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Activation of neuregulin -1/ErbB signaling promotes proliferation in human Schwann cell neoplasia.

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NOTE TO USERS

ACTIVATION OF NEUREGULIN-1/ERBB SIGNALING PROMOTES PROLIFERATION IN HUMAN SCHWANN CELL NEOPLASIA

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
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INFORMATION TO USERS

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

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Title Activation of Neuregulin-1/ErbB Signaling Promotes Proliferation in Human Schwann Cell Neoplasia

Neurofibromas, malignant peripheral nerve sheath tumors (MPNSTs), and schwannomas are three distinct types of peripheral nerve sheath tumors derived from Schwann cells, the myelinating glia of peripheral nerve. Dysregulated Schwann cell proliferation is a feature common to these neoplasms, and defining the molecules that promote inappropriate Schwann cell proliferation will provide important insights into the mechanisms underlying the formation of peripheral nerve sheath tumors. I have tested the hypothesis that neuregulin-1 (NRG-1) growth and differentiation factors, a group of developmentally regulated Schwann cell mitogens, promote neoplastic Schwann cell proliferation in neurofibromas, MPNSTs, and schwannomas. I first review the pathogenesis of Schwann cell neoplasms, with a focus on tumor suppressor gene inactivation (first article) and inappropriate growth factor signaling (second article). I then present my experimental work that examines the expression and actions of NRG-1 isoforms and their erbB receptors in neurofibromas and MPNSTs (third article) and schwannomas (forth article). The results indicate that NRG-1 isoforms (α and β transmembrane precursors from classes II and III) and NRG-1 receptors (erbB-2, -3 and -4) are expressed in neurofibromas and MPNSTs. Human MPNST cell lines express multiple NRG-1 isoforms, and erbB membrane tyrosine kinases that are constitutively

phosphorylated. Futhermore, pharmacological erbB kinase inhibition markedly reduced DNA synthesis in MPNST cells. These results suggest that autocrine, paracrine, and/or juxtacrine signaling promotes neoplastic Schwann cell proliferation and thus may be a therapeutic target. In the fourth article, I examine the expression pattern of NRG-1 isoforms and erbB receptors in surgically resected human schwannomas. I have found that neoplastic Schwann cells within schwannomas overexpress both α and β NRG-1 isoforms (classes II and -III) relative to normal eighth cranial nerve. These tumors also overexpress the NRG-1 related factor NRG-2 (α and β isoforms). Schwannomas almost uniformly express NRG-1 receptors (erbB-2 and erbB-3), with some tumors also expressing EGF receptor and erbB4. Taken together, these data support the hypothesis that NRG-1 and/or NRG-2, acting through erbB receptors, promotes schwannoma tumorigenesis. This work thus provides evidence for future investigations of NRG-1 isoforms and erbB kinases in peripheral nerve sheath tumorigenesis and their significance as a therapeutic target.

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LIST OF ABBREVIATIONS

ARIA	acetylcholine receptor inducing activity
AMP	adenosine-monophosphate
cAMP	cyclic adenosine-monophosphate
ATP	adenosine triphosphate
CDK	cyclin dependent kinase
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Erk	extracellular related kinase
EtNU	*N*-ethyl-*N*-nitrosourea
FBS	Fetal Bovine Serum
FISH	florescent in situ hybridization
GAP	GTPase activating protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GEF	guanine nucleotide exchange factors
GEM	genetically engineered murine
GFAP	glial fibrilary acidic protein

GGF	glial growth factor
GGF-β3	glial growth factor-β3
GTP	guanine triphosphate
GTPase	guanine triphosphate hydrolase
HGF	hepatocyte growth factor
HGFA	hepatocyte growth factor activator
HRP	horseradish oeroxidase
JNK	Jun N-terminal kinase
LOH	loss of heterozygosity
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MEKK	mitogen-activated protein kinase / extracellular related kinase kinase kinase
MPNST	malignant peripheral nerve sheath tumor
MTTs	malignant Triton tumor
NDF	neu differentiation factor
NF1	neurofibromatosis type 1
NF2	neurofibromatosis type 2
NRG-1	neuregulin-1
NSE	neuron specific enolase
Pak1	p21-activated kinase 1
PBS	phosphate-buffered-saline

PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PNST	peripheral nerve sheath tumor
PVDF	polyvinylidene fluoride
TK	tyrosine kinase
Rb	Retinoblastoma
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
RNAi	RNA-interference
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium-dodecyl-sulfate
SCF	stem cell factor
SEK	stress-activated protein kinase
SMDF	sensory and motor neuron-derived factor
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β

INTRODUCTION

NEUREGULIN-1/ERBB SIGNALING IN SCHWANN CELL NEOPLASMS: A RECAPITULATION OF DEVELOPMENT?

Dysregulated Schwann cell proliferation is a feature common to neurofibromas, malignant peripheral nerve sheath tumors (MPNSTs), and schwannomas; thus, identifying growth factors that promote neoplastic Schwann cell proliferation may provide important insights into the mechanisms that drive Schwann cell neoplasia. A consideration of the growth factors known to promote mitogenesis during Schwann cell development suggests that neuregulin-1 (NRG-1) growth and differentiation factors may be among the growth factors active in peripheral nerve sheath tumors (PNSTs) (1-11). This hypothesis is further supported by the previous identification of a unique mitogenic activity in human PNSTs, the role of NRG-1/erbB signaling in rodent MPNST cell lines, and the phenotype of a NRG-1 overexpressing transgenic mouse model recently developed in our laboratory (12-14).

During development, the proliferation of Schwann cells/Schwann cell precursors is dependent on axon-derived trophic signals (10). Purification of axon-derived extracts led to the partial purification of a Schwann cell mitogenic activity known as glial growth factor (GGF) (15-22). The partially purified GGF activity contained three proteins designated GGF-I, -II, and -III (23). Purification and molecular cloning identified gene products derived from the NRG-1 locus as the critical component of the axon-derived Schwann cell mitogenic activity and demonstrated that at least part of this ac-

tivity corresponded to GGF-II (24). In current use, NRG-1 is the consensus name for a series of molecules known by several names, including heregulin, neu differentiation factor (NDF), GGF, acetylcholine receptor inducing activity (ARIA), and sensory and motor neuron-derived factor (SMDF) (24-29). Although all NRG-1 isoforms share complete identity in some domains, it is now known that these molecules are structurally diverse. This structural diversity results from extensive alternative splicing and the use of at least three alternative promoters (29).

It has been established, both in vitro and in vivo, that developmental Schwann cell proliferation, survival, and differentiation is dependent on NRG-1 isoforms and their receptors, erbB2 and erbB3 (1-12, 30-34). For example, NRG-1β isoforms are potent mitogens for cultured neonatal Schwann cells (5-9). In addition, NRG-1 isoforms have been shown to drive development of neural crest cells toward a glial phenotype (11). In vivo studies of transgenic mice have elucidated the requirement for NRGs and their receptors in Schwann cell development. At E10.5, NRG-1 knockout mice are deficient in the number of Schwann cell precursors (30). ErbB2 and erbB3 knockouts have a similar phenotype (31-34). Taken together, these in vitro and in vivo experiments have established the role of NRG-1 in Schwann cell development.

SCHWANN CELL NEOPLASMS CONTAIN A GGF-LIKE ACTIVITY

In 1986, Brockes et al. reported the partial purification of a GGF-like activity in multiple schwannomas (also known as acoustic neuromas) and a single neurofibroma (12). As noted above, it has been established that NRG-1 gene products account for at least the GGF-II activity, suggesting that the GGF-like activity in human schwannomas

and neurofibromas is NRG-1. However, an alternative possibility is that the schwannoma- and neurofibroma-derived GGF-like activity represents another NRG-1-related molecule such as the recently discovered NRG-2, NRG-3, or NRG-4 growth factors (35-37). Thus, I consider the findings of Brockes et al (12) as strongly suggestive of the possibility that NRG-1 promotes neoplastic Schwann cell proliferation. Confirming this hypothesis required that we definitively establish the molecular identity of the GGF-like activity present in Schwann cell tumors. This was a major motivation for the studies delineated in the third and fourth articles in this dissertation.

THE ROLE OF NRG-1 IN NEOPLASIA: EVIDENCE FROM TRANSGENIC AND CELL CULTURE STUDIES

Studies in rodent models also support the hypothesis that inappropriate NRG-1/erbB signaling promotes the formation of PNSTs (13, 38-47). A series of studies over the last four decades have shown that treating rats, mice, and hamsters in utero with the chemical mutagen *N*-ethyl-*N*-nitrosourea (EtNU) results in the formation of neoplasms resembling human MPNSTs (38-47). These tumors frequently carry an activating mutation in erbB2, one of the NRG-1 receptor subunits (41, 45). Our laboratory has examined the role of NRG-1/erbB signaling in JS1 cells, a line derived from an EtNU-induced rat MPNST. We found that the JS1 cells express multiple NRG-1 isoforms in combination with the NRG-1 receptor erbB3 and a nonmutated form of erbB2. The erbB kinases expressed by JS1 cells were constitutively tyrosine phosphorylated, indicating that they were constitutively active. Pharmacological inhibition of erbB kinase action abolished this phosphorylation and markedly inhibited JS1 cell proliferation (13). Our laboratory has also generated transgenic mice overexpressing the NRG-1 iso-

form GGFβ3 under the control of a developmentally regulated Schwann cell promoter (the major myelin protein zero [P_0] promoter). P_0-GGFβ3 transgenic animals develop Schwann cell hyperplasia and neoplasms with the histologic, immunohistochemical, and ultrastructural features of human MPNSTs, indicating that overexpression of this NRG-1 isoform promotes MPNST development in this transgenic mouse model system (14). These compelling results were another strong impetus for the studies presented in this dissertation.

SCHWANN CELL NEOPLASMS ARE DEPENDENT ON NRG-1/ERBB SIGNALING

The body of work presented in this dissertation investigates the mechanisms of neoplastic Schwann cell proliferation in neurofibromas, MPNSTs, and schwannomas, with a specific focus on the trophic factor neuregulin-1 (NRG-1) and its receptors (erbB-2, -3, and -4). Based on the functions of NRG-1, I hypothesized that NRG-1 gene product(s) account for the GGF-like activity detected in Schwann cell neoplasms, and that this activity may act in an autocrine, paracrine, and/or juxtacrine fashion to promote proliferation in Schwann cell neoplasia. To investigate the mechanisms of NRG-1/erbB signaling in Schwann cell neoplasia, I have performed a series of analyses in human Schwann cell neoplasms. The results of these studies indicate that NRG-1 isoforms and the NRG-1 receptors (erbB -2, -3 and -4) are expressed in Schwann cell neoplasms, and contribute to peripheral nerve sheath tumorigenesis.

TUMOR SUPPRESSOR MUTATIONS AND GROWTH FACTOR SIGNALING IN THE PATHOGENESIS OF *NF1*-ASSOCIATED PERIPHERAL NERVE SHEATH TUMORS. I. THE ROLE OF TUMOR SUPPRESSOR MUTATIONS

by

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ABSTRACT

Patients with neurofibromatosis type 1 (*NF1*), a common autosomal dominant tumor predisposition syndrome, develop benign cutaneous, intraneural, and plexiform neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs), an aggressive form of Schwann cell neoplasm that frequently arises from plexiform neurofibromas. Impressive advances have been made in defining the molecular mechanisms responsible for neurofibroma and MPNST tumorigenesis, including the identification of key tumor suppressor gene mutations, an improved understanding of the functions of these tumor suppressors, and the production of transgenic mouse models in which tumor suppressor gene mutations predispose animals to the development of neurofibromas and MPNSTs. It has also become apparent that dysregulated growth factor signaling cooperates with tumor suppressor mutations to promote neurofibroma and MPNST tumorigenesis. In Part I of this two-part review, we consider findings demonstrating that Schwann cells are the primary neoplastic cell type in neurofibromas and MPNSTs and that specific tumor suppressor gene mutations promote the development of these tumors. In Part II, which will be published in a later issue, we will review evidence indicating that inappropriate growth factor signaling contributes to this process by stimulating the proliferation, survival, and migration of Schwann cells whose regulatory mechanisms have been crippled by a loss of tumor suppressor function.

INTRODUCTION

Neurofibromatosis type 1 (*NF1*) is an autosomal dominant tumor predisposition syndrome that occurs in approximately 1 in 3,500 newborn infants worldwide (1-3).

The manifestations of *NF1* are protean and include pigmentary lesions (café-au-lait macules, axillary freckling, and Lisch nodules), the development of several tumor types (optic gliomas, pheochromocytomas, and juvenile chronic myeloid leukemia), bony dysplasias, and learning disabilities. The hallmark of *NF1*, however, is the occurrence of multiple neurofibromas. Neurofibromas are benign PNSTs that present as fleshy nodules in skin (dermal neurofibromas), circumscribed masses in nerves (intraneural or nodular neurofibromas), or diffuse lesions spreading through multiple fascicles of large nerves or nerve plexuses (plexiform neurofibromas). These neoplasms, particularly the plexiform variants, can cause significant dysfunction in *NF1* patients, producing disfigurement, pain, and neurologic deficits (4).

Plexiform neurofibromas may also become quite large, sometimes involving entire body segments or limbs. These large plexiform neurofibromas develop an extensive vascular network and elaborate factors that stimulate the growth of adjacent bone and soft tissue, further impairing the function of the affected limb or body segment. Plexiform and, less commonly, intraneural neurofibromas may also undergo transformation to MPNSTs, the most common malignancy developing in *NF1* patients. Overall, *NF1* patients have a 10% lifetime risk of developing MPNSTs and *NF1* patients with symptomatic plexiform neurofibromas may have a risk as high as 30% (5). The prognosis for patients with MPNSTs is poor, with overall 5- and 10-year survival rates of 34% and 23%, respectively (6, 7). Surgery, ideally resulting in complete tumor resection, is the primary treatment for MPNSTs (8). Radiotherapy can help control local disease and delay recurrence, but has little effect on overall survival (5). Chemotherapy has, at best, a modest effect on the survival of patients with MPNSTs (8).

Since neurofibromas and MPNSTs produce considerable morbidity and mortality in *NF1* patients, there is great interest in elucidating the molecular mechanisms responsible for the development of these tumors. Over the past two decades, significant advances have been made in our understanding of the role tumor suppressor gene mutations play in the pathogenesis of neurofibromas and MPNSTs. However, it is widely held that tumor suppressor mutations alone are insufficient for the formation of PNSTs and that these mutations must cooperate with dysregulated growth factor signaling to promote the proliferation, survival, and migration of neoplastic Schwann cells. At present, our understanding of the role of dysregulated growth factor signaling in neurofibroma and MPNST pathogenesis is much more limited than our knowledge of the role tumor suppressor mutations play in their development. This is unfortunate, given that growth factor receptor inhibitors such as Herceptin (trastuzumab; an anti-erbB2 antibody useful in treating patients with c-*neu* overexpressing breast cancers) and Gleevec (STI-571, imatinib; used to treat chronic myeloid leukemia and gastrointestinal stromal tumors) have proven quite effective in treating other tumor types.

Our goals in the first part of this two-part review are to i) examine evidence that Schwann cells are the primary neoplastic cell type in neurofibromas and MPNSTs and, (ii) consider findings in human neoplasms and animal models that indicate that specific tumor suppressor mutations occur in neoplastic Schwann cells and promote neurofibroma and MPNST tumorigenesis. The second part of this review, which will be published in a subsequent issue, will examine evidence implicating specific growth factors and growth factor receptors in the pathogenesis of neurofibromas and MPNSTs, focusing particularly on findings that shed light on the action of each growth factor on neo-

plastic Schwann cells. We will also suggest some future lines of investigation that will likely be important for the development of new treatments for neurofibromas and MPNSTs.

EVIDENCE THAT SCHWANN CELLS ARE THE PRIMARY NEOPLASTIC CELL TYPE IN *NF1*-ASSOCIATED PERIPHERAL NERVE SHEATH TUMORS

Neurofibromas are composed of multiple cell types including Schwann cells, mast cells, perineurial cells, fibroblasts, and endothelial cells (see Fig. 1 for a comparison of the histology of normal nerve and the three major tumor types that develop in peripheral nerve). The histogenesis of neurofibromas has long been controversial, in large part because of the significant cellular heterogeneity observed in these neoplasms. However, it is now generally accepted that Schwann cells represent the primary neoplastic cell type in neurofibromas. Forty to 80% of the cells in neurofibromas are Schwann cells (9) and these glia demonstrate several functional abnormalities. Schwann cells isolated from neurofibromas, unlike normal Schwann cells or neurofibroma-derived fibroblasts, are invasive (10, 11) and promote angiogenesis (10). Neurofibroma-derived Schwann cells do not form colonies in soft agar (a measure of tumorigenicity and anchorage-independent growth) or masses when grafted subcutaneously into immunodeficient (nude) mice. Unlike normal Schwann cells, however, Schwann cells isolated from neurofibromas do survive and show infiltrative growth when xenografted into the sciatic nerve of nude mice (12). Cytogenetic abnormalities, when identified, are found in the Schwann cell component of neurofibromas (13). MPNSTs derived from plexiform neurofibromas (as well as sporadically occurring

MPNSTs) frequently demonstrate histologic features associated with Schwann cells, including immunoreactivity for the Schwann cell marker S100β, expression of long-spacing fibrous collagen (Luse bodies), and partial investment of tumor cells by basement membrane (14). Considered together with studies of tumor suppressor gene mutations (see below), these findings argue that Schwann cells are the primary neoplastic cell type in neurofibromas and MPNSTs.

Although the central role Schwann cells play in peripheral nerve sheath tumorigenesis is becoming increasingly clear, the origin of these tumor cells remains poorly understood. Given their morphologic and immunohistochemical features, it is tempting to speculate that these tumor elements are derived from mature Schwann cells or from immature Schwann cell-like elements persistently present in the peripheral nervous system. Alternatively, neoplastic Schwann cells in neurofibromas and MPNSTs may arise from multipotent neural crest stem cells, which have been identified in mammalian fetal peripheral nerve (15); this latter possibility is potentially consistent with the observation that some MPNSTs demonstrate focal divergent differentiation (e.g. the presence of a rhabdomyosarcomatous component in the MPNST variant known as a malignant Triton tumor). At present, our ability to distinguish between these possibilities is hampered by our limited understanding of normal Schwann cell development and a paucity of phenotypic markers that can be used to define neurofibroma- and MPNST-associated Schwann cells.

TUMOR SUPPRESSOR MUTATIONS IN PERIPHERAL NERVE SHEATH TUMORS

Mutations of the *NF1* Locus in Neoplastic Schwann Cells: A Key Early Step in the Pathogenesis of Neurofibromas and MPNSTs Resulting in Increased Ras Activity

NF1 patients carry a constitutional mutation in the *NF1* locus, a tumor suppressor gene that is located on the long arm of chromosome 17 (17q11.2). This mutation may be inherited from a parent, but the *NF1* locus has a relatively high mutation rate and up to 50% of *NF1* cases represent new mutations (4). In keeping with Knudson's "two-hit" hypothesis (9), it is believed that neoplasms develop in *NF1* patients when a somatic mutation disrupts the remaining functional copy of the *NF1* allele. Consistent with this postulate, loss of heterozygosity (LOH) of the *NF1* locus is evident in both neurofibromas and MPNSTs (2, 5, 9, 16-19), occurring in Schwann cells, but not fibroblasts, isolated from these neoplasms (13, 20-22). Fluorescent in situ hybridization (FISH) analyses of plexiform neurofibromas and MPNSTs confirm that *NF1* deletions are found in S100β-immunoreactive Schwann cells in vivo as well (23). The observation that biallelic inactivation of *NF1* occurs in Schwann cells in both neurofibromas and MPNSTs suggests that mutation of the remaining functional copy of the *NF1* gene is a key early step in the development of these neoplasms.

NF1 mutations have important functional consequences in Schwann cells. Neurofibroma derived Schwann cells lacking expression of neurofibromin, the product of the *NF1* locus, have a growth advantage over neurofibromin-positive Schwann cells in vitro and in vivo (12). Schwann cells derived from *Nf1* null mice (see below) also show increased chemotactic and chemokinetic migration relative to normal Schwann cells (24), consistent with the infiltrative behavior of neurofibromas and MPNSTs. As our

understanding of the functions of neurofibromin has grown, the mechanisms underlying these altered Schwann cell responses have become increasingly clear. Neurofibromin is a large (~220 to 250 kD) protein composed of multiple functional domains, including a GTPase activating protein (GAP)-related domain. Neurofibromin is thought to act, at least in part, by negatively regulating the activity of Ras proteins, a family of small G-proteins that act as a key regulators of mitogenesis and other cellular responses (19). Ras proteins are essentially binary switches which get turned "on" when they bind GTP. Neurofibromin turns this switch "off" by using its GAP-related domain to stimulate an intrinsic GTPase activity in Ras proteins, thereby accelerating the cleavage of GTP to GDP. Consistent with this action, loss of *NF1* function is associated with increased levels of activated (GTP-associated) Ras proteins in neurofibroma (22) and MPNST (25,26) cells.

The Increase in Ras Activity Resulting From Neurofibromin Loss Activates Key Signaling Cascades Mediating Mitogenesis, Migration, and Transformation

Three "classical" neurofibromin-regulatable Ras proteins (H-, N-, and K-Ras) are known. All three of these proteins are expressed in wild type and *Nf1* null (-/-) mouse Schwann cells (24) and likely contribute to neoplasia. H-Ras appears to play a particularly important role as farnesyltransferase inhibitors, which inhibit the activation of H-Ras while sparing N- and K-Ras, interfere with the abnormal proliferation of *Nf1*-/- Schwann cells (27). There is little evidence to indicate that activating Ras mutations occur in neurofibromas and MPNSTs. H-, N-, and K-Ras are instead likely activated when growth factors, such as those that will be discussed in Part II of this review, bind to and activate integral membrane tyrosine kinase receptors expressed by neoplas-

tic Schwann cells. Growth factor-activated Ras proteins in turn stimulate the activation of at least two mitogen-activated protein (MAP) kinase cascades (Fig. 2). The first of these cascades, the Ras-Raf-MEK1/2-ERK1/2 pathway, plays an essential and well-established role in promoting mitogenesis and the transformation of several cell types, including Schwann cells (28). Ras also activates Rac and Cdc42, two members of the Rho family of small G-proteins, leading to the sequential activation of p21-activated kinase 1 (Pak1), MAPK/ERK kinase kinase (MEKK), stress-activated protein kinase (SEK) and Jun N-terminal kinase (JNK; also known as stress-activated protein kinase or SAPK). JNK regulates the action of Jun and multiple other transcription factors that are essential for the control of mitogenesis and survival in nonneoplastic cells (29). Activation of JNK is required for Ras-induced transformation in several cell types (29) and for in vivo oncogenesis in at least some settings (30); although potentially important in the pathogenesis of neurofibromas and MPNSTs, the precise role JNK plays in these neoplasms remains to be defined.

One protein in the Rac signaling cascade, Pak1, is additionally notable for its ability to interact with other signaling cascades. Pak1 is clearly an essential mediator of Ras action in neoplastic Schwann cells as dominant negative Pak1 mutants block Ras-mediated transformation of T antigen-immortalized rat Schwann cells and inhibit the ability of human ST88-14 MPNST cells to form colonies in soft agar and tumors in nude mice (28). Although Pak1 activates JNK in these cells, its promotion of Ras-mediated transformation is dependent on "cross-talk" with the Ras-Raf-MEK1/2-ERK1/2 pathway rather than activation of JNK (28); this reinforcement of ERK signaling is thought to promote a persistent activation of ERK that is necessary for transfor-

mation. Pak1 also forms part of a "feedforward" signaling loop (Fig. 2) in which merlin, a tumor suppressor produced by the neurofibromatosis type 2 (*NF2*) locus, inhibits Pak1 action (31), and activated Pak1, in turn, inhibits merlin (32). The regulatory interactions of Pak1 and merlin are likely disrupted in at least a subset of MPNSTs, as deletions of the region of chromosome 22 encoding merlin (band 22q12.2) were detected in 30% (33) and 45% (34) of the MPNSTs studied in two series. Consistent with the hypothesis that *NF2* loss contributes to the pathogenesis of human MPNSTs, mice with Schwann cell specific ablation of *Nf2* develop MPNSTs at a low frequency (35) and animals lacking both *Nf2* and *p53* develop multiple MPNST-like neoplasms (36).

In contrast to its effects on proliferation, expression of an H-Ras dominant negative mutant (which inhibits the action of H-, N- and K-Ras) in *Nf1*-/- mouse Schwann cells does not reduce the enhanced migration evident in these glia (24). Based on this observation, it was concluded that classical Ras proteins make little contribution to enhanced migration in neoplastic Schwann cells and that other neurofibromin-regulated proteins must mediate these responses. Consistent with this hypothesis, neurofibromin also stimulates the GTPase activity of nonclassical Ras proteins such as R-Ras and TC21/R-Ras2 (37), two small G-proteins that have been implicated in the control of cell motility in other systems (38). Although the contribution R-Ras makes to the increased mobility of *Nf1*-/- Schwann cells remains poorly understood, TC21/R-Ras2 is essential for increased migration in Schwann cells lacking neurofibromin, acting through a signaling pathway that includes phosphatidylinositol 3-kinase to mediate this effect (Fig. 2). *Nf1*-/- Schwann cells also secrete an as yet unidentified factor that enhances the chemotaxis of neoplastic Schwann cells and do so at levels greater than ei-

ther wild-type or *Nf1* haploinsufficient (+/-) Schwann cells (24). Loss of neurofibromin expression therefore enhances the migration of neoplastic Schwann cells both by activating a TC21/R-Ras2-dependent signaling cascade that promotes migration and by enhancing the secretion of factors that act in an autocrine or paracrine fashion to promote Schwann cell migration.

At present, the functions of other neurofibromin domains are poorly understood. Neurofibromin has been shown to regulate cyclic AMP (cAMP) dependent signaling pathways (39), but the significance that loss of this activity has for Schwann cell neoplasia is unclear.

Mutations of *p53* and Cell Cycle Regulatory Pathway Genes Accumulate as Neurofibromas Progress to Become MPNSTs

As *NF1* deletions occur in both neurofibromas and MPNSTs, it is evident that *NF1* mutations alone are not sufficient for MPNST pathogenesis and that additional mutations are required for progression from neurofibroma to MPNST. In at least some MPNSTs, these additional mutations include loss-of-function mutations of the *p53* tumor suppressor, a molecule controlling cell cycle progression and cell death that is mutated in more than half of human cancers (40). Deletions and other mutations of the *p53* locus are common in MPNSTs, being found in 29% to 75% of the tumors studied in three series (41-43). Nuclear accumulation of *p53* protein, a finding that can be associated with ineffective *p53* action occurring secondary to mutations in *p53* functional domains (particularly the DNA binding domain), has been identified in MPNSTs in some studies (44-46); others, however, have found a poor correlation between *p53* immunoreactivity and mutations of this tumor suppressor (43).

Mutations of genes that regulate cell cycle progression also accumulate in MPNSTs. The *INK4A* (also known as *CDKN2A/p16*) gene is located on the short arm of chromosome 9 (9p21), a region that is deleted or otherwise altered in up to 75% of MPNSTs (47, 48). This locus encodes both $p16^{INK4A}$, a protein that arrests cells in the G1 phase of the cell cycle by inhibiting cyclin-dependent kinases 4 (CDK4) and 6 (CDK6), and $p19^{ARF}$, a polypeptide that binds to Mdm2 and prevents it from degrading *p53*. Immunoreactivity for $p16^{INK4A}$ is evident in virtually all neurofibromas (49). In contrast, the $p16^{INK4A}$ antigen is commonly undetectable in MPNSTs and deletions of the *INK4A* gene were identified in 50% of the MPNSTs studied in two series (49, 50). The expression of $p27^{kip1}$, a cyclin-dependent kinase inhibitor that controls G1/S progression, is also altered in MPNSTs. Nuclear $p27^{kip1}$ immunoreactivity is prominent in neurofibromas, while this protein accumulates in the cytoplasm of MPNST cells (46).

It has been suggested that this alteration results from epigenetic phenomena rather than mutations of the $p27^{kip1}$ gene. The Retinoblastoma (Rb) protein, a tumor suppressor that impedes cell cycle progression and becomes inactivated when phosphorylated by CDK4/6 kinases, may contribute to peripheral nerve sheath tumorigenesis in some cases. Rb protein is undetectable in a small subset of MPNSTs, with LOH of the Rb locus evident in some of these same tumors (45). It remains to be determined whether Rb mutations are more common than indicated by initial studies and at what stage they contribute to peripheral nerve sheath tumorigenesis.

Neurofibromas and MPNSTs Developing in Transgenic Mice with Null Mutations of the *Nf1* and *p53* Tumor Suppressor Genes.

Shortly after the identification of the *NF1* locus, several laboratories began to develop transgenic mice with null mutations of the murine Nf1 gene for the purpose of gaining further insight into neurofibroma and MPNST pathogenesis. Mice homozygous for a germline *Nf1* "knock-out" mutation (*Nf1*-/- mice) die in utero secondary to cardiac malformations (double outlet right ventricle and endocardial cushion defects) (51-53) and, in some cases, exencephaly (54). Mice heterozygous for the *Nf1* mutation (*Nf1*+/- mice) are viable, fertile and, like human *NF1* patients, develop pheochromocytomas and myeloid leukemias (52). They do not, however, develop neurofibromas, suggesting that the "second hit" of the *Nf1* locus in murine Schwann cells is rate-limiting, occurring infrequently relative to the occurrence of *Nf1* mutations in murine adrenal medulla and bone marrow. To overcome this difficulty, *Nf1*-/- mouse embryos have been fused with wild-type embryos to produce chimeric mice in which only a portion of their somatic cells are *Nf1*-/-. These chimeric mice, unlike animals with germline *Nf1* mutations, develop multiple tumors resembling human neurofibromas in association with dorsal root ganglia, nerve trunks within limbs or, less commonly, other sites such as the trigeminal nerve; *Nf1*-/- chimeric mice do not develop dermal neurofibromas (55). In tumors arising in chimeric mice, the overwhelming majority of the tumor cells are derived from the *Nf1*-/- embryonic stem cells, indicating that there is minimal recruitment of wild-type cells into these neoplasms.

Although experiments with *Nf1*-/- chimeric mice demonstrated that a loss of neurofibromin predisposes mice to the formation of lesions histologically similar to human neurofibromas, they did not establish the identity of the cell type key to tumor

initiation. In an elegant set of experiments, Parada et al addressed this issue by producing mice with a Schwann cell-specific ablation of the *Nf1* locus (56). Mice with *Nf1*-/- Schwann cells developed microscopic hyperplastic lesions in cranial nerves, but not the other histologic features of neurofibromas. However, when mice with *Nf1*-/- Schwann cells and *Nf1* haploinsufficiency (*Nf1*+/-) in all other cell types were produced, these animals developed neurofibroma-like neoplasms within cranial nerves and spinal cord nerve roots composed of mixtures of the same cell types evident in human neurofibromas. These findings indicate that neurofibroma tumorigenesis requires both a complete loss of neurofibromin in Schwann cells and *Nf1* haploinsufficiency in other, as yet unidentified, cellular elements (56). As a corollary, it is evident that interactions between *Nf1*-/- Schwann cells and other *Nf1*+/- cell types are required for neurofibroma formation.

In addition to the models described above, two laboratories have generated transgenic mice that carry *cis* linked null mutations of the *Nf1* and *p53* loci, rendering them haploinsufficient for both genes (55, 57). In addition to neoplasms previously observed in mice with knockouts of the *Nf1* and *p53* genes alone (lymphomas, osteosarcomas, and hemangiosarcomas), *Nf1/p53* haploinsufficient mice develop soft tissue sarcomas, the majority of which have the histologic and immunohistochemical characteristics of human MPNSTs and malignant Triton tumors (MTTs). Consistent with the hypothesis that *NF1* and *p53* mutations cooperate to promote the progression of neurofibromas to MPNSTs in humans, the MPNST-like tumors developing in mice with *cis* linked *Nf1* and *p53* null mutations demonstrate LOH of the remaining functional alleles of the *Nf1* and *p53* genes. To further investigate the origin of the soft tissue sarcomas

developing in *Nf1/p53* haploinsufficient mice, Vogel et al established permanent cell lines from approximately 70 of these neoplasms and examined their expression of mRNAs encoding neural crest, Schwann cell, and myogenic markers. These cell lines expressed, to varying degrees, all three sets of markers, leading these investigators to propose that the MPNST- and MTT-like neoplasms developing in mice carrying *cis* linked *Nf1* and *p53* null mutations are derived from a multipotent neural crest stem cell (57).

The transgenic neurofibromas and MPNSTs described above were among a group of genetically engineered murine (GEM) neoplasms whose histologic, immunohistochemical, and ultrastructural features were recently reviewed and compared to their human counterparts by a panel of pathologists with expertise in neuropathology and soft tissue neoplasms (58). Based on these comparisons, a grading scheme was proposed in which GEM neoplasms were classified as Grade I (tumors with low cellularity, bland/uniform cytology, and no mitoses or necrosis), II (tumors with increased cellularity, nuclear atypia and some mitotic activity), or III (tumors with marked cellularity, atypia, high mitotic activity, and areas of hemorrhage or necrosis). This group also specified that GEM PNSTs should be designated, based on their cellular composition, as GEM schwannomas, GEM neurofibromas, GEM perineuriomas, or GEM PNSTs (tumors arising in peripheral nerves with some immunohistochemical or ultrastructural evidence of schwannian differentiation that do not fit into the preceding categories). This group concluded that the pathology of the mouse neurofibromas and MPNSTs described above was highly similar to that of their human counterparts and classified them as GEM Grade I neurofibromas and GEM Grade III PNSTs, respec-

tively; the term "malignant" was not be applied to the MPNST-like neoplasms arising in transgenic mice as their clinical behavior has not yet been adequately defined.

SOME UNRESOLVED QUESTIONS AND CONCLUSIONS

Over the last 15 years, a coherent picture of the genetic abnormalities responsible for the pathogenesis of *NF1*-associated neurofibromas and MPNSTs has begun to emerge. There is now strong evidence that Schwann cells in *NF1* patients are the primary neoplastic cell type in neurofibromas. The Schwann cells in these tumors, which are already haploinsufficient for *NF1*, become neoplastic when they lose the function of their remaining *NF1* allele, an event that results in the activation of several Ras-dependent signaling pathways that regulate mitogenesis and migration and promote transformation. These Schwann cell changes are not themselves sufficient for the development of neurofibromas; NF1-/- Schwann cells must interact with other NF1+/- cell types (mast cells, perineurial cells, fibroblasts and/or Schwann cells) to form a neurofibroma. Abnormalities of additional tumor suppressor genes accumulate in MPNSTs, some of which (*p53*, *INK4A*, $p27^{kip1}$) have been identified. Considered together, the studies cited above support a multistage model of tumor progression in which mutation of both Schwann cell *NF1* alleles leads to the development of a neurofibroma and the subsequent accumulation of mutations or other abnormalities involving additional tumor suppressor genes (i.e., *p53*, *INK4A* and $p27^{kip1}$) is necessary for progression to MPNST (Fig. 3).

Although the evidence cited above is compelling, we must emphasize that this story is incomplete. It is likely that several other tumor suppressors and oncogenes

relevant to peripheral nerve sheath tumorigenesis remain to be identified. Cytogenetic analyses of MPNSTs have shown that these neoplasms frequently have near-triploid or hypodiploid karyotypes and show losses (1p, 9p, 11, 12p, 14q, 17q, 18, 22q, X, and Y) and gains (chromosome 7) in several chromosomal regions (47, 59); some of these alterations, as well as other abnormalities, have also been identified using comparative genomic hybridization (60-65). Although some of the regions of chromosomal loss contain known tumor suppressors (e.g. the *INK4A* locus in 9p, the *NF1* gene in 17q and the *NF2* gene in 22q), others do not and at least one chromosomal region, 1p, has been suggested to contain an unidentified tumor suppressor gene that contributes to MPNST pathogenesis (66). In addition, the chromosomal rearrangements, gains, and losses observed in MPNSTs are highly variable and differ between individual tumors (13), suggesting that there may be more than one pathway that can lead to the development and progression of these neoplasms. In support of this latter hypothesis, some human MPNST cell lines have been found to express functional *p53* (67); a subset of these lines express activated Notch receptors, suggesting the existence of an alternative, Notch-dependent, pathway for progression from neurofibroma to MPNST. Identifying other oncogenic molecules and tumor suppressors contributing to peripheral nerve sheath pathogenesis and deciphering alternative neoplastic pathways will be highly important if new, more effective therapies for neurofibromas and MPNSTs are to be developed.

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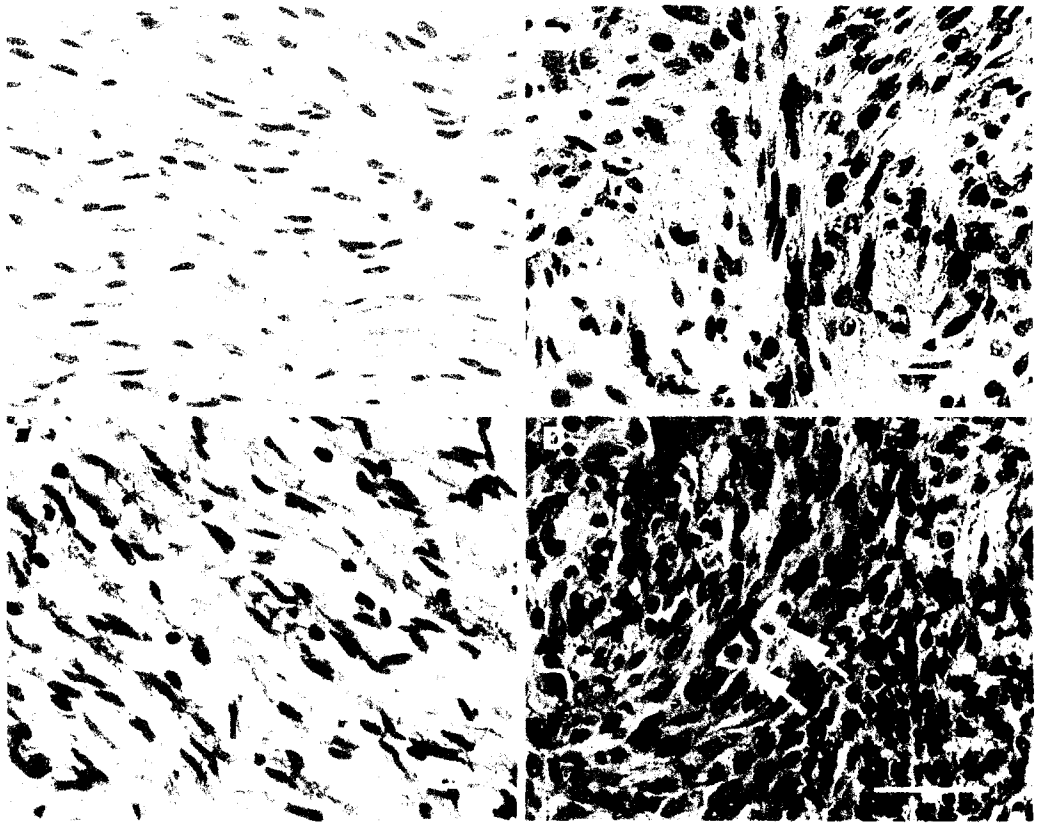


FIGURE 1. Comparison of the histology of normal human peripheral nerve, schwannomas, neurofibromas and MPNSTs. **(A)** Sensory nerve adjacent to a thoracic dorsal root ganglion showing an orderly arrangement of Schwann cells associated with and myelinating axons. **(B)** Schwannoma arising in a thoracic spinal nerve root from a 22 year old woman. Shown is an area of densely packed neoplastic Schwann cells (Antoni A pattern); unlike neurofibromas, schwannomas are almost completely composed of Schwann cells and compress, rather than infiltrate, the nerve in which they develop. **(C)** Neurofibroma developing in a lumbar spinal nerve root from a 56 year old woman with *NF1*. The infiltrative nature of this neoplasm is demonstrated by the presence of entrapped myelinated axons (indicated by the arrow; note the "neurokeratin" artifact [herringbone pattern] produced by formalin fixation of the myelin sheath). **(D)** MPNST resected from a 56-year-old man with *NF1*. Note the numerous mitotic figures evident in this tumor (arrows). Scale bar for A-D: 50 μm.

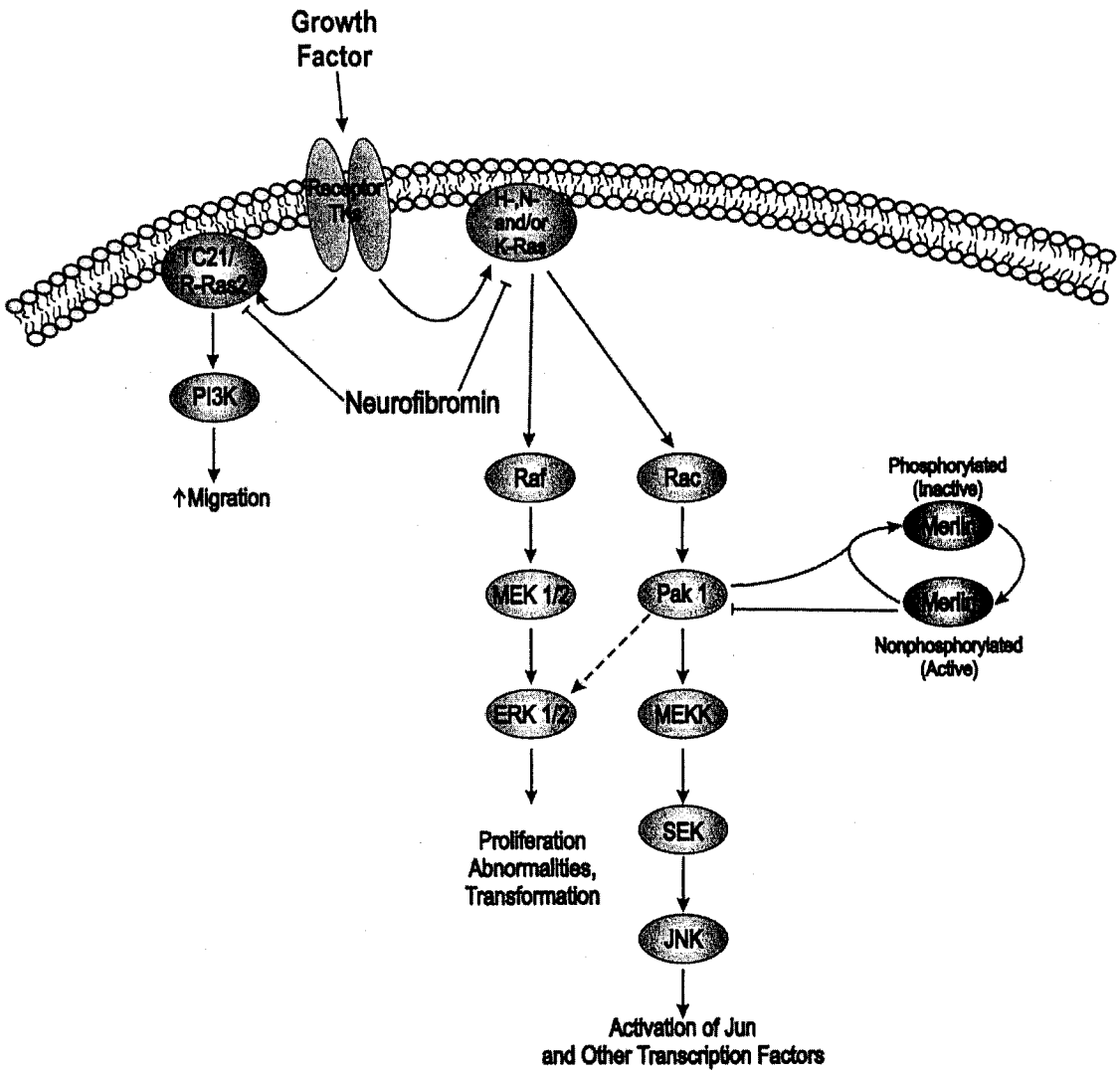
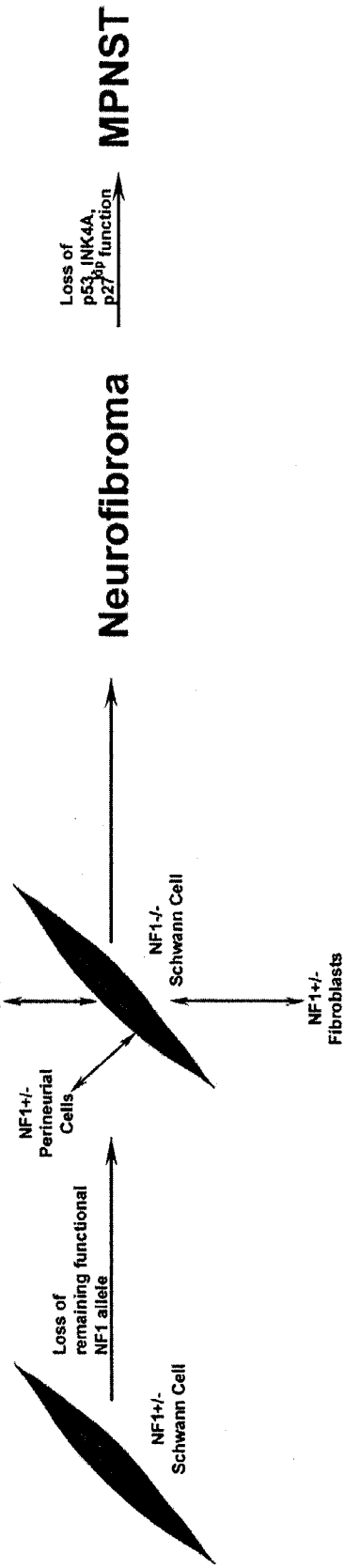


FIGURE 2. Ras-dependent signaling pathways affected by neurofibromin loss in neoplastic Schwann cells. Growth factors that may activate these signaling pathways include the molecules that will be discussed in Part II of this review. The cross-activation of ERK 1/2 by Pak1 is indicated by a dashed arrow as Pak1 is also capable of phosphorylating Raf and MEK 1/2 and the level at which Pak1 cross-talks with Ras-Raf-MEK-ERK cascade is not yet clear. We would also emphasize that it is likely that other, as yet unidentified, molecules participate in some of these signaling cascades. Abbreviations: Receptor TKs, receptor tyrosine kinases; PI3K, phosphatidylinositol 3-kinase; MEK, mitogen-activated kinase (MEK)/extracellular signal-regulated kinase (ERK) kinase; MEKK, MAPK/ERK kinase kinase; SEK, stress-activated protein kinase; JNK, Jun N-terminal kinase.

FIGURE 3. The development of neurofibromas and their subsequent progression to become malignant peripheral nerve sheath tumors is associated with a series of tumor suppressor gene mutations. The process is initiated when a Schwann cell or Schwann cell precursor (*NF1*+/- Schwann cell) in an *NF1* patient undergoes loss of the remaining functional *NF1* allele. A process involving as yet undefined interactions with other haploinsufficient cell types in the nerve then leads to the formation of a neurofibroma. The subsequent loss or abnormal function of other tumor suppressor genes including *p53*, *INK4A* and $p27^{kip}$ results in the malignant transformation of *NF1*-/- Schwann cells in the neurofibroma and the development of an MPNST.



TUMOR SUPPRESSOR MUTATIONS AND GROWTH FACTOR SIGNALING IN THE PATHOGENESIS OF *NF1*-ASSOCIATED PERIPHERAL NERVE SHEATH TUMORS II. THE ROLE OF DYSREGULATED GROWTH FACTOR SIGNALING

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ABSTRACT

Patients with neurofibromatosis type 1 (NF1), one of the most common genetic disease affecting the nervous system, develop multiple neurofibromas that can transform into aggressive sarcomas known as malignant peripheral nerve sheath tumors (MPNSTs). Studies of human tumors and newly developed transgenic mouse models indicate that Schwann cells are the primary neoplastic cell type in neurofibromas and MPNSTs and that development of these PNSTs involves mutations of multiple tumor suppressor genes. However, it is widely held that tumor suppressor mutations alone are not sufficient to induce PNST formation and that dysregulated growth factor signaling cooperates with these mutations to promote neurofibroma and MPNST tumorigenesis. In Part I of this review, we discussed findings demonstrating that a loss of *NF1* tumor suppressor gene function in neoplastic Schwann cells is a key early step in neurofibroma formation and that progression from neurofibroma to MPNST is associated with abnormalities of additional tumor suppressor genes, including *p53*, *INK4A* and $p27^{kip1}$. In Part II of this review, we consider evidence that dysregulated signaling by specific growth factors and growth factor receptors promotes the proliferation, migration, and survival of neoplastic Schwann cells in neurofibromas and MPNSTs.

INTRODUCTION

Neurofibromatosis type 1 (*NF1*) is one of the most common genetic diseases involving the nervous system, affecting approximately 1 in 3,500 infants (1-3). In addition to pigmentary lesions (café-au-lait macules, axillary freckling, and Lisch nodules), bony dysplasias, and learning disabilities, individuals with this autosomal dominant

tumor predisposition syndrome may develop several types of neoplasms, including pheochromocytomas, optic gliomas, and juvenile chronic myeloid leukemia. The most distinctive characteristic of *NF1* patients, however, is the occurrence of multiple neurofibromas. Neurofibromas are tumors of peripheral nerve and are composed of Schwann cells intermingled with smaller numbers of fibroblasts, mast cells, perineurial cells, and vascular elements. These nerve sheath tumors manifest themselves as fleshy nodules in skin (dermal neurofibromas), circumscribed masses in nerves (intraneural or nodular neurofibromas), or lesions growing diffusely through multiple fascicles of large nerves or nerve plexuses (plexiform neurofibromas). Although benign, neurofibromas cause significant disfigurement, pain, and neurologic deficits. Further, plexiform and, less commonly, intraneural neurofibromas can transform into highly aggressive sarcomas known as malignant peripheral nerve sheath tumors (MPNSTs). Beyond surgery, few therapeutic options are available for patients with MPNSTs; consequently, the prognosis for individuals with these neoplasms is poor. In light of the significant morbidity and mortality neurofibromas and MPNSTs produce in *NF1* patients, there is considerable interest in defining the molecular mechanisms underlying the pathogenesis of these tumors, with the hope that this will lead to the development of new, more effective therapies.

As discussed in Part I of this review (4), studies of human tumors and recently developed transgenic mouse models indicate that Schwann cells are the primary neoplastic cell type in neurofibromas and that the pathogenesis of neurofibromas and MPNSTs results, at least in part, from loss-of-function mutations of tumor suppressor genes in Schwann cells. However, a growing body of evidence indicates that dysregu-

lated signaling by growth factors and their receptors acts cooperatively with tumor suppressor mutations to promote PNST formation. Defining the precise role specific growth factors play in neurofibromas and MPNSTs has recently acquired practical importance as a number of growth factor receptor inhibitors have proved effective in treating other tumor types (e.g., Herceptin [trastuzumab; an erbB2 inhibitory antibody used to treat patients with *c-neu* overexpressing breast cancers) and Gleevec (STI-571, imatinib; used to treat chronic myeloid leukemia and gastrointestinal stromal tumors]) and could potentially be adapted to treat PNSTs arising in *NF1* patients. Our goal in Part II of this review is to examine evidence indicating that specific growth factors and growth factor receptors contribute to neurofibroma and MPNST tumorigenesis, focusing particularly on findings that shed light on the action each of these molecules has on neoplastic Schwann cells.

EVIDENCE SUGGESTING A ROLE FOR DYSREGULATED GROWTH FACTOR SIGNALING IN *NF1*-ASSOCIATED PERIPHERAL NERVE SHEATH TUMORS

Coincident with the search for the *NF1* tumor suppressor gene during the 1980s and early 1990s, several lines of investigation began to indicate that neurofibromas and MPNSTs elaborate growth factors that contribute to their own development. For instance, crude extracts of *NF1*-associated neurofibromas were found to contain an activity that promoted the growth of cultured neurofibroma explants (5), and plasma from *NF1* patients (but not normal controls) was demonstrated to stimulate the proliferation of cultured cells derived from neurofibromas (6). Spurred by observations such as these, multiple laboratories initiated studies that culminated in the identification of

growth factors and growth factor receptors that are aberrantly expressed in neurofibromas and MPNSTs and promote Schwann cell mitogenesis, migration, and/or survival (see below for details on specific factors). Depending on their cellular source, these growth factors may stimulate neoplastic Schwann cells in an autocrine, paracrine, or endocrine fashion.

The identification of the tumor suppressor genes that are mutated in neurofibromas and MPNSTs and a consideration of the functional requirements of the affected signaling pathways provided additional, albeit indirect, evidence that dysregulated growth factor signaling promotes peripheral nerve sheath tumorigenesis. *NF1* patients carry a constitutional mutation of the *NF1* tumor suppressor gene, and inactivation of the remaining functional *NF1* allele appears to be an essential early event in the development of neurofibromas (4). Neurofibromin, the product of the *NF1* locus, facilitates the inactivation of Ras proteins (Fig. 1), a family of small G-proteins that serve as key regulators of mitogenesis, migration, and other cellular responses. Neurofibromin loss in Schwann cells within neurofibromas thus promotes the increased activation of Ras and Ras-activated signaling cascades evident in these neoplasms. Loss-of-function mutations or functional abnormalities of other tumor suppressor genes such as *p53*, *INK4A* and $p27^{kip1}$ also accumulate as neurofibromas transform into MPNSTs and likely cooperate with *NF1* loss to produce the malignant Schwann cell phenotype (4, 7).

Although neurofibromin loss interferes with the cell's ability to inactivate activated Ras, it is clear that complementary mechanisms must exist that serve to activate these G-proteins in the first place. As activating mutations of Ras are rarely, if ever, detected in neurofibromas and MPNSTs, this cannot be the mechanism promoting Ras ac-

tivation in neoplastic Schwann cells. The identification of Schwann cell growth factors in neurofibromas and MPNSTs provided support for an alternative concept, namely, that dysregulated signaling by growth factors promotes tumorigenesis by stimulating the proliferation, migration, and/or survival of Schwann cells whose cytoplasmic signaling (i.e. Ras-dependent; Fig. 1) and cell cycle regulatory mechanisms are defective because of a loss of tumor suppressor gene function.

GROWTH FACTORS AND GROWTH FACTOR RECEPTORS IMPLICATED IN PERIPHERAL NERVE SHEATH TUMORIGENESIS

Over the last three decades, a number of growth factors have been suggested as candidate molecules promoting the development of neurofibromas and MPNSTs. To rigorously establish that these molecules do indeed promote Schwann cell neoplasia, there are criteria that each candidate growth factor should meet. First, the growth factor must be present in the neoplasm in a biologically active form. Second, neoplastic Schwann cells, alone or in combination with other cell types within the PNST, must express the receptor(s) necessary for responsiveness to the growth factor. Third, blocking signaling by the growth factor or its receptor (e.g. using pharmacologic inhibition [preferably with at least two functionally distinct inhibitors], expression of dominant negative mutants, "knock-down" by RNA interference or inhibition with neutralizing antibodies) should reduce the in vitro proliferation, survival, or migration of neoplastic Schwann cells isolated from human neurofibromas or MPNSTs. Fourth, interference with growth factor signaling should reduce the proliferation, survival, migration or metastasis of neoplastic human Schwann cells in in vivo model systems (e.g., tumor cells xenografted into nude mice). Finally, overexpression of the candidate growth factor or

growth factor receptor in Schwann cells of transgenic mice should induce the formation of neurofibroma- or MPNST-like neoplasms that meet the previous four criteria. Demonstrating that stimulation with exogenous growth factor promotes proliferation, migration or survival can also be useful, but is not strictly required as we have encountered situations in which levels of endogenously produced growth factor are sufficient to maximally stimulate tumor cells and addition of exogenous factor produces no further increase in the measured parameter (8). The criteria delineated above may require modification under some circumstances (e.g., neoplasms in which activating mutations or overexpression of growth factor receptors result in ligand-independent activation of the receptor).

At present, the criteria outlined above have not been fully satisfied for any of the growth factors discussed in this review. Below, we consider the evidence that is available for several candidate growth factors and growth factor receptors, beginning with the four systems that have been most extensively studied and then proceeding to several more molecules for which intriguing initial studies are available.

Neuregulin-1 and Its ErbB Receptors

In contrast to the other growth factors discussed below, the hypothesis that neuregulin-1 (NRG-1) and its erbB receptors play a role in peripheral nerve sheath tumorigenesis had its origins in studies of animal models rather than observations in human tumors. Beginning nearly 40 years ago, it was noted that rats (9, 10), mice (11, 12) and hamsters (13) transplacentally exposed to the alkylating agent *N*-ethyl-*N*-nitrosourea (EtNU) frequently developed malignant Schwann cell neoplasms that were

variously referred to as malignant peripheral nerve sheath tumors (14, 15) or malignant schwannomas [e.g., (16)]. Some investigators have described these EtNU-induced PNSTs simply as "schwannomas"; however, we think this designation inappropriate as our experience and that of others (17) is that cell lines derived from these tumors (e.g. JS1 [18] and RN22 [19] cells) proliferate very rapidly and form colonies in soft agar, thus behaving like MPNST cells rather than neurofibroma or schwannoma cells.

A search for oncogenes in EtNU-induced MPNSTs demonstrated the frequent expression of a constitutively activated form of the erbB2 (*c-neu*) membrane receptor tyrosine kinase carrying a point mutation in sequences encoding the transmembrane domain of this molecule (20-25). Consistent with the hypothesis that the mutant erbB2 kinase confers a proliferative advantage on immature Schwann cells (25), transfection of this oncogene into cultured embryonic Schwann cells immortalized and transformed these glia (26). Furthermore, a kinase-deficient erbB2 dominant negative mutant inhibited the proliferation and anchorage-independent growth of EtNU-induced MPNST cells (27). Mutation of erbB2 occurs very early in the development of EtNU-induced MPNSTs, with cells carrying the mutant allele being evident as soon as 7 days after exposure to the carcinogen (25). It is unclear whether tumor suppressor gene mutations occur in EtNU-induced MPNSTs and, if so, whether they mirror those found in human MPNSTs. Although two loci on rat chromosome 10 (which shares homology with the mouse chromosome carrying the *Nf1* and *p53* loci) have been associated with susceptibility to EtNU-induced tumorigenesis, these loci are distinct from the *Nf1* and *p53* genes (28).

When first identified as an oncogene in EtNU-induced MPNSTs, erbB2 was an "orphan" receptor with no known ligand. In the early 1990s, however, it was found that erbB2 serves as a coreceptor that is activated when the related kinases erbB3 and erbB4 bind proteins in the NRG-1 family of growth and differentiation factors. NRG-1 proteins (which include heregulin, neu differentiation factor, glial growth factor [GGF], acetylcholine receptor inducing activity [ARIA], and sensory and motor neuron-derived factor [SMDF]) are a family of structurally diverse polypeptides encoded by alternatively spliced mRNAs transcribed from a single gene (29). NRG-1 factors potently promote the proliferation, survival, and migration of Schwann cells during embryogenesis and early postnatal life (30-34), raising the question of whether they might have similar effects in PNSTs. To test this hypothesis, we produced transgenic mice in which expression of the NRG-1 isoform GGFβ3 is directed by regulatory elements of the Schwann cell-specific myelin protein zero promoter (P_0-GGFβ3 mice) (35). P_0-GGFβ3 mice develop prominent Schwann cell hyperplasia in their sciatic nerves, an abnormality that culminates in the development of a hypertrophic neuropathy resembling Charcot-Marie-Tooth disease. P_0-GGFβ3 mice also uniformly develop preneoplastic lesions in their trigeminal, dorsal root and sympathetic ganglia (Fig. 2A, B); in contrast to the diffuse hyperplasia seen in the sciatic nerve of these animals, the intraganglionic lesions in P_0-GGFβ3 mice are initially delimited from adjacent nerve (Fig. 2C). About 60% of these animals develop large tumor masses, most commonly in the trigeminal nerve, by 6 to 12 months of age. These tumors are markedly hypercellular neoplasms (Fig. 2D, E) with significant cellular atypia, brisk mitotic activity and foci of hemorrhage and necrosis. Their behavior is locally aggressive, as demonstrated by invasion of brain paren-

chyma (Fig. 2F) and other adjacent tissues. As the tumors arising in P_0-GGFβ3 mice also demonstrate immunohistochemical and ultrastructural evidence of schwannian differentiation (Fig. 2G-J), we have classified them as genetically engineered murine (GEM) Grade III peripheral nerve sheath tumors (PNSTs; see Part I of this review for a discussion of recent consensus recommendations for the classification of transgenic mouse peripheral nervous system tumors (4). NRG-1 expression is evident in these tumors and they overexpress NRG-1 receptors, suggesting that autocrine or paracrine signaling promotes their proliferation, survival, and/or other effects. We do not yet know whether the GEM Grade III PNSTs arising in P_0-GGFβ3 mice carry mutations of *Nf1*, *p53* or other tumor suppressor genes, as is seen in human MPNSTs.

There is also evidence that dysregulated NRG-1/erbB signaling contributes to peripheral nerve sheath tumorigenesis in humans. In 1986, Brockes et al reported that a subset of neurofibromas and the single MPNST they examined contained a GGF-like activity (36). We have recently determined that neurofibromas and MPNSTs do indeed express multiple α and β transmembrane precursors from the class II (GGF) and III (SMDF) NRG-1 subfamilies (M.S. Stonecypher, S.J. Byer and S.L. Carroll, submitted). Like the neoplasms developing in P_0-GGFβ3 mice, many human neurofibromas and MPNSTs also express the erbB receptors (erbB2, erbB3, and/or erbB4) mediating NRG-1 responsiveness (Fig. 2K, L). A similar pattern of NRG-1 and erbB expression is present in four human MPNST cell lines (NMS-2, NMS-2PC, Mash-1, and YST-1 cells), and treating these lines with two structurally and functionally distinct erbB inhibitors, PD158780 and PD168393, markedly reduces their proliferation. These observations indicate that activation of erbB kinases, potentially mediated by a NRG-1/erbB

autocrine loop, promotes the proliferation of human MPNST cells. It remains to be determined whether NRG-1/erbB signaling has other protumorigenic effects (e.g. increased survival and migration) and whether these erbB inhibitors or other anti-erbB agents (e.g. Herceptin) will prove to be effective for treating *NF1*-associated neurofibromas and MPNSTs in vivo.

Epidermal Growth Factor Receptor

The EGF receptor (EGFR; also known as erbB1), a membrane receptor tyrosine kinase closely related to the NRG-1 receptors (erbB2, erbB3, and erbB4) has also been implicated in the pathogenesis of neurofibromas and MPNSTs. Examining three human lines derived from *NF1*-associated MPNSTs (90-8, 88-14, and 88-3 cells) and one isolated from a sporadic MPNST (S-26T cells), DeClue et al found that all four lines expressed EGFR and responded to EGF with increased EGFR and MAP kinase phosphorylation (37). The proliferation of human 88-14 MPNST cells grown in the presence of limiting (0.1%) amounts of fetal calf serum is dependent on exogenous EGF and inhibition of EGF signaling with an anti-EGFR antibody (mAb225) or pharmacologic inhibitors of the EGFR (tyrphostins A-25 and AG-1478) significantly reduces this growth. When maintained in higher (2%) concentrations of fetal calf serum, human 88-14 and 90-8 MPNST cells proliferate independent of exogenous EGF, but their growth is still inhibited by EGFR antagonists, indicating that EGFR contributes to mitogenesis even in the absence of added EGF. EGFR is also expressed in human PNSTs in vivo. Although evident only in fibroblasts and perineurial cells in normal human nerves, a small subpopulation of EGFR+/S-100β+ cells is found in human neurofibromas. EGFR

expression is present in human MPNSTs as well but is variable, with some neoplasms containing strong EGFR immunoreactivity in nearly all cells and others containing only scattered weakly labeled cells (37). This variable EGFR expression has been suggested to reflect transient EGFR expression associated with specific stages in the pathogenesis of Schwann cell neoplasms. Alternatively, there may be more than one pathway to Schwann cell neoplasia, some of which do not require EGFR expression (37).

Prominent EGFR expression has also been found in 23 of 24 cell lines derived from soft tissue neoplasms (13 malignant Triton tumors [MTTs]), 1 MPNST, 1 leiomyosarcoma, 1 rhabdomyosarcoma, 1 sarcoma not otherwise specified and 7 with unknown diagnoses] developing in *Nf1/p53* haploinsufficient mice (38) and in in vitro transformed $Nf1^{-/-}$ Schwann cells isolated from embryonic day 12.5 mouse embryos (37). As with human MPNST cell lines, the growth of lines derived from tumors arising in *Nf1/p53* haploinsufficient mice is EGF-dependent when maintained in medium with limiting amounts of fetal calf serum (38). The EGFR can be activated by multiple ligands, including EGF, transforming growth factor-α, amphiregulin, heparin-binding EGF, epiregulin and β-cellulin (39). These mouse tumor lines express amphiregulin, heparin-binding EGF, and epiregulin; they do not, however, express EGF or β-cellulin (38). It is not yet known whether EGFR ligands are expressed in human neurofibromas and MPNSTs and, if so, whether they are required for tumorigenesis. It is conceivable that ligand expression may not be necessary for human peripheral nerve sheath tumorigenesis as ligand-independent activation of EGFR has been observed in other tumor types with high levels of EGFR expression (39). Consistent with this hypothesis, amplification of the EGFR locus, an event thought to be associated with EGFR overexpres-

sion, occurs in a subset of MPNSTs, being evident in 26% of human tumors in one series (40). Alternatively, EGFRs expressed by neoplastic Schwann cells in neurofibromas and MPNSTs may be transactivated by other growth factors (e.g., lysophosphatidic acid, cytokines) as described in several other cell types (39).

We have found that EGFR and NRG-1 receptors are coexpressed in some, but not all, human neurofibromas and MPNSTs (M.S. Stonecypher, S.J. Byer and S.L. Carroll, submitted). This observation is intriguing as the EGFR is capable of heterodimerizing and "cross-talking" with NRG-1 receptors (39). It will be interesting to determine whether coexpressed EGF and NRG-1 receptors enhance each other's signaling capabilities and produce novel responses in neoplastic Schwann cells.

Hepatocyte Growth Factor and its c-Met Receptor

Hepatocyte growth factor (HGF; also known as scatter factor) is a heparin-binding growth factor that is produced by a variety of mesenchymal cell types during development (41, 42) and released from these cells as an inactive zymogen. Following cleavage by a serine protease [the HGF activator (HGFA)], mature HGF promotes the proliferation, migration and morphogenesis of nearby epithelial cells expressing the c-Met membrane receptor tyrosine kinase. HGF activation of c-Met is frequently enhanced by CD44, a transmembrane glycoprotein implicated in the activation of several high-affinity growth factor receptors and in cell-cell and cell-matrix adhesion (43). Dysregulated HGF/c-Met signaling, facilitated in many instances by CD44 splice variants carrying variant exons 3 or 6 (CD44v3 or CD44v6), has been implicated in the

pathogenesis of a variety of carcinomas where these molecules enhance mitogenesis, invasion, metastasis, and angiogenesis.

Purified neonatal rat Schwann cells also express c-Met, and the mitogenesis of these glia is potently stimulated by HGF (44). Neonatal Schwann cells do not, however, themselves express HGF, suggesting that HGF released from another cell type acts on Schwann cells in developing peripheral nerve (44). In contrast, HGF and c-Met are co-expressed in human neurofibromas (45-47) and MPNSTs (45, 47), with immunoreactivity for both molecules being reportedly greater in MPNSTs than in neurofibromas (45). Based on these observations and the demonstration that HGF is a mitogen for neonatal rat Schwann cells, it was expected that HGF and c-Met would form an autocrine loop driving the proliferation of neoplastic Schwann cells in neurofibromas and/or MPNSTs. Consistent with this hypothesis, Su et al found that HGF, c-Met, HGFA, and CD44 colocalize in distinct regions within MPNSTs in vivo (48). However, these regions of colocalization did not correspond to areas demonstrating increased MIB-1 labeling indices, raising the question of whether HGF/c-Met autocrine signaling has effects other than stimulating mitogenesis. Examining three human MPNST cell lines, these investigators identified one line, ST8814 cells, which coexpressed HGF, c-Met, CD44, and the HGF activator. Ablation of c-Met expression in ST8814 cells with a ribozyme targeting c-Met mRNA had no effect on the proliferation of this line, confirming that this signaling pathway was not promitogenic in at least some MPNST cells. In contrast, knock-down of c-Met expression markedly inhibited ST8814 cell invasion in an in vitro assay (48), leading these authors to suggest that HGF/c-Met signaling promotes metastasis from MPNSTs. Curiously, ST8814 cell invasion promoted by

HGF/c-Met signaling was not inhibited by neutralizing anti-CD44v3, antiCD44v6, or anti-pan CD44 antibodies. It is not yet known whether HGF is widely required for MPNST invasion and metastasis in vivo and, if so, why CD44 is not required for these effects.

Stem Cell Factor and Its Receptor, c-Kit

Stem cell factor (SCF, Kit ligand), acting through its receptor, the c-Kit membrane tyrosine kinase, promotes mast cell precursor migration into normal tissues and the subsequent maturation, activation, and survival of these cells (49). The prominent population of mast cells present within neurofibromas likewise express the c-Kit receptor (50), and these neoplasms contain SCF (50) that is produced, at least in part, by neoplastic Schwann cells (51, 52). SCF expression is related to the level of neurofibromin activity in Schwann cells, with murine *Nf1*$^{-/-}$ Schwann cells secreting approximately sixfold more SCF than either wild-type or *Nf1*$^{+/-}$ Schwann cells (53). Further, *Nf1*$^{+/-}$ mast cells demonstrate increased proliferation (54, 55), survival (54, 55), and migration (53) in response to SCF. Considered together, these observations indicate that enhanced SCF expression by neoplastic Schwann cells is an important factor promoting mast cell accumulation in neurofibromas.

SCF may have other roles in PNST formation as well. Despite the fact that MPNSTs contain much smaller numbers of mast cells than are present in neurofibromas, human MPNST cell lines continue to express SCF (51, 52), suggesting that this growth factor acts on additional cell types in MPNSTs. These alternative SCF targets may include the neoplastic Schwann cells themselves. Some, but not all, MPNST cell

lines express high levels of c-Kit protein and stimulation with exogenous SCF increases the proliferation of these lines (56). Further, the basal proliferation of c-Kit expressing MPNST cell lines is inhibited by treatment with tyrphostin A9 (56), a pharmacologic inhibitor that targets platlet derived growth factor (PDGF)-related tyrosine kinases, the group of receptors to which c-Kit belongs. It is not yet clear whether autocrine or paracrine signaling through the SCF/c-Kit signaling pathway promotes the proliferation of only a subset of MPNSTs in vivo or is more widely used.

Other Growth Factors Potentially Promoting Peripheral Nerve Sheath Tumorigenesis

PDGF and its Receptors

PDGF BB, acting through its β receptor, is a potent mitogen for neonatal rat Schwann cells (57, 58). PDGF BB and the PDGF-β receptor are coexpressed in neurofibromas in vivo (59) and by cultured neurofibroma (60) and MPNST (61, 62) cells. MPNSTs and MPNST-derived cell lines also express the PDGF α receptor. The proliferation of cultured neurofibroma (60) and MPNST cells (61) is potently enhanced by stimulation with PDGF BB, a response that is associated with abnormal activation of calcium-dependent signaling pathways (62). These observations suggest that autocrine or paracrine signaling involving PDGF BB and its β- and/or α-receptors promotes the mitogenesis of neoplastic Schwann cells in neurofibromas and MPNSTs in vivo. This hypothesis has not yet been verified by approaches such as demonstrating that inhibition of PDGF or PDGF receptor action inhibits the basal proliferation of neurofibroma and MPNST cells or by showing that transgenic mice overexpressing PDGF in Schwann cells develop PNSTs.

Midkine

Midkine is a 13 kDa heparin-binding growth factor that is highly expressed during embryogenesis and is overexpressed in several human tumor types (63). Midkine is also overexpressed in the skin of *NF1* patients relative to normal controls and stimulates the proliferation of endothelial cells (64, 65), neurofibroma-derived fibroblastoid-like cells (64, 65) and ST8814 cells (65), an S-100-positive line derived from a human MPNST. Neoplastic Schwann cells likely are a major source of midkine in neurofibromas and MPNSTs in vivo, as this growth factor is expressed at higher levels in $NF1^{-/-}$ Schwann cells than in $NF1^{+/-}$ or wild-type Schwann cells.

Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) has complicated effects on normal Schwann cells, inducing proliferation and phenotypic alterations in some situations (66) and triggering apoptosis in others (67). Our knowledge of TGF-β action in neurofibromas and MPNSTs is intriguing but limited. TGF-β1 and its receptor are expressed in neurofibromas and MPNSTs, with higher levels of expression evident in the latter tumor type (47). This factor has been reported to stimulate DNA synthesis in cells derived from a human neurofibroma (60). However, a second group of investigators found that TGF-β1 inhibited the proliferation of a single cell line derived from an EtNU-induced rat MPNST (68). It is currently unclear whether these apparently contradictory findings reflect differences in neurofibroma and MPNST responses to TGF-β1 or other variables in these two experimental systems.

Sex Steroids

It is well known that new neurofibromas frequently appear at puberty and that existing neurofibromas increase in size during this period. Neurofibromas also increase in size and frequency in pregnant women and regress after parturition. These observations suggest that puberty- and pregnancy-associated changes such as alterations in blood levels of sex steroids promote neurofibroma growth. Despite these well-known clinical observations, information supporting this hypothesis is surprisingly sparse. In a series of 59 human neurofibromas, 75% of these neoplasms were found to express progesterone receptors (69), with expression of this steroid receptor being more common in dermal neurofibromas (which occur with increased frequency after puberty) than in plexiform neurofibromas (which are thought to be congenital). Interestingly, the neurofibroma cells expressing progesterone receptors were S-100 negative, indicating that they are not $NF1^{-/-}$ Schwann cells. Estrogen receptors are detectable in only a small number of neurofibromas (69, 70). At present, little information is available regarding the effects sex steroids or steroid hormone inhibitors have on human neurofibroma and MPNST cells in vitro or in vivo.

CONCLUSIONS AND FUTURE DIRECTIONS

As discussed in Part I of this review, considerable evidence has accumulated over the last 15 years indicating that loss of function of the *NF1* tumor suppressor gene is a key early step in the pathogenesis of *NF1*-associated neurofibromas and that subsequent progression from neurofibroma to MPNST is associated with abnormalities of additional tumor suppressor genes, such as *p53*, *INK4A* and $p27^{kip1}$. It has also becom-

ing increasingly evident that multiple aberrantly expressed growth factors and/or growth factor receptors contribute to peripheral nerve sheath tumorigenesis by promoting the proliferation, migration, and/or survival of neoplastic Schwann cells. However, several aspects of growth factor action in neurofibromas and MPNSTs remain poorly defined. First, our current understanding likely underestimates the complexity of the functions these factors perform in neurofibromas and MPNSTs. For instance, further study is needed to determine whether specific promitogenic growth factors contribute to tumorigenesis in alternative ways such as promoting survival, intraneural migration, and metastasis. As attested to by the observation that SCF targets both mast cells and neoplastic Schwann cells, it is also likely that several of these factors have important protumorigenic effects on more than one cell type within PNSTs. Finally, it must be determined whether each of the growth factors considered in this review is universally required for neurofibroma and/or MPNST pathogenesis or whether there are distinct subtypes of PNSTs, each relying on the action of a different growth factor or growth factor receptor for its development. Answering questions such as these will require investigations that go beyond simply demonstrating that neoplastic Schwann cells express a growth factor and its receptor and that stimulation with that growth factor promotes Schwann cell proliferation. In particular, it will be important to develop new transgenic mouse models in which candidate growth factors or growth factor receptors are overexpressed in Schwann cells; at present, P_0-GGFβ3 mice, in which NRG-1 is overexpressed in Schwann cells, represent the only reported model in which growth factor overexpression induces the formation of PNSTs. Once developed, transgenic mice overexpressing growth factors in Schwann cells can be crossed to animals carrying tar-

geted mutations of tumor suppressor genes (e.g., *Nf1*, *p53*), allowing the hypothesis that dysregulated growth factor expression and tumor suppressor mutations cooperatively promote peripheral nerve sheath tumorigenesis to be directly tested for the first time. Transgenic mouse models in which PNSTs result from growth factor overexpression will also serve as extraordinarily useful tools for assessing the therapeutic potential of existing agents that inhibit specific growth factors or growth factor receptors.

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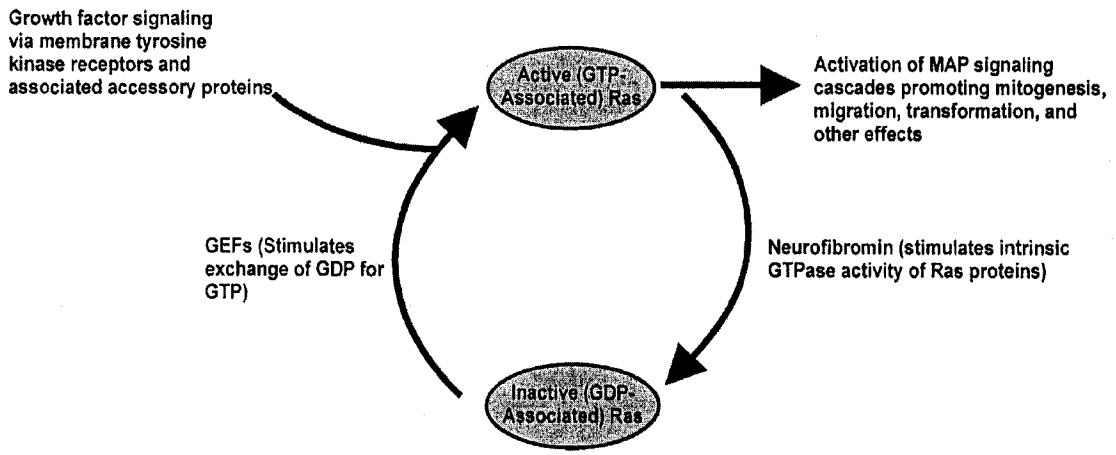
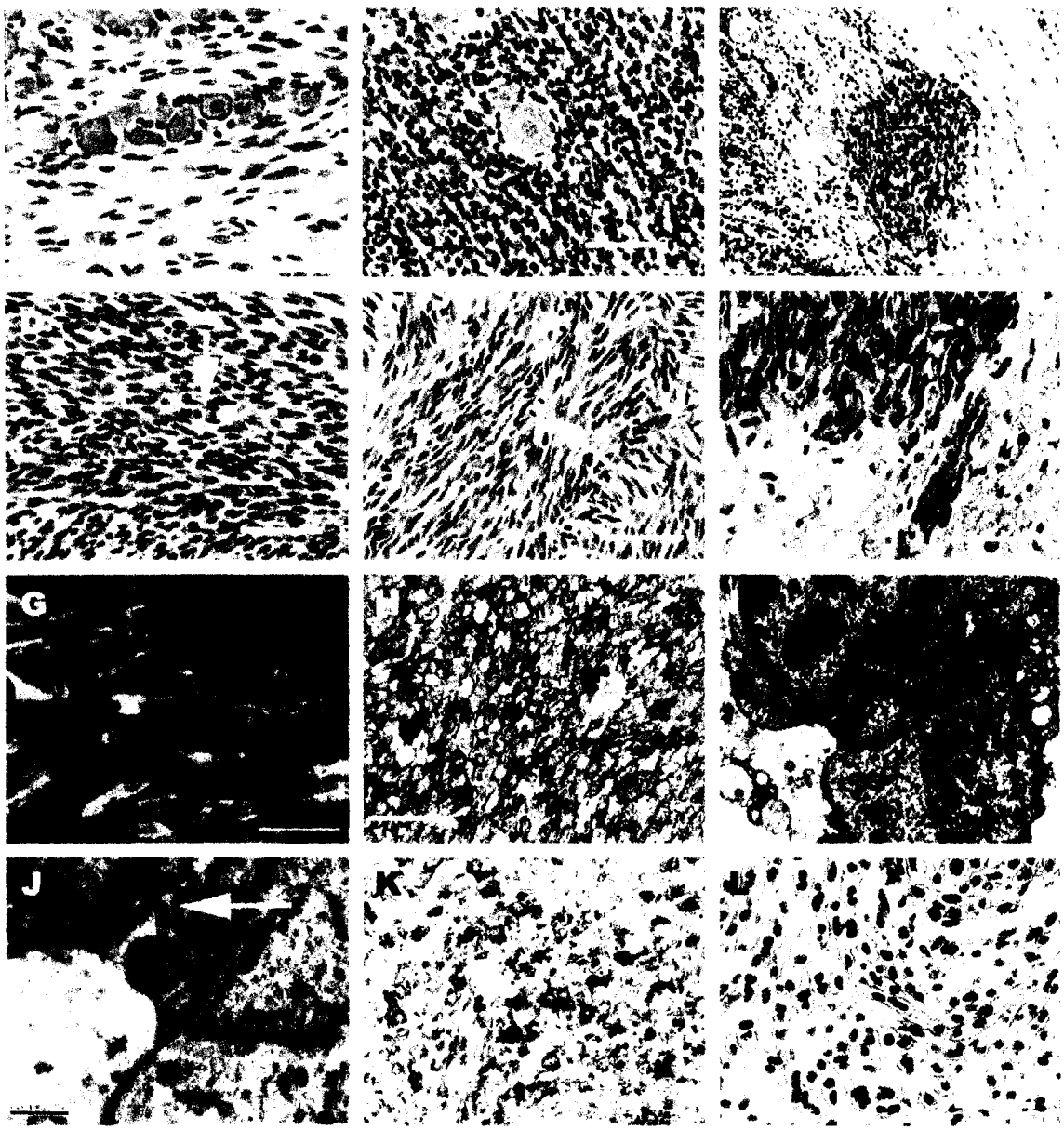


FIGURE 1. Schematic representation of the regulation of Ras activity in neoplastic Schwann cells. Ras proteins, a group of molecules that includes the neurofibromin-regulatable "classic" (H-Ras, N-Ras, and K-Ras) and "nonclassic" (R-Ras and TC21/R-Ras2) Ras proteins, are active when associated with GTP, leading to the activation of mitogen-activated protein (MAP) kinase cascades promoting mitogenesis, migration, transformation, and other effects. Neurofibromin inactivates Ras proteins by stimulating an intrinsic GTPase activity present in Ras proteins, resulting in hydrolysis of GTP to GDP; guanine nucleotide exchange factors (GEFs) promote an exchange of GDP for GTP, resulting in reactivation of Ras. Growth factors, commonly acting through membrane tyrosine kinase receptors and associated accessory proteins (e.g. Grb2 and Sos) are also required to stimulate Ras activation. For more detailed descriptions of the Ras proteins, the signaling events they control and the consequences of activating Ras-dependent signaling cascades, see Part I of this review (4).

FIGURE 2. MPNST-like neoplasms developing in transgenic mice expressing the NRG-1 isoform GGFβ3 in myelinating Schwann cells. (A, B) Comparison of the trigeminal ganglion of a wild-type mouse **(A)** and a mouse **(B)**, demonstrating the preneoplastic lesions uniformly present in the peripheral ganglia of P_0-GGFβ3 mice. **(C)** A preneoplastic lesion in the trigeminal ganglion of a P_0-GGFβ3 mouse, demonstrating the clear delineation of this lesion from adjacent trigeminal nerve (right side of the field). **(D, E)** Two examples of GEM grade III PNSTs developing in the trigeminal nerves of P_0-GGFβ3 mice. These neoplasms are markedly hypercellular lesions composed of highly anaplastic cells. Mitotic figures (arrows) are frequent in these tumors. These neoplasms, like their human counterparts, show some variability in their histologic appearance. **(F)** A third GEM grade III PNST invading and destroying brain parenchyma in a P_0-GGFβ3 mouse. **(G)** Immunoreactivity for S100β in a GEM grade III PNST developing in a P_0-GGFβ3 mouse. S100 immunoreactivity is indicated by Cy-3 labeling (red); the section has been counterstained with the nuclear dye bisbenzamide (blue). **(H)** Immunoreactivity for the basal lamina protein collagen type IV individually invests tumor cells in GEM grade III PNSTs developing in P_0-GGFβ3 mice. This section is from the same neoplasm shown in panel D and has been lightly counterstained with hematoxylin to highlight tumor cell nuclei. **(I, J)** Transmission electron micrographs of the tumor shown in D. Individual tumor cells are invested by a basal lamina, with loops of basal lamina material frequently seen extending away from the cells (arrow). **(K)** Membranous and cytoplasmic immunoreactivity for the NRG-1 receptor erbB3 in a neurofibroma from a human *NF1* patient. **(L)** Immunoreactivity for the NRG-1 receptor erbB4 in a MPNST from a human *NF1* patient. This antigen is evident in association with membranes as well as tumor cell nuclei; this latter distribution likely reflects the cleavage and internalization of a cytoplasmic fragment of erbB4, as described in other cell types (71). **A-H**, **K** and **L**: originnal magnification : 40x; scale bar, 50 μm. **(I)** original magnification : 10,000x;. **(J)** original magnification : 40,000x; scale bar = 0.5 μm.



ACTIVATION OF THE NEUREGULIN-1/ERBB SIGNALING PATHWAY PROMOTES THE PROLIFERATION OF NEOPLASTIC SCHWANN CELLS IN HUMAN MALIGNANT PERIPHERAL NERVE SHEATH TUMORS

by

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ABSTRACT

Patients with neurofibromatosis type 1 develop aggressive Schwann cell neoplasms known as malignant peripheral nerve sheath tumors (MPNSTs). Although tumor suppressor gene mutations play an important role in MPNST pathogenesis, it is likely that dysregulated signaling by as yet unidentified growth factors also contributes to the formation of these sarcomas. To test the hypothesis that neuregulin-1 (NRG-1) growth factors promote mitogenesis in MPNSTs, we examined the expression and action of NRG-1 in human MPNSTs and neurofibromas, the benign precursor lesions from which MPNSTs arise. Multiple α and β transmembrane precursors from the class II and III NRG-1 subfamilies are present in both tumor types. Neoplastic Schwann cells within these neoplasms variably express the erbB kinases mediating NRG-1 responses (erbB2, erbB3 and/or erbB4). Human MPNST cell lines (Mash-1, YST-1, NMS-2 and NMS-2PC cells) similarly coexpress multiple NRG-1 isoforms and erbB receptors. These MPNST lines are NRG-1 responsive and demonstrate constitutive erbB phosphorylation. Treatment with PD168393 and PD158780, two structurally and mechanistically distinct erbB inhibitors, abolishes erbB phosphorylation and reduces DNA synthesis in these lines. These findings suggest that autocrine and/or paracrine NRG-1/erbB signaling promotes neoplastic Schwann cell proliferation and may be an important therapeutic target in neurofibromas and MPNSTs.

INTRODUCTION

Neurofibromatosis type 1 (*NF1*) is an autosomal dominant tumor predisposition syndrome that affects 1 in 3500 individuals (1-3). *NF1* patients develop multiple types

of neoplasms, including benign peripheral nerve sheath tumors known as neurofibromas that occur in skin (dermal neurofibromas), spinal and cranial nerve roots (intraneural or nodular neurofibromas) and large nerve plexuses (plexiform neurofibromas). Neurofibromas are infiltrating neoplasms composed of multiple cell types, with Schwann cells representing 40-80% of the tumor, and fibroblasts, perineurial cells, mast cells and vascular elements making up the remainder (4). Although dermal neurofibromas rarely undergo malignant transformation, plexiform and, less frequently, intraneural neurofibromas may progress and become malignant peripheral nerve sheath tumors (MPNSTs), a highly aggressive form of Schwann cell neoplasm (5). The prognosis for patients with MPNSTs is poor, with only 23% of these individuals alive 10 years after diagnosis (6, 7). Beyond surgery, few therapeutic options are available for patients with MPNSTs (8). Postsurgical irradiation of the tumor bed helps control local disease and delays tumor recurrence, but has no effect on overall survival (5). At present, no effective chemotherapeutic regimens are available for MPNSTs (8). Because of these limitations, there is considerable interest in establishing the mechanisms responsible for MPNST tumorigenesis and using this information to develop new, more effective, therapies.

Mutations in tumor suppressor genes such as *NF1* (4, 5, 9-18), *p53* (18-21) and *INK4A* (also known as *CDKN2A/p16*) (22, 23) play an important role in the pathogenesis of neurofibromas and MPNSTs. However, it is likely that tumor suppressor mutations alone are not sufficient to induce PNST formation and that other abnormalities such as dysregulated growth factor signaling act cooperatively with these mutations to promote neurofibroma and MPNST tumorigenesis (24, 25). We have hypothesized that

inappropriate signaling by proteins in the neuregulin-1 (NRG-1) family of growth and differentiation factors contributes to the pathogenesis of PNSTs. NRG-1 proteins stimulate the proliferation, survival and migration of developing Schwann cells (26-28), and activating mutations of the NRG-1 receptor subunit erbB2 promote MPNST formation in rodents exposed to the mutagen *N*-ethyl-*N*-nitrosourea (EtNU) *in utero* (29-35). We have found that JS1 cells, a rat cell line derived from an EtNU-induced MPNST, lack this erbB2 mutation and express erbB2 in combination with the NRG-1 receptor subunit erbB3 and multiple NRG-1 isoforms. The NRG-1/erbB signaling pathway is constitutively activated in JS1 cells and the proliferation of this line is profoundly reduced by pharmacologic inhibition of erbB signaling (36). We have also generated transgenic mice expressing the NRG-1 isoform glial growth factor-β3 (GGFβ3) in myelinating Schwann cells (P_0-GGFβ3 mice) (37) and have found that these animals develop MPNST-like Schwann cell neoplasms with a latency of 6-12 months. GGFβ3 expression is maintained in these neoplasms and they markedly overexpress NRG-1 receptor subunits, suggesting that autocrine or paracrine NRG-1 signaling is essential for tumorigenesis in P_0-GGFβ3 mice. Considered together, these findings indicate that constitutive activation of the NRG-1/erbB signaling pathway promotes MPNST pathogenesis in rodents.

It is not yet known if the rodent models noted above are relevant to human neurofibroma and MPNST pathogenesis. To test the hypothesis that constitutive activation of the NRG-1/erbB signaling pathway promotes neoplastic Schwann cell proliferation in human PNSTs, we have examined human neurofibromas and MPNSTs to determine whether they express NRG-1 isoforms and the erbB kinases necessary for NRG-1 re-

sponsiveness and whether erbB signaling is essential for the proliferation of human MPNST cells.

MATERIALS AND METHODS

Study Cases

Protocols for studies with human tissue were approved by the University of Alabama at Birmingham Institutional Review Board for Human Use. Frozen neurofibroma and MPNST tissue was provided by the Southern Division of the Cooperative Human Tissue Network/University of Alabama at Birmingham Tumor Bank (Director: William Grizzle, MD, PhD). Paraffin-embedded blocks of neurofibroma and MPNST tissue were obtained from the files of the Department of Pathology of the University of Alabama School of Medicine (Birmingham, AL, USA). Patient ages, sex, race, *NF1* status, and tumor location for the fresh and paraffin-embedded tissues used in our studies are provided in Tables 1 and 2, respectively.

Antisera and Immunohistochemical Reagents

We have previously described the production and characterization of a rabbit polyclonal antibody that specifically recognizes the EGF-like common domain found in all biologically active NRG-1 isoforms (a 'pan'-NRG antibody) (38). A mouse monoclonal antibody recognizing an epitope in the EGFR (residues 996-1002) was obtained from MP Biomedicals (clone c11; Irvine, CA, USA). A mouse monoclonal antibody directed toward the C-terminus of human erbB2 (residues 1242-1255) was purchased from Oncogene Research Products (Ab-3; La Jolla, CA, USA). The mouse IgM mono

clonal antibody RTJ.1 recognizes the C-terminal domain of erbB3; this antibody and an isotype-matched negative control clone (C48-6) were obtained from BD Biosciences PharMingen (San Diego, CA, USA). Affinity-purified rabbit polyclonal antisera for EGFR (sc-03), erbB3 (sc-285), erbB4 (sc-283) and the NRG-1 'a'-carboxy-terminal domain (sc-348) were obtained from Santa Cruz Biotechnology (Santa Cruz, Ca, USA). Rabbit polyclonal antibodies recognizing S100β (#Z0311) and glial fibrillary acidic protein (GFAP) (#Z0334) and an anti-desmin mouse monoclonal antibody (clone D33)

Table 1. Characteristics of Nerve Sheath Tumors Used for Protein and RNA Analyses

Case no.	Diagnosis	Location	Age/race/gender	NF status
1	Neurofibroma	Back	53/U/F	*NF1*
2	Neurofibroma	Retroperitoneum	45/W/M	*NF1*
3	Neurofibroma	Sciatic	45/W/M	*NF1*
4	Neurofibroma	Left buttock	17/B/M	*NF1*
5	Neurofibroma	Cervical	27/W/M	*NF1*
6	Neurofibroma	Cervical	38/B/M	*NF1*
7	Neurofibroma	Cervical	22/B/F	*NF1*
8	Neurofibroma	Left buttock	24/U/F	*NF1*
1	MPNST (epithelioid)	Lower Abdomen/groin	54/W/M	Unknown
2	MPNST	Right peroneal nerve	40/U/F	*NF1*
3	MPNST	Brain metastasis	62/W/F	*NF1*
4	MPNST	Right posterior thigh mass	46/W/M	*NF1*
5	MPNST	Retroperitoneal	20/B/M	*NF1*
6	MTT	Peritoneum	40/W/F	*NF1*
7	MPNST	Median nerve, Right arm	16/B/M	*NF1*

Case numbers correspond to cases indicated in Figures 1-4.

were purchased from Dako (Carpinteria, CA, USA). The mouse anti-collagen type IV monoclonal antibody (clone PHM-12) used in our studies was from Ventana Medical Systems (Tucson, AZ, USA). A mouse monoclonal antibody recognizing smooth mus

Table 2. NRG-1 and ErbB Immunoreactivity in Human Neurofibromas and MPNSTs

Case no.	Diagnosis	Location	Age/Race/Gender	NF Status	ErbB-1	ErbB-2	ErbB-3	ErbB-4	NRG-1
9	Neurofibroma	C1-C2 nerve Root	38M	Unknown	-	+	-	-	-
10	Neurofibroma	C1-C2 nerve Root	63M	Unknown	-	+	-	-	+
11	Neurofibroma	Cervical nerve root	21F	*NF1*	-	+	+	-	+
12	Neurofibroma	Gastric Plexus	43/W/F	*NF1*	+	+	+	+	+
13	Neurofibroma	Left leg	43/W/F	*NF1*	+	+	+	+	+
14	Neurofibroma	Right neck	42/B/M	*NF1*	+	+	+	+	+
15	Neurofibroma	C1-C2 nerve Root	74M	Unknown	-	+	+	+	-
8	MPNST	Left buttock	33/B/F	Unknown	+	+	+	+	+
9	MPNST	Left buttock	33/B/F	Unknown	+	+	+	+	+
10	MPNST	Left thigh	31/B/F	*NF1*	+	+	+	-	+
11	MPNST	Brain Metastasis	62/W/F	*NF1*	-	+	+	+	+
12	MPNST	Right thigh	14/B/M	*NF1*	-	+	-	-	+

cle actin (clone 1A4) was purchased from NeoMarkers (Fremont, CA, USA). A rabbit anti-neuron-specific-enolase (NSE) antibody was obtained from Chemicon International (Temecula, CA, USA). The mouse monoclonal antibody recognizing GAPDH (clone 6C5) was purchased from Advanced ImmunoChemical Inc. (Long Beach, CA, USA). Nonimmune mouse and rabbit IgGs were obtained from Pierce (Rockford, IL, USA). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody was from Jackson Immunoresearch Inc. (West Grove, PA, USA). Tyramide signal amplification reagents, including streptavidin-HRP, biotinyl tyramide, amplification diluent, and blocking reagent were purchased from Perkin-Elmer Life and Analytical Science Products (Renaissance TSA-Indirect kit, Boston, MA, USA). DAB peroxidase substrate (SK-4100) was obtained from Vector Laboratories (Burlingame, CA, USA).

RT–PCR Analyses of NRG-1 Isoforms Expressed in Peripheral Nerve Sheath Tumors and MPNST Cell Lines.

Our designation of NRG-1 domains follows the nomenclature of Peles and Yarden (39). The common forward oligonucleotide used for amplification of sequences encoding the EGF-like and juxtamembrane domains of NRG-1 corresponds to nucleotides 631-648 of human NDF (also known as class I NRG-1 isoforms; GenBank accession no. U02326) (41). The reverse oligonucleotide for PCR of NRG-1 transmembrane precursor cDNAs represents nucleotides 811-828 (encoding a portion of the transmembrane domain sequence) of human NDF. This oligonucleotide pair will amplify sequences encoding all possible combinations of EGF-like and juxtamembrane sequences found in NRG-1 transmembrane isoform mRNAs. Secreted NRG-1 isoform cDNAs were amplified using the common forward oligonucleotide in combination with a re-

verse oligonucleotide corresponding to nucleotides 482-502 of a human NDFβ3 splice variant (GenBank accession no. U02327) (40). NDF amino-terminal sequences were amplified using oligonucleotides corresponding to nucleotides 484-504 and 887-867 of human NDFβ3 (GenBank accession no. BC006492). GGF (class II NRG-1 isoform) amino-terminal sequences were amplified using oligonucleotides representing nucleotides 429-452 and 900-877 of a human GGFβ3 cDNA (GenBank accession no. L12260). Sensory and motor neuron-derived factor (SMDF) (class III NRG-1 isoform) NRG-1 amino-terminal sequences were amplified using oligonucleotides corresponding to nucleotides 806-822 and 1231-1210 of a human SMDFβ3 cDNA (GenBank accession no. BC007675).

Single-stranded cDNA for use as a PCR template was synthesized from total cellular RNA isolated from tumor tissue or cultured tumor cells using Trizol reagent (Invitrogen). RNA was reverse transcribed in a 20 ul reaction using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Superscript Plus, Life Technologies, Gaithersburg, MD, USA). After completion of reactions, samples were diluted to 100 ul with distilled water, boiled for 5 min and stored at -80°C until use.

A 2 ul portion of each cDNA was used as a PCR template in reactions performed for 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. PCR products were ligated into pCR4-TOPO and ligations transformed into *Escherichi coli* (Top10 strain) as recommended by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA). The sequence of these clones was determined by cycle sequenc-

ing using an automated sequencer (ABI Model 373A DNA Sequencing System, Applied Biosystems Inc. Foster City, CA, USA).

Immunohistochemistry for ErbB Kinases and NRG-1 in Tumor Sections

Immunohistochemical detection of erbB and NRG-1 proteins was performed on 4- to 5-μm-thick sections of paraffin-embedded tissue using a modified protocol we have previously developed and validated (41). In brief, after deparaffinization of sections in xylenes and graded alcohols, antigen rescue was performed by gently boiling slides in 10 mM citrate buffer (pH 6.0) for 15 min and then allowing them to cool to room temperature for 15 min. Nonspecific binding was blocked by incubating sections for 15 min with TNB blocking buffer (0.1 M Tris-HCl (pH 7.5)/0.15 M NaCl/ 0.5% blocking reagent) at room temperature. Sections were then incubated overnight at 41°C with primary antibodies diluted in blocking buffer (1:100 dilution, EGFR clone c11; 1:100 dilution, erbB2 Oncogene Ab-3; 1:100-1:250 dilution, erbB3 monoclonal RTJ.1; 1:500 dilution, erbB4 rabbit polyclonal antibody; 1:250-1:500 dilution, pan NRG-1 rabbit polyclonal antibody). After three rinses in phosphate-buffered saline (PBS; 10 min per rinse), sections were incubated for 1 h at room temperature with HRP-conjugated secondary antibody (diluted 1:250-1:500 in blocking buffer). After three washes in PBS, biotinyl tyramide (diluted 1:50 in amplification diluent) was applied to tissue sections for 10 min at room temperature. Sections were again washed three times in PBS and then incubated with streptavidin-HRP (diluted 1:100 in TNB) for 45 min. After three final washes with PBS, immunoreactive structures were visualized by diaminobenzidine deposition. To confirm the specificity of staining, control slides were treated

identically except that primary antibody was replaced with blocking buffer alone. As an additional control, some sections were processed with nonimmune mouse IgG (for EGFR and erbB2 controls), nonimmune isotype-matched IgM (for erbB3 controls) or nonimmune rabbit IgG (for erbB4 and pan NRG controls) in place of the primary antibodies. No staining was observed in these experiments. For the erbB2, erbB4 and pan NRG-1 antigens, specificity of staining was further confirmed by preincubating antisera with either the immunizing peptide or a nonrelated peptide (10 ng/ml); in all instances, preincubation with the immunizing peptide, but not the nonrelated peptide, abolished the staining pattern observed with primary antibody alone. Following immunostaining, slides were lightly counterstained with hematoxylin, coverslipped and mounted with Permount (Fisher).

Immunoblotting

Lysates for immunoblotting were prepared by homogenizing specimens in Trizol reagent (Invitrogen) according to the manufacturer's protocol. Protein pellets were dissolved in 1% sodium-dodecyl-sulfate (SDS) supplemented with protease (Sigma #P8340) and phosphatase (Sigma #P5726) inhibitors diluted 1:100-1:250. Protein concentrations were determined with a modified Lowry method (*DC* Protein Assay; Bio-Rad, Hercules, CA, USA). Equivalent quantities of protein lysates were resolved on 8% SDS polyacrylamide gels. Membranes were blocked in 5% nonfat dry milk in TBST (0.15 M NaCl, 10 mM Tris (pH 8.0), 0.05% Tween 20) prior to incubation with primary antibody. The following primary antibody concentrations were used for immunoblotting: EGFR (Santa Cruz sc-03; 1:1000), erbB-2 (Oncogene Ab-3; 1: 500-

1:1000), erbB3 (Santa Cruz sc-285; 1:1000), erbB4 (Santa Cruz sc-283; 1:1000), pan NRG-1 (1:5000) and NRG-1 'a' carboxy terminus (Santa Cruz sc-348; 1:1000). HRP-conjugated donkey anti-rabbit and donkey anti-mouse secondary antibodies were used at a 1:10000 dilution in 5% milk/TBST. Immunoreactive species were detected by enhanced chemiluminescence (Pierce). In preliminary experiments, we examined the feasibility of using immunoreactivity for GAPDH, β-actin and ERK-2 to verify equivalent loading of neurofibroma and MPNST lysates. However, we found that the highly variable degree of collagenization in neurofibromas and MPNSTs rendered normalization to intracellular housekeeping protein levels problematic. Consequently, equivalent loading and transfer of tumor lysates was verified by Coomassie staining of the polyvinylidene fluoride (PVDF) membrane post-transfer, while equivalent loading and transfer of cell line lysates was verified by reprobing blots with an anti-GAPDH antibody. Specificity of immunoblotting was determined for each immunoblot by preincubating the blotting antibody with a 5-10-fold excess of the appropriate immunizing peptide.

MPNST Cell Lines, Neonatal Schwann Cells and Their Culture Conditions

The Mash-1 cell line is a human MPNST cell line derived from a skin/chest metastasis in a male *NF1* patient (42 and was obtained from the Cell Bank of the RIKEN BioResource Center (Ibaraki, Japan). The NMS-2 cell line was established from an MPNST arising in the right thigh of a 30-year-old man with *NF1* and the NMS-2PC cell line was derived from a retroperitoneal metastasis occurring 9 months later in the same patient (43). NMS-2 and NMS-2PC cells were generously provided by Dr Akira Ogose (Department of Orthopedic Surgery, Niigata University, Niigata, Japan). The

YST-1 cell line was derived from an epithelioid MPNST that occurred in the upper arm of an 8-year-old girl with no clinical evidence of *NF1* (44). YST-1 cells were kindly provided by Dr Yoji Nagashima (Department of Pathology, Yokohama City University School of Medicine, Yokohama, Japan). NMS-2, NMS-2PC and YST-1 cell lines were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 IU/ml penicillin and 10 μg/ml streptomycin. Mash-1 cells were maintained in a 1:1 mixture of Keratinocyte SFM medium (Invitrogen; supplemented with 5 ng/ml EGF and 50 μg/ml bovine pituitary extract) and DMEM (supplemented with 5% heat-inactivated FBS). Cultures of neonatal Schwann cells were established from the sciatic nerves of postnatal day 0-2 Sprague–Dawley rat pups using our previously described methodology (37) and maintained in DMEM supplemented with 10% FBS.

Immunostaining and RT–PCR Analyses of Marker Expression in MPNST Cell Lines

To detect S100β immunoreactivity, MPNST cells were plated at low density in chamber slides and allowed to adhere overnight. The next morning, slides were fixed for 30 min at room temperature with 4% paraformaldehyde and then rinsed three times with PBS (5 min per wash). Nonspecific binding was blocked by incubating cells for 30 min in blocking buffer (PBS containing 1% bovine serum albumin, 0.2% nonfat dry milk and 0.3% Triton X-100). Cells were then incubated overnight at 4°C with anti-S100β antibody diluted 1:200 in blocking buffer. The next morning, cells were rinsed three times with PBS and then incubated for 1 h at room temperature with Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch) diluted

1:250 in blocking buffer. Following three more rinses in PBS, slides were mounted using 1:1 PBS:glycerol and examined by fluorescence microscopy.

To construct tissue arrays, MPNST cells were grown to confluence and then removed from their substrate nonenzymatically using Cellstripper (Mediatech, Herndon, VA, USA). Cells were pelleted and mixed with an equal volume of HistoGel specimen processing gel (Richard-Allan Scientific, Kalamazoo, MI, USA). After coagulation at 4°C, cell pellets were transferred to cassettes and fixed in 10% formalin. Fixed pellets were then dehydrated through graded alcohols and xylenes and paraffin embedded. Then, 1.5 m cores were taken in triplicate from donor blocks of MPNST cell pellets and human controls (paraffin blocks of normal adrenal medulla, a breast carcinoma, normal pituitary, normal kidney, SKOV-3 ovarian carcinoma cells, MCF-7 breast carcinoma cells, PC-3 prostate carcinoma cells and DU145 prostate carcinoma cells) and used to construct tissue arrays. Sections (4-5 μm) were prepared, deparaffinized with xylene and rehydrated through graded ethanol to PBS. After blocking nonspecific binding by incubating rehydrated sections for 30 min in blocking buffer, sections were incubated with primary antibody or nonimmune IgG for 2 h at room temperature. Dilutions of primary antibodies were: collagen type IV, 1:50; GFAP, 1:500; NSE, 1:1000; smooth muscle actin, 1:1000; and desmin, 1:50. Following incubation with primary antibodies, sections were rinsed three times with PBS and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Following three more washes with PBS, immunoreactivity was detected by diaminobenzidine deposition. Sections were lightly counterstained with hematoxylin and mounted with Permount for light microscopic examination.

For RT-PCR analyses, 2 μl of cDNAs synthesized from total cellular RNA as described above was used as a PCR template in reactions performed for 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Primers were designed from the reference sequence deposited for each tested marker in Entrez Gene using PrimerSelect software (Version 3.10; DNAStar Inc.).

DNA Synthesis Assays

To assay DNA synthesis in human MPNST cell lines, 40 000 cells/well were plated in 24-well plates in RPMI medium containing 10% FBS and allowed to attach overnight. The next morning, PD168393 (Calbiochem, La Jolla, CA, USA), PD158780 (Calbiochem) or vehicle (DMSO) was added to each well. At 12 h after addition of vehicle or erbB inhibitors, 1 μCi/ml of [^{3}H]thymidine was added to each well. After 12 h, [^{3}H] thymidine incorporation was assayed using our previously described methodology (36, 45). All experiments were performed in triplicate, with six replicates/condition performed in each experiment. One-way ANOVA, followed by a Tukey *post hoc* test, was performed on the resulting data, with $P<0.05$ considered statistically significant.

RESULTS

Neurofibromas and MPNSTs Express Multiple NRG-1 Immunoreactive Proteins

We prepared lysates from a series of these tumors (Table 1) and probed immunoblots of the lysates with a rabbit polyclonal antibody that recognizes an epitope within the EGF-like common domain present in all functional NRG-1 isoforms (a 'pan' NRG-1 antibody) (38); a lysate of normal human sciatic nerve collected from an autop-

sied patient was included as a positive control. Multiple NRG-1 immunoreactive species ranging in size from 20 to 150 kDa were evident in all of the neurofibromas examined (Fig. 1a). The masses of the most abundant larger (120 and 150 kDa) species detected in these neurofibromas are consistent with the masses of NRG-1 transmembrane precursors we have previously detected in nervous system tissues using this antibody (38). Likewise, the smaller (20, 45 and 58 kDa) NRG-1 immunoreactive proteins migrate at the positions expected for the extracellular domains of proteolytically cleaved NRG-1 transmembrane precursors or directly secreted NRG-1 isoforms (38, 46, 47). Comparing the sizes of the NRG-1-like proteins detected in neurofibromas with those evident in normal sciatic nerve, a tissue type we have previously shown contains multiple NRG-1 isoforms (38), we found that the NRG-1 immunoreactive species detected in neurofibromas comigrated with those evident in sciatic nerve. To further establish the identity of the proteins detected with the pan NRG-1 antibody, these same lysates were also probed with a second antibody that recognizes a subset of NRG-1 isoforms (transmembrane precursors with an 'a' variant carboxy-terminus). This antibody, like the pan NRG-1 antibody, recognized a 120 kDa species in the majority of the neurofibromas, with a 150 kDa protein also evident in some tumors (Fig. 1b). The anti-'a' carboxy-terminus antibody also labeled a prominent 55 kDa protein in sciatic nerve and neurofibromas and a 65 kDa polypeptide in sciatic nerve. These latter polypeptides are too small to be full-length NRG-1 transmembrane precursor proteins and likely represent recently described NRG-1 proteolytic cleavage products thought to mediate 'back-signaling' into the interior of NRG-1-expressing cells (48). We conclude that neurofi-

bromas express multiple NRG-1 immunoreactive proteins, some of which are NRG-1 transmembrane precursors.

To ascertain whether MPNSTs also express NRG-1 proteins, lysates were prepared from seven surgically resected MPNSTs (Table 1), immunoblotted and probed with the pan NRG-1 antibody. We found that these neoplasms, like neurofibromas, express multiple NRG-1 isoforms (Fig. 1c) with masses similar to those of the NRG-1 immunoreactive species detected in neurofibromas. The only difference was that the masses of the higher molecular weight NRG-1 proteins present in MPNSTs were more variable than those evident in neurofibromas, with a 90 kDa protein (which was also evident in neurofibromas with long exposures (data not shown)) being much more prominent in MPNSTs. It was thus apparent that MPNSTs, like neurofibromas, express multiple NRG-1 immunoreactive proteins.

Neurofibromas and MPNSTs Express a Complex Mixture of α and β Transmembrane Precursors from the Class II and III NRG-1 Subfamilies

The *NRG1* locus, using a combination of alternative splicing and transcription from multiple promoters, produces at least 15 NRG-1 isoforms with distinct structural and functional characteristics (47). To determine whether the variably sized proteins detected by the pan NRG-1 antibody in human neurofibromas and MPNSTs represent distinct NRG-1 splice variants, we examined the structure of the NRG-1 transcripts expressed in these neoplasms. NRG-1 activation of erbB kinases is mediated by the EGF-like domain, which is composed of an amino-terminal invariant (common) domain coupled to α or β variant domains (Fig. 2a, left panel); NRG-1 α and β isoforms are functionally distinct, differing both in their affinities for NRG-1 receptors and their

ability to elicit at least some biologic responses (47). The EGF-like domain is followed by a structurally variable juxtamembrane domain, which determines whether the protein is synthesized as a directly secreted form or as a transmembrane precursor requiring proteolytic cleavage for release. As all functional NRG-1 isoforms must contain a receptor binding domain, we used PCR to amplify NRG-1 sequences encoding a region encompassing the EGF-like common, EGF-like variant (α or β), juxtamembrane (1, 2 or 4 variant) and transmembrane domains from six neurofibromas and six MPNSTs. These sequences were detectable in all of the neurofibromas and MPNSTs tested (data not shown). To further examine the protein structures predicted by these NRG-1 sequences, the PCR products amplified from two neurofibromas and two MPNSTs were cloned and multiple subclones sequenced. We found that the NRG-1 transmembrane precursors expressed in these neoplasms were structurally diverse and included NRG-1 β1, β2 and α2 variants (Fig. 2a). In contrast, sequences encoding the EGF-like domain of NRG-1 secreted isoforms (isoforms with a '3' variant juxtamembrane domain) could not be amplified from neurofibroma and MPNST cDNAs, although these primers did produce a PCR product of the appropriate size from control cDNAs (data not shown). Three NRG-1 subfamilies (class I, II, and III NRG-1 isoforms) are known, each defined by a unique amino-terminal sequence that confers distinctive biologic characteristics on the protein (47). To establish which NRG-1 subfamilies are expressed in human neurofibromas and MPNSTs, PCR was performed with cDNAs derived from six neurofibromas and six MPNSTs using primers specific for sequences encoding each of the three distinct NRG-1 amino-terminal domains. Class II (GGF) NRG-1 transcripts were detected in two of six neurofibromas and in all six MPNSTs (Fig. 2b). RNA encoding

class III (SMDF/cysteine-rich domain) NRG-1 isoforms was present in all neurofibromas and MPNSTs tested (Fig. 2c). In contrast, although readily amplified from control specimens, class I (NDF) NRG-1 transcripts were not detected in any of the neurofibromas and MPNSTs examined (data not shown). We conclude that human neurofibromas and MPNSTs express a complex mixture of α and β transmembrane precursors from the class II and III NRG-1 subfamilies.

Human Neurofibromas and MPNSTs Express the ErbB Membrane Tyrosine Kinases Necessary for NRG-1 Responsiveness

NRG-1 binds directly to the erbB3 (HER3) or erbB4 (HER4) membrane tyrosine kinases, an event that promotes homo- or heterodimerization with other erbB family members (49), with erbB2 (c-*neu*, HER2) being the preferred partner for both NRG-1 receptors (50, 51). The erbB kinases mediating NRG-1 responses are also capable of heterodimerizing to and thus 'crosstalking' with the EGFR receptor (erbB1), a molecule whose expression is increased (52) in many PNSTs (53). To determine whether NRG-1 receptors are expressed in PNSTs and to examine the relationship between the expression of receptors for NRG-1 and EGF, we immunoblotted lysates of surgically resected neurofibromas and MPNSTs and probed them with antibodies recognizing EGFR, erbB2, erbB3 or erbB4. In human neurofibromas, EGFR was detected in five of six tumors examined (Fig. 3) and was expressed at very high levels in two neoplasms and at moderate levels in several others. erbB2 (c-*neu*) was evident in two neurofibromas (Fig. 3), while erbB3 was present in all six neurofibromas, with strikingly high levels of expression in four tumors (Fig. 3). A protein of the expected 185 kDa mass was also detected in two neurofibromas with an anti-erbB4 antibody (Fig. 3). PCR

analyses of erbB expression in these neoplasms confirmed the expression patterns observed by immunoblot analyses and indicated that erbB2 and erbB3 mRNA was detectable in all six neurofibromas (data not shown), suggesting that some neoplasms expressed relatively low levels of these kinases or that erbB2 and erbB3 expression is confined to a subpopulation of cells within these tumors.

In human MPNSTs, EGFR was present in six of the seven tumors studied (Fig. 4); this kinase was expressed at very high levels in three neoplasms. ErbB2 was detected in five tumors (Fig. 4), with erbB2 levels in three of the neoplasms being comparable to those in JS1 MPNST cells, a line we have shown overexpresses both erbB2 and erbB3 relative to non-neoplastic adult Schwann cells (36). ErbB3 was readily detected in six of seven human MPNSTs, with strikingly high levels of expression observed in four tumors (Fig. 4). The anti-erbB4 antibody detected a protein of the expected 185 kDa mass in two MPNSTs (Fig. 4), with a slightly smaller erbB4 immunoreactive protein evident in a third tumor.

To establish whether erbB kinases and NRG-1 within PNSTs were predominantly associated with tumor cells or non-neoplastic elements such as the abundant vasculature characteristic of these neoplasms, sections of a second series of neurofibromas and MPNSTs (Table 2) were immunostained with the 'pan' NRG-1 antibody or antibodies recognizing EGFR, erbB2, erbB3 or erbB4. EGFR immunoreactivity was detected in a subset of neurofibromas (three of seven tumors; Fig. 5 and Table 2) and MPNSTs (three of five tumors; Fig. 5 and Table 2), while erbB2 labeling was uniformly present in neurofibromas and MPNSTS (Fig. 5 and Table 2). Expression of the direct NRG-1 receptor, erbB3, was detected in five out of seven neurofibromas and in

three out of five MPNSTs (Fig. 5 and Table 2). ErbB4 immunoreactivity was less common, being found in four neurofibromas and one MPNST. NRG-1 and each of the erbB receptors were predominantly associated with cells within the tumor mass, demonstrating distinct cellular distributions in neurofibroma and MPNST cells. EGFR, erbB2 and erbB3 immunoreactivity was evident as cytoplasmic and membranous staining. In contrast, erbB4 immunoreactivity was predominantly associated with nuclei and surface membranes; the presence of nuclear immunoreactivity is consistent with previous studies demonstrating that activated erbB4 is proteolytically cleaved and its carboxy terminus translocated to the nucleus (54). NRG-1 immunoreactivity was also associated with the cytoplasm and surface membranes of tumor cells and thus has a distribution similar to that of EGFR, erbB2 and erbB3. These results, considered together with our PCR and immunoblot analyses, indicate that the majority of human MPNSTs express NRG-1 in combination with the erbB kinases necessary for NRG-1 responsiveness.

MPNST Cell Lines Express Multiple NRG-1 Isoforms and the ErbB Membrane Kinases Mediating NRG-1 Responses

The coexpression of multiple NRG-1 isoforms and their receptors in neurofibromas and MPNSTs raised the question whether these molecules are expressed by the same cells and thus capable of stimulating proliferation or other effects by autocrine and/or paracrine signaling. To test this hypothesis, we assembled a panel of human MPNST cell lines derived from *NF1*-associated (Mash-1 (42), NMS-2 (43) and NMS-2PC (43) cell lines) or sporadically arising (YST-1 cells; 44) MPNSTs. Prior to examining NRG-1 and erbB expression in these lines, we verified their lineage by immu-

nostaining cells grown in chamber slides or embedded in tissue arrays with a panel of antibodies recognizing Schwann cell, neuronal or muscle markers. Mash-1 (Fig. 6a), YST-1 (Fig. 6b), NMS-2 (Fig. 6c) and NMS-2PC (Fig. 6d) cells all demonstrated cytoplasmic and nuclear immunoreactivity for the Schwann cell marker S100β, which was abolished when the primary antibody was replaced with nonimmune IgG (Fig. 6e). All four lines also showed cytoplasmic immunoreactivity for collagen type IV (data not shown), a basal lamina protein elaborated by Schwann cells. Immunoreactivity for GFAP, the neuronal marker NSE and the muscle marker desmin was not evident in any of these lines. However, a subpopulation of cells within three of the MPNST lines stained for smooth muscle actin (Fig. 6f), suggesting that these lines, like a subset of MPNSTs (e.g. malignant Triton tumors), have a potential for divergent differentiation. To further test this hypothesis, we examined the expression of mRNAs encoding 18 Schwann cell, neuronal and muscle markers in all four lines using RT-PCR (Fig. 6g). In addition to the markers noted above, we found that these lines uniformly express transcripts encoding the Schwann cell markers myelin protein zero (P_0), $p75^{LNTR}$, Sox10 and Krox 20, with several lines additionally expressing myelin basic protein, peripheral myelin protein-22 (PMP22), Pax3 and GAP43. mRNAs encoding the light neurofilament subunit were also present in all four lines, with transcripts for a second neuronal intermediate filament, peripherin, weakly detectable in YST-1 and NMS-2 cells. The muscle marker SM22α (transgelin) was additionally expressed by all four MPNST cell lines; other muscle markers were detectable only with difficulty in some lines (calponin-1, desmin) or completely undetectable (MyoD1). We conclude that Mash-1, YST-1, NMS-2 and NMS-2PC cells express multiple Schwann cell markers and varia-

bly express neuronal and muscle markers, as expected for neoplastic elements derived from MPNSTs. Of note, the pattern of marker expression observed in these four human MPNST cell lines is similar to that found in cell lines derived from the MPNST-like tumors that develop in *Nf1*+/-:*p53*+/- mice (55).

Probing lysates of the human MPNST cell lines with the 'pan' NRG-1 antibody, we found that multiple NRG-1 immunoreactive proteins were present in all four lines (Fig. 7a). The major NRG-1 like proteins evident in these lines had masses of 45, 58, 120 and 150 kDa and thus comigrated with several of the NRG-1 species detected in surgically resected human neurofibromas and MPNSTs. Probing these same lysates with the antibody recognizing the subset of NRG-1 transmembrane precursors with an 'a' carboxy-terminal domain, we found that this antibody recognized proteins migrating at the same position as the two larger (120 and 150 kDa) NRG-1 proteins detected by the pan NRG-1 antibody (Fig. 7b). As in tumor tissue and sciatic nerve, this antibody also labeled a 55 kDa protein in all four lines and a 65 kDa polypeptide present in YST-1 cells; the sizes of these immunoreactive species are consistent with those of intracellular domain fragments released from mature NRG-1 transmembrane precursors by proteolytic cleavage (48). Preincubation of the pan NRG-1 and 'a' carboxy-terminus antibodies with their immunizing peptide abolished or markedly diminished the NRG-1 immunoreactivity detected in these MPNST cell line lysates (Fig. 7a and b), while preincubation with an unrelated antigen had no effect on the detected signals (data not shown).

The presence of variably sized NRG-1 immunoreactive proteins in the human MPNST lines suggested that these cells, like neurofibromas and MPNSTs *in vivo*, ex-

press multiple NRG-1 isoforms. To test this hypothesis, we examined the structure of the NRG-1 mRNAs expressed in each MPNST cell line. Using primers that amplify sequences encoding the EGF-like common, EGF-like variant, juxtamembrane and transmembrane domains, we detected NRG-1 transmembrane precursor transcripts in all four MPNST cell lines (Fig. 7c, top panels). Expression of mRNAs encoding NRG-1 secreted isoforms was more limited, being evident only in YST-1 cells (Fig. 7c, bottom panels). PCR performed with primers hybridizing to sequences encoding the amino-terminal domain of class II (GGF) NRG-1 isoforms demonstrated that the NRG-1 transcripts detected in all four MPNST cell lines included members of this NRG-1 subfamily (Fig. 7d, top panels), with class III (SMDF) NRG-1 mRNAs also present in NMS-2, NMS-2PC and YST-1 cells (Figure 7d, bottom panel). In contrast, class I (NDF) NRG-1 sequences were detectable only in YST-1 cells (data not shown). We conclude that NMS-2, NMS-2PC and YST-1 MPNST cells, like MPNSTs *in vivo*, express predominantly class II and III NRG-1 transmembrane precursor proteins. The diversity of NRG-1 isoforms expressed in Mash-1 cells is more restricted, consisting solely of class II NRG-1 transmembrane isoforms.

To establish whether each of the four MPNST cell lines expressed the erbB receptors necessary for NRG-1 responsiveness, we probed lysates of these cells with antibodies recognizing EGFR, erbB2, erbB3 or erbB4. We found that all four of these MPNST cell lines express a combination of erbB receptors that renders them potentially NRG-1 responsive (Fig. 8a). Two lines (Mash-1 and NMS-2PC) express erbB2 together with erbB3, a combination of NRG-1 receptor subunits identical to that in non-neoplastic Schwann cells (38), while a third (NMS-2 cells) expresses erbB2, erbB3 and

erbB4. Although YST-1 cells did not contain detectable levels of erbB2, these cells co-express erbB3 and erbB4; erbB4 is capable of heterodimerization with erbB3 or homodimerization with itself to form a functional NRG-1 receptor (54). Two MPNST cell lines (NMS-2 and NMS-2PC cells) also expressed EGFR, indicating that erbB signaling in these lines is potentially enhanced by coactivation of EGFR. EGFR was undetectable in lysates of Mash-1 and YST-1 cells.

In at least some cell types, erbB4 undergoes proteolytic cleavage and fragments from its carboxy terminus are transported to the nucleus, where they are thought to participate in as yet undefined signaling events (54). Probing immunoblots of NMS-2 and YST-1 lysates with an antibody recognizing the erbB4 carboxy terminus, we found that this antibody recognized both the full-length 185 kDa species and several smaller proteins (Fig. 8b). These smaller polypeptides included a fragment similar in mass to a previously described erbB4 cleavage product (80 kD; 54) and a prominent 55 kDa species (Fig. 8b, left panel). Preincubation with the immunizing peptide abolished labeling of both the full-length erbB4 protein and the smaller proteins (Fig. 8b, right panel). Consistent with the hypothesis that erbB4 is proteolytically cleaved in MPNST cells and its carboxy terminus transported to the nucleus, prominent erbB4 immunoreactivity was found in the nuclei of YST-1 (Fig. 8c) and NMS-2 (Fig. 8d) cells, with lighter staining also evident in association with the cytoplasm and/or cellular membranes. As shown above, this pattern of erbB4 immunoreactivity is similar to that seen in neurofibromas and MPNSTs *in vivo* (see Fig. 5).

Constitutive Activation of Erbb Receptors Promotes DNA Synthesis in Human MPNST Cell Lines

To determine whether human MPNST cells are responsive to NRG-1, Mash-1 cells, which express erbB2 and erbB3 in the absence of EGFR expression, were stimulated for 15 min with vehicle or NRG-1β (1, 10 or 50 nM). The direct NRG-1 receptor erbB3 was then immunoprecipitated from lysates of these cells, immunoblotted and probed with an antibody recognizing phosphotyrosine. Phosphorylated erbB3 was detected in these experiments as a band of the expected 185 kDa mass that increased in intensity with increasing concentrations of NRG-1β (Fig. 9a). Pretreating cells with PD168393, a 6-acrylamido-4-anilinoquinazoline compound that irreversibly inhibits erbB receptors by forming a covalent bond with a critical cysteine residue in the tyrosine kinase domain (56), blocked NRG-1β-induced phosphorylation of this 185 kDa protein (Fig. 9a), confirming its identification as erbB3. The coexpression of NRG-1 and its receptors in human MPNST cell lines suggests that autocrine or paracrine NRG-1 signaling may promote constitutive activation of erbB receptors expressed by neoplastic Schwann cells in MPNSTs. To test this hypothesis, we immunoprecipitated erbB4 from unstimulated YST-1 cells (a line that expresses erbB3 and erbB4 in the absence of detectable EGFR) and probed the blotted immunoprecipitates with an anti-phosphotyrosine antibody. A phosphorylated protein of the expected 185 kDa mass was readily detectable in unstimulated YST-1 cells (Figure 9b, left panel). The presence of this phosphorylated species was abolished by pretreating YST-1 cells with PD168393 (Fig. 9b, left panel). Longer exposures of the blots shown in Fig. 9a likewise demonstrated the presence of a lower level of phosphorylated erbB3 in unstimulated Mash-1

cells (Fig. 9b, right panel). We conclude that erbB receptors expressed in Mash-1 and YST-1 cells are constitutively phosphorylated.

To test the hypothesis that human MPNST mitogenesis is dependent on constitutive activation of the NRG-1/erbB signaling pathway, we challenged Mash-1, NMS-2, NMS-2PC and YST-1 cells with 1, 10, 100 or 1000 nM PD168393. After 24 h of inhibition, [^{3}H]thymidine incorporation in cells receiving vehicle was compared to that in cells treated with PD168393. We found that treatment with PD168393 reduced DNA synthesis in all four MPNST cell lines in a concentration-dependent manner (Fig. 10), although some cell lines were more sensitive to this treatment than others (e.g., compare Mash-1 and YST-1 cells). We also examined the effects that treatment with 1, 10 or 20 μM concentrations of PD158780, a 4-[ar(alk)ylamino]pyridopyrimidine derivative that specifically inhibits erbB receptors by competing with ATP (adenosine triphosphate) for binding to the erbB tyrosine kinase domain (57), had on DNA synthesis in these human MPNST cell lines. Consistent with our previous demonstration that PD158780 effectively reduces erbB tyrosine phosphorylation and DNA synthesis in JS1 cells (37) (a line derived from a chemically induced rodent MPNST), we found that this mechanistically distinct erbB inhibitor also markedly reduced DNA synthesis in all four human MPNST cell lines (data not shown).

DISCUSSION

We have previously shown that constitutive activation of the NRG-1/erbB signaling pathway is essential for the proliferation of an MPNST cell line derived from an EtNU-induced rodent tumor (36) and that NRG-1 overexpression in the Schwann cells

of transgenic mice induces the formation of MPNST-like neoplasms (37). The results presented here demonstrate that human neurofibromas and MPNSTs likewise express multiple NRG-1 isoforms in combination with the erbB membrane tyrosine kinases mediating NRG-1 responses. We have also found that four human MPNST cell lines similarly coexpress multiple NRG-1 isoforms and erbB kinases, that erbB receptors expressed by these cells are constitutively phosphorylated and that two structurally and mechanistically distinct pharmacologic inhibitors of erbB kinases reduce erbB phosphorylation in these cells and inhibit their proliferation. Considered together, these observations support the hypothesis that inappropriate activation of the NRG-1/erbB signaling pathway promotes neoplastic Schwann cell proliferation in human MPNSTs. Our results also raise some intriguing new questions regarding the role specific NRG-1 isoforms and erbB receptors play in human peripheral nerve sheath tumorigenesis and the possible significance of their coexpression with the EGFR.

Three lines of evidence indicate that NRG-1 is locally synthesized by Schwann cells within neurofibromas and MPNSTs. First, using an antibody recognizing the EGF-like common domain present within all functional NRG-1 isoforms, we identified multiple NRG-1-like immunoreactive proteins in all of the neurofibromas and MPNSTs we examined. Since NRG-1 mRNAs are also present within neurofibromas and MPNSTs, it is likely that much of the NRG-1 detected within each tumor was locally synthesized rather than representing NRG-1 protein derived from the circulation. This hypothesis is further substantiated by our finding that a second antibody recognizing the carboxy terminus of NRG-1 isoforms with an 'a' variant carboxy terminus also labels a subset of the NRG-1 proteins detected by the pan NRG-1 antibody in neurofibromas and

MPNSTs; the 'a' variant carboxy-terminal domain is intracellular and is not thought to be released from cells synthesizing NRG-1 transmembrane precursors (48). Finally, we have demonstrated that multiple NRG-1 proteins and mRNAs are expressed by four permanent cell lines derived from human MPNSTs. Taken together, these findings support the hypothesis that neoplastic Schwann cells themselves produce NRG-1, which then acts in an autocrine and/or paracrine manner to promote neoplastic Schwann cell proliferation.

The structure of the NRG-1 isoforms predicted by neurofibroma- and MPNST-derived NRG-1 cDNAs also has important implications for the potential signaling capabilities of these proteins. We have found that mRNAs encoding NRG-1 transmembrane precursors predominate in PNSTs, with transcripts encoding directly secreted NRG-1 isoforms being undetectable in these neoplasms. NRG-1 transmembrane precursors are thought to act when they are proteolytically cleaved, releasing the NRG-1-extracellular domain containing the receptor-binding EGF-like domain. The released extracellular domain then binds to and activates erbB receptors expressed on the same or neighboring cells. It has recently been found that this proteolytic cleavage also releases the intracellular domain of NRG-1 transmembrane precursors from the surface membrane, allowing it to be translocated into the nucleus of the NRG-1-expressing cell, where this domain is thought to mediate poorly understood 'backsignaling' events (48). Although our demonstration that erbB signaling is required for the proliferation of MPNST cells argues that 'forward' signaling by the NRG-1 extracellular domain is essential for peripheral nerve sheath tumorigenesis, the preferential expression of NRG-1 transmembrane precursors in PNSTs raises the question of whether backsignaling by

the NRG-1 intracellular domain also makes an important contribution to this process. As NRG-1 signaling in PNSTs is potentially dependent on proteolytic cleavage of transmembrane precursors, inhibitors of the enzymes that cleave NRG-1 transmembrane isoforms (e.g., matrix metalloproteases; 58, 59) may be useful agents for the treatment of neurofibromas and MPNSTs.

In 1986, Brockes *et al.* (60) reported that a heparin-binding GGF-like activity was present in a single MPNST and that two of the five neurofibromas they studied contained intermediate to high levels of a similar activity. We have found that mRNAs encoding class II (GGF) NRG-1 isoforms are expressed in human neurofibromas and MPNSTs. We did not detect mRNA encoding NRG-2, a related factor with similar biochemical properties, in any of these tumors using RT-PCR (data not shown). Considered together, these findings suggest that the GGF-like proteins detected by Brockes *et al.* were class II (GGF) NRG-1 isoforms, a group of molecules that act as secreted factors and accumulate at high concentrations in structures such as Schwann cell basement membranes. We also found that the NRG-1 isoforms expressed by neurofibromas and MPNSTs include class III (SMDF/CRD-NRG) NRG-1 variants. Unlike GGF isoforms, class III NRG-1 proteins remain membrane-associated (47) and thus, likely function solely as juxtacrine signaling molecules. The differing phenotypes of transgenic mice overexpressing class II (37) and class III (61) NRG-1 isoforms in the peripheral nervous system also indicate that members of these NRG-1 subfamilies have distinct effects on the phenotype of Schwann cells. Given these functional differences, it will be of great interest to establish what role class II and III NRG-1 isoforms play in the pathogenesis of PNSTs.

Our findings demonstrate that cells within neurofibromas and MPNSTs express combinations of erbB receptors that render them potentially NRG-1 responsive. As the human MPNST cell lines we have studied also express these receptors, it seems likely that a major portion of the erbB protein expressed in neurofibromas and MPNSTs is associated with neoplastic Schwann cells. These observations, considered together with our findings that NRG-1 is expressed in neurofibromas and MPNSTs, that the erbB receptors expressed in human MPNST cell lines are constitutively phosphorylated and that two mechanistically distinct erbB kinase inhibitors inhibit DNA synthesis in these lines, support the hypothesis that neoplastic Schwann cell proliferation is driven by autocrine and/or juxtacrine signaling in these PNSTs. Interestingly, however, the particular combination of erbB kinases expressed in an individual tumor is variable. Since erbB2, erbB3 and erbB4 differ in their ability to activate specific cytoplasmic signaling pathways (49), it is likely that NRG-1 signaling will have overlapping, but distinct, effects on the phenotype of neoplastic Schwann cells expressing different profiles of erbB receptors. Consistent with previous observations in human neoplasms and transgenic mouse models (52, 62), we have also found that EGFR is present in many human neurofibromas and MPNSTs, commonly being coexpressed with NRG-1 receptors. Although the EGFR does not bind NRG-1, it is capable of interacting with other activated erbB receptors, allowing the EGF and NRG-1 receptor systems to 'crosstalk' and modify the cellular responses evoked by EGFR and NRG-1 receptor ligands (49). These findings thus suggest that effects mediated through the erbB kinases expressed by human neurofibromas and MPNSTs may be variable, with the phenotype of neoplastic

Schwann cells being determined, in part, by the combination of erbB receptors expressed by individual cells.

In summary, our findings support the hypothesis that constitutive activation of the NRG-1/erbB signaling pathway promotes the proliferation of neoplastic Schwann cells in human neurofibromas and MPNSTs. The effectiveness erbB neutralizing antibodies (e.g., trastuzumab (Herceptin)) and small molecular erbB inhibitors (e.g., PD168393 and PD158780) have shown in preclinical and/or clinical studies with other tumor types raises the intriguing question of whether agents such as these may be effective in treating human neurofibromas and MPNSTs. Determining the role specific NRG-1 isoforms and erbB kinases play in the pathogenesis of human PNSTs will also be essential for developing new, more effective treatments for these therapeutically challenging Schwann cell neoplasms.

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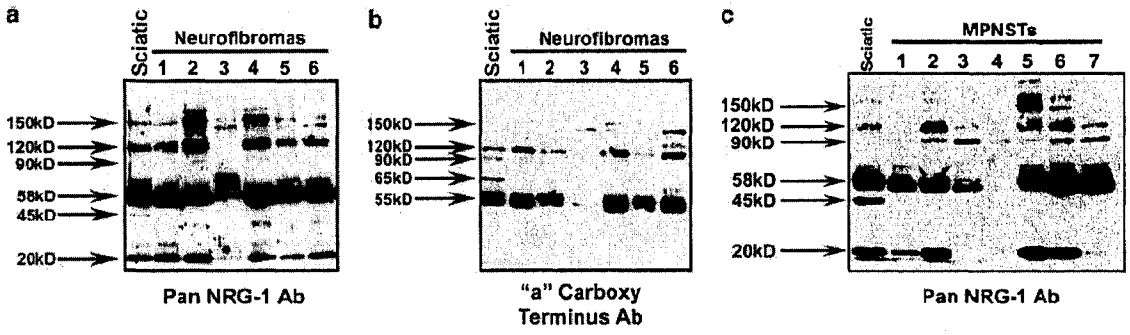


Figure 1. Neurofibromas and MPNSTs express multiple NRG-1 immunoreactive proteins. **(a)** Immunoblot of lysates of human sciatic nerve (Sciatic) and six human neurofibromas probed with a rabbit polyclonal antibody recognizing an epitope within the EGF-like common domain found in all NRG-1 isoforms. **(b)** Immunoblot of lysates of human sciatic nerve and human neurofibromas probed with a rabbit polyclonal antibody recognizing an epitope within the subset of NRG-1 transmembrane isoforms containing an 'a' variant carboxy terminus. **(c)** Immunoblot of lysates of human sciatic nerve (Sciatic) and seven human MPNSTs probed with a rabbit polyclonal antibody recognizing an epitope within the EGF-like common domain found in all NRG-1 isoforms. Arrows to the left of each panel indicate the major immunoreactive species and their molecular weights.

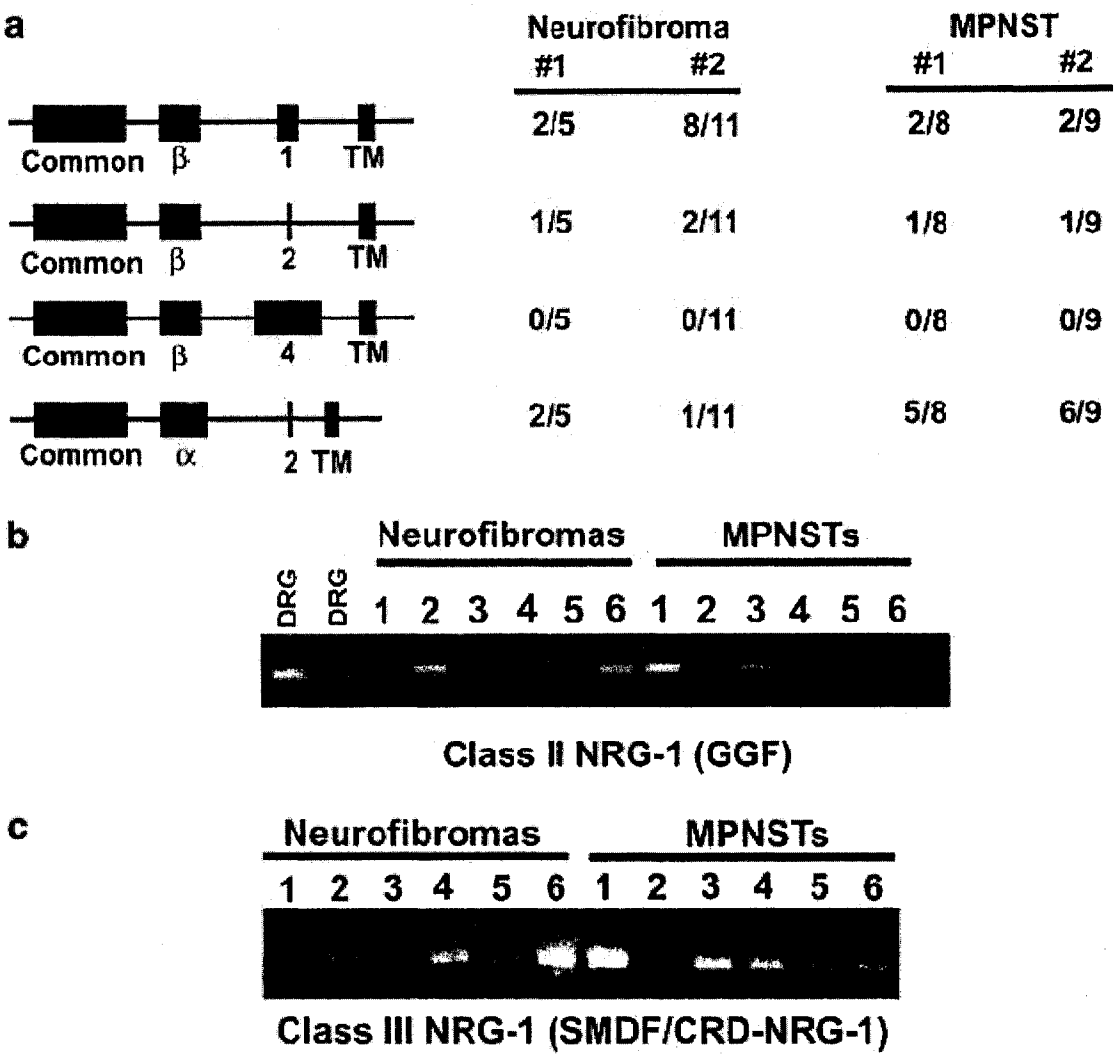


Figure 2. Neurofibromas and MPNSTs express mRNAs encoding a complex mixture of NRG-1 transmembrane precursors including α and β isoforms from the class II and III NRG-1 subfamilies. **(a)** Structures of the EGF-like and adjacent domains hypothetically possible in NRG-1 isoforms expressed in MPNSTs. Domain designations: Common, the NRG-1 EGF-like common domain; α or β, EGF-like variant domains; 1, 2 or 4, juxtamembrane domains; TM, transmembrane domain. Ratios to the right of each diagram indicate the number of times a structural variant was isolated divided by the total number of clones sequenced. **(b)** PCR analysis of class II NRG-1 (GGF) mRNA expression in human neurofibromas and MPNSTs. DRG cDNAs from two autopsied patients were included as positive controls. **(c)** PCR analysis of class III NRG-1 (SMDF/cysteine-rich domain NRG-1) mRNA expression in human neurofibromas and MPNSTs. Images in panels **(b)** and **(c)** are photographs of ethidium bromide-stained gels.

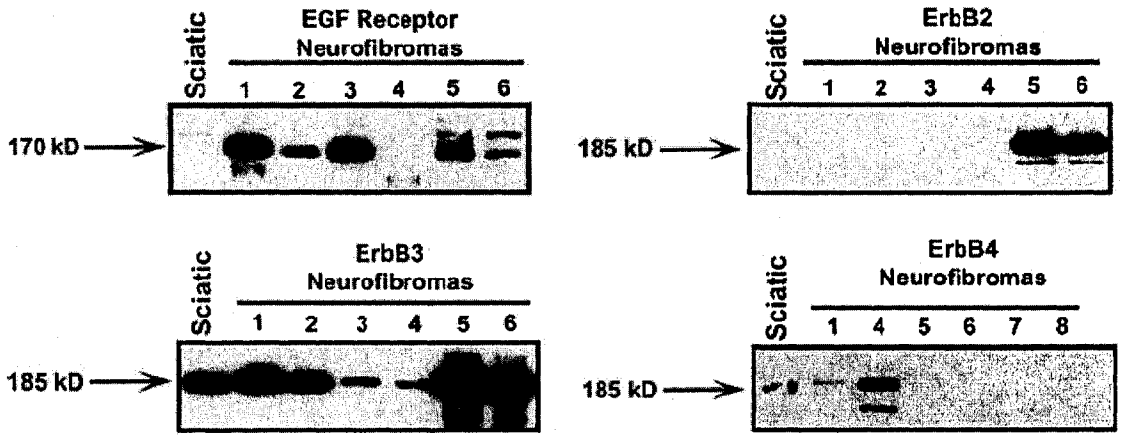


Figure 3. EGFR, erbB2, erbB3 and erbB4 protein expression in human neurofibromas. Arrows to the left of each panel indicate the expected position of each erbB kinase; in our experience, minor variability in the size of erbB kinases (potentially resulting from differences in post-translational modification) is frequently encountered in tumors overexpressing an erbB kinase. Lysates of human sciatic nerve and, in some blots JS1 MPNST cells, were included as controls. Numbers above each lane indicate the case number corresponding to the clinical information presented in Table 1. Longer exposures of the erbB2 blot showed that erbB2 expression was also present in sciatic nerve (data not shown; see also Fig. 4). The quantity of lysate available from tumors 2 and 3 was insufficient for examination of erbB4 expression, so two additional tumors (7 and 8) were obtained and probed for this receptor.

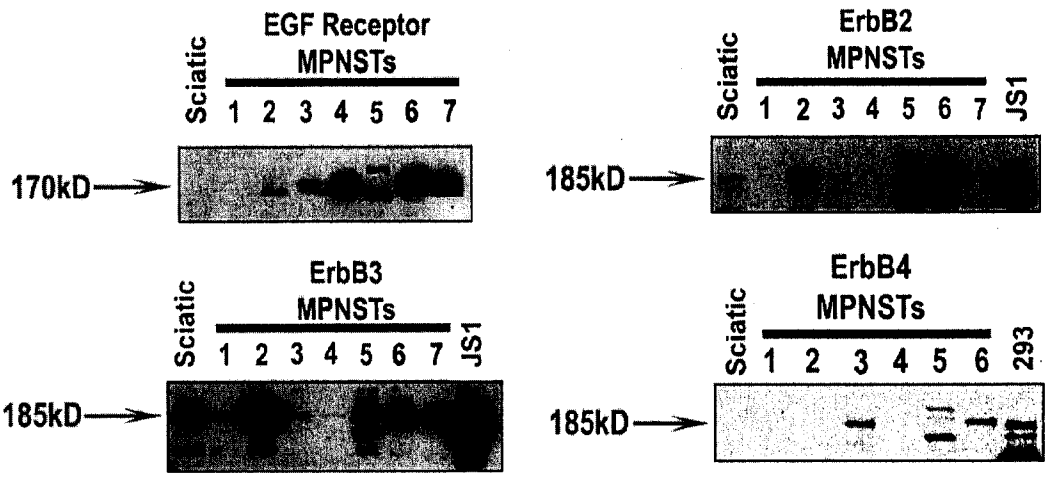


Figure 4. EGFR, erbB2, erbB3 and erbB4 protein expression in human MPNSTs. Arrows to the left of each panel indicate the expected position of each erbB kinase. Lysates of human sciatic nerve and, in some blots JS1 MPNST cells or 293 cells, were included as positive controls. Numbers above each lane indicate the case number corresponding to the clinical information presented in Table 1. Longer exposures of the erbB4 blot (not shown) showed low levels of erbB4 expression in sciatic nerve as were observed in Fig. 3.

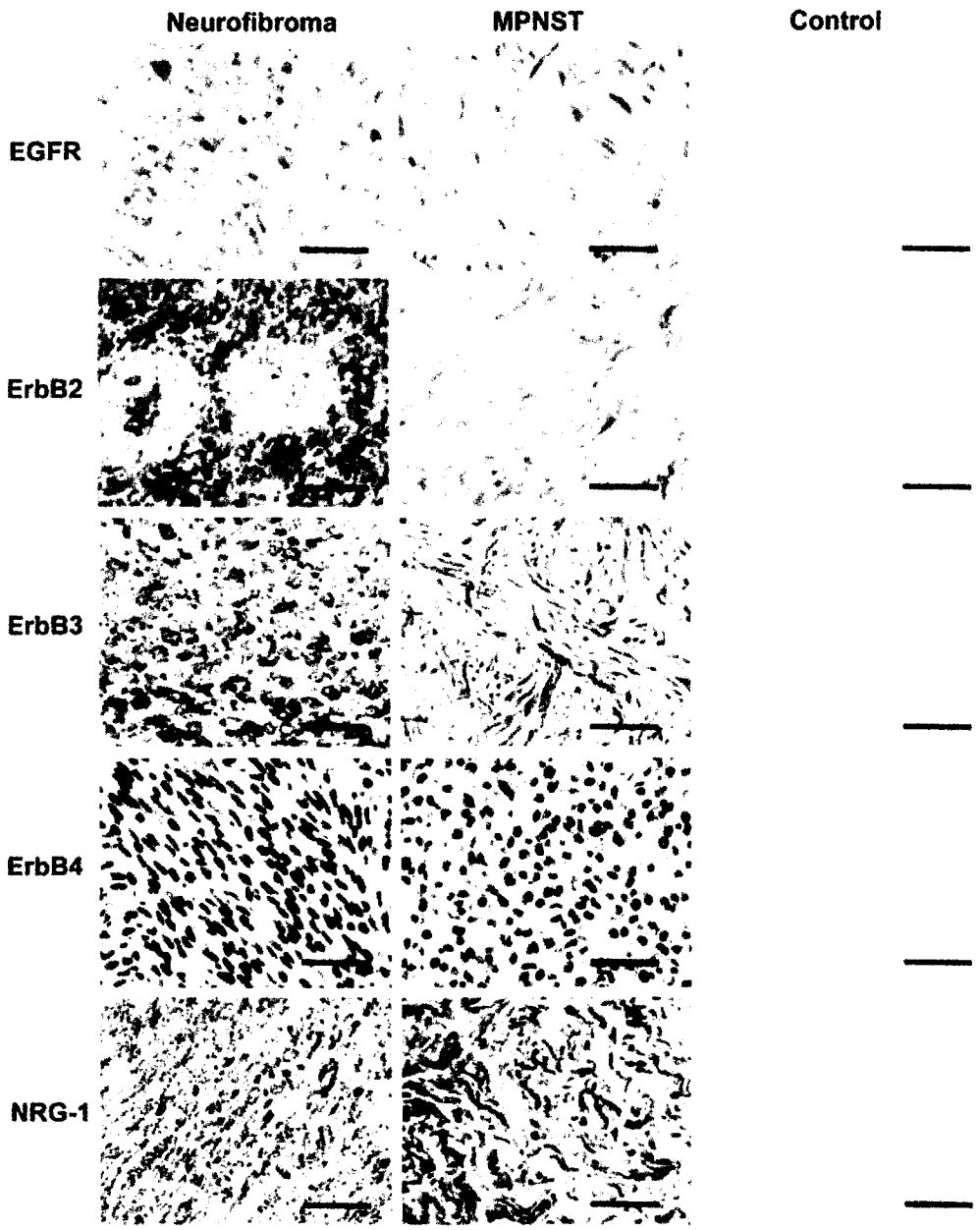


Figure 5. ErbB and NRG-1 immunoreactivity in human neurofibromas and MPNSTs. Shown are representative photomicrographs of neurofibromas (left column) and MPNSTs (middle column) immunostained for EGFR, erbB2, erbB3, erbB4 or the NRG-1 EGF-like common domain (indicated to the left of each row). The right column demonstrates the staining evident in control sections (either immunostains in which the primary antibody was replaced with nonimmune immunoglobulin (EGFR and erbB3) or immunostains in which the primary antibody was preincubated with the immunizing peptide (erbB2, erbB4 and NRG-1). These sections have been lightly counterstained with hematoxylin. Scale bar, 50 μm.

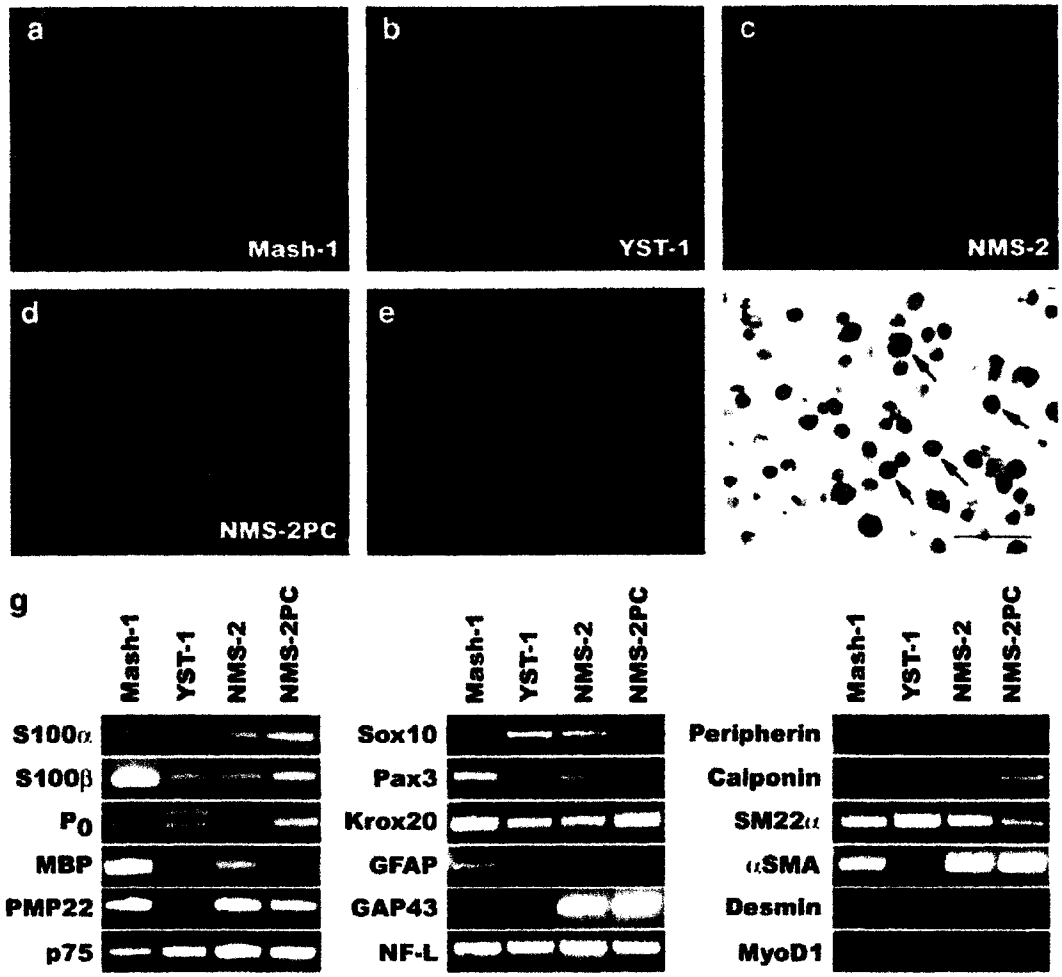
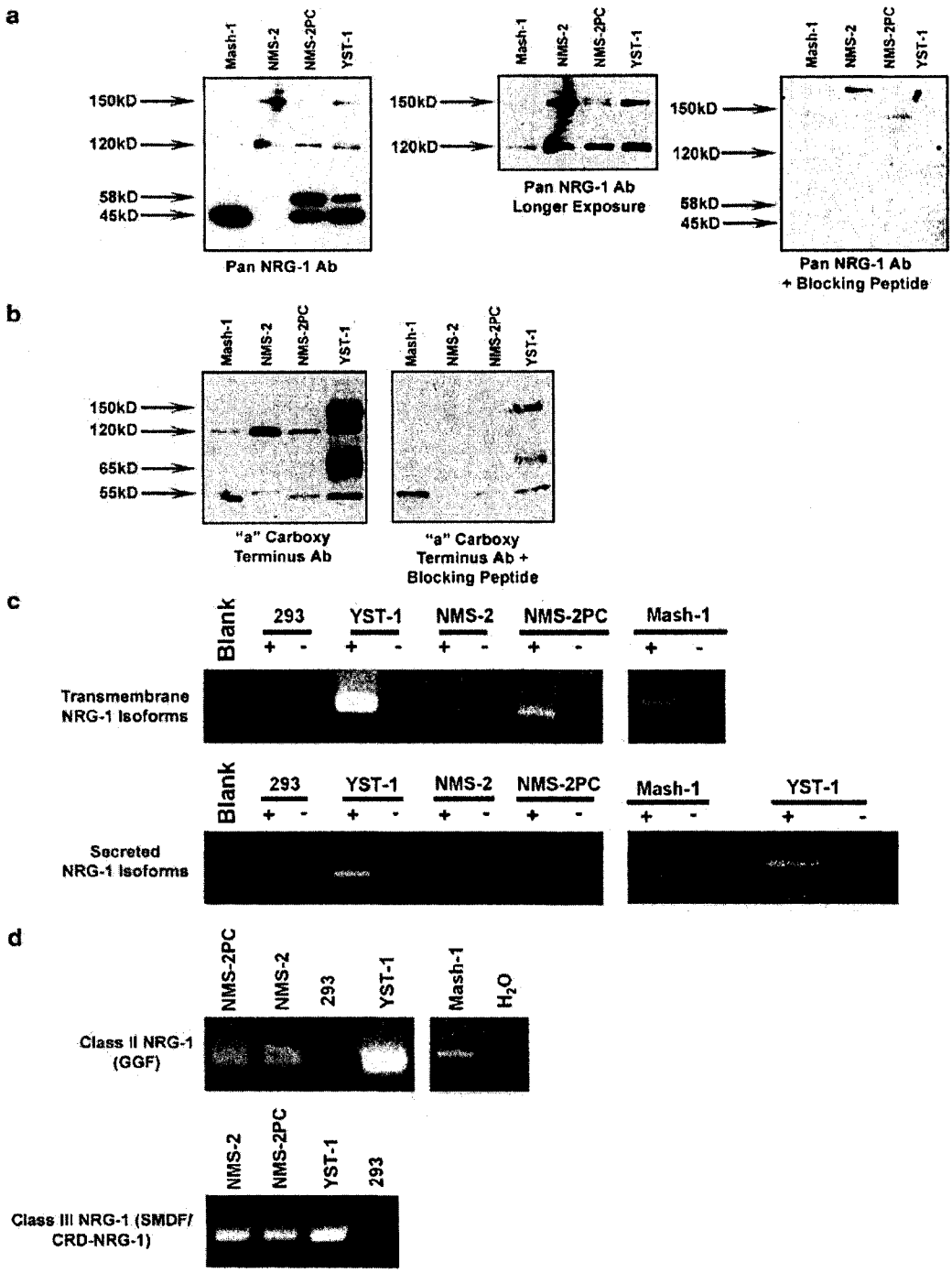


Figure 6. Human MPNST cell lines express multiple Schwann cell markers in combination with some neuronal and muscle markers. Shown are immunostains for the Schwann cell marker S100β in Mash-1 **(a)**, YST-1 **(b)**, NMS-2 **(c)** and NMS-2PC **(d)** cells. This immunoreactivity was not evident when the primary antibody was replaced with nonimmune IgG (**e**, representative photo of Mash-1 cells stained with nonimmune IgG). Tissue array section of NMS-2PC cells demonstrating immunoreactivity for smooth muscle actin in a subpopulation of these cells (**f**, arrows). Scale bar, 50 μm. **(g)**, RT–PCR analyses of the expression of mRNAs encoding Schwann cell, neuronal and muscle markers in human MPNST cell lines. The identity of the tested cell lines is indicated above each lane, with markers indicated to the left of each panel. P_0, myelin protein zero; MBP, myelin basic protein; PMP22, peripheral myelin protein-22; p75, the p75 low-affinity neurotrophin receptor; GFAP, glial fibrillary acidic protein; NF-L, light neurofilament subunit; αSMA, α-smooth muscle actin.

Figure 7. Human MPNST cell lines express multiple class II and III NRG-1 isoforms. **(a)**, Lysates of human MPNST cell lines (indicated above each lane) probed with an antibody recognizing an epitope in the EGF-like common domain found in all NRG-1 isoforms (left panel). A longer exposure (middle panel) is included to better demonstrate the higher molecular weight NRG-1 proteins in each cell line. Preincubation with the immunizing peptide abolished labeling of these proteins (right panel). Arrows to the left of these three panels indicate the position and molecular weight of each protein. **(b)** A second antibody recognizing an epitope at the carboxy terminus of a major subset of NRG-1 isoforms (transmembrane precursors with an 'a' carboxy terminus) labels proteins with molecular weights similar to some detected with the pan NRG-1 antibody (left panel) as well as potential cleavage products (55 and 65 kDa species). Preincubation with the immunizing peptide abolished or markedly reduced labeling with this antibody (right panel). **(c)** PCR analyses of the expression of transmembrane and secreted NRG-1 isoforms in human MPNST cell lines. The identity of the cell lines is indicated above each lane; reverse-transcribed RNAs and reverse transcription reactions performed identically except for the addition of reverse transcriptase are indicated as + and -, respectively. Blank is a PCR reaction performed with distilled water substituted for cDNA. YST-1 cDNA was included in the second set of PCR reactions for secreted NRG-1 isoforms (right panel) as a positive control. **(d)** PCR detection of sequences encoding class II (upper panels) and III (lower panel) NRG-1 isoforms. A cDNA produced by reverse transcription of 293 cells (293) and a water blank (H_2O) were included as negative controls.



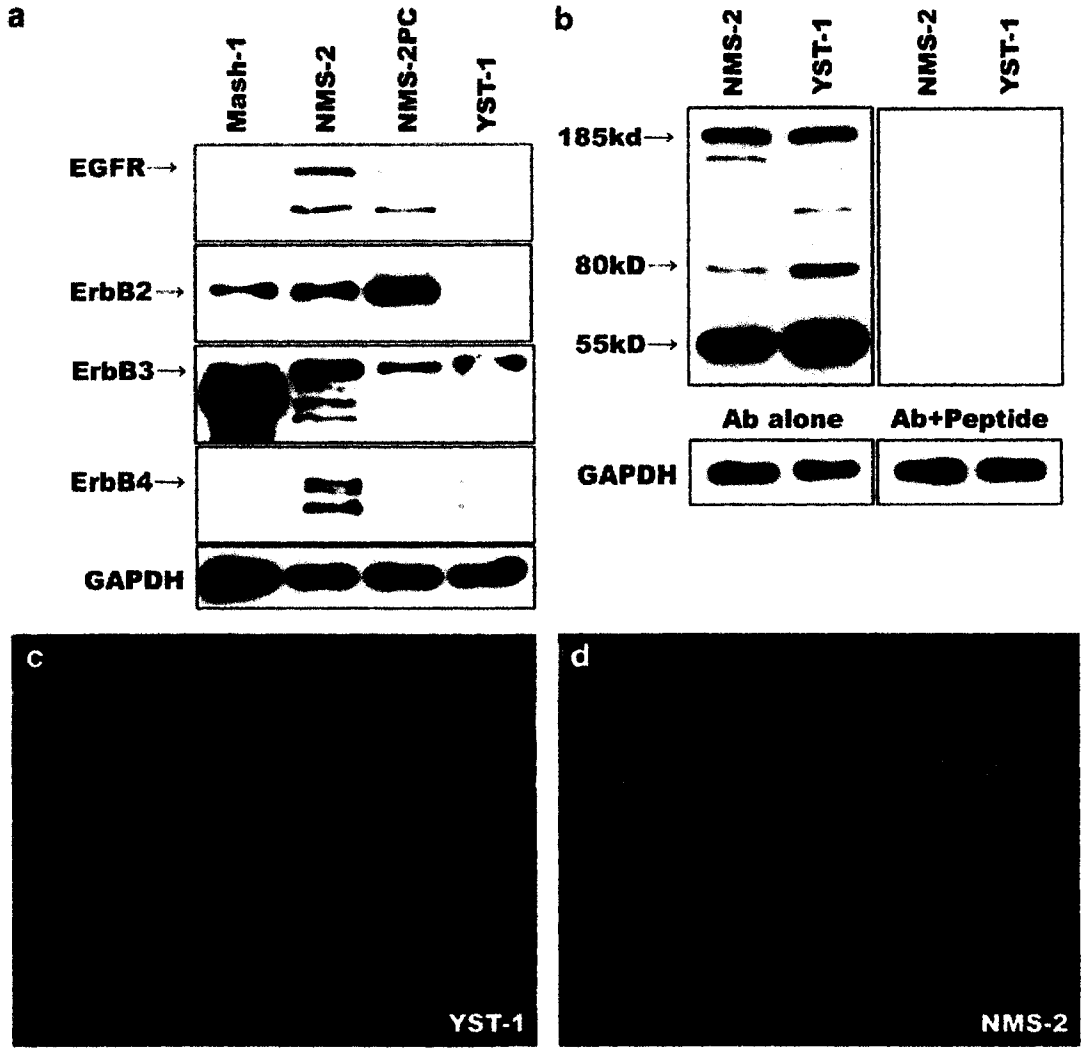


Figure 8. Human MPNST cell lines express varying combinations of the erbB kinases mediating NRG-1 responsiveness. **(a)** Immunoblot analyses of erbB expression in four human MPNST cell lines. The identity of the cell lines is indicated above each lane, with the specific full-length erbB receptor-(a 170 kDa protein for the EGFR; 185 kDa species for the other three erbB kinases) labeled by each antibody indicated to the left of the blots. GAPDH loading controls are shown below the erbB immunoblots. **(b)** Immunoblot analyses of the erbB4 immunoreactive species detectable in NMS-2 and YST-1 cells. Left panel, blots probed with anti-erbB4 antibody alone detect the 185 kDa full-length protein as well as some smaller species (indicated by arrows to the left of the panel). Right panel, preincubating the primary antibody with the immunizing peptide blocks the labeling of these species. GAPDH loading controls are indicated below each panel. **(c, d)**, YST-1 and NMS-2 cells immunostained with the erbB4 antibody demonstrate prominent nuclear labeling in combination with cytoplasmic and/or membranous labeling.

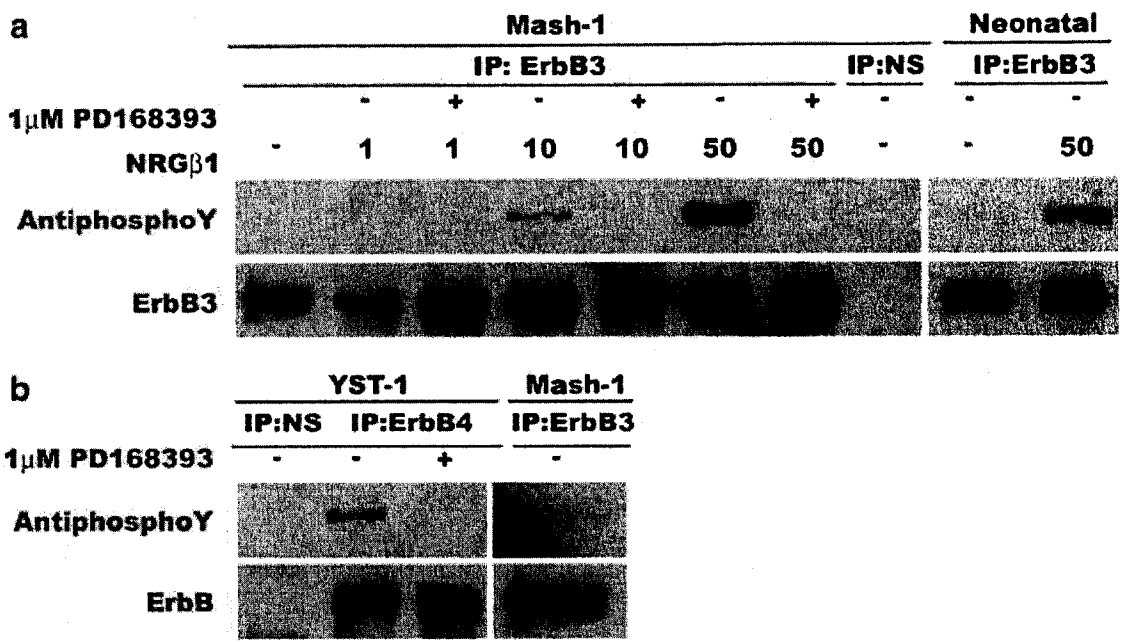


Figure 9. Human MPNST cells are NRG-1 responsive and demonstrate constitutive erbB tyrosine phosphorylation that is abolished by treatment with the pharmacologic inhibitor PD168393. **(a)** (Left panel), To examine NRG-1 responsiveness, Mash-1 cells were stimulated with vehicle (0) or 1, 10 or 50 nM recombinant NRG-1β in the presence (+) or absence (-) of 1 uM PD168393. ErbB3 was immunoprecipitated from lysates of these cells and the blotted immunoprecipitates probed with an anti-phosphotyrosine antibody (top panel), with phosphorylated erbB3 evident as a 185 kDa band. The bottom panel represents the top panel reprobed with an anti-erbB3 antibody. NS, control immunoprecipitation performed using nonimmune rabbit IgG. An induction of erbB phosphorylation is evident in cells stimulated with 10 or 50 nM NRG-1β. Right panel, control neonatal Schwann cells stimulated with NRG-1β. **(b)** Possible constitutive erbB tyrosine phosphorylation in YST-1 MPNST cells was examined by immunoprecipitating erbB4 from lysates of unstimulated YST-1 cells and probing immunoblotted immunoprecipitates with an anti-phosphotyrosine antibody (top panel). Phosphorylated erbB4 was evident as a 185 kDa species that was abolished by pretreating cells with PD168393. Longer exposures of the unstimulated Mash-1 immunoprecipitates illustrated in panel **(a)** likewise show evidence of constitutive erbB3 phosphorylation in this line (right panel). (Bottom panels), Reprobe of the blots shown in the top panels with an anti-erbB4 (left) or anti-erbB3 (right) antibody.

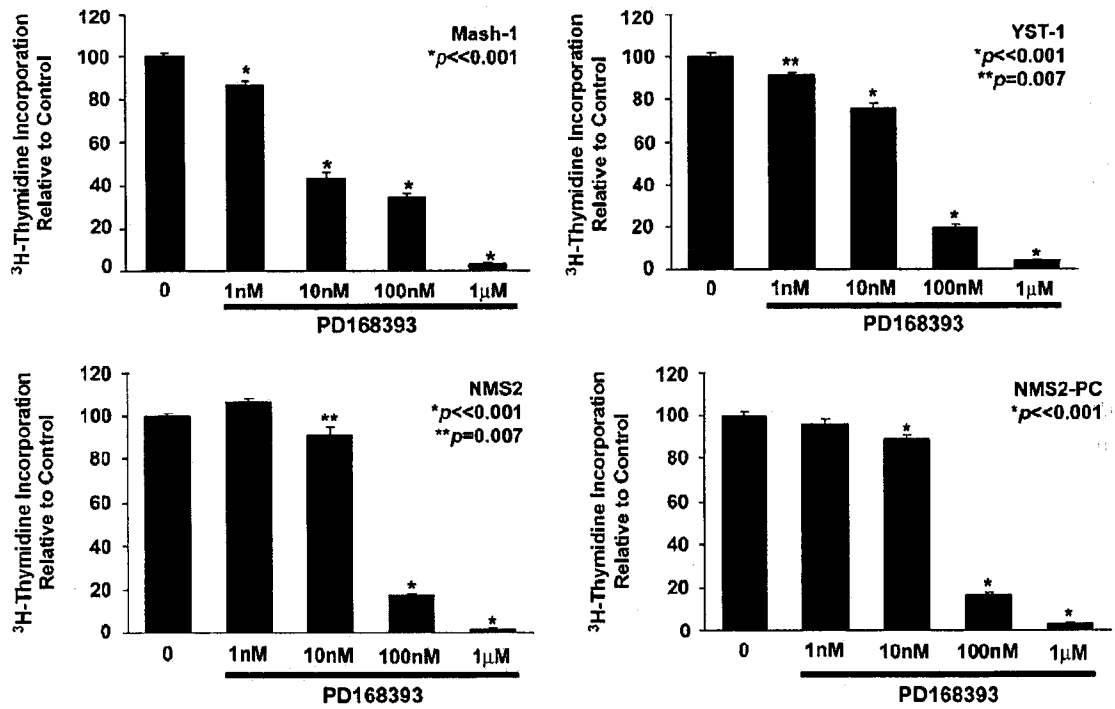


Figure 10. The erbB inhibitor PD168393 inhibits DNA synthesis in human MPNST cells in a concentration-dependent fashion. Bars indicate [^{3}H]thymidine incorporation relative to controls (untreated cells) in Mash-1, YST-1, NMS2 and NMS2-PC cells (indicated above each graph). Standard errors of the mean are indicated for each condition. *P*-values for the indicated treatment conditions are for comparisons to control. Experiments were performed in triplicate, with 6-8 replicates per condition performed in each experiment.

NEUREGULIN GROWTH FACTORS AND THEIR ERBB RECEPTORS FORM A POTENTIAL SIGNALING NETWORK FOR SCHWANNOMA TUMORIGENESIS

by

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ABSTRACT

Sporadic and neurofibromatosis type 2 (NF2)-associated schwannomas contain a glial growth factor (GGF)-like activity that has been hypothesized to promote neoplastic Schwann cell mitogenesis. It is not known whether this GGF-like activity is neuregulin-1 (NRG-1), an EGF-related molecule that regulates the proliferation, survival, and differentiation of developing Schwann cells; the related factor NRG-2; or another NRG/EGF ligand. Here we report that neoplastic Schwann cells within schwannomas overexpress multiple α and β transmembrane precursors from the class II and class III NRG-1 subfamilies. NRG-2 α and β transcripts are similarly overexpressed in these tumors. Of the other eight known NRG/EGF ligands, only heparin-binding EGF, epiregulin, and transforming-growthfactor-alpha (TGFα) are detectable in schwannomas. Neoplastic Schwann cells almost uniformly overexpress erbB2 and erbB3, two membrane receptor kinases mediating NRG-1 and NRG-2 action. Overexpression of the NRG receptor erbB4 and EGF receptor is also evident in schwannomas, but is more limited, only occurring in a subset of these tumors. ErbB2, the preferred dimerization partner for all erbB kinases, is constitutively phosphorylated in schwannomas. These observations suggest that autocrine, paracrine, and/or juxtacrine NRG-1/NRG-2 signaling promotes schwannoma pathogenesis and that this signaling pathway may be an important therapeutic target in schwannomas.

INTRODUCTION

Schwannomas, the third most common type of primary nervous system neoplasm, are derived from Schwann cells, the myelinating glia of peripheral nerves. These

benign peripheral nerve sheath tumors (PNSTs) arise most frequently from sensory nerves, with common sites of occurrence including nerves in the head and neck region, cranial and spinal nerve roots, and nerves along the extensor surfaces of the extremities (1). Schwannomas typically occur as single lesions in otherwise normal patients. However, individuals with the autosomal dominant tumor susceptibility syndrome neurofibromatosis type 2 (*NF2*) characteristically develop bilateral eighth cranial nerve (acoustic) schwannomas and multiple schwannomas of spinal dorsal nerve roots (2, 3). Multiple subcutaneous and spinal root schwannomas are also a feature of schwannomatosis, a rare genetic disorder distinct from *NF2* (4). Sporadic, *NF2*-associated and schwannomatosis-associated schwannomas frequently occur in confined spaces within the cranium and spinal column, where their impingement on nerves and the central neuraxis results in considerable patient morbidity. Surgical resection is the standard approach to treating these tumors, but can be rendered difficult by tumor size, multiplicity, or location. The alternative is radiotherapy or radiosurgery, both of which have shown limited success, particularly when larger neoplasms are being treated (5). Given these limitations, there is considerable interest in defining the molecular abnormalities promoting schwannoma tumorigenesis and using this information to develop new, more effective treatments for these tumors.

The genetic alterations involved in schwannoma pathogenesis are poorly understood. It is known that patients with *NF2* carry a germline mutation in the *NF2* tumor suppressor gene on chromosome 22q12 and, in keeping with the proposed function of this gene, develop schwannomas when the remaining functional copy of the *NF2* gene is lost (6, 7). Both sporadic and *NF2*-associated schwannomas demonstrate a nearly

universal loss of expression of merlin (8-10), the product of the *NF2* locus. Merlin (also known as schwannomin) is related to the ERM (ezrin, radixin, and moesin) proteins and has been found to modulate cell motility and to suppress mitogenesis (3); based on these actions, it is thought that a loss of merlin expression in Schwann cells leads to inappropriate Schwann cell growth and the formation of schwannomas. Other genetic abnormalities such as chromosome 1p loss (11) and 9q34 gain (12) have also been identified in small subsets of schwannomas, but the essential genes within these regions remain to be defined.

Overexpression of growth factors and/or their receptors may also contribute to the pathogenesis of sporadic and *NF2*-associated schwannomas. In 1986, Brockes et al reported that extracts from three *NF2*-associated acoustic schwannomas and 5 of 7 sporadic acoustic schwannomas contained a Schwann cell growth-promoting activity similar to that present in partially purified preparations of bovine pituitary glial growth factor (GGF) (13). Pituitary GGF was subsequently purified and cloned, leading to the finding that pituitary-derived GGF proteins were members of the neuregulin-1 (NRG-1) family of growth factors, a group of EGF-related molecules that act through erbB membrane tyrosine kinases (TKs) to stimulate the proliferation, survival, and migration of Schwann cells during development (14-16). The GGF-like activity present in schwannomas, however, has never been definitively identified. Although it is tempting to assume that this activity similarly represents NRG-1 proteins, other NRG-related molecules, including NRG-2, NRG-3, and NRG-4, have recently been identified. Some of these molecules have biochemical and functional characteristics similar to those of NRG-1, raising the question of whether the GGF-like activity in schwannomas instead

corresponds to one of these other growth factors. As an initial step toward defining the GGF-like activity present in schwannomas, we have systematically analyzed the expression of specific isoforms of NRG-1 and NRG-2 and of all the other known NRG and EGF family growth factors in human schwannomas. As each erbB kinase differs in its affinity for these ligands, we have also examined the expression of all four erbB receptors in schwannomas and ascertained whether there is evidence of constitutive erbB activation in these tumors.

MATERIALS AND METHODS

Study Cases

Experiments described in this manuscript were approved by the Institutional Review Boards for Human Use of the University of Alabama at Birmingham and Washington University School of Medicine. Paraffin blocks of human schwannoma cases were obtained from the files of the Departments of Pathology of Washington University School of Medicine (St. Louis, MO) and the University of Alabama School of Medicine (Birmingham, AL). Frozen surgically resected schwannoma tissue was provided by the Southern Division of the Cooperative Human Tissue Network/University of Alabama at Birmingham Tumor Bank and the University of Alabama Brain Tumor Bank.

Antisera and Immunohistochemical Reagents

We have previously described the production and characterization of a rabbit polyclonal antibody that recognizes the EGF-like common domain found in all biologi-

cally active NRG-1 isoforms (a "pan NRG-1" antibody) (17). A mouse monoclonal antibody recognizing an epitope in the EGF receptor (EGFR; residues 996-1002) was obtained from MP Biomedicals (clone c11; Irvine, CA). A mouse monoclonal anti-erbB2 antibody directed toward the C-terminus of human c-*neu* (residues 1,242-1,255) was purchased from Oncogene Research Products (Ab-3; La Jolla, CA). The mouse IgM monoclonal antibody RTJ.1 recognizes the C-terminal domain of erbB3; this antibody and an isotype-matched negative control clone (C48-6) were obtained from BD Biosciences PharMingen (San Diego, CA). Affinity-purified rabbit polyclonal antisera for EGFR (sc-03), erbB3 (sc-285), erbB4 (sc-283), and the NRG-1 "a"-carboxy-terminal domain (sc-348) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody recognizing erbB2 phosphorylated on Tyr^{1248} (clone PN2A) was purchased from Lab Vision Corporation (Fremont, CA). A rabbit anti-cow S100β antibody (antibody Z0311) was obtained from DAKO (Glostrup, Denmark). Nonimmune mouse and rabbit IgGs were obtained from Pierce (Rockford, IL). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit and donkey anti-mouse secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Tyramide signal amplification reagents, including streptavidin-HRP, biotinyl tyramide, amplification diluent, and blocking reagent, were purchased from Perkin-Elmer Life and Analytical Science Products (Renaissance TSA-Indirect kit; Boston, MA). DAB peroxidase substrate (SK-4100) was obtained from Vector Laboratories (Burlingame, CA).

Immunoblotting

Lysates for immunoblotting were prepared by homogenizing specimens in TRI-zol reagent (Invitrogen Life Technologies; Carlsbad, CA) according to the manufacturer's protocol. Protein pellets were dissolved in 1% sodium dodecyl sulfate (SDS) supplemented with protease (Sigma no. P8340) and phosphatase (Sigma no. P5726) inhibitors diluted 1:100-1:250. Protein concentrations were determined with a modified Lowry method (*DC* Protein Assay; Bio-Rad, Hercules, CA), and equivalent quantities of protein lysates were resolved using 8% SDS polyacrylamide gels. Proteins were transferred to PVDF membranes by electroblotting for 180 minutes at 0.5 A in transfer buffer (25 mM Tris [pH 8.3], 0.192 M glycine, 20% methanol). Nonspecific binding was blocked by incubating membranes in 5% nonfat dry milk in TBST (0.15 M NaCl, 10 mM Tris [pH 8.0], 0.05% Tween-20) prior to incubation with primary antibody. The following primary antibody concentrations were used for immunoblotting: pan NRG-1 (1:5,000), EGFR (sc-03; 1:1,000), erbB2 (Oncogene Ab-3; 1:500), erbB3 (sc-285; 1:1,000) and erbB4 (sc-283; 1:1,000). Horseradish peroxidase conjugated donkey anti-rabbit and donkey anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA) were used at a 1:10,000 dilution in 5% nonfat dry milk/TBST. Immunoreactive species were detected by enhanced chemiluminescence (Pierce).

Schwannomas commonly show highly variable degrees of collagenization. This variability can markedly alter the relative contribution intracellular and extracellular proteins make to the final protein composition of tumor lysates. In keeping with this, our preliminary experiments demonstrated that using GAPDH, β-actin, or ERK-2 immunoreactivity as a loading control for schwannoma lysates was problematic. Conse-

quently, we verified equivalent loading and transfer by Coomassie staining PVDF membranes after transfer. Specificity of immunoblotting was determined in parallel experiments in which primary antibodies were preincubated with a 5- to 10- fold excess of the appropriate immunizing peptide prior to probing blots.

Immunohistochemistry

Immunohistochemistry for NRG-1 and its erbB receptors was performed using a highly sensitive staining protocol we have previously developed and validated (18). In brief, after 4- to 5-μm sections were deparaffinized in xylenes and graded alcohols, antigen rescue was performed by gently boiling slides in 10 mM citrate buffer (pH 6.0) for 15 minutes and then allowing them to cool to room temperature for 15 minutes. Non-specific binding was blocked by incubating sections for 15 minutes with TNB blocking buffer (0.1 M Tris-HCl [pH 7.5]/0.15 M NaCl/ 0.5% blocking reagent [Perkin-Elmer]) at room temperature. Sections were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer (pan NRG-1, 1:250-1:500; EGFR [clone c11], 1:100; erbB2 [Oncogene Ab-3], 1:100; erbB3 [clone RTJ.1], 1:100; erbB4 [sc-283], 1:500 and phosphorylated erbB2 [clone PN2A], 1:500). Adjacent sections of each tumor were stained with anti-S100β antibody as a positive control. After three rinses in phosphate-buffered saline (PBS; 10 minutes per rinse), sections were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody (diluted 1:500 in blocking buffer). After three washes in PBS, biotinyl tyramide (diluted 1:50 in amplification diluent) was applied to tissue sections for 10 minutes at room temperature. Sections were again washed three times in PBS, follow-

ing which they were incubated with streptavidin-HRP (diluted 1:100 in TNB) for 45 minutes. After three final washes with PBS, immunoreactive structures were visualized by diaminobenzidine deposition. To confirm the specificity of staining, control sections were incubated with non-immune rabbit or mouse immunoglobulin in place of the primary antibodies; no staining was observed in these experiments. When the immunizing peptide was available, the specificity of staining was further confirmed by preincubating antisera with either the immunizing peptide or a nonrelated peptide (10 ng/ml); in all instances, preincubation with the immunizing peptide, but not the nonrelated peptide, abolished or markedly reduced the staining pattern observed with primary antibody alone.

RT-PCR Analyses of NRG-1 Isoforms Expressed in Schwannoma Biopsy Specimens

Our designation of NRG-1 domains follows the nomenclature of Peles and Yarden (19). The common forward oligonucleotide used for amplification of sequences encoding the EGF-like and juxtamembrane domains of NRG-1 corresponds to nucleotides 631-648 of human neu differentiation factor (NDF; GenBank accession no. U02326) (20). The reverse oligonucleotide used for PCR of NRG-1 transmembrane precursor cDNAs represents nucleotides 811-828 (encoding a portion of the transmembrane domain sequence) of human NDF. This oligonucleotide pair will amplify sequences encoding all possible combinations of EGF-like and juxtamembrane sequences found in NRG-1 transmembrane isoform mRNAs. Secreted NRG-1 isoform cDNAs were amplified using a reverse oligonucleotide corresponding to nucleotides 482-502 of

a human NDFβ3 splice variant (GenBank accession no. U02327) (20) in combination with the common forward oligonucleotide.

Total RNA was isolated from schwannomas using Trizol reagent according to the manufacturer's recommendations. Single-stranded cDNA for use as a PCR template was reverse transcribed from total cellular RNA in a 20-μl reaction containing random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Superscript Plus; Life Technologies, Gaithersburg, MD). After completion of reactions, samples were diluted to 100 μl with distilled water, boiled for 5 minutes, and stored at -80°C until use.

Two μl of each cDNA was used as a PCR template in reactions performed for 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. PCR products were ligated to pCR4-TOPO and ligations were transformed into *Escherichi coli* (TOP10 strain) as recommended by the manufacturer (Invitrogen). The sequence of these clones was determined by cycle sequencing using an automated sequencer (ABI Model 373A DNA Sequencing System, Applied Biosystems, Inc; Foster City, CA).

Quantitative RT-PCR

One μg of total RNA isolated from schwannomas as described above was reverse transcribed in a 20-μl reaction containing random hexamer primers and Moloney murine leukemia virus reverse transcriptase; a parallel reaction, performed identically except for the addition of reverse transcriptase, was used to verify an absence of genomic DNA contamination. After completion of reverse transcription, samples were di-

luted to 100 µl with distilled water, boiled for 5 minutes, and stored at –80°C until used in real-time PCR experiments.

Real-time quantitative PCR was performed using an ABI 7500 Real Time PCR System (Applied Biosystems, Inc.). Assays for NRG-1, NRG-2, and erbB cDNA levels were performed using TaqMan MGB probes labeled with FAM dye (ABI Assay-on-Demand Gene Expression assays) in a 20-µl reaction containing 2 µl of cDNA, ROX passive reference dye, and TaqMan Universal PCR mix (Applied Biosystems, Inc.); this reaction mix was supplemented with uracil *N*-glycosylase to minimize the possibility that spurious results could be produced by carryover of contaminating PCR products. The specific primer sets used and the regions hybridizing to the FAM-labeled probes are as follows:

1. EGFR: Hs00193306_m1, probe sequence starts at nucleotide 2,715 of NM005228.
2. ErbB2: Hs00170433_m1, probe sequence starts at nucleotide 2,139 of NM004448.
3. ErbB3: Hs00176538_m1, probe sequence starts at nucleotide 273 of NM001982.
4. ErbB4: Hs00171783_m1, probe sequence starts at nucleotide 1,524 of NM005235.
5. NRG-1α: Hs01103794_m1, probe hybridizes to sequences at the junction of the EGF-like common and α domains.
6. NRG-1β: Hs00247624_m1, probe hybridizes to sequences at the junction of the EGF-like common and β domains.
7. NRG-1 Class I/II isoforms: Hs00247620_m1, probe and amplicon are within sequences encoding the immunoglobulin-like domain.
8. NRG-1 Class III isoforms: Hs00247641_m1, probe hybridizes to sequences at the junction of the SMDF amino-terminal and EGF-like common domains.

9. NRG-2α: Hs00993410_m1, probe hybridizes to sequences at the junction of the EGF-like common and α domains.
10. NRG-2β: Hs00993401_m1, probe hybridizes to sequences at the junction of the EGF-like common and β domains.

Levels of 18S ribosomal cDNA were assayed in parallel reactions using TaqMan MGB probes labeled with VIC dye (Applied Biosystems no. 4319413E). Assays for 18S and target transcripts were performed in triplicate for each cDNA.

Experimental results were analyzed using Applied Biosystems Sequence Detection Software (Version 1.2.3). The relative quantity of each target transcript was established by normalizing the level of the corresponding cDNA to that of 18S cDNA in the same reverse transcription reaction. Controls lacking added template were performed in parallel with each primer set to verify an absence of contamination.

RT-PCR Analyses of NRG-3, NRG-4, and EGF Family Ligand Expression in Schwannomas

One μg of total RNA isolated from schwannomas as described above was reverse transcribed in a 20-μl reaction containing oligo-dT primer and Moloney murine leukemia virus reverse transcriptase; a parallel reaction, performed identically except for the addition of reverse transcriptase, was used to verify an absence of genomic DNA contamination. Two μl of each cDNA was used as a PCR template in reactions performed for 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The sequences of the primers used in these experiments correspond to the following nucleotides of the indicated archived sequences:

1. NRG-2: 926-949 and 1251-1230, GenBank accession no. NM013984

2. NRG-3: 169-189 and 607-587 (set 1); 79-102 and 529-510 (set 2), GenBank accession no. AK098823
3. NRG-4: 66-87 and 475-453, GenBank accession no. NM138573
4. Heparin-binding EGF: 753-776 and 1158-1135, GenBank accession no. NM001945
5. β-cellulin: 279-298 and 740-717, GenBank accession no. NM001729
6. Epiregulin: 2548-2571 and 3071-3049, GenBank accession no. NM001432
7. EGF: 3288-3311 and 3690-3671, GenBank accession no. NM001963
8. Transforming growth factor α: 881-897 and 1348-1325, GenBank accession no. NM003236
9. Amphiregulin: 574-597 and 1010-990 (set 1); 103-126 and 614-591 (set 2), GenBank accession no. NM001657

PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining. Parallel reactions lacking cDNA were performed to verify an absence of contamination in these experiments.

RESULTS

Human Schwannomas Express Multiple NRG-1-Immunoreactive Proteins

At least 15 structurally distinct NRG-1 proteins are produced from a single gene via a combination of alternative mRNA splicing and alternative promoter usage (21). All of these proteins contain an epidermal growth factor (EGF)-like domain that directly binds to and activates NRG-1 receptors (22). We therefore focused initially on determining whether the NRG-1 EGF-like domain was detectable in human schwannomas. Lysates prepared from six surgically resected human schwannomas (Table 1)

were immunoblotted and probed using a "pan NRG-1" antibody that recognizes the amino-terminal (common) portion of the NRG-1 EGF-like domain.

Table 1. Characteristics of Human Schwannomas Used for Protein and RNA Analyses

Case no.	Diagnosis	Location	Age/Race/Gender
-	Normal	Eighth cranial nerve	52/B/F
1	Schwannoma	Sacral spinal nerve	48/H/M
2	Schwannoma	Sacral spinal nerve	31/H/F
3	Schwannoma	Eighth cranial nerve	35/W/F
4	Schwannoma	Thoracic spinal nerve	31/W/F
5	Schwannoma	Eighth cranial nerve	38/W/F
6	Schwannoma	Thoracic spinal nerve	22/B/F

As all of the schwannomas examined were derived from either eighth cranial nerve or spinal nerve roots, the expression of NRG-1 in these schwannomas was compared to that evident in lysates of normal eighth cranial nerve collected from an autopsied patient. Multiple NRG-1-immunoreactive species were detectable in all six schwannomas, with sizes ranging from 150- to 58-kD (Fig. 1A). The masses of the larger (150- and 120-kD) NRG-1 proteins labeled by this antibody were similar to those of the NRG-1 isoforms we have previously detected in brain and spinal cord lysates (17) and likely represent NRG-1 transmembrane precursor proteins. The other NRG-1-immunoreactive species detectable in schwannomas (90-, 65-, 60- and 58-kD polypeptides) were too small to be transmembrane precursors and had masses consistent with either directly secreted NRG-1 isoforms or extracellular domain sequences released by proteolytic cleavage of NRG-1 transmembrane precursors (17, 21, 23). When comparing the NRG-1 proteins detected in schwannomas with those evident in normal eighth cranial nerve, we found that the most prominent (58-kD) NRG-1 protein in normal

nerve comigrated with a protein present in the majority of the schwannomas tested. However, schwannomas also consistently demonstrated prominent expression of several higher molecular weight NRG-1 isoforms that were detectable in normal nerve only with long exposures, suggesting that the tumors expressed distinct profiles of NRG-1 isoforms. In both schwannomas and eighth cranial nerve, labeling of NRG-1-immunoreactive proteins was abolished or markedly diminished by preincubating the primary antibody with immunizing peptide (data not shown).

To further establish the identity of the proteins labeled by the pan NRG-1 antibody, schwannoma lysates were probed with an antibody that recognizes an epitope within the carboxy-terminus of a major subset of NRG-1 isoforms (transmembrane precursors with an "a" variant carboxy-terminus). This antibody labeled several proteins in schwannomas that ranged in size from 55- to 150-kD (Fig. 1B). The larger polypeptides detected by the "a" carboxy-terminus antibody (150- and 120-kD) comigrate with the larger species labeled by the pan NRG-1 antibody, consistent with the notion that these molecules are NRG-1 transmembrane precursors. The anti-NRG-1 "a" carboxy-terminus antibody also labeled several smaller proteins in eighth cranial nerve and in a subset of schwannomas. These smaller (80-, 70-, 65-, and 55-kD) proteins are too small to be full-length NRG-1 transmembrane precursor proteins and likely represent proteolytic cleavage products of NRG-1 transmembrane precursors. Labeling of all of the polypeptides detected by the anti-"a" carboxy-terminus antibody in schwannomas and eighth cranial nerve was abolished or markedly diminished by preincubating the primary antibody with immunizing peptide (data not shown). Considered together, these

findings indicate that multiple NRG-1 proteins are present in schwannomas, at least some of which are NRG-1 transmembrane precursors.

NRG-1 protein in schwannomas could be primarily associated with neoplastic Schwann cells or alternatively be derived from nonneoplastic elements within the tumors (e.g. vasculature or cells in the tumor capsule). To establish the distribution of NRG-1 in schwannomas, we immunostained paraffin sections prepared from a second series of six tumors (Table 2) using the pan NRG-1 antibody. NRG-1 immunoreactivity was readily detectable in four of these neoplasms, where it was associated with neoplastic Schwann cells (Fig. 2A).

TABLE 2. ErbB Immunoreactivity in Human Schwannomas

Case no.	Location	Age/Race/Gender	ErbB-1	ErbB-2	ErbB-3	ErbB-4	PN2A
7	Eighth cranial nerve	37/W/F	+	+	+	+	-
8	Thoracic spinal nerve	22/B/F	-	+	-	-	+
9	Thoracic spinal nerve	71/W/M	+	+	+	-	+
10	Thoracic spinal nerve	71/W/M	+	+	+	-	-
11	Eighth cranial nerve	74/W/F	-	+	-	-	+
12	Eighth cranial nerve	57/B/M	-	+	+	+	+

In contrast, little immunoreactivity was evident in the nonneoplastic elements within the tumors. Immunostaining of neoplastic Schwann cells was eliminated when the pan NRG-1 antibody was replaced with nonimmune rabbit IgG (Fig. 2B) and was markedly diminished when the primary antibody was preincubated with the immunizing peptide prior to staining tumor sections. Immunostaining with the anti-"a" carboxy-

terminus antibody produced a similar distribution of NRG-1 immunoreactivity in schwannomas (data not shown), further confirming these observations.

Schwannomas Overexpress a Complex Mixture of α and β Transmembrane Precursors from the Class II and Class III NRG-1 Subfamilies

The presence of multiple variably sized NRG-1 proteins in schwannomas suggested that these tumors express several structurally and functionally distinct NRG-1 isoforms. Structural variation in the NRG-1 EGF-like domain and adjacent regions has particularly important functional implications. The NRG-1 EGF-like domain is composed of an invariant amino-terminal (common) domain that can be fused to either an α or a β domain (Fig. 3), resulting in the production of isoforms that differ in their affinity for the NRG-1 receptors and in their ability to elicit at least some responses such as mitogenesis (21). A structurally variable juxtamembrane domain is found immediately carboxy terminal to the EGF-like domain; one variant of this domain ("3" isoforms) contains a termination codon and thus allows the protein to be directly secreted, while the other three (1, 2, or 4 variants) are fused to membrane-spanning sequences in NRG-1 transmembrane precursors (Fig. 3). To determine whether the NRG-1 isoforms expressed in human schwannomas demonstrated structural variability in these regions, we used RT-PCR to amplify sequences encoding the EGF-like common, EGF-like variant, juxtamembrane, and transmembrane domains of NRG-1 transmembrane precursors. Using RNA isolated from five of the same tumors used for our immunoblot analyses, we found that these NRG-1 sequences were uniformly detectable in all five schwannomas (data not shown). To further examine the structure of these NRG-1 transmembrane precursor mRNAs, we cloned the PCR products derived from two tumors and se-

quenced 21 independent subclones. We found that the NRG-1 transmembrane precursor transcripts expressed in schwannomas were structurally diverse and included mRNAs encoding NRG-1 α2, β1, and β2 isoforms (Fig. 3). Using these same schwannoma cDNA templates, we also attempted to amplify sequences encoding the EGF-like common, EGF-like variant, and "3" juxtamembrane domains of directly secreted NRG-1 isoforms. Although these primers produced a PCR product of the expected size from control templates, we could not detect secreted NRG-1 isoform transcripts in any of the five schwannomas tested (data not shown).

The NRG-1 protein we detected in normal eighth cranial nerve and schwannomas by immunoblot analyses is potentially produced by cells endogenous to both tissue types. However, it is known that NRG-1 protein is transported anterogradely and retrogradely along axons in peripheral nerve (24), raising the question of whether much of the NRG-1 protein we detected in eighth cranial nerve was axon derived. As mRNAs are largely excluded from axons, measurements of NRG-1 mRNA levels are likely to produce a more accurate comparison of NRG-1 expression in normal and neoplastic Schwann cells. We therefore compared the levels of expression of NRG-1 α and β transcripts in normal eighth cranial nerve and schwannomas. Performing real-time quantitative PCR with TaqMan primers specific for NRG-1α or NRG-1β cDNAs and normalizing the resulting data to the levels of 18S ribosomal RNA present in the same cDNAs, we found that both types of transcripts were detectable in normal eighth cranial nerve, albeit at low levels (Fig. 4A). The expression of NRG-1α mRNA was consistently markedly elevated in schwannomas, being found at levels ranging from 66 to over 5,000 times greater than that found in normal eighth cranial nerve. NRG-1β mRNA ex-

pression was also increased in all five of the schwannomas we tested (Fig. 4A), with levels varying from 59- to 295-fold greater than the levels present in normal eighth cranial nerve. Comparing the increase in NRG-1β transcript levels to that for NRG-1α in these schwannomas, we found that four of the five tumors demonstrated greater increases in NRG-1α mRNA than in NRG-1β mRNA.

Three major NRG-1 subfamilies (class I [NDF], class II [GGF], and class III [SMDF/cysteine-rich domain] isoforms) have been identified, each of which is characterized by subfamily-specific amino-terminal sequences with distinct functional characteristics. To determine which of these three subfamilies are expressed in schwannomas, we performed RT-PCR analyses using primers specific for the amino-terminal sequences of each subfamily. We found that Class II and Class III transcripts were readily detected in schwannomas, while Class I mRNA expression was not evident in these tumors (data not shown). To compare the relative levels of expression of the major NRG-1 subfamilies in schwannomas to that in normal eighth cranial nerve, we performed real-time quantitative PCR using TaqMan primers specific for the immunoglobulin-like (Ig-like) domain present in Class II isoforms or primers specific for the Class III amino-terminal domain. Again, we found that both primer sets detected low levels of mRNA in eighth cranial nerve (Fig. 4B). The expression of Class II NRG-1 mRNA was consistently increased in the five schwannomas we examined, with levels ranging from 6- to 159-fold greater than that found in eighth cranial nerve. Class III NRG-1 transcript levels were likewise elevated in all five schwannomas, with levels 8- to 1,380-fold higher than the levels detected in eighth cranial nerve. We conclude that

human schwannomas overexpress a complex mixture of NRG-1 mRNAs encoding α and β transmembrane precursors from the Class II and III subfamilies.

Schwannomas Overexpress NRG-2 α and β Isoforms Relative to Normal Eighth Cranial Nerve

Neuregulin-2 (NRG-2 (25, 26); also known as *Don-1* (27) and neural- and thymus-derived activator for erbB kinases [NTAK (28)]) is an NRG-1-related growth factor represented by six alternatively spliced isoforms derived from a single gene. NRG-2 is heparin binding (28) and stimulates phosphorylation of the NRG-1 receptor subunits erbB2, erbB3, and erbB4 (25-28). As these features are shared with the GGF-like activity previously identified in human schwannomas (13), we examined the potential expression of NRG-2 transcripts in the same five schwannomas described above. In initial RT-PCR experiments performed using primers specific for amino-terminal domain sequences present in all NRG-2 isoforms, we readily detected NRG-2 mRNA in all five schwannomas (data not shown). To compare the levels of expression of NRG-2 transcripts in schwannomas to that in normal eighth cranial nerve, we then quantitated the levels of NRG-2 mRNA in these tissues. Since the EGF-like domain of NRG-2, like that of NRG-1, is alternatively spliced to produce isoforms with distinct effects on cell growth and erbB phosphorylation (29), we performed these analyses using RT-PCR with TaqMan primers specific for NRG-2α or NRG-2β cDNAs and normalized the resulting data to the levels of 18S ribosomal RNA present in the same reverse transcription reactions. We found that the expression of NRG-2α was consistently elevated in schwannomas, with levels of mRNA 1.9- to 14.1-fold higher than the levels found in eighth cranial nerve (Fig. 5). The expression of NRG-2β transcripts was upregulated

1.4- to 5.8-fold relative to normal eighth cranial nerve in four of the five schwannoma biopsies, with one tumor containing levels of NRG-2β mRNA lower than that in normal nerve. Comparing the expression of NRG-2α and NRG-2β transcripts in each tumor, we found that, as with NRG-1, α isoform mRNAs were overexpressed to a greater extent than β isoforms in four of the five schwannomas.

Expression of Other NRG and EGF Family Ligands in Human Schwannomas

The expression of erbB4 and EGFR in smaller subsets of schwannomas (see below) raised the question of whether other NRG and EGF family ligands capable of activating these receptors might be expressed in schwannomas. To test this hypothesis, we used RT-PCR to amplify sequences encoding neuregulin-3 (NRG-3), neuregulin-4 (NRG-4), heparin-binding EGF (HB-EGF), β-cellulin, epiregulin, EGF, transforming growth factor-α (TGF-α) and amphiregulin. Transcripts encoding HB-EGF and epiregulin, two ligands capable of activating either erbB4 or EGFR, were evident in four of the five schwannomas (Fig. 6). We also found evidence that the EGFR ligand TGF-α was expressed in all five of these tumors. In contrast, mRNAs for NRG-3, NRG-4, β-cellulin, EGF, or amphiregulin were undetectable in all of the schwannomas we examined.

Schwannomas Overexpress Multiple ErbB Kinases and Demonstrate Constitutive Activation of ErbB Signaling

NRG-1 and NRG-2 bind directly to the erbB3 and erbB4 membrane tyrosine kinases and subsequently induce heterodimerization with erbB2 or, alternatively, in the case of erbB4, homodimerization to form active signaling complexes (30, 31). To de-

termine whether human schwannomas express the erbB receptors necessary for responsiveness to NRG-1 or NRG-2, we immunoblotted lysates of the same schwannomas examined for NRG-1 expression (Table 1) and probed these immunoblots with antibodies recognizing erbB2, erbB3, or erbB4. ErbB2 and erbB3 were the most commonly expressed erbB kinases in schwannomas, with a protein of the expected 185-kD size being detected in all six schwannomas by the anti-erbB2 and anti-erbB3 antibodies (Fig. 7). Expression of erbB4 was more restricted, with an erbB4-immunoreactive protein being readily identifiable in three of the six schwannomas. The erbB2-, erbB3-, and erbB4-like immunoreactive polypeptides found in schwannomas comigrated with erbB proteins detected by these antibodies in normal eighth cranial nerve (Fig. 7 and longer exposures of these same blots [data not shown]). We conclude that one or more cell types within schwannomas expresses the complement of erbB receptors required for responsiveness to NRG-1 and NRG-2.

The EGFR (also known as erbB1) is capable of heterodimerizing with and "cross-talking" to the NRG-1 receptors. Furthermore, expression of the EGFR is increased in other types of PNSTs (32), including a subset of MPNSTs (33). To determine whether EGFR expression is similarly inappropriately expressed in schwannomas, we also probed the normal eighth cranial nerve and schwannoma lysates with an anti-EGFR antibody. With long exposures, a protein of the expected 170-kD size was weakly detectable in normal eighth cranial nerve using this antibody (data not shown). However, only one of the six schwannomas tested expressed detectable levels of EGFR (Fig. 7).

Our immunoblot analyses suggested that erbB2 and erbB3 (and, less frequently, erbB4) were overexpressed in schwannomas relative to normal eighth cranial nerve. To quantify these differences and to determine whether they were paralleled by increases in levels of erbB mRNAs, we performed real-time quantitative PCR assays on normal eighth cranial nerve and schwannoma cDNAs using TaqMan primer sets specific for each erbB kinase. The levels of erbB transcripts established in these assays were normalized to the levels of 18S ribosomal RNA in the same cDNA. As shown in Figure 8, all four transcripts were present to varying degrees in normal nerve and all of the tested schwannomas. Consistent with the immunoblotting data, levels of erbB2 and erbB3 mRNA were elevated in all six schwannomas relative to normal eighth cranial nerve (5- to 26-fold and 3- to 14-fold increased relative to nerve, respectively). Paradoxically, the expression of erbB4 transcripts was lower in all six schwannomas than in eighth cranial nerve; we did note, however, that erbB4 mRNA accumulated to relatively higher levels in the three schwannomas with the highest levels of erbB4 protein by immunoblot (tumors 3, 4, and 6). The expression of EGFR messenger RNA in schwannomas again paralleled the results of our immunoblot analyses, with almost all tumors showing levels of expression lower than or equivalent to that observed in normal eighth cranial nerve. The sole exception was schwannoma 4, the same tumor that exhibited increased levels of EGFR protein in immunoblot analyses.

To establish whether expression of each of the erbB kinases was associated with neoplastic Schwann cells, we immunostained sections of the schwannomas examined for NRG-1 expression (Table 2) using antibodies recognizing EGFR, erbB2, erbB3, or erbB4. Focal immunoreactivity for EGFR was found in three of the six tumors, being

evident as cytoplasmic staining associated with patches of tumor cells (Fig. 9). In contrast, strong erbB2 immunoreactivity was associated with the membranes and cytoplasm of tumor cells throughout all six of the tumors. The anti-erbB3 mouse monoclonal antibody RTJ.1 similarly produced strong staining of the membranes and cytoplasm of tumor cells in four of the six neoplasms. An anti-erbB4 rabbit polyclonal antiserum labeled tumor cells in two schwannomas, but did so with a pattern distinct from that seen with the other three anti-erbB antibodies. ErbB4 immunoreactivity in tumor cells was prominently associated with tumor cell nuclei, as well as with the cytoplasm and membranes of these cells; our findings of the presence of nuclear erbB4 immunoreactivity in schwannoma cells is consistent with previous reports demonstrating that erbB4 can be proteolytically cleaved and its carboxy-terminus translocated into the nucleus (31). In all instances, replacing the primary antibody with nonimmune immunoglobulin or, when possible, preincubating the primary antibody with the immunizing peptide abolished erbB immunoreactivity in schwannomas (Fig. 9).

ErbB2 is the preferred dimerization partner for all other erbB receptors. Dimerization is accompanied by phosphorylation of multiple tyrosine residues in the carboxy-terminal sequences of erbB2, including Tyr^{1248}, a residue whose phosphorylation is linked to activation of the ERK/MAPK (mitogen-activated protein kinase) signaling pathway. To determine whether erbB signaling is constitutively activated in schwannomas, we immunostained sections of these tumors with PN2A, a mouse monoclonal antibody that recognizes erbB2 phosphorylated on Tyr^{1248}. PN2A immunoreactivity was readily identified in four of the six schwannomas, producing a punctate pattern of cytoplasmic staining in a major subset of tumor cells (Fig. 9) that was not evident when

the primary antibody was replaced with nonimmune IgG. We conclude that erbB signaling is constitutively activated in at least some schwannomas.

DISCUSSION

There is extensive evidence indicating that inactivating and missense mutations of the *NF2* tumor suppressor gene play a key role in the pathogenesis of schwannomas. We have hypothesized that inappropriate signaling by as yet unknown growth factors also contributes to this process and, on the basis of the results presented here, propose that NRG-1 and/or NRG-2 proteins are among the growth factors promoting schwannoma tumorigenesis. In support of this hypothesis, we have found that neoplastic Schwann cells within schwannomas express multiple α and β transmembrane precursors from the class II and III NRG-1 subfamilies, as well as NRG-2 α and β isoforms. Furthermore, schwannomas express transcripts encoding NRG-1 and NRG-2 isoforms at levels much higher than those found in normal eighth cranial nerve. We have also found that schwannomas consistently overexpress erbB2 and erbB3, two receptor tyrosine kinases (RTKs) that mediate NRG-1 and NRG-2 responses. These observations, considered together with our demonstration of constitutive erbB2 phosphorylation in schwannomas, are consistent with the hypothesis that autocrine, paracrine, and/or juxtacrine NRG-1 and/or NRG-2 signaling in neoplastic Schwann cells promotes schwannoma tumorigenesis. Our results also raise intriguing questions about the role specific NRG-1 and NRG-2 isoforms play in this process, how NRG-1 and NRG-2 actions in schwannomas differ from the actions of these same growth factors in other types of PNSTs such as neurofibromas and MPNSTs and the potential for interactions between

NRG-1/NRG-2 signaling and signaling by other erbB ligands expressed in schwannomas.

Our real-time quantitative PCR analyses indicate that schwannomas overexpress both α and β isoforms of NRG-1 and, to a lesser extent, NRG-2. However, the majority of the neoplasms we examined showed relatively greater increases in the expression of mRNAs encoding NRG-1α and NRG-2α. These observations have important implications with regard to the actions of NRG-1 and NRG-2 in schwannomas. NRG-1β is a potent mitogen for nonneoplastic human (15, 34), monkey (35), and rat (16, 36) Schwann cells, whereas NRG-1α does not promote Schwann cell proliferation (36). The effects NRG-2 α and β have on Schwann cell mitogenesis have not yet been studied in detail. However, NRG-2 α and β isoforms do differ in their ability to stimulate the proliferation of breast cancer cell lines (29). The relative preponderance of non-mitogenic NRG α isoform expression in schwannomas is therefore consistent with the slow growth that is characteristic of these neoplasms. We would emphasize that this does not imply that NRG α proteins have no effects on neoplastic Schwann cells. In microarray experiments comparing the effects NRG-1α and NRG-1β have on the transcriptome of nonneoplastic Schwann cells, we have confirmed that NRG-1α stimulation triggers a number of transcriptional alterations in these glia (S.L. Carroll and M.S. Stonecypher, data not shown). It should also be noted that, although NRG-1 and NRG-2 act through the same receptors, these growth factors elicit distinct biological responses in at least some cell types (37). Considered together, these observations suggest that NRG-1α, NRG-1β, NRG-2α, and NRG-2β each have isoform-specific effects on neoplastic Schwann cells.

The expression of multiple transmembrane precursors from the Class II and Class III NRG-1 subfamilies likely further diversifies NRG-1 signaling in human schwannomas. Class II (GGF) NRG-1 proteins are heparin-binding molecules that are released from the cells synthesizing them. Following release, GGF proteins act as paracrine or autocrine signaling molecules that accumulate at high concentrations in structures such as the basement membranes that individually invest neoplastic Schwann cells within schwannomas. In contrast, Class III NRG-1 proteins remain membrane associated and likely function only as juxtacrine signaling molecules (21). Both Class II and Class III NRG-1 transmembrane precursors are thought to become active when they are proteolytically cleaved within the juxtamembrane domain (20), releasing the extracellular domain for secretion (Class II isoforms) or display as a membrane-associated ligand (Class III isoforms). This proteolytic cleavage also releases the NRG-1 intracellular domain from the inner surface of the cell membrane, allowing it to move into the cellular interior, where it has been postulated to serve as a "back-signaling" molecule (38). Using the anti-"a" carboxy-terminus antibody (see Fig. 1), we have found that probable proteolytic fragments derived from NRG-1 transmembrane precursors are indeed present in human schwannomas; this observation, considered together with our finding that schwannomas synthesize predominantly NRG-1 transmembrane precursors, raises the question of whether NRG-1 back-signaling events also make an important contribution to schwannoma pathogenesis. Our data thus support a model in which NRG-1 promotes schwannoma formation in a complex process that involves paracrine, autocrine, and/or juxtacrine forward signaling in combination with back-signaling events. These signaling events may be even more complex than indicated by

our initial studies because a recent exhaustive analysis of the structure of the NRG-1 gene suggests the existence of an additional six NRG-1 subfamilies, each characterized by its own unique amino-terminal domain (39).

Prior to this study, there have been conflicting reports as to whether NRG receptors are expressed in human schwannomas. Hansen and Linthicum found that vestibular schwannomas demonstrated immunoreactivity for erbB2, erbB3, and NRG-1 (40). However, two other groups recently reported that erbB2 (41, 42) and erbB3 (42) expression is absent or only very weakly detectable in schwannomas. Using immunoblot, RT-PCR, real-time quantitative PCR, and immunohistochemical analyses, we found that erbB2 and erbB3 were readily detectable in schwannomas. ErbB2 and erbB3 were also the most commonly expressed erbB kinases in schwannomas, with protein and mRNA for these receptors uniformly overexpressed in our first series of tumors (Table 1) and with four of six neoplasms demonstrating immunoreactivity for both kinases in our second series (Table 2). To further confirm the immunohistochemical findings described in this manuscript, we subsequently immunostained a third series of 29 eighth cranial nerve schwannomas (27 sporadic, 2 *NF2* associated) using different erbB2 and erbB3 antibodies (data not shown); immunoreactivity for both erbB2 and erbB3 was present in 27 of these tumors, including both *NF2*-associated schwannomas. We conclude that erbB2 and erbB3 are indeed expressed by neoplastic Schwann cells within the majority of schwannomas and that these glia are thus potentially responsive to NRG-1 and NRG-2 expressed endogenously in schwannomas. As overexpression of growth factor receptors promotes neoplastic growth in other tumor types, it is reason-

able to hypothesize that overexpression of erbB2 and erbB3 makes a similar contribution to schwannoma tumorigenesis.

In contrast to the relatively slowly growing benign schwannoma, neurofibromas can become quite large and transform into aggressive MPNSTs. We have recently demonstrated that autocrine and/or paracrine NRG-1/erbB signaling promotes neoplastic Schwann cell proliferation in human neurofibromas and MPNSTs (43) and that transgenic mice expressing the NRG-1 isoform GGFβ3 in myelinating Schwann cells develop neoplasms with morphologic, immunohistochemical. and ultrastructural features highly similar to those of human MPNSTs (44). As our earlier work and the findings described here indicate that inappropriate NRG/erbB signaling may be an important factor in the pathogenesis of neurofibromas, MPNSTs, and schwannomas, it must be asked why inappropriate NRG-1/erbB signaling in these three different types of PNSTs is associated with such major differences in clinical behavior. In part, this is likely explained by differences in the genetic abnormalities that accompany NRG/erbB signaling in each tumor type. As noted above, schwannomas typically develop mutations in the *NF2* tumor suppressor gene. In contrast, neurofibromas demonstrate loss of neurofibromin expression and, as they progress and become MPNSTs, abnormalities in additional tumor suppressor genes, including *p53*, *INK4A,* and $p27^{kip1}$ (45). Another possibility is that schwannomas, neurofibromas, and MPNSTs are derived from cells corresponding to different stages of differentiation in the Schwann cell lineage and that NRG/erbB signaling has distinct effects in these different contexts. This hypothesis is consistent with the observation that schwannomas typically show features highly similar to those of mature Schwann cells, while MPNSTs demonstrate a capacity for diver-

gent differentiation reminiscent of multipotent neural crest stem cells (45). Finally, it is evident that schwannomas and neurofibromas/MPNSTs have significant differences in their pattern of NRG and erbB expression. Of particular note, we have detected NRG-2 expression in schwannomas (this work), while we did not find evidence for NRG-2 expression in neurofibromas or MPNSTs (43). In addition, while we only occasionally found evidence of increased EGFR expression in schwannomas, many neurofibromas and MPNSTs express elevated levels of this receptor (32, 43).

Of the other eight NRG and EGF ligands, only HB-EGF, epiregulin and TGF-α were expressed in the majority of the schwannomas we examined. In contrast, increased expression of erbB4, which is activated by HB-EGF and epiregulin, was evident only in a subset of these neoplasms; overexpression of EGFR, which serves as a receptor for all three of these ligands, was found in only a single tumor. Nonetheless, we cannot exclude the possibility that HB-EGF, epiregulin, or TGF-α also plays an important role in the pathogenesis of at least the subset of schwannomas expressing erbB4 and EGFR. Both erbB4 and EGFR are capable of heterodimerizing with erbB2 and erbB3 (46), indicating that ligands activating erbB4 and EGFR have the potential to "cross-talk" with erbB2 and erbB3 and thus modify cellular responses triggered by NRG-1 or NRG-2 stimulation. In addition, NRG-1 and NRG-2 can also act through erbB4; since each of the four erbB kinases activates a distinct profile of cytoplasmic signaling effectors (46), NRG actions in erbB4-expressing schwannomas may differ from those evident in tumors lacking this kinase.

In summary, our findings support the hypothesis that autocrine, paracrine, and/or juxtacrine signaling by multiple NRG-1 and NRG-2 isoforms promotes the

pathogenesis of human schwannomas. As erbB neutralizing antibodies such as Herceptin and small molecular erbB inhibitors have been effective in treating other tumor types, these observations also suggest that inhibiting the NRG/erbB signaling pathway may be an effective means of treating patients with surgically unresectable schwannomas. Establishing the role individual erbB kinases and specific NRG-1 and NRG-2 isoforms play in schwannoma tumorigenesis will be important for furthering our understanding of how these PNSTs form and developing innovative new approaches to their treatment.

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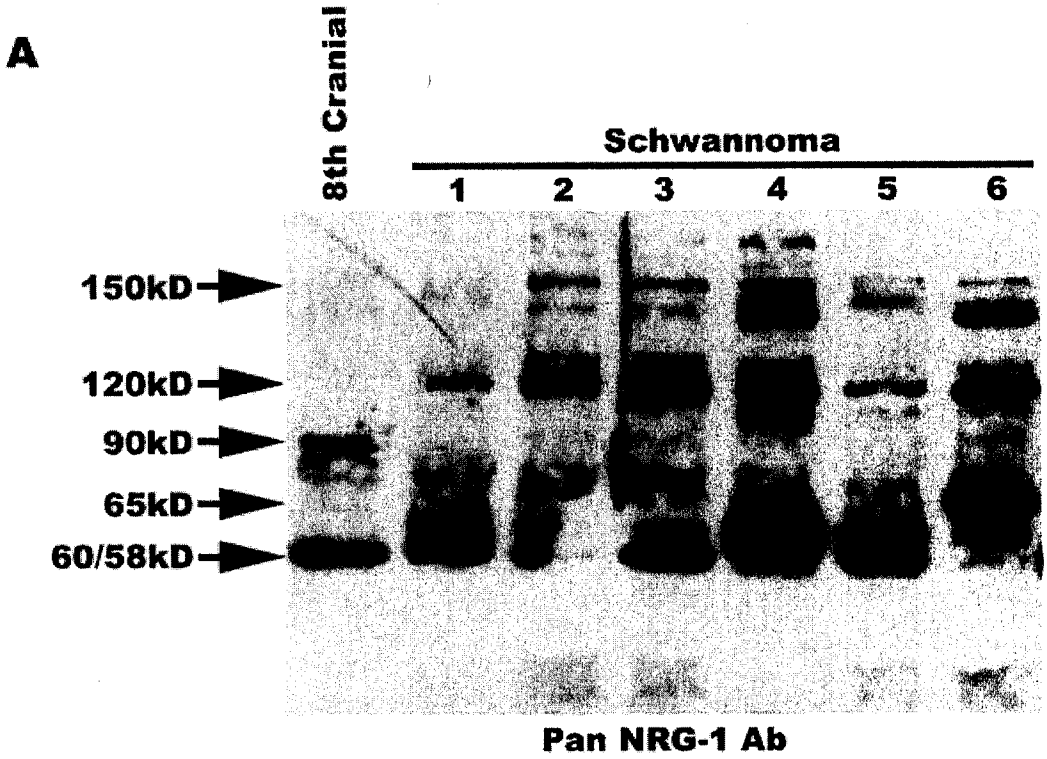
FIGURE 1. Multiple NRG-1 immunoreactive species are expressed in human schwannomas. (A) Lysates of normal eighth cranial nerve and six schwannomas probed with a "panNRG-1" antibody recognizing the EGF-like common domain found in all NRG-1 isoforms. Multiple NRG-1 proteins ranging in size from 150- to 58- kD are present in the schwannomas, with prominent 90- and 58- kD species evident in eighth cranial nerve. Longer exposures of this same blot show lower levels of the 120- and 150- kD NRG-1 polypeptides in eighth cranial nerve (data not shown). (B) An antibody recognizing the "a" variant carboxy terminus, a domain found in a major subset of NRG-1 transmebrane precursors, recognizes multiple proteins in human schwannomas and eighth cranial nerve. Like the pan NRG-1 antibody, this antibody labels 150- and 120- kD proteins as well as potential cleavage products derived from NRG-1 transmembrane precursor proteins (80-, 70-, 65-, and 55- kD proteins). The four smaller species are readily detectable in eighth cranial nerve; again, longer exposures of this blot (data not shown) demonstrate the presence of lower levels of the 150- and 120-kD proteins in eighth cranial nerve. Arrows to the left of these two panels indicate the immunoreactive species and their molecular weights. Numbers above each schwannoma lane correspond to the cases indicated in Table 1.



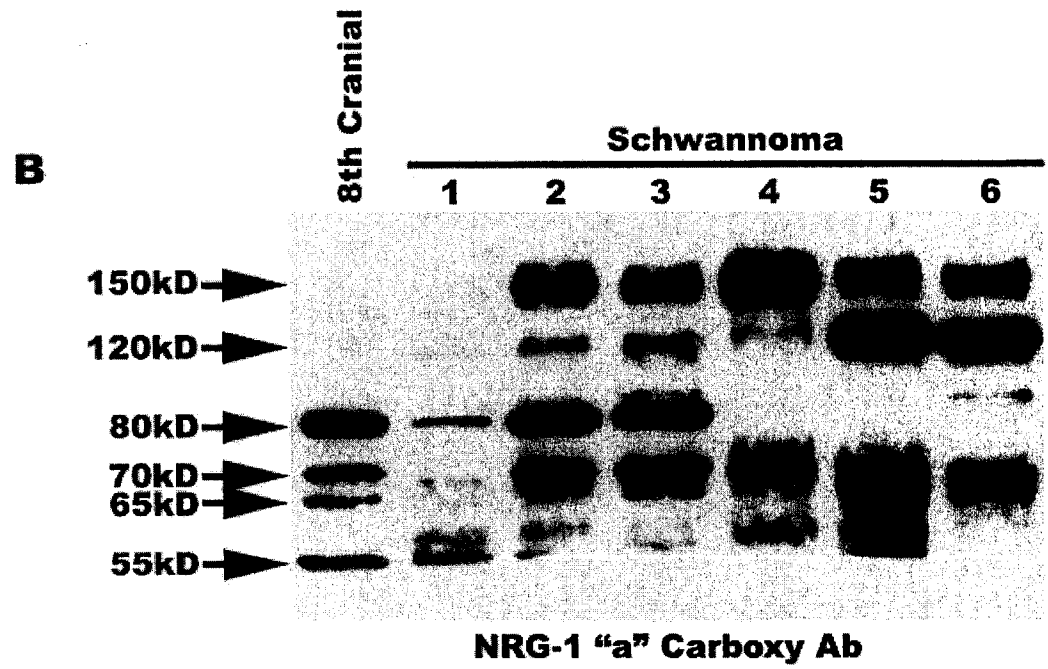


FIGURE 2. Immunohistochemical demonstration of NRG-1-like protein expression in a schwannoma labeled with the pan NRG-1 antibody (left panel). Replacing the antibody with nonimmune IgG abolishes this staining (right panel). Both sections have been lightly counterstained with hematoxylin. Scale bar, 50 μm.

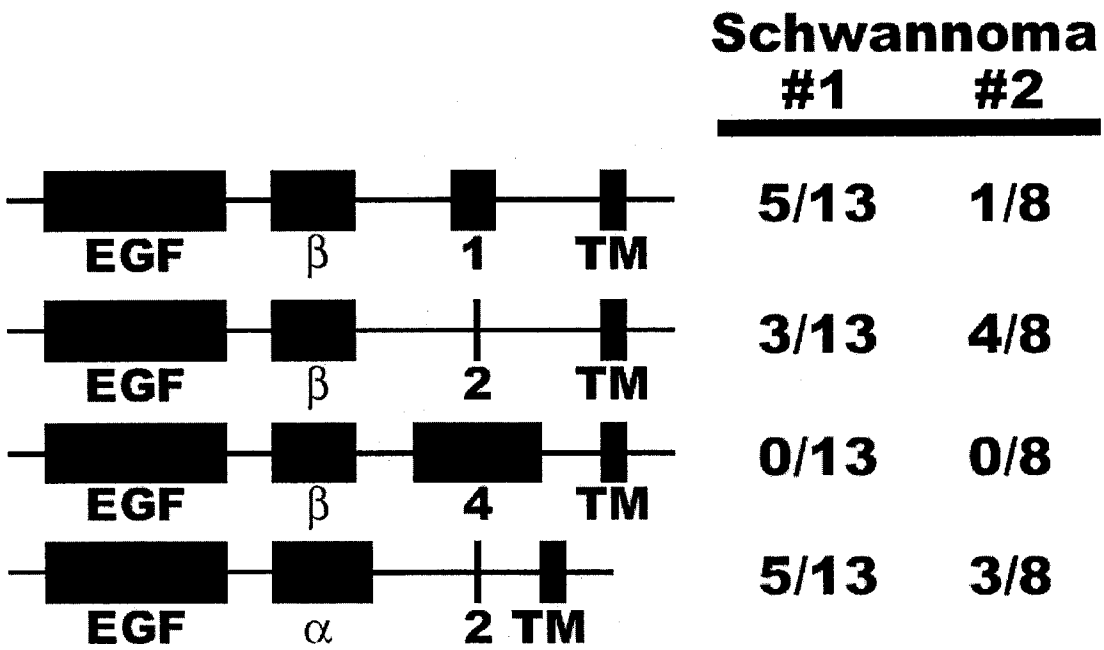
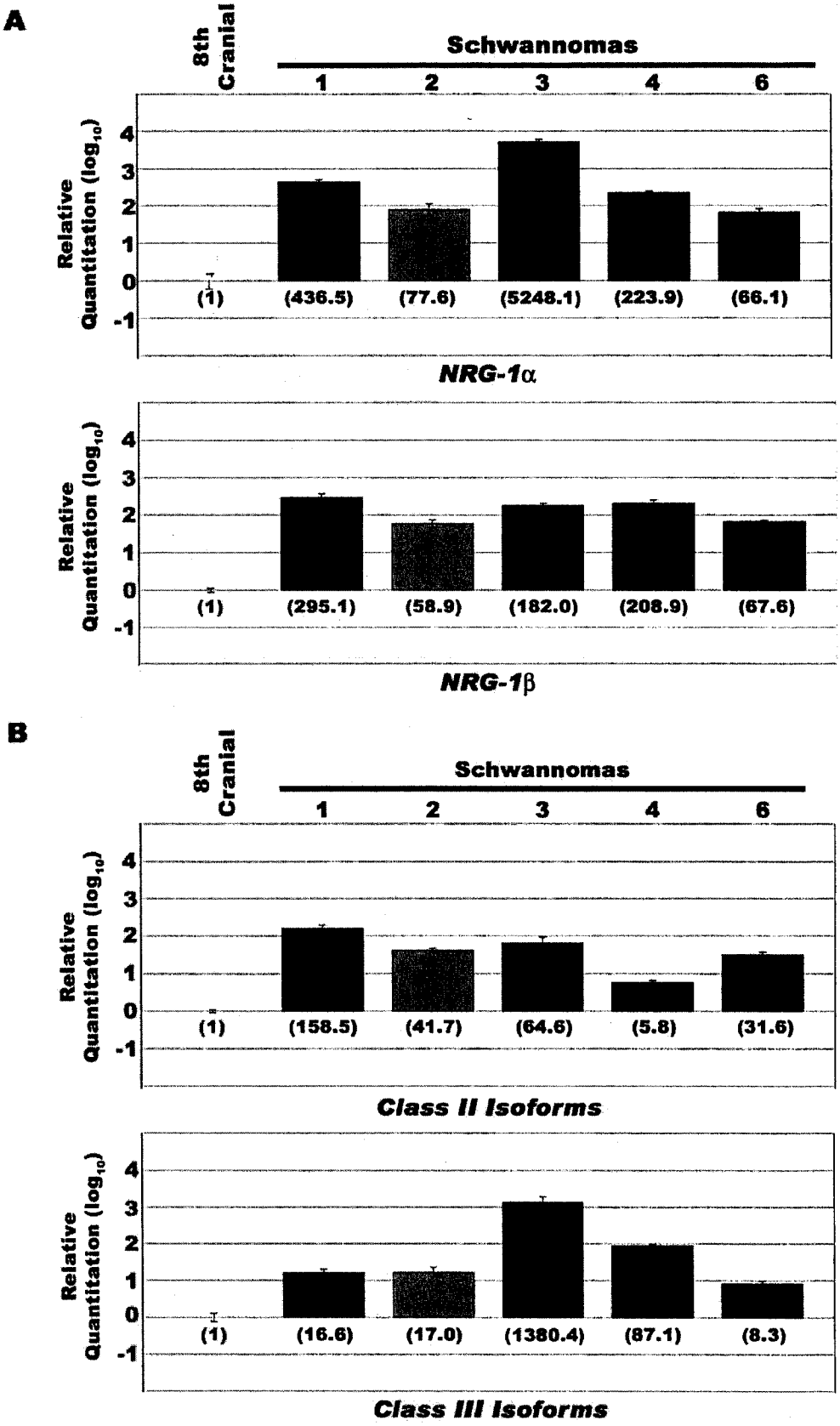


FIGURE 3. Schwannomas express mRNAs encoding a complex mixture of α and β NRG-1 isoforms. Structural diagrams of the protein structures predicted by partial NRG-1 cDNAs isolated from schwannomas is indicated to the left. Boxes in these structural diagrams indicate each protein domain, with the size of the box being directly proportional to the size of that domain; lines connecting boxes are included only to indicate structural linkage between domains. Domains are as follows: EGF, the NRG-1 EGF-like common domain; α or β, NRG-1 EGF-like α or β variant domains; 1, 2, or 4, variant juxtamembrane domains; TM, the initial portion of the transmembrane domain of NRG-1 transmembrane precursor proteins. Ratios indicated adjacent to each juxtamembrane domain indicate the frequency with which clones encoding that structural variant were identified when PCR-generated clones were sequenced.

FIGURE 4. Real-time quantitative PCR assays for mRNAs encoding specific NRG-1 isoforms in schwannomas and normal eighth cranial nerve. **(A)** Comparison of the levels of NRG-1α (top panel) and β (bottom panel) isoform mRNA in human schwannomas and normal eighth cranial nerve. **(B)** Comparison of the levels of mRNAs encoding class II NRG-1 isoforms (top panel) and class III NRG-1 isoforms (bottom panel) in human schwannomas and normal eighth cranial nerve. For both A and B, bars indicate the relative levels of expression, with expression in eight cranial nerve arbitrarily designated as 1; note that the values indicated by the bars are expressed on a log10 scale. Confidence intervals of 95% are indicated for each bar. Numbers in parentheses below the bars indicate the fold change converted from log10. Numbers above schwannoma bars correspond to the cases indicated in Table 1.



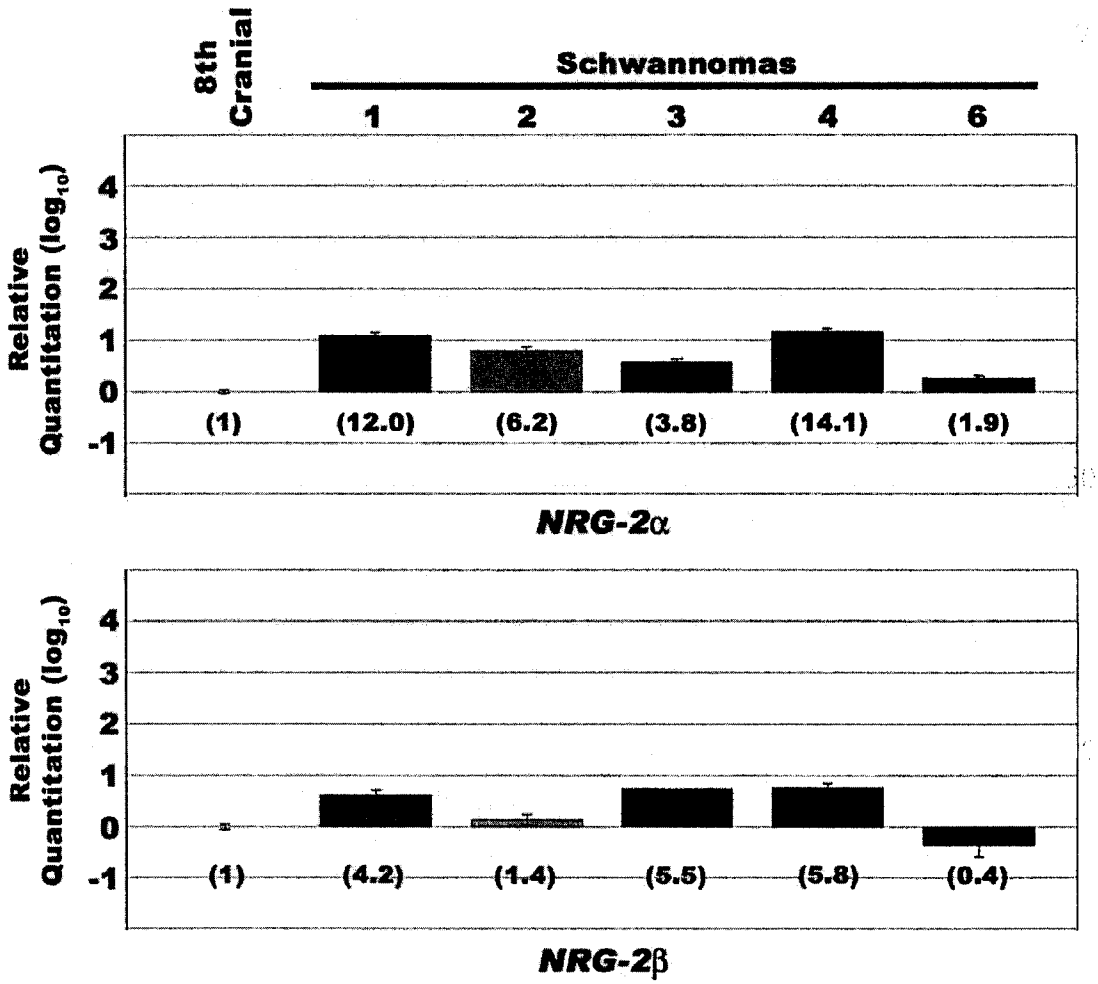


FIGURE 5. Real-time quantitative PCR assays comparing the level of expression of mRNAs encoding NRG-2 α (top panel) and β (bottom panel) isoforms in human schwannomas and normal eighth cranial nerve. Bars indicate the relative levels of expression, with expression in eight cranial nerve arbitrarily designated as 1; note that these values are expressed on a log10 scale. Confidence intervals of 95% are indicated for each bar. Numbers in parentheses below the bars indicate the fold change converted from log10. Numbers above schwannoma bars correspond to the cases indicated in Table 1.

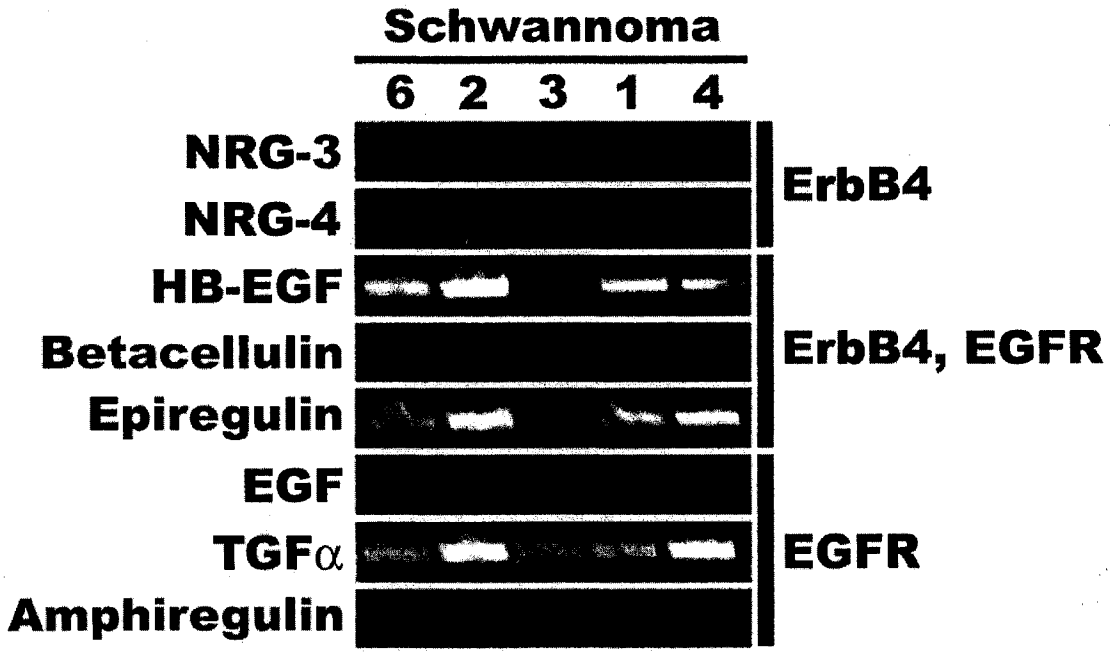


FIGURE 6. RT-PCR analyses of the expression of mRNAs encoding NRG-3, NRG-4 and multiple EGF family ligands in human schwannomas. The identity of each ligand is indicated to the left of the corresponding panel. Numbers above each lane indicate the case number corresponding to the clinical information presented in Table 1. Thick bars to the right of the panels indicate the receptors capable of binding each ligand. HB-EGF, heparinbinding epidermal growth factor; EGF, epidermal growth factor; TGFα, transforming growth factor α.

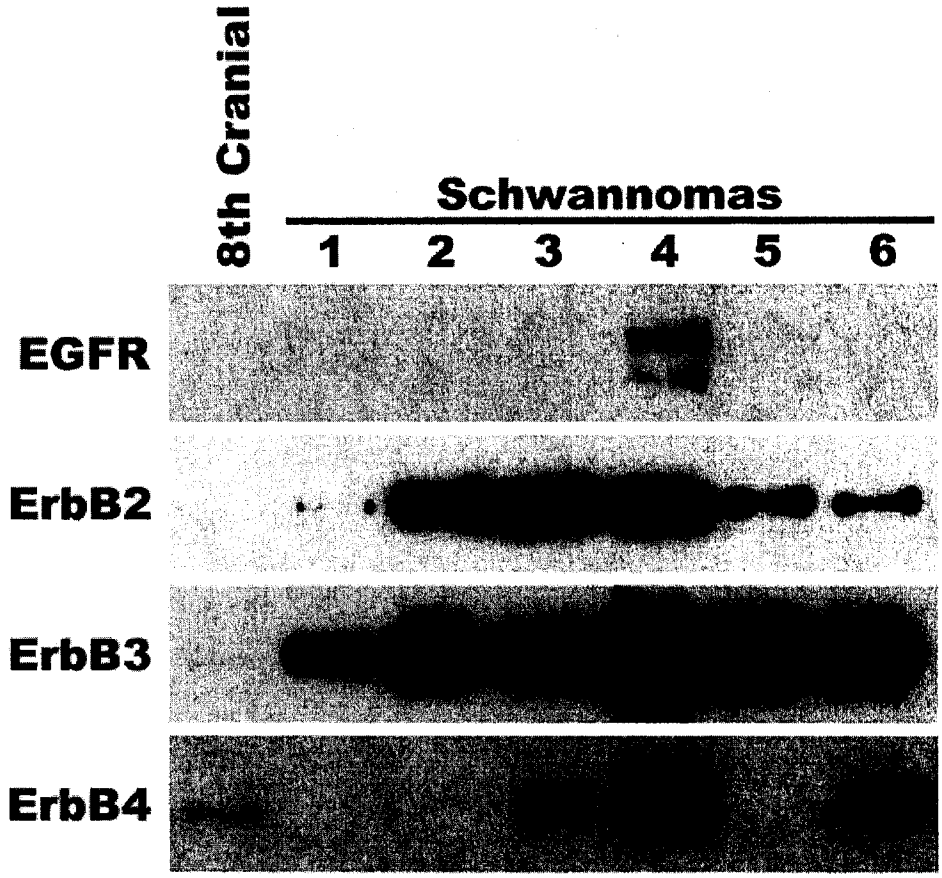
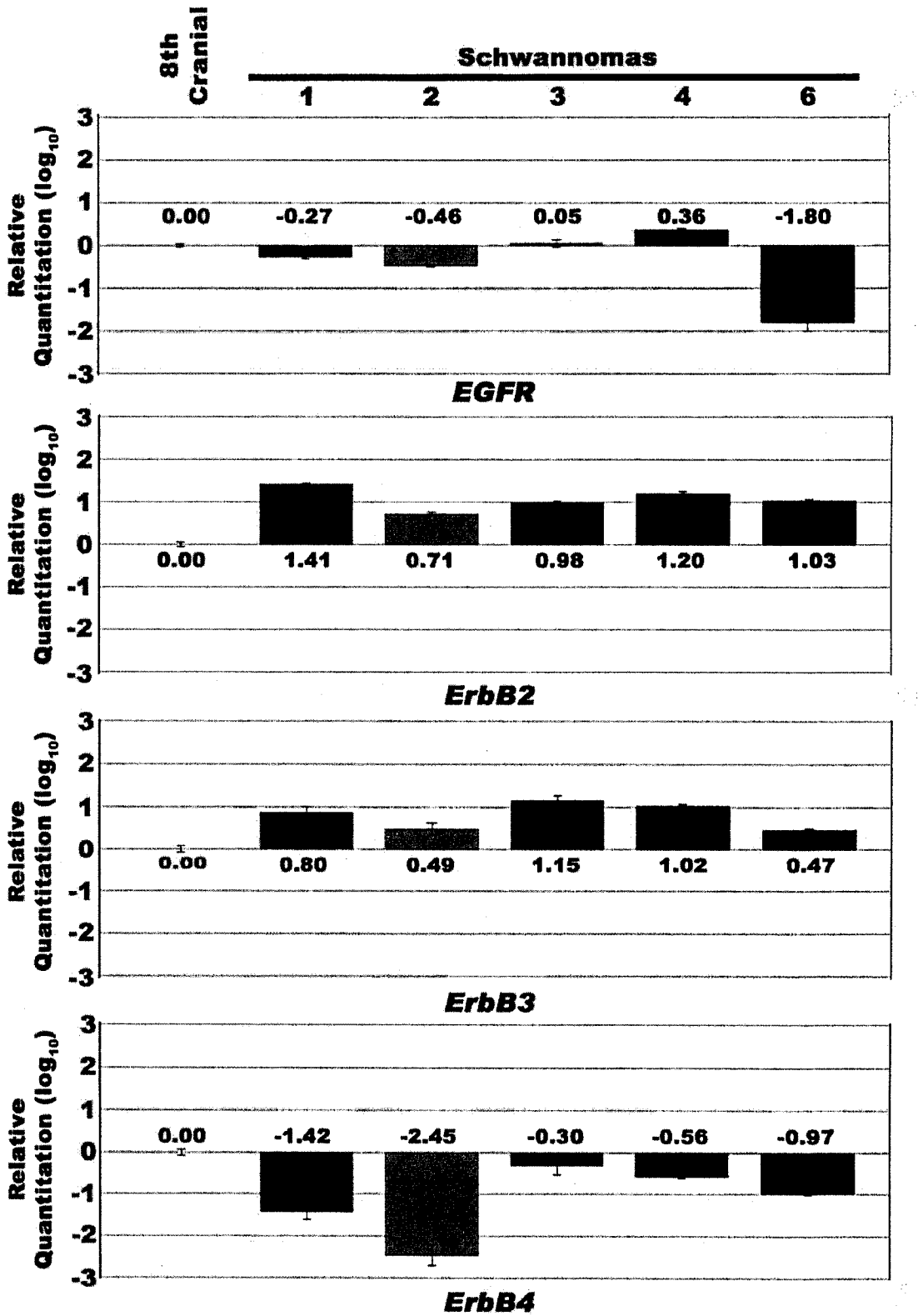


FIGURE 7. Western blot analyses of erbB expression in schwannomas. The specific erbB receptor examined is indicated to the left of each blot. Numbers above each schwannoma lane correspond to the cases indicated in Table 1. Longer exposures of the EGFR and erbB2 blots demonstrated the presence of a relatively low level of each of these receptors in normal eighth cranial nerve (data not shown).

FIGURE 8. Real-time quantitative PCR comparing the levels of expression of EGFR, erbB2, erbB3, and erbB4 mRNAs in schwannomas to that detectable in normal eighth cranial nerve. Bars indicate the relative levels of expression, with expression in eight cranial nerve arbitrarily designated as 1; note that these are values are expressed on a log10 scale. Confidence intervals of 95% are indicated for each bar. Numbers in parentheses below the bars indicate the fold change converted from log10. Numbers above each schwanoma bars correspond to the cases indicated in Table 1.



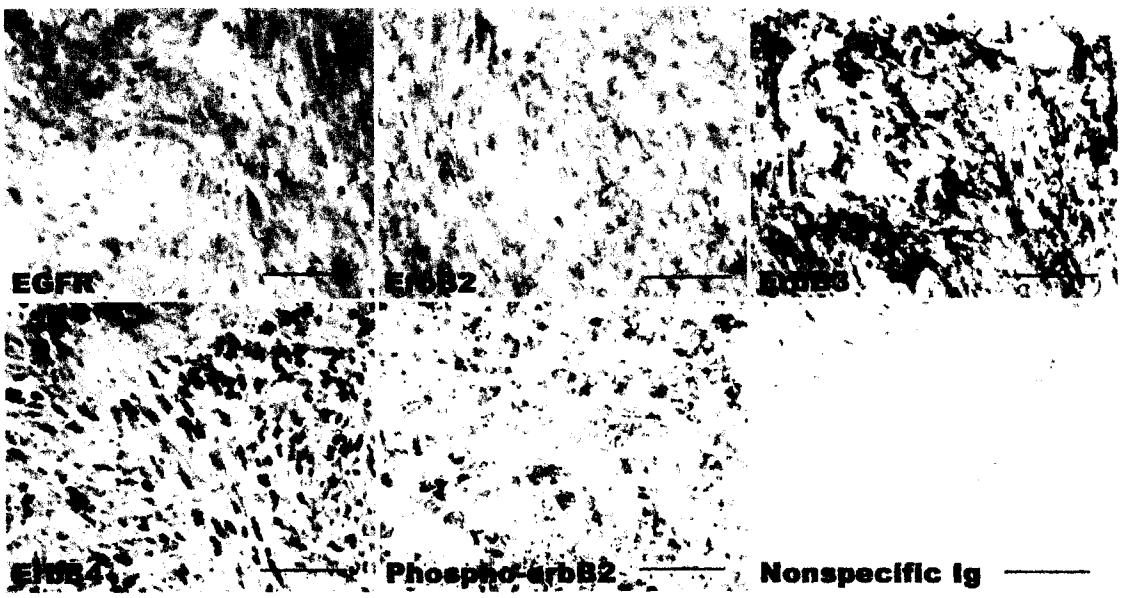


FIGURE 9. Immunohistochemical localization of EGFR, erbB2, erbB3, erbB4, and phosphorylated erbB2 in human schwannomas. Shown are representative sections of human schwannomas immunostained for EGFR, erbB2, erbB3, erbB4, and erbB2 phosphorylated on residue 1248 (pTyr1248; antigen indicated in the lower left corner of each panel). Staining for each of these antigens was abolished in control sections in which the primary antibody was replaced with nonimmune mouse antibody (EGFR, pTyr1248) or IgM nonspecific control antibody (erbB3) or when the primary antibody was preincubated with the immunizing peptide (erbB2, erbB4); an example of this is indicated in the last panel (Nonspecific Ig). All sections have been lightly counter-stained with hematoxylin. Scale bar, 50 μm.

CONCLUSIONS AND FUTURE DIRECTIONS

INAPPROPRIATE NRG-1/ERBB SIGNALING IS A COMMON FEATURE IN SCHWANN CELL NEOPLASIA

The findings described in this dissertation provide evidence that inappropriate NRG-1/ErbB signaling promotes tumorigenesis in the three major classes of Schwann cell neoplasms (schwannomas, neurofibromas, and MPNSTs). Although they share a common cell of origin, these Schwann cell neoplasms are highly variable in their phenotype (48, 49). The finding that activation of the NRG-1/erbB signaling pathway plays a role in the pathogenesis of all three types of these Schwann cell neoplasms thus raises several important questions. For example, if NRG-1/erbB signaling drives a pro-proliferative and protumorigenic phenotype, then why do Schwann cell neoplasms have such differences in proliferative rates? The first and perhaps most likely explanation is that NRG-1/erbB signaling can have distinct effects, depending on the properties of the cells responding to growth factor stimulation. Each of these tumor types typically carries a different background of tumor suppressor mutations; consequently, the impact of NRG-1/erbB signaling may become enhanced in the presence of specific tumor suppressor mutations, resulting in an increased proliferative rate. There are several potential methods that could be used to test this hypothesis experimentally. Specifically, cultured neonatal Schwann cells could be transfected with RNAi constructs that selectively target tumor suppressors such as neurofibromin. Under control and transfected conditions, neonatal Schwann cells could be stimulated with a pro-mitogenic concentration of NRG-1β, and the effects on proliferation could be assayed by [^{3}H]thymidine incorporation. This experimental approach could be applied to other tumor suppressors as

well, and would test the hypothesis that tumor suppressor inactivation enhances the proliferative effect of NRG-1.

SCHWANN CELL SUBPOPULATIONS

In addition to tumor suppressors, it is possible that other biological factors determine NRG-1 responsiveness. One possibility is that there are multiple subpopulations of Schwann cells that differ in their response to NRG-1 stimulation. Schwann cells are not a homogeneous cell population. In vivo, Schwann cells are postmitotic cells that facilitate nerve conduction (myelinating Schwann cells) and secrete tropic factors (nonmyelinating Schwann cells) (50-52). These broad classifications may not capture the full biological diversity that Schwann cells exhibit. One possibility is that, like other tissues, peripheral nerve contains a multipotent stem-cell like population that retains the ability to differentiate into Schwann cells, and possibly other cell types (53-61). Specific Schwann cell subpopulations may also exhibit differences in NRG-1 responsiveness, with an increased propensity for tumorigenesis. Such a mechanism may occur during MPNST formation in the P_0-GGFβ3-transgenic mouse. P_0-directed expression of the NRG-1 isoform GGFβ3 may act on a specific Schwann cell subpopulation and increase the Schwann cell precursor pool; with an increased number of Schwann cells or precursors, there is a corresponding increase in the probability that mutations will occur in tumor suppressor genes. The Schwann cell subpopulation hypothesis could be investigated through a detailed in vivo marker analysis of peripheral nerve(s), similar to that we have shown for human MPNST cell lines.

WHAT IS A NORMAL SCHWANN CELL?

The data presented in this dissertation raise the following question: are neoplastic Schwann cells sensitive to erbB kinase inhibition because of their properties as neoplastic cells or because of their properties as Schwann cells? This question is difficult to address because of the difficulty of defining a normal Schwann cell. In vivo, Schwann cells are postmitotic cells that facilitate nerve conduction (myelinating Schwann cells) and secrete tropic factors (nonmyelinating Schwann cells). Several methods have been described for the in vitro culture of Schwann cells (17, 59-62). The most widely used Schwann cell culture method was originally described by Martin Raff in the late 1970s (59). In this method, Schwann cells are harvested from neonatal nerve when Schwann cells are at an immature developmental stage. Schwann cells at this stage of development are critically dependent on axon-derived trophic signals for their survival. Therefore, by supplementing the media with axon-derived trophic factors, it is possible to expand this population of neonatal Schwann cells. The peripheral axotomy culture model derived in our laboratory is another example of a Schwann cell culture protocol that models a particular developmental state (60). In this culture model, Schwann cells are isolated from axotomized adult rats. Peripheral axotomy induces Schwann cells to dedifferentiate and proliferate, and these cells can further be expanded in culture (60). Significantly, these variations in culture conditions may produce phenotypic differences in the populations of Schwann cells that are cultured (13, 63). For example, neonatal Schwann cells cultured under the Raff protocol do not express EGF Receptor (EGFR) while Schwann cells cultured according to our laboratory's protocol do express EGFR (13). These data demonstrate that cultured Schwann cells possess

biological variability and/or plasticity of erbB expression. ErbB biological variability and/or plasticity may also occur in vivo with distinct Schwann cell subpopulations having unique erbB expression patterns. In this work we have analyzed a series of human MPNST cell lines and demonstrated that these cells consistently express schwannian markers and variably express muscle and neuron markers. Future experiments that utilize Schwann cell cultures and/or Schwann cell tumor biopsies could benefit from a detailed marker analysis similar to that we demonstrated for MPNST cell lines. Such a detailed marker analysis would provide a means of standardizing cell culture techniques and may have applicability in defining the developmental/differentiation status of Schwann cell neoplasms.

Schwann cell proliferation during development and nerve injury is unique in that it is normally a self-limiting process. Specifically, during development, non-axon-associated Schwann cell precursors undergo apoptosis; following nerve injury, Schwann cells dedifferentiate and proliferate. Schwann cell proliferation eventually ceases, with Schwann cells exiting the cell cycle and returning to the myelinating state (10). Schwann cell proliferation becomes pathological when proliferation is no longer self-limiting due to loss of inhibitory cell cycle control mechanisms. What molecular alterations abrogate the normally self-limiting process of Schwann cell proliferation and promote Schwann cell tumorigenesis? Based on current evidence (reviewed in the first article in this dissertation) and ongoing studies from our laboratory, tumor suppressor genes such as neurofibromin and *p53* are candidate molecules that may inhibit Schwann cell proliferation during both development and nerve injury.

WHY MAKE SO MANY NRG-1 ISOFORMS? MECHANISTIC AND FUNCTIONAL CONSEQUENCES OF NRG-1 ISOFORM DIVERSITY?

While all NRG-1 isoforms contain a conserved EGF-like domain that is both necessary and sufficient for receptor binding, significant isoform diversity exists within the family (29). Alternative splicing produces NRG-1α and NRG-1β isoforms, which differ in their affinity for erbB receptors. This difference in affinity may have a functional consequence (29). In vitro, NRG-1β isoforms promote Schwann cell proliferation, while the NRG-1α isoforms do not (64). However, data from our laboratory indicate that the NRG-1α2 isoform induces significant alterations in gene expression (described below). In addition, quantitative PCR data show that NRG-1α isoforms were significantly increased relative to normal eighth cranial nerve in human schwannoma biopsies. Taken together, these data indicate that NRG-1α isoforms have novel functions in normal and neoplastic Schwann cells that have not been fully appreciated. Given that NRG-1α and NRG-1β isoforms induce distinct patterns of gene expression, it will be useful to test the hypothesis that NRG-1 isoforms with distinct amino-termini (NDF, GGF, SMDF) likewise induce distinct changes in gene expression.

Global gene expression profiling is one technique that can be applied to determine isoform-specific effects (65). To interpret data from such expression profiling experiments, it is necessary to consider the prevailing hypothesis regarding how receptor tyrosine kinase (RTK) signaling affects gene expression (66-70). One theory holds that RTK signaling activates diverse signaling pathways and results in the activation of a defined set of genes. In this model, the growth factor serves as a trigger for downstream signaling events (66). Another theory holds that combinatorial tyrosine phosphorylation events result in unique patterns of gene expression (70-73). A host of evidence suggests

that the ligand itself encodes information for signaling specificity (71). For example, in cells expressing only erbB4 receptors, distinct EGF-like ligands (β-cellulin, NRG-1, NRG-2, and NRG-3) differentially activate erbB receptor phosphorylation, leading to specific activation of effector signaling pathways (72). Recently, the issue of isoform specific effects has been explored by analyzing the expression profiles of breast cancer cells stimulated with distinct EGF-like ligands (EGF, NRG-1β, and NRG-2β). These results showed that each growth factor activated distinct cytoplasmic signaling pathways and resulted in distinct patterns of gene expression (73). In this model, the ligand is more than a trigger; rather, it encodes specific information that results in unique patterns of gene expression. These results suggest that NRG-1 isoforms likely have distinct biological roles. Preliminary expression profiling experiments from our laboratory (described below) favor the hypothesis of qualitative differences in gene expression with distinct biological roles for NRG-1 isoforms.

NRG-1β1 and NRG-1α2 Alter Gene Expression in Adult Schwann Cells

To examine the role of NRG-1 isoform-specific effects, we have performed expression profiling studies using NRG-1 (NRG-1β1 or NRG-1α2) -stimulated cultured adult Schwann cells. In adult Schwann cells, the majority of genes induced by NRG-1β and NRG-1α were transcription factors, including a number of immediate-early genes. NGFI-A, NGFI-B, HZF-3, ANIA-6, PC3, Krox-24, and c-fos were induced by both NRG-1 isoforms; PRG1, Krox-20, and Nor-1 were induced by NRG-1β only; c-jun was induced by NRG-1α only. Notable among these transcription factors is Krox-20, a transcription factor that has been shown to be upregulated in response to NRG stimulation

(74). In addition to transcription factors, there are multiple genes that are transcriptionally regulated by NRG-1 treatment. For example, both isoforms induced the expression of HES-1, a transcription factor implicated in the Delta-Notch signaling pathway, and associated with maintenance of glial cell lineage (75, 76). Stimulation with NRG-1β induced the expression of the proinflammatory molecules IL-1α, IL-6, and cyclooxygenase-2; however, stimulation with NRG-1α2 did not induce these proinflammatory molecules. These gene expression profiles highlight the differential biological effects induced by NRG-1β1 and NRG-1α2. In summary, our laboratory's microarray data indicate that NRG-1 isoforms induce distinct, yet overlapping patterns in gene expression. The microarrary expression profiling data are detailed in Tables 1-4.

DIFFERENTIAL ERBB RECEPTOR ACTIVATION IN SCHWANN CELL NEOPLASIA

ErbB receptors integrate the inputs of all NRG-1 isoforms and other EGF-like ligands (69). As there are four erbB isoforms that are all capable of heterodimerization, erbB combinatorial events create yet another mechanism to generate signal diversity and/or specificity (69, 77-80). NRGs bind either erbB3 or erbB4 and induce homodimerization/heterodimerization events with other ErbB family members (79, 80). The ErbB expression profile that I have identified in human MPNST cell lines shows variable erbB expression; however, in all cases, the erbB receptor profile is consistent with NRG-1 responsiveness, and human MPNST cell lines are dependent on NRG-1/erbB signaling for maximal proliferation. Furthermore, I assayed erbB activation via tyrosine phosphorylation of the direct NRG-1 receptors. In these experiments, I assayed net levels of erbB tyrosine phosphorylation, specifically, erbB3 in Mash-1 cells and

Tables 1-4: NRG-1β1 and NRG-1α2 isoforms alter gene expression in adult Schwann cells. Adult Schwann cells were expanded and plated in a serum free Schwann cell defined media at a defined density (1 million cells/T185). After 24 hours, vehicle, 10 nM NRG-1β1, or 10 nM NRG-1α2 was added to the media, and the cells were stimulated for 24 hours. Following 24 hours of stimulation, adult Schwann cells were lysed in Trizol Reagent (Gibco), and the RNA was extracted. The RNA served as a template to generate cDNA, which was used as the template to generate biotinylated cRNA probes. These biotinylated cRNA probes were hybridized to high-density oligonucleotide microarrays (Affymetrix U34A Chip interrogating ~8,800 transcripts representing a collection of both annotated genes and expressed sequence tags). Hybridization was detected using a streptavidin-phycoerythrin conjugate and quantified with a high-resolution scanner. Microarrays were scaled to a uniform target intensity of 1,500. Hybridization to perfect match oligos, relative to mismatch oligo controls, was used to generate the average difference, a measure of gene expression. Unstimulated (control) flasks provided baseline levels of gene expression. Microarray experiments were performed in triplicate to determine statistically significant changes in gene expression ($p = 0.05$). Treated cells were compared to baseline levels in order to determine the fold changes in gene expression indicated in Tables 1-4.

TABLE 1. Genes Induced by NRG-1β1

GenBank Accession no.	Gene Description	Fold Change
M60921	Rat PC3 NGF-inducible antiproliferative putative secreted protein	4.17
M18416	Nerve growth factor-induced (NGFI-A) gene	3.05
AA891041	JunB	2.26
U75397	Krox-24	2.24
U17254	Immediate early gene transcription factor NGFI-B	2.21
AF030091	Activity and neurotransmitter-induced early gene (ania-6)	1.74
U01146	Nuclear orphan receptor HZF-3	1.69
D13417	Transcription factor HES-1	2.16
X06769	c-fos	2.10
X63594	RL/IF-1	2.48
L02896	Major heparin sulfate proteoglycan (glypican)	2.08
AF013144	MAP-kinase phosphatase (cpg21)	1.84
AF044201	Neural membrane protein 35	1.65
AA859585	EST	1.63
AA799448	EST	1.62
AA799788	*Rattus norvegicus* serine protease gene accession no. L38482	1.56
AA800669	Gene homologous to *Mus musculus*, protein phosphatase 2 (formerly 2A) regulatory subunit A (PR 65), alpha isoform, accession no. BC006606	1.55
H31144	Similar to *mus musculus* tyrosine kinase, non-receptor, 2 (Tnk2) accession no. NM_016788	1.54
U23769	CLP36 (clp36)	1.53

TABLE 2. Genes Induced by NRG-1α2

GenBank Accession no.	Gene Description	Fold Change
D13417	Transcription factor HES-1	7.38
M60921	Rat PC3 NGF-inducible antiproliferative putative secreted Protein	4.33
X96437	PRG1 (an early-response gene)	2.82
M18416	Nerve growth factor-induced (NGFI-A) gene	2.73
U01146	Nuclear orphan receptor HZF-3	2.63
U17254	Immediate-early gene transcription factor NGFI-B	2.45
U75397	Krox-24	2.45
X06769	c-fos	2.22
AI176710	NOR-1 neuron derived orphan receptor; thyroid/steroid receptor superfamily	2.09
AF030091	Activity and neurotransmitter-induced early gene 6 (ania-6)	1.97
L23148	Inhibitor of DNA binding 1, helix-loop-helix (Id1)	1.83
U78102	Krox-20	1.76
X63594	RL/IF-1 (encoding an IkappaB)	5.45
M26754	Interleukin-6 (IL-6)	3.19
D00403	Interleukin-1 alpha	3.09
L25925	Cyclooxygenase-2	2.82
AA800853	EST	1.67
AA799525	Gene similar to *Homo sapiens*, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9 (39 kD), Accession no. BC015837	1.64
D25543	Novel Golgi-associated protein GCP360	1.61
AF014009	Calcium-independent phospholipase A2	1.54
AI012942	Cdc25B	1.53

TABLE 3. Genes Repressed by NRG-1β1

GenBank Accession no.	Gene Description	Fold Change
M91234	Rat VL30 element	-2.15
U96490	Liver mRNA	-1.9
U17260	Arylamine *N*-acetyltransferase-1 (AT-1)	-1.82
AA894080	EST	-1.79
X62875	High Mobility Group Protein I (Y)	-1.74
AI102839	Gene homologous to *Mus musculus* Notch gene homolog 3, accession no. NM_008716	-1.64
U67994	DNA primase small subunit	-1.61
X97443	Transmembrane protein Tmp21-I	-1.59
AA859593	Gene homologous to mouse mRNA for estrogen-responsive finger protein, accession no. D63902	-1.57
D86297	*Rattus norvegicus* mRNA for rat erythroid-specific delta-aminolevulinate	-1.54
AA800169	EST	-1.53
AA875033	Rattus norvegicus fibulin 5 (Fbln5), accession no. NM_019153	-1.52
AF061971	Palmitoyl-protein thioesterase (PPT-2)	-1.51

TABLE 4. Genes Repressed by NRG-1α2

GenBank Accession no.	Gene Description	Fold Change
U71089	CXC chemokine receptor1-like protein (CXCR1)	-1.89
AA874926	Similar to KIAA1096 protein (*Homo sapiens*)	-1.79
D84418	Chromosomal protein HMG2 accession no. XM_043678	-1.72
AA893328	Gene homologous to *Mus muculus*, similar to calnexin, accession no. BC012408	-1.62
AI180288	*Rattus norvegicus* Caldesmon 1 (Cald1)	-1.58
AA819708	*Rattus norvegicus* cytochrome c oxidase subunit VIIa 3 (Cox7a3), accession no. NM_022503	-1.54
U09793	P21 (c-Ki-ras)	-1.50

erbB4 in YST-1. Phosphorylation of erbB receptors at critical tyrosine residues creates binding sites for docking proteins that couple to downstream signaling pathways.

Given the number of potential tyrosine phosphorylation events, numerous combinatorial possibilities exist, and more experimentation will be necessary to dissect the biological functions of combinatorial tyrosine phosphorylation. One methodological approach would be a tryptic phosphopeptide mapping strategy analogous to that of Sweeney et al (72). In Sweeney et al's methodology, breast cancer cell lines were stimulated with NRG-1 or NRG-2 isoforms, and erbB receptors were enriched by anti-phosphotyrosine immunoprecipitation and resolved using 2-D gel electrophoresis (72). Using this methodology, it was possible to define unique patterns of erbB tyrosine phosphorylation. This approach could be applied to NRG-1 signaling in Schwann cell biology (cultured cells and/or neoplastic cells). Specifically, this methodology could be applied to cultured neonatal Schwann cells, with cells being treated with promitogentic NRG-1β isoforms and compared with cells treated with non-mitogenic NRG-1α isoforms. Alternatively, a tryptic phosphopeptide mapping could be applied to neoplastic Schwann cell lines treated with erbB kinase inhibitors. By monitoring the erbB phosphopeptide map, it may be possible to identify key residues that are required to transmit pro-proliferative signal(s).

ERBB MUTATIONS AND OTHER STRUCTURAL VARIANTS

The data presented here demonstrate that human MPNSTs are dependent on erbB kinase signaling. An alternative interpretation of the data is that the cells are de-

pendent on baseline levels of erbB kinase activity in a manner that is ligand independent. On the basis of our current results, we cannot exclude this possibility. ErbB genetic and/or structural alterations are another possible mechanism of dysregulated erbB signaling that may be functioning in Schwann cell neoplasms. Specifically, erbB structural alterations can be generated by mutations, deletions, alternative splicing, and/or proteolytic processing (81-100). Such erbB genetic and/or structural alterations may be occurring in Schwann cell neoplasia and thus may result in aberrant erbB signaling. When they occur, erbB genetic alterations may provide a specific target for therapeutic intervention. Recently, multiple laboratories have identified mutations within the kinase domains of EGFR and erbB2 (84-86). ErbB receptor mutation may enhance the kinase activity of the mutated receptor and aberrantly activate signaling mechanisms that stimulate prosurvival and/or promitogenic pathways. As a result, it may be possible to preferentially target mutated kinase domain(s). Such an approach has been successfully used in other tumor types. One example is the erbB kinase inhibitor gefitinib, which has a higher response rate in pulmonary adenocarcinomas carrying EGFR kinase mutations than in tumors lacking this mutation (84, 85).

POSTRECEPTOR SIGNALING

As a consequence of NRG-1 stimulation, tyrosine phosphorylation of erbB receptors creates docking sites that create binding sites for adapter proteins through SH2 adaptor-protein interactions, thus initiating a series of events that result in activation of downstream signaling processes such as the Ras/Raf/Map kinase signaling pathway (69). In the neoplastic state, it is possible that negative regulatory signaling mechanisms

are removed, thus potentiating the effect(s) of growth factor stimulation. The tumor suppressor neurofibromin functions as an inhibitor of Ras activity (101); consequently, NRG-1 modulation of Ras/Raf/Map kinase signaling is a candidate mechanism in Schwann cell neoplasms. Removing neurofibromin's negative regulatory activity may make neoplastic Schwann cells hyperresponsive to their own growth factors such as NRG-1. To test this hypothesis, it would be useful to treat normal human Schwann cells and MPNST cell lines with erbB kinase inhibitors (e.g. PD168393) and subsequently monitor levels of Ras activity. Furthermore, normal human Schwann cells could be treated with NRG-1 isoform(s), and their Ras activity could be assayed and compared to that evident in NRG-1-stimulated MPNST cells. These experiments would test the effects of NRG-1/erbB signaling on Ras signaling and determine how changes in Ras activity correlate with changes in proliferation in normal and neoplastic Schwann cells.

MODELS OF NEOPLASTIC SCHWANN CELL GROWTH

To date, investigators have used multiple experimental models of Schwann cell neoplasia. The generation of transgenic mice carrying *cis*-linked *Nf1*-/+/*p53*-/+ double mutations has shown that inactivation of these two tumor suppressors can induce MPNST formation (102). In addition, the phenotype of the P_0-GGFβ3 transgenic mouse shows that overexpression of a Schwann cell mitogen GGFβ3 can induce Schwann cell hyperplasia and MPNST formation (14). These transgenic models of Schwann cell neoplasia demonstrate that genetic manipulation (either through tumor suppressor inactivation or through growth factor overexpression) can lead to Schwann cell neoplasia. At present, the extent of the similarities and differences that exist in these two trans-

genic models is difficult to ascertain. However, our preliminary experiments demonstrate that these two transgenic models have significant biological differences. For example, the frequency of EGFR overexpression is one difference between the two neoplasms. Cell lines derived from *Nf1/p53* knockout mice show frequent overexpression of EGFR (103, 104). In contrast, cell lines derived from P_0-GGFβ3 transgenic mice rarely express EGFR, while the majority of tumors express the direct NRG-1 receptors erbB3 and/or erbB4. The biological differences that occur between these two transgenic models raise the following questions: What class of Schwann cell neoplasms is being modeled? How well does a given model recapitulate the phenotype of human neoplasms? Do these models select for subsets of Schwann cell neoplasms? Are these models sufficiently similar to the human neoplasms to test potential therapies?

HOW ACCURATELY DO TRANSGENIC MODELS RECAPITULATE HUMAN SCHWANN CELL NEOPLASIA?

While tumors and cell lines derived from these animals (*Nf1/p53* knockout and the P_0-GGFβ3 transgenic) demonstrate histological and structural features resembling human MPNSTs, it is unclear how accurately these models recapitulate the biology of the human tumors. Tumor cells, but not nontransformed Schwann cells, from P_0-GGFβ3 mice demonstrate tumorigenic properties (growth in soft agar and subcutaneous growth in SCID and NIH III mice); consequently, it seems likely that other molecular events occur (inactivation of tumor suppressors) must occur for MPNST formation. Future experiments will address the following question: Do MPNST cells from P_0-GGFβ3 mice have tumor suppressor mutations analogous to human MPNSTs? Examining the status of neurofibromin and *p53* for genetic inactivations will determine how

closely these transgenic cell lines mimic human Schwann cell neoplasms. Another question to be addressed is whether persistent activation of NRG-1/erbB signaling is required to maintain proliferation in tumorigenic cells from P_0-GGFβ3 mice. Preliminary results from our laboratory indicate that tumor cells from P_0-GGFβ3 transgenic mice are dependent on NRG-1/erbB signaling as the erbB kinase inhibitors PD168393 and 158780 inhibit DNA synthesis in these cells.

CELL CULTURE MODELS OF HUMAN SCHWANN CELL NEOPLASMS

In addition, to examinations of transgenic models of Schwann cell neoplasia, studies of neoplastic human Schwann cell lines help to elucidate mechanisms of neoplastic Schwann cell growth. Several laboratories have examined MPNST cell lines for erbB expression (103, 105). When our laboratory's data are compared, several important differences emerge, most notably the frequency with which erbB3 and erbB4 expression is found in human neoplastic Schwann cells. Despite these differences, the four human MPNST cell lines that we analyzed all expressed NRG-1 receptors and required erbB kinase signaling for proliferation. Significantly, my experiments demonstrated that human MPNST cell lines that do not express EGFR (Mash-1 and YST-1) were sensitive to erbB kinase inhibition. Several potential mechanisms may explain the requirement for erbB kinase signaling in human MPNST cell lines. One possibility is that all neoplastic Schwann cells require a baseline level of erbB kinase signaling. Another possibility is that increased erbB expression by itself is sufficient to promote neoplastic transformation. A third possibility is that, through overexpression of erbB receptors, neoplastic Schwann cells become hyperresponsive to stimulation by EGF-like

ligands, most specifically NRG-1. The sections below present several potential methods to address these questions using multiple strategies to inhibit NRG-1/erbB signaling.

ANTAGONISM OF NRG-1/ERBB SIGNALING: SOLUBLE RECEPTORS AND NEUTRALIZING ANTIBODIES

Recently, clinical success has been achieved with small molecule erbB kinase inhibitors in a number of solid tumors (106). The small molecule erbB kinase inhibitors Iressa and Tarceva are relatively safe and have limited toxicity in humans (106). Before therapeutics can be tested in human clinical trials, it will be necessary to test specific compounds in preclinical models of Schwann cell neoplasms. Future experiments will examine the effects of small molecule erbB inhibitors on the growth of Schwann cell neoplasms in tumor xenograft models. The data presented here demonstrate that small molecule erbB kinase inhibition markedly inhibits the proliferation of human MPNST cell lines. In addition to small molecule kinase inhibitors, a number of strategies have been developed to antagonize NRG-1/erbB signaling. For example, neutralizing antibodies have been generated to antagonize specific erbB receptors (106-115). Among these, the best characterized is the humanized anti-erbB2 antibody Herceptin, which is an FDA-approved treatment for erbB2-positive breast cancer (106, 107). Multiple mechanisms have been proposed to account for Herceptin's in vivo effects, including enhanced receptor internalization/downregulation, activation of complement, and immune effector functions (108, 109). In addition to Herceptin, there are several strategies for therapeutic intervention using other anti-erbB receptor antibodies (106-115). Like Herceptin, the monoclonal antibody 2C4 targets erbB2; however, 2C4 targets an erbB2 epitope distinct from that of Herceptin (110, 111). 2C4 binds amino acids within

erbB2's dimerization domain and, by inhibiting this dimerization domain, prevents erbB2 from dimerizing with other erbB family members (111). Furthermore, there is a precedent for an erbB3 neutralizing antibody that blocks the interactions of ligand with erbB3 (112-115). In one series of experiments, this erbB3 neutralizing antibody has been used to block the binding of exogenous NRG-1 to erbB3 in ovarian cancer cell lines. Blocking the NRG-1/erbB3 interaction resulted in decreased NRG-1-stimulated erbB tyrosine phosphorylation, MAPK phosphorylation, and proliferation in ovarian cancer cells (113).

In addition to erbB receptor-targeted antibodies, several biologically based approaches could be utilized for NRG-1 ligand neutralization. Several laboratories have generated erbB-F_c fusion proteins that contain the extracellular domain of the erbB receptors recombinantly fused to the F_c region of human IgG1 (116). Preliminary results from our laboratory have indicated that treatment of a human MPNST cell line (YST-1) with an erbB4-F_c soluble receptor inhibits constitutive erbB4 tyrosine phosphorylation, while administration of an erbB2-F_c soluble receptor has no effect (Fig. 1). However, the use of soluble receptors can also have limitations that make data interpretation problematic. First, erbB soluble receptors would not distinguish the effects of NRG-1 from the effects of other EGF-like ligands that also signal through the direct NRG-1 receptors erbB3 and erbB4. Second, as soluble receptors are large molecules that cannot pass through the plasma membrane, soluble receptors may not be able to fully inhibit certain ligand/erbB interactions. Most notably, soluble receptors would be unable to inhibit the activity of internalized erbB receptors. These limitations of soluble receptors are impor-

tant considerations given that the expression of erbB4 is predominantly nuclear in erbB4 expressing MPNST cell lines (NMS-2 and YST-1).

Another possibility is to inhibit the actions of NRG-1 directly via an NRG-1-targeted neutralizing antibody. Antagonizing the effects of all NRG-1 isoforms would likely require an antibody with specificity for the EGF-like common domain. Alternatively, if specific NRG-1 isoforms are determined to be the optimal therapeutic target, it may be possible to target these isoforms selectively with specific neutralizing antibodies. All of these examples are potential biological interventions that can disrupt NRG-1/erbB signaling. Several recent experiments have provided encouraging results on combination targeted therapy. For example, combination therapy with a small molecule erbB kinase inhibitor and an EGFR neutralizing antibody resulted in an additive inhibition of tumor growth both *in vitro* and *in vivo* (117, 118). Consequently, a combination of small molecule erbB kinase inhibitors with biological agents that target NRG-1/erbB signaling may have similar effects in the treatment of human Schwann cell neoplasms. As with any therapeutic intervention, the challenge will be to maximize therapeutic efficiency while minimizing adverse effects.

FUTURE EXPERIMENTS: RNAI

As the role of NRG-1/erbB signaling emerges in Schwann cell neoplasms, it will be necessary to determine the precise contribution of each NRG-1 isoform and each erbB receptor in the development and maintenance of tumorigenesis. Treatment with short RNA oligonucleotides enables sequence-specific gene silencing through a process known as RNAi. RNA-mediated gene silencing could be utilized to knock-

down class specific NRG-1 isoforms, and test the effect(s) of NRG-1 isoform genetic knock down on cellular functions such as survival, proliferation, and migration. In addition, RNAi could be utilized to knock down individual erbB receptors so that the contribution of individual erbB kinases can be tested. RNAi knock-down of erbB isoforms has several specific advantages over other erbB inhibition strategies. Specifically, coexpression of erbB3 and erbB4 commonly occurs in the MPNST cell lines derived from P_0-GGFβ3 mice. NRG-1 isoforms can bind both erbB3 and erbB4, and a consequence of erbB3/erbB4 receptor coexpression is that it is difficult to ascertain the relative contribution of individual erbB receptors to mediating NRG-1 responsiveness. RNAi could be used to ablate expression of erbB3 and/or erbB4 individually, thus allowing the contribution of each of these receptors to be determined.

ARE THE EFFECTS OF NRG-1 UNIQUE?

Transgenic overexpression of the NRG-1 isoform GGFβ3 induces Schwann cell hyperplasia and MPNST tumorigenesis (14). It is not yet clear how generalizable these observations are. In principle, it is possible that the tumorigenic phenotype seen in P_0-GGFβ3 mice is completely specific to GGFβ3, a type II NRG-1 isoform that is directly secreted. The second possible scenario is that the P_0-GGFβ3 tumorigenic phenotype is a more general effect of overexpression of any of a number of NRG-1 isoforms. The third possibility is that the P_0-GGFβ3 tumorigenic phenotype can be reproduced by overexpression of other Schwann cell mitogens such as platlet-derived growth factor or FGF-2. Generating transgenic animals that express other NRG-1 isoforms or other Schwann cell mitogens may provide additional insights into these issues.

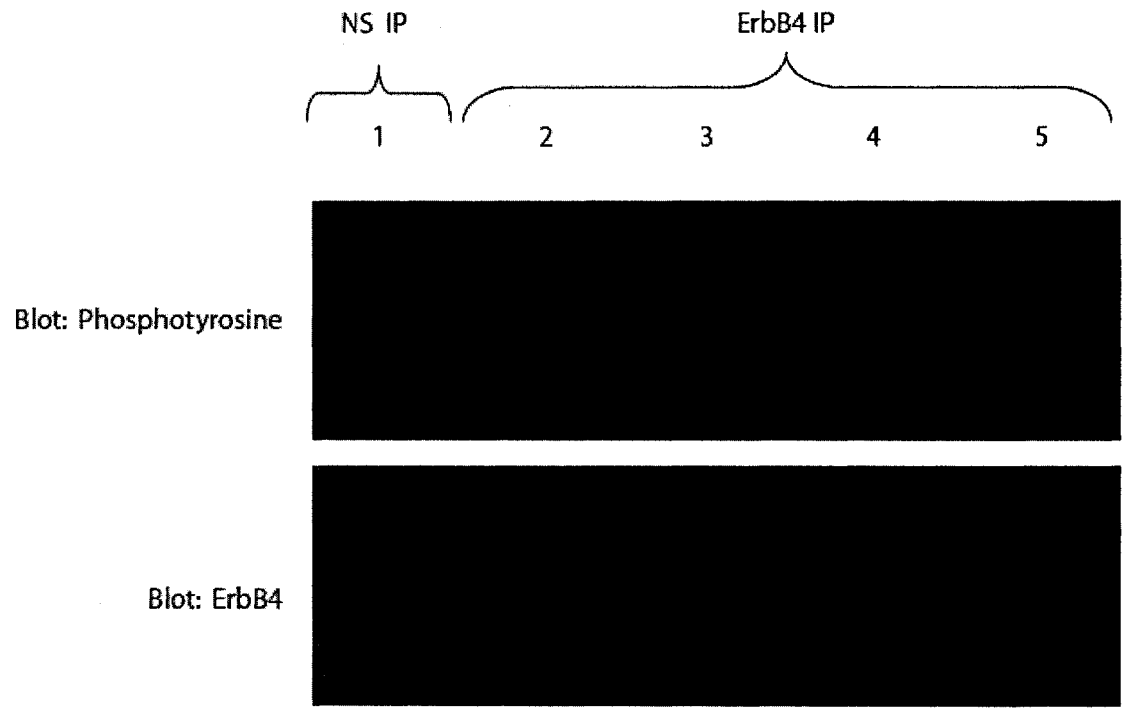


Figure 1. Treatment with an erbB4-Fc soluble receptor inhibits constitutive erbB4 tyrosine phosphorylation in the human MPNST cell line YST-1. To test the effect of ligand inhibition, YST-1 cells were treated for 24 hours with one of the following four conditions: the extracellular domain of erbB2 linked to the Fc region of human immunoglobulin (erbB2-Fc), ErbB4 recombinantly linked to the Fc region of human immunoglogulin (erbB4-Fc), the human Fc region alone (Hu-Fc), and a vehicle only control. The extracellular domain of erbB4 binds neuregulin isoforms with high affinity, whereas erbB2 has no known ligand. Cells were treated with these conditions for 24 hours, lysed, and immunoprecipitated with erbB4 or a nonspecific immunoglobulin control (NS) as described previously. Immunoprecipitates were resolved using SDS-PAGE, and probed for anti-phosphotyrosine (top panel) and erbB4 (bottom panel). Consistent with previous experiments, constitutive erbB4 tyrosine phosphorylation was detected at ~185 kD in YST-1 cells (lane 2). Treatment with erbB4-Fc (lane 4) resulted in a marked inhibition of erbB4 tyrosine phosphorylation, while treatment with either Hu-Fc (lane 3) or erbB2-Fc (lane 5) did not have an appreciable effect.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office of Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and the approval period is for three years. The Assurance number is FWA00005960.

Principal Investigator:	CARROLL, STEVEN L
Co-Investigator(s):	
Protocol Number:	**X021127007**
Protocol Title:	*Characterization of a Putative Neuregulin/erbB Receptor Autocrine Loop in Schwann Cell Neoplasia*

The IRB reviewed and approved the above named project on 9-10-04. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

3 Approval Date: 9-10-04

Date IRB Approval Issued: 9-10-04

HIPAA Waiver Approved?: No

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

470 Administration Building
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Fax 205.934.1301
irb@uab.edu

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APPENDIX B

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

NOTICE OF APPROVAL WITH STIPULATIONS

DATE: August 31, 2004

TO: Steven L. Carroll, M.D., Ph.D.
SC-843 0017
FAX: 975-6356

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Role of Neuregulin-1 in Schwann Cell Neoplasia
Sponsor: NIH
Animal Project Number: 040807321

On August 31, 2004, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	C	2000
Rats	B	200

Approval is granted with the following stipulation(s):

The Animal Use Safety Information Sheet (AUSIS) corresponding to this project was forwarded to Occupational Health and Safety (OH&S) for their review and authorization. When you receive the authorized AUSIS, please contact the ARP Health and Safety Specialist, Earle Durboraw (934-3538), to discuss specific safety precautions which may be necessary for the ARP care staff. Animal procurement and use of potentially hazardous agents in live animals in this project may not occur until Mr. Durboraw has informed the IACUC Office that a satisfactory discussion has occurred.

Animal use is scheduled for review one year from August 2004. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 040807321 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1717 7th Avenue South
205.934.7692 • Fax 205.934.1188
iacuc@uab.edu
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Mailing Address:
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1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

MEMORANDUM

DATE: August 31, 2004

TO: Steven L. Carroll, M.D., Ph.D.
SC-843 0017
FAX: 975-6356

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use COmmittee (IACUC) on August 31, 2004.

Title: Role of Neuregulin-1 in Schwann Cell Neoplasia

Sponsor: NIH

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

Institutional Animal Care and Use Committee
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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Mark S. Stonecypher

Graduate Program Cell Biology

Title of Dissertation Activation of Neuregulin-1/ErbB Signaling Promotes Proliferation in Human Schwann Cell Neoplasia

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that he may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
Steven L. Carroll	, **Chair**	
Stuart J. Frank		
Andra R. Frost		
G. Yancey Gillespie		
Kevin A. Roth		

Director of Graduate Program

Dean, UAB Graduate School

Date JAN 0 6 2006