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PRINCIPAL INVESTIGATOR: Nancy Ratner, Ph.D.

CONTRACTING ORGANIZATION: University of Cincinnati
Cincinnati, Ohio 45267-0553

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13. ABSTRACT (Maximum 200 Words) <p>Humans with mutations in the <i>NF1</i> gene develop benign peripheral nerve tumors comprised mainly of Schwann cells (neurofibromas) and hyperpigmented spots on the skin (CALM). Mice with mutations of the <i>Nf1</i> gene fail to develop CALM or neurofibromas. We cut the sciatic nerve of <i>Nf1/nf1</i> mice and induced frequent CALM- and rare neurofibroma-like lesions. We hypothesized that nerve lesion creates an environment that triggers abnormal behavior of heterozygous cells, including Schwann cells. We proposed to test this hypothesis using nerve grafting. Our data shows that mutant Schwann cells secrete factor(s) that cause melanocyte pigmentation, and that, in addition, subpopulations of mutant Schwann cells transdifferentiate into pigment-forming cells in the wound environment. We also wounded <i>Nf1/nf1</i> mice in a chemical carcinogenesis paradigm and obtained mice with increased pigmentation and keratinocyte tumors, further substantiating our hypothesis that a wound environment can trigger features of human NF1 disease. Finally, using transgenic mice, we proposed testing whether Ras activation in peripheral nerve Schwann cells is necessary and/or sufficient to promote wound-associated phenotypes of <i>Nf1/nf1</i> mice. Using a promoter that drives robust Schwann cell expression, we cloned constructs into the new promoter-driven plasmids, and have identified several founder mice.</p>			
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FOREWORD

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(5) Introduction:

Our goal is to understand the development of benign manifestations of NF1, especially hyperpigmented skin patches (cafe-au-lait macules) and neurofibromas. A link between wounding and both pigmentation and tumor formation was hypothesized for NF1 patients (Riccardi, 1990). Extensive evidence shows that irritation, inflammation, and/or wounding promote skin tumor development (Martins-Green et al., 1994). Mice treated with tar, TPA, or expressing viruses (bovine papilloma virus or the tat gene of T-cell retrovirus) or oncogenes (v-src, v-jun) develop tumors on wounding. Thus "involvement of wounding or wound repair in carcinogenesis may be a general phenomenon" (Dvorak, 1986; Martins-Green et al., 1994). Therefore, based solely on anecdotal evidence, we hypothesized that wounding mice with a single mutant *Nf1* allele might cause pigmentation and/or tumor formation. To test this hypothesis, we developed a model system in which transection of the sciatic nerve in mice with a single mutant *Nf1* allele led to consistent hyperpigmentation and rare tumor formation. The major goal of this grant is to clarify the mechanism(s) underlying these effects. This year we extended our studies on wounding on *Nf1/nf1* mutant mice. In a series of studies supported by the NIH, we carried out excisional skin wounding and showed that fibroblasts behaved abnormally in response to this sort of wounding; alterations in pigmentation did not develop (Atit et al., 1999). In the last year, we also exposed *Nf1/nf1* mutant mice DMBA + TPA in a standard mouse chemical carcinogenesis analysis. We report here (Task 3) that the *Nf1/nf1* mutant mice respond abnormally in this paradigm, demonstrating both benign tumor formation (papillomas) and increased pigmentation. We are convinced, based on all these studies, that wounding is sufficient to promote features of human NF1 disease in *Nf1/nf1* mutant mice.

(6) Body:

STATEMENT OF WORK (REVISED AS DIRECTED FOLLOWING 1998 REORT)

Wounding-Induced Manifestations of Type 1 Neurofibromatosis

Task 1, Analyze *Nf1/nf1* mice after nerve lesion.

- a. Lesion nerves by crush, nerve cut or nerve cut with deflection and analyze pigmentation (complete)
- b. Crush nerves of adult mice and define abnormalities in proliferation (complete).
- c. Wound nerves of 30 neonatal mice and define abnormalities (completed).
- d. Paralyze 20 mice with ricin and cut nerves; analyze for pigmentation (completed).

Task 2, Develop nerve grafting experiments for assessment of contribution of specific cell types to the *Nf1/nf1* wound-induced phenotype.

- a. Carry out graft surgeries on recipient mice and complete analysis of phenotypes of grafted cells (completed experiments; data analysis in progress).
- b. *In vitro* analysis of mutant cells for melanogenic potential (in progress).
- c. *In vitro* analysis of medium from mutant cells for stimulation of melanogenesis (completed).

Task 3, Test if another wounding paradigm, chemical carcinogen exposure, stimulates increased pigmentation or tumor formation in *Nf1/nf1* mice.

- a. Apply DMBA or DMBA and TPA to wild type and mutant mice (completed).
- b. Analyze resultant skin spots and tumors for Ras mutations (completed).

- c. Analyze skin for abnormal TPA response (completed).

Task 4, Develop transgenic mice with altered Ras-GTP levels in Schwann cells.

- a. Make and sequence v-Ha-*ras* and *GRD-1* and *GRD-2* constructs (completed).
- b. Test each construct in Schwann cells (completed) and inject each DNA into blastocysts (ongoing).
- c. Obtain 3-10 founder animals per construct; start breeding to obtain F₁ generation (ongoing).
- d. Characterize phenotypes of three transgenic strains; breed *GRD-1* and *GRD-2* strains to *Nf1/nf1* mice; (months 24 - 36). This will require an average census of 60 mice/strain.
- e. Lesion nerves of 20 v-Ha-*ras* expressing mice and analyze phenotype (months 24 - 36).
- f. Lesion nerves of 20 *GRD-1* and 20 *GRD-2* -expressing mice and 40 double transgenics (20 each *Nf1/nf1-GRD-1* and *Nf1/nf1-GRD-2*) and complete analysis (We hope to begin this by the end of the granting period).

Task 1: Define steps in wound healing that precede and correlate with hyperpigmentation.

In normal nerves, nerve cuts result in penetration of serum factors and inflammatory cells into the epineurium because the perineurium is disrupted (Olsson et al., 1973; Weerasuriya et al., 1980). Following peripheral nerve cut axons and myelin sheaths degenerate in the denervated distal stump, macrophages invade the nerve to remove debris (Griffin et al., 1993), and Schwann cells proliferate (Siironen et al., 1994). This process is known as Wallerian degeneration. Subsequently, axons emerge from the proximal stump and use Schwann cell surfaces and Schwann cell basal lamina tubes (Bands of Bungner) to re-innervate peripheral targets (Fawcett et al., 1990).

We hypothesized that one or more of these events must be altered in *Nf1/nf1* mice, resulting in the observed phenotypes (hyperpigmentation and tumor formation) after nerve cut, resembling human NF1. Schwann cells with *NF1* mutations might show altered responses to macrophage products released on nerve damage (Griffin et al., 1993; Saada et al., 1996). Effects of growth factors present in neurofibromas (e.g. Krasnoselsky et al., 1994) on Schwann cells might also be potentiated by macrophage products or hormones. In this way, paracrine signals can be envisioned to promote tumor formation.

Last year, we cut and deflected the sciatic nerve, rather than resuturing proximal and distal stumps. Pigmentation was greatly increased (Figure 1). This leaves distal Schwann cells separated from axons. This year, we repeated deflection experiments on more animals (total n = 9 +/+ and 9 +/-), with similar results. Thus it appears highly unlikely that neurons are required for hyperpigmentation. We continued to test the hypothesis that neurons are not required, in experiments proposed as 2b in the original grant. We killed neurons with ricin, then lesioned the nerve. We predicted a significant level of hyperpigmentation. However,

no pigmentation was detected. In the interim, we learned that ricin is also toxic to macrophages (Morrison et al., 1999). Therefore, this experiment is consistent with the idea that excess tissue damage mediated through macrophages is a key trigger for increased pigmentation (see also our results using macrophage conditioned medium in Atit et al., 1999).

We have also considered whether abnormal Schwann cell proliferation after nerve injury might contribute to the hyperpigmentation, even though it appears unlikely considering our recent findings (see below). To assess Schwann cell proliferation, we crushed nerves of adult wild type and mutant mice and analyzed BrdU incorporation into Schwann cells. No differences were detected between genotypes, indicating that abnormal proliferation is unlikely to account for abnormal response to nerve damage (Figure 2).

Task 2, Use nerve grafting experiments to assess the contribution of specific cell types to the *Nf1/nf1* wound-induced pigmentation phenotype.

We hypothesized that nerve-derived Schwann cells could transdifferentiate into melanocytes, accounting for hyperpigmented cells. To test this idea, we cut a length of mouse sciatic nerve and incubated it in Hoechst dye. The nerve piece was grafted into a recipient host by suturing to the proximal and distal stumps of the sciatic nerve. The dye-labeled piece was then cut in its center, and deflected proximally and distally. One month later, the host animal was perfused with fixative, sections were cut on a cryostat and blue (dye positive) cells were counted. If nerve-derived cells transdifferentiate, then blue cells with pigment granules should be detected. The results were striking. We detected numerous blue cells outside the nerve in surrounding epineurium, and within muscle layers under the skin. Of 8568 dye-positive nuclei counted, 506 were adjacent to pigment granules,

presumably within the cytoplasm of the grafted cells. Therefore we **have modified our interpretation from last year's report.**

Table 1. Pigmented cells derive from peripheral nerve.

Host Number	# dye-positive cells counted*	# pigmented cells dye-positive	# pigmented cells dye-negative
1	2264	118	95
2	2451	127	110
3	1817	171	129
4	2026	89	76

*Cells were counted in 10 – 20 sections from each host animal. The percentage of dye-labeled pigmented cells was 5.2; 5.3; 9.4; 4.3% of total dye-labeled cells in the four animals. Note that many dye-negative cells were also pigmented. These are likely cells that arose from the host nerve.

An image of a portion of a section from one animal is shown in figure 3.

We conclude from this experiment that one or more populations of cells within the sciatic nerve have the potential to form pigment-forming cells. This experiment strongly suggests that cells from the nerve itself contribute to the hyperpigmentation phenotype, rather than effects of the injury on melanocytes outside the nerve.

Schwann cells and melanocytes are each derived from the neural crest. Therefore it seemed much more likely that Schwann cells rather than fibroblasts could begin to form pigment. To begin to determine which population of nerve cells can generate pigmented cells, we individually grafted unlabeled Schwann cells of various genotypes at *Nf1* to cut sciatic nerves. The Schwann cells were purified in culture and 20,000 cell plated onto each matrigel coated 8 uM pore-size filters of a 2cm² transwell unit (Costar). Approximately

equal cell numbers were confirmed by MTT assay as described (Kim et al., 1997). Filters were wrapped around cut nerves after suture of proximal and distal stumps. We evaluated wild type, heterozygous, null and *Nf1*^{-/-}*TXF* cells grafted into wild type and heterozygous hosts. Three animals were studied in each group; total animals n = 24. Schwann cells mutant at *Nf1* when grafted into wild type or heterozygous hosts augmented the overall pigmentation observed in the limb. The most dramatic effect was seen when *Nf1*^{-/-}*TXF* Schwann cells were grafted into heterozygous hosts. Figure 4 shows a representative results from *Nf1*^{-/-}*TXF* Schwann cells, as compared to implantation of wild type Schwann cells. These results strongly suggest that the population of cells derived in our laboratory that we have called *Nf1*^{-/-}*TXF* have an enhanced capacity to contribute to the hyperpigmentation phenotype. These cells are present in cultures of embryonic dorsal root ganglia from null embryos. We have shown that they express the EGFR aberrantly (DeClue et al., in revision for J. Clin. Invest.) and proliferate in serum free medium (Kim et al., 1997) while normal and null Schwann cells do not. These cells do not preferentially attach to axons. Recently, Parada and co-workers reported some of these characteristics for Schwann cells acutely knocked-out for *Nf1* (Parada, personal communication). These *Nf1*^{-/-}*TXF* Schwann cells are easy to grow as compared to other Schwann cells. As shown above, the *Nf1*^{-/-}*TXF* Schwann cells may exhibit a key feature of progenitor cells, the ability to alter their lineage.

As an additional test of the hypothesis that *Nf1*^{-/-}*TXF* Schwann cells are capable of transdifferentiation, we conducted experiments in which *Nf1*^{-/-}*TXF* Schwann cells were grafted into heterozygous hosts, and tested for pigmentation. Cells were expanded in culture, plated onto filters, labeled with Hoescht dye, and transplanted into cut sciatic nerve. After one month, recipient animals were perfused, sections cut, and dye-positive cells evaluated for

pigmentation. As controls, wild type mouse Schwann cells and *Nf1*^{-/-} fibroblasts were similarly labeled and transplanted around cut nerves.

Table 2. Pigmented cells can derive from *Nf1*^{-/-}*TXF* Schwann cells.

Donor cells	# Recipient animals	# Dye-positive cells counted*	# Pigmented cells dye-positive	Pigmented cells dye-negative
+/+ Schwann Cells	3	3500	0	467
-/- Fibroblasts	3	3700	0	367
-/-TXF Schwann Cells	2	2402	68	430

*Fifteen – twenty sections containing dye-positive cells were evaluated from each host animal. In total 6.2 and 10.5% of the dye-positive -/-TXF Schwann cells were pigmented.

This result strongly suggests that pigmented cells can derive from *Nf1*^{-/-}*TXF* Schwann cells. As additional controls for this experiment, we grafted labeled wild type Schwann cells and null fibroblasts (n = 6 each) into heterozygous hosts. As expected we found pigmented cells from the wild type Schwann cells at exceeding low incidence, possibly below our level of detection. So far, 0 of 3500 dye positive cells were pigmented. This result is consistent with an increased propensity of the mutant cells to undergo pigmentation. The grafts containing fibroblasts showed a similar low to no dye-positive pigmented cells.

What are the *Nf1*^{-/-}*TXF* cells? We previously stained these cells for expression of the Schwann cell markers S100 and p75NGFR. Positive staining was reported (although not shown) in Kim et al. (1997). We have gone back and done a more detailed marker analysis. We stained normal mouse Schwann cells, *Nf1*^{-/-}*TXF* Schwann cells, and fibroblasts with

anti-S100, anti-p75NGFR, anti-GFAP and anti-SMA. GFAP is specific for glia (astrocytes and Schwann cells); S100 marks Schwann cells and perineurial cells; p75NGFR marks Schwann cells and some fibroblasts; SMA is an actin expressed in embryonic fibroblasts, embryonic muscle, and smooth muscle. The results are shown in Figure 4.

Many fibroblasts in our preparation were positive for SMA. Fibroblasts were negative for GFAP and expressed low levels of nuclear S100 and low levels of p75. Normal mouse Schwann cells were as anticipated robustly positive for cytoplasmic S100 and strongly expressed p75. The cells expressed GFAP at modest levels. *Nf1*^{-/-}*TXF* cells were negative for SMA, positive for S100 and p75 (at similar levels to normal Schwann cells). While all *Nf1*^{-/-}*TXF* cells expressed some GFAP, about half the cells showed strongly elevated GFAP staining. To summarize, *Nf1*^{-/-}*TXF* cells show marker expression consistent with Schwann cells.

These data strongly suggest that peripheral nerve cells, including subpopulations of Schwann cells we have called *Nf1*^{-/-}*TXF* can become pigmented under appropriate conditions *in vivo*. Because pigmented cells derive after nerve injury in wild type and heterozygous mice, it is likely that similar populations exist within even normal nerves. The idea that a plastic “neural crest like” population of cells exists in late embryonic nerve has recently been shown for rat (Morrison et al., 1999). Our data would be the first to demonstrate a plastic precursor-like cell in adult nerve. Our data strongly supports the idea that mutation at *Nf1* augments either the number of these cells and/or their ability to become pigmented.

One of the problems we have encountered is that the dye-labeled nuclei are adjacent to cytoplasm containing pigment granules. While unlikely, it remains possible that the pigmented cells are actually adjacent to the grafted cells. We have been unable to stain the

dye labeled cells with standard stains such as hematoxylin and eosin, as this procedure leaches out the dye. We therefore have expanded a line of *in vitro* investigation begun last year. On one hand, we have added medium from Schwann cells wild type or mutant at *Nf1* to normal mouse melanocytes. Conversely, we exposed *Nf1*^{-/-}*TXF* Schwann cells to melanogenic factors.

The conditioned medium experiments are complete. In three separate experiments, medium from *Nf1*^{-/-} and *Nf1*^{-/-}*TXF* Schwann cells contained more melanogenic activity than corresponding wild type or medium only controls. Data are shown in Figure 6, and are documented by photographs showing increased pigmentation of the cells and by tyrosinase activity; tyrosinase is an enzyme required for melanin production. These results demonstrate that part of the mutant phenotype is likely to arise through enhanced secretion of melanogenic factors by mutant cells.

We have also begun to determine if appropriate medium conditions will encourage pigmentation by *Nf1*^{-/-}*TXF* Schwann cells. This is the anticipated result if Schwann cells, under nerve lesion conditions, transdifferentiate into melanocytes. In initial experiments (n = 2) we cultured *Nf1*^{-/-}*TXF* on LabTek slides, in melanocyte differentiation medium containing bFGF, TPA, cholera toxin and IBMX. The preliminary data look encouraging. About 15% of the cells have flattened out and formed small cellular inclusions, possible pre-melanosome. After 3 days we stained for *trp-1*, a melanosome enzyme. Authentic C57Bl/6 melanocytes were positive, our cells negative. We have fixed cells now after 6 days, and will continue to monitor cultures at longer times and stain for *trp-1* as well as a histochemical stain using L-dopa. We will also try more complex media according to Rao et al., 1997.

Task 3, Test if another wounding paradigm, chemical carcinogen exposure, stimulates increased pigmentation or tumor formation in *Nf1/nf1* mice.

- a. Apply DMBA or DMBA and TPA to wild type and mutant mice (completed).
- b. Analyze resultant skin spots and tumors for Ras mutations (completed).
- c. Analyze skin for abnormal TPA response (completed).

This set of experiments was not proposed in the original grant or statement of work. However, because the initially proposed experiments were progressing slowly [as described in detail in last year's progress report], we had time and animals to carry out this study. In addition, Ms. Pawlus of the DOD encouraged us to submit a revised Statement of Work based on last year's report. The data enclosed was presented at the NNFF Consortium meeting at MIT, and a paper describing our findings submitted to Cancer Research. A grant proposing experiments to follow up the findings was submitted to the DOD in fall of 1999. The data substantiate our hypothesis that a wound environment can trigger features of human NF1 disease.

Rationale:

Neurofibromatosis type 1 patients, but not mice heterozygous for mutations in the *Nf1* tumor suppressor (*Nf1*+/-), develop hyperpigmented spots and benign tumors. *Nf1* mice were exposed to DMBA or DMBA + TPA to test for co-operation between *Nf1* mutation and carcinogen exposure. DMBA caused C57Bl/6 *Nf1*+/- mice to develop pigmented skin patches, with hair follicles in anagen phase, at 2-fold increased frequency. Papillomas developed in 75% of *Nf1*+/- mice treated with DMBA and TPA but never in wild type mice or *Nf1*+/- mice treated with TPA only. Loss of *Nf1* does not circumvent the requirement for

activating mutations in c-H-*ras* in this model; although neurofibromin is a GAP for Ras all papillomas analyzed had mutations in codon 61 of H-*ras*. *Nf1*^{+/-} mutants showed significant sustained increases in proliferation of keratinocytes in response to TPA. Thus, the *Nf1* gene is a modifier of carcinogen-induced phenotypes that acts by stimulating TPA-mediated pathways, co-operating with activating *ras* mutations in keratinocytes. The data are consistent with a role for *Nf1* in epidermal cell carcinogenesis and keratinocyte dysfunction in NF1 patients.

Neurofibromatosis type 1 (*NF1*) is a common inherited autosomal dominant human disease, affecting 1 in 3500 individuals worldwide (reviewed in Huson, 1998). *NF1* patients heterozygous for mutations in the *NF1* gene are predisposed to develop benign and malignant tumors, and to pigmentation defects. Pigmentation defects include patches of hyperpigmented skin called cafe-au-lait macules (Crowe and Schull, 1953; Benedict et al., 1968; Takahashi, 1976; Frenk and Marazzi, 1984) found in all *NF1* patients by five years of age (Korf et al., 1992). *NF1* patients also develop axillary and inguinal freckling (Crowe et al., 1964; Friedman and Birch, 1997), and patches of retinal melanocytes (Lisch nodules) (Lewis and Riccardi, 1981; Huson et al., 1987). While it is not understood how mutations at the *NF1* locus in specific skin cell type(s) cause these *NF1* skin manifestations, a role for the *NF1* gene product, neurofibromin, in the skin is suggested by this aberrant pigmentation.

The expression pattern of neurofibromin is consistent with *NF1* function in the skin. In adult human skin neurofibromin is readily detectable in melanocytes and keratinocytes, and at much lower levels in fibroblasts (Malhotra and Ratner, 1994; Hermonen et al., 1995). Wounding up-regulates neurofibromin expression in human skin fibroblasts (Yla-Outinen et al., 1998). In rodents, neurofibromin is expressed at very low levels in keratinocytes, melanocytes, and fibroblasts of the adult skin (Daston and Ratner, 1992), but shows high expression in keratinocytes during embryonic and early postnatal life as keratinocytes are differentiating (Malhotra and Ratner, 1994). Thus, the major cell types in the skin express neurofibromin and could be affected by loss of function at *NF1*. Melanocytes from *NF1* patients are subtly abnormal (Kaufmann et al., 1991); keratinocytes have not been studied.

NF1 is considered to be a tumor suppressor gene, as loss of heterozygosity at *NF1* has been demonstrated in *NF1* patient malignant tumors, in neurofibromas, and in myeloid

disease (Legius; 1993; Sawada et al., 1996; Side et al., 1998; Serra et al., 1997). NF1 patients are at 4-fold increased risk for malignant disease (Sorenson et al., 1986; Zoller et al., 1997). In a population-based study in Sweden, 16% of adult NF1 patients developed carcinomas, suggesting possible increased risk for epidermal tumors (Zoller et al., 1997).

Heterozygous *Nf1* mice do not spontaneously develop benign tumors or the pigment abnormalities seen in human NF1 patients; *Nf1* null mice die *in utero* so are unavailable for analysis (Brannan et al., 1994; Jacks et al., 1994). Wounding has been hypothesized to serve as a triggering event of neurofibroma formation and café-au-lait macules in human NF1 (Riccardi, 1992). To begin to test this hypothesis, we used mice with targeted mutations in the *Nf1* gene and found aberrant skin fibroblast function after excisional skin wounding (Atit et al., 1999). Like wounding, skin carcinogens elicit an inflammatory response and induce skin cells to proliferate (reviewed in DiGiovanni, 1992; Scribner and Suss, 1978).

We tested if treatment of *Nf1* mutant skin with dimethylbenzanthracene (DMBA) increases pigmentation. Topical application of DMBA induces patches of pigmentation in susceptible strains of mice (Klaus and Winkelmann, 1965; Forbes et al., 1965; Kanno et al., 1987). DMBA treated skin shows increased proliferation of normally dormant DOPA-inactive melanocytes and enhanced melanogenic activity (Tsambaos et al., 1989); melanin is transferred to adjacent keratinocytes leading to visible pigmentation.

We also challenged *Nf1* mutant mice in the two-stage carcinogenesis model, in which skin is initiated with a potent carcinogen like DMBA and then exposed to multiple treatments with a tumor promoter such as wounding or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Deelman et al., 1927; DiGiovanni, 1992). Susceptible strains of mice develop papillomas of the skin (Boutwell et al., 1964; Slaga et al., 1989).

Ras activation is an important step in tumor progression in response to skin carcinogens. After DMBA exposure, activating mutations in the c-Ha-*ras* proto-oncogene at codon 61 develop in keratinocytes (Quintinalla et al., 1986; Nelson et al., 1992). Activating mutations in *ras* can serve as an initiating event in the two stage tumorigenesis model, because targeting of the v-Ha-*Ras* gene to follicular keratinocytes causes development of papillomas at a very high frequency, after promotion with wounding or TPA (Leder et al., 1990; Hansen and Tennant 1994a,b). Targeting Ras to melanocytes results in increased pigmentation and susceptibility to melanoma (Powell et al., 1995; Gause et al., 1997).

The molecular pathways regulated by neurofibromin are not completely understood. Neurofibromin functions as a Ras-GTPase activating protein (Ras-GAP) in some mammalian cell types (De Clue et al., 1992; Basu et al., 1992; Kim et al., 1995, 1997; Largaespada et al., 1996; Bollag et al., 1996; reviewed in Kim and Tamanoi, 1998). Neurofibromin also has ill-defined non-Ras functions in mammalian cells (Johnson et al., 1994; Atit et al., 1999). In *Drosophila*, NF1 regulates a cyclic AMP-dependent protein kinase A pathway in a Ras-Raf independent manner (The et al., 1997; Guo et al., 1997). Functional effects *in vivo* of *Nf1* mutations in skin could occur through either of these pathways, or others.

We show in this report that DMBA treatment leads to an increased frequency of pigmented spots in *Nf1*^{+/-} as compared to wild type mice. Furthermore, initiation with DMBA followed by promotion with TPA stimulates papilloma formation in *Nf1*^{+/-} C57Bl/6 mice but not wild type mice. The data are particularly striking as the C57Bl/6 strain is noted for resistance to chemical carcinogens (Kiguchi et al., 1997; Reiners, 1984). The increase in papilloma incidence in *Nf1*^{+/-} mice suggests co-operation between Ras activation and *Nf1* inactivation. Our data indicate that *Nf1* is a strong modifier of responses induced by skin carcinogens.

Materials and Methods:

Chemicals and Reagents:

7,12-dimethylbenz[a]anthracene (DMBA), 12-*O*-tetradecanoyl-13-acetylphorbol (TPA), BrdU were purchased from Sigma Chemicals Co. (St. Louis, MO). Biotinylated monoclonal anti-PCNA and anti-BrdU was from Zymed Laboratories Inc. (San Francisco, CA).

Animals:

C57Bl/6 wild-type female mice (breeders) were obtained from Harlan (Indianapolis, IN). *Nf1* +/- mice were generated by targeting one allele of the *Nf1* gene (Brannan et al., 1994). For genotyping, DNA from a toe clip was isolated and the presence of the targeted allele determined by PCR as described in Brannan et al. (1994). Wild type and *Nf1*+/- mice were obtained by mating *Nf1*+/- C57Bl/6 males to C57Bl/6 breeder females. Mice were maintained on a 12-h light/dark cycle in a temperature and humidity-controlled room and were provided with reverse osmosis water and rodent chow (Purina, St. Louis, MO) ad libitum. Male mice were treated as described at 9-12 weeks of age. Dorsal skin hair was carefully shaved with surgical clippers one day before topical dosing was begun and mice with skin in anagen phase at the initial shaving were excluded from the analysis. Mice were housed four per cage until 20 weeks of tumor promotion treatment, or until papillomas formed. Subsequently mice were individually housed in polystyrene cages to avoid injury to newly formed papillomas.

Treatments:

Mice were initiated by application of 40ug of DMBA in 200ul acetone on Day 1 and Day 10. Mice were observed for 2-4 months after initiation. One week after initiation was complete one group of mice received topical applications of 0.2 ml of 6.4 nM TPA in acetone (0.8ug) delivered using a micropipette. Mice received TPA three times per week for 24 weeks. Control mice received 0.2 ml of acetone vehicle on the same schedule. Dorsal skin was shaved once every 4-6 weeks to maintain exposure of the skin to the treatments. The incidence of pigmentation and skin papillomas was recorded weekly. Tumor data are expressed as the percentage of mice with papillomas (tumor incidence) and the average number of papillomas per mouse (tumor multiplicity).

Histology:

Mice were euthanized in a chamber filled with CO₂. Treated skin from pigmented and unpigmented regions, and papilloma tissue with adjacent skin was excised from the dorsum. Specimens were fixed in 10% formalin, embedded in paraffin, and 6 microns sections cut and stained with hematoxylin and eosin. Some sections were immunostained with biotinylated anti-PCNA or anti-BrdU per directions from the manufacturer. Hair follicle number was counted on a light microscope in at least 5 different fields/section. Multiple sections were analyzed from two different specimens of treated skin per mouse.

H-ras codon 61 mutational analysis:

DNA was isolated from unpigmented and pigmented regions of the treated skin of three wild type animals and five *Nf1*^{+/-} animals after an overnight lysis in buffer (100mM Tris, 0.2% SDS, 200mM NaCl, 5mM EDTA) containing proteinase K (100ug/ml). DNA was precipitated in isopropanol and resuspended in TE. Genomic DNA was isolated

from papilloma laden mouse skin using Puregen reagents (Gentra Systems, Minneapolis, MN) and proteinase K. DNA was quantitated by UV spectroscopy at 260 nm. For *ras* mutational analysis, all PCR primers were based on the mouse *H-ras* genomic sequence of Brown et al. (1988). The enriched polymerase chain reaction (EPCR) method described in detail by Mitchell and Warshawsky (1998) was used to screen for codon 61 mutations in treated skin and papilloma-derived DNA. Amplification of a normal allele gives a 135 bp band and the allele with the codon 61 mutation gives a 162 bp fragment. Normal mouse liver DNA was used as the negative control and an *H-ras* codon 61 CAA-CGA DNA fragment was used as the positive control (Mitchell and Warshawsky, 1998). Reverse cyclic dideoxy sequencing was used to confirm the codon 61 mutations (Mitchell and Warshawsky, 1998).

Epidermal labeling index after TPA treatment

Wild type or *Nf1*^{+/-} mice were treated 4 times over two weeks with acetone, 0.8 μ g TPA, or 4 μ g TPA, and sacrificed 48 hours after the last treatment. In other experiments animals were treated once with acetone, 0.8 μ g TPA, or 4 μ g TPA, and sacrificed 48 hours after treatment. One hour before sacrifice, mice were injected intraperitoneally with 100mg/kg BrdU (Sigma) in saline. Two to three specimens from the treated area of the dorsal skin were collected into 10% normal buffered formalin, and processed for paraffin embedding. Immunohistochemistry to detect BrdU-labeled cells was carried out on deparaffinized sections according to manufacturer's instructions; sections were counterstained with hematoxylin. The percent of labeled basal keratinocytes (labeling index) was calculated after counting 1500 to 2000 basal keratinocytes per mouse as described in Naito et al. (1987).

Results

Increased incidence of pigmentation in *Nf1*^{+/-} skin after treatment with DMBA:

Twenty-two wild-type mice and 30 *Nf1*^{+/-} littermates were treated with DMBA on day 1 and day 10. Within three weeks after initiation, most *Nf1*^{+/-} (n=26/30) mice showed large patches of pigmentation, all greater than 1 cm². This phenotype was absent in untreated mice, and in mice treated with acetone only (n = 5-9/ genotype; Table 3).

<u>Treatment</u>	<u><i>Nf1</i>^{+/+}</u>	<u>% affected</u>	<u><i>Nf1</i>^{+/-}</u>	<u>% affected</u>
Acetone	0/5	0	0/9	0
TPA	0/8	0	0/15	0
DMBA	8/22	36	23/30	76
DMBA+TPA	6/6	100	12/12	100

Table 3. Incidence of pigmentation after treatment with skin carcinogens.

C57Bl/6 male mice were treated with 200µl topical acetone vehicle, TPA (0.8ug, 3 times per week), DMBA (40ug, 2 treatments), or DMBA+TPA (DMBA 40ug, 2 treatments followed by TPA, 0.8ug, 3 times per week). Animals exhibited that exhibited one or more patches of pigmentation larger than 1cm² were scored as positive. When patches appeared, they were always larger than 1cm². The first three groups were scored by 4 months after initiation of experiments. The DMBA+TPA group was scored 6 months after initiation of treatment.

Pigmented patches were also absent in wild type or *Nf1*^{+/-} exposed to TPA alone, even after 2 months of treatment (Table 3). Pigmented patches remained grossly visible for at least 4 months (Fig. 7B and C; Table 3). Most wild type mice did not develop pigmentation in response to DMBA (Fig. 7A), but 8 of 22 wild type mice did (Table 3). Thus, a significant increase in the incidence of pigmentation was observed in *Nf1*^{+/-} mice in response to DMBA.

Skin was analyzed by histology to define skin components contributing to the pigmentation. Wild type and *Nf1*^{+/-} skin had normal histology outside pigmented areas, with hair follicles in the resting (telogen) phase (Fig. 7D). In contrast, skin from pigmented patches of affected wild type and *Nf1*^{+/-} mice showed large numbers of hair follicles filled with melanin pigment (Fig. 7E and F, white arrows). Skin sections from wild type and *Nf1*^{+/-} mice were immunostained with anti-PCNA, a proliferation marker, to confirm that the hair follicles in pigmented spots were in anagen phase. The visible brown precipitate marks proliferating follicular keratinocytes (Fig. 7H and I). Hair follicles were found in multiple stages of differentiation in which different compartments of the follicle contain proliferating cells (not shown).

Estimates of hair follicle numbers were obtained from sections of unpigmented and pigmented skin of 4-6 wild type and *Nf1*^{+/-} mice two months after initiation (Fig. 8). Normal-appearing skin of wild type and *Nf1*^{+/-} mice had similar low numbers of hair follicles/field (Fig. 8A). When pigmented skin was analyzed (Fig. 8B) much higher numbers of follicles were present, but the numbers were indistinguishable in the two genotypes. Thus, visible pigmented spots are indistinguishable at the gross and histological levels between the wild type and *Nf1*^{+/-} groups. These data suggest that the *Nf1* gene is a modifier of the pigmentation response to DMBA in the C57Bl/6 strain of mice.

Papilloma formation in *Nf1*^{+/-} mice after DMBA and TPA treatment:

In a second group of mice, dorsal skin was initiated with DMBA and then promoted with multiple treatments of TPA to determine if *Nf1*^{+/-} skin would respond differentially to a tumor promoter. All animals in the wild type (n=6/6) and *Nf1*^{+/-} (n=12/12) groups developed large patches of pigmented skin (Table 3) after exposure to DMBA + TPA. Skin histology

from unpigmented (Fig. 9B) and pigmented (Fig. 9C) regions was indistinguishable from that observed when skin was treated with DMBA alone (Fig. 7D, F).

The C57Bl/6 mouse strain is extremely resistant to tumor formation in response to the DMBA and TPA regimen (Reiners et al., 1984; Naito et al., 1987; reviewed in DiGiovannini 1992; Kiguchi et al., 1997). In an initial study, none of 6 wild type mice analyzed developed papillomas. Yet, after 20 weeks of promotion, papillomas began to arise on the dorsum of most *Nf1*^{+/-} mice, which were littermates of the wild type mice analyzed (Fig. 10A). By the end of 24 weeks, 9/12 (75%) of *Nf1*^{+/-} animals (Fig. 10A) had developed papillomas. Most animals had 1-3 tumors; tumor ranged in volume from 1 to 80mm³ (Fig. 10B). In a second experiment none of 12 wild type mice developed papillomas in response to DMBA + TPA whereas 9 of 12 mutants developed papillomas by 20 weeks after promotion (not shown). None of 8 wild type or 15 *Nf1*^{+/-} mice developed papillomas in response to TPA alone. Acetone alone did not provoke papillomas in one wild type or 4 *Nf1*^{+/-} mice.

Papillomas were analyzed histologically in hematoxylin and eosin stained paraffin sections (Fig. 9D). Papillomas showed epithelial hyperplasia (*e* in Fig. 9E) and trapped sebaceous glands with follicular cysts (*s* and *c* respectively, Fig. 9F) characteristic of papillomas. Trapped sebaceous glands were a significant component of most of the papillomas examined. The hypothesis that the sebaceous glands are part of the neoplastic process was considered but excluded because the whole structure of nearby hair follicles was well preserved. It appears that sebaceous glands are trapped in the papillomas in *Nf1*^{+/-} mice even at stages when the papillomas are large (C. Conti, MD Anderson Cancer Center, Smithville, TX, personal communication). The reason for this is not known. Larger papillomas showed hyperkeratosis (not shown). These data demonstrate that loss of one *Nf1*

allele dramatically increases papilloma incidence in C57Bl/6 *Nf1*^{+/-} mice treated with DMBA + TPA. Thus, the *Nf1* gene acts as a modifier of the papilloma phenotype.

Ras mutational analysis of pigmented skin and papillomas

The c-H-*ras* gene is a target for activating mutations induced by DMBA and TPA (Quintanilla, 1986). In mouse skin papillomas, mutations in >90% of the tumors are detected at codon 61 of c-H-*ras* (reviewed in DiGiovannini, 1992). Because neurofibromin can act as a GAP for the Ras proteins, it seemed possible that *ras* gene mutations that frequently are detected in papillomas might not be detected in *Nf1* mutants. To test this idea, we used an enriched PCR analysis designed by David Warshawsky and Kent Mitchell of University of Cincinnati (see Methods). We carried out the analysis in collaboration with them. Treated mouse skin from unpigmented and pigmented regions from three different *Nf1*^{+/+} mice and five different *Nf1*^{+/-} mice did not show the c-H-*ras* codon 61 mutation (Fig. 11A lanes 1-4). We analyzed the DNA from 6 of the largest *Nf1*^{+/-} skin papillomas for c-H-*ras* codon 61 mutations. Results showed that 6 of 6 independent tumor specimens analyzed contain cells with the A to T transversion at codon 61 from the c-H-*ras* gene (Figure 11B, lanes 1-4). Thus, presence of *ras* mutations in addition to functional inactivation of a single allele of *Nf1* is required to overcome the resistance of C57Bl/6 mouse strain to papilloma formation.

Epidermal labeling index after TPA treatment of epidermis

We tested whether loss of *Nf1* alters keratinocyte proliferation in response to TPA, accounting for altered susceptibility of mutant animals to papilloma formation. Mice were treated with TPA once, or four times over two weeks. Forty-eight hours after the last TPA

treatment, mice were injected with BrdU for 1 hour. *In vivo* labeling indices of basal intra-follicular epidermal keratinocytes were calculated in tissue sections. Labeling indices were similar in wild type and mutant animals either untreated or treated with acetone. We also did not observe a significant difference between genotypes in labeling index after a single treatment with 0.8 μ g or 4 μ g of TPA (not shown). Even after 4 treatments of 0.8 μ g TPA over two weeks, no difference in labeling index was observed between genotypes. Because wild type mice had a high degree of proliferation ($37\pm 5.3\%$, n=4) under these conditions, we treated two sets of mice with a higher dose (4 μ g) of TPA over two weeks. Similar epidermal hyperplasia (7-9 layers) was observed in animals of both genotypes. Under these conditions a difference between the wild type and *Nf1*^{+/-} keratinocytes was revealed (Fig. 12). A consistent increase in epidermal labeling index was detected in mutant mice (7/7) as compared to 4 wild type animals. This difference was significant ($p < .0001$; Student's *t*-test). Our results on sections from wild type mice can be compared with Naito et al. (1987), and Kiguchi et al. (1997), who showed a $10.8\pm 2.1\%$ and $\sim 8\%$ labeling index using this protocol on C57Bl/6 mice. Values for individual wild type and mutant animals are shown in Fig. 6C. The results show that *Nf1*^{+/-} keratinocytes abnormally sustain proliferation in response to TPA.

Discussion

We demonstrated a cooperating effect of mutation in a single *Nf1* allele with activating *ras* mutations to override the genetic resistance of C57Bl/6 mice to skin tumor promotion. Thus, *Nf1* is one of the long-elusive modifier genes for epithelial tumorigenesis in mice. Our data are consistent with a role for *NF1* in human epidermal carcinogenesis. Indeed, regions of LOH including *NF1* have been reported in breast, ovarian and esophageal cancer (Wertheim et al., 1996; Dunn et al., 1999). In addition, *NF1* message is down-regulated in epithelial ovarian cancer (Iyengar et al., 1999) and neurofibromin expression is down-regulated in urinary bladder transitional cell carcinogenesis (Aaltonen et al., 1999).

DMBA-treated *Nf1*^{+/-} mice were twice as likely as wild type mice to develop pigmented skin patches. NF1 patients develop pigmented patches with greatly increased frequency as compared to normal humans (Riccardi, 1992; Korf et al., 1992). In humans, melanocytes and epidermal keratinocytes in café-au-lait macules contain "macromelanosomes" (Benedict et al., 1968; Takahashi, 1976; Malhotra and Ratner, 1994). In the mouse pigment was in keratinocytes in hair follicles, not in the epidermis, with no evidence for macromelanosomes. The storage of pigment and the location of the pigmented cells are different in mouse and human skin (Miller et al., 1993), possibly accounting for these differences.

DMBA was required for pigmented patch formation; patches were elicited neither by acetone nor by TPA alone. DMBA may have direct effects on skin cells, or act as a mutagen (reviewed in DiGiovanni, 1992; Quintanilla et al., 1986). Increased numbers of patches in *Nf1* mutants could be explained if mutants have increased numbers of stem cells that are targets for DMBA-induced mutation, or if the *Nf1* mutation increases the frequency of mutations in the stem cell population. However, wild type and mutant mice treated with

DMBA each had cells with the potential to form pigmented patches, because DMBA + TPA treatment caused patches to develop in all mice. Therefore we speculate that DMBA-initiated cells with reduced neurofibromin are more likely to proliferate and form pigmented patches in the absence of TPA than are wild type cells.

Keratinocytes and/or melanocytes may be responsible for increased pigmentation in response to DMBA. It is unlikely that pigmentation is due solely to elevated Ras-GTP in melanocytes. We failed to detect *H-Ras* mutation in pigmented patches, even though the assay used detects such mutations in as few as 1 in 10^8 cells (Quintinalla et al., 1986; Mitchell and Warshawsky, 1998), and Greisser et al. (1995) failed to find altered Ras-GTP levels in melanocytes from NF1 patients. Also, while hyperpigmentation does result in mice in which melanocytes express *v-H-Ras* (Powell et al., 1995), melanogenesis is present in hair bulbs and cells within the dermis and not almost exclusively associated with hair follicles as in *Nf1* mice.

In the mouse proliferation of follicular keratinocytes correlates with melanogenesis in the anagen phase of the hair follicle cycle (reviewed in Slominiski et al., 1993). Melanin produced in follicular melanocytes is transferred to precortical keratinocytes (Chase, 1954; reviewed in Slominski et al., 1993), leading to visible pigmentation. Our analysis of DMBA-treated pigmented patches revealed skin with features of the anagen phase of the hair cycle (Hansen, et al., 1984; Miller et al., 1993). Pigmented skin contained numerous large hair follicles with actively dividing follicular keratinocytes, significantly increased dermal thickness, and numerous enlarged sebaceous glands. We therefore hypothesize that the target cell of the pigmentation response stimulated by DMBA is the keratinocyte (or its stem cell).

Increased papilloma formation in *Nf1* mutants is also consistent with the affected cell being the keratinocyte or its precursor. Initiated cells proliferate during tumor promotion,

eventually forming papillomas with expansion of the epidermal keratinocyte population (Scribner et al., 1978; DiGiovanni, 1992). Both the initiated papilloma precursor and the pigmented spot precursor may be follicular bulge cells (Binder et al., 1997).

C57Bl/6 is a tumor-promoter resistant mouse strain (reviewed in DiGiovanni, 1992). While no previous studies utilized exactly the doses of DMBA and TPA used here, and none used male mice, using female mice investigators reported no papillomas (Kiguchi et al., 1997) or few papillomas (Reiners et al., 1984) in wild type C57Bl/6 mice treated with these carcinogens. Others reported low numbers of papillomas (Chouroulinkov et al., 1988; O'Brien et al., 1997). In our experiments none of the wild type animals, littermates of mutants and backcrossed at least 10 generations onto the C57Bl/6 background, treated with DMBA and TPA developed tumors. In contrast, papillomas arose in 75% of *Nf1* heterozygous mice treated using this paradigm, demonstrating that loss of one *Nf1* allele is sufficient to increase the frequency of papilloma formation in a resistant strain of mouse. The question of whether complete loss of *Nf1* is required for the papilloma formation described here remains open. Preliminary experiments (not shown) have failed to show LOH using PCR analysis. However, DMBA is a point mutagen and point mutations affecting *Nf1* cannot be excluded.

Mutational analysis revealed activating H-*Ras* mutations at codon 61 in all (n=6) of tested papillomas. These data are consistent with previous reports that have shown 90% of papillomas tested have an A to T transversion mutation in codon 61 of the c-H-*ras* gene (Quintanilla et al., 1986). Indeed, activated *ras* targeted to keratinocytes can serve as an initiating event in skin carcinogenesis models (Roop et al., 1986; Leder et al., 1990; Greenhalgh et al., 1993; Hansen and Tenant, 1994a,b; Brown et al., 1998). It is believed that populations of initiated cells expand during the promotion phase and manifest as papillomas

(DiGiovanni, 1992). We therefore have shown that functional inactivation of at least a single allele of the *Nf1* gene cooperates with activating mutations in the *ras* gene to enhance the papilloma phenotype in *Nf1*^{+/-} mice on a resistant genetic background.

The co-operative effects between *Ras* and *Nf1* that we have defined in keratinocytes could act downstream of Ras or independent of Ras. Neurofibromin may function in non-Ras pathways in keratinocytes, just as it appears to do in several other cell types (Johnson et al., 1994; Griesser et al, 1997; Guo et al., 1997; Kim et al., 1997; Atit et al., 1999). Alternatively, decreased levels of neurofibromin may increase Ras-GTP in keratinocytes, increasing signaling downstream of Ras-GTP and providing an effect additive with mutationally activated *H-Ras*. The idea that Ras-GTP higher than achieved by a single mutated *H-Ras* allele can contribute to epidermal tumorigenesis is consistent with results of Bremmer et al. (1994) who showed that most papillomas carrying mutant *H-Ras* alleles are trisomic for chromosome 7, increasing the copy number of mutant *H-Ras*. Manges et al. (1998) showed that *Nf1*^{+/-} mice over-expressing N-Ras driven by the MMTV promoter are at increased risk for developing lymphomas. Just as in our study, loss of *Nf1* co-operates with increased Ras activity (from over expression or activating mutations) to increase tumor incidence.

Nf1 mutant mouse keratinocytes show sustained proliferation in response to TPA. This result is consistent with data showing that strains of mice susceptible to papilloma formation have sustained epidermal proliferation in response to TPA (Naito et al., 1987; Kiguchi et al., 1997). TPA activates PKC, which is essential for regulation of genes involved in keratinocyte differentiation (Dlugosz and Yuspa, 1993, 1994; Lee et al., 1998). Our data suggest that neurofibromin normally down-regulates TPA-mediated signaling pathways in

keratinocytes. Our finding that *Nf1* hemizygous mouse keratinocytes have increased proliferative potential likely accounts for the observed papilloma formation.

Like mechanical wounding, topical application of skin carcinogens injures the skin and induces a wound-healing response (Scribner, 1978). Riccardi hypothesized a role for injury in pigmentation defects and tumor formation in NF1 patients (Riccardi, 1992). We have demonstrated that mutation in a single *Nf1* allele in mice alters the susceptibility of skin to pigmentation and tumors induced by carcinogens. Our data are consistent with a role for keratinocytes, and perhaps injury, in the abnormal skin pigmentation characteristic of NF1 patients.

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Task 4: Develop transgenic mice with altered Ras-GTP levels in Schwann cells to test the hypothesis that this alteration is necessary and/or sufficient to explain the nerve transection induced phenotype observed with *Nf1/nf1* mice.

Several lines of evidence suggest that increased levels of Ras-GTP contribute to cellular abnormalities that underlie features of type 1 neurofibromatosis disease. For example, NF1-deficient Schwann cells derived from a knock-out animal model have characteristics similar to those derived from neurofibromas, including elevated levels of Ras-

GTP. In addition, some of the abnormal phenotypes found in *Nf1*-deficient mouse Schwann cells can be reversed by an inhibitor of Ras activation, FPTI (Kim, et al., 1997). These observations support the idea that a normal function of neurofibromin in Schwann cells is regulation of Ras-GTP levels. *Nf1/nf1* heterozygous mouse nerves appear normal, however, upon nerve lesion they form rare Schwann cell tumors and show hyperpigmentation (see above). We postulate that altered Ras-GTP levels in Schwann cells are involved in the development of wound-related phenotypes in *Nf1/nf1* mice. To test this hypothesis, we have chosen to utilize Schwann cell-specific gene promoters to drive expression of activated Ras and the GAP-related domain (GRD) of neurofibromin in transgenic mice. Mice expressing constitutively active Ras may mimic the *Nf1* mutant mice, whereas expressing GRD may rescue the mutant phenotype.

4A. Creating Schwann cell-specific Ras and GRD constructs.

Previously, the P₀ promoter was the only Schwann cell-specific gene promoter available. Because we have experienced difficulty expressing transgenes with this promoter, we have chosen an alternative promoter derived from the 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) gene to drive expression in Schwann cells. CNP expression is restricted to oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. Unlike P₀ expression levels which fluctuate dependent upon myelination events, and in regeneration, CNP is expressed at a relatively constant level throughout development of the peripheral nervous system and after nerve injury (Edwards and Braun, 1988; Stahl, et al., 1990). Therefore, the CNP upstream sequence may serve as general, glial cell-specific promoter not dramatically influenced by Schwann cell differentiation. This promoter drove expression of a β -galactosidase-neomycin phosphotransferase fusion gene, called GEO, both *in vitro* and *in vivo* (Chandross, et al., 1999). To validate this construct in our laboratory, we

transiently transfected primary rat Schwann cells and stained with the β -galactosidase antibody; the construct expressed well. In contrast we were never able to detect expression of β -galactosidase after transient transfection of cultured Schwann cells with a P0-LacZ construct. Having this positive control, we have chosen to repeat the experiments described for the P0-Ras and P0-GRD constructs in the original grant utilizing the CNP promoter.

Driving expression of the GRD and Ras transgenes with the CNP promoter promoter will be a major advantage in the wounding experiments. Following nerve injury, P0 expression is downregulated (Gupta, et al., 1988). If the introduced transgene is under the control of the P0 promoter, it may not be expressed at a high enough level at this critical moment. In other words, by the time the transgene is expressed, events leading to the wounding-induced phenotype may have already occurred unaffected by the introduced gene. Therefore, absence of a change in phenotype induced by P0-driven mutant Ras and GRD may not necessarily indicate the Ras pathway is not involved. Unlike the P0 gene, CNP expression is slightly upregulated in response to nerve injury (LeBlanc, et al., 1992). Therefore, CNP-driven transgenes will be more likely to be expressed at sufficient levels after wounding.

Both isoforms of the GRD of human *NF1*, GRD1 and GRD2, were subcloned downstream of the functional CNP promoter fragment. Transcript level and GAP activity of GRD2 is reduced *in vivo* compared to GRD1 (Huynh, et al., 1994), however, the type 2 transcript predominates in differentiating Schwann cells (Gutmann, et al., 1993). Therefore, transgenic mice expressing GRD1 and GRD2 may have different phenotypes. Creating the CNP-GRD1 and CNP-GRD2 constructs was similar to the protocol described in our original grant for cloning downstream of the P0 promoter. Both GRD cDNA sequences were

amplified by PCR to include a 5' HindIII site, a 3' NcoI site, and a 5' HA tag for subsequent detection with an HA antibody. The HA tag was chosen because, unlike the myc antibody required to observe transgene expression in our original P₀ constructs, the HA antibody was not raised in mice. Therefore, less non-specific background signal should be detected in the transgenic mice with the HA antibody. The CNP-GEO plasmid was digested with HindIII and NcoI to remove the GEO sequence, allowing for directional cloning of the GRD PCR products downstream of the CNP promoter. Transformed bacterial colonies were screened by colony PCR followed by restriction mapping and sequencing. Extensive screening was required to identify positive clones, possibly due to an inherent susceptibility of the GRD fragment to rearrangement.

A mutated version of Ras, Ras12V (Capon et al., 1983), was also subcloned into a CNP expression vector. The Ras12V mutant activates all known pathways downstream of Ras. Because there is an internal HindIII site in the Ras cDNA, an alternate cloning strategy was conducted to create CNP-Ras12V. The original CNP constructs we received contained limited restriction sites for subcloning. Therefore, to accomplish the cloning of the Ras12V and to facilitate future cloning endeavors a CNP expression vector was created. We chose the pCMV-Script expression vector (Stratagene) as a template for several reasons: 1) The multiple cloning site (MCS) contains 15 restriction sites providing a simple cloning strategy for several different fragments. 2) Downstream of the MCS lies a termination sequence that can be utilized by the inserted gene. 3) The vector size is small (~ 4 kb), aiding in transfection efficiency. 4) The neomycin resistance gene is controlled by both a prokaryotic and a eukaryotic promoter, allowing for selection with kanamycin in bacterial cells and G418 in mammalian cells.

The CMV promoter was removed from pCMV-Script with VspI and SacI and replaced with a 50 bp polylinker, created by dual primed extension of oligonucleotides containing 8 additional restriction sites. This polylinker allowed for insertion of the CNP promoter while preserving restriction sites within the MCS. The modified Script construct was cut with Psp1406I, filled out with Klenow to create a blunt end, and then digested with SpeI. A pSKII vector containing the CNP promoter region was digested with HindIII, filled out with Klenow to create a blunt end, and then cut with XbaI to remove the functional promoter fragment used in CNP-GEO. SpeI and XbaI share compatible ends, allowing for semi-directional cloning of the CNP promoter into the Script backbone. The Ras cDNA sequences were amplified with primers containing a 5' ClaI site followed by an HA tag and a 3' XhoI site. PCR products were digested and cloned into these sites in the MCS, between the CNP promoter and the termination sequence. Positive clones were confirmed by restriction mapping and sequencing.

3B. Test Ras and GRD constructs *in vitro* and inject into blastocysts to create transgenic mice.

To show the CNP constructs were functional, transient transfections into primary rat Schwann cells were conducted this year. Expression was detected with the HA antibody (Figure 13). These constructs were then prepared for *in vivo* studies. The functional expressing unit containing the CNP promoter followed by the inserted gene sequence and the termination sequence was excised and purified for injection into blastocysts. Thus far, three possible founders have been identified from our injection of GRD1 into blastocysts.

Three injections into 120 embryos each were performed with the CNP-GRD1 and CNP-GRD2 constructs, and the CNP-Ras12V construct. Pups were characterized by PCR for the presence of the introduced transgene resulting in only four possible founders (Table 4).

Breeding was initiated to obtain an F1 generation for each of the four putative founders. To date, two of the three CNP-GRD1 possible founders transmitted the transgene to their offspring, as determined by PCR. Because only one possible founder containing CNP-GRD2 was identified and has yet to transmit the transgene, additional injections with this fragment are underway.

Table 4 : Summary of injection data for CNP constructs (1999).

Construct	Pups	Possible founders	Transmit transgene	Express Protein
Injection 1: CNP-GRD1	10	1	Yes (2/5 pups positive)	Test Ongoing
Injection 2: CNP-GRD1	19	1	Yes (5/8 pups positive)	Test Ongoing
Injection 3: CNP-GRD1	12	1	Breeding ongoing	
Injection 4: CNP-GRD2	21	1	No (0/8 pups positive)	
Injection 5: CNP-GRD2	22	0	-	
Injection 6: CNP-GRD2	10	0	-	
Injection 7: CNP-Ras12V	16	tail clip 10/19		
Injection 8: CNP-Ras12V	16	tail clip 10/20		
Injection 7: CNP-GRD2	Not yet born			

Because the CNP transgenes are HA-tagged, expression will be documented by HA staining of paraffin-embedded sciatic nerve, spinal cord, and brain. Kidney and heart will serve as negative controls. All tissues will be taken from 3 week-old pups positive for the transgene by PCR. Parallel staining will be performed on a non-transgenic littermate for additional negative controls. Sections of lung expressing a different HA-tagged transgene have been provided (Juliana Conklin/Tim Weaver TCHRF) for a positive control for staining. Once expression of the CNP transgene is confirmed, Southern analysis will be conducted with DNA from the founder and respective offspring to assess transgene copy number.

We would have preferred injection into C57Bl/6 mice to obtain transgenic mice with the same genetic background as the *Nf1/nf1* mice, allowing direct comparisons between transgenic and knockout lines. However, because technical difficulties are associated with injection into C57Bl/6 blastocysts, due to their small size, we have chosen to inject into a

C57Bl/6-129 hybrid mouse strain. Blastocysts from this line are larger than the pure C57Bl/6 and have produced better results at our transgenic core facility. F1 mice produced by founders crossed by C57Bl/6 mice are about 82% C57BL/6; a cross to *Nf1* mice on the C57 background will be nearly pure C57Bl/6.

3C. Characterize phenotypes of the transgenic strains and breed GRD strains to *Nf1/nf1* mice.

We have identified 3 PCR⁺ putative GRD1 founder mice and 1 PCR⁺ putative GRD2 mouse. Two of the 3 PCR⁺ GRD1 mice have transmitted the transgene to offspring, and offspring are now being tested for protein expression by immunostaining of tissue sections and Western analysis, both using antibody against the HA tag. We have obtained paraffin embedded tissue sections from a colleague in which a transgene is known to drive expression of an HA-tagged lung protein. Therefore we have a positive control for tag detection.

The putative founder GRD1 mice appear normal and healthy. This outcome was predicted in our original application.

Further mouse generation: We will continue to ask our core lab to carry out injections until we obtain at least two mouse lines per construct that show expression of the HA tag in Schwann cells. We anticipate that a second round of injection of GRD2 will be completed by the end of November.

Once we identify transgene expression in lines of mice using the HA tag, we will test if Ras-GTP is altered in activity due to GRD or RasV12 expression. RasV12 mice are predicted to have constitutively high Ras-GTP in all Schwann cells, even in the absence of mitogen stimulation. Expression of GRD in Schwann cells is predicted to decrease Ras-GTP.

However, as Ras-GTP is normally low, we predict detecting a decrease in Ras-GTP signaling in the cells only after mitogen addition, when Ras-GTP is normally elevated.

Our lab has recently developed an *in vitro* single cell Ras-GTP assay that will be perfect for analysis of these Schwann cells (Sherman et al., submitted). However because we do not know whether to expect a detectable change in transient elevation of Ras-GTP in the absence of loss of *Nf1*, we will breed expressing GRD animals to *Nf1* mutants regardless of the results of this assay.

3D. Lesion nerves of CNP-Ras12V transgenic mice and analyze phenotype.

Once we are convinced we have a functional Ras12V construct expressing in mice, we will lesion nerves. After a one to three month recovery period, nerves will be analyzed for wounding response and compared to wild type.

3E. Lesion nerves of GRD expressing mice and *Nf1/nf1*-GRD double transgenic mice and analyze phenotype.

We hope to begin these experiments late next year, assuming availability of mutants.

(7): Key Research Accomplishments

- We have made progress toward understanding how pigmentation arises after nerve lesion in *Nf1/nf1* mice. Our results suggest that mutant Schwann cells, together with a mutant environment, collaborate to make mutant animals become hyperpigmented. This result mimics the tendency for NF1 patients to develop café-au-lait macules and to have overall hyperpigmented skin. The results are to be presented at the Society for Neurotrauma and the Society for Neuroscience Meetings this month.
- We made a new CNPase promoter cassette and cloned all 3 tagged constructs into it; all constructs were tested *in vitro* and injected into blastocysts.
- We undertook and completed a study complementary to the original proposal, and showed that *Nf1/nf1* mice developed aberrant pigmentation and also benign keratinocyte tumors. This study was submitted for publication. This study is consistent with our hypothesis that wounding does abnormally affect *Nf1/nf1* mice and, significantly, demonstrates that effects of *Nf1* mutation synergize with Ras activation in cells.

(8) Reportable Outcomes

1. Atit, R.A., Mitchell, K. Nguyen, L. Warshawsky, D. and Ratner, N. The neurofibromatosis type 1 (*Nf1*) tumor suppressor is a modifier of carcinogen-induced pigmentation and papilloma formation in C57Bl/6 mice. Submitted to Cancer Research.
2. Ratner, N. and Daston, M.M. (1999) Neurofibromatosis type 1 and Neurofibromatosis type 2: Genetic and cellular mechanisms of peripheral nerve tumor formation. Glial Cell Development: Basic Principles and Clinical Relevance Jessen, K.R. and Richardson, W.D. Oxford University Press, in press.
3. Rizvi, T., Atit, R.A., and Ratner, N. (1999) Transdifferentiation of Neurofibromatosis type-1 mutant Schwann Cells into Melanocytes following wounding of *Nf1/nf1* mouse nerve. Soc. Neurosci., 25, 740a.
4. Ratner, N., Atit, R.A., and Rizvi, T. (1999) Transdifferentiation of Neurofibromatosis type-1 mutant Schwann Cells into Melanocytes following wounding of *Nf1/nf1* mouse nerve. J. Neurotrauma., (abstract) in press.
5. Oral Presentation of data from Tasks 1 – 3 at the International Consortium for the Molecular Biology of NF1 and NF2; MIT, Cambridge, MA Summer, 1999
6. A grant application to the DOD was submitted based on results in Task 3; the proposal will also be submitted to the NIH Nov. 1, 1999.

7. Shyra Cryder-Miller is being supported as a nested fellow by this grant. Shyra obtained her Ph.D. degree at the University of North Carolina and is trained as a human geneticist. Shyra attended a New York Academy meeting on Charcot-Marie-Tooth disease last year to learn some Schwann cell biology and she will attend the NNFF meeting in Aspen in June. She sat in on a Developmental Neuroscience Graduate Course, and has attended a bi-weekly Cancer Journal Club. Shyra made all the constructs in Task 4 and is beginning to evaluate resultant mice.

8. Radhika Atit carried out the work in Task 3. The experiments were made possible by this grant, although Radhika's salary was funded by other sources. Radhika received her Ph.D. degree in May 1999 and is now a postdoc at Sloan-Kettering in NYC.

(9) Conclusions

Importance of the studies:

Our studies were begun to test the hypothesis that wounding could initiate features of human NF1 in transgenic mice. We now feel confident that wounding can indeed cause features of human NF1 in mutant mice. We are eager to understand more of the mechanism underlying this effect, and how it may be relevant to human NF1.

(8): References

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(9): Appendices

Figure 1: Gross photograph of mice hindlimb area after 1 month of nerve transection and/or deflection. Skin has been removed to show pigmentation. (A): Wild type mouse with nerve transection shows little pigmentation. (B): Wild type mouse with nerve transection and deflection; shows little pigmentation. (C): *Nf1/nf1* mouse with nerve transection. (D): *Nf1/nf1* mouse with nerve transection and deflection shows clusters of highly pigmented cells and a larger pigmented area as compared to C.

Figure 2: No significant changes in percent BRDU positive cells after nerve crush injury in *Nf1*^{+/-} mice. Animals were injected with BRDU 1 hour prior to sacrifice. Nerves were fixed, embedded in paraffin, and sections stained with anti-BRDU using a kit (Zymed) or hematoxylin to label nerve nuclei. Data are shown from sciatic nerves that had been crushed 4 or 7 days prior to sacrifice; low percent BRDU-positive nuclei were present in uncrushed contralateral nerves from animals of both genotypes. The mean percentage of labeled cells is BRDU-positive cells over total number nuclei. For each set of data nerves from 4 – 7 animals were evaluated. Statistical analysis was using Student's t-test; p=0.18 for 4 days and p=0.044 for 7 days.

Figure 3: Cryosection containing nerve and muscle 1 month after nerve transection and graft of Hoescht labeled nerve fragment into a *Nf1*^{+/-} animal, followed by nerve transection. (A): Hoechst labeled cells (blue nuclei) were visualized using a DAPI filter. (B): Bright field. Note black pigmented cells. (C): Bright field and DAPI showing grafted nuclei in close proximity to pigment clusters.

Figure 4: Gross photograph of mouse hindlimb area 1 month after nerve transection and placement of grafts of $+/+$ Schwann cells or $-/-$ TXF cells on filters. Skin has been removed to show pigmentation. A and C are wild type animals; B and D are from *Nf1/nf1* animals. A and B represent grafting of $+/+$ Schwann cells. C and D represent grafts from $-/-$ TXF Schwann cells. Increased pigmentation is seen in *Nf1/nf1* mice after grafting of $-/-$ TXF Schwann cells (D).

Figure 5: Photomicrographs of immunostained $-/-$ TXF Schwann cells indicate that they are Schwann cells. $+/-$ Schwann cells and $-/-$ Fibroblasts are used as controls. A, D, G and J represent as $-/-$ TXF Schwann cells; B, E, H and K as $+/-$ Schwann cells while C, F, I and L are $-/-$ fibroblasts. A-C shows anti-P75 NGFr; D-F, S-100; G-I, GFAP and J-L, smooth muscle actin (SMA) immunostaining.

Figure 6: A) *In vitro* pigmentation assay using mouse cells. Cultured mouse melanocytes were exposed for 7 days to medium alone (left panel) or medium conditioned by *Nf1* $-/-$ mouse Schwann cells for 48h (right panel). Schwann cell medium was added to normal mouse melanocytes; medium was replenished after 3 days. The melanocytes in medium conditioned by *Nf1* mutant cells showed striking increases in pigmentation as compared to control medium conditioned by wild type cells (Rizvi et al., in preparation). This result has been replicated in three independent experiments. B) Tyrosinase assay. The amount of pigment in mouse melanocytes was quantitated by a tyrosinase enzyme assay (Zhao and Boissy, 1994). Wild type mouse melanocytes were exposed to medium conditioned by mouse *Nf1* Schwann cells $+/+$, $+/-$, $-/-$, or $-/-$ -TXF (see Kim et al., 1997). After 7 days,

triplicate wells of melanocytes were lysed and tyrosinase quantitated. Increased tyrosinase activity was noted when Schwann cells were null but not heterozygous at *Nf1*. Student's t-test confirmed statistical significance of the results. The Schwann cell conditioned medium appears to contain factor(s) that induce pigmentation in the melanocytes.

Fig. 7: Gross appearance and histology of DMBA treated wild type and *Nf1*^{+/-} skin.

A, *B*, and *C*, show gross photographs of shaved dorsum of mice two months after initiation with two doses of 40 μ g of DMBA. Treated areas are outlined in white. *A* is representative of a wild type animal after initiation; this animal shows no large pigmented spot. Other, affected, animals with large pigmented spots are shown in *B* and *C*. The mouse shown in *B* is wild type; in *C* is a heterozygous mouse. Pigmented areas are designated (P) and unpigmented areas designated (U). *D*, *E*, and *F* show H&E-stained sections of skin, all at the same magnification. *D* shows initiated skin from an unpigmented area of *Nf1*^{+/-} skin, identical in histology to wild type skin. *E* and *F* show sections from pigmented region of wild type and *Nf1*^{+/-} animals shown in *B* and *C*, respectively. White arrows point to hair follicles filled with pigment; sweat glands are pointed out with black arrowheads. *F* shows a section from a pigmented region with dermal pigmentation that was not cell-associated (black arrow). *G*, *H*, *I* show anti-PCNA immunostaining (brown precipitate shown by black arrowheads) with hematoxylin counterstain of sections from the regions shown in *D*, *E*, *F*. *H*, *I* melanin is present in follicles (black arrows). Bar = 50 μ m *D-F*, Bar = 10 μ m *G-I*.

Fig. 8: Numbers of hair follicles are increased in pigmented skin regions in both wild type and *Nf1* mutant mice. Numbers of hair follicles were counted in unpigmented, *A* and pigmented, *B* areas of skin 2 months after initiation. Each bar represents averages of the

number of hair follicles in 8-10 fields from at least 5 sections of individual wild type or *Nf1*^{+/-} mice.

Fig. 9: Gross and histological appearance of skin and papillomas from *Nf1*^{+/-} mice after tumor promotion. *A*, gross appearance of a representative affected *Nf1*^{+/-} mouse after initiation and 24 weeks of tumor promotion with 0.8 μ g of TPA three times per week. Hair was gently shaved to reveal pigmented areas, P and unpigmented areas, U. White arrowheads point to a papilloma on the dorsum of this mouse. *B-F*, are photographs of H&E stained sections. *B, C* show the histological appearance of unpigmented, *B* and pigmented, *C* skin from the mouse shown in *A*. Note the marked expansion of hair follicles in *C*. *D* shows a section through a typical exophytic papilloma with epidermal hyperplasia, centrally located follicular cysts and trapped sweat glands. At higher magnification, keratinocyte hyperplasia (*e*) is shown in *E* and follicular cysts (*c*) in *F*, as are the sebaceous glands prominent in most of the papillomas generated. Bar=50 μ m *B, C*; Bar = 125 μ m *D*; Bar = 10 μ m *E, F*.

Fig. 10: Papilloma incidence and volume in *Nf1*^{+/-} mice. (*A*) shows the incidence of papilloma development over weeks after promotion with TPA. Squares represent papillomas in *Nf1*^{+/-} mice; diamonds represent wild type mice, which never developed papillomas. (*B*) shows volumes of papillomas in *Nf1*^{+/-} mice 24 weeks after promotion with TPA. * = a 80mm³ papilloma.

Fig. 11: Mutational analysis of codon 61 from the H-*ras* gene in treated skin and papillomas *A, B*, PCR amplification of the A \rightarrow T transversion at codon 61 from the H-*ras* gene. Presence of the top band (162 bp) indicates the presence of the mutation (arrow). *A*, from

pigmented skin (lanes 1, 3) and unpigmented skin (2, 4) from the treated area of wild-type (lanes 1, 2) and *Nf1*^{+/-} (lanes 3,4) animals. Lane 5 and 6 represent negative control and positive controls respectively and M = marker. Data is representative of that collected from wild type (n=4) and *Nf1*^{+/-} (n=5) individual mice. *B*, Papillomas were isolated 24 weeks after tumor promotion with TPA. Lanes 1-4 show results of 4 representative papillomas analyzed from individual animals. Lane 5 shows a water control, lane 6 and 7 represent negative and positive controls respectively. M = marker. *C*, autoradiogram showing reverse cyclic dideoxy sequencing of the mutation within the second base of codon 61 (asterisk) from the *H-ras* gene. All 6 papillomas positive for mutations in the PCR screen were confirmed by sequencing.

Figure 12: TPA exposure results in enhanced epidermal labeling index in *Nf1*^{+/-} mice. Anti-BrdU staining of typical skin sections from wild type (*A*) and *Nf1*^{+/-} (*B*) mice 48hrs after the last of four treatments with TPA (4.0 μ g). Arrows designate positively stained basal keratinocytes. Bar = 10 μ m. *C*, Data from mice in 2 individual experiments is shown. The percent of BrdU- labeled basal keratinocytes was counted. Each bar (hatched = wild type; solid = *Nf1*^{+/-}) represents the mean percent labeled cells in 2-3 sections from each mouse. Error bars show standard error.

Figure 13: Cultured rat Schwann cells transfected with HA-tagged plasmids. For comparison, the top panel shows a Schwann cell transfected with a CMV-driven HA-tagged RasV12 construct from Adrienne Cox; the field is shown in phase microscopy at the right. The same field is shown in under fluorescein optics at the left; about 6% of the cells typically

express the transgenes. The bottom three sets of figures shown transient transfection of the HA-tagged CNP-RasV12 and GRD constructs, with matched phase images.

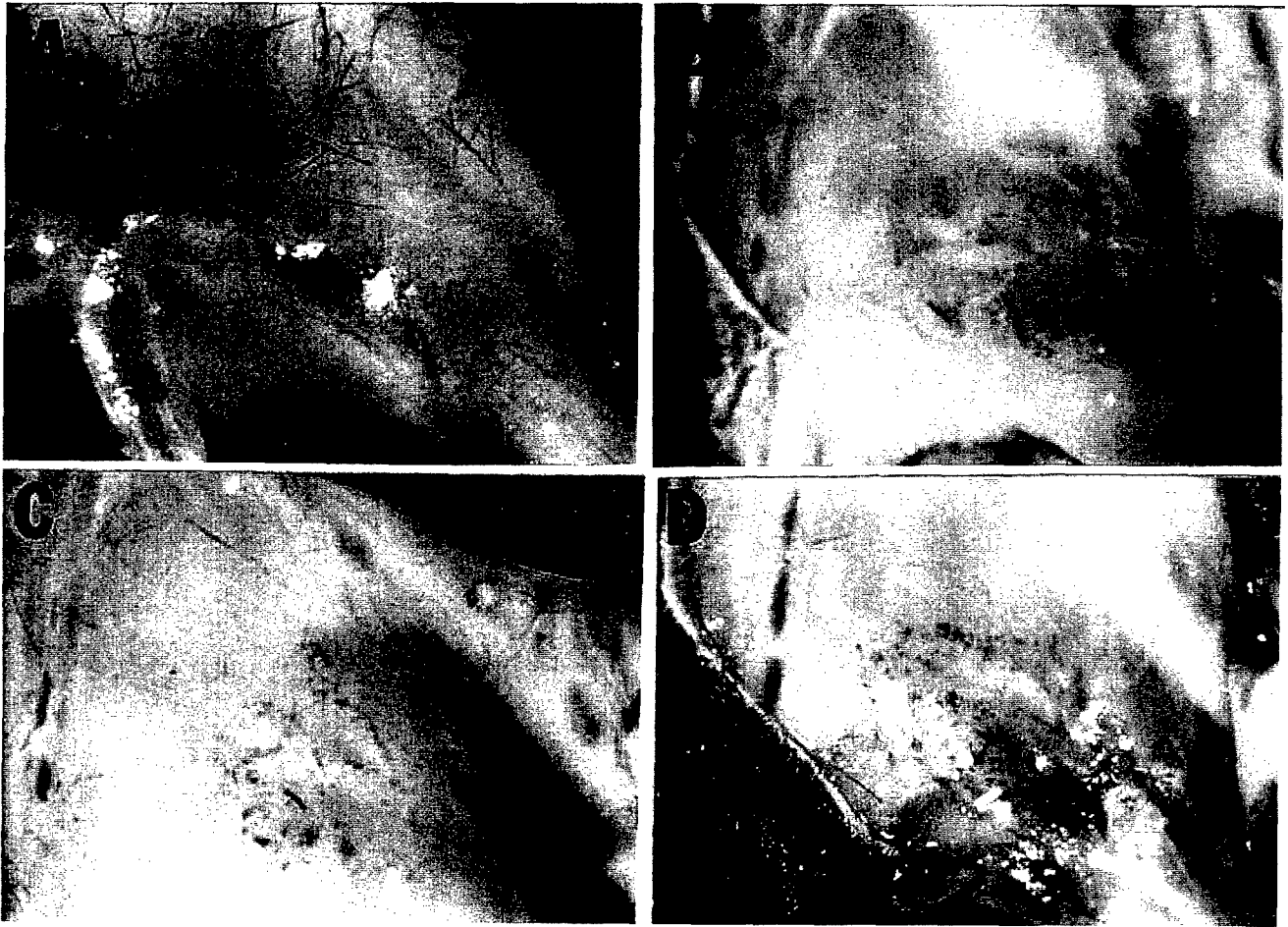


Figure 1
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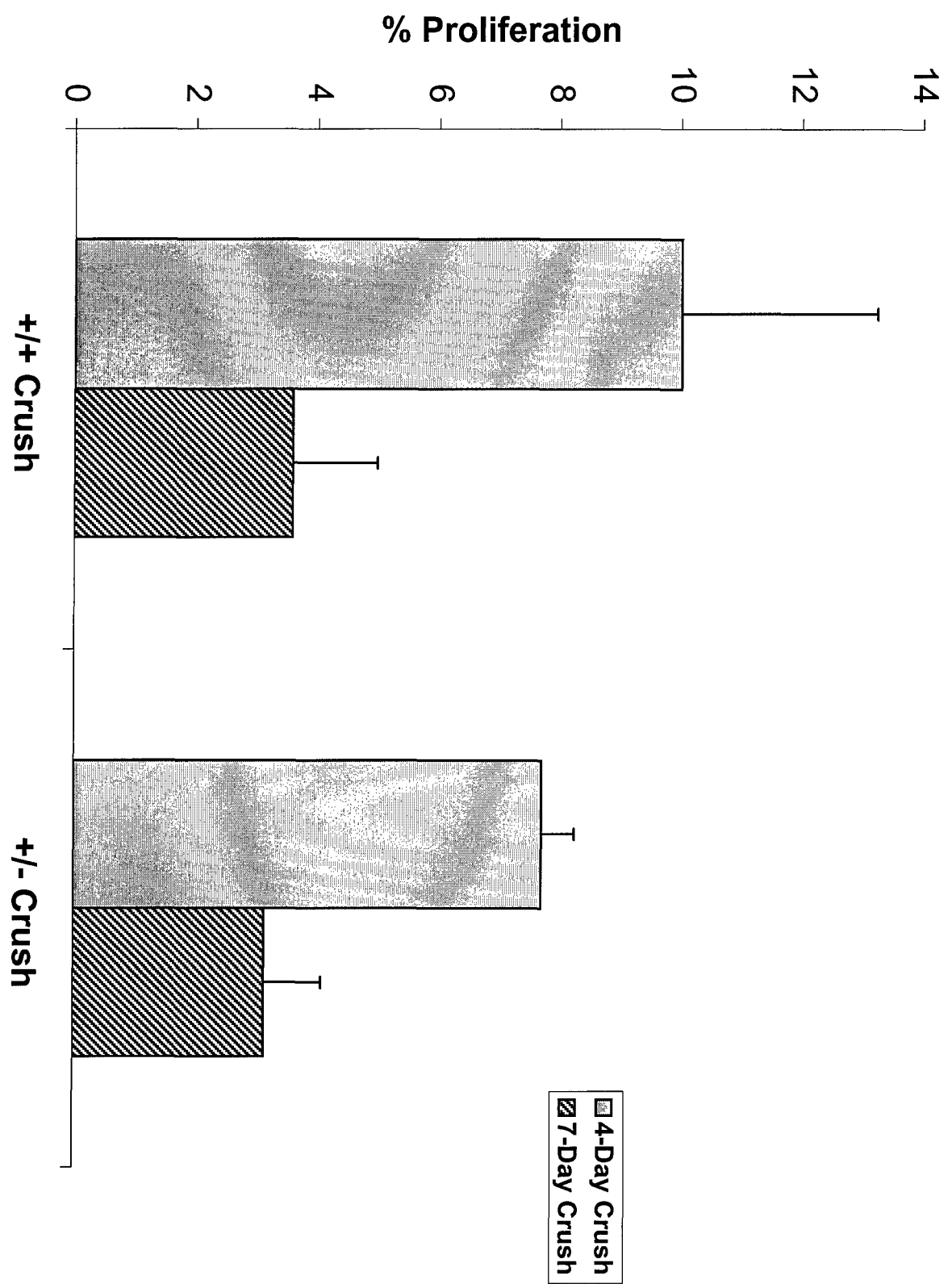


Figure 2
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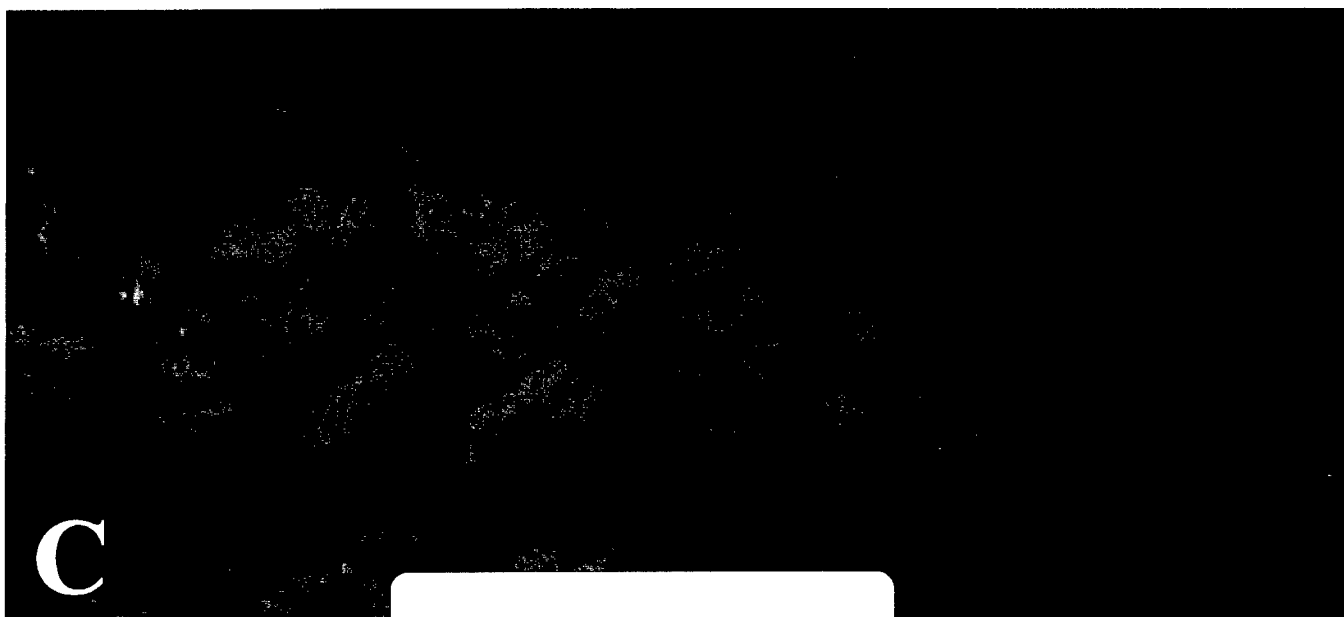
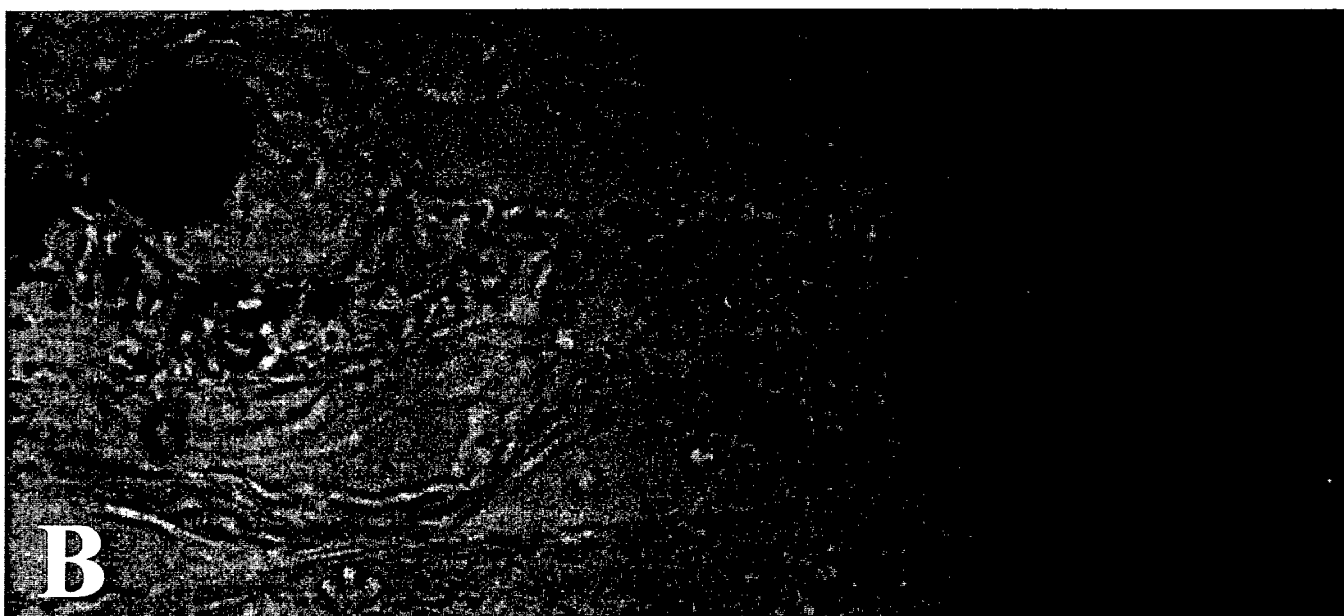


Figure 3
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Figure 4
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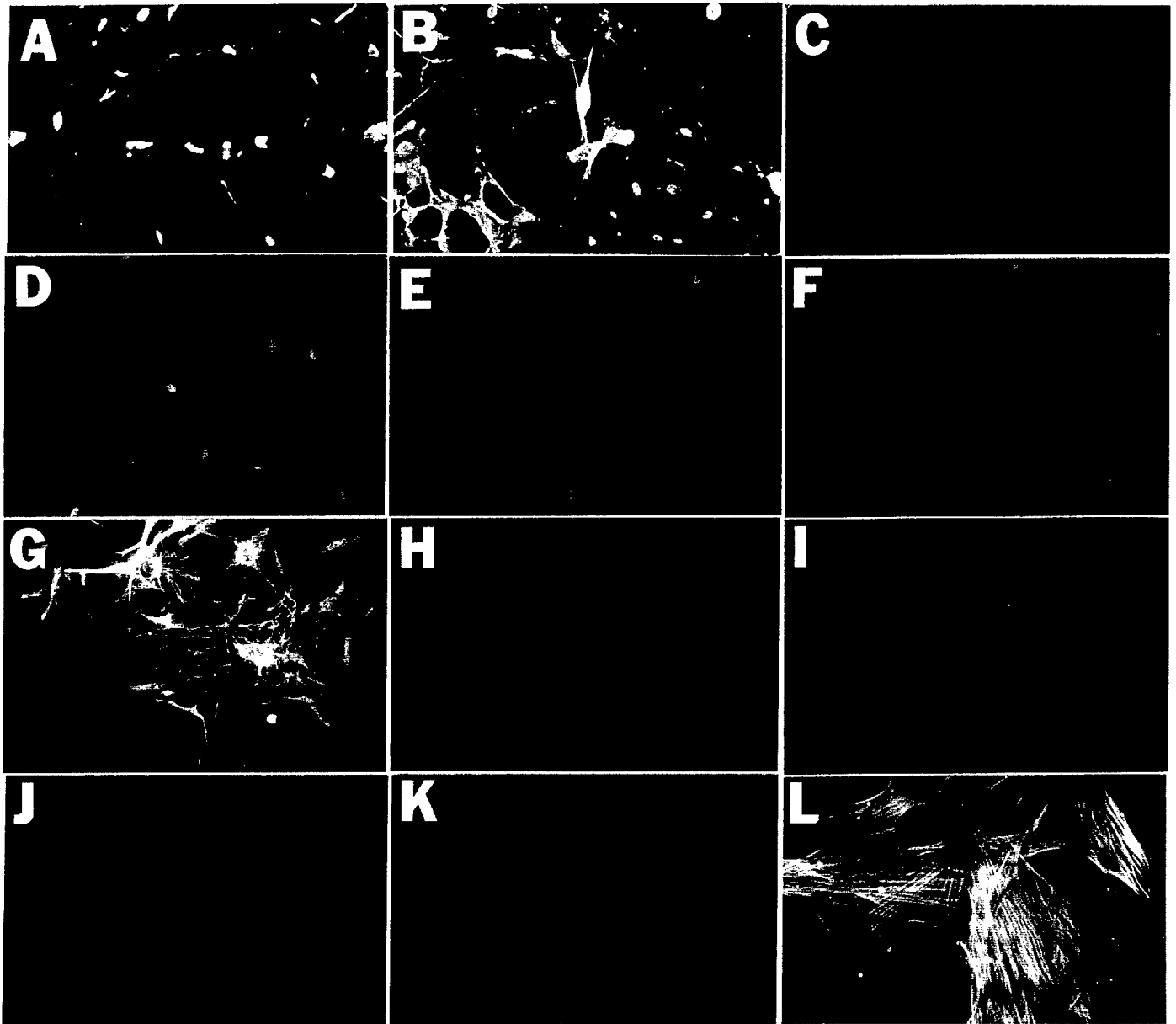


Figure 5
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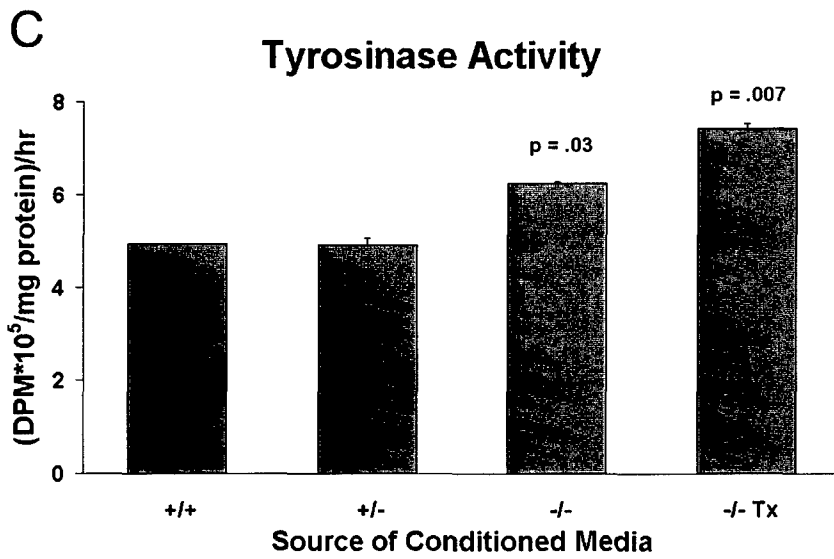
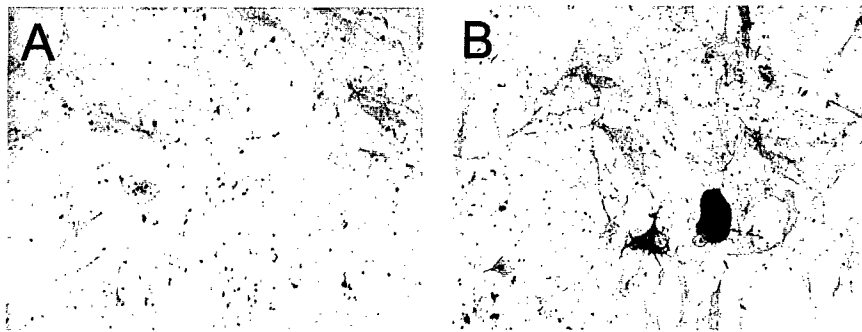


Figure 6
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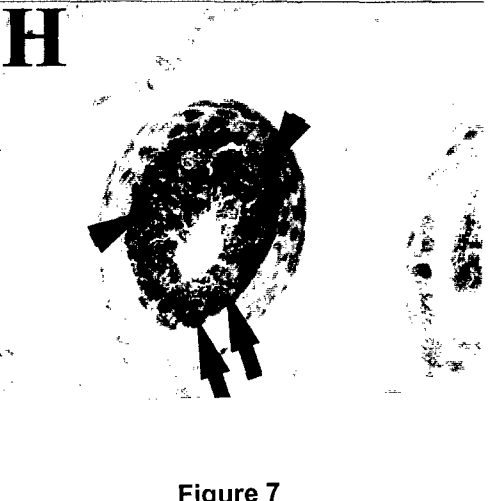
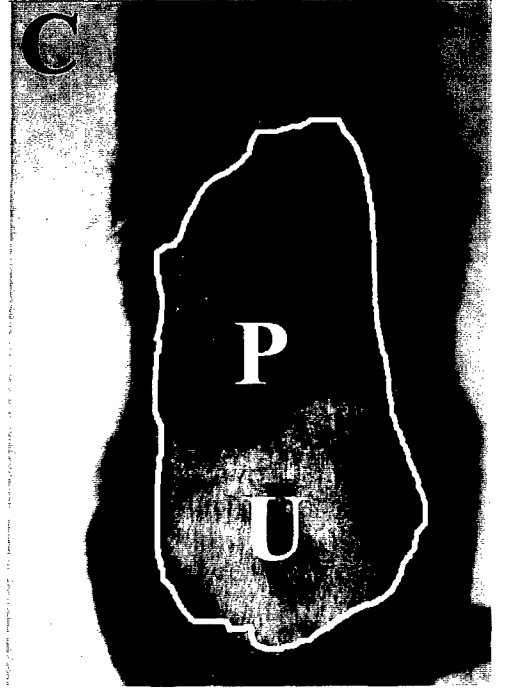
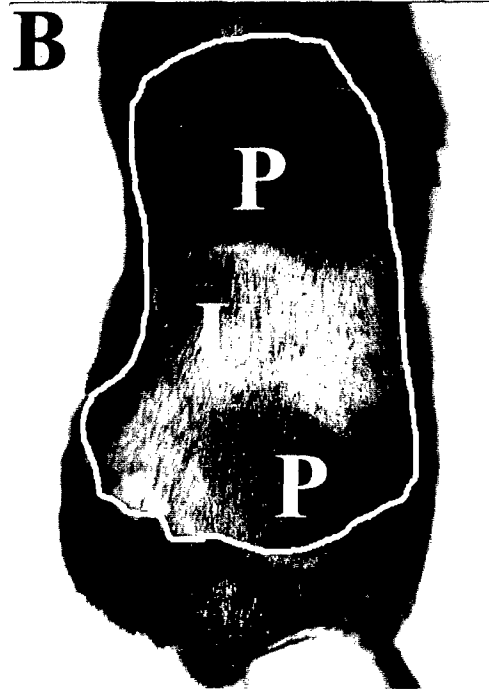
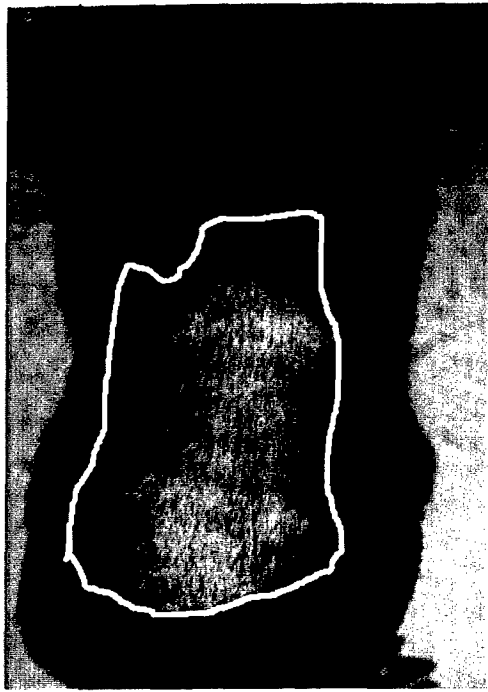


Figure 7
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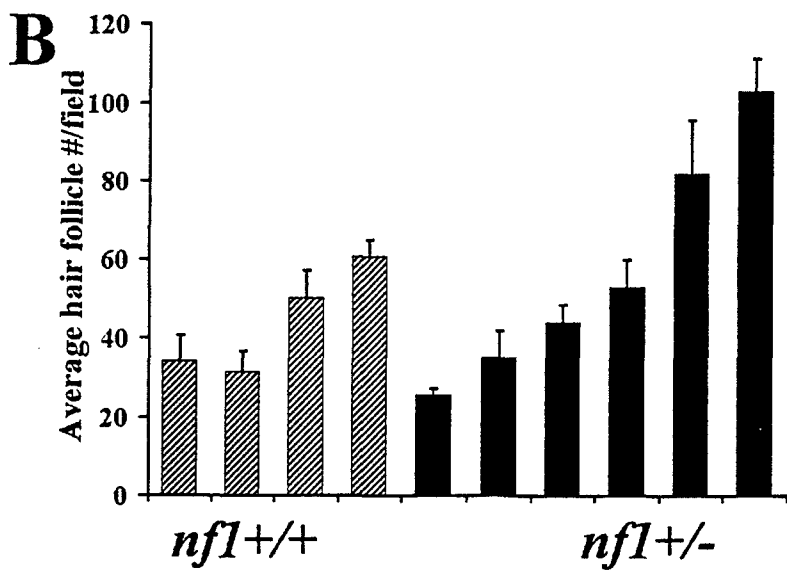
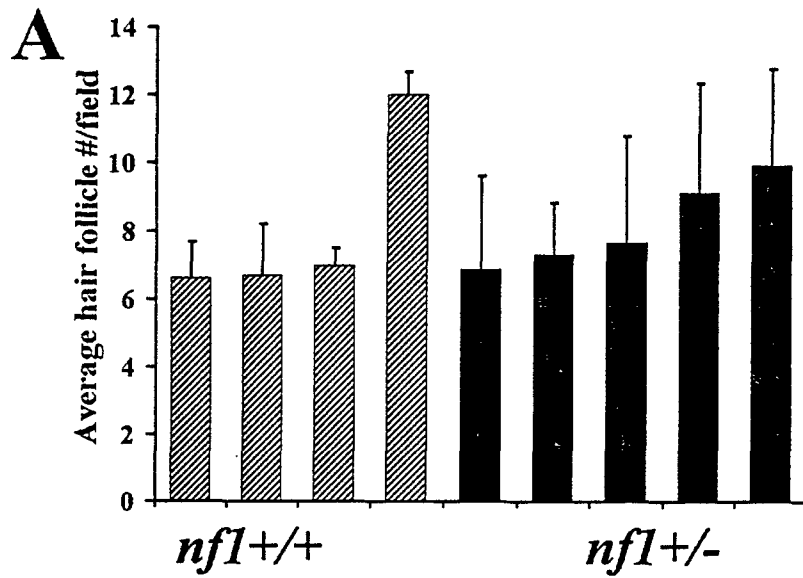


Figure 8
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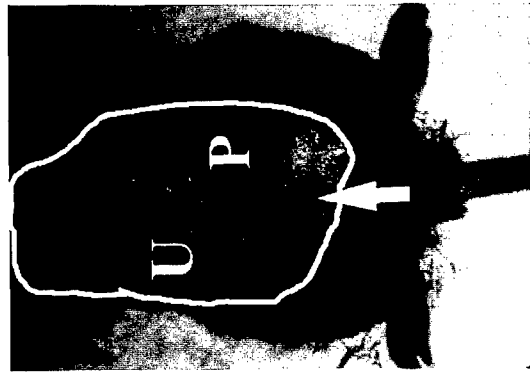
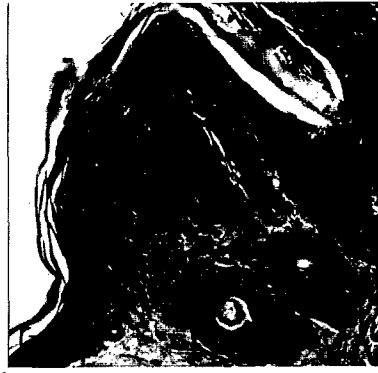
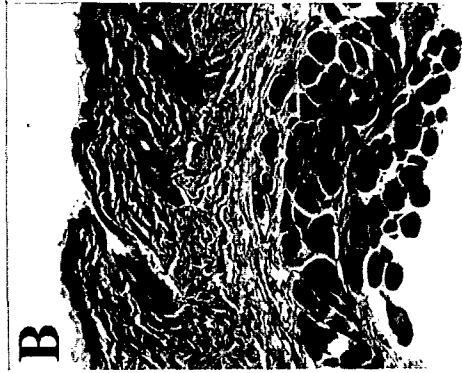
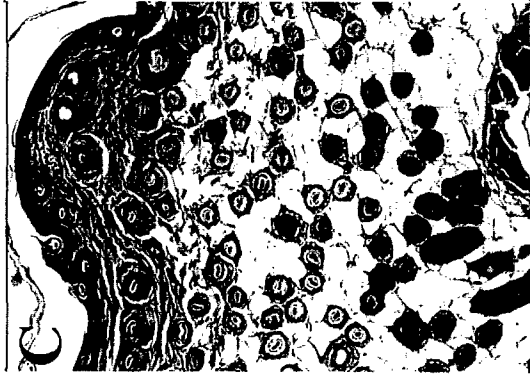


Figure 9
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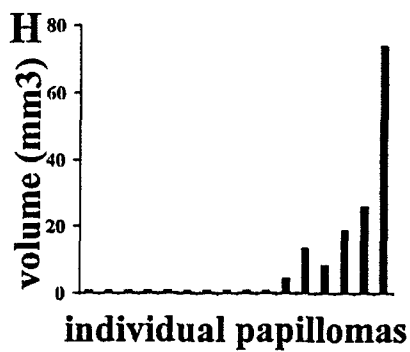
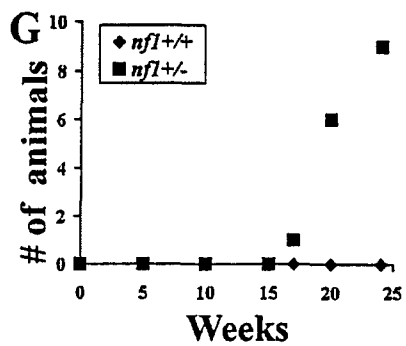


Figure 10
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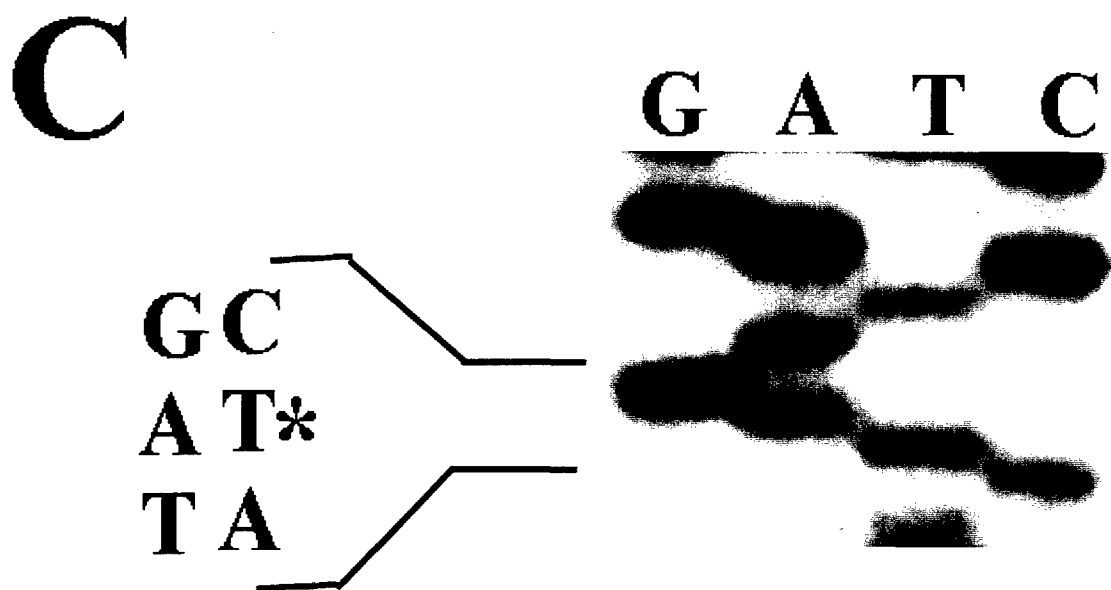
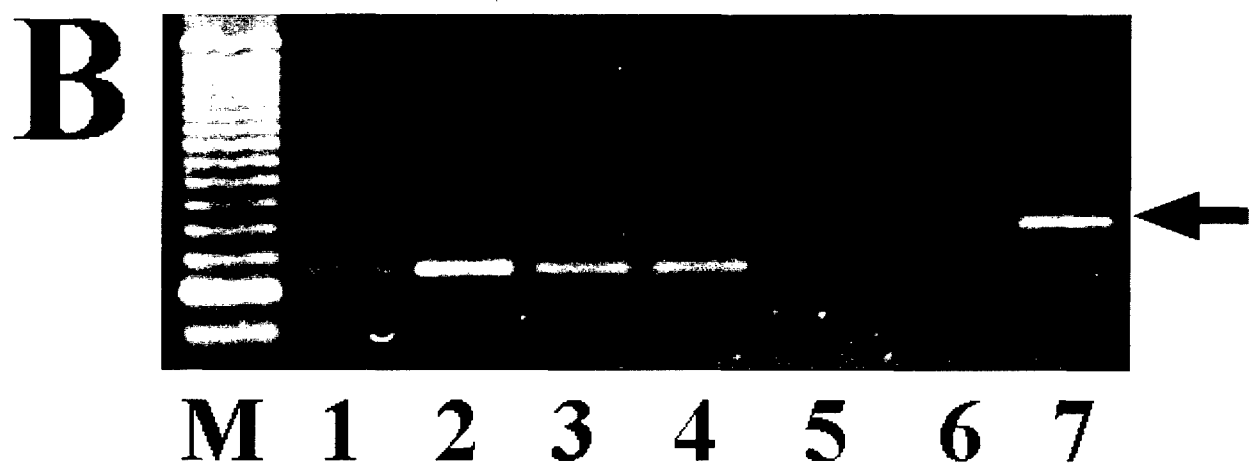
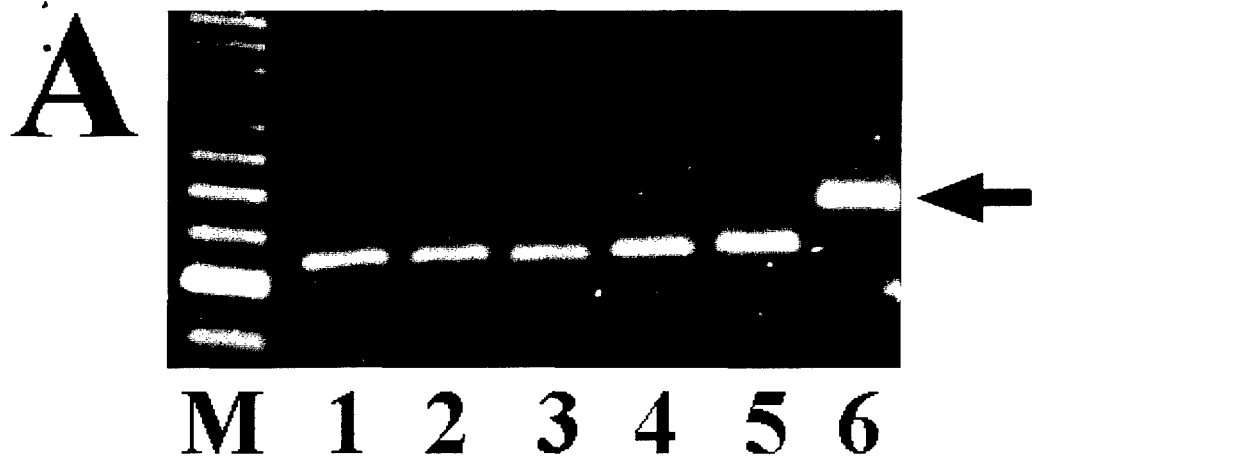


Figure 11
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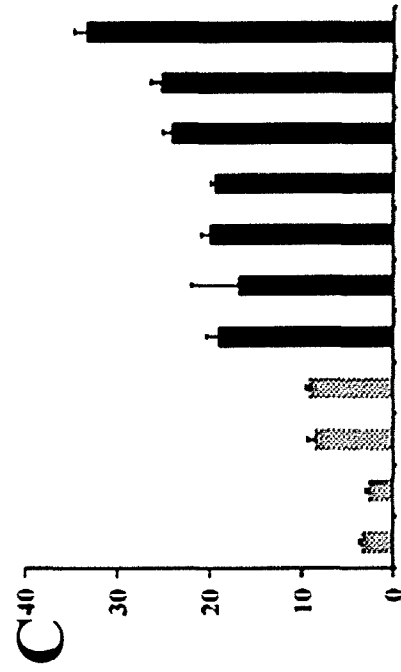
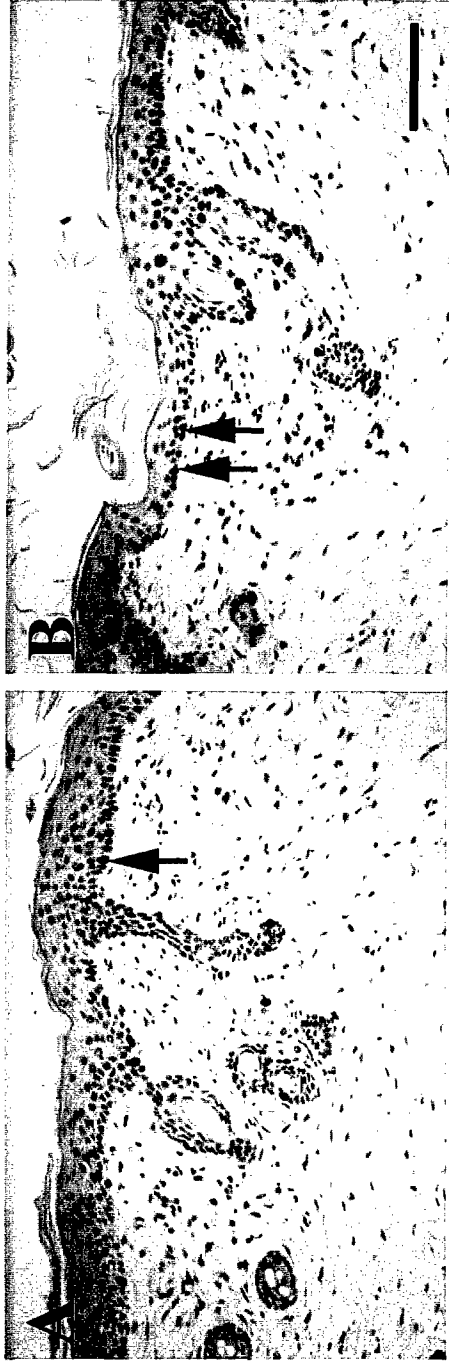


Figure 12
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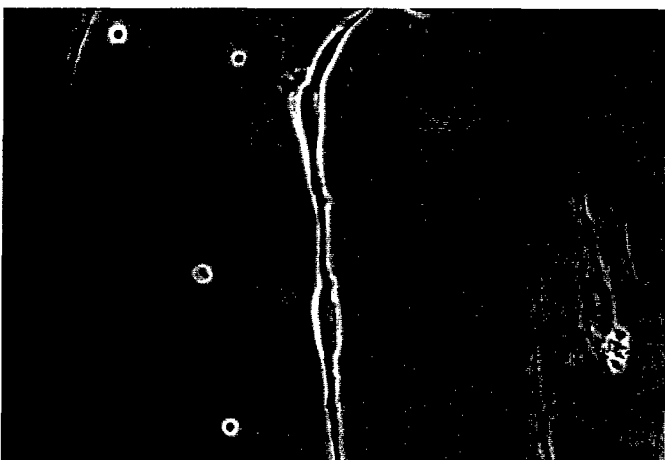
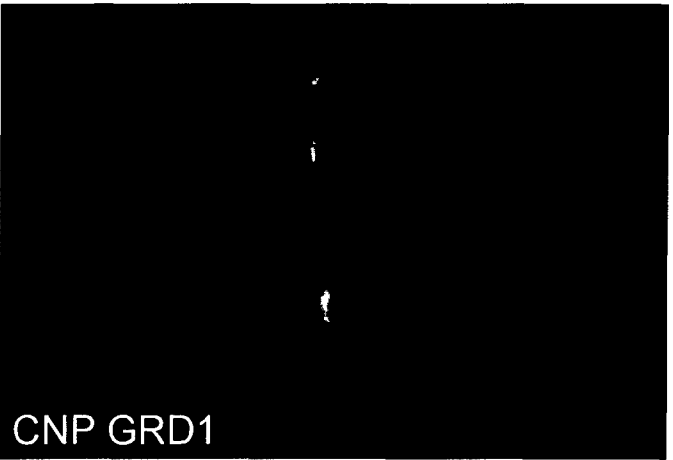
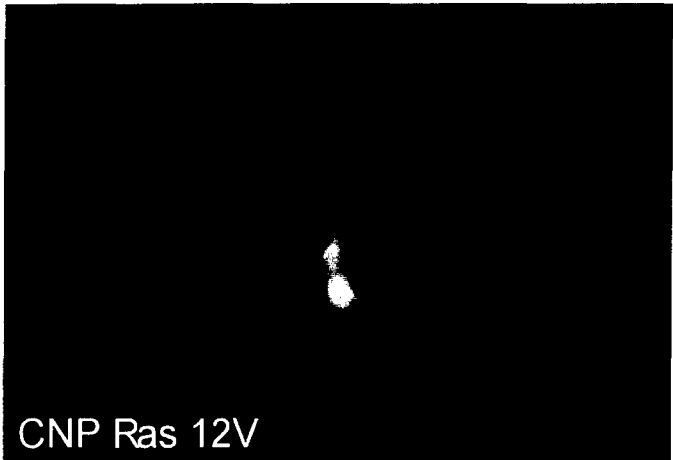
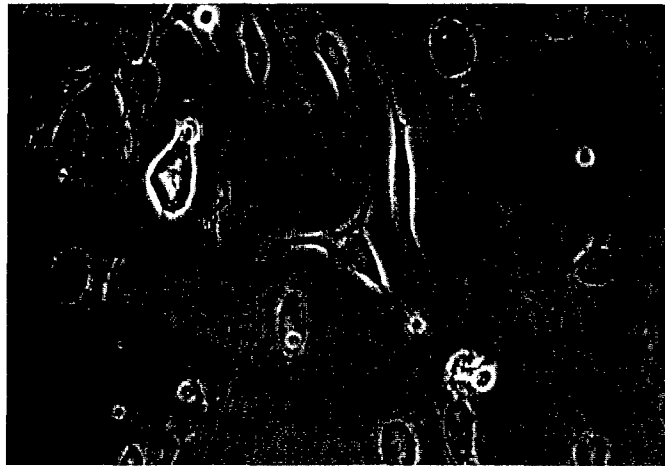


Figure 13
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TRANSDIFFERENTIATION OF NEUROFIBROMATOSIS-1 MUTANT SCHWANN CELLS INTO MELANOCYTES FOLLOWING WOUNDING OF *Nfl/nfl* MOUSE NERVE: Tilit Rizvi, Radhika Aili and Nancy Ratner, Dept Cell Biology, Neurobiology & Anatomy, Univ. Cincinnati, Coll. Med., Cincinnati, OH 45267

Patients with type I neurofibromatosis (NF1) develop peripheral nerve tumors and café-au-lait macules, hyperpigmented spots on the skin. Mice heterozygous for targeted mutations at *Nfl* fail to develop CALM or nerve tumors. To test if wounding induces NF1 manifestations, we cut the sciatic nerve of *Nfl/nfl* mice. Cut ends of transected sciatic nerve were rejoined with sutures or pulled proximally and distally in deflection experiments. *Nfl/nfl* and wild type mice (22/genotype) were evaluated 30 days after surgery. All injured *Nfl/nfl* animals developed patches of pigmented cells in muscles overlying the nerve, identified as melanocytes by EM. Pigmentation was more intense in animals with deflection as compared to animals with transection suggesting that axons are not required for the effect; wild type animals had little or no pigmentation.

Purified mutant *Nfl* Schwann cells were labeled with Hoechst dye and grafted around transected wild type nerves. Of 4000 labeled cells counted in 6 animals, 68 contained pigment granules; of pigmented cells counted, 12.6% were dye-positive. Thus *Nfl* mutant Schwann cells can transdifferentiate into pigmented melanocytes. We also tested if *Nfl*-mutant environment contributes to the phenotype. Schwann cells wild type or mutant at *Nfl* were transplanted into wild type or heterozygous mice. Pigmentation was maximal when both host and graft were mutant. Thus environmental factor(s) and cell autonomous Schwann cell abnormalities contribute to lesion-induced hyperpigmentation in *Nfl* mutants.

A few *Nfl/nfl* mice developed tumors by 3 months after nerve injury. 4/28 developed unencapsulated unpigmented tumors while 5/28 developed encapsulated pigmented tumors. Thus, wounding of the sciatic nerve in mice with a single mutant *Nfl* allele is sufficient to cause disruption of tissue organization with features resembling human NF1. Supported by NIH NS-28840 and the DOD.

292.17

MYELIN DEFECTS IN THE ANTERIOR MEDULLARY VELUM OF THE *TAIEP* MUTANT RAT. J. Song¹, S. Kirvell², A. M. Butt² and I. D. Duncan¹. ¹Department of Medical Science, University of Wisconsin, Madison, WI 53706; ²Division of Physiology, UMDS, Guy's and St. Thomas' Hospitals, London, United Kingdom.

The *taiep* rat is a myelin mutant in which hypomyelination and progressive demyelination of the central nervous system (CNS) are accompanied by an accumulation of microtubules within oligodendrocytes (OLs) (Duncan et al, 1992). *In vitro* study of *taiep* OLs suggests that the microtubule abnormality is directly responsible for the myelin defects (Song et al, 1999). Here we further examined the myelin defects in the *taiep* rat's anterior medullary velum (AMV), a CNS structure which enables visualization of single OLs and their processes *in vivo*. Immunocytochemical studies of myelin proteins (PLP, MAG, MBP) in the AMV have shown a significant myelin deficit in *taiep*. Small caliber axons are more severely affected than are larger caliber. Affected *taiep* axons have thinner myelin sheaths and frequently show a complete absence or discontinuity of myelin. PLP and MAG accumulate in the perinuclear area, but not in the processes, of the *taiep* OLs, suggesting that these myelin proteins are not transported properly from the cell body. This study also shows that while most OLs in the control AMV at two weeks of age are mature and myelinating axons, a number of the *taiep* OLs remain at an immature stage with abundant processes which do not myelinate any axons. These results suggest that the *taiep* mutation affects the normal distribution of myelin proteins, mainly in type I or II oligodendrocytes which myelinate the small diameter axons (Funded by NIH grant NS32361).

292.16

THE *IN VIVO* EFFECTS OF TNF- α ON OLIGODENDROCYTES IN THE RAT ANTERIOR MEDULLARY VELUM
F. Reddington¹, M. Berry, S. Kirvell, A.M. Butt
GKT School of Biomedical Sciences, Kings College, St Thomas' Campus, London, UK

Tumour necrosis factor (TNF- α) is implicated in the pathology of Multiple Sclerosis (MS) and Experimental Allergic Encephalomyelitis (EAE), autoimmune diseases in which oligodendrocytes and myelin are disrupted. *In vitro*, TNF- α has been shown to cause oligodendrocyte disruption and cell death but its actions *in vivo* have not been elucidated. To address the question of whether TNF- α affects oligodendrocytes *in vivo*, the cytokine was administered into the lateral ventricle and its effects were tested using our model tissue of the anterior medullary velum (AMV), which roofs the IVth ventricle. Three age groups were used, neonate (1-2 weeks), juvenile (3-6 weeks) and adult (160-200g) Rats were sedated with halothane and older animals were anaesthetized with hypnorm/hypnovel. Animals received either TNF- α or saline and AMV were analysed after 48 and 72 hours. Rats were sacrificed via lethal injection (0.7ml/kg lethalobarb) and perfusion fixed with 4% paraformaldehyde. AMV were removed intact and double immunofluorescence labelled with Rip/anti-Neurofilament or anti-MBP/anti-Neurofilament. There was no dramatic actions of TNF- α at any of the ages studied but in younger animals both Rip and MBP immunolabelling appeared abnormal. We conclude that TNF- α has very little acute effect *in vivo* but the altered Rip/anti-MBP immunolabelling may be indicative of early changes in oligodendrocytes, leading to demyelination in the longer-term. We are currently examining this possibility by looking at later time points.

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292.18

TEMPORAL PROGRESSION OF REACTIVE ASTROCYTOSIS IN SEVERAL BRAIN REGIONS OF THE MYELIN MUTANT "TAIEP" RAT. Leon Chávez, B.A., Galindo, S., Guevara, J., Villegas, O., Ugarte, A., Luna, J., Mena, R., and Martínez-Fong, D. Depto. Fisiol., Biofís. y Neuroci. "Pro. Multidisc. de Biomed. Mol., Biol. Cel. CINVESTAV, Inst. de Fisiol. de UAP.

Reactive astrocytosis reflects predominantly phenotypic changes of resident astroglia, rather than migration or proliferation of such cells has been suggested, *in vivo*. This process may contribute to maintain the neuronal surviving by eliminating toxic compounds produced by injured neurons, and by releasing trophic factors. In contrast, activated astrocytes may increase the neurological injury by releasing cytotoxic compounds such as cytokines, excitatory amino acids and free radicals (NO, OH, O₂⁻, etc.). We have previously reported evidence of astroglial activation in primary cultures from cerebral cortex and cerebellum of "taiep" rats. In these cultures, pro-inflammatory cytokines (IFN γ and TNF α) in presence of LPS increased NO accumulation. Besides, the cytokine-induced NO accumulation was less sensitive to the anti-inflammatory effect of TGF β 1. To demonstrate astroglial activation *in vivo*, we measured GFAP immunoreactivity in several brain regions of "taiep" rats by ELISA and indirect immunofluorescence. A rabbit polyclonal antibody anti-GFAP (1:3400) and a goat anti-rabbit IgG conjugated with either radish peroxidase or fluorescein were used. The absorbance was determined at 414 nm in an ELISA spectrometer. We found a significant increase in GFAP immunoreactivity only in cerebral stem of 15-days-old "taiep" rats. However, there was a significant increase in GFAP immunoreactivity in all studied regions since 1 month up to 3 months of age (end of the study). There was not statistic difference in diencephalon in the period studied. Increased GFAP immunofluorescence was confirmed in the same regions by confocal microscopy. These results show the presence of activated astroglial in myelin mutant "taiep" rat and suggest a possible contribution to the brain injury.

STAINING, TRACING AND IMAGING TECHNIQUES: OPTICS AND DYES

293.1

ACETYLCHOLINESTERASE BINDS TO DISSOCIATED NEURONS OF APLYSIA. M. Srivatsan¹. Dept. of Physiology, Univ. of Kentucky College of Medicine, Lexington, KY 40536-0298

Acetylcholinesterase (AChE) is present in hemolymph (HML) of adult *Aplysia*, a marine mollusc (Bevetagua et al., 1975). It promotes neurite growth in cultured *Aplysia* neurons (Srivatsan and Perez, 1997) and prompted us to examine AChE-neuronal interaction which may lead to enhanced neurite growth. AChE from HML was purified by edrophonium chloride affinity column and was coupled (100 μ g/ml) to Fluospheres (Molecular Probes). Enzyme activity of coupled AChE was assayed. Bovine serum albumin (BSA, 10mg/ml) was added to the coupled AChE to prevent nonspecific binding. Neurons were dissociated from pedal ganglia of *Aplysia* and were plated on polylysine coated glass cover slip -mounted dishes as before (Srivatsan and Perez, 1997). Two hours after plating, the medium was replaced with artificial sea water (ASW) and Fluosphere-coupled AChE was added (final conc. 100ng/ml) to the dishes and were incubated in the dark for 5 min. at 20°C followed by 30 min. at 4°C. Dishes to which (a) uncoupled AChE was added in excess prior to the addition of Fluosphere-coupled AChE and (b) Fluosphere-coupled BSA alone was added served as controls. After repeated rinsing with ASW to remove unbound AChE, examination of the dishes under fluorescent inverted microscope revealed fluorescent spots on the neuronal membrane in dishes to which Fluosphere-coupled AChE alone was added demonstrating the binding of AChE to the neuron. Fluorescent spots were absent in the control dishes indicating that the binding was specific. Neurons with three or more fluorescent spots were counted as positive for AChE binding. Initial results showed that AChE binds to ca 65% of pedal neurons in the culture dish. This result suggests that HML AChE specifically binds to a subset of dissociated pedal neurons of *Aplysia*. The role of sugar residues of AChE in its binding to neurons is currently being investigated.

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293.2

IMAGING REPETITIVE SPREADING DEPRESSION IN SUBMERGED NEOCORTICAL SLICES. T.R. Anderson, C.R. Jarvis and R.D. Andrew¹. Dept. of Anatomy & Cell Biology, Queen's University, Kingston, ON, Canada, K7L-3N6.

Spreading depression (SD) is a profound but transient depolarization of neurons and glia that arises focally and migrates across the cortical and subcortical gray. *In vivo* it occurs under normoxic conditions during migraine aura where it precedes migraine pain but does not damage tissue. During stroke however, SD can arise repeatedly near the ischemic core and may promote neuronal damage. We developed a brain slice preparation that repeatedly generates robust SD during superfusion with artificial CSF, thereby facilitating imaging and drug application during SD experiments. Rat neocortical slices were exposed to elevated K⁺ aCSF (26 mM KCl) for 2 min @ 3 ml/min. SD was measured as a negative DC shift recorded in layers 2/3 and as a front of elevated light transmittance using intrinsic optical signal imaging. Pretreatment with NMDA receptor antagonists (n=8) blocked SD, even with K⁺ exposure extended to 10 min. CNQX had no effect (n=6). Repeated SD episodes were imaged with full recovery of evoked synaptic responses. In contrast, SD evoked by metabolic inhibition (bath application of low O₂/glucose, ouabain or cyanide) was not blocked by glutamate antagonists, was not repeatable, and eliminated evoked synaptic responses post-SD. The findings support *in vivo* studies showing SD block by NMDA antagonists in normoxic, but not ischemic, neocortex. In conclusion, this slice paradigm is useful for studying regenerative SD with a spacial and temporal resolution not possible *in vivo*.

Supported by the Ontario Heart and Stroke Foundation and the Medical Research Council of Canada.

J Neurotrauma (1999)

TRANSDIFFERENTIATION OF NEUROFIBROMATOSIS-1 MUTANT SCHWANN CELLS INTO MELANOCYTES FOLLOWING WOUNDING OF *Nf1/nf1* MOUSE NERVE: Nancy Ratner*, Radhika Atit and Tilat Rizvi, Dept. Cell Biology, Neurobiology & Anatomy, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0521.

Patients with type 1 neurofibromatosis (NF1) develop peripheral nerve tumors and cafe-au-lait macules, hyperpigmented spots on the skin. Mice heterozygous for targeted mutations at *Nf1* fail to develop hyper-pigmentation or nerve tumors. Wounding has been proposed as an initiating event in human NF1. To test this hypothesis, we cut the sciatic nerve in *Nf1/nf1* mice. Cut ends of transected sciatic nerve were either rejoined with sutures or pulled proximally and distally. Mice (22/genotype) were evaluated 30 days after surgery. All injured *Nf1/nf1* animals developed patches of pigmented cells in muscles overlying the nerve, identified as melanocytes by electron microscopy. Pigmentation was more intense in animals with deflection as compared to transection suggesting that axons are not required for the effect; wild type animals had little or no pigmentation. A few *Nf1/nf1* mice developed tumors 3 months after nerve injury. 4/28 developed unencapsulated unpigmented tumors while 5/28 developed encapsulated pigmented tumors. Thus, wounding of the sciatic nerve in mice with a single mutant *Nf1* allele causes features resembling human NF1.

To define mechanisms underlying wound-induced hyperpigmentation, purified mutant *Nf1* Schwann cells were labeled with Hoechst dye and grafted around transected wild type nerves. Of 4000 labeled cells counted in 6 animals, 68 contained pigment granules; of pigmented cells counted, 12.6% were dye-positive. Thus *Nf1* mutant Schwann cells appear to transdifferentiate into pigmented melanocytes. We tested if the *Nf1* environment contributes to the phenotype. Schwann cells wild type or mutant at *Nf1* were transplanted into wild type or heterozygous hosts. Pigmentation was maximal when both host and graft were mutant. Thus environmental factor(s) and Schwann cell abnormalities appear to contribute to lesion-induced hyperpigmentation in *Nf1* mutants. Supported by NIH NS-28840 and a grant from the DOD.



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