Neuronal Differentiation of Human Embryonic Stem Cell-Derived Neural Crest Stem Cells by Pulsed Electrical Field

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ABSTRACT:

This work surveyed the potential of using exogenous pulsed electrical field stimuli to guide the differentiation of human embryonic stem cell (hESC) derived neural crest stem cells (NCSCs) towards their neuronal lineage. A vertical electrode bioreactor able to vary the parameters of direct current pulse frequency, width and intensity was fabricated for this purpose. Dendritic morphology was observed for hNCSCs cultured on cathodes subject to 1 Hz, 50 ms pulse at 150mV/mm and 200mV/mm for 24 hours. The morphological phenomena were observed in a dosage and polarity orientation dependent manner; however, significant apoptosis was observed post stimulation.

hNCSCs and hESCs were then cultured on cathodes and subject to 1 Hz, 50ms pulse at 150mV/mm and 200mV/mm for 1.5 hours/day and 3 hours/day for 8 days. The expression of neuron-specific class III beta tubulin (Tuj1) was observed through immunofluorescence post stimulation and the expression levels of Tuj1 were qualitatively higher for the stimulated hNCSCs and hESCs as compared to the controls. Apoptosis was only observed in hNCSCs subject to the harshest condition.

Ethylene glycol tetraacetic acid (EGTA) was used to sequester extracellular Ca\textsuperscript{2+} in the media during the stimulation of hNCSCs on cathodes subject to 1 Hz, 50ms pulse at 200mV/mm for 24 hours. As EGTA concentrations were increased from 0mM to 2mM, dendritic morphology was reduced. Thus, the morphological phenomena were correlated to the available free extracellular calcium concentrations. Further investigation into the intracellular calcium concentrations in stimulated hNCSCs and hESCs was accomplished through the Fluo-4 assay. hNCSCs and hESCs were stimulated on cathodes subject to 2 Hz, 50ms pulse at 200mV/mm for 5 hours. Increased chronic levels of intracellular calcium were observed for hNCSC and hESC samples post stimulation.
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1.1 Introduction

It has long been recognized that various living tissues exhibit electrical activities and that these activities can impact tissue remodeling, cell migration during wound healing, and cell proliferation and differentiation (1,2). Previous studies have also shown that various living tissues have naturally occurring electrical activities. Examples include: trans-epithelial potentials in glands and embryos (in mV range) (3), large electrical fields detected at wound sites (~2 V/cm) (4), and intracellular gradients of potential that segregate charged proteins within the cytoplasm (5). Inquiry into the impact of electrical field stimulation on tissue re-modeling has yielded, to some degree of success, clinical treatments for bone fracture, nerve fiber repair, and soft tissue regeneration (6-8). Furthermore, it has been demonstrated that electrical field stimulation has profound impact on cellular behaviors including cell migration, plurality, nerve cone growth, and ion channel distribution (2).

The goal of this study is to test the hypothesis that electrical stimulation can influence stem cell differentiation towards electrically active cell types, and to develop a new platform that might be applied to manipulate stem cell differentiation. In this study, we examined the effect of exogenous pulsed electrical potential on the differentiation of human embryonic stem cell (ESC)-derived neural crest stem cells (NCSCs) (9). The NCSCs were culture in an electrical bioreactor flexible to the variance of the parameters: orientation of polarity, intensity, pulse time and frequency. The electrodes used in this study were set perpendicular to the substrate to maximize the exposure of the cellular bodies to the electrical field.

In a 24 hr stimulation of hNCSCs cultured on cathodes subject to 1Hz, 50 ms pulse, apparent dendritic morphology was only observed for 150mV/mm and 200mV/mm conditions. Though extended culture of the hNCSCs post stimulation yielded significant cell death, the morphological phenomena observed were dosage and polarity orientation dependent and seemed to suggest differentiation towards the neuronal lineage. Further study of hNCSCs and hESCs was carried out with a lower acute stimulation dosage, so as to avoid apoptosis and/or serosis. Eight day stimulations were conducted at 1Hz, 50ms pulse at 150mV/mm and 200mV/mm for 1.5 hours/day and 3 hours/day for 8 days. Immunostaining of neuron-specific class III beta tubulin (Tuj1) revealed qualitatively higher expression levels for the stimulated hNCSCs and hESCs as compared to the controls. Induced dendritic morphology was observed in hNCSC samples but not hESC samples. Also, in contrast to the 24 hr study, significant cell death was only observed in hNCSCs subject to the harshest condition.

In order to probe into the mechanism of the observed electrical stimulation induced differentiation, the morphological phenomena observed in previous experiments was correlated to available free extracellular Ca\(^{2+}\) in the media. In this study, ethylene glycol tetraacetic acid (EGTA) was used to sequester extracellular Ca\(^{2+}\) in the media during the stimulation of hNCSCs on cathodes subject to 1Hz, 50ms pulse at 200mV/mm for 24 hours. As EGTA concentrations were increased from 0mM to 2mM during stimulation, dendritic morphology was reduced
indicating that the morphological change induced by stimulation is dependent on the availability of extracellular Ca\(^{2+}\), for either trans-membrane flux or triggering the release of internal Ca\(^{2+}\) storage.

Further investigation of calcium’s role in the induced differentiation was accomplished by use the Fluo-4 assay. The Fluo-4 assay was conducted on hNCSCs and hESCs stimulated on cathodes subject to 2Hz, 50ms pulse at 200mV/mm for 5 hours. Increased chronic levels of intracellular calcium were observed for hNCSC and hESC samples post stimulation. Again, morphological phenomena were observed in hNCSC samples but not hESC samples.

1.2 Materials and Methods

1.2.1 Bioreactor Design

A model of the electrical stimulation platform used in this study is illustrated in Figure 1. The platform creates a vertical electrical field through the polarization of top and bottom electrodes. In contrast to the conventional horizontal alternative (2, 10), we hypothesize that the vertical configuration serves to maximize the cell bodies’ exposure to the electrical field, which allows for more effective regulation of trans-membrane polarity. The top electrodes were made from 99.99% pure gold wire connected to printed circuit board. Figure 1 illustrates only one top electrode; however, up to three top electrodes can fit onto one 96 well plate.

The bottom electrodes were created using electron beam vapor deposition of chromium and gold onto glass. 4nm chromium and 6 nm gold were successively vapor deposited through a patterned mask onto the glass creating a 96 electrode array. The bottom electrode thickness and composition ensured sufficient transparency for optical and immunofluorescent transmission microscopy and adhesion of the gold onto the glass. Also, the high purity of the surface gold on both the bottom and top electrodes was necessary in order to prevent galvanic erosion of the gold at the experimental voltages applied.

Fig. 1. Top view of 96 well array bioreactor
1.2.2 Electrical Stimulation Parameters

![Diagram of electrode configurations]

**Fig. 2.** The electrical stimulation platform used in this study has an up/down electrode configuration to maximize cell bodies’ exposure to electrical field. The top electrodes are made of pure gold wires and the bottom electrodes are e-beam evaporated 6nm gold on 4nm chromium and glass. This culture platform can ensure appropriate transparency for optical and immunofluorescent transmission imaging and sufficient adhesion of the electrode to the glass substrate. To stimulate the cells, we designed a stimulator which can provide 5 individually adjustable stimulation outputs. Adjustable stimulation parameters includes: frequency, intensity, and pulse width. We chose pulsed DC stimulation to avoid electrolyte depletion at the electrode-medium interface and in order to reduce heat generated by electrical current. The parameters used in cellular studies were constrained by prior protein coagulation and current depletion experiments. (data not shown)

1.2.3 Chemicals

The sylgard 184 silicone elastomer kit was used in this study to create the polydimethylsiloxane (PDMS) polymer purchased from Ellsworth Adhesives. The 99.99% pure gold wire (0.635 mm diameter) used as the top electrode in this study was purchased from Surepure Chemetals and the ethylene glycoltraacetic acid (EGTA) E3889 was purchased from Sigma.

1.2.4 Cells and Reagents

The human neural crest stem cells used in this study came from the generous donation of Studer et al. In expansion, cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM)/Ham’s F12, N2, fibroblast growth factor-2 (FGF2), and epidermal growth factor (EGF). FGF2 was withdrawn and 1% fetal bovine serum (FBS) was added in order to promote differentiation. During culture, media was changed every other day. Neurobasal media (#21103049) was purchased from Invitrogen (Carlsbad, CA). Additionally, the human embryonic stem cells used in this study came from the generous donation of Lee et al. In expansion, hESCs were cultured in DMEM/F12 medial in 1% FBS.
All reagents for RNA isolation and quantitative PCR analysis were purchased from Applied Biosystems (Carlsbad, CA). These reagents are Magmax-96 RNA isolation kit (AM 1830), taqman primers (Hs99999905_m1, Hs00801390_s1), Taqman gene expression master mix (4331182), and high capacity cDNA reverse transcription kit with RNase inhibitor (4374966).

Fig. 3. Cells used in this study include (A) human ESC derived NCSCs and (B) human ESCs (line H7). Before stimulation, NCSCs were cultured in growth media containing DMEM/F12, N2, FGF2 and EGF. ESCs were cultured with feeder free mTESR media from STEMCELL Technologies Inc. Bars represent 200 µm.

1.2.5 Cell Seeding

NCSCs were expanded for 50 days, flow sorted with p75 and HNK1 and frozen for later use. Upon thawing, the NCSCs were brought up in expansion media for 5 days. For experiment, the NCSCs were cultured at a seeding density of $3 \times 10^4$/cm$^2$ on the matrigel coated gold surfaces (1:100 matrigel in PBS, 2hr incubation at 37ºC). Expansion media was changed to differentiation media before stimulation.

In the ESC study, frozen ESCs were thawed and seeded at a seeding density of $10^4$/cm$^2$ on matrigel coated gold surfaces (1:100 matrigel in PBS, 2hr incubation at 37ºC). Single cell colonies were cultured through use of Rho-associated kinase (ROCK) inhibitor.

1.3 Results

Past research has repeatedly shown that Ca$^{2+}$ ions can trigger many biological events when they bind to and activate regulatory proteins that mediate various biological functions within cells. We hypothesized that, by using pulsed electrical field stimuli, we can manipulate the intracellular calcium of cells and possibly influence their differentiation behavior. This hypothesis is based on the observation that regulation of ion-channel by depolarizing reagent, such as tetraethylammonium chloride, has shown great impact on lineage specification of neuronal progenitors (I, 11) and that electrical field stimulation has great influence over expression and distribution of various ion channels (I2).
To test this hypothesis, we cultured cells on cathodes and anodes separately with different time and electrical field intensities. We found that pulsed electrical stimulation can produce dendritic morphology in NCSCs and that this effect is polarity and dosage dependent (Fig. 4-5).

**Fig. 4.** Human NCSCs were cultured on cathodes (Bg) and anodes (Tg). The cells were pulsed stimulated for 24 hours and microscopy data was compared with no stimulation control. The results show that the cultured NCSCs are morphologically different from control only when they are cultured on cathodes and at exposed to an intensity range of 150-200 mV/mm. Because significant cell death was observed post stimulation, a smaller dose stimulation experiment was carried out. Results are presented in Fig. 5. Bars represent 200 µm.
To minimize the cellular death observed in Fig. 4, a multiple small dose stimulation experiment was carried out. NCSCs were stimulated at 200 mV/mm for 3 hours daily for three days. FGF2 and EGF were withdrawn from media and 2% FBS was added to provide nutrients for cells. After three days, the cells cultured on the cathodes showed neuron-like dendritic morphologies; whereas, the control cells and those cultured on the anode showed little morphological change. Bars represent 100 µm.

Experiments in Fig. 4 and 5 suggest that a proper dosage of electrical stimulation could elicit neuronal differentiation of human ESC derived NCSCs. Therefore, in order to investigate the long term effect of pulsed electrical field on NCSCs and ESCs we extended the daily stimulation to 8 days, observing the cells’ morphology daily. Post stimulation, TuJ1 expression was analyzed through immunofluorescence staining to prove the cells were differentiating towards neuronal lineage. The results showed that under stimulated conditions as compared to control, NCSCs and ESCs increased their TuJ1 expression. However, significant cell death was observed under the most extreme condition for NCSCs.
NCSCs cultured 1.5 hr or 3.0 hr daily stimulation at 150 or 200 mV/mm on cathode compared to control showed that NCSCs do not exhibit significant morphological change at 150 mV/mm but tend to die at 200 mV/mm after 8 daily treatments. Maximum change in morphology was observed at day 3 for 200 mV/mm as shown in Fig. 4. Tuj1 staining of these samples showed that electrically stimulated cells have more Tuj1 expression than the control sample in all but the 3 hr daily stimulation at 200 mV/mm, which resulted in significant cell death. Bars represent 200 µm.

Fig. 6. NCSCs cultured 1.5 hr or 3.0 hr daily stimulation at 150 or 200 mV/mm on cathode compared to control showed that NCSCs do not exhibit significant morphological change at 150 mV/mm but tend to die at 200 mV/mm after 8 daily treatments. Maximum change in morphology was observed at day 3 for 200 mV/mm as shown in Fig. 4. Tuj1 staining of these samples showed that electrically stimulated cells have more Tuj1 expression than the control sample in all but the 3 hr daily stimulation at 200 mV/mm, which resulted in significant cell death. Bars represent 200 µm.

Fig. 7. Human ESC were cultured for 1.5 hr or 3.0 hr daily stimulation at 150 or 200 mV/mm on the cathode. Compared to control, ESC do not have noticeable morphological change to the control in any of the conditions tested. However, ESC cultured with longer stimulation times per day were observed to exhibit increased levels of Tuj1 expression. PCR analysis is under way to quantify this change. Bars represent 200 µm.

1.3 Discussion

The observed morphological phenomena and increased Tuj1 expression were hypothesized to result from a change in intracellular calcium concentrations and subsequent secondary messenger activity. To prove the mechanism is calcium related, we used the calcium
chelator ethylene glycol tetraacetic acid (EGTA) to control the extracellular calcium concentration during stimulation. EGTA serves as a preferential and irreversible chelator for calcium and when exposed to cell media, it sequesters extracellular calcium rendering it inactive and impermeable to cellular membranes. Results, presented in Figure 8, showed that stimulation could not elicit morphological change if free calcium ions were sequestered from the system. Therefore, the dendritic morphological phenomena are Ca\textsuperscript{2+} dependent. Further study, utilizing a flou-4 assay, showed an increase in chronic intracellular calcium in both NCSCs and ESCs after exposure to the pulsed electrical field stimuli. Results are shown in Figure 9.

![Image](image.png)

**Fig. 8.** EGTA inhibits NCSCs’ morphological change induced by pulsed electrical stimulation. Cells were soaked in 0.5 or 2.0 mM EGTA for 5 hr. After stimulation, they were then cultured in media (DMEM/F-12/N2) supplemented with 5% FBS for 2 days. Only samples that had available free Ca\textsuperscript{2+} and were exposed to electrical stimulation (bottom ground 200mv/mm) showed neuronal morphology. (A) Cells were stimulated with 0.5mM EGTA (0.5mM available free Ca\textsuperscript{2+}). Red line highlights the neuron-like morphology observed for this sample. (B) shows NCSCs that were treated with 0.5mM EGTA (0.5mM available free Ca\textsuperscript{2+}) but were not exposed to the electrical field. (C) shows NCSCs that were stimulated with 2.0mM EGTA and had the least available free Ca\textsuperscript{2+} in the media. No dendritic morphology was observed in (C). Bars represent 100 µm.
Fluo-4 assays were conducted after NCSCs and ESCs were cultured for two days. Stimulations were given in 50 ms pulses, 4Hz for four hours daily over the course of two days. Both NCSCs and ESCs cultured on the cathode exhibited higher concentrations of chronic intracellular calcium as compared to those stimulated on the anode and the control cells. Phase images are thresholded with cytoimmunofluorescent calcium images to better show dendritic morphology. Bars represent 100 µm.

1.5 Conclusion

In summary, this work surveyed the potential of using pulsed electrical stimulation to facilitate ESC-derived NCSC differentiation towards its neuron lineage. The observed morphological changes and increased TUJ1 expression, in response to the stimulation of NCSCs, are polarity dependent and dosage dependent. Through the use of EGTA, this work found that the observed morphological phenomena correlate to bioavailability of free Ca$^{2+}$ in the media. Fluo-4 assay demonstrated that NCSCs and ESCs stimulated on cathodes had increased chronic concentrations of intracellular calcium as compared to controls.

1.6 Acknowledgements

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1.7 References

1.8 Adendums

Zachary Wach: Research Blurb

I have worked in Dr. Hai-Quan Mao’s laboratory since the beginning of my junior year. Dr. Mao’s lab resides in the Materials Science and Engineering Department in the Whiting School of Engineering and also holds affiliation with the Translational Tissue Engineering Center in the School of Medicine at The Johns Hopkins University. My research predominates in the guidance of human stem cell differentiation through topographical, material, chemical and other physical micro and nano-environmental cues. I received the PURA scholarship for my senior design work, which has shown great potential in provoking neuronal differentiation of human embryonic stem cell-derived neural crest stem cells by pulsed electrical field stimuli. Recently, I have been awarded the Senior Design Award and expect to publish this work by the summer of 2011.

My experience as a Johns Hopkins researcher has been immeasurably rewarding. The most notable general advantages I have observed in being a materials science undergraduate researcher at Hopkins as compared to a researcher in other undergraduate disciplines around campus are: the streamlined nature of entering a research group, the highly mentor-student based atmosphere, the independence and confidence one experiences in applying the rare knowledge of a materials science to problems and the warm, family-like nature of the lab groups. Also, in being a transfer student from the University of Utah, itself a Tier I research institution, I can say from my own anecdotal experience that the research at Hopkins, particularly that available to the undergraduates, exceeds expectation, especially in the Materials Science and Engineering Department.

Zachary Wach: Biography

Mr. Zachary Wach is currently pursuing a Bachelor of Science in Materials Science and Engineering with an emphasis in biomaterials and a Bachelor of Science in Applied Mathematics from The Johns Hopkins University. He has worked in Dr. Hai-Quan Mao's laboratory from the fall of 2009 to the current day where his research predominates in the guidance of stem cell differentiation. He has received the PURA scholarship to support his research in the neuronal differentiation of human embryonic stem cell derived-neural crest stem cells by pulsed electrical field and hopes to publish this work by the summer of 2011. He will reside in Baltimore for at least one more year as he finishes up his dual majors and prepares for the application process to MD/PhD combined programs.

Mr. Wach holds position as Chief Scientific Officer of Solar Systems Express LLC, an upstart privatized space company, and as Chief Financial Officer of My Favorite People LLC, an ecommerce ticketing marketplace that is planned to launch by July of 2011. Recently, Solar Systems Express LLC has received recognition for its inventions and ideas and has been invited to the Technology Symposium sponsored by the Whiting School of Engineering at The Johns Hopkins University.