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Tumorigenic Properties of Neurofibromin-Deficient Neurofibroma Schwann Cells

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Dermal and plexiform neurofibromas are peripheral nerve sheath tumors that arise frequently in neurofibromatosis type 1. The goal of the present study was to examine the tumorigenic properties of neurofibromin-deficient human Schwann cells (SCs) that were found to represent a subset of SCs present in approximately half of the total neurofibromas examined. Highly enriched SC cultures were established from 10 dermal and eight plexiform neurofibromas by selective subculture using glial growth factor-2 and laminin. These cultures had low tumorigenic potential in classical *in vitro* assays yet several unique preneoplastic properties were frequently observed, including delayed senescence, a lack of density-limited growth, and a strong propensity to spontaneously form proliferative cell aggregates rich in extracellular matrix. Western blot analysis failed to detect full-length neurofibromin in any of the neurofibroma SC cultures, indicating that neurofibromin-deficient SCs had a substantial growth advantage. Immunohistochemical staining of the originating tumors showed the majority were comprised principally of neurofibromin-negative SCs, whereas the remainder contained both neurofibromin-negative and neurofibromin-positive SCs. Lastly, engraftment of neurofibromin-deficient SC cultures into the peripheral nerves of *scid* mice consistently produced persistent neurofibroma-like tumors with diffuse and often extensive intraneural growth. These findings indicate that neurofibromin-deficient SCs are involved in neurofibroma formation and, by selective subculture, provide a resource for the development of an *in vivo* model to further examine the role of these mutant SCs in neurofibroma histogenesis. *(Am J Pathol 2001, 158:501–513)*

Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition with a high frequency of peripheral nerve sheath tumors called neurofibromas. Dermal neurofibromas usually develop during adolescence and adulthood. These small tumors involve terminal nerves and may be numerous, yet have no apparent risk of malignant transformation. In contrast, plexiform neurofibromas are usually congenital, typically involve deep or "named" nerves, can become very large, and may cause serious functional impairment. Because plexiform tumors often occur on critical nerves and are not discrete masses, surgical removal is rarely complete and recurrence is associated with increased morbidity and fatality. A recent study suggests that plexiform neurofibromas develop in the majority of NF1 patients.[1] Additionally, plexiform tumors may progress to malignancy, which occurs in an estimated 6% of NF1 patients.[2]

Unlike schwannomas, which consist predominantly of Schwann cells (SCs), neurofibromas show marked cellular heterogeneity. Nevertheless, SCs are the major cell type amplified in neurofibromas and typically comprise 40 to 80% of the tumor cells. Additionally, there is a substantial population of interspersed fibroblastic or perineurial cells, along with various vascular and inflammatory elements embedded in an extensive extracellular matrix.[3] Because of this cellular heterogeneity, the histogenesis of neurofibromas has been controversial. Although there is increasing evidence for a SC origin, some studies suggest the contribution of SCs and fibroblastic cells.[4–6] On the other hand, an emerging view proposes that all of the major cellular elements of neurofibromas are of SC lineage and that the fibroblastic/perineurial-like elements may be immature or variant SCs.[7–10]

NF1 is caused by disruptive mutations in the *NF1* gene, which encodes the GAP-related protein neurofibromin. Thus, all cells in an NF1 individual are initially haploinsufficient for neurofibromin activity(s). However, consistent with the tumor suppressor gene two-hit model, it seems that loss of function of the remaining *NF1* allele is associated with neurofibroma formation, as first observed by our lab as loss of heterozygosity.[11] Although there are several approaches to animal models of NF1, presently none exist in which neurofibromas can be readily induced using defined human cell populations deficient in neurofibromin. Gene targeting has been used to construct mouse strains harboring mutations in the *Nf1* gene.[12,13] The mouse knockout model, similar to the

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human NF1 condition, involves only a single constitutional mutation and homozygous mice (*Nf1*$^{-/-}$) die during gestation. Despite the high level of conservation between mouse and human neurofibromin, it is clear that *Nf1* knockout mice are not prone to the formation of neurofibromas. Thus, the inadequacy of the heterozygous mouse model may be attributed to a low mutation rate of the remaining wild-type *Nf1* allele within the relatively short murine life span. Original studies by Martuza and co-workers[14] demonstrated the growth of minced human neurofibromas in the subrenal capsule and sciatic nerve of immunodeficient mice that retained their morphological features and genomic identities. Thus, the use of defined neurofibroma cell populations in animal models will greatly enhance efforts to understand the histogenesis of neurofibromas.

Neurofibroma SCs have invasive and angiogenic properties, suggesting that these are genetically altered cells with tumorigenic properties.[15,16] Additionally, cytogenetic studies show that plexiform neurofibromas harbor genetically abnormal SCs and strongly implicate these cells as the central component in the development of these potentially progressive tumors.[17] Recently, somatic loss of heterozygosity was found in SCs, but not fibroblasts cultured from a neurofibroma, suggesting that genetic alterations of the *NF1* gene in SCs are involved in the development of neurofibromas.[18] In a more comprehensive study, Rutkowski and co-workers[19] further demonstrated that neurofibroma-derived SCs typically lacked *NF1* mRNA whereas fibroblasts isolated from neurofibromas expressed the *NF1* transcript. In the present study, SCs subcultured from numerous neurofibromas were examined for neurofibromin expression and tumorigenic properties *in vitro* and after intraneural engraftment. Our findings strongly implicate neurofibromin-deficient SCs in the histogenesis of at least a subset of neurofibromas.

Materials and Methods

SC Culture

Normal Adult Nerve SCs

All specimens included in this study were obtained in accordance with protocols approved by the University of Florida Institutional Review Board. Human SCs were isolated from normal adult sural nerves by modifications of methods described previously.[20,21] Briefly, segmented nerve fascicles were cultured for 10 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 5% calf serum, 2 μm forskolin, 25 ng/ml human recombinant glial growth factor-2 (GGF-2), and antibiotics (expansion medium). The tissue was then dissociated for 18 hours in medium 15% calf serum, 1.25 U/ml Dispase (Collaborative Research Inc., Bedford, MA), 300 U/ml collagenase (type XI; Sigma Chemical Co., St. Louis, MO) and antibiotics. The digested tissue was dispersed by trituration, passed through a 30-μm mesh nylon screen, and centrifuged (200 $\times$ *g*, 10 minutes). The cell pellet was resuspended (50 segments/2 ml) in medium containing N2 supplements[22] and 2 ml of the cell suspension was spread across the surface of a 75-cm^2 flask precoated sequentially with polyornithine (0.1 mg/ml) and laminin (10 μg/ml) (prepared as described by Muir[23]). After a 6-hour incubation, the medium was supplemented by the gentle addition of expansion medium (10 ml). The cultures were grown to near confluency and the SCs were isolated by differential detachment using mild trypsinization and gentle shaking. The highly enriched cultures were expanded in expansion medium in dishes coated with laminin only. All cultures were withdrawn from treatment with forskolin and GGF-2 before storage or use.

Neurofibroma SCs

All patients met recognized diagnostic criteria for NF1[24] and tumor specimens were characterized as neurofibromas by histopathological study. Patient ages ranged from 4 to 69 years (the majority were young to middle-aged adults) and the reasons for surgery included cosmetic, functional deficit, and tissue donation on NF1-related fatality. These factors were unrelated to the tissue culture outcome and phenotype. Any capsular material was removed and viable tumor isolated from surgically resected neurofibromas. Tumor tissue (1 cm^2) was finely minced and incubated at 37°C overnight in 10 ml of L-15 medium containing 15% calf serum, 1.25 U/ml Dispase, 300 U/ml collagenase, and antibiotics. The tissue was dispersed by trituration and strained through a 30-μm mesh nylon screen. The filtrate was diluted with L-15 and centrifuged (400 $\times$ *g*, 5 minutes). The cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 5% calf serum, and antibiotics (standard medium), and cells were seeded into tissue culture flasks ($\approx$10^6 cells/75 cm^2. After 4 days, cultures were detached with trypsin/ethylenediaminetetraacetic acid and passaged 1:4. Half of the passaged cells were grown in standard culture conditions; these were later harvested and stored in liquid nitrogen. The other half of the cells were seeded in flasks precoated with laminin (10 μg/ml) and grown in standard medium containing GGF-2 (25 ng/ml). GGF-2 treatment caused rapid proliferation of Schwann-like cells (SLCs). During subsequent passage, SLCs were enriched further by differential detachment using mild trypsinization and shaking. For the specified cultures, the combination of preferential laminin attachment, differential detachment, and selective mitogen treatment with GGF-2 yielded highly enriched (95 to 99.5%) SLC cultures within 3 to 4 passages. All tumorigenic and protein expression assays were performed using cultures at passage 3 to 4 that had been withdrawn from GGF-2 for at least 2 days. GGF-2 was generously provided by M. Marchionni (Cambridge Neuroscience, Cambridge, MA).

Anchorage and Serum Requirements

The growth of SLC-enriched cultures was assessed in serum-free and unattached conditions. Early (passage 2

Table 1. Characteristics of Neurofibroma-Derived Schwann Cell Cultures

SC Culture	Culture type*	S-100/p75†	NFn SC culture‡	NFn tumor§
Derived from dermal neurofibromas				
SC^+(cNF89.1)	type-2	+/+	−	n.d.
SC^+(cNF93.1a)	type-4	v/v	−	$-/^+$
SC^+(cNF93.1b)	type-4	v/v	−	$-/^+$
SC^+(cNF96.5f)	type-2	+/+	−	$-/^+$
SC^+(cNF96.5g)	type-2	+/+	−	$-/^-$
SC^+(cNF97.2a)	type-2	+/+	−	$-/^-$
SC^+(cNF97.2b)	type-2	+/+	−	$-/^-$
SC^+(cNF98.4a)	type-2	+/+	−	$-/^-$
SC^+(cNF98.4d)	type-2	+/+	−	$-/^-$
SC^+(cNF99.1)	type-2	+/+	−	$-/^-$
Derived from plexiform neurofibromas				
SC^+(pNF92.1)	type-3¶	+/−	−	$-/^-$
SC^+(pNF94.5)	type-2	+/+	−	$+/^-$
SC^+(pNF95.1)	type-2	+/+	−	$-/^-$
SC^+(pNF95.5)	type-3	+/±	−	$-/^+$
SC^+(pNF95.6)	type-3¶	+/±	−	$+/^-$
SC^+(pNF95.11b)	type-3¶	+/+	−	$+/^-$
SC^+(pNF97.9)	type-4	v/v	−	$-/^-$
SC^+(pNF98.3)	type-3	+/+	−	$-/^+$

*Growth classification specified in Results.
†Immunocytochemical expression of SC antigens, S-100 (cytoplasmic), and p75NGFR.
‡Expression of neurofibromin (+ or −) by NF1 SC culture determined by Western blot.
§Neurofibromin immunoreactivity for originating tumor as described in Results.
¶Proliferate without GGF2.
v, varied with density; ±, heterogeneous.

to 4) cultures were maintained in standard medium and detached from the culture dish with 0.5 mmol/L ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS). To examine anchorage dependency, cells were seeded in poly(HEMA)-coated culture wells at a density of 10^5 cells/well in Joklik medium (to minimize cell aggregation) supplemented with 10% serum. To examine serum dependency, cells were seeded in culture wells coated with laminin-1 (10 μg/ml) at a density of 10^5 cells/well and grown in serum-free N2 medium supplemented with 1% heat-inactivated bovine serum albumin. Cell viability was assessed at 0, 24, and 72 hours using a Trypan blue dye-exclusion assay. Counting chambers of a hemocytometer were filled with the cell suspension and viable cells (dye-excluding) as well as nonviable cells (dye-absorbing) were counted.

Growth in Soft Agarose

Anchorage-independent colony formation was determined by growing cells in soft agarose as described by Neugut and Weinstein.[25] A thin base-layer of 0.9% agarose was allowed to solidify for 1 hour in culture wells. A single-cell suspension in standard medium containing 0.4% agarose was layered over the solid agarose base and allowed to solidify. The cultures were grown for 2 to 8 weeks and viable colonies consisting of >25 cells were scored by phase-contrast microscopy. Percent colony formation was calculated as: (number of viable colonies/total viable cells seeded) × 100%.

Subcutaneous Engraftment

All animal procedures were performed in accordance with approved IACUC protocols. NF1 SC cultures were tested for their ability to form tumors after subcutaneous injection in immunodeficient *nude* mice. Cells grown on laminin in medium containing GGF-2 were harvested by trypsinization and resuspended in Hanks' balanced salt solution. Numerous subcutaneous injections were made using 2×10^6 cells/site. Mice were examined for development of palpable tumors for at least 3 months. Thereafter, animals were euthanized and the injection sites were surgically exposed and examined for signs of tumor formation. Because there were no signs of tumor growth no histology was performed.

Nerve Engraftment

Human neurofibroma-derived SC cultures (Table 1) from cryopreserved stocks were grown on laminin for 4 days in medium supplemented with GGF-2. Dissociated cells were collected, rinsed thoroughly, and resuspended as a dense slurry in Hanks' solution. Young adult *scid* mice were anesthetized and sciatic nerves of both legs were exposed at mid-thigh. A cell suspension (5×10^5 in 4 μl) was gradually injected intrafascicularly in both nerves through a fine needle (35 gauge) attached to a Hamilton syringe. The site was closed in layers with sutures and the revived mouse returned to specific pathogen-free housing. At 1 to 8 weeks after implantation, the animals were sacrificed under anesthesia and the nerves ($n = 4$

for each culture) were removed and fixed by immersion in 4% paraformaldehyde. Nerve segments were embedded in paraffin and sectioned for immunohistochemical staining.

Immunohistochemistry

Neurofibroma Cultures

Monolayer cultures were examined for immunoreactivity with antibodies to the SC antigens S-100 (DAKO, Carpinteria, CA) (1/300) and the low-affinity nerve growth factor receptor (p75) (4 μg/ml, hybridoma 200-3-G6-4; American Tissue Culture Collection, Rockville, MD). Cultures grown on laminin-coated chamber slides were fixed with 2% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2) for 20 minutes, then washed with PBS containing 0.5% Triton X-100. Nonspecific antibody binding was blocked with PBS containing 0.1% Triton and 10% normal serum (blocking buffer) for 1 hour. Primary antibodies were diluted in blocking buffer and applied to wells for 2 to 4 hours at 37°C. Bound antibodies were labeled with peroxidase-conjugated secondary antibodies for 1 hour at 37°C and chromogenic development was accomplished with 3,3′-diaminobenzidine-$(HCl)_4$ (0.05%) and hydrogen peroxide (0.03%) in PBS. Bromodeoxyuridine (BrdU) incorporation *in vitro* and immunolabeling of BrdU-DNA were performed as described previously.[26]

Nerve Grafts

Sciatic nerves engrafted with neurofibroma-derived SC cultures were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2), sectioned in paraffin, and stained with hematoxylin and eosin for routine light microscopic examination. To identify transplanted human neurofibroma-derived SCs, nerve sections were immunostained with polyclonal anti-GSTπ (DAKO) (1/100) (a human-specific antiserum to the ubiquitous cellular protein, glutathione *S*-transferase) and a monoclonal antibody to p75 (4 μg/ml, hybridoma 200-3-G6-4) (a primate-specific antibody to the low-affinity nerve growth factor receptor). Deparaffinized sections were pretreated with methanol containing 1% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. Nonspecific antibody binding was blocked with 10% normal serum in PBS containing 0.3% Triton X-100 for 60 minutes at 37°C. Primary antibodies were diluted in blocking buffer and applied to sections overnight at 4°C. Bound antibodies were labeled with biotinylated secondary antibodies for 4 hours at 37°C followed by the avidin-biotin-peroxidase reagent (DAKO) for 2 hours. Chromogenic development was accomplished with 3,3′-diaminobenzidine-$(HC1)_4$ (0.05%) and hydrogen peroxide (0.03%) in PBS.

Neurofibroma Tissue Specimens

Portions of the primary tumor used for cell culture were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2), sectioned in paraffin, and stained with hematoxylin and eosin for routine light microscopic examination. Sections were immunostained for neurofibromin with the NF1GRP(N) antibody (1 μg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against a peptide corresponding to residues 509 to 528 of the predicted *NF1* gene product. The specificity of antibody to this neurofibromin peptide was reported previously.[27] Serial sections were immunolabeled with polyclonal anti-S-100 (1:300, DAKO). Immunoperoxidase labeling with the avidin-biotin-peroxidase reagent was performed as described above, except to enhance neurofibromin staining, antigen retrieval was achieved by pretreating sections in 0.1% trypsin for 20 minutes at 37°C. Immunostained sections were lightly counterstained with hematoxylin. Negative controls used no primary antibody. Additionally, for the NF1GRP(N) antibody, preadsorption with a 10-fold molar excess of peptide antigen (SC-67P, Santa Cruz) was used to achieve complete blocking of neurofibromin immunoreactivity.

Western Immunoblotting

SC cultures were scraped from dishes and cell pellets were homogenized in ice-cold extraction buffer consisting of 50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and complete protease inhibitors (Boehringer-Mannheim, Indianapolis, IN). The soluble fraction was collected by centrifugation (10,000 × *g*, 20 minutes) and then was made 2 mol/L in urea. The extract was concentrated and fractionated by ultrafiltration using a 100-kd cut-off membrane. Total protein content of the high molecular mass retentate was determined using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). Samples were mixed with sodium dodecyl sulfate-containing electrophoresis sample buffer containing 2 mol/L urea and 5% 2-ME and then heated to 80°C for 2 hours. Samples (100 μg) were electrophoresed on 4 to 15% polyacrylamide gels and electroblotted to nitrocellulose sheets in transfer buffer containing 0.1% sodium dodecyl sulfate. Blots were rinsed in water and fixed in 25% isopropanol/10% acetic acid. Nitrocellulose sheets were washed with 0.05 mol/L Tris-HCl (pH 7.4) containing 1.5% NaCl and 0.1% Triton X-100 and then blocked in the same buffer with the addition of 5% dry milk (blocking buffer). The blots were incubated for 2 hours with anti-NF1GRP(N) antibody (1 μg/ml) in blocking buffer. Bound antibody was detected by peroxidase conjugated swine anti-rabbit IgG (affinity purified, DAKO) diluted 1/2,000 in blocking buffer. Immunoreactive bands were developed by chemiluminescent methods (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Relative molecular mass was determined using prestained markers including myosin (205 kd). Control samples were similarly processed from cell pellets obtained from normal human nerve SC cultures and SC cultures derived from embryonic homozygous *Nf1* knockout mice.[12]

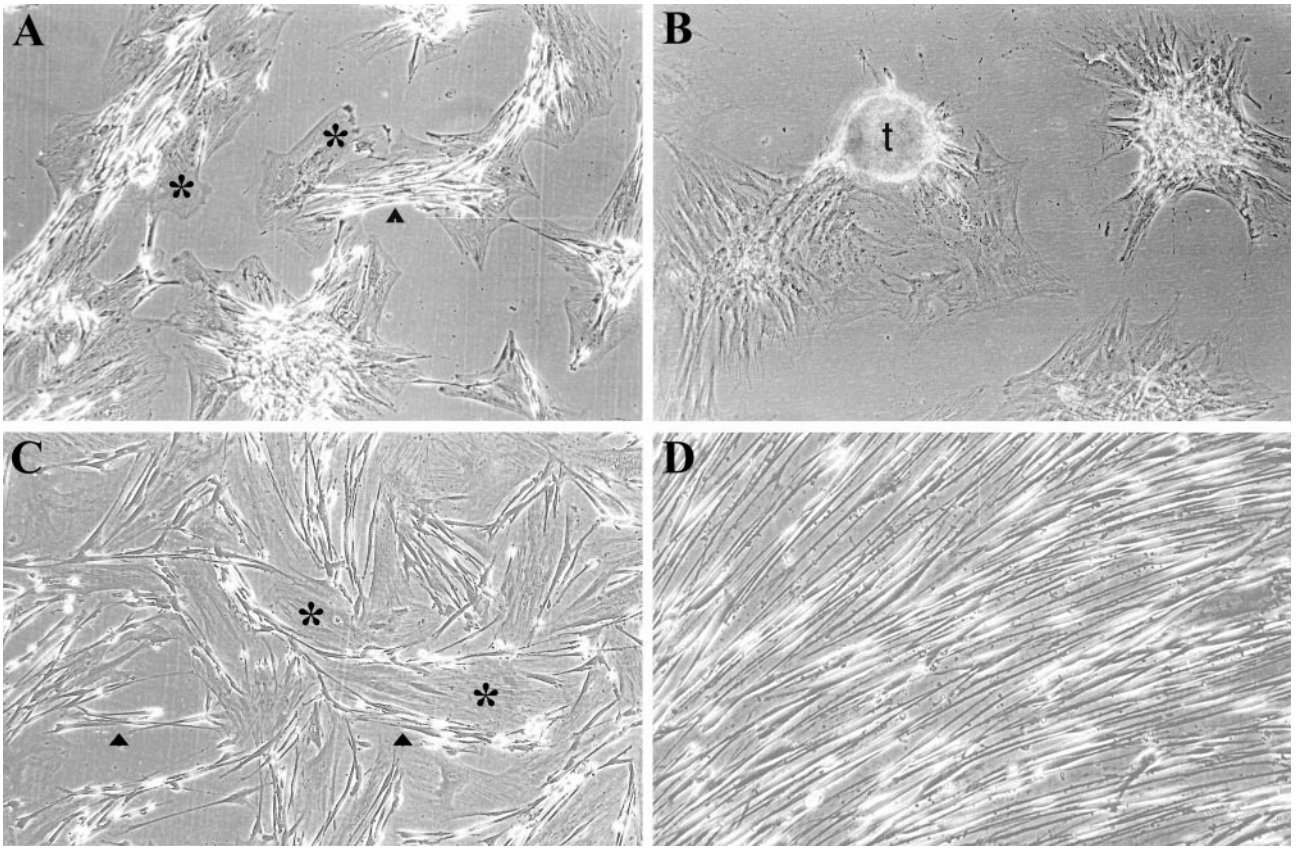

Figure 1. Subculture of neurofibroma-derived SCs. **A:** A typical primary neurofibroma culture containing mainly SLCs and FLCs. Seeded on tissue culture plastic, phase-bright SLCs (**arrowhead**) grew exclusively on patches of underlying FLCs (**asterisk**). **B:** SLCs proliferated in response to GGF-2 and formed dense cell aggregates (culture tumors, t) demonstrating their lack of contact-inhibited grow and continued association with the FLC sublayer. **C:** SLCs preferentially attached to a laminin substratum, which circumvented the association of SLC to the FLC layer and allowed expansion of the SLCs. **D:** A highly enriched SLC population was subcultured by growth on laminin in the presence of GGF-2 within three to four passages. Original magnification, ×200.

Results

Culture of Normal Human Nerve SCs

SCs do not proliferate in response to standard serum-supplemented medium and, until recently, methods for isolating normal human SCs were mostly unreliable. Based on recent advances,[20,21] we investigated various means to enrich and expand SCs from adult human nerve segments. Successful enrichment of SC cultures and depletion of fibroblastic cells was readily achieved by treatment with the SC mitogens GGF-2 and forskolin combined with differential cell detachment and preferential growth on a laminin-coated substratum. After 3 to 4 passages under these conditions, hundreds of millions of SCs were obtained nearly free of fibroblast contamination from several centimeters of adult tibial nerve. These cultures were homogeneous and contained highly elongated SCs that stained intensely for S-100 and p75, recognized markers for cells committed to a SC lineage.[28] The specificity of S-100 as a marker for cultured human SCs is shown in Figure 3A. After enrichment and expansion, SC division rapidly decreased in the absence of GGF-2 and forskolin. Growth on a laminin substratum was required at all stages to improve attachment and to minimize cell attrition. SC expansion was limited to ~10 population doublings before senescence regardless of mitogen stimulation, confirming the earlier findings by Rutkowski and colleagues.[29]

Culture of Neurofibroma SCs

Our goal was the enrichment and characterization of SLCs from dermal and plexiform neurofibromas. Monolayer cultures of neurofibromas were initiated by enzyme dissociation under standard culture conditions. The most frequent primary culture obtained from dermal and plexiform neurofibromas contained a sublayer of fibroblast-like cells (FLCs) admixed with numerous (20 to 60%) spindle-shaped SLCs (Figure 1). Numerous procedures to enrich and expand SLCs from the primary cultures were tested. It is notable that enrichment of SLCs was not improved by combined treatment with GGF-2 and agents that elevate cAMP. In particular, forskolin caused considerable heterogeneity in the SC population and hampered the development of the SLC cultures described below. Thus, unlike normal SC cultures, neurofibroma cultures were treated only with GGF-2 and were not exposed to forskolin.

Categorical responses to SC enrichment procedures (see Materials and Methods) emerged for the neurofibroma cultures. We defined four culture types based on prevalent cell morphologies, growth under standard cul-

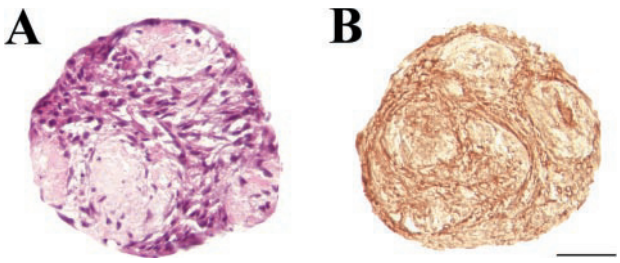

Figure 2. Neurofibroma SCs aggregated and formed culture tumors. Aggregates of SLCs, such as those shown in Figure 1B, were collected from early neurofibroma cultures and sectioned. **A:** H&E staining of culture tumors revealed areas of high cell density and cell-sparse regions of dense extracellular matrix similar to those seen in neurofibromas. **B:** Immunolabeling with laminin demonstrated a rich basement membrane network throughout the SC aggregates. Scale bar, 50 μm.

ture conditions, growth response to GGF-2 and laminin, and the onset of senescence. Findings were based on 40 robust cultures, 24 from dermal and 16 from plexiform neurofibromas.

A first type of neurofibroma culture (type-1) was distinguished by a poor response to the SC enrichment treatment with GGF-2 and laminin. These cultures (from dermal and plexiform tumors) were rapidly dominated by FLCs that often displayed abnormal growth characteristics (eg, rapid and protracted proliferation and loss of density-limited growth). The SLCs (immunopositive for S-100 and p75) failed to proliferate sufficiently and became increasingly diluted with repeated passage. Type-1 cultures accounted for 14 of 24 dermal and eight of 16 plexiform cultures, were deemed intractable to SLC enrichment, and were excluded from the analyses to follow.

A second subset, type-2 neurofibroma cultures, were amenable to enrichment of the SLCs. In most of these cultures the SLCs proliferated rapidly in the presence of GGF-2 and soon formed confluent islands on top of underlying FLCs (Figure 1A). Cultured on native tissue culture plastic, the SLCs attached exclusively to the FLCs. Despite the limited surface area of the underlying the FLCs, proliferation by the SLCs continued in the presence of GGF-2. As a result, the SLCs formed dense cellular masses or culture tumors (Figures 1B and 2A), indicating the absence of contact inhibition (density-limited growth) by neurofibroma SLCs. Immunolabeled sections of these culture tumors showed an extensive laminin-rich extracellular matrix (Figure 2B). These observations potentially relate to the adhesive mechanisms involved in the growth and development of neurofibromas.[30]

Growth on a laminin substratum, which circumvented the adhesion of the SLCs to the FLCs, was necessary for the subsequent enrichment and expansion of the SLCs (Figure 1C). Once outnumbered by the mitogen-driven SLCs, the FLCs were effectively diminished by differential detachment. Thereafter, the SLC population was readily enriched and expanded in large numbers within 3 to 4 passages (Figure 1D). Type-2 SLC cultures stained for S-100 and p75 and generally resembled highly spindled SCs obtained from normal nerve (Figure 3, A and B). However, purified SLCs from some neurofibromas, particularly those from plexiform tumors, were stubby and less elongated (Figure 3C). The SLCs derived from dermal neurofibromas had a limited proliferative capacity similar to that of normal adult SCs (10 doublings) and became senescent thereafter. By comparison, the plexiform SLCs were less restricted and often were passaged >20 times before showing signs of senescence. All type-2 SLC-enriched neurofibroma cultures grew very slowly when withdrawn from GGF-2, but were stable for months on a laminin substratum. SLC-enriched type-2 cultures accounted for eight of 24 dermal and two of 16 plexiform cultures.

A third type of neurofibroma culture was obtained exclusively from plexiform tumors and contained SLCs with several preneoplastic properties. Type-3 SLCs expanded rapidly without close association with the FLC sublayer and were easily enriched to near homogeneity. Five type-3 cultures were established from plexiform tumors; SLCs in two cultures had a stubby, spindle shape whereas the other three cultures were multipolar or polygonal (Figure 3D). In each culture, nearly all cells were stained for S-100, whereas p75 expression varied. Three multipolar/polygonal type-3 cultures grew particularly well (doubling times 2 to 4 days) in response to serum and GGF did not further increase their growth rates. These cultures also showed protracted expansion (>20 to 50 passages). The occurrence of these preneoplastic properties is almost certainly indicative of genetic abnormalities originating *in vivo* because growth factor-independent proliferation and expansion beyond 10 passages were never observed in cultures of normal human SCs (also see Rutkowski et al[29]). Furthermore, the three GGF-independent type-3 cultures were derived from sizable recurrent plexiform neurofibromas.

A fourth subset of neurofibroma culture, at low density, were pleomorphic, lacy and phase-pale, and stained faintly, or not at all, for S-100 and p75. However, with increasing density they became elongated spindle cells and grew in parallel arrays. Remarkably, when grown to confluency, these cultures formed dense ridges of S-100-expressing SLCs (not shown). The appearance of these S-100-positive SLCs did not decrease after numerous passages but, instead, continued to increase in number with increasing culture density. These cultures were expanded extensively (>50 passages) before showing signs of senescence. These observations suggest that these cultures contained a poorly differentiated cell type capable of giving rise to a reversibly differentiated SLC component. This type of pleomorphic property also was observed in several neurofibrosarcoma cultures (not shown).

In summary, 40 primary cultures were established from 24 dermal and 16 plexiform neurofibromas and subcultured for enrichment of SLCs. Twenty-two of 40 cultures did not respond to SLC enrichment or were predominantly fibroblastic (type-1). Ten dermal and eight plexiform tumor cultures yielded enriched SLC cultures (type-2, -3, and -4) suitable for additional genetic and biological studies (listed in Table 1). The type-2 and type-3 cultures (eight from dermal and seven from plexiform tumors) were the focus of subsequent studies.

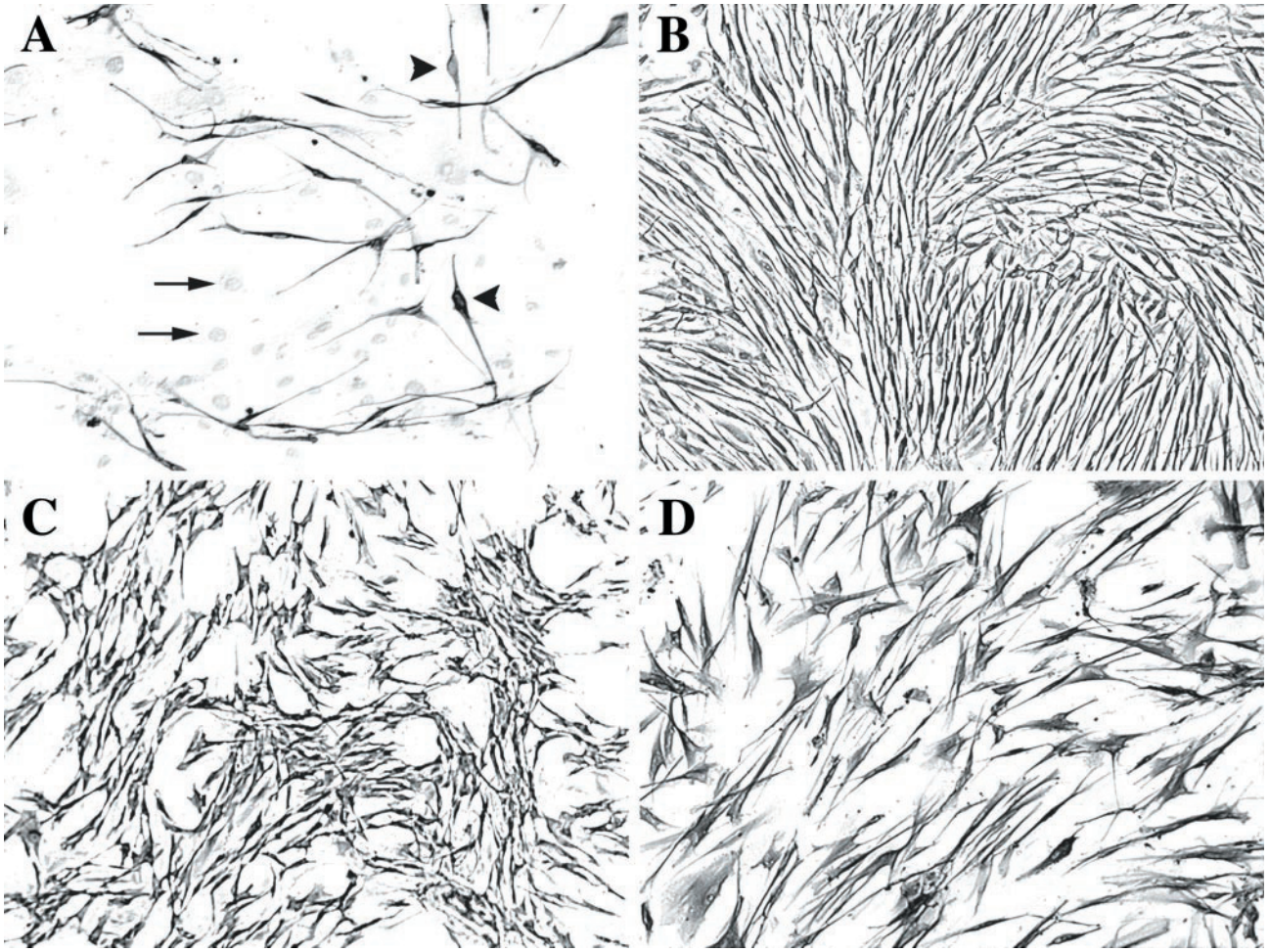

Figure 3. Morphology and S-100 immunostaining of nerve and neurofibroma-derived SC cultures. **A:** A primary culture established from normal adult human nerve, like those from neurofibromas, contained a mixture of S-100-positive SCs (**arrowheads**) and fibroblastic cells (**arrows**). SCs showed intense cytoplasmic S-100 immunoreactivity, while faint (if any) staining was visible in the nuclei of fibroblastic cells (contrast-enhanced image). After enrichment and expansion of SLCs from neurofibromas, the resulting SC cultures immunostained positive for S-100 yet often differed in morphology and growth properties (**B–D**). **B:** Type-2 cultures from dermal neurofibromas most often showed highly elongated spindle-shape cells similar to normal SCs. **C:** Type-2 and some type-3 cultures from plexiform neurofibromas generally contained multipolar and stubby spindled cells. **D:** Type-3 cultures were obtained exclusively from plexiform tumors and contained elongated but primarily polygonal cells. Original magnification, ×200.

Neurofibromin Expression by SCs in Neurofibromas

Before neurofibroma specimens were prepared for cell culture, representative specimens of tumor were fixed and processed for routine histology and immunohistochemistry. Paraffin sections were immunostained with an anti-neurofibromin peptide antiserum. Neurofibromin immunoreactivity in normal control tissues was similar to that reported by other laboratories.[31] In neurofibromas the SC elements were identified by their nuclear characteristics (elongated, wavy nuclei with pointed ends) and positive immunoreactivity for S-100 protein (Figure 4). Four basic patterns of neurofibromin immunoexpression in S-100-positive regions were observed: 1) neurofibromin-negative ($-/^-$); 2) predominantly neurofibromin-negative with focal areas of distinct positive staining ($-/^+$); 3) predominantly immunoreactive tumor with focal areas of distinct negativity ($+/^-$); and 4) neurofibromin-positive ($+/^+$). Immunoreactivity patterns for the surgical resection specimens corresponding to the SC cultures from which they were derived are summarized in Table 1. The majority (14 of 17) of the neurofibromas that gave rise to SC cultures were predominately neurofibromin-negative (designated $-/^-$ and $-/^+$ in Table 1). Moreover, more than one-half of these tumors were completely negative ($-/^-$), showing no neurofibromin immunostaining in any SC elements (Figure 4A). Furthermore, even in the few predominately neurofibromin-positive tumors ($+/^-$), many individual SCs were negative for neurofibromin (Figure 4C). In areas of positive immunoreactivity, tumor cells showed discrete, granular staining in the perinuclear cytoplasm and in delicate elongated processes. In tumors with positively and negatively stained regions, the regions of neurofibromin nonreactivity were histologically similar to immunoreactive areas. Overall, neurofibromin immunoexpression in most neurofibromas that gave rise to a SC culture was very low (see Discussion). The finding of both positive and negative areas of neurofibromin immunoreactivity in some neurofibromas could not be at-

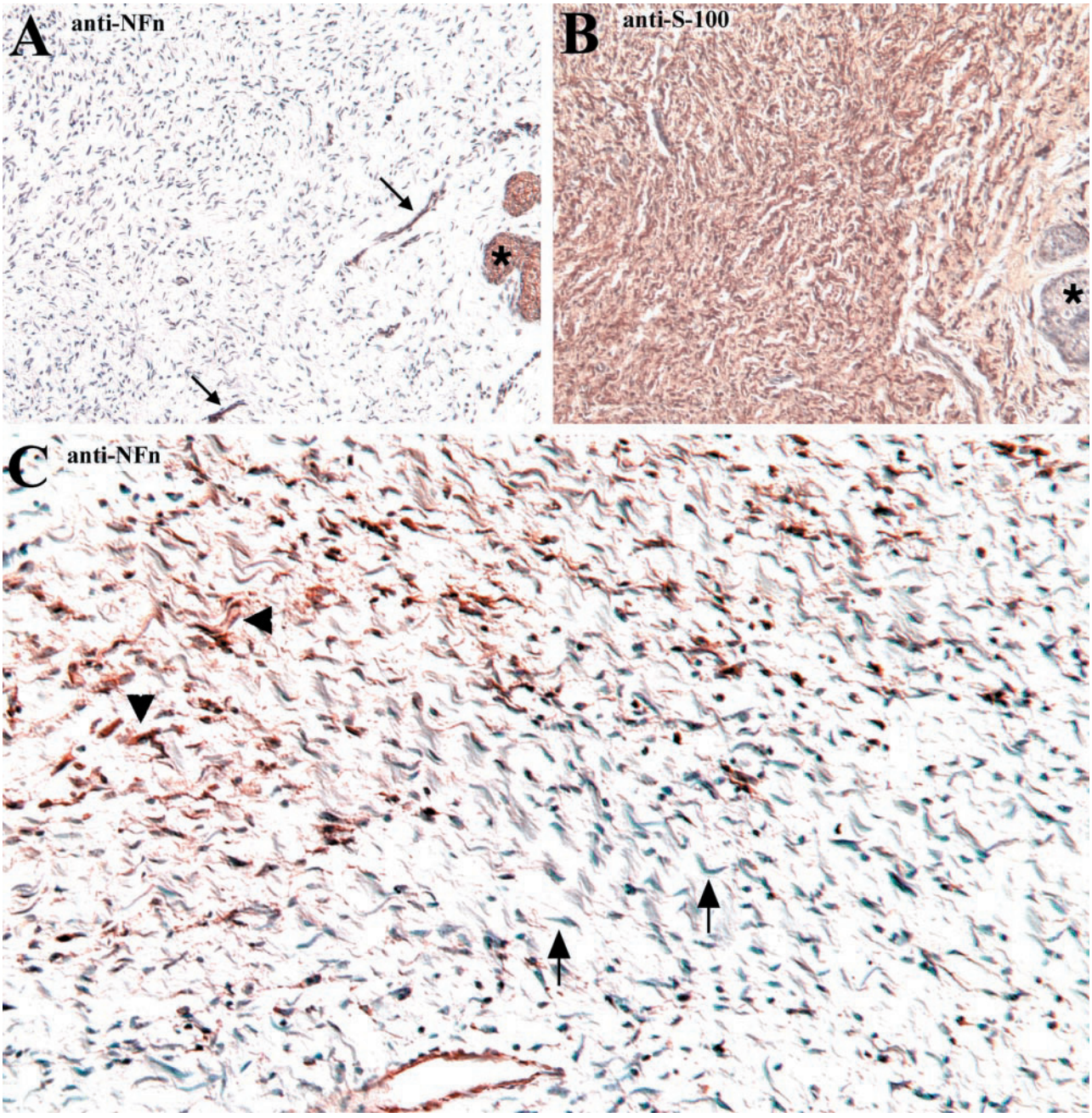

Figure 4. Neurofibromin immunoreactivity of SCs in neurofibromas. Tumor sections were immunoperoxidase stained for neurofibromin (**A** and **C**) and S-100 (**B**). **A:** Most neurofibromas that gave rise to SC cultures were either mainly or completely devoid of neurofibromin-positive SCs. Vascular elements (**arrows**) and sweat glands (**asterisk**) stained for neurofibromin and served as internal positive controls. **B:** A serial section of the neurofibroma shown in **A** showing strong and diffuse immunoreactive for the SC marker protein, S-100. **C:** A neurofibroma containing intermixed populations of neurofibromin-positive (**arrowheads**) and neurofibromin-negative SCs (**arrows**). In areas of positive immunoreactivity, tumor cells showed discrete, granular staining in the perinuclear cytoplasm and in delicate elongated processes. SC elements were identified by their nuclear characteristics (elongated, wavy nuclei with pointed ends) and positive immunoreactivity for S-100 protein. Sections were counterstained with hematoxylin. Original magnifications: ×200 (**A** and **B**), ×400 (**C**).

tributed to artifacts of fixation, tissue preparation, or regional differences in antibody concentration. Dermal and vascular elements were uniformly positive for neurofibromin in both immunoreactive and nonreactive regions of a given tumor (Figure 4). Also, tumors that were negative or that contained areas of neurofibromin nonreactivity were all strongly and widely immunoreactive for S-100 protein (Figure 4B).

Neurofibromin Expression by NF1 SC Cultures

We hypothesized that the abnormal growth properties of NF1 SC cultures, particularly the type-3 cultures, are the result of a severe deficiency in neurofibromin expression. Normal SCs and type-2, -3, and -4 NF1 SC cultures were examined for the expression of neurofibromin by Western immunoblotting. Results are shown in Figure 5. Antibody

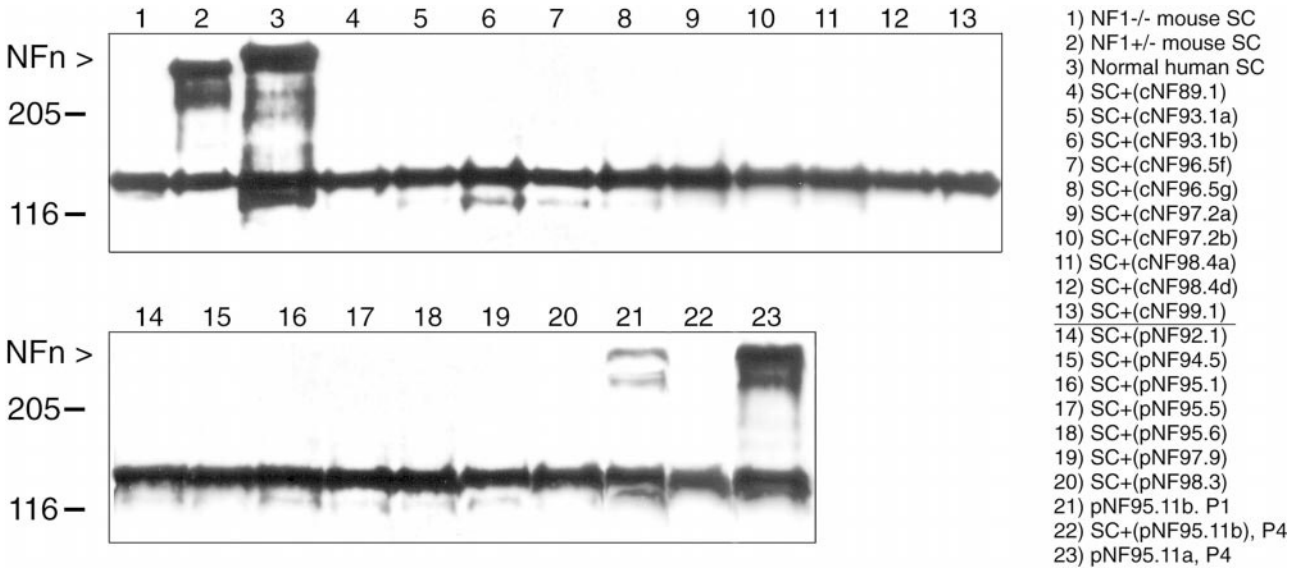

Figure 5. Western immunoblot analysis of the SC cultures for neurofibromin expression. Culture cell pellets were extracted and a high molecular mass fraction was obtained by ultrafiltration (>100 kd). Samples (100 μg of total protein) were electrophoresed and transferred to nitrocellulose sheets. The blots were stained for neurofibromin using antibody NF1GRP(N) and chemiluminescent detection. Antibody specificity was demonstrated on extracts of SC-enriched cultures from embryonic *Nf1* knockout mice. Full-length neurofibromin was absent from homozygous cultures ($Nf1^{-/-}$) (**lane 1**), whereas heterozygous cultures ($Nf1^{+/-}$) (**lane 2**) expressed a predominant immunoreactive band-pair with a M_r = 225 to 250 kd, slightly smaller than the M_r = 240 to 260 kd neurofibromin bands produced by normal human SCs (**lane 3**). A band at 140 kd appeared in each sample that, although undefined, indicated the consistency of total protein loaded in each lane. Full-length neurofibromin was absent in extracts of the 10 dermal SC cultures (**lanes 4** to **13**) and eight plexiform SC cultures (**lanes 14** to **20** and **22**). Also shown is the original mixed primary culture pNF95.11b (**lane 21**) from which the type-3 SC culture, SC+(pNF95.11b), was derived (**lane 22**) and the type-1 culture, pNF95.11a (an earlier specimen from the same patient).

specificity was examined on extracts of SC-enriched cultures from embryonic *Nf1* knockout mice. Full-length neurofibromin was absent from homozygous cultures ($Nf1^{-/-}$) (lane 1), whereas heterozygous cultures ($Nf1^{+/-}$) (lane 2) expressed a predominant immunoreactive band-pair with a M_r = 225 to 250 kd (slightly smaller than that produced by human cells). A band at 140 kd appeared in each sample that, although undefined, indicated the consistency of total protein loaded in each lane. Extracts of normal human SC cultures (lane 3) contained a predominant neurofibromin-immunoreactive band-pair with a M_r = 240 to 260 kd. In contrast, full-length neurofibromin was absent in extracts of the 10 dermal SC cultures (lanes 4 to 13) and eight plexiform SC cultures (lanes 14 to 20 and 22). As an example, we also show the mixed primary culture pNF95.11b (lane 21) from which the SC culture, SC+(pNF95.11b) (lane 22), was derived. The first passage of the culture pNF95.11b contained an admixture of cells (including numerous FLCs and perhaps diverse SC lineages). A modest band-pair corresponding to full-length neurofibromin was observed in this culture extract (lane 21), indicating the genetic heterogeneity in this early culture. This finding is consistent with the pattern of neurofibromin immunostaining ($+/^-$) observed in the originating tumor sections (Table 1). However, neurofibromin expression was undetectable in the derived type-3 SC culture (lane 22). The pNF95.11b cultures were established from a resection of a recurrent plexiform neurofibroma. Two years earlier, we established a culture from a specimen obtained from the initial resection. This culture, pNF95.11*a*, was type-1 (dominated by FLCs and intractable to SC enrichment) and expressed abundant neurofibromin (lane 23). Neurofibromin content in this culture extract was presumably contributed by the large population of fibroblasts, but we cannot exclude the contribution by *NF1* heterozygous SCs. From these results we conclude that neurofibromin-deficient SCs have the best long-term growth advantages and that our subculture methods to enrich for SCs from neurofibromas are highly selective for neurofibromin-deficient SCs.

Properties of NF1 SC Cultures in Classical Tumorigenic Assays

Neoplastic and tumorigenic properties of type-2 and -3 neurofibroma SC cultures were examined in several classical tests, including assays of serum and anchorage dependence, colony formation in soft agarose, and subcutaneous tumor formation in immunodeficient mice. The proliferative properties (normal and abnormal) of these cultures were described in a previous section. Although not all of the NF1 SC cultures listed in Table 1 were tested repeatedly, findings of tumorigenic properties were consistent for all type-2 and -3 cultures from dermal and plexiform tumors. Notably, all type-2 and type-3 NF1 SC cultures showed properties similar to normal human SCs. First, the survival of the NF1 SC cultures and normal SCs was not highly growth factor-dependent, as 85 to 95% of cells remained viable in serum-free medium for at least 72 hours (compared to 95% survival of the highly tumorigenic RN22 schwannoma and C6 glioma cell lines). Proliferation was not observed in any of the SC cultures in the absence of serum. Second, the survival of the SC cultures was anchorage-dependent. Under nonadherent

conditions the percentage of viable normal SCs dropped below 40% after 72 hours. Similarly the survival of NF1 SC cultures was 20 to 40% after 72 hours in suspension culture (compared to >95% in the RN22 and C6 lines).

Colony formation in soft agarose is an indicator of high tumorigenicity and anchorage-independent growth, properties of transformed cells and some, but not all solid tumor cultures. Suspended as single cells in soft agarose, 72% of RN22 and 88% of C6 cells proliferated and rapidly formed colonies that become visible to the eye within 2 weeks. In contrast, multicellular foci of normal SC or NF1 SC cultures were rarely observed and none reached the 25-cell colony criterion, even after cultured for 2 months in agarose.

NF1 SC cultures showed no subcutaneous tumorigenic growth. Millions of cells per site were injected subcutaneously in *nude* mice. No palpable tumors were found after 3 months and there were no subcutaneous foci visible in postmortem examinations. Taken together, these observations indicate that the neurofibroma SC cultures, even the type-3 cultures, had low tumorigenic potential in these classical assays.

Neurofibroma SC Cultures Grafted in the Mouse Nerve

The tumorigenic growth of selected neurofibroma SC cultures was examined as xenografts in the sciatic nerves of adult immunodeficient *scid* mice. Each NF1 culture was engrafted into four nerves and six nerves were engrafted with an equal number of normal human SCs. Engrafted nerves were examined by immunostaining with an antibody specific to human glutathione *S*-transferase (Figure 6). First, transplantation of normal SCs resulted in transient occupancy (Figure 6C) and survival appeared to be severely limited because in four of six of the nerves glutathione *S*-transferase labeling was absent after 8 weeks. In contrast, all neurofibroma SC cultures (seven of seven) showed persistent and diffuse intraneural growth throughout the same period. Typically, neurofibroma SCs emanated from glutathione *S*-transferase-positive foci and grew in extensive longitudinal streams that intermingled with the host nerve elements (Figure 6A). Tumor cell migration also was associated with the nerve sheaths. Some NF1 SC cultures developed sizable masses that displaced nerve elements and caused significant regional enlargement of the nerve diameter (Figure 6B). Tumor masses varied in size for the different culture grafts, but additional work is required to quantitate the size and distribution of the tumor grafts. It will also be important to determine whether growth rates of the cultures, grafts, and originating tumors are related. Nevertheless, all observations indicated that tumor development in the mouse nerve, like that of human neurofibromas, was relatively slow and benign. There were only sporadic signs of functional impairment associated with the largest tumors and no mortality was associated with the transplants for up to 8 weeks. These results demonstrate reliable and sustained tumor growth by neurofibroma-derived human SCs implanted in the mouse nerve.

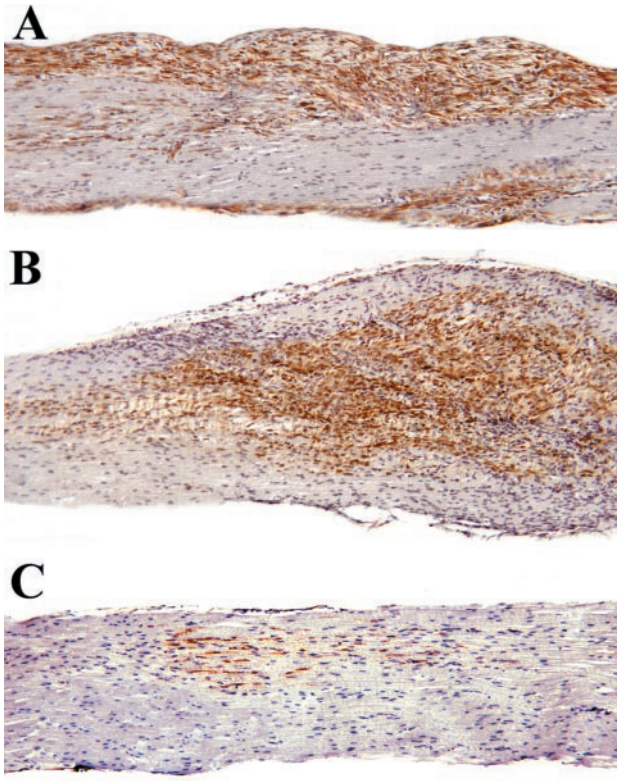

Figure 6. Tumor growth by human NF1 SC cultures xenografted into the nerves of *scid* mice. SC cultures from neurofibromas were transplanted into the nerves of immunodeficient mice. The growth and distribution of engrafted cells was traced by immunostaining with an antibody specific to human glutathione *S*-transferase. NF1 SC xenografts 4 weeks (**A**) and 8 weeks (**B**) after engraftment in the mouse sciatic nerve developed sizable masses and substantially increased the nerve diameter. NF1 SCs invaded along the longitudinal nerve axis between axons and along the epineurial sheath. Even the most extensive graft of normal SCs showed meager growth after 8 weeks, whereas most normal SC grafts failed to survive (**C**). Sections were counterstained with hematoxylin. Original magnification, ×100.

Discussion

In general, neurofibroma SCs, like their counterparts in normal nerves, fail to proliferate *in vitro* in the absence of specific mitogens.[5,7] Past efforts to establish models for human neurofibromas have relied on tissue explants and primary cultures of limited cell number with marked cellular heterogeneity. Only very recently have SCs been isolated from neurofibromas.[17–19] The lack of human neurofibroma-derived SC cultures and the low tumorigenic potential of neurofibroma cells in animal models has hampered the study of these prevalent tumors. Here we report the subculture of SCs from 10 dermal and eight plexiform NF1 tumors. All 18 of these NF1 SC cultures were found to be neurofibromin-deficient. Interestingly, many of the originating tumors contained both neurofibromin-negative and neurofibromin-positive SCs, indicating that SCs that lack functional *NF1* alleles have a distinct growth advantage *in vitro*. This advantage has been exploited by our subculture enrichment procedures using GGF-2 (without forskolin) and laminin.

Loss of *NF1* gene expression has been reported in malignant and benign SC tumors and pheochromocytomas from patients with NF1.[32,33] Based on the two-hit

hypothesis of tumor suppressor genes such as *NF1*, it is expected that neurofibromas should contain a supernumerary population of neurofibromin-deficient cells that are tumorigenic. Recent findings of cytogenetic alterations, loss of heterozygosity, and the absence of *NF1* mRNA expression in SCs cultured from neurofibromas strongly implicate SCs as the major neoplastic elements of dermal and plexiform neurofibromas.[17–19] In addition, in the present study we found that SCs isolated from numerous neurofibromas lack neurofibromin expression, confirming that *NF1* is inactivated in these SCs. Despite these persuasive findings, immunohistochemical evidence for a dominant population of neurofibromin-deficient cells has been highly ambiguous and scarcely reported. In an earlier study of numerous NF1 tumors, we concluded that a majority of neurofibromas consist mainly of SCs that express neurofibromin.[34] This antithetical observation was based on a large number of random archival specimens and, moreover, held true for a significant proportion of neurofibrosarcomas as well. However, on closer scrutiny, to examine the two-hit hypothesis at the single-cell level, it became evident that neurofibromin-negative SCs were present in most, if not all, neurofibromas. In the present study we focused on an independent group of neurofibromas from which SC subculture was successful. In most of the tumors in this group, neurofibromin labeling of tumoral elements was particularly sparse or absent. The remainder contained mixed populations of neurofibromin-negative and neurofibromin-positive SCs. The neurofibromin antibody used in these studies was raised against a peptide corresponding to amino acids 509 to 528 of the predicted *NF1* gene product. Directed against an N-terminal epitope, the antibody should bind to known neurofibromin splice variants as well as highly truncated (abnormal) forms. These results provide strong evidence that neurofibromin-negative cells of SC lineage contribute centrally to the formation of at least a significant subset of both dermal and plexiform neurofibromas. Nevertheless, there was notable variability in neurofibromin expression by SCs within many of the neurofibromas. The presence of neurofibromin expressing (*NF1* heterozygous) SCs suggests they too may perpetuate tumor formation, perhaps driven by the paracrine influence of the neurofibromin-deficient cell population. In this regard, Gutmann and co-workers[35] reported that neurofibromin expression by SCs in benign tumors may be down-regulated by factors produced within the tumor. Thus, paracrine influences may represent a novel mechanism for inactivating growth-suppressing genes and allowing for increased cell proliferation in tumors even in nonclonal cells. It is interesting that proliferation of neurofibromin-deficient SCs in response to GGF-2 was not enhanced by forskolin. Because forskolin increases SC expression of growth factor receptors including the GGF receptors erbB2 and erbB3,[36,37] this suggests that neurofibroma SCs express high levels of GGF receptors. Hyperexpression of erbB receptors has been reported in NF1 tumors and an inverse expression pattern of erbB2 and neurofibromin was shown for human SCs.[38,39] Additionally, SCs can express GGF and, at doses submaximal for proliferation, GGF-2 increases and directs the migration of SCs.[40–42] Taken together, these findings raise the possibility that GGF may function in an autocrine/paracrine mechanism that supports the continued growth of SCs in neurofibromas.

Despite considerable advances in the molecular genetics of NF1, the histogenesis of neurofibromas remains enigmatic. Dermal and plexiform neurofibromas contain a variety of cell types including SCs, perineurial cells, and fibroblasts. It is commonly held that despite their cellular complexity the histological features of neurofibromas are monotonously consistent.[43] In contradistinction, our studies of numerous neurofibromas and their derivative cell cultures indicate there are several levels of cellular and genetic diversity in this class of benign peripheral nerve sheath tumor. Cytogenetic abnormalities were identified in one of five of the dermal (our unpublished observation) and four of six of the plexiform SC cultures.[17] There were no consistent chromosomal regions involved in the abnormal karyotypes, suggesting that originating tumors are heterogeneous and may bear a variety of primary and/or secondary genetic changes. Additionally, the two plexiform cultures that displayed no cytogenetic rearrangements showed GGF-independent growth, suggesting that they either contain underlying genetic abnormalities not yet detected, or have expression abnormalities because of epigenetic influence. Clearly, neurofibromin deficiency did not confer GGF-2-independent growth on all of the developed SC cultures. Despite some differences in morphology, GGF-2 dependence, and karyotype, all NF1 SC cultures showed similarly low tumorigenic potential in several classical *in vitro* assays. However, the neurofibroma SC cultures showed a strong propensity to aggregate and form culture tumors. A similar growth pattern was observed for suspension cultures whereby NF1 SCs readily grew in aggregates reminiscent of tumor spheroids (an *in vitro* model for tumorigenic growth). Neurofibroma culture tumors contained an extensive laminin-rich extracellular matrix similar to that observed in neurofibromas.[30] These observations attest to a tumorigenic property of neurofibromin-deficient SC cultures that may be related to the adhesive mechanisms involved in the growth and development of neurofibromas. Sheela and co-workers[15] first demonstrated that NF1 SCs are angiogenic and invasive. In subsequent studies, which included two of the NF1 SC cultures used in the present report, we also concluded that NF1 SCs have a high invasive potential and a loss of negative autocrine growth control.[16] Despite these tumorigenic properties, our NF1 SC cultures, as well as the neurofibroma cultures used by Sheela and colleagues,[15] failed to form subcutaneous tumors in immunodeficient mice. Taken together, these findings imply that NF1 SCs have a tumorigenic potential that was not fully expressed in the *in vivo* model systems used previously.

Inceptive studies demonstrated the growth of implanted human neurofibroma tissue or SC preparations into the sciatic nerves of immunodeficient mice and the potential of this xenograft model for studying the tumorigenesis in NF1.[14,44] In the present study neurofibroma-like tumors resulted from the transplantation of neurofibromin-deficient NF1 SC cultures into the nerves of *scid*

mice. Extensive migration was consistently observed and many tumors were sizeable and substantially enlarged in the mouse nerve. There were apparent differences in the growth by the different transplanted NF1 SC cultures but overall tumor expansion was slow, suggesting that the growth rate of the developed tumors may reflect that of human neurofibromas. Additional studies are required to determine whether the growth patterns of the engrafted SC tumors correlate with those of the originating human tumors. This intraneural engraftment model is the first to achieve tumorigenic growth *in vivo* by human neurofibromin-deficient SCs and provides the means to study the histogenesis of neurofibromas in a relevant cellular environment. A further enhancement to this NF1 tumor model will be to transplant these neurofibroma SCs in the nerves of immunodeficient mice that are also heterozygous for *Nf1*. This highly relevant model of neurofibroma will also provide the opportunity to observe the interactions and contributions of engrafted ($Nf1^{-/-}$) cells and ($Nf1^{-/+}$) nerve elements with the same genetic background as those found in NF1 patients.

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References

1. Tonsgard JH, Kwak SM, Short P, Dachman AH: CT imaging in adults with neurofibromatosis-1—Frequent asymptomatic plexiform lesions. Neurology 1998, 50:1755–1760
2. Gutmann DH, Collins FS: Neurofibromatosis type 1. The Metabolic and Molecular Bases of Inherited Disease. Edited by CR Scriver, AL Beaudet, WS Sly, D Valle. New York, McGraw-Hill, 1995, pp 677–696
3. Peltonen J, Jaakkola S, Lebwohl M, Renvall S, Risteli L, Virtanen I, Uitto J: Cellular differentiation and expression of matrix genes in type 1 neurofibromatosis. Lab Invest 1988, 59:760–771
4. Erlandson RA, Woodruff JM: Peripheral nerve sheath tumors: an electron microscopic study of 43 cases. Cancer 1982, 49:273–287
5. Krone W, Mao R, Muhleck OS, Kling H, Fink T: Cell culture studies on neurofibromatosis (von Recklinghausen). Characterization of cells growing from neurofibromas. Ann NY Acad Sci 1986, 486:354–370
6. Atit RP, Crowe MJ, Greenhalgh DG, Wenstrup RJ, Ratner N: The Nf1 tumor suppressor regulates mouse skin wound healing, fibroblast proliferation, and collagen deposited by fibroblasts. J Invest Dermatol 1999, 112:835–842
7. Sobue G, Sonnenfeld K, Rubenstein AE, Pleasure D: Tissue culture studies of neurofibromatosis: effects of axolemmal fragments and cyclic adenosine 3′,5′-monophosphate analogues on proliferation of Schwann-like and fibroblast-like neurofibroma cells. Ann Neurol 1985, 18:68–73
8. Nakamura T, Hara M, Kasuga T: Early lesion of N-nitroso-N-ethylurea-induced hamster neurofibromatosis model. Cancer Res 1991, 51: 434–438
9. Kharbanda K, Dinda AK, Sarkar C, Karak AK, Mathur M, Roy S: Cell culture studies on human nerve sheath tumors. Pathology 1994, 26:29–32
10. Feany MB, Anthony DC, Fletcher CDM: Nerve sheath tumours with hybrid features of neurofibroma and schwannoma: a conceptual challenge. Histopathology 1998, 32:405–410
11. Colman SD, Williams CA, Wallace MR: Benign neurofibromas in type 1 neurofibromatosis (NF1) show somatic deletions of the NF1 gene. Nat Genet 1995, 11:90–92
12. Brannan CI, Perkins AS, Vogel KS, Ratner N, Nordlund ML, Reid SW, Buchberg AM, Jenkins NA, Parada LF, Copeland NG: Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Dev Rev 1994, 8:1019–1029
13. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA: Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nat Genet 1994, 7:353–361
14. Lee JK, Sobel RA, Chiocca EA, Kim TS, Martuza RL: Growth of human acoustic neuromas, neurofibromas and schwannomas in the subrenal capsule and sciatic nerve of the nude mouse. J Neurooncol 1992, 14:101–112
15. Sheela S, Riccardi VM, Ratner N: Angiogenic and invasive properties of neurofibroma Schwann cells. J Cell Biol 1990, 111:645–653
16. Muir D: Differences in proliferation and invasion by normal, transformed and NF1 Schwann cell cultures are influenced by matrix metalloproteinase expression. Clin Exp Metastasis 1995, 13:303–314
17. Wallace MR, Rasmussen SA, Lim IT, Gray B, Zori RT, Muir D: Culture of cytogenetically abnormal Schwann cells from benign and malignant NF1 tumors. Genes Chromosom Cancer 2000, 27:117–123
18. Kluwe L, Friedrich R, Mautner VF: Loss of NF1 allele in Schwann cells but not in fibroblasts derived from an NF1-associated neurofibroma. Genes Chromosom Cancer 1999, 24:283–285
19. Rutkowski JL, Wu K, Gutmann DH, Boyer PJ, Legius E: Genetic and cellular defects contributing to benign tumor formation in neurofibromatosis type 1. Hum Mol Genet 2000, 9:1059–1066
20. Rutkowski JL, Tennekoon GI, McGillicuddy JE: Selective culture of mitotically active human Schwann cells from adult sural nerves. Ann Neurol 1992, 31:580–586
21. Casella CTB, Bunge RP, Wood PM: Improved method for harvesting human Schwann cells from mature peripheral nerve and expansion in vitro. Glia 1996, 17:327–338
22. Bottenstein JE, Sato GH: Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc Natl Acad Sci USA 1979, 76:514–517
23. Muir D: Metalloproteinase-dependent neurite outgrowth within a synthetic extracellular matrix is induced by nerve growth factor. Exp Cell Res 1994, 210:243–252
24. Gutmann DH, Aylsworth A, Carey JC, Korf B, Marks J, Pyeritz RE, Rubenstein A, Viskochil D: The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. JAMA 1997, 278:51–57
25. Neugut AI, Weinstein IB: Use of agarose in the determination of anchorage-independent growth. In Vitro 1979, 15:351–358
26. Muir D, Varon S, Manthorpe M: An enzyme-linked immunosorbent assay for bromodeoxyuridine incorporation using fixed microcultures. Anal Biochem 1990, 185:377–382
27. Gutmann DH, Wood DL, Collins FS: Identification of the neurofibromatosis type 1 gene product. Proc Natl Acad Sci USA 1991, 88: 9658–9662
28. Zorick TS, Lemke G: Schwann cell differentiation. Curr Opin Cell Biol 1996, 8:870–876
29. Rutkowski JL, Kirk CJ, Lerner MA, Tennekoon GI: Purification and expansion of human Schwann cells in vitro. Nat Med 1995, 1:80–83
30. Leivo I, Engvall E, Laurila P, Miettinen M: Distribution of merosin, a laminin-related tissue-specific basement membrane protein, in human Schwann cell neoplasms. Lab Invest 1989, 61:426–432
31. Daston MM, Scrable H, Nordlund M, Sturbaum AK, Nissen LM, Ratner N: The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, Schwann cells, and oligodendrocytes. Neuron 1992, 8:415–428
32. Takahashi K, Suzuki H, Hatori M, Abe Y, Kokubun S, Sakurai M, Shibahara S: Reduced expression of neurofibromin in the soft tissue tumours obtained from patients with neurofibromatosis type 1. Clin Sci 1995, 88:581–585
33. Gutmann DH, Cole JL, Stone WJ, Ponder BA, Collins FS: Loss of

neurofibromin in adrenal gland tumors from patients with neurofibromatosis type I. Genes Chromosom Cancer 1994, 10:55–58
34. Rojiani AM, Rojiani MV, Wallace MR, Johnson J, Muir D: Expression of the NF1 gene product in peripheral nerve sheath tumors from NF1 patients. J Neuropathol Exp Neurol 1999, 58:524
35. Gutmann DH, Silos-Santiago I, Geist RT, Daras M, Rutkowski JL: Lack of NF1 expression in a sporadic schwannoma from a patient without neurofibromatosis. J Neurooncol 1995, 25:103–111
36. Weinmaster G, Lemke G: Cell-specific cyclic AMP-mediated induction of the PDGF receptor. EMBO J 1990, 9:915–920
37. Cheng LL, Esch FS, Marchionni MA, Mudge AW: Control of Schwann cell survival and proliferation: autocrine factors and neuregulins. Mol Cell Neurosci 1998, 12:141–156
38. Rowley PT, Kosciolek B, Bader JL: Oncogene expression in neurofibromatosis. Ann NY Acad Sci 1986, 486:327–332
39. Schlegel J, Muenkel K, Trenkle T, Fauser G, Ruschoff J: Expression of the ERBB2/neu and neurofibromatosis type 1 gene products in reactive and neoplastic schwann cell proliferation. Int J Oncol 1998, 13:1281–1284
40. Raabe TD, Clive DR, Neuberger TJ, Wen D, DeVries GH: Cultured neonatal Schwann cells contain and secrete neuregulins. J Neurosci Res 1996, 46:263–270
41. Carroll SL, Miller ML, Frohnert PW, Kim SS, Corbett JA: Expression of neuregulins and their putative receptors, ErbB2 and ErbB3, is induced during Wallerian degeneration. J Neurosci 1997, 17:1642–1659
42. Mahanthappa NK, Anton ES, Matthew WD: Glial growth factor 2, a soluble neuregulin, directly increases Schwann cell motility and indirectly promotes neurite outgrowth. J Neurosci 1996, 16:4673–4683
43. Riccardi VM: Neurofibromatosis: Phenotype, Natural History, and Pathogenesis, ed 2. Baltimore, Johns Hopkins University Press, 1992
44. Appenzeller O, Kornfeld M, Atkinson R, Snyder RD: Neurofibromatosis xenografts. Contribution to pathogenesis. J Neurol Sci 1986, 74:69–77