The role of Neurofibromatosis Type 1 in Schwann Cell development and tumor formation and the influence of steroid hormones

by

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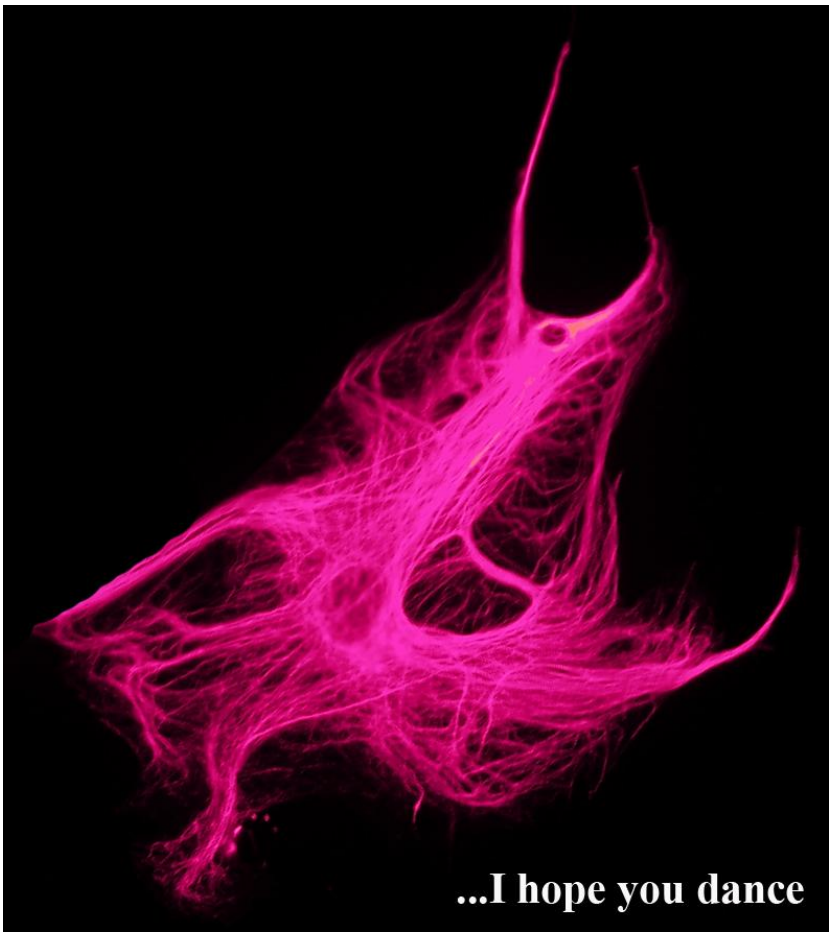
...I hope you dance

DEDICATION

To my husband Brad and youngest daughter Caitlin, who followed me in pursuing my dream and still love me.

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LIST OF ABBREVIATIONS

NF1	Neurofibromatosis Type 1
NF1+/+	cell with both functional NF1 alleles-expresses normal levels of neurofibromin
NF1+/-	cell with one functional NF1 allele-expresses low levels of neurofibromin
NF1-/-	cell with no functional NF1 alleles-expresses little/no neurofibromin
NC	neural crest
SC	Schwann Cell
mESC	mouse embryonic stem cell
D3	wild-type NF1+/+ mESC
D3SC	wild-type NF1+/+ SC-like differentiated mESC
SKO	heterozygous NF1+/- mESC
SKOSC	heterozygous NF1+/- SC-like differentiated mESC
DKO	mutant NF1-/- mESC
DKOSC	mutant NF1-/- SC-like differentiated mESC
SW10	mouse Schwann Cell with temperature-sensitive SV40 large T antigen
PNF	human benign plexiform neurofibroma tumor cell (pNF00.11)
ST	human malignant peripheral nerve sheath tumor cell (ST88-14)
MPNST	malignant peripheral nerve sheath tumor
E5	day 5 of embryonic development
SAG	E5 chick statoacoustic ganglion
PNS	peripheral nervous system
E2	17β-estradiol steroid hormone
2ME2	2-methoxyestradiol, an estrogen metabolite

ER	estrogen receptor
P4	progesterone steroid hormone
PR	progesterone receptor
T	testosterone (an androgen) hormone
AR	androgen receptor
ICC	immunocytochemistry
IHC	immunohistochemistry
RTqPCR	reverse transcription quantitative polymerase chain reaction

ABSTRACT

The role of Neurofibromatosis Type 1 in Schwann Cell development and tumor formation and the influence of steroid hormones

by

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Chair: Kate F. Barald

The Neurofibromatosis Type 1 (NF1) gene functions as a tumor suppressor gene. One known NF1 function is to turn off the p21ras pathway by accelerating Ras hydrolyzation of active rasGTP to inactive rasGDP. Loss of neurofibromin (the protein product of the NF1 gene) in the autosomal dominant disorder NF1 is associated with tumors of the peripheral nervous system, particularly neurofibromas in which the major cell type is the Schwann Cell (SC). We have developed an *in vitro* system for differentiating mouse embryonic stem cells (mESC) that are NF1 wild type (+/+), heterozygous (+/-), or null (-/-) into SC-like cells to study the role of NF1 in SC development and tumor formation. These SC-like cells, regardless of their NF1 status, express SC markers appropriate for their developmental stage, and support and preferentially direct neurite outgrowth from primary embryonic neurons. They are also capable of expressing myelin proteins. NF1 null and heterozygous SC-like cells proliferate at an accelerated rate compared to NF1 wild type; this growth advantage can

be reverted to wild type levels using a Mek inhibitor The mESC of all NF1 types can also be differentiated into neuron-like cells. The behavior and genetic repertoires of the cells under different developmental conditions can be compared, providing an ideal paradigm for studies of the role of NF1 in cell growth and differentiation of the different cell types affected by NF1.

The number and size of neurofibromas in NF1 patients has been shown to increase during pregnancy. SC-like differentiated mESC with varying levels of neurofibromin and NF1 tumor cell lines derived from a malignant and a benign human tumor, were used to study proliferation in response to hormones. Estrogen and androgen receptors showed very low/no expression in the NF1+/+ cells, low levels of expression in NF1+/- cells, and high levels of expression in NF1-/- cells. We have also found that an E2 metabolite, 2Methoxyestradiol, is cytotoxic to the NF1-/- malignant tumor cell line, and inhibits proliferation in the other cell lines. 2ME2 or its derivatives could provide new treatment avenues for NF1 hormone-sensitive tumors at times of greatest hormonal influence.

CHAPTER I

INTRODUCTION

HORMONE INVOLVEMENT IN NEUROFIBROMATOSIS TYPE 1

NEUROFIBROMATOSIS TYPE I (NF1) - ORIGIN OF NEUROFIBROMAS

The tumor suppressor gene *NF1*, which encodes the neurofibromin protein, is responsible for one of the most common autosomal dominant disorders, in which all cells of the affected individual are heterozygous for the *NF1* gene. This disorder, also called Neurofibromatosis type 1 (NF1), affects 1/3000 to 1/3500 individuals worldwide and occurs in equal frequency in males and females and across all ethnic groups (Friedman et al., 1999). NF1 is a progressive disorder that may be quiescent for long periods, and is 100% penetrant but demonstrates a wide range of severity that cannot be predicted even in family members who presumably carry the same mutation (Friedman et al., 1999). The human *NF1* gene, located on chromosome 17q11.2, is extremely large, 335 kb with 60 exons (figure 1.1). It is highly mutated even accounting for its large size; while 50% of NF1 patients inherit the mutant allele from a parent, 50% of the mutations are new, there are no "mutational hotspots" (Friedman et al., 1999). *NF1* encodes a more than 13 kb transcript, which translates into the 250 kDa neurofibromin protein. Expression of neurofibromin is ubiquitous, however, expression is highest in the nervous system (Friedman et al., 1999). Neurofibromin expression is associated with microtubules, from

which it disaggregates on exposure to anti-mitotic drugs, such as colcemid (Gregory et al., 1993). Following colcemid removal, cells recovered microtubule assembly and NF1 microtubule localization (Gregory et al., 1993). Mutations in *NF1* are responsible for widely varying phenotypes, most but not all affected tissues are from neural crest-derived cells (figure 1.2), including numerous benign tumors of the peripheral nervous system such as cutaneous neurofibromas, plexiform neurofibromas and optic gliomas (for reviews, see Viskochil et al., 1993; Gutmann et al., 1994; Blum and Kambich, 1997; Feldkamp et al., 1998; Packer et al., 2002; Zhu et al., 2002). NF1 is also known as von Recklinghausen's Disease: Fredrich Daniel van Recklinghausen was the first to describe the nervous tissue origin of the tumors in 1882 (Blickstein et al., 1988; Reynolds et al., 2003). Neurofibromas are the most commonly found and potentially debilitating manifestations of NF1. These benign growths, comprised mostly of Schwann cells (SC) or SC-like cells, but with substantive numbers of fibroblasts, mast cells, perineurial cells and vascular endothelial cells, arise on nerves that range in size from extremely small terminal branches to the spinal nerve roots. Mast cells are also found in other tumors, such as pancreatic β–cell tumors, mammary carcinomas, and squamous carcinomas (Roche, 1985; Soucek et al., 2007; Theoharides and Conti, 2007). Mast cells are required for tumor angiogenesis in mice, they secrete vascular endothelial growth factor (VEGF) and other cytokines necessary for angiogenesis (Roche, 1985; Soucek et al., 2007; Theoharides and Conti, 2007). NF1 has been reported to modulate mast cell growth (Ingram et al., 2001). NF1 patients cite such discrete neurofibromas as the single most significant burden of their disorder because of their disfiguring effects and associated physical and social discomfort (Wolkenstein et al., 2001; Page et al., 2006). Discrete

neurofibromas may cause job loss, discrimination, low self-esteem, and social and psychological consequences that can require psychiatric intervention (Wolkenstein et al., 2001; Page et al., 2006). Although most discrete neurofibromas are cutaneous, arising in the dermis or epidermis, subcutaneous discrete neurofibromas have also been reported. Discrete tumors are rarely present at birth, but are found in 48% of 10 yr olds (notably, precocious puberty is a common feature of NF1) (Virdis et al., 2003), 84% of 20 year olds, and virtually all NF1 patients over the age of 40. Hundreds and even thousands of these variably sized lesions have been found in adults with NF1. Karvonen et al. (2000) reported that many tiny neurofibromas have been found in the normal-looking skin of NF1 patients, indicating a more widespread occurrence than was previously understood. Many have speculated that the number and size of these neurofibromas may increase in response to hormonal stimuli as commonly described by women during pregnancy, but a direct correlation of neurofibroma growth and hormonal stimulation remains to be elucidated. Large neurofibromas can also arise from multiple nerves within plexuses. These are termed plexiform neurofibromas (Packer et al., 2002). Diffuse plexiform neurofibromas are frequently associated with significant morbidity and even mortality. There are histopathological dissimilarities between discrete and diffuse plexiform neurofibromas, suggesting that different mechanisms may exist for the formation of these different types of neurofibromas (Palmer et al., 2004). Plexiform neurofibromas, first arising in early childhood, are often relatively stable for a number of years but are capable of aggressive growth, particularly as puberty approaches or during pregnancy. Nodular plexiform neurofibromas can also cause serious neurological dysfunction. About 5% of plexiform neurofibromas undergo malignant transformation and eventually

become malignant peripheral nerve sheath tumors (MPNSTs) (Woodruff, 1999). A hormonal "trigger" may be responsible both for growth of the tumors and for such malignant transformation (Posma et al., 2003) but a direct causal relationship has not been firmly established. The physical and emotional burden of NF1 is therefore enormous (Wolkenstein et al., 2001; Sebold et al., 2004; Graf et al., 2006; Page et al., 2006).

NF1 IS A TUMOR SUPPRESSOR GENE; ROLE OF LOSS OF HETEROZYGOSITY

NF1 patients inherit only one functional allele (and are therefore genetically heterozygous for *NF1*), and the second allele is lost in most, but not all, cells in the tumors. This "second hit" results from loss of heterozygosity (LOH) in specific cells (figure 1.3), but it is not clear what role LOH actually plays in tumorigenesis, although it is thought to be essential. The lack of a functional gene product, the protein neurofibromin, is thought to cause the aberrant cellular proliferation characteristic of neurofibromatosis type 1 (Packer et al., 2002). However, loss of heterozygosity (LOH) has been found in some, but not all neurofibromas, and in some but not all cell types within the neurofibromas themselves (Zhu et al., 2002). Zhu and Parada (Zhu et al., 2002; Parada et al., 2005) hypothesized that LOH occurs in neural crest stem cells early in embryogenesis (figure 1.3). These stem cells then differentiate to form both Schwann cells (SC) and peripheral nervous system (PNS) nerve cells. Nerve cells induce proliferation of the $NF1^{-/-}$ SC, which then recruit both heterozygous fibroblasts and SCs. Therefore, SCs are probably the primary cells involved in the formation of neurofibromas, although the presence of two SC types, one with and one without LOH,

suggests that LOH may be the primary event triggering neurofibroma formation, but may not be the sole event (Fishbein et al., 2005). A major unanswered question is whether a decrease in neurofibromin as a result of haploinsufficiency can be sufficient to induce neurofibroma formation, or whether other mutations, at the NF1 locus or other loci (e.g. P53, retinoblastoma), or environmental conditions including trauma, altered blood supply and possibly the elevated levels of specific hormones, seen at puberty and during pregnancy, could also serve as necessary triggers (Horyn et al., 1988; Zhu et al., 2002; Parada et al., 2005).

PROLIFERATION VS. DIFFERENTIATION; THE ROLE OF *NF1*'S RAS-GAP FUNCTION

Cell differentiation is known to be important in suppression of both tumor growth and malignancy. Neurofibromin may suppress aberrant cellular proliferation by promoting cell differentiation, possibly through its interaction with Ras. Neurofibromin contains a domain homologous to the ras GTPase-activating protein, GAP (Weiss et al., 1999). NF1 can be considered a gene regulating programmed cell death, because one of neurofibromin's functions is to serve as a ras-GAP, which accelerates the conversion of active Ras-GTP to inactive Ras-GDP (figure 1.4). The ras-GAP region of the NF1 gene responsible for its tumor suppressor function is located at exons 21-27a, labeled the Ras-GRD (GAP-related domain) (figure 1.1). NF1's Ras-GTP suppression activity inhibits cell growth and promotes apoptosis. Mutations that inactivate NF1 reduce the suppressive activity of Ras-GTP and promote cellular proliferation. Ras-GTP levels are elevated in plexiform neurofibromas (Feldkamp et al., 1999), which could explain the pronounced hyperplasia seen in these tumors. Ras-GTP levels were found to be normal in

fibroblasts from four individual neurofibromas and a plexiform neurofibroma (Sherman et al., 2000). However, in the same tumors, Schwann cells were heterogeneous in their expression of Ras-GTP. The activity of p21ras is also known to be involved in many types of cell differentiation (Cichowski and Jacks, 2001; Packer et al., 2002) including *in vitro* neuronal differentiation. In neurons under normal conditions, it may also play a role in various neurotrophin-mediated neuronal differentiation and survival mechanisms (Klesse and Parada, 1998; Klesse et al., 1999), probably through interaction with phosphatidylinositol 3' kinase (PI3K) (Klesse and Parada, 1998; Klesse et al., 1999). The mTOR (target of rapamycin) pathway has also been implicated in NF1 (Dasgupta et al., 2005; Johannessen et al., 2005).

NF1's roles in proliferation, differentiation and apoptosis are context-dependent. Loss of NF1 expression could therefore result in either loss of proliferation or increased proliferation.

CELLULAR MECHANISMS; NF1'S ROLE AS A REGULATOR OF FINAL NEURONAL NUMBERS IN THE PERIPHERAL NERVOUS SYSTEM

The Neurofibromatosis 1 (*NF1*) gene and its protein product, neurofibromin, have been demonstrated to have important roles in regulating the normally occurring programmed cell death (apoptosis) that is vital to the correct formation of the nervous system and other developmental systems in the embryo (Li and White, 1996; Klesse and Parada, 1998; Lakkis and Epstein, 1998; Roth et al, 2007a). Apoptosis is a normal physiological process that regulates cell populations in both the developing embryo and the adult (Wyllie et al., 1980). It is particularly important in regulating the final cell

numbers of neurons and glial cells in the nervous system, because about twice as many cells are produced as are needed for the final functional system (reviewed by Heidenreich, 2003). *NF1* is hypothesized to be, by an as yet unknown mechanism but possibly through attenuated Ras signaling, responsible for promoting cell death of normal excess neurons and glial (Schwann) cells during development. In the absence of this tumor suppressor gene, cell types that are not postmitotic (as neurons are) can proliferate uncontrollably, producing both the plexiform and cutaneous benign neurofibroma tumors characteristic of the disorder (Feldkamp et al., 1998).

NEUROFIBROMATOSIS TYPE 1 (*NF1*) IN A CHANGING HORMONAL MILIEU: NF1 AND PREGNANCY

The incidence of pregnancy among NF1 patients is relatively low and is inversely correlated with the severity of the disease (from about 1/5000 to about 1/18,000 obstetrical patients has NF1, compared with 1/3000-1/3500 in the general population) (Blickstein et al., 1988; Weissman et al., 1993). Blum and Kambich (1997) stress that obstetrical complications are important considerations for NF1-affected women of childbearing age, particularly in certain societies and third-world countries. They speculate that women with very mild cases of NF1 are frequently not diagnosed and their disorder either does not complicate pregnancy or, if symptoms worsen during the pregnancy, NF1 is then diagnosed. They further comment that women with severe clinical manifestations of NF1 rarely elect to bear children except in underdeveloped countries. There has been a reported increase in the number and size of cutaneous and plexiform neurofibromas with pregnancy going back over 100 years (Brickner, 1906;

Dugoff and Sujansky, 1996; Isikoglu et al., 2002; Posma et al., 2003); reviewed in (Blum and Kambich, 1997). There have been reports that pregnant NF1 patients with giant "royal" tumors are more affected than patients with only nodular tumors, with possible reciprocal effects between NF1 and pregnancy (Blickstein et al., 1988). The earliest obstetrical description of NF1, termed "fibroma molluscum gravidarum" was in 1906, by Brickner, while the adverse associations between NF1 and pregnancy were first recognized by Hirst in 1911 (Brickner, 1906; Blickstein et al., 1988).

Only two studies of NF1 patients during pregnancy involved relatively large numbers of patients (Weissman et al., 1993; Dugoff and Sujansky, 1996). The Dugoff and Sujansky study reported on 105 women and 180 pregnancies: 60% of patients reported growth of new neurofibromas; 52% experienced enlargement of existing neurofibromas, sometimes with a decrease in size of nodules after delivery (Dugoff and Sujansky, 1996). However, multiparous women who had NF1 experienced new growth of neurofibromas only in some pregnancies (Dugoff and Sujansky, 1996), even though hormone levels should be similar for each. Growth of Schwannomas as well as neurofibromas was also reported in NF1 patients during pregnancy (Swapp and Main, 1973; Ribella, 1975). Obstetric complications are important considerations for affected women of childbearing age (Blum and Kambich, 1997). This is possibly due to mild cases remaining undiagnosed or severe cases more subject to report, as well as low marriage rates due to cosmetic disfigurement or voluntarily choosing to remain childless (Blickstein et al., 1988; Blum and Kambich, 1997).

Malignant degeneration of neurofibromas during pregnancy has also been reported (Ginsburg et al., 1981; Baker et al., 1989; Puls and Chandler, 1991; Posma et al.,

2003), as well as maternal death from an intracranial hemorrhage resulting from a glioblastoma that recurred during pregnancy (Hadi, 1995). A second pregnancy resulted in the overgrowth of a peripheral nerve sheath tumor that had initially arisen during a first pregnancy, which proved fatal to the patient (Posma et al., 2003). Another case report found that a patient with initially mild disease symptoms died of the results of multiple malignancies, which the physician believed arose and became worse during and after pregnancy and delivery (Heffner, 1969). Boiten et al. (1987) also reported a combination of central nervous system tumor enlargement, new tumor appearance, hydrocephalus and hypertension in a pregnant patient with NF1.

Weissman et al. (1993) also reported a higher than expected rate of first trimester spontaneous abortions (20.7%), still births (8.7%) and intrauterine growth retardation (13.0%), which were not seen or possibly not reported in the Colorado study (Dugoff and Sujansky, 1996). Additional studies have also reported growth retardation of the fetus in patients with NF1 (Belton et al., 1984; Blickstein and Lancet, 1987). An excellent review of all of these aspects can be found in Blum and Kambich (1997).

In patients with NF1, pregnancy can cause severe hypertension and/or blood vessel weakness. An increased risk of hypertension has been recognized in all adults with NF1, but severe hypertension has been specifically noted in pregnant patients with NF1 (Wiznitzer et al., 1986; Bertrand et al., 1992; Hagymasy et al., 1998) including some earlier studies of patients whose pregnancies had to be terminated because of life-threatening conditions (Edwards et al., 1983). The most common causes of hypertension in NF1 patients are renal artery stenosis or phaeochromocytoma, but possible mechanisms have not been reported (Humble, 1967; Criado et al., 2002). At least one

case was found in which the undiagnosed NF1 led to fatal complications under anesthesia (Humble, 1967). There have also been reports in the literature of blood vessel rupture during pregnancy in NF1 patients, including a report of a patient with fatal renal artery rupture (Tapp and Hickling, 1969) and another NF1 patient whose arm was amputated after brachial artery rupture (Tidwell and Copas, 1998). However, at least one report (Jarvis and Crompton, 1978) of a study of hypertension in ten pregnant patients with NF1 in the course of twenty-seven pregnancies found only two cases of pre-eclampsia, a still birth and four spontaneous abortions. The majority of pregnancies in this study demonstrated no evidence of hypertension; the number of hypertensive NF1 patients was also reportedly low in another study (Weissman et al., 1993), and no excessive hypertension was reported in the (Dugoff and Sujansky, 1996) study. No record was made of the patients' lesions, however, or whether they were affected by the pregnancies. Another study that attempted to relate hypertension to the number of neurofibromas found that development of hypertension and poor outcome in NF1 pregnancies is directly proportional to the number of lesions found in the patient (Sharma et al., 1991).

Prospective studies, such as those cited above, involved patients with relatively mild forms of the disorder whose pregnancies were obstetrically uneventful even if there are reported increases in size and number of their neurofibromas (Dugoff and Sujansky, 1996). In contrast, a large number of retrospective case studies by obstetricians and gynecologists tend to involve only a few women (Ansari and Nagamani, 1976) or even single patients, (Ribella, 1975) and report severe and therefore particularly notable effects.

Essentially all of these studies, both large and small, are descriptive, providing no significant insight to the pathogenesis of disease progression or mechanisms of complications such as the severe hypertension seen in some NF1 patients. None of these reports presents a functional study of the mechanism of new lesion growth or the enlargement of preexisting lesions during pregnancy. One study done on both male and female neurofibroma tumors found 75% expressed progesterone receptor (PR), while 5% expressed estrogen receptor (ER) (McLaughlin and Jacks, 2003). Both male and female tumor cells were tested, and, whether these tumors came from men or women was not significant with regard to PR and ER expression (McLaughlin, personal communication). A recent paper has been published that investigates the potential role of steroid hormone action on the growth, proliferation and tumorigenicity of cells that do or do not express the NF1 gene or its protein product, neurofibromin. It is the first published study of RNA levels of steroid hormone receptors in primary NF1 tumor cells, Schwann Cell (SC)-enriched NF1 tumor cells and normal SC, along with an analysis of proliferation and apoptosis. This study found a great deal of heterogeneity among the cell types, regardless of tumor origin or gender, suggesting the importance of each individual tumor's microenvironment (Fishbein et al., 2007).

THE HORMONAL MILIEU DURING PREGNANCY

Concentrations of the steroid hormones 17β-estradiol (E2), progesterone (P4) and testosterone (T) are upregulated during pregnancy (Witorsch, 2002; Fernandez-Valdivia et al., 2005; Okada et al., 2005; Rodriguez-Cuenca et al., 2006). Steroid hormones are synthesized from cholesterol, with P4 synthesized first, followed by T, which is a

precursor for E2, which is converted to E2 through the aromatase enzyme in the ovary (Gao et al., 2005) (figure 1.5) . E2 has been shown to be involved in cell proliferation, and is a ligand for the estrogen receptor (ER) (Revankar et al., 2005; Boonyaratanakornkit and Edwards, 2007). P4 is modified to E2 and is involved in both proliferation and differentiation (Fernandez-Valdivia et al., 2005); P4 is the ligand for the progesterone receptor (PR) (Fernandez-Valdivia et al., 2005; Boonyaratanakornkit and Edwards, 2007). The E2 regulates PR by transactivation through the ER (Fernandez-Valdivia et al., 2005; Okada et al., 2005). Steroids can, although rarely, cross-react and bind to receptors other than their native receptor, because of the similarity of their receptor conformation, even though the actual sequence identity may be low (Gao et al., 2005) (figure 1.6). Testosterone is the primary circulating androgen, even in women, and is the ligand for the androgen receptor (AR), although it can also cross-react with both the ER and PR, albeit with very low affinity (Gao et al., 2005; Boonyaratanakornkit and Edwards, 2007). Steroid receptors (ER, PR, and AR) belong to the nuclear receptor family of transcription factors, which are activated when their ligand (E2, P4 or T) binds to the cytoplasmic-envelope-anchored receptor and the complex subsequently enters the nucleus to bind to hormone response elements (HRE) in gene promoter regions, thereby activating transcription of downstream effector genes (Fernandez-Valdivia et al., 2005; Boonyaratanakornkit and Edwards, 2007; Gavrilova-Jordan and Price, 2007) (figure1.7). Breast cancer tumor cells which express both ER and PR have a better prognosis because they are more likely to respond to hormone antagonist treatment (Jacobsen et al., 2003), although tumors that express only ER or PR, but not both, have been reported to have a poorer outcome (Jacobsen et al., 2005).

Steroid hormone action is not mediated exclusively through classical pathways (McEwen and Alves, 1999; Ho and Liao, 2002; Zhang et al., 2004; Chen et al., 2005; Gao et al., 2005; Jacobsen et al., 2005; Revankar et al., 2005; Sonneveld et al., 2006; Gavrilova-Jordan and Price, 2007). There have been reports of steroid hormones (P4, E2, T) influencing cells independent of their classical receptors (PR, ER, AR) (McEwen and Alves, 1999; Ho and Liao, 2002; Zhang et al., 2004; Chen et al., 2005; Gao et al., 2005; Jacobsen et al., 2005; Revankar et al., 2005; Sonneveld et al., 2006; Gavrilova-Jordan and Price, 2007). Conversely, the receptors have also been shown to exert their influence without hormones (Chen et al., 2005; Jacobsen et al., 2005; Sonneveld et al., 2006). ER expression has been seen outside the nucleus (McEwen and Alves, 1999; Ho and Liao, 2002; Zhang et al., 2004; Revankar et al., 2005). E2 has also been shown to modulate secondary messengers, such as Ca++ and NO, and activate the PI3K/Akt and MAPK pathways (Ho and Liao, 2002; Boonyaratanakornkit and Edwards, 2007; Gavrilova-Jordan and Price, 2007). These effects are not inhibited by ER inhibitors, suggesting they are not mediated through the classical receptors (Ho and Liao, 2002) (figure 1.6).

We put forward the hypothesis that hormones, particularly those that rise during puberty and pregnancy, are responsible for the observed increase in both size and numbers of cutaneous and plexiform neurofibromas in both adolescent and pregnant patients with NF1. It has also been postulated that hormones are also involved in the progression of benign lesions (neurofibromas) to MPNST (Fishbein et al. 2007). A recent study describes RNA levels of steroid hormone receptors, along with the effects of the steroid hormones progesterone (P4), estrogen (E2), or testosterone (T) on primary and SC-enriched human benign and malignant NF tumor cell lines and normal SC (Fishbein

et al., 2007). Fishbein et al. found a great deal of heterogeneity in estrogen receptor, progesterone receptor, and androgen receptor expression in normal SC and most NF1 tumor samples tested, with no clear division based on tumor type or gender (Fishbein et al., 2007). The greatest variation in hormone receptor levels occurred between primary tumor cells versus SC-enriched tumor cell cultures, suggesting that other cell types other than the SC have steroid hormone receptors. There were also some statistically significant differences in proliferation and apoptosis in cells exposed to steroid hormones or their antagonists, but these differences were also heterogeneous. The data reported did not fit into a simplistic or global model to explain the functional role of hormonal pathways in the progression of NF1. The data did suggest that the surrounding cells unique to each tumor's microenvironment were important to tumorigenicity, and that classical steroid receptor effects may only apply to a subset of NF1 tumors, regardless of gender (Fishbein et al., 2007). There is also work in progress from our laboratory studying Schwann Cell-like (SC-like) differentiated mouse embryonic stem cells that have been engineered to lack neurofibromin (Roth et al., 2007a; Roth et al., 2007b).

The most potent hormone effector in pregnancy is estrogen in the form of 17βestradiol (E2). It promotes the proliferation of endometrial cells in the uterus, and is essential for maintaining pregnancy. It is initially produced by the corpus luteum and later by the placenta. Additional estradiol species are also produced, including 17αestradiol (also called 17αE2), which is a weak estrogen that induces some estrogen responsive genes, including vascular endothelial growth factor (VEGF), which promotes angiogenesis--new blood vessel formation. However, its action in rat pituitary tumor cells is independent of the estrogen receptors (ER), but acts through the PI3Kinase-AKT

signaling pathway. There is also evidence that this is the pathway through which the NF1 protein product, neurofibromin, acts (Klesse and Parada, 1998).

E2 also has proliferative effects on estrogen-responsive tumor cells. A report by Ahmad et al., (1999) demonstrated that in the MCF-7 breast carcinoma cell line, E2 upregulates AKT activity and increases cell proliferation. Both ICI 182, 780 (ICI), a pure antiestrogen and wortmannin (a PI3K inhibitor) block this effect (Ahmad et al., 1999). In addition, Wilson et al., (2002) have demonstrated that estradiol treatment reduced the number of neuronal cells undergoing apoptotic cell death. They examined the level of activation of Akt kinase that mediates the antiapoptotic signals. Explants treated with estradiol had elevated levels of pAkt and treatment with ICI prevented the effect of estradiol, suggesting that estradiol prevents injury-induced apoptosis in neurons and that Akt activation is probably responsible for this effect (Wilson et al., 2002). Although the evidence at this time is only circumstantial, there is a good possibility that the two pathways, the NF1-dependent pathway and the estrogen-dependent pathway, both interact through effects on PI3 Kinase.

Progesterone (P4) is also critical for maintaining pregnancy. P4 can be involved in cell differentiation and modulation of E2 proliferative effects. E2 and P4 can have complementary functions, with P4 inhibiting the ER (Jacobsen et al., 2003). P4 and E2 can be used in combination to stimulate tumor development by simulating pregnancy in mice with dormant mammary tumors (Gattelli et al., 2004). The combination of E2 and P4 caused the tumor cells to break dormancy and begin proliferating. The classical progesterone receptor (PR) inhibitor RU486 also has inhibitory effects on the glucocorticoid receptor (GR) (Ghoumari et al., 2003; Zhang et al., 2006).

There are two distinct forms of the estrogen receptor (ER), ERα and ERβ, which are transcribed from different genes (Boonyaratanakornkit and Edwards, 2007). Both subtypes bind estrogens as well as selective ER modifiers (SERMs) including the anti-cancer agent tamoxifen and a number of other anti-cancer ER inhibitors (Cummings, 2002; Boonyaratanakornkit and Edwards, 2007). ER receptors are classically located in the nucleus although they can also be located in the nuclear or mitochondrial membrane (Gavrilova-Jordan and Price, 2007). PRs and androgen receptors (AR), have also been found on mitochondrial membranes, and are very similar/identical to classical nuclear steroid receptors (Gavrilova-Jordan and Price, 2007). At high concentrations, E2 is also able to bind to the AR (Yeh et al., 1998).

Classical steroid receptors form dimers, interact with basal transcription factors and coactivators and bind to hormone response elements in DNA, leading to the modulation of target gene transcription. The distribution of the ER and PR subtypes varies among tissues and among cells in a specific tissue, and their expression also varies during development (Van Den Bemd et al., 1999; Boonyaratanakornkit and Edwards, 2007).

ICI 182,780 (ICI) at 10^{-7} M is a pure antiestrogen that leads to the downregulation and subsequent loss of the estrogen receptors themselves (Wakeling et al., 1991; Van Den Bemd et al., 1999; Cicatiello et al., 2000). It also has estrogen receptor antagonistic effects and inhibits estrogen binding to both the α and β forms of the human estrogen receptor (ER) (Robertson et al., 2001; Garnier et al., 2003). ICI has been shown to inhibit the growth of breast cancer cells and cell lines (Robertson et al., 2001). It has also been shown to be effective against tamoxifen-resistant breast cancer in post- menopausal

women (Robertson, 2001). ICI induces a conformational change in the ER and induces a ligand concentration-dependent increase in its proteolytic degradation. (Hyder and Stancel, 2002) also showed that ICI has antiprogestin effects in a human breast cancer cell line, blocking progesterone responsive genes such as vascular endothelial growth factor (VEGF), which is a potent angiogenic growth factor. ICI blocked progestin-induced VEGF mRNA and protein synthesis (Hyder and Stancel, 2002), although it did not block binding of progestin to the PR or down-regulate the endogenous PR. Estrogen does not upregulate VEGF in these breast cancer cells. Therefore ICI could block angiogenesis in the breast cancer cell line through a mechanism unrelated to its ER inhibition and the authors suggest that either ICI binds to a site in the PR different from its ligand binding site and/or ICI binds to a co-activator or co-repressor or to yet another protein involved in the transcriptional complex associated with responsive genes such as VEGF. ICI also effectively inhibits the ER in the hippocampus but not the frontal cortex of the brain (Mize et al., 2003). Studies in both rodents and primates have shown that progestins can counteract the antiatherogenic effects of estrogens. ICI inhibits these effects demonstrating that the antiatherogenic effects of E2 are mediated through ER and studies with ER knockout mice also demonstrate that ERs mediate these protective effects of estrogen on the vasculature (reviewed by Karas, 2002).

2-Methoxyestradiol (2ME2) is a natural estrogen metabolite of E2 that has no estrogenic effects itself but has both antiangiogenic and antitumorigenic effects (Fotsis et al., 1994; Lin et al., 2001; Schumacher and Neuhaus, 2001; Carothers et al., 2002), including effects on osteosarcoma (Maran et al., 2002) and Ewing sarcoma (Djavaheri-Mergny et al., 2003). Both microvasculature and large vessel endothelial cell

proliferation are also blocked by 2ME2 (Fotsis et al., 1994). Proliferation of many types of normal cells, including thyroid cells (Wang et al., 2000) and chondrocytes in the growth plate (Sibonga et al., 2002) are inhibited by 2ME2. However, 2ME2-induced apoptosis is independent of the ER α and β (LaVallee et al., 2002). 2ME2 anti-angiogenic activity occurs through impaired hypoxia inducible factor-1 (HIF-1) accumulation, which results in inhibited vascular endothelial growth factor (VEGF) expression (Mabjeesh et al., 2003). Analogues of 2ME2 have been made and tested for their enhanced inhibitory abilities and one analogue, 14-Dehydro-2-ME-2, was 16-fold more potent than 2-ME2 against various tumor cell lines, including prostate cancer cell lines (Tinley et al., 2003). An important study of 2ME2 published in 2001 by Brueggemeier et al. (2001) demonstrated that 2ME2, which has only weak affinity for the ER, not only has antiproliferative activity but also alters tubulin dynamics. A variant of this metabolite, 2-methoxymethylestradiol, was more effective than the native metabolite in producing cytotoxicity and altering tubulin dynamics in intact cells (Brueggemeier et al., 2001).

During pregnancy, 2ME2 may put a growth check on estrogen-stimulated cell growth. We hypothesize that (1) levels of both estrogens and 2ME2 may be different in women with NF1 from those in women without NF1 and (2) 2ME2 levels might be lower in NF1 patients. If 2ME2 levels are lower in individuals with NF1, 2ME2 may be unable to compensate for estrogen levels that promote the growth of neurofibromas and might also be involved in the conversion of benign to malignant tumors that has also been reported in some pregnant NF1 patients (Posma et al., 2003). However, there are no studies in the literature examining the levels of hormones or hormone metabolites in pregnant women with NF1. Almost all of the studies published to date about women

with NF1 are descriptive. There are no studies of the mechanism by which any of these hormones or metabolites act on cells that do or do not make neurofibromin. Studies on the effects of 2ME2 derivatives on NF1 tumor cell growth can be found in the appendix at the end of this dissertation.

NEUROGENERATIVE AND NEUROPROTECTIVE EFFECTS OF STEROID HORMONES

In a recent study (MacLusky et al., 2003), the neuroprotective effects of estradiol and progesterone were examined in a model of neuronal apoptosis induced by growth factor insufficiency. Estradiol and progesterone were able to maintain neuronal phenotypes in PC12 cells induced to become neuron-like by nerve growth factor (NGF), but not in naïve PC12 cells that were not NGF treated. The E2 protective effect could be inhibited by ICI, indicating that the effect was probably mediated through the ER. It was also dependent on RNA and protein synthesis (MacLusky et al., 2003). It is thought that ERα is particularly important in mediating this effect. (Ivanova et al., 2002) found that estrogens stimulated the differentiation of neuronal and neural networks in the central nervous system. E2 was found to increase the expression of glial cell-derived neurotrophic factor (GDNF) in developing hypothalamic cells in culture. An additional report (Brannvall et al., 2003) demonstrated that both embryonic and adult rat neural stem cells (NSC) have both ERs and that E2 treatment decreased the proliferation of NSC stimulated by epidermal growth factor (EGF). This was EGF receptor (EGFR) dependent, necessitating the examination of the mouse embryonic stem cells used in these studies for all three receptors (EGFR, and ER α and β). This report further demonstrated that E2 increased the ratio of neurons to glial cells in embryonic NSC but

not in the adult NSC, suggesting that E2 influences neurogenesis only during embryonic development. However, neuronal regeneration in areas of the brain known to regenerate neurons (e.g. olfactory bulb, hypothalamus) was not examined. Estrogen (E2) upregulates the retinoic acid receptor a (RARa) in human breast cancer cells at both the RNA and protein levels. Two regions of the promoter sequence appear to be sensitive to the estrogen stimulation; however, ER DNA binding ability is not required for the estrogen-induced increases in this transcriptional activity (Elgort et al., 1996). Nerve growth factor levels were measured in the serum of NF1 patients before pregnancy, during the course of pregnancy and post-pregnancy. There were no significant differences among the levels measured at any stage or between NF1 patients and control subjects who do not have NF1 (Elgort et al. 1996).

POSSIBLE ROLE OF STEROIDS IN HYPERTENSION AND VASCULAR STRUCTURE ALTERATIONS SEEN IN PREGNANT PATIENTS WITH NF1

Vascular endothelial growth factor (VEGF) is a major inducer of tumor angiogenesis (Ruohola et al., 1999). It is expressed in endothelial cells, and its expression in cancer cells, such as breast cancer cells indicates a poor prognosis for tumor progression. Neurofibromas tend to be highly vascular (Thomas and De Vries, 2007). 17βestradiol (E2) upregulates VEGF mRNA in many cancer cells (Ruohola et al., 1999), leading to subsequent angiogenesis. This E2-mediated angiogenesis could also be responsible for the growth of neurofibromas, which need vascularization to survive beyond microscopic sizes. This increased growth could also result in the appearance of new tumors during pregnancy, if previously microscopic tumors grow to detectable sizes.

ICI 182, 780 (ICI) inhibits the E2-mediated upregulation, indicating that it probably acts by an ER-dependent mechanism (Ruohola et al., 1999). In contrast, tamoxifen also upregulates VEGF mRNA on its own and has no inhibitory effect like that of ICI (Ruohola et al., 1999).

E2 inhibits vascular smooth muscle cell proliferation whereas ICI causes proliferation of these cells (Lavigne et al., 1999). Teoh et al. (1999) reported that short-term exposure to pharmacological levels of 17α or 17βestradiol (E2) produced relaxation in pig coronary artery in organ culture. However, physiological concentrations of estradiol also enhanced the endothelium-independent relaxation of pig coronary artery produced by other agents. This relaxation was not blocked either by tamoxifen or by ICI, suggesting that the effect is mediated through a different ER and that 17βestradiol may be responsible for acute affects on blood flow by a different mechanism than that involved in ER downregulation. Additional effects on vasculature include effects on the integrity of the endothelial cell lining of blood vessels that is affected by E2 (Cho et al., 1998). Tight junctions between the endothelial cells are critically important for maintaining the integrity of the blood vasculature. Estradiol increases the cation specificity of the tight junctions. In addition, E2 also acts as an antioxidant at the genomic level. By improving the NO/O_2^- balance; E2 keeps the expression of proatherosclerotic gene products low and regulated in endothelial cells (Wagner et al., 2001).

Extracellular Ca^{++} is essential for maintaining occlusion of the paracellular space in endothelial cells by tight junctions (Cho et al., 1998). If extracellular calcium is lowered, cells separate weakening blood vessel walls and leading to rupture (Cho et al., 1998). Estrogen modulates tight junctional resistance across the endothelial cells (Teoh et

al., 1999). Estrogen increases the tight junctional permeability to ions (Teoh et al., 1999). A nuclear receptor, possibly other than ER, mediates the effect of estrogen on cation selectivity (Revankar et al., 2005).

POSTULATED INTERACTIONS OF NF1, ESTROGENS AND APOPTOSIS PATHWAYS

We hypothesize that hormones influence NF1-mediated apoptotic events through the phosphatidylinositol 3 Kinase (PI3K) pathway, which has been implicated in NF1 signal transduction (Yao and Cooper, 1995; Klippel et al., 1996; Rodriguez-Viciana et al., 1996; Kulik et al., 1997; Parrizas and LeRoith, 1997; Stokoe et al., 1997; Crowder and Freeman, 1998; Klesse and Parada, 1998; Klesse et al., 1999). A report also suggests that the PI3K cascade mediates the neuroprotective effects of estrogen on cortical neurons (Honda et al., 2000).

Over the past decade, the scientific community has gained significant insight into the genetic mechanisms underlying NFI. However, to date the hormonal pathways modulating NF1 phenotypes have not been well elucidated. Over the years, patients with NF1 have reported a dramatic change in tumor number and size during puberty as well as pregnancy, suggesting hormonal regulation of tumor growth (Ansari and Nagamani, 1976; Blickstein and Lancet, 1987; Dugoff and Sujansky, 1996; Isikoglu et al., 2002). While this possibility has always been viewed as being quite plausible given our recognition of hormonal responsiveness in many tumor types, little scientific evidence has supported this clinical observation. Recently, new insights into the hormonal regulation of neurofibromas' growth have been reported as summarized above. We also

hypothesize that the effect of hormones on neurofibromas may be indirect through their effects on angiogenesis. Increased blood vessel formation during pregnancy could allow existing tumors to grow, as well as increase microscopic tumors to grow to detectable sizes. Further studies into the molecular mechanisms by which hormonal stimulation can regulate the growth of neurofibromas or blood supply is necessary to provide a more accurate understanding of the natural history of the disease, as well as for the development of innovative tools for the management of patients who have NF1. Recognition by clinicians of the hormonal impact of NF1 is essential to provide care of patients. Ideally, further knowledge about the pathways and modifying facts of NF1 will be instrumental in improving the prognostic and therapeutic management of the disease as well.

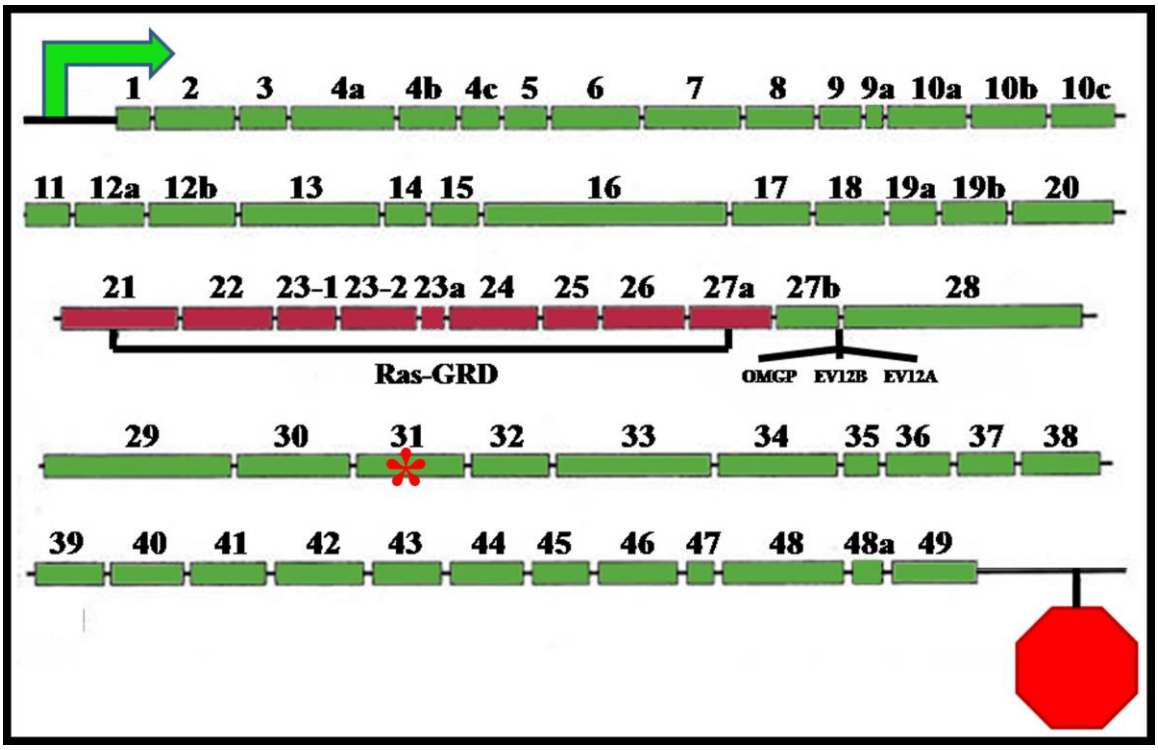


Figure 1.1: NF1 structure. NF1 is a very large gene, spanning 350 kb and comprised of 60 exons. The best characterized area of the gene is the Ras-GAP related domain (Ras-GRD) encoded by exons 21-27a. This area is responsible for the tumor suppressor function of NF1. There are also 3 genes with unknown functions encoded in the reverse direction between exons 27b and 28. This dissertation uses mouse embryonic stem cells that are wild type, heterozygous or null for the NF1 gene through homologous recombination that replaced exon 31 (red asterisk) with a neo cassette. Neurofibromin is the name of the 250 kDa protein encoded by the NF1 gene (Based on Friedman, et al., 1999).

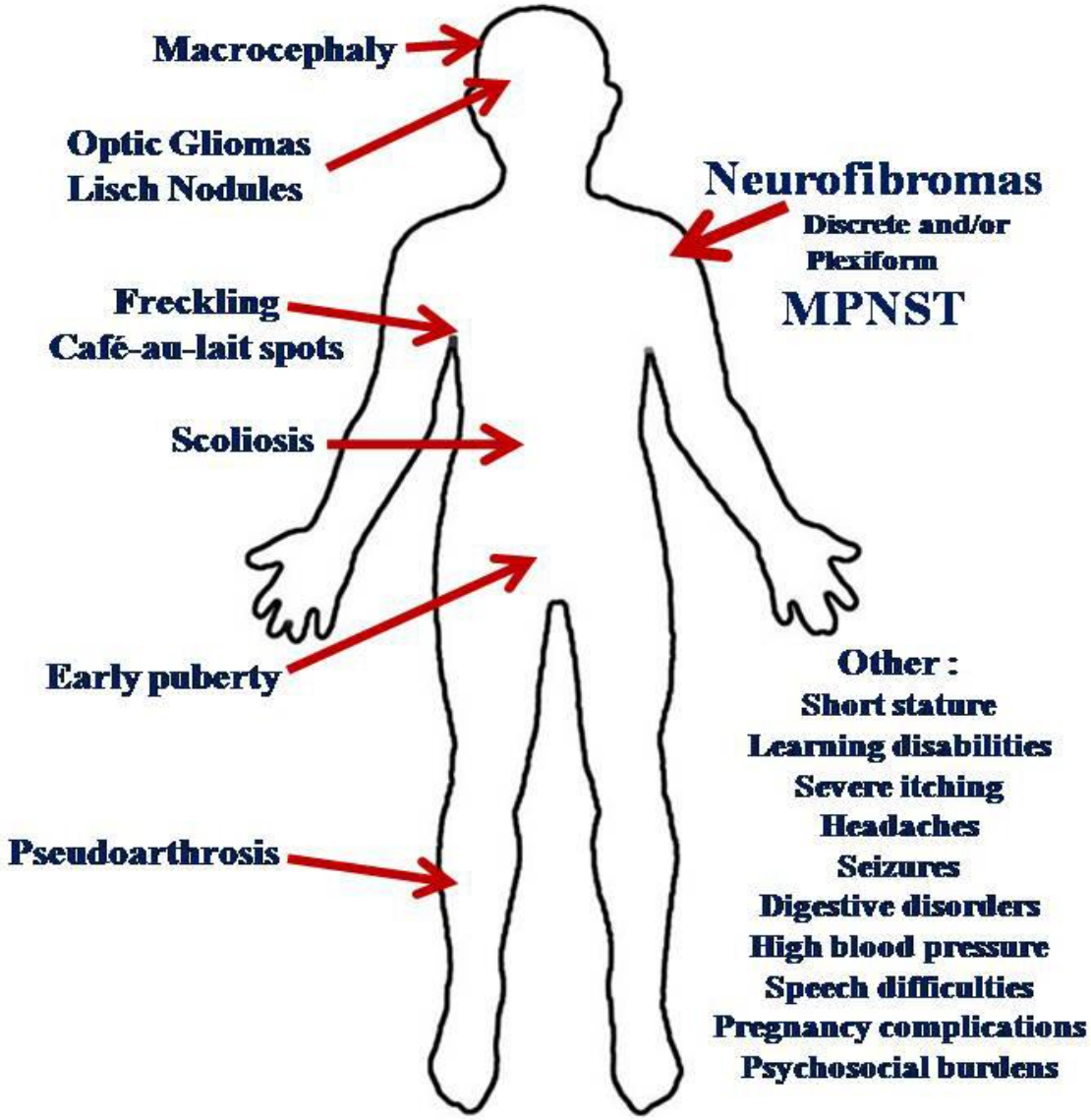


Figure 1.2: NF1 manifestations. The disease Neurofibromatosis Type 1 (NF1) has very pleiotropic phenotypes, ranging from café au lait spots, bone abnormalities, Lisch nodules, optic gliomas, early puberty, and learning disabilities as well as the hallmark tumors called neurofibromas. Neurofibromas are usually benign, and can be discrete or plexiform (along the length of the nerve). Approximately 5% of plexiform tumors progress to malignancy, becoming malignant peripheral nerve sheath tumors (MPNST). This dissertation focuses on the role of NF1 in Schwann Cell development and tumor formation.

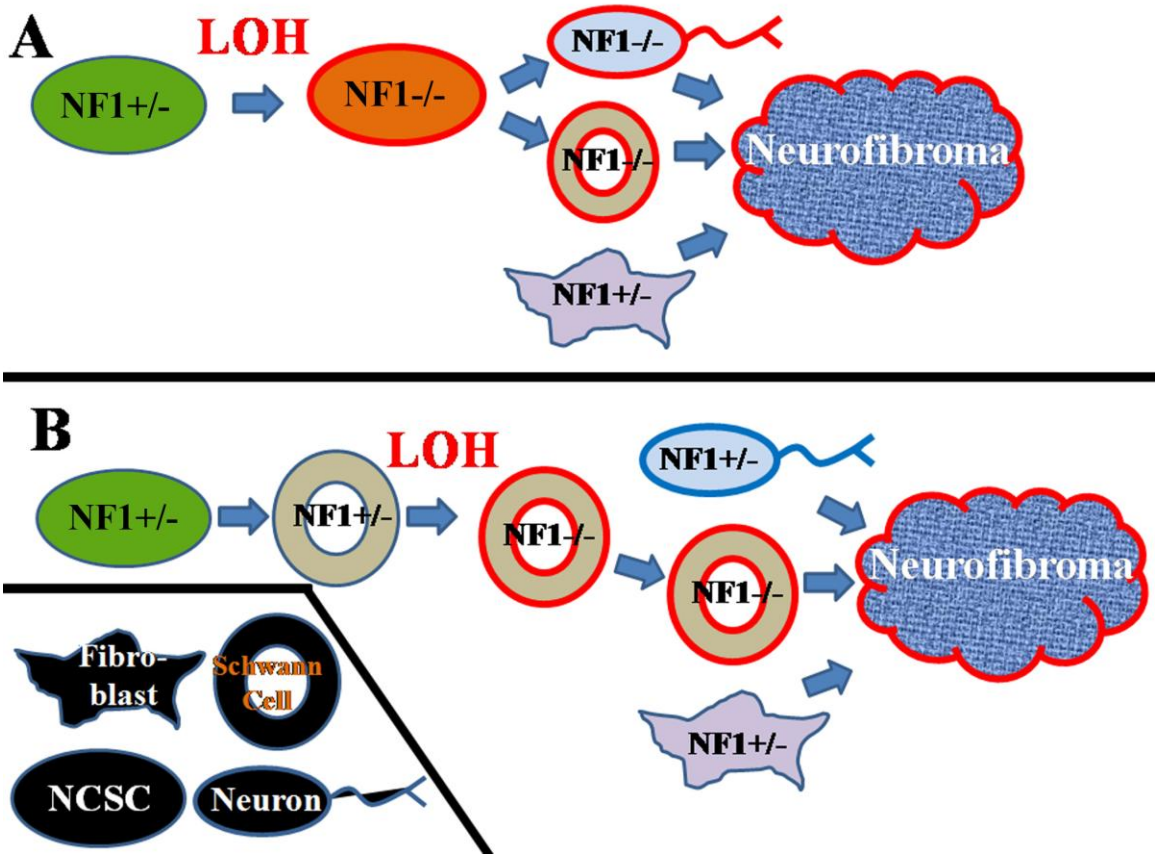


Figure 1.3: NF1 and loss of heterozygosity (LOH) in neurofibromas. Schwann Cells (SC) have been found to be the initiating cell types in neurofibromas, but these tumors are a heterogeneous mixture of NF1+/- and NF1-/- cells, including SC, neurons, neural crest stem cells (NCSC), and fibroblasts, with their accompanying signals. LOH can occur at different points along the SC differentiation pathway. **A.** Model for LOH in NCSC. Both neurons and SC, downstream of NCSC differentiation, are NF1-/-, while other cell types in tumors remain NF1+/-. **B.** Model for LOH in SC. Only SC are NF1-/-, while other cell types in the neurofibromas remain NF1+/-. (Based on Zhu, et al., 2002). Not all SC in tumors have LOH, however, suggesting that LOH may not be the trigger for tumor formation. Mutations in other genes (for e.g. p53 or retinoblastoma), injury or hormonal stimulation may be necessary factors.

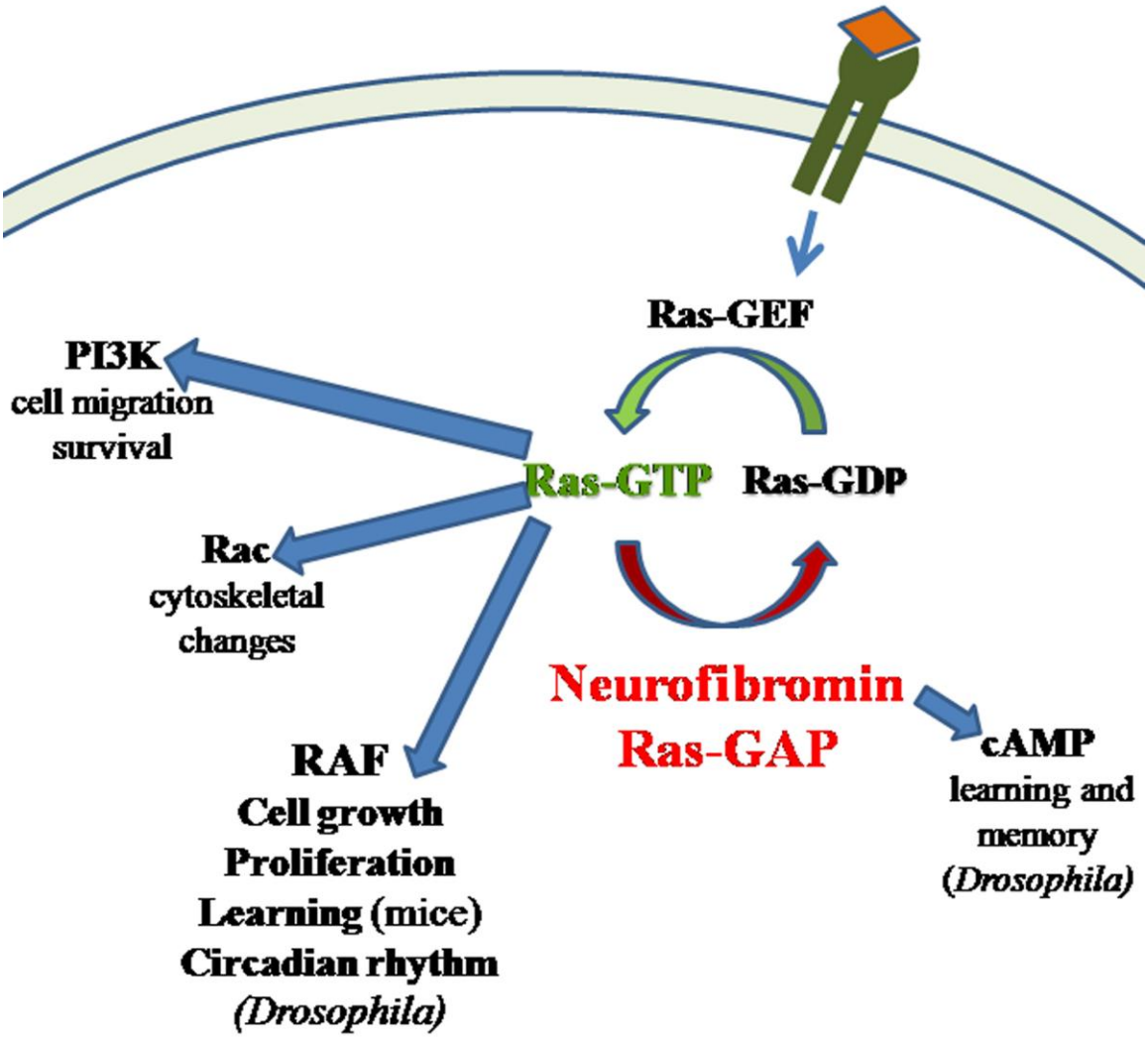
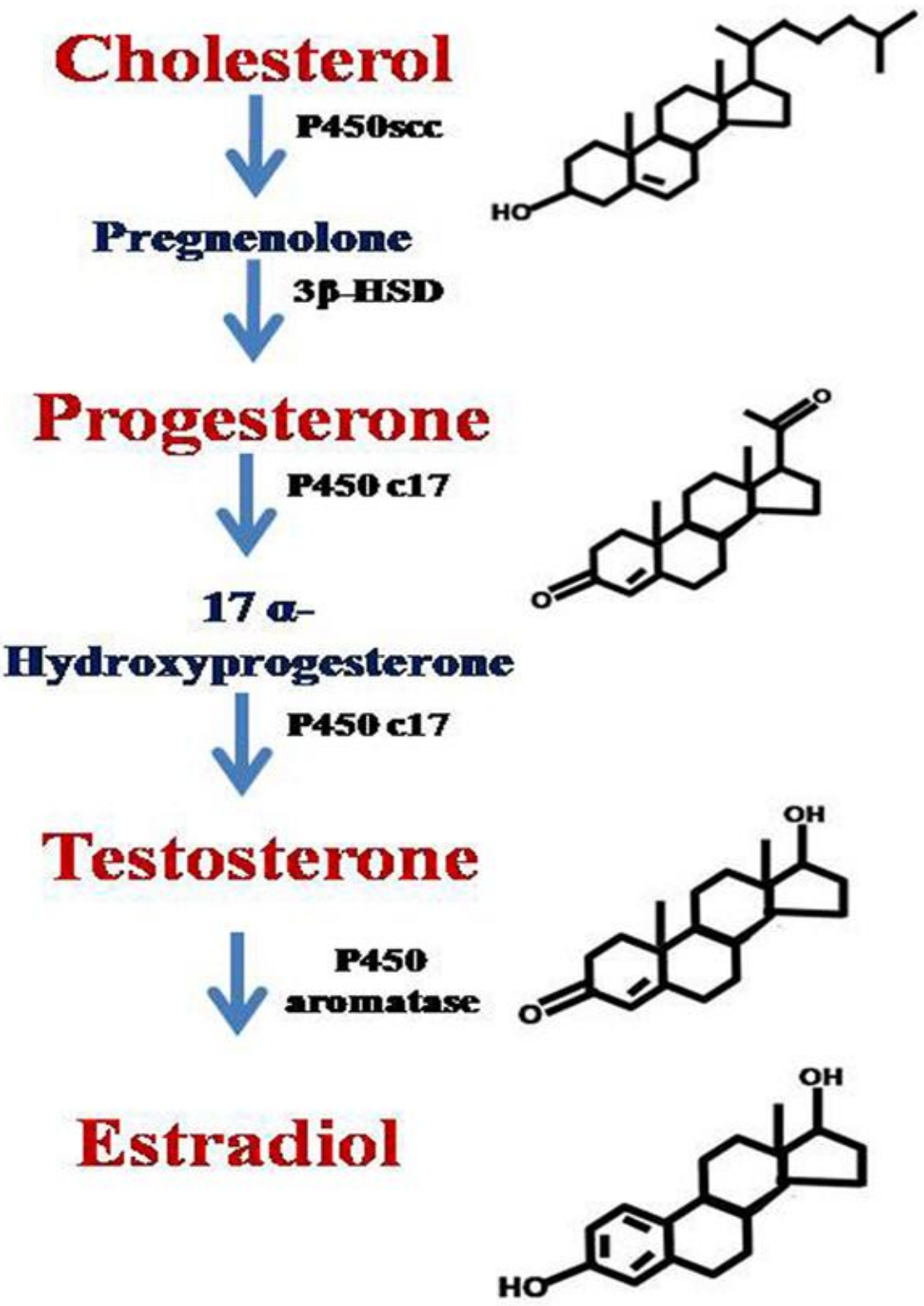


Figure 1.4: NF1 pathways. While neurofibromin has been shown to be part of the cAMP pathway in *Drosophila*, its most well-characterized function is as a Ras-GAP tumor suppressor. Following ligand/receptor binding and the resulting signal, neurofibromin accelerates the hydrolyzation of active Ras-GTP to inactive Ras-GDP, thus turning off multiple downstream pathways, such as PI3K, Rac and RAF. When the NF1 gene is missing or mutated, neurofibromin is not available to shut down these pathways, leaving them constitutively active. This can result in uncontrolled cell growth and formation of peripheral nerve tumors called neurofibromas.

Figure 1.5: Steroid hormone synthesis. The steroid hormones progesterone, testosterone, and 17β-estradiol are upregulated during pregnancy. All of these steroid hormones are originally synthesized from cholesterol and converted to the respective hormones through enzymatic action. Steroid hormones are ligands for classical steroid receptors that activate downstream genes, promoting proliferation and differentiation. Progesterone is the ligand for the progesterone receptor, estrogen for the estrogen receptor, and testosterone for the androgen receptor, although they can also rarely cross-react with low affinity. There are also other, non-classical steroid hormone pathways that allow for quick response to hormonal stimulation through secondary messengers and small G-proteins.


Cholesterol
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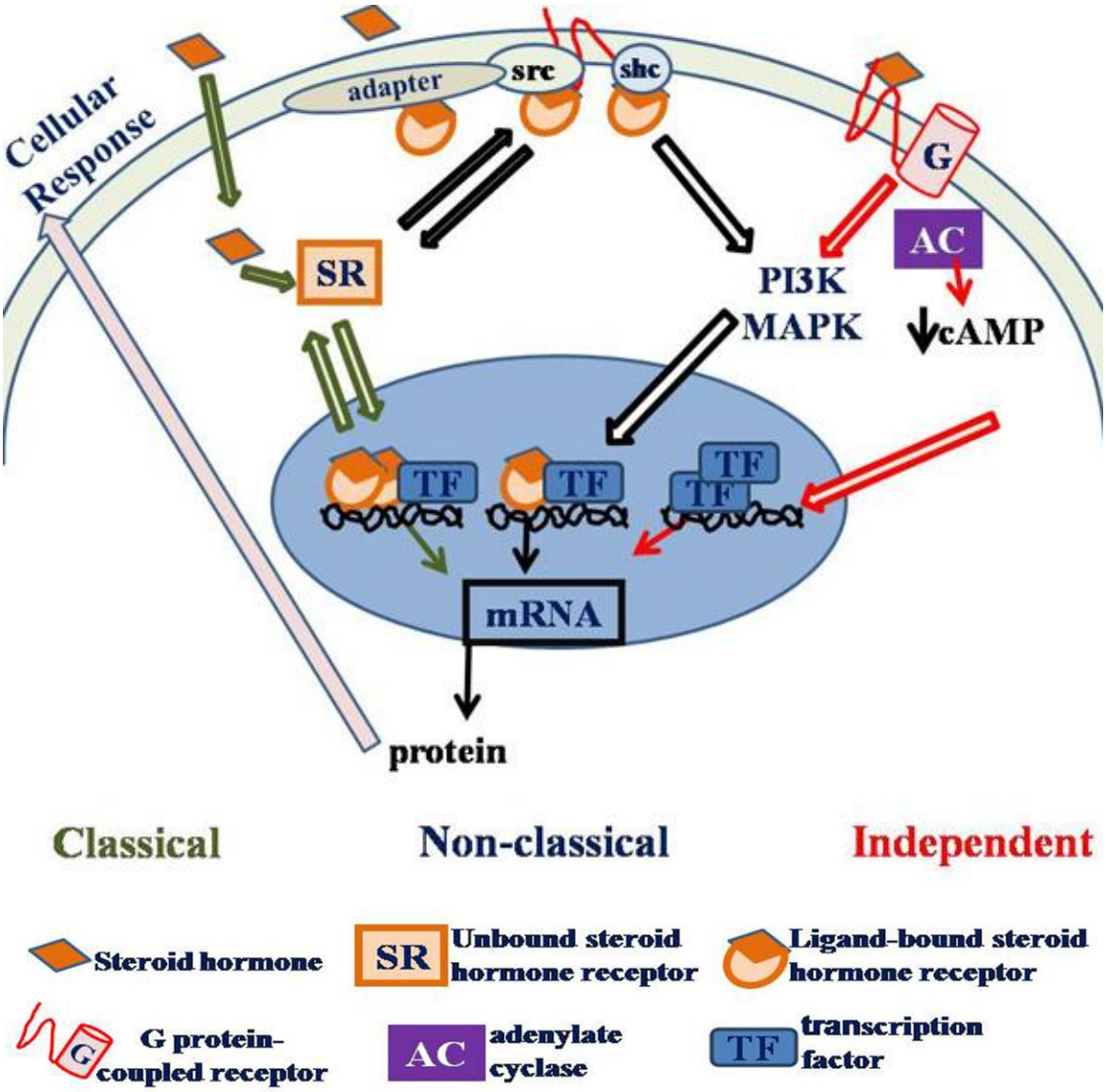


Figure 1.6: Steroid hormone pathways. In the classical pathway (left side), steroid hormones bind to classical receptors in the cell, where they induce conformational changes. The steroid/receptor complex then translocates to the nucleus where it dimerizes, binds to genes via transcription factors and hormone response elements and activate transcription, leading to protein production and cellular responses. In the non-classical pathway (middle), membrane-bound or cytoplasmic steroid receptors associate with signaling molecules, leading to activation of PI3K or MAPK signaling cascades. In the steroid receptor-independent pathway (right side), novel G protein-coupled receptors that are unrelated to classical nuclear steroid receptors, mediate rapid, non-genomic signaling cascades through adenylate cyclase inhibition, cAMP production and MAPK activation. Other transcription factors are also involved in these pathways. (Based on Boonyaratanakornkit and Edwards, 2007).

REFERENCES

Ahmad S, Singh N, Glazer RI. 1999. Role of AKT1 in 17beta-estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. Biochem Pharmacol 58:425-430.

Ansari AH, Nagamani M. 1976. Pregnancy and neurofibromatosis (von Recklinghausen's disease). Obstet Gynecol 47:25S-29S.

Baker VV, Hatch KD, Shingleton HM. 1989. Neurofibrosarcoma complicating pregnancy. Gynecol Oncol 34:237-239.

Belton SR, Ferguson JE, 2nd, Catanzarite VA. 1984. Neurofibromatosis and pregnancy: report of a case complicated by intrauterine growth retardation and oligohydramnios. Am J Obstet Gynecol 149:468-469.

Bertrand C, Ville Y, Fernandez H. 1992. [Hereditary hypertension in Recklinghausen's disease during pregnancy. A family]. Presse Med 21:2142-2144.

Blickstein I, Lancet M. 1987. [Neurofibromatosis and pregnancy]. Harefuah 112:392-393.

Blickstein I, Lancet M, Shoham Z. 1988. The obstetric perspective of neurofibromatosis. Am J Obstet Gynecol 158:385-388.

Blum K, Kambich MP. 1997. Maternal genetic disease and pregnancy. Clin Perinatol 24:451-465.

Boiten J, Jansen EN, de Graaff R. 1987. Von Recklinghausen neurofibromatosis (VRNF) and pregnancy. A single case study. Clin Neurol Neurosurg 89:181-184.

Boonyaratanakornkit V, Edwards DP. 2007. Receptor mechanisms mediating non-genomic actions of sex steroids. Semin Reprod Med 25:139-153.

Brannvall K, Korhonen L, Lindholm D. 2003. Estrogen-receptor dependent regulation of neural stem cell proliferation and differentiation. Mol Cell Neurosci:512-520.

Brickner S. 1906. Fibroma molluscum gravidarum: A new clinical entity. Am J Obstet Gynecol 53.

Brueggemeier RW, Bhat AS, Lovely CJ, Coughenour HD, Joomprabutra S, Weitzel DH, Vandre DD, Yusuf F, Burak WE, Jr. 2001. 2-Methoxymethylestradiol: a new 2-methoxy estrogen analog that exhibits antiproliferative activity and alters tubulin dynamics. J Steroid Biochem Mol Biol 78:145-156.

Carothers AM, Hughes SA, Ortega D, Bertagnolli MM. 2002. 2-Methoxyestradiol induces p53-associated apoptosis of colorectal cancer cells. Cancer Lett 187:77-86.

Chen Y, Zajac JD, MacLean HE. 2005. Androgen regulation of satellite cell function. J Endocrinol 186:21-31.

Cho MM, Ziats NP, Abdul-Karim FW, Pal D, Goldfarb J, Utian WH, Gorodeski GI. 1998. Effects of estrogen on tight junctional resistance in cultured human umbilical vein endothelial cells. J Soc Gynecol Investig 5:260-270.

Cicatiello L, Addeo R, Altucci L, Belsito Petrizzi V, Boccia V, Cancemi M, Germano D, Pacilio C, Salzano S, Bresciani F, Weisz A. 2000. The antiestrogen ICI 182,780 inhibits proliferation of human breast cancer cells by interfering with multiple, sequential estrogen-regulated processes required for cell cycle completion. Mol Cell Endocrinol 165:199-209.

Cichowski K, Jacks T. 2001. NF1 tumor suppressor gene function: narrowing the GAP. Cell 104:593-604.

Criado E, Izquierdo L, Lujan S, Puras E, del Mar Espino M. 2002. Abdominal aortic coarctation, renovascular, hypertension, and neurofibromatosis. Ann Vasc Surg 16:363-367.

Crowder RJ, Freeman RS. 1998. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 18:2933-2943.

Cummings FJ. 2002. Evolving uses of hormonal agents for breast cancer therapy. Clin Ther 24 Suppl C:C3-25.

Dasgupta B, Yi Y, Chen DY, Weber JD, Gutmann DH. 2005. Proteomic analysis reveals hyperactivation of the mammalian target of rapamycin pathway in neurofibromatosis 1-associated human and mouse brain tumors. Cancer Res 65:2755-2760.

Djavaheri-Mergny M, Wietzerbin J, Besancon F. 2003. 2-Methoxyestradiol induces apoptosis in Ewing sarcoma cells through mitochondrial hydrogen peroxide production. Oncogene 22:2558-2567.

Dugoff L, Sujansky E. 1996. Neurofibromatosis Type 1 and pregnancy. American Journal of Medical Genetics (Semin. Med. Genet.) 66:7-10.

Edwards JN, Fooks M, Davey DA. 1983. Neurofibromatosis and severe hypertension in pregnancy. Br J Obstet Gynaecol 90:528-531.

Elgort MG, Zou A, Marschke KB, Allegretto EA. 1996. Estrogen and estrogen receptor antagonists stimulate transcription from the human retinoic acid receptor-alpha 1 promoter via a novel sequence. Mol Endocrinol 10:477-487.

Feldkamp MM, Angelov L, Guha A. 1999. Neurofibromatosis type 1 peripheral nerve tumors: aberrant activation of the Ras pathway. Surg Neurol 51:211-218.

Feldkamp MM, Gutmann DH, Guha A. 1998. Neurofibromatosis type 1: piecing the puzzle together. Can J Neurol Sci 25:181-191.

Fernandez-Valdivia R, Mukherjee A, Mulac-Jericevic B, Conneely OM, DeMayo FJ, Amato P, Lydon JP. 2005. Revealing progesterone's role in uterine and mammary gland biology: insights from the mouse. Semin Reprod Med 23:22-37.

Fishbein L, Eady B, Sanek N, Muir D, Wallace MR. 2005. Analysis of somatic NF1 promoter methylation in plexiform neurofibromas and Schwann cells. Cancer Genet Cytogenet 157:181-186.

Fishbein L, Zhang X, Fisher LB, Li H, Campbell-Thompson M, Yachnis A, Rubenstein A, Muir D, Wallace MR. 2007. In vitro studies of steroid hormones in neurofibromatosis 1 tumors and schwann cells. Mol Carcinog 46:512-523.

Fotsis T, Zhang Y, Pepper MS, Adlercreutz H, Montesano R, Nawroth PP, Schweigerer L. 1994. The endogenous oestrogen metabolite 2-methoxyoestradiol inhibits angiogenesis and suppresses tumour growth. Nature 368:237-239.

Friedman JM, Gutmann DH, MacCollin M, Riccardi VM. 1999. Neurofibromatosis: Phenotype, Natural History, and Pathogenesis. Johns Hopkins University Press. 381 p.

Gao W, Bohl CE, Dalton JT. 2005. Chemistry and structural biology of androgen receptor. Chem Rev 105:3352-3370.

Garnier M, Giamarchi C, Delrieu I, Rio MC, Chinestra P, Bayard F, Poirot M, Faye JC. 2003. Insulin and estrogen receptor ligand influence the FGF-2 activities in MCF-7 breast cancer cells. Biochem Pharmacol 65:629-636.

Gattelli A, Cirio MC, Quaglino A, Schere-Levy C, Martinez N, Binaghi M, Meiss RP, Castilla LH, Kordon EC. 2004. Progression of pregnancy-dependent mouse

mammary tumors after long dormancy periods. Involvement of Wnt pathway activation. Cancer Res 64:5193-5199.

Gavrilova-Jordan LP, Price TM. 2007. Actions of steroids in mitochondria. Semin Reprod Med 25:154-164.

Ghoumari AM, Dusart I, El-Etr M, Tronche F, Sotelo C, Schumacher M, Baulieu EE. Mifepristone (RU486) protects Purkinje cells from cell death in organotypic slice cultures of postnatal rat and mouse cerebellum. PROC Natl Acad Sci USA 7953-7958.

Ginsburg D, Hernendez E, Johnson J. 1981. Sarcoma complicating von Recklinghausen disease in pregnancy. Obstetrics & Gynecology 58:385-387.

Graf A, Landolt MA, Mori AC, Boltshauser E. 2006. Quality of life and psychological adjustment in children and adolescents with neurofibromatosis type 1. J Pediatr 149:348-353.

Gregory PE, Gutmann DH, Mitchell A, Park S, Boguski M, Jacks T, Wood DL, Jove R, Collins FS. 1993. Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. Somat Cell Mol Genet 19:265-274.

Gutmann DH, Cole JL, Collins FS. 1994. Modulation of neurofibromatosis type 1 gene expression during in vitro myoblast differentiation. J Neurosci Res 37:398-405.

Hadi HA. 1995. Clinical significance of neurofibromatosis in pregnancy. Am J Perinatol 12:459-461.

Hagymasy L, Toth M, Szucs N, Rigo J, Jr. 1998. Neurofibromatosis type 1 with pregnancy-associated renovascular hypertension and the syndrome of hemolysis, elevated liver enzymes, and low platelets. Am J Obstet Gynecol 179:272-274.

Heffner RR, Jr. 1969. Hydromyelia in Von Recklinghausen's disease with neurofibrosarcoma. Conn Med 33:311-313.

Heidenreich KA. 2003. Molecular mechanisms of neuronal cell death. Ann N Y Acad Sci 991:237-250.

Ho KJ, Liao JK. 2002. Nonnuclear actions of estrogen. Arterioscler Thromb Vasc Biol 22:1952-1961.

Honda K, Sawada H, Kihara T, Urushitani M, Nakamizo T, Akaike A, Shimohama S. 2000. Phosphatidylinositol 3-kinase mediates neuroprotection by estrogen in cultured cortical neurons. J Neurosci Res 60:321-327.

Horyn G, Bourgeois-Dujols P, Palaric JC, Giraud JR. 1988. Recklinghausen's disease and vascular complications during pregnancy. J Gynecol Obstet Biol Reprod (Paris) 5:641-645.

Humble RM. 1967. Phaeochromocytoma, neurofibromatosis and pregnancy. Anaesthesia 22:296-303.

Hyder SM, Stancel GM. 2002. Inhibition of progesterone-induced VEGF production in human breast cancer cells by the pure antiestrogen ICI 182,780. Cancer Lett 181:47-53.

Ingram DA, Hiatt K, King AJ, Fisher L, Shivakumar R, Derstine C, Wenning MJ, Diaz B, Travers JB, Hood A, Marshall M, Williams DA, Clapp DW. 2001. Hyperactivation of p21(ras) and the hematopoietic-specific Rho GTPase, Rac2, cooperate to alter the proliferation of neurofibromin-deficient mast cells in vivo and in vitro. J Exp Med 194:57-69.

Isikoglu M, Has R, Korkmaz D, Bebek N. 2002. Plexiform neurofibroma during and after pregnancy. Arch Gynecol Obstet 267:41-42.

Ivanova T, Karolczak M, Beyer C. 2002. Estradiol stimulates GDNF expression in developing hypothalamic neurons. Endocrinology 143:3175-3178.

Jacobsen BM, Richer JK, Sartorius CA, Horwitz KB. 2003. Expression profiling of human breast cancers and gene regulation by progesterone receptors. J Mammary Gland Biol Neoplasia 8:257-268.

Jacobsen BM, Schittone SA, Richer JK, Horwitz KB. 2005. Progesterone-independent effects of human progesterone receptors (PRs) in estrogen receptor-positive breast cancer: PR isoform-specific gene regulation and tumor biology. Mol Endocrinol 19:574-587.

Jarvis GJ, Crompton AC. 1978. Neurofibromatosis and pregnancy. Br J Obstet Gynaecol 85:844-846.

Johannessen CM, Reczek EE, James MF, Brems H, Legius E, Cichowski K. 2005. The NF1 tumor suppressor critically regulates TSC2 and mTOR. Proc Natl Acad Sci U S A 102:8573-8578.

Karas RH. 2002. Animal models of the cardiovascular effects of exogenous hormones. Am J Cardiol 90:22F-25F.

Karvonen SL, Kallioinen M, Yla-Outinen H, Poyhonen M, Oikarinen A, Peltonen J. 2000. Occult neurofibroma and increased S100 protein in the skin of patients with neurofibromatosis type 1: new insight to the etiopathomechanism of neurofibromas. Arch Dermatol 136:1207-1209.

Klesse LJ, Meyers KA, Marshall CJ, Parada LF. 1999. Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. Oncogene 18:2055-2068.

Klesse LJ, Parada LF. 1998. p21 ras and phosphatidylinositol-3 kinase are required for survival of wild-type and NF1 mutant sensory neurons. J Neurosci 18:10420-10428.

Klippel A, Reinhard C, Kavanaugh WM, Apell G, Escobedo MA, Williams LT. 1996. Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. Mol Cell Biol 16:4117-4127.

Kulik G, Klippel A, Weber MJ. 1997. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol 17:1595-1606.

Lakkis MM, Epstein JA. 1998. Neurofibromin modulation of ras activity is required for normal endocardial-mesenchymal transformation in the developing heart. Development 125:4359-4367.

LaVallee TM, Zhan XH, Herbstritt CJ, Kough EC, Green SJ, Pribluda VS. 2002. 2-Methoxyestradiol inhibits proliferation and induces apoptosis independently of estrogen receptors alpha and beta. Cancer Res 62:3691-3697.

Lavigne MC, Ramwell PW, Clarke R. 1999. Inhibition of estrogen receptor function promotes porcine coronary artery smooth muscle cell proliferation. Steroids 64:472-480.

Li Y, White R. 1996. Suppression of a human colon cancer cell line by introduction of an exogenous NF1 gene. Cancer Res 56:2872-2876.

Lin HL, Liu TY, Wu CW, Chi CW. 2001. 2-Methoxyestradiol-induced caspase-3 activation and apoptosis occurs through G(2)/M arrest dependent and independent pathways in gastric carcinoma cells. Cancer 92:500-509.

Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW, Giannakakou P. 2003. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 3:363-375.

MacLusky NJ, Chalmers-Redman R, Kay G, Ju W, Nethrapalli IS, Tatton WG. 2003. Ovarian steroids reduce apoptosis induced by trophic insufficiency in nerve growth factor-differentiated PC12 cells and axotomized rat facial motoneurons. Neuroscience 118:741-754.

Maran A, Zhang M, Kennedy AM, Sibonga JD, Rickard DJ, Spelsberg TC, Turner RT. 2002. 2-methoxyestradiol induces interferon gene expression and apoptosis in osteosarcoma cells. Bone 30:393-398.

McEwen BS, Alves SE. 1999. Estrogen actions in the central nervous system. Endocr Rev 20:279-307.

McLaughlin ME, Jacks T. 2003. Progesterone receptor expression in neurofibromas. Cancer Res 63:752-755.

Mize AL, Young LJ, Alper RH. 2003. Uncoupling of 5-HT1A receptors in the brain by estrogens: regional variations in antagonism by ICI 182,780. Neuropharmacology 44:584-591.

Okada A, Sato T, Ohta Y, Iguchi T. 2005. Sex steroid hormone receptors in the developing female reproductive tract of laboratory rodents. J Toxicol Sci 30:75-89.

Packer RJ, Gutmann DH, Rubenstein A, Viskochil D, Zimmerman RA, Vezina G, Small J, Korf B. 2002. Plexiform neurofibromas in NF1: toward biologic-based therapy. Neurology 58:1461-1470.

Page PZ, Page GP, Ecosse E, Korf BR, Leplege A, Wolkenstein P. 2006. Impact of neurofibromatosis 1 on Quality of Life: a cross-sectional study of 176 American cases. Am J Med Genet A 140:1893-1898.

Palmer C, Szudek J, Joe H, Riccardi VM, Friedman JM. 2004. Analysis of neurofibromatosis 1 (NF1) lesions by body segment. Am J Med Genet A 125:157-161.

Parada LF, Kwon CH, Zhu Y. 2005. Modeling neurofibromatosis type 1 tumors in the mouse for therapeutic intervention. Cold Spring Harb Symp Quant Biol 70:173-176.

Parrizas M, LeRoith D. 1997. Insulin-like growth factor-1 inhibition of apoptosis is associated with increased expression of the bcl-xL gene product. Endocrinology 138:1355-1358.

Posma E, Aalbers R, Kurniawan YS, van Essen AJ, Peeters PM, van Loon AJ. 2003. Neurofibromatosis type I and pregnancy: a fatal attraction? Development of malignant schwannoma during pregnancy in a patient with neurofibromatosis type I. Bjog 110:530-532.

Puls LE, Chandler PA. 1991. Malignant schwannoma in pregnancy. Acta Obstet Gynecol Scand 70:243-244.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307:1625-1630.

Reynolds RM, Browning GG, Nawroz I, Campbell IW. 2003. Von Recklinghausen's neurofibromatosis: neurofibromatosis type 1. Lancet 361:1552-1554.

Ribella E. 1975. Su di un caso di gravidanza in donna affecta da neurofibromatosi. Minerva Ginecologica:607-613.

Robertson JA, Zhang Y, Ing NH. 2001. ICI 182,780 acts as a partial agonist and antagonist of estradiol effects in specific cells of the sheep uterus. J Steroid Biochem Mol Biol 77:281-287.

Roche WR. 1985. Mast cells and tumors. The specific enhancement of tumor proliferation in vitro. Am J Pathol 119:57-64

Rodriguez-Cuenca S, Gianotti M, Roca P, Proenza AM. 2006. Sex steroid receptor expression in different adipose depots is modified during midpregnancy. Mol Cell Endocrinol 249:58-63.

Rodriguez-Viciana P, Warne PH, Vanhaesebroeck B, Waterfield MD, Downward J. 1996. Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. Embo J 15:2442-2451.

Roth TM, Ramamurthy P, Ebisu F, Lisak RP, Bealmear BM, Barald KF. 2007a. A mouse embryonic stem cell model of Schwann cell differentiation for studies of the role of neurofibromatosis type 1 in Schwann cell development and tumor formation. Glia 55:1123-1133.

Roth TM, Ramamurthy P, Muir D, Wallace M, Barald KF. 2007b. Influence of hormones and hormone metabolites on the growth of Schwann Cells derived from mouse embryonic stem cells and tumor cell lines expressing variable levels of neurofibromin. In revision.

Ruohola JK, Valve EM, Karkkainen MJ, Joukov V, Alitalo K, Harkonen PL. 1999. Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. Mol Cell Endocrinol 149:29-40.

Schumacher G, Neuhaus P. 2001. The physiological estrogen metabolite 2-methoxyestradiol reduces tumor growth and induces apoptosis in human solid tumors. J Cancer Res Clin Oncol 127:405-410.

Sebold CD, Lovell A, Hopkin R, Noll R, Schorry E. 2004. Perception of disease severity in adolescents diagnosed with neurofibromatosis type 1. J Adolesc Health 35:297-302.

Sharma JB, Gulati N, Malik S. 1991. Maternal and perinatal complications in neurofibromatosis during pregnancy. International Journal of Gynecology & Obstetrics 34:221-227.

Sherman LS, Atit R, Rosenbaum T, Cox AD, Ratner N. 2000. Single cell Ras-GTP analysis reveals altered Ras activity in a subpopulation of neurofibroma Schwann cells but not fibroblasts. J Biol Chem 275:30740-30745.

Sibonga JD, Sommer U, Turner RT. 2002. Evidence that 2-methoxyestradiol suppresses proliferation and accelerates apoptosis in normal rat growth plate chondrocytes. J Cancer Res Clin Oncol 128:477-483.

Sonneveld E, Riteco JA, Jansen HJ, Pieterse B, Brouwer A, Schoonen WG, van der Burg B. 2006. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. Toxicol Sci 89:173-187.

Soucek L, Lawlor ER, Soto D, Shchors K, Swigart LB, Evan GI. 2007. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. Nat Med 13:1211-1218.

Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT. 1997. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science 277:567-570.

Swapp GH, Main RA. 1973. Neurofibromatosis in pregnancy. Br J Dermatol 88:431-435.

Tapp E, Hickling RS. 1969. Renal artery rupture in a pregnant woman with neurofibromatosis. J Pathol 97:398-402.

Teoh H, Leung SW, Man RY. 1999. Short-term exposure to physiological levels of 17 beta-estradiol enhances endothelium-independent relaxation in porcine coronary artery. Cardiovasc Res 42:224-231.

Theoharides TC, Conti P. 2004. Mast cells: the JEKYLL and HYDE of tumor growth. Trends in Immunol 25:235-241.

Thomas SL, De Vries GH. 2007. Angiogenic expression profile of normal and neurofibromin-deficient human Schwann cells. Neurochem Res 32:1129-1141.

Tidwell C, Copas P. 1998. Brachial artery rupture complicating a pregnancy with neurofibromatosis: a case report. Am J Obstet Gynecol 179:832-834.

Tinley TL, Leal RM, Randall-Hlubek DA, Cessac JW, Wilkens LR, Rao PN, Mooberry SL. 2003. Novel 2-methoxyestradiol analogues with antitumor activity. Cancer Res 63:1538-1549.

Van Den Bemd GJ, Kuiper GG, Pols HA, Van Leeuwen JP. 1999. Distinct effects on the conformation of estrogen receptor alpha and beta by both the antiestrogens ICI 164,384 and ICI 182,780 leading to opposite effects on receptor stability. Biochem Biophys Res Commun 261:1-5.

Virdis R, Street M, Bandello M, Tripodi C, Donadio A, Villani A, Cagozzi L, Garavelli L, Bernasconi S. 2003. Growth and pubertal disorders in neurofibromatosis type 1. Journal of Pediatric Endocrinology & Metabolism 16:289-292.

Viskochil D, White R, Cawthon R. 1993. The neurofibromatosis type 1 gene. Annu Rev Neurosci 16:183-205.

Wagner AH, Schroeter MR, Hecker M. 2001. 17beta-estradiol inhibition of NADPH oxidase expression in human endothelial cells. Faseb J 15:2121-2130.

Wakeling AE, Dukes M, Bowler J. 1991. A potent specific pure antiestrogen with clinical potential. Cancer Res 51:3867-3873.

Wang SH, Myc A, Koenig RJ, Bretz JD, Arscott PL, Baker JR. 2000. 2-Methoxyestradiol, an endogenous estrogen metabolite, induces thyroid cell apoptosis. Mol Cell Endocrinol 165:163-172.

Weiss B, Bollag G, Shannon K. 1999. Hyperactive Ras as a therapeutic target in neurofibromatosis type 1. Am J Med Genet 89:14-22.

Weissman A, Jakobi P, Zaidise I, Drugan A. 1993. Neurofibromatosis and pregnancy. An update. J Reprod Med 38:890-896.

Wilson ME, Liu Y, Wise PM. 2002. Estradiol enhances Akt activation in cortical explant cultures following neuronal injury. Brain Res Mol Brain Res 102:48-54.

Witorsch RJ. 2002. Low-dose in utero effects of xenoestrogens in mice and their relevance to humans: an analytical review of the literature. Food Chem Toxicol 40:905-912.

Wiznitzer A, Katz M, Mazor M, Pinku A, Hagay ZJ. 1986. Neurofibromatosis in pregnancy. Four case reports and review of the literature. Isr J Med Sci 22:579-581.

Wolkenstein P, Zeller J, Revuz J, Ecosse E, Leplege A. 2001. Quality-of-life impairment in neurofibromatosis type 1: a cross-sectional study of 128 cases. Arch Dermatol 137:1421-1425.

Woodruff JM. 1999. Pathology of tumors of the peripheral nerve sheath in type 1 neurofibromatosis. Am J Med Genet 89:23-30.

Wyllie AH, Kerr JF, Currie AR. 1980. Cell death: the significance of apoptosis. Int Rev Cytol 68:251-306.

Yao R, Cooper GM. 1995. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003-2006.

Yeh S, Miyamoto H, Shima H, Cahng C. 1998. From estrogen to androgen receptor: a new pathway for sex hormones in prostate. Proc Natl Acad Sci USA 95:5527-5532.

Zhang J, Tsai FTF, Geller DS. 2006. Differential interaction of RU486 with the progesterone and glucocorticoid receptors. J Mol Endocrinol 37:163-173.

Zhang Z, Cerghet M, Mullins C, Williamson M, Bessert D, Skoff R. 2004. Comparison of in vivo and in vitro subcellular localization of estrogen receptors alpha and beta in oligodendrocytes. J Neurochem 89:674-684.

Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF. 2002. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science 296:920-922.

CHAPTER II

A MOUSE EMBRYONIC STEM CELL MODEL OF SCHWANN CELL DIFFERENTIATION FOR STUDIES OF THE ROLE OF NEUROFIBROMATOSIS TYPE 1 IN SCHWANN CELL DEVELOPMENT AND TUMOR FORMATION

ABSTRACT

The Neurofibromatosis Type 1 (NF1) gene functions as a tumor suppressor gene, of which one known function is to turn off the p21ras pathway by accelerating Ras hydrolyzation of active rasGTP to inactive rasGDP. Loss of neurofibromin (the protein product of the NF1 gene) in the autosomal dominant disorder NF1 is associated with tumors of the peripheral nervous system, particularly neurofibromas, benign lesions in which the major cell type is the Schwann Cell (SC). NF1 is the most common cancer predisposition syndrome affecting the nervous system. We have developed an in vitro system for differentiating mouse embryonic stem cells (mESC) that are NF1 wild type (+/+), heterozygous (+/-), or null (-/-) into SC-like cells to study the role of NF1 in SC development and tumor formation. These SC-like cells, regardless of their NF1 status, express SC markers, and support and preferentially direct neurite outgrowth. They are also capable of expressing myelin proteins. NF1 null and heterozygous SC-like cells proliferate at an accelerated rate compared to NF1 wild type; this growth advantage can be reverted to wild type levels using an inhibitor of MAP kinase kinase (Mek). The

mESC of all NF1 types can also be differentiated into neuron-like cells and the behavior and genetic repertoires of the cells under different developmental conditions can be compared. This system provides an ideal paradigm for studies of the role of NF1 in cell growth and differentiation of different cell types affected by NF1.

INTRODUCTION

NF1 is the most common human cancer predisposition syndrome affecting the nervous system (Gutmann et al. 2001). Hallmarks of NF1 include a range of symptoms from café au lait spots of the skin to tumors of the peripheral nervous system. Schwann Cells (SC) have been found to be the originating cell type in most of the benign tumors (Zhu et al. 2002) and can also contribute to malignant peripheral nerve sheath tumors (Lee et al. 2004; Miller et al. 2006). Loss of heterozygosity (LOH) is often seen in NF1 tumors, particularly in the SC population (Zhu et al. 2002), although not all SCs in the tumors showed LOH (Rutkowski et al. 2000; Sawada et al. 1996; Serra et al. 2000).

Neurofibromin is the protein product of the Neurofibromatosis Type 1 (NF1) gene. One known function of the NF1 tumor suppressor gene is to turn off the p21ras pathway by accelerating Ras hydrolyzation of active rasGTP to inactive rasGDP (Friedman et al. 1999). When NF1 is mutated, Ras is constitutively active, enabling affected cells to proliferate uncontrollably. This allows tumor formation, particularly in neural crest derived cells, such as those in the peripheral nervous system, which are highest in neurofibromin expression (Cichowski and Jacks 2001; Daston et al. 1992; Friedman et al. 1999).

Studies have shown that Neuregulin-1 (Nrg-1) promotes differentiation of neural crest stem cells into Schwann Cells (Sieber-Blum et al. 2004). Neural crest (NC) cells are embryonic transitory migratory cells that differentiate into many cell types (Dorsky et al. 2000; Kalcheim and Burstyn-Cohen 2005; Le Douarin et al. 2004; Lobsiger et al. 2002), including those of the peripheral nervous system affected in NF1, such as Schwann Cells and neurons (Cichowski and Jacks 2001; Daston and Ratner 1992; Vogel et al. 1995). Before becoming mature SC, which can be either myelinating or nonmyelinating, NC cells pass through SC precursor and immature SC stages. Myelination of SC requires the presence of large diameter neurites (Bunge et al. 1982; Bunge et al. 1980; Ratner et al. 1986).

We have used media containing neuregulin to produce a SC differentiation model using mouse ES (mES) cells that have one (NF1+/-), both (NF1+/+) or neither (NF1-/-) functional allele, which have the potential to differentiate into many different cell types, including neuron-like cells (figure 2.1A) and Schwann-like cells (figure 2.1B). They provide an ideal system to study the effects of the environment on the gene expression cascades involved in the differentiation of different cell types that make variable levels of neurofibromin.

MATERIALS AND METHODS

Cell Types: The cell lines used for these studies include: D3 (NF1+/+) mES cell line (Doetschmann 1985), SKO (NF1+/-) mES cell line (Jacks et al. 1994), DKO (NF1-/-) mES cell line with the NF1 gene disrupted by insertion of a neo cassette replacing exon

31 (Jacks et al. 1994) (figure1.1), the SW10 (NF1+/+) mouse Schwann Cell line which carries a temperature sensitive SV40 large T antigen and which is grown at 37°C (the non-permissive temperature for transgene expression) for differentiation (ATCC, Manassas, VA), primary rat Schwann Cells, grown as previously described (Lisak and Bealmear 1991).

Media: Proliferating (ES) consisted of 81% DMEM without Phenol Red, 1% L-Glut, 1% Pen/Strep, 1% non-essential amino acids (Gibco, Carlsbad, CA), 15% FBS (Atlanta Biological, Norcross, GA), 1% Sodium Pyruvate(2% stock), 7 µL/L βMercaptoethanol (Sigma, St. Louis, MO), 1000 U/mL ESGRO (Chemicon, Temecula, CA). **Neuronal Differentiation** consisted of 95%F-12, 1% Pen/Strep, 1% N2, 2% B27 (Gibco), 2% Sodium Pyruvate (2% stock), 0.5 µg/L bFGF, 1 µg/L IL-7, 5 µg/L IGF-1 (Sigma), either 1 µg/L CNTF (R+D) or 1 µg/L NGF (Chemicon). **Schwann Cell Differentiation (SC)** consisted of 84% α-mod. MEM without Phenol Red, 1% Pen/Strep (Gibco), 10% FBS (Atlanta Biological), 5% 11-day chick embryo extract, 10 ng/mL NRG-1 (R&D systems, Minneapolis, MN). **SW10 media** consisted of 88% DMEM without Phenol Red, 1% L-Glutamine, 1% Pen/Strep, 10% FBS (Atlanta Biological). **Primary rat Schwann Cell media** consisted of 90% DMEM (Gibco), 10% FBS (Atlanta Biological). **Minimal media** consisted of 87% DMEM without Phenol Red, 1% Pen/Strep, 1% L-Glutamine (Gibco), 10% FBS (Atlanta Biological), 1% Sodium Pyruvate (Gibco).

Antibodies used included NF1 (rabbit polyclonal 1:50), Oct 4 (goat polyclonal 1:50) (Santa Cruz, CA), Tuj1 (mouse monoclonal 1:500) (Covance, Berkeley, CA), Neurofilament (rabbit polyclonal 1:400) (Chemicon, Temecula, CA), S100 (rabbit polyclonal 1:200 Novocastra, Newcastle Upon Tyne, UK or mouse monoclonal 1:50

Abcam, Cambridge, MA), GFAP (mouse monoclonal 1:400) (Chemicon), peripheral myelin basic protein (mouse monoclonal 1:10), Ki67 (rabbit polyclonal 1:100), (Abcam), αtubulin (mouse monoclonal 1:200), AlexaFluor 350, 488, 594, DAPI (Molecular Probes, Eugene, OR), goat α rabbit-HRP (1:500) Zymed, San Francisco, CA).

Reagents used included Histomouse (Zymed), Prolong Gold anti-fade (Molecular Probes), Porcine gel (Sigma), CellTiter96 cell proliferation assay (Promega). All antibodies were diluted in 10% donkey serum (Chemicon) in 0.1% Tween20/Phosphate Buffered Saline (PBS) (Sigma).

Differentiation of mES cells into neuron-like cells: mES cells in ES medium were plated onto Primaria (Falcon) plates and allowed to attach overnight. After 24 hr, cells were washed 1x with PBS and the medium was changed to neuronal differentiation medium containing either 1 µg/L CNTF or 1 µg/L NGF. Undifferentiated mES cells were fed daily, while cells in neuronal differentiation media were fed every other day. After 5 days' growth, cells were labeled for expression of NF1, Oct4 (self-renewing mES cells), and Tuj1 (neuronal differentiation).

Differentiation of mES cells into SC-like cells: mES cells in ES medium were plated onto Primaria (Falcon) plates and allowed to attach overnight. After 24 hr, cells were washed 1x with PBS and the medium was changed to SC medium. Cells were fed every other day and passed when confluent (~every 5-6 days). After one week, cells had the appearance of SC-like cells (flat and spindly), and proliferation rate was assessed and found to have decreased. After 2 weeks in SC-differentiation media cells were examined by RTqPCR for changes in NF1 expression and immunoassayed for S100 and GFAP

expression. After one month, cells were assayed for the ability to initiate and direct neurite outgrowth and to produce myelin. Cells have been frozen and thawed and remain proliferative and SC marker-expressing over at least 20 passages.

Immunocytochemistry (ICC): 40,000 cells were plated onto 0.1% porcine gel-coated (Sigma) coverslips (Corning, Corning, NY) and grown overnight. Cells were then fixed in 4% paraformaldehyde (Sigma), permeabilized in 0.2% TritonX100 (Sigma), stained and mounted onto Superfrost plus slides (Fisher, Pittsburgh, PA). The Zymed Histomouse kit was used according to the manufacturer's instructions for the non-fluorescent ICC using mouse monoclonal antibodies. Fluorescence micrographs were taken with an Olympus BX-51 microscope, and non-fluorescent micrographs with Nikon ACT-1 software on a Leitz Diavert inverted microscope.

RTqPCR: RTqPCR was performed using primer pairs designed using the Beacon designer program (BioRad, Hercules, CA), with target Ta at 55º C, length 18-22 bp and amplicon size 100 – 200. SW10 gene expression was normalized to GAPDH, while ES cell fold changes with SC-like differentiation were compared by dividing SC differentiated cell expression by proliferative cell expression. Quantification of SC marker expression was assayed at the time points listed. To assay SC-like cells myelin gene upregulation in the presence of neurons, day E5 chick SAGs were excised and added at time points listed and grown an additional seven days before RTqPCR analysis. Total RNA from the samples was extracted using Qiagen mRNA Kit 74106 (Qiagen, CA). RT-PCR was performed as follows: cDNA was synthesized from 2 µg total RNA by reverse transcription using Super Script III transcriptase (Invitrogen, Carlsbad, CA) and oligo dT primer. The PCR conditions consisted of an initial denaturation at 94°C for

1min, 94°C for 1 min, 55°C for 30 sec and 74°C for 30 sec and 34 cycles and final extension at 72°C for 5 min and 4°C holding temperature. The PCR products were separated on 2.0% agarose gels and visualized using ethidium bromide (staining) under UV light.

PCR primers: **NF1**: forward AGTTTCTCTCCTCGCTGGTCTTC reverse CGTTTCCTGCCACCCGTTTG; **Oct4**: forward CCAACGAGAAGAGTATGAGG reverse CTGAGTAGAGTGTGGTGAAG. **α4integrin**: forward GAATCTCCTCCACCTACTCACAG reverse CCAACGGCTACATCAACATATCC; **Cad19**: forward GGAGGAGACAGACAAGATG reverse TGAAGGAGTGATGGTAGATG; **PMP22**: forward GCTCTGTTCCTGTTCTTCTG reverse TGCCTCACTGTGTAGATGG; **MBP**: forward AGACCCTCACAGCGATCCAAG reverse AGTCAAGGATGCCCGTGTCTC; **α1β1integrin**: forward CAGTGAATGGCAACAATGAAG reverse ATCAGCAGCAAGGCAAGG

Proliferation rates of mES cells during SC-like differentiation: 30,000 cells per well were counted and plated onto Primaria (Falcon) 24-well plates. After indicated days' growth, proliferation was assayed using immunocytochemistry with the Ki67 antibody, which recognizes cells in active cell cycle. If indicated, E5 chick statoacoustic ganglions (SAGs), cells which are destined to innervate the inner ear, were excised and added to the plate and grown an additional three days before assay was performed. The percentage of Ki67 positive cells as compared to total cell number was used to determine proliferation rate. Microsoft Excel was used to graph and analyze the data. Data are expressed as mean +/- SD.

Proliferation rates of mES cells after addition of U0126 Mek inhibitor: To determine if the Ras pathway is involved in overproliferation of NF1+/- and NF1-/- SC-like cells 10,000 cells per well were plated onto 96-well Primaria plates. After one hour, either 4 μM U0126 (Mek inhibitor) or ethanol (vehicle control) were added to wells and cells were allowed to grow three days. Proliferating cells were determined using the CellTiter96 (Promega) proliferation assay and absorbance was measured at 492 nm by microplate reader (Fisher). Absorbance is directly proportional to number of actively metabolizing cells.

Statoacoustic ganglion-supported neurite outgrowth and directional outgrowth: To test for SAG-supported neurite outgrowth, statoacoustic ganglions (SAGs) from E5 chick embryos (Bianchi et al. 2005a; Bianchi et al. 2005b) were excised and plated onto undifferentiated mES cells (D3, SKO, or DKO), SC-differentiation mES cells (D3SC, SKOSC, DKOSC), or SW10SC cells. After 5 days, cultures were assayed for presence or absence of neurite outgrowth (Neurofilament positive) as well as the percentage of the area occupied by SAG-neurite positive cells. The total area of SAG neurite positive cells and outgrowth was quantified using Metamorph software (Universal Images Corp) threshold image analysis by determining the percent of Neurofilament-stained cell area compared to the total area. To determine if SAG neurite outgrowth was preferentially directed towards SC-differentiated cells, we excised and plated SAGs on culture dishes to which undifferentiated mES cells and SC-like cells were subsequently added on coverslips in two different quadrants of the plate equidistant from the SAG center. We first allowed the SAGs to attach overnight and then added two coverslips, one containing undifferentiated mES cells and the other coverslip containing SC-like cells,

with the coverslips placed equidistant from the SAG center. These cultures were grown for five days and assessed for a) preferential direction of neurite outgrowth, b) any contact between the mES cells (SC-differentiated or undifferentiated) and the neurites. Neurites in each half of the plate were assessed for extension toward each cell type (undifferentiated vs. SC-differentiated).

Quantification of SAG neurite outgrowth branching and intersections: In order to differentiate and quantify any differences between NF1+/+ WT, NF1+/- heterozygous and NF1-/- null SC-like cells' affect on SAG neurite branching, we overlaid a grid onto SAG/SC-like cell co-culture fluorescent figures (n=12) stained with Tuj1 for neurite outgrowth and counted the number of intersections in random sections. Microsoft Excel was used to graph and analyze the data. Data are expressed as mean +/- SD.

RESULTS

mES cells assume a SC-like morphology and sequentially express SC lineage markers following growth in neuregulin-containing SC media, regardless of their NF1 expression level that increased in NF1+/+ and NF1+/- SC-like cells.

We exposed the (D3 NF1+/+, SKO NF1+/-, and DKO NF1-/-) mES cells to a medium (SC media) designed to enhance Schwann Cell differentiation. After growth for one week in SC media, fed every second day, all cell types (D3 NF1+/+, SKO NF1+/-, and DKO NF1-/-) flattened out and took on the spindly appearance characteristic of SC and tested positive by immunocytochemistry (ICC) for the expression of two different SC

proteins-S100 and GFAP, as did the SW10 mouse SC and the primary rat SC (figure 2.1B, compare with mouse SC line SW10 and primary rat SC in bottom rows). Hereafter, SC-like differentiated mES cells will be referred to as D3SC, SKOSC, and DKOSC, for D3 (WT-NF1+/+), SKO (single knock out-that is missing one NF1 allele; NF1+/-), or DKO (double knock out; NF1-/-).

Since exon 31 was replaced by a neo cassette in these mutant NF1 alleles (Jacks et al. 1994) (figure 1.1), we were able to target this area to design primers to quantify NF1 expression. RTqPCR confirmed NF1 expression in undifferentiated D3, and SKO cells, while NF1 was not expressed in DKO cells (figure 2.1C). NF1 expression levels were set at 100% in D3 cells. This level was used to determine NF1 expression levels of SKO (55%) and DKO (0%) cells, and all SC-differentiated cells. NF1 expression was upregulated ~150% after two weeks in SC-differentiation media in D3SC (from 100% to 152%) and SKOSC (from 55% to 85%) cells, while remaining at undetectable levels in DKOSC (figure 2.1C). NF1 was also expressed in SW10 SC and primary mouse SC (not shown).

To determine whether differentiating cells followed all the characteristic stages of differentiation into mature SC, we also tested gene expression at various stages of differentiation from undifferentiated ES cells to mature SC, using as markers genes characteristic for these stages according to reports in the literature (Jessen and Mirsky 1991; Lobsiger et al. 2002). These included the following stages: Undifferentiated ES (Oct4), neural crest (alpha4integrin), SC progenitor (Cad19), immature SC (PMP22), to mature myelinating SC (myelin basic protein-MBP) and unmyelinating SC (alpha1beta1integrin). We found that Oct4 (figure2.2A) was most highly expressed on

day 0 of differentiation in culture, becoming undetectable with culture in SC-differentiation media. DKO and SKO cells expressed lower levels of Oct4 on day 0 than D3 cells. By contrast, markers along the SC-differentiating pathway, such as alpha4integrin (figure 2.2B) and Cad19 (figure 2.2C) were more gradually upregulated, while genes characteristic of more mature SCs, including PMP22 (figure 2.2D) whose expression persists through myelination, MBP (figure 2.2E) and alpha1beta1integrin (figure 2.2F) generally needed the presence of SAG neurites to stimulate their highest expression levels (figure 2.2 and table 2.1). An exception to this was in DKOSC (NF1-/-) cells, in which MBP was seen to be downregulated in the presence of SAG neurites.

Overproliferation of NF1-deficient or null SC-like cells can be reduced by Mek inhibition

NF1+/+ mES cells had higher proliferation rates than NF1 deficient mES cells (figure 2.3A, day 0), but after day 9 in SC-differentiating conditions NF1 deficient cells had higher proliferation rates than NF1+/+ cells (figure 2.3A, days 18, 28), presumably due to constitutively active Ras. D3SC cells' proliferation rate was comparable to SW10 SC (47%). As quiescence is expected for SC in the presence of neurites, following chick statoacoustic ganglion (SAG) addition to the culture, NF1+/+ cells (D3SC, SW10) became less proliferative, with 34% and 29% actively cycling cells, respectively (figure 2.3A, rightmost set). By contrast, most SKOSC (70%-60%) and DKOSC (77%-66%) cells remained in active cell cycle (figure 2.3A). Ki67 expression revealed non-axonal associated clumps of proliferative SKOSC (NF1+/-) and DKOSC (NF1-/-) cells among the SAG neurites (figure 2.3B).

Overproliferation of SKOSC and DKOSC cells could be reversed to WT levels by the addition of 4 μM U0126, a Mek inhibitor (Favata et al. 1998), which is a component of the Ras pathway. Since this pathway is constitutively active following the loss of NF1 function, our results indicate that the Ras pathway is likely to be involved in this overproliferation (figure 2.3C).

SC-like differentiated mES cells can support and direct neurite outgrowth from primary chick statoacoustic ganglia

We found that neurite outgrowth and complexity of neurite formation and branching in chick statoacoustic ganglia (SAG) was increased in the presence of mES cells that had become SC-like (figure 2.4). Excised SAGs from E5 chick embryos plated onto undifferentiated mES (D3, SKO or DKO) cells, SC-differentiated (D3SC, SKOSC, or DKOSC) mES cells or SW10 SC cells had different neurite outgrowth patterns. We found that undifferentiated mES cells, regardless of their NF1 genotype and NF1 expression level, did not support neurite outgrowth or myelin production, while SAGs grown on any of the SC-differentiated cells (figure 2.4A) supported neurite outgrowth over 5 days, with branching processes never seen in cultures of SAGs alone (figure 2.5B), or on defined substrates such as poly-L-lysine, even in neurite outgrowth promoting medium (Bianchi et al. 2005b). Sometimes outgrowth was so extensive, especially in NF1 deficient and NF1 null cells, that it obscured our ability to discern the shape of the original SAG (especially see SKOSC figure 2.4A middle row to the right). We next determined if the SAGs needed physical cell-cell contact with the SC-like cells or if the cells could affect the directionality of the neurite outgrowth without direct contact. Excised SAGs were plated on culture dishes on which undifferentiated mES cells

(figure 2.4C, on the left) and SC-like cells (figure 2.4C, on the right) were subsequently added equidistant from the SAG center on coverslips in two different quadrants of the plate. After 5 days growth, in every case the SAGs preferentially extended neurites toward/over the SC-like mES cells, and almost invariably veered around any undifferentiated colonies without making contact (figure 2.4C). Although undifferentiated ES cells did not seem to inhibit the initiation of neurite outgrowth, neurites that had begun to grow out from the side of the explant nearer the undifferentiated ES cells, turned and extended towards the differentiated SC-like mES cells (n=4 for each cell type). Over the 5 day observation period, the cells of both types continued to proliferate (ES cells at a faster rate than the SC-like cells) (figure 2.4C), with cells migrating off the coverslip, so that the undifferentiated cells sometimes grew very near to the SAGs, causing the neurites to veer around the colonies (purplish mounds) of undifferentiated mES cells (figure 2.4). When neurite outgrowth in the half plate that contained SC-differentiated mES cells was compared to that in the other half of the plate, occupied by undifferentiated mES cells, the direction of neurite outgrowth was always found to be towards the SC-like cells; neurites that began by traveling toward the undifferentiated cells changed course and veered towards the SC-like cells in every case (n=5). Even though the cells were grown without LIF (ES) or Nrg-1 (SC) supplements to the medium, they kept their respective morphological appearance over the 5 days of the assay.

All SC-like mES cells expressed myelin in cells near axons, while only NF1-deficient cells showed plate overgrowth and greater SAG neurite extension and branching

To assay whether the SC-like cells were maturing, we determined if the SC-like mES cells would express myelin protein in the presence of SAG neurons. We plated and cultured the SAGs and either SC-like cells or SW10 SC for five days, after which the cells were labeled for either PNS myelin protein expression using a mouse monoclonal antibody to peripheral myelin and the Histomouse kit (figure 2.5A, left column), or analyzed for a combination of PNS myelin (for SC), Neurofilament (for neurites) and DAPI (nuclear stain) (figure 2.5A, right column). With the Histomouse kit, we were able to demonstrate that the SC-like cells and SC that expressed myelin clustered around what appear to be axons (figure 2.5A, left column). In SKOSC and DKOSC, cells farther away from the neurites did not stain for myelin expression and became very confluent. In the two NF1+/+ cell lines (D3 and SW10), myelin positive cells associated with the axons, but there was very little growth on the rest of the plate. We wanted to visualize the association of SC-differentiated cells to the SAG neurites in a single preparation. Therefore, fluorescent secondary antibodies to a neural marker, Tuj1, for SAG neurites and DAPI nuclear stain for SC-differentiated cells were used concomitantly. In these cultures, NF1+/+ cells (D3SC, SW10) associated only with SAG neurites, while clusters of non-axon associated NF1+/- (SKOSC) and NF1-/- (DKOSC) SC-like cells were also seen (figure 2.5A, right column). To be sure that cells that may have been excised with the SAG were not those producing the observed myelin expression, we also grew SAGs on plates without any additional cells in the same medium used to culture SC or SW10 cells. SAGs or associated cells did not express myelin under any of these conditions

(figure 2.5B). Most SAGs did not extend any neurites at all under these conditions (figure 2.5B, right); occasionally neurite extension was initiated, but these neuritic processes were confined to areas around the SAG, without extending onto the plate (figure 2.5B, left). An example of extensive SAG neurite branching seen exclusively over SC-like differentiated cells is also shown (fig, 2.5B, bottom).

Next, we wanted to determine if there were differences in the area of the plate occupied by neurons or neurites that varied with levels of NF1 expression. To quantify the amount of neurite outgrowth, we used the Metamorph software program to measure the percentage of the area covered by neurite outgrowth, as represented by Tuj1 or neurofilament antibody staining, to the total area. We found that D3SC cells stimulated SAGs and their associated neurites to cover significantly more area than SKOSC cells or DKOSC cells (figure 2.5C). Although the D3SC cells stimulated more total neuron/neurite-positive area, much of this area was seen to be in clumps of SAGs themselves rather than in neurite extensions over the SC lawns. SAGs grown in the presence of SKOSC and DKOSC cells visually appeared to have more neurite branching over the areas of the plate with underlying SC-like cells (figure 2.5B, bottom). When clumps of somata were subtracted from the total area and only the extent of neurite outgrowth and branching was measured, DKOSC and SKOSC cells had higher percentages of neurite positive area compared to the total area occupied by antibody-positive neuronal cells/and/or neurites (figure 2.5C). They also had more SC-like mES cell proliferation not associated with SAG neurites (figure 2.5A). In order to differentiate between NF1+/+ WT, NF1+/- heterozygous and NF1-/- null SC-like cells' effect on SAG neurite branching, we overlaid a grid onto various figures and counted the number of

SAG neurite intersections in random sections. Visually, NF1+/+ SC-like cells appeared to elicit thicker, more parallel SAG neurite outgrowth than either NF1+/- or NF1-/- SC-like cells, which appeared more highly branched (figure 2.6A). Quantification of intersecting neurites revealed that NF1+/- and NF1-/- SC-like cells elicited significantly greater primary neurite branching compared to NF+/+ SC-like cells, but not between NF1+/- and NF1-/- SC-like cells (figure 2.6B).

DISCUSSION

We sought a model for the role of NF1 in SC development in which the level of neurofibromin could be controlled and comparisons made among cells with different level. mES cells that were wild type, heterozygous or mutant for the NF1 gene were chosen as the cell source (Jacks et al. 1994). In this study, mES cells were differentiated into SC-like cells in order to more closely approximate the originating tumor cell type (Zhu et al. 2002). Since Neuregulin-1 (Nrg-1) had been found to promote differentiation of neural crest stem cells into Schwann Cells (Sieber-Blum et al. 2004), we used a modified version of Nrg-1 containing media to preferentially direct these mES cells into SC-like cells.

By the end of one week in Nrg-1-containing SC media, the mES cells had flattened out and changed their shape to more closely resemble SC morphologically. All cell types also slowed their rate of proliferation, suggesting a change from a more rapidly growing undifferentiated ES cell rate to cells with a similar growth pattern to SCs. To

distinguish the undifferentiated mES cells from the SC-like cells, we refer to SC-differentiated cells as D3SC (NF1+/+), SKOSC (NF1 +/-), or DKOSC (NF1-/-).

After two weeks in SC medium, D3SC, SKOSC, and DKOSSC all expressed two different SC proteins-S100 and GFAP, demonstrated by ICC. NF1 expression in undifferentiated ES cells (D3-NF1+/+, and SKO-NF1+/-) was upregulated upon differentiation into SC-like differentiated cells, consistent with the known higher expression of NF1 in SC, while DKO (NF1-/-) cells' level of NF1 expression remained undetectable. To determine if differentiation from undifferentiated ES cells to SC required passage through intermediate stages, we tested these cells over a three week period in culture for expression of stage-specific markers of the SC differentiation lineage.

Oct 4, a marker of undifferentiated ES cells, was most highly expressed at day 0 of differentiation in all cell types, although expression was highest in D3 (NF1+/+) cells, and was then downregulated to undetectable levels. This may be because SKO and DKO mES cells seem to be more difficult to retain as undifferentiated in culture, with colonies forming a more irregular border than D3 (NF1+/+) mES cells (figure 2.1A and personal observation).

By contrast, alpha4integrin (neural crest), Cad19 (SC precursors), PMP22 (immature SC), MBP (myelinating SC) and alpha1beta1integrin (unmyelinating SC) were expressed at undetectable/very low levels in undifferentiated ES cells, but became more highly expressed as differentiation progressed.

Earlier SC lineage markers reached their highest level of expression over time when grown in SC-differentiation medium, although all the cells on the culture plate did not appear to differentiate at the same time. This could be due to the observation that undifferentiated mES cells grow in large clumped colonies rather than in flat monolayers. This could cause the outer cells, with greater exposure to the differentiation media, to differentiate more rapidly than the inner cells of the mounds.

In vivo, there is a close relationship between neurons and SCs (Bunge et al. 1980; Lobsiger et al. 2002; Wood 1976). Myelinating SC wrap around one or more individual large-diameter axons; non-myelinating SCs insulate small-diameter neuron bundles that are imbedded in the cytoplasm and surrounded by plasma membrane (Lobsiger et al. 2002). SCs are unlikely to express myelin unless they are in the presence of neurons (Coman et al. 2005; Lobsiger et al. 2002).Genes that are upregulated later in SC development (PMP22), as well as mature SC genes expressed in myelinating (MBP) and unmyelinating (alpha1beta1integrin) SC were expressed at their highest levels following exposure to SAG neurites, with the exception of DKOSC cells' MBP expression, which was decreased in the presence of SAGs. This could be due to overproliferation and aberrant association of NF1-/- SC with axons, and because SAGs showed greater branching of neurites when grown in co-culture with DKOSC cells, which may result in smaller diameter axons that are not myelinated.

In agreement with NF1's function as an inactivator of the Ras pathway (Friedman et al. 1999; Kogut et al. 2007; Mattingly et al. 2006), DKOSC (NF1-/-) and SKOSC (NF1+/-) cells showed significantly increased cell proliferation rates compared to D3SC cells, and aberrant proliferative cells unassociated with SAG neurites in co-culture. When

we inhibited this pathway using U0126, a Mek inhibitor (Favata et al. 1998; Kogut et al. 2007; Mattingly et al. 2006), we were able to return proliferation rates to WT levels. This indirectly indicates that the inability to hydrolyze Ras effectively resulted in overproliferation. D3SC (NF1+/+) cells, already able to hydrolyze and inactivate Ras, were unaffected by U0126 addition.

To stimulate myelin expression and to determine axonal association, we examined the ability of the mES-derived SC-like cells to myelinate the chick statoacoustic ganglion (SAG), which innervates the inner ear, the individual neurites of which are myelinated (Bianchi et al. 2005b; Whitlon et al. 2006). It has been suggested that direct interaction with SC serves as a substrate for neuron attachment and growth, such as with SAGs (Whitlon et al. 2006). We found that neurite outgrowth in SAGs was increased in the presence of mES cells that had become SC-like, as compared to undifferentiated mES cells, MEF cells (not shown) or in media alone without an underlying cell layer. This was true regardless of their NF1 genotype and NF1 expression level, although NF1 deficient cells exhibited more elaborate branching. The SAGs grown on SC-like cells supported neurite outgrowth over the 5 day culture period, sometimes in NF1 deficient and null cells even to the extent that we were unable to discern the shape of the original SAG explant. Directional neurite outgrowth was also extensive when we excised and plated the SAGs on culture dishes on which undifferentiated mES cells and SC-like cells were subsequently added on coverslips in two quadrants of the plate equidistant from the SAG. The SAGs in every case (n=5) extended neurites toward/over the SC-like mES cells, regardless of their NF1 status. While undifferentiated ES cells did not seem to inhibit the initiation of neurite outgrowth, neurites that had begun to grow out from the side of the

explant nearer the undifferentiated ES cells, turned and extended towards the differentiated SC-like mES cells, suggesting either a chemoattractant signal from the SC-like cells or a repulsive signal from the undifferentiated mES cells. Even though the cells were grown without LIF (ES) or Nrg-1 (SC) supplements to the medium, they kept their respective morphological appearance over the 5 days of the assay.

We then determined that SC-like mES cells could express myelin protein and associate with axons in the presence of SAG neurons. SC-like cells and SC that expressed myelin clustered around the neurite processes. In SKOSC and DKOSC, in addition to neurite-associated cells, non-associated proliferative cells farther away from the axons did not express myelin and became very confluent. In the two NF1+/+ cell lines (D3 and SW10), myelin-positive cells clustered around the axons, but there was very little cell growth on the rest of the plate, again suggesting a possible chemoattractant component produced by the SC-like cells or the SAG itself. Axonal association was also seen in SC-like cells closer to the neurites when we used fluorescent secondary antibodies to visualize both the neurites and the SC-like cells. In addition, large numbers of NF1-deficient cells also grew without axonal association. Although during this short culture period we could not document SC-like cells wrapping around neurites, that is most likely due to the plates becoming very confluent by day 5, since undifferentiated mES cells had to be plated at a high enough density to avoid spontaneous differentiation, and did not allow for the one month or more that must ensue before wrapping has been documented in culture (Ratner et al. 1986). When SAGs were grown on plates without any additional cells, the chick SAGs or associated non-neuronal cells did not express myelin under any of the conditions tested in these experiments. This was done to confirm that the myelin

protein was expressed from the SC-like cells rather than any cells excised with the SAG. Occasionally neurites were initiated, but they were mostly confined to areas around the SAG, without extending into the plate, also suggesting that a chemoattractant could be secreted from the SC-like cells. Cells grown on mouse embryonic fibroblasts also did not support neurite outgrowth or myelination (not shown).

When we quantified neurite outgrowth, we found that while NF1+/+ (D3SC) SC-like cells had larger total neurite-positive areas, much of this was due to SAG clumps. When neurites only were measured, NF1+/- (SKOSC) and NF1-/- (DKOSC) SC-like cells elicited greater SAG neurite outgrowth and considerably greater branching of SAG neurites. More neurites intersecting would leave thinner diameter axons that are more characteristic of a non-myelinating phenotype. SC that wrap around neurons greater than 1μM diameter myelinate, while SC associated with axons smaller than 1μM diameter are non-myelinating. This could explain why MBP (myelinating SC) expression was higher in D3SC/SAG co-cultures and alph1beta1 (associated with non-myelinating SC) expression was higher in SKOSC/SAG and DKOSC/SAG co-cultures.

From our experiments we conclude that NF1 (+/+, +/- or -/-) mESC can differentiate *in vitro* into Schwann Cell (SC)-like cells that express appropriate SC genes in the expected order of expression for SC maturation, support and preferentially direct primary ganglion neurite outgrowth, and express myelin in cells near neurites. *In vitro*, NF1+/+ SC-like cells (D3SC) are more likely to express myelinating SC genes, and become quiescent in the presence of SAG neurites at a rate similar to SW10 mouse SC. NF1+/- (SKOSC) and NF1-/- SC-like cells (DKOSC) are more likely to stimulate greater neuron migration, neurite outgrowth and branching, express non-myelinating genes, and

slightly reduce proliferation in the presence of SAG neurites. Overproliferation of NF1 deficient (SKOSC) and null (DKOSC) SC-like cells can be reduced to NF+/+ (D3SC) levels by the addition of a Mek inhibitor, indirectly indicating Ras pathway involvement. This paper is the first of which we are aware that shows that mES cells can be preferentially directed down the Schwann Cell pathway. Since all three mES cell lines, regardless of their NF1 status, can respond by differentiating into SC-like cells, these cells can be used in further studies of the function of SC with varying levels of neurofibromin in tumor formation and growth.

Figure 2.1: Undifferentiated mES cells that express various levels of neurofibromin grow in mounds and can be differentiated into neuron-like cells and SC-like cells. NF1 expression is upregulated following SC differentiation. Cell Types are listed in the leftmost box of each row. **A**: Self-renewing mES cells express Oct 4 (blue). NF1 expression (green) is strong in D3 (NF1+/+) cells, weakly expressed in SKO (NF1+/-) cells, and not expressed in DKO (NF1-/-) cells. Tuj1 (red), a neuronal marker, was weakly expressed around the edges of colonies, showing some differentiation in peripheral cells (left). After 5 days growth in neuronal differentiation media, Tuj1 expression was strongly observed in the neurites of neuronal-like cells, while Oct 4 expression decreased and was only seen in some clumps, perhaps due to the inability of CNTF or NGF to diffuse into the middle of these large colonies (center and right). **B**: Left column: morphology of mES cells, which take on a flat and spindly appearance after one week's growth in SC differentiation media, similar to the morphology of the SW10 SC line and primary rat SC. (32x magnification with phase 1 filter). Middle column shows expression of S100 and right column shows GFAP expression (40x magnification). **C**: RTqPCR shows NF1 expression in SKO cells is 55% that of D3 cells, while expression in DKO cells is undetectable. After SC differentiation, NF1 expression increased ~150% in both D3SC cells and SKOSC cells, while remaining undetectable in DKOSC cells

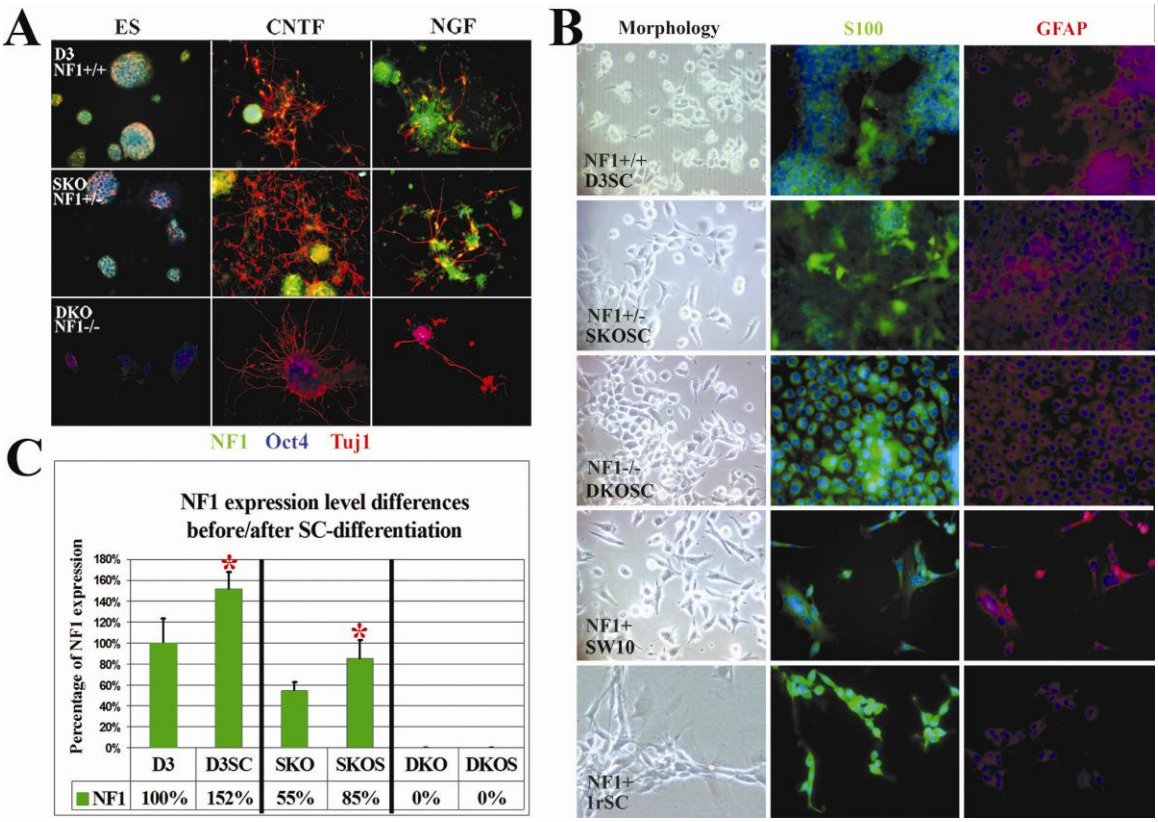
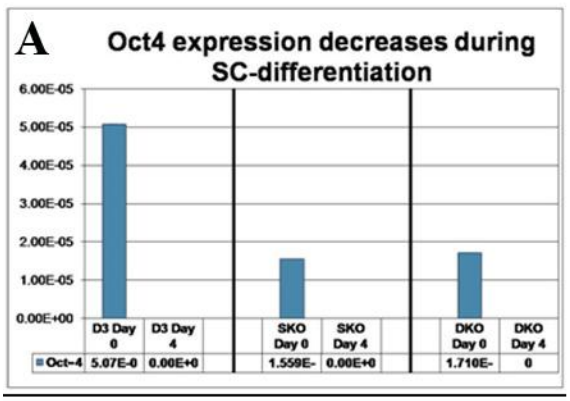
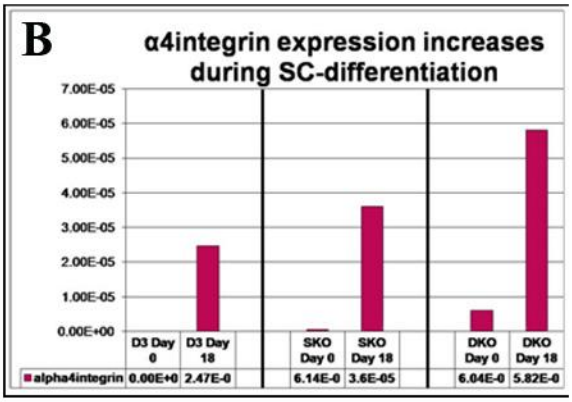

A
ES
CNTF
NGF
D3
NF1+/+
SKO
NF1+/-
DKO
NF1-/-
NF1 Oct4 Tuj1
B
Morphology
S100
GFAP
NF1+/+
D3SC
NF1+/-
SKOSC
NF1-/-
DKOSC
NF1+
SW10
NF1+
1rSC
C
NF1 expression level differences
before/after SC-differentiation
Percentage of NF1 expression
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
D3
D3SC
SKO
SKOS
DKO
DKOS
NF1
100%
152%
55%
85%
0%
0%

Figure 2.2: Expression of SC lineage markers are upregulated with time. **A**: Oct4, expressed in undifferentiated ES cells, is highly expressed on Day 0 in all cell types, especially D3 (NF1+/+) but is not detectable with SC-differentiation. **B**: α4integrin, expressed in neural crest and SC precursors, **C:** Cad19, expressed in SC precursors, and **D:** PMP22, expressed in immature SC, as well as myelinating and non-myelinating SC, are either undetectable or expressed at very low levels in undifferentiated ES cells but are all detectable following culture in SC differentiation media. **E, F**: Two markers of mature SC are upregulated in the presence of SAG neurites (starred columns). MBP, expressed in myelinating SC, is more highly upregulated in D3SC (NF1+/+) cells, while α1β1integrin, expressed in non-myelinating SC, is most highly expressed in DKOSC (NF1-/-) cells.


A
Oct4 expression decreases during SC-differentiation
6.00E-05
5.00E-05
4.00E-05
3.00E-05
2.00E-05
1.00E-05
0.00E+00
D3 Day 0
D3 Day 4
SKO Day 0
SKO Day 4
DKO Day 0
DKO Day 4
Oct-4
5.07E-0
0.00E+0
1.559E-
0.00E+0
1.710E-
0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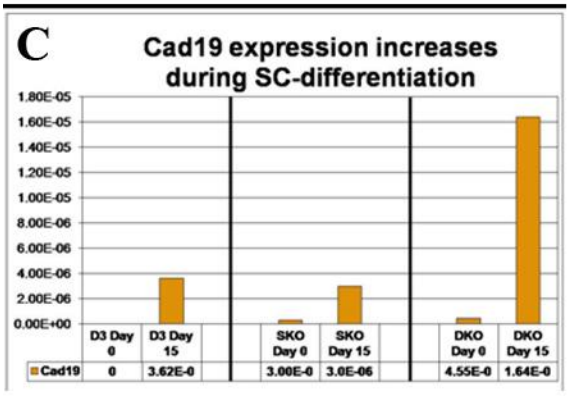
α4integrin expression increases during SC-differentiation
7.00E-05
6.00E-05
5.00E-05
4.00E-05
3.00E-05
2.00E-05
1.00E-05
0.00E+00
D3 Day 0
D3 Day 18
SKO Day 0
SKO Day 18
DKO Day 0
DKO Day 18
alpha4integrin
0.00E+0
2.47E-0
6.14E-0
3.6E-05
6.04E-0
5.82E-0


C
Cad19 expression increases during SC-differentiation
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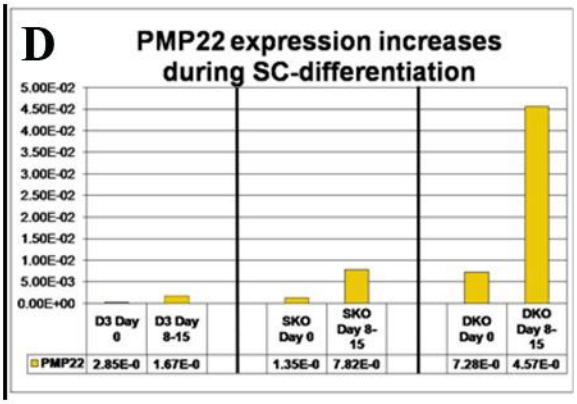
1.60E-05
1.40E-05
1.20E-05
1.00E-05
8.00E-06
6.00E-06
4.00E-06
2.00E-06
0.00E+00
D3 Day 0
D3 Day 15
SKO Day 0
SKO Day 15
DKO Day 0
DKO Day 15
Cad19
0
3.62E-0
3.00E-0
3.0E-06
4.55E-0
1.64E-0


D
PMP22 expression increases during SC-differentiation
5.00E-02
4.50E-02
4.00E-02
3.50E-02
3.00E-02
2.50E-02
2.00E-02
1.50E-02
1.00E-02
5.00E-03
0.00E+00
D3 Day 0
D3 Day 8-15
SKO Day 0
SKO Day 8-15
DKO Day 0
DKO Day 8-15
PMP22
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1.67E-0
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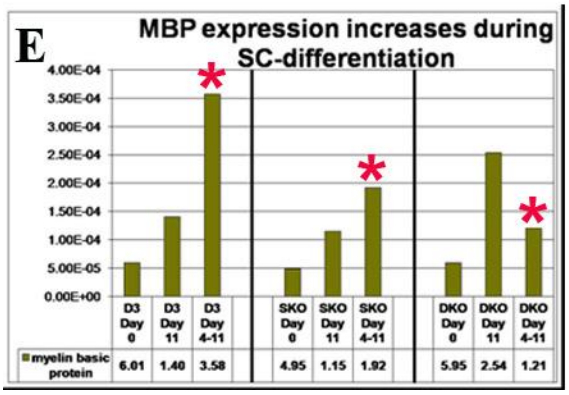
7.28E-0
4.57E-0


E
MBP expression increases during SC-differentiation
4.00E-04
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
D3 Day 0
D3 Day 11
D3 Day 4-11
SKO Day 0
SKO Day 11
SKO Day 4-11
DKO Day 0
DKO Day 11
DKO Day 4-11
myelin basic protein
6.01
1.40
3.58
4.95
1.15
1.92
5.95
2.54
1.21

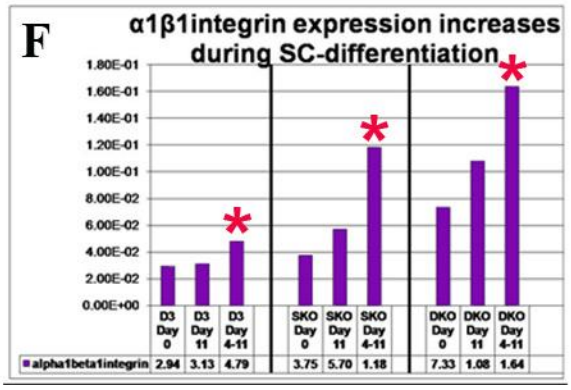

F
α1β1integrin expression increases during SC-differentiation
1.80E-01
1.60E-01
1.40E-01
1.20E-01
1.00E-01
8.00E-02
6.00E-02
4.00E-02
2.00E-02
0.00E+00
D3 Day 0
D3 Day 11
D3 Day 4-11
SKO Day 0
SKO Day 11
SKO Day 4-11
DKO Day 0
DKO Day 11
DKO Day 4-11
alpha1beta1integrin
2.94
3.13
4.79
3.75
5.70
1.18
7.33
1.08
1.64

Figure 2.3: Proliferation rates differ between cell types based on NF1 genotype. **A:** NF1+/+ mES cells have higher proliferation rates, determined by percentage of cells in active cell cycle (Ki67+) than NF1 deficient mES cells (day 0). By contrast, SC-like differentiated NF1 deficient cells had higher proliferation rates than NF1+/+ cells. By 28 days in SC-differentiation media, D3SC cells had the same percentage (47%) of cells in cell cycle as SW10 SC, while SKOSC and DKOSC cells were hyperproliferative (70% and 77%, respectively). Following addition of SAG cells to the culture, NF1+/+ cells (D3SC, SW10) showed reduced proliferation, with only about 30% of their cells in cell cycle, while NF1+/- (SKOSC) and NF1-/- (DKOSC) still had over 60% of cells in cell cycle. **B:** Staining for Ki67-positive cells reveals proliferative clumps of non-axon associated SKOSC and DKOSC cells (100x magnification) **C:** Overproliferation of NF1+/- and NF1-/- SC-differentiated cells is dependent on Ras signaling. SKOSC (NF1+/-) and DKOSC (NF1-/-) cells proliferate at a significantly increased rate over that of D3SC (NF1+/+) cells (green columns), presumably due to the unavailability of NF1 to accelerate inactivation of the Ras pathway. This overproliferation can be decreased to wild type levels through application of 4 μM U0126, an inhibitor of the Mek component of the Ras pathway (red columns).

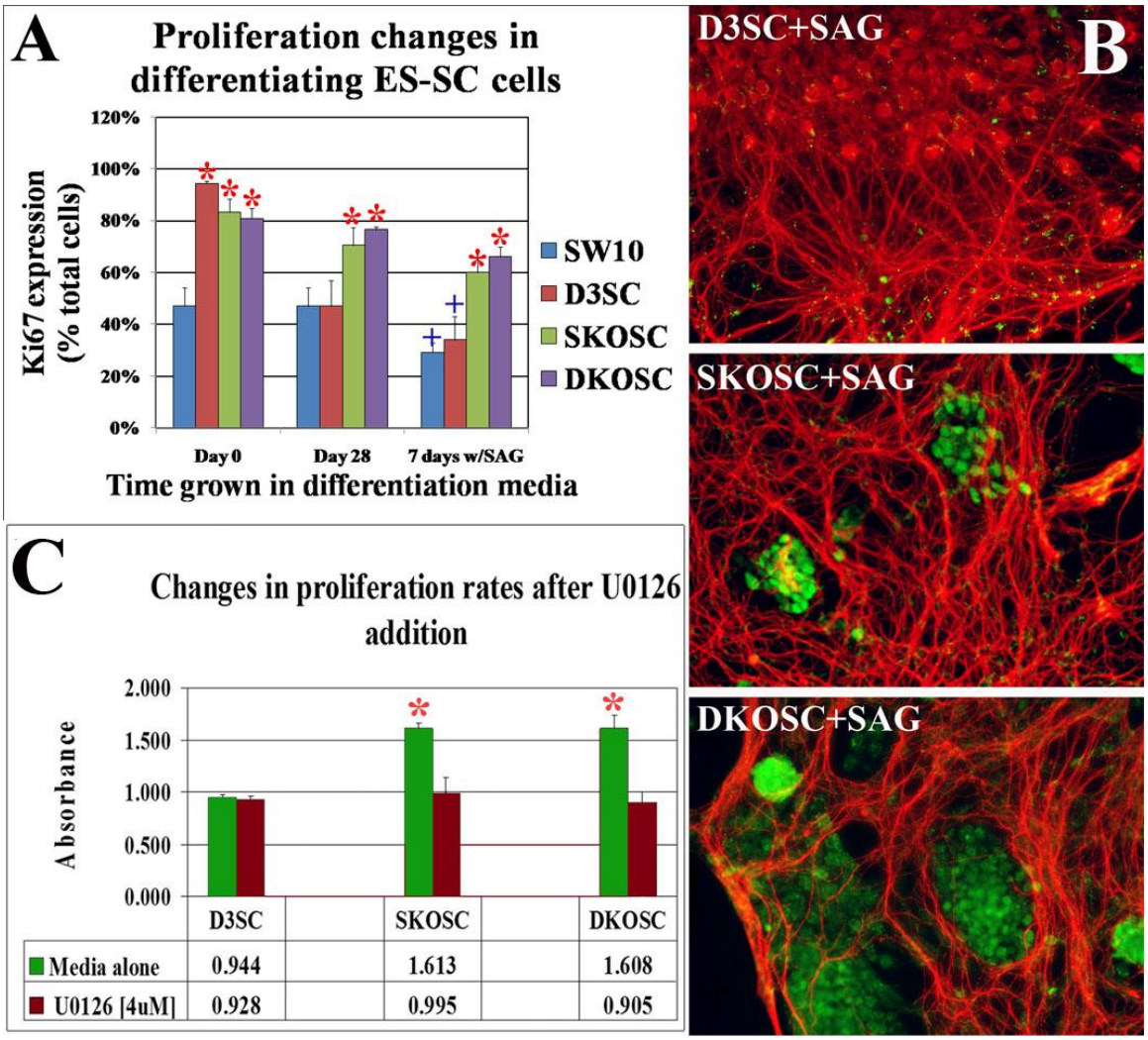

A
Proliferation changes in differentiating ES-SC cells
Ki67 expression (% total cells)
120%
100%
80%
60%
40%
20%
0%
Day 0
Day 28
7 days w/SAG
Time grown in differentiation media
SW10
D3SC
SKOSC
DKOSC
B
D3SC+SAG
SKOSC+SAG
DKOSC+SAG
C
Changes in proliferation rates after U0126 addition
Absorbance
2.000
1.500
1.000
0.500
0.000
D3SC
SKOSC
DKOSC
Media alone 0.944 1.613 1.608
U0126 [4uM] 0.928 0.995 0.905

Figure 2.4: mES cells which had been differentiated into SC-like cells, but not undifferentiated mES cells, attracted neurite outgrowth from primary neurons. Cell types are listed in each box. **A**: E5 chick SAGs were excised and plated onto the cell types listed, grown for 5 days and labeled with antibodies to Tuj1. There was little or no neurite outgrowth toward undifferentiated mES cells, but extensive outgrowth toward SC-like cells (Photographs of undifferentiated cells are at 20x and SC-like cells are at 10x magnification) **B**: When SAGs were plated on the TC substrate equidistant between coverslips plated with undifferentiated mES cells (left side) and SC-like differentiated mES cells (right side), neurite outgrowth was preferentially directed towards the SC-like cells. When undifferentiated mES cells migrated off the coverslips towards the SAGs, the neurites actively avoided the colony (seen as purple mounds). When neurite outgrowth was initiated on the side on which the undifferentiated cells were plated, the neurites turned and continued growing towards the SC-like cells (4x magnification).

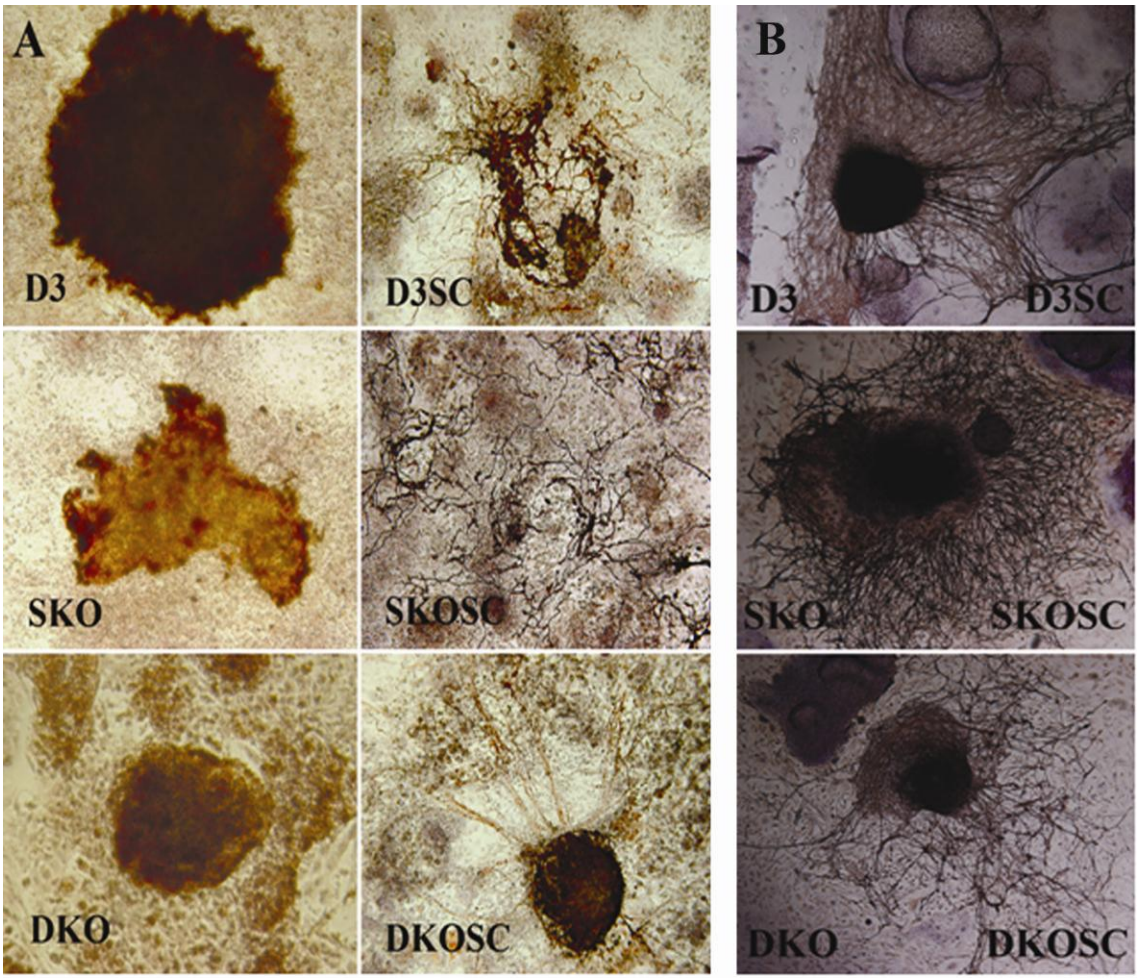

A
D3
D3SC
SKO
SKOSC
DKO
DKOSC
B
D3
D3SC
SKO
SKOSC
DKO
DKOSC

Figure 2.5: SC-like cells stimulate neurite outgrowth, express myelin protein and associate with neurites when grown in the presence of primary neurons. **A**: E5 chick SAGs were plated onto SC-like mES cells or SW10 SC and grown for 5 days, after which they were fixed and stained. Cells were observed to cluster around neurites and expressed myelin protein (left column). While D3SC and SW10 cells (both NF1+/+) associated only with the neurites, SKOSC (NF1+/-) and DKOSC (NF1-/-) cells grew to confluency over the entire plate (left column, 10x magnification, right column 100x magnification). Some SAG neurites grown with SC-like cells showed extensive branching (B, bottom right) **B**: Excised SAGs grown in media alone without an underlying cell layer did not express myelin. Neurite outgrowth was either confined around the SAG (left) or was not observed (right) (40x magnification) **C**: Amount of SAG neurite outgrowth is dependent on NF1 status of underlying cells. Total positive area is greater in SAGs grown with D3SC (NF1+/+) cells, but neurite extension area is greatest in DKOSC cells. This is attributed to greater migration and neurite outgrowth and branching of the SAG cells in the presence of SKOSC (NF1+/-) and DKOSC (NF1-/-) cells. Areas of neurite-positive cells were measured using Metamorph threshold image analysis.

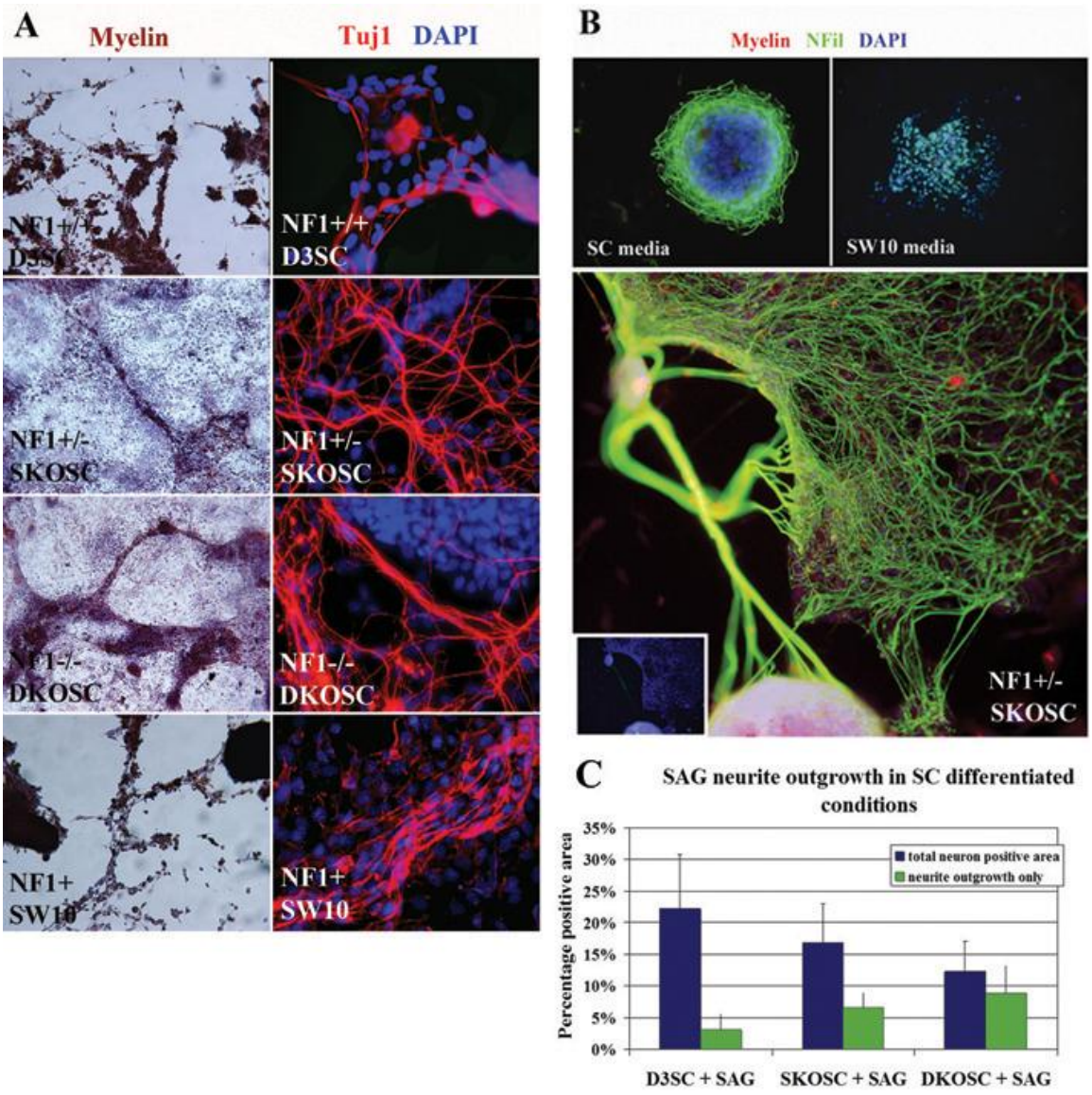

A
Myelin
Tuj1 DAPI
NF1+/+
D3SC
NF1+/+
D3SC
NF1+/-
SKOSC
NF1+/-
SKOSC
NF1-/-
DKOSC
NF1-/-
DKOSC
NF1+
SW10
NF1+
SW10
B
Myelin NFil DAPI
SC media
SW10 media
NF1+/-
SKOSC
C
SAG neurite outgrowth in SC differentiated conditions
Percentage positive area
35%
30%
25%
20%
15%
10%
5%
0%
total neuron positive area
neurite outgrowth only
D3SC + SAG
SKOSC + SAG
DKOSC + SAG

Figure 2.6: Primary neuron processes intersect more frequently when grown in the presence of NF1-deficient SC-like cells. **A.** In order to differentiate between NF1+/+ WT, NF1+/- heterozygous and NF1-/- null SC-like cells' effect on SAG neurite branching, we overlaid a grid onto various figures (Tuj1-stained for visualization of neurite outgrowth after 5 days' co-culture, 100x magnification) and counted the number of SAG neurite intersections in random sections. NF1+/+ SC-like cells elicited thicker, more parallel SAG neurite outgrowth more characteristic of myelinating SC. **B.** Quantification of these calculations revealed that NF1+/- and NF1-/- SC-like cells elicited greater primary neurite branching. More neurites intersecting would leave thinner diameter axons that are more characteristic of a non-myelinating phenotype. There were significantly greater neurite intersections on SAGs grown on NF1+/- and NF1-/- SC-like cells compared to NF+/+ SC-like cells, but not between NF1+/- and NF1-/- SC-like cells.

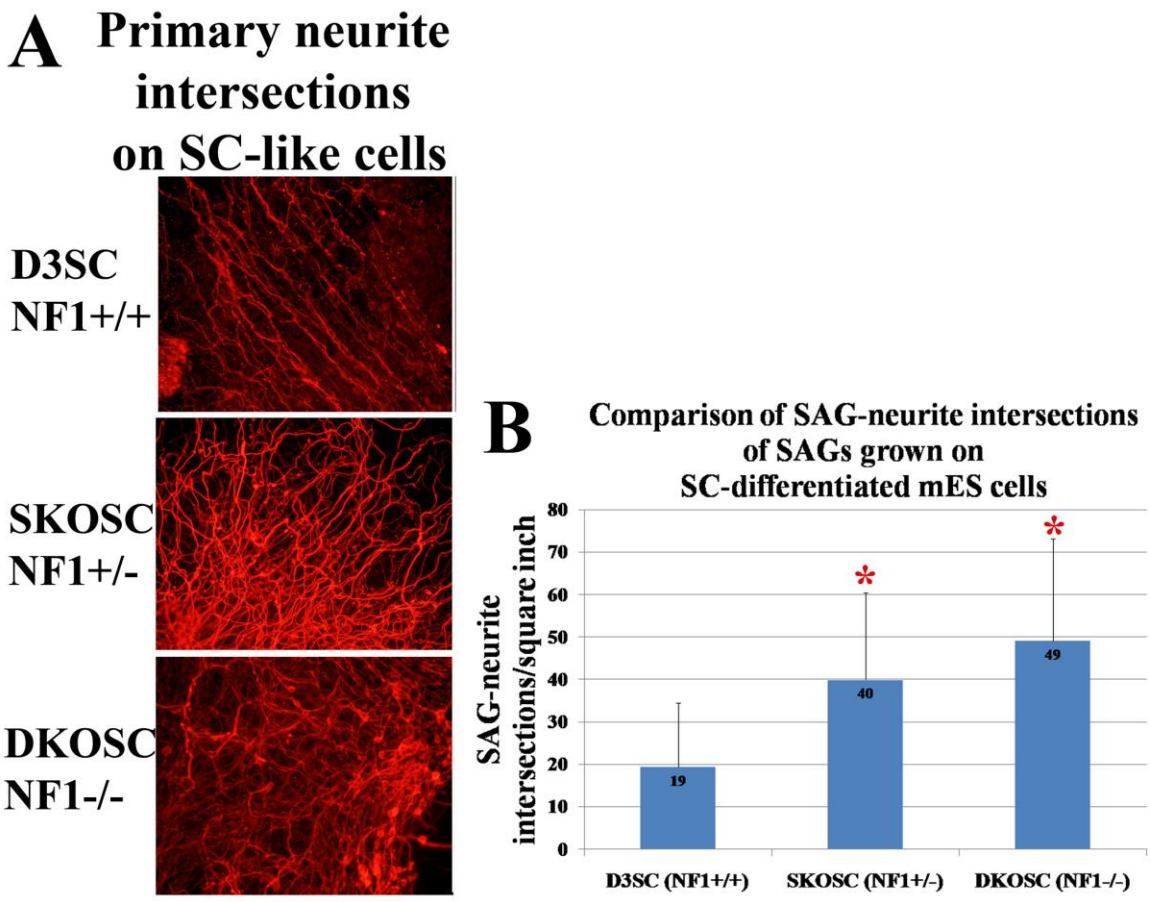

A
Primary neurite intersections on SC-like cells
D3SC NF1+/+
SKOSC NF1+/-
DKOSC NF1-/-
B
Comparison of SAG-neurite intersections of SAGs grown on SC-differentiated mES cells
SAG-neurite intersections/square inch
0
10
20
30
40
50
60
70
80
19
40
49
*
*
D3SC (NF1+/+)
SKOSC (NF1+/-)
DKOSC (NF1-/-)

Table 2.1: The expression levels of genes involved in differentiation from ES cells to SC change over time. D3 (NF1+/+), SKO (NF1+/-), and DKO (NF1-/-) cells were tested at the time points listed with or without the presence of chick statoacoustic ganglions (SAGs). Timing of greatest expression for each of the genes tested is listed in the indicated middle column. Undifferentiated mES marker Oct4 was expressed most highly on differentiation day 0, and remained undetectable thereafter. Genes involved in NC differentiation (alpha4integrin), and SC precursors (Cad19) increased expression during exposure to SC-differentiation media, while PMP22, expressed in immature SC throughout myelination, as well as mature markers of myelinating (MBP) and unmyelinating (alpha1integrin) SC were expressed more highly in the presence of chick SAG neurites. An exception to this was reduced MBP expression in DKOSC cells grown with SAGs.

D3 cells: SC-differentiation gene expression										D3 cells + SAG for 7 days: SC-differentiation gene expression					
		Day 0	Day 1	Day 4	Day 8	Day 11	Day 15	Day 18	highest expression	Day 1-8 +SAG	Day 4-11 +SAG	Day 8-15 +SAG	Day 15-22 +SAG		
Expressed in...	Gene								day					Gene	Expressed in...
Undifferentiated	**Oct4**	5.07E-05	NE	NE	NE	NE	NE	NE	0	NE	NE	NE	NE	**Oct4**	Undifferentiated
NC, SCprecursors	**α4integrin**	NE	2.85E-06	1.79E-06	2.10E-06	1.03E-05	9.12E-06	2.47E-05	18	1.11E-05	9.96E-06	6.84E-06	3.13E-06	**α4integrin**	NC, SCprecursors
SC precursors	**Cad19**	NE	4.84E-06	5.77E-07	9.35E-07	7.13E-06	3.62E-06	8.54E-06	18	4.03E-06	1.30E-06	2.78E-06	7.30E-06	**Cad19**	SC precursors
SC precursors, Immature SC, Myelinating SC	**PMP22**	2.85E-03	1.06E-03	3.81E-04	2.51E-04	8.71E-04	8.25E-04	3.81E-03	15-22 +SAG	1.08E-03	1.48E-03	1.67E-03	4.29E-03	**PMP22**	SC precursors, Immature SC, Myelinating SC
Myelinating SC	**MBP**	6.01E-05	4.35E-05	1.61E-05	4.13E-05	1.40E-04	6.65E-05	1.40E-04	4-11 +SAG	1.07E-04	3.58E-04	8.30E-05	9.06E-05	**MBP**	Myelinating SC
Non-myelin SC	**α1β1integrin**	2.94E-02	1.98E-02	9.91E-03	1.80E-02	3.13E-02	2.20E-02	5.69E-02	15-22 +SAG	3.54E-02	4.79E-02	2.12E-02	5.84E-02	**α1β1integrin**	Non-myelin SC

SKO cells: SC-differentiation gene expression										SKO cells + SAG for 7 days: SC-differentiation gene expression					
		Day 0	Day 1	Day 4	Day 8	Day 11	Day 15	Day 18	highest expression	Day 1-8 +SAG	Day 4-11 +SAG	Day 8-15 +SAG	Day 15-22 +SAG		
Expressed in...	Gene								day					Gene	Expressed in...
Undifferentiated	**Oct4**	1.56E-05	NE	NE	NE	NE	NE	NE	0	NE	NE	NE	NE	**Oct4**	Undifferentiated
NC, SCprecursors	**α4integrin**	6.14E-07	3.67E-06	5.9E-06	1.49E-05	2.45E-05	4.6E-05	3.6E-05	15	5.20E-05	2.64E-05	3.27E-05	7.3E-05	**α4integrin**	NC, SCprecursors
SC precursors	**Cad19**	3.00E-07	7.63E-07	1.4E-06	2.49E-06	6.96E-06	3.0E-06	2.1E-06	11	3.56E-07	7.06E-07	1.27E-07	1.7E-06	**Cad19**	SC precursors
SC precursors, Immature SC, Myelinating SC	**PMP22**	1.35E-03	3.67E-03	1.7E-03	2.09E-03	1.92E-04	1.35E-03	3.85E-03	4-11 +SAG	7.81E-03	9.33E-03	7.82E-03	6.2E-03	**PMP22**	SC precursors, Immature SC, Myelinating SC
Myelinating SC	**MBP**	4.95E-05	3.79E-05	5.8E-05	1.09E-04	1.15E-04	4.99E-05	3.22E-05	4-11 +SAG	1.65E-04	1.92E-04	1.04E-04	1.0E-04	**MBP**	Myelinating SC
Non-myelin SC	**α1β1integrin**	3.75E-02	4.45E-02	4.8E-02	2.14E-02	5.70E-02	3.75E-02	1.94E-02	4-11 +SAG	5.20E-02	1.18E-01	3.35E-02	3.9E-02	**α1β1integrin**	Non-myelin SC

DKO cells: SC-differentiation gene expression										DKO cells + SAG for 7 days: SC-differentiation gene expression					
		Day 0	Day 1	Day 4	Day 8	Day 11	Day 15	Day 18	highest expression	Day 1-8 +SAG	Day 4-11 +SAG	Day 8-15 +SAG	Day 15-22 +SAG		
Expressed in...	Gene								day					Gene	Expressed in...
Undifferentiated	**Oct4**	1.71E-05	NE	NE	NE	NE	NE	NE	0	NE	NE	NE	NE	**Oct4**	Undifferentiated
NC, SCprecursors	**α4integrin**	6.04E-06	7.01E-07	2.0E-06	2.06E-06	1.26E-05	2.27E-05	5.82E-05	18	6.02E-06	1.65E-05	6.87E-06	2.85E-05	**α4integrin**	NC, SCprecursors
SC precursors	**Cad19**	4.55E-07	3.19E-07	1.6E-06	2.51E-06	1.20E-05	1.64E-05	3.64E-06	15	3.41E-06	1.10E-06	5.49E-06	5.00E-06	**Cad19**	SC precursors
SC precursors, Immature SC, Myelinating SC	**PMP22**	7.28E-03	4.15E-03	1.3E-03	1.05E-02	3.10E-02	1.33E-02	3.22E-04	8-15 +SAG	4.10E-02	2.64E-02	4.57E-02	5.24E-03	**PMP22**	SC precursors, Immature SC, Myelinating SC
Myelinating SC	**MBP**	5.95E-05	6.64E-05	3.9E-05	1.80E-04	2.54E-04	1.77E-04	4.73E-04	18	3.57E-05	1.21E-04	1.12E-04	3.27E-04	**MBP**	Myelinating SC
Non-myelin SC	**α1β1integrin**	7.33E-02	2.00E-02	1.9E-02	7.96E-03	1.08E-01	4.43E-02	5.69E-02	8-15 +SAG	1.16E-01	1.64E-01	2.30E-01	3.49E-02	**α1β1integrin**	Non-myelin SC

REFERENCES

Bianchi LM, Daruwalla Z, Roth TM, Attia NP, Lukacs NW, Richards AL, White IO, Allen SJ, Barald KF. 2005a. Immortalized mouse inner ear cell lines demonstrate a role for chemokines in promoting the growth of developing statoacoustic ganglion neurons. J Assoc Res Otolaryngol 6(4):355-67.

Bianchi LM, Daruwalla Z, Roth TM, Attia NP, Lukacs NW, Richards AL, White IO, Allen SJ, Barald KF. 2005b. Immortalized Mouse Inner Ear Cell Lines Demonstrate a Role for Chemokines in Promoting the Growth of Developing Statoacoustic Ganglion Neurons. J Assoc Res Otolaryngol:1-13.

Bunge MB, Williams AK, Wood PM. 1982. Neuron-Schwann cell interaction in basal lamina formation. Dev Biol 92(2):449-60.

Bunge MB, Williams AK, Wood PM, Uitto J, Jeffrey JJ. 1980. Comparison of nerve cell and nerve cell plus Schwann cell cultures, with particular emphasis on basal lamina and collagen formation. J Cell Biol 84(1):184-202.

Cichowski K, Jacks T. 2001. NF1 tumor suppressor gene function: narrowing the GAP. Cell 104(4):593-604.

Coman I, Barbin G, Charles P, Zalc B, Lubetzki C. 2005. Axonal signals in central nervous system myelination, demyelination and remyelination. J Neurol Sci 233(1-2):67-71.

Daston MM, Ratner N. 1992. Neurofibromin, a predominantly neuronal GTPase activating protein in the adult, is ubiquitously expressed during development. Developmental Dynamics 195:216-26.

Daston MM, Scrable H, Nordlund ML, Sturbaum AK, Nissen LM, Ratner N. 1992. The protein product of the Neurofibromatosis Type 1 gene is expressed at highest abundance in neurons, Schwann cells, and oligodendrocytes. Neuron 8:415-28.

Doetschmann T. 1985. J Emb Exp Morph 87:27-45.

Dorsky RI, Moon RT, Raible DW. 2000. Environmental signals and cell fate specification in premigratory neural crest. BioEssays 22:708-16.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F and others. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273(29):18623-32.

Friedman JM, Gutmann DH, MacCollin M, Riccardi VM. 1999. Neurofibromatosis: Phenotype, Natural History, and Pathogenesis: Johns Hopkins University Press. 381 p.

Gutmann DH, Wu YL, Hedrick NM, Zhu Y, Guha A, Parada LF. 2001. Heterozygosity for the neurofibromatosis 1 (NF1) tumor suppressor results in abnormalities in cell attachment, spreading and motility in astrocytes. Hum Mol Genet 10(26):3009-16.

Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. 1994. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nat Genet 7(3):353-61.

Jessen KR, Mirsky R. 1991. Schwann cell precursors and their development. Glia 4(2):185-94.

Kalcheim C, Burstyn-Cohen T. 2005. Early stages of neural crest ontogeny: formation and regulation of cell delamination. Int J Dev Biol 49(2-3):105-16.

Kogut MH, Genovese KJ, He H. 2007. Flagellin and lipopolysaccharide stimulate the MEK-ERK signaling pathway in chicken heterophils through differential activation of the small GTPases, Ras and Rap1. Mol Immunol 44(7):1729-36.

Le Douarin NM, Creuzet S, Couly G, Dupin E. 2004. Neural crest cell plasticity and its limits. Development 131(19):4637-50.

Lee PR, Cohen JE, Tendi EA, Farrer R, GH DEV, Becker KG, Fields RD. 2004. Transcriptional profiling in an MPNST-derived cell line and normal human Schwann cells. Neuron Glia Biol 1(2):135-147.

Lisak RP, Bealmear B. 1991. Antibodies to interleukin-1 inhibit cytokine-induced proliferation of neonatal rat Schwann cells in vitro. J Neuroimmunol 31(2):123-32.

Lobsiger CS, Taylor V, Suter U. 2002. The early life of a Schwann cell. Biol Chem 383(2):245-53.

Mattingly RR, Kraniak JM, Dilworth JT, Mathieu P, Bealmear B, Nowak JE, Benjamins JA, Tainsky MA, Reiners JJ, Jr. 2006. The mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD184352 (CI-1040) selectively induces apoptosis in malignant schwannoma cell lines. J Pharmacol Exp Ther 316(1):456-65.

Miller SJ, Rangwala F, Williams J, Ackerman P, Kong S, Jegga AG, Kaiser S, Aronow BJ, Frahm S, Kluwe L and others. 2006. Large-scale molecular comparison of

human schwann cells to malignant peripheral nerve sheath tumor cell lines and tissues. Cancer Res 66(5):2584-91.

Ratner N, Elbein A, Bunge MB, Porter S, Bunge RP, Glaser L. 1986. Specific asparagine-linked oligosaccharides are not required for certain neuron-neuron and neuron-Schwann cell interactions. J Cell Biol 103(1):159-70.

Rutkowski JL, Wu K, Gutmann DH, Boyer PJ, Legius E. 2000. Genetic and cellular defects contributing to benign tumor formation in neurofibromatosis type 1. Hum Mol Genet 9(7):1059-66.

Sawada S, Florell S, Purandare SM, Ota M, Stephens K, Viskochil D. 1996. Identification of NF1 mutations in both alleles of a dermal neurofibroma. Nat Genet 14(1):110-2.

Serra E, Rosenbaum T, Winner U, Aledo R, Ars E, Estivill X, Lenard HG, Lazaro C. 2000. Schwann cells harbor the somatic NF1 mutation in neurofibromas: evidence of two different Schwann cell subpopulations. Hum Mol Genet 9(20):3055-64.

Sieber-Blum M, Grim M, Hu YF, Szeder V. 2004. Pluripotent neural crest stem cells in the adult hair follicle. Dev Dyn 231(2):258-69.

Vogel KS, Brannan CI, Jenkins NA, Copeland NG, Parada LF. 1995. Loss of neurofibromin results in neurotrophin-independent survival of embryonic sensory and sympathetic neurons. Cell 82(5):733-42.

Whitlon DS, Ketels KV, Coulson MT, Williams T, Grover M, Edpao W, Richter CP. 2006. Survival and morphology of auditory neurons in dissociated cultures of newborn mouse spiral ganglion. Neuroscience 138(2):653-62.

Wood PM. 1976. Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. Brain Res 115(3):361-75.

Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF. 2002. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science 296(5569):920-2.

CHAPTER III

INFLUENCE OF HORMONES AND HORMONE METABOLITES ON THE GROWTH OF SCHWANN CELLS DERIVED FROM MOUSE EMBRYONIC STEM CELLS AND TUMOR CELL LINES EXPRESSING VARIABLE LEVELS OF NEUROFIBROMIN

ABSTRACT

Loss of neurofibromin, the protein product of the tumor suppressor gene Neurofibromatosis Type 1 (NF1), is associated with neurofibromas, which are comprised largely of Schwann Cells. The number and size of neurofibromas in NF1 patients has been shown to increase during pregnancy. Mouse embryonic stem cells with varying levels of neurofibromin were differentiated into Schwann-like cells. These cells and NF1 tumor cell lines derived from a malignant and a benign human tumor were used to study proliferation in response to hormones. Estrogen (ER) and androgen (AR) receptors showed very low/no expression in the NF1+/+ cells, low levels of expression in NF1+/- cells, and more intense expression in NF1-/-cells. Assays on mouse tissues in which cre recombinase targeted the loss of NF1 in Schwann Cell lineage cells showed similar ER and AR expression. Expression of progesterone receptor (PR), however, was also robust in NF1-deficient tissues, suggesting an important role for the tumor microenvironment. We have also found that an E2 metabolite that is not ER-dependent, 2Methoxyestradiol (2ME2), is cytotoxic to the NF1-/- malignant tumor cell line, and inhibits proliferation in

the other cell lines. 2ME2 or its derivatives could provide new treatment avenues for NF1 hormone-sensitive tumors at times of greatest hormonal influence.

INTRODUCTION

Neurofibromatosis type 1 Origin and Loss of Heterozygosity

Neurofibromatosis type 1 (NF1) is the most common human cancer predisposition syndrome affecting the nervous system, affecting 1/3000 to 1/3500 live births world-wide. NF1 tumors are heterogeneous, and Schwann Cells (SC) or SC precursors have been shown to be the initiating cell type (Serra et al., 2001; Zhu et al., 2002). Loss of heterozygosity has been found in some, but not all neurofibromas, and in some but not all cell types within the neurofibroma (Menon et al., 1990; Daschner et al., 1997; Rasmussen et al., 2000).While neurofibromin is generally a microtubule-associated cytoplasmic protein (Gregory et al., 1993), it has also been found to be actively transported to the nucleus (Vandenbroucke et al., 2004).

The manifestations of NF1 are highly variable, even among members of the same family with the same mutation. However, hallmarks of NF1 include tumors of the peripheral nervous system, which are usually benign and often first appear at puberty (McLaughlin and Jacks, 2003; Fishbein et al., 2007). Although they are rarely present at birth, they are found in 48% of 10 yr olds (notably, precocious puberty is a common feature of NF1 (Virdis, 2000)), 84% of 20 year olds, and virtually all NF1 patients over the age of 40, (McGaughran et al., 1999; DeBella et al., 2000). Neurofibromas can also arise from multiple nerves within plexuses, which are termed plexiform neurofibromas

(Woodruff, 1999; Gutmann and Giovannini, 2002). Plexiform neurofibromas are first seen in early childhood and are capable of aggressive growth, particularly as puberty approaches or during pregnancy (Dugoff and Sujansky, 1996). About 5% of plexiform neurofibromas undergo malignant transformation and eventually become malignant peripheral nerve sheath tumors (MPNSTs) (Korf, 1999; Woodruff, 1999). Contributions from other mutations, at *NF1* or other loci, environmental conditions including trauma and the elevated levels of specific hormones seen at puberty and during pregnancy, may also be "triggers" for tumorigenesis or enlargement. Such stimuli could be responsible for initial growth of the tumors, for increases in tumor size and number during pregnancy, puberty or exogenous hormonal stimulation and for malignant transformation (Posma et al., 2003). Reports indicate that up to 80% of pregnant women with NF1 experience an increase in tumor size and/or number during pregnancy, with one third of these lesions regressing in the postpartum period, suggesting a hormonal influence (Dugoff and Sujansky, 1996).

Hormonal Influence on NF1 tumor growth and progression

A recent study of 59 NF1 patients who were taking either oral contraceptives or who had contraceptive implants, found that only those women with contraceptive subdermal implants containing high doses of synthetic progesterone (150 mg medroxyprogesterone acetate or 200 mg norethisteron enanthat) experienced an increase in the size and numbers of their tumors; those on combined oral contraceptives experienced no significant effects on neurofibroma growth (Lammert et al., 2005).

Prospective studies, such as cited above, involved patients with relatively mild forms of the disorder whose pregnancies were obstetrically "uneventful" even if there were reported increases in the size and number of their neurofibromas (Dugoff and Sujansky, 1996). In contrast, a large number of "retrospective" case studies by obstetricians and gynecologists tended to involve only a few women (Ansari and Nagamani, 1976), or even single patients, (Posma et al., 2003) and reported severe and therefore particularly notable effects. A second pregnancy resulted in one case in the overgrowth of a peripheral nerve sheath tumor that had initially arisen during a first pregnancy, which proved fatal to the patient in the second pregnancy (Posma et al., 2003). Another case report found that a patient with initially mild disease symptoms died of the results of multiple malignancies, which the physician believed arose and became worse during and after pregnancy and delivery (Heffner, 1969; Boiten et al., 1987).

The Hormonal Milieu During Pregnancy

Concentrations of the steroid hormones 17β-estradiol (E2), progesterone (P4) and testosterone (T) increase during pregnancy (Witorsch, 2002; Fernandez-Valdivia et al., 2005; Okada et al., 2005; Rodriguez-Cuenca et al., 2006). E2 has been shown to be involved in cell proliferation, and is a ligand for the Estrogen Receptor (ER) (Revankar et al., 2005). P4 is modified to E2 and is involved in both proliferation and differentiation (Fernandez-Valdivia et al., 2005); P4 is the ligand for the Progesterone Receptor (PR) (Fernandez-Valdivia et al., 2005). The PR is regulated by E2 by transactivation through the ER (Fernandez-Valdivia et al., 2005; Okada et al., 2005). Testosterone is the primary circulating androgen, even in women, and is the ligand for the androgen receptor (AR), although it can rarely cross-react with both the ER and PR at very low affinity (Gao et al.,

2005). Steroids can rarely cross-react and bind to receptors other than their native receptor, because of the similarity of their receptor conformation, even though the actual sequence identity may be low (Gao et al., 2005). Hormone receptor-positive breast cancer cells were shown to be more likely to respond to hormone antagonist treatment (Jacobsen et al., 2003; Jacobsen et al., 2005). A study done on both male and female neurofibroma tumors found 75% expressed PR, while 5% expressed ER, regardless of the sex of the patient, although neither co-expression nor expression of AR was tested (McLaughlin and Jacks, 2003). Recently, Fishbein et al. found that tumor-derived SC cultures showed heterogeneous results for proliferation and apoptosis using steroid hormone ligands and receptors. Statistically significant changes applied to only a subset of tumor cells, regardless of gender (Fishbein et al., 2007).

Antimetabolites/Antagonists for Hormones and Angiogenesis

2-methoxy-estradiol (2ME2) is a naturally occurring E2 metabolite that rises in serum during pregnancy (Wang et al., 2000) and is a potent antiangiogenic factor, although it is not ER-dependent (Wang et al., 2000; Dingli et al., 2002). 2ME2 has been found to inhibit tumor cells (e.g. breast cancer, prostate cancer, and ovarian carcinoma) by destabilizing and depolymerizing microtubules (MT) and impairing hypoxia inducible factor 1 (HIF-1) accumulation in the nucleus (Wang et al., 2000; Mabjeesh et al., 2003; Ireson et al., 2004). HIF-1 also induces vascular endothelial growth factor (VEGF) expression, therefore inhibition of HIF-1 results in inhibition of VEGF expression that is required for angiogenesis (Mabjeesh et al., 2003). Tumors require angiogenesis to grow larger than 1 mm in size due to the limits of nutrient diffusion to cells, therefore 2ME2 may be acting indirectly to inhibit tumor growth through inhibition of angiogenesis,

although that cannot be the reason 2ME2 has apoptotic effects on cultured tumor cells (Wang et al., 2000; Mabjeesh et al., 2003). In such cells, 2ME2 acts by disrupting microtubules, with prolonged (24-72 hours) exposure to malignant tumor cells resulting in apoptosis (Wang et al., 2000; Mabjeesh et al., 2003).

An embryonic stem cell-based model of SC differentiation for studies of NF1 tumorigenesis

We recently reported a new stem cell based model for studies of tumorigenesis in NF1 (Roth et al., 2007). This *in vitro* system has been used to differentiate mouse embryonic stem cells (mESC), which are NF1 wild type (+/+), heterozygous (+/-), or null (-/-) into Schwann Cell-like cells for studies of NF1 in SC development and tumor formation. Regardless of their NF1 status, all of these cells were shown to express SC markers appropriate for their maturation stage, including myelin proteins. These stem cell-derived SC (mESC) can also preferentially direct neurite outgrowth from primary peripheral neurons. We reported that the NF1 -/- and +/- SC-like stem cell-derived cells proliferated more rapidly than NF1 (+/+) cells but that proliferation rates reverted to wild type levels after treatment with an inhibitor of MAP kinase kinase (Mek) (Roth et al., 2007). We further demonstrated that mESC of all NF1 types could be differentiated into neuron-like cells. This novel stem cell based model system provides an ideal model to study of the role of hormones on the cell growth and differentiation of the different cell types affected by NF1 in cells with differing levels of neurofibromin that are neither transformed nor malignant. In this study, we have focused on the SC-like cells derived from these stem cells.

MATERIALS AND METHODS

Cell Types: The cell lines used for these studies include the NF1+/+ (D3) mES cell line (Doetschmann, 1985), NF1+/- (SKO) mES cells (Jacks et al., 1994), NF1-/- (DKO) mES cell line (Jacks et al., 1994), SW10 (NF1+/+) mouse Schwann Cell line with a temperature sensitive SV40 large T antigen, grown at 37°C (the non-permissive temperature for transgene expression) for differentiation (ATCC) (Hai et al., 2002), pNF00.11 (referred to as PNF cells in this report) (NF1-/-) human plexiform neurofibroma (Muir et al., 2001), ST88-14 (referred to as ST cells in this report) (NF1-/-) human MPNST (gift of Dr. Larry Sherman, Oregon Health Sciences University) (Su et al., 2004). Positive control cell lines for hormone receptors were: ER+ and PR+ MCF-7 cells (gift of Dr. Dorraya El-Ashry, Univ. of MI) and AR+ LNCaP cells and mouse breast and prostate tissues (gift of Dr. Diane Robins, Univ. of MI).

Differentiation of mES cells to SC-like cells*:* We had previously found that mES cells that were wild type, heterozygous or homozygous for the NF1 gene could be differentiated into neuron-like cells and SC-like cells, (Roth et al., 2007) the latter of which more closely approximate the initiating tumor cell type in NF1. SC-like differentiated mES cells will be referred to as D3SC (NF1+/+), SKOSC (NF1+/-) and DKOSC (NF1-/-), depending on the number of NF1 alleles expressed.

Media: Proliferating (ES) cells medium consisted of 81% DMEM without Phenol Red, 1% L-Glut, 1% Pen/Strep, 1% non-essential amino acids (Gibco, Carlsbad, CA), 15% fetal bovine serum (FBS) (Atlanta Biological, Norcross, GA), 1% Sodium Pyruvate(2% stock), 7 μL/L βMercaptoethanol (Sigma, St. Louis, MO), 1000 U/mL ESGRO (Chemicon, Temecula,

CA). **Schwann Cell Differentiation (SC) medium** consisted of 84% a-mod. MEM without Phenol Red, 1% Pen/Strep (Gibco), 10% FBS (Atlanta Biological), 5% 11-day chick embryo extract, 10 ng/mL neuregulin NRG-1 (R&D systems, Minneapolis, MN). **Tumor cell medium for growth of PNF(Muir et al., 2001) and ST(Su et al., 2004) cell lines** contained 84% DMEM without Phenol Red, 1% Pen/Strep (Gibco), 25 ng/mL NRG-1 (R&D systems), 15% FBS. **SW10 SC cell line medium** consisted of 88% DMEM without Phenol Red, 1% L-Glutamine, 1% Pen/Strep, 10% FBS (Atlanta Biological). Note: Regular FBS was replaced by charcoal-stripped FBS (Valley Biomedical, Winchester, VA) in all media used for hormone assays. **Minimal medium** consisted of 87% DMEM without Phenol Red, 10% FBS, 1% Pen/Strep, 1% L-Glutamine, 1% Sodium Pyruvate (2% stock).

Antibodies: All antibodies were diluted in 10% donkey serum (Chemicon International, Temecula, CA) in 0.1% Tween20/Phosphate-Buffered Saline (TBST) (Sigma, St. Louis, MO). ERα (rabbit polyclonal 1:50), AR (rabbit polyclonal 1:50) NF1 (rabbit polyclonal 1:50) (Santa Cruz, Santa Cruz, CA), Tuj1 (mouse monoclonal 1:500) (Covance, Berkeley, CA), Neurofilament (rabbit polyclonal 1:400) (Chemicon International), S100 (rabbit polyclonal 1:200 Novocastra, Newcastle Upon Tyne, UK or mouse monoclonal 1:50 Abcam, Cambridge, MA), GFAP (mouse monoclonal 1:400) (Chemicon), myelin (mouse monoclonal 1:10) (Abcam), PR (mouse monoclonal 1:50) (Abcam), αtubulin (mouse monoclonal 1:200), Caspase-3 (mouse monoclonal 1:1000) (BD Transduction, San Jose, CA), AlexaFluor 350, 488, 594, DAPI (Molecular Probes, Eugene, OR), goat α rabbit-HRP (Zymed, San Francisco, CA). Note: We also looked at ERβ expression, but did not see expression in any of the cells or tissues tested. Therefore ER=ERα in this dissertation. **Hormones and receptor inhibitors** used included progesterone, RU486, 17β-estradiol, 2ME2 (Sigma) ICI 182,780 (Tocris),

testosterone and flutamide (gift of Dr. Diane Robins, University of MI). All were diluted in 100% ethanol, which was also used for vehicle wells. **Reagents** used included CellTiter96 cell proliferation assay kit (Promega), Histomouse (Zymed), Prolong Gold anti-fade (Molecular Probes), Porcine gel (Sigma), In situ cell death detection kit, fluorescein (TUNEL) (Roche, Indianapolis, IN), Citrisolv, Ethanol, Sodium citrate dihydrate (Fisher Scientific, Fair Lawn NJ).

Immunocytochemistry (ICC): On day 0, 40,000 cells were plated onto 0.1% porcine gel-coated coverslips (Corning, Corning, NY) and grown overnight. For microtubule visualization in the 2ME2 assay, varying concentrations of 2ME2 were added to the media; cells were grown an additional 24 hours. Cells were then fixed in 4% paraformaldehyde (Sigma), permeabilized in 0.2% TritinX100 (Sigma), stained and mounted on Superfrost plus slides (Fisher, Pittsburgh, PA). Fluorescence micrographs photographs were taken with an Olympus BX-51 microscope, and non-fluorescent micrographs with Nikon ACT-1 software on a Leitz Diavert inverted microscope.

Hormone Receptor Immunohistochemistry (IHC) on mouse tissue: The control mouse tissues used in this study were from a pool of phenotypically indistinguishable mice with four genotypes: NF1flox/+;P0A-cre+, NF1flox/flox;P0A-cre-, NF1flox/-;P0A-Cre-, and NF1+/-. The mutant mice used were of the genotype, NF1flox/-;P0A-cre+ (mutants). Twelve to twenty months old mice were monitored until signs of distress appeared, at which time they were subjected to necropsy. The P0A-cre transgenic strain was initially generated on the FVB background (Giovannini et al., 2000). After five generations of being backcrossed to the 129 Svj background, the P0A-cre transgenic mice were crossed to the NF1flox/- mice that were maintained on the 129 Svj background. Subsequent crosses generated control and

mutant mice for analysis (Zhu et al, 2002, Zheng et al, submitted). All mice in this study were cared for according to the guidelines that were approved by the Animal Care and Use Committees of the University of Michigan at Ann Arbor. For histological analysis, control and mutant littermates at 12-22 months of age were perfused with 4% paraformaldehyde (PFA). Sciatic nerves were dissected, post-fixed overnight for 2 hours at room temperature and transferred to 30% sucrose overnight. Sciatic nerves were embedded using OCT medium and sectioned longitudinally at 65 μm thicknesses using a cryostat. Mouse tissue slides were deparaffinized in Citrisolv and rehydrated in successive dilutions of ethanol. Antigen retrieval was performed by boiling slides for 10 min. in 0.01M sodium citrate buffer pH 6.0, followed by IHC at room temperature in a humidified chamber. Non-specific binding was blocked with 10% donkey serum, incubated with primary antibodies listed followed by secondary antibody incubation for 20 minutes and 5 minute DAPI staining. Washes between steps were done in 0.1% PBST. After coverslips were applied using Prolong Gold anti-fade mounting medium and dried in the dark overnight, photographs were taken at 40x magnification using an Olympus BX-51 microscope.

Measures of Cell Proliferation: A) Hormones and receptor inhibitors: Cells were grown overnight in hormone-free phenol red-free media. The next day, cells were counted, plated and allowed to attach for two hours in SC differentiation medium (containing hormone receptor inhibitor if specified) before hormone was added in the concentrations indicated. Inhibitors and hormones were replenished after 48 hours. At four days growth, proliferation was assayed using a Beckman Z1 Particle Coulter counter (Beckman Coulter, Inc., Fullerton, CA). Cell numbers were converted to percentages using proliferation of cells in ethanol-containing (vehicle) wells as 100%. Statistical analysis: data are expressed as mean +/- SD,

and the Student's t-test was used to gauge significance, which was ($p<0.05$). B) 2ME2: Cells were counted, plated, and allowed to attach overnight in hormone-free medium and phenol-red free medium. Varying concentrations of 2ME2 were added to the media; cells were grown an additional two days, proliferation was assayed using the CellTiter96 (Promega) proliferation assay and absorbance was measured by microplate reader (Fisher). Absorbance was converted to percentages using absorbance of cells grown in vehicle (EtOH) as 100%. Equation used: treated well/vehicle well x 100.

RTqPCR: RTqPCR was performed using primer pairs designed using the Beacon designer program (BioRad, Hercules, CA), target with Ta at 55º C, a length 18-22 bp and amplicon size 100 – 200. Gene expression was normalized to GAPDH. Total RNA from the samples was extracted using a Qiagen RNeasy Kit (Kit 74106, Qiagen, CA). RT-PCR was performed as follows: cDNA was synthesized from 2 µg total RNA by reverse transcription using Super Script III transcriptase (Invitrogen, Carlsbad, CA) and oligo dT primer. A 2 µL aliquot of the cDNA of each sample was used for PCR with the hormone receptor primers. The PCR conditions included an initial denaturation at 94°C for 1min, followed by 94°C for 1 min, 55°C for 30 sec and 74°C for 30 sec and 34 cycles and final extension at 72°C for 5 min with a 4°C holding temperature. The PCR products were separated on 2.0% agarose gels and visualized using ethidium bromide under UV light.

PCR primers: NF1: forward AGTTTCTCTCCTCGCTGGTCTTC reverse CGTTTCCTGCCACCCGTTTG; AR: Forward: GCGGTCCTTCACTAATGTCAACTC Reverse: TGCCTCATCCTCACACACTGG; PR: Forward: CTGGATGAGCCTGATGGTGTTTG Reverse: GGCACAGCGAGTAGAATGACAG; ER

Forward: GAAAGGCGGCATACGGAAAGAC Reverse:

TCAAGGACAAGGCAGGGCTATTC.

TUNEL assay: Apoptosis was measured by TUNEL assay. PNF and ST cells were plated, grown, fixed and permeabilized as in the 2ME2 ICC assay. The TUNEL assay was performed according to Roche kit specifications with the following modifications. Caspase-3 antibody was added at 1:1000 to the TUNEL reaction mixture and incubated at 37°C for 60 minutes in the dark. After PBS rinses, DAPI was added at 1:1000 for 3 minutes. Coverslips were mounted on slides with Molecular Probes' Antifade medium and dried 24 hours in the dark before fluorescence visualization.

RESULTS

The expression of steroid hormone receptors is correlated with NF1 expression in SC-like mES-derived cells

To determine whether the mES-derived SC-like cells or human SC tumor cell lines responded differently to the steroid hormones that are upregulated during pregnancy (P4, E2, and T), we first determined whether these cells expressed the appropriate receptors (PR, ER, AR) for the relevant classical steroid receptor pathways. We found that the level of classical receptor expression varied depending on the level of neurofibromin expressed by the cell type/line. While PR (figure 3.1A, first column) was not intensely expressed in any of the cell lines, the NF1-/- (DKOSC, PNF, ST) cells had the most intense staining, although the Schwann cell line SW10 also showed some PR expression (figure 3.1A, bottom row, left). ER expression (figure 3.1A, third column) and AR expression (figure 3.1A, fifth column)

followed similar patterns to each other, with little/no expression in the NF1+/+ (D3SC, SW10) cells, low levels of expression in NF1+/- (SKOSC) cells, and more intense levels of expression in NF1-/- (DKOSC, PNF, ST) cells. While PR and AR expression was nuclear, ER localization was seen in both the nucleus and the cytoplasm, as has been previously noted (Ho and Liao 2002; Revankar et al. 2005; Zhang et al. 2004). The differences in expression levels were confirmed in mouse SC-like cells by RTqPCR (figure3.1B). In RTqPCR assessments, D3SC cells expressed 20% of the amount of PR, 0.5% of the ER and 9.4% of the AR seen in DKOSC cells (set at 100%), while SKOSC cells expressed 24% of the amount of PR, 0.25% of the ER and 41.6% of the AR expressed by DKOSC cells (figure 3.1C). We then co-stained for both steroid hormone receptors and SC markers in the same cells in order to verify co-expression levels (figure 3.2A-C).

In order to assay whether *in vivo* mouse tissue with or without NF1 expression show similar patterns of classical hormone receptor expression as mouse cells *in vitro* did, we obtained tissue from mice in which cre transgene targeted the loss of NF1 in cells only from the Schwann Cell lineage and stained for classical hormone receptor expression. Mice which carried the cre transgene (cre+) developed hyperplasia in trigeminal nerves and neurofibroma tumors in dorsal root ganglia (DRG) (figure 3.3). High levels of ER (green, middle columns) and AR (green, right columns) were seen in NF1-deficient mouse tissues (cre+), but not in mouse tissue with normal NF1 expression levels (cre-), similar to the expression patterns seen in cultured mouse cells (figure 3.3). In contrast to the *in vitro* assays, however, high levels of PR (red, left columns) expression were also seen in NF1-deficient mouse tissues (cre+) (figure 3.3).

Hormonal Effects on Cell Proliferation

Because expression of hormone receptors was found to be associated with the level of NF1 expression in the cells tested, with DKOSC cells expressing much more intense levels of the receptors than either the D3SC or SKOSC cells (figure 3.1B, figure 3.2A-C), we assayed the cells for hormonal effects on cell proliferation. Concentrations of the hormones were chosen based on dose response curves that determined the optimal doses that induced proliferation rather than differentiation (not shown). After growing the cells overnight in hormone-free phenol-red free media, we added one of the ligands for the steroid hormone receptors (P4, E2, and T) and/or its respective nuclear receptor inhibitor (RU486, ICI 182, 780 or Flutamide) (Wang et al., 2000; Witorsch, 2002) (figure 3.4). Addition of P4 (figure 3.4A) increased proliferation significantly only in SW10, ST and DKOSC cells. The effect of the PR inhibitor RU486 was cell type-specific, ranging from significant inhibition even below control levels (SW10 and PNF) to no significant effect (ST, D3SC, SKOSC, DKOSC) (figure 3.4A) n=5. Addition of E2 (figure 3.4B) or T (figure 3.4C) significantly increased proliferation of malignant NF1 tumor cells (ST) and DKOSC and D3SC cells. However, the NF1-/- cell lines ST and DKOSC cells were the only cell lines that showed a significant increase in proliferation with all three of the hormones tested. There was generally a slight trend, although not a significant decrease, seen when their respective inhibitors for the classical receptors (ICI 182, 780 for ER, Flutamide for AR) were used to preincubate cells for 2 hours.

2-Methoxy-estradiol (2ME2) effects

In order to determine whether 2ME2, an estrogen metabolite that has been found to be upregulated during normal pregnancies (Wang et al., 2000), affected the growth of NF1-/- mES cells, we grew the SC-like mES cells, SW10 and the tumor cell lines for 3 days in 2ME2 concentrations between 0.1 μM and 100 μM. We determined that 10 μM was the threshold for significant changes in growth rates in all cells and cell lines tested (not shown). To determine if the effect was cytotoxic (causing apoptosis) or only to decrease proliferation, we grew all cells for two days in 10 μM 2ME2. Malignant ST cells were the only cell type to show a significant *decrease* in cell number when compared to the initial cell number (figure 3.5A). All other cells had either static cell numbers (PNF, DKOSC, and D3SC), or merely decreased proliferation rates (SKOSC, SW10) compared to control cells grown without 2ME2 addition.

Effects of 2ME2 Exposure on Microtubules

The mechanism of 2ME2 action in cultured cells is reported to be through disruption of microtubules (Mabjeesh et al., 2003), and not through effects on the classical ER itself, for which it has very low affinity (Ireson et al., 2004). We therefore examined tumor cells grown with or without added 2ME2 for expression of αtubulin to visualize any changes in these cytoskeletal elements with increasing concentrations of 2ME2. We found that with the addition of 10 μM 2ME2 (figure 3.5B, middle column), the PNF cells rounded up but their nuclei (stained with DAPI) remained intact and the cells retained some cytoskeletal structure. In the ST cells, however, cells appeared to have the classic apoptotic profile (Edinger and Thompson, 2004). The cells rounded up, nuclei were “broken up” and appeared as clusters of

nucleic acid-containing material, and cell membranes were blebbed (Edinger and Thompson, 2004). The microtubules no longer were found in long extensions (figure 3.5B, left column), and tubulin protein was more diffuse in the cytoplasm. With 100 μM 2ME2 (figure 3.5B, right column), PNF cells also began to lose microtubule extensions, tubulin expression was diffuse, and 5-10% nuclei were becoming clustered and fragmented. ST cells became reduced in size (less than half the normal diameter) and apoptotic, with very little cytoplasm extending beyond the nucleus, thus visually exhibiting a much stronger effect from the 2ME2 on the cytoskeleton. To confirm that these malignant ST cells were becoming apoptotic in 2ME2, we assayed them for both caspase-3 expression and TUNEL staining. While less than 1% of benign PNF tumor cells were TUNEL+ and Caspase-3+, approximately 20% of ST cells stained positive for these apoptotic markers after 24 hours (figure 3.5C).

DISCUSSION

Because NF1 tumors often grow in size and increase in number during pregnancy (Ansari and Nagamani, 1976; Dugoff and Sujansky, 1996), suggesting a hormonal influence, we conducted experiments to determine whether the mES-derived SC-like cells or human SC tumor cell lines with variable levels of neurofibromin responded differently to the steroid hormones that are upregulated during pregnancy (P4, E2, and T) (Fernandez-Valdivia et al., 2005; Okada et al., 2005; Rodriguez-Cuenca et al., 2006). We first determined that these cells expressed the appropriate receptors (PR, ER, AR) for the relevant classical steroid receptor pathways (Chen et al., 2005; Okada et al., 2005; Ozawa, 2005; Sonneveld et al., 2006). We found that the level of receptor expression

varied depending on whether the cell type/line expressed neurofibromin and at what levels. NF1-/- (DKOSC, PNF, ST) cells had the most intense levels of PR expression, while ER and AR expression followed similar patterns to each other, with very low/no expression in the NF1+/+ (D3SC, SW10) cells, low levels of expression in NF1+/- (SKOSC) cells, and most intense expression in NF1-/- (DKOSC, PNF, ST) cells. The differences in expression levels were confirmed in the mouse SC-like cells by RTqPCR. These findings suggest that NF1 mutant cells can more readily respond to hormones that rise during pregnancy. Since at least a part of this increase is inhibited by the classical receptor inhibitors, some of this increase can be attributed to these classical pathways. Other possible non-genomic pathways include second messenger cascades involving G-protein coupling, cAMP and MAPK pathway activation that affects Ca^{++} channels, and PI3K and Akt pathways (Zhang et al., 2004). PR and AR localization was exclusively nuclear, and ER was seen in both the nucleus and the cytoplasm, in accord with the findings of others (Ho and Liao 2002; Revankar et al. 2005; Zhang et al. 2004). Breast cancer tumor cells that express both ER and PR have been found to have a better prognosis because they are more likely to respond to hormone antagonist treatment (Jacobsen et al., 2003), although tumors that express only ER or PR, but not both, have been reported to have a poorer outcome (Jacobsen et al., 2005). McLaughlin and Jacks (2003) suggest an important role of P4 in tumor development regardless of the patient's sex; however, tumor cell type was not identified and tumors were not tested for co-expression of ER and PR; AR expression was not tested or not reported in their study. Positive expression of tumors was defined in their study as slides with 5 or more positive cells per 10 high power fields, with virtually none of the sections containing more than

one hundred positive cells (McLaughlin and Jacks, 2003). We assayed for in vivo expression of classical hormone receptors (PR, ER, AR) in mouse tissues engineered to target loss of NF1 in Schwann Cell lineage cells carrying a cre transgene (Zhu et al., 2002, Zheng et al. submitted), which subsequently developed hyperplasias (trigeminal nerve) or neurofibroma tumors (dorsal root ganglia). We found high levels of expression of all three classical receptors (ER, AR, PR) in NF1-deficient tissues (cre+), and low/no expression in tissues with normal levels of NF1 expression (cre-). This is in contrast to our *in vitro* findings, where ER and AR were upregulated in NF1-/- cells, but PR was not. We attribute this difference to the importance of the microenvironment in tumorigenesis, since surrounding cells and their resultant signals with and in addition to the NF1-deficient cells could also be affecting hormone receptor expression. Fishbein et al. found differing heterogeneous levels of ER, PR and AR in most NF1 tumor samples and normal SCs tested. Primary tissue samples they tested showed higher variation than cultured samples, implying that cells other than SC express hormone receptors (Fishbein et al., 2007). Our results indicate that E2 is likely also to be an important hormone, although its effects may be enhanced or influenced by P4. Since T is a precursor for E2, proliferative effects on cells could be mediated through aromatase activity (Gao et al., 2005), or through non-classical indirect pathways by increasing intracellular Ca^{++} (Chen et al., 2005), rather than through the AR itself. For example, anabolic effects of androgens in muscle may be mediated through insulin-like growth factor-1, perhaps through upregulation of IGF receptors. Androgens can also oppose the action of glucocorticoids or have a dominant negative effect on glucocorticoid receptors (GR) either by

downregulating GR or competing for GR binding, and glucocorticoids can also bind to the AR (Chen et al., 2005).

Expression of hormone receptors was found to be associated with the level of NF1 expression in the cells tested in the stem cell model of SC differentiation, with NF1-/- (DKOSC) SC-like cells expressing much higher levels (up to 400x) of the receptors than either the NF1+/+ (D3SC) or NF1+/- (SKOSC) SC-like cells. We therefore assayed the cells for hormonal effects on cell proliferation. Concentrations were chosen based on dose response curves that determined the optimal proliferation response vs. differentiation (not shown). Although the increases in proliferation we found were modest, they were significant. Addition of P4 (figure 3.4A) increased proliferation significantly only in SW10, ST and DKOSC cells, possibly because P4 can also be involved in cell differentiation and modulation of E2 proliferative effects (Jacobsen et al., 2003). E2 and P4 can have complementary functions, with P4 inhibiting the ER (Jacobsen et al., 2003). P4 and E2 can be used in combination to stimulate tumor development by simulating pregnancy in mice with dormant mammary tumors (Gattelli et al., 2004), where the combination of E2 and P4 caused the tumor cells to break dormancy and begin proliferating. P4 may need to be tested in combination with E2 to see a synergistic effect on proliferation, although we first wanted to determine each hormone's effect individually. Also, PR had the least expression of any of the receptors we tested in any of the SC-like mES cells with variable neurofibromin levels or in the tumor cell lines, even though it had previously been found that 75% of neurofibromas tested expressed PR and 5% expressed ER (McLaughlin and Jacks, 2003). Perhaps this is because here we are testing cell lines, rather than the cellularly heterogeneous tumors

assayed in the McLaughlin and Jacks paper. McLaughlin and Jacks also did not assay for the number of receptors per cell, but just the presence of PR and ER in neurofibromas. We found that the effect of the PR inhibitor RU486 was variable, ranging from significant inhibition of cell proliferation even below control levels in some cell types (SW10 and PNF) to no significant effect (ST, D3SC, SKOSC, DKOSC). RU486 is also an inhibitor of AR and GR that could also influence the results, although since these cells were grown in hormone-free media with only P4 added, this influence should be minimized. Addition of E2 or T significantly increased proliferation of malignant NF1 tumor cells (ST) and DKOSC and D3SC cells. However, the NF1-/- cell line ST, derived from the human malignant tumor and the mES-derived NF1-/- (DKOSC) SC-like cells were the only cell lines that showed a significant increase in proliferation with all three of the hormones tested. There was generally a slight trend, although not a significant decrease in proliferation when their respective inhibitors (ICI 182, 780 for ER, Flutamide for AR) were used to preincubate cells for 2 hours. This may be because the hormone action is not mediated exclusively through classical pathways (McEwen and Alves, 1999; Ho and Liao, 2002; Zhang et al., 2004; Chen et al., 2005; Gao et al., 2005; Jacobsen et al., 2005; Revankar et al., 2005; Sonneveld et al., 2006). There have been reports of steroid hormones (P4, E2, T) influencing cells independent of their classical receptors (PR, ER, AR) (McEwen and Alves, 1999; Ho and Liao, 2002; Zhang et al., 2004; Chen et al., 2005; Gao et al., 2005; Jacobsen et al., 2005; Revankar et al., 2005; Sonneveld et al., 2006). E2 has also been shown to modulate secondary messengers, such as Ca^{++} and NO, and activate the PI3K/Akt and MAPK pathways (McEwen and Alves, 1999; Ho and Liao, 2002; Revankar et al., 2005). These effects are not inhibited by ER inhibitors,

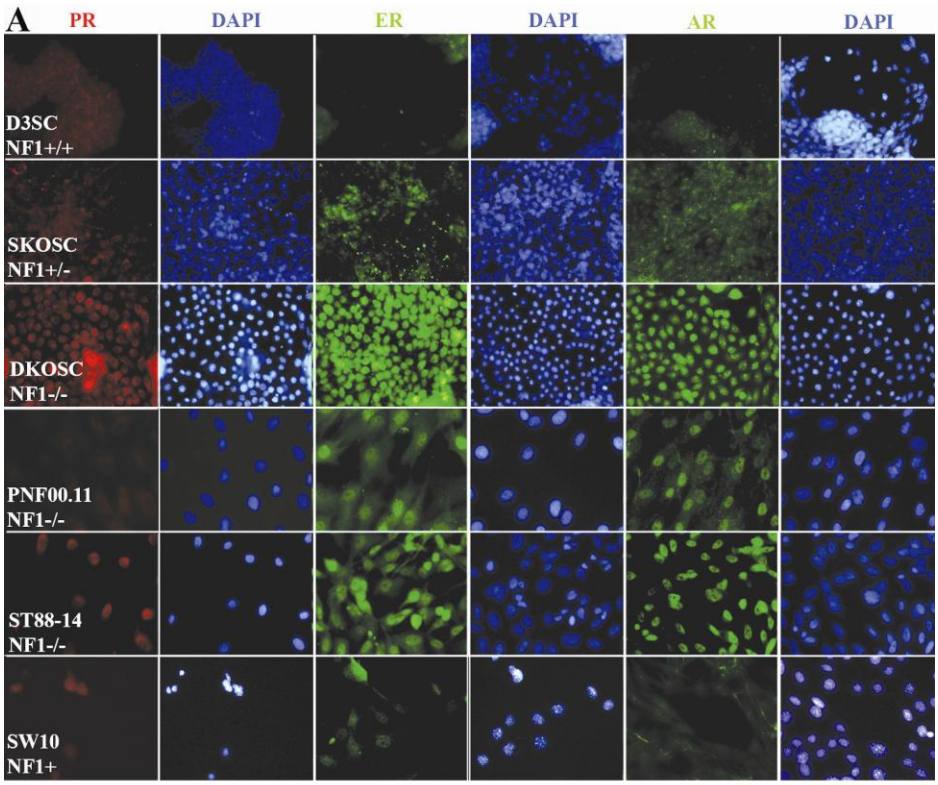
suggesting they are not mediated through the classical receptors (Ho and Liao, 2002). Many of the E2-stimulated pathways are initiated at the plasma membrane, suggesting that they could be mediated by an unidentified G-protein coupled receptor (Ho and Liao, 2002). Nongenomic actions of androgen with plasma membrane-associated signalling pathways involve activation of kinase signalling cascades or modulation of intracellular Ca^{++} thought to be mediated through interaction of AR and cytosolic pathway proteins (Gao et al., 2005). Conversely, steroid receptors have been shown to exert their influence without hormone stimulation (Chen et al., 2005; Jacobsen et al., 2005; Sonneveld et al., 2006). Genes regulated by unliganded PR encode membrane associated, cell-cycle regulatory, DNA repair and apoptosis proteins (Jacobsen et al., 2005). ERα has been found to activate the PI3K/Akt pathway in an E2-independent manner by binding constitutively to the p85 subunit of PI3K and activating PI3K/Akt pathway (McEwen and Alves, 1999; Ho and Liao, 2002). ER expression has been seen outside the nucleus (McEwen and Alves, 1999; Ho and Liao, 2002; Zhang et al., 2004; Revankar et al., 2005), for example, ERβ localized to the cytoplasm and plasma membrane of neurons and astrocytes, and was also seen in the myelin of oligodendrocytes and glia (Zhang et al., 2004). PR also regulates a cluster of G-protein signalling pathways, ras and multiple kinase pathways (Jacobsen et al., 2003).

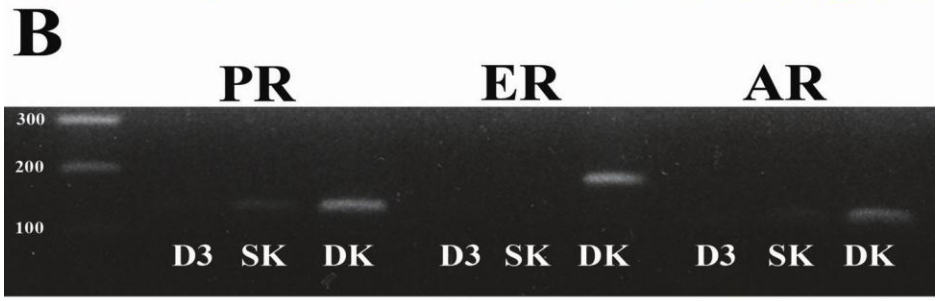
We also hypothesize that the complex hormonal milieu during pregnancy or the response to these hormones may be different in women with NF1. For example, 2-methoxy-estradiol (2ME2), which is an estrogen metabolite, has been found to be upregulated during pregnancy (Wang et al., 2000). We hypothesize that 2ME2 may either be present at lower levels systemically in women with NF1, resulting in tumor

overgrowth, or cell receptors for 2ME2 may be lacking in NF1-/- cells. 2ME2 has been found to be cytotoxic to numerous tumor cells as well as some other rapidly-growing cells (Mabjeesh et al., 2003), but not to normal or quiescent cells, by inducing apoptosis (Wang et al., 2000; Ireson et al., 2004). Wang et al. (Wang et al., 2000) found that normal thyroid cells first underwent cell cycle arrest with apoptosis initiated after prolonged (6 days) exposure. 2ME2 has no E2-like activity and has very low affinity for the ER; suggesting it is not ER-dependent (Wang et al., 2000; Ireson et al., 2004). The mechanism of 2ME2 action is reported to be through disruption of microtubules by destabilization and disassembly, and impairment of hypoxia inducible factor-1 (HIF-1) nuclear accumulation, thus inhibiting vascular endothelial growth factor (VEGF) expression (Mabjeesh et al., 2003). VEGF is required for angiogenesis, which in turn is needed for tumor growth beyond the size at which nutrients can diffuse. *In vitro*, 2ME2 competes with colchicine for tubulin binding sites and disrupts interphase microtubules, resulting in cell death (Mabjeesh et al., 2003). Neurofibromin has been found to associate and co-purify with microtubules, suggesting NF1 involvement in microtubule-mediated pathways (Gregory et al., 1993). We determined that 10 μM was the threshold for significant changes in growth rates in all cells and cell lines tested (not shown). We found that 2ME2, a naturally occurring estrogen metabolite, disrupts microtubules and is cytotoxic to malignant NF1 tumor cells while its effect is to slow or halt the growth of the other cell lines tested, regardless of their NF1 status. This suggests that perhaps additional mutations, for example p53, which has been associated with MPNSTs (Menon et al., 1990), are needed for 2ME2 cytotoxic effects specific to malignant tumors, since non-malignant NF1 tumor cells did not undergo apoptosis with 2ME2 treatment.

Future studies will focus on the effects of these hormones separately and in combination, on these stem cell derived SC-like cell lines and other cells whose NF1 status is known, including other malignant NF1 tumor cell lines. Because of the complex hormonal milieu during pregnancy, it will be necessary to look for synergy among hormone effects. We will also investigate the mechanisms of these hormones' effects through other mechanisms than their classical receptors. We also hypothesize that the effect of hormones on neurofibromas may be indirect through hormonal effect on angiogenesis. Increased blood vessel formation during pregnancy could allow existing tumors to grow, as well as increase microscopic tumors to grow to detectable sizes. It is also worth noting that while most of the cultured cells in this study are mouse cells, the tumor cell cultures are from human neurofibromas, which may explain some of the differing results.

Figure 3.1: A: SC-like cells and NF1 tumor cells express varying levels of steroid hormone receptors. DAPI staining of cell nuclei is shown to the right of the hormone receptor assayed. Left column, **PR**: Low levels of PR expression were seen in all cell types, with some upregulation in NF1-/- (DKOSC, PNF, ST) cells. Third column, **ER** and fifth column **AR**: Very low levels of both ER and AR seen in NF1+/+ (D3SC, SW10) cells, a slightly higher level in NF1+/- (SKOSC) cells, and most intense levels in NF1-/- (DKOSC, PNF, ST) cells suggests a stronger ability of NF1-/- cells to respond to hormones upregulated during pregnancy. Some cell types displayed both nuclear and cytoplasmic expression of ER. Photographs were taken at 40x magnification. **B:** Using gel visualization, expression of hormone receptors was verified by RTPCR (D3=D3SC, SK=SKOSC, DK=DKOSC). **C:** RTqPCR shows percentage of hormone receptor expression of D3SC and SKOSC cells in comparison to DKOSC cells (DKOSC expression set at 100%). D3SC cells expressed 20% the amount of PR, 0.5% ER and 9.4% PR of DKOSC cells, while SKOSC cells expressed 24% the amount of PR, 0.25 ER and 41.6% AR of DKOSC cells.


A
PR
DAPI
ER
DAPI
AR
DAPI
D3SC
NF1+/+
SKOSC
NF1+/-
DKOSC
NF1-/-
PNF00.11
NF1-/-
ST88-14
NF1-/-
SW10
NF1+


B
PR
ER
AR
300
200
100
D3 SK DK
D3 SK DK
D3 SK DK

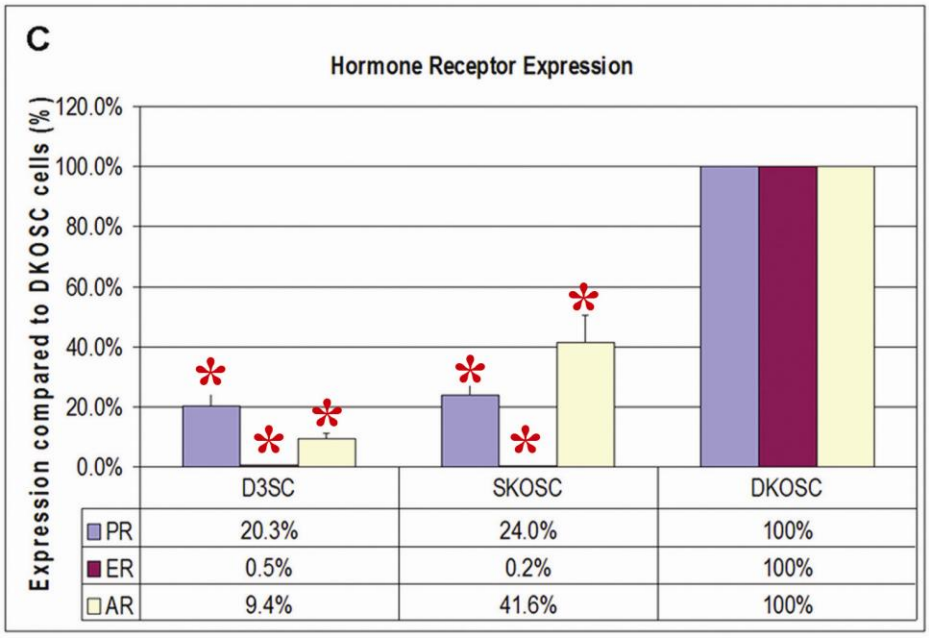

C
Hormone Receptor Expression
Expression compared to DKOSC cells (%)
120.0%
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
D3SC
SKOSC
DKOSC
PR 20.3% 24.0% 100%
ER 0.5% 0.2% 100%
AR 9.4% 41.6% 100%

Figure 3.2: Co-expression levels of steroid hormones and SC markers in SC-like differentiated mES cells. This verifies the co-expression levels of S100 and PR, GFAP and ER or S100 and AR in **A:** D3SC cells **B:** SKOSC cells and **C:** DKOSC cells. The staining for all three hormone receptors (PR, ER, AR) was most intense in the DKOSC (NF1-/-) cells (**C**), while all three cell types (D3 NF1+/+, SKOSC NF1+/-, and DKOSC NF1-/-) expressed the SC markers, S100 or GFAP. Photographs were taken at 40x magnification.

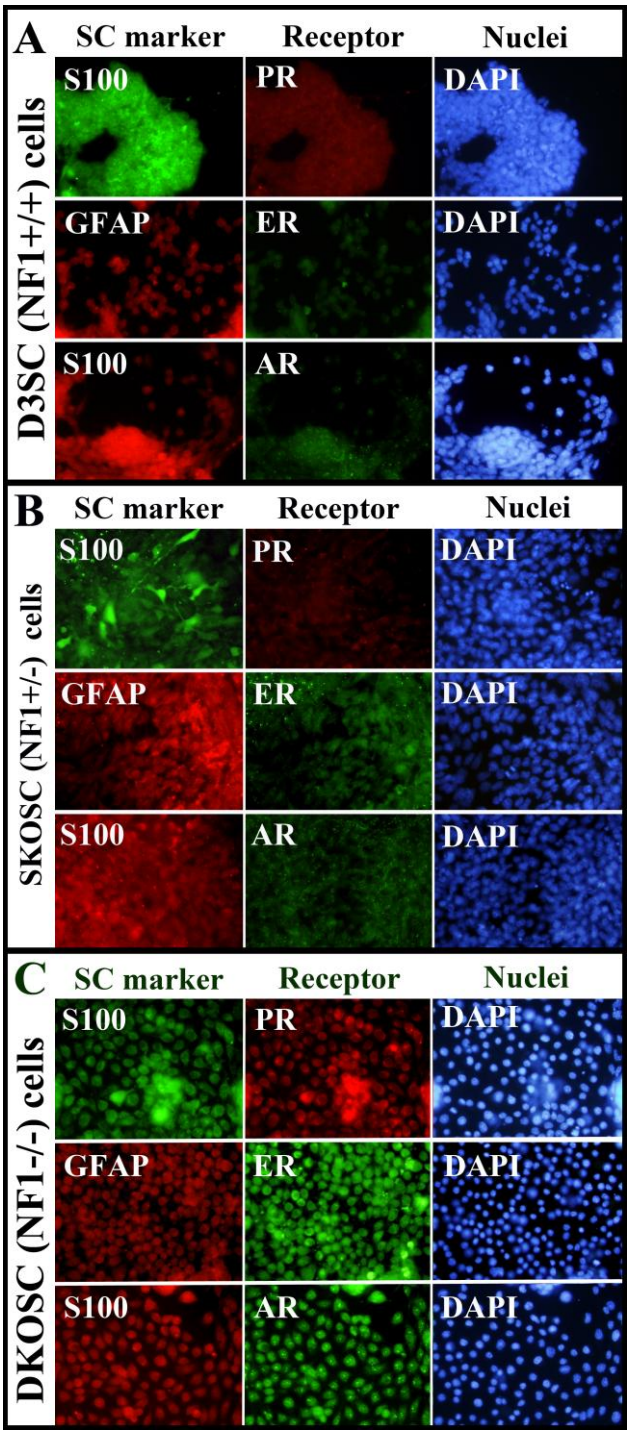

A
SC marker
Receptor
Nuclei
D3SC (NF1+/+) cells
S100
PR
DAPI
GFAP
ER
DAPI
S100
AR
B
SC marker
Receptor
Nuclei
SKOSC (NF1+/-) cells
S100
PR
DAPI
GFAP
ER
DAPI
S100
AR
DAPI
C
SC marker
Receptor
Nuclei
DKOSC (NF1-/-) cells
S100
PR
DAPI
GFAP
ER
DAPI
S100
AR
DAPI

Figure 3.3: Hormone receptor expression in mouse tissue. In order to assay for classical hormone receptor expression *in vivo*, we obtained mice in which cre recombinase expression targets loss of floxed NF1 expression to cells in the Schwann Cell lineage (Zhu et al., 2002, Zheng et al., submitted). Mice transgenic for the cre transgene developed hyperplasia in the trigeminal nerve and neurofibroma tumors in dorsal root ganglia (DRG), while mice without the cre transgene expressing normal NF1 levels did not. *In vivo*, mouse tissues with or without NF1 expression showed similar patterns of classical ER (green, middle columns) and AR (green, right columns) steroid hormone receptor expression as mouse cells *in vitro*. The NF1-deficient tissues (cre+) had intense expression of ER and AR, while the NF1+ (cre-) did not. PR expression (red, left columns), however, was much more robust in NF1-deficient tissues (cre+) than that seen in cultured NF1-/- cells. This could be attributed to signaling from the surrounding cells in the tumor tissue micrownvironment, in contrast to the homogeneous NF1-/- cells in culture. DAPI staining of cell nuclei is shown in blue. Micrographs were taken at 40x magnification.

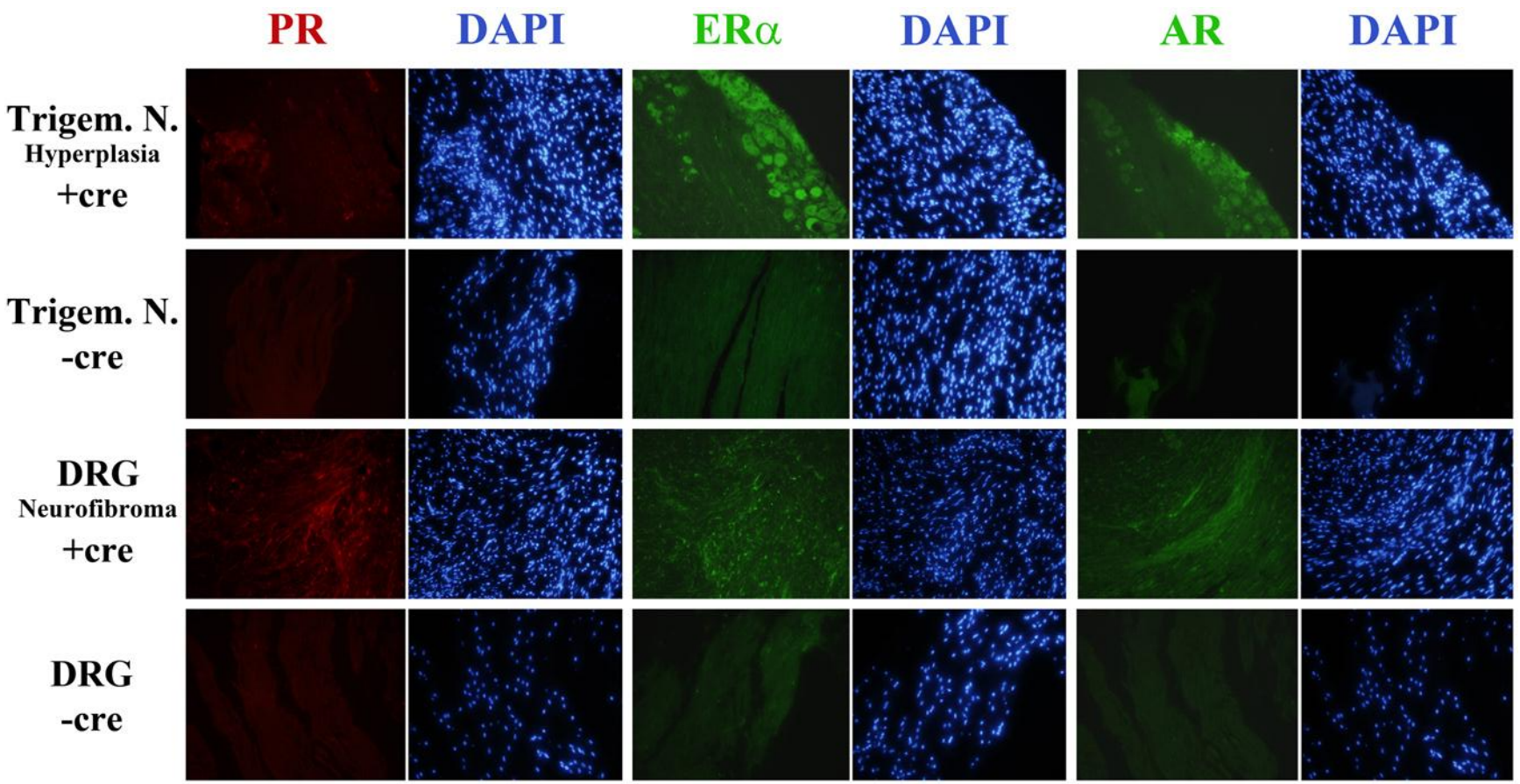

PR
DAPI
ERα
DAPI
AR
DAPI
Trigem. N.
Hyperplasia
+cre
Trigem. N.
-cre
DRG
Neurofibroma
+cre
DRG
-cre

Figure 3.4: Hormones that are increased during pregnancy affect the proliferation of SC-like cells and NF1 tumor cells differently, depending on the level of neurofibromin expression. **P4**: Addition of 0.1 µM P4 increased proliferation significantly only in SW10, ST and DKOSC (asterisk), while 1.0 µM PR inhibitor RU486 returned proliferation rates to control levels (plus sign). **E2**: 0.1 µM E2 increased proliferation significantly in malignant NF1 tumor cells (ST), DKOSC, and D3SC cells (asterisk), while addition of 1.0µM ER inhibitor ICI 182, 780 reduced proliferation rates to control levels only in D3SC cells, which had a small but significant difference (plus sign). **T**: Addition of 1.0 µM T also significantly increased proliferation only in malignant NF1 tumor cells (ST), DKOSC and D3SC SC-like differentiated mES cells (asterisk); addition of 10 µM of the AR inhibitor Flutamide returned proliferation rates to control levels (plus sign).

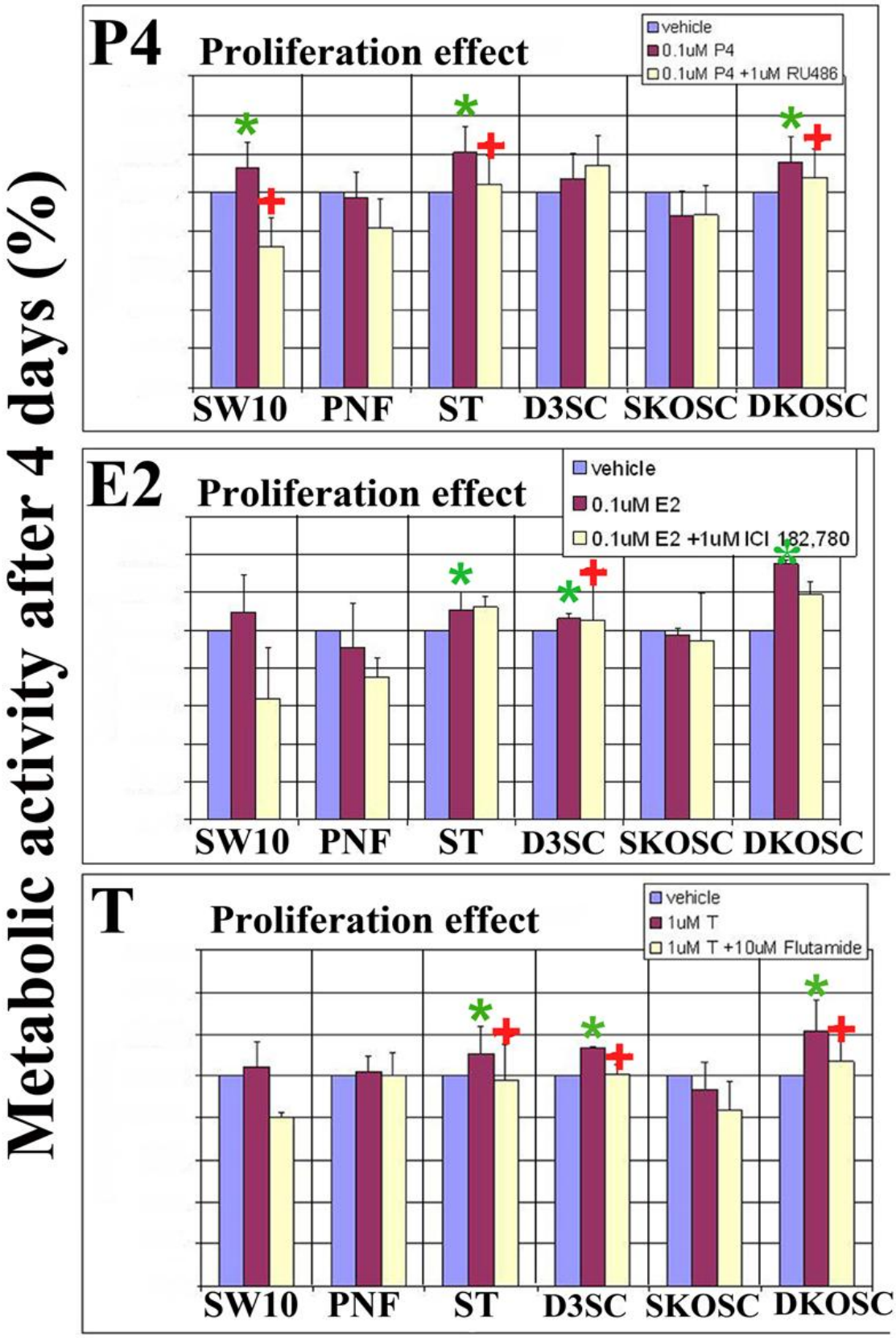
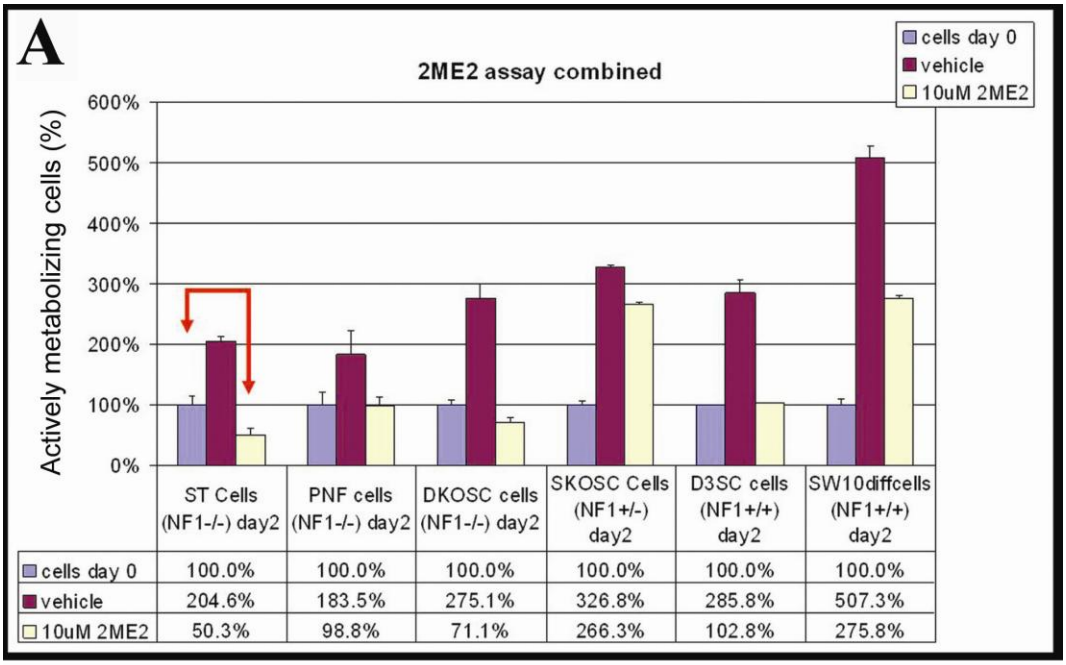
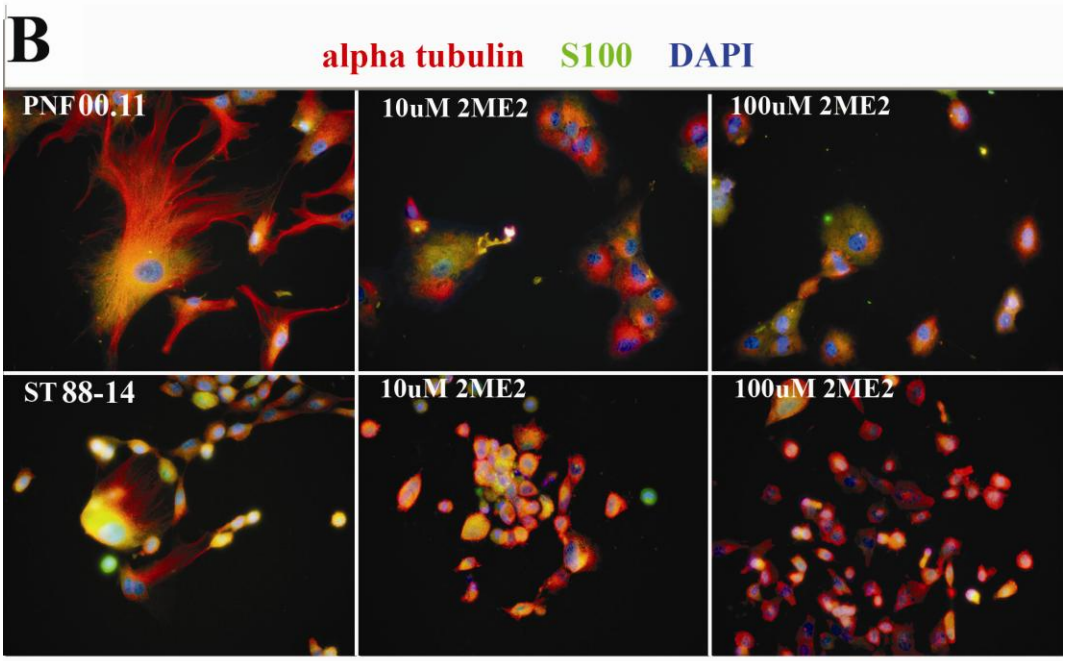
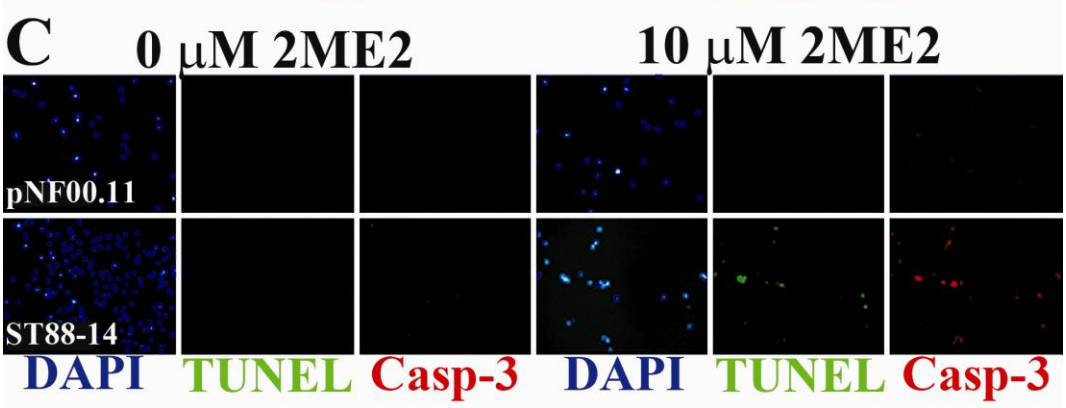

Metabolic activity after 4 days (%)
P4
Proliferation effect
vehicle
0.1uM P4
0.1uM P4 +1uM RU486
SW10
PNF
ST
D3SC
SKOSC
DKOSC
E2
Proliferation effect
vehicle
0.1uM E2
0.1uM E2 +1uM ICI 182,780
SW10
PNF
ST
D3SC
SKOSC
DKOSC
T
Proliferation effect
vehicle
1uM T
1uM T +10uM Flutamide
SW10
PNF
ST
D3SC
SKOSC
DKOSC

Figure 3.5: 2ME2, a naturally occurring E2 metabolite, is cytotoxic to a malignant NF1 tumor cell line, but only slows or halts growth in other cell lines tested. Microtubules are disrupted in this process. **A**: When cells were grown for 2 days in 10 μM 2ME2, malignant ST cells were the only cells that showed significant cell death compared to beginning cell numbers. All other lines (including the benign PNF tumor cells) either had static numbers (PNF, DKODC, D3SC), perhaps due to cell cycle arrest, or merely decreased proliferation (SKOSC, SW10) **B**: Tumor cells were plated and attached overnight before the listed concentration (0 μM left, 10 μM middle, 100 μM right) was added to the media. After an additional 24 hours incubation, cells were fixed and stained for α-tubulin (microtubules), S100 (SC origin of cells) and DAPI (nucleus), 40x magnification. Tumor cells grown without 2ME2 (left) had extensive tubulin networks. Addition of either 10 μM (middle) or 100 μM (right) 2ME2, however, caused the cells to round up and appear apoptotic-nuclei were breaking up and appeared as clusters, and the cells showed blebbing. The microtubules no longer stained as strands, but tubulin showed a more diffuse expression. All of these effects were more pronounced in the malignant ST cells, which showed very little cytoplasm beyond the nucleus in the presence of 10 μM or 100 μM 2ME2. **C**: Tumor cells were grown as described and stained for TUNEL and caspase-3 expression. While approximately 20% of the malignant ST cells (bottom row) were positive for apoptosis, only about 1% of the benign PNF cells were positive (top row).



	ST Cells (NF1-/-) day2	PNF cells (NF1-/-) day2	DKOSC cells (NF1-/-) day2	SKOSC Cells (NF1+/-) day2	D3SC cells (NF1+/+) day2	SW10diffcells (NF1+/+) day2
cells day 0	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
vehicle	204.6%	183.5%	275.1%	326.8%	285.8%	507.3%
10uM 2ME2	50.3%	98.8%	71.1%	266.3%	102.8%	275.8%





REFERENCES

Ansari AH, Nagamani M. 1976. Pregnancy and neurofibromatosis (von Recklinghausen's disease). Obstet Gynecol 47:25S-29S.

Boiten J, Jansen EN, de Graaff R. 1987. Von Recklinghausen neurofibromatosis (VRNF) and pregnancy. A single case study. Clin Neurol Neurosurg 89:181-184.

Chen Y, Zajac JD, MacLean HE. 2005. Androgen regulation of satellite cell function. J Endocrinol 186:21-31.

Daschner K, Assum G, Eisenbarth I, Krone W, Hoffmeyer S, Wortmann S, Heymer B, Kehrer-Sawatzki H. 1997. Clonal origin of tumor cells in a plexiform neurofibroma with LOH in NF1 intron 38 and in dermal neurofibromas without LOH of the NF1 gene. Biochem Biophys Res Commun 234:346-350.

DeBella K, Szudek J, Friedman JM. 2000. Use of the national institutes of health criteria for diagnosis of neurofibromatosis 1 in children. Pediatrics 105:608-614.

Dingli D, Timm M, Russell SJ, Witzig TE, Rajkumar SV. 2002. Promising preclinical activity of 2-methoxyestradiol in multiple myeloma. Clin Cancer Res 8:3948-3954.

Doetschmann T. 1985. J Emb Exp Morph 87:27-45.

Dugoff L, Sujansky E. 1996. Neurofibromatosis Type 1 and pregnancy. American Journal of Medical Genetics (Semin. Med. Genet.) 66:7-10.

Edinger AL, Thompson CB. 2004. Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16:663-669.

Fernandez-Valdivia R, Mukherjee A, Mulac-Jericevic B, Conneely OM, DeMayo FJ, Amato P, Lydon JP. 2005. Revealing progesterone's role in uterine and mammary gland biology: insights from the mouse. Semin Reprod Med 23:22-37.

Fishbein L, Zhang X, Fisher LB, Li H, Campbell-Thompson M, Yachnis A, Rubenstein A, Muir D, Wallace MR. 2007. In vitro studies of steroid hormones in neurofibromatosis 1 tumors and schwann cells. Mol Carcinog 46:512-523.

Gao W, Bohl CE, Dalton JT. 2005. Chemistry and structural biology of androgen receptor. Chem Rev 105:3352-3370.

Gattelli A, Cirio MC, Quaglino A, Schere-Levy C, Martinez N, Binaghi M, Meiss RP, Castilla LH, Kordon EC. 2004. Progression of pregnancy-dependent mouse mammary tumors after long dormancy periods. Involvement of Wnt pathway activation. Cancer Res 64:5193-5199.

Gregory PE, Gutmann DH, Mitchell A, Park S, Boguski M, Jacks T, Wood DL, Jove R, Collins FS. 1993. Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. Somat Cell Mol Genet 19:265-274.

Gutmann DH, Giovannini M. 2002. Mouse models of neurofibromatosis 1 and 2. Neoplasia 4:279-290.

Hai M, Muja N, DeVries GH, Quarles RH, Patel PI. 2002. Comparative analysis of Schwann cell lines as model systems for myelin gene transcription studies. J Neurosci Res 69:497-508.

Heffner RR, Jr. 1969. Hydromyelia in Von Recklinghausen's disease with neurofibrosarcoma. Conn Med 33:311-313.

Ho KJ, Liao JK. 2002. Nonnuclear actions of estrogen. Arterioscler Thromb Vasc Biol 22:1952-1961.

Ireson CR, Chander SK, Purohit A, Perera S, Newman SP, Parish D, Leese MP, Smith AC, Potter BV, Reed MJ. 2004. Pharmacokinetics and efficacy of 2-methoxyoestradiol and 2-methoxyoestradiol-bis-sulphamate in vivo in rodents. Br J Cancer 90:932-937.

Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. 1994. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nat Genet 7:353-361.

Jacobsen BM, Richer JK, Sartorius CA, Horwitz KB. 2003. Expression profiling of human breast cancers and gene regulation by progesterone receptors. J Mammary Gland Biol Neoplasia 8:257-268.

Jacobsen BM, Schittone SA, Richer JK, Horwitz KB. 2005. Progesterone-independent effects of human progesterone receptors (PRs) in estrogen receptor-positive breast cancer: PR isoform-specific gene regulation and tumor biology. Mol Endocrinol 19:574-587.

Korf BR. 1999. Plexiform neurofibromas. Am J Med Genet 89:31-37.

Lammert M, Mautner VF, Kluwe L. 2005. Do hormonal contraceptives stimulate growth of neurofibromas? A survey on 59 NF1 patients. BMC Cancer 5:16.

Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW, Giannakakou P. 2003. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 3:363-375.

McEwen BS, Alves SE. 1999. Estrogen actions in the central nervous system. Endocr Rev 20:279-307.

McGaughran JM, Harris DI, Donnai D, Teare D, MacLeod R, Westerbeek R, Kingston H, Super M, Harris R, Evans DG. 1999. A clinical study of type 1 neurofibromatosis in north west England. J Med Genet 36:197-203.

McLaughlin ME, Jacks T. 2003. Progesterone receptor expression in neurofibromas. Cancer Res 63:752-755.

Menon AG, Anderson KM, Riccardi VM, Chung RY, Whaley JM, Yandell DW, Farmer GE, Freiman RN, Lee JK, Li FP, et al. 1990. Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. Proc Natl Acad Sci U S A 87:5435-5439.

Muir D, Neubauer D, Lim IT, Yachnis AT, Wallace MR. 2001. Tumorigenic properties of neurofibromin-deficient neurofibroma Schwann cells. Am J Pathol 158:501-513.

Okada A, Sato T, Ohta Y, Iguchi T. 2005. Sex steroid hormone receptors in the developing female reproductive tract of laboratory rodents. J Toxicol Sci 30:75-89.

Ozawa H. 2005. Steroid Hormones, their receptors and neuroendocrine system. J Nippon Med Sch 72:316-325.

Posma E, Aalbers R, Kurniawan YS, van Essen AJ, Peeters PM, van Loon AJ. 2003. Neurofibromatosis type I and pregnancy: a fatal attraction? Development of malignant schwannoma during pregnancy in a patient with neurofibromatosis type I. Bjog 110:530-532.

Rasmussen SA, Overman J, Thomson SA, Colman SD, Abernathy CR, Trimpert RE, Moose R, Virdi G, Roux K, Bauer M, Rojiani AM, Maria BL, Muir D, Wallace MR. 2000. Chromosome 17 loss-of-heterozygosity studies in benign and malignant tumors in neurofibromatosis type 1. Genes Chromosomes Cancer 28:425-431.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307:1625-1630.

Rodriguez-Cuenca S, Gianotti M, Roca P, Proenza AM. 2006. Sex steroid receptor expression in different adipose depots is modified during midpregnancy. Mol Cell Endocrinol 249:58-63.

Roth TM, Ramamurthy P, Ebisu F, Lisak RP, Bealmear BM, Barald KF. 2007. A mouse embryonic stem cell model of Schwann cell differentiation for studies of the role of neurofibromatosis type 1 in Schwann cell development and tumor formation. Glia 55:1123-1133.

Serra E, Rosenbaum T, Nadal M, Winner U, Ars E, Estivill X, Lazaro C. 2001. Mitotic recombination effects homozygosity for NF1 germline mutations in neurofibromas. Nat Genet 28:294-296.

Sonneveld E, Riteco JA, Jansen HJ, Pieterse B, Brouwer A, Schoonen WG, van der Burg B. 2006. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. Toxicol Sci 89:173-187.

Su W, Gutmann DH, Perry A, Abounader R, Laterra J, Sherman LS. 2004. CD44-independent hepatocyte growth factor/c-Met autocrine loop promotes malignant peripheral nerve sheath tumor cell invasion in vitro. Glia 45:297-306.

Vandenbroucke I, Van Oostveldt P, Coene E, De Paepe A, Messiaen L. 2004. Neurofibromin is actively transported to the nucleus. FEBS Lett 560:98-102.

Virdis R. 2000. Neurofibromatosis type 1 and precocious puberty. Journal of Pediatric Endocrinology & Metabolism 13:841-844.

Wang SH, Myc A, Koenig RJ, Bretz JD, Arscott PL, Baker JR. 2000. 2-Methoxyestradiol, an endogenous estrogen metabolite, induces thyroid cell apoptosis. Mol Cell Endocrinol 165:163-172.

Witorsch RJ. 2002. Low-dose in utero effects of xenoestrogens in mice and their relevance to humans: an analytical review of the literature. Food Chem Toxicol 40:905-912.

Woodruff JM. 1999. Pathology of tumors of the peripheral nerve sheath in type 1 neurofibromatosis. Am J Med Genet 89:23-30.

Zhang Z, Cerghet M, Mullins C, Williamson M, Bessert D, Skoff R. 2004. Comparison of in vivo and in vitro subcellular localization of estrogen receptors alpha and beta in oligodendrocytes. J Neurochem 89:674-684.

Zheng H, Chang L, Patel N, Yang J, Lowe L, Burns DK, Zhu Y. Abnormal differentiation of stem/progenitor cells triggers tumor formation in peripheral nerves. Submitted.

Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF. 2002. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science 296:920-922.

CHAPTER IV

CONCLUSION

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder of the nervous system, resulting in neurofibromas of the peripheral nervous system (Friedman et al., 1999; Roth et al., 2007a). These mostly benign tumors are thought to originate from Schwann Cells (SC) and their precursors (SCP) (Zhu et al., 2002; Parada et al., 2005), and tend to increase in size and number at puberty and pregnancy (Posma et al., 2003; Virdis et al., 2003), suggesting hormonal influences. Neurofibromas contain a heterogeneous population of cell types, including SC, SCP, neurons, fibroblasts, and mast cells (Gutmann et al., 1994; Blum and Kambich, 1997; Feldkamp et al., 1998; Friedman et al., 1999).

While mast cells are commonly seen upregulated in response to tissue inflammation (Galli and Wershil, 1996; Theoharis and Conti, 2004; Marone et al., 2002), they are also found in other tumors, such as mammary adenocarcinomas and melanoma (Theoharis and Conti, 2004). The presence of mast cells in neurofibromas is perivascular in close proximity to neurons, and NF1 has been shown to modulate mast cell growth (Theoharis and Conti, 2004, Ingram et al., 2000).There also appear to be functional connections in interactions between nerve endings and mast cells when grown in co-culture (Marone et al., 2002).

The cell types found in NF1 tumors of both the benign and malignant types are heterogeneous and LOH is variable even among a specific cell type contributing to the tumors. Furthermore, although increases have been found in both size of tumors and numbers of tumors (or enlargement of microscopic tumors to visible size) during times of puberty and pregnancy, times at which hormones may be playing a role, a rigorous examination of the effects of hormones on cells with and without neurofibromin has been difficult. Such studies require a homogeneous population of cells of a given cell type that have the same known NF1 status. This dissertation provides the first study of which we are aware that has devised an *in vitro* system of differentiating mouse embryonic stem (mES) cells of known NF1 status preferentially into Schwann Cell-like (SC-like) cells or other cell types affected in NF1. The SC-like cells derived from mES cells, regardless of NF1 status, express appropriate SC genes in the expected order of expression for SC maturation, support and preferentially direct primary ganglion neurite outgrowth, and express myelin in cells near neurites (Roth et al., 2007b).

mES cells provide a good model system for studying NF1 because they represent a homogenous population of clonally-derived cells that are available in all NF1 genotypes +/+, +/-, -/- (representing LOH), contain a known mutation site (Jacks et al., 1994) and are neither transformed nor malignant. Also, NF1 tumors are initiated from SC/SCP cells and the mES cells can be differentiated into cell types all along the SC pathway from ES → neural crest →SCP → immature SC → mature myelinating or non-myelinating SC. These differentiated cells of known NF1 status can then be used both *in vitro* and *in vivo* to test effects of hormones, hormone metabolites or other compounds on proliferation, tumor formation and growth, differentiation, apoptosis, and cell functions. Other cell

types found in neurofibromas, such as neurons, can also be differentiated from mES cells of all NF1 genotypes and studied in contrast to or in combination with SC-like cells (Roth et al., 2007b; Roth et al., 2007c).

In contrast to *in vivo* studies, where tumor phenotypes are only seen after NF1 LOH, we found several phenotypic responses in the NF1+/- (SKOSC) SC-like cells as well as NF1-/- (DKOSC) SC-like cells as compared to the NF1+/+ (D3SC) SC-like cells. Our hypothesis is that this is due to the homogeneous *in vitro* culture system, without signals from surrounding NF1+/- cells and other micro-environmental factors. These surrounding signals could rescue in vivo tissues from displaying an NF1 haplo-insufficient phenotype. In our studies, all of the SC-like cells, regardless of NF1 genotype, were morphologically similar to SC, expressed SC protein markers S100 and GFAP, and supported neurite outgrowth. We also noticed some differences in the SC-like differentiated cells. We found that both NF1+/- and NF1-/- SC-like cells were over-proliferative in culture, even in the presence of primary neurons. This overproliferation in both NF1+/- and NF1-/- cells could be reduced to normal levels with the addition of a Mek inhibitor, indirectly suggesting Ras pathway involvement.

In vitro, all mES cell lines, regardless of NF1 genotype, recapitulated the progression of cell types all along the SC pathway from ES → neural crest →SCP → immature SC. In contrast, we found that NF1+/+ SC-like cells (D3SC) are more likely to express myelinating SC genes, become quiescent in the presence of SAG neurites and associate with SAG neurites similarly to SW10 mouse SC. NF1+/- (SKOSC) and NF1-/- (DKOSC) SC-like cells are more likely to stimulate greater neuron migration, neurite outgrowth and branching, express non-myelinating genes, slightly reduce proliferation in

the presence of SAG neurites and do not associate properly with SAG neurites. In addition to overproliferation, this result could be a proximity issue, since there is a reduced need for the close proximity of neurons to SC *in vitro* compared to the close quarters of *in vivo* neuron/SC interaction. We would also like to see what effects, if any, are seen on neurite/SC interaction if Mek is over-stimulated in the SC-like cells. Overproliferation of NF1 deficient (SKOSC) and null (DKOSC) SC-like cells can be reduced to NF+/+ (D3SC) levels by the addition of a Mek inhibitor, indirectly indicating Ras pathway involvement (Roth et al., 2007b). In order to more directly assay Ras pathway involvement, we would need to look at the effects on Mek phosphorylation when U0126 (Mek inhibitor) is added.

In studies of classical steroid hormone pathways, we found that SC-differentiated NF1-/- cells (DKOSC) and NF1-/- tumor cells (ST, PNF) express higher levels of classical hormone receptors tested than either NF1+/- (SKOSC) or NF1+/+ (D3SC, SW10) cells, suggesting that NF1-/- cells would be more likely to respond to steroid hormones. Estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) were all expressed in highest levels in NF1-/- cells, while expression was greatly reduced/null in NF1+/- and NF1+/+ cells. Although PR expression was highest in NF1-/- cells, overall expression was lower than ER and AR (Roth et al., 2007b).

When we examined the effect that steroid hormone addition had on cell proliferation, we found a modest but significant increase in cell proliferation in DKOSC (NF1-/-) and ST88-14 (malignant NF1-/-) cells with all the hormones tested. Estrogen (E2) is the ER ligand, testosterone (T) is the AR ligand, and progesterone (P4) is the PR ligand, although they can rarely cross-react with the other receptors to a much lesser

extent than that with which they bind to their classical receptor. These modest proliferation increases were mainly returned to normal levels with the addition of classical hormone receptor inhibitors (ICI 182, 780 for ER, Flutamide for AR, and RU486 for PR), suggesting that this effect was at least partially mediated through classical hormone pathways. In some cases, however, increases were not returned to normal levels, suggesting other, non-classical pathway involvement (Roth et al., 2007b).

Future studies will focus on the effects of these hormones separately and in combination, on these stem cell-derived cell lines and other cells whose NF1 status is known, including other malignant NF1 tumor cell lines *in vitro* and *in vivo*. We would like to see if there are effects such as downregulation, on *NF1* expression in the presence of steroid hormones and/or inhibitors. Because of the complex hormonal milieu during pregnancy (Witorsch, 2002; Fernandez-Valdivia et al., 2005; Okada et al., 2005; Rodriguez-Cuenca et al., 2006), it will be necessary to look for synergy among hormone effects. Tumor growth response to steroid hormones may also be due to the effects on other NF1+/- cells in the tumor microenvironment, which may need to be studied *in vivo* in order to determine the many interactions that influence the effect of hormones on mouse tissues and hormone receptors. NF1 silencing and re-expression studies would be difficult due to the large transcript size of the *NF1* gene (Friedman et al., 1999).

We will also investigate the mechanisms of these steroid hormones' effects through mechanisms other than their classical nuclear receptors (figure 1.6). These include steroid hormone receptors localized to the plasma membrane and cytoplasm, including G protein-coupled receptors, c-src, and shc, which lead to MAPK and P13K signaling cascades activation (Honda et al., 2000; Pietras et al., 2001; Razandi et al.,

2002; Levin, 2005; Levin and Pietras, 2007; Pedram et al., 2007). There have also been reports of novel G protein-coupled receptors unrelated to the classical nuclear steroid hormone receptors that can mediate rapid non-genomic effects of steroid hormones through adenylyl cyclase inhibition and cAMP production that activates the MAPK pathway (Levin, 2001; Pedram et al., 2002; Revankar et al., 2005; Boonyaratanakornkit and Edwards, 2007). Non-classical effects of E2 have been better characterized than those of T and P4, including through possible mitochondrial non-genomic membrane-bound E2 receptors (Chen et al., 2005; Gavrilova-Jordan and Price, 2007). ER, as well as AR and PR, have been found in both the plasma membrane as well as the cell cytoplasm (Razandi et al., 2002; Levin, 2005; Levin and Pietras, 2007; Pedram et al., 2007).

In this dissertation, we also hypothesized that the complex hormonal milieu during pregnancy (Grow, 2002; Fishbein et al., 2007) or the response to these hormones may be different in women with NF1. For example, 2-methoxy-estradiol (2ME2), which is an estrogen metabolite, has been found to be upregulated during pregnancy (Wang et al., 2000), but may either be present in lower levels systemically in women with NF1, resulting in tumor overgrowth, or cell receptors for 2ME2 may be lacking in NF1-/- cells. 2ME2 has been found to be cytotoxic to numerous tumor cells as well as some other rapidly-growing cells (Mabjeesh et al., 2003), but not to normal or quiescent cells, by inducing apoptosis (Wang et al., 2000; Ireson et al., 2004). 2ME2 has no E2-like activity and has very low affinity for the ER; its effects are not classical ER-dependent (Wang et al., 2000; Ireson et al., 2004). In a study using prostate cancer cells grown in culture, it was found that the effect of 2ME2 was to block cell-cycle progression at the G_2/M stage (Kumar et al., 2001). The mechanism of 2ME2 action in cultured cells is reported to be

through dissociation of microtubules (Mabjeesh et al., 2003). Neurofibromin has been found to associate and co-purify with microtubules, suggesting *NF1* involvement in microtubule-mediated pathways (Gregory et al., 1993). It would therefore be interesting to see whether 2ME2 addition would change *NF1* expression patterns, since the effect of 2ME2 is to disaggregate microtubules, and other anti-mitotic drugs (e.g. colcemid) have been found to disaggregate neurofibromin staining, which recovered after drug removal (Gregory et al., 1993). We found that 2ME2, a naturally occurring estrogen metabolite, disrupted microtubules and was cytotoxic to malignant NF1 tumor cells while its effect was to slow or halt the growth of the other cells lines tested, regardless of their NF1 status. This suggests that perhaps additional mutations, for example p53, which has been associated with MPNSTs (Menon et al., 1990), are needed for 2ME2 cytotoxic effects specific to malignant tumors, since non-malignant NF1 tumor cells did not undergo apoptosis with 2ME2 treatment.

Neurofibromas tend to be highly vascular (Thomas and De Vries, 2007). Therefore, we also hypothesize that the effect of hormones on neurofibromas may be indirect through hormonal effect on angiogenesis (Ruohola et al., 1999; Roth et al., 2007a), due to the heterogeneous effect of hormones on SC-like and NF1 tumor cells. Mast cells, which are found in neurofibromas as well as various other tumors such as mammary carcinomas and melanomas, express factors which could be tumor-beneficial (Theoharis and Conti, 2004; Marone et al., 2002). Some of these factors function to induce neovascularization, such as heparin, interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) (Theoharis and Conti, 2004; Marone et al., 2002). Mast cells accumulate at sites of tumors in response to chemoattractants implicated in tumor growth,

such as Regulated upon Activation, Normal T-cell Expressed, and Secreted **(**RANTES) and Monocyte chemotactic protein-1 (MCP-1) (Theoharis and Conti, 2004). It is of interest to note that we have found increased MCP-1 mRNA expression in NF1-/- (DKOSC) SC-like cells using RTqPCR (not shown). Increased blood vessel formation during pregnancy, initiated by mast cell accumulation, could allow existing tumors to grow as well as increase microscopic tumors to grow to detectable sizes.

It is also worth noting that while most of the cultures used in these studies are mouse cells, the tumor cell cultures are from human neurofibromas, which may explain some of the disparate results. With conditional mouse NF1 knockout models, we are getting closer to being able to recapitulate the human NF1 condition using mouse systems.

REFERENCES

Boonyaratanakornkit V, Edwards DP. 2007. Receptor mechanisms mediating non-genomic actions of sex steroids. Semin Reprod Med 25:139-153.

Chan CC, Lao TT, HO PC, Sung EO, Cheung AN. 2003. The effect of mifepristone on the expression of steroid hormone receptors in human decidua and placenta: a randomized placebo-controlled double-blind study. J Clin Endocrinol Metab 88:5846-5850.

Chen JQ, Yager JD, Russo J. 2005. Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. Biochim Biophys Acta 1746:1-17.

Criado E, Izquierdo L, Lujan S, Puras E, del Mar Espino M. 2002. Abdominal aortic coarctation, renovascular, hypertension, and neurofibromatosis. Ann Vasc Surg 16: 363-367.

Fernandez-Valdivia R, Mukherjee A, Mulac-Jericevic B, Conneely OM, Demayo FJ, Amato P, Lydon JP. 2005. Revealing Progesterone's Role in Uterine and Mammary Gland Biology: Insights from the Mouse. Semin Reprod Med 23:22-37.

Fishbein L, Zhang X, Fisher LB, Li H, Campbell-Thompson M, Yachnis A, Rubenstein A, Muir D, Wallace MR. 2007. In vitro studies of steroid hormones in neurofibromatosis 1 tumors and schwann cells. Mol Carcinog 46:512-523.

Friedman JM, Gutmann DH, MacCollin M, Riccardi VM. 1999. Neurofibromatosis: Phenotype, Natural History, and Pathogenesis. Johns Hopkins University Press. 381 p.

Galli SJ, Wershil BK. 1996. The two faces of the mast cell. Nature 381:21-22.

Gavrilova-Jordan LP, Price TM. 2007. Actions of steroids in mitochondria. Semin Reprod Med 25:154-164.

Gregory PE, Gutmann DH, Mitchell A, Park S, Boguski M, Jacks T, Wood DL, Jove R, Collins FS. 1993. Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. Somat Cell Mol Genet 19:265-274.

Grow DR. 2002. Metabolism of endogenous and exogenous reproductive hormones. Obstet Gynecol Clin North Am 29:425-436.

Honda K, Sawada H, Kihara T, Urushitani M, Nakamizo T, Akaike A, Shimohama S. 2000. Phosphatidylinositol 3-kinase mediates neuroprotection by estrogen in cultured cortical neurons. J Neurosci Res 60:321-327.

Ingram DA, Yang FC, Travers JB, Wenning MJ, Hiatt K, New S, Hood A, Shannon K, Williams DA. 2000. Genetic and biochemical evidence that haploinsufficiency of the Nf1 tumor suppressor gene modulates melanocyte and mast cell fates in vivo. J Exp Med 191:181-188.

Clapp, D. W.Ireson CR, Chander SK, Purohit A, Perera S, Newman SP, Parish D, Leese MP, Smith AC, Potter BV, Reed MJ. 2004. Pharmacokinetics and efficacy of 2-methoxyoestradiol and 2-methoxyoestradiol-bis-sulphamate in vivo in rodents. Br J Cancer 90:932-937.

Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA. 1994. Tumour predisposition in mice heterozygous for a targeted mutation in Nf1. Nat Genet 7:353-361.

Kumar AP, Garcia GE, Slaga TJ. 2001. 2-Methoxyestradiol blocks cell-cycle progression at G_2/M phase and inhibits growth of human prostate cancer cells. Mol Carcinog 31:111-124.

Levin ER. 2001. Cell localization, physiology, and nongenomic actions of estrogen receptors. J Appl Physiol 91:1860-1867.

Levin ER. 2005. Integration of the extranuclear and nuclear actions of estrogen. Mol Endocrinol 19:1951-1959.

Levin ER, Pietras RJ. 2007. Estrogen receptors outside the nucleus in breast cancer. Breast Cancer Res Treat.

Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW, Giannakakou P. 2003. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 3:363-375.

Marone G, Galli SJ, Kitamura Y. 2002. Probing the roles of mast cells and basophils in natural and acquired immunity, physiology and disease. Trends Immunol 23:425-427.

Menon AG, Anderson KM, Riccardi VM, Chung RY, Whaley JM, Yandell DW, Farmer GE, Freiman RN, Lee JK, Li FP, et al. 1990. Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. Proc Natl Acad Sci U S A 87:5435-5439.

Okada A, Sato T, Ohta Y, Iguchi T. 2005. Sex steroid hormone receptors in the developing female reproductive tract of laboratory rodents. J Toxicol Sci 30:75-89.

Parada LF, Kwon CH, Zhu Y. 2005. Modeling neurofibromatosis type 1 tumors in the mouse for therapeutic intervention. Cold Spring Harb Symp Quant Biol 70:173-176.

Pedram A, Razandi M, Aitkenhead M, Hughes CC, Levin ER. 2002. Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. J Biol Chem 277:50768-50775.

Pedram A, Razandi M, Sainson RC, Kim JK, Hughes CC, Levin ER. 2007. A conserved mechanism for steroid receptor translocation to the plasma membrane. J Biol Chem 282:22278-22288.

Pietras RJ, Nemere I, Szego CM. 2001. Steroid hormone receptors in target cell membranes. Endocrine 14:417-427.

Posma E, Aalbers R, Kurniawan YS, van Essen AJ, Peeters PM, van Loon AJ. 2003. Neurofibromatosis type I and pregnancy: a fatal attraction? Development of malignant schwannoma during pregnancy in a patient with neurofibromatosis type I. Bjog 110:530-532.

Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER. 2002. ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. Mol Endocrinol 16:100-115.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. 2005. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science 307:1625-1630.

Rodriguez-Cuenca S, Gianotti M, Roca P, Proenza AM. 2006. Sex steroid receptor expression in different adipose depots is modified during midpregnancy. Mol Cell Endocrinol 249:58-63.

Roth TM, Petty EM, Barald KF. 2007a. Hormone Involvement in Neurofibromatosis Type 1. In preparation.

Roth TM, Ramamurthy P, Ebisu F, Lisak RP, Bealmear BM, Barald KF. 2007b. A mouse embryonic stem cell model of Schwann cell differentiation for studies of the role of neurofibromatosis type 1 in Schwann cell development and tumor formation. Glia 55:1123-1133.

Roth TM, Ramamurthy P, Muir D, Wallace M, Barald KF. 2007c. Influence of hormones and hormone metabolites on the growth of Schwann Cells derived from mouse embryonic stem cells and tumor cell lines expressing variable levels of neurofibromin In revision.

Ruohola JK, Valve EM, Karkkainen MJ, Joukov V, Alitalo K, Harkonen PL. 1999. Vascular endothelial growth factors are differentially regulated by steroid

hormones and antiestrogens in breast cancer cells. Mol Cell Endocrinol 149:29-40.

Sharma JB, Gulati N, Malik S. 1991. Maternal and perinatal complications in neurofibromatosis during pregnancy. International Journal of Gynecology & Obstetrics 34: 221-227.

Soucek L, Lawlor ER, Soto , Shchors K, Swidart LB, Evan GI. 2007. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. Nat Med 13:1211-1218.

Theoharides T, Conti P. 2004. Mast cells: the JEKYLL and HYDE of tumor growth. Trends in Immunology 25:235-341.

Thomas SL, De Vries GH. 2007. Angiogenic expression profile of normal and neurofibromin-deficient human Schwann cells. Neurochem Res 32:1129-1141.

Virdis R, Street M, Bandello M, Tripodi C, Donadio A, Villani A, Cagozzi L, Garavelli L, Bernasconi S. 2003. Growth and pubertal disorders in neurofibromatosis type 1. Journal of Pediatric Endocrinology & Metabolism 16:289-292.

Wang SH, Myc A, Koenig RJ, Bretz JD, Arscott PL, Baker JR. 2000. 2-Methoxyestradiol, an endogenous estrogen metabolite, induces thyroid cell apoptosis. Mol Cell Endocrinol 165:163-172.

Witorsch RJ. 2002. Low-dose in utero effects of xenoestrogens in mice and their relevance to humans: an analytical review of the literature. Food Chem Toxicol 40:905-912.

Zhu Y, Ghosh P, Charnay P, Burns DK, Parada LF. 2002. Neurofibromas in NF1: Schwann cell origin and role of tumor environment. Science 296:920-922.

APPENDIX

SYNTHESIZED ESTROGEN HORMONE METABOLITES HAVE HETEROGENEOUS EFFECTS ON CELL PROLIFERATION

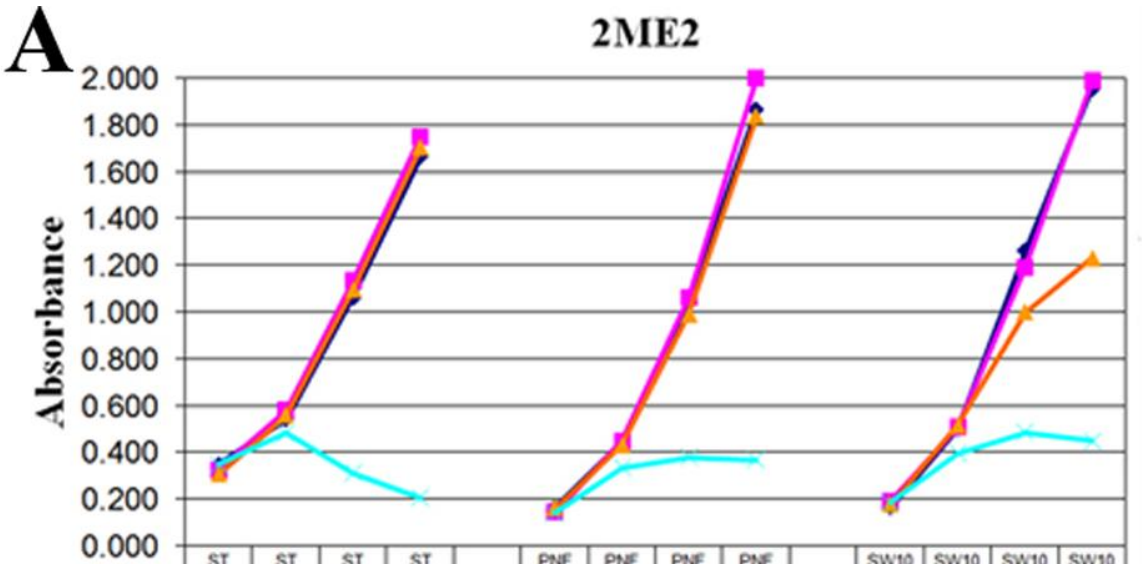
Following the discovery that the naturally occurring estrogen metabolite 2ME2 was cytotoxic only to malignant NF1-/- ST88-14 MPNST tumor cells, we sought to find another estrogen derivative that could be effective at inducing apoptosis in tumor cells at lower concentrations. Twelve estrogen metabolites were synthesized by Dr. Douglas Jewett in our lab and assayed for anti-proliferative or cytotoxic effects on hormone receptor-positive tumor cell lines (table A.1). The effect of these compounds was not consistent-none tested was cytotoxic to all tested cell lines. This is most likely because the cell lines expressed a variety of different hormone receptors. Regarding the malignant NF1-/- ST88-14 MPNST tumor cells, both 2MeO2bisMATE and 2ME2disulfamate in addition to 2ME2 were cytotoxic at 10μM (table A.1). A follow-up assay suggested that 2MeO2bisMATE, but not 2ME2 or 2ME2disulfamate, was cytotoxic to malignant (ST88-14) NF1-/- tumor cells at 1μM, one-tenth the concentration needed for cytotoxicity (figure A.1, 2ME2disulfamate not shown). A benign (pNF00.11) NF1-/- tumor cell line and a mouse NF1+ SC line (SW10) reduced/halted proliferation in response to either 2ME2 or 2MeO2bisMATE (figure A.1). Additional estrogen compounds tested had limited/no effect on cell proliferation even at 10μM (table A.1, figure A.2).

Further assays were then performed to compare the effects of 2ME2bisMATE to 2ME2 on cell proliferation and cytotoxicity in the malignant NF1-/- ST88-14 MPNST tumor cells and NF1+/+, NF1+/- or NF1-/- SC-like cells. We found that the number of

proliferating cells decreased in all cell lines tested after 2 says' exposure to either 2ME2 or 2MeO2bisMATE (figure A.3), however, only malignant NF1-/- ST88-14 MPNST tumor cells showed significant apoptosis at 1μM 2MeO2bisMATE (figure A.4). This data suggests that therapeutic compounds specifically cytotoxic at low concentrations to malignant tumor cells could possibly be synthesized.

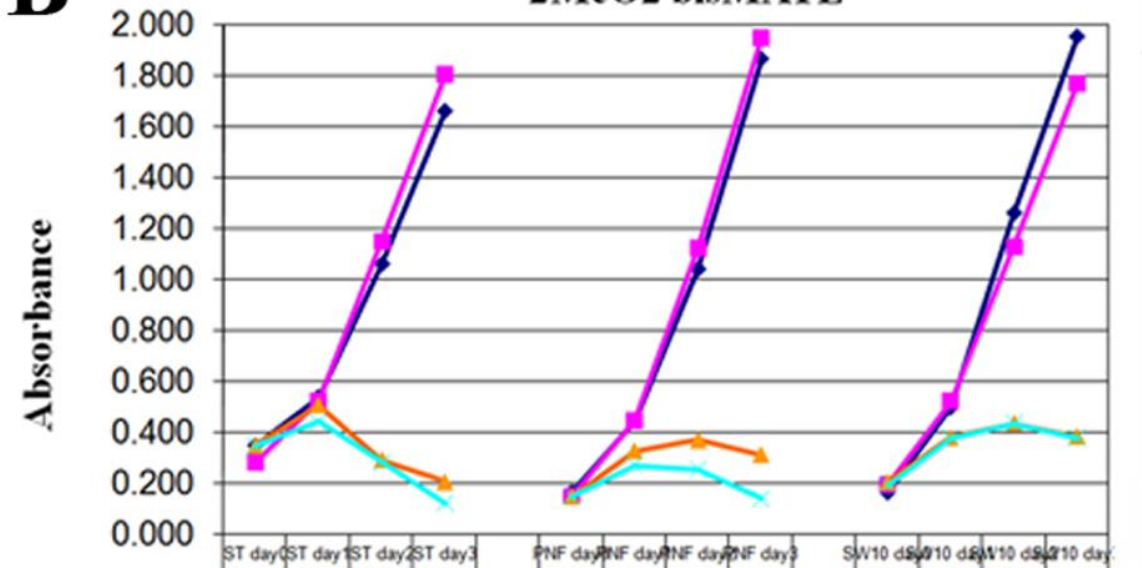
Figure A.1: Cytotoxic effects of synthesized estrogen metabolites. Application of 2MeO2bisMATE (1.0 µM) is cytotoxic to NF1-/- malignant peripheral nerve sheath (MPNST) (ST) cells at one-tenth the concentration of 2ME2 (10 µM) .A: Decreased numbers of metabolizing cells from the original number was seen only in a malignant NF1-/- cell line tested, while a benign human plexiform neurofibroma (PNF) cell line and a mouse NF1+ SC line (SW10) only showed decreased proliferation. B: 2MeO2-bisMATE (1.0 µM) showed this effect using one-tenth the concentration of 2ME2 (10 µM).

A

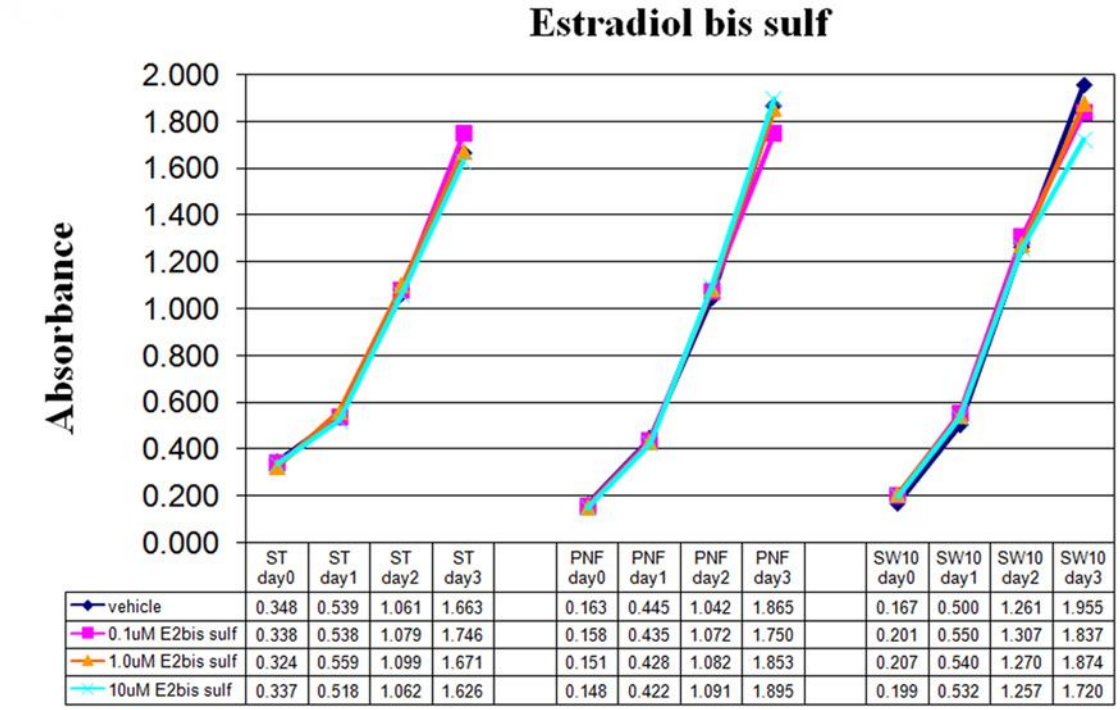


	ST day0	ST day1	ST day2	ST day3		PNF day0	PNF day1	PNF day2	PNF day3		SW10 day0	SW10 day1	SW10 day2	SW10 day3
vehicle	0.348	0.539	1.061	1.663		0.163	0.445	1.042	1.865		0.167	0.500	1.261	1.955
0.1uM 2ME2	0.326	0.580	1.133	1.750		0.144	0.448	1.059	1.998		0.190	0.507	1.191	1.986
1.0uM 2ME2	0.305	0.556	1.094	1.702		0.159	0.431	0.991	1.835		0.176	0.516	0.999	1.231
10uM 2ME2	0.347	0.481	0.311	0.203		0.140	0.334	0.378	0.366		0.185	0.395	0.485	0.449

B



	ST day0	ST day1	ST day2	ST day3		PNF day0	PNF day1	PNF day2	PNF day3		SW10 day0	SW10 day1	SW10 day2	SW10 day3
vehicle	0.348	0.539	1.061	1.663		0.163	0.445	1.042	1.865		0.167	0.500	1.261	1.955
0.1uM 2MeO2bisMATE	0.283	0.526	1.152	1.806		0.148	0.449	1.123	1.950		0.197	0.524	1.129	1.770
1.0uM 2MeO2bisMATE	0.351	0.508	0.292	0.207		0.149	0.327	0.371	0.314		0.207	0.382	0.436	0.384
10uM 2MeO2bisMATE	0.349	0.443	0.283	0.123		0.147	0.272	0.255	0.142		0.195	0.380	0.435	0.380



	ST day0	ST day1	ST day2	ST day3		PNF day0	PNF day1	PNF day2	PNF day3		SW10 day0	SW10 day1	SW10 day2	SW10 day3
vehicle	0.348	0.539	1.061	1.663		0.163	0.445	1.042	1.865		0.167	0.500	1.261	1.955
0.1uM E2bis sulf	0.338	0.538	1.079	1.746		0.158	0.435	1.072	1.750		0.201	0.550	1.307	1.837
1.0uM E2bis sulf	0.324	0.559	1.099	1.671		0.151	0.428	1.082	1.853		0.207	0.540	1.270	1.874
10uM E2bis sulf	0.337	0.518	1.062	1.626		0.148	0.422	1.091	1.895		0.199	0.532	1.257	1.720

Figure A.2: Not all synthesized estrogen metabolites affected cell proliferation. For example, E2 bis sulfamate showed no effect on cell proliferation after 2 days' application of up to 10 µM.

Figure A.3: SC-like and malignant peripheral nerve sheath tumor (MPNST) cells grown in the presence of 2ME2 or 2MeO2bisMATE decrease proliferation. Application of 1.0 µM 2MeO2bisMATE to all cells, regardless of NF1 status, showed a decrease in proliferation over two days at, one-tenth the concentration of 2ME that decreased cell proliferation at 10 µM. Cell types tested inlcuded the SC-like **A.** D3SC (NF1+/+), **B.** SKOSC (NF1+/-), **C.** DKOSC (NF1-/-) and **D.** MPNST (ST) cells. Decreased cell proliferation could be the result of cytotoxicity or cell cycle arrest. 40x magnification.

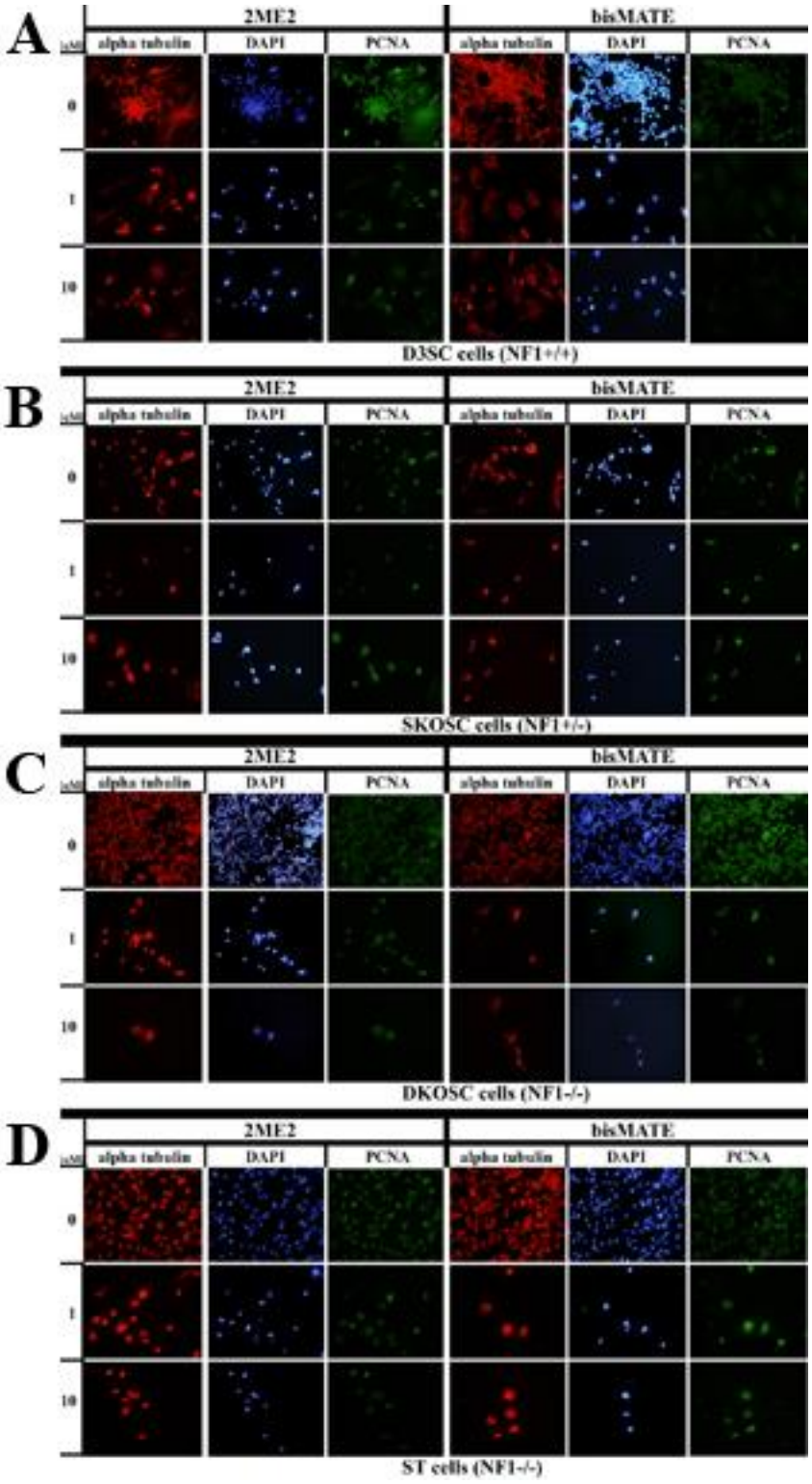
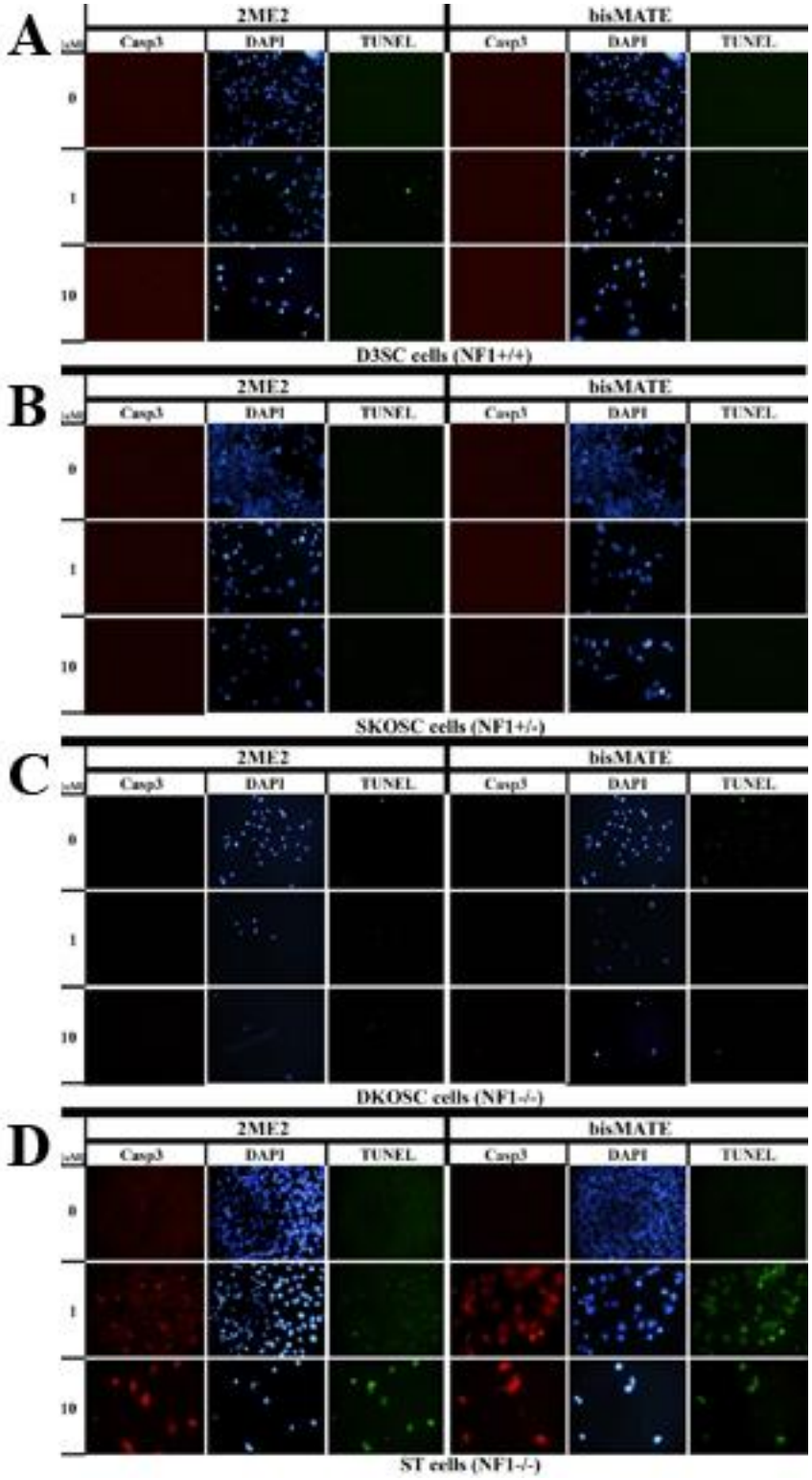

A
2ME2
bisMATE
uM
alpha tubulin
DAPI
PCNA
0
1
10
D3SC cells (NF1+/+)
B
SKOSC cells (NF1+/-)
C
DKOSC cells (NF1-/-)
D
ST cells (NF1-/-)

Figure A.4: 2MeO2bisMATE causes apoptosis in malignant NF1-/- MPNST cells at one-tenth the concentration of 2ME2. Decreased cell proliferation (see figure A.4) could be the result of apoptosis or cell quiescence. Two day application of 2MeO2bisMATE resulted in a considerable percentage of apoptotic cells only in MPNST (ST) cells at 1.0 µM, one-tenth the concentration of 2ME that resulted in apoptotic ST cells at 10 µM. SC-like cells **A.** D3SC (NF1+/+), **B.** SKOSC (NF1+/-), and **C.** DKOSC (NF1-/-) cells showed very little apoptosis (~1%), while MPNST cells **D.** ST (NF1-/-) cells showed a considerable percentage of apoptotic cells (~20%). 40x magnification.


A
2ME2
bisMATE
μM
Casp3
DAPI
TUNEL
Casp3
DAPI
TUNEL
0
1
10
D3SC cells (NF1+/+)
B
2ME2
bisMATE
μM
Casp3
DAPI
TUNEL
Casp3
DAPI
TUNEL
0
1
10
SKOSC cells (NF1+/-)
C
2ME2
bisMATE
μM
Casp3
DAPI
TUNEL
Casp3
DAPI
TUNEL
0
1
10
DKOSC cells (NF1-/-)
D
2ME2
bisMATE
μM
Casp3
DAPI
TUNEL
Casp3
DAPI
TUNEL
0
1
10
ST cells (NF1-/-)

.

Table A.1: The effect of estrogen metabolites is not consistent across cancer cell lines. We tested a range of synthesized hormones at concentrations of either 0 µM or 10 µM on a variety of hormone receptor-positive cancer cells. Only 2ME2, 2MeO2-bisMATE and 2ME2-disulfamate were cytotoxic at 10 µM to malignant NF1-/- ST cells. Only2MeO2-bisMATE was cytotoxic at 1.0 µM (not shown). Synthesized hormone metabolites' effects were heterogeneous among the hormone receptor-positive cell lines tested-none was cytotoxic to all cell types.

Effect of 10uM estrogen metabolites on various cancer cell lines' proliferation over 3 days				
cell line	ST88-14 (MPNST)	N15C6 AR+ (LNCaP)	PR+ (MCF-7)	ER+ (MCF-7)
metabolite				
2ME2	**D**	**P**	**L**	**L**
2MeO2bisMATE	**D**	**P**	**L**	**L**
2-OH E2 Trisulfamate	**NE**	**L**	**L**	**NE**
4-Sulfamyl Tamoxifen	**NE**	**D**	**D**	**P**
E10 2500 Trisulfamate	**L**	**L**	**L**	**NE**
Resvemtrol Sulfamate	**L**	**L**	**NE**	**S**
C 16 Disulfamate	**NE**	**L**	**NE**	**NE**
2ME2 Disulfamate	**D**	**S**	**S**	**S**
4-OH Tamoxifen	**NE**	**D**	**D**	**D**
E 2510 Sulfamate	**P**	**S**	**P**	**P**
Sulfamyl Raloxifene	**NE**	**NE**	**NE**	**NE**
C 18 Disulfamate	**NE**	**S**	**NE**	**NE**
NE=no effect, L=significantly less than control, S=static numbers, P=Partial cell death (some cells undergo apoptosis), D=cell death				