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14. ABSTRACT

Neurofibromatosis type 2 (NF2) is an autosomal dominant genetic disorder with a penetrance of nearly 100% by age 601. NF2 is characterized by multiple tumors to the nervous system and skin, with the most common being a vestibular schwannoma2. Hearing loss is the most common presenting symptom in adults, becoming life-threatening as the tumor impinges on vital structures1. Patients demonstrate considerable heterogeneity in tumor onset and growth and novel tools to explore patient variability are needed. The complex local microenvironment consists of the nerve, schwannoma and vasculature, largely endothelial cells, is not typically modeled as a composite in vitro. Animal models capture this crosstalk but are limited (e.g. Avastin is specific to human, but not murine VEGF)3. *The development of a 3D in vitro NF2 model using iPSCs to generate both Schwann cells and endothelial cells would enable screening of novel* `therapeutics, as well as a better understanding of current and novel treatments.`

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Abstract for Form 298

NF2 is a genetic disorder with increased propensity to form Schwannomas. Current treatment options are limited. Chemotherapy is not appropriate due to the slow tumor growth and tumors are often considered inoperable due to their number, location and risk of nerve damage. Anti-VEGF antibodies that slow tumor growth by reducing new blood vessels that vascularize the tumors are only effective in some patients. Thus, there is a need for novel therapies with low toxicity, as patients tend to be younger, and to therefore require long-term treatment options. NF2 Schwannomas are most commonly found on the vestibulocochlear nerve and their frequency decreases in lower parts of the body, including the lower limbs; why the tumors form in this pattern is not understood. The vestibulocochlear nerve fires at a much higher frequency than more distal nerves. This raises the hypothesis that higher electrical activity contributes to the formation of Schwannomas in NF2 patients. In this project we investigated the effect of electrical activity on tumor formation in *in vitro* models of Schwannoma. To better model the disease we generated human induced pluripotent stem cells (iPSCs) from patients. Following, we derived iPSC-Schwann cells to model the disease *in vitro. The model we designed include the important element of electrical stimulation to mimic nerve activity.* We then investigated the impact of electrical stimulation on cell proliferation and DNA damage.

In this project, after all IRB paperwork and agreements were in place, Dr. Plotkin recruited patients with germline NF2 that either responded or did not respond to anti-VEGF treatment and collected fibroblast samples that were transported to NSCI. NSCI generated a bank of fibroblasts from these iPSCs and then produced and quality controlled six iPSC lines from NF2 patient samples exceeding the original plan to produce 4. NSCI also had 3 patient lines in the lab to use as control. Each of these iPSC lines were used to produce first Schwann progenitor cells that were then differentiated into Schwann cells expressing the relevant markers (SOX10, MPZ, S100B, CD271) and morphology. In parallel, we made significant progress in developing the 2D and 3D NF2 'disease in a dish' models, using primary Schwann cells, endothelial cells and electrical stimulation. We established a workflow utilizing flow cytometric methods to examine cell cycle status and DNA damage after electrical stimulation. A key finding from this modeling is that primary human Schwann cells do exhibit increased proliferation with increased electrical stimulation. This is significant as it indicates that the higher frequency electrical stimulation could be in part responsible for the increased growth of tumors in the vestibular location. Further we demonstrate that primary human Schwann cells secrete angiogenic factors that stimulate endothelial migration in the *in vitro* model. This is promising progress towards developing an assay for NF2 angiogenesis compatible with moderate throughput drug testing.

1. Introduction

Neurofibromatosis type 2 (NF2) is a genetic disorder with mutations in the tumor suppressor protein Merlin (Fong et al., 2011). Current treatment options are limited; chemotherapy is not appropriate due to the slow tumor growth and tumors are often considered inoperable due to their number, location and risk of nerve damage (Balasubramaniam et al., 2007; Evans, 2009). Thus, there is a need for novel therapies with low toxicity, as patients tend to be younger, requiring long-term treatment. Avastin, a human vascular endothelial growth factor (VEGF) antibody, has exhibited some efficacy in the clinic and is generally well tolerated (Fong et al., 2011). Avastin counteracts VEGF, which acts on endothelial cells to form new blood vessels and promotes endothelial proliferation, migration, and survival. VEGF has been shown to be expressed by Schwann cells (Brushart et al., 2013). The loss of a functional merlin protein in NF2 upregulates VEGF, which spurs angiogenesis (London and Gurgel, 2014). NF2 schwannomas most commonly are found on the vestibulocochlear nerve that fires at a much higher frequency (0.5-10 kHz) than the more distal nerves (in the tens of Hz) (Carney and Yin, 1988; Searchfield et al., 2004). Schwann cells are known to be responsive to electrical activity, and differences in activity may alter cytokine production in the NF2 microenvironment, contributing to angiogenesis and tumor growth (Koppes et al., 2011; Koppes et al., 2014). In this project, we sought to investigate the effect of electrical activity on an NF2 Schwannoma cell line, quantifying cell proliferation, DNA damage, and cytokine production by using patient-specific NF2 Schwann cells derived from human induced pluripotent stem cells (iPSCs) to model the disease *in vitro*.

2. Keywords

Neurofibromatosis type 2, induced pluripotent stem cells, angiogenesis, electrical stimulation, personalized medicine

3. Accomplishments

Major goals of the project

Aim 1: Generate 4 patient-specific iPSC Avastin-responsive and control cell lines to produce human Schwann cells and endothelial cells for use in the 3D microenvironment.

Aim 2: Develop a 3-dimensional (3D) model of the acoustic nerve microenvironment to evaluate changes in Schwann cell proliferation and angiogenesis in response to tonic electrical stimulation in the vestibulocochlear nerves.

Aim 3: Evaluate Avastin in the 3D model of the acoustic nerve microenvironment using NF-2 patient derived Schwann cells and endothelial cells; test combined with anti-IGF-1R and/or anti-PDGFR.

Table 1 Summary of the final progress toward Project Aims

TASKS	Site 1	Site 2	% completion
Specific Aim 1			
Local IRB/ISCRO and HRPO Approval	Temple	Thompson	100%
Derive iPSC lines from NF2 (4 NF2 patients proposed 6 done); 3controls already in hand, See Table 2	Temple		100%
NF2 Genetic Analysis	Temple		100%
Differentiate iPSC into Schwann cells, purify, characterization	Temple		100%
Modeling Schwannoma loss of NF2 via Knockdown experiments	Temple		50%
Differentiate iPSC into endothelial cells, purify, characterization	Temple		75%
AIM 1: Milestone(s): >Develop and characterize NF2 iPSC lines >Differentiate to Schwann cells or Endothelial cells	Temple		100%
Specific Aim 2			
HEI-193 response to electrical stimulation: > magnitude > frequency		Thompson	100%
Development of an NF2 in vitro model to examine both changes in Schwannoma proliferation/viability and changes in angiogenesis		Thompson	90%
Development changes in Schwannoma proliferation/viability and changes in angiogenesis due to tonic electrical stimulation	Temple	Thompson	90%
AIM 2Milestone(s): >Develop a 3D model of vasculature and Schwannoma to quantify changes in Schwannoma proliferation and angiogenesis >Evaluate HEI-193 Schwannoma response to electrical stimulation and any electrically mediated changes to angiogenesis using HUVEC as model human lines	Temple	Thompson	90%
Specific Aim 3			NA
Sensitivity of iPSC derived Schwann cell proliferation and Endothelial angiogenesis to Co-culture	Temple	Thompson	10%
Sensitivity of iPSC derived Schwann cell proliferation and Endothelial angiogenesis to electrical stimulation	Temple	Thompson	15%
Sensitivity of iPSC derived Schwann cell proliferation and Endothelial angiogenesis to electrical stimulation and Avastin	Temple	Thompson	0%
Sensitivity of iPSC derived Schwann cell proliferation and Endothelial angiogenesis to electrical stimulation and Avastin + anti-IGF	Temple	Thompson	0%
Sensitivity of iPSC derived Schwann cell proliferation and Endothelial angiogenesis to electrical stimulation and Avastin + anti-PDGFR	Temple	Thompson	0%
Sensitivity of iPSC derived Schwann cell proliferation and Endothelial angiogenesis to electrical stimulation and effective combinations of drugs (Avastin, anti-IGF, anti-PDGFR)	Temple	Thompson	0%
AIM 3 Milestone(s) Achieved: Evaluate sensitivity of iPSC Schwann cell proliferation and iPSC endothelial angiogenesis to one or more of the following in the novel 3D platform: > tonic electrical stimulation; Avastin; Anti-IGF; Anti-PDGFR	Temple	Thompson	0%
Manuscript preparation	Temple	Thompson	Planning

Some milestones in the original SOW were given at 9 months, however this is an error on our part as the timeline for aim 3 does not start until month 19. Based on the aim 3 work proposed, the timeline for achieving these milestones should be 35 months.

Accomplishments towards these goals.

Aim 1.

1.1 Agreements and IRB approval

We completed all the IRB approvals with Massachusetts General Hospital (MGH) and Albany Medical College (AMC) and Rensselaer Polytechnic Institute (RPI). After extensive discussions, we moved ahead with two separate approvals: one for the work done at MGH with Dr. Plotkin and the other for work done at NSCI with Dr. Temple. AMC's IRB served as the sole IRB overseeing the work being done at NSCI and by Dr. Thompson at RPI.

To identify the specific NF2 mutation in each line, we used a core facility expert in NF2 genetic analysis: The University of Alabama at Birmingham Medical School Medical Genomics Laboratory and they genotyped the six iPSC lines we produced, as indicated in **Table 2.**

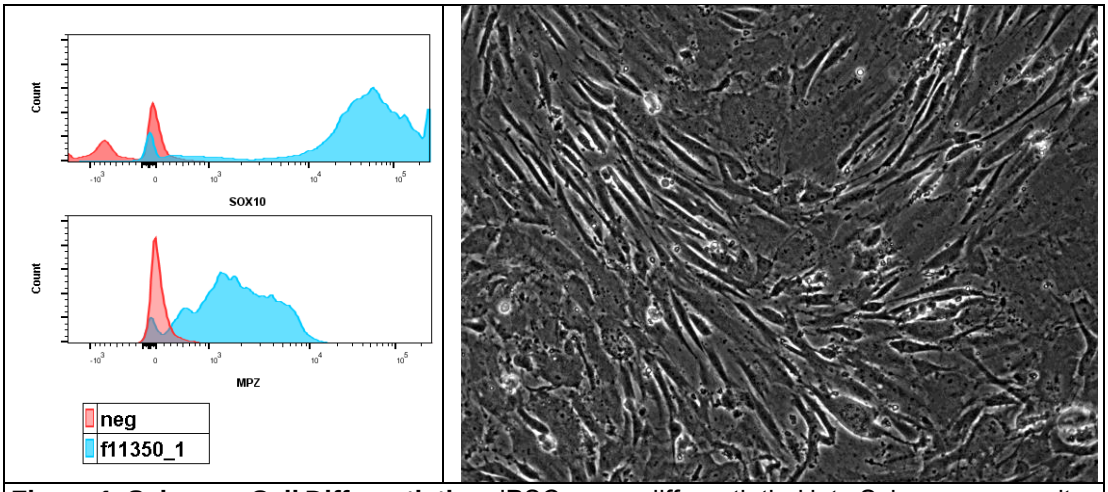

Figure 1. Schwann Cell Differentiation. iPSCs were differentiatied into Schwann progenitor Cells (SCP) and then into Schwann cells. (Left) Marker analysis of the SCP shows the cells are positive for SCP markers SOX10 and MPZ - shown is a typical example from control cells. (Right) Phase contrast image of fully differentiated Schwann cells. The image is from NF2 line NCNF2-051817_clone1.

1.2 Differentiation of pluripotent stem cells into Schwann cells

We received patient samples from MGH and produced and QCd iPSCs from 6 samples, more than the original 4 lines planned. **Table 2** summarizes the final progress of each line and the associated NF2 genotype.

Overall, we tested 4 different methods to produce the Schwann cells in 2 wild type lines and all 3 clones of NCNF2033117 and NCNF2040517. Of the four methods tested, we had little success with several prior protocols (Kim et al., 2017; Lee et al., 2010) but the fourth method we tested yielded Schwann progenitor cells (SCP) and Schwann cells at a high efficiency (Kim et al., 2017) (**Figure 1**). In parallel, we established positive control Schwann cell cultures at NSCI. During this process we developed efficient methods for QCing the differentiation of iPSCs into SCP and Schwann cells using antibodies and flow methods, including the surface receptor p75, the cytoplasmic marker S100beta, CD56, MPZ, and the nuclear transcription factor SOX10 (Liu et al., 2015). Using this method, we successfully produced SCPs for all the lines for distribution and we demonstrated their capacity to produce terminally differentiated Schwann cells. Utilizing a previously published protocol (Hollmann et al., 2017) we were able to successfully generate endothelial cells (**Figure 2**), however we were unable to generate an intermediate endothelial progenitor cell suitable for cell banking which greatly increased the costs of this approach. Due to cost restraints, we chose not to generate endothelial cells for use in modeling aims 2 and 3.

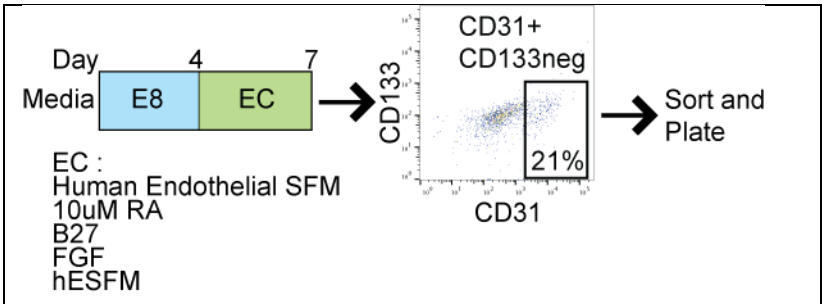

Figure 2. Endothelial Differentiation Protocol. The differentiation protocol takes 7 days after which a clear population CD31+ Cd133- endothelial cells can be seen.

Because loss of heterozygosity is frequent in NF2 tumors, we attempted to model this. Knockdown of NF2 using gapmer technology had little success, with either of two different gapmers tested. In each case, a low transfection rate for the NF2 gapmers limited our ability to measure the levels of NF2 within the cultures. One caveat to knocking down NF2 in our iPSC-derived cells is that NF2 knockouts are embryonic lethal. The iPSC derived Schwann cells may be more embryonic-like or immature than adult Schwann cells, which could lead to lethality in the cells with knockdown of NF2 and impede our ability to knockdown NF2 successfully. Indeed, we observed some toxicity. Due to these issues, we chose not to precede with the NF2 knockdown characterization and instead devoted resources and time to other key aspects of NF2 tumor model development.

Table 2. Summary of patient-derived NF2 iPSCs produced for this project and the differentiation of these and control iPSCs into Schwann cell products.

iPSC ID	Clones Available	Sex	Age	Treatment*	Genotyping	Karyotype	QC#	SCP
NCNF2-033117	3	M	40	NR	c.[592=/C>T];[592C=]	Normal	Tri-Diff	Yes
NCNF2-040517	3	F	30	R	c.[(1371_1502)_(1503_1628)del];[1371_1628=]	Normal	Tri-Diff	Yes
NCNF2-051817	3	F	40	NR	c.[447G>A];[447G=]	Normal	Tri-Diff	Yes
NCNF2-053117	3	F	32	R	c.[1079dupT];[1079=]	Normal	Tri-Diff	Yes
NCNF2-071417	1	F	29	R	c.[(45_185)_(412_472)dup];[45_472=]	Normal	Tri-Diff	Yes
NCNF2-102017	3	F	28	R	c.[1397_1398insTC];[1397_1398=]	Normal	Tri-Diff	Yes
F11350.1	1	M				Normal	Tri-Diff	Yes
F12468.13	1	F				Normal	Tri-Diff	Yes
GiH161	1	F				Normal	Tri-Diff	Yes

*Patient was treated with anti-VEGF therapy and either responded ® or didn't respond (NR)

#iPSC lines were fully QCd to our core facility standard. Tests for QC included analysing the karyotype and testing whether the iPSCs could differentiate into endoderm, ectoderm and mesoderm (Tridiff= this was successful, i.e. the lines are pluripotent).

Aim 2.

Development of the 3D model.

2.1 Effect of tonic stimulation on normal primary human Schwann cells

In order to continue the development of the 3D model, we sought to determine the effect of tonic stimulation on human primary Schwann cells in anticipation of the iPSC-derived Schwann cells. Primary human Schwann cells were obtained from a commercial source and used in the stimulation model. Primary cells are known to be more sensitive to environmental factors *in vitro* than, for example, cell lines. Because iPSC-cells are more like primary cells, this transition to a primary cell source was an important step to prepare for the iPSC-derived Schwann cell products.

Table 3. iPSC derived Schwann cells.

Cell Line	NF2 line	Viability following ES
NCNF2 040517 Clone2	Y	>98%
NCNF2 040517 Clone3	Y	>98%
NCNF2 033117 Clone1	Y	TBD
NCNF2 033117 Clone2	Y	>98%
NCNF2 051817 Clone1	Y	>98%
NCNF2 051817 Clone2	Y	>98%
NCNF2 053117 Clone1	Y	>98%
NCNF2 053117 Clone2	Y	TBD
F11350.1	N	>98%

Briefly, primary human Schwann cells were seeded onto poly-l-lysine coated glass coverslips at a density of $5x10^4$ cells/cm^2 and cultured overnight at 37°C. The coverslips were placed in a custom rectangular poly(dimethyl siloxane) (PDMS) chamber with a defined geometry to provide a constant electric field. Biological-grade platinum electrodes were placed at both ends of the chamber, along with reference electrodes, to enable us to measure the voltage drop across the

chamber using LabVIEW. AC stimulation was applied at low (20 Hz) and high (1 kHz) frequency via a pulse generator. Human Schwann cell viability did not appear to be influenced by electrical stimulation (ES) (**Table 3**).

Human Schwann Cell Proliferation after Electrical Stimulation: As a baseline for the iPSC Schwann cells, Human Schwann cell proliferation was assessed in response to electrical stimulation at 24 hours and at 3 days. For a screen to evaluate a larger experimental space (stimulation magnitude, duration and drug concentration), it is important to detect changes early. We evaluated cell number and proliferation at 1 versus 3 days in culture. First, the number of cell nuclei was calculated and compared to the number in the unstimulated control. This was accomplished using Cell Profiler Image Analysis Software. Previously we saw an effect of stimulation on the HEI-193 NF2 Schwannoma cell line and the primary human Schwann cells at 3 days post-stimulation, as described in prior reports. Here, we plated primary human Schwann cells onto poly-l-lysine-coated glass coverslips, cultured overnight to adhere to the surface and stimulated for 4 hours at 50 mV/mm at either low (20 Hz) or high (1 kHz) frequency. Primary human Schwann cells were cultured in commercially available complete Schwann cell medium (ScienCell Research Labs, Carlsbad CA). After stimulation, the medium was changed, and the cells were incubated for 24 hours or 3 days. The cells were then fixed with paraformaldehyde and stained with 4', 6'-diamidino-2-phenylindole (DAPI) to identify all nuclei. In addition, the cell conditioned medium was collected immediately after the 4 hour stimulation and at 24 and 72 hours post-stimulation. During stimulation, to measure proliferation, human Schwann cells were treated with an EdU pulse for either the duration of culture or during the electrical stimulation treatment. The collected medium was stored at -80°C for future analysis.

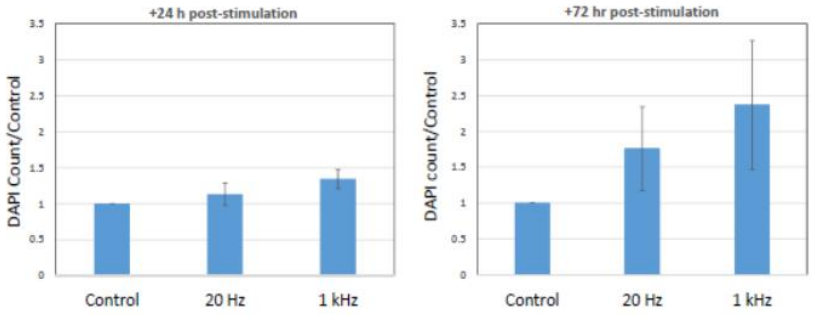

Figure 3: Human Schwann cell count post-stimulation. A) At 24 hr post-stimulation, there is no significant increase in human Schwann cell number, while at 72 hr post-stimulation significant increases in Schwann

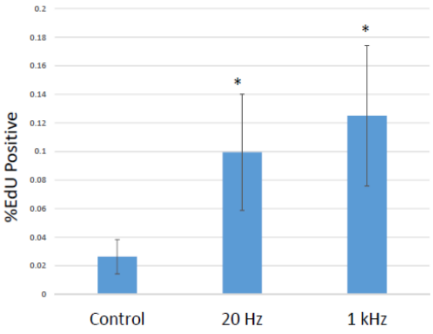

Figure 4: Human Schwann Proliferation post-stimulation. EdU treatment during 4 hr pulse at 0, 20 Hz or 1 kHz, resulted in significant increases in EdU+DAPI labeled nuclei

We observed a significant increase in cell number as measured by the number of DAPI-labeled nuclei at 3 days in culture but not by 24 hours (**Figure 3**). Primary human Schwann cells divide more slowly than cell lines, which is expected. Long EdU pulses of 72 hours resulted in similar rates of proliferation regardless of stimulation treatment condition (0 Hz, 20 Hz or 1 kHz) as most (70-80%) of the cells had proliferated within the 3-day window (not shown). In contrast, EdU treatment during stimulation, results in a higher percentage of EdU^{+}-DAPI labeled nuclei. This is in agreement with our 72 hour cell count data, so increased cell numbers can be attributed, at least in part, to increased proliferation (**Figure 4**). We conclude that primary Schwann cells demonstrate an increase in cell number due to electrical stimulation, as we found previously for an immortalized Schwann cell line. This is a novel finding that may be applicable to areas of regenerative medicine where increased Schwann cell production is needed, for example, for injuries to the peripheral nerve/spinal cord, during use of cell-mediated therapies. The information on primary human Schwann cell behavior was incorporated into our workflow for iPSC-derived Schwann cell NF2 tumor modeling. iPSC-derived human Schwann cells were seeded in culture and stimulated in the presence of EdU and cultured overnight before changes in proliferation were assessed.

Incorporation of Human iPSC derived Schwann cell lines: *Passaging iPSC Schwann cells*: The Temple Lab generated iPSC-Schwann cells for both control and NF2 lines (**Table 2,3**). The iPSC Schwann cells were cultured on poly-L-lysine coated tissue plastic in commercially available Schwann Cell Medium (ScienCell) with 200 ng/ml of Neuregulin (NRG1) added to the medium. NRG1 is important for these iPSC-derived Schwann cells while it is not required by primary human Schwann cells. The iPSC-derived Schwann cells were fed every other day and were passaged with Accutase to maintain high viability and minimize cell loss. iPSC Schwann cell proliferation rates were found to depend on both the passage number and cell density. The cells are typically cultured for several weeks in a single t-12.5 flask before reaching 90-100% confluence. Upon passaging, the cells detach as sheets and a longer Accutase incubation time applied (>5 min) to break up the cell sheets into single/smaller clusters of cells. Accutase was found preferable to trypsin for iPSC-Schwann cell passaging.

iPSC-Schwann cells were received in the Thompson lab on passage 2, and there was a noticeable change in growth rate by passage 4-5. Due to these problems and extensive culture time needed to produce enough cells for both the short term and long term experiments, along with the increased cost of utilizing NRG1 throughout the time course, we opted to use the Schwann cell progenitor (SCP) cells in our experiments with all culture being maintained in the Temple lab. These cells are rapidly growing and able to produce sufficient numbers to

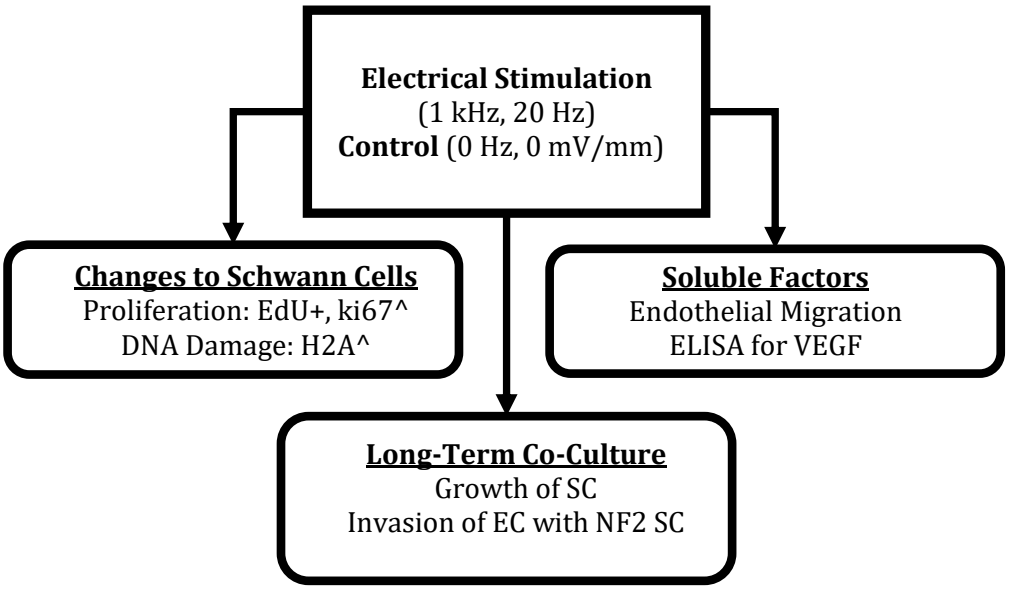

Figure 5: Work Flow for iPSC-Derived Schwann Cell Screening Samples were separated into three paths: A) Changes to SCPs via destructive but rapid screening via FACS to quantify (Ki67) and DNA damage (H2A), and non-destructive analysis of proliferation and morphometrics followed by quantification of EdU+DAPI+ nuclei using microscopy with image analysis. B) Analysis of SCP-produced soluble factors via Endothelial Migration Assay and ELISA and 3) Long-term 3D model with iPSC-SCPs.

support our experiments in a cost effective and timely manner. In reviewing the literature, Zheng et al 2008 and Kuo et al 2010 suggest that the tumors are characterized by the expansion of non-myelinating Schwann cells, which supports our concept of studying SCPs.

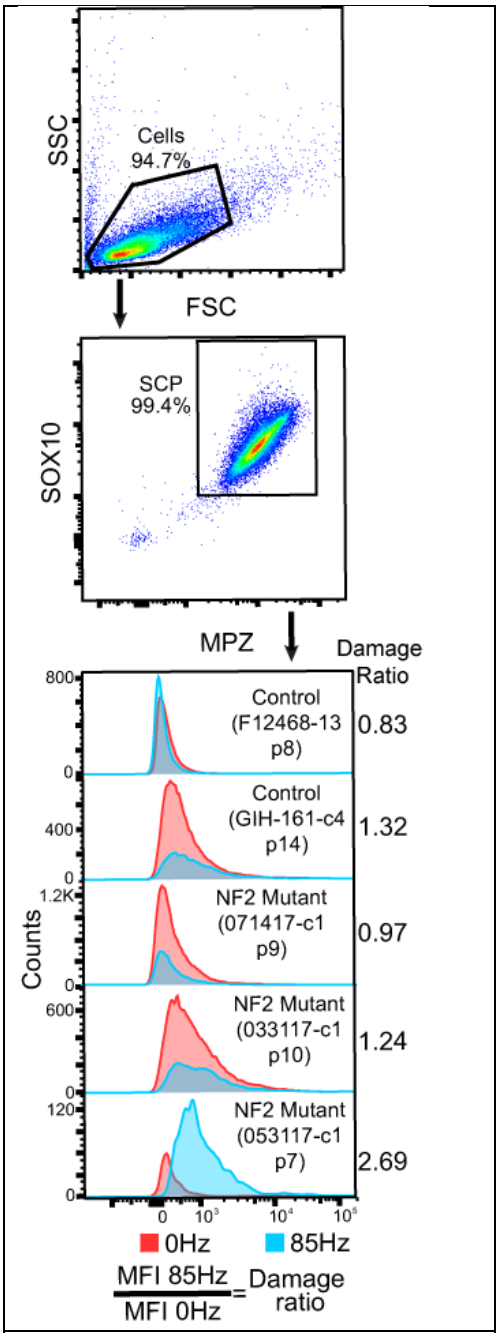

Figure 6. Flow Cytometric analysis of gamma-H2AX. DNA damage before and after stimulation in SCP was examined using gamma-H2AX levels by calculating the ratio of the MFI of the gamma-H2AX channel. In general, electrical stimulation generated a DNA damage response and in one of the NF2 mutants the response was twice as strong.

1:2-3. *Workflow.* Our team worked with the NF2 cell line (HEI 193) and with primary human Schwann cells to develop a work flow for the iPSC derived SCP analysis. Given the analysis time required for multiple samples with multiple labels (EdU, Ki67 and H2A), FACs protocols were developed (**Figure 5,6**). Briefly, samples were seeded in tissue culture and the experiment was run in the Thompson Lab; culture medium was collected prior to stimulation, after stimulation and at 18 hours post-stimulation. The fixed cells were collected and processed in the Temple lab for FACs analysis. Dr. Boles worked with students in the Thompson lab to develop an effective working protocol to evaluate DNA damage and cell proliferation using gamma-H2AX levels and EdU incorporation, respectively, via Flow analysis. As noted above, no significant changes in viability were observed following electrical stimulation (**Table 3**).

9 x 22 mm cover glass were cleaned, sterilized and coated with Matrigel and stored at 37°C for 24 hours. iPSC-derived SCPs were seeded onto these Matrigel-coated cover glass using specialized inserts to seed directly onto the culture surface at 5.75 x10^4 cells/cm^2. SCPs were treated with ROCK inhibitor and cultured for 3 days. The SCP-coated cover glass were transferred into the custom electrical stimulation chambers for stimulation. Samples were given 0 Hz (unstimulated control) or 85 Hz (stimulated). Following stimulation, samples were incubated in fresh medium overnight. Medium was then collected on ice and stored at -80°C for ELISA. Samples were collected for quantification of DNA damage by Flow analysis, quantification of cell proliferation via EdU staining, and for isolation of RNA.

Normal human Schwann cells were used as a negative control for DNA damage, and these cells were exposed to 2x15 sec pulses with a UV X-linker to serve as the positive control for the DNA damage measurement; together these controls helped to identify the appropriate gating for the Flow experiment.

DNA damage in response to electrical stimulation

Assay development centered on the optimization of the antibodies and dealing with autofluorescence from the iPSC derivatives. After analyzing 5 of the 9 lines, we found that, somewhat surprisingly, each line had a different degree of autofluorescence, which prevented us from achieving consistent thresholds to quantify cell proliferation using EdU incorporation. Thus, we adopted a two-pronged approach: samples were seeded, treated and then divided for examination of DNA damage by FACS, and separately, proliferation was assessed by staining cells for EdU incorporation with image quantification.

For examining DNA damage, cells were stained for the SCP markers SOX10 and MPZ and for gamma-H2AX using the Median Fluorescence Intensity (MFI) measurements. The ratio of the MFI before and after stimulation was calculated to determine the extent of the DNA damage (**Figure 6**).

To quantify proliferation, the SCP lines were assessed before and after electrical stimulation to determine: 1) the total cell count to normalize protein production for ELISA and 2) quantify any differences due to stimulation. Samples were fixed at various time points, stained, imaged and analyzed (**Figure 7**). To date, 5 lines have been screened which include 2 responder NF2 lines (the patient responded to anti-VEGF treatment), 1 non-responder NF2 line, and 2 control lines. One control line (C3) was excluded from the analysis: In this control line, the initial cell attachment after seeding was similar to the other lines, however there was little growth and then detachment. After the analysis, we found that electrical stimulation activated a DNA damage response in 3 of the 5 cell lines tested, however one NF2 mutant (NSCI-053117-c1, a

responder) had a much stronger proliferation response than the other cell lines (**Figure 7)**, which could be due to that particular NF2 mutation or to the genetic background or another feature of that iPSC line.

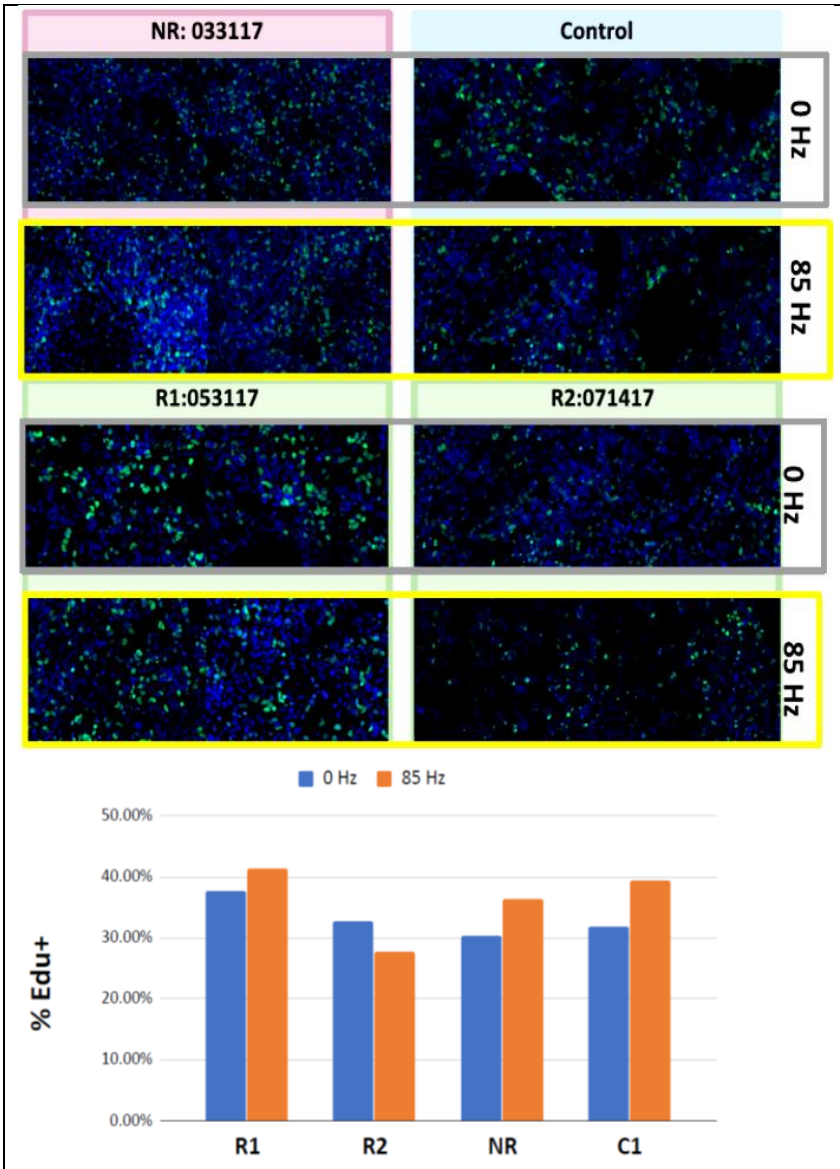

Figure 7: SCP proliferation in response to Electrical Stimulation. The cells were treated with EdU during the 4 hr pulse, cultured for 24 hrs and then fixed with paraformaldehyde. To identify all cells, nuclei were stained with 4', 6'-diamidino-2-phenylindole (DAPI - blue) and proliferating cells are EdU+ (green) cells. Image processing was performed to determine EdU+/DAPI+ cells. Regardless of stimulation (0 or 85 Hz), SCP are proliferating during the experiment. Electrical stimulation may modestly increase following 3 of the 4 cell lines. A decrease was observed for R2, which appears consistent with the images.

Cell proliferation in response to electrical stimulation

Previously we saw an effect of stimulation on proliferation of the HEI-193 NF2 Schwannoma cell line and the primary human Schwann cells at 1-day post-stimulation, as described in the prior reports. Here, we plated iPSC-SCP onto Matrigel coated glass coverslips, cultured for 3 days to adhere to the surface and stimulated for 4 hours at 50 mV/mm at 85 Hz frequency or 0 Hz (unstimulated mock control). The iPSC-SCPs were also treated with EdU during the stimulation and transferred to fresh medium post-stimulation. The cells were then fixed with paraformaldehyde and stained with 4', 6'-diamidino-2-phenylindole (DAPI) to identify all nuclei. First, the number of cell nuclei was calculated and compared to the number in the unstimulated control. This was accomplished using Cell Profiler Image Analysis Software. EdU+/DAPI+ cells were quantified and compared to representative images. All samples exhibited cell proliferation over the 24 hour period ranging from 27%-42%. For most lines there was a small increase in proliferation due to the electrical stimulation, with the exception of R2 (NSCI-071417-c1) line, where the unstimulated control exhibited greater proliferation. It is difficult to draw any conclusions based on the small dataset, however these data do suggest that ,as with the prior Schwann cells tested, SCPs can respond to electrical stimulation with an increase in proliferation. In the future, given the length of the experiment and the rate of proliferation, we plan to reduce the seeding density or the duration of plating prior to stimulation from 3 days to 2 days, to allow more room in the plate for cell growth, thus avoiding the complication of contact inhibition that can stop cells dividing.

We are in the process of completing and validating these results and plan on applying repeated exposure of electrical stimulation over several days. Although we were on track to complete these experiments within the funding period, progress was impeded due to a recent power outage that resulted in damaged instrumentation that took time to restore. Due to this, we have to regrow and retreat the cell samples.

After completion of these experiments, we will use ELISA to examine the effect of electrical stimulation on VEGF production. It will be interesting to determine whether VEGF production is different between responder and non-responder lines, and while our sample numbers are small, we will track production relative to R or NR status.

2D-Angiogenesis Assay for SCP-derived factors.
An important goal of this project was to assess the angiogenic factors produced by Schwann cells with NF2 mutations obtained from different individuals vs controls.

Schwann cells are known to increase VEGF production *in situ* following a nerve crush injury and transplanted Schwann cells following a myocardial infarct are thought to be responsible for increased VEGF in the heart (Gupta et al., 2005; Zhang et al., 2010). Clearly, evidence exists to support the hypothesis that VEGF expression is influenced via normal Schwann cell-endothelial interactions. Significant changes in VEGF expression have been reported in patients with Neurofibromatosis. NF1 knockdown increases Schwann cell-released VEGF(Kawachi et al., 2013) and in NF2, the loss of functional merlin protein inhibits expression of SEMA3F (an anti-angiogenic protein) allowing VEGF to spur angiogenesis (London and Gurgel, 2014). We know that some patients respond to anti-VEGF therapy more than others, and one explanation could be that some patients have an increased propensity to produce VEGF. This could be NF2-mutation specific and/or due to genetic background.

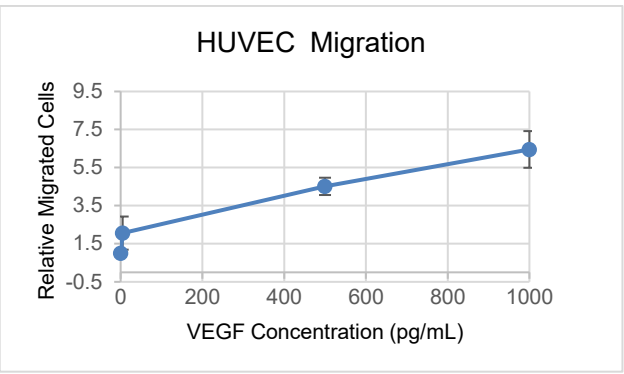

Figure 8: HUVEC migration increases with VEGF concentration in Schwann cell medium. Using 8 mm ThinCert Membranes, HUVEC migration to the VEGF stimulus was quantified and increases linearly from 5-1000 pg/mL VEGF. p >0.05 (n=3)

In addition to VEGF, cells can release other angiogenic molecules that stimulate blood vessel growth to sustain the tumor. Hence in our models we wanted to assess the level of VEGF and of other angiogenic factors produced from the patient-derived and control Schwann cells.

A common assay used to indicate angiogenesis is to visualize the early reorganization of human umbilical vein endothelial cells (HUVECs) in the 2D Angiogenesis assay (EGM625 Angiogenesis Kit, Millipore Sigma). In establishing the assay in the Thompson lab, reproducibility over a wide range of VEGF concentrations was obtained but reproducibly was affected when the VEGF was added to Schwann cell medium. This medium contains Fetal Bovine Serum (5%) and Schwann cell growth supplement and there is likely interference due to these supplements that affects HUVEC migration and re-organization. The variability of the HUVEC morphology was highly dependent on the local density of seeded endothelial cells.

A different screen was therefore introduced that assesses endothelial migration. Briefly, serum-starved HUVECs were placed in a 8μm ThinCert membrane. The medium to be tested was loaded under the membrane (600uL) and 200 uL of HUVEC cell suspension was placed above the membrane. Given the medium surrounding the ThinCert was equivalent to the medium within the insert, a gradient was generated, and cells that responded migrated into the lower chamber. In agreement with other studies, we found that an incubation time of 4 hours was optimal, while at later incubation times, cell migration with respect to VEGF concentration was not significant between 5-1000 pg/mL.

The membranes were incubated at 37°C with 5% CO_2 for 4 hours. At the end of the incubation period, the seeded HUVECs on the upper side of the membrane were wiped away with a Q-tip and the cells that had migrated through the membrane and had adhered to the underside of the membrane were rinsed with PBS and fixed for quantification. The cells were labeled with DAPI and the ThinCert membranes were excised and mounted on slides. Multiple images were taken and stitched together for analysis; DAPI+ nuclei were counted.

Using this assay, we successfully observed a linear increase in HUVEC migration over 5-1000 pg/mL of VEGF even in the background of Schwann cell medium (**Figure 8**). Further, as this experiment is of short duration, tests can be done in batches for efficiency. In the future we anticipate being able to use iPSC endothelial cells

in this assay which will allow us to assess whether NF2 mutation alters endothelial responses to angiogenic factors.

Once experimental parameters are assessed with this rapid assay, an angiogenic cytokine array can be run on the conditioned media samples to determine (1) what combination of factors are produced and (2) differences in factor production (if any) after electrically stimulating the NF2 vs control iPSC-derived Schwann cells.

The samples of conditioned media from the stimulated and non-stimulated iPSC-SCP have been collected and are stored at -80°C. Once the remaining samples have been collected, as described above, our plan is to run all the conditioned medium samples, assessing them for angiogenic factors with the 2D assay we have established and with VEGF ELISA.

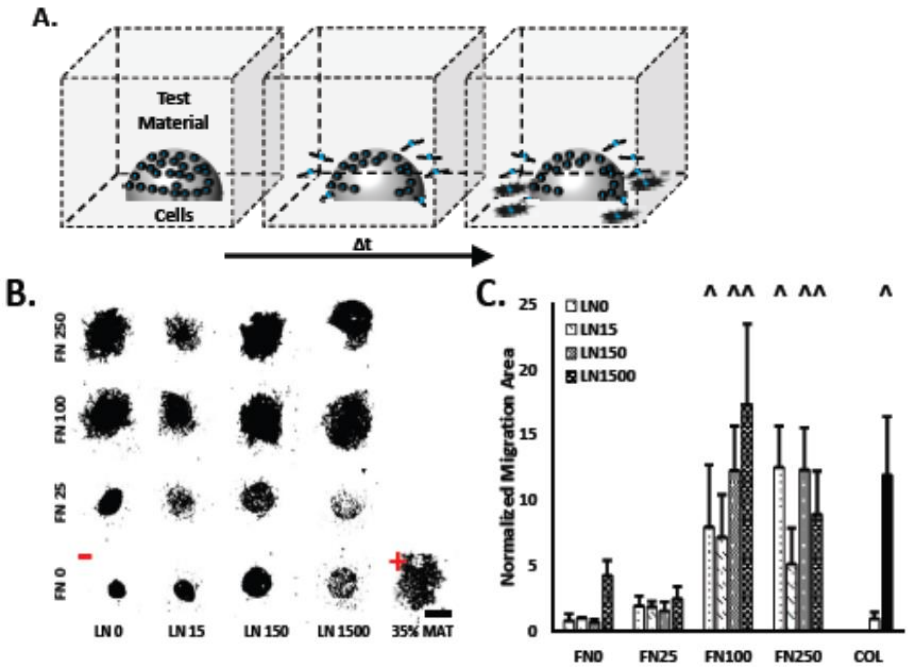

Figure 9: Schwann cells in collagen composite gels. (A.) Schematic depicting SC migration in composite gels. Briefly, SCs are seeded in (Collagen-Matrigel) COL-MAT droplets and allowed to partially gel for 5 minutes prior to addition of test composite biomaterial. The test biomaterial should be screened to support viability and migration. Over time, cells migrate through the test material, and upon reaching the gel edge, they spread and proliferate. (B) Flatbed fluorescent scans of F-actin show base migration in COL-alone or COL-LN-FN after 4 days in culture (scale bar = 1mm, red markers indicate control materials). (C.) Significant increases in SC migration occurred within materials that included 100 or 250 μg/mL FN (mean ± s.d., n>3, $p<0.05$, data normalized to migration through COL-only) (*: significantly greater than COL-only; ^: greater than COL-only and similar to COL-MAT)

3D Angiogenesis Assay for SCP-derived factors

The overarching goal of this project was to develop an NF2 tumor model that included an ability to assess vascularization. Given that tumors are 3D structures with complex vessel ingrowth, we also made progress on a novel 3D model. In collaboration with Professor Pankaj Karande at Rensselaer Polytechnic Institute, we have developed a 3D platform to assay endothelial angiogenesis over time. While the 2D assay will provide a rapid 6-12 hour long readout, true angiogenesis that includes the stable formation of microvessels is a lengthier and more complex process. Select conditions/lines that produce greater levels of angiogenic factors can be assessed further in 3D co-culture with microvessels. This will provide the means to evaluate NF2 SCP angiogenesis more fully, as they migrate toward or within the NF2 SCP seeded constructs.

The HUVEC-produced microvessel work was presented in the last update. In this approach, the 3D printed Schwann cell microcultures were assessed over a week in culture for health and viability. Schwann cells are sensitive to matrix composition. Collagen1-only constructs are not supportive over 5-14 days in culture and decreases in cell number are observed. Inclusion of laminin and fibronectin into the Collagen biomaterial does support Schwann cell survival (**Figure 9**) and migration. Experiments are underway to combine the 3D cultured Schwann cells and the HUVEC microvessels, to improve 3D modeling of NF2 tumors.

Expression profiling after electrical stimulation

Using funding outside this grant, we plan on examining the changes in RNA expression after stimulation between NF2 mutants and WT SCP cells using the RNA collected during the above experiments. This will allow us to more fully characterize the effects of NF2 mutation in SCP. We have already generated RNA-sequencing data for a control and NF2 line before and after electrical stimulation, and at the completion of our experiments in Jan.

2020 we will obtain RNA-seq data for the rest of our lines. These data will allow us to better interpret the results from Aim 2 and allow us to probe the changes in the secretome induced by electrical stimulation at the RNA expression level, including monitoring known angiogenic factors.

Training and Professional Development Opportunities

Mr. Tyree Williams re-joined the Thompson laboratory in July 2018, returning to Rensselaer Polytechnic Institute as a PhD Student. He previously worked as an REU student at Rensselaer visiting from Fisk University. Ms. Chidambaram, while making strides on the project and in the PhD program, decided to leave the PhD program in June 2018 completing her MS to pursue a career in industry. Both students have a number of professional development opportunities both at Rensselaer Polytechnic Institute within the department of Biomedical Engineering as well as at the Neural Stem Cell Institute (NSCI). Clearly, this was not ideal transition point, but Dr. Thompson, Mr. Williams and undergraduate Alyssa Quezada were able carry the work forward while training Mr. Williams during Summer 2018.

Overall Summary

For Aim 1, after a delay due to IRB processing of over a year, we successfully obtained patient samples from Dr. Plotkin and generated multiple iPSC lines with different germline NF2 mutations from patients, producing more lines (6) than originally planned (4). These iPSC lines are a valuable resource and will be made available to the NF2 research community. Using these lines, we established an efficient differentiation protocol to generate Schwann cell progenitor cells suitable for cell banking. These are also available for distribution from our core facility Neuracell. Using the iPSC-SCP cells, we were able to generate terminally differentiated Schwann cells. We also demonstrated the ability to differentiate these lines into endothelial cells using an established protocol, albeit at low efficiency. There have been recent advances reported in iPSC-brain endothelial cell generation, so we will incorporate these advances in future work.

For Aim 2, we developed an efficient workflow and several protocols to assess the effects of electrical stimulation on cell proliferation and DNA damage. We also created a 2D angiogenesis assay compatible with Schwann cell culture medium and a novel 3D angiogenesis assay to observe an effect on NF2 Schwann cell factors on endothelial organization and vessel formation. These assays are ready and assessment of several iPSC-SCPs has been done, as reported above. Our work indicates that, at least for some lines, electrical stimulation at high frequency, similar to that of the vestibular nerve, increases DNA damage and cell proliferation of Schwann cells. If this result is confirmed after completing more lines, it will be an important conclusion that could explain why NF2 tumors are prone to form around this high frequency nerve. Several of the assays required collection of iPSC-SCP conditioned medium before and after stimulation, including for assessment of VEGF and other angiogenic factor production. These media were collected and stored, and after all the iPSC-SCP lines have been run, the conditioned media samples will be assayed together for angiogenic factors. Due to circumstances beyond our control (a power surge that damaged our instrumentation) we were unable to complete these sets of experiments in the original time frame. However, we plan to complete this work in Jan. 2020 and then prepare a manuscript to report our work.

Upon completing this work, we will be able to establish whether iPSC-SCPs proliferation, DNA damage and VEGF and angiogenic factor production are altered with mutation and with stimulation at high frequency mimicking the NF2 tumor environment. We will then be able to move to test anti-VEGF and other drugs as originally proposed in Aim 3. Unfortunately, the initial long delay in IRB approval, the time to establish iPSC-Schwann cell products and assays using this novel iPSC-SCP cell type, and instrument failures delayed progress. However, we have accomplished our main goal to advance NF2 models and hope to secure additional funding to utilize these ready assays more broadly to enable drug development for NF2 tumor treatment in the future.

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4. Impact

Impact on the Development of the Principal Disciplines of the Project

Our collection of NF2 iPSCs is a valuable product of this project that we will make available, positively impacting the NF2 field. Our finding that electrical stimulation levels comparable to that of the vestibulocochlear nerve increase primary Schwann cell number more than distal-nerve-level stimulation is novel and strengthens our findings reported previously on Schwannoma cell lines. If the same is found with NF2 iPSC-NF2 Schwann cells, this would advance our understanding as to why NF2 tumors form predominantly in that location. This will open new avenues of exploration into the underlying mechanisms, which will expand the NF2 research field.

Given the dependence of these tumors on vasculature for growth, improving our understanding of what types of angiogenic factors are produced from Schwann cells under different stimulation conditions and with/without NF2 mutations has translational value.

Our work on developing a 3D model incorporating NF2 mutant versus control cells, vascular endothelial cells and electric stimulation to mimic nerve activity should improve our understanding of NF2 tumor development and growth. Given we have now made considerable progress in iPSC NF2 Schwann cell product differentiation, assays to assess the effects of stimulation, our goal is to complete these studies and prepare a publication on NF2 modeling in 2020. We anticipate that this novel iPSC-based NF2 tumor model will have impact in the field and towards developing therapeutics that slow or prefent NF2 tumor formation.

Impact on Other Disciplines

Electrical stimulation might affect the growth of other tumors that form in close proximity to neurons. Our results provide incentive to examine the effect of electric stimulation on tumors, such as those occurring due to NF1 mutations. Studies to determine if electrical stimulation has an impact on normal cells or cells following injury may be relevant for expanding cells ex vivo for cell-mediated therapy or promoting regeneration in vivo. Understanding what mechanism mediate these changes is important and will be explored in future studies. We also note that these parameters that take into account specific patterns of electrical activity could be applied *in vitro/in vivo* in the field of regenerative medicine to promote the revascularization/repair of a tissue.

Impact on Technology Transfer

Nothing to report.

Impact on Society beyond Science and Technology

A major goal is to use iPSC-derived products to provide information about disease, and our application of this technology should improve public knowledge about the potential of this new technology. Our hope is that our work will also stimulate public interest in NF2, which is a rare and devastating condition, to help support further work in this area.

5. Changes/Problems

The applications for the IRB approvals were more complex and time-consuming than originally anticipated and this led to significant delays of over a year. Given that iPSC-cells grow slowly, on human time-frames not mouse time-frames, we found testing of different protocols to find the optimal Schwann cell differentiation took longer than expected. Together, these problems were a significant impediment to achieving all aims of the project. Nevertheless, we surmounted these hurdles and plan to complete the project and publication in the coming year.

In our original submission, we had proposed to use the Live/Dead assay to determine cell death, and the Cy-Quant DNA proliferation assay. We have replaced these assays with the EthD-III/DAPI and DAPI staining, respectively. The reason for this change, in the case of the Live/Dead assay, was because EthD-III/DAPI preserved the natural state of the stained cells more accurately, as determined by phase microscopy. The Cy-Quant DNA proliferation assay was replaced with DAPI staining as we were able to perform this more rapidly.

In our original submission, we proposed to evaluate endothelial response to the electrically stimulated Schwann cells in a 3D assay. Given the prolonged time required to generate microvessels, we sought an assay that had a more rapid read-out. The 2D angiogenesis assay can be run in co-culture with transwells, or using cell – conditioned medium, producing results within a few hours. We therefore included this assay as a rapid way to

screen the complex conditioned medium samples. Given the departure of Dr. Guohao Dai to Northeastern University, we have been working with Dr. Pankaj Karande, a long standing collaborator of Dr. Thompson, with expertise in 3D models of microvasculature in the skin and blood brain barrier. His research laboratory generously shared protocols and technical expertise on the development of the 3D model of microvessels. They identified the needed growth factors and seeding density to support microvessel growth. Hence, Drs. Thompson, Karande and Temple have adjusted the 3D culture model to enable improved modeling of the Schwann cell-blood vessel interaction while providing a readout at early (around 7 days) and later (3 week) timepoints.

In our original submission, we proposed to evaluate HEI-193 Schwannoma lines as well as the iPSC-derived Schwann cells. We added primary human Schwann cells to provide a control for the HEI lines while the Human iPSC-derived Schwann cells were being generated. Comparison between the groups is being performed to evaluate both 2D angiogenesis and 3D models and determine how robust our models are for drug testing.

6. Products

NF2 iPSC line collection and Schwann Cell Progenitors from this are available to other researchers with a UBMTA.

Publications are in planning stages.

7. Participants and Other Collaborating Organizations

Individuals Worked on the Project

Name: Sally Temple, PhD
Position: Principal Investigator
Months Worked: 1.0 calendar months
Contribution: Overall direction of the research activities.
Support: CDMRP NF140040 award.

Name: Deanna Thompson, PhD
Position: Co-Investigator
Effort: 1.0 summer months
Contribution: Development and testing of the 3D NF model.
Support: CDMRP NF140040 award.

Name: Nathan Boles, PhD
Position: Research Scientist
Months Worked: 2.5 calendar months
Contribution: Design and conduct of the planned experiments.
Support: CDMRP NF140040 award.

Name: Thomas Kiehl, PhD
Position: Bioinformatician
Months Worked: 1.25 calendar months
Contribution: Statistical and bioinformatics support
Support: CDMRP NF140040 award.

Name: Yangzi Tian
Position: Research Technician
Months Worked: 3.3 calendar months
Contribution: Laboratory technical Support
Support: CDMRP NF140040 award.

Name: Tyree Williams
Position: Graduate Student
Months Worked: 2.0 academic months
Contribution: Laboratory technical support
Support: RPI institutional funding.

Changes in Support of PD/PIs or other Senior/Key Personnel

Dr. Temple received a new award from the NYS-DOH for spinal cord research. The award began June 1, 2018 and is titled, *The role of zinc in axon regeneration following spinal cord injury*. This funding is included in the revised funding table included in the Appendix section.

Other Organizations Involved as Partners

Name: Rensselaer Polytechnic Institute
Address: 110 Eighth Street
Troy, NY 12180-3522
Facilities: Dr. Thompson laboratory is located at RPI.
Collaboration: Dr. Thompson serves as the Co-Investigator on this collaborative project. Experiments are conducted at both the principal site, Regenerative Research Foundation, and the facilities at RPI.

8.Special Reporting Requirements

None

9. Appendix

OTHER SUPPORT

SALLY TEMPLE, PH.D.

ACTIVE:

W81XWH-15-1-0152 (Temple) 09/15/2015 – 09/14/2019 1.2calendar
CDMRP-USAMRMC $ 674,211
Modeling NF2 Tumors for Drug Screening Using Induced Pluripotent Stem Cells
We will develop NF2 iPSC lines for use in investigations of Schwann cell – endothelial cells relevant to NF2 disease and testing of patient-specific drug therapies.

DOH01-C30605GG-3450000 (Temple) 11/01/2015 – 10/31/2019 1.2 calendar
NYS-DOH SCIRB $ 1,097,684
Sustained delivery of IL10 and SHH to promote spinal cord regeneration after injury
Here we propose to test whether a combination of sustained IL-10 and sustained SHH delivered via microbeads to the injury site will counteract inflammatory processes, promote a regenerative environment and improve recovery better than either alone. This is a collaborative project involving Aileen Anderson, Ph.D. through a subaward with UC Irvine.

R35NS097277 (Temple) 12/01/2016 – 11/30/2024 6.0 calendar
NIH/NINDS Res. Program Award $ 350,000
Defining Characteristics of Cortical Progenitor Cells over Time in Mouse and Human
This Research Program Award will support Dr. Temple's related to cortex development and application of stem cell technologies to understand and potentially treat developmental and neurodegenerative disorders.

R01AG056293 (Temple) 08/01/2017 – 04/30/2022 1.8 calendar
NIH/NIA $ 125,000
iPSC Modeling of AD Using Progerin
In this project, we will increase the level of the protein progerin in human iPSC lines with the goal of defining how abnormalities associated with aging contribute to the onset and progression of AD pathology.

DOH01-2017-00002 (Temple) 03/01/2018 - 02/29/2020 1.2 calendar
NYS-SCIRB $ 75,000
The role of zinc in axon regeneration following spinal cord injury
The objective of this proposal is to develop a novel approach to improving axon regeneration and behavioral recovery after SCI using Zinc chelating agents.

PENDING:

NONE