Montana-Based Inquiry into Neurodegeneration: Engrailed Proteins and Parkinson's Disease

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INTRODUCTION

An estimated 10 million people worldwide have Parkinson's disease (1). Parkinson's disease is a chronic neurological disorder that is characterized by neuron degeneration in the midbrain, leading to progressive involuntary muscle movement. However, the cause of it is not yet known, and it is currently incurable. Dopaminergic neuron degeneration leads to a scarce supply of dopamine, which causes the individual to exhibit involuntary movement (2). A defining pathological feature of Parkinson's disease is the aggregation of alpha-synuclein in dopaminergic neurons, which is believed to result in the loss of beneficial alpha-synuclein function. The Engrailed proteins are believed to protect the mesencephalic dopaminergic (mDA) neurons during development and are actively added to protect mDA neurons in Parkinson's disease experiments. Studying this disease *in vivo* would require access to the human brain, which is difficult to do. Therefore, *in vitro* techniques are used. Stem cells can be derived from human skin cells and then changed into dopaminergic neurons to model Parkinson's disease. Through my research, I am testing my hypothesis that Engrailed proteins protect neurons from cell death. The Reijo Pera lab has evidence that Engrailed proteins and alpha-synuclein interact physically. We believe Parkinson's disease may be in part due to high levels of alpha-synuclein having an inhibitory effect on the Engrailed proteins. To test this hypothesis, I am overexpressing alpha-synuclein and Engrailed proteins in mDA neuron cell cultures. I am also testing cell viability and the function of the Engrailed proteins.

METHODS

Lentivirus Overexpression: Lentiviruses are used as a gene delivery vector to deliver viral RNA into the DNA of the host cell. It is a type of retrovirus that can infect dividing and nondividing cells. Once the cell is infected, the DNA is made through reverse transcriptase. We use a modified lentiviral vector because it can infect and express its genes in human cells. By using this method, we successfully overexpressed the EN1, EN2, and GFP genes in our cells.

Alternative Approach: To overexpress alpha-synclein, we took the alpha-synuclein protein from bacteria, purified it, and then added it to the cell culture.

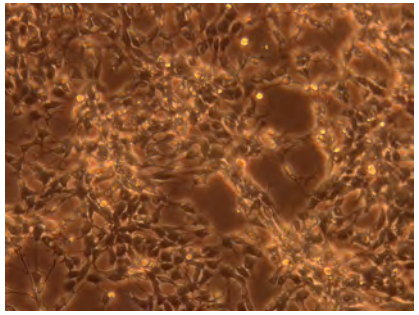

Figure 1: mDa progenitor cells that are used to create neurons. It is easier to create neurons using these cells rather than stem cells.

Immunofluorescence General Protocol: This procedure uses primary and secondary antibodies to tag a protein of interest. After washing cells, a blocking buffer is added to block free sites on the membrane. After an hour, the blocking buffer is removed and a primary antibody is added. The primary antibody finds and attaches to the protein of interest. Then to be able to see the protein of interest under a fluorescent microscope, a secondary antibody, that has a fluorescent tag, is added and it attaches to the primary antibody. To tag many different proteins in a cell culture, there are different primary and secondary antibodies that can be added.

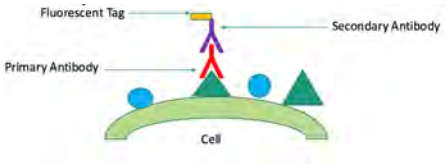

Figure 2: Immunostaining diagram that shows how the primary and secondary antibody attaches to the protein of interest in the cell culture.

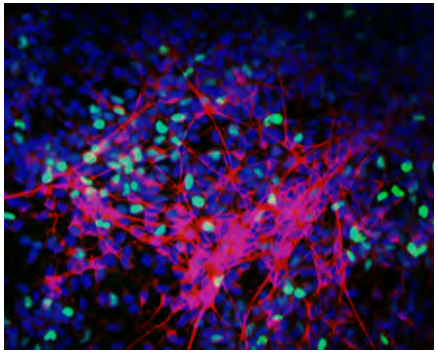

Figure 3: Immunostaining results that shows B3 tubulin as red, the nucleus as blue, and EN1 protein as green. Different primary and secondary antibodies were added to the cell culture, and the secondary antibodies gave the proteins their respective colors.

Cell Differentiation: The degeneration of A9 Dopaminergic (DA) neurons in the substantia nigra pars compacta part of the brain, leads to Parkinson's disease (PD). Human pluripotent stem cells (hPSCs) can be used to derive A9 DA neurons for PD regenerative cell replacement therapy, which is one of the most promising PD treatment strategies. I have followed a protocol that transforms hPSCs to A9 DA neurons using a stepwise differentiation method. A9 DA neurons originate from floor plate precursors found in the ventral midline of the central nervous system. The culture conditions were optimized by mimicking this environment. Floor plate precursor cells were generated by using small molecule CHIR99021 to activate the Wnt signaling pathway. Then these FP cells were specified to DA neurons with the growth factors BDNF, GDNF, etc. The generated DA neurons will be of A9 cell type and they can be derived in less than 4 weeks.

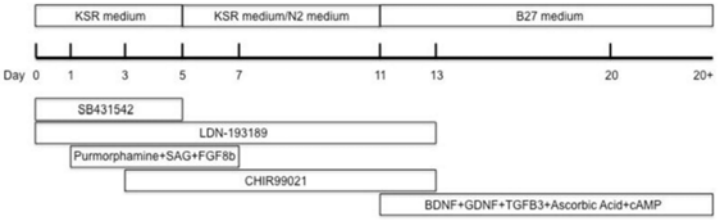

Figure 4: Overview of the differentiation protocol that shows culture medium growth factors and small molecules that were used each day. *Overview taken from Zhang et al. 2015*

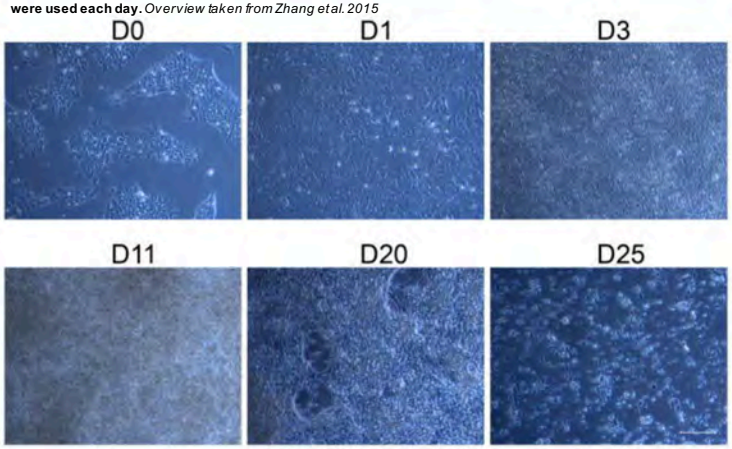

Figure 5: Morphological changes of the cell during differentiation. H9 hESCs were plated as single cells in the appropriate cell density. 48 hours later, these cells reached about 70% confluence in the plate as colonies (D0). The cells expanded rapidly during the first few days of differentiation, reaching over 90% confluence after 24 hours of differentiation (D1), and then becoming confluent 48 hours later (D3). However, most of these cells still exhibit typical hESC morphology and as differentiation continues, the cells expand more and gradually lose hESC morphology. At the end of D11 of differentiation, there were more than one cell layer in many parts of the plate, as evidenced by the darker color of the cells. Starting from about D17 to D20, some cells died, leaving spaces in the plate. Also, in these spaces and sometimes under the cell layer, some neuron projections could already be observed (D20). To make more space for cells to grow and differentiate, the cells were replated at the end of D20. At this time, cells aggregated to form small clusters and a lot more axons/neurites emerged from these clusters. Also, axons/neurites from different clusters formed some connections in as short as 4-5 days (D25). Scale bar, 200 µm.

EXPERIMENTAL DESIGN

There will be three parts to the experiment. First, we hope to find whether or not the Engrailed proteins protect TH neurons from alpha-synuclein. To test this, we will use different genotypes of cells to create 3 different conditions: GFP (control), Engrailed protein-1 (EN-1), and Engrailed protein-2 (EN-2) and then we will add alpha-synuclein protein to those cell culture dishes. We will create these different conditions and then differentiate the cells into neurons and compare the percentage of the TH neurons that survive.

The second part of the experiment will be to see if alpha-synuclein inhibits the translational function of the Engrailed proteins. Previous studies show that the Engrailed proteins regulate the translation of Ndufs1 and Ndufs3 genes. We will test this by adding alpha-synuclein to the cell and using Western Blotting to see if the translation of Ndufs1 and Ndufs3 will change.

The third part of the experiment, will be to see if alpha-synuclein inhibits the transcriptional regulatory function of the Engrailed protein. Engrailed protein is a well known transcriptional regulator. We will test this by adding alpha-synuclein to the cell cultures and using ChIP-seq to map if there are any changes to the Engrailed proteins binding to the genome.

ACKNOWLEDGMENTS

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