Running head: **Schwann cell hormone sensitivity**

The effect of estradiol, testosterone and human chorionic gonadotropin on the proliferation of Schwann cells with *NF1*$^{+/-}$ or *NF1*$^{-/-}$ genotype derived from human cutaneous neurofibromas

Paula Pennanen[1*], Sirkku Peltonen[2], Roope A. Kallionpää[1], Juha Peltonen[1a]

Address: [1]Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland, [2]Department of Dermatology, University of Turku and Turku University Hospital, PO BOX 52, 20521 Turku, Finland

[a]Corresponding author: Juha Peltonen, Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland, juhpel@utu.fi, Fax. +358 2 231 0320

Abstract

Dermal neurofibromas are the hallmarks of neurofibromatosis type 1 (NF1). Neurofibromas harbor Schwann cells with two different genotypes: Schwann cells which carry the germline mutation and a healthy *NF1* allele ($NF1^{+/-}$), and a subpopulation of Schwann cells which harbor the so called second hit leading to inactivation of both *NF1* alleles ($NF1^{-/-}$). The second hit in the *NF1* gene of Schwann cells is considered to be the initial step in the development of neurofibromas. Dermal neurofibromas typically start to grow in puberty, and their number and size increase during pregnancy, indicating hormone responsiveness. This is the first study to address the effect of human chorionic gonadotropin (hCG) on the proliferation of human $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cells in vitro. In addition, the effects of estradiol and testosterone were also investigated. The results showed that $NF1^{-/-}$ Schwann cells were more sensitive to estradiol, testosterone and human chorionic gonadotropin than $NF1^{+/-}$ cells. Specifically, the proliferation of $NF1^{-/-}$ Schwann cells was increased by up to 99 %, 110 % and 170 % compared to vehicle control when treated with estradiol, testosterone and hCG, respectively. Interestingly, no effect of estradiol, testosterone or hCG on the proliferation of the cells with $NF1^{+/-}$ genotype was observed. To conclude, the somatic second hit in the *NF1* gene sensitizes Schwann cells to sex hormones resulting in a highly increased proliferation. Our results highlight the significance of sex hormones in the regulation of neurofibroma growth.

Key words: Neurofibromatosis 1, NF1, neurofibroma, Schwann cell, sex hormone, estradiol, testosterone, human chorionic gonadotropin, hCG

Introduction

Neurofibromatosis 1 (NF1) is a neurocutaneous cancer predisposition syndrome with an incidence of 1/2000 [1]. NF1 is caused by mutations in the *NF1* gene located on chromosomal region 17q11.2. The gene encodes a tumor suppressor protein neurofibromin which is involved in controlling cell proliferation. Neurofibromin is a GTPase activating protein (GAP), which facilitates conversion of active Ras-GTP to inactive Ras-GDP. Thus, loss of neurofibromin leads to over activation of the Ras pathway. Neurofibromin is ubiquitously expressed in normal tissues including peripheral nerves and their Schwann cells [2-4].

Cutaneous neurofibromas are the hallmarks of NF1 and cause the main disease burden in adults affecting the quality of life [5]. Adult NF1 patients may have hundreds or thousands of neurofibromas in their skin. These tumors consist of Schwann cells, perineurial cells, fibroblasts, endothelial cells, and mast cells [6]. Each neurofibroma contains a clonal subpopulation of Schwann cells with double inactivation of the *NF1* gene, resulting in $NF1^{-/-}$ genotype [7, 8]. The formation of a neurofibroma requires the $Nf1^{-/-}$ Schwann cells on an otherwise $Nf1^{+/-}$ cellular background as demonstrated in an experimental mouse model [9].

Clinical findings show that NF1 is a hormone-dependent disease since neurofibromas arise and grow during puberty and the number and size of neurofibromas increase especially during pregnancy [10-13]. However, surprisingly few reports on steroid hormone action on Schwann cells have been published. Expression of steroid

hormone receptors is a prerequisite for the action of steroid hormones. Schwann cells originating from rat sciatic nerves have been studied by Jung-Testas et al. (1993, 1996) who showed the expression of progesterone and estrogen receptors [14, 15]. McLaughlin and Jacks (2003) found that the majority (86 %) of human cutaneous neurofibroma tissue samples expressed progesterone receptors [16]. Fishbein et al. (2007) reported heterogeneity between patients and individual tumors: estrogen, progesterone and androgen receptors were differentially expressed in neurofibromas, neurofibroma derived Schwann cell cultures, and Schwann cell cultures from sporadic neurofibromas of otherwise healthy persons. They also reported hormone ligand-mediated cell growth of neurofibroma derived Schwann cells suggesting association between hormones and neurofibroma development and growth [17].

Overdiek et al. (2008) demonstrated that 60 % of the Schwann cells cultured from cutaneous neurofibromas expressed progesterone receptors and progesterone increased the proliferation of Schwann cells [18]. Specifically, the proliferative response to progesterone was significantly enhanced in $NF1^{-/-}$ cells compared to $NF1^{+/-}$ or normal human Schwann cells cultured from peripheral nerves of healthy control persons. In genetically normal rodent Schwann cells, estradiol was shown to protect the cells from oxidative stress, and increase Schwann cell proliferation, differentiation, and survival [19, 20]. Our recently published dataset (accessible at NCBI GEO database, accession GSE32029) demonstrates the expression of estrogen receptors α and β, and receptors for androgens, progesterone, and luteinizing hormone/chorionic gonadotropin in human $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cells derived from NF1 associated neurofibromas.

Collectively, these findings suggest that steroid hormones have a role in the formation of cutaneous neurofibromas

Malignant peripheral nerve sheath tumors (MPNSTs) are tumors originating from Schwann cells of large peripheral nerves. NF1 patients have approximately 16 % lifetime risk of MPNST and the 5-year survival is only 30-50 % [21, 22]. Estradiol has been reported to increase the cell proliferation in mouse xenograft models of human MPNSTs, while hormone deprivation inhibits the proliferation [23, 24].

The action of sex hormones is mediated through receptors which ultimately regulate gene expression [25]. Human chorionic gonadotropin (hCG) plays an important role in the reproductive physiology. hCG is structurally and functionally similar to luteinizing hormone (LH) and both bind to the same receptor, LH/CG-R [26]. hCG is important for the maintenance of pregnancy following the implantation of the embryo. The levels of hCG increase during the first months of pregnancy from 5 IU/ml up to 100 000 IU/ml. Estradiol is synthesized in ovaries, testes, and adrenal cortex. In women, the level of estradiol varies during the menstrual cycle between 0.1 – 2 nmol/l, increases during pregnancy, and decreases during menopause below the level of 0.13 nmol/l. In men, the level of estradiol is up to 0.1 nmol/l. Also testosterone is produced by the adrenal glands, testes and ovaries. The testosterone levels vary with age between 0.1 – 40 nmol/l in men and between 0.1-2 nmol/l in women.

A method to culture *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells separately from neurofibromas has previously been described [7, 27] and is based on the observation

that the presence of forskolin promotes proliferation of cells bearing only the first hit of the *NF1* gene (*NF1*$^{+/-}$) while the Schwann cells with two hits (*NF1*$^{-/-}$) are able proliferate without forskolin. The protocol has successfully been used in further characterization of the second hit mutations of the *NF1* gene in Schwann cells cultured from cutaneous neurofibromas in NF1 [28]. The cell culture method provides an opportunity to selectively study the effect of one or two hits of the *NF1* gene on hormone sensitivity of Schwann cells. Specifically, the aim of this study is to elucidate the roles of estradiol, testosterone and hCG in the regulation of Schwann cell proliferation.

Materials and Methods

Patients and neurofibroma tissue samples

The study was approved by the Ethics Committee of Southwest Finland Hospital District, Turku, Finland. Cutaneous neurofibromas were obtained from the Department of Dermatology at Turku University Hospital with informed written consent of each patient. Patients fulfilled the NIH diagnostic criteria for NF1 [29] and they came for removal of disturbing cutaneous neurofibromas. Tumors were removed with CO_2 laser and immersed immediately into RPMI medium (Gibco, Grand Island, NY, USA). Eight cutaneous neurofibromas from 2 patients were used in the study. The tissue donors were a female aged 37-39 years (germline *NF1* mutation c.3868A>T, p.K1290X) and a 36-year-old male (germline *NF1* mutation c.1246C>T, p.Arg416X).

Preparation of tumor tissue for cell culture

NF1$^{+/-}$ and *NF1*$^{-/-}$ cells were cultured essentially as described by Serra et al. [7], with minor modifications: relatively small (≃ 5-15 mm) neurofibromas (Fig.1 a) were used for establishing cell cultures because they showed the greatest growth potential. The neurofibroma tumor tissue was dissected from surrounding dermis and subcutaneous fat, cut into 1x1 to 2x2 mm pieces, and immersed into preincubation medium containing D-MEM, inactivated 10 % fetal bovine serum (iFBS; Invitrogen, USA), 2 µmol/L forskolin (Merck, Darmstadt, Germany), and antibiotics (Penicillin G, 100 U/mL, Gibco; Amphotericin B 1.25 µg/mL, Gibco) for 3 to 7 days at 37 °C and 5 % CO_2.

Enzymatic and mechanical dissociation of the tumors

The tumor pieces were enzymatically digested using 160 U/mL collagenase type 1 (Merck) and 0.8 U/mL dispase grade 1 (Roche, Indianapolis, Indiana, USA) at 37 °C and 5 % CO_2 for 18 – 24 h. After the incubation, tissue pieces were dissociated mechanically with pipette tips. The cell suspension was centrifuged at 1200 rpm for 10 min, suspended in fresh D-MEM with 10 % iFBS for a quick wash, and centrifuged again. At this point the cells could be frozen or continued to cultivation. The cells were cultured on cell culture plastics coated with 1 mg/mL poly-L-lysine (Sigma-Aldrich, St. Louis, Missouri, USA) and 4 µg/mL laminin (Invitrogen). During the first two passages, the cells were cultured on coated 6-well plates and later on coated cell culture flasks.

Schwann cell cultivation and enrichment of NF1$^{+/-}$ cells

To enrich *NF1*$^{+/-}$ cells, half of the tumor cell suspension was plated in proliferation medium containing D-MEM, 10 % fetal calf serum (Invitrogen), 100 U/mL penicillin

G, 0.5 mM 3-iso-butyl-1-methylxanthine (IBMX, Sigma, St. Louis, Missouri, USA), 10 nM β1-heregulin (Peprotech, NJ, USA), 2.5 µg/mL insulin (Sigma-Aldrich) and 0.5 µM forskolin. Half of the medium volume was replaced with fresh medium every 3 to 4 days and the cells were passaged with trypsin when they reached 60 – 70 % confluency.

Schwann cell cultivation and enrichment of $NF1^{-/-}$ cells

To enrich $NF1^{-/-}$ cells, half of the cell suspension from a tumor was treated with proliferation medium containing forskolin, prepared as described above, for 24 hours, followed by serum-free N2 medium [30] containing 73 % D-MEM, 25 % F12 nutrient mix (Gibco), 100 U/mL penicillin G, 1 % N2-supplement (Gibco) and 0.5 mM IBMX for 24 h. The cells were then cultured in proliferation medium without forskolin, which is a crucial difference from the protocol of culturing $NF1^{+/-}$ cells. Half of the medium volume was changed to fresh medium every 3 to 4 days. If $NF1^{-/-}$ cells looked non-viable or had to be further divided, the cells were treated as above: with proliferation medium containing forskolin for 24 hours, followed by serum-free N2 medium for 24 h, and then proliferation medium without forskolin.

Incubation of Schwann cells with sex hormones and measurement of cell proliferation

The effect of sex hormones on human $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cell proliferation was quantified with 5-bromo-2-deoxyuridine (BrDU) colorimetric ELISA immunoassay (Roche Diagnostics, Mannheim, Germany). Schwann cells, of passage 5 or greater, were plated on coated 96-well plates at a density of 6000 cells per well in 100 µl of proliferation medium without forskolin. Colorless D-MEM (Gibco) was used to prepare

the proliferation medium for hormone testing. Each experiment contained four replicate wells. Estradiol (Sigma), testosterone (Sigma) and hCG (Sigma) were added as ethanol solutions on day 0 at concentrations of 0.001 nM – 100 nM for 48 (Day 2) or 96 (Day 4) hours. For controls, 1 % ethanol was added instead of hormones. Ten µM BrDU was added to the medium for 18 hours before the cells were fixed with ethanol and excess background was blocked with blocking reagent (Roche Diagnostics) for 30 min. Cells were then incubated with Anti-BrDU antibody for 1 hour, followed by washing. After 30 min incubation with substrate and the development of the color, stop solution was added into the wells. Absorbance at 450 nm was measured with the Hidex microplate reader (The Plate Chameleon Multilabel Counter, Hidex Oy, Turku, Finland) to determine BrDU incorporation in the cells, reflecting cell proliferation during the BrDU incubation period.

Statistical analysis

Blank measurements were subtracted from the absorbance readings of the cell proliferation assays, and the resulting numbers were normalized relative to the vehicle controls specific to the assay day and cell line. To account for correlation between assays from the same cell line and cells from the same patient, linear mixed effects regression with random intercepts for tumor and patient was used for statistical analysis. Comparisons were made between the vehicle control and hormone treatments as well as between genotypes. Statistical software R version 3.3.2 with lmerTest package version 2.0-33 was used for the analysis.

Results

The two genotypically different ($NF1^{+/-}$ and $NF1^{-/-}$) Schwann cell populations displayed different phenotypes as illustrated in Figure 1. More specifically, $NF1^{-/-}$ cell cultures were homogenous and contained typical spindle shaped Schwann cells [7] (Fig. 1c) which grew tail to tail and formed lattices. The morphology and growth pattern of $NF1^{+/-}$ cells were clearly different. The cell cultures were dense and the cells formed linear bundles and swirls (Fig. 1b). Both $NF1^{+/-}$ and $NF1^{-/-}$ cells were S100 positive (not shown).

Human Schwann cell cultures with $NF1^{+/-}$ and $NF1^{-/-}$ genotypes were treated with estradiol, testosterone and hCG at concentrations ranging from 0.001 nM to 100 nM for two or four days. The tested hCG concentrations correspond to 0.53 IU/l – 53000 IU/l. The effect of sex hormones on human $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cell proliferation was quantified with BrDU colorimetric ELISA immunoassay.

Addition of estradiol (Fig. 2), testosterone (Fig. 3) or hCG (Fig. 4) had no consistent effect on the proliferation of the cells with $NF1^{+/-}$ genotype after two or four days of treatment. In contrast, testosterone and hCG increased the proliferation of the $NF1^{-/-}$ Schwann cells already after two days by 15 – 22 % (Fig. 3) and 29 – 51% (Fig. 4), respectively. The effect on $NF1^{-/-}$ cells was markedly more pronounced after four days ($P < 0.001$), testosterone yielding up to 110 % and hCG up to 170 % increase in cell proliferation compared to vehicle control. The highest concentration, 100 nM, of hCG failed to produce a significant increase in cell proliferation. Estradiol resulted in a

significant 86 – 99 % increase in proliferation at concentrations 0.001–10 nM on day four, but only minor effect was observed on day two (Fig. 2).

Direct comparisons between *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells at specific hormone concentrations and time points indicated that the difference between genotypes was statistically significant for hCG at the lowest concentrations on day 4 (Fig. 4). Assays with estradiol and testosterone showed high variation on day 4, since one cell line with *NF1*$^{-/-}$ genotype responded more strongly to estradiol and testosterone treatment than the other cell lines. Exclusion of this outlier reduced the increase in cell proliferation to 40 – 56 % and 49 – 60 % compared to vehicle control at estradiol and testosterone concentrations 0.001 – 10 nM, respectively ($P < 0.001$ compared to vehicle for all). Despite the reduction in effect size, the differences between genotypes were significant with $P < 0.05$ at 0.001 – 10 nM estradiol and 0.001–100 nM testosterone, indicating that the responses to hormone treatments are robust for the exclusion of the exceptionally sensitive outlier cell line.

Discussion

The findings of this study provide new information about the behavior of Schwann cells of two different genotypes, *NF1*$^{+/-}$ and *NF1*$^{-/-}$, when exposed to estradiol, testosterone or human chorionic gonadotropin (hCG). Moreover, this is the first study to address the effect of hCG on the proliferation of human *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells in vitro. The hormone concentrations used in the current study were based on the physiological

range of the tested hormones. These results demonstrate that the hormones can act directly on Schwann cells, and offer a feasible explanation for clinical observations which show an increase in the number and size of neurofibromas in association with puberty and pregnancy. The results also point out that Schwann cells with two hits in the *NF1* gene are more prone to the effects of sex hormones than the cells with one mutation only. Specifically, the sex hormones used in this study increased the proliferation of *NF1*$^{-/-}$ Schwann cells by up to 99 % – 170 % compared to vehicle control after 4 days of treatment. Estradiol and hCG stimulated Schwann cell proliferation at concentrations 0.001–10 nM while the 100 nM concentration did not show significant effect.

Since neurofibromin inhibits the proliferation controlling Ras pathway, its complete loss in *NF1*$^{-/-}$ Schwann cells may allow more pronounced proliferative responses to mitogens such as the sex hormones estradiol, testosterone and hCG. In addition, the receptors for these hormones directly interact with the Ras pathway, possibly increasing its activation [31-33]. Therefore, the hormone sensitivity of *NF1*$^{-/-}$ Schwann cells may be due to aberrant signal transduction resulting from complete loss of neurofibromin.

By the age of 8, nearly all NF1 patients have 2 or more of the features included in the NIH Diagnostic Criteria. Neurofibromas are present in half of the 10-year-old patients and an increase in their number occurs during puberty and later during pregnancy. Over 80 % of 20-year-old NF1 patients have neurofibromas [34, 35]. In addition to the effect of sex hormones on the natural course of neurofibroma growth,

another clinically interesting question is whether oral contraceptives promote the growth of benign neurofibromas. The only study published on this issue suggested that low doses of estrogen and progesterone did not seem to stimulate the growth of neurofibromas in most of the 59 NF1 patients studied. However, tumor growth was observed in two patients taking high doses of progesterone [36].

The results showed that relatively small (⌀ 5-15 mm) neurofibromas (Fig.1a) were optimal sources for cell cultures with respect to growth potential. $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cells were grown in culture medium containing phenol red which is known to bind to estrogen receptors [37, 38]. Culturing in a medium devoid of phenol red was tested but the cells were not sufficiently viable and proliferated very slowly. Thus, the red culture medium was used for expansion of the cultures but the hormone testing was carried out in colorless medium. Culture medium also contained FBS which contains hormones. Culturing without FBS was tested but the cells did not survive without it.

The crucial difference between $NF1^{+/-}$ and $NF1^{-/-}$ Schwann cell cultures is the proliferation medium with ($NF1^{+/-}$) or without ($NF1^{-/-}$) forskolin, which allows segregating the genotypes from each other. In vitro studies of a genetically normal peripheral nerve injury mouse model demonstrated that estradiol promoted Schwann cell proliferation in the presence of forskolin, and differentiation in the absence of forskolin leading to early remyelination of the injured peripheral nerve [20]. Forskolin is known to be a unique diterpene activator of cyclic AMP (cAMP) production [39]. Serra et al. (2000) noted that removal of forskolin enriched $NF1^{-/-}$ Schwann cells, whereas addition led to enrichment of $NF1^{+/-}$ Schwann cells. Nevertheless, $NF1^{-/-}$ cells

were growing best when forskolin was present in the culture medium for 24 hours after the cells were plated. They indicated that the stimulation of cAMP pathway was important for *NF1*$^{-/-}$ cells, yet even more important to *NF1*$^{+/-}$ cells [7].

Our recently published dataset (accessible at NCBI GEO database, accession GSE32029) together with earlier literature show that *NF1*$^{+/-}$ and *NF1*$^{-/-}$ Schwann cells express estrogen, androgen and LH/CG-R receptors. The current study demonstrates that the somatic second hit in the *NF1* gene sensitizes Schwann cells to sex hormones resulting in a highly increased proliferation which explains, at least in part, the growth of neurofibromas in puberty and pregnancy.

Conclusions

Neurofibroma-derived Schwann cells whose both *NF1* alleles were mutated responded to sex hormones by increased proliferation. The results elucidate the proliferation of the *NF1*$^{-/-}$ Schwann cells and may pave the way for future therapeutic approaches.

Abbreviations

NF1	neurofibromatosis type 1
NF1	human *NF1* gene
NF1$^{+/-}$	cells carrying the constitutional *NF1* mutation only
NF1$^{-/-}$	cells with the *NF1* second hit
NIH	National Institutes of Health
PBS	phosphate buffered saline
BSA	bovine serum albumin
MPNST	malignant peripheral nerve sheath tumor
hCG	human chorionic gonadotropin

Availability of data

Raw data is available on request**.**

Ethics and consent to publish

This study has been performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Southwest Finland Hospital District, and patients gave their informed written consents. The study was carried out at Turku University Hospital and the University of Turku.

Conflict of Interest

The authors declare no conflict of interest.

Authors' contributions

JP conceived the study. JP and SP made a significant contribution to writing the paper. SP collected the neurofibroma samples. RAK analyzed the data, drafted and revised the paper. PP designed the experiments, cultured Schwann cells, performed the assays, analyzed the data and wrote the paper. All authors read and approved the final version of the manuscript.

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Titles and legends to figures

Fig. 1 The two neurofibroma-derived Schwann cell populations. Relatively small (⌀ 5-15 mm) cutaneous neurofibromas were used as starting material for two different Schwann cell populations (**a**). *NF1*$^{+/-}$ cells were dense and formed linear bundles and swirls (**b**). *NF1*$^{-/-}$ cell cultures were homogenous and contained typical spindle shaped Schwann cells (**c**). Scale bars 50 µm.

Fig. 2 The effect of estradiol on the proliferation of human Schwann cells after 2 and 4 days. Estradiol concentrations 0.001–10 nM resulted significant increases in *NF1*$^{-/-}$ cell proliferation on day 4. The values are normalized relative to the vehicle control. Error bars show standard error of mean. Asterisks denote statistical significance of the comparison to the vehicle control of the respective genotype, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 3. The effect of testosterone on the proliferation of human Schwann cells after 2 and 4 days. Testosterone concentrations 0.001–100 nM yield significant increases in *NF1*$^{-/-}$ cell proliferation on days 2 and 4. The values are normalized relative to the vehicle control. Error bars show standard error of mean. Statistical significance of the comparison to the vehicle control of the respective genotype is marked with asterisks, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4. The effect of human chorionic gonadotropin (hCG) on the proliferation of human Schwann cells after 2 and 4 days. hCG concentrations 0.001–10 nM demonstrated significant increases in *NF1*$^{-/-}$ cell proliferation on days 2 and 4. The values are normalized relative to the vehicle control. Error bars show standard error of mean. Asterisks denote statistical significance of the comparison to the vehicle control of the respective genotype (above each bar), or between genotypes, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.