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Mutation analyses of the NF2 gene from NF2 patients suggest that the inactivation of the NF2 gene, and the consequent lack of gene product (protein known as Merlin/Schwannomin) is the primary cause of this disease. Within the three years period, we plan to develop a laboratory protocol to establish Schwann cell culture using surgical specimens from NF2 patients, and compare the tumor cells from patients with different NF2 gene mutations. In addition, we want to extend the life span of primary culture cells by immortalizing them using a retrovirus which we engineered. This will not only ensure the reproducibility of results within the NF2 community, but also provide scientists greater access to certain materials for the study of biologic function of Schwann cell and also important for testing therapeutic approaches.

During the first 12 months, an additional 4 normal Schwann cells cultures were added to the previously banked cultures. One addition of primary human vestibular schwannoma culture from a NF2 patients and one normal Schwann cell culture are in the process of immortalization.

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FOREWORD

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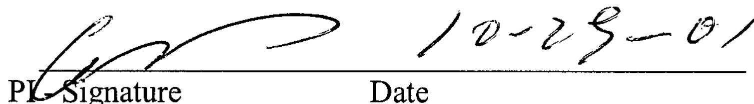

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Introduction

In the Eight years since the NF2 gene was identified, NF2 research has been divided into four main areas: 1) Natural history; 2) NF2 gene function; 3) *In vitro* and *in vivo* models; and 4) Therapeutic intervention. Although there have been some important discoveries, we are still unable to answer key questions about what factors predict the tumor growth rate in patients, the pathogenesis of NF2, whether the current NF2 mouse knock-out model can be used to represent human NF2, and whether gene therapy is the future therapy for NF2. Most researchers feel that the lack of an *in vitro* model system has limited their research progress and development of such a model should be a high priority. The purpose of this study is to develop a NF2 gene deficient *in vitro* model that can be used to further understand NF2 gene function and to facilitate development of new treatments. In this research study, we propose to develop a primary and permanent human Schwannoma cell culture system, and with this *in vitro* model, to test the **hypothesis** that different NF2 gene mutations result in different degrees of loss in NF2 gene function and the loss in NF2 gene function directly controls the tumor growth rate."

To test the hypothesis, we propose the following **Specific Aims**:

1) Establish a reproducible protocol for the primary culture of human Schwann and schwannoma cells and characterize NF2 gene function by studying cytoskeleton organization and tumor cell growth rate in culture.

2) Immortalize one normal Schwann cell culture and two schwannoma cell cultures with different mutations and phenotypes and characterize the cell lines.

Successful completion of these aims will allow us to: 1) better understand NF2 gene function in Schwann cells at the cell and molecular level and 2) obtain a useful *in vitro* tool for screening of new therapeutic agents for NF2.

Body

STATEMENT OF WORK

Establish an in vitro model for the study of NF2 gene function.

Specific Aim 1: Establish a reproducible protocol for the primary culture of human Schwann and schwannoma cells and characterize NF2 gene function by studying cytoskeleton organization and tumor cell growth rate in culture.

Task 1: Months 1-20 Establish primary culture protocol by collecting normal vestibular nerve tissues and schwannoma tissues and store all cultures in liquid nitrogen at their passage 3.

Report:

The bank of human normal nerve cells and schwannoma and their primary cultures:
During the twelve month period, 7 NF2 patients underwent tumor removal surgery at House Ear Clinic. All vestibular schwannoma tissues were collected. Of those 7 tumors, 5 tumors were processed for primary cultures. In addition, 6 normal human sciatic nerve tissues were also collected and 4 Of them were cultured.

Specific Aim 2: Immortalize one normal Schwann cell culture and two schwannoma cell cultures with different mutations and phenotypes and characterize the cell lines

Task 1: Primary culture cell immobilization by retrovirus.

In addition to the previously immortalized 2 primary human schwannoma cultures and one normal Schwann cell cultures, one vestibular schwannoma culture from a NF2 patient and one normal human Schwann cell culture were transduced by retroviral vectors carrying HPV E6-E7 genes and neo^r gene. All cultures are growing under the selection drug G418 (0.4 mg/ml) since 4 days post viral transduction. The drug-resistant cells from Passage 0 and each successive passage were stored and tested for schwannal cell marker S100 expression (Table 1).

Table 1:

Primary cultures	Cell type	passage	S100 positive cells at latest passage
476	schwannoma	6	>99%
SN#1	Schwann	3	Weakly positive

Progress on Task 1: In progress.

Task 2. Characterization of the stable long term cell line.

In order to evaluate the usefulness of the cell line, using an adenoviral vector mediated gene transfer technique, changes in the phenotypic characteristics after NF2 gene restoration in NF2 gene-mutated human schwannoma cell line (HEI 193) were investigated. The overexpression of Merlin/Schwannomin in HEI 193 led to an inhibition of cell proliferation under serum-free conditions (Figure 1). Upon PDGF stimulation in culture, Merlin/Schwannomin appeared to inhibit the activation of the MAPK and PI3K signaling pathways, impinging on the phosphorylation of Erk 1/2 and Akt, respectively. The data also show that PDGFR is more rapidly internalized by the schwannoma cells overexpressing NF2. Therefore, this process is suggested as a model for a mechanism of Merlin/Schwannomin tumor suppressor function, which intermediates acceleration of the cell surface growth factor degradation.

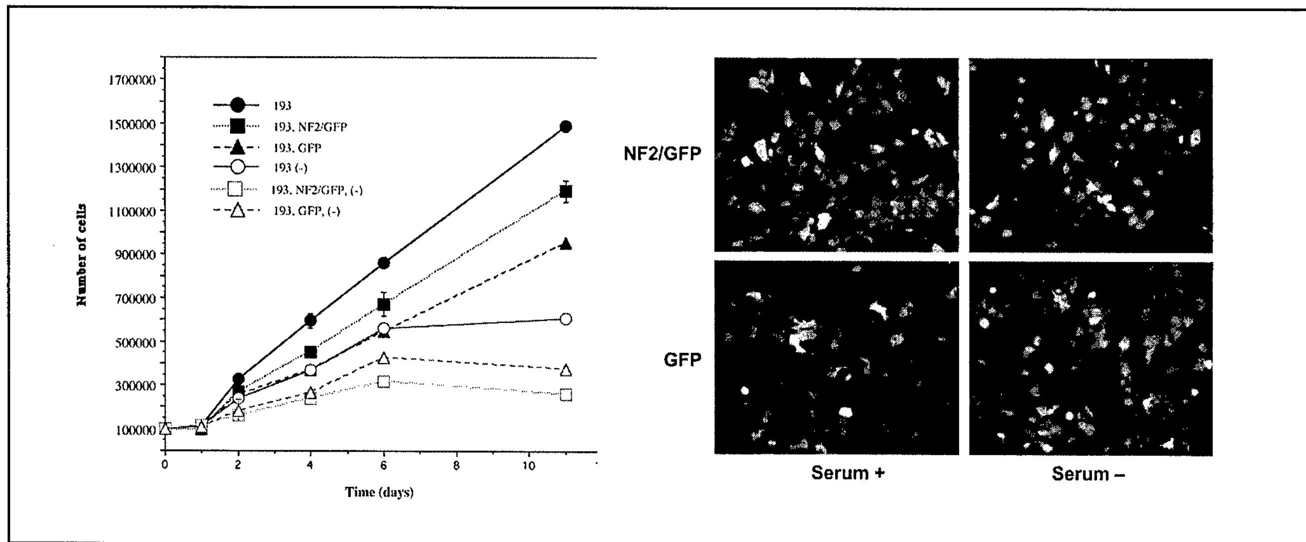

Figure 1. Cell proliferation of HEI 193, HEI 193 overexpressing NF2/GFP, HEI 193 overexpressing GFP. In medium containing 10% serum, the NF2 overexpressing cells proliferate faster than only GFP transduced ones. Under serum-free conditions the growth speed converts. Under both conditions,

untransduced schwannoma HEI 193 cells grow at the most rapid rate. In the photo is to observe in all four investigated conditions the same level of fluorescence (HEI 193 cells showed no flurorescence).

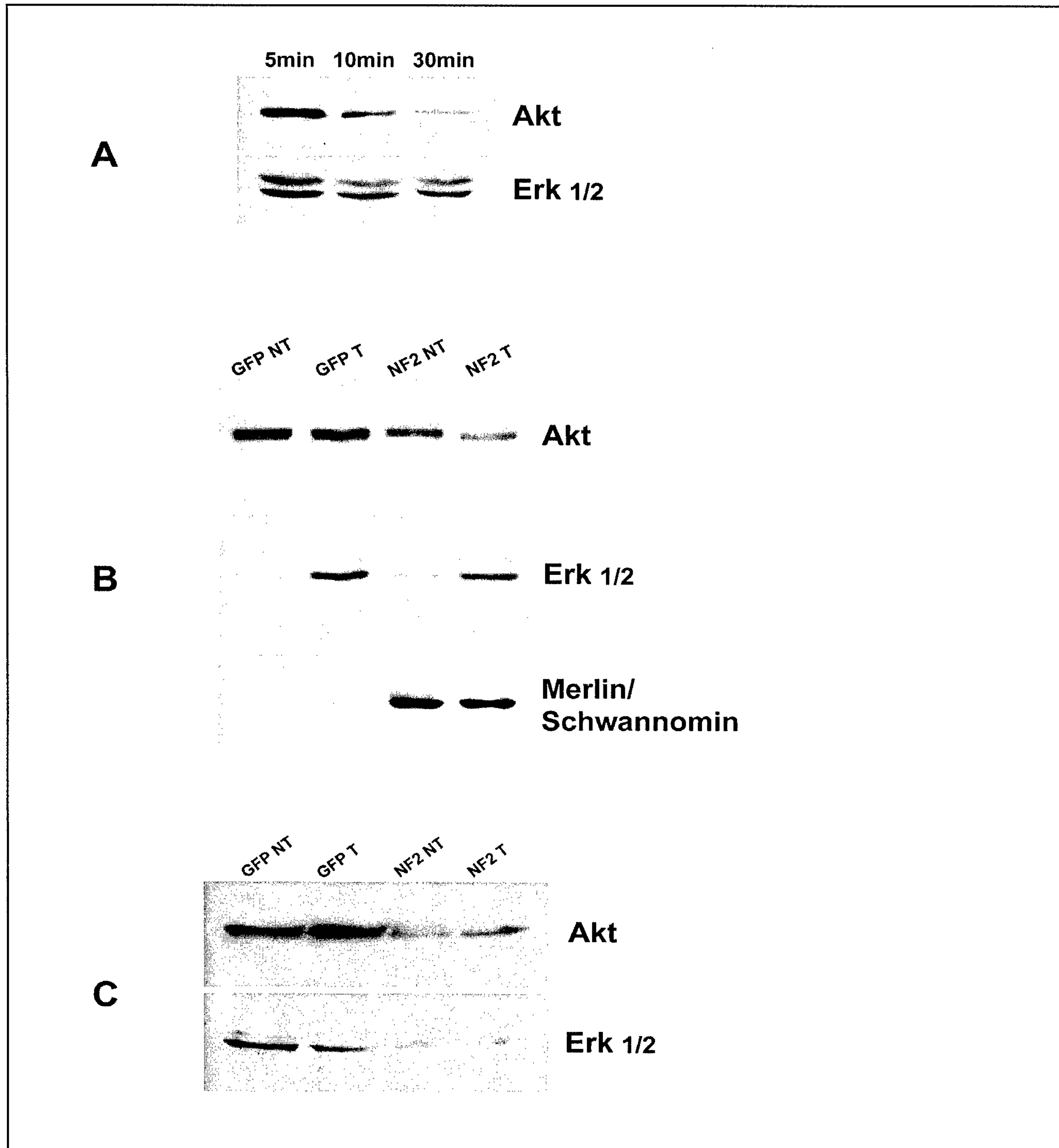

Figure 2. The influence of ligand stimulation on human schwannoma (T treated, NT non treated with the ligand). A The shortest stimulation with PDGF (5 minutes) showed the highest activation of Erk 1/2 and Akt. B In HEI 193 NF2 transduced cells there is a different effect on the activity of Erk 1/2 and Akt to see. In these cells the activity of PDGF-treated ones is drastically reduced compared to GFP-

transduced ones. C The same experiment as in B, with heregulin used as ligand for stimulation of PDGFR internalization.

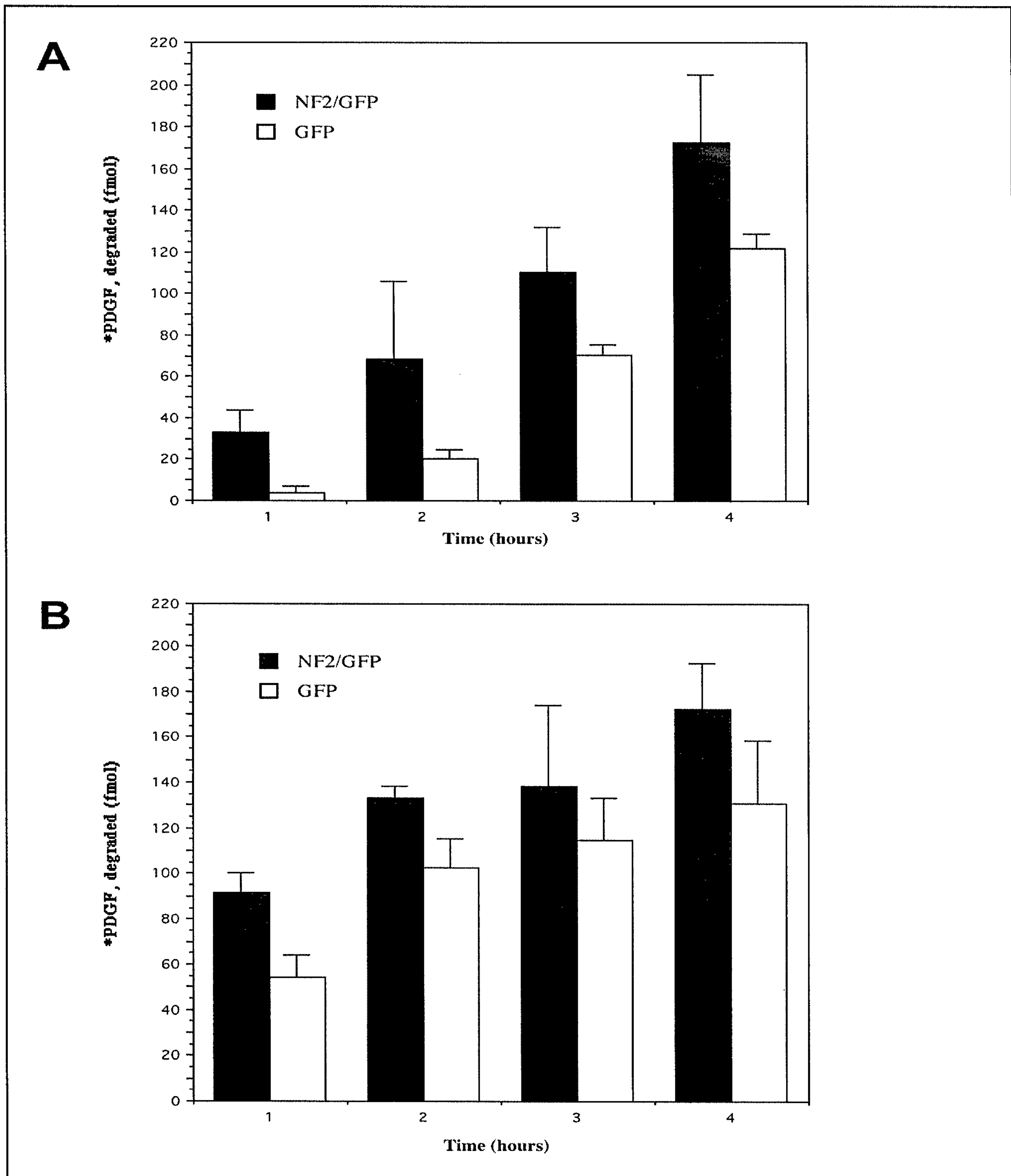

Figure 3. The PDGF-mediated degradation rate of PDGFR in NF2-transduced HEI 193 cells is highly accelerated. Under different conditions (A: plated at 500000 cells/well and 2 days starvation followed; B: plated

at 250000 cells/well and 4 days starvation followed) the PDGF stimulation leads to a similar increase of degradation in NF2-overexpressing cells compared to GFP-overexpressing ones.

Progress on Task 2: In progress.

Key Research Accomplishments

- Establishment of NF2 specific vestibular schwannoma primary culture bank.
- Establishment of human normal Schwann culture bank
- Partially immortalized a normal Schwann cell culture.
- Overexpression of NF2 gene able to inhibit NF2 gene mutated schwannoma cell proliferation under serum free condition

Reportable Outcomes

- Society for Neuroscience Meeting Nov. 2001 San Diego, California

Abstracts for " Overexpression of the NF2 gene inhibits schwannoma cell proliferation through promoting PDGFR degradation" will be submitted for presentation

- Establishment and characterization of a schdwannoma cell line from a patient with NF2 Manuscript submitted to J of international Oncology.

- Overexpression of the NF2 gene inhibits schwannoma cell proliferation through promoting PDGFR degradation. Manuscript in preparation

-Human primary vestibular schwannoma tissue and culture bank

-Establish a retroviral vector transduced potential human Schwann cell line.

Conclusions

The goals of this project are to develop a methodology to establish a reliable *in vitro* system and test its credibility for the study of NF2 gene function in Schwann cells. Over the second twelve months of the project, we have established a standard method for culturing primary schwannoma cells and normal Schwann cells. In addition to two previously immortalized cultures, one primary schwannoma cultures and one normal Schwann cell culture were transduced by retrovirus and one has shown signs of immortalization. We have begun to show the characteristics of the cell lines using different molecular approaches.