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13. ABSTRACT *(Maximum 200*

We developed methods to purify Schwann cells from mouse embryos mutant at *NF1* and compared mutant cells to wild type Schwann cells. *NF1*-deficient cells showed abnormalities in proliferation, differentiation, ability to stimulate angiogenic reactions and invade basement membranes. A gene dosage effect was confirmed; Schwann cells heterozygous at *NF1* were intermediate in phenotype to wild type or null cells. Activation of Ras in Schwann cells correlated with loss of *NF1*, phenotypes of Schwann cells deficient in *NF1* were mimicked in Schwann cells expressing activated Ras, and drugs that inhibit Ras function reversed some phenotypes of *NF1*-deficient cells. Together, the data demonstrate that a major NF1 function in Schwann cells is regulation of Ras activation. We propose that protein farnesyl transferase inhibitors might be useful to correct phenotypes of benign, *NF1*-deficient, Schwann cells in human patients. Additional studies defined the first cellular abnormality in fibroblasts lacking *NF1*, a failure to form perineurium; fibroblast abnormalities occurred mainly or solely in null cells. Experiments aimed at testing if *NF1*-deficient fibroblasts have calcium handling abnormalities were inconclusive. The data demonstrate that both Schwann cells and fibroblasts manifest cell autonomous abnormalities as a consequence of *NF1* mutations.

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CHAPTER 1: INTRODUCTION

This report documents identification of cellular abnormalities in cells derived from the peripheral nervous system of mouse embryos mutant at the *NF1* locus, provided to us by C. Brannan and N. Copleland at the National Cancer Institute. Our goal was to identify cell-autonomous phenotypes resulting from *NF1* mutations in mouse cells that might aid in understanding formation of peripheral nerve sheath tumors, neurofibromas, in the human inherited disease type 1 neurofibromatosis.

There are several major roadblocks to understanding tumor formation in human NF1. One of the chief problems is the lack of an animal model; while cattle develop neurofibromas the genetic defect has not been identified and tumors occur only in a few cows. Similarly damselfish develop neurofibromas but the etiology of tumor formation differs from that of NF1, which is inherited in an autosomal dominant fashion. A third possible model, mice that have targeted mutations at the NF1 locus do not develop tumors as heterozygotes, unlike their human counterparts. Mice null at the NF1 locus die between embryonic days E11 and E14, so that tumor formation cannot be assessed (Brannan et. al., 1994). To circumvent these difficulties we hypothesized that cells from mouse embryos lacking neurofibromin could characterized in tissue culture and provide an <u>in vitro</u> model of tumor development.

A second major problem is that it is not clear which cell type, or types, are defective in NF1 and underlie tumor formation. Candidates include fibroblasts, neurons, and Schwann cells (the major cell types present in normal peripheral nerves). Although Schwann cells are frequently considered the pathogenic cell type in NF1, little data links primary Schwann cell dysfunction to Schwann cells. To define cell-autonomous abnormalities in these three cell types that might contribute to neurofibroma formation, we hypothesized that by mixing and matching normal and NF1-deficient cells in culture, we could define abnormalities.

Analysis of NF1-deficient Schwann cells required pure Schwann cell cultures. We succeeded in developing a method to purify mouse Schwann cells from mutant embryos (Chapter 2; in press in Cell Biology and Pathology of Myelin, Devon R.M., ed., 1996). This method has already been requested from laboratories worldwide.

The effect of loss of neurofibromin on Schwann cells was analyzed. Proliferation in response to several growth factors decreased when Schwann cells lacked neurofibromin, but not when neurons lacked neurofibromin. In parallel experiments, the level of ras-GTP was elevated by reduction in neurofibromin, consistent with neurofibromin acting as an off switch for ras in Schwann cells (Chapter 3; Kim et al., 1995). This an important result as neurofibromin is a large protein with only a small GAP-related domain predicted to modulate Ras-GTP levels. Nonetheless, our data support the idea that modulation of Ras-GTP is a major function of neurofibromin in Schwann cells.

Since preparation of the midterm report, we demonstrated that mouse Schwann cells lacking NF1 show features of human neurofibroma Schwann cells in that they stimulate angiogenic responses on the chorioallantoic membrane and invade basement membranes. Further, mutant Schwann cells can be induced to hyperproliferate (a phenotype predicted when cells lose a tumor supressor) in response to activation of PKA or by removal of serum from the culture medium. Thus, we have developed two models for Schwann cell hyperplasia in the absence of NF1 that can be tested in human, neurofibroma-derived, cells. Proliferative abnormalities in mutant Schwann cells were reversed by a Ras inhibitor (protein farnesyltransferase) obtained from Merck Pharmaceuticals. This data provides additional evidence that modulation of Ras-GTP levels is a primary function of neurofibromin in Schwann cells. The data support the hypothesis that drugs such as farnesyl transferase inhibitors could be used to treat human NF1 patients. This work is presented in Chapter 4

and has been submitted for publication to Molecular and Cellular Biology.

Chapter 5 describes studies examining effects of lack of neurofibromin on Schwann cell differentiation that have been submitted to Molecular and Cellular Neuroscience. As lack of neurofibromin in Schwann cells was corellated with decreased Schwann cell proliferation (except under special conditions noted above) we hypothesized that Schwann cells lacking NF1 would show a more differentiated phenotype than wild type cells. This idea was borne out by Western blotting and myelin formation assays using mutant Schwann cells. Strikingly, however, organotypic cultures (containing neurons, Schwann cells and fibroblasts lacking NF1) showed the oppposite phenotype: a decrease in number of myelin segments of 70% was observed in the absence of neurofibromin. We hypothesize that interactions among multiple NF1-deficient cell types regulate Schwann cell phenotype: experiments are in progress to test this hypothesis.

We tested the hypothesis that loss of neurofibromin in fibroblasts causes cell autonomous abnormalities. Chapter 6 (Rosenbaum et al., 1995) documents analysis of fibroblast function in cell culture. Fibroblasts lacking NF1 hyperproliferate in response to serum. Strikingly null fibroblasts fail to organize neuron-Schwann cell groups into fascicles. The data show that NF1-deficient fibroblast from the mouse recapitulate the abnormal blood-nerve barrier present in human neurofibromas. Together with our data showing that Schwann cells lacking NF1 manifest cell-autonomous abnormalities, the data are consistent with the hypothesis that both fibroblast and Schwann cell abnormalities could contribute to human neurofibroma formation.

An additional goal of this grant was to test the hypothesis that calcium handling is abnormal in cells lacking neurofibromin. A major effort was made to substantiate preliminary results, described in the midterm report, suggesting that uptake of calcium (assessed by $^{45}Ca^{2+}$ uptake into whole cells or permeabilized cells)

showed reductions in cells without neurofibromin. While results are described in Chapter 7, we have been unable to confirm preliminary data in a reproducible fashion, and we remain uncertain as to the validity of the original observations. Experiments on fibroblasts without neurofibromin showed that release of calcium (using FURA-2 as a fluorescent indicator of intracellular free calcium) after exposure to agonists (bombesin, bradykinin, ionomycin, thapsigargin) showed normal kinetics. Similarly, the kinetics of efflux of $^{45}Ca^{2+}$ from mutant cells was identical to wild type cells. Thus, we tentatively conclude that calcium handling is not reproducibly different in wild type and mutant cells.

CHAPTER 2: A PROCEDURE FOR ISOLATING SCHWANN CELLS DEVELOPED FOR ANALYSIS OF THE MOUSE EMBRYONIC LETHAL MUTATION NF1

INTRODUCTION

The study of Schwann cell proliferation and differentiation has been facilitated by the availability of cultured Schwann cells that faithfully mimic Schwann cell in vivo maturation, growth, and differentiation. Transgenic mouse models and naturally occurring mouse mutants serve as increasingly important tools for the study of Schwann cell biology. We have developed methods to purify Schwann cells from single embryonic day 12.5 (E12.5) mutant mouse embryos in order to define abnormalities caused by mutations at the type 1 neurofibromatosis (NF1) locus. This method can be used to study Schwann cells from any mutant mouse that survives until day 12 of embryonic life.

NF1 null Schwann cells provide a potential mouse model for Schwann cell tumor formation in the common inherited disease, neurofibromatosis type 1 (NF1) (Riccardi, 1991) . Adult mice heterozygous for the NF1 mutation do not show identified peripheral nerve abnormalities (Brannan et al., 1994; Jacks et al., 1994) and homozygous mutants die in utero between embryonic day 11-15, prior to formation of mature peripheral nerve, making it impossible to use peripheral nerves or neonatal DRG as sources for null Schwann cells. Homozygous NF1 mutant embryos are obtained by mating heterozygous mice: genotypes of each embryo are not known at the time of dissection. Separate cultures, each derived from a single embryo, have to be established for purification of Schwann cells of identified genotype. Further, some types of experiments require highly purified Schwann cells. Our method generates homogeneous (≥ 99.5% pure) Schwann cells from single embryos.

In vitro studies have used Schwann cells from rat, chicken

mouse, and human peripheral nerves or dorsal root ganglia (DRG) (Askanas et al., 1980; Bhattacharyya et al., 1993; Cochran and Black, 1985; Kim et al., 1989; Kuhlengel et al., 1990; Levi et al., 1994; Morrissey et al., 1991; Rutkowski et al., 1992; Wood, 1976; Wood and Bunge, 1975; Yong et al., 1988; Rutkowski et al., 1995) . A recurrent problem in preparing Schwann cell cultures is connective tissue contamination (fibroblastic cells) and overgrowth of Schwann cells by fibroblasts in long-term culture. Two methods (Brockes et al., 1979; Wood and Bunge, 1975) were developed to obtain viable Schwann cells essentially free of contaminating fibroblasts from rat peripheral nervous system, and most Schwann cell purification methods now used are based on either of these two procedures. The original method developed by Wood and Bunge (1975) used dorsal root ganglia as a source of neurons to expand Schwann cell numbers: our method uses features of this technique. A dissociation procedure, developed by Brockes (Brockes et al., 1979) , used newborn rat sciatic nerve; fibroblasts were reduced by treating the primary cultures with antimitotic agents, followed by complement-dependent immunocytolysis of residual fibroblasts by using anti-Thy-1. Purified Schwann cells can be propagated in subsequent culture in the presence of growth factors. In recent years, availability of recombinant neuregulins, including glial growth factor (Lemke and Brockes, 1984; Levi et al., 1995; Marchionni et al., 1993; Rutkowski et al., 1995) have enabled significant and selective expansion of Schwann cell numbers. We tested combinations of these methods for use in embryonic mouse Schwann cells.

Methods for preparing Schwann cells from normal or neurological mouse mutants have been reported. Cornbrooks et al (1983) and Cochran and Black (1985) used prenatal DRG and the explant method to generate enriched Schwann cell preparations in 4 weeks, but did not report Schwann cell yields. Shine and Sidman (1984) used sciatic nerves from 1 day old mice as a source for Schwann cells but did not attempt purification of Schwann cells.

Krikorian et al (1982) and Manthorpe et al (1980) used differential adhesion to enrich for Schwann cells from normal mouse neonatal DRG; maximal purification of 97% Schwann cells was achieved. Difficulties were reported in removing contaminating fibroblasts from mouse Schwann cell cultures due to limitations in utilizing antimitotics or complement mediated lysis (Kalderon, 1984; Krikorian et al., 1982; Manthorpe et al., 1980; Seilheimer and Schachner, 1987) . Unlike rat Schwann cells, mouse Schwann cell proliferation can be stimulated with serum (Komiyama and Suzuki, 1991; Krikorian et al., 1982; Seilheimer and Schachner, 1987) so that attempts to selectively eliminate fibroblasts using antimitotic drugs also results in Schwann cell death. Kalderon (1984) achieved 95% purity from new-born mouse sciatic nerve by complement-mediated lysis; Seilheimer and Schachner (1987) reported 99.5% purity from new-born sciatic nerve by combining complement mediated lysis using both anti Thy-1.1 and anti MESA-1. None of these protocols began with early embryonic DRG and at the same time generated nearly homogeneous Schwann cell cultures.

Here we describe a method to generate highly purified populations of mouse Schwann cells from single E12.5 mouse embryos by a procedure that includes three steps : i) culture of Schwann cell precursors derived from embryonic DRG in the presence of neurons in serum-free medium on tissue culture plastic to selectively stimulate Schwann cell maturation and proliferation; ii) mechanical separation of the Schwann cell-associated neuronal network from underlying fibroblasts; iii) enzymatic dissociation of Schwann cells from neurons and subsequent expansion in secondary culture in rhGGF2-supplemented serum-containing media. A schematic illustration of the procedure is shown in Figure 1.

MATERIALS AND METHODS

*Solutions and Media**

10% serum medium: Dulbecco's modified Eagle's medium (DMEM) with high glucose (GIBCO cat # 11965-050) supplemented with 10 vol.% heat inactivated fetal bovine serum and penicillin/streptomycin (0.1 mg/ml)

C medium : DMEM with high glucose (GIBCO) supplemented with 10 vol.% human placental serum (provided by local hospital delivery rooms) and nerve growth factor (NGF) (50 ng/ml) (Harlan cat # BT-5017).

N2 medium: 1:1 ratio of DMEM and F-12 supplemented with Na-selenite (5 ng/ml) (Sigma cat # S-526), putrescine (16 ug/ml) (Sigma cat # P-7505), progesterone (125 ng/ml) (Sigma cat # P-0130), transferrin (0.2 mg/ml) (Sigma cat # T-2252), insulin (0.4 ug/ml) (Sigma cat # I-5500), NGF (50 ng/ml) and gentamycin (2.5 ug/ml) (GIBCO cat # 15710-015)).

Leibowitz-15 (L-15) medium (GIBCO)

Trypsin-collagenase: 0.05% trypsin (GIBCO) and 0.1% collagenase (Worthington Biochemical) in Hank's balanced salt solution (HBSS) (GIBCO).

Poly-L-lysine solution: 0.05 mg/ml poly-L-lysine (Sigma cat # P-7890) prepared in 0.15 M sodium borate pH 8.0, filter sterilized.

*All media was pre-warmed to 37°C before use

23G1 sterile syringe needles (Becton Dickinson); 6-well plastic culture dishes (Falcon); 35 mm and 100 mm culture dishes (Falcon); 4 ml and 15 ml poly-styrene tubes (Falcon); Stereoscopic dissecting microscope; Sterile cotton-plugged glass pasteur pipettes (Fisher)

Schwann Cell Isolation from E12.5 Mouse Embryos

Step 1 Mouse embryos are sterilely removed from 12.5 day

pregnant female mice under anesthesia by Cesarean section and maintained during the dissection procedure under sterile conditions in pre-warmed L-15 medium. Each embryos is placed in a separate 35 mm culture dish and processed individually. Embryos are killed by decapitation and the head transferred to prelabeled 1.5 ml eppendorf tubes to be used for genotyping. The spinal cord of each embryo is removed and transferred to a new dish, and DRG are dissected from the vertebral column as described for rat DRG at a similar stage (Salzer et al., 1980) . We have been unable to collect significant numbers of DRG from mouse spinal cords prior to day 12. The spinal cord is discarded and L-15 medium carefully removed using a glass pasteur pipette. DRG are enzymatically dissociated by adding 20-25 drops (about 1.5 ml) of 0.25% trypsin and incubated at 37°C for 40 min with gentle swirling (40 rpm) in an air incubator. It is important not to over-incubate cells in trypsin. Dissociated cells are transferred to 15 ml tubes containing 10 ml of 10% FBS media, using a separate glass pasteur pipette for each embryo, and centrifuged for 5 min at 80xg. Pelleted cells are collected by submerging a pasteur pipette under the medium and lifting out the pellet. Cells are transferred to a 4 ml tube containing approximately 0.5 ml C medium. A single cell suspension is generated by triturating the pellet 20-25 times through a glass pipette, being careful to avoid generation of air bubbles. A new glass pipette is used for each embryo. Resuspended cells derived from a single embryo are plated in two wells of a uncoated 6-well plastic culture dish each containing 2 ml of C medium. This is done by dropping same number of cell suspension drops on each well. Culture dishes are then placed at 35°C in a 7.5% CO2 humidified atmosphere. At this point, cultures are composed of neurons, fibroblasts and Schwann cell precursors. After 24 hours, serum-medium is removed and 2 ml of fresh serum-free N2 medium supplemented with NGF (50 ng/ml) and gentamycin (0.5 ug/ml) added; plates are transferred to 10% C02 at 37oC. Cells are observed frequently but the medium is not

changed. It is important to use uncoated plastic dishes, as when embryonic cells are plated onto collagen-coated substrates, fibroblasts become bundled together with neurites and Schwann cells, making the subsequent Schwann cell separation difficult.

Figure 2 illustrates the typical appearance of DRG cultures after various times in serum-free defined N2 media. By 24 hours after plating, most cells attach to the plastic (not shown) and by 48 hours (panel A), many neurons extend neurites. Phase-bright neuronal bodies are observed. Spindle shaped Schwann cells are present in apparent association with the growing neurites. By day 4 (panel B), extensive neuronal networks develop. Schwann cells greatly increase in number and a great proportion of Schwann cells become associated with developing neurites. By 6 days (panel C), virtually the entire neurite network is occupied by Schwann cells. Large flat cells (fibroblasts) are also seen in culture with no obvious association to neurons.

Step 2 On day 6 or 7, each well is marked with the genotype of the embryo from which the cells are derived, and placed under a dissecting microscope in a sterile environment. Under 500X magnification, the neurites and underlying fibroblast layers are visible. Neuron-associated Schwann cells are visible as beaded structures along neurites. Starting from a corner of the plate where few fibroblasts are present, Schwann cell-neuron networks are gently lifted up using a 23G1 needle, and slowly peeled off toward the middle of the plate. Usually, neuronal networks that are being peeled off can be distinguished from the fibroblast layer left behind at this magnification. If DRG are dissociated well initially, almost complete separation of neuron-Schwann cell networks can be achieved essentially free of fibroblasts. However, if dense populations of fibroblasts are observed in the direction from which neurons are being peeled off, neurons are cut off at that point and the neuronal network remaining on the plate lifted starting from a new point. Sometimes, fibroblasts that are

condensed underneath neuronal bodies are difficult to separate from the neurons. In this case, these areas are first excised from the plate using a surgical razor blade, and the separation procedure proceeds. It is crucial to obtain Schwann cell-neuronal networks that are free of contaminating fibroblasts at this point, because removal of fibroblasts from the later Schwann cell cultures is more difficult. Attempts to remove fibroblasts from Schwann cell cultures by complement mediated lysis using several different commercial anti -mouse Thy1.1 antibodies were unsuccessful. Thus, it is important not to over-grow the fibroblasts in the initial DRG cultures; fibroblast overgrowth occurs when DRG cells remained in culture for longer than 7 days prior to purification. After successfully removing the neuron-Schwann cell network from the plates, neurons and Schwann cells are transferred to 15 ml tubes containing 7 ml 10% FBS-DMEM. Cells from a single embryo can be maintained separately, or cells from embryos of the same genotype pooled.

Step 3 Schwann cells, associated with neurons, are centrifuged for 5 min at 80xg and Schwann cells are dissociated from neurons by enzymatically dissociating the resulting pellets in trypsin-collagenase solution in a volume of approximately 0.5 ml per embryo. For example, if cells from 6 embryos (12 wells) of the same genotypes are pooled, 3 ml of the enzyme solution is used to dissociate Schwann cells. If cells from a single embryo are processed separately, a minimum amount of 1.5 ml is used per embryo (corresponding to 2-wells of the DRG culture). Cells are then incubated at 37°C for 30 min with gentle rotation (30-40 rpm) in an air incubator, with tubes laid on their side, not vertically placed. This allows even access of enzymes to the cells. The enzyme reaction is stopped by adding DMEM plus 10% FBS, cells centrifuged, resuspended in 2 ml of DMEM+10% FBS and counted in a hemacytometer. On average, 0.3 X 106 cells are obtained from one embryo. Cells are plated onto poly-L-lysine-coated 100 mm plates and cultured in DMEM+10%FBS supplemented with glial growth factor

(rhGGF2, 10 ng/ml) (Cambridge Neuroscience) and 2 uM forskolin to suppress contaminating fibroblast growth. After 5-6 days, plates become confluent with approximately 3 X 106 cells/plate (Figure 2 panel D). The majority of cells have the spindle-shape appearance and oval nucleus characteristic of cultured Schwann cells. When cultures are examined for the expression of a Schwann cell antigen, low affinity NGF receptor (NGFR) using a rat anti mouse NGFR antibody (obtained from Dr. David Anderson, Caltech), a minimum of 99.5% of the cells are positive for NGFR expression. In three separate experiments, 99.5%, 99.9% and 99.6% of cells were NGFR positive; most of the NGFR positive cells had spindle shaped morphology
(Figure 3, panel A). Large flat cells (fibroblasts) are negative for the staining. NGFR expressing cells were also positive for S100 expression (not shown).

After a plate becomes confluent with proliferating mouse Schwann cells, cells are trypsinized and replated onto new poly-L-lysine coated plates at a density of 0.5 X 106 cells/100 mm plate in 10% serum containing media. Next day, the medium is changed to 10% serum medium supplemented with rhGGF2 and forskolin (2 mM). If the culture appears contaminated with a significant numbers of fibroblasts, serum-free N2 medium can be used during the subsequent Schwann cell expansion. As the passage number increases, each passage being one week in culture, mouse Schwann cells tend to become less responsive to growth factors and begin to die (in contrast to rat Schwann cells). The demise of cells occurs faster in serum-free media than in serum containing media. We recommend use of Schwann cells which have gone through less than 4 passages for analysis.

RESULTS

Characterization of Mouse Schwann Cells

Proliferation in Response to Soluble Mitogens and axons.

Double-label immunocytochemistry assay was used to investigate cell division of isolated mouse Schwann cells in response to rhGGF2 (Figure 3). In control cultures, most NGFR positive cells remained quiescent. Upon rhGGF2 stimulation, the majority of NGFR positive cells incorporated [^{3}H]-thymidine. Quantitation of results is shown in Figure 4; rhGGF2 treatment resulted in proliferation of 50-60% of NGFR positive cells (Figure 3. panel B). In rat Schwann cells, mitogenic effects of GGF are potentiated by agents that increase intracellular cAMP. When purified mouse Schwann cells were treated with rhGGF2 in combination with forskolin, no synergy between two factors was observed. In some experiments, forskolin even slightly inhibited GGF induced mouse Schwann cell proliferation. Forskolin alone had no effect on mouse Schwann cell proliferation, similar to results reported for rat Schwann cells.

Basic fibroblast growth factor (bFGF) stimulates rat Schwann cell proliferation only when it is accompanied by agents that increase cAMP in the cells and by itself, has no effect on cell division. In contrast, when mouse Schwann cells were treated with bFGF in serum-free medium, 18% of NGFR positive cells were labeled. Thus, bFGF, by itself, is a mitogen for mouse Schwann cells. Addition of forskolin potentiated the mitogenicity of bFGF (labeling index: 42%). It has been shown previously that in serum containing media, bFGF stimulates proliferation of mouse Schwann cells prepared from neonatal mice DRG (Krikorian et al., 1982) . In our analyses, mouse Schwann cells showed slightly increased basal level proliferation in serum containing media (data not shown).

One of the characteristic features of Schwann cells is increased cell proliferation in association with axons. Isolated

mouse Schwann cells were added to purified rat DRG neuronal cultures (Wood, 1975; Wood and Bunge, 1976) and incorporation of [^{3}H]-thymidine was examined (Figure 3, panel C). Cells established association with axonal membranes, and nuclear labeling was greatly enhanced in cells with association with axons. Contaminating fibroblasts with flat morphology showed no obvious relationship with axons.

Po Expression and Myelin Formation. Differentiation of rat Schwann cells can be assessed in isolated cultures. Agents that increase intracellular cAMP elevation upregulate expression of myelin proteins (Lemke and Chao, 1988; Morgan et al., 1991; Sobue and Pleasure, 1984) . One of these proteins is Po, which has been used as a general marker for differentiating Schwann cells (Morgan et al., 1991) . To examine whether mouse Schwann cells also display this phenotype, cells were treated with forskolin, an activator of adenylate cyclase, each day for 5 days, cell extracts prepared and expression of Po analyzed by SDS-PAGE followed by Western blot analysis. As shown in Figure 5, while control cells show no expression of the protein, forskolin treatment induced about a 20 fold increase in Po expression (analyzed by densitometric scan of blots).

Specific association with axons is necessary for the formation of myelin by Schwann cells (Duncan, 1934; Friede, 1972; Matthews, 1968; Voyvodic, 1989). In culture, Schwann cells can be induced to form myelin around axons by co-culturing Schwann cells with neurons (Eldridge et al., 1987). We showed recently that these mouse Schwann cells can also form myelin around axons (Rosenbaum et al., 1996).

Characterization of Phenotypes of Schwann Cells Derived from NF1 Knock-Out Mice

To study the effect of loss of NF1 gene product, neurofibromin, in Schwann cells, Schwann cells were isolated from

wild type, heterozygous or null mouse embryos at E12.5 using the method described here and phenotypes characterized. The high purity of these cultures enabled us to carry out biochemical experiments to measure levels of Ras-GTP in normal and mutant cells (Kim et al., 1995) ; GTP-Ras is significantly increased in the absence of NF1, substantiating the idea that NF1 is the major Ras-GAP in Schwann cells (Basu et al., 1992; DeClue et al., 1992) . Null Schwann cells also show altered morphology as compared to wild type cells, and decreased cell proliferation in response to axons or rhGGF2 (Kim et al., 1995) . Recently, we have used mouse Schwann cells from mutants in studies of perineurium formation (Rosenbaum et al., 1995) , of myelination (Rosenbaum et al., 1996) and of cell transformation (Kim et al., 1996) . Our results confirm that alterations in mouse Schwann cells resulting from mutations in a single gene can be detected using the purification methods described.

SUMMARY

Several purification methods did not yield satisfactory results for Schwann cells of the mouse. A new method was developed that generates a large quantity of essentially pure mouse Schwann cells. Because fibroblasts are removed at early stage of the isolation procedure, Schwann cells can be maintained in serum containing media, greatly improving survival and proliferation. The method generates 2-3 X 106 Schwann cells/embryo in less than two weeks, providing a powerful new tool for analysis of mouse Schwann cells in vitro. Several properties were used to identify isolated cells as Schwann cells. First, the cells are characteristically spindle-shaped in culture. Second, they express immunocytochemical markers, including S100 and p75NGF receptor, characteristic of Schwann cells (Jessen and Mirsky, 1991; Schachner et al., 1981; Stefansson et al., 1982) . Third,

the cells adhere to rat axons and proliferate in response to axonal contact (Aguayo et al., 1976; McCarthy and Partlow, 1976; Salzer et al., 1980; Wood and Bunge, 1975) . The cells also proliferate in response to known Schwann cell mitogens, GGF and bFGF. Regulation of mouse Schwann cell proliferation differs from that in rat (Davis and Stroobant, 1990; Raff et al., 1978; Stewart et al., 1991) . Response of mouse Schwann cells to bFGF is independent of cAMP elevation, and increasing cAMP levels in mouse Schwann cells also fails to synergize with GGF mitogenicity. Finally, mouse Schwann cells formed myelin around axons when cultured with rat peripheral neurons and expressed the myelin protein Po after exposure to forskolin. Thus, mouse Schwann cells isolated from E12.5 embryos show only subtle differences from better-studied rat cells.

FIGURE LEGENDS

Figure 1. :A schematic diagram of a procedure for isolating mouse Schwann cells from E12.5 embryo. (A)-(D): Dorsal root ganglia (DRG) are removed from a E12.5 mouse spinal cord and cells are enzymatically dissociated. The cell suspension, mostly composed of neurons, fibroblasts and Schwann cell precursors, are plated onto uncoated 6-well culture dish and maintained in a serum-free medium supplemented with NGF. (D)-(F) Schwann cells are allowed to expand until the neuritic networks are populated (6-7 days), then subsequently, Schwann cell-neuron networks are mechanically separated from fibroblasts by peeling them off from the dish using a needle. Next, the Schwann cells are enzymatically dissociated from the neurons and plated on a poly-L-lysine coated plate in DMEM+10% FBS containing rhGGF (10 ng/ml) and forskolin (2 mM). After 1 week in culture, ≥ 99.5% of the cells is positive for both S100 and NGFR.

Figure 2. Phase-contrast micrographs of mouse dorsal root ganglion (A-C) and purified Schwann cell (D) cultures prepared from a E12.5 embryo. A: Embryonic mouse DRG cultures 48 hours in vitro. Dissociated mouse DRG were plated onto a uncoated 6-well culture dish and maintained in a serum-free medium supplemented with NGF. Most of the NGF supported neurons (arrow head pointing to a neuronal cell body) had extended neurites. Note that the entire neuritic territory appears occupied by tightly associated spindle shaped Schwann cells (arrows). Large flat fibroblasts (F) are also present with no obvious association with neurons. B: Four days in vitro. An extensive neuronal networks are being established. Neuron-associated Schwann cells are increased in numbers (arrows). C: Six days in vitro. Most of the culture area is populated with extended neurites and virtually the entire neuritic network is occupied with Schwann cells. Fibroblasts (F) had also increased in numbers but with no association with

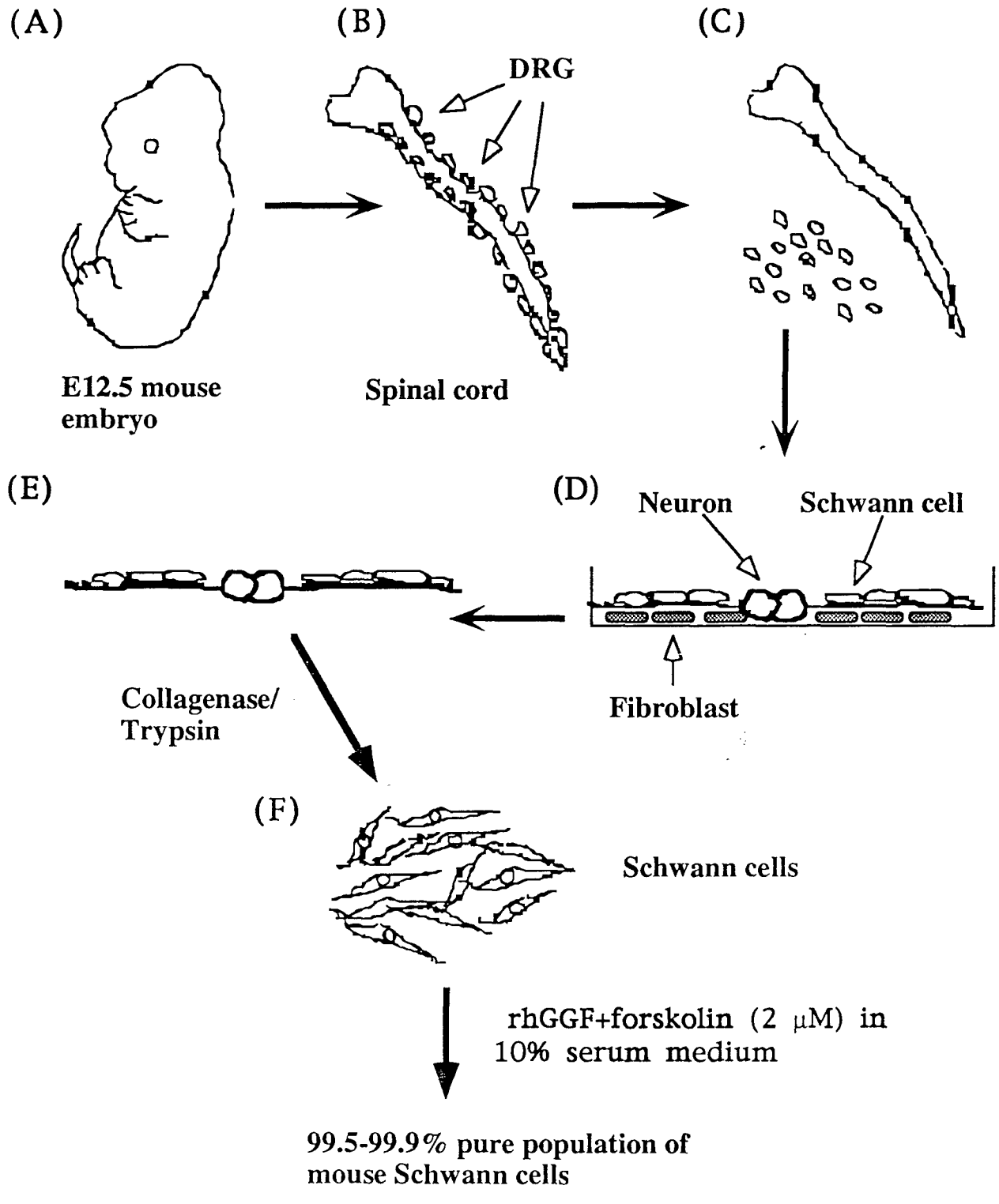
neurons. D: A confluent culture of purified Schwann cells. Neuron-Schwann cell networks were separated from the underlying fibroblasts layer as described in the text. Subsequently, Schwann cells were dissociated from the neurons and expanded in the presence of rhGGF2 and forskolin in DMEM+10% FBS. The majority of cells have the spindle-shape appearance characteristic of Schwann cells. Arrow points to a mitotically active, dividing Schwann cell. Bar = 140 mm

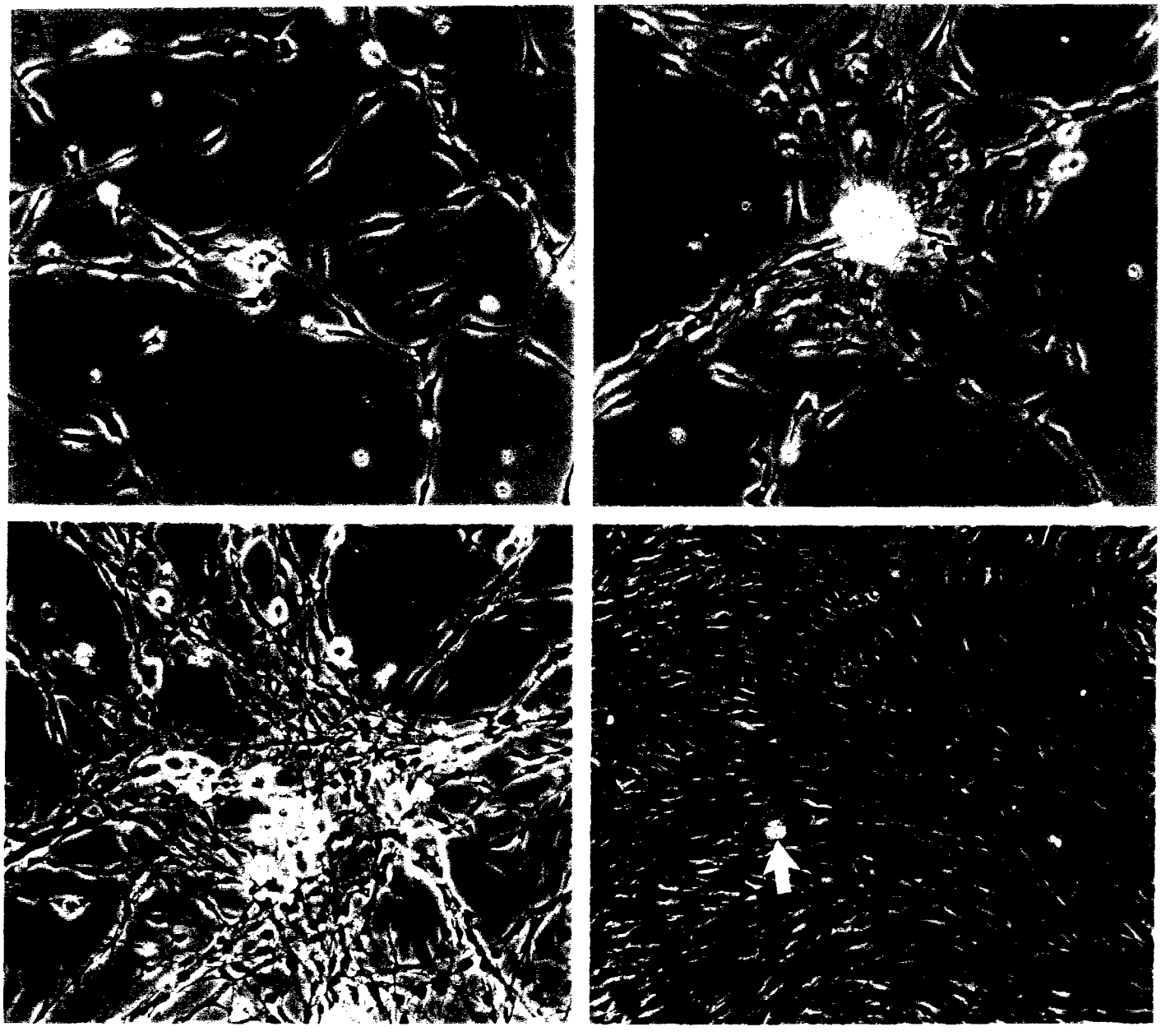
Figure 3. Expression of NGFR by purified mouse Schwann cells. Autoradiograph of Schwann cells stimulated by a soluble growth factor (rhGGF2) or neuronal contacts. A: Purified mouse Schwann cells were fixed in culture and processed for immunostaining with NGFR. DAB stained NGFR positive Schwann cells are shown. B: Schwann cells were stimulated with rhGGF and exposed to 0.5uCi/ml [^{3}H]-thymidine for 24 hours, fixed, stained with NGFR and processed for autoradiography. Most of the NGFR positive cells (Schwann cells) are overlaid with silver grains upon rhGGF2 stimulation (compare with Schwann cells in panel A (untreated with rhGGF). C: Purified mouse Schwann cells seeded onto cultures of E16 rat DRG neurons. After 24 hours, [^{3}H]-thymidine was added and the cultures were incubated for an additional 24 hours prior to fixation and autoradiography. Cultures were counter-stained with toluedine blue. Neuron-associated Schwann cells with labeled nuclei are shown (arrow heads). Contaminating fibroblast with no obvious association with neurons are also present (F). Bar = 263 mm

Figure 4. Proliferation of mouse Schwann cells purified from E12.5 embryos in response to forskolin, rhGGF2 or combination of the two. Cells were plated on poly-L-lysine coated 8-chamber glass Lab-Tek slides (25,000 cells/well) in DMEM+10% FBS. After 48 hours, medium was switched to serum-free N2 and cells were stimulated with either forskolin (5 mM), rhGGF2 (10 ng/ml), bFGF (10 ng/ml) or forskolin in combination with rhGGF2 or bFGF. Cells

were pulsed for 24 hours with 0.5uCi/ml [^{3}H]-thymidine, fixed, immunostained with NGFR and processed for autoradiography. Schwann cell proliferation was determined by the percentage of NGFR positive cells with labeled nuclei.

Figure 5. Po expression of mouse Schwann cells purified from E12.5 embryos. Schwann cells were incubated for 4 days in either rhGGF2 (10 ng/ml) or forskolin (5 mM) in serum-free N2 medium and cell lysate prepared. Equal amounts of each lysate was separated on a 10% SDS gel and Po expression analyzed by Western blot using polyclonal antibodies raised against rat Po. Induction of Po expression is seen in forskolin (F) treated mouse Schwann cells, while no Po expression is seen in rhGGF2 (G) treated cells.


(A)
(B)
(C)
DRG
E12.5 mouse embryo
Spinal cord
(E)
(D)
Neuron
Schwann cell
Collagenase/ Trypsin
Fibroblast
(F)
Schwann cells
rhGGF+forskolin (2 μM) in 10% serum medium
99.5-99.9% pure population of mouse Schwann cells



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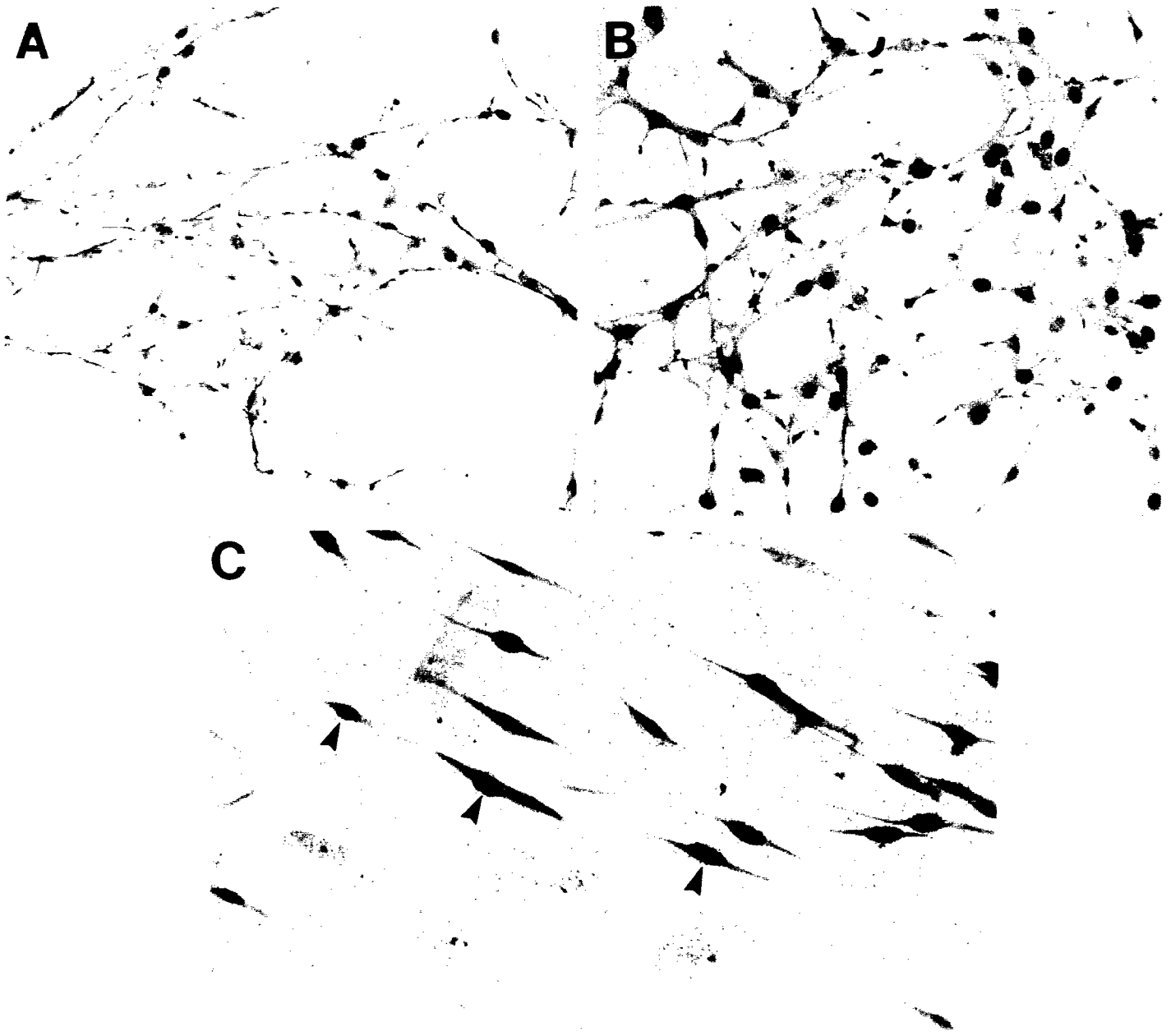
A
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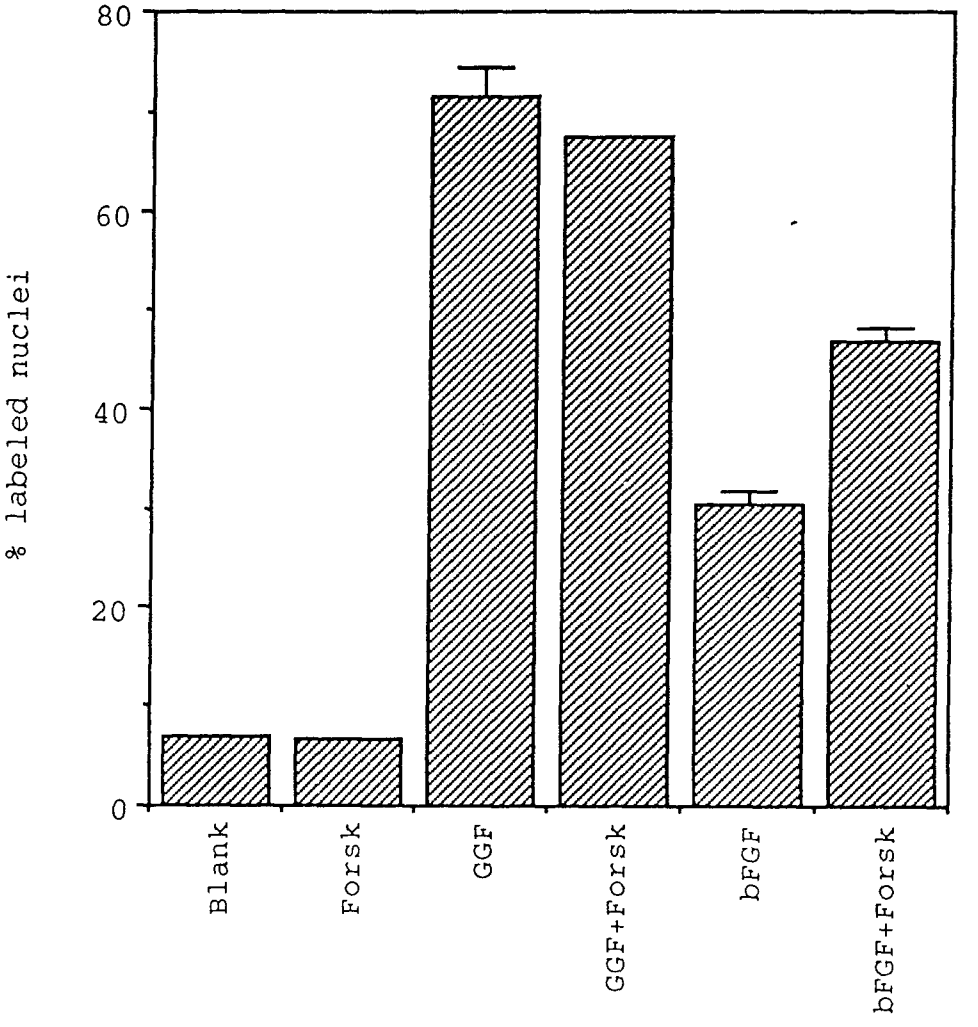

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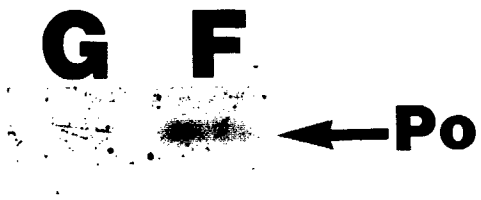
G
F
Po

CHAPTER 3. SCHWANN CELLS FROM NEUROFIBROMIN DEFICIENT MICE EXHIBIT ACTIVATION OF $p21^{ras}$, INHIBITION OF CELL PROLIFERATION AND MORPHOLOGICAL CHANGES.

ABSTRACT

Schwann cells are thought to be abnormal in type 1 neurofibromatosis (NF1) and to contribute to the formation of benign and malignant tumors in this disease. To test the role of the NF1 gene product neurofibromin as a Ras-GTPase activating protein in Schwann cells, and to study the effect of the loss of neurofibromin on Schwann cell proliferation, we isolated Schwann cells from mice with targeted disruption of NF1. The properties of these neurofibromin deficient cells were strikingly similar to those of v-ras expressing rat Schwann cells with normal levels of neurofibromin. The similarities included: growth inhibition, noted as a decrease in cell division in response to glial growth factor 2 (GGF2) and of neuronal contact; morphological changes such as the appearance of elaborated processes; and elevated levels of Ras-GTP. Furthermore, Ras-GTP levels in the neurofibromin deficient Schwann cells were consistently elevated in response to GGF2 treatment. In contrast to these results, introduction of v-ras into a Schwannoma cell line (RN22) led to cell transformation. We conclude that neurofibromin functions as a major regulator of Ras-GTP in Schwann cells; however, mutation in NF1 by itself is unlikely to explain the hyperplasia observed in Schwann cell tumors in NF1 disease.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is an inherited human autosomal disease affecting approximately 1 in 3500 individuals around the world (Riccardi, 1991). The disease affects many organ systems including the peripheral nervous system; NF1 patients develop benign peripheral nerve tumors called neurofibromas. The majority (65-80%) of the cells in neurofibromas are Schwann cells, suggesting that Schwann cells are the abnormal cell type in these tumors. Consistent with this idea, Schwann cells, but not fibroblasts, from neurofibromas show angiogenic and invasive properties (Sheela et al., 1990) ; and patients with NF1 are also at risk for development of malignant Schwann cell tumors called neurofibrosarcomas (Riccardi and Eichner, 1986) .

The NF1 gene, mutated in NF1 disease, encodes an 11-13kb mRNA (Marchuk et al., 1991; Wallace et al., 1990) . The NF1 gene product, neurofibromin, is a 220-250kD protein expressed ubiquitously during development and present in highest abundance in adult in cells of the nervous system, including Schwann cells (Bollag et al., 1990; Daston et al., 1992; DeClue et al., 1991b; Gutmann et al., 1991) . Neurofibromin contains a region homologous to yeast IRA1 and IRA2, which are negative regulators of RAS in yeast. A domain within this region is also homologous to mammalian GTPase-activating proteins (GAPs) (Ballester et al., 1990; Xu et al., 1990a; Xu et al., 1990b) . GAPs bind to mammalian Ras proteins and stimulate their intrinsic GTPase activity, accelerating the conversion of Ras from an active, GTP-bound form to an inactive, GDP-bound form (Trahey and McCormick, 1987; Vogel et al., 1988; Wigler, 1990) . The GAP related domain (GRD) of neurofibromin corresponds to the catalytic region of GAP. Expression of the GRD of neurofibromin or of GAP complements ira mutations in yeast, and this domain functions as a negative regulator of mammalian Ras in vitro (Ballester et al., 1990; Golubic et al., 1992; Xu et al., 1990a) . Because of the GAP-like activity of neurofibromin, it has been suggested that disease

manifestations of NF1 patients may result primarily from abnormal regulation of Ras in affected cells (Basu et al., 1992; DeClue et al., 1992) .

It is possible that mutations at the NF1 locus in Schwann cells could directly trigger abnormal cell growth, perhaps due to inadequate regulation of p21ras. Supporting this hypothesis, it has been shown that malignant Schwann cell lines derived from NF1 patients contain very low levels of neurofibromin and increased levels of GTP-bound Ras proteins (Basu et al., 1992; DeClue et al., 1992) . Lowering Ras-GTP in these cells catalytically, or introducing a Ras-specific neutralizing antibody inhibited the growth of the cells. However, while in many cell types high Ras activity enhances cell proliferation or transformation (Bourne et al., 1990; Downward, 1992b; Gibbs et al., 1985) , in other cells (such as PC12) high Ras-GTP induces differentiation. Ridley et al. (1988) found that introduction of activated ras into cultured rat Schwann cells resulted in inhibition of DNA synthesis and growth arrest, unless accompanied by a second (nuclear) oncogene, in which case the ras gene transformed the cells. Thus the effect of Ras activity in Schwann cells depends on the status of other genes in the cells.

Schwann cell proliferation has been studied in well-defined culture systems. Although normal Schwann cells from perinatal sciatic nerves are quiescent, even in the presence of serum, they can be stimulated to enter the cell cycle by contact with cultured neurons (Wood, 1975, Brockes, 1979) . This model is thought to reflect the axon-regulated proliferation of Schwann cells in the developing nerve; in the normal adult nerve Schwann cell, proliferation is very low. The division of cultured Schwann cells can also be induced by several defined mitogens. Products of the neuregulin gene, including the glial growth factors (GGFs), are potent Schwann cell mitogens (Lemke and Brockes, 1984) , and expression of neuregulin by neurons is likely to underlie the mitogenic effect of neurons for Schwann cells (Marchionni et al.,

1993; Orr-Urtreger et al., 1993) . Exposure of Schwann cells to neuregulin activates tyrosine phosphorylation of 185 kd receptors HER2, HER3 and HER4 (Levi et al., 1994) ; one or both of these receptors is likely to transduce neuregulin signals.

Targeted disruption of the NF1 gene in mice, by homologous recombination, has recently been reported (Brannan et al., 1994; Jacks et al., 1994). Although heterozygous mice were phenotypically normal at birth and did not develop neurofibromas, they did demonstrate elevated tumor formation in various organs (Jacks et al. 1994). In contrast, homozygous mutant mice died in utero by day 14.5, with death apparently resulting from cardiac abnormalities. The lethality for the homozygotes occurs prior to formation of mature peripheral nerves. To test the effect of loss of neurofibromin on Schwann cells, we developed a method to culture, expand and purify Schwann cells from wild-type and mutant mouse embryo dorsal root ganglia prior to death, at day 12.5. We have used these Schwann cells to analyze Ras-GTP levels, cell morphology, and the response to growth factors. These results have been compared with those obtained after expression of oncogenic ras in primary rat Schwann cells.

RESULTS

Expression of activated ras or loss of neurofibromin expression in cultured Schwann cells results in growth inhibition

To investigate the effect of high Ras activity in Schwann cells, we introduced a retroviral shuttle vector encoding v-rasH (pBW1423), which also contains the neomycin resistance gene) by retroviral infection into primary rat Schwann cell cultures. G418 selection produced clonal lines of slow growing cells expressing v-rasH. Due to difficulties in expanding individual clones to obtain sufficient numbers of cells for the assays, multiple clones were pooled, and the population of cells were compared to uninfected controls (maintained in vitro for similar time as v-rasH expressing cells). When cultured in the presence of recombinant human GGF2 (rhGGF2) and forskolin (the mitogenic activity of GGF is potentiated by agents that elevate intracellular cAMP levels in Schwann cells), uninfected Schwann cells grew rapidly (Figure 1), doubling about every two days. By contrast, the v-ras expressing Schwann cells grew much more slowly, and displayed a lag of several additional days. Cells remained adherent to the culture dish, and cell death was not evident over the course of the experiment.

In a related experiment, the synthesis of DNA in response to rhGGF2 and/or forskolin was determined for the v-rasH expressing rat Schwann cells by measuring the uptake of [^{3}H]thymidine. Schwann cells infected with a retrovirus encoding G418 resistance only (pBW1594) served as the controls for this experiment. As expected, the control cells showed a proliferative response to rhGGF2 and forskolin, with the forskolin potentiating the stimulatory activity of rhGGF2 on DNA synthesis about 8-fold. Although a similar degree of cooperation between rhGGF2 and forskolin was seen for the v-ras H expressing (1423) Schwann cells, but DNA synthesis in response to mitogenic stimulation was reduced 4-5 fold compared to controls.

To investigate the effect of neurofibromin on Schwann cell proliferation, highly enriched (80-95% pure) populations of mouse Schwann cells were grown, as described in the Materials and Methods, from 12.5 day embryos that were wild-type (+/+), heterozygous mutant (+/-), and homozygous mutant (-/-) for NF1. Cells were treated with rhGGF2, pulse-labeled with [^{3}H]thymidine, and subjected to autoradiography. Schwann cells were distinguished from residual fibroblasts by concurrent immunostaining with rat anti-mouse low affinity NGF receptor (NGFR) antibody. The percentage of Schwann cells that incorporated [^{3}H]thymidine after rhGGF2 stimulation is shown in Figure 3. (In contrast to rat Schwann cells, forskolin does not potentiate GGF mitogenicity in mouse Schwann cells {H.K. and N.R., unpublished observations}). In cultures from wild-type mice, 44% of the NGFR positive cells showed nuclear labelling. A 50% reduction in labeling was observed in mutant (nf/nf) Schwann cells, compared to wild-type(+/+); heterozygous (+/nf) cells exhibited an intermediate reduction in labeling. The latter result appears to correlate with the observation that cells from (+/nf) mice express about one-half as much neurofibromin as wild-type cells (Brannan et. al., 1994).

Expression of activated ras or loss of neurofibromin in Schwann cells induces morphological changes, accompanied by elevated levels of Ras-GTP

The previous experiments demonstrated a similar decrease in growth in Schwann cells that expressed v-ras or lacked neurofibromin. The cultures were also found to have similar morphologic alterations (Figure 4, Figure 5). Expression of v-ras in primary rat Schwann cells caused elongation of the cells (Figure 4). The cells also exhibited increased numbers of long processes (arrows), and the cell bodies became brighter and hyper-refractile under phase-contrast microscopy. Ridley et al. (1988) also reported increased elongation and refractility in Schwann

cells following micro-injection of oncogenic p21ras protein. Analogous changes in morphology were also observed in Schwann cells from homozygous mutant mice lacking neurofibromin (Figure 5). The cells were more elongated and refractile than cells from (+/+) mice, and many cell bodies showed outgrowth of long processes; heterozygous mutant cells displayed an intermediate phenotype with respect to these changes. Schwann cells from (-/-) mice also were less adherent than controls. Morphological effects were most pronounced in cultures just prior to confluence.

The similarity between the effects of activated ras and loss of neurofibromin in Schwann cells is consistent with the possibility that neurofibromin may be a physiological regulator of Ras-GTP in Schwann cells. To examine this question, we analyzed the level of GTP bound to p21ras in the Schwann cell cultures (Table 1; Table 2). Uninfected rat Schwann cells grown for 2-3 days in the absence of GGF contained about 5% GTP bound to Ras. When cells were cultured in the presence of rhGGF2 during the labelling period, the proportion of Ras-GTP was higher, about 9%. This result indicated that treatment of cells with GGF, which is known to activate a receptor tyrosine kinase(s), leads to increased Ras activity in the Schwann cells. The v-ras expressing (1423) Schwann cells contained 25% Ras-GTP, consistent with the presence of oncogenic Ras protein in the cells. v-ras transfected fibroblast lines often display higher Ras-GTP levels, such as 60% in NIH 3T3 (Table 1). This difference is likely due to lower expression of the v-rasH encoded protein in the 1423 Schwann cells, as these cells were isolated by retroviral infection, rather than by DNA transfection.

When Schwann cells from neurofibromin deficient mouse embryos were examined, a significant elevation of Ras-GTP was reproducibly observed in the (-/-) cells, in multiple independent experiments (Table 2). The level of GTP in these cells was about 25%, compared with about 10% in (+/+) cells. Furthermore, a dosage effect was noted in that cells from (+/-) mice reproducibly had

intermediate levels of Ras-GTP (approximately 15%). These results demonstrate that neurofibromin is an important physiological regulator of Ras-GTP in Schwann cells, and that even a 50% decrease in the level of the protein is reflected in higher Ras-GTP levels and phenotypic changes, as described above. As was true for the rat Schwann cells, Schwann cells from mouse embryos of all NF1 genotypes contained higher levels of Ras-GTP when grown in the presence of rhGGF2 than without. This result makes it unlikely that neurofibromin is involved in the mechanism by which GGF elevates Ras-GTP levels in the Schwann cells.

Overexpression of ras or loss of neurofibromin inhibits Schwann cell proliferation in respond to neuronal contact; loss of neurofibromin in Schwann cells, but not in neurons, is responsible for the growth inhibition.

During peripheral nerve development, direct interactions between axons and adjacent Schwann cells are thought to be crucial for Schwann cell division. A Schwann cell-neuron co-culture system, in which Schwann cells adhere to axons and proliferate only when in contact with axons, is thought to reflect the in vivo situation (Wood, 1975) . To investigate the role of Ras in Schwann cell proliferation triggered by neuronal contact, control and v-rasH expressing (1423) Schwann cells were seeded onto purified rat neurons prepared from embryonic dorsal root ganglia. Both populations of Schwann cells attached normally and spread onto sensory neuron processes (not shown). After 16-18 hours, cultures were pulsed with [^{3}H]thymidine-containing media for 17 hours and the proliferating Schwann cells (distinguished by nuclear labeling after autoradiography) were quantified and expressed as a percentage of total Schwann cells in contact with axons. Introduction of v-rasH (1423) into Schwann cells led to a reduction in Schwann cell proliferation in the Schwann cell-neuron co-cultures (Figure 6). The proportion of labeled 1423 cells was only one-third that of the control (1594) cells.

The effect of neurofibromin deficiency on Schwann cell proliferation was also studied in Schwann cell-neuron co-cultures. Schwann cells from wild type (+/+), heterozygous mutant (+/-) or homozygous mutant (-/-) mouse embryos were seeded onto normal rat neurons. Schwann cells of all three genotypes adhered to neurons and spread normally (not shown). The proliferation of (+/-) and (-/-) Schwann cells was significantly reduced as compared to wild-type cells. While 57% of (+/+) Schwann cells incorporated [^{3}H]thymidine, only 45% of (+/-) and 36% of (-/-) Schwann cells incorporated label (Table 3). This result was observed in 4 separate experiments using Schwann cells from different embryos. Loss of neurofibromin expression and introduction of an activated ras thus cause a similar inhibitory effect on neuron-triggered Schwann cell proliferation.

It has been suggested that neurofibromin deficient neurons might provide Schwann cells with a hyperproliferative signal in neurofibromas. To test this possibility in the mouse model system, dorsal root ganglion neurons were purified from wild-type or neurofibromin deficient mouse embryos and seeded with normal rat Schwann cells. Resulting co-cultures were analyzed for [^{3}H]thymidine incorporation into normal rat Schwann cells (Table 4). No significant difference was observed between control and mutant neurons, suggesting that loss of neurofibromin in neurons does not alter the neuron's ability to stimulate Schwann cell proliferation, at least in this model system.

Expression of activated ras causes morphological transformation of a rat Schwannoma cell line

Although the effect of high Ras-GTP in the Schwann cells described here is to inhibit their growth, the opposite result has been observed for cells from malignant Schwannomas of NF1 patients (Basu et al., 1992; DeClue et al., 1992) . In those cells, Ras-GTP provided a positive signal for growth. Also, the experiments of Ridley et al. (see Introduction) suggested that activated ras

could induce transformation in Schwann cells that had other genetic alterations. To further explore this point, we have investigated the effect of v-rasH on the RN-22 rat Schwannoma line, a derivative of RN-2 (Pfieffer and Wechsler, 1972). Although this line, derived from a chemically-induced malignant tumor, undoubtedly has a number of genetic mutations, we observed no alteration in neurofibromin expression or in the regulation of Ras-GTP in RN-22 cells (DeClue et al., 1992).

The v-rasH (pBW1423) gene was introduced into the RN-22 line by retroviral infection and G418 selection (Figure 7). While the RN-22 line displays a flat, adherent, non-refractile morphology, the v-ras expressing cells became rounded and refractile, and were no longer monolayer-restricted (Figure 7A). When seeded in soft agar, the parental line could form a few small colonies; in contrast, the v-ras RN-22 line grew into very large colonies under these conditions (Figure 7B). We conclude that the transforming effect of activated ras on the rat Schwannoma line is similar to the role of high Ras-GTP in NF1 patient tumor cells, and thus differs from the effect seen in primary Schwann cells. It is therefore likely that other genetic alterations, in addition to inactivation of the normal NF1 allele, are required during the course of NF1 malignant tumor development. These other mutations may allow the effect of high Ras-GTP to be altered from growth-inhibitory to transforming in the Schwann cells of the developing tumor.

DISCUSSION

The current studies have shown that reduced expression of neurofibromin in Schwann cells leads to decreased cell proliferation and morphological changes which are accompanied by increased levels of Ras-GTP in the cells. Since Schwann cells with normal levels of neurofibromin that express a mutationally activated ras gene display a similar phenotype, our observations provide experimental support for the idea that neurofibromin in Schwann cells acts, primarily or solely, as a physiological negative regulator of Ras by stimulating the intrinsic GTPase activity of Ras. It has been speculated that neurofibromin may, in addition to its regulation of GTP-RAS, serve as an effector protein for Ras-induced growth inhibition (Bollag et al., 1990). This does not, however, seem to be the case for the Schwann cells described here, since cells lacking NF1 expression respond to high Ras-GTP in a manner that is similar to that of wild-type cells into which v-ras has been introduced.

Although similar conclusions have been drawn for neurofibromin in malignant schwannoma cell lines derived from patients with NF1 (Basu et al., 1992; DeClue et al., 1992), it was unclear whether this characteristic would also apply to normal Schwann cells. For example, we have not observed alterations of Ras-GTP in fibroblast cells isolated from the mice lacking neurofibromin expression (J.E.D., unpublished data). In addition, a function that is independent of its Ras-GAP activity has been reported in neuroblastoma and melannoma cell lines, in which neurofibromin does not appear to have GAP-like activity (Johnson et al., 1994; Johnson et al., 1993) . However, a GAP-dependent function has been demonstrated for neurofibromin during muscle differentiation (Gutmann et al., 1994). Taken together, these results indicate that neurofibromin is especially important as a regulator of Ras-GTP in some cells (e.g., Schwann cells) but not in others.

The Ras-GAP activity of neurofibromin has been proposed as

the basis for its tumor supressor function (Xu et al., 1990). In this model, activation of the Ras signaling pathway would lead directly to Schwann cell hyperplasia in NF1 patients. However, this view is incompatible with our results, as decreased levels of neurofibromin and elevated levels of Ras-GTP resulted in inhibition of Schwann cell growth. Schwann cell growth arrest by Ras has been reported previously by Ridley et. al. (1988); under their growth conditions, v-rasH expressing Schwann cells were arrested in either the G1 or G1/M phase of the cell cycle. In those studies, Schwann cells re-entered cell division and became transformed by co-expression of another nuclear oncogene, such as SV40 large T antigen or the c-myc oncogene together with v-rasH. A similar result was obtained in the present study with the Schwannoma cell line, RN22. RN22 is a derivative of a chemically-induced malignant Schwann cell tumor line, but has no alteration in neurofibromin or in the regulation of Ras-GTP (DeClue et. al., 1992). Introduction of v-rasH into this cell line caused cell transformation. Taken together these studies suggest that activation of ras, as occurs with neurofibromin deficient Schwann cells, is insufficient by itself to cause Schwann cell hyperplasia. However, gain of function mutations in other oncogene(s), loss of function mutations in tumor suppressor genes other than NF1, or possibly epigenetic alterations with similar properties can alter the response of Schwann cells to activated ras from growth inhibition to transformation. Thus, abnormal growth of Schwann cells in malignant and possibly also in benign Schwann cell tumors in NF1 patients may require additional mutations that reverse the growth inhibitory effect of ras.

An alternative possibility is that Schwann cell hyperplasia in NF1 tumors may result from an altered susceptibility of neurofibromin deficient Schwann cells to growth factors unique to the in vivo tumor environment. It is possible that the in vitro models we have utilized do not adequately reflect the proliferation of neurofibromin deficient Schwann cells in the

whole animal. For example, while mouse Schwann cells mutant at the PMP22 locus (the Trembler mutation) are hyperproliferative in situ, they remain quiescent in vitro unless stimulated by mitogens (Do Thi et al., 1993) . However, neurofibroma-derived Schwann cells from NF1 patients do not display growth factor independence (S. Sharma and N. Ratner, unpublished observations) nor do they form tumors in nude mice, in spite of being provided with an in vivo environment (Sheela et. al., 1990). Furthermore, neurofibroma-derived cells also interact normally with rat sensory neurons (Baron, 1991) , as described here for neurofibromin deficient rodent Schwann cells. Therefore, it remains a possibility that specific cues present in the tumor environment stimulate Schwann cell division, while in vitro decreased mitogenic response is observed in cells with reduced neurofibromin expression. For example, putative abnormalities in other cell types in the peripheral nerve might make growth factors accessible to NF1 Schwann cells, promoting their growth and possibly making them susceptible to mutations at the NF1 locus or at other loci. Neurofibromas contain mitogens including hepatocyte growth factor (Krasnoselsky, 1994) , basic fibroblast growth factor (Ratner, 1990) and insulin-like growth factor-2.

The current studies have demonstrated that rhGGF2 stimulation of Schwann cells results in increased Ras-GTP levels. The contribution of neuregulin receptor tyrosine kinase(s) HER2, 3 or 4 to increases in Ras-GTP are uncertain at this time, although this result is consistent with neuregulin receptors transmitting signals utilizing the Ras signaling pathway (Wen et al., 1992) . Activation of HER2 (neu in rat) has been shown to cause increase in tyrosine kinase activity and protein phosphorylation (Lonardo et al., 1990; Pandiella et al., 1989; Peles et al., 1991; Yarden, 1990) . Activating mutations in neu are correlated with formation of Schwann cell tumors in ethyl nitrosourea (ENU)-treated prenatal hamsters and rats (Schubert et al., 1974) . Paradoxically, although rhGGF2 stimulates growth of Schwann cells and increases

Ras-GTP levels, constitutive overexpression of v-ras inhibits Schwann cell growth in the presence or absence of GGF. It is likely that small increases of Ras-GTP play a role in growth stimulation, while prolonged abnormally high Ras-GTP results in growth inhibition. Activation of non-Ras signals, such as PKC-gamma, or other HER ligands, may also contribute to Schwann cell growth (Peles and Yarden, 1993) .

Modulation of intracellular cAMP levels is critical in the regulation of Schwann cell proliferation and differentiation (LeBlanc et al., 1992; Pleasure et al., 1985; Raff et al., 1978; Sobue and Pleasure, 1984; Yamamoto et al., 1993) . Interaction between the cAMP-dependent pathway and the Ras signalling pathway has been reported in other mammalian systems, where PKA activation inhibits the Ras pathway at the level of Raf activation (Cook and McCormick, 1993; Graves et al., 1993; Marx, 1993; Wu et al., 1993) . Thus it is possible that in v-ras-expressing or neurofibromin deficient Schwann cells, inhibition of cell division might be overcome by PKA-dependent inhibition of the Ras pathway. This hypothesis could explain our observation that addition of forskolin to v-ras-expressing Schwann cell potentiates proliferation in response to rhGGF2, although we can not rule out the possibility that the cAMP and Ras signaling pathways are not connected in Schwann cells.

Ras activation and reduced neurofibromin expression in Schwann cells leads to morphological changes in Schwann cells, which become elongated, hyper-refractile, tend to grow across each other and send out long processes. These morphological alterations were previously reported in Schwann cells microinjected with oncogenic Ras proteins (Ridley et al., 1988) . Strikingly, neurofibroma-derived fibroblasts lack organized actin stress-fibers (Peltonen et al., 1984) . In 3T3 fibroblasts, expression of oncogenic ras induces reorganization of cytoskeletal architecture, including depolymerization of the actin stress fiber network, loss of focal adhesions, and disorganization of cell

surface fibronectin; these are characteristics of many transformed cells (Dartsch et al., 1994; Paterson et al., 1990; Stacey and Kung, 1984) . In Schwann cells, activation of Ras induces growth inhibition, yet a similar shape change is observed, suggesting that cytoskeletal reorganization is not a secondary consequence of cellular transformation, but rather is a specific cellular response induced by Ras. In addition to changes in the actin cytoskeleton, the observed morphological changes could be due, at least in part, to alterations of the microtubule network. In this regard, it is interesting to note that neurofibromin has been reported to co-localize and associate with microtubules (Gregory et al., 1993; Bollag et al., 1990).

Cessation of Schwann cell proliferation in the developing nerve is correlated with cell differentiation as assessed by expression of myelin markers (Morgan et al., 1991; Webster and Favilla, 1984) . The growth inhibition seen in Schwann cells expressing oncogenic ras or lacking neurofibromin may be related to increased Schwann cell differentiation. A function for Ras in Schwann cell differentiation has been suggested (Gutmann et al., 1993) ; in the neuronal PC12 cell line increased Ras-GTP levels are sufficient to inhibit cell division and promote differentiation (Bar-Sagi and Feramisco, 1985; Muroya et al., 1992) . The oncogenic ras-expressing Schwann cells and neurofibromin deficient cells described here will facilitate further investigation of the effects of Ras and neurofibromin on Schwann cell differentiation.

MATERIALS AND METHODS

Preparation of rat primary Schwann cells

Primary Schwann cells were purified from sciatic nerves from 1-2 day-old neonatal rat pups as described previously (Brockes et al., 1979) . For plating Schwann cells, culture plates were coated with poly-L-lysine as following; plates were washed 2X with Hank's balanced salt solution (Hank's BSS) then bottom of the plates were covered with poly-L-lysine (0.05 mg/ml) prepared in 0.5 M sodium borate bffer (pH7.4). After 30 min, poly-L-lysine was removed and the plates were washed 3 X with Hank's BSS, 1X in DMEM and 1X in DMEM+10 % FBS media. Just prior to plating, medium was removed from the plates and cell were plated. Purified population of Schwann cells were expanded and maintained in DMEM with 10 % FBS supplmented with rhGGF2 or crude embryonic cow brain extract and 2 uMforskolin.

Glial Growth Factors (GGF)

Both purified and crude forms of recombinant human GGF2 (rhGGF2) were titrated for their activities in Schwann cell proliferation. Here we determined that purified rhGGF2 (2ng/ml) and crude conditioned media diluted 1:100 resulted in maximum Schwann cell proliferation. No difference in cellular responses were observed using purified or crude rhGGF2; both forms are simply referred to as rhGGF2 or GGF.

Schwann cell Infection

Recombinant retroviruses stocks (provided by Dr. Doug Lowy) were used to introduce v-rasH into primary rat Schwann cells. v-rasH virus was generated from the plasmid pBW1423, a retroviral vector that encodes wild-type Val14p21rasH from the Moloney murine sarcoma virus strain mH1 LTR and neo from the simian virus 40 early promoter (Willunsen et al., 1986; Willunsen et al., 1991) . As a control for infection, virus stocks carrying the vector

plasmid (pBW1594) encoding only G418-resistance were used. For infection, quiescent primary Schwann cells were plated on poly-L-lysine coated 35 mm culture dishes in DMEM with 10% FBS at a density of 105 cells/dish. The next day, quiescent cells were stimulated to grow by adding rhGGF2 or embryonic cow brain extract (Nordlund et al., 1992) in combination with 2 uMforskolin (Calbiochem). Following a 20-24 hour incubation, Schwann cells were infected by addition of 1694 or 1423 (8 x 105 ffu/ml) viral stock at 1:50 to the Schwann cell culture dishes. Twenty-four hours later, media was switched to selective medium containing 400 ug/ml G418. After 2 weeks in culture, populations of G418 resistant clones were pooled and maintained in Schwann cell growth medium in the continuous presence of G418.

Growth Curves

Quiescent Schwann cells were plated on poly-L-lysine coated 6 well plates in DMEM with 10% FBS, at a density of 1x104 cells/well. Two days after plating, cells were stimulated to grow by switching media to DMEM with 10% FBS supplemented with rhGGF2 and 2 uMforskolin. Every second day, cell numbers were determined after trypsinization by counting duplicate samples in a hemocytometer.

Schwann cell Proliferation Assay

Quiescent Schwann cells were plated on 96-well culture dishes in DMEM with 10% FBS at a density of 1x104 cell/well. Two days after plating, the medium was changed to serum-free defined N2 medium (Ratner et al., 1986) containing rhGGF2. Following 18 hours of incubation, cells were pulsed with 0.5 mCi/well [^{3}H]thymidine for another 24 hours. Schwann cell DNA synthesis was measured by monitoring the incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material by liquid scintillation counting as described (Nordlund et.al., 1992).

For Schwann cell-dorsal root ganglion (DRG) co-culture, quiescent Schwann cells (25,000 cells/coverslip) were plated onto

rat DRG neurons on collagen coated coverslips in serum-free defined N2 media and were incubated for 16-17 hours. Then, 2uCi/ml of [^{3}H]thymidine was added to each culture and 17 hours later, the cells were fixed and processed for autoradiography as described (Ratner et al., 1985). Schwann cell DNA synthesis was determined by counting the per-cent labeled nuclei of neuron-associated Schwann cells.

PCR conditions

For genotyping the NF1 mice, heads of E12.5 embryos were removed and lysed in lysis buffer (Laird, 1991) overnight at 55oC, and DNA was isolated. One µl of head DNA was added to 25 µl of PCR reaction mixture containing 2.5 µl 10x reaction buffer (Perkin-Elmer Cetus), 200 ng of each oligonucleotide primer (NeoTkp, NF31a, NF31b) (Brannan et al., 1994) , 0.25 units of Taq polymerase (Perkin-Elmer Cetus), and a mixture of dATP, dCTP, dGTP and dTTP each at a final concentration of 0.2 mM. PCR was performed for 40 cycles using the following conditions: 94°C for 1 min., 55°C for 1 min., 72°C for 2 min. About 8 µl of each amplication product was used for electrophoresis on a 1% agarose gel. A 194 bp band indicated a wildtype allele, a 340 bp band the mutant allele as described previously (Brannan et al., 1994).

Dorsal Root Ganglion (DRG) culture

DRG cultures were obtained from E12.5 mouse or E15.5 rat embryos essentially as described for E15 rat embryos (Bunge, 1983a; Kleitman, 1991; Wood, 1976). Briefly, whole embryos from pregnant female animal were taken out, decapitated, and the spinal cord with dorsal root ganglia (DRG) still attached was dissected out. DRG were removed from the spinal cord and enzymatically dissociated in 0.25% trypsin in Hanks' BSS (Gibco) at 37°C for 40 minutes. Cells were centrifuged in DMEM containing 10% FBS at 800 rpm for 5 minutes and the pellet was resuspended in approximately 0.5 ml of DMEM based C medium composed of 10% human placental

serum (provided by the delivery room of a local hospital) and 50 ng/ml nerve growth factor (2.5s NGF, Harlan Bioproducts). Cells were mechanically dissociated using a narrow bore Pasteur pipette. About 5-6 drops of this cell suspension containing neurons, Schwann cell precursors, and fibroblasts were plated as 5 single drops on 35 mm dishes (Falcon) coated with ammoniated rat tail collagen and incubated overnight at 37°C with 7.5% CO2. The following day, viable cells were firmly attached to the substrate and dishes were flooded with C medium. In order to obtain a purified neuronal cell population cultures were treated with the antimitotics 5-fluoro-2'-deoxyuridine and uridine (Sigma), which were added to the C medium to a final concentration of 10 uM final concentration (CF medium) (Bunge, 1983b). Cultures were cycled through two passages of C/CF; each medium being replaced every 3 days.

Isolation of mouse Schwann cells from embryonic DRG cultures
Dorsal root ganglia (approroximately 50 ganglia/embryo) were removed from E12.5 mouse spinal cords and cells dissociated in 0.25% trypsin as described above. A single cell suspension was generated by trituration through a narrowed-bore pipettes, and one-third of the cells from each embryo were dispersed in a single well of a 6-well plastic culture plate in 3 ml DMEM+10% human placental serum+50 ng/ml nerve growth factor (C medium). Two to three days later, medium was replaced with serum-free defined N2 medium. At the end of the total culture period (8-10 days), we observed that neurons with long processes were covered by Schwann cells and some fibroblasts were attached to the plastic dish. At this point, the cells were incubated in 1 ml of Hank's BSS containing 0.025 % collagenase for 4-5 min at 37°C with gentle shaking. This light collagenase treatment allowed the Schwann cell-neuron network to detach from the plate leaving most of the fibroblast layer behind. Schwann cell-neuron layers were gently transferred to 15 ml tubes and centrifuged for 5 min at 800 rpm.

Schwann cells and neurons were dissociated from each other by incubation in 1ml of Hank's BSS containing 0.05% collagenase+ 0.05% trypsin for 30 min at 37 oC. Remaining fibroblasts were removed using complement-mediated lysis with monoclonal antibody (1:1000) against mouse/rat fibroblast surface antigen Thy 1.2 (Biosource). Cells were plated on poly-L-lysine coated 35mm culture dish in DMEM+10% FBS, and after 3-4 hours, the medium was switched to N2 supplemented with rhGGF2. After reaching near confluence, cells were harvested and treated with another cycle of anti Thy1.2-complement step before plating for experiments.

Immunostaining of Schwann cells

Schwann cell cultures on collagen-coated coverslips were rinsed twice with Leibovitz's L-15 media and fixed in 4% paraformaldehyde for 20 min. Cells subsequently were rinsed three times, 5 min each, with phosphate buffer saline (PBS) containing 10% normal goat serum (NGS). Rat anti mouse low affinity NGF receptor antibody (the gift of M. Rao and D. Anderson) was diluted to 1:10 in PBS+10% NGS (PBS-NGS), added to the coverslips and incubated overnight at 4oC. The primary antibody was removed by washing the coverslips three times with PBS-NGS. Biotinylated secondary antibody (VECTASTAIN 1:200 in PBS-NGS) was added and after 30 min at room temperature, the coverslips were washed three times with PBS-NGS. Prewarmed Avidin and Biotinylated horseradish peroxidase macro molecular Complex (ABC, VECTASTAIN) solution was added as described by the manufacturer, and after 30 min incubation at room temperature, coverslips were washed three times with PBS. DAB-H2O2 substrate (1 mg/ml DAB+1:1500 H2O2 in PBS) was added for 10 min or until the color reaction develops. The reaction was stopped by washing the coverslips twice with PBS and after the final wash in water, the coverslips were air dried and DPX mounted on glass slides, and processed for autoradiography.

In vivo guanine nucleotide binding analysis

In vivo guanine nucleotide binding was assayed essentially as described (DeClue et al., 1991). 1-2 x 106 rat or mouse Schwann cells were plated on poly-L-lysine coated T-25 flasks, and cultured in the absence of rhGGF2 for 2-3 days, then where indicated, grown with (2 ng/ml) rhGGF2 during the labeling period (10 hr). Thin-layer chromatographs were analyzed on an AMBIS scanner, and the levels of GTP bound were calculated after subtracting background levels of radioactivity and correcting for the different phosphorus content of GDP and GTP.

FIGURE LEGENDS

Figure 1. Growth of uninfected and v-ras expressing Schwann cells (Control; 1423) in culture. On day 0, quiescent Schwann cells were plated on poly-L-Lysine coated 6-well culture plates in DMEM with 10% FBS, at a density of 1x104 cells/well. 48 hr after plating, cells were stimulated by the addition of rhGGF2 and 2 uM forskolin. Cell numbers were determined by counting of duplicate samples.

Figure 2. DNA synthesis by control (1694) or v-ras expressing (1423) Schwann cells in serum-free defined media supplemented with either forskolin (2 uM), rhGGF2 (2 nM) or both. Quiescent Schwann cells were plated on 96-well culture dishes in DMEM with 10% FBS at a density of 1x104 cell/well. 48 hr after plating, the medium was changed to serum-free defined medium containing rhGGF2 or forskolin. Cells were incubated for 18 hr, then pulsed with 0.5 uCi/well [^{3}H]thymidine for 24 hr. Schwann cell DNA synthesis was measured as incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material by liquid scintillation counting.

Figure 3. Proliferation of Schwann cells from NF1 mutant mice in response to rhGGF2. Mouse Schwann cells prepared from wild type (+/+), heterozygous (+/-) or homozygous (-/-) mutant animals were plated on collagen-coated coverslips in 10% serum in the presence of rhGGF2. Cells were pulsed for 6 hr with [^{3}H]thymidine, fixed, and incubated with rat anti-mouse NGFR antibody, and the percentage of Schwann cells (NGFR+) that incorporated radiolabel after rhGGF2 stimulation were determined following autoradiography.

Figure 4. Expression of v-ras result in morphological changes in Schwann cells. A) normal rat Schwann cells; B) v-ras (1423) infected Schwann cells.

Figure 5. Decrease in neurofibromin expression result in morphological cahnges in mouse Schwann cells. A) and C) wild-type mouse Schwann cells; B) and D) heterozygous NF1 mutant (+/-) mouse Schwann cells; C) and E) homozygous NF1 mutant (-/-) mouse Schwann cells. Cells were grown on poly-L-Lysine coated tissue culture dishes at either low (A, B, and C) or high (D, F, E) desities in DMEM with 10% FBS, supplemented with 2 nM rhGGF2 and 2 uM forskolin. Arrows in each panel designate cell processes originating from a single cell body.

Figure 6. DNA synthesis of v-ras expressing Schwann cells in neuron-Schwann cell co-culture. Quiescent Schwann cells (2.5x104 cells/coverslip) were plated onto rat dorsal root ganglia neurons on coverslips in serum-free defined media. 16-18 hr post-plating, 2 uCi/ml of [^{3}H]thymidine was added to each culture. Following 17 hours of incubation, cells were fixed and processed for autoradiography. Schwann cell DNA synthesis was determined by counting the per-cent of radiolabeled nuclei of neuron-associated Schwann cells.

Figure 7. Cellular morphology (A) and anchorage-independent growth (B) of the RN-22 line, before (left panels) or after (right panels) infection with the 1423 (v-ras) virus and G418 selection. (A) Cells were plated at 5x105/60mm dish two days prior to microscopy. Original magnification was 85 X. (B) Cells (7x104/ml) were seeded in 0.4% agar in a 60 mm dish and grown for 15 days prior to microscopy.

Table 1. In vivo GTP Binding of $p21^{ras}$ in Rat Embryo Schwann Cells and in Control Lines[a,b]

Line	GGF	% GTP bound	n=[c]
Uninfected	-	5.2 (+/- 0.5)	2
Uninfected	+	9.5 (+/- 4.1)	2
v-*ras*(1423)-infected	+	25.5 (+/- 5.0)	3
NIH 3T3 Lines			
c-*ras*-overexpressing		1.1 (+/- 0.3)	2
v-*ras*-transformed		61.2 (+/- 1.5)	2

[a]Cell labelling, lysis, immunopecipitation and thin-layer chromatography were carried out as described in Experimental Procedures. Plates were scanned with *AMBIS* before the numbers above were calculated.
[b]The v-*ras* expressing Schwann cells were derived by infection with amphotropic helper virus; NIH 3T3 lines were derived by transfection.
[c]Cells were isolated and infected in two independent experiments, and the values obtained were averaged.

Table 2. In vivo GTP Binding of $p21^{ras}$ in Schwann Cells From Wild-type and Neurofibromin-Deficient Mouse Embryos[a]

Experiment[b]	*NF1* Genotype[c]	rhGGF2	% GTP bound
1	+/+	-	8.9 (+/- 0.4)
	+/+	+	12.1 (+/- 0.2)
	+/-	-	13.8 (+/- 0.2)
	+/-	+	17.1 (+/- 1.2)
	-/-	-	16.5 (+/- 0.5)
	-/-	+	24.3 (+/- 2.3)
2	+/+	-	8.9
	+/+	+	11.5
	+/-	-	14.2
	+/-	+	17.8
3	+/+	+	8.4 (+/- 3.4)
	+/-	+	9.3 (+/- 0.9)
	-/-	+	27.4

[a]Values were determined as for Table 1.
[b]Different preparations of embryo Schwann cell cultures were analyzed in each experiment. Values shown for Experiment 1 and control lines are the averages of two samples; for Experiment 3 the (+/+) and (+/-) are averages of three. Others are single determinations.
[c]Genotype of each embryo was verified by PCR.

Table 3. Wild -type and Neurofibromin-Deficient Mouse Schwann Cell Proliferation in Response to Normal Rat Neurons[a]

Genotypes (Schwann cells)	Schwann cell labeling index (n)	% of control proliferation
+/+	57.3 ± 1.4	100
+/-	45.4 ± 0.8	79.2
-/-	36.5 ± 4.6	63.4 (p=0.003)

[a]Wild type (+/+), heterozygous (+/-) or homozygous (-/-) mouse Schwann cells (20,000 cells/ coverslip) were seeded onto normal rat neurons; and pulsed with ^{3}H-thymidine to label proliferating Schwann cells. At the end of incubation period, cells were fixed and processed for autoradiography. Schwann cell DNA synthesis was determined by counting the % labeled nuclei of neuron-associated Schwann cells.

Table 4. Normal Rat Schwann Cell Proliferation in Response to Wild-type and Neurofibromin-Deficient Mouse Neurons[a]

Genotypes (Neurons)	Schwann cell labeling index (n)	% of control proliferation
+/+	46.7 ± 5.5 (3)	100
+/-	49.6 ± 0.5 (3)	106
-/-	51.9 ± 5.7 (3)	111

[a]Primary rat Schwann cells (20,000 cells/coverslip) were seeded onto either wild type (+/+), heterozygous (+/-) or homozygous (-/-) mouse Schwann cell and the DNA synthesis was determined as described in Table 3.

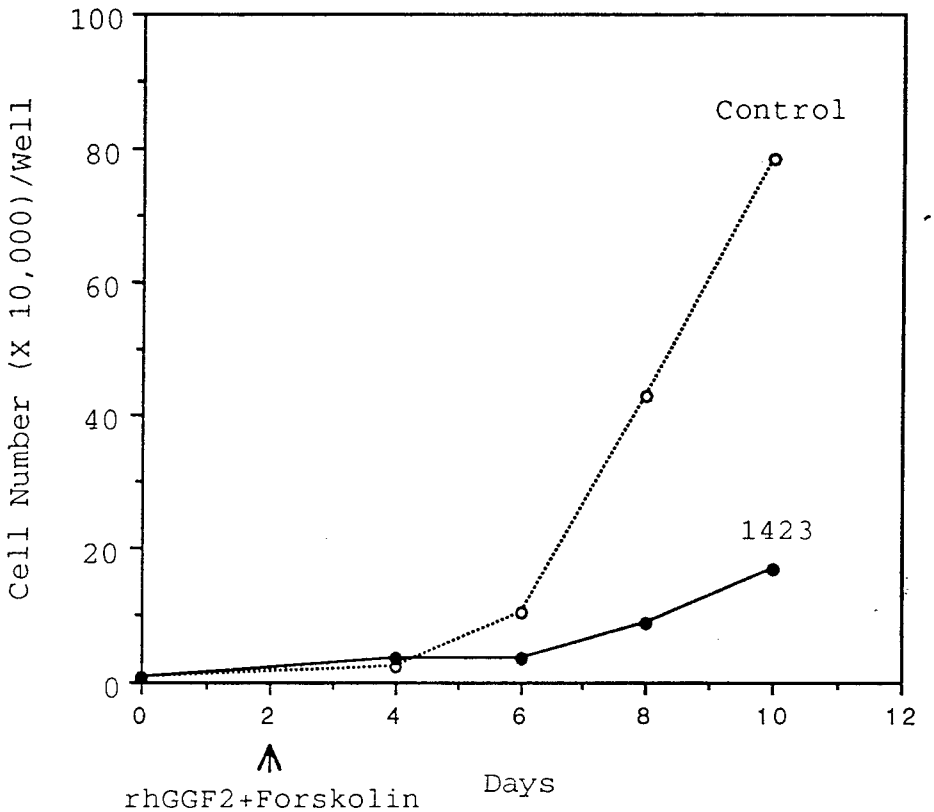

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Figure 1
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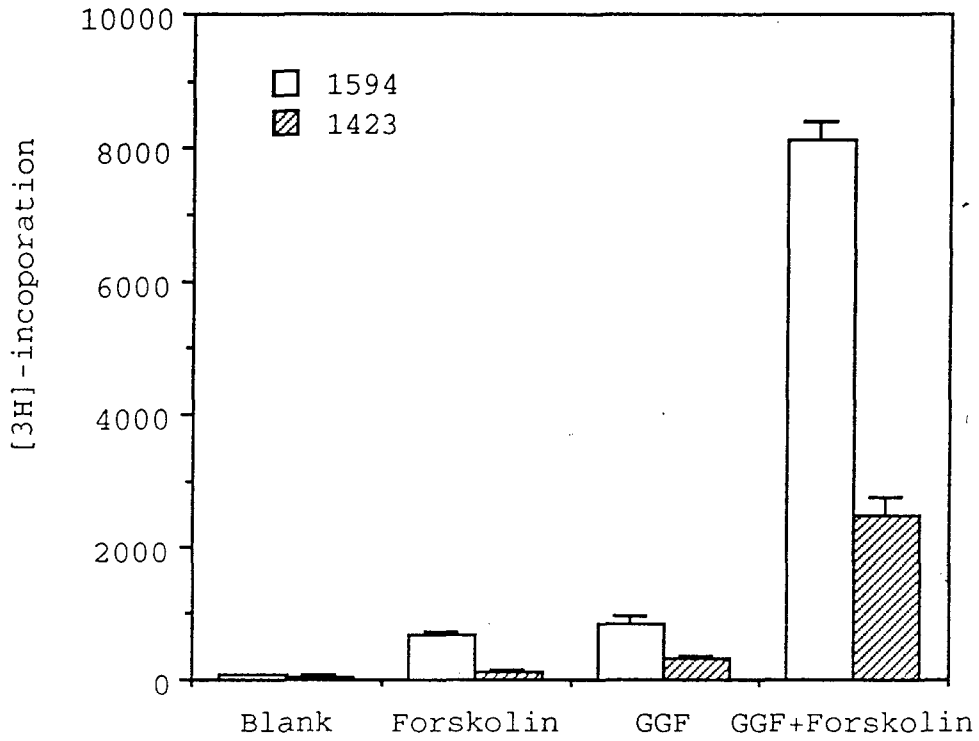

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Figure 2
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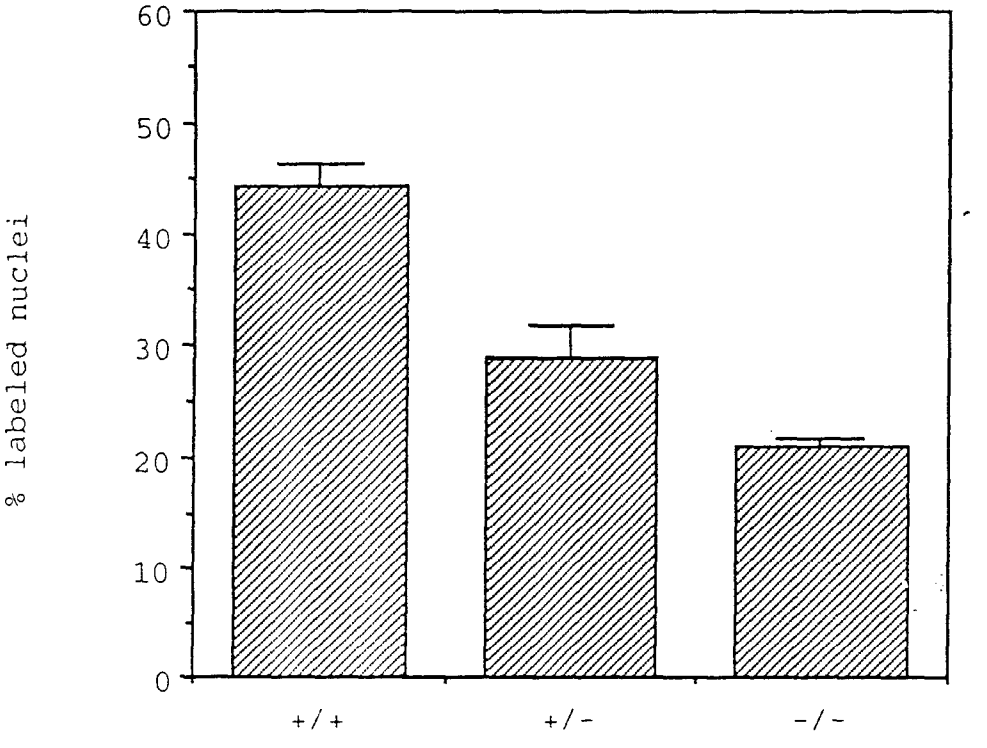

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Figure 3
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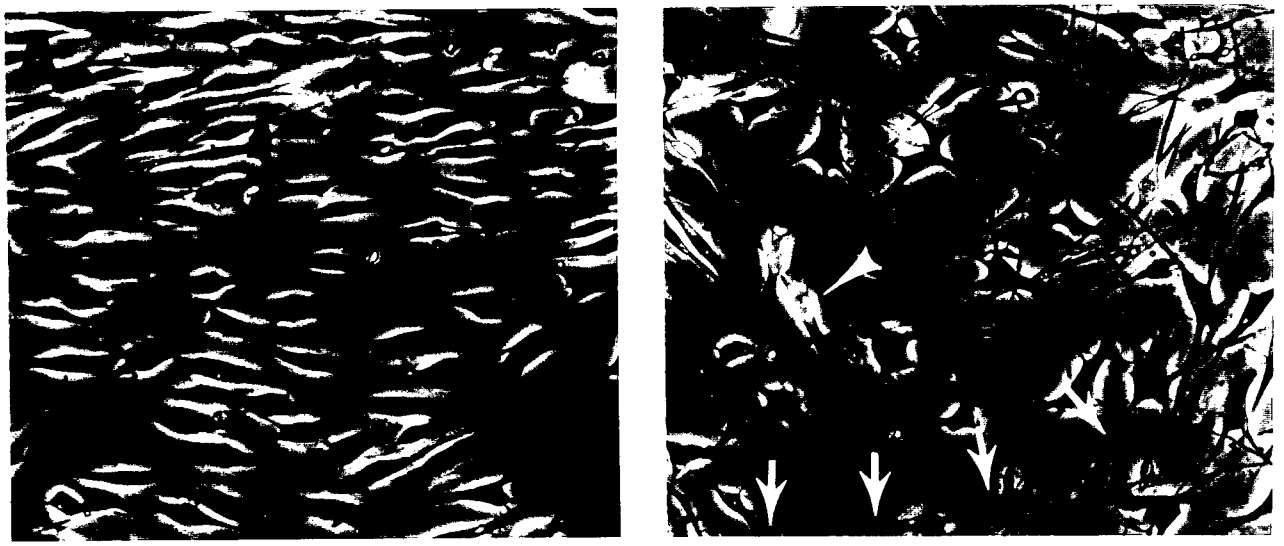

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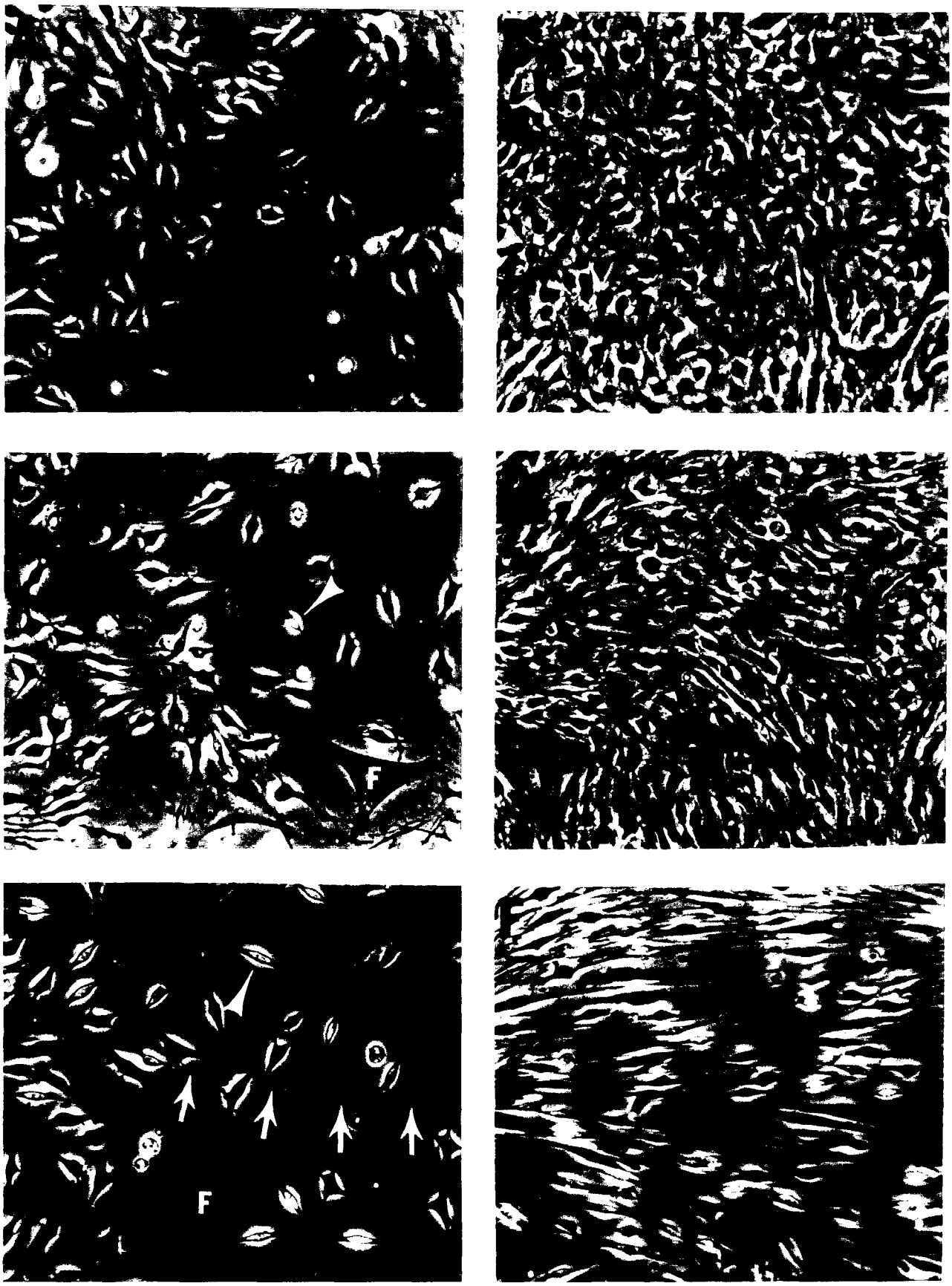

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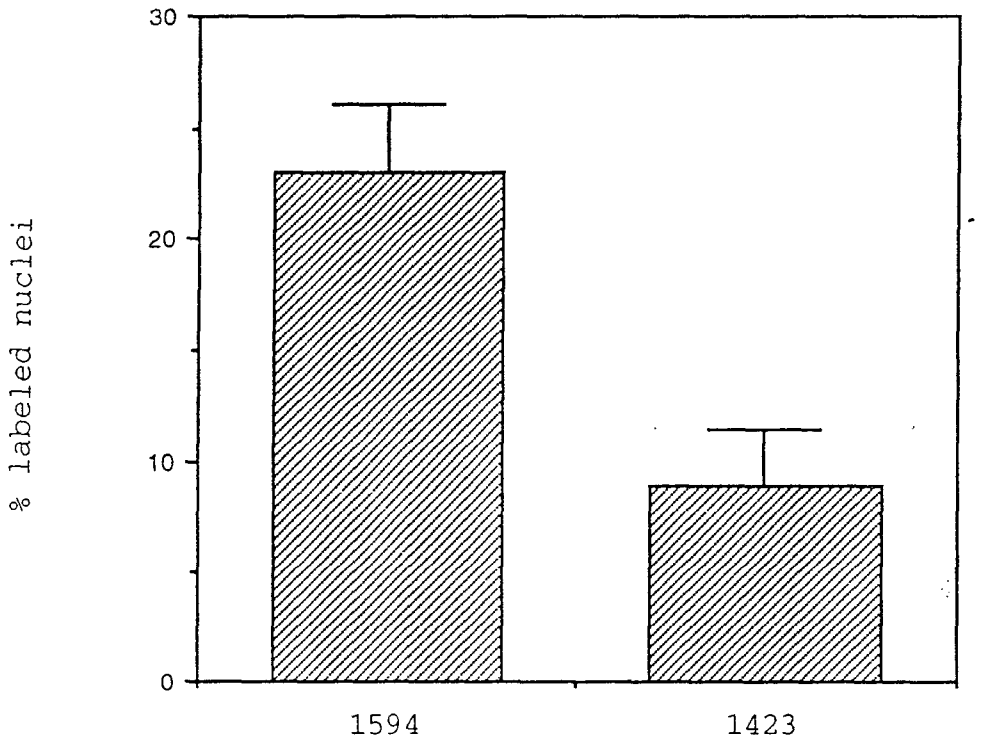
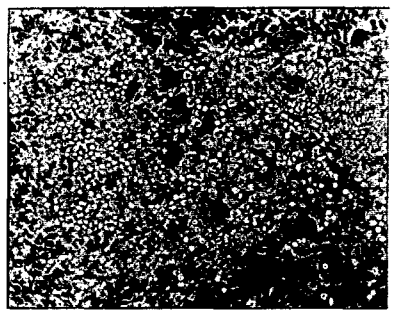

% labeled nuclei
30
20
10
0
1594
1423

A) Morphology

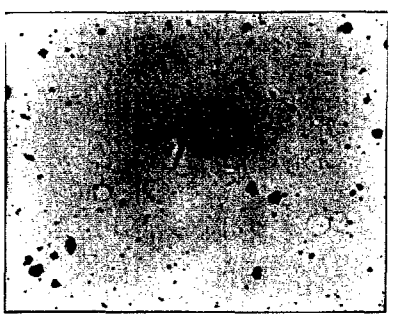
RN-22

v-*ras* RN-22

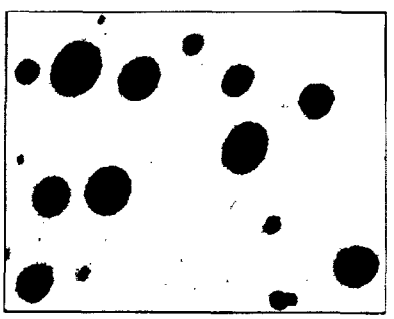


B) Agar Colony Formation

RN-22



v-*ras* RN-22



CHAPTER 4. NF1-DEFICIENT MOUSE SCHWANN CELLS ARE ANGIOGENIC, INVASIVE AND CAN BE INDUCED TO HYPERPROLIFERATE: REVERSION OF SOME PHENOTYPES BY AN INHIBITOR OF FARNESYL PROTEIN TRANSFERASE

ABSTRACT

We developed a model of Schwann cell tumor formation in type 1 neurofibromatosis (NF1). As do Schwann cells from human neurofibromas, mouse Schwann cells heterozygous or null at NF1 show angiogenic and invasive properties. Mutations at NF1 are insufficient to promote Schwann cell hyperplasia which we show can be induced by PKA activation or removal of serum from the culture medium: after serum removal, clones of hyperproliferating Schwann cells develop growth-factor independent proliferation, lose contact with axons in vitro and decrease expression of the cell-differentiation marker P0; hyperproliferating cells develop after a 1-week lag in Schwann cells heterozygous at NF1. An anti-Ras farnesyl protein transferase inhibitor inhibits clone formation and hyperproliferation of null mutant cells, but not invasion, and could be useful in treating benign manifestations of NF1.

INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the most common inherited human autosomal dominant diseases, with a world wide incidence of one in 3,500 individuals (Riccardi, 1991). Benign manifestations frequently associated with NF1 include pigmented lesions of the skin (cafe au lait spots), hamartomas of the iris (Lisch nodules), learning disabilities, formation of optic pathway gliomas and neurofibromas (Huson, 1994). Neurofibromas are one of the major defining features of NF1. Cutaneous neurofibromas, benign peripheral nerve sheath tumors, are associated with small nerve branches, while plexiform neurofibromas develop along major peripheral nerves and progress into malignant neurofibrosarcomas in about 4% of NF1 patients (Riccardi, 1992). Genetic studies show that malignant tumors in NF1, including neurofibrosarcomas, contain cells with mutations in both the constitutionally inactivated and previously normal somatic allele consistent with NF1 acting as a tumor suppressor (Glover et al., 1991; Legius et al., 1993; Skuse et al., 1989). The mechanism of neurofibroma formation is less clear. While a recent study demonstrated loss of heterozygosity (LOH) at the NF1 locus in some neurofibromas (Colman et al., 1995) , 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 (40-85%) of cells in neurofibromas are Schwann cells (Ducatman et al., 1984; Peltonen et al., 1988) and, in contrast to Schwann cells in normal nerve, neurofibroma Schwann cells are found without association with axons (Kamata, 1978; Poirier et al., 1968). Furthermore, Schwann cells, but not fibroblasts, from neurofibromas show angiogenic and invasive properties (Sheela et al., 1990). 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 IRAs and mammalian Ras-GTPase activating proteins (GAPs) (Ballester et al., 1990; Xu et al., 1990a; Xu et al., 1990b) that function as negative regulators of Ras by accelerating the conversion of Ras-GTP to Ras-GDP. The GAP related domain of neurofibromin complements ira mutations in yeast and functions as a negative regulator of Ras in vitro (Ballester et al.,, 1990; Golubic et al., 1992; Xu et al.,, 1990a). Cell lines derived from malignant neurofibrosarcomas have little neurofibromin and high levels of Ras activation (Basu et al., 1992; DeClue et al., 1992). Lowering Ras-GTP in these cells inhibits cell growth. Expression of an oncogenic Ras in cultured rat Schwann cells, however, is correlated with decreased cell proliferation (Kim et al., 1995; Ridley et al., 1988) and transformation of Ras-expressing Schwann cells requires introduction of a second oncogene (Kim et al., 1995; Ridley et al., 1988). It is not known whether phenotypes of neurofibroma Schwann cells might result from loss of NF1 alone or require additional gain of function mutation in other oncogene(s), loss of function mutation in tumor suppressor genes other than NF1, or epigenetic alterations.

To address these issues, we have taken advantage of mice with targeted mutations in the NF1 gene (Brannan et al., 1994; Jacks et al., 1994). 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 methods to isolate Schwann cells from E12.5 NF1 null mouse embryos prior to death, and showed that NF1 null Schwann cells do not hyperproliferate or lose contact with axons in vitro (Kim et al.,, 1995; Rosenbaum et al., 1995). Heterozygous and null Schwann cells derived from NF1-deficient mice do show increased ratios of Ras-GTP to Ras-GDP (Kim et al.,, 1995) but also exhibit decreased proliferation in response to axonal signals and increased expression of protein zero (Po), a marker of Schwann cell differentiation (Rosenbaum et al., 1996). 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. 1) NF1 null mutant Schwann cells undergo hyperplasia when exposed to forskolin, an agent that increases cAMP in cells. 2) Hyperplasia and dissociation from axons is 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 post-translationally for membrane targeting and activation by farnesyl-protein transferase (FPTase). FPTase inhibitors (FPTIs) reverse Ras transformed phenotypes in cultured cells (Prendergast et al., 1994) and growth of malignant tumors in vivo (Kohl et al., 1995; Nagasu et al., 1995) . FPTIs inhibited growth of malignant Schwannoma cell lines derived

from an NF1 patient (Yan et al., 1995). We now show, using the FPTI, L-739,749 (Prendergast et al.,, 1994) , 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.

RESULTS

NF1 mutant mouse Schwann cells are both angiogenic and invasive in vitro

Schwann cells derived from neurofibromas of NF1 patients are abnormal as, unlike normal Schwann cells or neurofibroma derived fibroblasts, they promote angiogenesis and invade basement membranes in the chick chorioallantoic membrane (CAM) model system (Sheela et al.,, 1990). 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 (Kim et al.,, 1995). After growth in culture in serum containing medium supplemented with recombinant human glial growth factor 2 (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 2-3 days after seeding the cells on the CAM, while wild type (+/+) Schwann cells did not promote angiogenic responses (Table 1). 57% (4 out of 7) eggs tested with +/- Schwann cells were positive for angiogenesis as were 66% (6 out of 9) 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 invasion rate (Ir) 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 (Figure 1A), while +/+ mouse Schwann

cells showed Ir of 1-2%, -/- Schwann cells showed a 7-fold increase in their invasiveness compared to +/+ cells. +/- cells had an intermediate Ir of 5-7%. By 5 days (Figure 1B), 10% of wild type cells invaded the Matrigel while 23% of +/- cells and 35% -/- cells had crossed the Matrigel-coated filters. This result, which was consistent in 4 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.

Invasive properties of NF1 deficient mouse Schwann cells are independent of elevated Ras activity in the cells

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 establish whether the invasive properties of Schwann cells lacking neurofibromin is 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 (Kohl et al., 1993) was carried out on neurofibromin deficient (-/-) Schwann cells. Cells were pretreated with 10 uM L-739,749 for 2 days and metabolically labeled with [^{35}S]-methionine during the last 20 hour of incubation in the presence or absence of the drug. The amount of processed Ras was monitored by immunoprecipitation and SDS-PAGE (Figure 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 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 in the continuous presence of the drug. As shown in Figure 1B, pretreatment with the drug failed to inhibit the invasion of +/- or -/- Schwann cells through Matrigel-coated basement membrane. Similar results were obtained 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 is independent of the elevated level of activated Ras in these cells. Another possibility is that Ras activation causes long-term changes in Schwann cells that can not be reversed by FPTI exposure, or that drug treatment is insufficient to reverse the NF1 phenotype. To rule out this last concern, we tested FPTI in another Schwann cell bioassay.

We showed previously that NF1 mutant mouse Schwann cells proliferate less than wild type cells in response to contact with axons or exposure to a soluble growth factor (rhGGF2) (Kim et al.,, 1995). When rhGGF2 treated neurofibromin deficient (-/-) Schwann cells were exposed with L-739,749, there was a dose dependent increase in cell proliferation up to a level comparable to that of wild type cells (Figure 3A). Thus, FPTI can reverse some effects of NF1 loss in Schwann cells. These results indicate that growth inhibition in NF1-deficient Schwann cells is due to the increased Ras activity consistent with the demonstration that Ras-overexpression in normal rat Schwann cells also show decreased proliferation (Kim et al.,, 1995; Ridley et al.,, 1988).

Forskolin induces hyperplasia in mouse Schwann cells deficient for neurofibromin: inhibition of the forskolin effect by farnesyl protein transferase inhibitors.

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 NF1-deficient (-/-) Schwann cell response to two growth factors present in neurofibromas, bFGF and hepatocyte growth factor (HGF). Cells were exposed to growth factor, incubated with [^{3}H]-thymidine and subjected to autoradiography, and the percent 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 Schwann cell response to intracellular cAMP elevation, both wild type and -/- mouse Schwann cells were treated with forskolin, an activator of adenylate cyclase, 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 (Stewart et al., 1991). Normal mouse Schwann cells also did not show any proliferative response to forskolin (Figure 3B). In contrast, proliferation of -/- Schwann cells was increased about 40 fold compared to the untreated control even at the lowest concentration (2 mM) of forskolin tested. Like wild type Schwann cells, +/- Schwann cells did not show any response to forskolin (data not shown). Treatment of cells with dideoxy forskolin, a forskolin analog which lacks the function to activate adenylate cyclase, 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 loss of both alleles is required for this phenotype.

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 mediated proliferation (Figure 3C). Drug treatment had no effect on wild type 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 the surrounding cells (Figure 4 panel B). Unlike typical -/- Schwann cell morphology, distinguished from wild type cells (panel A) by narrowed and bright cell bodies with elongated processes (Kim et al.,, 1995) , these cells were flattened, round and shorter in length. 100% of these cells were immunoreactive with antibodies recognizing S100 and NGF receptor (NGFRp75), 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 (panel D). In confluent cultures, these cells formed foci (panel D inset) whereas wild type cells (panel C) 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 media (N2) for 1 week after the first passage of the cells (Table 2A). While no clones of transformed cells arose in cultures maintained in 10% FBS containing media, clones were observed in -/- Schwann cell culture kept in N2. Transformed cell clones also developed in +/- cultures in N2 after a 1-week lag compared to the time of appearance of clones in -/- cultures.

To test the proliferative properties of morphologically transformed -/- Schwann cells, we pooled clones of these cells from a confluent culture (TXF-/-) 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 (Kim et al.,, 1995) (Figure 5A). In contrast, TXF-/- Schwann cells showed high basal level proliferation under serum-free conditions in the absence of rhGGF2, indicating growth factor independence (Figure 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 appearance of transformed cell clones in serum-free cultures and inhibits the growth of transformed -/- Schwann cells

In order to determine if the transformation of NF1 mutant Schwann cells was related to increased Ras activity, -/- Schwann cells were kept in either N2 or N2 with 10 uM L-739, 749 for 10 or 16 days, and the number of TXF-/- cell clones

that arose in cultures were counted (Table 2B). 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 size of 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 (Figure 4 panel F).

In order 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 uM L-739,749 for 4 days and growth in N2 was compared to that of untreated TXF-/- cells by counting cells every 2 days for 6 days (Figure 5C). Increase in 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 5-6 days after dissociation of Schwann cells from the neurons present in these cultures. However, when we carefully examined Schwann cells in original DRG cultures 4

days after removal from -/- embryos, we detected clones of both S100 and NGFRp75 positive cells with transformed morphology. Strikingly, these cells had lost contact with neurons (Figure 6). This result indicates that during the process of morphological transformation Schwann cells acquire growth factor independence, hyper-proliferate and show one of the characteristic features of human neurofibromas, loss of contact with axons.

Po 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 Po protein, normally a marker for Schwann cells stimulated to differentiate (Rosenbaum et al.,, 1996). In order to determine if the differentiated phenotype is maintained in TXF-/- cells, confluent cultures were grown in N2 supplemented with either rhGGF2 or 5 uM forskolin for 5 days, conditions under which Schwann cells lacking neurofibromin express Po protein. Figure 7 shows Western blots probed for Po protein from protein extracts prepared from -/- or TXF-/- Schwann cells. As shown before, -/- cells express Po in both rhGGF2 and forskolin conditions. In TXF-/- Schwann cells, basal levels of Po was completely lost (GGF lane) and cells did not express Po even in the presence of forskolin, conditions that induce Po expression in wild type cells. This result suggests that the TXF-/- not only increase proliferation, but also have lost their ability to differentiate.

DISCUSSION

The basis of tumor formation in NF1 is not understood. In part, this is 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, angiogenic, lose contact with axons, and 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 (Sheela et al.,, 1990). We have also shown that when loss of NF1 is accompanied by epigenetic changes, such as removal of serum components, or activation of 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. 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 (Auerbach and Auerbach, 1994; Schott and Morrow, 1993). 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 and HGF, are present in neurofibromas

(Krasnoselsky et al., 1994; Ratner et al., 1990). As mutations at the NF1 locus in Schwann cells result in increased activation of Ras, it is of same interest that constitutive activation of Ras can facilitate tumor angiogenesis. Activated Ras upregulates expression of angiogenic factors, including HGF, bFGF and VEGF, in fibroblasts (Coffer et al., 1991; Goldberg et al., 1990; Iberg et al., 1989; Powell and Klagsbrun, 1993). In a tumor cell line, VEGF secretion is suppressed by disruption of mutant Ras activity by farnesyl protein transferase inhibitor (Rak et al., 1995). Thus, it is possible that in NF1 deficient Schwann cells, induction of angiogenesis might be due to secretion of 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 (Krone et al., 1986) and neurofibroma Schwann cells invade chicken CAM basement membranes (Sheela et al., 1990). Invasive potential of tumor cells is often correlated with overexpression and secretion of variety of enzymes that degrade extracellular matrix components of basement membrane (Goldberg et al., 1990; Liotta et al., 1988). These

proteolytic enzymes include members of metalloproteinases (MMPs). In tumors with Ras mutations, oncogenic Ras contribute to invasive behavior of tumor cells by upregulating the production MMPs (Ballin et al., 1988; Ballin et al., 1991; LoSardo et al., 1995; Spinucci et al., 1988) . 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 collagen barrier resembling basement membrane (Muir, 1995). It has been suggested that increased Ras activity in neurofibroma derived Schwann cells might responsible for 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 GAP-related domain (GRD) of NF1 are required for this phenotype, or that Ras activation prior to drug treatment causes long term changes in gene expression that can not be reversed by drug exposure.

Hyperplasia of NF1 null Schwann cells was obtained by exposure of cells to forskolin, an activator of protein kinase A. In contrast, several tyrosine kinase receptor ligands, including glial growth factor (Kim et al., 1995) , HGF, and bFGF reduce 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 first phenotype identified to date that affects only null Schwann cells. 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 reported in other systems, in

which PKA inhibits Ras signaling at the level of Raf activation (Cook and McCormick, 1993; Wu et al., 1993). A neuropeptide, calcitonin gene related peptide (CGRP), has been proposed as a 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 (Cheng et al., 1995). CGRP is expressed by sensory and motor neurons (Arvidsson et al., 1990; Noguchi et al., 1990) and is expressed by axons within neurofibromas (Vaalasti et al., 1990). 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. We favor the alternative idea that one or a few cells sustained 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 mutational event 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 (Kim et al., 1995; Ridley et al., 1988). Loss of the p53 gene is

frequently detected in malignant neurofibrosarcomas from NF1 patients; p53 mutations could explain development of the hyperproliferating cells we have detected (Legius et al., 1994; Menon et al., 1990). 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 (Denko et al., 1994; Ichikawa et al., 1990; Stenman et al., 1987; van den Berg et al., 1991). 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 out of 4 known malignant neurofibrosarcoma Schwann cells grow in soft agar (Yan et al., 1995).

NF1 heterozygous mutant Schwann cells become hyperproliferative in serum-free media 7-10 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 demonstrated LOH in these cells. A high spontaneous mutation rate in the NF1 gene (1/10,000 alleles/generation) has been reported (Crowe et al., 1956). We can not 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 neurofibrosarcomas that arise in the context of benign neurofibromas 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 multi-polar), and decreases proliferation of hyperplastic cells. It is possible that factor(s) present in serum affect Ras activity in NF1 mutant Schwann cells through activation of the serum response factor (SRF). In fibroblasts SRF, 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 (Kim et al., 1994). FPTI also reverses Ras transformation, and causes actin re-organization (Prendergast et al., 1994). 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 defective perineurium formation around axon-Schwann cell units (Rosenbaum et al., 1995). 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 we have defined in Schwann cells lacking NF1 will provide insights into Schwann cell tumor pathogenesis in human NF1.

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 (Kim et al., 1995; Kim and Ratner, 1996). Dorsal root ganglia of the embryos were enzymatically dissociated and cells from single embryos plated onto 2 wells of 6-well culture plates in 10% human placental serum containing DMEM supplemented with NGF. Next day, the medium was switched to N2 medium (Ratner et al., 1986) containing NGF and gentamycin (5 ug/ml). After 5-6 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 enzymatically dissociated from the neurons in 0.01% collagenase. Cells were centrifuged, resuspended in DMEM with 10% FBS and plated on poly-L-lysine coated 100 mm cell culture plates at a density of approximately 1 x 106 cells/plate. These cells were considered passage "0". Next day, cells were switched to Schwann cell growth media containing rhGGF2 (10ng/ml) (Cambridge Neuroscience) and 10% FBS, with 2 uM forskolin was added to suppress fibroblast growth. After one week, cells were trypsinized and replated at the plating density (passage "1"). 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 media supplemented with GGF and forskolin from passage "0" throughout the culture period. For experiments where L-739, 749 (Merck Pharmaceutical) was used, cells were preincubated with 10 uM L-739, 749 for 3-5 days in Schwann cell growth media prior to experiments, unless otherwise indicated.

Chicken Chorioallantoic Membrane Assay for Angiogenesis

Chorioallantoic membranes (CAM) of day 6 postfertilization standard chicken eggs (Spafas, Inc., Roanoke, IL) were dropped and exposed by cutting a window (1 cm2) on one side of the egg using the false air sac technique (Asprunk et al., 1975; Sheela et al., 1990). After exposing the membrane, windows were sealed using Transpore tape (3M) and the egg put into humid incubator at 35-37oC. Three days later, cultured cells were trypsinized, washed three times in DMEM and seeded onto the CAM at a density of 1-1.5 x 106 cells/10 ml /egg. Windows were sealed again and eggs incubated for another 48 hours. Forty-eight hours later, eggs were removed from incubator for observation using 6 X 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

Invasion capacity of cells through Matri-gel was evaluated using a vital dye method (Boghaert et al., 1992). The polycarbonate filter of 8 mm pore size 2-cm2 transwell unit (Costar) was coated with 30 ug Matri-gel (Collaborative Biomedical Products). Frozen Matri-gel was thawed over-night at 4°C and reconstituted in cold PBS to obtain the a concentration of 0.3 ug/ml. To each chamber, 100 ml of diluted Matri-gel was added and air-dried over-night. The next day, the membranes were reconstituted with 200 ml serum-free DMEM and after removal of the excess medium, 200 ml cell suspension in 10% FBS (400,000 cells/ml) were loaded on the membrane (in triplicate). The transwells were then placed in 24-well dishes containing 800 ml culture medium per well. After 3 or 5 days incubation at 37oC, 20 ml and 80 ml of MTT (5 mg/ml) in PBS was added to the transwells and the bottom

wells, respectively. Metabolic conversion of MTT to formazan was allowed for at least 2 hours at 37oC. Cells and formazan crystals were scraped from the bottom of the membrane with precut Wattman 3M filter papers and placed in 150 ml n-butanol. After removing 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 ml n-butanol. Subsequently, the formazan was allowed to dissolve at 4°C for 24 hours and the additional 100 ml n-butanol was added. 150 ml from each tube was transferred to individual wells of 96-multiwell dish and the optical density of the formazan product was measured at OD540. Invasion rate (Ir) was determined by the ratio of the OD from the bottom of the filter to those of 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 (10ng/ml) and grown to near confluence. Cells were then treated with or without 10 uM L739,439 for two days in the absence of growth factor. At the end of the incubation period, cells were switched to 5 ml labeling media containing 200uCi/ml ^{35}S-methionine and 10% dialyzed FBS with or without L-739,749. After 16-18 hours, cell lysates were prepared by adding 1 ml of lysis buffer (20 mM Tris HCl pH 7.5, 100 mM NaCl, 5 mM MgCl2, 2 mM EDTA, 1% NP-40, 0.1% SDS, 16 mg/ml aprotinin, 1 mM DTT, 1 uM leupeptin, 0.7 ug/ml pepstatin, 0.2 mM PMSF, 0.5% Na-deoxycholate and 0.8 mM NEM) to each flask. Cells were scrapped off from the flasks and incubated on ice for 30 min then centrifuged for 10 min at 14,000 rpm. Equal amounts of TCA precipitable counts of each extract was precipitated with 10 ug of Y13-259 anti Ras antibody (Oncogene Science) conjugated to 10 ug rabbit anti rat IgG complexed with protein G agarose. For the peptide

competition control, Y13-259 was preincubated with 100 ug of ras peptide for 1 hour at 4°C prior to use. After 3 hours of incubation at 4oC, 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 and 0.3% SDS), twice in wash buffer II (1 M NaCl, 0.1 M Tris HCl pH 8.0 and 0.1% NP-40) and three times in wash buffer III (10 mM Tris HCl pH 8.0 and 0.1% NP-40). Beads were then boiled for 5 min and samples were run on a 15% SDS-acrylamide gel and gel processed for autoradiography.

Schwann Cell Proliferation Assay

Schwann cells (25,000 cells/well) were plated on poly-L-lysine coated 8 chamber well Lab-Tek glass slides in DMEM plus 10% FBS. Two days after plating, the media were switched to serum free media (N2) supplemented with appropriate growth factors. Cells were incubated in the presence or absence of growth factors for another 48 hours and [^{3}H]-thymidine (1uCi/ml) was added during the last 24 hour incubation period. Cells were fixed in 4% paraformaldehyde and immunostained with a Schwann cell specific antigen, NGF receptor and processed for autoradiography (Kim et al., 1995). 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 assayed using a conventional 96-well plate assay as described previously (Nordlund et al., 1992).

Po Extraction and Western Blot

Schwann cells isolated from wild type or null NF1 mouse embryos were plated (1-1.5 x 106 cells/dish) onto poly-L-lysine-coated 60 mm culture dishes in DMEM with 10% fetal calf serum. After 24 hours, dishes were treated with rhGGF2 or with 5 uM forskolin, both in serum-free media (N2). Cells were cultured for another 4 days; medium was changed each

day. At the end of the culture period, cells were lysed in 50 ml of buffer (2% SDS, 2 mM EGTA, 2 mM EDTA, 5 mM Tris HCl pH 6.8 and 2 mM PMSF), boiled for 5 min and centrifuged for 15 min at 14,000 rpm (Morgan et al., 1991). Amount of proteins in each sample was determined from the resulting supernatant by Lowry assay (Markwell et al., 1978) and an equal amount of protein per sample loaded and separated in 10% SDS polyacrylamide gels. Proteins were then transferred onto nitrocellulose and Po detected by Western blot analysis using a polyclonal antibody (1:500) raised against rat Po protein from D. Colman.

FIGURE LEGENDS

Figure 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 (+/-, -/-) Schwann cells grown in DMEM+10% FBS supplemented with rhGGF and forskolin were preincubated with or without FPTI (10 mM) for 3-5 days. Cells (80,000 cells/chamber) were then plated on matrigel coated invasion chamber membrane (polycarbonate filter, 8 mm) in DMEM+10% FBS without growth factors, and incubated for another 3 or 5 days. Invasion rate (Ir) of cells were determined by ratio of cells from bottom of the filter to those of bottom and top of the filter combined. This is a representative result of 4 separate experiments with each value run in triplicate.

Figure 2. Inhibition of Ras processing in NF1 deficient mouse Schwann cells by L-739,749.

Cells were preincubated with 10 uM L-739,749 and metabolically labeled Ras proteins were immunoprecipitated and Ras processing was analyzed on 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) are shown in lane 2 (no drug treatment). In lane 3, multiple bands of slower mobility than in lane 2 indicate the presence of unprocessed forms of Ras.

Figure 3. L-739,749 reverses the growth response of NF1 deficient Schwann cells to rhGGF2 and forskolin.

(A) L-739,749 on rhGGF2 stimulated growth of wild type (closed circle) or NF1 null mutant (open circle) mouse Schwann cells. (B) Forskolin treatment on NF1 null mouse Schwann cells results in hyperproliferation while it has no effect on wild type Schwann cells (C) Partial inhibition of forskolin induced NF1 deficient mouse Schwann cell growth by L-739,749. Cells were plated on poly-L-lysine coated 8-chamber glass Lab-Tek slides (25,000 cells/chamber) and preincubated with (A and C) or without (B) different doses (0-25 mM) of L-739,749 in DMEM+10% FBS for 2 days. Medium was switched to serum-free N2 containing either rhGGF2 (10ng/ml), different doses of forskolin (0-25 mM) or 10 uM forskolin and after 16-18 hours, cells were labeled with [^{3}H]-thymidine (0.5 uCi/ml) for another 24 hours. Proliferation of Schwann cells was determined by the percentage of NGFR positive cells with labeled nuclei; 800-1000 cells were counted in each condition and each point is an average of duplicate sample. This is an representative results of 3-4 separate experiments.

Figure 4. Growth of NF1 deficient mouse Schwann cells in a continuous serum-free condition results in morphological transformation and focus formation.
Phase contrast micrographs were taken from wild type (A, C and E) and NF1 mutant (B, D and F) Schwann cell cultures grown in serum-free condition. Cells with their morphologies distinct from surrounding cells start to appear in NF1 mutant cell cultures (panel B), where as wild type cells remained unchanged (panel A). As cultures become confluent, morphologically transformed NF1 mutant cells take over the whole growth area and foci are formed (arrow, inset) whereas wild type cells show contact inhibition of growth (panel C). In the presence of 10 uM L-739,749, growth of transformed NF1 mutant cells is inhibited and no focus formation is observed

(panel F). L-739,749 has no effect on wild type cell growth (panel E). Contaminating fibroblasts (F) are also indicated. Bar = 135 mm

Figure 5. DNA synthesis of (A) wild type (+/+) or (B) morphologically transformed NF1 mutant (-/- TXF) Schwann cells in response to rhGGF2 and forskolin; effect of serum factors on mutant Schwann cell growth.
Cells were plated on 96-well plate in DMEM with 10% FBS at a density of 1 X 104/well. 48 hours 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 (1 mM), or combination of both. After 18 hours, [^{3}H]-thymidine (2 uCi/ml) was added and cells were incubated for another 24 hours and DNA synthesis was measured as incorporation of [^{3}H]-thymidine into trichloroacetic acid-insoluble material by liquid scintillation counting. (C) Growth of transformed -/- (TXF-/-) cells (solid line) and L-739,749 treated TXF-/- cells (dotted line) in N2. Cells were pretreated with or without 10 uM 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. 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.

Figure 6. Morphologically transformed NF1 deficient Schwann cells appear early in neuron co-culture 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 condition, morphologically altered Schwann cells (S100+, NGFRp75+) appear within 2-3 days after plating and subsequently form colonies (S) away from axons (arrows).

Morphology of these cells are also distinct from that of fibroblasts (F) (b) Another NF1 null DRG culture showing a Schwann cell colony (S). A mitotically active, dividing Schwann cell independent of axonal contact can be seen (arrow head). Normally, Schwann cells only grow in direct contact with axons (arrows).

Figure 7. Po expression in wild type, NF1 null, and morphologically transformed NF1 null Schwann cells in the presence of rhGGF2 or forskolin.
Schwann cells were incubated for 4 days in either rhGGF2 or 5 uM forskolin in serum-free medium and cell lysate prepared. Equal amounts of each lysate were separated on a 10% SDS gel and Po expression analyzed by Western blot using polyclonal antibodies raised against rat Po. Po expression was absent in wild type (+/+) mouse Schwann cells unless forskolin was added. NF1 null (-/-) Schwann cells expressed Po both in rhGGF2 and in forskolin, while Po expression was lost in morphologically transformed null (-/-TXF) Schwann cells even in the presence of forskolin.

Table 1. Angiogenic response of the chicken CAM to mouse Schwann cells isolated from wild type (+/+), NF1 heterozygous (+/-) or homozygous (-/-) mutant mouse embryos.

Cell types	Genotypes	# of eggs tested	Positive for Angiogenesis
Fibroblasts	+/+	3	0/3
	+/-	5	0/5
	-/-	6	0/6
Schwann cells	+/+	8	0/8
	+/-	7	4/7
	-/-	9	6/9

Dissociated fibroblasts or Schwann cells (1 X 10^6) prepared from wild type (+/+), NF1 heterozygous (+/-) or homozygous (-/-) mutant mice were placed onto the 9 day post-fertilization chick CAM. After 2-3 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.

Table 2A. Colony formation (# of colonies/cm^2) in NF1 deficient mouse Schwann cell cultures in serum-free defined medium; inhibition of colony formation by serum

	7 days[a]		14 days[b]
Genotypes	N2	10% FBS	N2
+/+	0	0	0
+/-	0	0	47.75
-/-	115.7	0	577.5

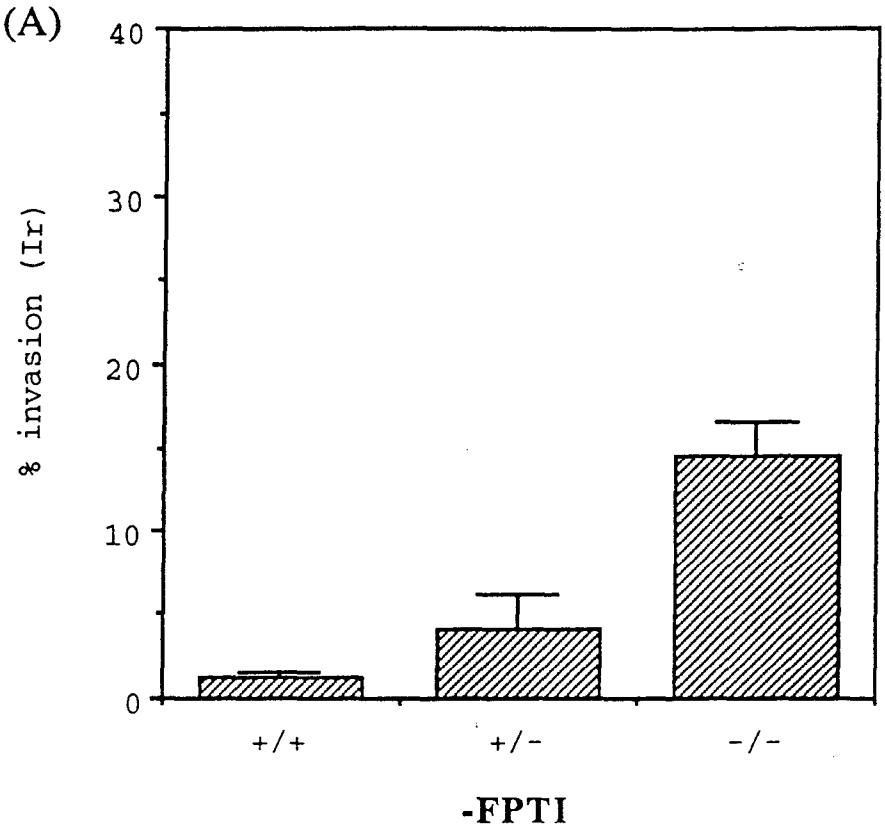
[a] Schwann cells from wild type (+/+), NF1 heterozygous (+/-) or null (-/-) mouse embryos were expanded in DMEM plus 10% FBS supplemented with rhGGF2 (10 ng/ml) and forskolin (2 μM). After 3-4 days, cells were replated on poly-L-lysine coated 60 mm culture plates at a density of 0.5 X 10^6 cells/plate in the presence of growth factors in serum-free N2 or 10% FBS-DMEM. Seven days later, the number of cell colonies (>4 cells) were counted.

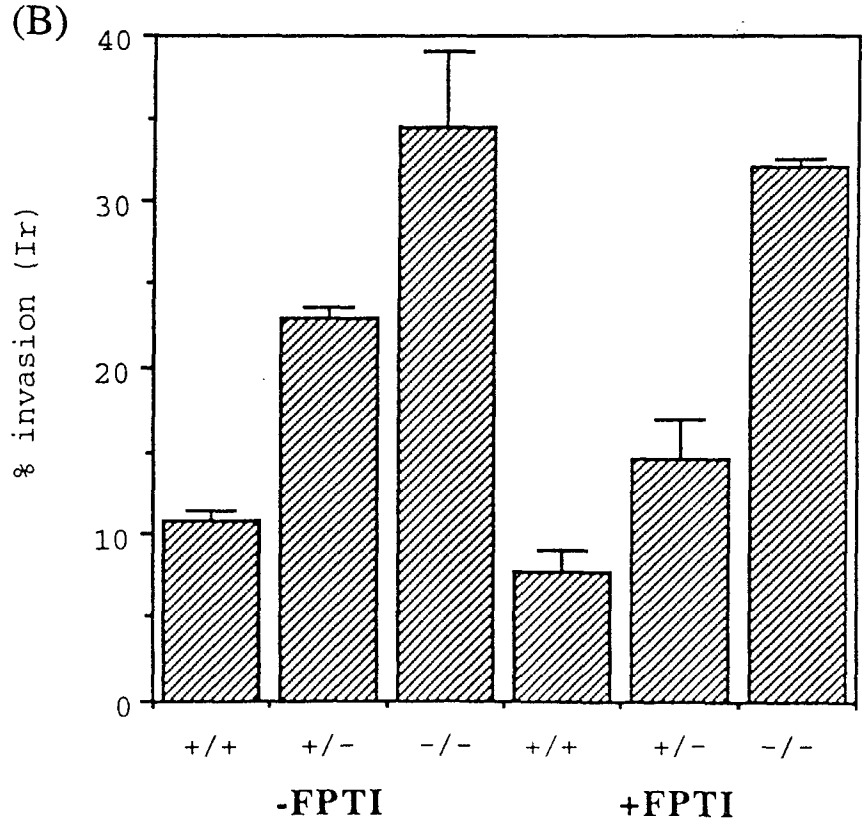
[b] In a separate experiment 7 days after initial plating on 60 mm plates in N2, cultures became confluent and no morphological transformation was observed in +/- cultures. Cells were trypsinized and replated at a density of 0.5 X 10^6 cells/plate. A week later, the number of colonies was counted.

Table 2B. Colony formation (# of colonies/60 mm plate) in NF1 null mutant mouse Schwann cell cultures in serum-free defined medium; inhibition of colony formation by FPTI (10 μM)[a]

	10 days		16 days	
Size of colonies	-FPTI	+FPTI	-FPTI	+FPTI
< 10 cells	10	2	15	0
< 40 cells	8	0	8	0
> 40 cells	14	1	14	0

[a]Schwann cells isolated from NF1 null mutant mouse embryos were expanded in DMEM+10% FBS supplemented with rhGGF2 (10ng/ml) and forskolin (2 μM). At the second passage, cells were plated onto poly-l-lysine coated 60 mm plates (0.5 X 10^6/plate) and medium was switched to serum-free N2 medium containing rhGGF2 and forskolin, with or without FPTI (10 μM). Medium was changed every 5 days, and number of colonies of varying size was counted on the 10th and 16th day.





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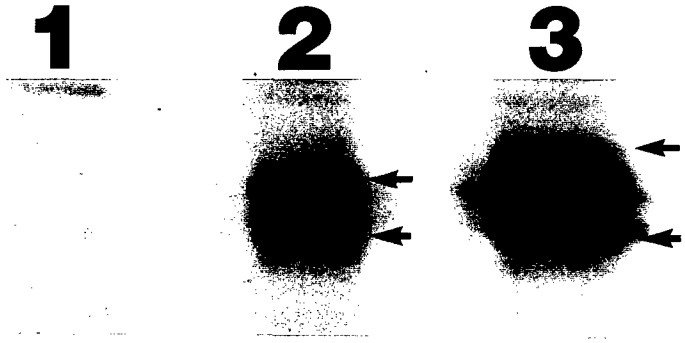

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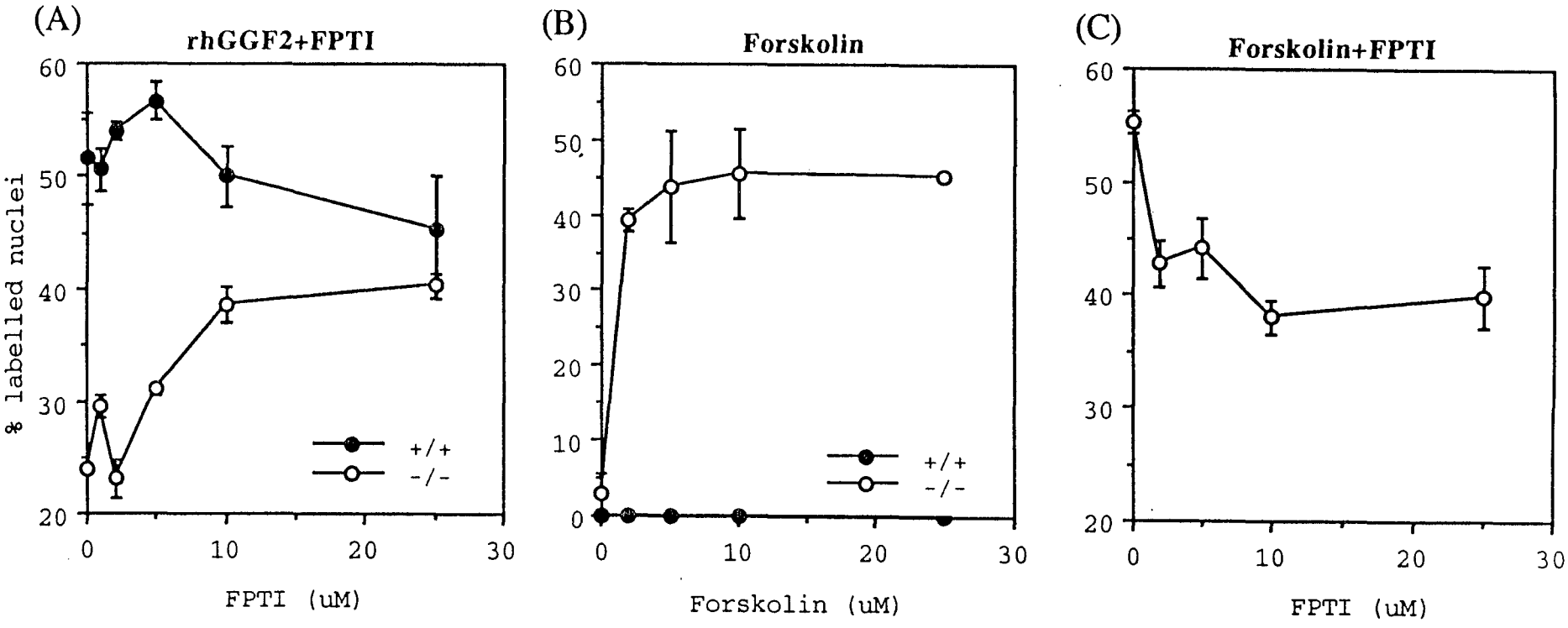
(A)
rhGGF2+FPTI
% labelled nuclei
60
50
40
30
20
0
10
20
30
FPTI (uM)
+/+
-/-
(B)
Forskolin
60
50
40
30
20
10
0
0
10
20
30
Forskolin (uM)
+/+
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(C)
Forskolin+FPTI
60
50
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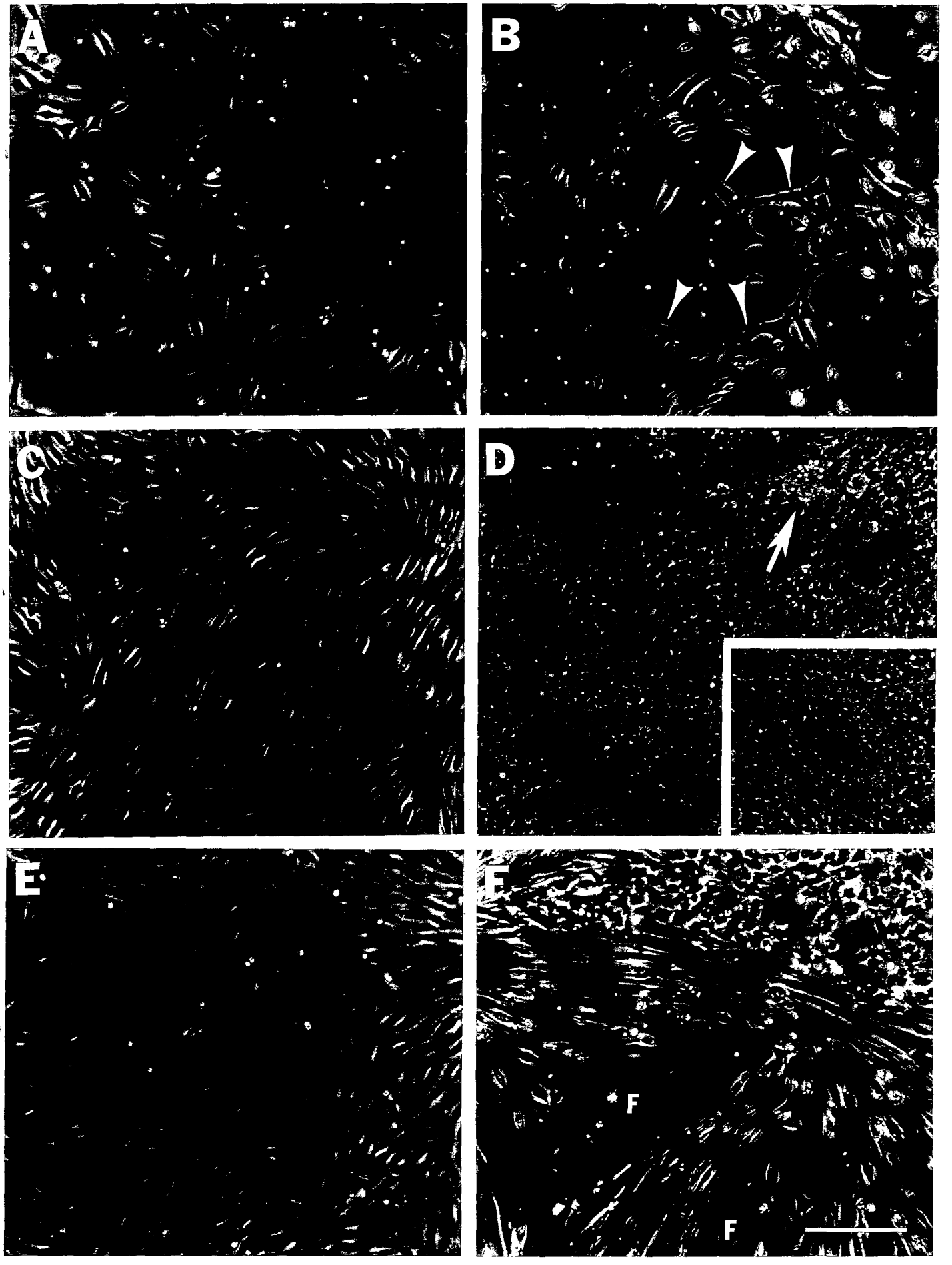

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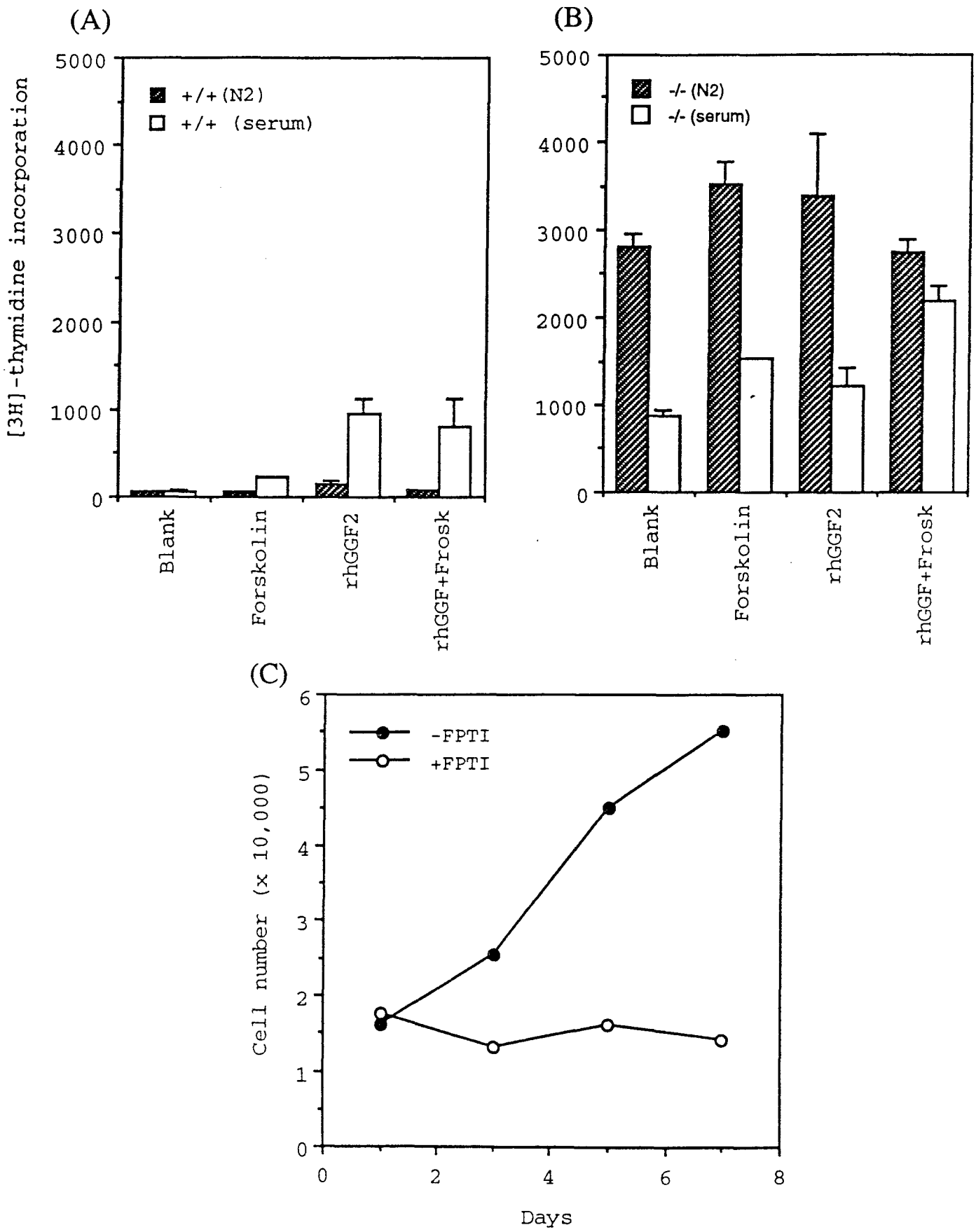

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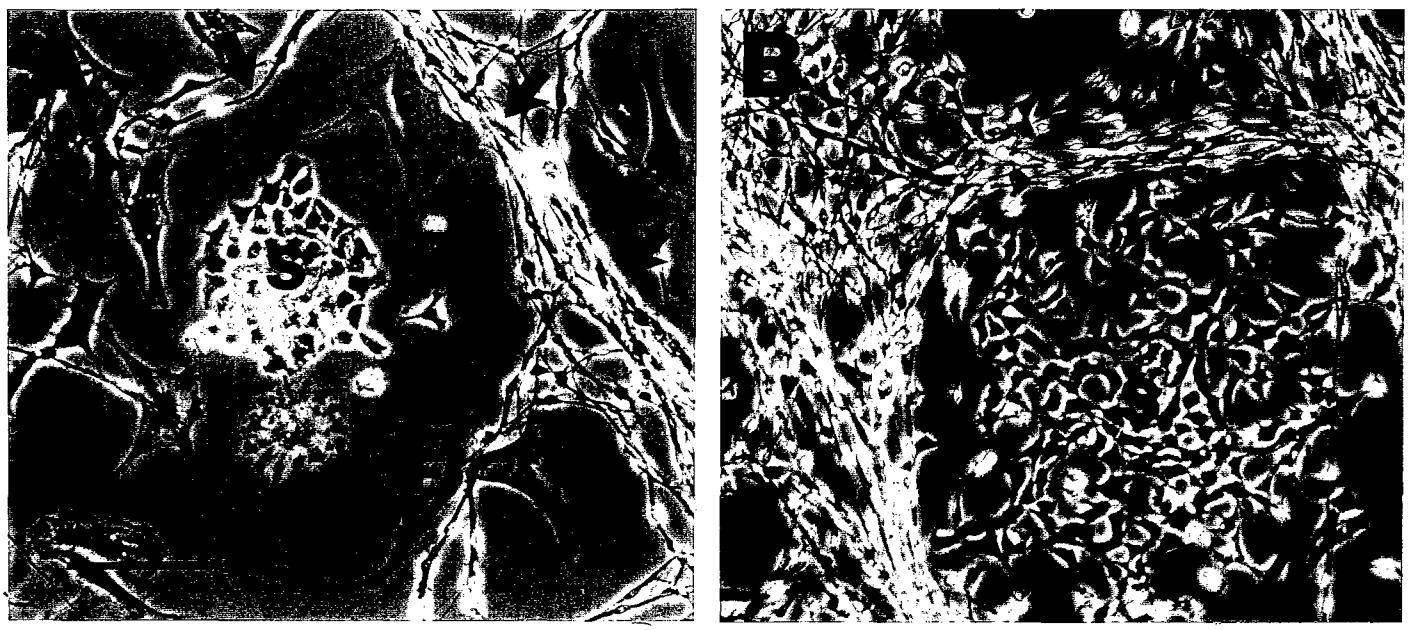

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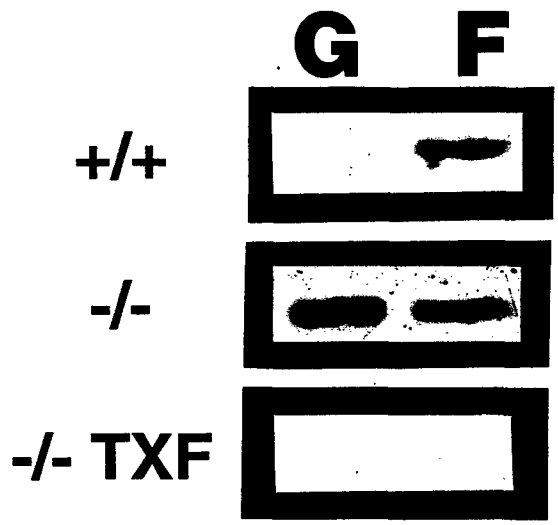
G
F
+/+
-/-
-/- TXF

CHAPTER 5. NEUROFIBROMIN IS REQUIRED FOR APPROPRIATE P_0 EXPRESSION AND MYELINATION

ABSTRACT

Loss of the NF1 gene product, neurofibromin, has been shown to activate Ras in Schwann cells. Using in vitro techniques we now show that Schwann cells purified from mouse embryos with null mutations in the *NF1* gene upregulate expression of the major myelin glycoprotein P_0 and form increased numbers of Sudan black stained myelin segments in co-culture with wild type neurons and fibroblasts. Absence of neurofibromin in neurons or fibroblasts alone does not influence myelination by normal Schwann cells. In contrast, in co-cultures of neurons, Schwann cells, and fibroblasts without neurofibromin, myelination is dramatically reduced. Thus, neurofibromin in multiple peripheral nerve cell types is required for normal myelination. We propose a model in which loss of neurofibromin in Schwann cells causes hyper-differentiation that can be over-ridden by unidentified stimuli from neurofibromin-deficient neurons and\or fibroblasts.

INTRODUCTION

During the first few postnatal weeks Schwann cells associated with large axons cease proliferation, upregulate expression of myelin-associated lipids and proteins, and manufacture a myelin sheath that speeds the conduction velocity of nerve impulses (reviewed in Webster and Favilla, 1984). Several Schwann cell membrane proteins and lipids are enriched in myelin and are diagnostic of myelin membrane production (reviewed in Lemke, 1993); these include the major myelin glycoprotein P_0. P_0 expression is detected in pre-myelinating Schwann cells, but is dramatically upregulated by development of the myelinating phenotype (Bhattacharyya et al., 1991; Baron et al., 1991; Trapp et al., 1988; Lemke and Chao, 1988).

Molecules that induce the myelinating phenotype are largely unknown. Myelination occurs only when Schwann cells interact with axons of large diameter (Duncan, 1934; Friede, 1972; Matthews, 1968; Voyvidic, 1989). Prolonged and continuous axon-Schwann cell interaction is also required for maintenance of myelin gene expression (Wu et al., 1994). The extent of myelin formation can be stimulated by increasing levels of the hormone progesterone (Koenig et al., 1995). In vitro, factors shown to control myelin deposition, and P_0 expression, include axonal contact, serum factors, and deposition of basal lamina (Eldridge et al., 1987; Fernandez-Vallee et al., 1993). Factors secreted by neurons can also upregulate expression of P_0 (Bolin and Shooter, 1993).

Exposure of cultured Schwann cells to growth factors exerts a negative influence on P_0 expression, (Morgan et al., 1991, 1994). In the nerve, Schwann cell division ceases prior to upregulation of P_0 expression (Stewart et al., 1993). Modulation in second messenger systems likely underlies the

complex regulation of the process of myelination. The transcription factor Krox-20 is required for myelination (Topilko et al., 1994). Cyclic AMP is a putative second messenger that may be required for myelination, as exposure of cultured Schwann cells to cAMP and its analogs, or to the adenylate cyclase activators forskolin or cholera toxin, causes increased expression of myelin proteins and lipids including galactocerebroside (Sobue and Pleasure, 1984, Sobue et al., 1986), P_0 (Morgan et al., 1991; 1994), and myelin basic protein (Lemke and Chao, 1988). Mice that lack cyclic AMP inducible transcription factor, SCIP, fail to myelinate axons (Bermingham et al., 1995) consistent with regulation of transcription of myelin genes through a cAMP pathway requiring neuron-glial interactions. However, cAMP elevation is insufficient to upregulate myelin gene expression in vivo (Poduslo et al., 1995). Therefore, additional signalling pathways are probably involved in induction and maintenance of myelin.

The Ras signalling pathway is a candidate regulator of Schwann cell phenoytpe. Neurofibromin, the protein product of the *NF1* locus, shares sequence homology with Ras GTPase activating proteins (GAP), IRA1 and IRA2 in yeast (Buchberg et al., 1990, Tanaka et al., 1990, Xu et al., 1990b) and $p120^{gap}$ in mammals (Trahey and McCormick, 1987, Xu et al., 1990a). Neurofibromin functions as a Ras GAP in vivo and in vitro (Ballester et al., 1990, Martin et al., 1990, Golubic et al., 1992; Xu et al., 1990a). Mutations at the *NF1* locus are predicted to increase active, GTP-bound, Ras in target cells. Human patients with mutations at the *NF1* gene develop peripheral nerve sheath tumors (neurofibromas) (Huson, 1994, Riccardi, 1992) that contain large numbers of Schwann cells (Stefansson et al., 1982; Peltonen et al., 1988) with altered phenotype (Sheela et al., 1990), but also contain fibroblasts and axons (Peltonen et al., 1988; Waggener, 1966; Vaalasti et

al., 1990); the relative contribution of neurons, Schwann cells and fibroblasts to neurofibroma formation is unknown. As predicted if *NF1* is a negative regulator of Ras activation, neurofibromin in malignant Schwann cell tumors from NF1 patients is dramatically reduced while GTP-bound p21 Ras is increased (Basu, 1992; DeClue et al., 1992). Primary mouse Schwann cells lacking neurofibromin also show elevation of Ras-GTP, and exhibit decreased proliferation (Kim et al., 1995). Decreased Schwann cell division or proliferation arrest are observed when rat Schwann cells express oncogenic, constitutively activated Ras (Ridley et al., 1988; Kim et al., 1995). Decreased cell division is consistent with the hypothesis that neurofibromin-deficient Schwann cells exhibit enhanced differentiation. Neurofibromin is expressed in sciatic nerve throughout development; there is little change in the overall expression level of *NF1* message during different stages of peripheral nerve development (Wrabetz et al., 1995) but neurofibromin is enriched in non-myelinating Schwann cells as compared to those that are associated with myelin sheaths (Daston et al., 1992), consistent with a role in some aspects of Schwann cell differentiation.

To test the role of neurofibromin in Schwann cell differentiation Schwann cells were isolated from mice in which the *NF1* locus was disrupted (Brannan et al., 1994, Jacks, 1994). Mice in which only one allele of the *NF1* locus is disrupted do not show peripheral nerve dysfunction or develop neurofibromas (Brannan et al., 1994, Jacks, 1994). Mice homozygous for the *NF1* mutation die in utero around mid-gestation apparently due to cardiac malformations (Brannan et al., 1994). Sensory ganglia from NF1 mice can be obtained prior to the death of homozygous mutant embryos and purified peripheral nerve cells, including Schwann cells, characterized in culture (Kim et al., 1995, Rosenbaum et al., 1995, Vogel et al., 1995). Homozygous embryos contain no

neurofibromin, while heterozygous embryos contain about one-half the levels of neurofibromin as wild type embryos (Brannan et al., 1994). We have used a culture system using neurons, Schwann cells, and fibroblasts from mice that lacked neurofibromin and analyzed these cultures for myelin formation. Our results demonstrate that Schwann cells without neurofibromin are more differentiated than wild type cells but that this phenotype can be overriden by a cellular environment in which neurons and fibroblasts in addition to Schwann cells are neurofibromin-deficient, suggesting a role for the Ras pathway in Schwann cells and in other peripheral nerve cells in regulation of myelination.

RESULTS

Organotypic cell cultures containing neurons, Schwann cell precursors, and fibroblasts were obtained by dissociating dorsal root ganglia from E12.5 mouse embryos wild type, heterozygous, or null at the *NF1* locus. Cells were grown on a substrate of type 1 collagen in medium containing serum and nerve growth factor. As in vivo, under these culture conditions neurons extend processes from cell bodies; processes provide a proliferative stimulus for Schwann cells, which cover neurites. Fibroblasts form perineurium-like layers surrounding fascicles of neuronal processes and Schwann cells. Cells from wild type embryos, heterozygous embryos, or embryos lacking neurofibromin were cultured for 18 days, then fixed and stained with Sudan black to highlight lipid-rich myelin segments. In two separate experiments 2-5 cultures of different genotype were analyzed for the presence of myelinated axons. Figure 1 shows results of a representative experiment in which the numbers of myelin segments were reduced in cultures lacking neurofibromin, although some myelin segments were present in these cultures. Cultures from heterozygous embryos were intermediate in number of myelin segments.

To quantitate the reduction in myelin segment number, each field of replicate cultures was assessed for number of myelin segments. Results are shown in Figure 2. While only 28% of the 2097 fields counted showed no myelin segments in wild type cultures, 80% of the 652 fields counted showed no myelin in cultures without neurofibromin. Cultures from heterozygous embryos were intermediate. Similarly, while more than 25% of fields contained more than 10 myelin segments in wild type cultures, only 3% of fields contained more than 10 myelin segments when neurofibromin was absent.

One explanation for the reduction in myelin formation was that very thin myelin segments, or abnormally compacted

myelin segments, were present in cultures without neurofibromin and that this myelin was not detectable by Sudan black staining. To test this hypothesis, cultures of all three genotypes were embedded in plastic and ultrathin sections examined by electron microscopy. No abnormal compaction was noted in cultures lacking neurofibromin. Rather, these cultures contained many examples of Schwann cells that had established 1:1 relationships with axons, yet had formed no myelin. In contrast, wild type cultures contained many Schwann cells in 1:1 relationships with axons and also many normally myelinated axons. Typical electron micrographs are shown in Figure 3. On occassion, a rare myelin sheath was observed in a culture lacking neurofibromin. When present, these sheaths showed apparently normal ultrastructure, with normal inner and outer mesaxons and normal compaction (Figure 3B, insert). These experiments suggest that lack of neurofibromin allows normal maturation of axon-Schwann cell interactions through ensheathment. Further, when myelin segments do form, they form normally, suggesting that lack of neurofibromin does not result in abnormal compaction or wrapping of axons by myelin lamellae.

Axonal diameter is a crucial regulator of the decision to myelinate (Duncan, 1934; Friede, 1972; Matthews, 1968; Voyvidic, 1989). We tested if the diameter of processes emanating from neurons without neurofibromin was smaller than the diameter of wild type neurites, resulting in lower level of myelination. The diameter of several hundred neurites in 1:1 relationship with Schwann cells was measured in wild type cultures and cultures without neurofibromin in two separate experiments. Results are shown in Table 1. No difference in fiber diameter was observed between genotypes, ruling out a contribution of axon diameter to the observed failure of myelination.

Abnormally low numbers of myelin segments in cultures that

contained neurons, Schwann cells, and fibroblasts lacking neurofibromin might be ascribed to one or more of these cell types. To test whether defects one or more of these cell types could reproduce the myelination failure observed in organotypic cultures, neurons, Schwann cells or fibroblasts without neurofibromin were added to cultures containing wild type cells of the other two cell types. These mixing experiments were facililated by use of readily obtained rat neurons or Schwann cells in lieu of mouse cells when possible, made possible by the cross-species conservation of neuron-glial-fibroblast signals. It has thus far proved impossible to obtain sufficient numbers of primary mouse neuron and Schwann cell cultures of differing genotype to carry out mixing experiments entirely using mouse cells.

To determine if myelination failure was due to the lack of neurofibromin in Schwann cells, rat neurons, normal mouse fibroblasts and Schwann cells of the three genotypes from NF1 mice were co-cultured. By 30 days in vitro, myelin sheaths were detectable in cultures containing Schwann cells of all three genotypes upon examination by phase-contrast microscopy; cultures were fixed and stained with Sudan black. Compared to cultures in which all three cell types derived from dorsal root ganglia of the same animal (Figures 1 & 2) the overall amount of myelin in these mixed (wild type) cultures was reduced. This may be due to age of these cultures, which take many weeks after dissection from embryos for cell purification and differentiation. It is also possible that by artificially mixing the three types of cells, the ratio among different cell types is not optimal to promote maximal myelination. Surprisingly, when the number of myelin sheaths per field was assessed in several cultures per genotype, cultures containing Schwann cells which lacked neurofibromin showed a significant increase in myelin formation as compared to cultures containing wild type

Schwann cells (Figure 4A). In 80% of the 1676 counted fields in wild type cultures no myelin segments could be found whereas only 36% of the 1618 fields in cultures containing Schwann cells without neurofibromin showed no myelin. About 8% of all fields in cultures with Schwann cells lacking neurofibromin contained more than 10 myelin segments in contrast to only 0.2% of all fields in wild type cultures. Cultures containing heterozygous Schwann cells also exhibited increased myelination as compared to wild type cultures. There was no statistically significant difference in the number of myelin segments between cultures containing heterozygous Schwann cells and those containing homozygous mutant Schwann cells. Cultures for this experiment derived from 3-4 different animals per genotype.

To reconcile these results with the lack of myelin formation present in organotypic cultures, we tested if neurons or fibroblasts without neurofibromin, when cultured with wild type Schwann cells, by themselves caused hypo-myelination in vitro. To analyze the potential influence of neurofibromin-deficient neurons on myelination, normal Schwann cells were added to 2 weeks old cultures of neurons; each culture contained neurons derived from a single mouse embryo. Cultures from up to 6 different embryos per genotype were analyzed in 3 different sets of experiments. When these cocultures were fixed and Sudan black stained after 3-4 weeks in vitro, myelin sheaths were present in all cultures but no difference in number of myelinated axons were detected (Figure 4B). Therefore, neurons mutant at *NF1* do not alter the extent of myelin formation by wild type Schwann cells in this assay.

To test the hypothesis that neurofibromin-deficient fibroblasts cause reduction in myelination by Schwann cells, fibroblasts of different genotype were added to co-cultures of wild type rat neurons and wild type rat Schwann cells

(Figure 4C). Two different experiments were performed in which myelin formation was analyzed after 30 and 37 days in vitro, respectively. Neither of the two experiments in which myelin segments were detected showed a significant difference in the number of myelinated axons. However, these experiments showed only a low overall amount of myelination; multiple additional experiments resulted in no myelination using fibroblasts of any genotype. These experiments suggest that fibroblasts mutant at *NF1* do not alter the extent of myelin formation by wild type Schwann cells in this assay.

Increased numbers of myelin segments in cultures containing neurofibromin-deficient Schwann cells with normal neurons and fibroblasts was contradictory to the decreased myelination in organotypic cultures from neurofibromin-deficient mice (Figures 1 & 2). Therefore, the differentiation state of neurofibromin-deficient Schwann cells was analyzed using a different experimental approach. Schwann cells were isolated from mouse embryos, purified, expanded and then treated in defined medium (N2) over a period of 4 days with either rhGGF2 or 4 _M forskolin. After protein extraction the relative amount of P_0 was determined by Western blot. Results are shown in Figure 5. As expected, normal rat Schwann cells showed increased P_0 expression upon stimulation with forskolin (Morgan et al., 1991, Sobue et al., 1986). Wild type mouse Schwann cells (MSC +/+) showed similar increase in P_0 expression on forskolin stimulation. In contrast, mouse Schwann cells without neurofibromin (MSC -/-) showed high levels of P_0 expression in rhGGF2; levels were similar or slightly higher than the ones expressed by forskolin treated cells in different experiments (n=4). Densotometric scans of resultant western blots in two experiments revealed a 11.7 and 12.8-fold increases in P_0 in null Schwann cells in rhGGF2 as compared to wild type Schwann cells. Schwann cells

heterozyous for *NF1* (MSC +/-) showed a P_0 expression pattern similar to that of wild type cells. These findings confirm that null Schwann cells differ from wild type cells in response to differentiation stimuli, as predicted by hypermyelination in neuron-Schwann cell co-cultures.

To begin to investigate the underlying basis for the hyperdifferentiation of Schwann cells lacking neurofibromin, we tested if the effect could be mimicked by Ras activation in Schwann cells. Activated Ras (GTP-Ras) is increased in Schwann cells lacking neurofibromin (Kim et al., 1995). Thus, upregulation of P_0 in neurofibromin-deficient Schwann cells might be due to increased GTP-Ras. Rat Schwann cells were infected with retroviruses containing cDNA encoded constitutively activated H-Ras (RSC v-Ras) and P_0 expression analyzed (figure 5). Basal levels of P_0 were increased in v-*Ras* expressing cells as compared to normal rat Schwann cells; desotometric scan showed a 4.6-fold increase in P_0 levels in v-*Ras* expressing cells as compared to normal rat Schwann cells. Thus, v-*Ras* expressing Schwann cells mimic the hyper-differentiation phenotype observed in the absence of neurofibromin.

DISCUSSION

The present study was performed to determine if Schwann cell differentiation is altered in the absence of the NF1 gene product, neurofibromin. To characterize properties of neurofibromin-deficient Schwann cells a complex cell culture system was analyzed containing neurons, Schwann cells, and\or fibroblasts from mice in which the *NF1* locus was disrupted by homologous recombination. The techniques we have described for isolation of transgenic mouse Schwann cells and myelination assays using mouse cells will be useful for study of many transgenic mouse mutants now available.

Myelination was dramatically reduced when neurons, Schwann cells and fibroblasts all lacked neurofibromin. In contrast, Schwann cells in isolation exhibited elevated P_0 expression in the absence of neurofibromin and formed more myelin segments as compared to wild type Schwann cells when cultured together with normal neurons and fibroblasts. A possible explanation of these results is that younger Schwann cells, present in organotypic cultures, fail to differentiate while older cells in mix-and match cultures show increased differentiation. However, isolated Schwann cell preparations from 1 week to 1 month after isolation always showed increased P_0 expression relative to wild type control cells. It is also possible that regulation of mouse Schwann cell differentiation by mouse neurons (present in organotypic cultures) differs from that by rat neurons (present in mix-and-match cultures). While we cannot formally exclude this possibility, it is unlikely as even xenografts of human and rat nerve can be myelinated in vivo by mouse axons (Steinmuller, 1970). We favor the explanation that, by itself, loss of neurofibromin in Schwann cells leads to a more differentiated phenotype, but that increased differentiation can be over-ridden when neurons and/or fibroblasts also lack neurofibromin. A direct test of this possibility using mix-and-match cultures in

which two of three cell types lack neurofibromin has not yet been technically feasible because of the limited life-spans of both neuronal and Schwann cell cultures combined with the limited number of mutant embryos obtained.

Schwann cells without neurofibromin upregulate the expression of myelin P_0 protein, independent of axonal (or other exogenous) stimuli. We have documented increases by heterozygous as well as null cells. This gene dosage effect is consistent with previous observations that Schwann cells heterozygous at *NF1* are intermediate between wild type and null cells in proliferation and in expression of Ras-GTP (Kim et al., 1995). However, increased P_0 expression was detected only in null cells, not in heterozygous Schwann cells. It is likely that in vitro conditions (exposure to rhGGF2 in defined medium) do not mimic all cues provided by axons in driving myelination in heterozygous cells.

The mechanism underlying increased P_0 expression in isolated Schwann cells lacking neurofibromin is likely to be increased Ras-GTP levels. Expression of activated Ras increased P_0 expression in a Schwann cell line (Gutmann et al., 1993), and we have shown here that expression of oncogenic ras in Schwann cells also upregulates P_0 expression. In normal Schwann cells, increasing intracellular cAMP augments Schwann cell differentiation (Morgan et al., 1991, Pleasure et al., 1985, Raff et al., 1978, Sobue and Pleasure, 1984) as assessed by increased P_0 expression. Activation of protein kinase A by cAMP inhibits the Ras pathway at the level of Raf activation in some cell types (Cook and McCormick, 1993). If this were true in normal Schwann cells, treatment with forskolin, by inactivating the Ras pathway, would be predicted to decrease differentiation. An alternative possibility consistent with our data is that Ras activation in Schwann cells causes activation of adenylate

cyclase, causing increases in P_0 (proposed by Lemke [1990]). However, while Ras activation of adenylate cyclase occurs in yeast, activation has not been detected in mammalian cells.

Why is myelination decreased when Schwann cells lacking neurofibromin are cultured together with neurons and fibroblasts that also lack neurofibromin? The ability of Schwann cells to associate with axons is not affected by the absence or presence of neurofibromin (Rosenbaum et al., 1995). The few myelin segments present in cultures without neurofibromin showed normal ultrastructure. Thus the processes of myelination involving upregulation of myelin proteins, wrapping of Schwann cell membrane around axons, and compaction of myelin lamellae can proceed normally. Loss of neurofibromin in neurons does not affect the axonal diameter so that the myelination failure in cultures without neurofibromin cannot be attributed to smaller axons.

We hypothesize that signals produced by neurofibromin-deficient axons and\or fibroblasts influence the behavior of Schwann cells lacking *NF1* (Figure 6). Loss of neurofibromin causes neurotrophin independence for survival and neurite outgrowth (Vogel et al., 1995). Cell autonomous abnormalities in fibroblasts lacking neurofibromin lead to failure to form perineurium in vitro (Rosenbaum et al., 1995). Therefore, both cell types display cell-autonomous changes due to loss of function at *NF1* that could result in abnormal (increased or decreased) production of particular factors. This idea is consistent with increased growth factor production by fibroblasts that express oncogenic *ras*: these cells produce growth factors including NDF (Peles et al., 1992). Both neurons and fibroblasts influence myelination; fibroblasts promote basal lamina deposition by Schwann cells required for myelination by Schwann cells (Obremski et al., 1993a,b; Bunge, 1993), which neuronal contact is a prerequisite for myelin

formation.

If abnormal axon- or fibroblast-derived signals exist, they do not influence the phenotype of normal Schwann cells, but only affect Schwann cells which themselves lack neurofibromin. This can be explained by assuming that Schwann cells react differently to neuron or fibroblast-derived signals in the absence of neurofibromin. For example, neurofibromin-deficient Schwann cells might hyper-respond to growth factor (or other types of signals) because of constitutive activation of the Ras pathway.

In summary, our findings suggest that abnormal cues from axons or fibroblasts combined with a modified Schwann cell response might result in a Schwann cell phenotype different from the one caused by lack of neurofibromin in Schwann cells alone; the decrease in myelination in cell cultures containing neurofibromin-deficient neurons, Schwann cells and fibroblasts might not be ascribed to one single cell type but rather be the consequence of abnormal cell-cell signalling events. An implication of this study for tumor formation in human patients with mutation in the *NF1* gene is that mutations in multiple heterozygous cell types could provide an environment in which loss of function mutations in a single cell type are manifested. Alternatively, multiple heterozygous cell types may be sufficient to develop peripheral nerve tumors together with undefined precipitating second events.

MATERIALS AND METHODS

Transgenic NF1 mice

C57BL/6J mice in which one allele of the *NF1* gene has been targeted were generated as described elsewhere (Brannan et al., 1994). In order to obtain NF1 (-/-) embryos, male and female *NF1* (+/-) mice were mated for 12-16 hours and checked for presence of a copulatory plug. Pregnant mice were sacrificed to obtain embryos after 12.5 days, with the day the plug was observed designated day 0.5. Genotyping of embryos was carried out as described (Rosenbaum et al., 1995; Brannan et al., 1994).

Dorsal root ganglion organotypic cultures and neurons from NF1 mice and embryonic rats

Dorsal root ganglia were obtained from E12.5 mouse and E15 rat embryos as described (Rosenbaum et al., 1995). A single cell suspension (containing neurons, Schwann cell precursors, and fibroblasts) was generated and plated in single drops onto collagen-coated 35 mm dishes (Falcon). 5-6 individual drops of cell suspension were separately placed onto the hydrophobic collagen-substrate so that drops did not flow together. Cells were allowed to attach overnight at 35°C and 7.5% CO_2. The following day dishes were flooded with "C" (DMEM with 10% human placental serum) medium. To obtain purified neuronal cell populations, cultures were treated with the antimitotics 5'-fluoro-2'-deoxyuridine and uridine (Sigma) added to "C" medium at 10^{-5} M final concentration ("CF" medium) (Bunge et al., 1983). Cultures were cycled through two-three passages of C/CF; each medium was replaced after 3 days.

Preparation of rat primary Schwann cells

Primary Schwann cells were purified from sciatic nerve from

postnatal day 1 rats as described previously (Brockes et al., 1979). Following isolation of Schwann cells by antimitotic treatment and complement-mediated lysis of residual fibroblasts, cells were maintained in DMEM plus 10% fetal bovine serum until plating onto neurons. In some experiments, Schwann cells were amplified for 1 week in recombinant human glial growth factor (rhGGF2, Cambridge Neuroscience) and 2 _M forskolin prior to addition to neurons.

Schwann Cell Infection

Recombinant retrovirus stocks (provided by Dr. Doug Lowy) were used to introduce v-*Ras*^{H} into primary rat Schwann cells as described (Kim et al., 1995). Two weeks after infection, G418 resistant clones were pooled and maintained in Schwann cell growth medium in the continuous presence of G418.

Isolation of mouse Schwann cells from embryonic DRG cultures

Dorsal root ganglia (approximately 50 ganglia/embryo) were removed from E12.5 mouse spinal cords and cells dissociated in 0.25% trypsin. A single cell suspension was generated, plated on plastic in N2 plus nerve growth factor (Harlan Bioproducts) for one week. Schwann cells were isolated by physical removal of neurons and Schwann cells from the fibroblast monolayer, followed by growth in rhGGF2 (Kim et al., 1995). Schwann cells were >99.5% pure as judged by immunolabelling with rat anti-mouse low affinity nerve growth factor receptor antibody (D. Anderson, Caltech) and rabbit anti-S100 (Dakopatts).

Preparation of mouse fibroblasts

Fibroblasts from E12.5 mouse embryos were obtained when embryos were dissected for DRG preparation as described (Rosenbaum et al., 1995). Fibroblasts were used within 3

passages for experiments.

Myelination Assay

Oranotypic cultures containing neurons, Schwann cells, and fibroblasts were maintained in "C" medium for about 1 week, then ascorbic acid was added to the medium to a final concentration of 50 _g/ml ("C+AA" medium) to enhance myelination (Eldridge et al., 1987). After 18 days in vitro, cultures were fixed and processed for Sudan-black staining. For analysis of effects of specific cell types on myelination, 2 week old rat or mouse-derived neuron cultures were seeded with mouse or rat-derived Schwann cells at a density of 0.4 x 10^6 cells per 35 mm dish. Cells were seeded in "C" medium, after 1 day in coculture the medium was changed to defined medium (N2) (Bottenstein and Sato, 1979) which allows Schwann cell proliferation but not myelination. Cultures were maintained at 37°C and 10% CO_2. When Schwann cells were evenly distributed over the culture (5-7 days) medium was changed to"C+AA" and cultures kept at 35°C and 7.5% CO_2. Mouse fibroblasts ($0.4x10^6$) were added to neuron-Schwann cell cultures at this time. Cultures were fixed 3-4 weeks later for 30 minutes at room temperature in 0.05 M sodium phosphate buffer pH 7.4 supplemented with 2% glutaraldehyde and 100 mM sucrose. Cultures were washed with 0.1 M sodium phophate buffer 3 times for 5 minutes each, treated with PBS containing 1% OsO_4 and 1.5% potassium ferrocyanide for 30 minutes at room temperature, washed again 3 times for 5 minutes each then placed into 0.1 M sodium phosphate buffer at 4°C overnight. The next day cultures were dehydrated in 30%, 50%, and 70% ethanol, 5 minutes each, and were then stained with 0.5% sudan-black in 70% ethanol for 60 minutes at room temperature. Cultures were destained in 70% ethanol for 30 seconds at room temperature, rehydrated in 50% and 30% ethanol for 5 minutes each, and mounted in glycerol gelatin

(Sigma) on glass slides. Sudan-black stained cultures were analyzed for the presence of myelin on a Leitz light microcope at 400X final magnification. The number of myelin segments per microscopic field was counted, with several hundred fields per culture analyzed. The number of myelin segments were expressed as 0, 1-9, 10-19, 20-29, or more than 30 myelin segments per field. The number of fields in each category was expressed as percentage of the total number of fields.

Electron Microscopy

Cultures were fixed for 30 minutes at room temperature in 0.1 M sodium cacodylate buffer pH 7.4 containing 2% paraformaldehyde, 1.25% glutaraldehyde, and 100 mM sucrose. Washes, postfixation, and embedding have been described (Rosenbaum et al., 1995). For Organotypic cultures derived from 4 wild type, 14 heterozygous and 7 null embryos were evaluated; 1-2 areas/culture were analyzed. Each block was selected 2 mm outside areas containing neuronal cell bodies, areas that are maximally differentiated (Obremski et al., 1993a). Ultrathin sections were stained with lead citrate and uranyl acetate and photographed on a JOEL-100 CX electron microscope.

P_0 extraction and Western Blot

Rat Schwann cells, v-^{H}*Ras*-infected rat Schwann cells or Schwann cells isolated from wild type or null NF1 mouse embryos were plated (1-1.5 x 10^6 cells/dish) onto poly-L-Lysine-coated 60 mm culture dishes in DMEM with 10% fetal calf serum. After 24 hours, dishes were treated with rhGGF2 or with 5 _M Forskolin, both in serum free media (N2). Cells were cultured for another 4 days; medium was changed each day. At the end of the culture period, cells were lysed in 50 _l of buffer (2% SDS, 2 mM EGTA, 2mM EDTA, 5 mM Tris

HCl pH6.8 and 2 mM PMSF), boiled for 5 min and centrifuged for 15 min at 14,000 rpm (Morgan et al., 1994). Amount of proteins in each sample was determined from the resulting supernatant by Lowry assay (Markwell, 1978) and an equal amount of protein per sample loaded and separated in 10% SDS polyacrylamide gels. Proteins were then transferred onto nitrocellulose and P_0 detected by Western blot analysis using a polyclonal antibody (1:500) raised against rat Po protein from D. Colman.

Figure Legends

Table 1. Lack of neurofibromin does not affect the area of ensheathed axons. Axon areas were measured in several hundred axons in electron micrographs using a computerized imaging system (Zeiss Videoplan). Data represent means +/-SEM. In two different experiments the cross-sectional axonal area was almost identical in wild type and neurofibromin-deficient cell cultures.

Figure 1, Sudan black visualization of myelin segments in organotypic DRG cultures from NF1 mice. Myelin is present in cultures of all genotypes; individual sudan-black stained myelin sheaths are denoted by arrows. The number of myelinated sheaths is reduced in *NF1* (-/-) cultures (C) as compared to *NF1* (+/-) (B) and wild type (A) cultures.

Figure 2. Reduction in number of myelin segments in organotypic cultures from embryos lacking neurofibromin. Sudan-black stained and whole mounted DRG-cultures were analyzed for the presence of myelin. The number of myelin segments was counted in microscopic fields covering the entire surface of each culture. The number of fields containing 0, 1-9, 10-19, 20-29, 30-39, 40-49, or more than 50 myelin sheaths was determined and expressed as a percentage of the total number of fields. Data represent means +/- SEM. 3 wild type, 5 *NF1* (+/-), and 2 *NF1* (-/-) DRG-cultures were evaluated in this experiment. Cultures were derived from at least two different embryos. Data are representative of 2 independent experiments.

Figure 3. Electron microscopic analysis of axon-Schwann cell interactions in organotypic cultures from wild type and mutant embryos. Organotypic cultures from wild type (A) and

NF1 (-/-) (B) mice were fixed and analyzed by electron microscopy. Samples for analysis were selected 2mm outside the region of neuronal cell bodies. Many axons (*) of large diameter were in 1:1 relationship with Schwann cells in both wild type (A) and *NF1* (-/-) (B) cultures. Myelin sheaths, although infrequent, showed normal morphology in NF1 (-/-) cultures (insert, Panel B). Data are representative of 4 independent experiments.

Figure 4. Quantitation of sudan black stained myelin segments in cultures containing neurofibromin-deficient neurons, Schwann cells, or fibroblasts. The number of myelin segments in sudan-black stained and whole mounted cultures was determined as described for figure 2. In the top panel, wild type neurons (rat), and wild type fibroblasts (mouse) were combined with Schwann cells from mice of different genotype. Per genotype 3-4 cultures were analyzed, each culture contained Schwann cells derived from a different embryo. Cultures were fixed after 30 days in vitro. Cultures containing neurofibromin-deficient Schwann cells showed increased myelination as compared to cultures which contained wild type Schwann cells. In the middle panel, wild type Schwann cells (rat) were combined with neurons from mice of different genotypes. Cultures were derived from 3-4 different embryos per genotype and analyzed for the presence of myelin after 25 days in vitro. No significant difference in myelin formation could be found among cultures containing neurons of different genotype. The experiment was repeated twice with similar results. In the bottom panel, wild type neurons (rat) and wild type Schwann cells (rat) were combined with mouse fibroblasts of different genotype. One set of cultures was analyzed after 30 days in vitro, another one after 37 days in vitro with similar results. For each set, one culture per genotype was fixed and the number of myelin segments determined as described for figure 2. No

significant difference in myelin formation was detected among cultures containing fibroblasts of different genotype.

Figure 5. P_0 expression in wild type, NF1 mutant mouse Schwann cells, and v-Ras expressing rat Schwann cells Schwann cells were incubated for 4 days in either rhGGF2 or 5 _M forskolin in serum-free medium and cell lysate prepared. Twenty _g of each lysate was separated on a 10% SDS gel and P_0 expression analysed by Western blot using polyclonal antibodies raised against rat P_0. P_0 expression was low in normal rat Schwann cells (RSC), wild type (+/+) mouse Schwann cells (MSC), and in heterozygous (+/-) mouse Schwann cells unless forskolin was added. *NF1* (-/-) mouse Schwann cells (MSC) and v-Ras expressing rat Schwann cells (RSC) expressed significant levels of P_0 both in rhGGF2 and in the presence of forskolin.

Figure 6. Model: Effects of loss of neurofibromin on Schwann cell proliferation and differentiation
(A) During normal Schwann cell development, neurofibromin functions as a negative regulator of cellular Ras and plays a crucial role in maintaining a balance between the levels of GTP-bound (active) and GDP-bound (inactive) Ras. Proper balance between active and inactive Ras determines whether Schwann cells proliferate or differentiate. **(B)** Mutation at the NF1 locus results in an increased ratio of GTP to GDP-bound Ras, activating the Ras mediated signalling pathway (Kim et al., 1995). As a result, Schwann cell proliferation decreases and differentiation (detected as upregulation of P_0 expression and increased numbers of myelin segments) increases. **(C)** Effects of NF1 mutations in Schwann cells (differentiation of Schwann cells) are overridden by NF1 mutations in fibroblasts and/or neurons. Decreased Schwann

cell differentiation might increase proliferation (broken arrow), but Schwann cell proliferation has not been tested under these conditions. Neurofibromin-deficient neurons or fibroblasts could produce increased or decreased levels of hypothetical factor(s) ("X"). Decreased differentiation of Schwann cells which themselves lack neurofibromin is explained by altered response of these mutant Schwann cells to factor X.

Genotype	Axonal area (number of axons measured)	
	exp. 1	exp. 2
+/+	1.13 + 0.75 (344)	0.93 + 0.90 (480)
- / -	1.13 + 0.86 (120)	0.91 + 0.83 (549)

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Figure 1
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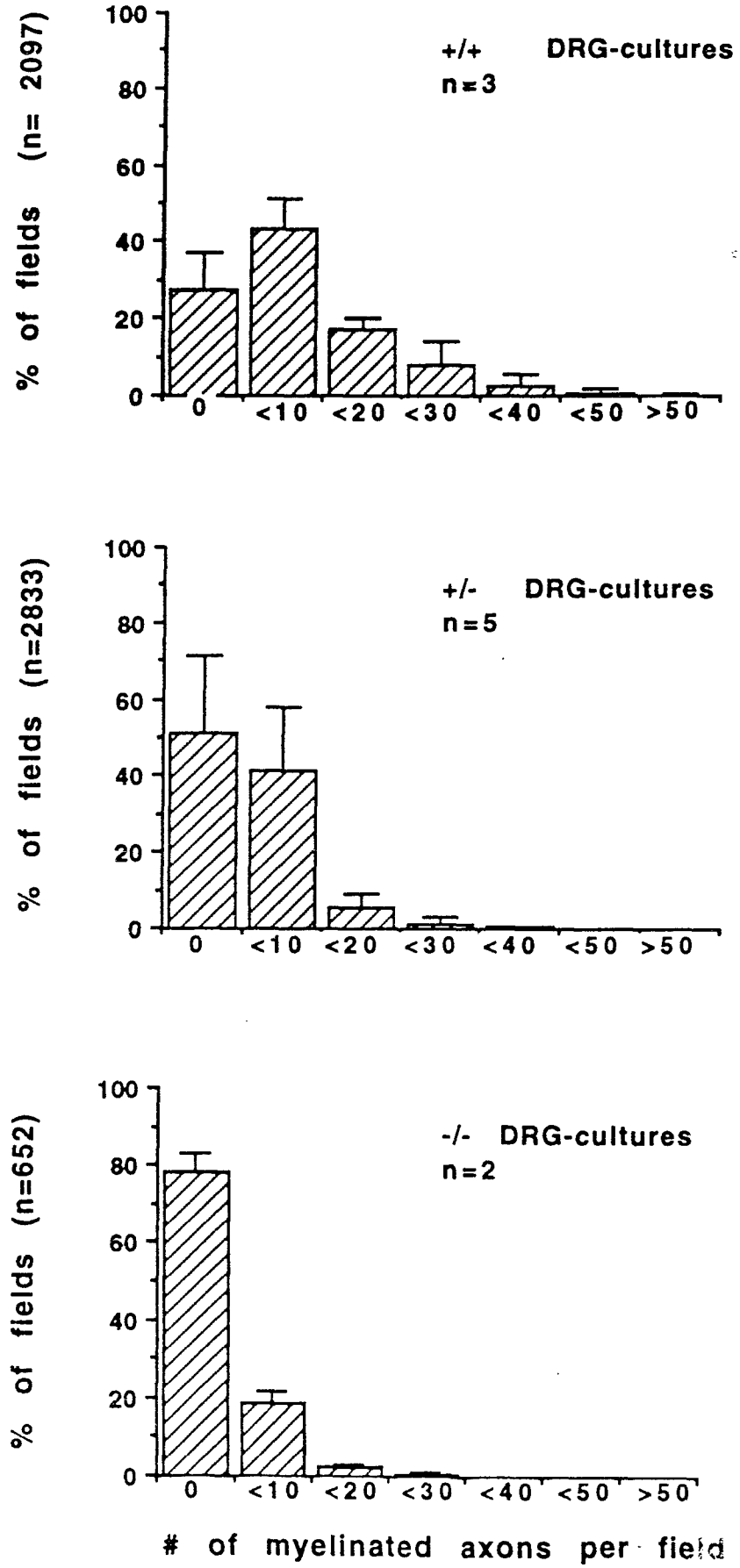

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Figure 2
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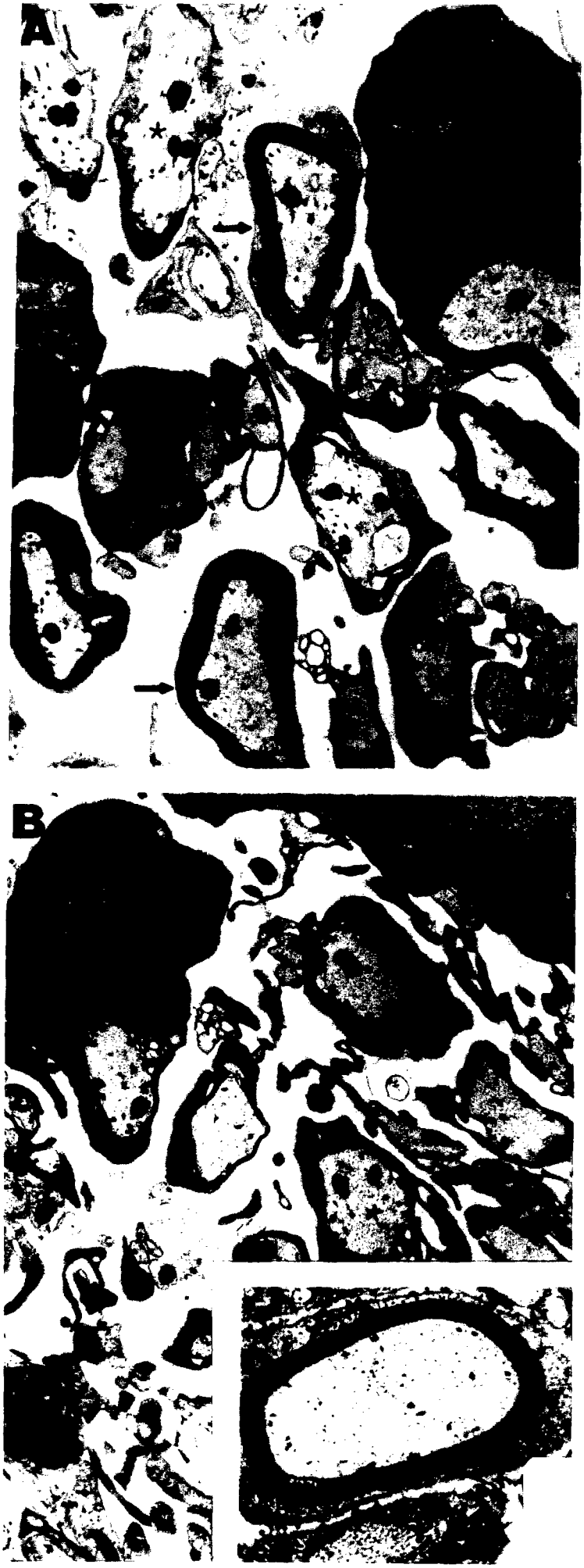
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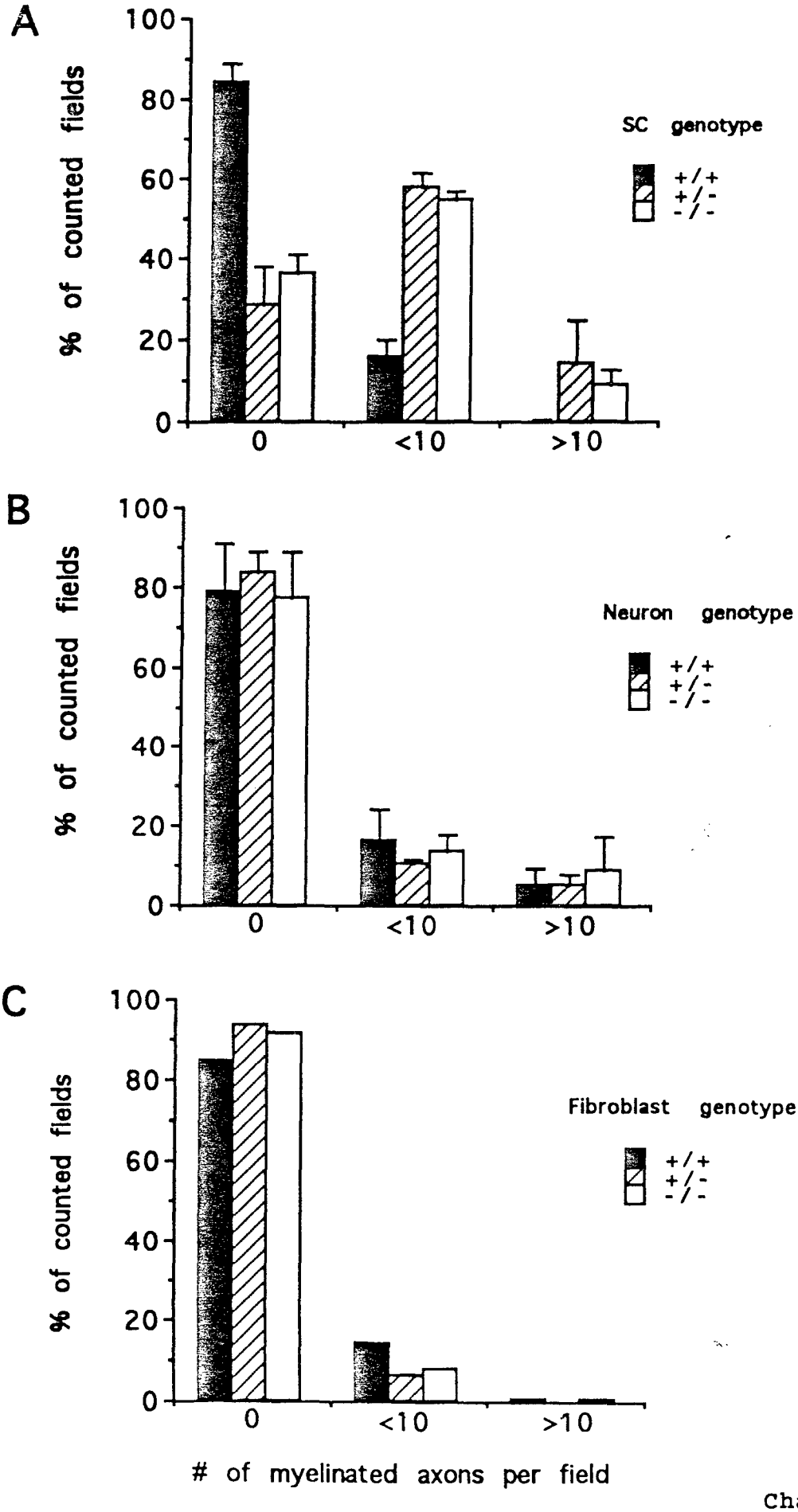
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Fibroblast genotype
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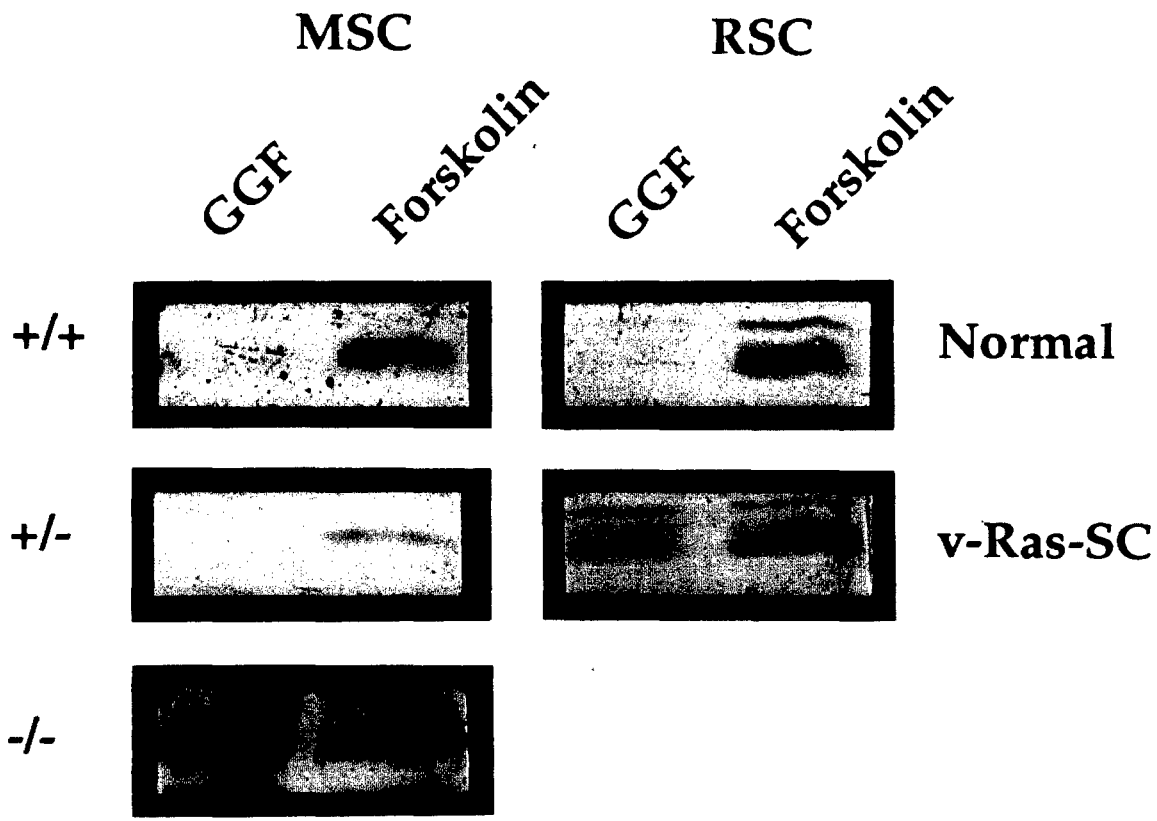
# of myelinated axons per field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Normal
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v-Ras-SC
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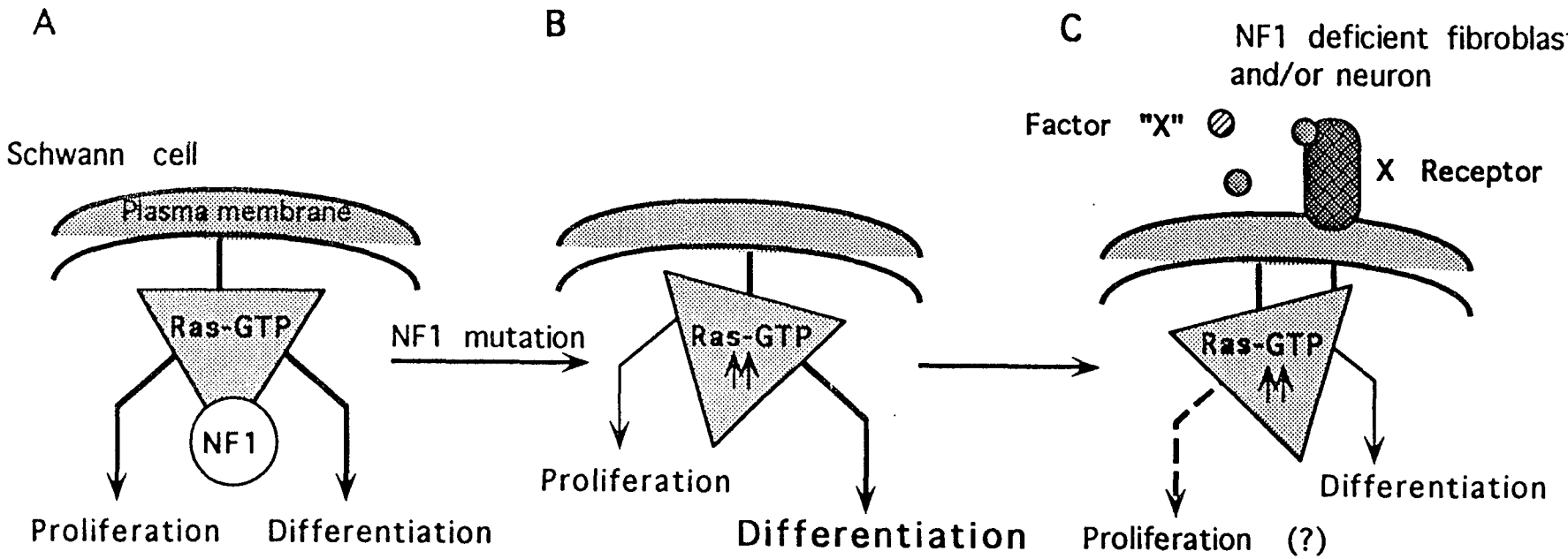
A
B
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NF1 deficient fibroblast
and/or neuron
Factor "X"
X Receptor
Schwann cell
Plasma membrane
Ras-GTP
NF1
NF1 mutation
Ras-GTP
Ras-GTP
Proliferation
Differentiation
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CHAPTER 6 NEUROFIBROMIN DEFICIENT FIBROBLASTS FAIL TO FORM PERINEURIUM IN VITRO

ABSTRACT

To identify the cell type(s) that give rise to nerve sheath tumors (neurofibromas), we have generated cell cultures containing neurons, Schwann cells and fibroblasts from transgenic mouse embryos in which the NF1 gene was disrupted by homologous recombination (Brannan et. al., 1994). All three cell types survived in the absence of the NF1 gene product, neurofibromin. Analysis of cultures by light and electron microscopy showed that normal fascicle formation by perineurial cells (stimulated by interaction of fibroblasts with axons and Schwann cells) failed to occur in the absence of neurofibromin. Fascicles were reduced in number and showed abnormal morphology in cultures when normal neurons and Schwann cells were cultured up to 37 days with NF1(-/-) fibroblasts. Cultures in which only one of the two NF1 alleles was mutant often showed an intermediate phenotype, suggesting a gene dosage effect. These observations suggest that mutations in the NF1 gene affect fibroblast behavior and might contribute to neurofibroma formation in NF1.

INTRODUCTION

Patients with the inherited disease type 1 neurofibromatosis (NF1) develop benign peripheral nerve sheath tumors called neurofibromas , described as the hallmark feature of the disease by von Recklinghausen (von Recklinghausen, 1882; Riccardi, 1981). Neurofibromas occur as small, usually multiple, tumors associated with nerve branches or as tumor masses associated with major peripheral or visceral nerve trunks (plexiform neurofibromas) (Riccardi, 1981; Wiestler, 1994). It remains unclear how different cell types contribute to formation and growth of neurofibromas. In normal peripheral nerves, contact between axons and Schwann cells is essential for Schwann cell proliferation and differentiation (Webster, 1984) , and Schwann cells are not observed dissociated from axons except after injury to a peripheral nerve. Co-culture of rat neurons and Schwann cells has shown that neurons stimulate Schwann cell proliferation and control assembly of Schwann cell basal lamina and myelin formation by Schwann cells (Wood, 1975, Bunge, 1983b, Eldridge, 1987, Bunge, 1993). Fibroblasts differentiate into perineurial cells when cultured in proximity to neurons and Schwann cells; inclusion of fibroblasts in neuron-Schwann cell co-cultures leads to formation of fascicles that closely resemble those observed in vivo (Williams et. al., 1982; Bunge et. al., 1989; reviewed in Bunge, 1993). Neurofibromas are charicatures of the normal organization of peripheral nerves. Immunohistochemical studies and ultrastructural analysis revealed that neurofibromas contain a mixed population mainly composed of Schwann cells, fibroblasts, perineurial cells, and axons (Fisher, 1968; Stefansson et. al., 1982; Peltonen, 1988; Hirose et. al., 1986; Nakajima, 1982), embedded in abundant extracellular matrix (Penfield, 1932). Schwann cells make up 40-80% of the cells in neurofibromas as defined by positive staining for the S-100 protein (Peltonen, 1988); many Schwann cells within neurofibromas show no apparent

contact to axons (Stefansson, 1982; Waggener, 1966; Poirier, 1968). Both von Recklinghausen (1882) and Penfield (1932) suggested that the primary abnormal cell in neurofibromas are mesodermal cells. Cells that morphologically resemble fibroblasts, containing dilated cisternae of rough endoplasmic reticulum and no basal lamin, and perineurial cells which show multiple smooth vesicles called caveolae and patchy basal lamina (Gamble, 1964, Thomas, 1984, Ushigome, 1986, Peters, 1991), are present in neurofibromas. Fibroblasts might play a role in the histogenesis of neurofibromas, as they produce extracellular matrix that is abundant in neurofibromas (Peltonen, 1984; Peltonen, 1988). Neurofibroma-derived fibroblasts have been reported to show differences in expression of adrenergic receptors (Kaila, 1988) , epidermal growth factor receptors (Zelkowitz, 1981b) , differential response to mitogens (Kadono, 1994), and altered organization of actin stress fibers (Peltonen, 1984, Hayashi, 1990). However, other investigators have stressed the Schwann cell origin of neurofibroma cells (Fisher, 1968; Waggener, 1966; Murray, 1941; Kharbanda, 1994). Schwann cells are abundant in neurofibromas and exhibit angiogenic and invasive behavior while normal Schwann cells do not, suggesting that defective Schwann cells might contribute to tumor formation (Sheela, 1990).

In 1990, the gene on the proximal long arm of human chromosome 17 responsible for causing NF1 was cloned (Cawthon, 1990; Viskochil, 1990; Wallace, 1990). The 220-280 kd protein product of the NF1 locus (neurofibromin) was subsequently identified (DeClue, 1991; Gutmann, 1991) and shown to be most abundantly expressed in the nervous system (Golubic et. al., 1992; Buchberg et. al., 1991; Daston et. al., 1992), especially in neurons, Schwann cells, and oligodendrocytes (Daston et. al., 1992; Huynh, 1994). The NF1 gene shares sequence homology with ras GTPase activating proteins in Saccharomyces cerevisiae (IRA1 and IRA2) (Buchberg, 1990; Tanaka, 1990; Xu, 1990) and mammals (GAP)

(Trahey, 1987; Xu, 1990) suggesting that neurofibromin is a part of the ras signal transduction pathway. As ras has been shown to have multiple effects on cell proliferation and differentiation (Barbacid, 1987; Satoh, 1992), tumor formation in neurofibromatosis patients could be explained by deregulation of intracellular signaling events secondary to NF1 mutations. Evidence in support of this hypothesis is the observation that neurofibromin levels are highly reduced while concentrations of GTP-bound p21ras are elevated in cells derived from malignant neurofibrosarcomas. (Basu, 1992; DeClue, 1992).

The role of mutations in the NF1 gene remains to be determined for benign lesions of NF1, including neurofibroma formation. Markers on chromosome 17 on which the NF1 allele is localized, are not deleted in DNA from neurofibromas (Menon, 1990; Skuse et. al., 1991), in contrast to results in maligant neurofibrosarcoma (Menon, 1990; Skuse et. al., 1991; Marchuk, 1993). If mutations in the NF1 gene are recessive at the cellular level, so that tumors arise only when both alleles acquire mutations, then the NF1 gene could act as a tumor suppressor, in a group of genes including RB, WT1, p53, and NF2 (Marshall, 1991).

In an attempt to define cells in which NF1 mutations might cause neurofibroma formation, and to test if alterations in both NF1 alleles are required for abnormal behavior, we have begun to study cell-cell interactions among cells from mice in which the NF1 locus was disrupted by homologous recombination (Brannan, 1994). Mice heterozygous for the NF1 mutation do not develop neurofibromas. Mice in which both alleles of the NF1 gene have been disrupted die in utero between embryonic days 11.5 - 14.5 (Brannan, 1994; Jacks, 1994), so that tumor formation cannot be analyzed directly. To obtain cells deficient in neurofibromin, we have generated mixed cell cultures from embryonic day 12.5 mouse embryos of all three genotypes. Here, we report that neuron, Schwann cell, and fibroblast co-cultures from

neurofibromin deficient mice imitate some of the features of the histologic phenotype of neurofibromas in human NF1 patients. While wildtype cultures show a high level of organization, with formation of fascicles composed of axons and Schwann cells surrounded by perineurial fibroblasts, neurofibromin-deficient cultures exhibit a highly disrupted pattern of organization. Cultures derived from heterozygous mice show an intermediate phenotype. We present evidence that neurofibromin deficiency in fibroblasts alone is sufficient to recapitulate this neurofibroma-like pattern of organization, consistent with the hypothesis that fibroblasts contribute to neurofibroma formation.

RESULTS

Cell cultures containing neurons, Schwann cell precursors, and fibroblasts were obtained by culturing dissociated cells from sensory (dorsal root) ganglia from embryonic day 12.5 mice. These organotypic cell cultures were prepared without prior knowledge of the genotype from crosses of heterozygous males and females. Within 16-24 hours after plating, observation of cell suspensions by phase contrast microscopy showed that viable cells had attached to the collagen substrate. Neurons, identified as round phase bright cells, had extended processes. Phase-dark cells (presumptive Schwann cell precursors and fibroblasts) were located underneath the neuronal cell layer (not shown). After 2-3 days in vitro, neurons became progressively more phase dark and a population of cells attached to neuronal processes and could be defined, based on attachment to neurites, as Schwann cells. Lack of neurofibromin did not by itself appear to cause changes in neuron-Schwann cell adhesion or in neuronal differentiation in response to nerve growth factor under these culture conditions, although subtle abnormalities cannot be ruled out. Fibroblasts continued to form a cellular background layer. No obvious difference in viability or cell number was noted in many cultures containing cells lacking neurofibromin as compared to wild type cultures until 9 days in vitro. However, by 9 days in vitro, in some (60%) of null cultures axons covered by Schwann cells appeared more obscured (Figure 1C) than in wildtype or heterozygous cultures (Figure 1A,B) and medium began to acidify more quickly than in cultures from other genotypes. A possible explanation for these results was increased cell number in some cultures lacking neurofibromin. To test this hypothesis we cultured dissociated dorsal root ganglion cells for up to 21 days and then counted the number of non-neuronal cells in a hemocytometer. In 2 of 3 experiments the number of non-neuronal cells (fibroblasts, macrophages and/or Schwann cells) was 2-3

fold increased in neurofibromin deficient cells compared to wildtype controls; a third experiment did not reveal a difference in cell number among different genotypes (Table 1). As we have shown that Schwann cell proliferation decreased when null cells are in contact with axons (Kim, 1995), it seemed unlikely that Schwann cell proliferation underlied increased cell number. Therefore, we tested whether fibroblast proliferation increased in the absence of neurofibromin. Growth curves were constructed using fibroblasts of different genotype. Western blot analysis confirmed that fibroblasts from null embryos contained no detectable neurofibromin, while heterozygous cells showed a reduction as compared to wild type cells (not shown). In 8 of 10 cell strains increased proliferation was detected in null cells as compared to wildtype cells (Figure 2). In the remaining experiments, no difference in growth rate was detected. Thus, fibroblast proliferation is increased in cell cutures from some but not all embryos.
After 18 days in vitro, cell cultures containing neurons, Schwann cells, and fibroblasts of each genotype were fixed and analyzed in semi-thin plastic sections to determine if normal organization of axons, Schwann cells and fibroblasts had occured. Cultures derived from 4 wildtype, 14 heterozygous and 7 NF1(-/-) embryos were evaluated in a total of 4 experiments. As neuronal somata had been plated in drops on collagen substrata, each sample contained discrete spots containing neuronal cell bodies; neuritic processes extended from each group of neuronal cell bodies radially. Cultures were fixed and embedded in plastic; samples for analysis were selected 2 mm outside the central area containing neuronal cell bodies in regions that are maximally differentiated (Obremski and Bunge, 1993a). Examination of semi-thin plastic sections by light microscopy revealed a striking difference among cultures containing cells of different genotypes. Wildtype cultures were very well organized, with a band of loosely packed axon-Schwann

cell units sandwiched between two compact multi-layers of elongated spindle-shaped fibroblasts (Figure 3A). Typically the bottom layer of fibroblasts, associated with the substratum of collagen, contained 3-5 fibroblasts and was much thinner than the top layer, which contained up to 8 cell layers. Layers of fibroblasts were orientated parallel to the collagen substrate and had narrow, fusiform processes. In contrast, neurofibromin-deficient cultures typically contained loosely arranged layers of fibroblasts sandwiching the axon-Schwann cell units and more space was evident between adjacent fibroblasts (Figure 3C). In some NF1 (-/-) sections even this pattern of organization was missing (Figure 3D), and fibroblasts were intermingled with axon-Schwann cell units, and not confined to two separate layers surrounding axons and Schwann cells. Sections from NF1 (+/-) cultures had an intermediate phenotype (Figure 3B).

As the fibroblasts in neurofibromin-deficient cultures appeared to behave abnormally, it seemed plausible that loss of neurofibromin in fibroblasts themselves might underlie this organizational deficit. Alternatively, it was possible that abnormal Schwann cells were unable to send correct signals for organization to surrounding fibroblasts. To discriminate between these alternatives, fibroblasts were expanded from E12.5 day embryos and 0.3x106 added to cultures of normal rat dorsal root ganglion neurons that were fully populated with rat Schwann cells. 17, 30, or 37 days after addition of fibroblasts of different genotype, culture organization was analyzed by assessment of semi-thin plastic sections. At 17 days in vitro sandwiching of groups of axons and Schwann cells between multiple layers of fibroblasts was observed in all sections from cultures containing wildtype fibroblasts (Figure 4A). Sections from cultures with neurofibromin deficient fibroblasts did not show this organizational pattern and resembled those sections in which all three cell types were neurofibromin deficient (Figure 4C). Cultures containing NF1 (+/-) fibroblasts exhibited an

intermediate phenotype (Figure 4B). By 30 and 37 days in vitro, fibroblasts in wildtype cultures appeared increasingly flattened and had formed perineurium, defined as concentric layers of elongated, spindle-shaped fibroblasts surrounding groups of axons and Schwann cells (Figure 4D-F) while in wildtype cultures and those containing NF1(+/-) fibroblasts most groups of axons and Schwann cells were organized into these fascicle-like groups, and were partially or completely surrounded by fibroblasts. In contrast, when cultures consisted of neurofibromin deficient fibroblasts together with normal neurons and Schwann cells, fascicles and perineurium formation could be found only rarely; most semithin cross sections did not show any fascicle formation at all. Even those fascicles present differed morphologically from those found in wildtype cultures: increased endoneurial space was evident and fibroblasts less frequently completely enclosed groups of axons and Schwann cells. In order to quantitate the relative number of fascicles in cultures containing fibroblasts of different genotypes, the number of perineurium-surrounded fascicles per culture was counted in 1 _m cross sections (TABLE II). At 30 days in vitro NF1 (-/-) cultures showed 1% as many as those from wildtype sections. Cultures with NF1 (+/-) fibroblasts contained 60% as many fascicles as wildtype cultures. In two experiments the number of fascicles was counted after 37 days in vitro. Cultures containing fibroblasts without neurofibromin showed 17.7% and 23.8% as many fascicles as those with wildtype fascicles. As the number of fascicles in cultures containing NF1 (-/-) fibroblasts was significantly higher after 37 days compared to only 30 days in vitro, it seems possible that the organizational deficit secondary to neurofibromin deficiency in fibroblasts might further improve over a longer incubation period. By 37 days, fascicle number in cultures containing NF1 (+/-) fibroblasts was similar to that in control cultures. When sections containing null fibroblasts were evaluated using

the electron microscope, it became apparent that even those regions of sections scored as containing fascicles at the light microscope level were in fact strikingly different from wild type controls (Figure 5). In cultures containing wildtype fibroblasts neurons and Schwann cells were grouped tightly together and surrounded closely by rings of fibroblasts that were further enclosed by multiple layers of fibroblasts (Figure 5A); few axon-Schwann cell units were excluded from these fascicles. In contrast, in cultures containing fibroblasts without neurofibromin even the most differentiated regions observed (Figure 5C) contained only a few axon-Schwann cell bundles separated by a single fibroblast process. More typically, axon-Schwann cell units were distant from the multiple fibroblast layers, and were not collected into bundles (Figure 5B). +/- cultures? Myelinated and non-myelinated axons were present in all cultures, independent of fibroblast genotype (Figure 5). Schwann cells were identified by association with axons. No evidence for Schwann cells that lost axonal contact could be found, either in wildtype cultures or in cultures containing NF1 (+/-) or NF1 (-/-) fibroblasts.

To define the phenotype of cells encircling bundles of axons and Schwann cells more definitively, sections of the same cultures were analyzed by electron microscopy at higher magnification. Perineurial cells are defined as elongated cells containing clear vesicles called caveolae and covered by basal lamina on both surfaces; this basal lamina may be patchy (Thomas, 1984; Peltonen, 1987). Cells with these characteristics were identified in cultures containing both neurofibromin-deficient and wild-type fibroblasts (Figure 6A,B), suggesting that even neurofibromin-deficient fibroblasts are capable of responding to cues from neurons and Schwann cells that result in perineurial cell differentiation. In addition, collagen fibers with similar diameter (nm) were present in wildtype and null cultures; fiber diameter is a characteristic of normal cultures containing

fibroblasts together with axons and Schwann cells. However, in cultures with wildtype fibroblasts cells with characteristics of perineurial cells were the same cells that ringed bundles of axons and Schwann cells; fibroblasts distant from axons and Schwann cells were progressively less differentiated. In contrast, in cultures containing fibroblasts lacking neurofibromin perineurial cell differentiation was observed in cells throughout the sections, with no preferential differentiation in cells close to axons and Schwann cells.

DISCUSSION

We have used cultures derived from neurofibromin deficient mice as a model system to study interactions among cells of the peripheral nerve that occur in the absence of the NF1 gene product, neurofibromin. While heterozygous mice in which the NF1 locus was targeted by homologous recombination do not develop neurofibromas (Brannan, 1994; Jacks, 1994), we have now shown that in homozygous null cultures derived from these mice several features of human neurofibromas are recapitulated. Cultures containing normal fibroblasts and Schwann cells form cordons with Schwann cells inside a sleeve of fibroblasts (Obremski, 1993b) , and fibroblasts form fascicles around axon-Schwann cell bundles when neurons are also present (Bunge, 1989; Williams, 1982; Williams, 1983). In contrast, as in human neurofibromas (Poirier, 1968, Hirose, 1986, Ushigome, 1986) , cultures consisting of normal neurons and Schwann cells together with neurofibromin deficient fibroblasts contain cells with the typical morphology of fibroblasts and cells with characteristics of perineurial cells that are close to but do not contact or segregate Schwann cells. In this sense the culture system we have developed models cellular behavior in neurofibromas. It has been suggested (Brannan et. al., 1994) that a feature of mice lacking neurofibromin is developmental delay. It remains possible that abnormalities we have observed in neurofibromin-deficient fibroblasts would be ameliorated with sufficient time in vitro.

Proximity to Schwann cells (and neurons) causes fibroblasts to differentiate into perineurial cells (Bunge, 1989) , and fibroblasts are known to stimulate Schwann cell basal lamina deposition (Obremski, 1993b) suggesting that reciprocal fibroblast-Schwann cell signals underlie nerve fascicle formation. It is possible that fibroblasts deficient in neurofibromin fail to recognize and/or respond to necessary signals from Schwann cells. However, fibroblasts lacking

neurofibromin must respond to signals from Schwann cells sufficiently to differentiate into perineurial cells as assessed by expression of cavelolae and basal lamina.

Alternatively, fibroblasts might recognize Schwann cell signals but fail to send additional information to Schwann cells that results in fascicle formation. The mechanisms underlying the abnormal fibroblast behavior we have observed remain unknown. Reciprocal signals between Schwann cells and fibroblasts that underlie peripheral nerve organization are also unknown (Bunge, 1993). We conclude from our results that neurofibromin is required for fibroblasts to form perineurium-like sheaths around axons and Schwann cells, and that the absence of neurofibromin in fibroblasts alone is sufficient to recapitulate the phenotype observed when neurons, Schwann cells and fibroblasts all lack neurofibromin. Our data suggest that molecules in the ras signalling pathway might be crucial for neuron-Schwann cell-fibroblast interactions, as the only known function of neurofibromin is as a modulator (or possibly effector) of ras signals (McCormick, 1994). It is currently not known if levels of ras-GTP are altered in the populations of neurofibromin-deficient fibroblasts studied here. While in some cells loss of neurofibromin is correlated with increased GTP-bound ras (Basu, 1992, DeClue, 1992, Kim, 1995) in other cell types such as melanoma and neuroblastoma cells (Johnson, 1994) and myeloid leukemia cells (Largaespada et. al., in preparation) it is not. Our data suggest that growth of mouse fibroblasts lacking neurofibromin is frequently, but not always, increased as compared to wild-type cells. Fibroblasts may be especially sensitive to fluctuations in levels of other gene products; this would result in increased growth only in certain cell populations. Differences between fibroblasts from different transgenic embryos might also reflect the influence of modifier genes which have been shown to affect the expression of the NF1 phenotype in humans (Easton, 1993), even on the relatively

homogenous C57 background. Several studies using human fibroblasts from neurofibromas reported altered growth as compared to normal fibroblasts. Krone et al. (1981) reported enhanced growth potential more often for NF fibroblast strains (in response to serum); Kadono et al. (1994) reported enhanced proliferation in response to TGFb1 and PDGF BB. Others reported no change in growth rate (Riccardi, 1981b) or even reduced growth (Zelkowitz, 1981b) , suggesting that heterogeneity may be characteristic of human as well as mouse fibroblasts with lowered neurofibromin.

Mouse fibroblasts heterozygous at the NF1 locus showed a phenotype intermediate between normal and null cells in cell growth and in at least early stages of fascicle formation. It is not known if abnormal cellular behavior in neurofibromas occurs in heterozygous cells or if loss of the previously normal somatic allelle of the NF1 locus is required. Loss of heterozygosity on human chromosome 17 was not detected in neurofibromas (Menon et. al, 1990; Skuse et. al., 1991); however, these data are inconclusive because if mutations at the NF1 locus were solely in fibroblasts they might be obscured by normal NF1 alleles present in the more abundant Schwann cells. While data presented here suggest that loss of one wildtype allele at the NF1 locus could contribute to functional deficits in fibroblasts resulting in disturbed perineurium function, genetic tests are required to ascertain if mutations are present in both NF1 allelles in neurofibroma fibroblasts.

The perineurial sheath acts as a perfusion barrier which regulates access of endoneurial cells to serum factors (Thomas, 1984). A failure of NF1 fibroblasts to form perineurium in vivo is predicted to result in a breakdown of this barrier function. Consistent with this view, plasma-derived fibronectin is present in neurofibromas (Peltonen, 1988). Breakdown of the perineurium could stimulate proliferation of endoneurial cells (fibroblasts and Schwann cells) by providing access to growth factors. We

have shown that hepatocyte growth factor is the major Schwann cell mitogen in neurofibromas (Krasnoselsky, in press) ; HGF is present in human and rodent serum (Hansson, 1988, Michalopoulos, 1992). Basic fibroblast growth factor (Ratner et. al., 1990) and IGF-1 (Schumacher, 1993) are also present in neurofibromas and are mitogenic for Schwann cells and for fibroblasts. Increased cell proliferation in response to serum growth factors might facilitate accumulation of mutations (at the NF1 locus or at other loci), contributing to neurofibroma formation. Thus, abnormalities in NF1 fibroblasts could provide the "ground" on which additional mutations lead to tumor formation.

While the behavior of fibroblasts in culture is strikingly similar to that of fibroblasts in neurofibromas, behavior of Schwann cells in the cultures does not mimic behavior in tumors. In neurofibromas Schwann cells occur without apparent contact with axons (Waggener, 1966, Poirier, 1968, Stefansson, 1982) , while we have not observed any Schwann cells lacking axonal contact in electron micrographs from many sets of cultures from cells without neurofibromin (Rosenbaum et. al., in preparation). This suggests that while loss of neurofibromin is sufficient to develop abnormalities in fibroblasts, it is insufficient to explain Schwann cell behavior in neurofibromas. Neurofibroma-derived human Schwann cells, like those from mice, associate with neurons and assemble basal lamina (Baron, 1991). It is possible that epigenetic events modulate Schwann cell behavior in neurofibromas. In contrast, genetic changes are likely to underlie formation of neurofibrosarcomas. Activation of ras in Schwann cells cooperates with other oncogenes to transform Schwann cells (Ridley, 1988) and malignant neurofibrosarcomas in NF1 frequently show mutations in p53 as well as at the NF1 locus (Menon et. al., 1990; Skuse et. al., 1991).

We conclude that fibroblasts might play an important role in the

formation of human neurofibromas. Our findings corroborate that neurofibromatosis should not be defined as a neurocristopathy (originally proposed by Bolande [1974]) as we have clearly demonstrated that loss of neurofibromin affects fibroblasts, cells of mesenchymal origin. Similarly, loss of neurofibromin is correlated with development of myeloid leukemia in humans and mice (Jacks, 1994; Shannon, 1994). However, we do not believe that neurofibroma formation can solely result from fibroblast abnormality. Neurofibroma derived Schwann cells clearly exhibit properties different from normal Schwann cells (Sheela, 1990, Kim, 1995) ; it is thus likely that Schwann cells also contribute to neurofibroma formation. We expect that use of the described cell culture system will enable further, mechanistic, studies of the roles of fibroblasts and Schwann cells in neurofibroma formation.

MATERIALS AND METHODS

Transgenic NF1 mice

C57BL/6J mice in which one allele of the NF1 gene has been targeted were generated as described elsewhere (Brannan, 1994). In order to obtain NF1 (-/-) embryos, male and female NF1 (+/-) mice were mated for 12-16 hours, then separated and female mice checked for the presence of a copulatory plug. Pregnant mice were sacrificed to obtain embryos after 12.5 days, with the day the plug was observed being day 0.5.

PCR conditions

For genotyping, embryo heads were removed, placed into 500 ul of lysis buffer (Laird, 1991), and incubated overnight at 55°C on a shaker. An equal volume of isopropanol was added and the mixture centrifuged for 10 minutes at approximately 10,000 rpm. The pellet was resuspended in 500 ul of TE (10 mM Tris.HCl, Ph 8.0; 1 mM EDTA, pH 7.5) and again incubated overnight at 55oC. After the DNA was completely dissolved 1 ul of the solution was used as a template for PCR together with 16 ul autoclaved H2O and 8 ul reaction mixture containing the following: 200 ng of each oligonucleotide primer (NeoTkp, 5'-GCGTGTTCGAATTCGCCAATG-3'; NF31a, 5'-GTATTGAATTGAAGCACCTTTGTTTGG-3'; NF31b, 5'-CTGCCCAAGGCTCCCCC AG-3'), 2.5 ul of 10x reaction buffer, 2 mM MgCl2 ,a mixture of dATP, dCTP, dGTP, and dTTP, each at a concentration of 0.2 mM, and 0.25 units Taq polymerase (Perkin-Elmer Cetus). Amplification was performed for 40 cycles using the following conditions: 94°C for 1 min., 55°C for 1 min., 72°C for 2 min. 8 ul of each amplication product was used for electrophoresis on a 1% agarose gel. A 194 bp band indicated a wildtype allele, a 340 bp band the mutant allele (Brannan et. al., 1994).

Tissue preparation from NF1 mice and embryonic rats

Tissue cultures were obtained from E12.5 mouse embryos and from E15 rat embryos essentially as described (Bunge, 1983; Kleitman, 1991; Wood, 1976). Pregnant females were anaesthesized, using methoxyflurane (MethofaneTM, Pitman-More), a hysterectomy performed and embryos dissected out of amniotic sacs. Embryos were decapitated in Leibovitz's L15 medium (Gibco). The spinal cord with dorsal root ganglia (DRG) attached was removed, and DRG enzymatically dissociated in 0.25% trypsin in Hanks' Balanced Salt Solution (Gibco) at 370 C on an orbital shaker at 40 rpm for 40 minutes at 37oC. Ganglia were transfered to approximately 10 ml of Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) and centrifuged at 800 rpm for 5 minutes. The pellet was resuspended in approximately 0.5 ml of "C medium" (DMEM containing 10% human placental serum [provided by the delivery room of a local hospital] and 50 ng/ml nerve growth factor [2.5S NGF, Harlan Bioproducts]) and mechanically dissociated using a narrowed bore Pasteur pipette. This cell suspension (containing neurons, Schwann cell precursors, and fibroblasts) was plated in single drops onto collagen-coated glass coverslips in 24 well plates, or on 35 mm dishes (Falcon), coated with ammoniated rat tail collagen. 5-6 individual drops of cell suspension were separately placed onto the hydrophobic collagen-substrate so that drops did not flow together. Cells were allowed to attach overnight at 35^{0}C and 7.5% C02. The following day dishes/wells were flooded with "C" medium. Medium was replaced at least twice per week.

Purified neuronal cultures

In order to obtain purified neuronal cell populations, rat cultures were treated with the antimitotics 5'-fluoro-2'-deoxyuridine and uridine (Sigma) added to "C" medium at 10-5 M

final concentration ("CF" medium) (Bunge, 1983). Cultures were cycled through two-three passages of C/CF; each medium was replaced after 3 days.

Preparation of rat primary Schwann cells

Primary Schwann cells were purified from sciatic nerve from postnatal day 1 rats as described previously (Brockes, 1979). Following isolation of Schwann cells by antimitotic treatment and complement-mediated lysis of residual fibroblasts, cells were maintained in DMEM plus 10% fetal bovine serum until plating onto neurons. In some experiments, Schwann cells were amplified for 1 week in recombinant human glial growth factor (rhGGF2, Cambridge Neuroscience) and 2 μM forskolin prior to addition to neurons.

Preparation of NF mouse fibroblasts

Fibroblasts from E12.5 mouse embryos were obtained when embryos were dissected for DRG preparation. After removal of internal organs, head, and spinal cord, remaining tissue of each embryo was transferred to a tube containing 2 ml of ice-cold 0.05% trypsin in 0.6 mM EDTA in Hanks' Balanced Salt Solution and incubated overnight at 4oC. The following day, tubes were incubated for 20 minutes at 37oC, approximately 10 mls of DMEM containing 10% FBS added and the cell suspension centrifuged for 5 minutes at 800 rpm. Cells were resuspended in 1 ml DMEM with 10% FBS then mechanically dissociated with a narrowed bore Pasteur pipette. The whole cell suspension was plated onto one 100mm tissue culture plastic dish containing 10-15 mls of DMEM, 10% FBS, 1% penicillin-streptomycin and maintained at 35°C and 7.5% C02 for several days until the cells were nearly confluent. Fibroblasts were used immediately or at up to 3 passages for experiments.

Preparation of neuron, Schwann cell, fibroblast co-cultures

Two week old purified rat neuronal cultures on a collagen-coated 35 mm dish were seeded with 0.3-0.5 x 10^6 rat Schwann cells in "C medium" and kept at 35°C and 7.5% CO2 overnight. After 24h cultures were switched to a defined "N2"-medium (Bottenstein, 1979) which stimulates Schwann cell proliferation but not myelination (Moya et. al., 1980) and maintained at 37°C and 10% CO2. After 3-7 days Schwann cells were distributed evenly over the whole neurite outgrowth. At this time medium was shifted to "C" supplemented with 50 µg/ml ascorbic acid (Sigma) to enhance Schwann cell differentiation (Eldridge, 1987). At the same time 0.3-0.5 x 10^6 NF mouse fibroblasts per dish were added; cultures were maintained at 35°C and 7.5% CO2.

Preparation of semithin sections

Cultures were fixed for 30 minutes at room temperature in 0.1 M sodium cacodylate buffer pH 7.4 containing 2% paraformaldehyde, 1.25% glutaraldehyde, and 100 mM sucrose. After 3 washes for 5 minutes each with 0.2 M cacodylate buffer, cultures were postfixed for 60 minutes at room temperature with 0.1 M cacodylate buffer containing 1% OsO4 and 1.5% potassium ferrocyanide, washed again and kept overnight at 4°C in 0.2 M cacodylate buffer. Cultures were dehydrated through 30%, 50%, and 70% ethanol (5 minutes each), stained en bloc for 30 minutes in 0.5% uranylacetate in 75% ethanol, further dehydrated in 85%, 95%, and 100% ethanol and embedded in Epon.

Culture analysis

For EM analysis ultrathin sections were stained with lead citrate and uranyl acetate and photographed on a JOEL-100 CX electron microscope at 4-50,000 magnification. Organotypic cultures derived from 4 wildtype, 14 heterozygous and 7 null embryos were evaluated; 1-2 areas/culture were analyzed. For experiments in which fibroblasts of different genotype were added to normal neurons and Schwann cells for quantitative

analysis, 3-8 different areas of each culture were evaluated. Cultures from a total of 4 different embryos were analyzed for each genotype. For all cultures, each block was selected 2 mm outside areas containing neuronal cell bodies, areas that are maximally differentiated (Obremski, 1993a). Semithin plastic sections were analyzed at 400x magnification for fascicle number. A fascicle was defined as a group of axons and Schwann cells encircled for at least 75% of its circumference by one or more elongated fibroblasts.

FIGURE LEGENDS

Table 1. Increased numbers of non-neuronal cells are present in some -/- DRG cultures. Dissociated dorsal root ganglian cells were plated onto plastic (24 well) dishes (2 ganglion-equivalents/well) in DMEM + 10% human placental serum + 50 ng/ml NGF. After the designated number of days in vitro (days i.v.). Cells were rinsed with PBS and dissociated in 0.25% trypsin (exp. 1) or collagenase followed by trypsin (exps. 2 + 3). In 2 of 3 experiments an increased number of non-neuronal cells was recovered from -/- cultures; these are cells that include fibroblasts, macrophages and Schwann cells. 1n = number of different embryos; 2 cultures from each embryo were evaluated; 2Total cell number (+/- standard deviation) was obtained by counting cells in a hemocytometer.

Table 2. Reduced fascicle formation in cultures of normal rat neurons and Schwann cells cultured with mutant mouse fibroblasts. Rat sensory neurons were fully populated with rat Schwann cells. Mouse fibroblasts of different genotypes were added and cultures maintained for the designated number of days in differentiation-promoting medium. After fixation and embedding fascicle formation was evaluated in semithin plastic sections. Within each experiment, fascicle number was averaged over all blocks analyzed (3-9 blocks/embryos). Note that variability in number of fascicles per section generated large standard errors.

Figure. 1 Morphology of 9 day old organotypic cell cultures from neurofibromin deficient mice by phase-contrast microscopy. Cell suspensions containing neurons, Schwann cell precursors, and fibroblasts were obtained from embryonic day 12.5 mice and plated onto collagen-coated 35 mm dishes. Neurons were identified as round phase dark cells (white arrowheads)

with prominent nuclei and nucleoli that extended processes. Schwann cells were defined based on their attachment to neuronal processes (arrow),fibroblasts formed a cellular background layer (black arrowhead). While neuronal processes and accompanying Schwann cells were easily identified in wildtype (A) and heterozygous (B) cultures., in many homozygous null cultures (C) Schwann cell-covered axons appeared more obscured.

Figure 2. Increased proliferation of neurofibromin-deficient fibroblasts. Fibroblasts isolated from E12.5 mouse embryos were plated onto 24 well plates in DMEM with 10% FBS at a density of 0.4x104 cells/well. Every second day after plating, cells were trypsinized and cell numbers were determined by counting duplicate samples in a hemocytometer. Data are representative of results from 8 of 10 embryos of different litters.

Figure 3. Failure of organization in cultures lacking neurofibromin.Cell cultures containing neurons, Schwann cells, and fibroblasts were obtained from wildtype, NF1 (+/-) or NF1 (-/-) mouse embryos, fixed after 18 days in vitro and analyzed in semi-thin plastic sections. Wildtype cultures showed a high level of organization with axon-Schwann cell units sandwiched between multiple layers of elongated fibroblasts (A). In most neurofibromin-deficient cultures fibroblasts were loosely arranged in two multi-layers sandwiching axon-Schwann cell units (C). In some neurofibromin deficient cultures this organizational pattern was completely disrupted (D) and fibroblasts were intermingled with axon-Schwann cell units. Cultures from NF1 (+/-) animals exhibited an intermediate phenotype (B). Arrows point to layers of fibroblasts above and below axon-Schwann cell units, arrowheads to axons associated with Schwann cells. In C, * denotes spaces between adjacent fibroblasts layers.

Figure 4. Fibroblasts lacking neurofibromin fail to fasciculate axons and Schwann cells. Fibroblasts derived from E 12.5 mouse embryos were added to cultures of normal rat neurons fully populated with rat Schwann cells. 37 days after addition of fibroblasts, cultures were fixed and analyzed in semi-thin plastic sections. In cultures containing wildtype fibroblasts (A) axon-Schwann cell units were grouped together in compact bundles and surrounded by flattened fibroblasts (arrows). In cultures containing neurofibromin deficient fibroblasts (C) fascicle-formation was incomplete and endoneurial space was increased (asterisk). Cultures with NF1(+/-) fibroblasts (B) appeared well organized but had a slightly increased endoneurial space.

Figure 5. Cultures were prepared as described in Fig. 4. In cultures containing wildtype fibroblasts (A) axon-Schwann cell units (arrowhead)were largely organized into compact fascicles enclosed by multiple layers of flattened fibroblasts (arrows); occassional axons ensheathed by Schwann cells were outside fascicles (curved arrow). Fascicles were sandwiched between layers of fibroblasts separated by collagen fibers (open arrowheads). In cultures with fibroblasts without neurofibromin (B), axons esheathed by Schwann cells (arrowheads) were not collected in groups but were enclosed by single fibroblast processes (arrows) or remained distant from overlying fibroblasts (C).

Figure 6. Perineurial cells differentiate in the absence of neurofibromin. Cultures were prepared as described in Fig. 4. Both wildtype (A) and neurofibromin-deficient (B) mouse fibroblasts exhibit characteristics of perineurial cell differentiation. Cells become elongated, are covered with patchy basal lamina (open arrows) on both sides and contain caveolae (arrows). Collagen fibers of similar diameter are

present in both cultures (arrowheads). Basal lamina also covered Schwann cells in cultures (curved arrows). In neurofibromin-deficient cultures, however, relatively undifferentiated fibroblasts (with distended rER) occurred adjacent to axon-Schwann cell units (double arrow).

	Genotype	Days i.v.	n_1	Total cell # x 10^5 $_2$
Experiment 1	+ / -	21	2	2.1
	- / -	21	1	5.7
				(2.7x fold increase)
Experiment 2	+ / +	12	6	5.3 ± 0.3
	+ / -	12	6	4.2 ± 0.3
	- / -	12	2	12.8 ± 5.0
				(3.6x fold increase)
Experiment 3	+ / +	19	1	1.3
	+ / -	19	2	1.1
	- / -	19	2	1.0
				(no increase)

	Experiment 1		Experiment 2
	No. of fascicles ± SEM		No. of fascicles ± SEM
	30 days	37 days	37 days
Genotype			
+ / +	9.3 ± 5.7	12.4 ± 14.0	8.0 ± 7.7
+ / -	5.5 ± 4.9	not done	10.7 ± 3.7
- / -	0.1 ± 0.2	2.2 ± 4.4	1.9 ± 2.7

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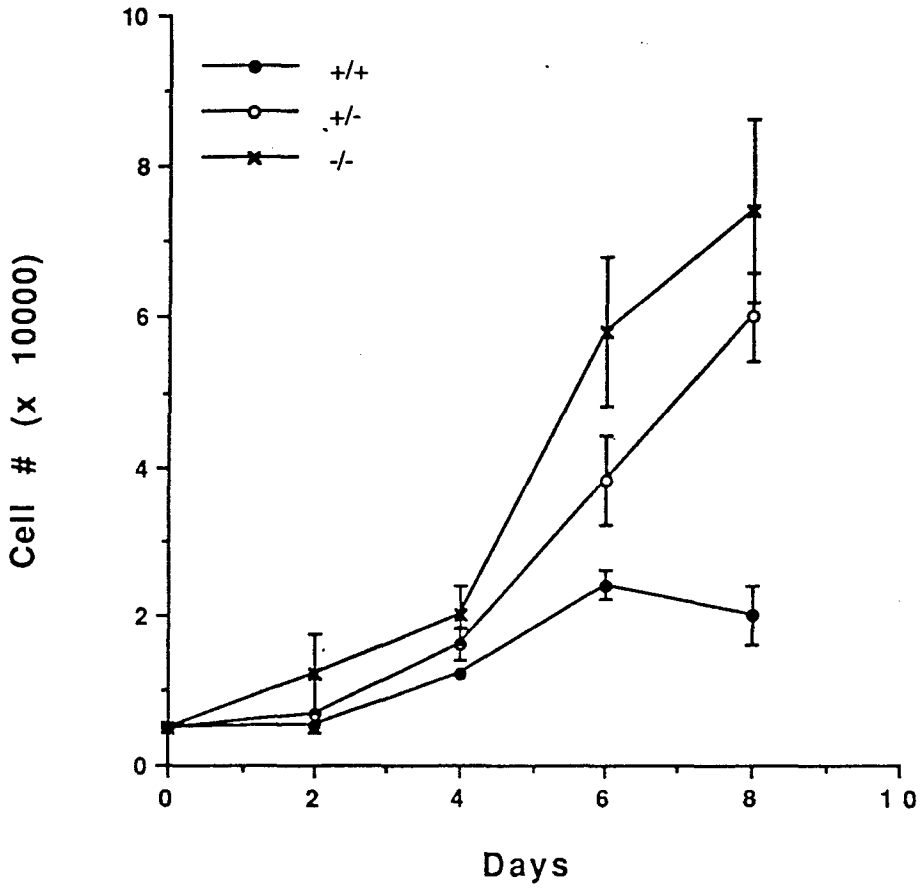

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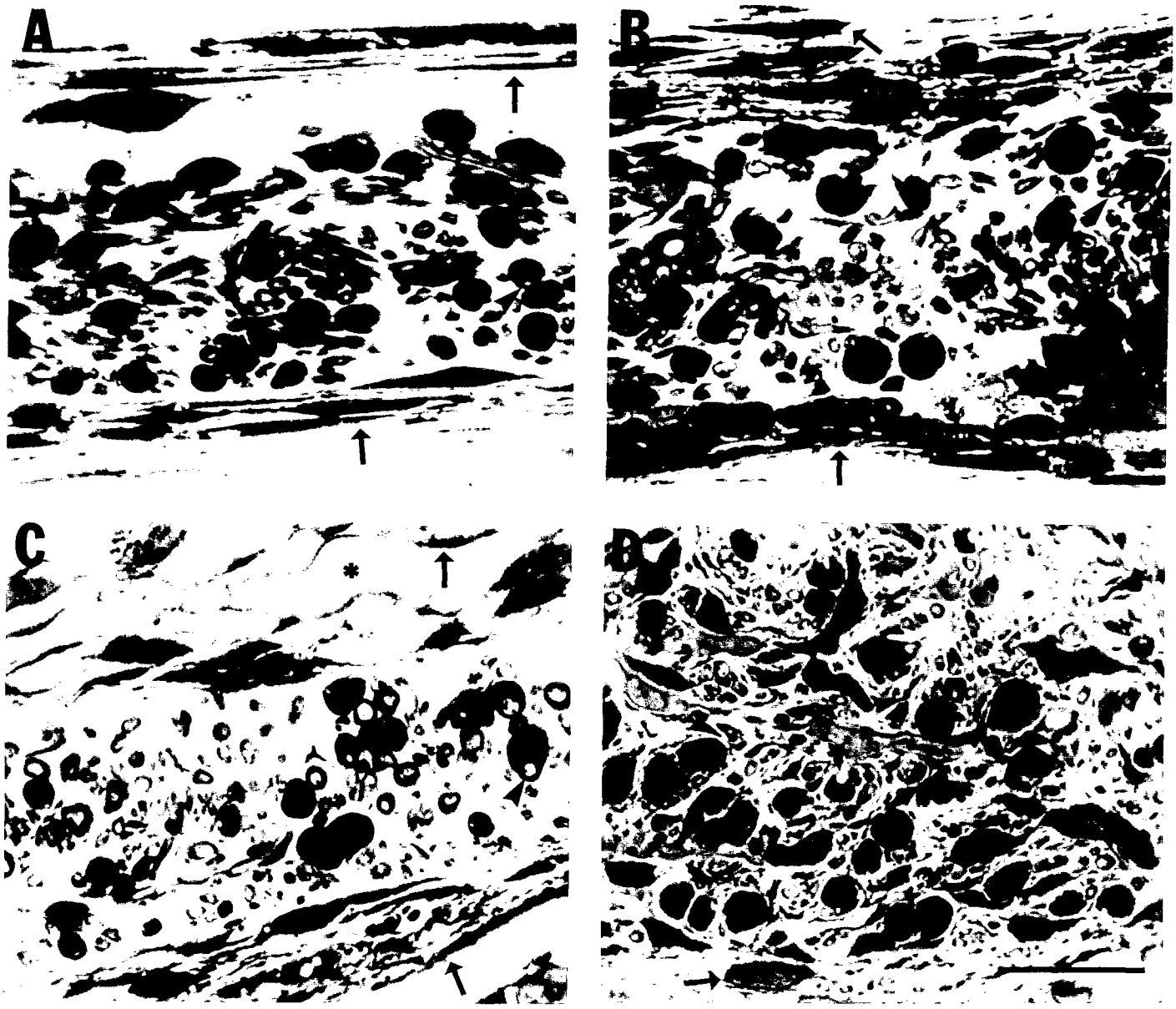

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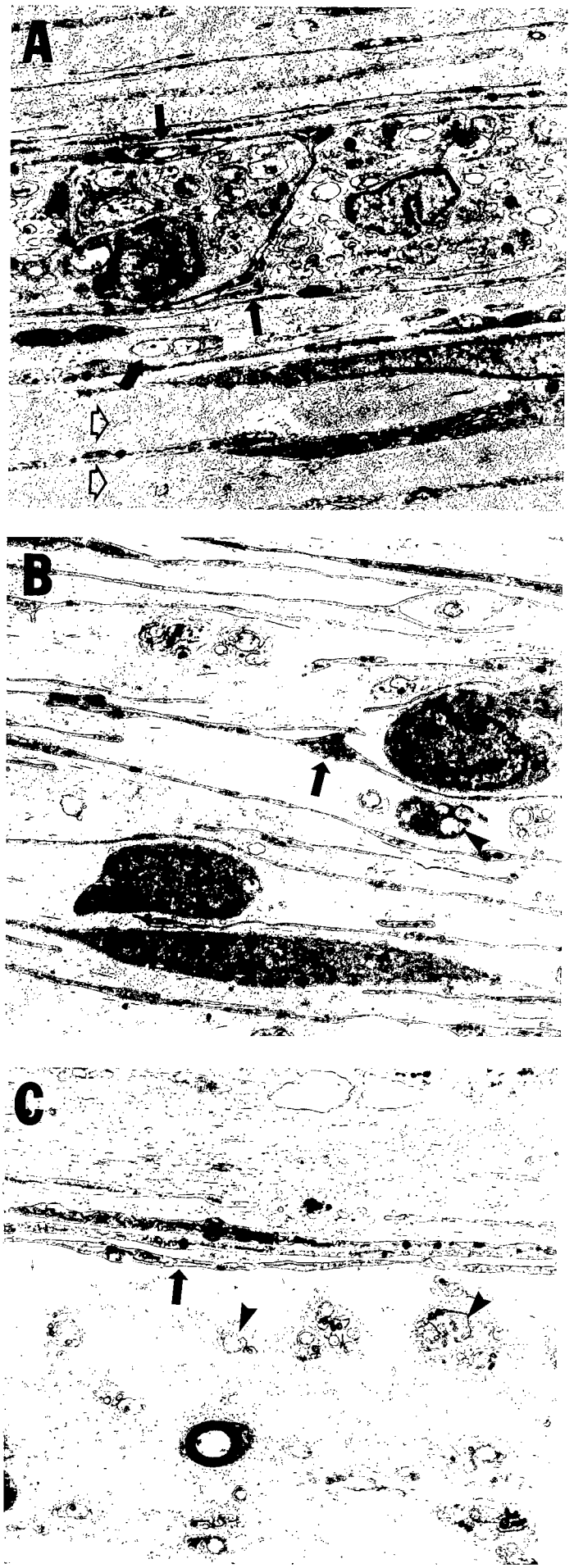
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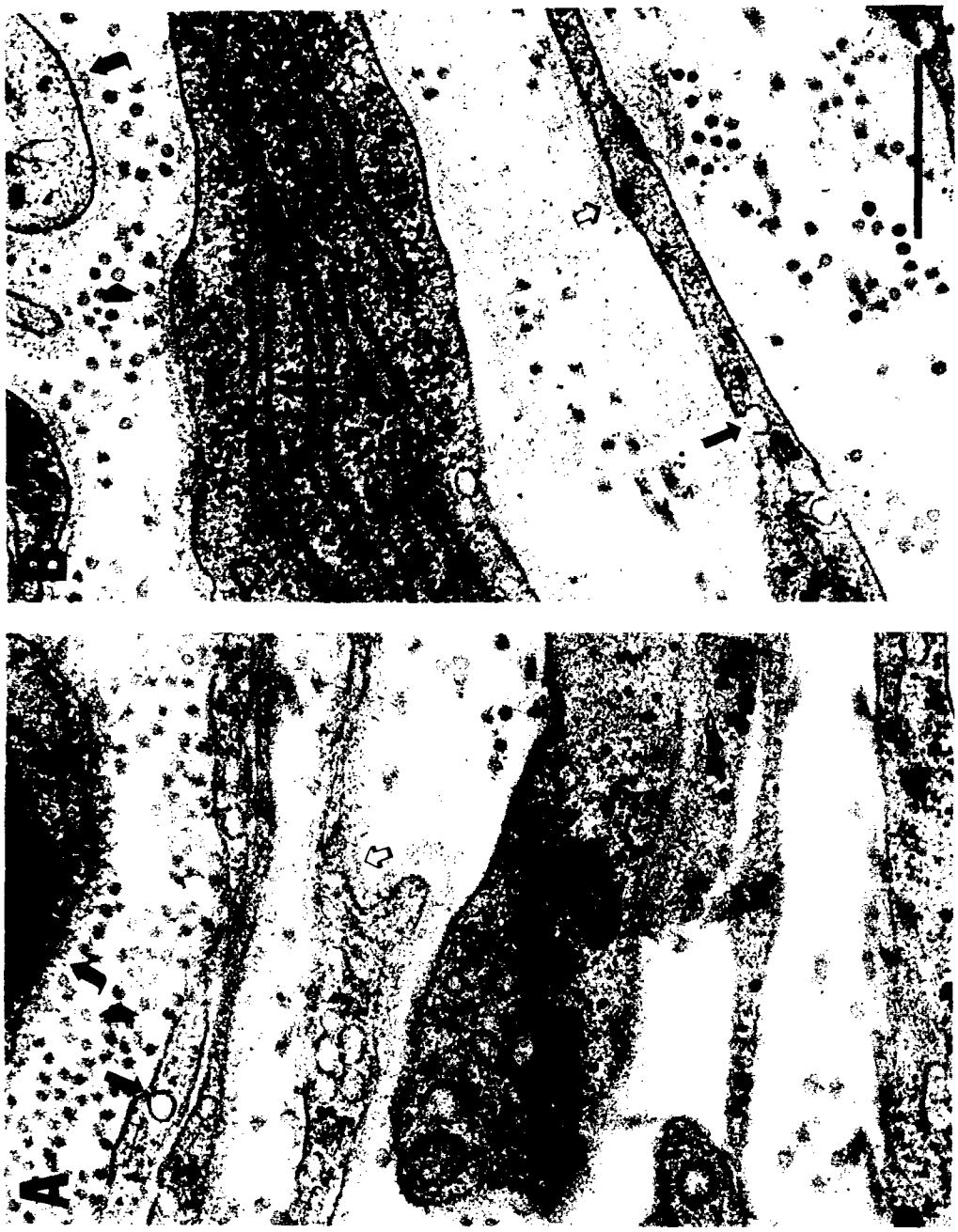


CHAPTER 7: NEUROFIBROMIN AND CALCIUM HOMEOSTASIS IN EMBRYONIC FIBROBLASTS

INTRODUCTION

von Recklinghausen's Neurofibromatosis (NF-1) is an autosomal dominant inherited disorder affecting 1 in 3500 humans (Riccardi, 1981). The hallmark of the disease is the development of neurofibromas which are peripheral nerve sheath tumors composed chiefly of fibroblasts, Schwann cells, and axons. The role of the different cell types in neurofibroma formation is unclear.

The gene that confers predisposition to NF-1 has been cloned and sequenced. The gene product, neurofibromin is a 220kDa protien and contains a region homologous to the mammalian GTPase activating protein (GAP) (Xu, et al., 1990). A fragment of neurofibromin contains the GAP-related domain (GRD) which can stimulate the activity of N-ras (Martin, et al., 1990) and H-ras (Ballester and Collins, 1990) *in vitro*. In addition, higher basal levels of ras-GTP are present in Schwann cells (Kim, 1995) and fibroblasts (van der Geer, 1995) lacking neurofibromin. The details of the putative connections between the loss of neurofibromin, deregulated ras, and neurofibroma formation are vaguely understood.

Neurofibromin is enriched in endoplasmic reticulum (ER) of the central nervous system neurons (Nordlund, et al., 1993). The ER contains important components of the Ca^{2+} regulatory system. It is the major site for intracellular calcium storage. These pools are targets of extracellular signals that release Ca^{2+} via the action of second messengers such as the binding of inositol 1,4,5-trisphosphate (IP3) on its receptor located in the ER membrane (Berridge, 1989). Extracellular signals leading to calcium release can also be transduced via pathways involving

p-21 ras. It is known that ras-GTP is essential for the release of calcium from intracellular stores in NIH3T3 cells (Lloyd, 1989, Maly, 1991). Based on the localization and the p21-ras-GAP activity, we hypothesized that neurofibromin might indirectly modulate intracellular calcium homeostasis.

To test this hypothesis, we prepared day 12.5 embryonic fibroblasts from the NF-1 knockout mice (Brannan, 1994) and wild-type day 12.5 mouse embryos. Using single cell imaging of fura-2AM loaded cells, we have measured release of calcium from intracellular stores in response growth factors and thapsigargin, an endoplasmic reticulum (ER) calcium ATPase inhibitor. We have also used ^{45}Calcium ($^{45}Ca^{2+}$) to monitor uptake and efflux across the plasma membrane (PM) and E.R. membranes in intact and permeablized fibroblasts. We have found variable differences in cellular uptake of calcium across the PM and the ER. Effects of loss of NF1 on the efflux of $^{45}Ca^{2+}$ in the null fibroblasts were measured also measured in collaboration with Mitch Villereal who is an expert in these assays; no differences in the kinetics of relase were observed from the wild-type cells.

RESULTS

^{45}Calcium Uptake:

Calcium uptake across the plasma membrane and into intracellular compartments was measured by incubating intact adherent fibroblasts for various time points in HBSS with 2μCi/ml of $^{45}Ca^{2+}$. Dishes were quickly rinsed three times in either isotonic $MgCl_2$-EGTA or $LaCl_3$-HBSS buffers to chelate any unincorporated $^{45}Ca^{2+}$ and block any further exchange of calcium. In four experiments done using $MgCl_2$-EGTA buffer for washes, we did not observe any differences in uptake. However, in the presence $LaCl_3$-HBSS buffer, we observed lower uptake in -/- fibroblasts in comparison to +/+ fibroblasts in 3 out of five experiments (Fig.1). In the remaining two experiments, we did not observe any differences between genotypes. In seven additional experiments, we observed low total uptake (less than 4 nmol $^{45}Ca^{2+}$/mg of protein) in wild-type cells, but further decrease in uptake by -/- fibroblasts in five of those experiments. Data from three representative experiments, showing calcium uptake by 20 minutes are shown in Table 1.

In an attempt to understand why we observed lower uptake in 60% of the experiments and no difference in other experiments, we measured uptake under variable conditions. The following variables were tested: no shaking and different speeds on the orbital shaker at room temperature and 37 degrees. None of these variables systematically improved the reliability of the assay or the extent of the total calcium uptake.

It is possible that failure to observe differences in calcium uptake between genotypes represents intra-embryonic differences. In all experiments, cell from at least 2 different embryos were pooled to generate cells for experiments. The variation in total uptake in wild type cells between experiments remains unexplained

To define the cellular compartments that show abnormal

calcium uptake and in an attempt to develop a more reliable assay, cells of all three genotypes were permeabilized with saponin and treated with mitochondrial inhibitors to block calcium uptake into mitochondria. In the presence of an an exogenous ATP regenerating system, the major uptake of calcium from the media is into endoplasmic reticulum compartments (Chueh and Gill, 1986). $^{45}Ca^{2+}$ was added to cells gentle stirring and aliquots removed at intervals; all-associated radioactivity was removed from free calcium by filtration. The result from a representative experiment is shown in Figure 2. While permeabilized wild type cells efficiently took up calcium, heterozygous cells were less efficient, and cells null at the NF1 locus took up only 20% as much calcium as did wild type cells at 5 minutes. While significant variability was again evident in the absolute maximum in uptake between experiments, in 6 experiments null cells took up about 25% as much calcium as did wild type cells. Note that total uptake into permeablized +/+ cells varied from a low of 4 to a high of 10 nm/mg of protein in different experiments. These studies suggest that uptake of calcium into one or more endoplasmic reticulum stores is deficient in cells lacking neurofibromin.

We also examined $^{45}Ca^{2+}$ uptake across the E.R. membrane, in saponin-permeablized adherent fibroblast. Experiments done with adherent permeablized fibroblasts did not yield reliable data; the cells consistently detached upon the addition of the buffer.

Finally, to elucidate any differences in uptake at the plasma membrane, we conducted additional $^{45}Ca^{2+}$ uptake experiments for 20 minutes with the +/+ and -/- fibroblasts in the presence of E.R. calcium ATPase inhibitor, thapsigargin. In the presence of thapsigargin, the calcium excanging ability of the plasma membrane can be effectively tested. Results from five experiments, demonstrated a similar percentage decrease in uptake by the +/+ and -/- fibroblasts preincubated with thapsigargin in comparison to control conditions (Table 2).

Once again, there was considerable variability in the level of total $^{45}Ca^{2+}$ uptake observed in both +/+ and -/- cells in control and thapsigargin pretreated conditions. Thus, the experiments done under thapsigargin preincubation conditions did not elucidate any differences in uptake across the plasma membrane.

Different techniques used in quantitating $^{45}Ca^{2+}$ uptake in the fibroblasts suggests that upake at the endoplasmic reticulum may be altered in -/- fibroblasts, but the variability in total calcium uptake by the cells of the same genotype make drawing strong conclusions difficult.

Calcium Release:

If calcium is taken up more slowly in cells without neurofibromin, then either stores may contain less calcium at steady state in these cells or stores may fill to the same magnitude but a longer time is required to reach steady state. To distinguish these hypotheses, we analyzed release of calcium from intracellular stores using the fluorescent dye FURA-2AM. In the mid-term report, we reported that no significant differences were observed in intracellular calcium basal levels, amount of calcium released, and the rate of release in response to growth factors and thapsigargin (Fig. 3-5). For further details, refer to the mid-term report.

In order to complete the characterization of intracellular calcium pool sizes, $^{45}Ca^{2+}$ efflux assays were perfomed to determine any difference in the sizes of the intracellular calcium pools and rate of intracellular calcium release. Wild-type and null fibroblasts were loaded with $^{45}Ca^{2+}$ (1uCi/ml) for 24 hours and then $^{45}Ca^{2+}$ efflux assays and calculations were performed as described in the methods. Kinetic analysis of the $^{45}Ca^{2+}$ efflux curves showed no significant difference in release of $^{45}Ca^{2+}$ from the fast (K_1), and slow (K_2) exchanging compartments (Borle, 1970, Owen and Villereal, 1983) of the +/+

and -/- fibroblasts (Fig. 6). This result suggests that the intracellular calcium pool sizes are not detecably different.

However, In 4/10 experiments, we observed a significant difference in the level of intracellular $^{45}Ca^{2+}$ in the -/- fibroblasts in the begining of the experiment. (Fig 6), even though kinetic analysis of these curves did not show differences in $^{45}Ca^{2+}$ release between the genotypes. The source of this extremely rapidly exchanging calcium pool is unknown. Additional experiments were performed in an attempt to characterize this initial difference in intracellular $^{45}Ca^{2+}$ levels between the genotypes. However, we were unable to reliably detect this difference under different rinsing, shaking, and temperature conditions.

We also examined the levels of one of the calcium binding proteins which have been postulated to contribute to the rapidly exchanging calcium pools in the cell. Calreticulin has been shown to be the most abundant calcium binding protein in fibroblasts (Opas, 1991). Western blot analysis of calreticulin protein levels in +/+ and -/- fibroblasts did not reveal any significant differences (data not shown). Thus, the major calcium binding protein pool is not the major contributor to the difference observed in the extremely rapidly exchanging calcium pool.

The data suggest that +/+ and -/- fibroblasts cultured under similar conditions do not show any reproducible alterations in intracellular calcium pool sizes, suggesting that differences in uptake could reflect that longer times are required to reach steady state.

DISCUSSION

$^{45}Ca^{2+}$ uptake and efflux assays with fibroblasts revealed the following: fibroblasts lacking neurofibromin appear to have altered ability to take up calcium, but are comparable to wild type fibroblasts in intracellular calcium pool sizes and their ability to release calcium.

In the mid-term report, we reported that in comparison to wild-type fibroblasts there was a significant decrease in uptake by permeablized and intact null fibroblasts. We have conducted additional experiments to confirm the preliminary result, but have been discouraged by the inability to achieve consistent uptake in fibroblasts. Furthermore, there are inherent varibles in the different techniques that have not as yet been adequately controlled. It remains possible that intraembryonic differences may also contribute to the variability that was observed between experiments. We have invested considerable effort to isolate the source(s) of the variability in these experiments under the guidance of our two collaborators, Dr. Stauderman and Dr. Villereal who are experts in the field and we have been unsuccessful.

Based on the data obtained to date we postulate the following tentative model: neurofibromin might function in cordination with other protein(s) involved in calcium exchange; consequently, in the absence of neurofibromin calcium uptake into intracellular stores is slower, but ultimately fill to normal levels.

MATERIALS AND METHODS:

Transgenic NF1 mice:

C57BL/6J mice in which one allele of the NF1 gene has been targeted were generated as described (Brannan, 1994). In order to obtain NF1 (-/-) embryos, male and female NF1 (+/-) mice were mated for 12-16 hours, then separated and female mice checked for the presence of a copulatory plug. Pregnant mice were sacrificed to obtain embryos after 12.5 days, with the day the plug was observed being day 0.5.

PCR conditions:

For genotyping, embryo heads were removed, placed into 500 µl of lysis buffer (Laird, et al., 1991), and incubated overnight at 55°C on a shaker. An equal volume of isopropanol was added and the mixture centrifuged for 10 minutes at approximately 10,000 rpm. The pellet was resuspended in 500 µl of TE (10 mM Tris.HCl, Ph 8.0; 1 mM EDTA, pH 7.5) and again incubated overnight at 55°C. After the DNA was completely dissolved 1 µl of the solution was used as a template for PCR together with 16 µl autoclaved H2O and 8 µl reaction mixture containing the following: 200 ng of each oligonucleotide primer (NeoTkp, 5'-GCGTGTTCGAATTCGCCAATG-3'; NF31a, 5'-GTATTGAATTGAAGCACCTTTGTTTGG-3'; NF31b, 5'-CTGCCCAAGGCTCCCCC AG-3'), 2.5 ml of 10x reaction buffer, 2 mM MgCl2 ,a mixture of dATP, dCTP, dGTP, and dTTP, each at a concentration of 0.2 mM, and 0.25 units Taq polymerase (Perkin-Elmer Cetus). Amplification was performed for 40 cycles using the following conditions: 94°C for 1 min., 55°C for 1 min., 72°C for 2 min. 8 ul of each amplication product was used for electrophoresis on a 1% agarose gel. A 194 bp band indicated a wildtype allele, a 340 bp band the mutant allele (Brannan, 1994).

Preparation of Fibroblasts:

Cells were obtained from 12.5 day mouse embryo. The head, internal organs, limbs, tail, and spinal cord were dissected out of each each embryo and the remaining torso was used as the primary tissue source. The tissue was transferred to individual 15 ml centrifuge tubes (FISHER) containing 2 mls of .25% trypsin-EDTA (GIBCO) and incubated at 4 degrees overnight. To activate the trysin, the tubes were incubated in a 37 degree environmental incubator and shaken at 75rpm for 30 min. The tissue was triturated approximately 15 times with a glass pasteur pippette to create a single-cell suspension. The trypsin was inactivated by adding 6 mls of Delbecco's Modified Eagles Media (DMEM, GIBCO) with 10% fetal bovine serum (Harlan Inc.). The cell suspension was centrifuged at 800 rpm in a clinical centrifuge. After removing the trypsin containing media, the pellet was resuspended in fresh DMEM with 10%FBS, subcultured in individual 100mm plates, and maintained at 35 degrees in 7.5% CO_2. After determining the genotype of the embryos (Rosenbaum, 1995), identical genotype cells were pooled and used for experiments between passage 2 to 4.

Growth factor stimulated calcium relase:

Loading of Fibroblasts with FURA-2AM:

10,000 cells were plated onto round glass coverslips (Fisher) glued into holes bored into 35mm tissue culture dishes (Curtin Matheson Scientific, Florence,KY). 24-72 hours later, cells were washed (1 ml each time) twice with Hepes-bufferred Krebs buffer (HBK) (118mM NaCl, 4.6mM KCl, 10mM glucose, 25mM Na-Hepes (pH=7.4), 1.2mM MgSO4, 1.1mM CaCl2, 0.1% BSA) (Stauderman, 1990). Cells were loaded in HBK containing 3uM FURA-2AM (Molecular Probes, Eugene,OR) for 30 min. at 37°C, then washed twice with HBK again (Stauderman, 1990). Cells were imaged at room temperature within 30 minutes after loading. Cells imaged in the presence of external calcium were bathed in HBK excluding added calcium and 300μM EGTA.

Imaging of Fibroblasts:

Single cell imaging analysis was perfomed using the INCA Plus Plus imaging system developed at University of Cincinnati. The cells were excited alternately with 340 and 380 nM wavelengths by computer controlled switching of filters in front of a 300 watt Xenon arc lamp. Emitted fluorescence was directed through dichroic mirror and barrier filter in the macular objective then to a Cohu Integrating CCD camera. Image paris were routinely captured every 5-8 seconds.

Estimation of Intracellular free calcium:

Solutions with known calcium concentration (0-650nM) in 2uM Fura-2 free acid (Molecular Probes,Oregon) were imaged as above. 340/380 ratio values were used to derive a standard curve, and used to calculate average calcium in each selected cell as previously described (Grynkiewicz, 1985, Wahl, 1990)

Drug additions:

Bradykinin (Sigma), bombesin (Sigma),PDGF (R&D Systems,Minneapolis, MN) thapsigargin (Sigma) in 1ml were each delivered with a pipetteman to the edge of the coverslip containing adherent cells and 1ml buffer.

Image Analysis:

In each field chosen, 4-8 cells were selected and continously analyzed using Inca Graphics. This program calculates and displays the intracellular calcium concentration for each time point of each selected cell.

^{45}Calcium Uptake with adherent intact cells:

300,000 cells were subcultured in 35mm tissue culture dishes (FISHER) 24 hours prior to the start of the experiment.

The cells were rinsed twice in Hepes-buffered Hanks' balanced salt solution (Hepes-HBSS, pH 7.45, bicarbonate-free) and then incubated for an appropriate time in Hepes-HBSS and 2uCi/ml $^{45}Ca^{2+}$ (DUPONT/NEN) for the indicated time at 37 degrees. The experiment was terminated by aspirating the assay media and rinsing dishes four times in ice cold wash buffer (118mM NaCl, 4mM KCl, 1.2mM $MgCl_2$, 1mM $LaCl_3$ (FLUKA)). Cells were extracted in .2% SDS and scintillation fluid (Scintiverse BD, FISHER) was added to the alliquot of cell extracts before counting on Beckman scintillation counter. Each time point was measured in triplicates.

To test the uptake of cells in the presence of calcium ATPases inhibitors, such as thapsigargin, cells were maintained in Hepes-HBSS for 10 min. at 37 degrees and then pretreated with 1uM of thapsigargin (SIGMA) in Hepes-HBSS for 20 min at 37 degrees. Finally, the cells were rinsed twice in Hepes HBSS and immediately used in the uptake assay as described above. Control cells were incubate in Hepes HBSS for 30 minutes at 37 degrees prior to the addition of $^{45}Ca^{2+}$.

$^{45}Ca^{2+}$ influx is expressed as nmol/mg protein and was calculated by dividing the total $^{45}Ca^{2+}$ uptake by the specific acitivity of the uptake buffer and the protein concentration of each sample. Protein concentrations were measured using the Lowry method (Lowry et al, 1951) or the DCII Protein Assay kit (BIORAD).

^{45}Calcium Uptake with Permeablized Adherent cells :
200,000 cells were subcultured in 35mm tissue culture dishes for 48 hours prior to the start of experiment. The cells were permeablized with saponin in Intracellular Buffer (IB) (140mM KCl, 10mM NaCl, 2.5mM $MgCl_2$, 10mM Hepes, pH 7.05) for 5 minutes at 37 degrees. After the permeablization, cells were gently rinsed in IB with mitochondrial inhibitors (1mM NaN3 and 1ug/ml oligomycin, SIGMA) incubated for 15 minutes in the same buffer

at 37°C. The cells are incubated in uptake buffer (IB, 2uCi/ml $^{45}Ca^{2+}$, 150uM $CaCl_2$, 250um EGTA, and mitochondrial inhibitors) with or without the ATP-regenerating compounds (40mM phosphocreatine, 12.5 U/mL creatine phosphokinase, 2mM ATP, SIGMA) for the indicated time. The experiment is terminated by aspirating the uptake buffer and gently rinsing cells three times in ice-cold wash buffer (IB, 1mM $LaCl_3$, FLUKA). Calculations are performed as described above.

^{45}Calcium uptake with permeablized suspended cells :
Plates of embryonic fibroblast were washed, removed from dishes with trypsin-EDTA, centrifiged, rapidly resuspended in 1-2 ml "intracellular buffer" (140mM KCl, 10mM NaCl, 2.5mM $MgCl_2$, 10mM HEPES pH=7.05), the recentrifuged (3 minutes at 800rpm) and resuspended in 1ml intracellular buffer. Cells were diluted to 1x106 cells\ml in the same buffer then saponin (SIGMA) added from a 0.5% stock (in H_2O) to a final concentration of 0.01%. Cells were incubated in saponin for 5 min. at 37oC, immediately centrifuged, and resuspended in 1ml intracellular buffer. Under these conditions, 95% of cells were permeabilized as assessed by exclusion of trypan blue. The pellet was washed with 12 ml intracellular buffer, centrifuged again, and the pellet resuspended in 1ml intracellular buffer (room temperature) with 10ug\ml oligomycin and 1mM sodium azide. Cells were counted again and diluted to 0.2 - 0.5 x 10^6 in the same buffer, placed into an ice bath with gentle stirring using a small stirring bar in a scintillation vial. Cells were used for experiments within 2 hours after addition of mitochondrial inhibitors; essentialy as previously described (Chueh and Gill, 1986).

To initiate uptake, cells were warmed to 37°C for exactly 2 minutes, diluted 2-fold with the same buffer to which was added 40mM phosphcreatine, 12.5 units\ml creatine phosphokinase, 2mM ATP, 500uM EGTA, 100uM $CaCl_2$ and 6% polyethylene glycol (PEG 6000), and 16uCi\ml $45CaCl_2$ (320Ci\mol final) (freshly prepared)

at room temperature. At 1 minute intervals, aliquots of cells were removed and filtered onto Scleicher and Scheull #31 glass filters. Filters were counted in Scintiverse; protein concentrations were calculated from residual cells in the final mixture using 200ul aliquots.

^{45}Calcium efflux with adherent intact cells:

Efflux experiments and calculations were performed as described in Owen et. al. Briefly, cells were used 48hrs following subculture and while they are still in the logarithmic phase of growth. The cells were loaded with $^{45}Ca^{2+}$ (1uCi/ml) for 24 hours in DMEM +10%FBS. Efflux is initiated by removing the labelled medium, followed by 2 brisk rinses and the addition of fresh isotope free medium containing 1.3mM calcium. At indicated times, the medium was removed and replaced with an equal amount of fresh medium. Throught out the assay, dishes were kept at 37 degrees and shaken at 100rpm. Radioactivity in the alliquots was quantitated in a scintillation counter. At the end of the experiment, the cells were extracted with 0.2% sodium dodecyl sulfate and the protein content was determined using the Lowry assay (Lowry, 1951).

FIGURE LEGENDS

Figure 1 and Table 1. $^{45}Ca^{2+}$ Uptake into intact embryonic fibroblasts is reduced in the absence of neurofibromin. Intact fibroblasts were loaded with labelled calcium as described in materials and methods. Then celluar proteins were extracted and labelled calcium uptake was quantitated as explained in methods. Triplicate wells were analyzed at each time point. The table indicates data from representative experiments with "low" uptake.

Table 2. $^{45}Ca^{2+}$ Uptake into intact embryonic fibroblasts is unaltered after pretreatment with thapsigargin. Adherent fibroblasts were pretreated with 1uM thapsigargin for 20 min. Then, the fibroblasts were loaded with labelled calcium as described in materials and methods. Triplicate wells were analyzed at each time point.

Figure 2. $^{45}Ca^{2+}$ Uptake into permeabilized embryonic fibroblasts is reduced in the absence of neurofibromin. Fibroblasts were permeabilized and loaded with labelled calcium as described in methods. Cells were gently stirred during uptake; 200 ul aliquots were taken at designated intervals and calcium remaining within cells was determined by rapid lanthanum quenching and filtration as described in methods. Each point shows the mean of duplicate determinations. This data is representative of 10 experiments.

Figure 3. Distribution of maximum response amplitude, recovery time and nm away from basal after recovery upon exposure to different physiological agonists. The graphs represent data from 3-5 experiments and 20-35 cells for each agonist. The values were calculated from individual cell traces generated from data obtained every 5-8 seconds. The

solid bars indicate null cells and open bars indicate wild type cells. The figures indicate that there are no significant differences in the overall distribution of the maximum response amplitude (A, B, C), recovery time (D, E, F), or nM away from basal calcium levels after recovery (G, H, I) upon exposure to these physiological agonists.

Figure 4. Response to physiological agonists. Each figure depicts representative traces of wild type (+/+) and null cells'(-/-) response after exposure to different agonists. The x-axis indicates time (sec.) and y-axis indicates concentration of intracellular calcium (nM). The arrow indicates the time of drug addition. The traces of wild type and null cells represent the similarity and the heterogeneity of responses to different agonists. The nM away from basal after recovery and time to recover after agonist exposure are also similar between wild type and null cells. These traces are representative of several experiments. Response to bradykinin (A), Bombesin(B), PDGF (C).

Figure 5. Response to Thapsigargin (1uM). Each graph is a representative trace of wild type (+/+) and null cells' (-/-) response to thapsigargin in the presence of extracellular calcium (A) and in the absence of extracellular calcium (B). The x-axis indicates time (seconds) and y-axis indicates concentration of intracellular calcium (nM). The arrow indicates the time of drug addition. In the presence of external calcium, intracellular levels of calcium remain elevated in wild type and null cells due to capacitive calcium entry. In the absence of calcium, there is no capacitive calcium entry and the plasma membrane Na+/Ca2+ exchangers restore steady state calcium levels in wild type and null cells.

Figure 6. $^{45}Ca^{2+}$ efflux in intact adherent fibroblasts is

comparable in wild-type and null fibroblasts. +/+ and -/- adherent fibroblasts were loaded with $^{45}Ca^{2+}$ to steady state equilibrium and efflux assay was conducted as described in materials. Data is represented as nmol of $^{45}Ca^{2+}$/ g of protein remaining in the cells. K-values indicate rate of $^{45}Ca^{2+}$ release in the fast and slow exchanging phases.

Figure 1:

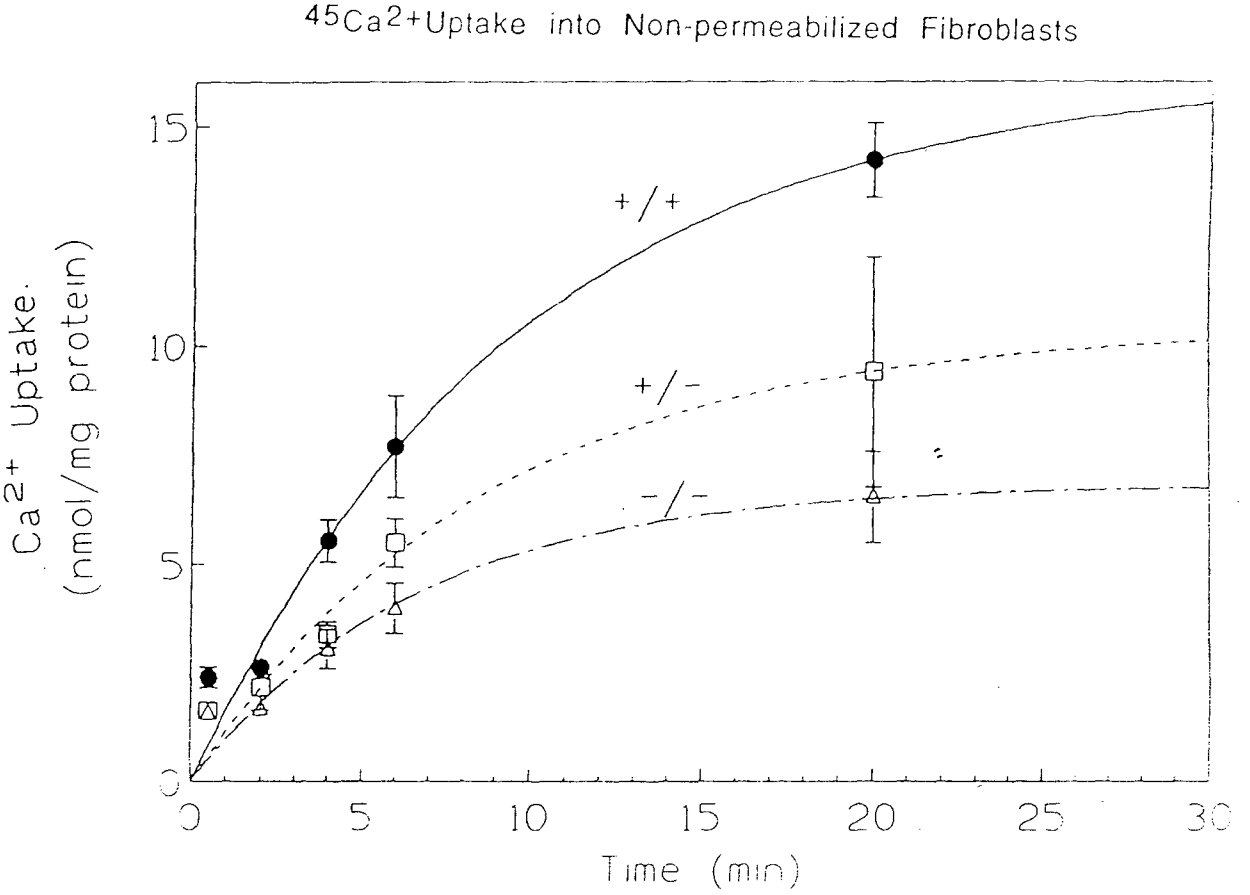


Table 1: "Low" $^{45}Ca^{2+}$ Uptake into intact embryonic fibroblasts within 20 minutes

	+/+	-/-
Exp. 1	3.38 ± .2	1.37 ± .007
Exp. 2	3.69± .12	2.0±.7
Exp. 3	3.43 ± .5	1.4 ± .3

Table 2: $^{45}Ca^{2+}$ uptake into intact embryonic fibroblasts after pretreatment with thapsigargin(TG).

	+/+	-/-
Exp. 1		
Control	3.76 ± .3	3.0 ± .37
TG	1.69 ± 1.2	2.3 ±.25
Exp. 2		
Control	3.43 ± .5	1.4 ± .3
TG	1.17 ± .39	.322 ± .04

Figure 2:

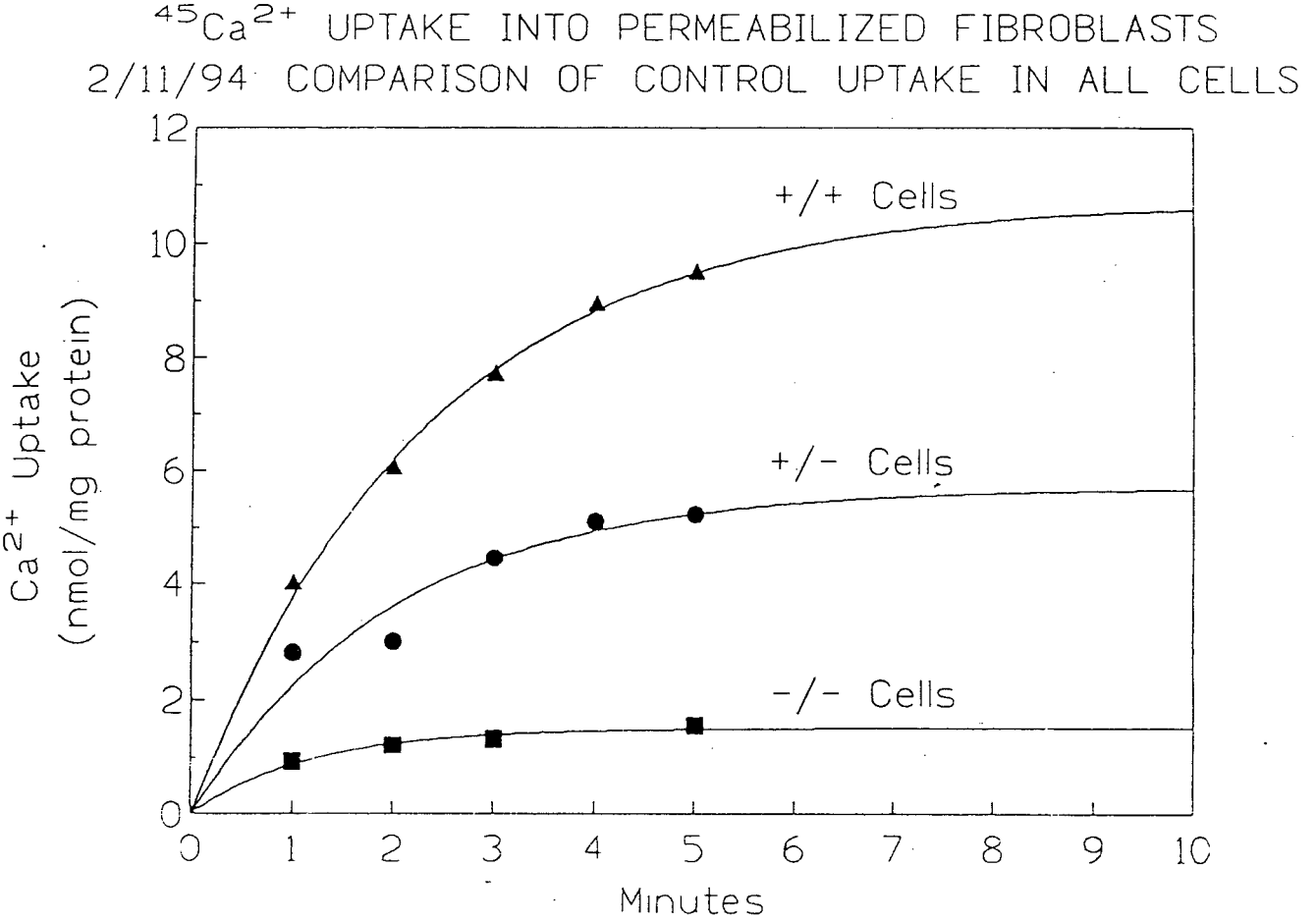

$^{45}Ca^{2+}$ UPTAKE INTO PERMEABILIZED FIBROBLASTS
2/11/94 COMPARISON OF CONTROL UPTAKE IN ALL CELLS
+/+ Cells
+/- Cells
-/- Cells
Ca^{2+} Uptake
(nmol/mg protein)
Minutes
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8
9
10

Figure 3:

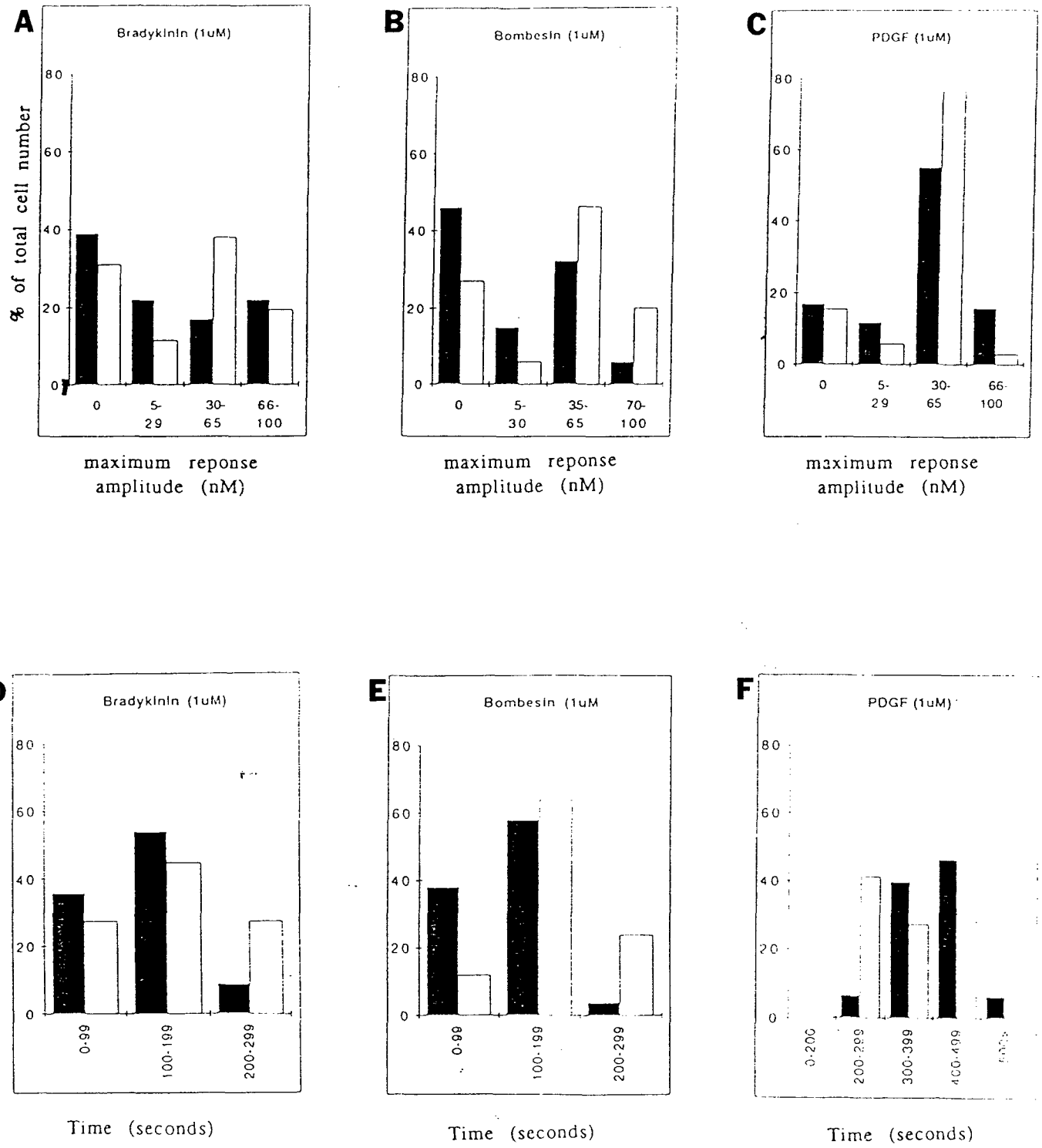

A
Bradykinin (1uM)
% of total cell number
80
60
40
20
0
0
5-29
30-65
66-100
maximum reponse amplitude (nM)
B
Bombesin (1uM)
80
60
40
20
0
0
5-30
35-65
70-100
maximum reponse amplitude (nM)
C
PDGF (1uM)
80
60
40
20
0
0
5-29
30-65
66-100
maximum reponse amplitude (nM)
D
Bradykinin (1uM)
% of total cell number
80
60
40
20
0
0-99
100-199
200-299
Time (seconds)
E
Bombesin (1uM
80
60
40
20
0
0-99
100-199
200-299
Time (seconds)
F
PDGF (1uM)
80
60
40
20
0
0-200
200-299
300-399
400-499
Time (seconds)

Figure 3 cont.

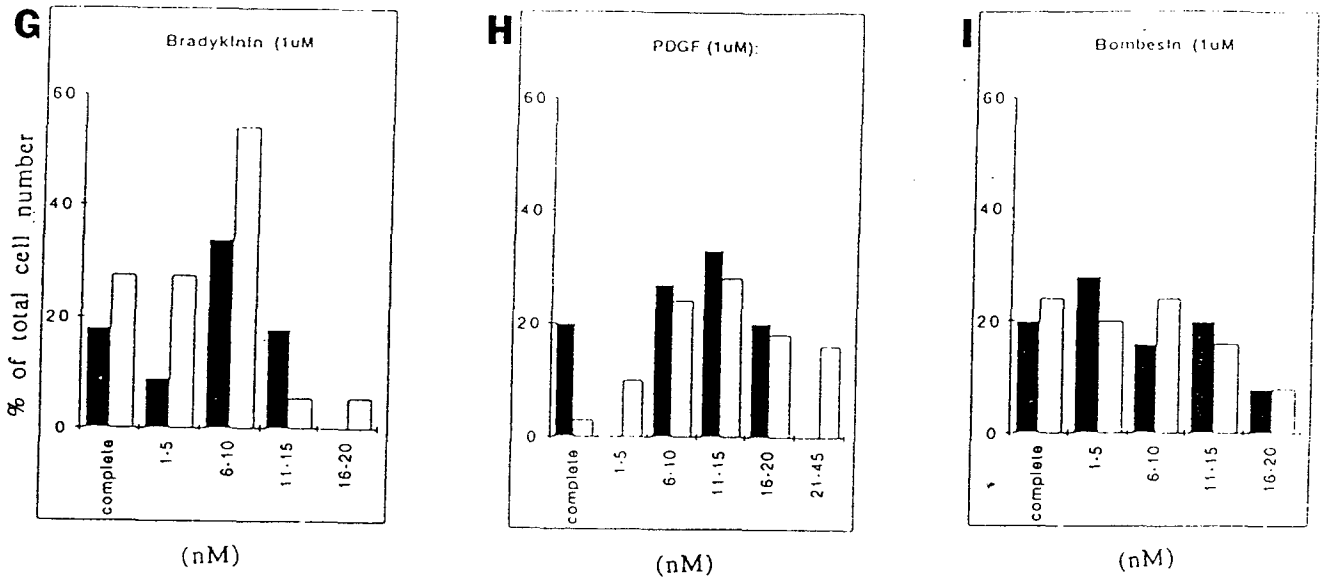


Figure 4:

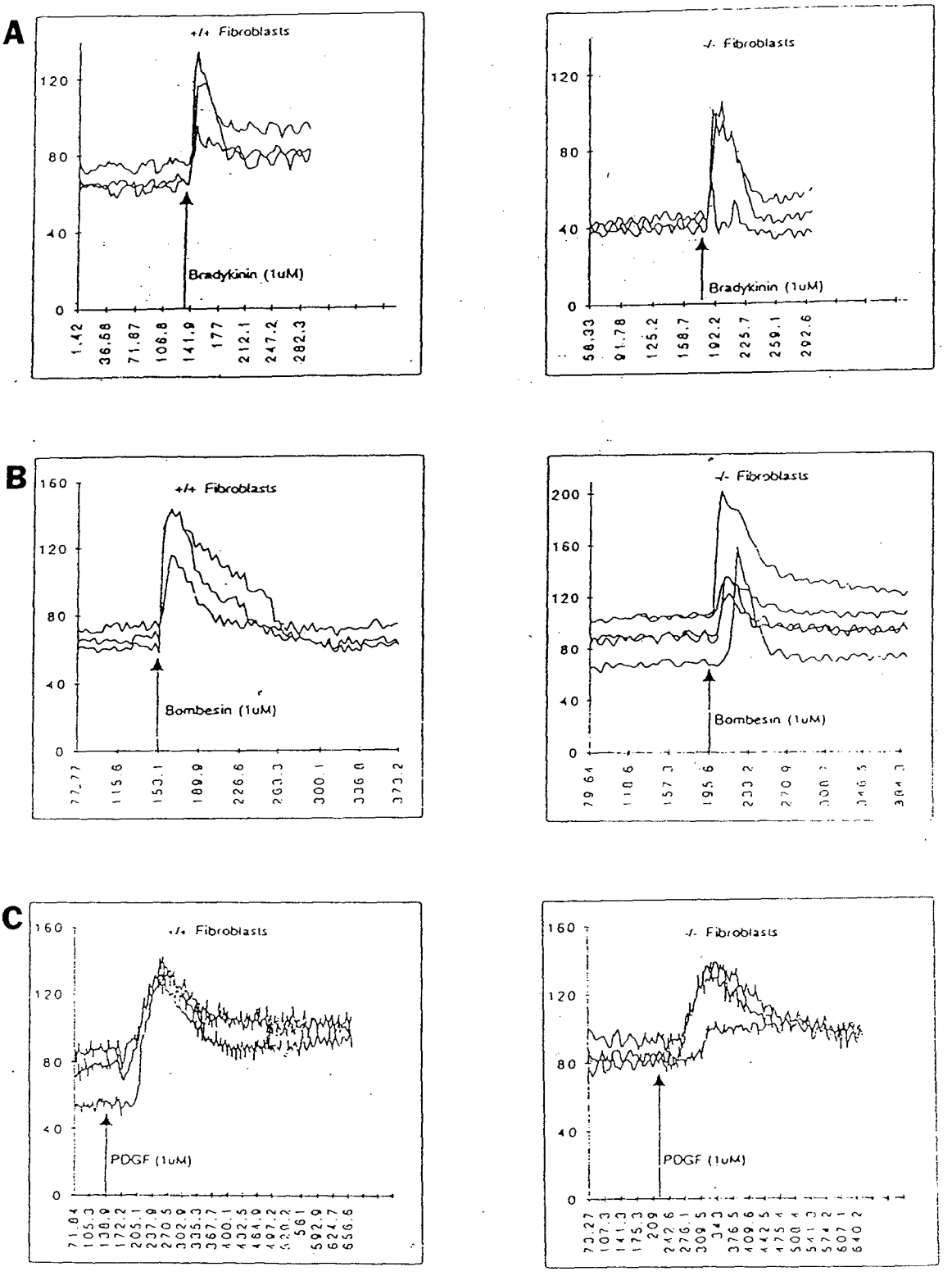

A
+/+ Fibroblasts
Bradykinin (1uM)
-/- Fibroblasts
Bradykinin (1uM)
B
+/+ Fibroblasts
Bombesin (1uM)
-/- Fibroblasts
Bombesin (1uM)
C
+/+ Fibroblasts
PDGF (1uM)
-/- Fibroblasts
PDGF (1uM)

Figure 5:

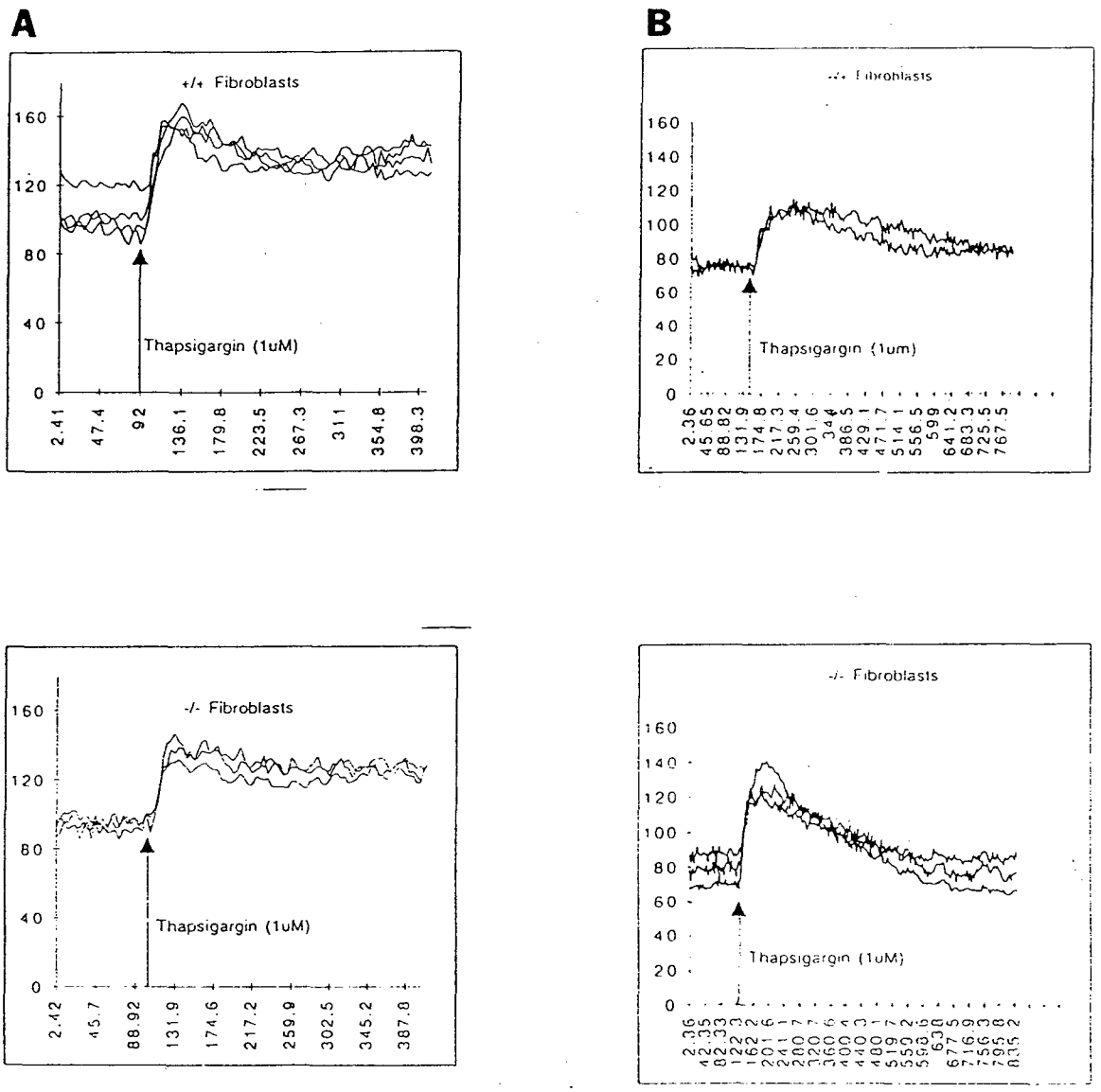
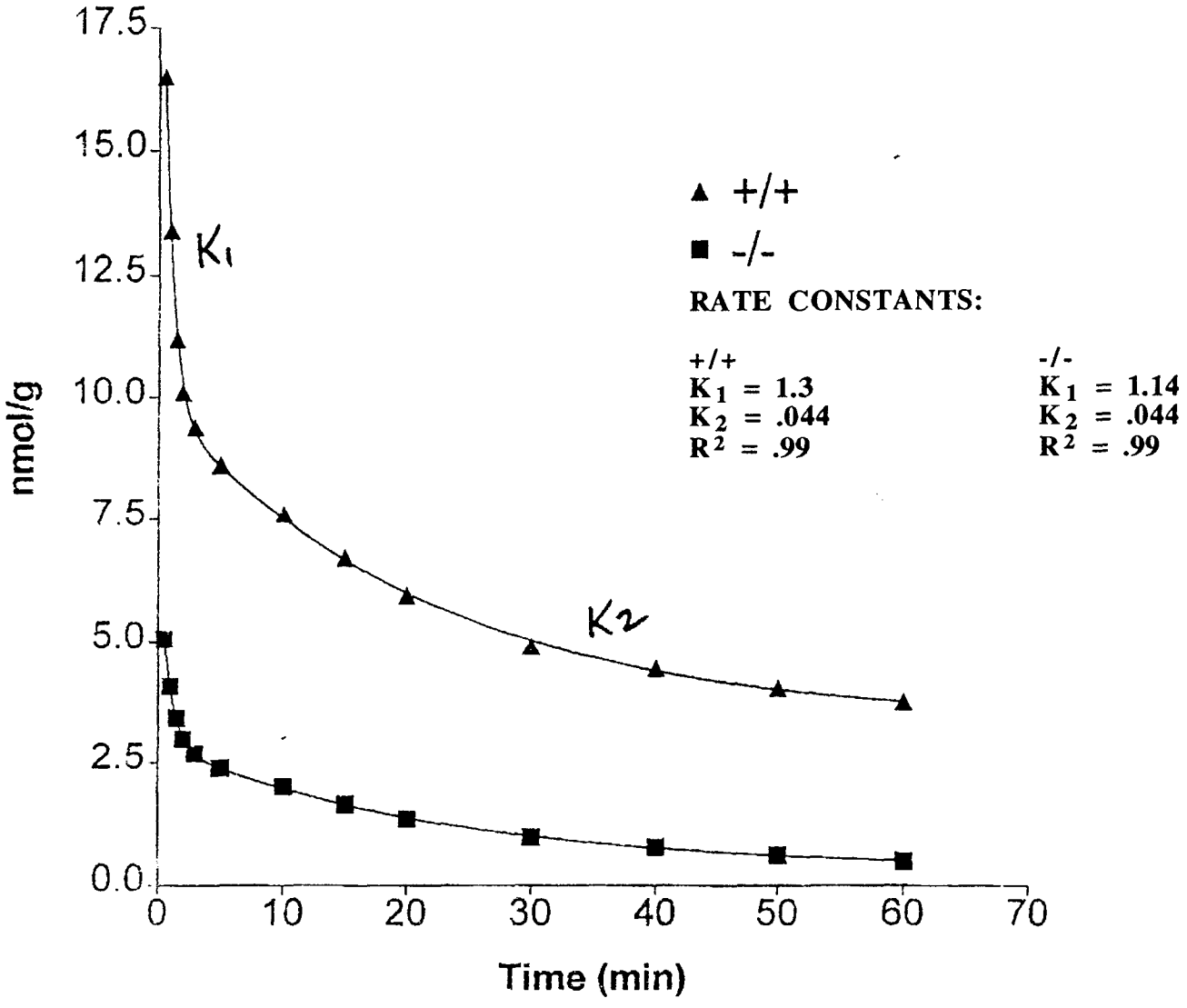

A
+/+ Fibroblasts
160
120
80
40
0
Thapsigargin (1uM)
2.41
47.4
92
136.1
179.8
223.5
267.3
311
354.8
398.3
B
-/- Fibroblasts
160
140
120
100
80
60
40
20
0
Thapsigargin (1um)
-/- Fibroblasts
160
120
80
40
0
Thapsigargin (1uM)
2.42
45.7
88.92
131.9
174.6
217.2
259.9
302.5
345.2
387.8
-/- Fibroblasts
160
140
120
100
80
60
40
20
0
Thapsigargin (1uM)

FIGURE 6:


17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
nmol/g
K1
K2
▲ +/+
■ -/-
RATE CONSTANTS:
+/+
K1 = 1.3
K2 = .044
R2 = .99
-/-
K1 = 1.14
K2 = .044
R2 = .99
0
10
20
30
40
50
60
70
Time (min)

CHAPTER 8 CONCLUSIONS

1. We have defined cell-autonomous abnormalities in mouse Schwann cells and in mouse fibroblasts lacking NF1. Fibroblast abnormalities are detected mainly or only in null cells and include hyperproliferation and failure to form perineurium. Schwann cells, either heterozygous or null at NF1, show multiple abnormalites. These include alterations previously defined in human neurofibroma Schwann cells (angiogenesis and invasion), indicating that mouse cells (of defined genotype) can be used as a model for the human cells. Schwann cells lacking NF1 also show either reduced response to growth factors or increased proliferation, depending on culture conditions.

2. We showed that loss of NF1 in mouse Schwann cells results in accumulation of activated, GTP-bound, Ras. This result suggested that drugs designed to inhibit Ras function could be used to reverse the phenotypes of NF1-deficient Schwann cells. Our definition of specific phenotypic changes in NF1-deficient Schwann cells allowed us to test this hypothesis: a protein farnesyl transferase reversed abnormalities in Schwann cell division (both hypo- and hyper-proliferation) characteristic of the mutant cells. We conclude that the mouse system may be useful as a first-stage screen to test drugs that are suggested to have utility in treating human NF1 patients.

3. Studies of Schwann cell differentiation in the absence of NF1 suggest the tantalizing hypothesis that multiple NF1-deficient cell types, acting together, cause phenotypic changes not observed when only one cell type is NF1-deficient.

4. Identification of altered phenotypes in Schwann cells and fibroblasts in <u>in vitro</u> systems will enable us to begin to elucidate molecular alterations that underlie these abnormalities (i.e. differential expression of growth factors or receptor).

5. Efforts to identify specific abnormalities in calcium

handling in cells lacking NF1 have not yielded reproducible results. While it remains possible that update of calcium in NF1-deficient fibroblasts is diminished as compared to wild-type cells, these data remain preliminary.

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1. Rosenbaum, T., Kim, H.A., Ling, B., and Ratner, N. (1996) Neurofibromin is required for appropriate P0 expression and myelination., submitted to Molecular and Cellular Neuroscience.
2. Kim, H., Ling, B. and Ratner, N. (1996) Farnesyl transferase inhibitors reverse hyperproliferation but not invasion in mouse Schwann cells lacking NF1, submitted to Molecular and Cellular Biology.

Published Abstracts:

1. Kim, H., DeClue, J.E., Rizvi, T.A. and Ratner, N. (1994) Effects of ras gene products on Schwann cell proliferation and differentiation. J. Neurosci. 20:299.14.
2. Rosenbaum, T., Kombrinck, K.W., Brannan, C.I., Perkins, N.A., Copeland, N.G. and Ratner, N. (1994) Abnormal proliferation and myelination in neuron-Schwann cell cultures from neurofibromin deficient mice. J. Neurosci. 20:299.15.
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1. Kim, H.A. and Ratner, N. (1996) A procedure for isolating Schwann cells developed for analysis of the mouse embryonic lethal mutation NF1. in Cell Biology and Pathology of Myelin, ed. Devon, R.M., et al., Plenum Press.

Patent Application:

1. Inventor: Nancy Ratner, Ph.D.
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