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13. ABSTRACT *(Maximum 200 Words)*

This is the first annual report from 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. In the first year of its existence, this Consortium has 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 innovative strategies are being deployed to make optimal use of these resources. The investigators have collaborated closely and have shared expertise and reagents extensively. This NF Consortium has been admitted to the Moue Models of Hman Cancer Consortium of the National Cancer Institute and is participating fully in the activities of the group.

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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 the tumors that arise in patients with 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. We have recently made 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. Improved mouse models would provide an invaluable resource 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 last year 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 this year, and Dr. Shannon is one of two Co-Chairs selected to lead the MMHCC for the next 2-3 years. 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 purposes of this consortium are to develop and validate murine models of NF-associated tumors, and to perform biologic and preclinical therapeutic studies. Toward this end, we are focusing on achieving three technical objectives (aims):

(1) To enhance existing lines of *Nf1* and *Nf2* mutant mice and to develop new *in vivo* models of NF-associated tumors. We are working to fully characterize lesions that arise in these mice focusing 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 has shown 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 first year of support, we have focused most of our efforts on sharing reagents, on enhancing existing mouse models of NF-associated tumors, and on generating and characterizing new models. Specific research goals in this area for year 1 included crossing the *Nf1*^{flox} allele with other transgenic lines, performing subarchnoid injections of *Ad-Cre* constructs to develop a meningioma model in *Nf2* mutant mice, constructing *P0 Cre tv-a* mice, and developing colonies of *Nf1*^{flox} mice at UCSF and MIT. Our progress and the current status of in 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

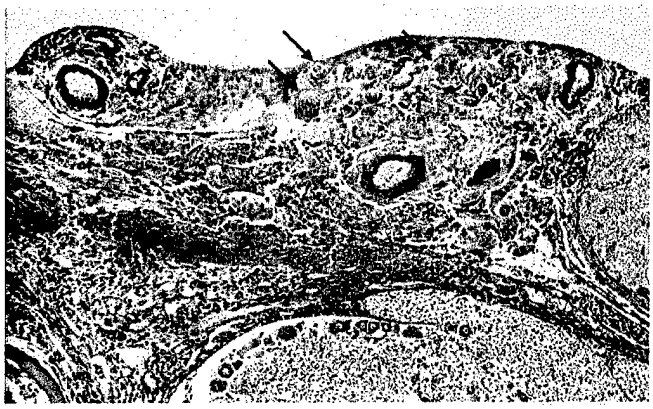
granulocyte-macrophage (CFU-GM) progenitor colonies in methylcellulose cultures exposed to low concentrations of granulocyte-macrophage colony stimulating factor GM-CSF (9). *Nf1*$^{-/-}$ fetal hematopoietic cells demonstrate a similar pattern of hypersensitive growth (11, 12), and adoptive transfer 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. The *Nf1*^{flox} strain generated by Dr. Parada is being deployed to circumvent these problems. Dr. Shannon is investigating inducible alleles for tissue specific expression of Cre and retroviral transduction as complimentary approaches for inducing somatic inactivation of *Nf1 in vivo.*

Dr. Shannon obtained *Nf1*$^{flox/flox}$ mice from Dr. Parada shortly after this award was funded. However, animals within the mouse barrier facility at UT Southwestern are potentially infected with *Helicobacter pylori,* while the UCSF facility is free of this pathogen. Dr. Shannon was therefore required to import male *Nf1*$^{flox/flox}$ mice into a non-barrier facility at UCSF, and subsequently performed embryo transfers to move the *Nf1*^{flox} allele into the barrier facility. Dr. Shannon s laboratory also obtained *Mx1 Cre* mice. In this line, the Cre recombinase is expressed from the interferon-inducible *Mx1* promoter (15). Dr. Shannon has generated colonies of both *Nf1*$^{flox/flox}$ and of *Mx1-Cre Nf1*$^{flox/flox}$ mice that will be used for studies that are described below.

Schwannoma Model. Dr. Giovannini and his colleagues recently demonstrated that conditional mutation of the *Nf2* gene in Schwann cells leads to hyperplasia and to the development of benign and malignant Schwannomas (8). A line of transgenic mice was generated in which Cre recombinase was expressed from the rat P0 promoter. Four independent transgenic lines (P0*Cre*$^{A-D}$) 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. A strategy similar to that utilized for the schwannoma model could not be used to produce a murine meningioma model, as there is no arachnoid cell-specific promoter available at this time. However, arachnoid cells are in contact with the cerebrospinal fluid (CSF) that circulates in the subarachnoid spaces. The alternative approach Dr. Giovannini has taken to target leptomeningeal cells is to deliver the recombinase directly into the CSF circulation. To investigate whether an adenovirus could express functional Cre protein in leptomeningeal cells *in vivo*, Dr. Giovannini constructed an E1-deleted *ad-Cre* recombinant adenovirus that was injected into *Nf2*$^{flox2/flox2}$ mice trans-orbitally. Three days later, tissues were collected and recombination at the *Nf2*^{flox2} locus was detected by PCR. A fragment corresponding to the recombined *Nf2*$^{\Delta 2}$ allele was detected in meningeal cells near the trigeminal nerves, ipsilateral and contralateral

cerebral cortex, brainstem, and spinal cord (data not shown). These data indicate that *ad-Cre* efficiently transduces cells in the neuraxis by diffusing through the CSF. After trans-orbital administration of *ad-Cre,* a cohort of 17 $Nf2^{flox2/flox2}$ mice was observed for 24 months and their survival compared to that of non-injected or *ad-lacZ*-injected controls. The percentage of surviving *ad-Cre* injected $Nf2^{flox2/flox2}$ mice was significantly reduced compared to those of *ad-lacZ*- injected and non-injected $Nf2^{flox2/flox2}$ animals (Kaplan-Meier Test: $p < 0.0001$). Four of 14 *ad-Cre* injected $Nf2^{flox2/flox2}$ mice (29%) developed meningeal tumors in the vicinity of the injection site at a mean age of 11 months. The tumors showed the histological features of benign meningothelial or transitional meningiomas (Fig. 1). Meningiomas were analyzed for recombination of the $Nf2^{flox2}$ allele using laser capture microdissection to obtain pure tumor DNA samples. A 124 bp fragment corresponding to the recombined *Nf2* allele(s) was detected in three tumors (Fig. 1). The fragment corresponding to the $Nf2^{flox2}$ allele(s) was not amplified in the tumors (not shown) indicating that recombination of the two $Nf2^{flox2}$ alleles was complete. Normal surrounding tissues showed no recombination of the $Nf2^{flox2}$ allele. Together, these findings suggest that Cre-*loxP*-mediated inactivation of both $Nf2^{flox2}$ alleles is rate-limiting for meningioma development, and they provide the first model of this important complication of NF2 diseae.



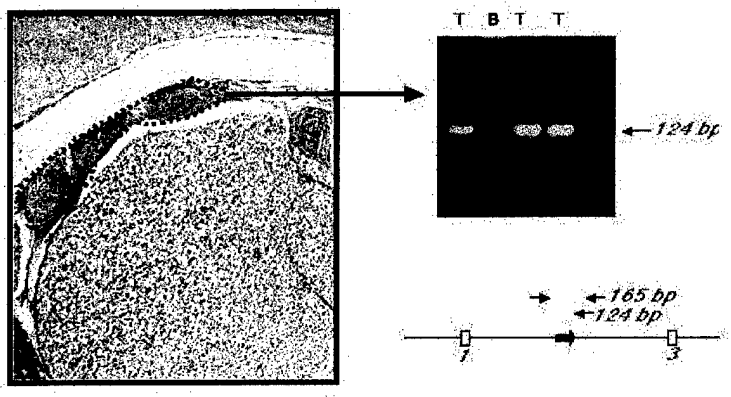


Figure 1. Histological and molecular analysis of phenotypic abnormalities in *ad-Cre* treated $Nf2^{flox2/flox2}$ mice. Left. A transitional meningioma with numerous, concentric, onion-bulb structures (arrows) is seen emanating from the leptomeninges covering the trigeminal nerve. Right. Tumors from *ad-Cre* treated $Nf2^{flox2/flox2}$ mice show *Nf2* gene inactivation. PCR analysis of DNA prepared from three laser-microdissected meningioma tumors (T) demonstrates *Nf2* gene inactivation, while normal brain (B) does not.

Sarcoma Models. Plexiform neurofibromas progress to MPNSTs in 5-10% of NF1 patients, suggesting that mutations at additional loci are required (16). 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 (17, 18). 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 will be a focus of preclinical therapeutic studies in the Parada and Jacks laboratories.

Recent evidence shows that mutations at the *INK4A* locus are also common in human MPNST (19). 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 (20). 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 (21). 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 (21). 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, 22), 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, they 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. Of particular interest is a high incidence of diffuse CNS tumors that resemble anaplastic astrocytomas (data not shown). Together, the efforts of the Jacks and Parada labs supported by other awards are yielding models that can be utilized to test promising therapeutic strategies using approaches described in Aim 3.

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, 23). 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 (24), 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* (25, 26). These data support the idea that Schwann cells are a critical target for *NF1* mutations, although direct experimental proof is still lacking. Dr. Parada s laboratory 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 (27). 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* $Nf1^{fl\$ox/flox}$ 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. However, *Krox20* $Nf1^{flox/flox}$ mice aged for 12-14 months develop multiple abnormal growths that histopathologically resemble 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.

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

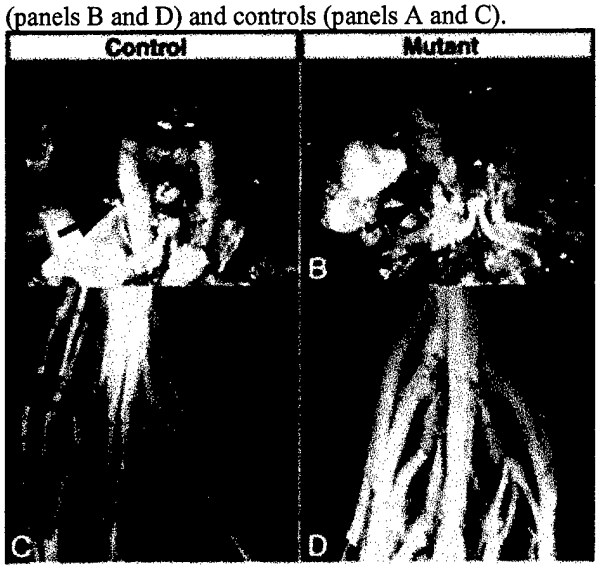
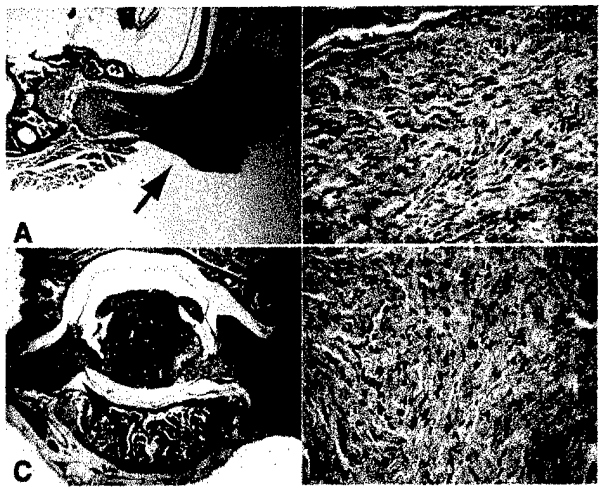


Figure 3. Neurofibromas from *Krox20 Nf1*$^{flox/flox}$ mice.



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 P0*Cre*^{C}; *Nf2*$^{flox2/flox2}$ mice (35%), and the lowest in P0*Cre*^{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, P0*Cre*^{B}; *Nf2*$^{flox2/flox2}$ mice were selected to address whether cooperation of *Nf2* and *Nf1* mutations occurs specifically in Schwann cells. P0*Cre*^{B}; *Nf2*$^{flox2/flox2}$ and *Nf1*$^{+/-}$ mice (2) were crossed to generate P0*Cre*^{B}; *Nf2*$^{flox2/flox2}$; *Nf1*$^{+/-}$ mice, that were monitored over time for the appearance of tumors. P0*Cre*^{B}; *Nf2*$^{flox2/flox2}$; *Nf1*$^{+/-}$ mice exhibited reduced survival compared to P0*Cre*^{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 P0*Cre*^{B}; *Nf2*$^{flox2/flox2}$; *Nf1*$^{+/-}$ mice developed peripheral nerve tumors (96%). By contrast, only one of 27 P0*Cre*^{B}; *Nf2*$^{flox2/flox2}$; *Nf1*$^{+/+}$ mice acquired a schwannoma by 9.5 months of age. Macroscopically, tumors usually emanated from the peripheral nerves of the tongue, peritoneum, limbs, and from spinal ganglia. Histological analyses of these tumors and those derived from *cis Nf2*$^{+/-}$; *Nf1*$^{+/-}$ mice are ongoing in collaboration with Drs. McClatchey and James Woodruff (Memorial Sloan-Kettering Cancer Center). 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 P0*Cre*^{B}; *Nf2*$^{flox2/flox2}$; *Nf1*$^{+/-}$ mice displayed deletion 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 $S100^{-}/p75^{+}$.

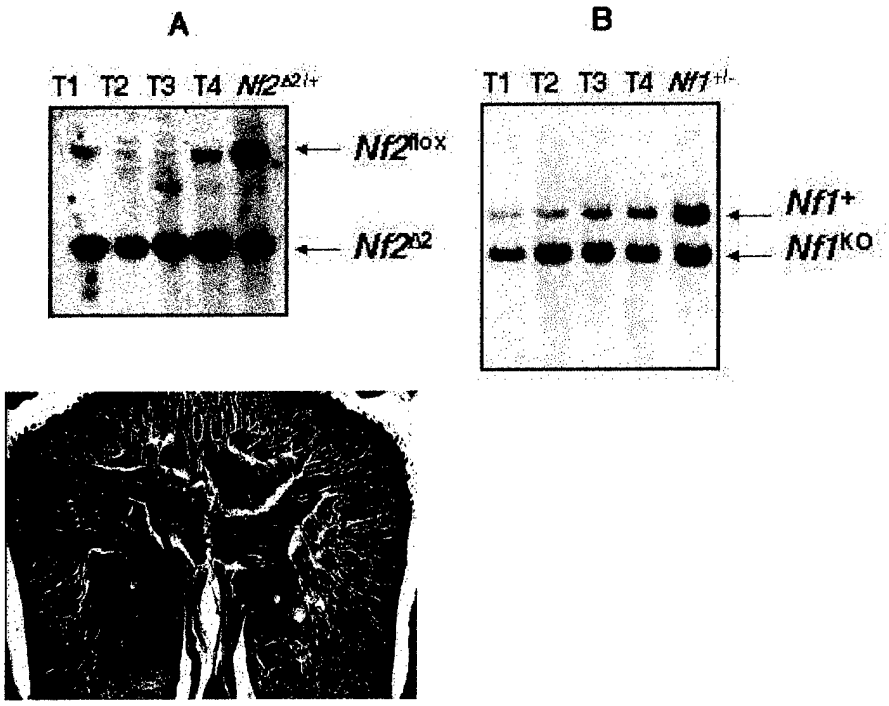


Figure 4. (A) Tumors of P0Cre^{B};$Nf2^{flox2/flox2}$; $Nf1^{+/-}$ mice show Cre-mediated *Nf2* gene inactivation. Southern blot analysis (probe B) of *Xba*I-*Bam*HI-digested DNAs from four representative tumors (lanes 1-3: MPNST; lane 4: neurofibroma). Bands corresponding to the $Nf2^{flox2}$ and $Nf2^{-2}$ 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 and are characterizing mouse models of almost all of the tumors that arise in individuals with NF1 and NF2. We have undertaken each element of the approved Statement of Work for this project, and have also generated models that can be used for biologic and preclinical therapeutic studies using funds provded by other awards. During the next year of funding, we will continue to prosecute the

experiments proposed in our application and will also exploit new opportunities based on our progress to date.

Research Plan

Leukemia Model. Dr. Shannon s group is pursuing two experimental approaches that Dr. James Downing (St. Jude Children s Research Hospital) has exploited successfully in a line of *AML1-ETO* knock in mice (28). The first strategy involves treating mice with the *Mx1-Cre Nf1*$^{flox/flox}$ genotype with interferon to induce expression of Cre recombinase in primitive hematopoietic cells. A few of these animals will be sacrificed one week later to ascertain if *Nf1* inactivation has occurred in bone marrow cells. If these studies demonstrate a high rate of Cre-mediated recombination, he will follow a second cohort of mice for evidence of disease after interferon treatment. These mice will have complete blood counts (CBCs) measured every 2 weeks. Animals that appear ill or develop pronounced leukocytosis will be sacrificed and the spleens will be weighed and sectioned. Flow cytometry will be performed to quantify the percentage of myeloid and lymphoid cells in the marrow and spleen, myeloid progenitor colony growth will be assayed, and biochemical analysis of Ras signaling will be carried out as described previously (12, 13, 29). These studies will rigorously ascertain if *Mx1-Cre Nf1*$^{flox/flox}$ mice provide a robust model that recapitulates features of human JMML and of the MPD that arises after adoptive transfer of *Nf1* mutant fetal liver cells. Dr. Shannon s lab has generated a colony of *Mx1-Cre Nf1*$^{flox/flox}$ mice, and these studies will begin in the next few weeks.

An alternative strategy involves collecting *Mx1-Cre Nf1*$^{flox/flox}$ fetal liver cells at E15, inducing Cre recombinase expression *in vitro,* and transplanting these cells into irradiated recipients. Dr. Shannon s lab has generated a retroviral vector in the murine stem cell virus (MSCV) backbone (30) that expresses both Cre recombinase and green fluorescent protein (GFP), and has generated high titer stocks for infecting fetal liver cells or bone marrow cells from *Nf1*$^{flox/flox}$ mice. This system allows cells that have been infected with the virus to be isolated by FACS. This strategy would represent a major improvement over our current procedures as fetal liver cells do not have to be genotyped before transfer, and there is >100-fold increase in the number of hematopoietic cells in the fetal liver between E12 and E15.

Plexiform Neurofibroma Formation in *Krox20 Nf1*$^{flox/flox}$ Mutant Mice. Preliminary data from Dr. Parada s laboratory indicate that 2 of 2 aged *Krox20 Nf1*$^{flox/flox}$ mice >12 months old developed plexiform neurofibromas. Although not as common as dermal neurofibromas, these lesions affect about 30% of NF1 patients, and are though to be the precursor of MPNST (31). Dr. Parada will age additional *Krox20 Nf1*$^{flox/flox}$ mutant mice, and will perform careful necropsies with histologic analysis to identify and characterize any neurofibromas. S100 will be used as a Schwann cell marker, EMA (epithelial membrane associated antigen) as a perineurial cell marker, and NF160 (neurofilament 160) as a neuronal marker. In addition, EM (electronic microscopy) will be performed to confirm the presence of multiple cell types (e.g. Schwann cells, perineurial cells, axon/neurons) in these plexiform neurofibromas. If *Krox20 Nf1*$^{flox/flox}$ mice develop neurofibromas with high penetrance, this will not only prove Schwann cells are the primary target for *Nf1* mutations that initiate tumor formation, but also will provide an excellent mouse model for studying plexiform neurofibromas. While preliminary, the data presented in Figures 2 and 3 suggest that a model for neurofibroma has been achieved.

Use of Avian Leukosis and Sarcoma Virus to Target Mature Schwann Cells *In vivo*. Dr. McClatchey will use the transgenic mice that express the tv-a receptor under the control of the P0 promoter described above. Expression of tv-a renders murine cells susceptible to infection by recombinant avian retroviruses that have been engineered to express many genes of interest, including *Cre*. Such recombinant viruses have been shown to replicate to a very high titer, allowing for efficient infection *in vitro* and *in vivo*. Initially, optimal conditions for expression of a lacZ reporter in mature Schwann cells will be ascertained by introducing lacZ-expressing avian retroviruses into a site adjacent to the sciatic nerve with and without nerve transection to induce Wallerian degeneration and proliferation of mature Schwann cells. These experimental conditions will subsequently be employed to infect mature Schwann cells with a Cre-expressing virus. This strategy has the added advantage of producing focal loss of *Nf2*, thus more closely mirroring the context of schwannoma development in humans. If this strategy proves successful in $Nf2^{flox}$ mice, the tv-a line can be used to inactivate *Nf1* in foci of cells that express P0. Avian retroviruses will also be used to introduce various dominant-acting signaling molecules (such as activated Rac) into t-va-expressing Schwann cells *in vivo* and *in vitro* (see below).

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. Thus, inhibitors of Ras processing (such as farnesylation, geranylgeranylation endopeptidase cleavage and methylation) and 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 (32). It is important to note 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. However, recent work has demonstrated physical association between merlin and ERM proteins as well as with several other cellular proteins. Merlin also binds and appears to regulate signaling from the transmembrane hyaluronic acid receptor CD44 (33), which has been implicated in tumor development and metastasis. Drs. McClatchey and Jacks have recently published that the small GTPases Rac1 and Cdc42 are capable of inducing the phosphorylation and presumed inactivation of merlin (34). Moreover, they have shown that loss of *Nf2* function in turn leads to activation of Rac1 signaling. $Nf2^{-/-}$ fibroblasts exhibit molecular and cellular signatures that are consistent with hyperactive Rac signaling, while overexpression of merlin can block Rac-induced transformation and signaling (34). Together, 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.

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 (17, 19, 35). 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. In previous studies, Dr. Shannon's laboratory generated breeding stocks and intercrossed mice heterozygous for a loss-of-function mutation in the gene encoding the Ras endoprotease (*Rce1)* and for *Nf1*. However, none of 65 embryos recovered at E13.5 contained the desired $Nf1^{-/-}$; $Rce1^{-/-}$ genotype. These data suggest a reduction in viability, which was unanticipated as the *Nf1* and *Rce1* mutations are expected to have opposite effects on Ras signaling. Dr. Shannon is utilizing the conditional *Nf1* allele developed by Dr. Parada to overcome this problem. As described above, his laboratory rederived the $Nf1^{flox}$line for these studies, and the initial crosses have been performed. These ongoing studies will therefore allow Dr. Shannon to ascertain if inhibiting this Ras processing enzyme might selectively impair the growth of *Nf1*-deficient cells *in vivo.*

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 nu/nu mice. The Parada lab has generated an efficient adenovirus expression system to express genes of interest in cell lines and primary (36). 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).

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 (37). Under each of these conditions, the total levels and particularly the ratio of hypo- to

hyperphosphorylated forms of merlin were elevated. Dr. McClatchey s laboratory has shown that *Nf2*$^{-/-}$ fibroblasts fail to undergo proper growth arrest under each of these conditions. Drs. McClatchey and Jacks have also found that *Nf2*$^{-/-}$ cells exhibit strikingly accelerated motility compared to wild-type cells in scrape-wounding and Boyden chamber assays. Fibroblast motility is positively regulated by Rac activation (38). Together, these preliminary observations of the growth and motility of *Nf2*$^{-/-}$ fibroblasts will form the basis for an interrogation of *Nf2*$^{-/-}$ and of *Nf1*$^{-/-}$; *Nf2*$^{-/-}$ Schwann cells (see below).

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 differences in their growth properties. For example, *Nf1*$^{-/-}$ fibroblasts grow to a higher saturation density than wild-type cells, but eventually arrest. 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. 5). This does not appear to be due to increased growth factor-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 (39). 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 (40). 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.

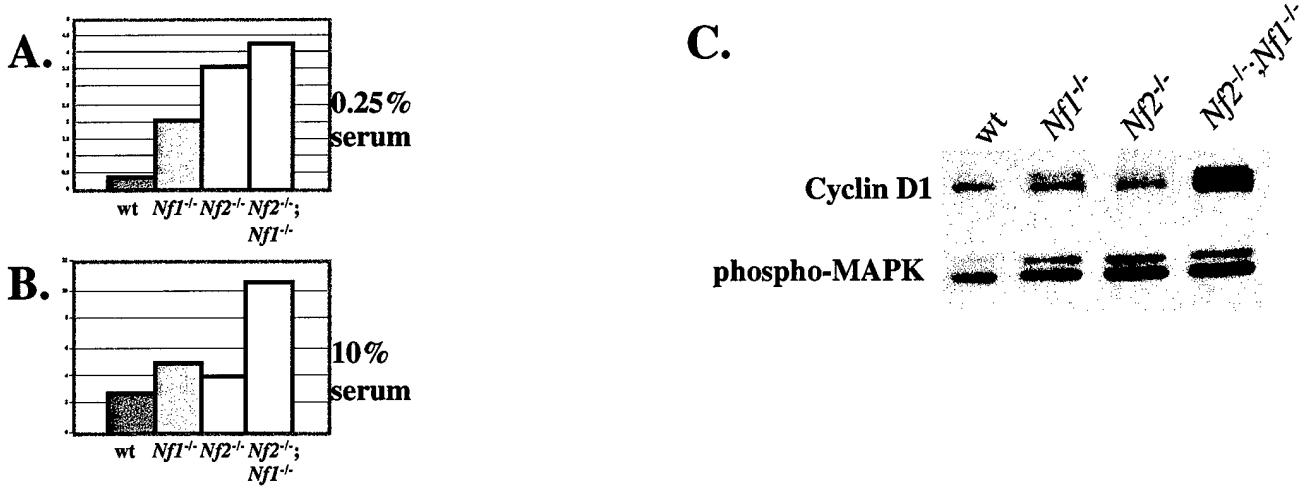


Figure 5: *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ fibroblasts exhibit a growth advantage compared to either *Nf1*$^{-/-}$ or *Nf2*$^{-/-}$ fibroblasts in the presence of growth factors. **A,B**, Fold increase in cell number after 7 and 5 days in 0.25% and 10% serum, respectively. **C**, Levels of cyclin D1 and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

A Cell-Based System for Testing Therapeutics in *Nf2*-Mutant Cells. D. 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 growth and viability of *Nf2*$^{-/-}$ cells under two conditions: exponential growth in 10% serum and growth 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 two different commercially available MEK inhibitors have established labeling conditions and validated the assay in immortalized fibroblasts. As shown in Figure 6, both U0126 (17.5μM) and PD98059 (100μM) efficiently caused growth arrest in cycling wild-type and *Nf2*$^{-/-}$ fibroblasts, although PD98059 inhibition of *Nf2*$^{-/-}$ fibroblast growth was considerably greater than its effects on wild-type cells. These studies are now being conducted in primary MEF cultures.

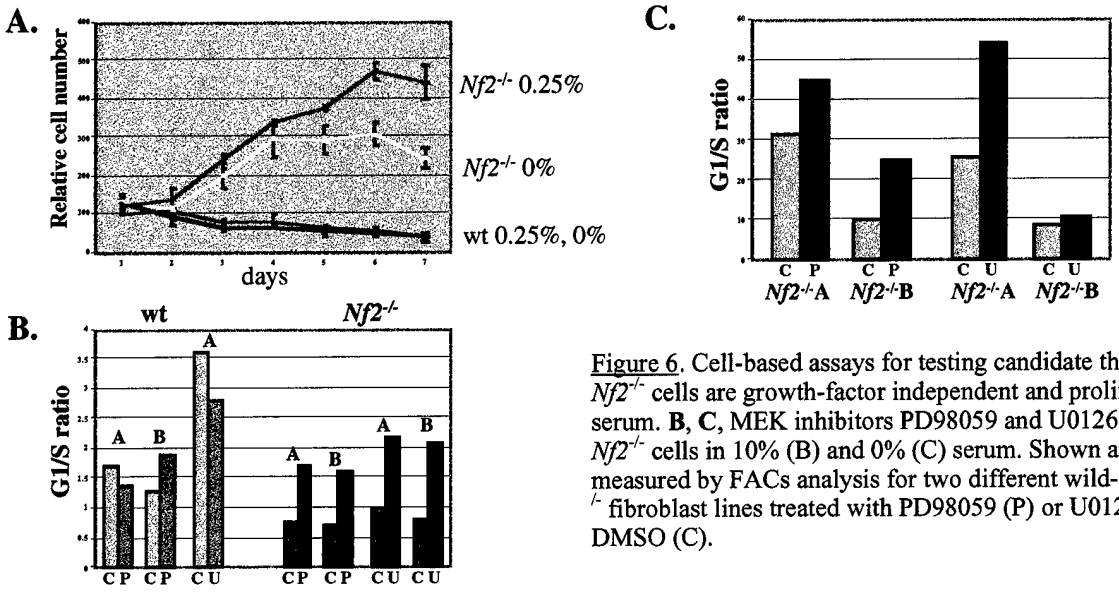


Figure 6. Cell-based assays for testing candidate therapeutic targets. **A**, *Nf2*$^{-/-}$ cells are growth-factor independent and proliferate in the 0% serum. **B, C**, MEK inhibitors PD98059 and U0126 inhibit the growth of *Nf2*$^{-/-}$ cells in 10% (B) and 0% (C) serum. Shown are the G1/S ratios measured by FACs analysis for two different wild-type (B only) and *Nf2*$^{-/-}$ fibroblast lines treated with PD98059 (P) or U0126 (U) compared to DMSO (C).

Research Plan

Cooperative Effects of *p53* Mutations on Tumorigenesis in the *Krox20-Cre* Background. Over the next year, Dr. Parada will establish cohorts with the following genotypes: *Krox2-Cre; Nf1*$^{flox/flox}$*; p53*$^{+/+}$ (control), and *Krox2-Cre; cis Nf1*$^{flox/flox}$*; p53*$^{+/-}$. These studies will address two questions. First, Dr. Parada will determine the incidence and latency to the development of neurofibroma in each group. These studies will allow him to determine if inactivating *p53* in the Schwann cell lineage cooperates with inactivation of *Nf1* in neurofibroma formation. Dr. Parada will also examine if MPNSTs arise in these mice. *Krox2-Cre; Nf1*$^{flox/flox}$ mice did not develop malignant tumors over a period of 14 months, suggesting that loss of *Nf1* in the Schwann cell lineage is insufficient for malignant transformation. He hypothesizes that mice in which both *Nf1* alleles and one *p53* allele are mutant will have a high likelihood of developing neurofibromas that progress to MPNSTs. If this proves true, this stain will be extremely valuable for molecular studies as well as for evaluating therapeutics.

Rce1 x *Nf1* Cross and Analysis of Cellular and Biochemical Phenotypes. Dr. Shannon s laboratory is crossing the conditional *Nf1*^{flox} allele into heterozygous *Rce1* mutant background to generate *Rce1*$^{+/-}$, *Nf1*$^{flox/+}$ animals. These mice will be backcrossed with *Nf1*$^{flox/flox}$ mice to

generate founders with the desired *Rce1*$^{+/-}$, *Nf1*$^{flox/flox}$ genotype, which will then be intercrossed. Pregnant females will be sacrificed ~E15 to obtain an optimal number of fetal hematopoietic cells for infection with *Cre-GFP* retroviral stocks. After 48 hours in culture, GFP-expressing cells will be isolated by FACS and injected into syngeneic recipients conditioned with 1000 cGy or total body irradiation (14). Based on colony assays, 60-80% of GFP-positive cells transduced with this vector undergo *Cre*-mediated recombination (data not shown). Because *Nf1*$^{-/-}$ fetal hematopoietic cells have a proliferative advantage *in vivo* when they are coinjected with *Nf1*$^{+/+}$ cells (14), we expect that recipients will develop MPD. We hypothesize that inactivation of *Rce1* will attenuate the MPD associated with adoptive transfer of *Nf1*-deficient fetal liver cells (i.e. that recipients of *Nf1*$^{-/-}$, *Rce1*$^{-/-}$ cells will have lower white blood cell counts, smaller spleens, and enhanced survival compared with control recipients. CFU-GM colony growth and MAPK/PI3K activation will be measured in response to GM-CSF in recipient bone marrow. If inactivation of *Rce1* reduces the abnormal growth of *Nf1*$^{-/-}$ hematopoietic cells *in vivo* and *in vitro,* these data would validate the general strategy of inhibiting Ras signaling by interfering with membrane targeting.

Growth and Survival of *Nf2*$^{-/-}$ and *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ Schwann Cells. Schwann cells play a central role in many of the pathologic complications of NF2. It is therefore essential to rigorously interrogate any possible merlin-regulated signaling pathways in this cell type. To examine the consequences of *Nf2* and *Nf1* inactivation on Schwann cell proliferation, Dr. McClatchey's laboratory will generate primary cultures from the conditional *Nf2* and *Nf1* mutant mice generated by Drs. Giovannini and Parada. Primary Schwann cell cultures will be prepared from sciatic nerves of 3-week-old mice. The purity of the cultures will be assessed by protein S-100 immunocytochemistry. The cultures will then be incubated with experimentally determined titers of *ad-Cre* or *ad-lacZ* (control) viruses to inactivate *Nf2* and/or *Nf1*. Recombination of the conditional alleles will be confirmed by PCR (5, 8). Using their studies of *Nf2*$^{-/-}$ fibroblasts as a guide, Dr. McClatchey's laboratory will examine proliferation rates, contact inhibition, growth factor dependence, anchorage independence and motility in Schwann cell cultures of various genotypes. Proliferation rates, contact inhibition, growth factor dependence, anchorage independence and motility will then be measured in Schwann cell cultures of various genotypes, and results will be compared to findings in fibroblasts. Transformed cells are able to achieve anchorage-independent growth in soft agar and subcutaneous tumor formation in nude mice. Although neither *Nf2*$^{-/-}$ nor *Nf2*$^{-/-}$; *Nf1*$^{-/-}$ fibroblasts are transformed by these criteria, it is possible that the requirements for anchorage-dependent growth are different for Schwann cells. Dr. McClatchey will therefore determine whether *Nf2*- and/or *Nf1*-deficient Schwann cells are capable of anchorage-independent growth in soft agar or nude mice. Given links between merlin function, the actin cytoskeleton, and Rho GTPases, her lab will also examine the integrity of the actin cytoskeleton in *Nf2*$^{-/-}$ Schwann cells. She has shown that *Nf2*$^{-/-}$ fibroblasts can form actin containing stress fibers, membrane ruffles, microspikes and focal adhesions. However, *Nf2*$^{-/-}$ fibroblasts exhibit many features of cells that overexpress Rac 1 including excessive membrane ruffling in response to replating and PDGF treatment during 'wound-healing' (34). To determine whether loss of merlin and/or neurofibromin results in increased cell motility of Schwann cells, Dr. McClatchey will measure cell motility by 'wound-healing' and Boyden chamber assay. The integrity of the actin cytoskeleton in *Nf2*- and/or *Nf1*-deficient Schwann cells will also be examined.

Biochemical Analysis of Potential Targets for *Nf2* Therapeutics. Although the molecular effects of merlin deficiency remain elusive, the link to Rac signaling provides potentially important clues. Accordingly, investigating the requirement for this signaling cascade in *Nf2*-associated Schwann cell tumorigenesis is a high priority. Drs. McClatchey and Giovannini will collaborate to examine the integrity of the Rac pathway in Schwann cells and in schwannomas from the $Nf2^{flox2/flox2}$ mice. Dr. McClatchey will examine basal and stimulable Rac and JNK activity in these cells by Pak binding assay and Western blot analysis using a phospho-JNK-specific antibody, respectively. They will also measure basal and stimulable levels of the downstream targets c-fos, SRE and AP1 by Western blot and sensitive luciferase reporter assays, respectively.

The collaborative studies of Drs. McClatchey, Giovannini, and Jacks suggest three possible therapeutic strategies for *Nf2*-associated tumors. Over the next year, we will begin to evaluate each of these in cell-based assays. The first involves inhibiting Rac signaling, either directly or indirectly. Like Ras, the biologic activity of Rac depends on post-translational processing (41). However, Rac is modified by geranylation rather than farnesylation; this reaction is catalyzed by geranylgeranyltransferase type I (GGTase)(42, 43). Specific GGTase inhibitors (GGTIs) have been developed and shown to interfere with processing (44, 45). In addition, Rac signaling is activated by Ras-GTP is some cell types. Thus, inhibitors of Ras processing as described above for NF1 may also be beneficial in NF2. Similarly, specific inhibitors of PI3K are rational therapeutics, because PI3K is instrumental in coupling Ras-GTP with Rac. Finally, many studies now indicate context-dependent cross-talk between Rac and the Ras-Raf-MEK-MAPK cascade. As for NF1, specific inhibitors of MAPK signaling may therefore be of therapeutic value. A second potential approach to treating NF2-associated tumors involves modulating PKA output. Several cell-permeable compounds that specifically stimulate or inhibit the PKA pathway have been developed, including forskolin and IBMX (activators), as well as H-89 and 8-bromo-cAMP (inhibitors). Given data indicating that the ERM proteins can control PKA signaling, the possible link between PKA and merlin phosphorylation, and the requirement for PKA signaling in Schwann cell proliferation, an exploration of the effects of these compounds on $Nf2^{-/-}$ cell growth and viability is warranted. Finally, many chemotherapeutic agents induce JNK activation and this is thought to promote apoptosis (46-48). Thus, $Nf2^{-/-}$ cells, which exhibit hyperstimulable JNK, may be also be hypersensitive to drugs that induce JNK-mediated apoptosis. Drs. McClatchey and Giovannini will continue to interrogate these pathways in *Nf2*-deficient cells over the next year.

The observation that cyclin D1 levels are specifically elevated in cycling $Nf2^{-/-}$; $Nf1^{-/-}$ fibroblasts is intriguing. Moreover, Kim et al. recently showed that while Schwann cells in cyclin D1-deficient mice develop normally, proliferation of mature Schwann cells was markedly impaired *in vitro* and *in vivo* (39). This suggests that cyclin D1 activity is required for the proliferation of mature, but not immature Schwann cells and provides a potential means of discriminating between these populations. Cyclin D1 is controlled at the level of both protein stability and mRNA transcription. Ongoing studies in Dr. McClatchey's laboratory aim to measure cyclin D1 protein stability and mRNA levels in $Nf2^{-/-}$; $Nf1^{-/-}$ fibroblasts by Northern blot and pulse-chase analyses to discriminate between these two. These studies will form the basis of a parallel investigation of signaling to cyclin D1 in $Nf2^{-/-}$ and $Nf2^{-/-}$; $Nf1^{-/-}$ Schwann cells that will be initiated over the next year.

Introduction of Signaling Molecules into Schwann Cells from P0-tv-a Mice. As described above, tv-a receptor expression renders murine cells susceptible to infection by avian retroviruses

engineered to drive the expression of signaling molecules *in vitro* and *in vivo*. In addition to the *in vivo* delivery of Cre expression into mature Schwann cells described above, Dr. McClatchey will begin using this system to introduce alleles of various activated and dominant-negative alleles signaling molecules into Schwann cells *in vitro*. The ability of wild-type and various mutant forms of *Nf2* to correct the aberrant growth of *Nf2* mutant Schwann cells will be assessed initially. Subsequently, activated forms of the small GTPases Rac, Rho, Cdc42 and Ras itself will be introduced into wild-type and $Nf2^{-/-}$ Schwann cells. The growth properties and cytoskeletal integrity of these cells will be evaluated as described above. The versatility of this system for the introduction of a variety of signaling molecules into Schwann cells *in vitro* will permit studies of multiple putative effectors of merlin function. These studies will be initiated over the next few months.

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 (49).

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 over the past year to address some of the practical issues involved in using these mouse models to test therapeutics and to initiate preclinical studies.

Progress

Preclinical Evaluation of a MEK Inhibitor in the JMML Model. 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 (50). 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 (50). 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. This work is funded by a grant from the American Cancer Society. 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). The Shannon lab is currently testing this agent *in vivo*. 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 PD studies, the Shannon lab has developed a robust assay 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 murine marrow cells 2, 4, and 8, hours after intraperitoneal injection (Fig. 7). The Shannon lab recently completed a 4 week toxicity study in wild-type mice in which treated animals received twice daily doses of 100 mg/kg of PD184352 without adverse effects. Thus, in contrast to FTI, PD184352 markedly inhibits a relevant biochemical target in primary *Nf1* mutant cells at tolerable doses.

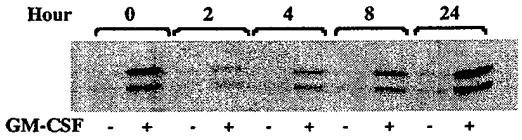


Figure 7. Phospohorylated ERK Levels in Marrow Cells after Treatment with PD184352. A single dose of 100 mg/kg was injected at hour 0, and mice were sacrificed 2, 4, 8, or 24 hours later. Primary marrow cells were maintained in serum only (-) exposed to GM-CSF for 10 min (+) before lysis. Equivalent 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 8).

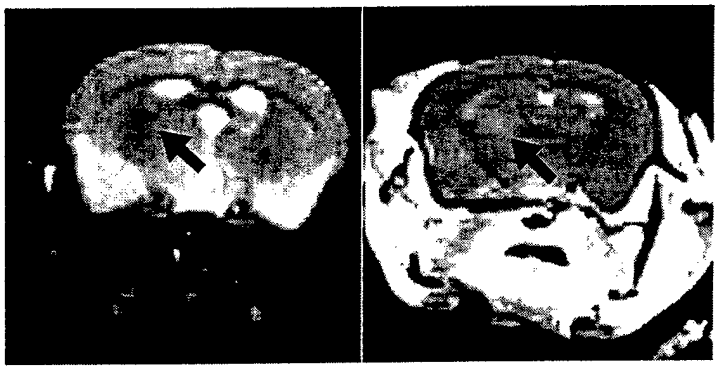


Figure 8. *Nf1*$^{+/-}$;*p53*$^{+/-}$ cis mouse on a C57BL/6 X A/J F1 background imaged by MRI with two different imaging agents. (Left) MION-enhanced T2-weighted MRI, dark region to left of midline indicative of tumor by this imaging method. (Right) Gd-enhanced T1-weighted MRI of same animal 21 days later, the same region to left of midline is brighter indicative of tumor by this imaging method.

Research Plan

General Strategy for Screening Therapeutics. The JMML-like MPD that develops in recipients of *Nf1*-deficient fetal liver cells is the most tractable mouse model of an NF1-associaed cancer. Advantages of this system include: (1) uniform disease onset and a subacute clinical course; (2) recipient mice are immunocompetent; (3) therapeutic effects can be assessed easily by measuring blood counts; (4) PD endpoints can be determined in primary target cells and correlated with clinical responses; and, (5) the specificity of a given therapeutic for *Nf1*-deficient cells can be quantified by transplanting *Nf1* mutant and wild-type cells into the same hosts (14). Given this, we will emphasize the myeloid leukemia model in pilot studies over the next year. However, it is likely that different tumor types will not respond in the same way to specific drugs, and it will therefore be essential to evaluate each therapeutic in a number of tumor systems. In general, agents that demonstrate a promising PD profile in the JMML model (e.g. PD184352) will be prioritized for testing in other tumor models. These proposed studies are beyond the one year duration of this award. At present, MPNSTs arising in *cis Nf; /p53* mice provide the best *de novo* solid tumor model. Cell lines derived from these tumors and from murine astrocytomas can also explanted into nu/nu mice to screen novel agents and to obtain PD data. In addition to testing therapeutics, we will continue to develop enhanced techniques for imaging mouse nervous system tumors. These studies and experience gained from conducting preclinical trials in *Nf1* mutant mice will be invaluable for subsequent testing in models of NF2-asociated tumors. We anticipate that the experiments proposed under aim 2 will uncover targets for testing therapeutics in *Nf2* mutant mice.

Evaluating Therapeutics in the JMML Model. In performing preclinical trials, we will follow the paradigm we developed previously to test the farnesyltransferase inhibitor L744,832 (14). During this fund year, Dr. Shannon will complete his studies of PD18532 and will initiate studies of a small molecule inhibitor of c-kit, a receptor tyrosine kinase that is activated by the stem cell factor (SCF). A potential interaction between signaling initiated by binding to the c-kit receptor and neurofibromin was suggested by the observation that mice with mutations at the white spotted *(W)* locus have defects in two lineages (hematopoietic cells and melanocytes) that are affected in NF1 patients. The *W* locus encodes the murine c-kit receptor (51-53). Drs. David Ingram and Wade Clapp collaborated with Drs. Shannon and Jacks on studies in which they

intercrossed W^{41} and *Nf1* mice. The W^{41} mutation is associated with a reduction in c-kit receptor tyrosine kinase activity to approximately 20% of wild-type levels. Surprisingly, haploinsufficiency at *Nf1* attenuated the melanocyte and mast cell defects in homozygous W^{41} mice; this partial phenotypic rescue was associated with an increase in SCF-induced MAPK activation to near wild type levels in homozygous W^{41} mast cells (54). Together these studies suggest that SCF and GM-CSF might act synergistically to induce MPD seen in the recipients of $Nf1^{-/-}$ fetal liver cells. Furthermore, mast cells, which represent an important cellular component of neurofibromas, express c-kit and are exquisitely sensitive to the activation status of the SCF-c-kit pathway for survival and cytokine release. Schwann cells also express c-kit. COR Therapeutics has developed a small molecule inhibitor of c-kit called KN2941 that is being moved into phase 1 human clinical trials. Dr. Shannon has obtained this inhibitor and will soon initiate preclinical studies.

Under the current protocol, recipient mice reconstituted with wild type ($Nf1^{+/+}$) or with $Nf1^{-/-}$ fetal liver cells are used to assess efficacy and toxicity. The proposed studies involve determining an appropriate dose of KN2941 followed by a preclinical trial. Dr. Shannon first measures PD endpoints in primary marrow cells from wild-type mice to demonstrate that KN2941 is inhibiting c-kit signaling *in vivo*. The design of these experiments involves injecting mice with a single dose of 60 mg/kg (2 mg/mouse), followed by sacrifice and biochemical studies at defined time points. Efforts are directed toward showing that marrow cells from treated mice were unable to activate c-kit signaling in response to SCF. MAPK activation are used as one readout and c-kit receptor phosphorylation and kinase activity will also be assessed in treated versus untreated cells. To test efficacy, lethally irradiated recipients that have developed MPD after receiving $Nf1^{-/-}$ fetal liver cells and control mice transplanted with wild-type cells are randomly assigned to receive either KN2941 or no treatment. Initially, 5-10 mice will be assigned to each of 4 experimental groups and will be treated for 8 weeks. Mice are weighed weekly and the drug dose adjusted accordingly. Blood counts are obtained every 2 weeks during treatment and, if clinical responses are seen, monthly for 3 months after the end of the treatment phase. Animals are sacrificed at the end of the experiment. At that time, livers and spleens are weighed; slides are prepared from bone marrow, liver and spleen for morphologic evaluation; and CFU-GM colonies are enumerated from the spleens. If the strategies proposed under aim 1 using Dr. Parada's $Nf1^{flox}$ mice provide a more tractable model, Dr. Shannon's lab will modify their current strategy to use this new system. In future years, Drs. Shannon, Jacks, and Parada hope to closely to extend this work to solid tumor models.

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 (55) 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

- The investigators established this Consortium and have extensively shared research reagents.
- Our NF Modeling Group was admitted to the NCI MMHCC and is participating actively in this mouse cancer modeling consortium.
- We have established two novel models of plexiform neurofibroma as well as a new model of MPNST.
- Lines of transgenic tv-a mice have been generated that will facilitate in vivo and in vitro studies of Schwann cell biology and tumorigenesis.
- Models of meningioma and astrocytoma have been generated and are being characterized.
- A recombinant chromosome that carries both the $Nf1^{flox}$ allele and a *p53* mutation has been generated to address cooperativeity between these tumor suppressor genes in tumorigenesis.
- Robust assays have established for investigating the growth of *Nf1* and *Nf2* deficient cells *in vitro* to discover targets for therapeutic interventions.
- A preclinical trial of a MEK inhibitor has been launched in the JMML model with correlative biochemical (pharmacodynamic) monitoring.
- Techniques are being developed to image mouse tumors in vivo in order to examine the efficacy of therapeutic interventions in solid tumor models.

REPORTABLE OUTCOMES

(a) Publications

There are no publications yet.

(b) Model Development

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.

(c) Employment and Research Opportunities

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

CONCLUSIONS

In the first year of its existence, this Consortium has made impressive 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 innovative strategies are being deployed to make optimal use of these resources. The investigators have collaborated closely and have shared expertise and reagents extensively. This NF Consortium has been admitted to the MMHCC and is participating fully in the activities of the group.

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