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13. ABSTRACT (Maximum 200 Words)

This work is testing the hypothesis that human NF1 neurofibroma (and/or MPNST) Schwann cells have increased growth or decreased apoptosis in response to estrogen and progesterone. Specific Aim 1 measured steroid hormone receptor expression in human normal, NF1 neurofibroma and MPNST Schwann cells. We found less than 2-fold difference in these receptor transcripts in tumor versus normal Schwann cells, however this may still be of biological significance. There is no detectable estrogen receptor in normal Schwann cells or NF1 tumors by immunohisto-chemistry (IHC). However, IHC assay for progesterone receptor was weakly positive in a minority of neurofibromas, consistent with transcript data in primary tumors (implicating non-Schwann cell). Specific Aim 2 tested in vitro response of tumor cells to hormones. RT-PCR, proliferation assays, and apoptosis assays showed a few significant responses of the neurofibroma/MPNST Schwann cell cultures to hormones or antagonists, but no global patterns, indicating tumors behave individually as expected. Specific Aim 3 tests in vivo hormone response of tumor cells xenografted into Nf1 mouse nerve. One MPNST has shown a dramatic effect with estrogen, with other cultures still under analysis. Preliminary data support an in vivo effect of hormones consistent with tumorigenesis (accumulated cell numbers) in at least some tumors.

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INTRODUCTION

Clinical literature suggests that steroid hormones may play a role in neurofibromatosis 1 (NF1) since neurofibroma growth shows some parallels with hormonal changes (Dugoff and Sujansky, 1996). For example, many patients develop neurofibromas at puberty, pregnancy often increases tumor size/number, women with NF1 may have a higher risk of malignancy and a higher neurofibroma burden, and neurofibroma development often slows in older adults. The steroid hormone field is being intensely researched in many types of cancer, but virtually nothing is known about these pathways in normal or NF1-tumor derived Schwann cells. Schwann cells comprise the bulk of these tumors, in which a large percentage are somatically mutated and clonally expanded (Rutkowski et al., 2000; Wallace et al., 2000; Serra et al., 2000).

Our goal is to characterize the growth responses of human normal and NF1 tumor Schwann cells (neurofibromin-negative) to steroid hormones, focusing on estrogen and progesterone. The hypothesis is that human neurofibroma (and MPNST, malignant peripheral nerve sheath tumor) Schwann cells have increased hormone responsiveness compared to normal Schwann cells, leading to tumor growth. The role of the hormone receptors is a growing topic in cancer research, but little is known about these in normal or NF1 Schwann cells.

Specific Aim 1 set out to determine steroid hormone receptor expression in human normal, NF1 neurofibroma, and NF1 MPNST Schwann cells, pre- and post-hormone treatment by studying archived material and cell culture materials. We have a number of NF1 tumor-derived enriched Schwann cell cultures that have been found to lack neurofibromin and will be the basis for this work (Muir et al., 2001). In Specific Aim 2, *in vitro* responses (proliferation and cell survival) of cultured human Schwann cells from normal nerve, NF1 neurofibromas and NF1 MPNSTs, to estrogen and progesterone and their antagonists has been tested. Both of these Aims are completed and are part of the Ph.D. thesis of my graduate student Lauren Fishbein (graduation August 2004), and we have combined these data for a manuscript that is under review (in Appendix). Specific Aim 3 is still ongoing, testing *in vivo* responses (proliferation and survival) of NF1-derived tumor Schwann cell lines to estrogen and progesterone, through a xenoplant model in *Nf1/scid* mice.

The data thus far suggest that in some tumors, steroid hormones play a role in tumor progression. However there are no clear patterns of tumor type or gender that can predict which tumors are responsive. Furthermore, it appears that tumor steroid receptor repertoire may vary in the patient temporally, based on PR immunostaining. This heterogeneity was expected and suggests that future treatments may have to be individualized for each tumor/patient, and that therapies might take advantage of the steroid hormone system when it is implicated in a certain case (or activate the system to employ a targeted therapy). This is an important step in scientific knowledge about the role of these hormones in NF1.

Task 1. To characterize the steroid hormone receptor profile in 24 normal and NF1 tumor Schwann cell cultures pre- and post-hormone treatment (months 1-30).

Progress on Task 1a: "To measure initial expression, RNA from cells grown under normal culture conditions will undergo semi-quantitative RT-PCR to evaluate transcript levels of steroid receptors, a quantitation control, and selected downstream targets. Primary tumor samples corresponding to cultures will be surveyed as well. Immunohistochemistry of primary tumor sections will also be done with for receptors showing transcripts (months 1-12)."

Real time PCR analysis for basal levels of estrogen receptor alpha (ERa), estrogen receptor beta (ERb), progestrone receptor (PR), and androgen receptor (AR) expression was performed on the following RNA samples: two normal (non-NF1) Schwann cell cultures, 17 primary dermal neurofibromas, 8 dermal Schwann cell cultures, 10 primary plexiform neurofibromas, 10 plexiform Schwann cell cultures, 2 primary MPNSTs, and 2 MPNST cultures. The data are shown in the manuscript in the Appendix (Table 1, Fishbein et al., submitted), where the numbers represent normalization to HPRT and the average of normal Schwann cell values. To summarize the key points of the RT-PCR data, there was very little ERb in normal Schwann cells, but statistically less so in tumor cultures (not primary tissue though). This would fit with our original hypothesis that ER beta, albeit at low levels, has tumor suppressor function in Schwann cells which is decreased in NF1 tumor Schwann cells. Overall the tumor samples showing the greatest difference from normal were the primary samples, with the cultures having less difference. The differences were not dramatic, with the most being an increase in PR of 1.9 fold over normal Schwann cells, and a general trend of increased PR in all tumor types. There was just as much variation between primary and culture material as between tumor types. Thus, the steroid receptor profile does not stratify the tumor types, nor did it correlate with gender of the patient. The increase in PR and AR was statistically significant in primary dermal and plexiform tumors compared to normal cultures. Since the cultures did not show significant change, this suggests that cells other than Schwann cells are responsible for the PR (and possibly AR) expression in neurofibromas. This supports the notion that a combined effect of mutated Schwann cells and other (probably not mutated) cells result in neurofibroma formation/progression.

To examine this at a protein level, immunohistochemistry was performed. There were no detectable levels of estrogen receptors at the level of this sensitivity (which is less sensitive than real time PCR). This is consistent with the data of McLaughlin and Jacks (2003). However, PR in 63 tumor sections (32 dermal, 26 plexiform, 5 MPNST) was weakly positive in 21 dermal and plexiform neurofibromas, with scattered positive cells that do not appear to be Schwann cells (Fig.1, Appendix manuscript, Fishbein et al submitted). This is consistent with the RT-PCR data above, and the findings of McLaughlin and Jacks (2003). The positive samples corresponded to 7 females and 4 males, thus no gender correlation. There are several other interesting observations. The positive samples were all from adult patients (some congenital tumors, some adult-onset), while none of the tumor samples from children under age 16 were positive (n=17

plexiform tumors). Also, 5 dermal neurofibromas from a pregnant woman were negative, however three tumors removed 2 months post-partum were all positive for PR. Furthermore, 2 other sets of dermal tumors from 2 other patients (male and female) also showed the same result, and these tumors had been removed at the same time. This could be coincidence, or related to technical handling of samples obtained at the same time, but might also suggest that PR expression might vary within a tumor temporally. The pregnancy case might reflect hormonal changes post-partum that affected the tumors. This is an interesting observation that elicits the idea that a tumor's cell behavior/gene expression may vary over time (epigenetic changes in one or more cell types, in addition to the true genetic changes in the Schwann cells). Thus, Task 1a is complete.

Progress on Task 1b: "Cultures treated in Task 2 will be analyzed with RT-PCR as above. (months 3-30)."

Task 1b is complete. Real-time PCR for the steroid receptors and putative downstream gene targets (ER alpha and beta, PR, ERBB2 (the neu oncogene), EGFR (epidermal growth factor receptor), and VEGF (vascular endothelial growth factor)) is completed for the normal and tumor Schwann cell cultures treated with the hormones or SERMs in Task 2. RNA samples collected at day 0, 1, 3, and 7 after drug treatment were used (and those of positive control samples such as MCF7 breast cancer cells). The data are shown in Table 1 below, as values normalized to no-treatment.

Table 1. Real-time PCR data for downstream target genes.

Normal Schwann Cell Cultures							
	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
male							
pn02.8	0d	1.00	1.00	--	1.00	1.00	1.00
	1dF	0.94	1.06	--	1.01	1.01	1.02
	1dE	0.88	1.17	--	0.99	1.00	1.00
	1dP	1.01	1.01	1.09	1.00	0.99	1.01
	3dF	1.03	1.01	1.08	0.99	1.00	0.98
	3dE	1.04	1.17	--	0.98	1.03	1.00
	3dP	1.02	1.15	--	0.99	1.01	0.98
	7dF	1.01	--	1.15	0.97	1.01	0.94
	7dE	0.92	--	--	0.98	1.01	0.95
	7dP	1.04	1.00	1.18	0.98	1.03	0.96
pn02.8	culture	1.03	1.18	--	0.96	0.97	1.02
male	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
pn97.4	0d	1.00	--	1.00	1.00	1.00	1.00
	1dF	0.99	--	0.97	0.99	1.00	0.92
	1dE	0.98	--	0.97	0.99	1.00	0.90
	1dP	0.99	--	0.98	1.01	0.99	0.91
	3dF	0.98	1.13	0.99	0.95	1.04	0.88
	3dE	1.02	1.20	1.02	0.99	1.04	0.91
	3dP	1.00	--	1.00	0.94	1.03	0.89
	7dF	1.00	--	1.03	0.96	1.04	0.92
	7dE	1.00	--	1.02	0.95	1.04	0.93

	7dP	1.00	--	1.02	0.94	1.04	0.91
pn97.4-1	culture	0.96	1.39	1.00	0.94	0.98	0.91
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
pn02.3	0d	--	--	1.00	1.00	1.00	1.00
	1dF	--	--	0.86	1.02	1.00	1.00
	1dE	--	--	0.85	1.01	1.00	0.99
	1dP	--	--	1.00	1.02	1.00	0.99
	3dF	--	--	0.86	1.00	1.02	1.02
	3dE	1.34	--	0.85	1.00	1.04	0.96
	3dP	--	--	--	0.99	1.04	1.08
	7dF	--	--	0.87	0.97	1.03	1.08
	7dE	1.35	--	0.86	0.97	1.04	1.02
	7dP	1.15	--	0.87	0.64	1.04	1.02
pn02.3-1	culture	1.21	1.40	0.94	1.02	0.99	0.89

Table 1. Continued.

Dermal Cultures							
male	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
cNF97.5	0d	--	--	1.00	1.00	1.00	1.00
	1dF	--	--	0.98	1.02	0.99	0.91
	1dE	--	--	0.98	1.02	0.99	0.89
	1dP	--	--	--	1.03	1.00	0.94
	3dF	1.16	--	1.00	1.02	1.02	0.94
	3dE	1.15	--	0.88	1.03	1.02	0.94
	3dP	1.15	--	0.99	1.02	1.01	0.93
	7dF	1.15	--	1.02	1.05	1.02	0.92
	7dE	1.13	--	1.00	1.00	1.01	0.91
	7dP	1.31	--	1.01	1.06	1.01	0.89
cNF97.5	culture	1.22	nd	--	1.06	0.99	0.97
male	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
cNF98.4d	0dF	1.00	1.00	1.00	1.00	1.00	1.00
	1dF	0.99	0.98	1.13	1.01	0.97	0.99
	1dE	0.90	0.98	1.15	1.01	1.00	1.02
	1dP	1.00	0.98	1.15	1.01	1.00	0.98
	3dF	1.01	1.02	1.17	0.99	1.02	1.01
	3dE	0.99	1.00	1.14	0.98	1.01	1.00
	3dP	1.02	0.85	1.13	0.99	1.02	1.00
	7dF	0.91	0.85	1.17	1.00	1.03	1.07
	7dE	1.06	0.87	1.18	1.01	1.03	1.09
	7dP	0.88	0.96	1.15	0.99	1.01	0.97
cNF98.4d-1	culture	1.05	0.73	1.15	1.08	1.06	1.13
UF 505T7-2	solid	1.20	1.24	1.37	0.89	1.07	1.02
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF

cNF99.1	0dF	--	--	1.00	1.00	1.00	1.00
	1dF	--	--	0.99	1.01	1.00	0.93
	1dE	1.11	--	1.00	1.01	0.99	0.92
	1dP	1.07	--	1.00	1.00	1.00	0.92
	3dF	1.09	1.13	1.01	0.97	1.04	0.91
	3dE	1.23	--	1.02	0.98	1.03	0.93
	3dP	1.24	1.12	1.01	0.98	1.04	0.92
	7dF	nd	nd	nd	nd	nd	nd
	7dE	nd	nd	nd	nd	nd	nd
	7dP	nd	nd	nd	nd	nd	nd
cNF99.1	culture	1.29	1.14	1.00	1.15	1.01	1.00

Table 1. Continued.

Dermal Cultures							
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
cNF96.5f	0dF	1.00	--	1.00	1.00	1.00	1.00
	1dF	0.98	1.32	1.02	1.00	0.98	0.98
	1dE	--	1.13	1.01	1.01	1.00	0.97
	1dP	--	1.35	--	1.03	1.00	0.99
	3dF	--	1.15	1.04	1.02	1.02	1.00
	3dE	1.01	1.41	1.04	1.03	1.03	1.03
	3dP	--	1.38	1.05	1.03	0.99	1.02
	7dF	nd	nd	nd	nd	nd	nd
	7dE	nd	nd	nd	nd	nd	nd
	7dP	nd	nd	nd	nd	nd	nd
UF 328T7	solid	--	1.37	1.27	0.94	1.18	--
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
cNF97.2a	0dF	1.00	--	1.00	1.00	1.00	1.00
	1dF	1.08	1.34	0.90	1.00	1.00	0.95
	1dE	0.96	1.12	0.86	0.99	0.97	0.95
	1dP	--	1.29	0.98	1.00	0.99	0.94
	3dF	1.09	1.15	1.02	0.99	1.01	1.00
	3dE	0.97	1.11	0.99	0.97	1.01	0.96
	3dP	1.08	1.11	0.97	0.99	1.00	0.95
	7dF	1.07	1.31	1.00	1.00	1.01	0.98
	7dE	0.96	1.29	1.00	1.01	1.00	0.93
	7dP	0.96	1.10	0.99	0.99	1.01	0.97
cNF97.2a-1	culture	nd	nd	nd	nd	nd	nd
UF 470T1-2	solid	nd	nd	nd	nd	nd	nd
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
cNF97.2b	0d	--	1.00	1.00	1.00	1.00	1.00
	1dF	--	0.99	0.90	1.01	0.97	0.99
	1dE	--	1.00	--	1.01	0.98	1.01
	1dP	1.16	0.99	0.97	1.01	0.97	0.99
	3dF	1.18	1.01	0.90	1.00	0.98	0.97

	3dE	1.06	0.99	0.88	1.00	1.00	0.97
	3dP	1.02	1.02	0.97	1.00	1.02	0.98
	7dF	--	1.02	0.90	1.00	1.01	0.98
	7dE	1.22	1.02	0.90	0.97	1.01	1.01
	7dP	--	1.01	1.02	0.97	1.01	1.02
cNF97.2b	culture	nd	nd	nd	nd	nd	nd
	solid	nd	nd	nd	nd	nd	nd

Table 1. Continued.

Plexiform Cultures							
male	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
pNF00.11	0dF	1.00	--	1.00	1.00	1.00	1.00
	1dF	0.97	--	1.02	1.02	1.00	0.99
	1dE	0.97	--	1.02	1.02	1.00	1.00
	1dP	1.00	--	1.03	1.04	1.02	1.01
	3dF	0.95	1.36	1.01	1.00	1.00	0.98
	3dE	0.98	1.20	1.06	1.02	1.00	1.01
	3dP	0.99	1.17	1.00	1.01	1.01	1.04
	7dF	nd	--	nd	nd	nd	nd
	7dE	nd	--	nd	nd	nd	nd
	7dP	nd	--	nd	nd	nd	nd
UF 609Tc	culture	--	--	--	1.08	1.03	1.07
UF 609T	solid	1.11	1.76	1.20	1.13	1.10	1.06
male	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
pNF95.11b	0dF	--	--	1.00	1.00	1.00	1.00
	1dF	1.28	--	1.04	1.03	1.03	0.90
	1dE	1.27	1.19	1.04	1.02	1.01	0.95
	1dP	1.24	--	1.03	1.02	1.02	0.94
	3dF	1.26	1.17	1.04	0.99	1.02	0.96
	3dE	1.27	1.34	1.04	1.00	1.02	0.93
	3dP	1.27	--	1.00	1.01	1.02	0.90
	7dF	1.24	1.38	1.05	1.02	1.01	0.92
	7dE	1.12	1.39	1.05	1.01	1.01	0.93
	7dP	1.23	1.14	1.03	1.02	1.01	0.89
pNF95.11b	culture	1.13	1.38	1.02	1.09	1.03	0.97
UF 378T2b	solid	1.48	nd	1.21	1.13	1.15	1.01
male	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
pNF95.6	0d	1.00	1.00	1.00	1.00	1.00	1.00
	1dF	0.98	0.84	1.02	1.00	1.00	0.98
	1dE	0.97	0.97	1.00	1.00	1.00	0.98
	1dP	1.01	0.98	0.91	1.00	1.00	0.96
	3dF	1.12	0.99	1.01	1.00	0.99	0.99
	3dE	1.00	0.99	1.01	1.04	1.01	0.98
	3dP	1.00	0.99	1.01	1.03	1.00	0.98
	7dF	0.99	--	1.01	1.02	0.98	0.96

	7dE	--	--	1.02	0.98	0.98	1.01
	7dP	--	--	1.01	0.98	0.96	1.01
pNF95.6	culture	nd	nd	0.92	1.00	1.00	1.19

Table 1. Continued.

Plexiform Cultures							
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
pNF00.13	0dF	--	--	1.00	1.00	1.00	1.00
	1dF	--	--	1.01	1.04	0.99	0.99
	1dE	--	--	1.13	0.98	1.00	0.97
	1dP	--	--	1.13	0.99	0.99	0.97
	3dF	--	--	1.15	0.98	1.00	1.03
	3dE	--	--	1.14	0.95	0.96	0.89
	3dP	--	--	1.14	0.95	0.96	0.92
	7dF	--	--	1.05	0.98	1.00	0.94
	7dE	1.19	--	1.13	1.01	0.97	0.91
	7dP	--	--	1.14	1.02	0.89	0.94
pNF00.13-1	culture	1.09	--	0.99	1.00	0.94	0.90
UF 632T1-2	solid	nd	nd	nd	nd	nd	nd
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
pNF01.1	0dF	--	1.00	1.00	1.00	1.00	1.00
	1dF	--	0.89	0.97	0.98	0.99	0.90
	1dE	--	0.93	0.97	0.98	0.98	0.94
	1dP	--	0.91	0.97	0.99	0.98	0.91
	3dF	--	0.92	0.97	0.93	1.00	0.91
	3dE	--	0.98	1.01	0.97	1.04	0.96
	3dP	--	0.93	0.95	0.97	1.00	0.92
	7dF	--	0.96	0.99	1.00	1.02	0.96
	7dE	--	0.93	0.99	0.97	1.02	0.93
	7dP	--	0.95	0.98	1.02	1.02	0.95
	3dICI	--	0.95	0.96	0.97	1.00	0.95
	3dMife	--	0.91	0.97	0.97	1.00	0.91
pNF01.1-2	culture	1.39	0.76	0.97	0.93	1.03	0.93
UF 746T1-2	solid	1.56	0.86	1.20	0.91	1.11	0.95
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
pNF99.5	0d	1.00	1.00	--	1.00	1.00	1.00
	1dF	1.02	1.18	1.15	0.98	--	0.97
	1dE	1.02	1.17	--	0.98	1.00	0.97
	1dP	--	1.16	--	0.98	0.92	0.99
	3dF	1.05	1.03	--	0.99	1.05	0.98
	3dE	1.03	--	--	0.99	1.01	0.97
	3dP	--	--	--	0.97	--	0.99
	7dF	1.12	1.18	1.15	0.95	1.01	0.95
	7dE	1.03	1.18	--	0.95	0.94	0.93
	7dP	--	1.15	1.28	0.95	0.91	0.95
	3dICI	--	--	--	0.95	--	1.07

UF 554T1c-1	culture	0.97	0.96	1.11	0.94	0.99	0.93

Table 1. Continued.

MPNST cultures							
male	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
sNF02.2	0d	1.00	1.00	1.00	1.00	1.00	1.00
	1dF	1.03	1.03	1.01	1.04	1.01	1.03
	1dE	1.02	0.99	1.01	1.02	1.00	0.99
	1dP	1.02	0.99	0.98	1.05	1.00	1.02
	3dF	1.07	0.89	1.07	1.04	1.00	1.05
	3dE	1.07	1.03	1.07	1.04	1.02	1.02
	3dP	1.07	1.04	1.04	1.05	1.00	1.02
	7dF	1.07	0.88	1.05	1.05	0.99	1.01
	7dE	1.05	1.03	1.02	1.05	1.00	1.00
	7dP	1.06	1.01	1.02	1.05	1.01	1.02
UF 820T-2	solid	1.03	1.13	1.08	1.22	1.13	1.25
male	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
sNF96.2	0d	--	1.00	--	1.00	1.00	1.00
	1dF	nd	nd	nd	nd	nd	nd
	1dE	nd	nd	nd	nd	nd	nd
	1dP	nd	nd	nd	nd	nd	nd
	3dF	--	0.99	--	1.03	1.03	0.98
	3dE	--	0.99	--	1.03	1.02	0.98
	3dP	--	0.99	1.11	1.01	1.02	0.99
	7dF	--	1.02	--	0.98	1.02	1.08
	7dE	--	1.03	--	0.98	1.01	1.07
	7dP	--	1.01	1.10	0.99	1.00	1.03
sNF96.2-1	culture	--	--	--	--	--	--
female	samples	ER alpha	ER beta	PR	ERBB2	EGFR	VEGF
sNF94.3	0dF	--	--	1.00	1.00	1.00	1.00
	1dF	--	--	1.00	1.00	1.01	0.97
	1dE	--	--	1.00	1.03	1.01	0.92
	1dP	--	--	0.99	1.03	1.02	0.94
	3dF	--	--	1.02	1.04	1.03	0.95
	3dE	--	--	1.02	1.06	1.03	0.95
	3dP	--	--	1.02	1.06	1.03	0.94
	7dF	--	--	1.02	1.09	1.04	0.92
	7dE	--	--	1.02	1.07	1.01	0.92
	7dP	--	--	1.03	1.08	1.03	0.93
sNF94.3-1	culture	--	--	0.93	0.92	0.92	0.80

-- means sample not detected
nd means sample not done

The levels of the receptors or downstream target genes are within the range 0.64 to 1.76 (with the vast majority in the 0.9-1.1 range, with 1.0 being the same as no treatment). Thus, there are not fold-change level differences in any samples. Furthermore, some changes were also seen in the level of untreated cells over time, making interpretation of treated cell data difficult, and indicating that time in culture may influence this variable, and that statistics of these data would be unreliable. This is not surprising given the low levels of these messages that we discovered—even small random variation in measurement would cause too much background error. Only three cultures showed alterations that were of possible interest because of related data in Task 2, and the fact that the untreated cells didn't show a parallel change. Three female cultures (normal Schwann cell culture pn02.3, dermal culture cNF99.1 and plexiform culture pNF00.13) showed an increase in *ERα* RNA expression after 3 or 7 days of treatment with 17β-estradiol (ratio compared to untreated cells of 1.4, 1.2 and 1.2, respectively). These cultures showed statistically significant changes in proliferation (decrease, decrease, increase, respectively) in response to ER ligands (ICI 182,780 and/or tamoxifen) (see Task 2). Progesterone treatment also related to increased *ERα* expression in pn02.3 and cNF99.1 (compared to untreated cell ratios of 1.2 for both cultures). After one day of treatment, progesterone was associated with a decrease in *ERα* expression to undetectable levels in the female plexiform culture pNF99.5, which was sustained across the 7 days of the assay. This culture was the only sample to show a statistically significant increase in apoptosis with 17β-estradiol treatment (Task 2). The male plexiform culture pNF95.6, showed a decrease in *ERα* expression to undetectable levels with both 17β-estradiol and progesterone, but only after 7 days of treatment. Finally, the male dermal culture cNF97.5 showed a transient decrease in *PR* expression to undetectable levels after one day of treatment with progesterone. The level returned to that of the untreated cells by day 3. Our conclusion is that, for these genes, there are no consistent or dramatic downstream effects of the treatments in Schwann cells, regardless of gender or tumor type. It is possible that small changes could be associated with a significant biological effect in a few cases, but this is also not necessarily gender or tumor-type specific. This heterogeneity is consistent with the other measures from our work on the project.

Progress on Task 1c: "Cultures positive for receptors by RT-PCR (pre- and/or post-treatment) will be analyzed by Western blot and immunofluorescence (months 3-30)."

Based on the Task 1a and 1b results, Task 1c was deemed no longer applicable because of the extremely low levels of the molecules, even in response to treatment. Reliable quantitative protein level analyses cannot be done with the techniques available.

Task 2. To measure cultures' proliferative and apoptotic response to hormone and SERM/antagonist treatment (months 1-30).

Progress on Task 2a: "Cultures will be treated with hormones/SERMs separately and proliferative response measured with BrdU assay, and cell survival measured by counting and TUNEL assay. (months 3-18)."

Task 2a is completed, and the data are summarized in Tables 2-4 in the attached manuscript in the Appendix (Fishbein et al, submitted). Four normal Schwann cell cultures, 7 plexiform-derived Schwann cell cultures, 6 dermal-derived Schwann cell cultures and 3 MPNST-derived Schwann cell cultures were analyzed for effects of steroid hormones on growth and survival. Figure 2 of the manuscript shows examples of cultures after the TUNEL assay (to measure apoptosis) and after staining for BrdU incorporation (to measure proliferation). Results are listed as the percent change of control to reflect alterations seen in cells with treatment relative to the same culture without treatment. Within each culture, proliferation index (PI) and apoptotic index (AI) differences due to treatment were examined with a one-way ANOVA (n=32). For one MPNST culture, sNF94.3, the proliferation and TUNEL assays could not be completed as the cells proliferated too quickly for the assays. Overall, there were only seven statistically significant differences in proliferation or apoptosis associated with the receptor ligand treatments. These changes are summarized in Table 2.

ICI 182,780, an ER antagonist, decreased proliferation ($p<0.05$) in two cultures (both female): normal Schwann cell culture pn02.3 and dermal neurofibroma-derived Schwann cell culture cNF99.1. Tamoxifen, a SERM, caused a decrease in proliferation ($p<0.05$) in two cultures: the same normal Schwann cell culture (pn02.3) and a male plexiform neurofibroma-derived Schwann cell culture (pNF00.11). Tamoxifen also increased proliferation ($p<0.05$) in the female plexiform culture pNF00.13. Lastly, mifepristone, typically a PR antagonist, increased proliferation ($p<0.05$) in the male dermal culture cNF97.5. When analyzing apoptotic indices, only one plexiform culture showed a statistically significant response (17β-estradiol increased apoptosis ($p<0.05$) in the female culture pNF99.5). Cultures which have a statistically significant response to a specific treatment did not necessarily show a corresponding increase in receptor RNA expression in the real-time PCR profile. For example, the dermal neurofibroma-derived culture cNF97.5 had no detectable *PR* expression, although this culture demonstrated an increase in proliferation with mifepristone treatment. There is precedent for this situation in the literature as tamoxifen, for example, has been shown to inhibit proliferation and induce apoptosis in ovarian cancer cell lines negative for ER [22, 23].

Tables 3 and 4 show proliferation and apoptosis assay data for cultures which show changes in PI and AI, respectively, by more than 10% compared to the no-treatment control. With this analysis, some trends were found in all tumor types and both genders despite the lack of statistical significance. For example, 3 of 6 dermal cultures displayed increases in proliferation in response to mifepristone, and 3 of 7 plexiform cultures showed decreases in response to tamoxifen. Some cultures showed an increase in proliferation with 17β-estradiol and a corresponding response to the SERM tamoxifen, either an increase (pNF02.6-female) or decrease (pn97.4-male) in proliferation. Similarly, progesterone increased proliferation in the female culture cNF96.5f, while mifepristone caused a decrease in the same culture. Also in cNF96.5f, progesterone was related to a trend toward increased proliferation and decreased apoptosis, as was mifepristone in cNF98.4d (male culture). This pattern is consistent with cell accumulation in the presence of these ligands. All of the average PIs (regardless of treatment conditions) for the normal Schwann cells as well as the dermal and plexiform cultures were within the range of 1-33% (median: 4.5%, 7.7%, and 9.7%, respectively), while the MPNST cultures ranged from 37-67% (median: 54.0%).

Only one plexiform culture with one treatment (pNF00.11/mifepristone) showed a decrease in apoptosis; all other significant changes or trends were toward increased apoptosis in the presence of ligand. Neither MPNST culture (both male) showed a

significant increase (or trend) in proliferation in response to any of the ligands, probably due to the already extremely high PI for these malignant cultures; however, they both showed increased apoptosis. This could be an interesting observation for future therapeutic research. Although many cultures showed at least a 10% change in AI in response to various ligands, all the average apoptotic rates (normal and tumor-derived) were under 7% (with most below 5%); therefore, the biological significance in alterations in these low rates is difficult to interpret. There were no trends or significant outcomes that seemed related to gender of the patient. One-way ANOVA to examine treatment effects on tumor type showed that the treatments did not affect one tumor group more than another for either PI or AI, although the MPNST cultures consistently had a statistically significantly higher PI regardless of treatment condition ($p<0.05$), as expected due to their malignant nature. No single culture sample showed significant results for both proliferation and apoptosis. There was no indication that gender played a role in determining response to steroid hormone receptor ligands for either proliferation or apoptosis. Thus, we do not see dramatic effects on Schwann cells in vitro, but the data suggest that specific tumors respond more to the steroid hormone system.

Progress on Task 2b: "Cultures showing a response will have dose response testing, and assays in (a) will be repeated using combinations of appropriate hormones/SERMs/antagonists. (months 12-30)."

Based on our data, dose response and combinatorial testing are no longer feasible as part of this pilot study due to the heterogeneity (and general lack) of initial responses. Furthermore, the most robust doses were used initially, suggesting the non-responding samples needn't be studied further. However, we hope to obtain future funding to study the few samples that did show response of some sort, to do combinatorial work and more functional assays to test hypotheses specific for those samples, and see if they represent paradigms of steroid hormone role in neurofibromas.

Progress on Task 2c: "Statistical analysis (months 24-36)."

Analyses have been completed and the methods are described in the attached manuscript in the Appendix (Fishbein et al, submitted). Significant results are described and interpreted under the other tasks as relevant to those experiments.

Task 3. To assay for *in vivo* proliferative or survival response of tumor cells to estrogen and progesterone. (months 12-36).

Progress on Task 3a: "Select 6 tumor and 2 normal Schwann cell cultures (in part based on *in vitro* results)."

Two MPNST cultures were originally chosen, but since one showed a dramatic phenotype with estrogen treatment, a third one was added. Because of the heterogeneity from the in vitro work, we chose to do the in vivo assay on more neurofibroma cultures as well, to get a better idea of pattern consistency within a tumor type. Four plexiform

cultures were selected. Since the two dermal cultures first attempted are both from males, we are planning to study 1-2 more dermal cultures from females. As indicated in previous progress reports, it was deemed un-necessary to test normal Schwann cell cultures based on existing data on these cultures from Dr. Muir (Muir et al., 2001).

Progress on Task 3b: "For each culture, sterilize 15 *scid* mice heterozygous for an *Nf1* mutation, inject cells, and treat mice with estrogen, progesterone, or placebo."

The colony of Nf1/scid mice, which always had poor fecundity, essentially died out in February 2004 (no more litters were being produced, even from 15 mating cages). This was apparently due to inbreeding effects and the fact that scid mice have a shortened lifespan and are generally less healthy (most die by 12 months). No other reasons for the breeding failure were found. We still need to perform surgeries on 2 more cell lines, and so will generate the heterozygous female mice again. We have purchased new scid mice from Jax Labs, and have set up the breeding scheme from the top. As we indicated last year, we have now chosen to operate on 3 mice for each culture for each condition (a total of 9 rather than 15), examining more cultures. This means that we can use the same preparation of cells for the whole experiment, keeping at least one variable constant (and also, some of the chosen cultures are almost gone, being able to divide only until P6-8; we couldn't generate enough cells for 15 mice). Since both sciatic nerves are being injected, this is a total of 6 nerves per treatment per culture. We have observed consistent growth patterns within each treatment/culture, and thus data from 6 nerves rather than 10 will adequately answer questions about effects of the hormones. Thus far, we have completed the surgery and harvesting of nerves from three MPNST cultures, 4 plexiform cultures, and 2 dermal cultures. This is summarized in Table 2 immediately below.

Table 2. Xenografts completed (N = number of mice)

CELL CULTURE	PLACEBO	ESTROGEN	PROGESTERONE
MPNST sNF9.43	N=3	N=3	N=3
MPNST sNF96.2	N=3	N=3	N=3
MPNST sNF02.2	N=3	N=3	N=3
Plexi pNF00.13	N=3	N=3	N=3
Plexi pNF95.11b	N=3	N=3	N=3
Plexi pNF01.1	N=3	N=3	N=3
Plexi pNF95.6	N=3	N=3	N=3
Dermal cNF97.5	N=3	N=3	N=3
Dermal cNF98.4d	N=3	N=3	N=3

Progress on Task 3c: "Perform proliferation assay (Ki67 staining) and TUNEL assay on xenografts by analysis of tissue section, and statistical analysis."

Most of the sectioning of these nerves and staining (H/E for general histology, Ki67 for proliferation, TUNEL for apoptosis, hGST for human cell identification) has been completed. However, we perform these analyses on 3 sets of serial sections throughout

the nerve. Thus, the entire nerve is embedded longitudinally and completely sectioned (about 40 slides). The widest tumor section is identified and the serial sets chosen based on that location, to keep the analyses as consistent as possible for each nerve. Thus, it is very time consuming to gather and analyze the data, so that is still in progress from the mice above, and will need to be done for the few remaining surgeries.

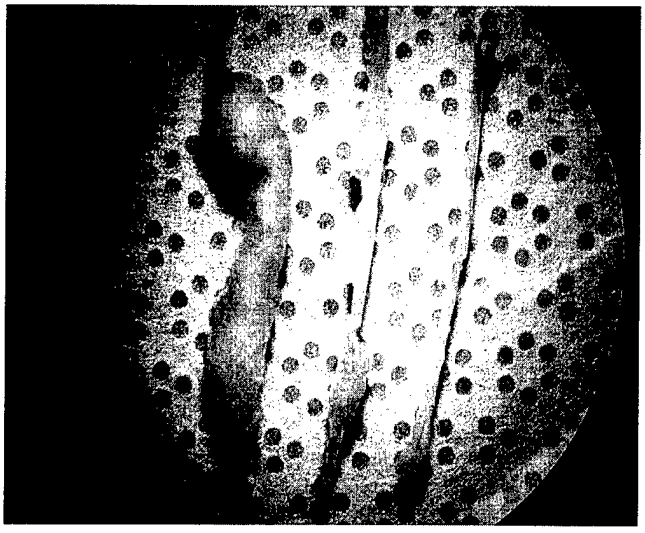
Originally we attempted to measure size of xenograft, however it became apparent that this was too difficult to do reliably given the variable 3-D shapes of the final clusters of human cells. This was also prone to error involving number of cells injected, such as if some leaked back out the fascicle. Furthermore, since the hypothesis is related to hormone response, we based the analysis on the Ki67 and TUNEL staining, comparing treatment to placebo, regardless of the exact number of cells present. The GST staining revealed the presence and boundaries of the xenograft. In the larger xenografts (extending beyond one microscopic field at 40X), Ki67 and TUNEL positive cells were counted in three equal size squares within the tumor area, and averaged. For the smaller tumors (in which the entire xenograft was visible within the microscope field), all the positive cells were counted. This was done at three separate sets of serial sections (thickest part of xenograft, and one set on either side), such that xenografts were being evaluated consistently. There are several types of statistical analyses that we are still considering, given that we are still generating and collecting data. In one method we've applied thus far, averages are taken of the measurements for each nerve and each treatment, and the ratio (# of Ki67 positive cells / #TUNEL positive cells) is normalized to the placebo average being set to 1 to make the interpretation simpler. Thus, a final ratio of 1 indicates very similar rates of proliferation and apoptosis in the tumor as compared to placebo; less than 1 indicates relatively more apoptosis occurred, and greater than 1 indicates that more proliferation was present under that treatment. We will employ tests such as T tests or ANOVAs to test for significant changes and do the comparisons needed. Table 3 below shows some preliminary data with current numbers, in average ratio. For example, the data suggest that the MPNSTs had relatively more proliferation than apoptosis under estrogen treatment, consistent with accumulated cell mass. One of the MPNSTs had increased apoptosis with progesterone, but the other two showed decreased apoptosis, also consistent with cell accumulation but perhaps not as strong an effect as estrogen. This was consistent with our visual observation of the sNF96.2 cell line (Fig. 1). This is the only culture thus far to show dramatic growth in vivo. This tumor responded to estrogen, with less of an effect with progesterone (Fig. 1). The tumor cells grew virtually the entire length of the sciatic nerve compartment, including up to the spinal nerve root. Xenograft of sNF96.2 in intact male mice showed that the culture xenograft grew well, but not as dramatically as in the sterilized female/estrogen-treated nerve compartment (data not shown). This suggests that gender of the xenograft may contribute to the xenograft growth in general, but we have too few cultures to answer this question. We also need to consult with a statistician to determine if/how to deal with outliers—although most data were quite consistent within a mouse and a treatment and a cell culture, there were occasional observations that were anomalous relative to the other measures of that culture.

Table 3. Xenograft proliferation/TUNEL (apoptosis) data, normalized to placebo.*

CELL CULTURE	Gender	Ave. ratio estrogen	Ave. ratio progesterone
MPNST sNF94.3	F	5.03	2.29
MPNST sNF96.2	M	1.86	0.375
MPNST sNF02.2	M	3.9	0.86
Plexiform pNF00.13	F	0.24	0.10
Plexiform pNF95.11b	M	0.09	0.33
Plexiform pNF01.1	F	2.002	1.84
Plexiform pNF95.6	F	1.1	0.88
Dermal neurofibroma cNF97.5	M	3.31	0.58
Dermal neurofibroma cNF98.4d	M	1.6	3.95

*preliminary data—does not include data from all nerves yet.

The sNF96.2 culture did not show significant alterations of proliferation or apoptosis in the in vitro analysis, suggesting that the in vivo situation is an important additional assay for measuring hormone effects. All of the cultures injected have shown viability of the human cells in the nerve compartment, and thus we are obtaining a good tumor model for testing treatments such as these. At the moment there are predominantly reproducible effects, but they appear heterogeneous between at least cultures and ligand. Full analysis and interpretation will be done during this next year, at which time we can examine statistical significance and trends that may shed light on mechanisms, in particular in comparison to data from Tasks 1 and 2.



A B C

Figure 1. sNF96.2 gross results. A and B represent nerves taken from xenografted mice treated with estrogen or placebo, respectively. C is a nerve from a PBS injected mouse for control. Dramatic growth of the tumor cells is evident with estrogen. Progesterone-treated nerves were very similar in size to placebo (data not shown).

KEY RESEARCH ACCOMPLISHMENTS

- Finished a large scale progesterone receptor immunohistochemistry analysis, which showed very similar results to a paper published in 2003, implicating PR in non-Schwann cells in neurofibromas.
- Completed proliferation and apoptosis assays for all tumor and normal cultures, in duplicate. Performed data analysis and submitted a manuscript.
- Completed most of the xenograft surgeries and nerve harvests, and have done most of the immunostaining and cell counting of these data.
- Set up the breeding colony again to produce the last set of Nf1 +/- scid mice needed to complete the study.
- Tasks 1 and 2 comprised part of the Ph.D. thesis of MD-PhD student Lauren Fishbein; her PhD was obtained this month (August 2004) from the University of Florida. Title of thesis: "Genetic and Epigenetic Studies of Neurofibromatosis 1 (NF1) Tumors".

REPORTABLE OUTCOMES

We presented most of the in vitro work at the NNFF International Consortium on the Molecular Biology of NF1 and NF2, June 2003 in Aspen, and have submitted these data as a manuscript to *Molecular Carcinogenesis* (attached in Appendix, cited below). This represents one of the first (and most recent) scientific papers to test the effects of steroid hormones and antagonists on NF1 and non-NF1 derived Schwann cells, in terms of proliferation and apoptosis (key phenomena in neoplasia).

Fishbein L, Zhang X, Fisher LB, Li H, Campbell-Thompson M, Yachnis A, Rubenstein A, Muir D, Wallace MR. *In vitro* studies of steroid hormones and their receptors in neurofibromatosis 1 tumors and Schwann cells. Submitted.

CONCLUSIONS:

Analyses of in vitro data suggest that steroid hormone receptor levels in normal and NF1 tumor-derived Schwann cells are low and do not differ in a multiple-fold fashion, even in response to in vitro hormone treatment. There is heterogeneity in the data that do not correlate with tumor type or gender, as originally predicted, but we are finding some significant results in the in vitro work. We anticipate publishing data from Tasks 1 and 2 during the coming year, and finishing work on Task 3 and submitting a manuscript on that work. We expect that some cultures will show significant effects in vivo, once data collection and statistical analysis are complete. The combination of in vitro and in vivo work will provide very complementary data that will shed light on positive, biologically significant effects of these hormones in some tumors. The ultimate data may help physicians predict which patients/tumors might respond to hormone therapy, and perhaps develop or apply some new therapies along these lines for such cases.

REFERENCES:

Dugoff L, Sujansky E. Neurofibromatosis type 1 and pregnancy. Am J Med Genet 66:7-10, 1996.

McLaughlin ME, Jacks T. Progesterone receptor expression in neurofibromas. Cancer Res 63:752-5, 2003.

Muir D, Neubauer D, Lim IT, Yachnis AT, Wallace MR. Tumorigenic properties of neurofibroma Schwann cells. Am J Pathol 58:501-513, 2001.

Rutkowski JL, Wu K, Gutmann DH, Boyer PJ, Legius E. Genetic and cellular defects contributing to benign tumor formation in neurofibromatosis type 1. Hum Mol Genet 9:1059-1066, 2000.

Serra E, Rosenbaum T, Winner U, Aledo R, Ars E, Estivill X, Lenard H-G, Lazaro C. Schwann cells harbor the somatic NF1 mutation in neurofibromas" evidence of two different Schwann cell subpopulations. Hum Mol Genet 9:3055-3064, 2000.

Wallace MR, Rasmussen SA, Lim IT, Gray BA, Zori RT, Muir D. Culture of cytogenetically abnormal Schwann cells from benign and malignant NF1 tumors. Genes Chrom Cancer 27:117-123, 2000.

APPENDICES: Attached manuscript that is under review at *Molecular Carcinogenesis.*

APPENDIX

In Vitro Studies of Steroid Hormones and Their Receptors in Neurofibromatosis 1 Tumors and Schwann Cells

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Abbreviations

ER estrogen receptor
PR progesterone receptor
AR androgen receptor
NF1 neurofibromatosis 1
MPNST malignant peripheral nerve sheath tumor
PCR polymerase chain reaction
BrdU bromodeoxyuridine

Running Title: Steroid Hormones in NF1

Key Words: NF1, steroid hormone receptors, Schwann cell, neurofibroma, apoptosis

Abstract

The most common NF1 feature is the benign neurofibroma, which consists predominantly of Schwann cells. Dermal neurofibromas usually arise during puberty and increase in number throughout adulthood. Plexiform neurofibromas, associated with larger nerves, are often congenital and can be life-threatening because of extensive growth. Malignant peripheral nerve sheath tumors in NF1 are believed to arise from plexiforms and occur in 5-10% of patients. Some female patients have reported an increase in number and size of neurofibromas during pregnancy. In addition, there are reports of an increased potential for malignant transformation of plexiform tumors during pregnancy. These observations suggested that steroid hormones influence growth of neurofibromas. To investigate this in a scientific manner, we first characterized the steroid hormone expression in neurofibromas. Immunohistochemistry and real-time PCR assays showed that estrogen receptors alpha and beta, progesterone receptor and androgen receptor are differentially expressed in primary neurofibromas and in NF1 tumor-derived Schwann cell cultures compared to normal Schwann cells, although there is some heterogeneity. This work then examined the *in vitro* effect of steroid hormone receptor ligands on proliferation and survival of NF1 tumor-derived Schwann cell cultures compared to normal Schwann cell cultures. The changes in proliferation and apoptosis are generally small, but some are statistically significant, and trends are consistent with increased cell accumulation. Our data suggest that steroid hormones can directly influence neurofibroma initiation or progression by acting through their cognate receptor, but that these effects may only apply to a subset of tumors, and are not limited by gender.

Introduction

Neurofibromatosis 1 (NF1) is an autosomal dominant disorder which affects about 1 in 3500 individuals worldwide. The NF1 tumor suppressor gene is located on chromosome 17q11.2 and spans 260kb of genomic DNA [1, 2, 3]. Despite full penetrance, there is variable expressivity seen even within families with the same constitutional mutation. The most characteristic feature of NF1 is the neurofibroma. Dermal (cutaneous and subcutaneous) neurofibromas are often size-limited and are mainly of cosmetic significance. Interestingly, these tumors often first develop during puberty and continue to increase in number throughout life. The plexiform neurofibromas form along deeper nerves in the body; and although they are slow-growing, they can become large and even life threatening. Nevertheless, many patients have internal plexiform tumors which are asymptomatic. In addition, there is a 5-10% chance of malignant potential for the plexiform tumors to become malignant peripheral nerve sheath tumors (MPNSTs). Many plexiform tumors are thought to be congenital. Some patients have reported an increase in size and number of both dermal and plexiform neurofibromas during pregnancy [4] or while taking birth control pills (Wallace, unpublished data). There have even been reports of increased malignant potential of plexiform tumors during pregnancy [5, 6]. This information suggests that steroid hormones may play a role in neurofibroma development, growth and/or survival.

Neurofibromas are heterogeneous, consisting of fibroblasts, mast cells, perineural cells and a majority of Schwann cells (some of which are clonally derived with somatic mutations). Nuclear steroid hormone receptors commonly regulate transcription of genes involved in the cell proliferation and apoptosis pathways, although this has never been tested in Schwann cells. Little research has been done on steroid hormone effects on Schwann cells. Jung-Testas et al. found low levels of estrogen receptor (ER) on cultured rat Schwann cells using binding assays

[7]. These cells showed a proliferative response to estradiol but only in the presence of forskolin, suggesting that increased cAMP is required for the effect. The same group demonstrated the presence of progesterone receptor (PR) in rat Schwann cells using ligand binding assays and receptor immunofluorescence [8]. In addition, PR is known to have a role in glial cells in activating genes involved in myelin production [9, 10]. Interestingly, estrogen treatment had no effect on PR level [11], in contrast to reciprocal regulation observed in reproductive organs of the rat. Furthermore, estrogen treatment of these cells did not increase ER binding or immunofluorescence. Another study examined estrogen and progesterone responsiveness in cultured Schwann cells derived from various aged rodents, and found that high levels of 17β-estradiol and progesterone had small proliferative effects in Schwann cells from males and newborns only [12].

While steroid hormones are involved in a number of tumors (e.g. breast and ovarian carcinoma, prostate carcinoma and benign meningiomas), very little work has been done to analyze the effects of steroid hormone receptors in human Schwann cell based tumors. Martuza et al. found evidence of ERs in schwannomas, but only in 1 of 6 neurofibromas, via ligand binding assays of dissociated tumor cells [13]. There was no progestin binding in 2 of 2 neurofibromas. Furthermore, Chaudhuri et al. failed to show any ER or PR binding in 5 neurofibromas and 4 neurofibrosarcomas [14]. A more recent study found PR immunoreactivity in some unidentified cells within human neurofibroma tissue sections (apparently not Schwann cells) but found no significant ER immunostaining [15]. Thus far, no human neurofibromas or MPNSTs have been studied *in vivo* or *in vitro* for transcript levels of *ER* and *PR*, or cellular response to the steroid hormones.

Overall, the literature suggests that ER and PR might be present in human Schwann cells, but the status of these in NF1-related neurofibromas is essentially unknown. Due to the limited

and somewhat conflicting data reported in the literature, it is unclear if or how human Schwann cells (normal or neurofibroma-derived) respond to steroid hormones. Based on the clinical data suggesting that neurofibromas are aggravated in some patients during times of hormonal surges, we hypothesized that steroid hormone receptors would be differentially expressed in NF1 neurofibromas and that neurofibroma-derived Schwann cells would have an altered sensitivity to ligands for these receptors, compared to normal (non-NF1) Schwann cells. Here we describe the first molecular study of the presence of steroid receptors and effects of their agonists/antagonists at the cellular level in neurofibroma and normal Schwann cells.

Materials and Methods

Sample Collection and Total Cellular RNA Isolation

Under IRB approval, normal (non-NF1) and neurofibroma Schwann cells were cultured and enriched from primary tissue as previously described [16]. Most of the primary tumor and tumor-derived Schwann cell cultures were from patients who met diagnostic criteria for NF1 [17], although a few were sporadic neurofibromas. Histologically, the primary samples contained ~70% Schwann cells (fibroblasts, mast cells, nerve axons, perineural cells and vascular endothelial cells making up the rest), and the tumor-derived Schwann cell cultures were enriched to >95% purity. RNA isolation from cultured cells and primary tissue was performed using TRIzol (Invitrogen, Carlsbad, CA). The positive control samples included Caco-2 (ERα.-/ERβ+ human colon cancer cell line [18]), MCF-7 (ERα.+/ERβ- human breast cancer cell line [19]), and human testis RNA (AR+ [20]).

Real-Time PCR

Reverse transcription reactions of 2μg of total RNA used random hexamers and Superscript II (Invitrogen, Carlsbad, CA) in a 20μl volume. Diluted (1:5) cDNA (1μl) was used as a template in a LightCycler rapid real-time thermal cycler system (Roche, Indianapolis, IN) as

follows: 10 mins. at 95°C, then cycling for 5 secs. at 94°C, 5 secs. at primer specific annealing temperature, 30 secs. at 72°C for 48 cycles in a 10μl volume using the Fast Start DNA Master SYBR GreenI mix (Roche, Indianapolis, IN). Fluorescent product was detected at the end of each extension step. Primers were designed to span at least one large intron (sequences are available upon request). A melting curve analysis and native polyacrylamide gel electrophoresis were performed for each reaction to confirm amplification specificity. A standard curve was generated by amplifying serial dilutions of a purified PCR product specific for each gene of interest. The data points were transformed to a log format and a linear regression line y=ax+b was used to determine values for each sample. Calculated values were then taken as a ratio compared to the average value from two separate normal Schwann cell culture samples. All reactions were repeated in duplicate and normalized to *HPRT* gene expression (a housekeeping gene commonly used as a control in such experiments, including in neuronal tissue [21]).

Progesterone Receptor Immunohistochemistry

Sixty-three formalin-fixed paraffin-embedded human neurofibroma sections (8μm) were stained with the monoclonal anti-progesterone receptor antibody (DAKO, Carpinteria, CA; Clone PgR 636, 1:50). Endogenous peroxidases were blocked with 1% hydrogen peroxide, and the sections were heated at 95°C for 30 mins in antigen retrieval buffer (DAKO, Carpinteria, CA) to unmask receptor epitopes. The antibodies were detected with a biotinylated goat anti-mouse secondary antibody (DAKO, Carpinteria, CA; 1:500) and ABC kit (DAKO, Carpinteria, CA), using 3,3-diaminobenzidine (DAB) (Sigma, St. Louis, MO) as a substrate.

Proliferation and TUNEL Assays

Human normal Schwann cells and neurofibroma-derived Schwann cell cultures were grown as previously described [16]. The cells were trypsinized, neutralized with serum-containing medium, and washed in phenol red free DMEM. The cells were then plated and

incubated for 48 hours in phenol red free DMEM with 1% dextran-coated charcoal-treated fetal bovine serum (removes endogenous steroid hormones). Then, for three days cells were treated in replicate chambers with EtOH carrier alone, 100nM 17ß-estradiol or progesterone (agonists for ER and PR, respectively), or 1μM ICI 182,780 (antagonist for ER), tamoxifen (mixed ER agonist/antagonist, SERM), or mifepristone (antagonist for PR) (Sigma, St. Louis, MO). These concentrations were chosen based on literature providing physiological levels for analysis of hormone effects. Cells for the proliferation assay were treated with 1μM BrdU (Sigma, St. Louis, MO) added for the final 24 hours and then fixed in 70% EtOH for 30 mins. at 4°C. Immunohistochemistry was completed on these cells using a mouse anti-BrdU primary antibody (DAKO, 1:200) and a secondary goat anti-mouse HRP antibody (DAKO, Carpinteria, CA; 1:500) and visualized with DAB (Sigma, St. Louis, MO). Cells to be used for the TUNEL assay were fixed in 4% paraformaldehyde for 25 mins. at room temperature, and immunohistochemistry was completed using the Promega DeadEnd Colorimentric Kit (Madison WI). Assays were done in replicate, and pictures of the slides from both assays were taken under a microscope with 200X magnification. Eight pictures were taken in a linear fashion across each chamber. Proliferation and apoptosis indices (PI or AI) are reported as a percent of stained cells.

Downstream Gene Expression Analysis

For each culture, the cells were treated (after the 48 hour washout period) on day 0, 3 and 6 with 100nM 17ß-estradiol or progesterone or ethanol carrier alone in replicate wells. The cells were harvested for RNA on day 0, 1, 3 and 7 to be used in real-time PCR analysis for expression of putative downstream targets of steroid hormone receptors (including *ERα, ERβ, PR, ERBB2, EGFR* and *VEGF*) as described above. The samples were normalized to *HPRT* and calculated values were taken as a ratio of the hormone treated sample compared to the same culture on day

0 before hormone treatment. For some cultures, RNA collection was only done on days 0, 1 and 3 due to an inadequate number of cells.

Statistics

For the real-time PCR data analysis from the steroid hormone receptor profile, a one-way analysis of variance (ANOVA) followed by Tukey's post-test analysis (GraphPad Prism software, San Diego, CA) was used to test for significant differences in expression for each gene between the tumor types.

Proliferation and apoptosis rates were analyzed using a one-way ANOVA followed by a Dunnett's post-test analysis (GraphPad Prism software, San Diego, CA) for comparison between control and experimental treatments within each culture. For an alternative analysis, the percentage of stained cells from each picture per treatment was considered as an individual data entry point (n=32; 8 pictures per chamber/ 2 chambers per replicate). For other analyses, the average percent of stained cells from each replicate assay was counted as a data entry point (n=2). For all of these analyses, data points were entered as either percent of stained cells or as a percent change from the average of the no-treatment control's PI or AI for each culture. Additional one-way ANOVA analysis was performed to determine any significant differences between proliferation or apoptosis within each class of tumor cultures compared to the normal Schwann cells for each treatment. A standard $p \leq 0.05$ was used to determine statistical significance. Finally, "trends" in proliferation and apoptosis for each treatment were reported for changes greater than or equal to 10% of the average no-treatment control value for each culture (but not meeting statistical significance), using n=2 as described above.

Results

Steroid Hormone Receptor Profile

Two independent normal Schwann cell cultures, pn97.3 and pn97.4, were analyzed for RNA expression of the *ERα*, *ERβ*, *PR* and *AR* genes. The tumor samples analyzed were grouped as follows (with some tumors having both primary and culture samples): 17 primary dermal neurofibromas and 8 dermal tumor-derived Schwann cell cultures, 10 primary plexiform neurofibromas and 10 plexiform tumor-derived Schwann cell cultures, and 2 primary MPNSTs and 2 MPNST tumor-derived Schwann cell cultures. The results are shown in Table 1, and the values are normalized to 1.0 based on the average of the normal Schwann cells.

Most samples had detectable expression of all four genes. Receptor expression was low overall, as is often found for transcription factors in general, with the cycle number for linear detection here typically >30 cycles. Many tumor samples had differential expression compared to normal Schwann cell cultures, consistent with our hypothesis, with the largest differences seen in primary tumor RNAs. In fact, when examining differences between tumor types for expression of each gene, *ERβ*, *PR* and *AR* showed statistically significant differences ($p<0.05$ with one-way ANOVA), but there was greater variation between primary versus culture samples than between tumor type. Thus, the expression data did not stratify the types of neurofibromas. For *ERβ*, the expression was undetectable in most of the culture samples, whereas the primary tissue samples had expression often equal to that in the normal Schwann cell cultures. For *PR*, the expression was significantly ($p<0.05$) increased in the primary tissue sections from dermal and plexiform tumors compared to the tumor cultures (which showed expression levels similar to the normal cultures). The primary MPNST samples also had an increase in *PR* expression although it did not reach statistical significance. *AR* expression was significantly ($p<0.05$) raised in dermal and plexiform primary tissue samples compared to dermal and plexiform cultures. Although the MPNST samples did not show statistical significance, the cultures had higher expression compared to the primary tissue samples.

Overall, *PR* seemed to have the largest differential expression within these samples (1.3-1.9 fold for most), and it was typically greater than normal, with 9 samples being as high or higher than the positive control (MCF-7). MCF-7 and Caco-2 are cell lines that are considered to have significant levels of the respective receptors; human testis tissue also has a high level of steroid hormone receptor expression. These control samples showed less than a two-fold difference in RNA expression of the steroid hormone receptors when compared to the average of the two normal Schwann cell cultures. Some of the tumor RNAs, especially *PR* in primary specimens, had similar levels of differential expression. Lastly, no obvious relationship between patient gender and expression levels of receptors was seen in any tumor type.

To follow up the *PR* RNA results, histological sections from 63 neurofibromas (32 dermal, 26 plexiform and 5 MPNSTs) were immunostained for PR. Interestingly, half of the women contributing tumors to this immunohistochemical analysis reported neurofibroma growth in response to pregnancy or birth control pills. Overall, there was no obvious correlation with PR positive sections and gender, hormone-response or family history of NF1, type of tumor, or location of tumor. Twenty-one neurofibromas (from 7 female and 4 male patients) showed scattered individual cells staining positive for PR (Figure 1). This included 13 dermal neurofibromas and 8 plexiforms (2 of which were sporadic isolated neurofibromas in individuals otherwise not meeting NF1 criteria). Of these 21 samples, 5 were tested with real-time PCR and 4 of them showed an increase in *PR* RNA expression for either the primary and/or culture samples (UF 378T, UF 537T1, UF 328T7, and UF 505T3). The rest of the tumors were negative for PR staining, including 5 dermal neurofibromas from a pregnant NF1 subject. Interestingly, this same subject subsequently had three additional dermal neurofibromas removed postpartum and all three showed scattered positive staining. ERα and ERβ immunohistochemistry was

performed on 10-15 neurofibroma sections, all of which showed no reactivity (UF Clinical Pathology Lab Core, data not shown).

Proliferation and TUNEL Assays

Functional assays tested the hypothesis that neurofibroma-derived Schwann cells have an altered sensitivity to steroid hormones. Four normal Schwann cell cultures, 7 plexiform-derived Schwann cell cultures, 6 dermal-derived Schwann cell cultures and 3 MPNST-derived Schwann cell cultures were analyzed for effects of steroid hormones on growth and survival. Figure 2 shows examples of cultures after the TUNEL assay (to measure apoptosis) and after staining for BrdU incorporation (to measure proliferation).

The proliferation and apoptosis results are reported as the percent change of control to reflect any alterations seen in cells with treatment relative to the same culture without treatment. Within each culture, proliferation index (PI) and apoptotic index (AI) differences due to treatment were examined with a one-way ANOVA (n=32). For one MPNST culture, sNF94.3, the proliferation and TUNEL assays could not be completed as the cells proliferated too quickly for the assays. Overall, there were only seven statistically significant differences in proliferation or apoptosis associated with the receptor ligand treatments. These changes are summarized in Table 2.

ICI 182,780, an ER antagonist, decreased proliferation ($p<0.05$) in two cultures (both female): a normal Schwann cell culture (pn02.3) and a dermal neurofibroma-derived Schwann cell culture (cNF99.1). Tamoxifen, a SERM, caused a decrease in proliferation ($p<0.05$) in two cultures: the same normal Schwann cell culture (pn02.3) and a male plexiform neurofibroma-derived Schwann cell culture (pNF00.11). Tamoxifen also increased proliferation ($p<0.05$) in the female plexiform culture pNF00.13. Lastly, mifepristone, typically a PR antagonist, increased proliferation ($p<0.05$) in the male dermal culture cNF97.5. When analyzing apoptotic

indices, only one plexiform culture showed a statistically significant response (17β-estradiol increased apoptosis ($p<0.05$) in the female culture pNF99.5). Cultures which have a statistically significant response to a specific treatment did not necessarily show a corresponding increase in receptor RNA expression in the real-time PCR profile. For example, the dermal neurofibroma-derived culture cNF97.5 had no detectable *PR* expression, although this culture demonstrated an increase in proliferation with mifepristone treatment. There is precedent for this situation in the literature as tamoxifen, for example, has been shown to inhibit proliferation and induce apoptosis in ovarian cancer cell lines negative for ER [22, 23].

Tables 3 and 4 display the results from the proliferation and apoptosis assays for cultures which show changes in PI and AI, respectively, by more than 10% compared to the no-treatment control. With this analysis, some trends were found in all tumor types and both genders despite the lack of statistical significance. For example, 3 of 6 dermal cultures displayed increases in proliferation in response to mifepristone, and 3 of 7 plexiform cultures showed decreases in response to tamoxifen. Interestingly, some cultures showed an increase in proliferation with 17β-estradiol and a corresponding response to the SERM tamoxifen, either an increase (pNF02.6-female) or decrease (pn97.4-male) in proliferation. Similarly, progesterone increased proliferation in the female culture cNF96.5f, while mifepristone caused a decrease in the same culture. Also in cNF96.5f, progesterone was related to a trend toward increased proliferation and decreased apoptosis, as was mifepristone in cNF98.4d (male culture). This pattern is consistent with cell accumulation in the presence of these ligands. All of the average PIs (regardless of treatment conditions) for the normal Schwann cells as well as the dermal and plexiform cultures were within the range of 1-33% (median: 4.5%, 7.7%, and 9.7%, respectively), while the MPNST cultures ranged from 37-67% (median: 54.0%).

Only one plexiform culture with one treatment (pNF00.11/mifepristone) showed a decrease in apoptosis; all other significant changes or trends were toward increased apoptosis in the presence of ligand. Neither MPNST culture (both male) showed a significant increase (or trend) in proliferation in response to any of the ligands, probably due to the already extremely high PI for these malignant cultures; however, they both showed increased apoptosis. This could be an interesting observation for future therapeutic research. Although many cultures showed at least a 10% change in AI in response to various ligands, it must be noted that all the average apoptotic rates (normal and tumor-derived) were under 7% (with most below 5%); therefore, the biological significance in alterations in these low rates is difficult to interpret. There were no trends or significant outcomes that seemed related to gender of the patient.

A final analysis examined effects from treatments on tumor type using a one-way ANOVA analysis. This analysis found that the treatments did not affect one tumor group more than another for either PI or AI, although the MPNST cultures consistently had a statistically significantly higher PI regardless of treatment condition ($p<0.05$), as expected due to their malignant nature.

Downstream Gene Expression Analysis

Functional effects of steroid hormones in the cell can also be tested by assaying changes in transcription levels of downstream target genes of the receptors. Since downstream targets are not known for Schwann cells, genes of interest which are known to be affected in other cell types were analyzed. However, real-time PCR results showed no significant alterations in expression of *ERBB2*, *EGFR* or *VEGF* in any of the cell cultures under any treatment conditions (two cultures were not tested due to an inadequate number of cells, pn02.7 and pNF02.6). In some samples, *ERα*, *ERβ*, and *PR* had RNA level alterations up or down compared to day 0 untreated cells. However, these changes were often paralleled by an equal change in the untreated cells at

the corresponding time point, making the interpretation difficult and suggesting that the changes in expression were not due to treatment but rather to time spent in culture. Nevertheless, data from a few cultures showed alterations in expression (most quite small), which are not seen in the untreated cells at those time points (data not shown). Three female cultures (normal Schwann cell culture pn02.3, dermal culture cNF99.1 and plexiform culture pNF00.13) showed an increase in *ERα* RNA expression after 3 or 7 days of treatment with 17β-estradiol (ratio compared to untreated cells of 1.4, 1.2 and 1.2, respectively). Interestingly, these cultures showed statistically significant changes in proliferation (decrease, decrease, increase, respectively) in response to ER ligands (ICI 182,780 and/or tamoxifen) (Table 2). Progesterone treatment also increased *ERα* expression in pn02.3 and cNF99.1 (ratio compared to untreated cells of 1.2 for both cultures). After one day of treatment, progesterone caused a decrease in *ERα* expression to undetectable levels in the female plexiform culture pNF99.5, which was sustained across the 7 days of the assay. This culture was the only sample to show a statistically significant increase in apoptosis with 17β-estradiol treatment (Table 2). The male plexiform culture pNF95.6, showed a decrease in *ERα* expression to undetectable levels with both 17β-estradiol and progesterone, but only after 7 days of treatment. Finally, the male dermal culture cNF97.5 showed a transient decrease in *PR* expression to undetectable levels after one day of treatment with progesterone. The level returned to that of the untreated cells by day 3.

Discussion

Steroid Hormone Receptor Profile

Characterization of the steroid hormone receptor profile in primary neurofibromas and tumor-derived Schwann cell cultures showed low levels of expression of *ERα*, *ERβ*, *PR* and *AR* RNA, with some differential expression of the receptors seen upon comparison of the tumor samples to normal Schwann cell cultures, consistent with our hypothesis. However, the largest

and most consistent differences appeared within the primary RNA samples rather than the neurofibroma-derived Schwann cell culture RNAs (Table 1). This suggests that the mixture of cells found in the primary neurofibroma together contribute to the steroid hormone RNA expression. This is consistent with a study in the literature showing that PR expression via immunohistochemistry is found in cells which express neurofibromin and do not express S-100, suggesting that PR expression is much less in the clonal Schwann cell population [15]. The low RNA expression levels are confirmed by the very low to absent PR protein seen after immunohistochemical staining of neurofibroma sections (Figure 1). It is interesting to note that the positive control samples for the real-time PCR analysis showed less than a two-fold difference in receptor expression compared to normal Schwann cell cultures, yet these tissues are generally considered responsive. This suggests that small levels and changes in receptor expression could be significant functionally. *ERα* and *ERβ* had lower differential RNA expression across the tumor panel. This is consistent with the finding that ER immunostaining was negative in a small number of neurofibroma tissue sections examined (data not shown). The study by McLaughlin and Jacks discussed above also found a lack of ER immunostaining in the same tumor panel which showed some PR immunoreactivity [15]. Thus, although *ERα* and *ERβ* transcripts are present, the protein is undetectable via immunostaining.

Proliferation and TUNEL Assays

Despite heterogeneity, even within normal Schwann cells, a few statistically significant differences were seen in the rate of proliferation or apoptosis of untreated cells compared to hormone receptor ligand treated cells (Table 2). Notably, other trends are evident despite the relatively low number of statistically significant differences (Tables 3 and 4). 17β-estradiol increased proliferation in 4 of 6 cultures responding to the ligand. Meanwhile, other ER ligands showed decreases in proliferation in 4 of 6 cultures with a response to ICI 182,780, and 4 of 6

cultures with a response to tamoxifen. Similarly, progesterone decreased proliferation in 4 of 6 cultures showing a response, while mifepristone, usually an antagonist for PR, increased proliferation in 4 of 7 cultures responding to this ligand. The overall rates of apoptosis were quite low (most ranging from 1-5%) making statistical differences in the AI difficult to assess. No one culture sample showed significant results for both proliferation and apoptosis. In addition, there was no indication that gender played a role in determining response to steroid hormone receptor ligands for either proliferation or apoptosis. Since only a few statistically significant *in vitro* effects were seen with steroid hormones on neurofibroma-derived Schwann cell cultures, perhaps the tumor environment and/or the surrounding cells are necessary to contribute to more dramatic steroid hormone effects on neurofibromas *in vivo*.

Downstream Gene Expression Analysis

Based on the literature, six genes were chosen as possible target genes for steroid hormone receptors in Schwann cells. For example, *EGFR* transcript levels can be regulated by ERα expression, which can be a direct or inverse relationship depending on cell type [24], and EGFR expression has been identified in distinct foci with immunohistochemistry on neurofibromas [25]. *ERBB2* is an oncogene regulated via ER in many cell types. This receptor dimerizes with ErbB3 in Schwann cells [26] to bind neuregulin, a potent mitogen for Schwann cells. Interestingly, overexpression of *ERBB2* can transform Schwann cells [27], and ENU-treated rodents develop neurofibromas with *ERBB2* activating point mutations [28]. *VEGF* has been shown to be a target gene for ER in breast cancer cell lines, while estradiol induced expression of VEGF depends on ER subtype and homo- or heterodimerization [19]. Neurofibromas are highly vascular tumors with current research focusing on changes in genes and proteins involved in angiogenesis. In addition, as previously mentioned, estrogen and progesterone can regulate the expression of their own and each others receptors in other cell

types. However, real-time PCR analysis of the six genes showed that RNA expression is negligibly altered in most of the neurofibroma-derived Schwann cell cultures after treatment with 17β-estradiol or progesterone.

Overall, these data support the hypothesis that steroid hormone receptors are moderately differentially expressed in neurofibromas compared to normal Schwann cell cultures. In addition, there are some effects from treatment with ligands for these receptors on neurofibroma-derived Schwann cell proliferation and survival *in vitro* but these are small. However, it is conceivable that over time these minor effects might accumulate to contribute to tumor growth. Given the clinical data, we hypothesized that 17β-estradiol and progesterone would cause significant increases in proliferation or decreases in apoptosis. With a few exceptions, most of the statistically significant proliferation changes were in the decreasing direction with receptor antagonists. Perhaps these ligands can be used in therapeutic design to slow or prevent tumor growth. The one significant apoptosis result was an increase, and most of the trends are towards the same direction. These observations do not fit a simplistic or global model very well; however, the data support that these receptors are present and active at low levels in normal Schwann cells and neurofibroma-derived Schwann cells and have the potential to contribute to tumor progression. These responses appear to be patient- and possibly tumor-specific. This is also consistent with anecdotal clinical data that not all patients develop neurofibromas during puberty and not all neurofibromas change during hormonal alterations. It is also possible that the Schwann cell does not act alone in response to the hormones. The tumor environment and/or surrounding cells may be important for that effect, and there are likely also other factors unique to each tumor (not unexpected, given that other genetic and epigentic alterations are believed to exist in many of these samples). However, it is feasible that the small changes we observe do contribute to biological effects, which are not quite as dramatic as seen in studies such as in

breast cancer. Further work, such as *in vivo* analysis, will help elucidate the effect of steroid hormones on Schwann cells and neurofibromas, leading to a better understanding of the association between these hormones and neurofibroma development and growth.

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References

1. Wallace, MR, Marchuk, DA, Anderson, et al. Type 1 Neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. Science 1990; 249(4965): 181-186.

2. Viskochil, D, Buchberg, AM, Xu, G, et al. Deletions and a translocation interrupt a cloned gene at the Neurofibromatosis type 1 locus. Cell 1990; 62: 187-192.

3. Cawthon, RM, Weiss, R, Xu, G, et al. A major segment of the Neurofibromatosis type 1 gene: cDNA sequence, genomic structures, and point mutations. Cell_1990; 62: 193-201.

4. Dugoff, L and Sujansky, E. Neurofibromatosis type 1 and pregnancy. Am J Med Genet 1996; 66(1): 7-10.

5. Puls, LE and Chandler, PA. Malignant schwannoma in pregnancy. Acta Obstet Gynecol Scand 1991; 10(3): 243-244.

6. Posma, E, Aalbers, R, Kurniawan, YS, van Essen, AJ, Peeters, PMJG and van Loo, AJ. Neurofibromatosis type 1 and pregnancy: a fatal attraction? Development of malignant schwannoma during pregnancy in a patient with neurofibromatosis type 1. BJOG: Intern J Obstet Gyn 2003; 110: 530-532.

7. Jung-Testas, I, Schumacher, M, Bugnard, H, and Baulieu, EE. Stimulation of rat schwann cell proliferation by estradiol: synergism between the estrogen and cAMP. _Dev Brain Res 1993; 72: 282-290.

8. Jung-Testas, I, Schumacher, M, Robel, P and Baulieu, EE. Demonstration of progesterone receptors in rat Schwann cells. J Steroid Biochem Mol Biol 1996; 58(1): 77-82.

9. Koenig, HL, Schumacher, M, Ferzaz, B, et al. Progesterone synthesis and myelin formation by Schwann cells. Science 1995; 268: 1500-1503.

10. Chan, JR, Rodriguez-Waitkus, PM, Ng, BK, Liang, P and Glaser, M. Progesterone synthesized by Schwann cells during myelin formation regulates neuronal gene expression. Mol Biol Cell 2000; 11: 2283-2295.

11. Jung-Testas, I and Baulieu, EE. Steroid hormone receptors and steroid action in rat glial cells of the central and peripheral nervous system. J Steroid Biochem Mol Biol_1998; 65(1-6): 243-251.

12. Svenningsen, AF and Kanje, M. Estrogen and progesterone stimulate Schwann cell proliferation in a sex- and age-dependent manner. J Neurosci Res 1999; 57: 124-130.

13. Martuza, RL, MacLaughlin, DT and Ojemann, RG. Specific estradiol binding in schwannomas, meningiomas and neurofibromas. Neurosurgery 1981; 9(6): 665-670.

14. Chaudhuri, PK, Walker, MJ, Das Gupta, TK and Beattie, CW. Steroid receptors in tumors of nerve sheath origin. J Surg Oncol 1982; 20: 205-206.

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

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

17. Stumpf, DA, Alksne, JF, Annegers, JF, et al.. Neurofibromatosis. Conference Statement. National Institutes of Health Consensus Development Conference. Arch Neurol 1988; 45: 575-578.

18. Campbell-Thompson, M, Lynch, IJ, and Bhardwaj, B. Expression of estrogen receptor (ER) subytpes and ERbeta isoforms in colon cancer. Cancer Res 2001; 61(2): 632-640.

19. Buteau-Lozano, H, Ancelin, M, Lardeux, B, Milanini, J and Perrot-Applanat, M. Transcriptional regulation of vascular endothelial growth factor by estradiol and tamoxifen in breast cancer cells: A complex interplay between estrogen receptors α and β. Cancer Res 2002; 62: 4977-4984.

20. Namiki, M, Yokokawa, K, Okuyama, A, et al. Evidence for the presence of androgen receptors in human Leydig cells. J Steroid Biochem Mol Biol 1991; 38(1): 79-82.

21. Pernas-Alonso, R, Morelli, F, di Porzio, U and Perrone-Capano, C. Multiplex semi-quantitative reverse transcriptase-polymerase chain reaction of low abundance neuronal mRNAs. Brain Res Protocols 1999; 4: 395-406.

22. Mabuchi, S, Ohmichi, M, Kimura, A, et al. Tamoxifen inhibits cell proliferation via mitogen-activated protein kinase cascades in human ovarian cancer cell lines in a manner not dependent on the expression of estrogen receptor or the sensitivity to cisplatin. Endocrinology 2004; 145(3): 1302-1313.

23. Ercoli, A, Scambia, G, Fattorossi, A, et al. Comparative study on the induction of cytostasis and apoptosis by ICI 182,780 and tamoxifen in an estrogen receptor-negative ovarian cancer cell line. Int J Cancer 1998; 76: 47-54.

24. Briand, P, Lundholt, BK, Skouv, J and Lykkesfeldt, AE. Growth response of breast epithelial cells to estrogen is influenced by EGF. Mol Cell Endocrinol 1999; 153(1-2): 1-9.

25. DeClue, JE, Heffelfinger, S, Benvenuto, G, et al. Epidermal growth factor receptor expression in neurofibromatosis type 1- related tumors and NF1 animal models. J Clin Invest 2000; 105: 1233-1241.

26. Rosenbaum, C, Karyala, S, Marchionni, MA, et al. Schwann cells express NDF and SMDF/n-ARIA mRNAs, secrete neuregulin, and show constitutive activation of erbB3 receptors: evidence for a neuregulin autocrine loop. Exp Neurol 1997; 148: 604-615.

27. Sherman, L, Sleeman, JP, Hennigan, RF, Herrlich, P and Ratner, N. Overexpression of activated neu/erbB2 initiates immortalization and malignant transformation of immature Schwann cells in vitro. Oncogene 1999; 18: 6692-6699.

28. Nakamura, T, Ushijima, T, Ishizaka, Y, et al. neu proto-oncogene mutation is specific for the neurofibromas in a N-Nitroso-N-ethylurea-induced hamster neurofibromatosis model but not for hamster melanomas and human Schwann cell tumors. Cancer Res 1994; 54: 976-980.

Table 1. Characterization of the steroid hormone receptor profile in neurofibromas.

Tumor Type	Samples	ER alpha	ER beta	PR	AR
Dermal cultures					
F*	UF 328T8c (cNF96.5g)	1.0	-†	1.1	1.1
F	UF 470T1c (cNF97.2a)	-	-	-	1.2
M	UF 505T3c (cNF98.4a)	0.9	1.0	1.1	1.1
M	UF 505T7c (cNF98.4d)	1.0	0.9	1.1	1.1
M	UF 475Tc (cNF97.5)	0.9	1.0	-	1.0
F	UF 532T1c (cNF99.1)	1.1	-	1.2	1.2
M	UF 17T1c (cNF00.10a)	1.0	-	-	0.9
M	UF 17T2c (cNF00.10b)	1.0	-	-	0.8
Plexiform cultures					
M	UF 378T2bc (pNF95.11b)	0.8	1.0	1.0	1.1
M	UF 362T1c (pNF95.6)	-	-	0.8	1.1
F	UF 440Tc (pNF97.9)	1.0	-	1.2	1.1
F	UF 511T2c (pNF98.3)	1.0	-	0.9	0.9
F	UF 554T1c (pNF99.5)	-	-	-	0.8
F	UF 572Tc (pNF00.6)	1.0	-	1.0	0.9
F	UF 593T1c (pNF00.8)	1.0	-	1.1	1.0
F	UF 836Tc (pNF02.6)	1.0	-	1.0	1.1
F	UF 550Tc (pNF99.4)	1.1	-	1.1	1.4
F	UF 746Tc (pNF01.1)	-	-	1.0	1.1
MPNST cultures					
M	UF 459Tc (sNF96.2)	-	-	-	1.2
F	UF 302Tc (sNF94.3)	0.7	0.9	1.2	1.3
Controls					
	MCF7	1.5	1.0	1.6	ND‡
	Testis	ND	ND	ND	1.2
	Caco2	ND	1.2	ND	ND

*M/F means derived from a male or female patient, respectively

†(-) means no expression detected

‡ND means not done

ER, PR, AR=estrogen, progesterone, and androgen receptors, respectively; MPNST=malignant peripheral nerve sheath tumor

Table 1. Continued.

		ER						
Tumor Type	Samples	alpha		beta		PR		AR
Primary dermal tumors								
F*	UF 328T7	1.1		-†		1.9		1.4
M	UF 17T1	1.1		1.1		1.2		0.9
M	UF 17T2	1.1		1.1		1.3		1.1
F	UF 80T2	1.3		-		1.7		1.4
F	UF 80T6	1.3		-		1.6		1.5
M	UF 158T3	1.2		1.2		1.4		1.2
M	UF 486T1	1.1		1.0		1.5		1.0
M	UF 486T5	1.3		1.1		1.7		1.1
F	UF 495T2	1.3		1.2		1.5		1.2
F	UF 495T3	1.3		1.2		1.4		1.2
M	UF 505T3	-		1.1		1.5		1.3
M	UF 505T7	1.3		1.1		1.6		1.4
F	UF 532T1	-		-		-		1.6
F	UF 549T	-		-		1.6		1.4
M	UF 743T1	1.2		1.2		1.4		1.2
M	UF 743T2	1.3		1.2		1.5		1.2
M	UF 743T4	1.2		1.2		1.4		1.1
Primary plexiform tumors								
M	UF 362T	-		1.2		1.5		1.6
F	UF 154T	1.3		1.1		1.6		1.3
F	UF 429T	1.2		1.2		1.4		1.2
F	UF 537T	-		-		1.3		1.0
F	UF 714T	1.2		1.2		1.3		1.2
F	UF 746T1	1.4		1.1		1.7		1.5
M	UF 787T1	1.2		-		1.6		1.4
M	UF 378T2b	-		1.0		1.4		1.4
F	UF 572T1	1.1		1.2		-		1.0
F	UF 550T1	-		1.1		1.4		1.1
Primary MPNSTs								
M	UF 459T	1.1		1.1		1.1		1.0
M	UF 820T	1.0		1.1		1.3		1.1

*M/F means derived from a male or female patient, respectively

†(-) means no expression detected

‡ND means not done

Table 2. Statistically significant alterations in proliferation and apoptosis in normal Schwann cells and neurofibroma-derived Schwann cell cultures in response to steroid hormone ligands.

Gender	Sample	17β-estradiol	Progesterone	ICI 182,780	Mifepristone	Tamoxifen
M	pn02.8					
M	pn97.4					
F	pn02.3			--P*(52.2%)		--P(61.0%)
F	pn02.7					
M	cNF97.5				+P(136.3%)	
M	cNF98.4d					
F	cNF99.1			--P(28.5%)		
F	cNF96.5f					
F	cNF97.2a					
F	cNF97.2b					
M	pNF00.11					--P(53.0%)
M	pNF95.11b					
M	pNF95.6					
F	pNF00.13					+P(155.5%)
F	pNF01.1					
F	pNF99.5	+A(280.0%)				
F	pNF02.6					
M	sNF02.2					
M	sNF96.2					

*+/--P, A(average % of no-treatment control)=increase or decrease in PI or AI (as a percent of no-treatment control) with $p<0.05$

pn=normal Schwann cell cultures; cNF=dermal cultures; pNF=plexiform cultures; sNF=MPNST cultures; M, F=derived from male or female patient, respectively

Table 3. Trends in proliferation in normal Schwann cells and neurofibroma-derived Schwann cell cultures in response to steroid hormone ligands.

Gender	Sample	17β-estradiol	Progesterone	ICI 182,780	Mifepristone	Tamoxifen
M	pn02.8		--*			
M	pn97.4	+			+	--
F	pn02.3					
F	pn02.7	--			--	+
M	cNF97.5				+	
M	cNF98.4d			+	+	
F	cNF99.1			--	+	
F	cNF96.5f	--	+		--	
F	cNF97.2a		--			
F	cNF97.2b					
M	pNF00.11		--			--
M	pNF95.11b	+	--			
M	pNF95.6			--	--	--
F	pNF00.13	+				
F	pNF01.1			--		--
F	pNF99.5					
F	pNF02.6	+	+	+		+
M	sNF02.2			--		
M	sNF96.2					

*+/--=increase or decrease ≥10% relative to no-treatment control

pn=normal Schwann cell cultures; cNF=dermal cultures; pNF=plexiform cultures; sNF=MPNST cultures; M, F=derived from male or female patient, respectively

Table 4. Trends in apoptosis in normal Schwann cells and neurofibroma-derived Schwann cell cultures in response to steroid hormone ligands.

Gender	Sample	17β-estradiol	Progesterone	ICI 182,780	Mifepristone	Tamoxifen
M	pn02.8			+*		--
M	pn97.4				+	+
F	pn02.3				--	--
F	pn02.7					
M	cNF97.5	--		+		
M	cNF98.4d		--		--	
F	cNF99.1				+	+
F	cNF96.5f		--		--	
F	cNF97.2a					
F	cNF97.2b		--		--	
M	pNF00.11				--	
M	pNF95.11b					+
M	pNF95.6			+	+	+
F	pNF00.13	+	+		+	
F	pNF01.1					
F	pNF99.5	+	+	+		+
F	pNF02.6	+				
M	sNF02.2	+		+	+	
M	sNF96.2		+		+	+

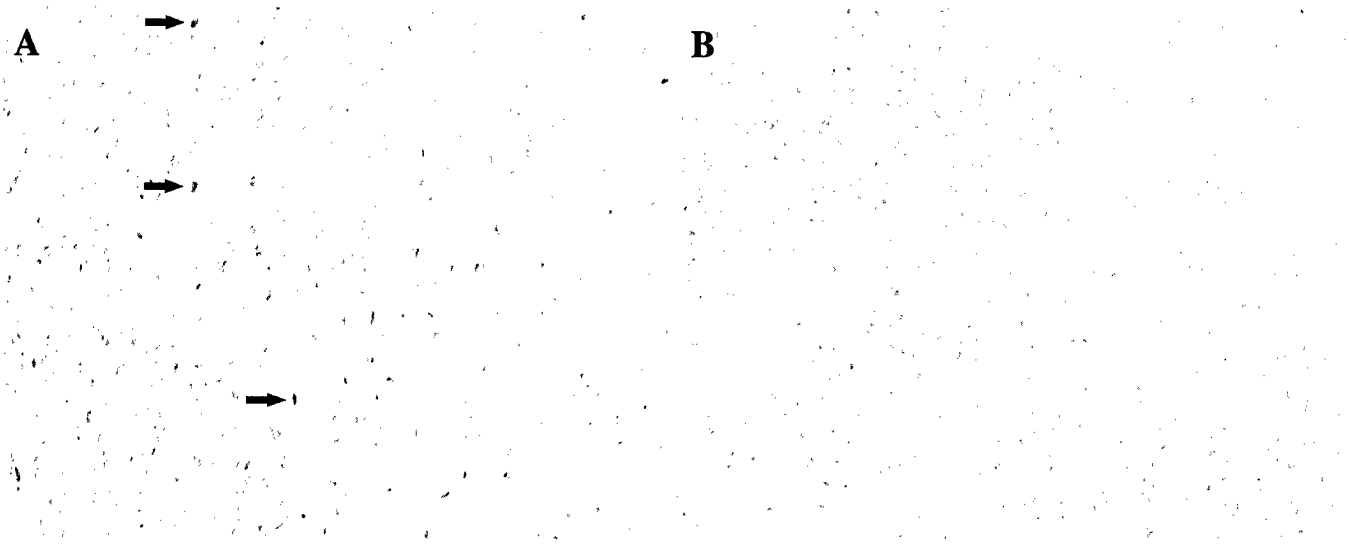
*+/--=increase or decrease ≥10% relative to no-treatment control

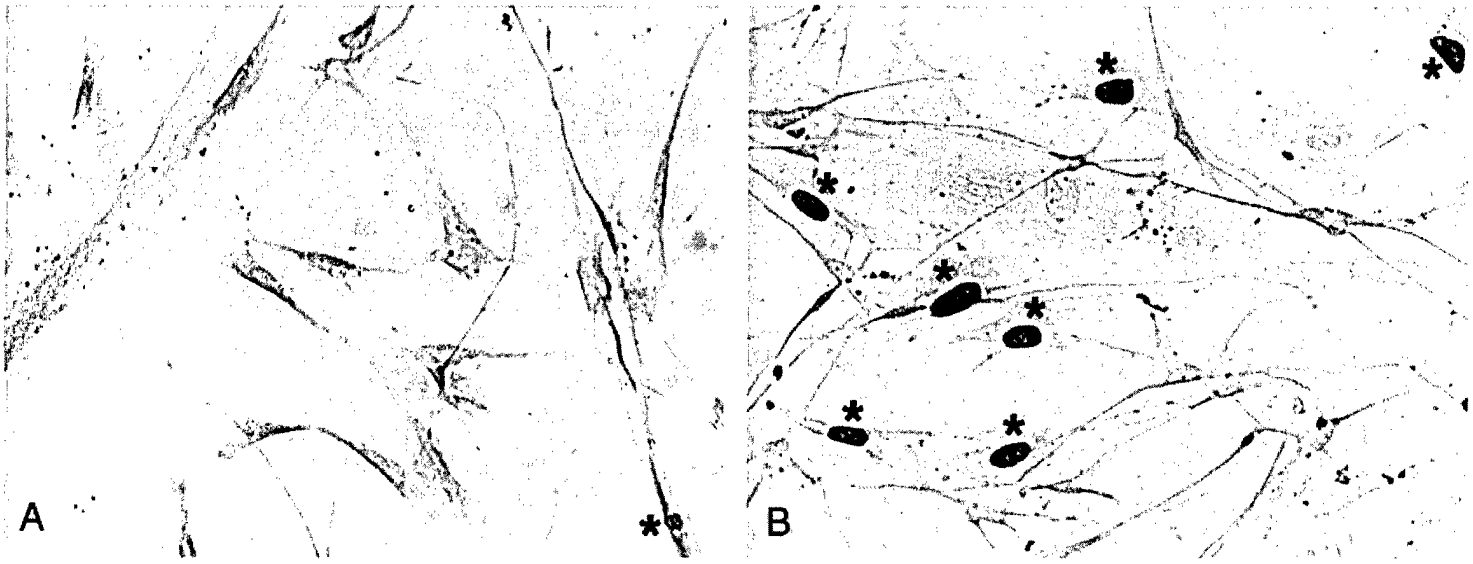
pn=normal Schwann cell cultures; cNF=dermal cultures; pNF=plexiform cultures; sNF=MPNST cultures; M, F=derived from male or female patient, respectively

Legends for Illustrations

Figure 1. PR immunohistochemistry (200x magnification). Panel A represents one of 21 neurofibromas which showed scattered individual cells positive for PR (see arrows). Panel B represents a negative control of neurofibroma tissue.

Figure 2. TUNEL and BrdU staining. Pictures show samples (200x magnification) from the normal Schwann cell culture pn97.4 for the (A) TUNEL assay to detect apoptosis (AI=5.6%) and the (B) BrdU proliferation assay (PI=30.4%). Asterisks (*) denote positively stained cells.


A
B


A
B