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Analysis of steroid hormone effects on xenografted human NF1 tumor Schwann cells

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Abbreviations: MPNST, malignant peripheral nerve sheath tumor; NF1, neurofibromatosis 1; SC, schwann cell; PR, progesterone receptor; ER, estrogen receptor; hGST, human glutathione S transferase; TUNEL, terminal deoxynucleotidal transferase dUTP nicked end labeling; DAB, 3,3'-diaminobenzidine; scid, severe combined immunodeficiency

The neurofibroma, a common feature of neurofibromatosis type 1 (NF1), is a benign peripheral nerve sheath tumor that contains predominantly Schwann cells (SC). There are reports that neurofibroma growth may be affected by hormonal changes, particularly in puberty and pregnancy, suggesting an influence by steroid hormones. This study examined the effects of estrogen and progesterone on proliferation and apoptosis in a panel of NF1 tumor xenografts. SC-enriched cultures derived from three human NF1 tumor types [dermal neurofibroma, plexiform neurofibroma and malignant peripheral nerve sheath tumor (MPNST)] were xenografted in sciatic nerves of ovariectomized *scid/Nf1*$^{-/+}$ mice. At the same time, mice were implanted with time-release pellets for systemic delivery of progesterone, estrogen or placebo. Proliferation and apoptosis by the xenografted SC were examined two months after implantation, by Ki67 immunolabeling and TUNEL. Estrogen was found to increase the growth of all three MPNST xenografts. Progesterone was associated with increased growth in two of the three MPNSTs, yet decreased growth of the other. Of the four dermal neurofibroma xenografts tested, estrogen caused a statistically significant growth increase in one and progesterone did in another. Of the four plexiform neurofibroma SC xenografts, estrogen and progesterone significantly decreased growth in one of the xenografts, but not the other three. No relationship of patient age or gender to steroid response was observed. These findings indicate that human NF1 Schwann cells derived from some tumors show increased proliferation or decreased apoptosis in response to particular steroid hormones in a mouse xenograft model. This suggests that anti-estrogen or anti-progesterone therapies may be worth considering for specific NF1 neurofibromas and MPNSTs.

Introduction

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder with a wide variety of features, the hallmark feature being neurofibromas.[1] Neurofibromas are benign peripheral nerve tumors that contain more than 50% Schwann cells (SC). Smaller, cutaneous or subcutaneous neurofibromas are termed "dermal" here and have no potential for malignancy. However, neurofibromas involving larger nerves can become large and have a risk of transformation to malignant peripheral nerve sheath tumor (MPNST).[1] Some of the neurofibroma SCs are clonally expanded from a SC whose normal *NF1* gene allele was mutated, fulfilling the tumor suppressor two-hit system. Neurofibromas often first appear around puberty and develop throughout adulthood. Pregnancy is often associated with increased size, number and malignant potential of neurofibromas.[2-6] Lammert et al.[7] reported a survey of 59 NF1 patients that found that while oral contraceptive pills did not stimulate subjective growth of neurofibromas in the majority of patients, two receiving high dose Depo Provera contraceptive did report significant tumor growth. Thus, neurofibroma growth often parallels hormonal changes. McLaughlin and Jacks[8] examined 59 human neurofibromas for the expression of estrogen (ER alpha) and progesterone receptors (PR) with immunohistochemistry. They found that the majority (75%) of neurofibromas had at least a few cells that expressed PR, but these cells were S100 negative (therefore not Schwann cells). They did not see detectable ER alpha staining. A similar study in our lab showed that 54% of neurofibromas were PR-positive and that cultured tumor Schwann cells do express steroid hormone receptor transcripts.[9]

Steroid hormones are cholesterol derivatives with important roles in growth, metabolism, differentiation and reproduction.

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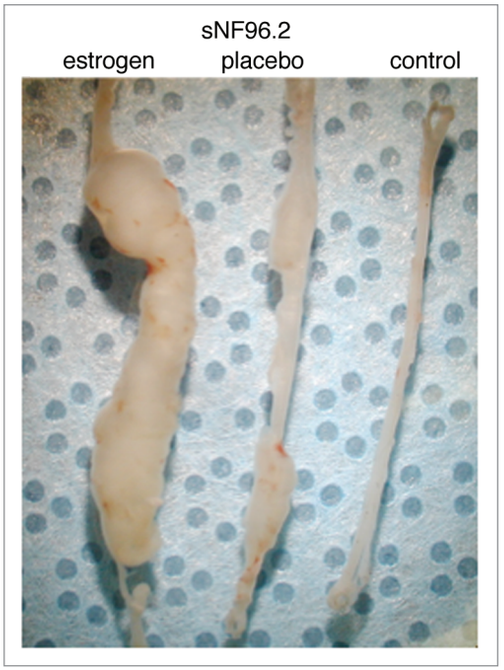

Figure 1. Representative sNF96.2 xenograft whole sciatic nerves show visible additional growth under influence of estrogen. The leftmost nerve is from a mouse treated with estrogen. The middle nerve is from a mouse treated with placebo pellet. The rightmost nerve is from an untreated mouse, where the nerve was injected with PBS as injection control.

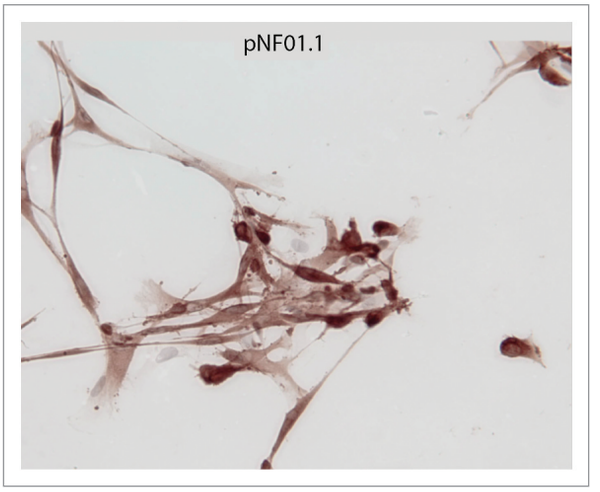

Figure 2. Cultured plexiform pNF01.1 Schwann cells are shown, stained with S-100, at x40 magnification. This culture shows characteristic enrichment for SC.

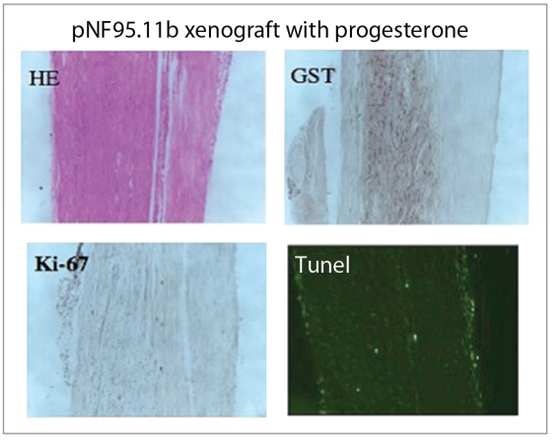

Figure 3. Immunohistochemical staining of plexiform pNF95.11b xenograft with progesterone hormone treatment. (A) H/E stain. (B) hGST immunostain (brown cells are engrafted human SC). (C) Ki-67 immunostain for proliferation. (D) TUNEL stain for apoptosis. Magnification x200.

They have been implicated in some cancers, especially breast and prostate cancer. A few biochemical studies have been done on rodent SCs,[10,11] however, only recently has data been published regarding hormone effects in normal human or NF1-tumor derived Schwann cells. Recent in vitro data indicated that steroid hormone receptor expression correlated with neurofibromin levels in mouse ES-derived Schwann cells and two human tumor cultures (from our group).[5,6] The plexiform culture did not significantly respond to hormones in a proliferation assay, but the MPNST line showed increase in proliferation rate in response to estrogen and progesterone. Our group showed that neurofibromin-deficient tumor SC responded to hormones in culture.[9] Overdiek et al.[12] also showed an increase in proliferation in neurofibroma SC in response to progesterone. To survey the effect of estrogen and progesterone on proliferation and apoptosis in a series of human neurofibroma Schwann cells in vivo, we employed a sciatic nerve xenograft model in ovariectomized *scid/Nf1* mice treated with progesterone or estrogen. Human SC derived from three NF1 tumor types (dermal neurofibroma, plexiform neurofibroma and MPNST) were tested by assaying proliferation and apoptosis of the xenografted SC using immunohistochemistry. This is the first such in vivo NF1 study.

Results

Eleven SC cultures (from four dermal neurofibromas, three MPNSTs and four plexiform neurofibromas) (five from females, six from males) were tested using a total of 104 sciatic nerve xenografts. The xenograft results were consistent for each culture; there were virtually no statistical outliers. As indicated previously, MPNST sNF96.2 grew the most dramatically (**Fig. 1**) with the sciatic nerve massively enlarged under estrogen treatment (and still visibly enlarged in placebo and progesterone treatment).[17] However, most of the xenografts did not produce grossly enlarged nerves. **Figure 2** shows an example of one of the plexiform SC cultures and **Figure 3** shows an example of xenograft immunostaining. hGST staining was detected in multiple cells in each xenograft, indicating that human cells were present in the sciatic nerve 2 months after the xenograft injection. Proliferation and apoptosis were measured by Ki67 staining and TUNEL staining of xenograft sections, respectively. All xenografts had at least some Ki67-positive hGST-positive cells except for cNF04.9a, a dermal neurofibroma-enriched SC culture which

failed to form a mass in one nerve each in two mice (one placebo, one estrogen). For all xenografts, the average ratios of Ki67/TUNEL positive cells in the same size area from serial sections were compared between the treatment groups (estrogen versus placebo, progesterone versus placebo). Results are illustrated by graphs in **Figure 4**. An increase in this ratio (i.e., increased survival and/or increased proliferation) is consistent with increased cell mass in treatment relative to placebo. Similarly a decrease indicates reduction in cell mass relative to placebo.

In all four dermal-derived SC culture xenografts (where human cells were evident), there is suggestion of increased tumor cell mass in presence of estrogen and/or progesterone (**Fig. 4A**). This met statistical significance ($p \leq 0.05$) in dermal culture cNF97.5 with estrogen ($p = 0.0189$) and dermal culture cNF99.1 with progesterone ($p = 0.0108$) (**Fig. 4A**). In the presence of progesterone, cNF97.5 had a growth trend but it was not statistically significant ($p = 0.895$). **Figure 5** shows sections of the dermal neurofibroma cNF97.5 xenograft, illustrating the significant increase in cell mass with estrogen but not progesterone. For the plexiform neurofibroma SC xenografts, the ratio of proliferation: apoptosis showed a decrease trend in the presence of estrogen (for pNF00.13, pNF95.6 and pNF95.11b) and progesterone (same three cultures) (**Fig. 4A**). This was statistically significant for estrogen and progesterone in pNF95.11b, while both hormones were associated with increase in pNF01.1 (not statistically significant, but the number of surviving cells for pNF01.1 was very small and thus most subject to error) (**Fig. 4A**). For MPNST-derived SC xenografts, there was a trend of

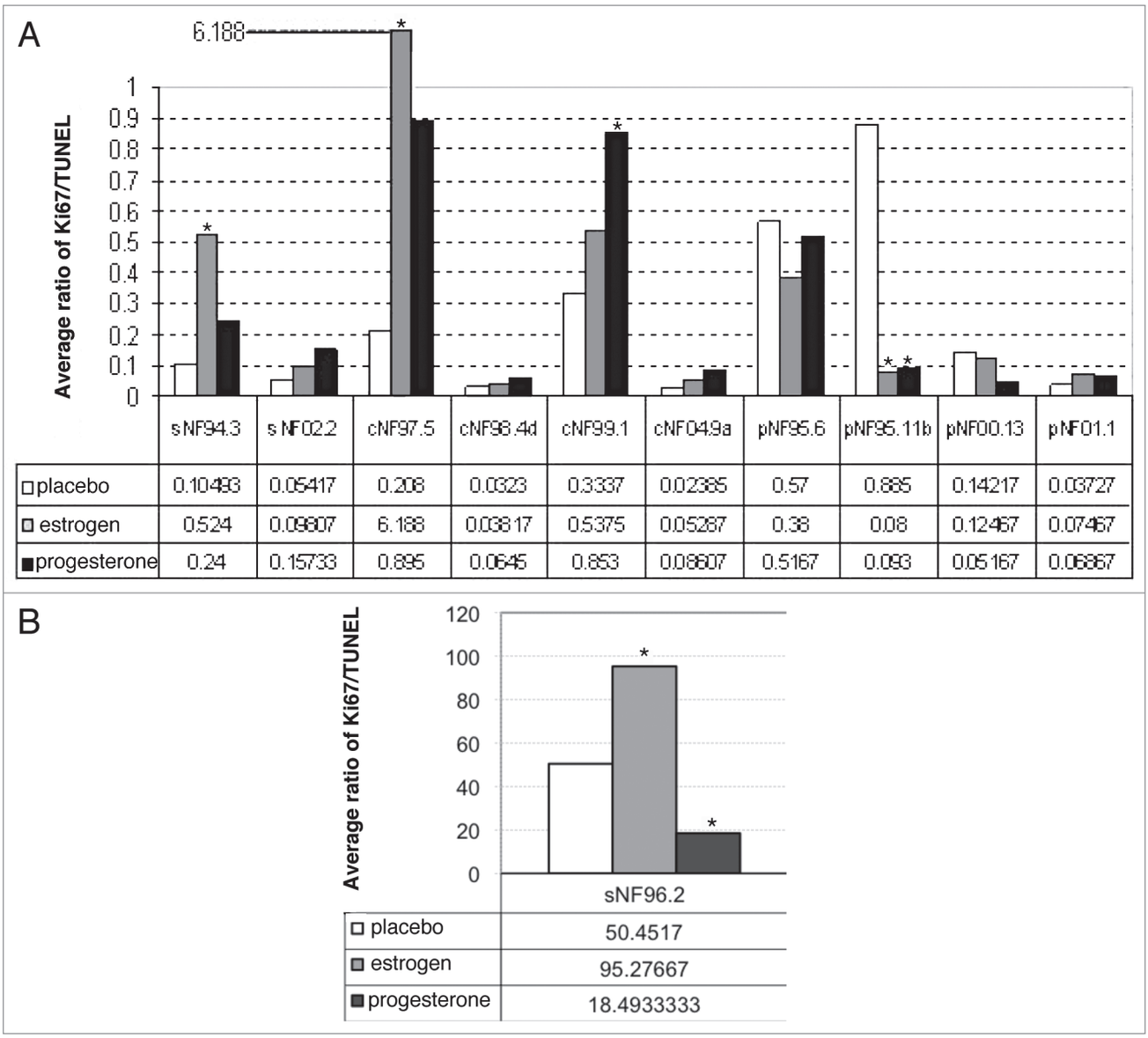

Figure 4. (A) Effect of hormones on xenograft compared to placebo. The star indicates $p \leq 0.05$. Open bar, placebo; gray bar, estrogen; black bar, progesterone. Xenografts of sNF94.3 and cNF97.5 showed statistically significant increase in response to estrogen, whereas the pNF95.11b xenograft showed significant inhibition with both hormones, compared to placebo. Progesterone was associated with an increase in the cNF99.1 xenograft. (B) Xenograft of sNF96.2 showed statistically significant inhibition in response to progesterone, but increase in xenograft size in response to estrogen.

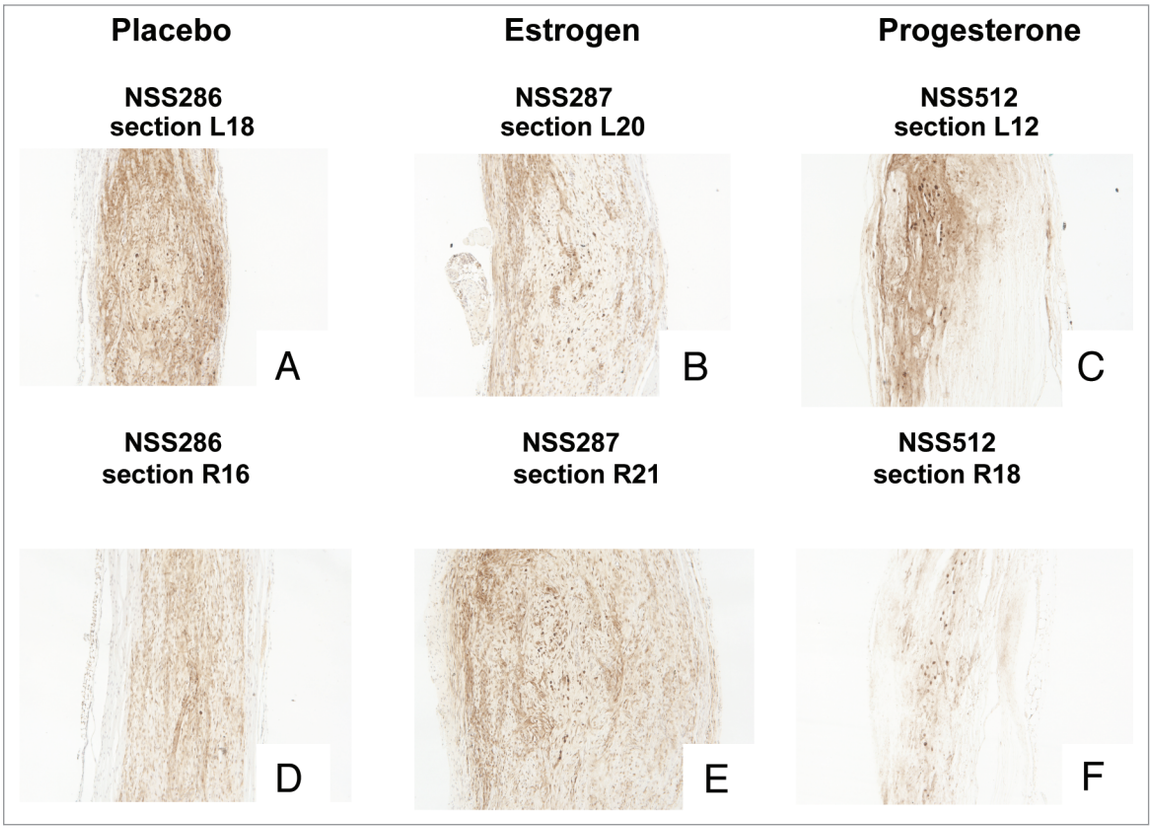

Figure 5. Example of hormone effects on xenografts at a gross morphology level. Photomicrographs are shown for the dermal neurofibroma cNF97.5 xenograft immunostained with hGST antibody to show cells of human origin. Photos (A and D) are placebo sections from left side and right side nerves, respectively; (B and E) are estrogen-associated sections; (C and F) are progesterone-associated sections. An increase in xenograft size can be seen in the presence of estrogen, but not progesterone. The magnification is x200.

increasing cell mass in the presence of estrogen for all three cultures, which was statistically significant in sNF96.2 and sNF94.3 (**Fig. 4**). Progesterone was associated with a trend of increasing cell mass in sNF94.3 and sNF02.2 xenografts relative to placebo, but decreasing cell mass compared to placebo in sNF96.2 (**Fig. 4**).

MPNST sNF96.2 produced the most interesting results due to its massive expansion in vivo. This xenograft best resembles an MPNST,[17] while another MPNST xenograft (sNF94.3, which did not grow as robustly) better fits histology criteria for a plexiform neurofibroma.[16] This culture was remarkable in that estrogen was associated with tremendous growth, greater than under placebo conditions (**Figs. 1 and 4B**). Yet progesterone was associated with failure of the xenografts to achieve placebo-level growth (**Fig. 4B**). Immunohistochemistry of sNF96.2 xenografts for progesterone receptor (PR) and estrogen receptor (ERα & ERβ) showed a negative result for all three receptors with DAB detection, compared to positive control slides (tonsil for PR, breast cancer for ERα, colon cancer for ERβ, data not shown). In addition, we also xenografted sNF96.2 into ovariectomized/pellet-implanted female *scid* mice lacking the *Nf1* heterozygous background. In comparison to the *Nf1(+/-)/scid* results, the *scid*-only xenografts grew less robustly, regardless of hormone treatment (statistically significant) (**Fig. 6**).

Discussion

The neurofibroma clonal tumorigenic cell is the Schwann cell, containing somatic loss of function of the remaining *NF1* allele.[21-23] Thus, these tumors (and MPNSTs) are heterogeneous in terms of different combinations of germline/somatic *NF1* (and in some cases, other genes) mutations, cytogenetics, expression array results and presence of a number of antigens.[8,9,14,21,22,24-46] Given this, and variable patient reports about whether their neurofibromas were affected by pregnancy, it is not surprising that this study found heterogeneous results.

MPNST sNF96.2 showed robust response to estrogen. The MPNST sNF96.2 data suggest that at least for this culture, a germline background of heterozygosity for a disruptive *Nf1* mutation in the recipient mouse is more permissive for increased growth under hormone influence compared to the *scid*-only background (wild-type at *Nf1* locus). Interestingly, this MPNST xenograft grows less robustly in male recipient mice, even smaller than female-placebo.[17] Estrogen induced a greater xenograft growth than placebo and progesterone actually somewhat inhibited growth compared to placebo. Thus, this cell line is clearly differentially responsive to hormones and genetic background. Such complexity may be true of many neurofibroma clonal SC, but the sNF96.2 cell line amplified sufficiently in vivo to clearly

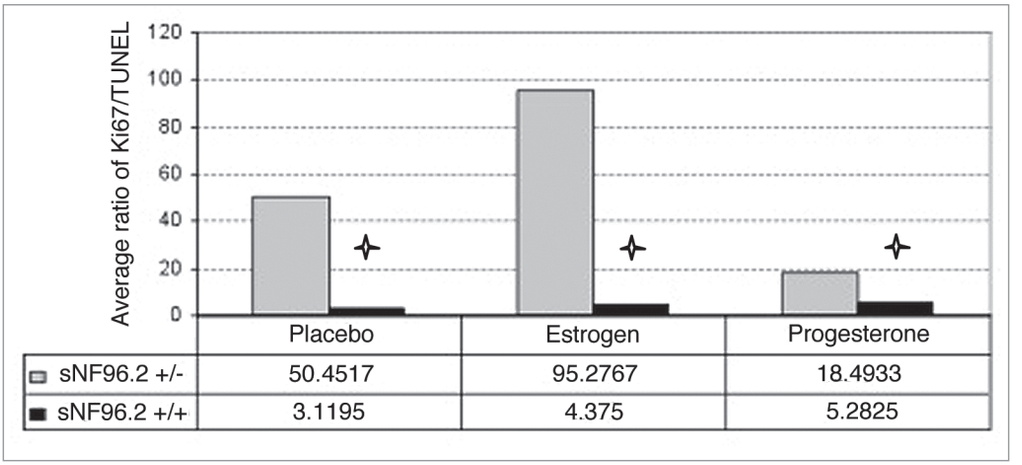

Figure 6. Comparison of the effects of hormone on sNF96.2 xenograft in *Nf1*$^{+/-}$, *scid* mice versus *Nf1*$^{+/+}$, *scid* mice. The star * indicates statistically significant results with $p \leq 0.05$. Gray bar, *Nf1*$^{+/-}$; black bar, *Nf1*$^{+/+}$.

observe these properties. Although progesterone and estrogen receptors were not detected in the xenograft, they may still be present at low levels as seen in some SC in vitro previously (or there may be additional receptors not yet identified).[5] Consistent with this is the previous observation that these receptor transcripts are undetectable in this cell line and barely detectable in RNA from primary tumor (using real-time PCR) and treatment with ligands had no effect on proliferation or apoptosis in culture.[9] Alternatively, it may be that the hormones are exerting effects through non-genomic mechanisms, regardless of karyotype, a phenomenon increasingly under study.[47]

Some xenografts of all three tumor types respond to hormones: clinical significance. Although estrogen and progesterone showed variable effects on xenograft growth, there were consistent results specific to each tumor culture. Responses are not related to patient age or tumor type, although most of the significant responding cultures were from males, and/or were from MPNSTs. The data support a functional role of steroid hormones in tumor growth in some individuals and/or tumors (3/11 positive for response to estrogen; 2/11 positive response to progesterone; 1/11 negative response to estrogen; 2/11 negative response to progesterone). These responses were in independent cell cultures except for sNF96.2 (described above) and pNF95.11b, which showed inhibition by both hormones compared to placebo. Overall, these observations suggest that hormone-related therapies may be of potential use in controlling growth of some NF1 tumors. This is feasible even in the absence of hormone receptors since some such agents (e.g., tamoxifen) can exert effects independent of estrogen receptor status.[48-51] Future work could involve expression array or proteomic studies comparing responding and non-responding tumors, to develop a profile that might predict response to hormones and thus suggest possible efficacy of anti-hormone therapies. If the mechanisms through which the hormones act in these tumors are characterized, we may be able to identify alternative therapeutic targets that are not directed at the hormones/receptors themselves, such as underway in breast cancer.[52] Although the two month interval was sufficient to show proof of principle in this experiment, extended xenograft time beyond 2 months would yield larger tumors, which could be useful for preclinical studies to measure treatment efficacy. This xenograft system could also be used to measure effects of hormone-related (or other) therapies, especially those aimed at the hormones known to stimulate certain tumor cell cultures. This work contributes to the growing evidence that steroid hormones can play an important role in NF1 tumorigenesis.

Materials and Methods

Neurofibroma SC culture. NF1 tumor-derived Schwann cells were isolated from surgical specimens of neurofibromas or MPNSTs, from patients (age 1–35 years) meeting NF1 diagnostic criteria, under IRB approval.[13] Our SC culture protocol that favors *Nf1*$^{-/-}$ cells has been described previously.[14] To briefly review, viable tumor was isolated from surgically resected neurofibromas and single cell suspensions were created. The cells were then cultured in Dulbecco's Modified Eagle's Medium supplemented with 15% fetal bovine serum, 50 ng/ml neuregulin (GGF2) and antibiotics (Pen/Strep) on laminin-coated plates. Schwann cells were enriched to 70–95% by differential detachment over several passages, using mild trypsinization. Cultures were characterized with S-100, P-75 (NGFR) and Ki-67 immunostaining. The three MPNST cultures we established are available at ATCC and do not require laminin or neuregulin.

Xenograft. The *Nf1*^{Fcr} mutation on a C57BL/6 background[15] was bred to B6.CB17 immunodeficient *scid* mice (Jackson Laboratories # 001913) through several generations to produce the desired "NSS" mice (*Nf1* heterozygote and *scid* homozygote) for xenograft. This xenograft system is well-established in our lab.[16,17] As approved by UF IACUC, bilateral ovariectomy, hormone pellet implant and sciatic nerve xenograft were performed on 8-week-old female mice. Under anesthesia, a 1.0 cm incision was made in the skin to expose the back muscles. A small 0.2 cm incision was made in the muscles overlying the ovaries on both sides. The ovaries were isolated, tied off with sterile sutures

and removed. The muscles and the skin were sutured separately. Ovariectomized females were then implanted with 60-day release pellets of either17β-estradiol (0.72 mg/pellet, cat # SE-121), progesterone (25 mg/pellet, SP-131) or placebo (SC-111) (Innovative Research of America). This is the established system for providing steady-state physiologic blood levels of these hormones in isolation.[18-20] Human tumor derived Schwann cells [5 x 10^5/5 ul (see below)] were then injected intra-fascicularly in the surgically-exposed sciatic nerve of each leg. This was sutured and the animals were returned to SPF housing for 2 months. We used three mice per treatment group, for a total of six xenografts for each treatment. All xenografts were performed on the same day for each culture, using the same batch of cells.

Analysis of xenograft. Sciatic nerves were harvested two months after surgery, fixed in 4% paraformaldehyde and paraffin embedded longitudinally. Serial sections (7 μm) were cut from each block through the entire nerve, and staining/immunohistochemistry was performed with H&E (hematoxylin and eosin staining), hGST (human glutathione transferase), Ki67 and TUNEL as described below. Every 7th section was H&E stained for light microscopy examination. Based on this, three sets of three serial sections across the widest area of the graft were used to determine proliferation-to-apoptosis ratio.

For hGST staining (using human-specific antibody), endogenous peroxidases were quenched with 1% hydrogen peroxide in methanol for 30 minutes. After washing in PBS, the rabbit polyclonal anti-human hGST antibody (Cat# 107; Dako, Carpinteria, CA) was added at a 1:100 dilution. Staining was detected with 1:500 dilution of swine anti-rabbit biotinylated conjugated-secondary antibody and ABC, 3,3'-diaminobenzidine as a substrate for peroxidase. For Ki67 staining, to identify proliferating cells, endogenous peroxidases were quenched with 1% H_2O_2 in methanol for 30 minutes. To unmask antigens, samples were pre-treated in Target Retrieval Solution (10 mM citrate buffer, pH 6.0, Dako Cat# S-1700; Carpinteria, CA) at 95–99°C for 30 minutes. The rabbit polyclonal anti-human Ki67 antibody (Cat # 21174418, Zymed, San Francisco, CA) was used at a 1:100 dilution. Staining was detected with a 1:500 dilution of swine anti-rabbit biotinylated conjugated-secondary antibody and ABC, 3,3'-diaminobenzidine as a substrate for peroxidase. For TUNEL (TdT-mediated dUTP Nick-End Labeling) staining of cells undergoing apoptosis, the DeadEnd™ Fluorometric Tunel System Kit (Cat# G3250, Promega, Madison, WI) was used according to the manufacturer's instructions. This system used fluorescent detection. PR immunohistochemical staining has been previously described.[9] ERalpha and ERbeta staining (DAB detection) were done by the Shands Diagnostic Resource Lab and the UF Molecular Pathology Core, respectively.

Statistical analysis. For most of the xenografts, sciatic nerves weren't visibly enlarged or enlarged in a uniform shape, so volumetric analysis was not possible. Instead, human (hGST positively-stained) cells within entire xenograft sections were counted. However, for the large xenograft developed from culture SNF96.2, we chose three representative non-adjacent areas in the GST-positive region for counting, at x400 magnification. The ratio of Ki67/TUNEL positive staining cells in the same area was compared from serial sections in each group (estrogen, progesterone, placebo). The average number of Ki67-positive cells and TUNEL-positive cells from three slides each was calculated for each sample. Data from all six xenografted nerves were averaged for each culture and condition, with and without Dixon outlier analysis. Ki67/TUNEL ratios were calculated for each group (placebo, estrogen and progesterone). The results were analyzed with a two-tailed unpaired t-test to compare effects for estrogen versus placebo and progesterone versus placebo.

Acknowledgements

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