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PRINCIPAL INVESTIGATOR: Jonathan Chernoff, M.D., Ph.D.

CONTRACTING ORGANIZATION: Institute for Cancer Research
Philadelphia, PA 19111

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**14. ABSTRACT**

In this project, we are seeking to use induced pluripotent stem (iPS) cell technology as a potential therapy in NF1. In the first years, we have successfully produced iPS cells from fobroblasts from Nf1 +/-mice and characterized the properties of these cells, which include growth in clusters, expression of stem cell markers, normal karyotype, and the ability to form teratomas in mice. We also created a targeting "gene-repair" vector and have used this vector to to replace the defective Nf1 allele in these iPS cells. We then showed that we could differentiate iPS cells into Schwann-like cells in vitro, enabling drug sensitivity studies.

**15. SUBJECT TERMS**
NF1, iPS, Schwann cells, gene targeting, differentiation

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**INTRODUCTION:**

The goal of this project was to use induced pluripotent stem (iPS) cells and homologous recombination as the basis for therapy in neurofibromatosis 1 (NF1). Our hypothesis was that we could reprogram skin cells derived from NF1 heterozygous to become iPS cells, then repair the damaged NF1 allele, differentiate these iPS cells to hematopoietic precursors, and reintroduce such cells into irradiated *Krox20-Cre*; $Nf1^{flox/-}$ mice. The theory behind these experiments is that NF1-related tumors require not only $Nf1^{-/-}$ Schwann cells, but also $Nf1^{+/-}$ mast cells, and these could be replaced by the repaired iPS cells.

**BODY:** We set ourselves four specific tasks. These were:

Task 1. **Create iPS cells from $Nf1^{+/-}$ keratinocytes.** In this step, we will obtain keratinocytes from $Nf1^{+/-}$; Oct4-GFP mice, which should express GFP when converted to iPS cells.

1a. Cross $Nf1^{+/-}$ mice with Oct4-GFP mice to obtain $Nf1^{+/-}$; Oct4-GFP mice (months 1-4).
1b. Isolate and expand keratinocytes from 1-2 month old mice (months 4-6).
1c. Infect with four adenoviruses (encoding Oct4, Klf4, Sox2, and c-Myc, isolate and characterize iPS cells (months 6-12).

Task 2. **Repair the damaged *Nf1* allele.** We will use standard methods of homologous recombination to repair the damaged *Nf1* allele in $Nf1^{+/-}$ iPS cells.

2a. Construct *Nf1* (re)targeting vector (months 1-6).
2b. Transfect $Nf1^{+/-}$ iPS cells and identify targeted, $Nf1^{+/+}$ iPS cells. (months 12-15).

Task 3. **Convert the iPS cells to hematopoietic precursors.** To convert the undifferentiated iPS cells to a transplantable state, we will use HoxB4 to drive the cells towards a hematopoietic lineage.

3a. Infect iPS cells with GFP-HoxB4 retrovirus (month 15).
3b. Isolate embryoid bodies and grow cells on OP9 stromal feeder layer. (months 15-18).

Task 4. **Transplant NF1 mouse with iPS-derived $Nf1^{+/+}$ or $Nf1^{+/-}$ cells.** We will replace the bone marrow cells of Krox20-Cre; $Nf1^{flox/-}$ mice with marrow derived from repaired iPS cells and observe the animals for signs of disease.

4a. Irradiate Krox20-Cre; $Nf1^{flox/-}$ and transplant with hematopoietic precursors derived from iPS cells. (months 18).
4b. Observe mice for signs of disease. (months 18-24).

**Progress**

By the end of the second year, we achieved aims one and two, as detailed below.

Task 1. **Create iPS cells from *Nf1*$^{+/-}$ keratinocytes.** As noted in last year's progress report, we made several technical changes in the course of achieving this aim.

1a. Cross *Nf1*$^{+/-}$ mice with Oct4-GFP mice to obtain *Nf1*$^{+/-}$; Oct4-GFP mice (months 1-4). We omitted this step, as efficiencies of iPS production are now such we did not need to use an Oct4 reporter. We therefore did not carry out this cross.

1b. Isolate and expand keratinocytes from 1-2 month old mice (months 4-6). We also omitted this step, as we found that we could efficiently reprogram mouse fibroblasts.

1c. Infect with four adenoviruses (encoding Oct4, Klf4, Sox2, and c-Myc, isolate and characterize iPS cells (months 6-12). We used retroviruses instead, as adenoviral transduction was not efficient in our hands. We used both the classic four factor (Oct4, Klf4, Sox2, c-Myc) and a three factor (Oct4, Klf4, Sox2) combinations to induce iPS formation. We found that the three-factor combination, which omits the potential oncogene c-Myc, gave robust, stem-cell appearing colonies (Fig. 1). Colony 3F-11, shown here, has a normal karyotype (not shown). This clone showed reactivation of endogenous stem cell genes, and formed embryoid bodies *in vitro* and teratomas *in vivo* (not shown).

Task 2. **Repair the damaged *Nf1* allele.** We have completed this task, using standard methods of homologous recombination to repair the damaged *Nf1* allele in *Nf1*$^{+/-}$ iPS cells.

2a. Construct *Nf1* (re)targeting vector (months 1-6). We used recombineering to create a "repair" allele (Fig. 2). This allele uses hygromycin for selection, and G418 sensitivity for counter-selection. That is, correctly targeted alleles, in which the damaged, neo-containing NF1 allele are replaced with our floxed hygromycin cassette, are identified as colonies of iPS cells that are hygromycin resistant but G418 sensitive.

2b. Transfect *Nf1*$^{+/-}$ iPS cells and identify targeted, *Nf1*$^{+/+}$ iPS cells. (months 12-15). This subtask has recently been completed. We found that 3 of 225 hygromycin resistant clones showed concomitant loss of G418 resistance. Southern blot and PCR analysis revealed replacement of the neo cassette and restoration of the NF1 reading frame (data not shown). The floxed hygromycin cassette was subsequently removed by infection with Adeno-Cre virus.

Task 3. **Convert the iPS cells to hematopoietic precursors.** We have recently begun this task, which could not be initiated until Task 2 was complete. We obtained OP9 stromal cells and are preparing for *Nf1*$^{+/-}$ and *Nf1*$^{+/+}$ iPS co-culture to obtain

hematopoietic precursors, as outlined in our initial proposal.

In addition to this work, we also have attempted to differentiate the iPS cells to Schwann cells. Such cells would be useful for drug sensitivity studies as well as for evaluating the effect of NF1 lesions on Schwann cell differentiation. We modeled our approach (with some modifications) on that of Kate Barald, who had successfully differentiated mouse ES cells to Schwann-like cells *in vitro* (Roth et al, Glia, 55:1123, 2007). In brief, we plated iPS cells in Schwann cell differentiation media (84% a-modified MEM without phenol red, 1% Pen/Strep, 10% FBS, 5% 11-day chick embryo extract, 10 ng/mL NRG-1). After 25 days in this media, the cells flattened out and became spindly (Fig. 3A), expressing both S100 and GFAP (Fig. 3B).

Task 4. **Transplant NF1 mouse with iPS-derived *Nf1*$^{+/+}$ or *Nf1*$^{+/-}$ cells.** We have not initiated this task yet, as it cannot be begun until task three is complete.

**KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research.

- Constructed pluripotent iPS cell lines from *Nf1*$^{+/-}$ cells.
- Completed retargeting construct to repair NF1 allele in these cells.
- Used homologous recombination to repair NF1 allele.
- Differentiated both WT and *Nf1*$^{+/-}$ iPS cells into Schwann-like cells *in vitro*.

**REPORTABLE OUTCOMES:**

None

**CONCLUSION:**

While we had to make a number of changes to our protocols, we made the first *Nf1*$^{+/-}$ iPS cells. In addition, we have successfully constructed a gene repair plasmid, and used it to repair the damaged *Nf1* allele.

The production of iPS cells from NF1 animals also allows us to differentiate these cells *in vitro* to Schwann cells. While not a part of the initial proposal, such cells should be a useful resource for studying the role of NF1 in Schwann cell differentiation and in drug sensitivity studies.

This project was designed for two years of funding, which is now complete. We intend to complete the project using institutional funds and/or any additional grant support we may obtain.

**REFERENCES:**

None

## BIBLIOGRAPHY OF PUBLICATIONS:

None

## LIST OF PERSONNEL:

1. Jonathan Chernoff, M.D., Ph.D. – Principal Investigator
2. Olga Villamar-Cruz, M.S., Scientific Technician I

## APPENDICES:

1) Figures 1 – 3

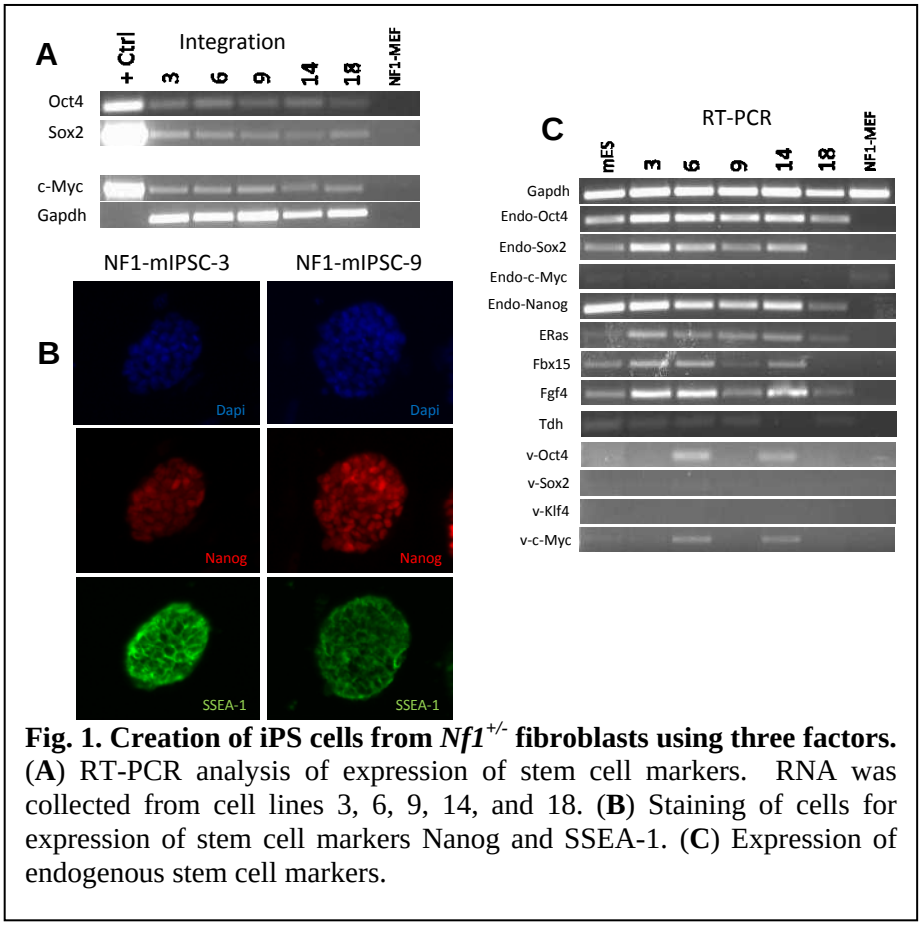


**Fig. 1. Creation of iPS cells from *Nf1*$^{+/-}$ fibroblasts using three factors.** (**A**) RT-PCR analysis of expression of stem cell markers. RNA was collected from cell lines 3, 6, 9, 14, and 18. (**B**) Staining of cells for expression of stem cell markers Nanog and SSEA-1. (**C**) Expression of endogenous stem cell markers.

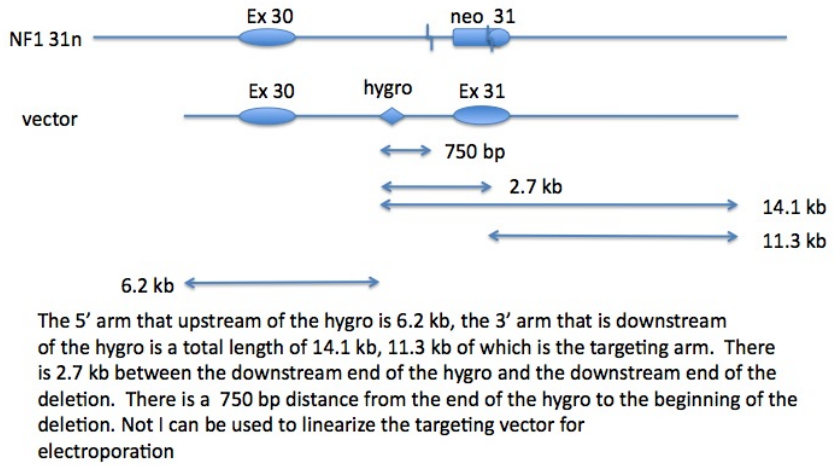


**Figure 2. NF1 gene repair vector.** The vector is designed to replace the disrupted NF1 allele in this mouse model, restoring exon 31.

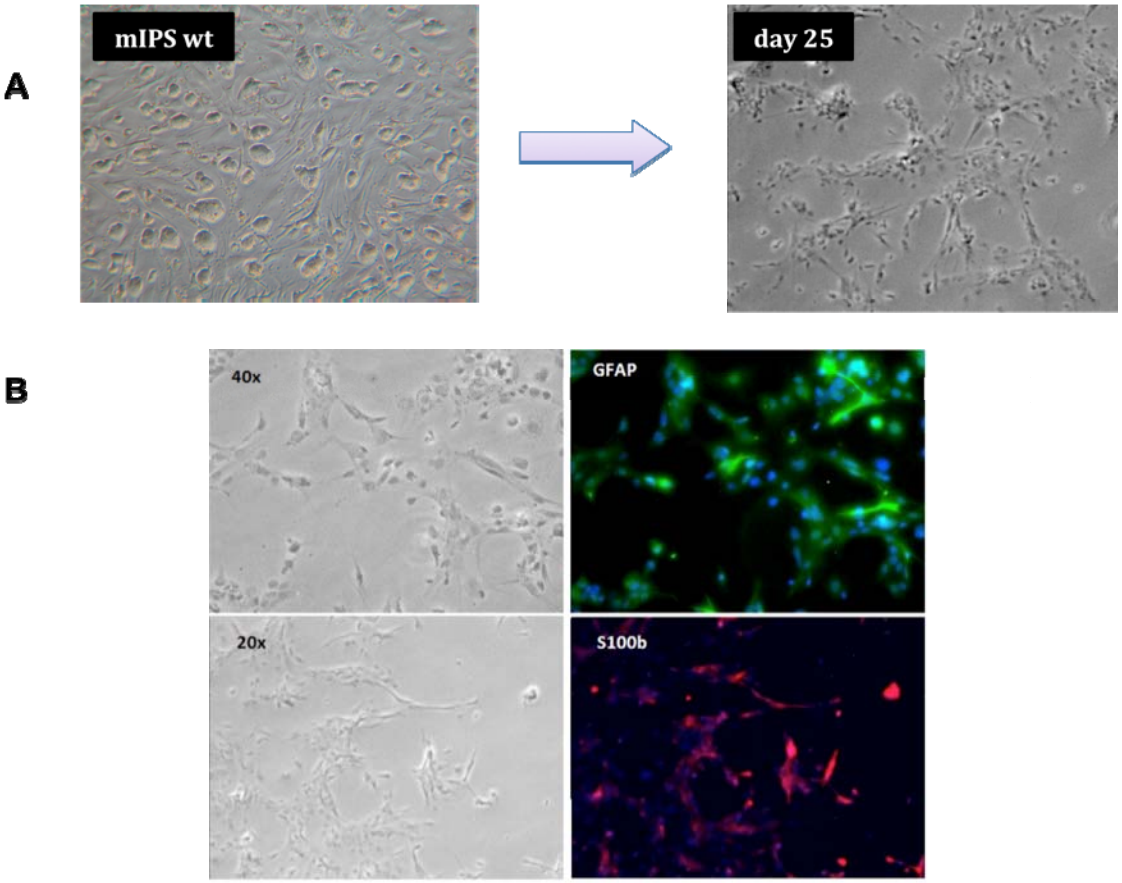


**Figure 3.** ***In vitro*** **differentiation of iPS cells to Schwann-like cells.** WT mouse iPS cells were differentiated and purified as described in the text. A) Morphology of iPS cells before and after growth in Schwann Cell differentiation media. B) staining with GFAP and S100 markers. DAPI staining shows feeder-layer cells.