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13. ABSTRACT ***(Maximum 200 Words)***

This is the final report for a two year award made to a Consortium of investigators who are working together to develop, characterize and utilize strains of mice that accurately model tumors that develop in persons with NF1 and NF2. This Consortium accomplished its primary goal of generating and characterizing mouse models of NF1 and NF2-associated tumors for biologic and preclinical therapeutic trials. A number of novel strains were developed, and shared widely with the research community. The investigators have collaborated closely and have shared expertise and reagents extensively. This NF Consortium has been admitted to the Moue Models of Human Cancer Consortium of the National Cancer Institute and is participating fully in the activities of the group. This Consortium received a grant from the U.S. Army Neurofibromatosis Program to continue our collaborative work through 2005.

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INTRODUCTION

Benign and malignant tumors are a major cause of morbidity and mortality in individuals afflicted with NF1 and NF2. The *NF1* and *NF2* genes function as tumor suppressors in humans and in mice. Although a great deal has been learned about the genetics, biochemistry, and cell biology of NF1 and NF2-associated tumors, it has proven difficult to translate these advances into new treatments. The development of accurate, well-characterized mouse models of NF-associated tumors NF1 and NF2 would provide an invaluable resource for bringing improved treatments to NF patients. The purpose of this consortium is to develop and validate such models, and to make these strains available to the scientific community for biologic studies and preclinical therapeutic trials. This effort is timely for a number of reasons.

First, recent advances in gene targeting technologies have made it is feasible to introduce many types of alterations into the mouse germline. Members of this research consortium have developed lines of *Nf1* and *Nf2* mutant mice, which have provided important insights into a number of the complications seen in human patients. During the two year funding duration of this project, we made dramatic progress in improving and extending these models. Second, much has been learned about the genetic and biochemical basis of deregulated growth in *NF1* and *NF2*-deficient human cells and in cells derived from *Nf1* and *Nf2* mutant mice. Genetic analysis of human and murine tumors has provided compelling evidence that *NF1/Nf1* and *NF2/Nf2* function as tumor suppressor genes (TSGs) *in vivo.* Biochemical data have suggested target proteins and pathways for rational drug design. The improved mouse models developed by this consortium now provide an invaluable platform for rigorous preclinical trials of these innovative approaches. Third, new therapies are urgently needed for many of the tumors that arise in individuals with NF1 and NF2. The current treatments for neurofibroma, optic nerve glioma, vestibular schwannoma, and for NF1 and NF2-asociated malignancies are frequently ineffective and carry a substantial risk of long term morbidity. This consortium is highly complimentary to the ongoing efforts to undertake human clinical trials because it will facilitate testing novel agents and approaches in a controlled preclinical setting. The quantity of drug required, expense, and potential liability are all either greatly reduced or eliminated when mouse models are used for preclinical studies. This will facilitate testing a wide range of new therapies that might benefit NF patients. Finally, the Mouse Models of Human Cancer Consortium (MMHCC) of the National Cancer Institute (NCI) is providing a historic opportunity for interactions among 20 research groups that are working to develop, validate, and enhance models of a variety of human cancers. NF is the only inherited cancer predisposition represented within the MMHCC as a discrete disease entity. Our group was admitted to the MMHCC in 2000 and has been participating in its activities. Drs. Jacks, Parada, and Shannon are members of the MMHCC Steering Committee, with Dr. Parada serving as the designated representative of the NF Consortium. Dr. Jacks was Co-Chair of the Steering Committee from its inception until last year, and Dr. Shannon is one of two Co-Chairs currently leading the MMHCC. Thus, this award has provided the NF research community with an exceptional level of representation within the mouse modeling community. The MMHCC is spearheading efforts in areas such as building repositories, devising pathologic classification schemes, imaging mouse tumors, and stimulating interactions with industry in the area of preclinical therapeutics that are of general importance to NF research. The laboratory researchers in this consortium are working closely with the National Neurofibromatosis Foundation (NNFF). This interaction facilitates research in NF1 and NF2 and links basic and clinical researchers with patients. The work initiated under this award will

continue through 2005 through a grant made by the U.S. Army Neurofibromatosis Research Program that commenced in October 2002.

To achieve our overall research goals, we focused on achieving the technical objectives (aims) listed below during the two years of funding provided by this award:

(1) To enhance existing lines of *Nf1* and *Nf2* mutant mice and to develop new *in vivo* models of NF-associated tumors. As new models of NF-associated tumors have been developed, we have fully characterized these lesions with an emphasis on how closely they reproduce the phenotypic, genetic, and biochemical alterations seen in comparable human tumors.

(2) To perform *in vitro* and *in vivo* experiments to elucidate biochemical pathways that are essential for the *in vivo* proliferative advantage of *Nf1* and *Nf2*-deficient cells as a way of identifying molecular targets for therapeutic interventions.

(3) To use these models to rigorously test the clinical and biochemical effects of rational therapies for tumors that arise in individuals with NF1 and NF2 in controlled preclinical trials.

BODY

Technical Objective (Aim) 1: To produce and characterize models of NF-associated tumors

Background

Production of *Nf1* Mutant Mice. Drs. Jacks and Parada independently disrupted *Nf1* by inserting a neomycin *(neo)* cassette into exon 31 (1, 2). Homozygous *Nf1* mutant ($Nf1^{-/-}$) embryos die *in utero* with cardiac anomalies. Heterozygous *Nf1* mutant mice ($Nf1^{+/-}$) display learning disabilities that are reminiscent of children with NF1 (3), but do not develop optic tract gliomas, Lisch nodules, or neurofibromas. These mice are predisposed to some of the same tumors as humans with NF1 including fibrosarcoma, pheochromocytoma, and a myeloproliferative disorder (MPD) that resembles juvenile myelomoncytic leukemia (JMML). Tumors arising in heterozygous *Nf1* mutant mice frequently show loss of the normal allele (2), a finding that is concordant with data from human patients. However, while human and murine *NF1* function as TSGs in some cell lineages, $Nf1^{+/-}$ mice are of limited value for preclinical studies because tumors arise unpredictably in a minority of the mice beginning around one year of age. The failure of these strains to develop neurofibroms was particularly disappointing, as this represents a major burden for NF1 patients.

To test the possibility that a mutation in the wild-type *Nf1* allele is required and rate-limiting in the formation of neurofibromas, the Jacks' laboratory performed blastocytst injections to generate chimeric mice that were partially composed of $Nf1^{-/-}$ cells (4). Multiple tumors (10-100) per mouse were detected in liveborn chimeras, usually emanating from the dorsal root ganglia or peripheral nerves in the limbs. These experiments provide "proof of principle" that inactivation of *Nf1* in cells of the dorsal root ganglia results in the frequent appearance of hyperplastic lesions that bear all the characteristics of neurofibromas. However, this methodology is impractical as an experimental model. The embryonic-lethal phenotype of $Nf1^{-/-}$ embryos restricted analysis of *Nf1* function to early development. To circumvent this problem and begin to address issues relevant to NF1 disease, Dr. Parada's laboratory employed *Cre-loxP* technology to create a conditional *Nf1* allele (5). Importantly, the Parada's lab found that the $Nf1^{flox}$ allele functions as a wild-type allele in spite of harboring *loxP* sites and a *neo* gene within

its intronic sequences. The $Nf1^{flox}$ allele is readily recombined *in vivo* to make a null allele through coexpression of *Cre* recombinase (5).

Production and Characterization of *Nf2* Mutant Mice. The role of loss of *Nf2* function in development and tumorigenesis has been studied in various mutant mouse models. Embryos homozygous for a *Nf2* mutation fail to initiate gastrulation (6). Although cancer prone, heterozygous *Nf2* mutant mice ($Nf2^{+/-}$) do not develop schwannoma or meningioma. Similarly, heterozygous *Nf2* mutant mice do not show Schwann cell hyperplasia or other manifestations of NF2 disease such as cataracts or cerebral calcifications. Thus, although these lines of *Nf2* mutant mice are useful for investigating merlin function, they do not accurately model important complications of NF2. The $Nf2^{+/-}$ mutant mice generated independently in Cambridge and Paris are predisposed to a number of malignant tumors including osteosarcomas, fibrosarcomas and hepatocellular carcinoma (7). These tumors frequently exhibit loss of the wild-type *Nf2* allele, confirming that *Nf2* functions as a TSG. Dr. McClatchey has shown that these tumors exhibit a remarkably high rate of metastasis (7). These results, together with the observations of Dr. Giovannini that *Nf2* inactivation is a rate-limiting step in murine Schwann cell tumorigenesis (described below), suggest that the study of *Nf2* may have broad implications for the study of cancer development and progression in humans.

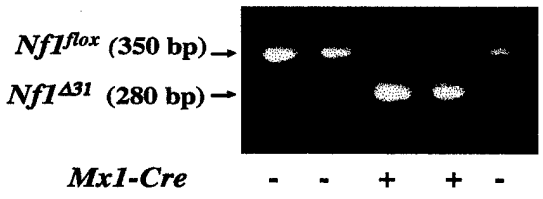
To circumvent the early embryonic-lethal phenotype associated with homozygous inactivation of *Nf2* and to test the hypothesis that the tumor spectrum might be modulated by the rate of the loss of the normal allele in specific tissues, Dr. Giovannini and his colleagues generated a conditional mutant *Nf2* allele (8). A two-step strategy was utilized to construct a mutant $Nf2^{flox2}$ allele characterized by the presence of *loxP* sites in the intronic regions flanking exon 2. As expected, mice homozygous for the $Nf2^{flox2}$ mutant allele ($Nf2^{flox2/flox2}$) were viable and fertile suggesting that the introduction of *loxP* sites did not hamper *Nf2* expression. Furthermore, phenotypic analysis over a period of 24 months showed that the spontaneous tumor spectrum of $Nf2^{flox2/flox2}$ and $Nf2^{+/+}$ mice of the same genetic background did not differ significantly. Induced expression of *Cre* recombinase in $Nf2^{flox2/flox2}$ mice results in biallelic inactivation of *Nf2* in specific tissue (8).

Progress Report

Overview. During the two year period of support, we focused most of our efforts on sharing reagents, on enhancing existing mouse models of NF-associated tumors, and on generating and characterizing new models. Our progress and the current status of specific NF1 and NF2 tumor models is summarized below.

Myeloid Leukemia Model. JMML is characterized by over-production of myeloid cells that infiltrate hematopoietic and non-hematopoietic tissues (9, 10). The hematopoietic system offers a number of advantages as an experimental model including well-defined culture systems to assay the proliferative potential of progenitor cells, techniques that permit adoptive transfer into irradiated recipients, and the ability to perform biochemical assays on primary cells. A hallmark of human JMML cells is that they selectively form excessive numbers of colony forming unit granulocyte-macrophage (CFU-GM) progenitor colonies in methylcellulose cultures exposed to low concentrations of granulocyte-macrophage colony stimulating factor GM-CSF (9). $Nf1^{-/-}$ fetal hematopoietic cells demonstrate a similar pattern of hypersensitive growth (11, 12), and

adoptive transfer of *Nf1*-deficient fetal liver cells into irradiated recipients induces a JMML-like MPD with hyperactive Ras (12, 13). These mice provide an *in vivo* model for correlating the clinical and biochemical effects of targeted therapeutics on the growth of *Nf1* deficient cells (14). However, this system is cumbersome and expensive because it requires maintaining a large breeding colony, performing multiple timed matings followed by embryo dissections around E12.5, genotyping by PCR, and injecting fetal liver cells into irradiated hosts. To circumvent these problems, Dr. Shannon obtained $Nf1^{flox/flox}$ mice from Dr. Parada, performed embryo transfers to move the $Nf1^{flox}$ allele into the UCSF barrier facility, and bred $Nf1^{flox}$ and *Mx1 Cre* mice. In the *Mx1 Cre* strain, Cre recombinase is expressed from the interferon-inducible *Mx1* promoter (15). *Mx1-Cre* $Nf1^{flox/flox}$ and control $Nf1^{flox/flox}$ mice were injected with polyinocinic-polycytidylic acid (pIpC) at 2 days of age to induce endogenous interferon production. A genotyping scheme based on use of the polymerase chain reaction was then used to document Cre-mediated excision of exon 31 in blood leukocytes (Fig. 1). Serial blood counts and smears were obtained every 4 weeks to follow mice for evidence of MPD. Somatic inactivation of *Nf1* consistently induces MPD in *Mx1-Cre* $Nf1^{flox/flox}$ mice, but not in control littermates, that is associated with leukocytosis, splenomegaly with myeloid infiltration, and the presence of increased numbers of $Gr1^{lo}/Mac1^{+}$ cells in the bone marrow (Fig. 1). These findings are similar to what has been reported in recipients of *Nf1-/-* fetal liver cells (12, 16), and these data indicate that our goal of developing a tractable model of a myeloid malignancy induced by somatic inactivation of *Nf1* in hematopoietic cells has been achieved.



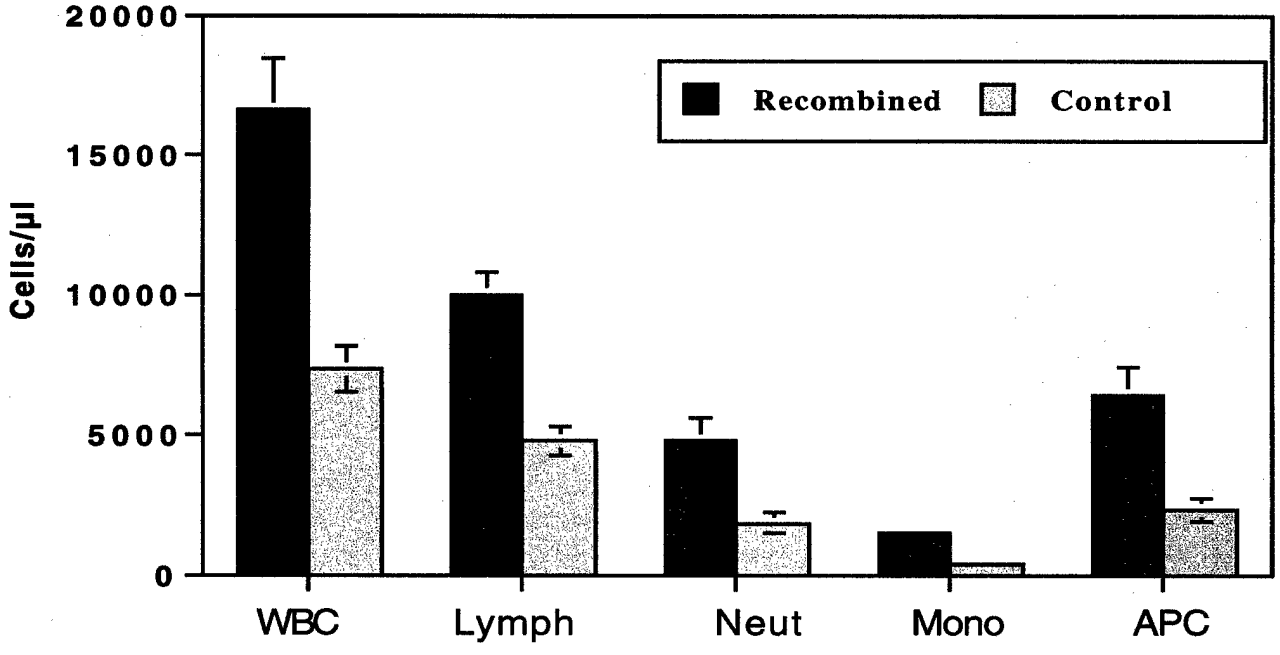


Figure 1. Somatic Deletion of *Nf1* in Hematopoietic Cells Induces MPD. Left. $Nf1^{flox/flox}$ pups were injected with pI-pC, then genotyped for co-inheritance of the *Mx1-Cre* transgene. The mice were bled 5 weeks later and the status of *Nf1* exon 31 was assessed by performing PCR on leukocyte DNAs. A 280 bp band corresponding to a deletion of exon 31 (Δ31) is visible in 2 pups that inherited the *Mx1-Cre* transgene (+) but not in 3 that did not. Below. White blood cell counts (WBC) are markedly elevated in these mice 3 months after pIpC injection. Total numbers of neutophils (Neut), monocytes (Mono), and lymphocytes (Lymph) are all increased. APC = absolute phagocyte counts (neutrophils + monocytes).

Schwannoma Model. Dr. Giovannini and his colleagues demonstrated that conditional mutation of the *Nf2* gene in Schwann cells leads to hyperplasia and to the development of benign and malignant Schwannomas (8). Four independent transgenic lines (P0Cre^{A-D}) were then crossed a conditional *Nf2* mutation ($Nf2^{flox2}$). Young P0Cre; $Nf2^{flox/flox}$ mice developed Schwann cell hyperplasia, predominantly in basal and spinal ganglia. Benign and malignant Schwann cell tumors were observed in various anatomic sites in significant percentage of older animals. Deletion of *Nf2* exon 2 in tumors and other lesions was demonstrated by Southern blotting. Thus, directed mutation of *Nf2* to Schwann cells leads to tumors and pre-neoplastic lesion that are highly related to those seen in human NF2. Together, these studies indicate that *Nf2* mutation is a rate-limiting step for both Schwann cell tumorigenesis and Schwann cell hyperplasia. This model will be valuable for evaluating therapies for NF2-associated tumors (8).

Meningioma Model. Meningioma is a common nervous system tumor that affects older adults (particularly women) and is often associated with significant morbidity. Though most lesions are hostologically benign (grade I), a significant proportion demonstrate aggressive features. In this regard, meningiomas can invade brain tissue, recur after resection, and spread along the leptomeninges to involve multiple regions. Individuals with NF2 are at significantly elevated risk for developing meningiomas, suggesting that the *NF2* gene might play a central role in regulating leptomeningeal cell proliferation. Biallelic inactivation of the *NF2* gene has been identified in 30-70% of sporadic meningiomas, leading to loss of expression of the *NF2* gene product, merlin. In addition, *NF2* inactivation is likely an early event in sporadic meningioma pathogenesis and is observed as frequently in grade I meningiomas as it is in high-grade tumors. Current animal models of meningiomas have relied on implantation of human meningioma cells in immunocompromised mice. Grade I meningiomas grow slowly *in vitro* and rarely survive as explants *in vivo*. Only a few high-grade malignant human meningioma cell lines grow as explants in immunocompromised mice *in vivo*, with tremendous variability and success. Based on these limitations, the availability of an *in vivo* model system in which meningiomas arise from normal arachnoidal cells would be a major advance.

Although cancer prone, heterozygous *Nf2* mutant mice ($Nf2^{+/-}$) do not develop meningioma (7, 8), but rather die with osteosarcomas and other tumor types not found in humans with NF2. Dr. Giovannini previously demonstrated that *Nf2* inactivation is a rate-limiting step in murine Schwann cell tumorigenesis using the *P0* promoter to express Cre recombinase in Schwann cells (8). Remarkably, meningioma was not observed in these mice, suggesting that Cre recombinase expressed from a Schwann cell-specific promoter does not affect meningioma progenitor cells. Electron microscopy and immunophenotypic studies show that meningiomas originate from arachnoidal cells of the meningeal coverings of the brain and spinal cord that are in contact with the cerebrospinal fluid (CSF). An alternative approach for the delivery of Cre recombinase into specific target tissues involves the use of a recombinant adenovirus (*adCre*). This approach also has the advantage of targeting the initiating genetic lesion (in this case, homozygous inactivation of *Nf2)* to a small population of susceptible cells, which is likely to model human cancer more accurately than when all of the cells in a target tissue are mutated (17).

To determine whether *Nf2* disruption is sufficient for meningioma formation, Dr. Giovannini inactivated *Nf2* in homozygous conditional knockout mice by *ad*Cre delivery. Thirty percent of mice with *ad*Cre-mediated excision of *Nf2* exon 2 developed a range of meningioma

subtypes histologically similar to the human tumors beginning at 4 months of age. Additional hemizygosity for *p53* did not modify meningioma frequency or progression suggesting that *Nf2* and *p53* mutations do not synergize in meningeal tumorigenesis. These results demonstrate that *Nf2* loss in arachnoidal cells is rate-limiting for meningioma formation. The availability of this first mouse model initiated with a genetic lesion found in familial and sporadic human meningiomas provides a powerful tool for investigating meningioma progression and for the preclinical evaluation of potential therapeutic interventions.

Sarcoma Models. Plexiform neurofibromas progress to MPNSTs in 5-10% of NF1 patients, suggesting that mutations at additional loci are required (18). A candidate for a cooperating TSG is *p53,* which is located on the same chromosome as *Nf1* in mouse and man. Indeed, *p53* mutations have been demonstrated in a subset of human tumors (19, 20). Therefore, to recapitulate the simultaneous loss of *Nf1* and *p53* seen in human cancers, the Jacks and Parada laboratories independently crossed *Nf1* and *p53* mutant mice to generate recombinant founders that carried both mutant alleles on the same chromosomal homolog (i.e. in *cis* configuration). Both the *cis* and *trans* $Nf1^{+/-}$; $p53^{+/-}$ mice began to develop tumors (primarily sarcomas) at 15 and 25 weeks, respectively. Thus, a heterozygous *Nf1* mutation, which is weakly tumorigenic, cooperates with a *p53* mutation to accelerate tumor formation and modify tumor spectrum. Molecular analysis of the normal *p53* and *Nf1* alleles in the *cis* $Nf1^{+/-}$; $p53^{+/-}$ mice revealed loss of heterozygosity (LOH) at both loci in at least 70% of the soft tissue tumors. Importantly, the predominant tumor type closely resembles MPNST. Because of the high penetrance of this phenotype, the fact that the tumors are generally visible and palpable, and the clear relevance to human NF1, these MPNST models are tractable for performing preclinical therapeutic studies.

In collaboration with Dr. Jeff DeClue (National Cancer Institute), the Parada lab has neurofibrosarcoma cell lines derived from *cis* $Nf1^{+/-}$; $p53^{+/-}$ mice to elucidate signaling pathways that might contribute to aberrant growth. These studies revealed abnormal signaling from the EGF receptor via the PI3 kinase pathway. This work has been published in *Cancer Research.*

Recent evidence shows that mutations at the *INK4A* locus are also common in human MPNST (21). The $p16^{INK4a}$/$p19^{ARF}$ locus encodes two TSGs: $p16^{INK4a}$ is a potent inhibitor of D-type cyclin-Cdk4/6 complex activity, while $p19^{ARF}$ stabilizes p53 via several mechanisms in response to oncogenic signals such as Ras activation (22). As the mutations identified in MPNSTs are not limited to either $p16^{INK4a}$ or $p19^{ARF}$, it is uncertain which alteration contributes to progression. Over the past year, the Jacks laboratory has continued to characterize the tumor spectrum in *cis* $Nf1^{+/-}$; $p53^{+/-}$ mice and has created mice carrying mutations at the *Nf1* and *Ink4A* loci. Consistent with studies in human tumors, $Nf1^{+/-}$; $Ink4A^{-/-}$ mice develop MPNSTs at high frequency with short latency (data not shown). Importantly, the *Ink4A* mutation itself causes a mild tumor phenotype (in contrast to germline mutations in *p53),* and the $Nf1^{+/-}$; $Ink4A^{-/-}$ animals appear to have a more narrow tumor spectrum than *cis Nf; p53* mice. This is an important consideration for the design, execution, and interpretation of studies evaluating novel therapeutics.

Astrocytoma Models. As discussed above, *cis* $Nf1^{+/-}$; $p53^{+/-}$ mice on a mixed C57Bl/6 x 129/Sv genetic background develop primarily soft tissue sarcomas, including a large percentage of MPNSTs. The Jacks lab is performing a screen to discover genetic modifiers of the tumor phenotype caused by the *cis* $Nf1^{+/-}$; $p53^{+/-}$ mutation. In the course of these studies, the *cis Nf1*; *p53* mutant homolog was crossed onto a pure C57Bl/6 background as well as onto a variety of

F1 genetic backgrounds (23). Although MPNSTs arise in *cis* $Nf1^{+/-}$; $p53^{+/-}$ mice on these other genetic backgrounds, a large percentage of these animals develop brain tumors. Up to 75% of *cis* $Nf1^{+/-}$; $p53^{+/-}$ mice (depending on the background) develop some form of brain lesion ranging from aberrant nuclear morphology to glioblastoma multiforme (23). Tumor cell lines have been established from several lesions to date, and all show loss of the wild-type copies of both *Nf1* and *p53*. These cell lines are capable of forming tumors upon subcutaneous and intracerebral injection into nude mice (data not shown). In recent studies, the Jacks laboratory has continued to investigate strain-specific differences in the predisposition to astrocytoma by manipulating the genetic background. In particular, given the absence of brain tumors in the original cohorts of *cis Nf1*; *p53* compound mutant mice studied on a mixed C57Bl/6 and 129/Sv genetic background (4, 24), they have analyzed the brain tumor phenotype of *cis Nf1*; *p53* mice on a pure 129/Sv background. Consistent with the presence of one or more tumor resistance alleles, the incidence of brain tumors/lesions on the 129/Sv background was approximately 10% (compared to 75% on a C57Bl/6 background), and the lesions that were detected were of early stage. Backcross and mapping studies aimed at identifying the relevant modifier alleles in the 129/Sv background are being pursued with other funds, but it is expected that the identification of such alleles will suggest targets for chemopreventive or therapeutic intervention in NF1-associated brain tumors.

Dr. Parada's lab has exploited the conditional *Nf1* allele as an alternative strategy for generating models of NF1-associated brain tumors. In particular, his laboratory crossed the $Nf1^{flox/flox}$ mutation onto a strain in which the Cre recombinase is expressed from a GFAP that is active in all neural cells (GFAP*). *GFAP*-Cre* $Nf1^{flox/flox}$ mice exhibit severe neurological dysfunction as a consequence of inactivating *Nf1* throughout the CNS. The Parada lab has generated considerable *in vivo* data from studies in which *Nf1* was inactivated in distinct cell compartments within the nervous system. In one study, ablation of *Nf1* in neurons resulted in severe global astrogliosis in the brains of the resultant mice. This astrogliosis was shown to be non-cell autonomous as the *Nf1* gene was intact in the astrocytes despite their increased size and GFAP overexpression. This work was published in *Genes & Development.* In two studies performed in collaboration with Dr. David Gutmann (Washington University), the Parada lab analyzed the *in vitro* properties of astrocytes lacking *Nf1* and assessed the *in vivo* consequences of ablating *Nf1 in vivo.* This work has resulted in two papers (see publications list below). These studies are presently being continued and additional *in vivo* models will be pursued in further studies that should continue to elucidate the role of *Nf1* inactivation in the development of optic gliomas, pilocytic astrocytomas and glioblastoma multiforme.

Neurofibroma Models. We identified the absence of neurofibroma formation in *Nf1* mutant mice as a major shortcoming of existing strains, and therefore placed a high priority on modeling this important complication of NF1 disease. Within the past year, we produced two neurofibroma models that are described here and in the following section. A hallmark of NF1 disease, the neurofibroma, is a complex neoplasm that contains multiple cell types including Schwann cells, perineurial cells, fibroblasts, neurons, and mast cells (5, 25). Schwann cells, which comprise 40 to 80% of neurofibromas, are the predominant cell type in these lesions. Cultured Schwann cells isolated from neurofibromas exhibit angiogenic and invasive properties (26), suggesting that *NF1* mutations in this cell type may be a critical event in tumorigenesis. Indeed, several recent studies reported that Schwann cells (but not fibroblasts) from neurofibromas show biallelic inactivation of *NF1* (27, 28). These data support the idea that Schwann cells are a critical target

for *NF1* mutations. Dr. Parada s laboratory therefore used the conditional $Nf1^{flox}$ allele to study the role of *Nf1* in Schwann cell development and neurofibroma formation.

To examine whether loss of *Nf1* in Schwann cells confers a growth advantage that is sufficient to induce neurofibroma formation *in vivo*, Dr. Parada s group exploited a strain of mice in which the Cre recombinase is expressed from the *Krox20* promoter. Previous studies have shown that the expression of Cre recombinase in this strain precisely mimics the endogenous *Krox20* gene, whose expression is restricted to Schwann cell lineage in the peripheral nervous system (29). Peripheral nerves from three 6-month-old *Krox20* $Nf1^{flox/flox}$ mutant mice and from control littermates have been examined in detail. The nerves of these *Krox20* $Nf1^{fl\S ox/flox}$ mice were uniformly enlarged when compared to the control nerves, including trigeminal (Fig. 2B, arrows), spinal roots (Fig, 2D), and sciatic nerves (data not shown). Histologic analysis indicates that the *Krox20* $Nf1^{flox/flox}$ nerves display Schwann cell hyperplasia although they are apparently tumor free at the age of 6 months. Together, these observations suggest that *Nf1* regulates Schwann cell development. *Krox20* $Nf1^{flox/flox}$ mice aged for 12-14 months develop multiple abnormal growths that model plexiform neurofibromas. These lesions emerge along cranial (Figure 3A, arrow and 3B) and spinal roots (Figure 3C, arrows and 3D), whereas none were found along peripheral nerves (data not shown). Thus, ablation of *Nf1* in Schwann cell precursors is a critical event in the eventual development of neurofibromas. In further studies, the Parada lab made the seminal observation that haploinsufficinecy for other cells in the stromal microenvironment (e.g. mast cells and fibroblasts) strongly potentated the formation of neurofibromas when *Nf1* was selectively ablated in Schwann cells. This work appeared in *Science* earlier this year. In summary, a relevant model in mice for plexiform neurofibromas has been developed. The availability of these mice will not only permit the testing of hypoytheses regarding the sequence of events that lead to neurofibromas, but also to develop further models that progress to neurofibrosarcomas and for testing of therapeutics.

Figure 2. Peripheral nerves from *Krox20* $Nf1^{flox/flox}$ mice (panels B and D) and controls (panels A and C).

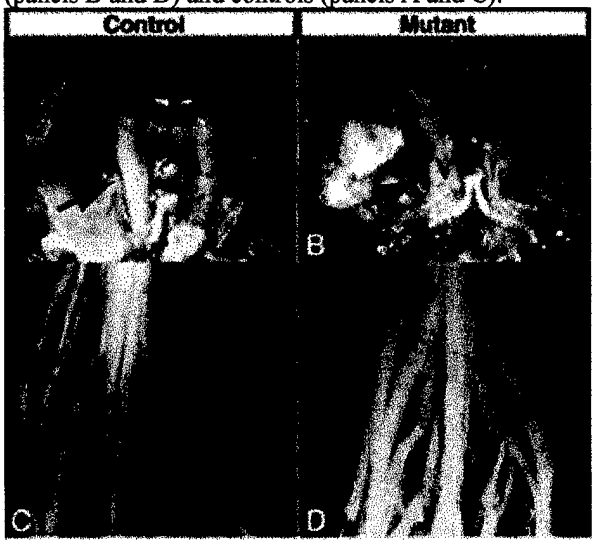
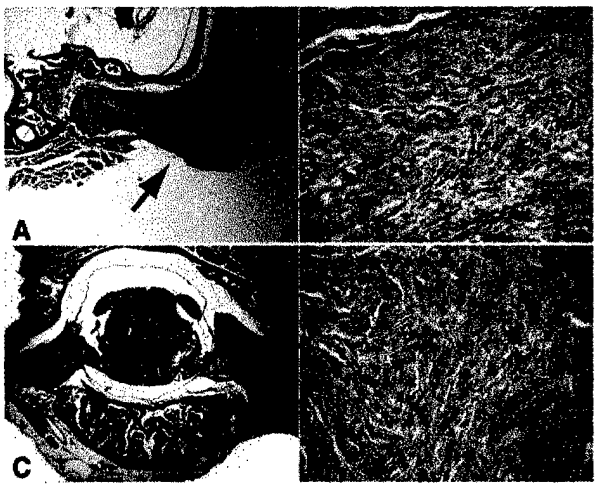


Figure 3. Neurofibromas from *Krox20* $Nf1^{flox/flox}$ mice.



Neurofibromas and MPNSTs in cis $Nf1^{+/-}$; $Nf2^{+/-}$ and P0Cre^{B}; $Nf2^{flox2/flox2}$; $Nf1^{+/-}$ Mice. Dr. McClatchey has found that *Nf1* and *Nf2* mutations cooperate in MPNST development in cis $Nf1^{+/-}$; $Nf2^{+/-}$ mice (data not shown). In addition, Dr. Giovannini has reported the phenotypes in *Nf2* conditional mutant mice that have been generated with four different P0*Cre* transgenic lines (A-D) (8). The highest incidence of benign Schwann cell tumors and MPNSTs was found in P0Cre^{C}; $Nf2^{flox2/flox2}$ mice (35%), and the lowest in P0Cre^{B}; $Nf2^{flox2/flox2}$ animals (4%). Schwann cell hyperplasia occurred at a high frequency in all four types of P0*Cre*; $Nf2^{flox2/flox2}$ mice (75-100%). Based on the lowest percentage of Schwann cell tumors combined with a high percentage of Schwann cell hyperplasia, P0Cre^{B}; $Nf2^{flox2/flox2}$ mice were selected to address whether cooperation of *Nf2* and *Nf1* mutations occurs specifically in Schwann cells. P0Cre^{B}; $Nf2^{flox2/flox2}$ and $Nf1^{+/-}$ mice (2) were crossed to generate P0Cre^{B}; $Nf2^{flox2/flox2}$; $Nf1^{+/-}$ mice that were monitored over time for the appearance of tumors. P0Cre^{B}; $Nf2^{flox2/flox2}$; $Nf1^{+/-}$ mice exhibited reduced survival compared to P0Cre^{B}; $Nf2^{flox2/flox2}$ littermates (Kaplan-Meier Test: p=<0.0001), and generally died during the first year of life. Twenty-seven of 28 histologically examined P0Cre^{B}; $Nf2^{flox2/flox2}$; $Nf1^{+/-}$ mice developed peripheral nerve tumors (96%). By contrast, only one of 27 P0Cre^{B}; $Nf2^{flox2/flox2}$; $Nf1^{+/+}$ mice acquired a schwannoma by 9.5 months of age. Tumors usually emanated from the peripheral nerves of the tongue, peritoneum, limbs, and from spinal ganglia. Microscopically, nearly all lesions occurred as independent primary tumors that did not metastasize but aggressively infiltrated adjacent tissues. They often encased nerves and were composed of cells with endoneural, perineural, epineural and/or Schwann cell characteristics. Both benign Schwann cell tumors with features of neurofibroma and MPNSTs were seen. Tumor DNAs were assayed by Southern blot analysis for Cre-mediated recombination of *Nf2* exon 2 and for loss of the wild-type *Nf1* allele. Four Schwann cell tumors from P0Cre^{B}; $Nf2^{flox2/flox2}$; $Nf1^{+/-}$ mice displayed deletions of *Nf2* exon 2 and loss of the *Nf1* wild-type allele (Figs. 4A and B). Interestingly the tongue was a frequent site of tumor development (Fig. 4C) and a continuum from hyperplasia to overt benign and malignant neoplasia could be observed at this site. Further characterization of the tumors by immunohistochemistry showed that 3 MPNSTs and 2 neurofibromas analyzed were S100^{-}/p75^{+}.

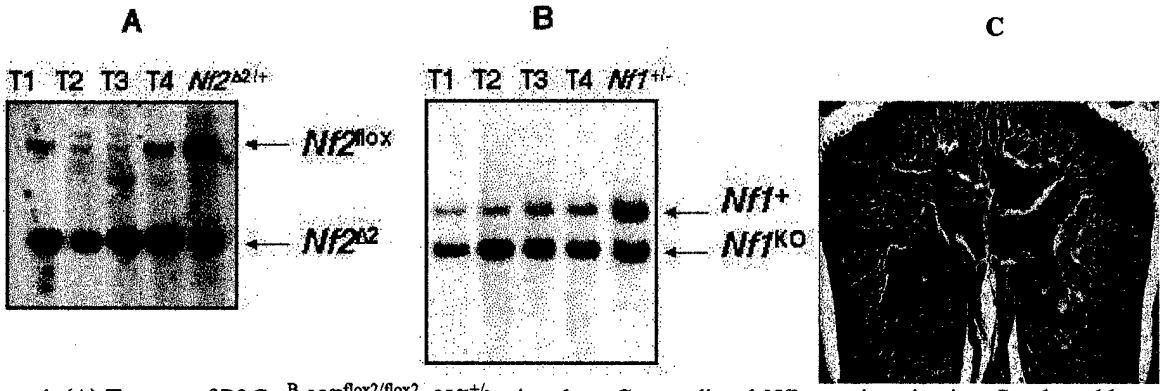


Figure 4. (A) Tumors of P0Cre^{B};$Nf2^{flox2/flox2}$; $Nf1^{+/-}$ mice show Cre-mediated *Nf2* gene inactivation. Southern blot analysis (probe B) of *Xba*I-*Bam*HI-digested DNAs from four representative tumors (lanes 1-3: MPNST; lane 4: neurofibroma). Bands corresponding to the $Nf2^{flox2}$ and $Nf2^{-2}$ alleles are indicated by arrows. (B) Tumors of P0Cre^{B}; $Nf2^{flox2/flox2}$; $Nf1^{+/-}$ mice show *Nf1* gene inactivation. Loss-of-heterozygosity (LOH) analysis: Southern blot analysis of *Pst*I-digested DNA of the four tumors in A. Note the under-representation of the wild-type allele (*Nf1*+). (C) Tongue neurofibromas in P0Cre^{B};$Nf2^{flox2/flox2}$; $Nf1^{+/-}$ mice (H&E staining).

Production of Mice Expressing Avian Leukosis and Sarcoma Virus Under Control of the P0 Promoter. The P0*Cre*; $Nf2^{flox2/flox2}$, *cis* $Nf1^{+/-}$; $Nf2^{+/-}$, and P0*Cre*; $Nf2^{flox2/flox2}$; $Nf1^{+/-}$ models establish *Nf1* and *Nf2* as TSGs for Schwann cells. However, it is uncertain whether the loss of *Nf1* and/or *Nf2* function must occur in a Schwann cell precursor or in a mature Schwann cells because the P0 promoter drives expression in both neural crest precursors and in mature myelinating Schwann cells during embryonic development. It is important to make this distinction in order to appropriately recognize the target cell for therapeutic strategies for human NF1 and NF2. Dr. McClatchey has generated transgenic mice that express the avian leukosis and sarcoma virus type a (tv-a) receptor under the control of the P0 promoter described above. Her laboratory is currently in the process of verifying the levels and distribution of t-va expression in Schwann cells in these transgenic lines. These mice can be used to achieve inactivation of *Nf1* or *Nf2* in foci of Schwann cells at various stages of development.

Summary. The members of this Consortium have generated novel mouse models of almost all of the tumors that arise in individuals with NF1 and NF2. We accomplished each element of the approved Statement of Work for this project, and have generated models that can be now exploited for biologic and preclinical therapeutic studies by our laboratories and by other researchers.

Technical Objective (Aim) 2: To perform in vitro and in vivo experiments that will elucidate molecular targets for therapeutic interventions.

Background

In addition to generating mouse models for testing potential therapeutic agents for NF1 and NF2-associated tumors, the identification and validation of molecular targets remains a significant rate-limiting step in the discovery of effective therapies for these diseases. Considerable work from our laboratories and others has demonstrated that loss of *NF1/Nf1* function leads to hyperactivation of the Ras signaling pathway.

Post-translational processing of Ras proteins has attracted considerable interest as a potential target for anticancer drug discovery. Ras proteins undergo posttranslational modification at a common C-terminal C*AAX* sequence (reviewed in 30-32). Processing is initiated by farnesyltransferase (Ftase), which attaches a farnesyl lipid to the thiol group of the cysteine (the "C" of the CAAX motif). Prenylation targets Ras to membranes, and is required for the biologic activity of normal and oncogenic Ras. Ftase inhibitors have shown promise as anticancer agents (31-34), and are currently being tested in NF1 patients with plexiform neurofibroma. However, K-Ras and N-Ras are substrates for geranylgeranyltransferase 1 (GGTase 1) and are processed by this alternative pathway when Ftase is inhibited. Indeed, extensive data now support the view that non-Ras C*AAX* proteins are critical *in vivo* targets of the Ftase inhibitors (reviewed in 31, 32, 35). After prenylation, the carboyxl terminal three amino acids are released by Rce1, an integral membrane endoprotease of the endoplasmic reticulum. The final step in Ras processing involves methylation of the prenylcysteine by isoprenylcysteine carboxyl methyltransferase.

In addition, agents that interfere with various signaling pathways downstream of Ras (such as the Raf1-MEK-MAPK and phosphoinositide-3-OH kinase (PI3K)-protein kinase B

(PKB; also known as Akt) cascades) are of obvious interest in the treatment of NF1-associated tumors. Upstream receptor tyrosine kinases and their ligands may also be required for the growth of specific *NF1/Nf1* mutant tumors, including GM-CSF in the case of myeloid leukemia and epidermal growth factor receptor for MPNSTs (36). It is important to note that genetic experiments in *Drosophila* have also demonstrated link between loss of neurofibromin function and PKA signaling.

The effects of *NF2/Nf2* mutation of intracellular signaling are less clear. Drs. McClatchey and Jacks have recently reported a reciprocal relationship between merlin and the small GTPase Rac1. Thus activation of Rac1 effects phosphorylation and presumed inactivation of merlin; in turn, merlin can negatively regulate the activity of endogenous Rac1. These data provide strong evidence that merlin controls cell proliferation, at least in part, through regulation of Rac output and suggest that therapeutic strategies that target the Rac signaling pathway may be beneficial in patients with NF2 disease. More recently, through the study of *N2*-deficient fibroblasts, Dr. McClatchey's laboratory has found that the primary cellular consequence of *Nf2*-deficiency is loss of contact-dependent inhibition of proliferation and loss of cadherin-mediated cell:cell junctions [Lallemand et al., submitted]. Merlin appears to play a direct role in establishing cadherin-mediated cell:cell communication, suggesting that cadherin-mediated signaling represents an additional important therapeutic target for NF2 and suggesting additional approaches to studying $Nf2^{-/-}$ and $Nf2^{-/-}$; $Nf1^{-/-}$ Schwann cells under the renewal of this proposal.

We have pursued a number of genetic, biochemical, and cell biologic experiments in an effort to uncover genes that cooperate in generating NF-associated tumors and to elucidate biochemical pathways that might be amenable to therapeutic intervention.

Progress Report

Cooperative Effects of *p53* Mutations on Tumorigenesis in the *Krox20-Cre* Background. Mutations at the *p53* and $p16^{INK4a}/p19^{ARF}$ loci have been reported in MPNSTs, but not in benign neurofibromas (19, 21, 37). These data suggests that these genetic lesions are involved in malignant progression rather than tumor initiation. As proposed in our initial application, Dr. Parada crossed $Nf1^{flox}$ and $p53^{+/-}$ mice and has identified founders in which an interchromosomal recombination event yielded a *cis* $Nf1^{flox}$; *p53*- chromosome 11 homolog.

Rce1 x *Nf1* Cross and Analysis of Cellular and Biochemical Phenotypes. As the only known *CAAX* protease in mammalian cells, Rce1 represents an attractive target for discovering drugs for treating disorders associated with hyperactive Ras such as NF1. The murine *Rce1* gene was disrupted by Dr. Stephen Young to elucidate its role in development and tumorigenesis. Genetic ablation eliminates Ras endoproteolytic activity, which results in mislocalization of ~50% of Ras away from the plasma membrane. Importantly, *Rce1*-deficient cells are unable to process either farnesylated or geranylgernaylated substrates(38). Homozygous mutant embryos *(Rce1-/-)* demonstrated late embryonic lethality with normal organogenesis (38). Dr. Shannon's laboratory performed adoptive transfer, biochemical, and competitive repopulation experiments to define the importance of *Rce1* in the growth of hematopoietic cells.

Fetal liver cells of all *Rce1* genotypes efficiently rescued hematopoiesis in irradiated recipients. Surprisingly, mice engrafted with *Rce1-/-* fetal liver cells developed leukocytosis by 3 months that persisted until they were killed at 6 months (Fig. 5A). This was due to increased numbers of mature myeloid cells in the *Rce1-/-* recipients (Fig. 5B). These mice remained well,

and demonstrated normal spleen sizes and splenic architecture at sacrifice (data not shown). However, FACs analysis of marrow and spleen from recipients of *Rce1-/-* fetal liver cells demonstrated an increase in the percentage of myeloid cells (Gr1 and/or Mac1-positive) and a commensurate reduction in B lymphocytes (B220-positive) (Fig. 5C). Since GM-CSF promotes the growth of myelomonocytic cells, CFU-GM growth was assessed in methylcellulose over a range of GM-CSF concentrations. Although there was some variability between individual experiments, colony growth was similar for wild-type, *Rce1+/-*, *Rce1-/-* cells. ERK kinase activities were measured in bone marrow collected 3–6 months after adoptive transfer. In multiple experiments, wild-type and *Rce1-/-* cells demonstrated equivalent basal and GM-CSF-stimulated ERK kinase activities (data not shown). Wild-type and *Rce1-/-* fetal liver tester cells were injected into irradiated hosts with the same reference population of BoyJ competitor cells to directly compare their repopulating potentials. Cells of both genotypes demonstrated equivalent repopulating potentials over a dose range that produced 10 - 70% donor cell chimerism (data not shown). These data provide strong evidence that inactivation of *Rce1* does not impair the proliferative capacity of normal hematopoietic cells. This work has been accepted for publication in *Blood.*

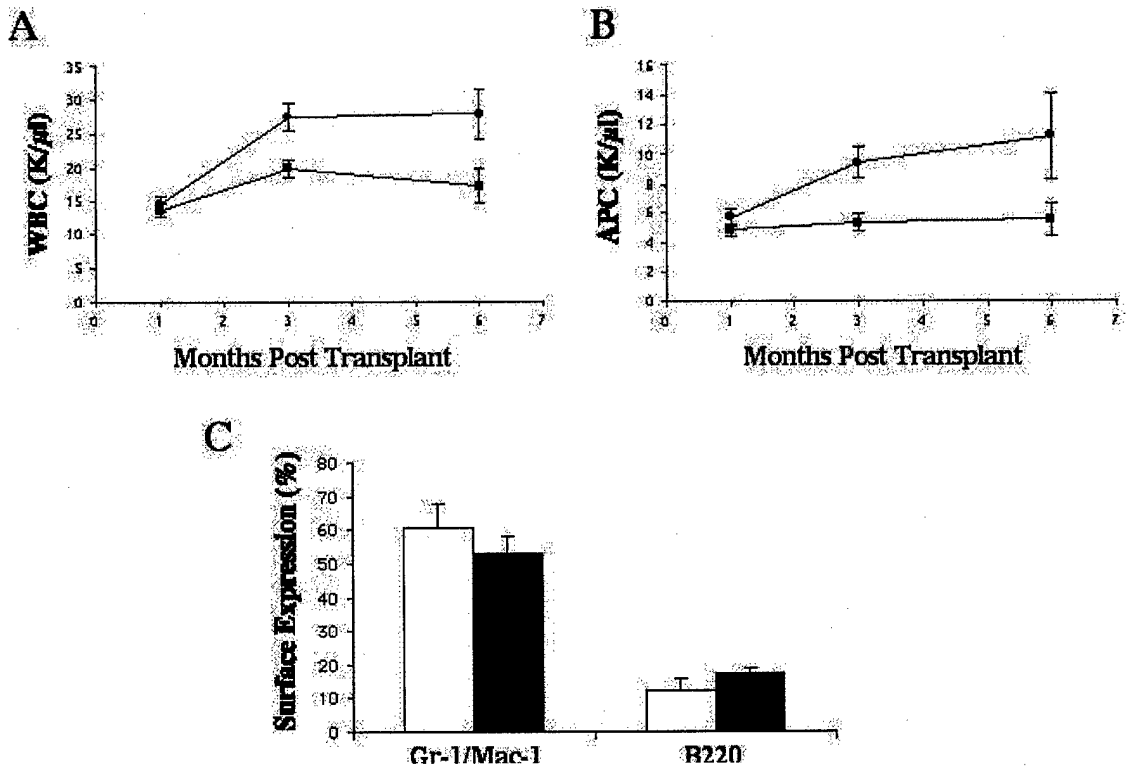


Figure 5. Hematologic parameters in recipients of *Rce1-/-*, *Rce1+/-*, and wild-type fetal liver cells. Recipients of *Rce1-/-* cells (n = 32) are shown as filled circles in panels A and B and as open bars in panel C. We observed no differences in recipients of *Rce1+/-* and wild-type fetal liver cells so the data from these mice were pooled (n = 40) and are shown as filled squares in panels A and B and as a filled bar in panel C. Panel A. White blood cell counts (WBC). Panel B. Absolute phagocyte counts (APC; monocytes + neutrophils). Panel C. Relative numbers of bone marrow cells expressing the myeloid markers Gr1 and Mac1 and the B lymphocyte marker B220 six months after adoptive transfer.

At first glance, these results argue that Rce1 represents a poor therapeutic target for NF1-associated tumors. However, lack of toxicity to normal cells represents a distinct advantage and it is noteworthy that STI-571 (Gleevec) is remarkably selective for cells that express the *BCR-ABL* oncogene despite fully inhibiting other growth factor receptors (39). Similarly, it is possible that blocking Rce1 will differentially affects cells that are dependent upon hyperactive Ras for survival and growth. Studies in tissue culture and in transgenic mice have shown that transformed cells select for higher levels of oncogenic Ras (40, 41); this may render them sensitive to a modest reduction in Ras signaling.

In previous studies, Dr. Shannon's laboratory generated compound heterozygous breeding stocks by intercrossing *Rce1* and *Nf1* mice. However, none of 65 embryos recovered at E13.5 contained the desired *Nf1*$^{-/-}$; *Rce1*$^{-/-}$ genotype. The *Mx1-Cre Nf1*$^{flox/flox}$ model of MPD that we have developed and a conditional mutant allele of *Rce1* produced by Dr. Young (42) now provide reagents to circumvent this problem. Dr. Shannon's lab in planning to intercross these mice to continue these studies with funding provided through the renewal award for this modeling consortium.

Analysis of Tumor-Derived Cell Lines from *cis Nf1/p53* Mutant Mice. The Parada and Jacks labs have generated a large series of clonal cell lines from a variety of tumors isolated from *cis Nf1; p53* mice, including MPNSTs and astrocytomas. These were characterized to assess growth potential, including doubling rates, ability to form colonies in soft agar, and ability to form solid tumors in immunologically compromised (nu/nu) mice. All sarcoma-derived lines exhibited rapid doubling times (mean 15 hours), and all tumor-derived lines formed multiple colonies in soft agar, and were tumoigenic in nude mice. The Parada lab has generated an efficient adenovirus expression system to express genes of interest in cell lines and primary (43). The virus itself does not significantly alter growth rates *in vitro.* In preliminary studies, expression of dominant-negative forms of Ras (dn-Ras) or MEK (dn-MEK) significantly slowed the proliferation of a line derived from a malignant triton tumor (data not shown). Expression of dn-Ras alone significantly decreased the number of colonies formed in soft agar assays and a dn-p53 allele has synergistic effects (data not shown). This work has essentially been completed and is in the process of being prepared into manuscript form. The data indicate that in early stages of malignancy, complete loss of *Nf1* and *p53* is sufficient to cause neoplasia. Thus, reintroduction of the *Nf1* and *p53* functions is sufficient to regress the tumor tissue. However, upon passage of these cell lines, additional "hits" occur within the malignant tumors or cell lines and they can no longer be regressed by restoring wild-type *Nf1* and *p53* functions (Klesse et al., in preparation).

Growth and Survival of Wild-Type and *Nf2*-Deficient Cells. To address the relevance of Rac-mediated signaling in *Nf2*-mutant cells and to define how merlin regulates growth, Dr. McClatchey's lab has been analyzing *Nf2*-deficient fibroblasts. Drs. McClatchey and Jacks previously found that merlin phosphorylation was regulated under conditions of growth arrest in cell culture including contact inhibition, growth factor deprivation and loss of adhesion (44). Dr. McClatchey's laboratory has now shown that *Nf2*$^{-/-}$ fibroblasts fail to undergo proper growth arrest under conditions of growth factor deprivation and high cell density. The most remarkable phenotype exhibited by *Nf2*-deficient primary cells is the inability to downregulate mitogenic signaling and undergo growth arrest upon high cell density (Lallemand et al., submitted). Moreover, Dr. McClatchey's found that merlin colocalizes and associates with adherens junction

components in confluent wild-type cells suggesting that merlin normally functions to directly control cell:cell communication. Importantly, loss of contact-dependent inhibition of growth and loss of adherens junctions are signatures of *Nf2*-deficiency that are also exhibited by $Nf2^{-/-}$ osteoblasts, osteosarcoma cells and Schwann cells (see below). Together, these preliminary observations of $Nf2^{-/-}$ fibroblasts form the basis for interrogating $Nf2^{-/-}$ and $Nf1^{-/-}$; $Nf2^{-/-}$ Schwann cells.

Primary mouse Schwann cells can be isolated from embryonic dorsal root ganglia (DRG) or from adult sciatic nerve. Both methods require labor-intensive methodologies and neither yields large quantities of Schwann cells for study. Although isolation of primary Schwann cells from embryonic (E12.5) DRG is the more complex method, the resulting cells can be differentiated *in vitro,* allowing an evaluation of *Nf2* function during Schwann cell differentiation and of the importance of the timing of *Nf2* loss during the differentiation program. An important achievement during this part year has been the establishment of reproducible isolation protocols for primary Schwann cells. Drs. McClatchey and Giovannini have isolated Schwann cells from $Nf2^{loxP/loxP}$ mice using both methods, achieving relatively pure p75+/S-100+ Schwann cell populations. The cultures were infected either with Ad-GFP (control) or with Ad-Cre to eliminate *Nf2* expression. Their initial observations indicate that *Nf2* loss in embryonic Schwann cells leads to dramatic growth advantages that parallel those seen in $Nf2^{-/-}$ fibroblasts. Thus $Nf2^{-/-}$ Schwann cells exhibit increased survival, hypersensitivity to glial growth factor (GGF), persistent mitogenic signaling and loss of contact-dependent inhibition of growth (Fig. 6). Wild-type and $Nf2^{-/-}$ Schwann cells express normal to elevated levels of both N- and E-cadherin, respectively; experiments underway aim to determine whether $Nf2^{-/-}$ Schwann cells form adherens junctions in culture. In fact, preliminary coculture experiments reveal that $Nf2^{-/-}$ Schwann cell:neuron interactions are markedly defective; normal Schwann cell:neuron interaction is thought to be cadherin-dependent. Notably, preliminary examination of mature sciatic nerve-derived $Nf2^{-/-}$ Schwann cells indicates that they do not exhibit a marked growth advantage relative to wild-type cells. Thus there may be a developmental window during which *Nf2* loss confers a distinct growth advantage upon Schwann cells.

Dr. Jacks' laboratory has extended the finding that Rac1 induces the phosphorylation of merlin on Serine 518 and has shown that the downstream effector of Rac, p21 activated kinase (Pak), is the responsible kinase. They have also developed an antibody that selectively recognizes merlin phosphorylation at Serine 518. In addition, the Jacks group has investigated a potential dominant-negative allele of *Nf2* in mouse fibroblasts. Expression of this allele (termed $Nf2^{BBA}$) caused transformation of established fibroblast cell lines, and the phenotype of these cells was similar to that reported in *Nf2-/-* fibroblasts. Articles describing these data were published in *The Journal of Biological Chemistry* and *Oncogene* earlier this year.

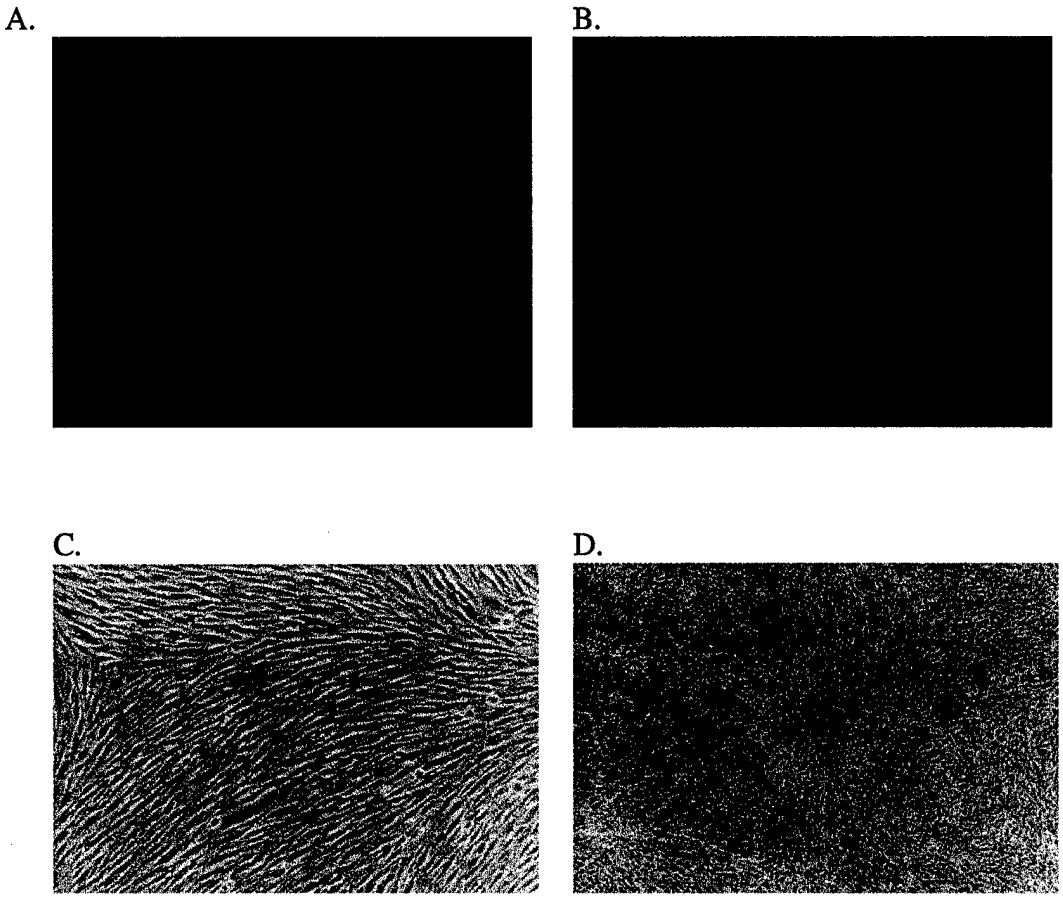


Figure 6. Primary *Nf2*$^{-/-}$ Schwann cells exhibit growth advantages that parallel those seen in *Nf2*$^{-/-}$ fibroblasts. A,B: Wild-type and *Nf2*$^{-/-}$ primary Schwann cells derived from E12.5 DRGs. Under conditions of limiting growth factors, *Nf2*$^{-/-}$ Schwann cells (green, labeled with p75 Schwann cell marker) continue to incorporate BrdU (red). In contrast, similarly isolated wild-type Schwann cells exhibit very low levels of BrdU incorporation. C,D: *Nf2*$^{-/-}$ Schwann cells do not undergo contact-dependent inhibition of growth, pile up on tope of one another and occasionally form foci.

Drs. McClatchey and Giovannini are pursuing the molecular basis for cooperativity between *Nf2* and *Nf1*. Preliminary studies of wild-type, *Nf2*$^{-/-}$, *Nf1*$^{-/-}$, and compound *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ fibroblasts have revealed important differences in their growth properties. For example, *Nf1*$^{-/-}$ fibroblasts grow to a higher saturation density than wild-type cells, but do undergo contact-dependent inhibition of growth and do form numerous adherens junctions. In contrast *Nf2*$^{-/-}$ fibroblasts and *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ fibroblasts, like *Nf2*$^{-/-}$ cells, do not saturate. Also, in contrast to *Nf2*$^{-/-}$ fibroblasts, *Nf1*$^{-/-}$ fibroblasts undergo growth arrest under serum free conditions. *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ fibroblasts are growth factor independent but do not exhibit an additional proliferative advantage in serum-free medium compared to *Nf2*$^{-/-}$ cells. However, under conditions of either limiting or added growth factors, *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ fibroblasts exhibit enhanced proliferation compared to *Nf2*$^{-/-}$ or *Nf1*$^{-/-}$ cells. Dr. McClatchey has examined various signaling pathways in these cells, and found that the levels of cyclin D1 are dramatically elevated specifically in cycling *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ fibroblasts (Fig. 7). This does not appear to be due to increased Ras-MAPK signaling as the levels of phosphorylated (active) MAPK are not selectively elevated in these cells. Interestingly,

in Schwann cells cyclin D1 levels are particularly sensitive to cooperation between growth factors and protein PKA signaling (45). Like Rac, PKA expression can induce merlin phosphorylation (A.I.M., unpublished), while ezrin has been reported to bind to and control the activity of PKA (46). These observations suggest that the pathways controlling cyclin D1 levels, particularly downstream of growth factor receptors and PKA, deserve investigation as potential targets for *Nf2* therapeutics.

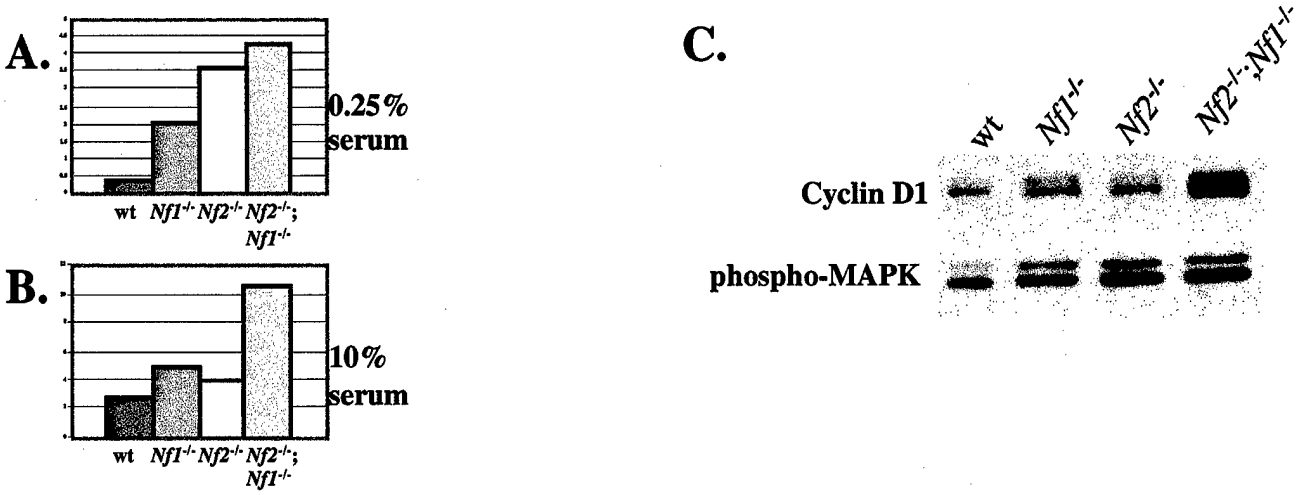


Figure 7. *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ fibroblasts exhibit a growth advantage compared to either *Nf1*$^{-/-}$ or *Nf2*$^{-/-}$ fibroblasts in the presence of growth factors. **A,B**, Fold increase in cell number after 7 and 5 days in 0.25% and 10% serum, respectively. **C**, Levels of cyclin D1 and phospho-MAPK in wt, *Nf1* $^{-/-}$,*Nf2*$^{-/-}$ and *Nf2*$^{-/-}$;*Nf1*$^{-/-}$ cells.

Dr. McClatchey s laboratory has extended their studies of *Nf2*$^{-/-}$ fibroblasts to develop cell-based assays for testing candidate therapeutic compounds. In pilot studies, they have used FACS analysis to examine the effects of several compounds on the persistent growth and viability of *Nf2*$^{-/-}$ cells under serum-free conditions. Propidium iodide (PI)/8-bromo-deoxyuridine (BrdU) labeling and FACS analysis allows a sensitive, reproducible, relatively high throughput method for measuring both changes in cell cycle characteristics such as induction of growth arrest and survival in cells exposed to various compounds. Preliminary studies using a panel of compounds that are known to block certain signaling molecules revealed that at a single, standard concentration virtually all of the compounds were able to inhibit the persistent proliferation of *Nf2*$^{-/-}$ cells under serum-free conditions. This panel included: MEK inhibitors U0126, PD98059; PI3K inhibitors wortmannin, LY294002; geranylgeranyltransferase inhibitors (GGTIs); farnesyltransferase inhibitors (FTIs) and activators of PKA (H89; IBMX). These data suggest that a number of signaling pathways contribute to the persistent proliferation of *Nf2*$^{-/-}$ cells under serum-free conditions. This is consistent with the observation of persistent activation of a number of signaling molecules in the membrane of confluent *Nf2*$^{-/-}$ cells and indicates broad consequences of *Nf2*-deficiency (data not shown). Dr. McClatchey is currently expanding this work to evaluate various concentrations of these compounds in both fibroblasts and Schwann cells; this work will be done under the renewal of this consortium.

Aim 3. Preclinical Studies of Experimental Therapeutics in Mouse Models

Background

Elucidating the biochemical consequences of inactivating *Nf1* or *Nf2* in susceptible target cells has begun to uncover rational targets for drug development. The mouse models we are generating provide systems for rigorously evaluating the efficacy of therapeutic strategies *in vivo.* An advantage of these models is that tissues can be collected before and after treatment to perform pharmacodynamic studies of target inhibition, which can then be correlated with clinical responses. The first example illustrating the feasibility of this approach in *Nf1* mutant mice involved a report from Dr. Shannon's lab in which a FTase inhibitor (FTI) was tested in the JMML model (14). Models with predictable onset of tumor formation also provide exceptional opportunities to test therapeutics as preventive agents or in early intervention trials. Along these lines, a recent study of angiogenesis inhibitors in a mouse model of pancreatic cancer elegantly demonstrated that the efficacy of specific therapeutics is dependent upon whether they were used to block tumor formation or to inhibit established lesions (47).

While the idea of using genetically engineered mouse models to evaluate therapeutics has intrinsic appeal, performing preclinical trials poses challenges. First, pharmaceutical companies are developing most of the promising agents. Intellectual property and data disclosure issues frequently impede transferring these compounds to academic laboratories. Second, pharmacokinetic and phamracodynamic (PK and PD) data may be lacking in the relevant mouse strains, and drug metabolism and distribution may differ in mouse and man. Third, serially imaging mouse tumors such as astrocytomas is difficult. Finally, developing reproducible assays for measuring the biochemical effects of a specific treatment in primary tissues involves considerable effort. Many of these issues are common to the general question of using mouse models to test cancer therapeutics and, as such, is a major focus of the MMHCC. Thus, our efforts in NF-associated tumors are benefiting from interactions with other MMHCC researchers. Here we describe our work since the inception of this modeling Consortium to address some of the practical issues involved in using these mouse models to test therapeutics and to initiate preclinical studies. Preclinical therapeutics will be a major focus of our future collaborative research.

Progress

<u>Preclinical Evaluation of a MEK Inhibitor in the JMML Model</u>. MEK is a dual specificity kinase that catalyzes the phosphorylation of $p44^{MAPK}$ (ERK1) and of $p42^{MAPK}$ (ERK2). In myeloid cells, MEK is directly activated by Raf and by cross-cascade signaling from the PI3K pathway (data not shown). PD184352 was identified in a screen for small molecule inhibitors of MEK (48). Biochemical studies infer an allosteric mechanism of action. PD184352 is a potent inhibitor of MAPK activation in cancer cell lines, and it induced regression of explanted tumors in nude mice that correlated with *in vivo* effects on MAPK phosphorylation (48). PD184352 is undergoing phase 1 testing in refractory malignancies. Dr. Shannon obtained PD184352 from Pfizer, Inc., and is currently studying this agent in the JMML model. Dr. Shannon has shown that 0.01 μM to 10 μM of PD184352 abrogates CFU-GM colony formation in response to GM-CSF from normal murine bone marrow as well as from wild-type and $Nf1^{-/-}$ fetal livers (data not shown). Although basal levels of MAPK activity in bone marrow cells collected from recipients

engrafted with *Nf1*$^{-/-}$ cells are consistently elevated above wild-type marrow, activation is modest (13). To overcome this potential impediment to phamracodynamic studies, a robust assay was developed that is based on the ability of PD184352 to inhibit GM-CSF-induced activation of MAPK in primary marrow cells. A single PD18532 dose of 100 mg/kg markedly attenuates MAPK activation in primary bone marrow cells 2, 4, and 8, hours after intraperitoneal injection (Fig. 8). The Shannon lab first completed a 4 week toxicity study in wild-type mice in which treated animals received twice daily doses of 100 mg/kg of PD184352. Thus, in contrast to FTI, PD184352 markedly inhibits a relevant biochemical target in primary *Nf1* mutant cells at tolerable doses. There were no adverse effects, and we then performed a controlled preclinical study in which this schedule was administered to *Mx1-Cre Nf1*$^{flox/flox}$ mice with MPD. Although PD184352 was well tolerated and we could demonstrate ERK kinase inhibition 2 and 4 hours after treatment, there was no beneficial effect on the MPD. We believe that higher doses of the inhibitor may be required for efficacy and we are currently developing methods for administering PD184352 and other compounds continuously through implantable pumps. In summary, while testing targeted therapeutics in genetically engineered mice is in its infancy, we have made considerable progress to date. In particular, *Mx1-Cre Nf1*$^{flox/flox}$ mice with MPD now provide a trractable system for administering targeted agents with pharmacodynamic monitoring of biochemical endpoints. We are committed to extending this strategy for evaluating compounds that might benefit patients with neurofibromatosis.

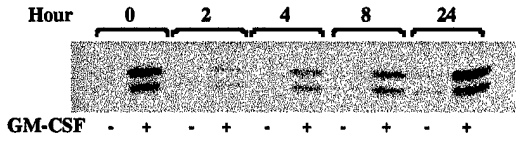


Figure 8. Phospohorylated ERK Levels in Marrow Cells after Treatment with PD184352. A single dose of 100 mg/kg was injected at hour 0, and mice were sacrificed 2, 4, 8, or 24 hours later. Primary marrow cells were maintained in serum only (-) exposed to GM-CSF for 10 min (+) before lysis. Equivalent amounts of MAPK protein were present in each lane (data not shown). Treatment with PD184352 reduced basal MAPK level and partially suppressed GM-CSF-induced activation at 2, 4, an 8 hours.

Imaging Nervous System Tumors in Mice. A major obstacle for the use of the astrocytoma, meningioma, and neurofibroma models for evaluating therapeutics is the inability to readily identify tumor-bearing animals or to accurately measure responses to therapy. Therefore, the Jacks laboratory is collaborating with Drs. Peter Black, Rona Carroll, and Mitchell Albert (Harvard Medical School) to use magnetic resonance imaging (MRI) techniques to measure the kinetics of astrocytoma growth in *Nf1* mutant mice and to optimize the use of various contrast agents. Preliminary results indicate that these tumors can be imaged using both gadolinium (Gd) or monocrystalline iron oxide nanoparticles (MION), and a single mouse can be studied multiple times to assess tumor kinetics and response to therapy (Figure 9).

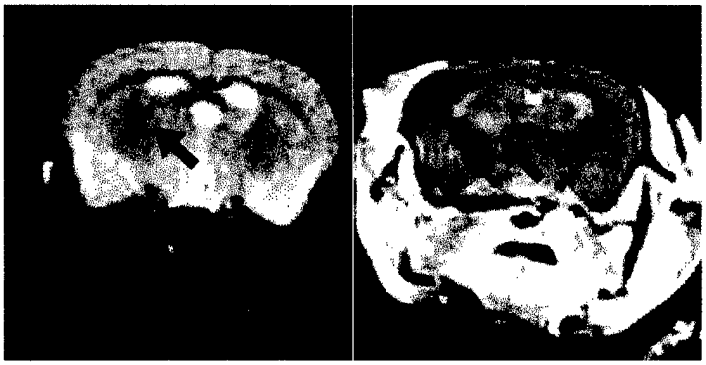


Figure 9. *Nf1*$^{+/-}$;*p53*$^{+/-}$ cis mouse on a C57BL/6 X A/J F1 background imaged by MRI with two different imaging agents. (Left) MION-enhanced T2-weighted MRI, dark region to left of midline indicative of tumor by this imaging method. (Right) Gd-enhanced T1-weighted MRI of same animal 21 days later, the same region to left of midline is brighter indicative of tumor by this imaging method.

Imaging Nervous System Tumors. The Jacks lab will extend their studies in the astrocytoma model by: 1) rigorously assessing the kinetics of tumor growth by imaging the same mouse at weekly timepoints; 2) optimizing the imaging of diffuse astrocytomas by experimenting with different contrast agents; and 3) assessing the efficacy of experimental therapeutics *in vivo*. Mice are injected with either Gd or MION (49) and imaged on a Bruker 8.5T Biospec, using T1 or T2 weighted imaging, respectively. Importantly, once optimal procedures and parameters are defined in the astrocytoma model, we will extend our analysis to mouse models of meningioma, schwannoms, and neurofibroma.

KEY RESEARCH ACCOMPLISHMENTS

(c) The investigators established this Consortium and have extensively shared research reagents.

(d) Our NF Modeling Group was admitted to the NCI MMHCC and is participating actively in this national mouse cancer modeling consortium.

(e) We have established two novel models of plexiform neurofibroma, a new model of MPNST, and a model of meningioma.

(f) Studies in *Krox20 Nf1*$^{flox/flox}$ mice demonstrated that a heterozygous mutant environment plays a major role in neurofibroma formation.

(g) Lines of transgenic tv-a mice have been generated that will facilitate *in vivo* and *in vitro* studies of Schwann cell biology and tumorigenesis.

(h) Models of astrocytoma have been generated and are being characterized.

(i) Recombinant chromosomes that carry both the *Nf1*^{flox} allele and a *p53* mutation or the *Nf2*^{flox} allele and a *p53* mutation have been generated to address cooperativeity in tumorigenesis.

(j) A highly tractable experimental model of JMML was developed by generating *Mx1-Cre Nf1*$^{flox/flox}$ mice and inducing somatic inactivation of *Nf1* in hematopoietic cells. This model is being used for preclinical testing of novel therapeutics.

(k) Robust assays have established for investigating the growth of *Nf1* and *Nf2* deficient cells *in vitro* to discover targets for therapeutic interventions.

(l) A comprehensive *in vivo* analysis of *Rce1*-deficient hematopoietic cells demonstrated that inactivtion of this Ras processing enzyme is not associated with impaired growth.

(m) A preclinical trial of a MEK inhibitor is underway in the *Mx1-Cre Nf1*$^{flox/flox}$ model of JMML model with correlative biochemical (pharmacodynamic) monitoring.

(l) Techniques are being developed to image mouse tumors in vivo in order to examine the efficacy of therapeutic interventions in solid tumor models.

(m) Stains of mutant mice have been shared widely with the NF research community (see list below in Reportable Outcomes). Through these collaborative experiments, the scientific value of this Consortium has extended well beyond the studies being pursued in the participant's laboratories.

REPORTABLE OUTCOMES

(a) Research Articles and Reviews

Kalamarides M, Niwa-Kawakita M, Leblois H, Abramowski V, Perricaudet M, Janin A, Thomas G, Gutmann D, Giovannini M. *Nf2* gene inactivation in arachnoidal cells is rate-limiting for meningioma development in the mouse. *Genes & Development* 2002; 16:1060-1065.

Gautreau A, Manent J, Fievet B, Louvard D, Giovannini M, Arpin M. Mutant products of the NF2 tumor suppressor gene are degraded by the ubiquitin-proteasome pathway. *J Biol Chem.* 2002; 277:31279-82.

Gutmann D and Giovannini M. Mouse models of neurofibromatosis type 1 and 2. *Neoplasia,* 2002; 4: 279-290.

Kumar S.M., Tang Y., Giovannini M., Bronson R., Weissleder R., Breakefield X.O. Detection of spontaneous schwannomas by MRI in a transgenic murine model of neurofibromatosis type 2. *Neoplasia* (in press)

Sun CX., Haipek C., Scoles DR, Pulst SM, Giovannini M., Komada M., and GutmannDH. Functional analysis of the relationship between the neurofibromatosis 2 (NF2) tumor suppressor and its binding partner, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS/HGS) *Human Molecular Genetics* (in press)

Denisenko-Nehrbass N., Goutebroze L., Galvez T., Bonnon C., Stankoff B., Ezan P., Giovannini M., Faivre-Sarrailh C., Girault J.A. Association of Caspr/paranodin with tumor suppressor schwannomin/merlin and β1 integrin in the CNS. *J. Neurocytochemistry* (in press)

Manent J., Oguievetskaia X., Bayer J., Ratner N., Giovannini M. Magnetic cell sorting for enriching Schwann cells from adult mouse peripheral nerves. *J. Neuroscience Meth.* (accepted in principle)

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Li, H., Velasco-Miguel, S., Vass, W.C., Parada, L.F., and DeClue, J.E. Epidermal growth factor receptor (EGFR) signaling pathways are associated with tumorigenesis in *Nf1:p53* mouse tumor-derived cell lines. *Cancer Res.,* 2002; 8:616-26.

Bajenaru, M.L.*, Zhu, Y.*, Hedrick N.M., Donahoe, J., Parada, L.F., and Gutmann, D.H. Astrocyte-specific inactivation of the neurofibromatosis 1 (Nf1) gene is insufficient for astrocytoma formation. *Mol. Cell Biology* 2002; 22:5100-5113 (*co-first authors).

Zhu, Y., Ghosh, P., Charnay, P., Burns, D.K., and Parada, L.F. Neurofibromas in NF1:Schwann cell origin and role of tumor environment. *Science* 2002; 296: 920-2.

Gitler, A.D.*, Zhu, Y.*, Lu, M.M., Parada, L.F., and Epstein, J.A. The Type 1 Neurofibromatosis (Nf1) gene product has distinct and essential roles in neural crest and endothelial cells. *Nature Genetics* (in press) (*co-first authors).

Zhu, Y. and Parada, L.F. The molecular and genetic basis of neurologic tumours. *Nature Reviews on Cancer.* 2002; 8:616-26.

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Aiyagari A, Taylor B, Aurora V, Young SG, Shannnon KM. Hematologic effects of inactivating the Ras processing enzyme *Rce1*. *Blood* (in press).

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Shaw RJ, Paez JG, Curto M, Yaktine A, Pruitt WM, Saotome I, O'Bryan JP, Gupta V, Ratner N, Der CJ, Jacks T, McClatchey AI. The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling. *Dev Cell* 2001; 1:63-72.

Lalemand, D, Curto, M, Saotome, I, Giovannini, M and McClatchey, AI. Nf2-deficiency promotes tumorigenesis and metastasis by destabilizing adherens junctions. Submitted.

(b) Model Development and Distribution to the Research Community

(c)

As described in the Body of this application, studies conducted to date have established a number of novel models of NF1 and NF2-associated tumors and have generated several new strains of mice. *Nf1* and *Nf2* mutant mice have been deposited in the MMHCC Repository where they are readily available to the research community. In addition, the participants in this Consortium have provided strains directly to the investigators listed below.

Karlene Reilly (National Cancer Institute)
Jeffrey DeClue (National Cancer Institute)
Jonathan Epstein (University of Pennsylvania)
D. Wade Clapp (Indiana University)
David Guttmann (Washington University)
David Largaespada (University of Minnesota)
Jeffrey Lawrence (UCSF)
Alcino Silva (UCLA)
Gerard Karsenty (Baylor)
Shaojun Tang (UC Irvine)
Shalom Avraham (Beth Israel)
James Bieker (Mount Sinai, New York)
Abhijit Guha (Labatt Brain Tumor Research Center, Tornoto)
Andreas Kurtz, (Harvard)
Jim Gussela (Harvard)
Dan Haber (Harvard)
Antonio Chiocca (Harvard)
Isidro Sanchez-Garcia (IBMCC)
Victor Tybulewicz (National Institute for Medical Research, London)
Lindsay Hinck (UC Santa Cruz)
Keqiang Ye (Emory University School of Medicine)
Lynda Chin (Dana Farber Cancer Institute)

(c) Employment and Research Opportunities

This award has provided salary support for technical personnel in each of participating labs.

CONCLUSIONS

During this two year award, this consortium made progress in accomplishing its primary goal of generating and characterizing mouse models of NF1 and NF2-associated tumors for biologic and preclinical therapeutic trials. A number of novel strains have been developed and reported, innovative strategies were deployed to make optimal use of these resources, and our research has provided a number of novel insights. The investigators have collaborated closely and have shared expertise and reagents extensively. This NF Consortium, which was admitted to the MMHCC and is participating fully in the activities of the group, will continue under a new 3 year award from the U.S. Army Neurofibromatosis Research Program.

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