced. Decreased proliferation in response to rhGGF2 and hyperplasia induced by removal of serum factors can be reversed using FPTI, implicating a role for Ras activation in these Schwann cell abnormalities. FPTIs diminish Ras activation, as assessed by mitogen-activated protein kinase activation, even in cell lines where Ras activation is increased 10- to 100-fold (14, 27). However, invasiveness of *Nf1*-deficient Schwann cells is not inhibited by FPTI. These results suggest the possible utility of FPTI in treating some, but not all, manifestations of human NF1.

Angiogenesis is induced when tumor cells secrete polypeptides mitogenic for endothelial cells, inducing blood vessel formation and tumor vascularization. Numerous angiogenic factors have been identified (3, 61). Which growth factor(s) are responsible for induction of angiogenesis by *Nf1*-deficient Schwann cells has not been addressed in this study. However, we have shown previously that two angiogenic factors, bFGF

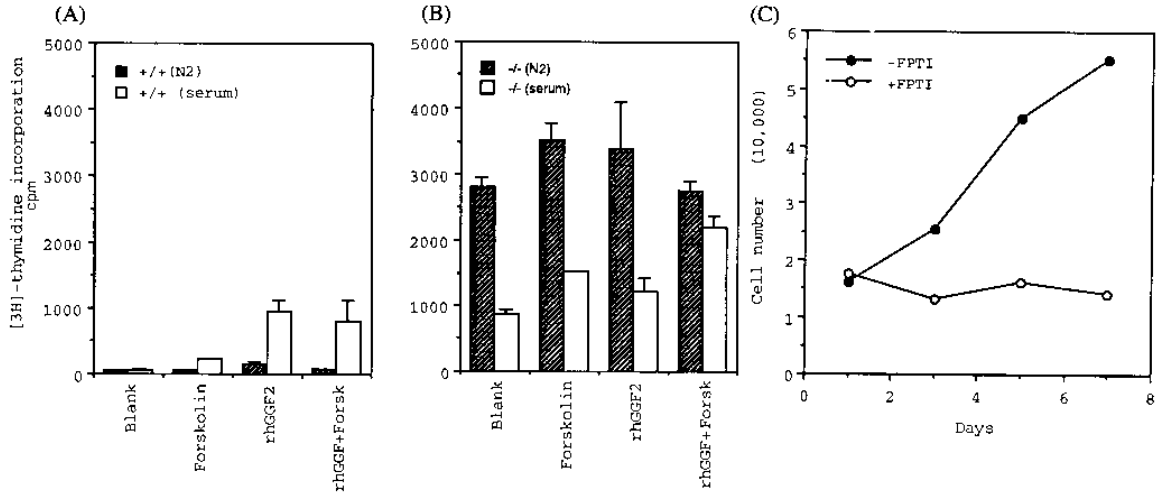


FIG. 5. DNA synthesis of +/+ (A) and TXF−/− (B) Schwann cells in response to rhGGF2 and forskolin and effect of serum factors on mutant Schwann cell growth. Cells were plated on 96-well plates in DMEM with 10% FBS at a density of 10^4/well. At 48 h after plating, the medium was changed to either serum-free defined medium (N2) or fresh DMEM–10% FBS supplemented with rhGGF2 (10 ng/ml), forskolin (Forsk) (1 μM), or a combination of both. After 18 h, [^{3}H]thymidine (2 μCi/ml) was added, cells were incubated for another 24 h, and DNA synthesis was measured as incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material by liquid scintillation counting. (C) Growth of TXF−/− cells (solid line) and L-739,749-treated TXF−/− cells (dotted line) in N2 medium. Cells were pretreated with or without 10 μM L-739,749 for 4 days and plated on poly-L-lysine-coated 24-well culture plates in DMEM with 10% FBS at a density of 25,000 cells/well. The next day, cells were switched into serum-free defined N2 medium and cell numbers were determined every other day by counting of triplicate samples in a hemocytometer.

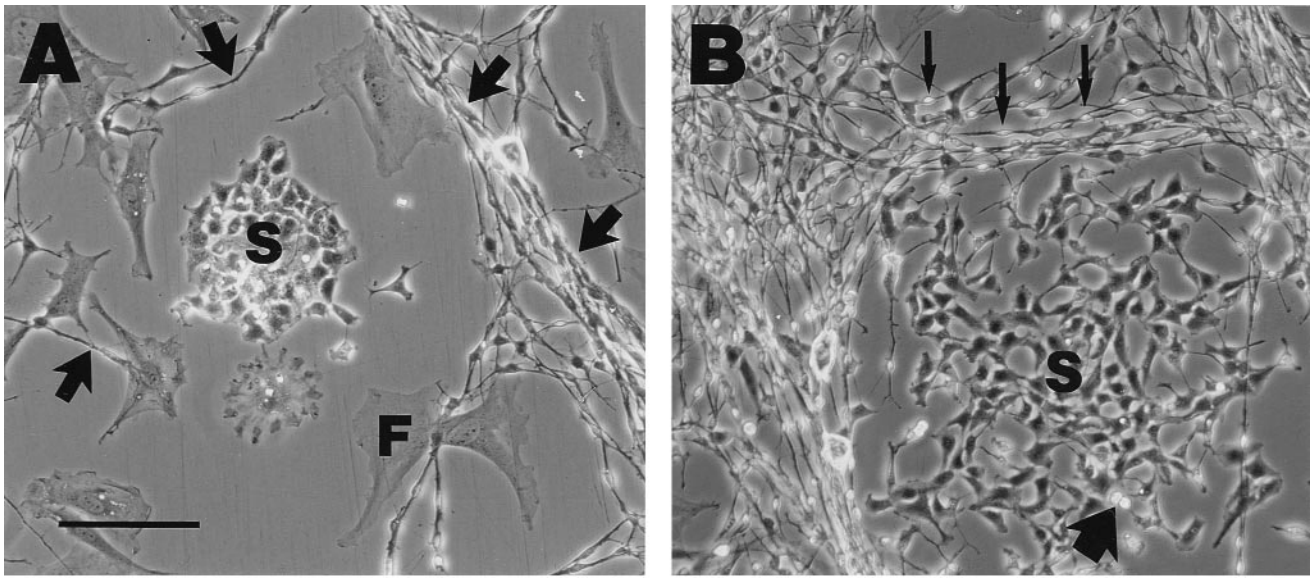


FIG. 6. Morphologically transformed *Nf1* −/− Schwann cells appear early in neuron coculture and grow independent of axonal contact. (A) Dorsal root ganglia from a single *Nf1*-null mutant embryo were dissociated and plated on plastic culture dish. In serum-free medium, morphologically altered Schwann cells ($S100^+$ $NGFR^{p75+}$) appear within 2 to 3 days after plating and subsequently form colonies (S) away from axons (arrows). The morphology of these cells is distinct from that of fibroblasts (F). (B) Another *Nf1*-null dorsal root ganglion culture showing a Schwann cell colony (S). A mitotically active, dividing Schwann cell independent of axonal contact can be seen (arrowhead). Normally, Schwann cells only grow in direct contact with axons (arrows).

and HGF, are present in neurofibromas (33, 55). As mutations at the *Nf1* locus in Schwann cells result in increased activation of Ras, it is of some interest that constitutive activation of Ras can facilitate tumor angiogenesis. Activated Ras upregulates expression of angiogenic factors, including HGF, bFGF, and vascular endothelial growth factor (VEGF), in fibroblasts (11, 20, 23, 51). In a tumor cell line, VEGF secretion is suppressed by disruption of mutant Ras activity by FPTI (53). Thus, it is possible that in *Nf1*-deficient Schwann cells, induction of angiogenesis is due to secretion of an angiogenic factor(s) resulting from increased Ras activity in the cells. We did not address whether FPTI is able to inhibit *Nf1* Schwann cell-induced angiogenesis on CAM, as FPTI treatment of the CAM itself stimulated angiogenesis (not shown). Strikingly, not only Schwann cells null at *Nf1* but also heterozygous cells stimulated angiogenic responses on the CAM, suggesting that in vivo, heterozygous Schwann cells could contribute to this feature of neurofibromas.

Loss of *Nf1* is also sufficient for Schwann cells to acquire an invasive phenotype. Heterozygous cells were intermediate between wild-type and null cells in their invasive potential. Although in human neurofibromas Schwann cells do not metastasize, Schwann cells in the tumors do invade extracellular matrices. Neurofibromas contains higher levels of proteolytic activity than normal nerve cells (34), and neurofibroma Schwann cells invade chicken CAM basement membranes (63). The invasive potential of tumor cells is often correlated with overexpression and secretion of variety of enzymes that degrade extracellular matrix components of basement membranes (20, 38). These proteolytic enzymes include members of metalloproteinases (MMPs). Ras may contribute to the invasive behavior of tumor cells by upregulating the production of MMPs. Fibroblasts transformed with oncogenic Ras show increased production of MMPs (5, 39, 65). Tissues from rat mammary carcinomas, known to express activated c-H-*ras*, show increased MMP production compared to normal breast tissue (5). In recent studies, Schwann cells derived from neurofibromas have been shown to secrete high levels of MMP-1 (collagenases) and MMP-9 (gelatinase B) and also to invade a collagen barrier resembling basement membranes (44). It has been suggested that increased Ras activity in neurofibroma-derived Schwann cells might be responsible for the invasiveness of the cells through stimulation of MMP production. In the present study, however, pretreatment of *Nf1*-null mutant mouse Schwann cells with FPTI failed to abolished the invasive phenotype in vitro. It is possible that regions outside the GRD of *Nf1* are required for this phenotype or that Ras activation prior to drug treatment causes long-term changes in gene expression that cannot be reversed by drug exposure. Furthermore, the possibility that residual Ras activity remains even in Schwann cells treated with FPTI for 5 to 8 days cannot be excluded. Additional experiments will be required to conclusively distinguish between these alternatives.

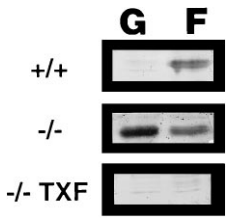


FIG. 7. P0 expression in +/+, −/−, and TXF−/− Schwann cells in the presence of rhGGF2 or forskolin. Schwann cells were incubated for 4 days in either rhGGF2 or 5 μM forskolin in serum-free medium, and cell lysate was prepared. Equal amounts of each lysate were separated on SDS–10% gels, and P0 expression was analyzed by Western blotting using polyclonal antibodies raised against rat P0. P0 expression was absent in +/+ mouse Schwann cells unless forskolin was added. −/− Schwann cells expressed P0 both in rhGGF2 and in forskolin, while P0 expression was lost in TXF−/− Schwann cells even in the presence of forskolin.

Hyperplasia of *Nf1*-null Schwann cells was obtained by exposure of cells to forskolin, an activator of PKA. In contrast, several tyrosine kinase receptor ligands, including glial growth

factor (29), HGF, and bFGF, reduced proliferation of these cells. Forskolin-induced hyperproliferation did not occur in *Nf1* heterozygous mutant Schwann cells, suggesting that this phenotype requires loss of the second allele at the *Nf1* locus. This is the only phenotype identified to date that affects only null Schwann cells. Forskolin effects are likely to be mediated through increases in intracellular cAMP levels, as forskolin is a potent activator of adenylate cyclase (62), and an analog of forskolin, dideoxy-forskolin, which lacks the ability to activate adenylate cyclase (62) was ineffective at stimulating *Nf1*-null Schwann cell proliferation. FPTI failed to completely reverse forskolin-stimulated proliferation, suggesting, as noted above, a GRD-independent function of neurofibromin; but it remains possible that prolonged FPTI treatment could reverse hyperplasia. Interaction between the cAMP and Ras signaling pathways has been found in other systems, in which PKA inhibits Ras signaling at the level of Raf activation (13, 71). A neuropeptide, calcitonin gene-related peptide (CGRP), has been proposed as an in vivo candidate for cAMP control of Schwann cell development. In culture, exposure to CGRP increases intracellular cAMP in Schwann cells and synergizes with a Schwann cell mitogen to stimulate proliferation (10). CGRP is expressed by sensory and motor neurons (1, 46) and is expressed by axons within neurofibromas (68). CGRP or other endogenous ligands might serve as signals that increase cAMP in *Nf1* Schwann cells, resulting in Schwann cell hyperplasia.

When cultured in the absence of serum, a subpopulation of mouse Schwann cells null at *Nf1* rapidly developed morphological changes and acquired a growth factor-independent phenotype. Morphologically transformed cells appeared by 3 days after isolation of cells from null embryos. It is possible that *Nf1*-deficient cells are highly susceptible to mutations and that each identified colony of altered cells represents a mutational event. It is also possible that alteration in the environment (loss of serum) facilitates altered behavior in a few but not all cells, perhaps in a subpopulation of immature Schwann cells. An alternative idea is that one or a few cells sustained a mutation(s) within the embryo and that removal of serum revealed phenotypic differences in these cells. In this view, multiple clones arise from a single (or a few) mutational events and are separated physically because of cell dissociation used to set up cultures. Incubation of rapidly proliferating Schwann cells with FPTI decreased transformed cell colony number and size, suggesting a requirement for Ras for this phenotype. Previous studies showed that activation of Ras in Schwann cells is insufficient to cause transformation (29, 57). Loss of the p53 gene is frequently detected in MPNSTs from NF1 patients; p53 mutations could explain development of the hyperproliferating cells that we have detected (37, 42). Mutations might occur as a consequence of Ras-induced genomic instability; in NIH 3T3 cells, Ras activation is directly associated with genomic instability resulting in chromosome aberrations and gene amplifications within one cell cycle, leading to cell transformation (17, 24, 66, 69). Consistent with this idea, a high frequency of microsatellite alterations in DNAs from NF1 neurofibromas has been reported (48). It is also possible that subpopulations of cells develop mutations by other mechanisms or that epigenetic thresholds are crossed, enabling hyperproliferation in subpopulations of Schwann cells.

Are rapidly proliferating colonies of Schwann cells transformed? Characteristics of transformed cells in colonies derived from *Nf1*-null Schwann cells include stimulation of angiogenesis, invasion of matrices, growth factor independence, and lack of contact inhibition. While we have been unable to demonstrate growth of these cells in soft agar (data not shown), only one of four known MPNST Schwann cell strains grows in soft agar (74).

Nf1 heterozygous mutant Schwann cells become hyperproliferative in serum-free media 7 to 10 days later than null Schwann cells. It is likely that these cells lose their normal *Nf1* allele before changing phenotype, but we have not yet analyzed LOH in these cells. A high spontaneous mutation rate in the *NF1* gene (1/10,000 alleles/generation) has been reported (15). We cannot rule out the possibility that phenotypic changes in heterozygous cells are due not to LOH but to other changes. The development of hyperproliferating colonies of Schwann cells among heterozygous cells might represent a model for transient hyperplasia of benign Schwann cells in human NF1. It seems more likely that this phenotype models steps toward development of MPNSTs that can arise in human NF1.

Addition of serum suppresses development of hyperplasia in *Nf1* mutant Schwann cells, alters the phenotype of hyperproliferating Schwann cells (from round to flat and multipolar), and decreases proliferation of hyperplastic cells. It is possible that factors present in serum affect Ras activity in *Nf1* mutant Schwann cells through activation of the serum response factor. In fibroblasts, serum response factor, a nuclear transcription factor, binds to the serum response element in the promoters of several growth factor-inducible genes and can reverse Ras transformation at least in part by activating actin expression (30). FPTI also reverses Ras transformation and causes actin reorganization (52). In human NF1, neurofibromas show periods of growth and periods when they stop growing. Suppression of NF1 Schwann cell growth could be explained by serum factors that have gained access to Schwann cells either after Schwann cells migrate away from axons or after defective perineurium formation around axon-Schwann cell units (59). Serum also contains mitogens for Schwann cells. Thus, serum exposure might both positively and negatively regulate neurofibroma growth.

In summary, using an in vitro model, we have demonstrated that phenotypes of Schwann cells lacking neurofibromin mimic those identified in NF1 patients. Cells heterozygous at *Nf1* display some phenotypes (angiogenesis and invasion), but these phenotypes are enhanced in cells null at *Nf1*. *Nf1* loss is by itself not sufficient for clonal hyperproliferation, which is likely to require additional events. FPTI may be useful for arresting growth of benign and malignant Schwann cells in NF1. We expect that mechanistic analysis of phenotypes that we have defined in Schwann cells lacking *Nf1* will provide insights into Schwann cell tumor pathogenesis in human NF1.

ACKNOWLEDGMENTS

We are indebted to Mark Marchionni (Cambridge Neuroscience) for providing rhGGF2, to Jackson Gibbs and Nancy Kohl (Merck Sharp and Dohme Research Laboratory) for the FPTI (L-739,749), and to David Colman (Columbia University) for P0 antibodies. We also thank Jackson Gibbs, James Fagin, and Peter Stambrook for review of the manuscript and Radhika Atit for assistance with maintenance of our *Nf1* mouse colony.

This work was supported by grants NIH-NS28840 and DAMD17-J3030 to N.R. H.A.K. was the recipient of a fellowship from NCI training grant CA59268.

REFERENCES

1. **Arvidsson, U., H. Johnson, F. Piehl, S. Cullheim, T. Hokfelt, M. Risling, L. Terenius, and B. Ulfhake.** 1990. Peripheral nerve section induces increased levels of calcitonin gene-related peptide (CGRP)-like immunoreactivity in axotomized motoneurons. Exp. Brain Res. **79:**212–216.
2. **Asprunk, K. H., D. R. Knighton, and J. Folkman.** 1975. Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. Am. J. Pathol. **79:**597–618.
3. **Auerbach, W., and R. Auerbach.** 1994. Angiogenesis inhibition: a review. Pharmacol. Ther. **63:**265–311.

4. **Ballester, R., D. Marchuk, M. Boguski, A. Saulino, R. Letcher, M. Wigler, and F. Collins.** 1990. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell **63:**851–859.
5. **Ballin, M., A. R. Mackay, J. L. Hartzler, A. Nason, M. D. Pelina, and U. P. Thorgeirsson.** 1991. Ras levels and metalloproteinase activity in normal versus neoplastic rat mammary tissues. Clin. Exp. Metastasis **9:**179–189.
6. **Ballin, M., D. E. Gomez, C. C. Sinha, and U. P. Thorgeirsson.** 1988. Ras oncogene mediated induction of a 92 kDa metalloproteinase; strong correlation with the malignant phenotype. Biochem. Biophys. Res. Commun. **154:**832–838.
7. **Basu, T. N., D. H. Gutmann, J. A. Fletcher, T. W. Glover, F. S. Collins, and J. Downward.** 1992. Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients. Nature **356:**713–715.
8. **Boghaert, E. R., J. F. Simpson, and S. G. Zimmer.** 1992. Invasion of malignant hamster brain tumor cells is influenced by the number of cells and the mode of malignant progression. Invasion Metastasis **12:**12–23.
9. **Brannan, C. I., A. S. Perkins, K. S. Vogel, N. Ratner, M. L. Nordlund, S. W. Reid, A. M. Buchberg, N. A. Jenkins, L. F. Parada, and N. G. Copeland.** 1994. Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Dev. **8:**1019–1029. (Erratum, **8:**2792.)
10. **Cheng, L., M. Khan, and A. W. Mudge.** 1995. Calcitonin gene-related peptide promotes Schwann cell proliferation. J. Cell Biol. **129:**789–796.
11. **Coffer, A., J. Fellows, S. Young, D. Pappin, and D. Rahman.** 1991. Purification and characterization of biologically active scatter factor from ras-transformed NIH 3T3 conditioned medium. Biochem. J. **278:**35–41.
12. **Colman, S. D., C. A. Williams, and M. R. Wallace.** 1995. Benign neurofibromas in type 1 neurofibromatosis (NF1) show somatic deletions of the NF1 gene. Nat. Genet. **11:**90–92.
13. **Cook, S. J., and F. McCormick.** 1993. Inhibition by cAMP of Ras-dependent activation of Raf. Science **262:**1069–1072.
14. **Cox, A. D., A. M. Garcia, J. K. Westwick, J. J. Kowalczyk, M. D. Lewis, D. A. Brenner, and C. J. Der.** 1994. The CAAX peptidomimetic compound B581 specifically blocks oncogenic ras signaling and transdormation. J. Biol. Chem. **269:**19203–19206.
15. **Crowe, C. A., W. J. Schull, and J. V. Neel.** 1956. A clinical, pathological and genetic study of multiple neurofibromatosis. Charles C Thomas, Springfield, Ill.
16. **DeClue, J. E., A. G. Papageorge, J. A. Fletcher, S. R. Diehl, N. Ratner, W. C. Vass, and D. R. Lowy.** 1992. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell **69:**265–273.
17. **Denko, N. C., A. J. Giaccia, J. R. Stringer, and P. J. Stambrook.** 1994. The human Ha-ras oncogene induces genomic instability in murine fibroblasts within one cell cycle. Proc. Natl. Acad. Sci. USA **91:**5124–5128.
18. **Ducatman, B. S., B. W. Scheithauer, D. G. Piepgras, and H. M. Reiman.** 1984. Malignant peripheral nerve sheath tumors in childhood. J. Neuro-Oncol. **2:**241–248.
19. **Glover, T. W., C. K. Stein, E. Legius, L. B. Andersen, A. Brereton, and S. Johnson.** 1991. Molecular and cytogenetic analysis of tumors in von Recklinghausen neurofibromatosis. Genes Chromosomes Cancer **3:**62–70.
20. **Goldberg, G. I., S. M. Frisch, C. He, S. M. Wilhelm, R. Reich, and I. E. Collier.** 1990. Secreted proteases. Regulation of their activity and their possible role in metastasis. Ann. N.Y. Acad. Sci. **580:**375–384.
21. **Golubic, M., M. Roudebush, S. Dobrowolski, A. Wolfman, and D. W. Stacey.** 1992. Catalytic properties, tissue and intracellular distribution of neurofibromin. Oncogene **7:**2151–2159.
22. **Huson, S. M.** 1994. Neurofibromatosis: historical perspective, classification and diagnostic criteria, p. 1–22. *In* S. M. Huson and R. A. Hughes (ed.), The neurofibromatoses. Chapman & Hall, London, England.
23. **Iberg, N., S. Rogelj, P. Fanning, and M. Klagsbrun.** 1989. Purification of 18- and 22-kDa forms of basic fibroblast growth factor from rat cells transformed by the ras oncogene. J. Biol. Chem. **264:**19951–19955.
24. **Ichikawa, T., N. Kyprianou, and J. T. Isaacs.** 1990. Genetic instability and the acquisition of metastatic ability by rat mammary cancer cells following v-H-ras oncogene transfection. Cancer Res. **50:**6349–6357.
25. **Jacks, T., T. S. Shih, E. M. Schmitt, R. T. Bronson, A. Bernards, and R. A. Weinberg.** 1994. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nat. Genet. **7:**353–361.
26. **James, G. L., J. L. Goldstein, M. S. Brown, T. E. Rawson, T. C. Somers, R. S. McDowell, C. W. Crowley, B. K. Lucas, A. D. Levinson, and J. C. Masters, Jr.** 1993. Benzodiazepine peptidomimetrics: potent inhibitors of Ras farnesylation in animal cells. Science **260:**1937–1942.
27. **James, G. L., M. S. Brown, M. H. Cobb, and J. L. Goldstein.** 1994. Benzodiazepine peptidomimetic BZA-5B interrupts the MAP kinase activation pathway in H-Ras-transformed rat-1 cells, but not in untransformed cells. J. Biol. Chem. **269:**27705–27714.
28. **Kamata, Y.** 1978. On the ultrastructure and acetylcholinesterase activity in von Recklinghausen's neurofibromatosis. Acta Pathol. Jpn. **28:**393–410.
29. **Kim, H. A., T. Rosenbaum, M. A. Marchionni, N. Ratner, and J. E. DeClue.** 1995. Schwann cells from neurofibromin deficient mice exhibit activation of p21ras, inhibition of cell proliferation and morphological changes. Oncogene **11:**325–335.
30. **Kim, J. H., F. E. Johansen, N. Robertson, J. J. Catino, R. Prywes, and C. C. Kumar.** 1994. Suppression of Ras transformation by serum response factor. J. Biol. Chem. **269:**13740–13743.
31. **Kohl, N. E., C. A. Omer, M. W. Conner, N. J. Anthony, J. P. Davide, S. J. deSolms, E. A. Giuliani, R. P. Gomez, S. L. Graham, and K. Hamilton.** 1995. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. Nat. Med. **1:**792–797.
32. **Kohl, N. E., S. D. Mosser, S. J. deSolms, E. A. Giuliani, D. L. Pompliano, S. L. Graham, R. L. Smith, E. M. Scolnick, A. Oliff, and J. B. Gibbs.** 1993. Selective inhibition of ras-dependent transformation by a farnesyltransferase inhibitor. Science **260:**1934–1937.
33. **Krasnoselsky, A., M. J. Massay, M. C. DeFrances, G. Michalopoulos, R. Zarnegar, and N. Ratner.** 1994. Hepatocyte growth factor is a mitogen for Schwann cells and is present in neurofibromas. J. Neurosci. **14:**7284–7290.
34. **Krone, W., R. Mao, O. S. Muhleck, H. Kling, and T. Fink.** 1986. Cell culture studies on neurofibromatosis (von Recklinghausen). Characterization of cells growing from neurofibromas. Ann. N.Y. Acad. Sci. **486:**354–370.
35. **Largaespada, D. A., C. I. Brannan, N. A. Jenkins, and N. G. Copeland.** 1996. Nf1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukaemia. Nat. Genet. **12:**137–143.
36. **Legius, E., D. A. Marchuk, F. S. Collins, and T. W. Glover.** 1993. Somatic deletion of the neurofibromatosis type 1 gene in a neurofibrosarcoma supports a tumour suppressor gene hypothesis. Nat. Genet. **3:**122–126.
37. **Legius, E., H. Dierick, R. Wu, B. K. Hall, P. Marynen, J. J. Cassiman, and T. W. Glover.** 1994. TP53 mutations are frequent in malignant NF1 tumors. Genes Chromosomes Cancer **10:**250–255.
38. **Liotta, L. A., U. Wewer, N. C. Rao, E. Schiffmann, M. Stracke, R. Guirguis, U. Thorgeirsson, R. Muschel, and M. Sobel.** 1988. Biochemical mechanisms of tumor invasion and metastases. Prog. Clin. Biol. Res. **256:**3–16.
39. **LoSardo, J. E., B. S. Goggin, O. Bohoslawec, and A. Neri.** 1995. Degradation of endothelial cell matrix collagen is correlated with induction of stromelysin by an activated ras oncogene. Clin. Exp. Metastasis **13:**236–248.
40. **Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **82:**206–210.
41. **Martin, G. A., D. Viskochil, G. Bollag, P. C. McCabe, W. J. Crosier, H. Haubruck, L. Conroy, R. Clark, P. O'Connell, and R. M. Cawthon.** 1990. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. Cell **63:**843–849.
42. **Menon, A. G., K. M. Anderson, V. M. Riccardi, R. Y. Chung, J. M. Whaley, D. W. Yandell, G. E. Farmer, R. N. Freiman, J. K. Lee, F. P. Li, et al.** 1990. Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. Proc. Natl. Acad. Sci. USA **87:**5435–5439.
43. **Morgan, L., R. K. Jessen, and R. Mirsky.** 1991. The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04^+) to a myelin phenotype ($P0^+$, $GFAP^-$, NGF^- receptor$^-$) depends on growth inhibition. J. Cell Biol. **112:**457–467.
44. **Muir, D.** 1995. Differences in proliferation and invasion by normal, transformed and NF1 Schwann cell cultures are influenced by matrix metalloproteinase expression. Clin. Exp. Metastasis **13:**303–314.
45. **Nagasu, T., K. Yoshimatsu, C. Rowell, M. D. Lewis, and A. M. Garcia.** 1995. Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. Cancer Res. **55:**5310–5314.
46. **Noguchi, K., E. Senba, Y. Morita, M. Sato, and M. Tohyama.** 1990. Co-expression of alpha-CGRP and beta-CGRP mRNAs in the rat dorsal root ganglion cells. Neurosci. Lett. **108:**1–5.
47. **Nordlund, M., D. Hong, X. Fei, and N. Ratner.** 1992. Schwann cells and cells in the oligodendrocyte lineage proliferate in response to a 50,000 dalton membrane-associated mitogen present in developing brain. Glia **5:**182–192.
48. **Ottini, L., D. L. Esposito, A. Richetta, M. Carlesimo, R. Palmirotta, M. C. Veri, P. Battista, L. Frati, F. G. Caramia, S. Calvieri, et al.** 1995. Alterations of microsatellites in neurofibromas of von Recklinghausen's disease. Cancer Res. **55:**5677–5680.
49. **Peltonen, J., S. Jaakkola, M. Lebwohl, S. Renvall, L. Risteli, I. Virtanen, and J. Uitto.** 1988. Cellular differentiation and expression of matrix genes in type 1 neurofibromatosis. Lab. Invest. **59:**760–771.
50. **Poirier, H., R. Escourolle, and P. Castaigne.** 1968. Les neurofibromes de la maladie de Recklinghausen. Acta Neuropathol. **10:**279–294.
51. **Powell, P. P., and M. Klagsbrun.** 1993. Regulation of basic fibroblast growth factor mRNA expression in rat C6 glioma cells. Exp. Cell Res. **209:**224–230.
52. **Prendergast, G. C., J. P. Davide, S. J. deSolms, E. A. Giuliani, S. L. Graham, J. B. Gibbs, A. Oliff, and N. E. Kohl.** 1994. Farnesyltransferase inhibition causes morphological reversion of *ras*-transformed cells by a complex mechanism that involves regulation of the actin cytoskeleton. Mol. Cell. Biol. **14:**4193–4202.
53. **Rak, J., Y. Mitsuhashi, L. Bayko, J. Filmus, S. Shirasawa, T. Sasazuki, and R. S. Kerbel.** 1995. Mutant ras oncogenes upregulate VEGF/VPF expres-

sion: implications for induction and inhibition of tumor angiogenesis. Cancer Res. **55:**4575–4580.
54. **Ratner, N., A. Elbein, M. B. Bunge, S. Porter, R. P. Bunge, and L. Glaser.** 1986. Specific asparagine-linked oligosaccharides are not required for certain neuron-neuron and neuron-Schwann cell interactions. J. Cell Biol. **103:** 159–170.
55. **Ratner, N., M. A. Lieberman, V. M. Riccardi, and D. M. Hong.** 1990. Mitogen accumulation in von Recklinghausen neurofibromatosis. Ann. Neurol. **27:**298–303.
56. **Riccardi, V. M.** 1991. Neurofibromatosis: past, present, and future. N. Engl. J. Med. **324:**1283–1285. (Editorial.)
57. **Ridley, A. J., H. F. Paterson, M. Noble, and H. Land.** 1988. Ras-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation. EMBO J. **7:**1635–1645.
58. **Rosenbaum, T., H. A. Kim, B. Ling, and N. Ratner.** Neurofibromin is required for appropriate P0 expression and myelination. Submitted for publication.
59. **Rosenbaum, T., Y. L. Boissy, K. Kombrinck, C. Brannan, N. A. Jenkins, N. G. Copeland, and N. Ratner.** 1995. Neurofibromin deficient fibroblasts fail to form perineurium in nitro. Development **121:**3583–3592.
60. **Sawada, S., S. Florell, S. M. Purandare, M. Ota, K. Stephens, and D. Viskochil.** 1996. Identification of NF1 mutations in both alleles of a dermal neurofibroma. Nat. Genet. **14:**110–112.
61. **Schott, R. J., and L. A. Morrow.** 1993. Growth factors and angiogenesis. Cardiovasc. Res. **27:**1155–1161.
62. **Seamon, K. B., and J. W. Daly.** 1981. Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. **7:**201–224.
63. **Sheela, S., V. M. Riccardi, and N. Ratner.** 1990. Angiogenic and invasive properties of neurofibroma Schwann cells. J. Cell Biol. **111:**645–653.
64. **Skuse, G. R., B. A. Kosciolek, and P. T. Rowley.** 1989. Molecular genetic analysis of tumors in von Recklinghausen neurofibromatosis: loss of heterozygosity for chromosome 17. Genes Chromosomes Cancer **1:**36–41.
65. **Spinucci, C., S. Zucker, J. M. Wieman, R. M. Lysik, B. Imhof, N. Ramamurthy, L. A. Liotta, and H. Nagase.** 1988. Purification of a gelatin-degrading type IV collagenase secreted by ras oncogene-transformed fibroblasts. J. Natl. Cancer Inst. **80:**1416–1420.
66. **Stenman, G., E. O. Delorme, C. C. Lau, and R. Sager.** 1987. Transfection with plasmid pSV2gptEJ induces chromosome rearrangements in CHEF cells. Proc. Natl. Acad. Sci. USA **84:**184–188.
67. **Stewart, J. S., P. A. Eccleston, K. R. Jessen, and R. Mirsky.** 1991. Interaction between cAMP elevation, identified growth factors, and serum components in regulating Schwann cell growth. J. Neurosci. Res. **30:**346–352.
68. **Vaalasti, A., H. Suomalainen, K. Kuokkanen, and L. Rechardt.** 1990. Neuropeptides in cutaneous neurofibromas of von Recklinghausen's disease. J. Cutaneous Pathol. **17:**371–373.
69. **van den Berg, S., B. Kaina, H. J. Rahmsdorf, H. Ponta, and P. Herrlich.** 1991. Involvement of fos in spontaneous and ultraviolet light-induced genetic changes. Mol. Carcinog. **4:**460–466.
70. **Vogel, K. S., C. I. Brannan, N. A. Jenkins, N. G. Copeland, and L. F. Parada.** 1995. Loss of neurofibromin results in neurotrophin-independent survival of embryonic sensory and sympathetic neurons. Cell **82:**733–742.
71. **Wu, J., P. Dent, T. Jelinek, A. Wolfman, M. J. Weber, and T. W. Sturgill.** 1993. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′,5′-monophosphate. Science **262:**1065–1069.
72. **Xu, G. F., B. Lin, K. Tanaka, D. Dunn, D. Wood, R. Gesteland, R. White, R. Weiss, and F. Tamanoi.** 1990. The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of S. cerevisiae. Cell **63:**835–841.
73. **Xu, G. F., P. O'Connell, D. Viskochil, R. Cawthon, M. Robertson, M. Culver, D. Dunn, J. Stevens, R. Gesteland, R. White, et al.** 1990. The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell **62:**599–608.
74. **Yan, N., C. Ricca, J. Fletcher, T. Glover, B. R. Seizinger, and V. Manne.** 1995. Farnesyltransferase inhibitors block the neurofibromatosis type I (NF1) malignant phenotype. Cancer Res. **55:**3569–3575.