

# Influence of Hormones and Hormone Metabolites on the Growth of Schwann Cells Derived From Embryonic Stem Cells and on Tumor Cell Lines Expressing Variable Levels of Neurofibromin<sup>†</sup>

Therese M. Roth,<sup>1</sup> Poornapriya Ramamurthy,<sup>1,2</sup> David Muir,<sup>3</sup> Margaret R. Wallace,<sup>4</sup> Yuan Zhu,<sup>1,5</sup> Lou Chang,<sup>1</sup> and Kate F. Barald<sup>1,2\*</sup>

Loss of neurofibromin, the protein product of the tumor suppressor gene neurofibromatosis type 1 (NF1), is associated with neurofibromas, composed largely of Schwann cells. The number and size of neurofibromas in NF1 patients have been shown to increase during pregnancy. A mouse embryonic stem cell (mESC) model was used, in which mESCs with varying levels of neurofibromin were differentiated into Schwann-like cells. NF1 cell lines derived from a malignant and a benign human tumor were used to study proliferation in response to hormones. Estrogen and androgen receptors were not expressed or expressed at very low levels in the NF1+/+ cells, at low levels in NF1+/- cells, and robust levels in NF1-/- cells. A 17 $\beta$ -estradiol (E2) metabolite, 2-methoxy estradiol (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 great hormonal influence. *Developmental Dynamics* 237:513–524, 2008. © 2008 Wiley-Liss, Inc.

**Key words:** Schwann cells; mouse ES cells; pregnancy hormones; steroid receptors

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## INTRODUCTION

### Neurofibromatosis Type 1 Origin and Loss of Heterozygosity

Neurofibromatosis type 1 (NF1) is the most common human tumor predisposition syndrome of the nervous system, affecting 1/3,000 to 1/3,500 live

births worldwide. Neurofibromas, the cardinal feature of NF1, are heterogeneous and composed of all the cellular components of peripheral nerves, including Schwann cells (SCs), fibroblasts, perineurial cells, axons, and mast cells. SCs or SC precursors have been shown to be the initiating cell type for tumorigenesis (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; Dachsenner et al., 1997; Rasmussen et al., 2000). Although neurofibromin is generally a microtubule-associated cytoplasmic protein (Gregory et al., 1993),

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<sup>1</sup>Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, Michigan

<sup>2</sup>Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, Michigan

<sup>3</sup>Department of Pediatrics, Neurology Division, University of Florida College of Medicine, Gainesville, Florida

<sup>4</sup>Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, Florida

<sup>5</sup>Department of Internal Medicine-Molecular Medicine and Genetics, University of Michigan Medical School, Ann Arbor, Michigan

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\*Correspondence to: Kate F. Barald, Department of Cell and Developmental Biology, University of Michigan Medical School, 3053 BSRB, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200. E-mail: kfbalard@umich.edu

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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, who presumably have the same mutation. Discrete cutaneous neurofibromas are usually benign and often first appear at puberty (McLaughlin and Jacks, 2003; Fishbein et al., 2007). Although these tumors are rarely present at birth, they are found in 48% of 10 year 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 (McGaughan et al., 1999; DeBella et al., 2000). Neurofibromas can also arise from multiple nerves within nerve 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). Approximately 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, enlargement, or tumor progression. 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 Milieu During Pregnancy

Concentrations of the steroid hormones 17 $\beta$ -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 the PR with 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, 2005) than hormone receptor-negative breast cancer cells.

### 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, so that 2ME2 may be acting indirectly to inhibit tumor growth through inhibition of angiogenesis, although that can-

not be the reason 2ME2 has apoptotic effects on cultured tumor cells (Mabjeesh et al., 2003; Wang et al., 2000). In such cells, 2ME2 acts by disrupting microtubules, with prolonged (24–72 hr) treatment of malignant tumor cells resulting in apoptosis (Wang et al., 2000; Mabjeesh et al., 2003).

### Embryonic Stem Cell-Based Model of SC Differentiation for Studies of NF1 Tumorigenesis

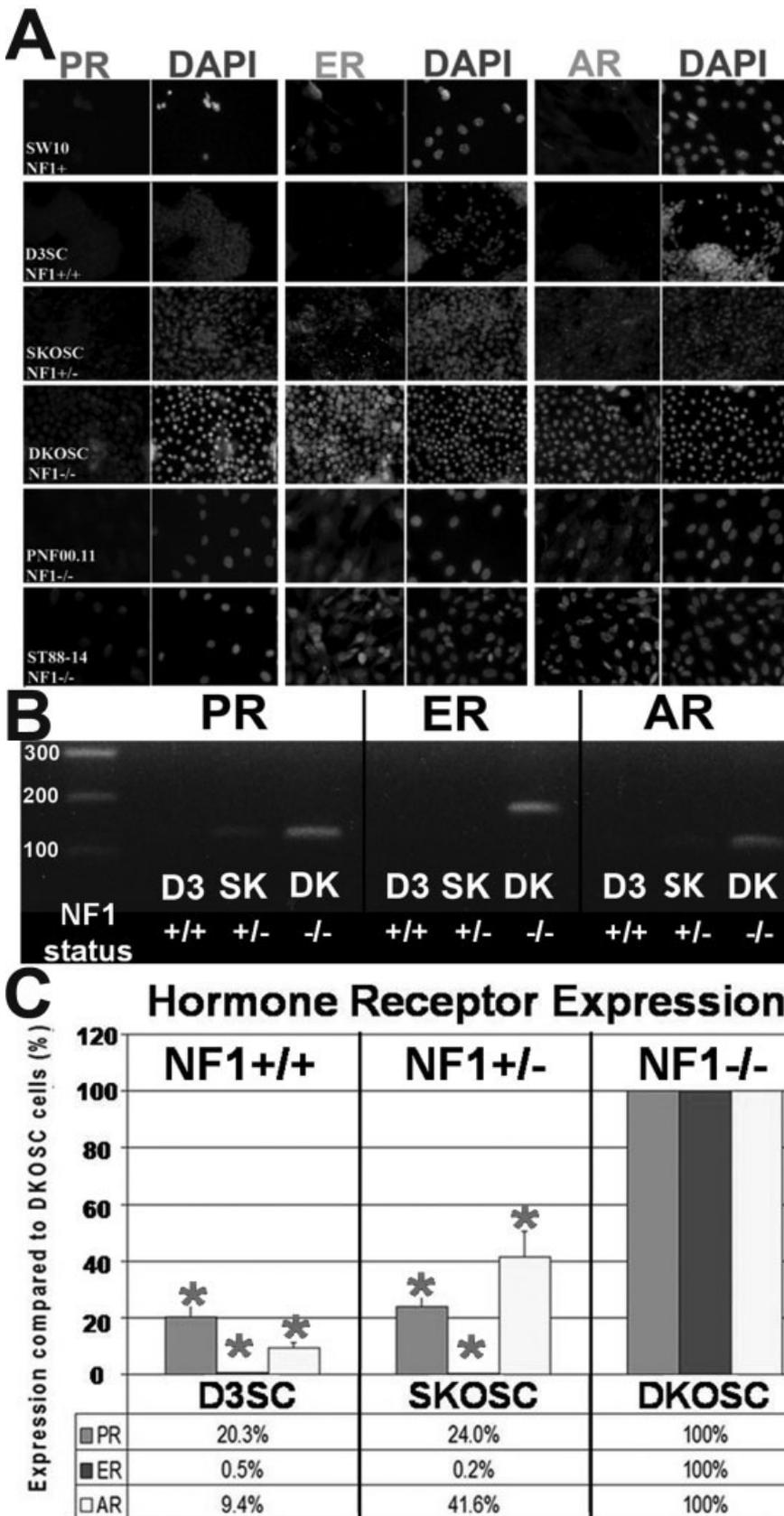
We recently reported a new stem cell-based model for studies of tumorigenesis in NF1 (Roth et al., 2007). An in vitro system was used to differentiate mouse embryonic stem cells (mESCs), which are NF1 wild-type (+/+), heterozygous (+/-), or null (-/-) into SC-like cells for studies of NF1. In this hormone study, we have focused on the SC-like cells derived from these mouse embryonic stem cells.

### RESULTS

The cell lines used for these studies include the NF1+/+ (D3) mES cell line (Doetschman, 1985), NF1+/- (SKO) mES cells (Jacks et al., 1994), NF1-/- (DKO) mES cell line (Jacks et al., 1994), SW10 (NF1+/+) mouse SC line that harbors a temperature sensitive SV40 large T antigen. When these cells are grown at 37°C (the nonpermissive temperature for transgene expression), they differentiate (ATCC; Hai et al., 2002). We also examined two human NF1 cell lines, pNF00.11 (referred to as PNF cells in this report; NF1-/-) a human plexiform neurofibroma SC-enriched culture (Muir et al., 2001), and ST88-14 (referred to as ST cells in this report; NF1-/-) derived from a human MPNST (a gift of Dr. Larry Sherman, Oregon Health Sciences University; Su et al., 2004). 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.

### 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

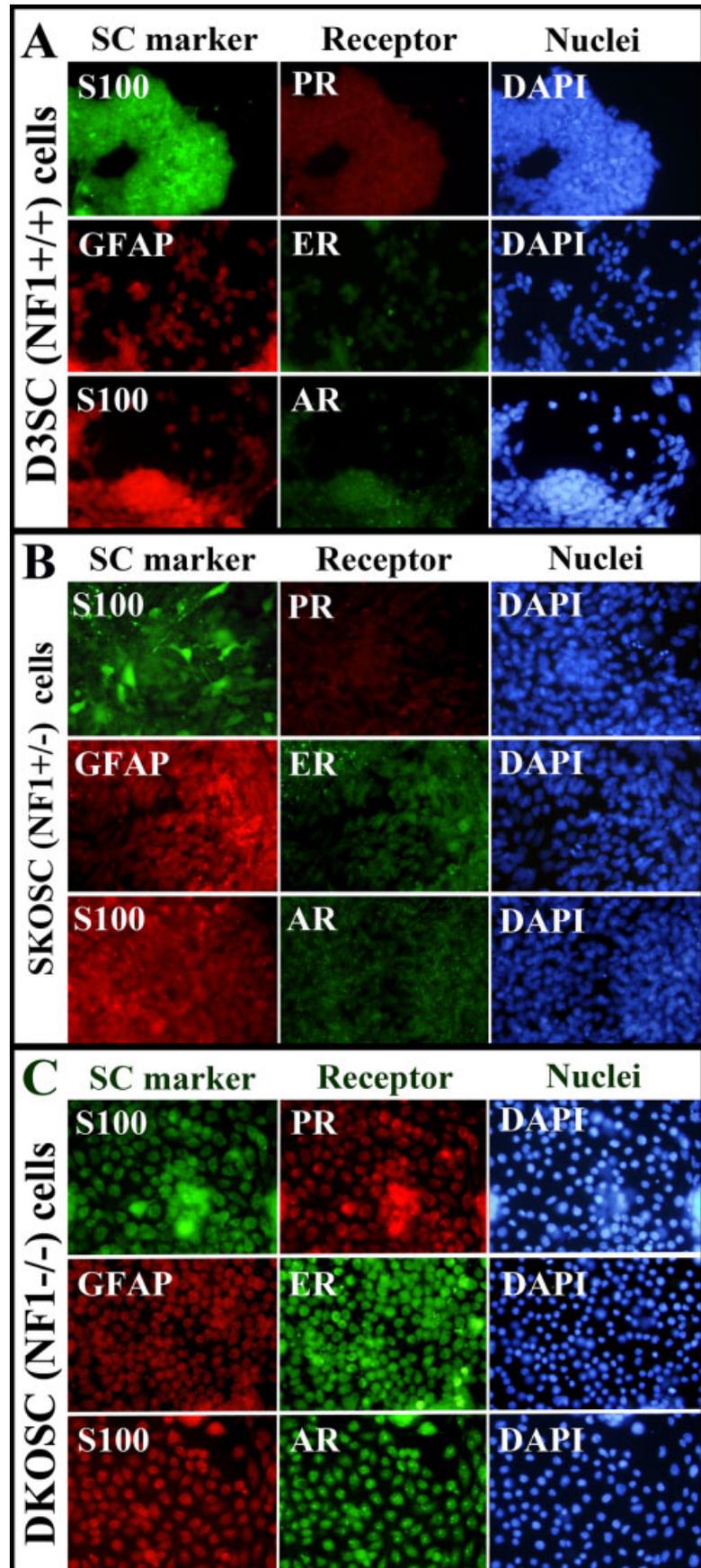


cell lines responded differently to the steroid hormones that are up-regulated during pregnancy (P4, E2, and T), we first determined whether these cells expressed the appropriate receptors (PR, ER, AR) for the relevant classic steroid receptor pathways at the protein level. We found that the level of classic receptor expression varied depending on the level of neurofibromin expressed by the cell type/line. Whereas PR (Fig.1A, first column) was not highly expressed in any of the cell lines, the NF1<sup>-/-</sup> (DKOSC, PNF, ST) cells had the most intense staining in the immunocytochemical experiments, although the SC line SW10 also showed some PR expression (Fig. 1A, bottom row, left). ER expression (Fig. 1A, third column) and AR expression (Fig. 1A, fifth column) followed similar patterns to each other, with little/no expression in the NF1<sup>+/+</sup> (D3SC, SW10) cells, low levels of expression in NF1<sup>+/-</sup> (SKOSC) cells, and higher levels of expression in NF1<sup>-/-</sup> (DKOSC, PNF, ST) cells. Immunocytochemical analysis demonstrated that, although PR and AR expression was exclusively nuclear, ER

**Fig. 1. A:** Schwann cell (SC) -like cells and neurofibromatosis type 1 (NF1) tumor cells express varying levels of steroid hormone receptors. 4',6-Diamidino-2-phenylidole-dihydrochloride (DAPI) staining of cell nuclei is shown to the right of the hormone receptor assayed. Left column, progesterone receptor (PR): Low levels of PR expression were seen in all cell types, with some up-regulation in NF1<sup>-/-</sup> (DKOSC, PNF, ST) cells. Third column, estrogen receptor (ER) and fifth column androgen receptor (AR): Very low levels of both ER and AR seen in NF1<sup>+/+</sup> (D3SC, SW10) cells, a slightly higher level in NF1<sup>+/-</sup> (SKOSC) cells, and most intense levels in NF1<sup>-/-</sup> (DKOSC, PNF, ST) cells suggests a stronger ability of NF1<sup>-/-</sup> cells to respond to hormones up-regulated during pregnancy. Some cell types displayed both nuclear and cytoplasmic expression of ER. Photographs were taken at  $\times 40$  original magnification. **B:** Using gel visualization, expression of hormone receptors was verified by reverse transcriptase-polymerase chain reaction (RT-PCR; D3=D3SC, SK=SKOSC, DK=DKOSC). **C:** Real-time quantitative PCR (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% AR of DKOSC cells, while SKOSC cells expressed 24% the amount of PR, 0.25 ER, and 41.6% AR of DKOSC cells.

localization was seen in both the nucleus and the cytoplasm, as has been previously noted (Ho and Liao, 2002; Zhang et al., 2004; Revankar et al., 2005). The differences in expression levels were confirmed in mouse SC-like cells by real-time quantitative polymerase chain reaction (RTqPCR; Fig. 1B). In RTqPCR assessments, with the level of expression in DKOSC cells set at 100%, D3SC cells expressed 20% of the amount of PR, 0.5% of the ER, and 9.4% of the PR seen in DKOSC cells, whereas SKOSC cells expressed 24% of the amount of PR, 0.25% of the ER, and 41.6% of the AR expressed by DKOSC cells (Fig. 1C). We then costained for both steroid hormone receptors and SC markers in the same cells, which verified coexpression levels (Fig. 2A–C).

To assay whether *in vivo* mouse tissue with or without NF1 expression showed similar patterns of classic hormone receptor expression as mouse cells *in vitro* did, we obtained tissue from mice in which a P0-driven cre transgene targeted the loss of NF1 in cells only from the SC lineage (Zheng et al., in revision) and stained for classic hormone receptor expression. In this model, NF1 mutant mice (cre+) develop neurofibroma tumors in dorsal root ganglia (DRG; Fig. 3). High levels of ER (green, middle columns) and AR (green, right columns) were seen in NF1-deficient mouse tissue (cre+), but not in mouse tissue with normal NF1 expression levels (cre-), similar to the expression patterns seen in the cultured NF1-deficient mouse cells (Fig. 3). In contrast to the *in vitro* assays, however, high levels of PR (red, left columns) expression were



**Fig. 2.** Coexpression levels of classic hormone receptors and Schwann cell (SC) markers in SC-like differentiated mouse embryonic stem cells (mESCs). **A–C:** This finding confirms the coexpression levels of S100 and progesterone receptor (PR), glial fibrillary acidic protein (GFAP) and estrogen receptor (ER) or S100 and androgen receptor (AR) in NF1<sup>+/+</sup> D3SC cells (A), NF1<sup>+/-</sup> SKOSC cells (B), and NF1<sup>-/-</sup> DKOSC cells (C). The staining for all three hormone receptors (PR, ER, AR) was most intense in the DKOSC (NF1<sup>-/-</sup>) cells (C), whereas all three cell types (D3SC NF1<sup>+/+</sup>, SKOSC NF1<sup>+/-</sup>, and DKOSC NF1<sup>-/-</sup>) expressed the SC markers S100 or GFAP. Photographs were taken at  $\times 40$  original magnification.

also seen in NF1-deficient mouse tissues (cre+; Fig. 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 higher levels of the receptors than either the D3SC or SKOSC cells (Figs. 1B, 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 medium, 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; Fig. 4). Addition of P4 (Fig. 4 P4) 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; Fig. 4, P4;  $n = 5$ ). Addition of E2 (Fig. 4, E2) or T (Fig. 4, T) significantly increased proliferation of malignant NF1 tumor cells (ST) and both DKOSC and D3SC cells. However, the NF1<sup>-/-</sup> 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 the respective inhibitors for the classic receptors (ICI182, 780 for ER, flutamide for AR) were used to block these receptors by preincubating cells with the inhibitors for 2 hr.

### 2ME2 Effects

To determine whether 2ME2, an estrogen metabolite that has been found to be up-regulated during normal pregnancies (Wang et al., 2000) and which has been found to affect the growth of various tumor cells (Fotsis et al., 1994; Maran et al., 2002; Dja-

vaheri-Mergny et al., 2003; Mabjeesh et al., 2003), affected the growth of NF1<sup>-/-</sup> mES cells, we grew the SC-like mES cells, SW10, and the tumor cell lines for 3 days in 2ME2 concentrations between 0.1  $\mu$ M and 100  $\mu$ M. We determined that 10  $\mu$ M was the threshold for significant changes in growth rates in all cells and cell lines tested (not shown). To determine whether the effect was cytotoxic (causing necrosis), apoptotic, or only decreased proliferation, we grew all cells for 2 days in 10  $\mu$ M 2ME2. Malignant ST cells were the only cell type to show a significant decrease in cell number when compared with the initial cell number (Fig. 5A). All other cells had either static cell numbers (PNF, DKOSC, and D3SC, or merely decreased proliferation rates (SKOSC, SW10) compared with control cells grown without 2ME2 addition. We did not observe cell necrosis.

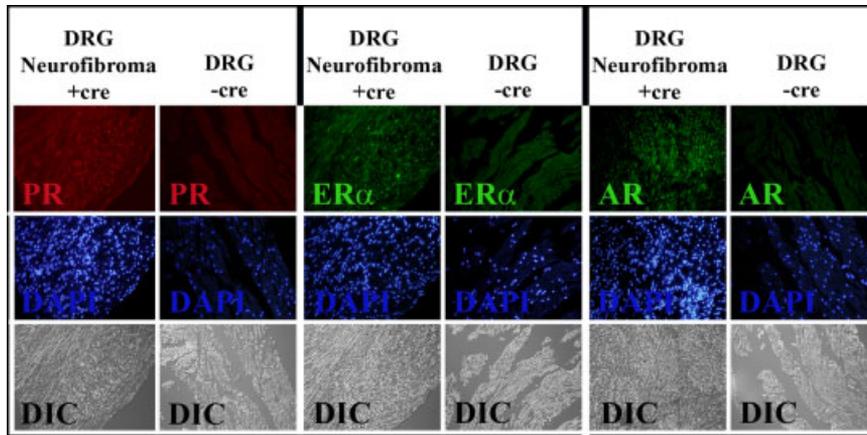
### Effects of 2ME2 Exposure on MTs

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 classic 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  $\alpha$ tubulin to visualize any changes in these cytoskeletal elements with increasing concentrations of 2ME2. We found that with the addition of 10  $\mu$ M 2ME2 (Fig. 5B, middle column), the PNF cells rounded up but their nuclei (stained with 4',6-diamidino-2-phenylidole-dihydrochloride [DAPI]) remained intact and 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 (Fig. 5B, left column), and tubulin protein was more diffuse in the cytoplasm. With 100  $\mu$ M 2ME2 (Fig. 5B, right column), PNF cells also began to lose microtubule extensions, tubulin ex-

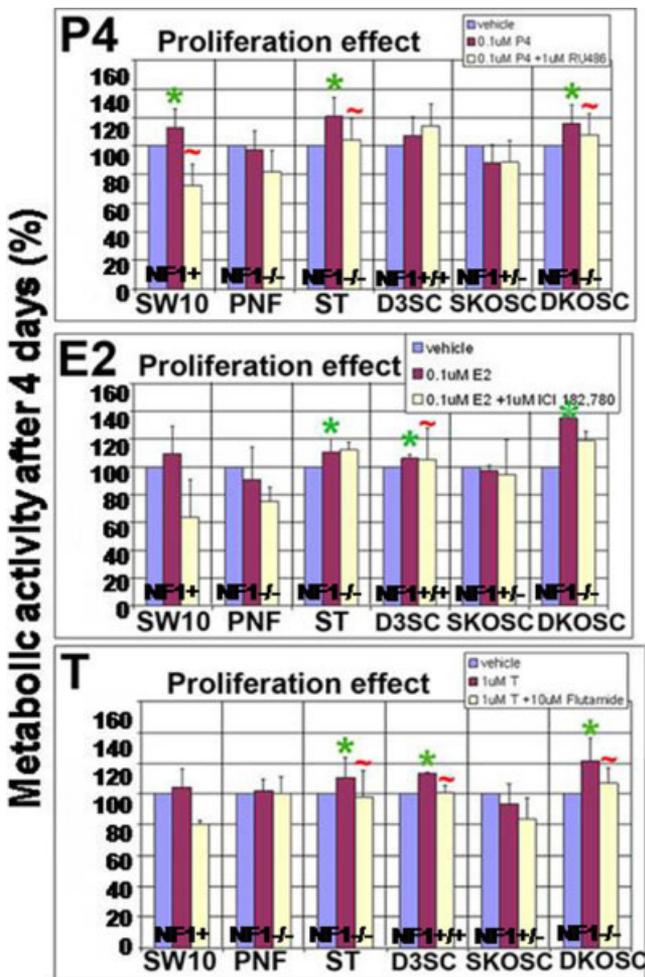
pression was diffuse, and 5–10% of the nuclei were becoming clustered and fragmented. ST cells became reduced in size (less than half the normal cell diameter) and apoptotic, with very little cytoplasm extending beyond the nucleus, thus visually exhibiting a much stronger effect from the 2ME2 on the cytoskeleton. To confirm that these malignant ST cells were becoming apoptotic in 2ME2, we assayed them for both caspase-3 expression and terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) staining. Although 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 hr (Fig. 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 cell model of NF1 or human SC tumor cell lines with variable levels of neurofibromin responded differently to the steroid hormones that are up-regulated 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 classic 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<sup>-/-</sup> (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<sup>+/+</sup> (D3SC, SW10) cells, low levels of expression in NF1<sup>+/-</sup> (SKOSC) cells, and most intense expression in NF1<sup>-/-</sup> (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



**Fig. 3.** Classic hormone receptor expression in mouse tissue. To assay for classic hormone receptor expression *in vivo*, we used mice in which cre recombinase expression targeted loss of floxed neurofibromatosis type 1 (NF1) expression to cells in the Schwann cell (SC) lineage (Zheng et al., in revision; Zhu et al., 2002). Mice transgenic for the cre transgene developed neurofibroma tumors in dorsal root ganglia (DRG), whereas mice without the cre transgene expressing normal NF1 levels did not. *In vivo*, mouse tissues with or without NF1 expression showed similar patterns of classic estrogen receptor (ER, green, middle columns) and androgen receptor (AR, green, right columns) steroid hormone receptor expression as SC-like mouse cells *in vitro*. The NF1-deficient tissues (cre+) had intense expression of ER and AR, whereas the NF1+ (cre-) did not. Progesterone receptor (PR) expression (red, left columns), however, was much more robust in NF1-deficient tissues (cre+) than that seen in cultured NF1-/- SC-like cells. This finding could be attributed to signaling from the surrounding cells in the heterogeneous, hypercellular tumor microenvironment, in contrast to the homogeneous NF1-/- SC-like cells in culture. 4',6-Diamidine-2-phenylidole-dihydrochloride (DAPI) staining of cell nuclei is shown in blue, and differential interference contrast (DIC, aka Nomarski) images are seen in the bottom row. Photographs were taken at  $\times 40$  original magnification.



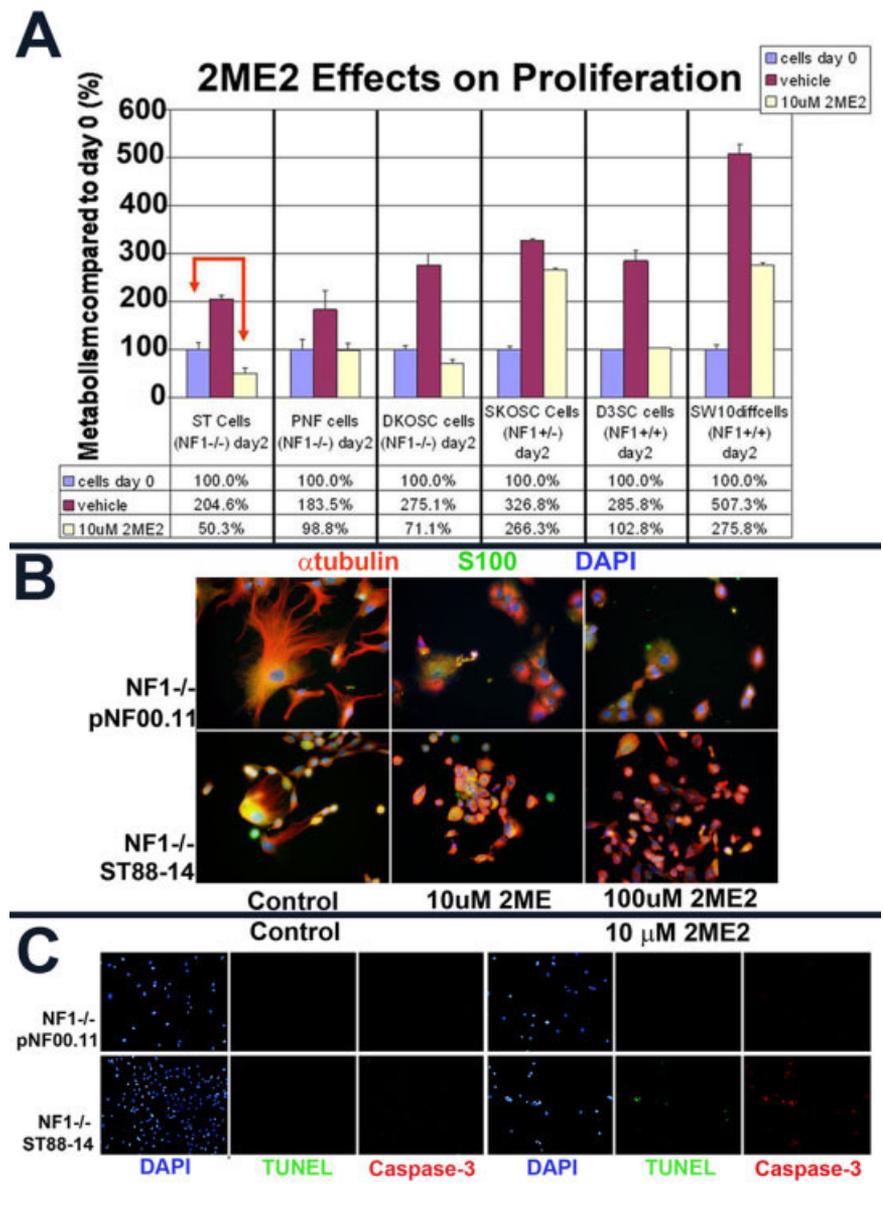
**Fig. 4.**

readily respond to hormones that rise during pregnancy. Because at least a part of this increase in cell numbers is inhibited by the classic receptor inhibitors, some of this increase must be mediated through these classic pathways. However, 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), which are known to be involved in NF1. PR and AR localization was exclusively nuclear, and ER was seen in both the nucleus and the cytoplasm, in accordance with the findings of others (Ho and Liao, 2002; Zhang et al., 2004; Revankar et al., 2005). Breast cancer tumor cells

**Fig. 4.** Hormones that are increased during pregnancy affect the proliferation of Schwann cell (SC)-like cells and neurofibromatosis type 1 (NF1) tumor cells differently, depending on the level of neurofibromin expression. Progesterone (P4): Addition of P4 increased proliferation significantly only in SW10, ST, and DKOSC (asterisk), whereas the progesterone receptor (PR) inhibitor RU486 returned proliferation rates to control levels (plus sign). 17 $\beta$ -Estradiol (E2): E2 increased proliferation significantly in malignant NF1 tumor (ST), DKOSC, and D3SC cells (asterisk), whereas addition of estrogen receptor (ER) inhibitor ICI 162,780 reduced proliferation rates to control levels only in D3SC cells, which had a small but significant difference (plus sign). Testosterone (T): Addition of T also significantly increased proliferation only in malignant NF1 tumor (ST), DKOSC, and D3SC SC-like differentiated mES cells (asterisk); addition of the AR inhibitor flutamide returned proliferation rates to control levels (plus sign).

that express both ER and PR have been found to have a better prognosis than those that do not express these receptors because they are more likely to respond to hormone 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).

A study done on both male and female neurofibroma tumors found that 75% expressed PR, while 5% expressed ER, regardless of the sex of the patient (McLaughlin and Jacks, 2003). This finding suggests an important role of P4 in tumor development; however, the tumor cell type was not identified and tumors were not tested for coexpression of ER and PR; neither AR expression nor number of receptors per cell was tested or reported in the study. Also, positive receptor expression of tumors was defined in their study as slides with 5 or more positive cells per 10 high power fields, and virtually none of the sections tested contained more than one hundred positive cells (McLaughlin and Jacks, 2003). We assayed for *in vivo* expression of classic hormone receptors (PR, ER, AR) in mouse tissues that had been engineered to target loss of NF1 in SC lineage cells carrying a cre transgene (Zheng et al., 2008; Zhu et al., 200), which subsequently developed neurofibroma tumors in the DRG. We found high levels of expression of all three classic receptors (PR, ER, AR) in NF1-deficient tissues (cre+) and low/no expression in tissues with normal levels of NF1 expression (cre-). This finding is in contrast to our *in vitro* findings, where ER and AR alone were up-regulated in NF1-/- cells, but PR was not. We attribute this difference to the importance of the tumor microenvironment in tumorigenesis, because surrounding cells and their resultant signals with and in addition to the NF1-deficient cells could also be affecting hormone receptor expression. Notably, DAPI staining and differential interference contrast visualization of the tissues demonstrated hypercellularity in the NF1-deficient cre+ tissues when compared with the cre- tissues with normal levels of NF1 expression, which could result in increased signaling in the tissues. Re-



**Fig. 5.** 2-Methoxy estradiol (2ME2), a naturally occurring  $17\beta$ -estradiol (E2) metabolite, is cytotoxic to a malignant neurofibromatosis type 1 (NF1) tumor cell line, but only slows or halts growth in other cell lines. Microtubules are disrupted in this process. **A:** When cells were grown for 2 days in  $10\ \mu\text{M}$  2ME2, malignant NF1 tumor cells (ST) cells were the only cells that showed significant cell death compared with 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). The y-axis indicates the percentage of cells actively metabolizing at day 2, compared with the number of cells actively metabolizing at day 0, which was set at 100%. **B:** Tumor cells were plated and attached overnight before the listed concentration ( $0\ \mu\text{M}$  left,  $10\ \mu\text{M}$  middle,  $100\ \mu\text{M}$  right) was added to the medium. After an additional 24 hr incubation, cells were fixed and stained for  $\alpha$ -tubulin (microtubules), S100 (SC origin of cells), and 4',6-diamidino-2-phenylidole-dihydrochloride (DAPI) (nucleus),  $\times 40$  original magnification. Tumor cells grown without 2ME2 (left) had extensive tubulin networks. Addition of either  $10\ \mu\text{M}$  (middle) or  $100\ \mu\text{M}$  (right) 2ME2, however, caused the cells to round up and appear apoptotic, that is, 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  $10\ \mu\text{M}$  or  $100\ \mu\text{M}$  2ME2. **C:** Tumor cells were grown as described and stained for terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) and cleaved caspase-3 expression. Whereas approximately 20% of the malignant ST cells (bottom row) were positive for apoptosis, only approximately 1% of the benign PNF cells were positive (top row).

cently, Fishbein et al. found differing heterogeneous levels of ER, PR, and AR in most NF1 tumor-derived SC samples and the normal SCs tested. Primary tissue samples they tested showed greater variation than cultured samples, suggesting that cells other than SC express hormone receptors (Fishbein et al., 2007). They also reported that tumor-derived SC cultures showed variable results for proliferation and apoptosis using steroid hormone ligands and receptors. Statistically significant changes were found in only a subset of tumor cells, regardless of gender (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. Because T is a precursor for E2, proliferative effects on cells could be mediated through aromatase activity (Gao et al., 2005), or through nonclassic indirect pathways by increasing intracellular  $\text{Ca}^{++}$  (Chen et al., 2005), rather than through the AR itself.

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<sup>-/-</sup> DKOSC cells expressing much higher levels (up to 400 $\times$ ) of the receptors than either the NF1<sup>+/+</sup> D3SC or NF1<sup>+/-</sup> SKOSC 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 statistically significant. Addition of P4 (Fig. 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 act synergistically with E2 to af-

fect proliferation, although in these studies we determined each hormone's effect individually. Also, PR had the lowest 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. Perhaps this is because in this assay we were testing cell lines, rather than the cellularly heterogeneous tumors assayed in the McLaughlin and Jacks paper (McLaughlin and Jacks, 2003) and their criteria for positive expression depended on only a small number of cells. 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 the glucocorticoid receptor, which could also influence the results (Ghoumari et al., 2003; Zhang et al., 2006). However, because these cells were grown in hormone-free medium with only P4 added, this influence should be minimal in these experiments. Addition of E2 or T significantly increased proliferation of the malignant NF1 tumor cells (ST) and both DKOSC and D3SC cells. However, the NF1<sup>-/-</sup> cell line ST, derived from the human malignant tumor and the mES-derived DKOSC were the only cell lines that showed a significant increase in proliferation with all three of the hormones tested. There was a slight trend or significant decrease in proliferation when their respective classic receptor inhibitors (ICI 182, 780 for ER, flutamide for AR) were used to preincubate cells for 2 hr. This may be because the hormone action is not mediated exclusively through classic 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 classic 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 messen-

gers, such as  $\text{Ca}^{++}$  and NO, and activate the PI3K/Akt and MAPK pathways (McEwen and Alves, 1999; Ho and Liao, 2002; Revankar et al., 2005), which are known to be involved in NF1 (Klesse and Parada, 1998; Klesse et al., 1999). These effects are also not inhibited by ER inhibitors, suggesting they are not mediated through the classic 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 signaling pathways involve activation of kinase signaling cascades or modulation of intracellular  $\text{Ca}^{++}$  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). ER $\alpha$  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, ER $\beta$  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 signaling pathways, ras, and multiple kinase pathways (Jacobsen et al., 2003). ER $\beta$  expression was not seen in any of the cell lines or tissues we tested (not shown), suggesting that ER $\beta$  is not a significant influence in the cells/tissues in this study.

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, 2ME2, which is an estrogen metabolite, which appears to hold overproliferation of cells

in check, has been found to be up-regulated during a normal 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<sup>-/-</sup> 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, suggesting that its mechanism of action 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 impairing HIF-1 accumulation in the nucleus thus inhibiting 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  $\mu$ M was the threshold at which 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 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 involved in 2ME2 cytotoxic effects specific to malignant tumors, because nonmalignant NF1<sup>-/-</sup> tumor cells did not undergo apoptosis with 2ME2 treatment.

Another hypothesis we posit is that the effect of hormones on neurofibromas may be indirect through hormonal effects on angiogenesis. Increased blood vessel formation during pregnancy could allow existing tumors to be supplied with additional nutrients and gas exchange as they grow, as well as to induce previously

microscopic tumors to grow to a size at which they are detectable. It is also worth noting that, although most of the cultures on which these studies were performed are of mouse cells, the tumor cell cultures are from human neurofibromas, which may explain some of the disparate results.

On the basis of these studies, we conclude that NF1<sup>-/-</sup> SC-like differentiated mES cells, as well as NF1-deficient mouse tissues and NF1<sup>-/-</sup> human tumor cells, express higher levels of classic steroid hormone receptors than cells and tissues with full expression of neurofibromin. Hormonal addition had a heterogeneous effect on cell proliferation, with a small but significant direct effect on the classic pathway in the NF1<sup>-/-</sup> SC-like and malignant NF1<sup>-/-</sup> human tumor cell lines tested. A naturally occurring estrogen metabolite, 2ME2 was able to induce apoptosis only in the malignant NF1<sup>-/-</sup> human tumor cells.

We have shown in a previous study that these SC-like differentiated mES cell lines (SKOSC, NF1<sup>+/-</sup> and DKOSC, NF1<sup>-/-</sup>) as well as two human NF1 tumor cell lines, are deficient in NF1 expression (Roth et al., 2007). We cannot, however, draw the general conclusion that increased steroid hormone receptor expression would be seen in all SC-like differentiated NF1-deficient mouse ES cell lines, because there are no other NF1<sup>+/-</sup> or NF1<sup>-/-</sup> mES cell lines available for comparison of which we are aware and the production of additional "knockout" ES cell lines is beyond the scope of this study. The effects of NF1 knockdown through RNA silencing and subsequent rescue by insertion of an NF1 construct into the knocked-down cells could also be used to verify the respective induction or suppression of steroid hormone receptors in NF1<sup>+/+</sup> D3SC cells. However, interpretation of such experiments would be difficult, because silencing is seldom complete and the huge size of NF1 makes re-expression experiments extremely difficult. These steps are beyond the scope of this current analysis, which is intended to examine the effects of hormonal treatment on proliferation in a homogeneous NF1<sup>-/-</sup> cell population as a "proof of principle" that ES knockout cell lines

can be used to test effects of neurofibromin loss, rather than a focus on the ES cell properties themselves.

Future studies will focus on the effects of these hormones separately and in combination on these stem cell-derived cell lines and other cells and tissues 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 classic receptors. Future analyses will also include in vivo assays that study the effect of steroid hormones on NF1-deficient tissues in the cre mouse models used in this study, which are also beyond the scope of the current study.

## EXPERIMENTAL PROCEDURES

### Cell Types

The cell lines used for these studies include the NF1<sup>+/+</sup> (D3) mES cell line (Doetschman, 1985); NF1<sup>+/-</sup> (SKO) mES cells (Jacks et al., 1994); NF1<sup>-/-</sup> (DKO) mES cell line (Jacks et al., 1994); SW10 (NF1<sup>+/+</sup>) mouse Schwann Cell line with a temperature sensitive SV40 large T antigen, grown at 37°C (the nonpermissive temperature for transgene expression) for differentiation (ATCC; Hai et al., 2002); pNF00.11 (referred to as PNF cells in this report; NF1<sup>-/-</sup>) human plexiform neurofibroma (Muir et al., 2001); and ST88-14 (referred to as ST cells in this report; NF1<sup>-/-</sup>) human MPNST (gift of Dr. Larry Sherman, Oregon Health Sciences University; Su et al., 2004). Positive control cell lines for hormone receptors were ER<sup>+</sup> and PR<sup>+</sup> MCF-7 cells (gift of Dr. Dorraya El-Ashry, Univ. of MI) and AR<sup>+</sup> LN-CaP 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% nonessential amino acids (Gibco, Carlsbad, CA), 15% fetal bovine serum (FBS; Atlanta Biological, Norcross, GA), 1% sodium pyruvate (2% stock), 7  $\mu$ L  $\beta$ mercaptoethanol (Sigma, St. Louis, MO), and 1,000 U/ml ESGRO (Chemicon, Temecula, CA). Schwann cell differentiation (SC) medium contained 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 medium used for hormone assays. Minimal medium contained 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 (Sigma). ER $\alpha$  (rabbit polyclonal 1:50); AR (rabbit polyclonal 1:50) and 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); glial fibrillary acidic protein (mouse monoclonal 1:400; Chemicon); myelin (mouse monoclonal 1:10; Abcam); PR (mouse monoclonal 1:50; Abcam);  $\alpha$ tubulin (mouse monoclonal 1:200); Caspase-3 (mouse monoclonal 1:1,000; BD Transduction, San Jose, CA); AlexaFluor 350, 488, 594, DAPI (Molecular Probes, Eugene, OR), goat  $\alpha$  rabbit-horseradish peroxidase (Zymed, San Francisco, CA). Note: We also looked at ER $\beta$  expression, but did not see expression in any of the experimental cells or tissues tested. Therefore, ER = ER $\alpha$  in this study. Hormones and receptor inhibitors used included progesterone, RU486, 17 $\beta$ -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 mounting medium (Molecular Probes), Porcine gel (Sigma), in situ cell death detection kit, fluorescein (TUNEL) (Roche, Indianapolis, IN), Citrisolv, ethanol, and sodium citrate dihydrate (Fisher Scientific, Fairlawn, NJ).

## Immunocytochemistry

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 medium; cells were grown an additional 24 hr. Cells were then fixed in 4% paraformaldehyde (Sigma), permeabilized in 0.2% Triton X-100 (Sigma), stained and mounted on Superfrost plus slides (Fisher, Pittsburgh, PA). Fluorescence micrographs were taken with an Olympus BX-51 microscope, and nonfluorescent micrographs with Nikon ACT-1 software on a Leitz Diavert inverted microscope.

## Hormone Receptor Immunohistochemistry on Mouse Tissue

The control mice used in this study are 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 20-month-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 (Zheng et al., in revision; Zhu et al., 2002). The Rosa26-LacZ allele was maintained on the mixed 129 Svj and C57Bl6 backgrounds. The mutant mice with or without the Rosa26-LacZ allele exhibited similar phenotypes. 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. Tissues were dissected, post-fixed overnight for 2 hr at room temperature, and transferred to 30% sucrose overnight. Tissues were embedded using OCT medium and sectioned longitudinally at 65  $\mu$ m 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 0.01 M sodium citrate buffer pH 6.0, followed by immunohistochemistry at room temperature in a humidified chamber. Nonspecific binding was blocked with 10% donkey serum, incubated with primary antibodies listed followed by secondary antibody incubation for 20 min and 5 min DAPI staining. Washes between steps were done in 0.1% PBST. After coverslips were applied using Prolong Gold antifade mounting medium and slides were dried overnight, photographs were taken at  $\times$ 40 magnification using an Olympus BX-51 microscope.

## Measures of Cell Proliferation

### *Hormones and receptor inhibitors.*

Cells were grown overnight in hormone-free phenol red-free medium. The next day, cells were counted, plated, and allowed to attach for 2 hr 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 hr. At 4 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  $\pm$  SD, and the Student's *t*-test was used to gauge significance, which was  $P < 0.05$ .

### 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 2 days, proliferation was assayed using the Cell-Titer96 (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  $\times$  100.

### RTqPCR

RTqPCR was performed using primer pairs designed using the Beacon designer program (Bio-Rad, Hercules, CA), target with Ta at 55°C, a length 18–22 bp, and amplicon size 100–200. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (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  $\mu$ g of total RNA by reverse transcription using Super Script III transcriptase (Invitrogen, Carlsbad, CA) and oligo dT primer. A 2- $\mu$ l 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 1 min, 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 ultraviolet light.

### PCR Primers

NF1: forward AGTTTCTCTCCTCG-CTGGTCTTC reverse CGTTTCTGCG-CACCCGTTTG; AR: forward GCG-GTCCTTCACTAATGTCAACTC reverse TGCCTCATCCTCACACTGG; PR: forward CTGGATGAGCCTGATG-GTGTGTTG reverse GGCACAGCGAG-TAGAATGACAG; ER forward GAA-AGGCGGCATACGGAAAGAC reverse TCAAGGACAAGGCAGGGCTATTC.

### TUNEL Assay

Apoptosis was measured by TUNEL assay. PNF and ST cells were plated, grown, fixed, and permeabilized as in the 2ME2 immunocytochemical assay. The TUNEL assay was performed according to Roche kit specifications with the following modifications. Caspase-3 antibody was added at 1:1,000 to the TUNEL reaction mixture and incubated at 37°C for 60 min in the dark. After PBS rinses, DAPI was added at 1:1,000 for 3 min. Coverslips were mounted on slides with Molecular Probes' Antifade medium and dried 24 hr in the dark before fluorescence visualization.

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