

Dopaminergic neuron regeneration using cell therapy in a rat model of Parkinson's disease

Submitted by

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Abstract

Parkinson's disease (PD) is a chronic neurodegenerative movement disorder caused by the loss of dopaminergic (DA) neurons located within the substantia nigra pars compacta (SNpc) of the midbrain. Current treatments involving drug therapy only temporarily alleviate the symptoms and does not treat the etiology of the disease. Human umbilical cord mesenchymal stem cells (MSCs) hold vast promise in cell therapy due to their multipotency and immunomodulatory properties. We hypothesize that MSCs can be induced to differentiate into DA neuron progenitors which can be transplanted into the SNpc to treat PD. In this project, we propose to develop a method to differentiate MSCs into DA neuron progenitors, as well as investigate their safety and efficacy when injected into a neonatal animal model of PD developed in our lab. The results of this study will aid in devising strategies to replace degenerated neurons, and to reverse and/or halt the progression of PD.

Keywords: Mesenchymal stem cells (MSCs), Parkinson's disease (PD), cell therapy, umbilical cord

Current Research and Knowledge

Parkinson's disease (PD) is a chronic neurodegenerative movement disorder caused by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) of the midbrain. PD is the second most common neurodegenerative disorder, and is expected to be diagnosed in nearly 1.2 million people in the United States by 2040⁶. This disease substantially impacts quality of life, activities of daily living, and causes a socioeconomic burden, amounting to nearly 14.4 billion dollars annually in the United States alone⁶. The current standard of treatment involving drug therapy only temporarily alleviates symptoms and does not treat the etiology of the disease. A neurosurgical procedure called deep brain stimulation is also performed to improve specific motor impairments, but it does not cure the disease. The procedure is invasive, and only a small population of patients qualify to have it performed.

Mesenchymal stem cells (MSCs) are an attractive source for cell therapies because they can be isolated without raising moral or ethical concerns, in contrast to embryonic stem cells. Embryonic stem cells are pluripotent and the most primitive, however, their potential use for cell therapy is compromised due to their ability to form teratomas. MSCs can be isolated from many sources, including bone marrow (BM), umbilical cord (UC) tissue, adipose tissue, and umbilical cord blood (UCB)³. Isolation of MSCs from BM involves an invasive procedure called a lumbar puncture⁷. In contrast, MSCs can be isolated non-invasively from an abundant and desirable source, UC, which is routinely discarded after delivery. UC is not only a rich source, but can also provide a plentiful number of cells often needed for cell therapy³. MSCs from UC hold vast potential in regenerative medicine and tissue engineering because they can be expanded to greater numbers than MSCs derived from other sources¹.

We hypothesize that UC-MSCs can be induced to differentiate into DA neuron progenitor cells *in vitro*, which can be augmented to treat PD². Our lab has developed a neonatal rat model to test the safety and efficacy of injecting UC-MSCs and their DA neuron progenitors into the SNpc for the treatment of severe PD. We determined the neonatal rat model to be a better model of PD because it is more representative of the severe DA alterations seen in humans⁵. Additionally, cell transplantation will not alter survivability, as well as, sensorimotor or cognitive function⁵. In this study, we propose to investigate the therapeutic potential of human UC-MSCs and DA neuron progenitors in a neonatal rat model of severe PD. The results obtained will lay the foundation for translational stem cell research to reverse and/or halt PD.

Aims and Objectives

Introduction

PD is a chronic neurodegenerative movement disease. The symptoms of PD include tremor, ataxic gait, bradykinesia, changes in speech, impaired balance, and rigidity. These symptoms negatively impact the quality of life and the ability to carry out activities of daily living for patients. Risk factors for acquiring PD include age, heredity, sex, and environmental exposure to toxins. Additionally, there is not a cure for the disease. Currently, the only options available are pharmaceutical drugs, the most commonly used is Levodopa. However, as the disease progresses the effects of Levodopa wear off and the symptoms can no longer be controlled. Without an effective therapy to treat PD, the disease can progress to cause difficulty swallowing, sleep disorders, incontinence, depression, and cognitive problems. The aims and objectives below outline UC-MSCs as a potential source of cell therapy to halt and/or reverse the progression of PD.

Aims

1. To induce differentiation of UC-MSCs into DA neuron progenitors.
2. To transplant UC-MSCs and DA neuron progenitors into the SNpc in an animal model of severe PD.
3. To determine functional improvement of PD after cell injection using neurobehavioral tests.
4. To investigate neuroprotection and neuroregeneration resulting from cell transplantation using biochemical, histological, and molecular analyses.

Objectives

1. Stem cells have been differentiated into DA neuron progenitors using a variety of cytotoxic chemicals and animal products. The use of cytotoxic chemicals decreases cell viability *in vitro*, as well as survivability *in vivo*. If differentiation includes the use of animal products, then they cannot be used in humans due to risk of a xenogenic reaction. Therefore, this study will follow a differentiation protocol strictly using cytokines and growth factors in the absence of animal products.
2. The proper injection technique of UC-MSCs and DA neuron progenitors is one of the most critical aspects of this study. Successful injection technique will allow for further understanding the regenerative mechanism of UC-MSCs and DA neuron progenitors.
3. Symptomatic or functional improvement is critical to treating PD due to the hindrance these symptoms have on patient's quality of life. This study will evaluate functional improvement in rats, and therefore, be able to determine if the cell injection was successful or not in providing therapeutic relief.

4. Biochemical analysis will allow for the quantification of dopamine being secreted *in vivo*, as well as determine the restoration of normal physiologic processes. Histological analysis is important in revealing how the cells migrate and integrate into the neural network. Histological analysis will also evaluate protein expression and tissue structure in order to determine the extent of DA neuron regeneration. Molecular analysis is required to ascertain the levels of DA neuron and neuronal gene expression in the tissues. All of the analyses above will collectively define the functionality and survivability of the cells injected into the SNpc.

Methodology

Human Umbilical Cord Tissue Samples and Isolation of Cells

Human UC tissue was collected from Providence Hospital under an approved protocol from consenting donors. Tissue was processed within a couple hours of delivery. The tissue was rinsed with phosphate buffered saline (PBS) to remove any remaining blood and blood clots. Then the UC tissue was carefully dissected to separate the different parts of the tissue, such as placenta, UC, and Wharton's jelly. Tissue pieces were minced into 1-2 mm pieces. Human UC tissue was plated in 75 cm² flasks and cultured in DMEM/F12 medium. Culture medium was changed every three days until cells grew. After cells began growing, the cell culture medium was changed every 1-2 days. Tissue and cell culture was kept in a 37°C with 5% CO₂ incubator. When cells grew to 70% confluence in tissue flasks, they were passaged and subcultured into new culture plates for future studies.

Dopaminergic Induction and Characterization

UC-MSCs will be plated directly in F12/DMEM medium for six hours, followed by neural induction towards DA neuronal progenitors. UC-MSCs will be induced towards a DA neuron fate using a variety of cytokines and growth factors in three stages: neural adaptation, neuronal priming, and neuronal maturation. During the neural adaptation phase, cells were plated in basic fibroblast growth factor (bFGF). Neuronal priming used FGF-8, sonic hedgehog (SHH), and transforming growth factor beta-3 (TGF β -3). Cells in the neuronal maturation stage were treated with TGF β -3, ascorbic acid, brain derived neurotrophic factor (BDNF), and glial derived neurotrophic factor (GDNF). All three stages last seven days each and were cultured in neurobasal medium and B27. In total, this differentiation protocol lasts for three weeks. Flow cytometry will be performed to quantify the expression of five MSC surface markers: CD90, CD105, CD73, CD44, and CD29. Undifferentiated cells should express all five markers in order to be classified as MSCs. In summary, immunophenotyping will be performed when cells are grown to 70% confluence. Cells will then be trypsinized, washed in PBS, and stained with FITC conjugated antibodies. The differentiation process will be analyzed with immunocytochemistry for expression of neural and DA neuron proteins, such as TH, TUJ-1, DAT, and MAP-2. Briefly, cells will be cultured on cover slips. On harvest day, they will be fixed in 4% paraformaldehyde for 10 minutes at room temperature. Cells will then be permeabilized with 0,5% Triton X-100 and blocked in 2% bovine serum albumin in PBS for 1 hour. Cells will be stained with primary antibody at dilutions according to the manufacturer, following staining with secondary antibody. Lastly, cells will be counterstained with DAPI at room temperature and then mounted for microscopic analysis. RNA from differentiated cells will be isolated to confirm DA neuron

specific lineage markers, specifically TH, Pitx3, DAT, Nurr1, En1, and Lmx1b. Cell viability will be analyzed with trypan blue staining. Cells that stain blue will be considered nonviable and cells that do not pick up the stain will be considered viable. Functionality of DA neuron progenitors will be determined using an enzyme-linked immunosorbent assay (ELISA) to quantify the amount of dopamine secreted *in vitro*. A whole-cell patch clamp assay will be performed to determine the ability of DA neuron progenitors to conduct an action potential.

6-Hydroxydopamine Induction and Cell Transplantation

Sprague Dawley neonate rats, three days postnatal, will be pretreated with an intraperitoneal (IP) injection of desipramine HCl (20 mg/kg) one hour before injection to protect noradrenergic nerves and allow 6-hydroxydopamine (6-OHDA) to become relatively selective, producing predominately DA neuron denervation. Before injection of 6-OHDA, rat pups will be individually immersed in ice for 60 seconds to induce cold-anesthesia. The neonate will be placed flat under a bright light to allow visualization of the sagittal sinus, transverse sinus, bregma, and lambda. Area of injection will be disinfected prior to the procedure by repeating three applications of povidone iodine, followed by 70% ethanol. A 26-gauge needle, attached to a microliter syringe, will be positioned relative to bregma (left side of the brain) -1.5 mm anterior posterior, ± 1.8 mm medial lateral, and -7.5 mm dorsal ventral. The needle will be lowered into the tissue and deliver 5 μ L of 6-OHDA in 0.1% saline ascorbic acid solution. The needle will be left in place for 30 seconds to ensure 6-OHDA settles into the tissue. All experimental rats will have to undergo this procedure once.

Immediately after 6-OHDA administration or on postnatal day five, UC-MSCs or DA neuron progenitors (1×10^6) in 0.1% saline ascorbic acid solution will be delivered in the same area. DA neuron progenitors will undergo the first two stages of differentiation and then be transplanted. After injection, rats will be slowly warmed to promote recovery and then returned to the litter with their mother.

The procedure will take approximately 30 minutes from anesthetic induction to recovery of the rats. Rats will be monitored daily for evident distress or behavioral changes until the end point for harvesting. Rimadyl will be administered if the rats are in any pain or distress. All animals will be monitored by a veterinarian in accordance to the Animal Welfare Act. All procedures explained will be performed following the guidelines of the Institutional Animal Care and Use Committee at Oakland University and Standard Operating Procedures.

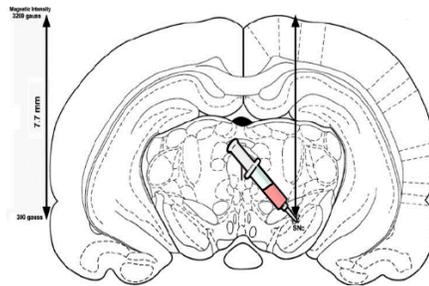


Image 1. Schematic demonstrating the site of injection of 6-OHDA and UC-MSCs or DA neuron progenitor cells.

Neurobehavioral Analysis

The contralateral rotation assay will be conducted once a week, beginning five to seven days after DA neuron degeneration. This assay is used to determine the efficacy of 6-OHDA to cause DA neuron cell death. Furthermore, if the animal rotates contralateral to the side of the 6-

OHDA injection, this is indicative that the 6-OHDA injection was successful and there was DA neuron degeneration. Animals will receive a subcutaneous injection of a DA agonist, apomorphine (0.5 mg/kg). The rats will be placed in a box to monitor contralateral rotations. Animals will have a five minute habituation period after being moved to an area for testing. After the adjustment period, rotations will be recorded for five minutes. Animals will be returned to their normal housing 30 minutes following the assay. More than seven contralateral rotations per minute is indicative of 80% DA neuron denervation in the SNpc. Post injection, if the number of rotations continually decreases, it will indicate the regeneration of DA neurons. Thus, the degree of recovery from PD symptoms will be determined by calculating the amount of contralateral rotations per minute.

The rotarod test will be conducted once a week, beginning five to seven days following 6-OHDA induction. For this assay, each rat will be placed on a horizontal, rotating rod that is suspended above a cage floor, but low enough to not cause any injury to the rat, should it fall from the rotating rod. Under normal conditions, the rat will try to stay on the rod to avoid falling off. The length of time the rat is able to stay on the rod will measure their balance, coordination, and ability to plan gross motor movements. This assay is important to the study because it will monitor the motor function in rats since DA neuron denervation causes motor impairments and seizures. Rats will be trained on the rotarod apparatus for three sessions of two minutes at speeds of 5, 10, and 15 rpm. After acclimation to the rotarod, tests will be performed at 15 rpm for 180 seconds. The number of times the rats fall from the rod will be measured, thus, the extent of recovery from PD symptoms will be determined by calculating the total duration of time on the rotarod.

Biochemical, Histological, and Molecular Analysis

For every implantation time point, there will be three tissue assays performed. The biochemical analysis will include determination of dopamine levels by liquid chromatography/mass spectroscopy. Quantitating the amount of dopamine readily available in the tissue will draw conclusions on the functionality and survivability of cells in host tissue. The second set of brain tissue will be used for histological analysis. The tissues will be processed with OCT medium or paraffin and then sectioned using a microtome. Brain tissue will be cut into 30 μM or 6 μM sections. Sections will be stained with hematoxylin and eosin and analyzed under light microscopy to observe tissue architecture. In addition, tissues will be investigated using immunohistochemistry with staining for DA neuron and neuronal cell-specific antibodies including TH, TUJ-1, and DAT. Stained tissue sections will be examined with confocal and fluorescent microscopy. The third set of rats will be used to analyze the gene expression of specific DA neuron markers, TH, Pitx3, DAT, Nurr1, En1, and Lmx1b, by quantitative reverse transcriptase- polymerase chain reaction (qRT-PCR).

Outcomes

Prior studies have used inefficient and cytotoxic methods for deriving DA neuron progenitors to be used for *in vivo* animal studies of PD. The proposed study seeks to devise a method for efficiently deriving functional DA neuron progenitors from UC-MSCs. After, UC-MSCs or DA neuron progenitors will be injected into a neonatal animal model. This will be the first time UC-MSCs and DA neuron progenitors will be injected into a neonatal animal model of severe PD. Furthermore, the results of the proposed project will determine the safety and efficacy of using

UC-MSCs as a source for cell therapy to treat PD. Ideally, we hope to study the mechanism of DA neuron regeneration, learn more about the regenerative properties of UC-MSCs and DA neuron progenitors, and to reverse and/or halt the progression of PD. The results obtained from this study hold the potential of contributing to human clinical trials using UC-MSCs as a form of cell therapy to treat PD, and also impacting the lives of millions of people.

Results

Flow Cytometry

MSCs were characterized with the five MSC surface markers: CD90, CD105, CD73, CD44, and CD29. Expression of these five surface markers deems the cells as MSCs. Flow cytometry experiments were performed in triplicate. MSCs significantly expressed all five surface five surface markers: 96.9%, 95.5%, 97.1%, 96.3%, and 98.2%, respectively. DA neuron progenitors were also subjected to characterization by flow cytometry to monitor the differentiation process. Most notably, CD90 and CD105 expression levels dropped. After undergoing differentiation towards a DA lineage, CD90 expression levels decreased to 72.6% and CD105 expression levels decreased to 59.0%. Furthermore, these results demonstrate that MSCs underwent differentiation.

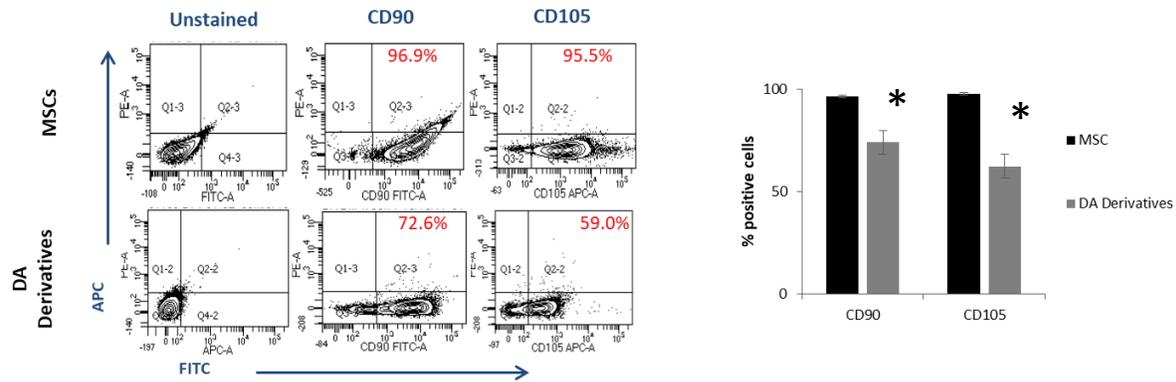


Figure 1. Flow cytometry results comparing MSCs to DA neuron progenitors. **A.** Flow cytometry graphs demonstrating the changes in the cell population as MSCs were differentiated towards a DA lineage. **B.** Graph illustrating the significant change in surface marker expression in CD90 and CD105.

Light Microscopy Analysis

Light microscopy analysis was performed in order to observe for significant changes in morphology. Light microscopy analysis was monitored daily using 5x, 10x, 20x, and 40x magnification. UC-MSCs have a characteristic fibroblast morphology in their immature, undifferentiated state. DA neurons have a neural, dendritic branching morphology. After week three of differentiation, neuronal priming, the DA neuron progenitor cells had demonstrated neural network morphology. This is a significant finding because there is a strong relationship between neural structure and functionality.

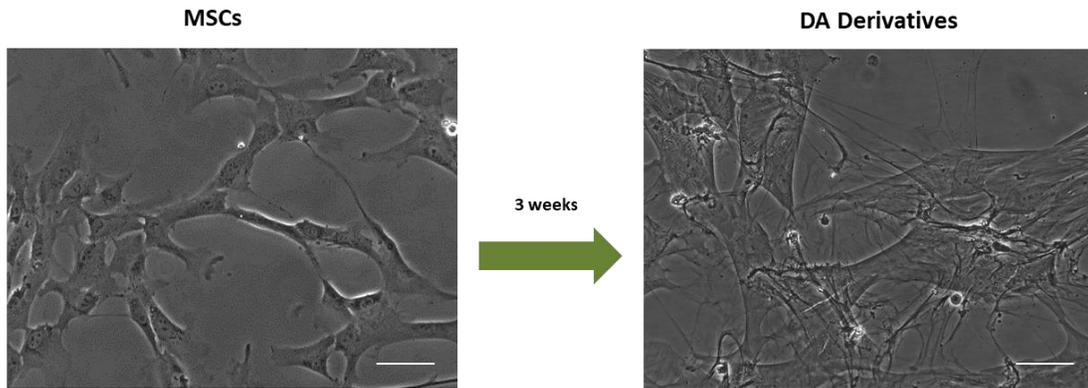


Figure 2. Morphological analysis comparing MSCs before and after neural induction. Images were taken using light microscopy at 20x magnification.

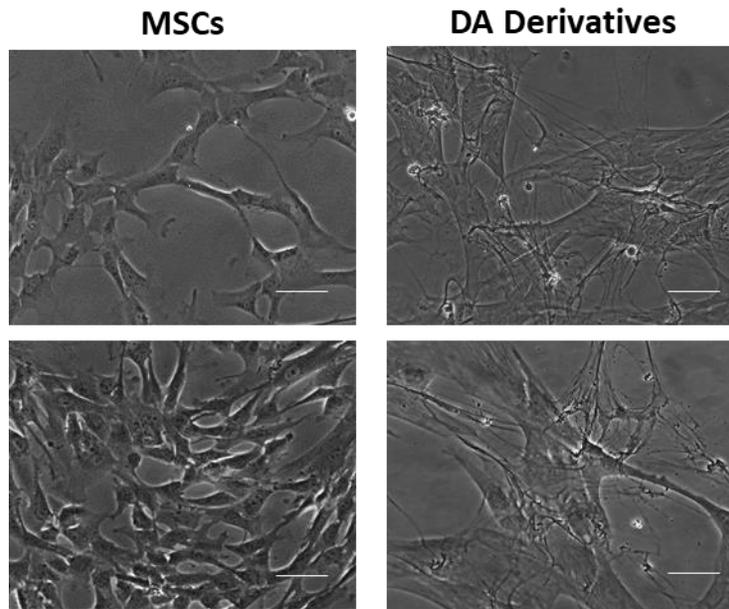


Figure 2.2. Morphological analysis using light microscopy after three weeks of DA differentiation. Images were taken at 20x magnification.

Immunocytochemistry

Both MSCs and DA neuron progenitors were stained with neural and DA proteins. Cells that are positive for the proteins will fluoresce because they have the antigens to bind the antibody used for staining. Immunocytochemical experiments were performed in triplicate. Confocal and fluorescent microscopy was used to detect expression. TH, TUJ-1, NCAM, and MAP-2 were used to stain both MSCs and DA derivatives. MSCs stained negative for all of the proteins previously listed. The DA derivatives stained positive for TH, TUJ-1, NCAM, and MAP-2. These results indicate that the DA differentiation protocol used was successful and generated DA derivatives that expressed proteins particular to the DA phenotype.

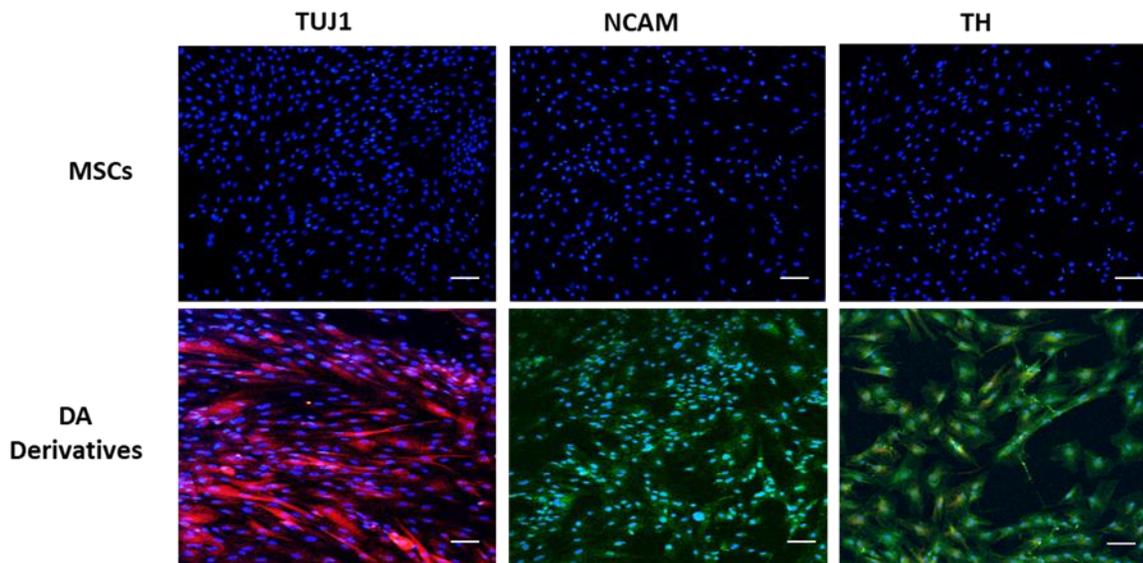


Figure 3. Confocal microscopy was used to capture images of cells after immunocytochemistry. MSCs stained negative for TUJ-1, NCAM, and TH. DA derivatives expressed TUJ-1, NCAM, and TH. Images were captured at 10x.

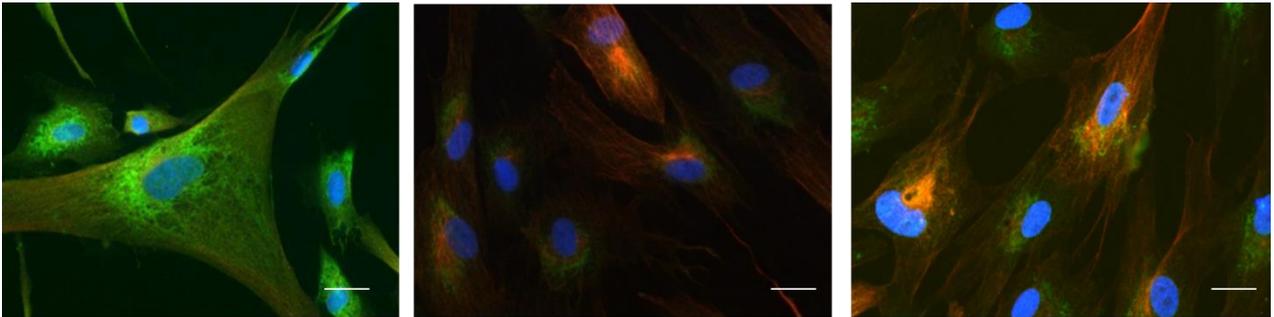


Figure 4. Fluorescent images of immunocytochemical staining of DA derivatives show positive expression for both TH and TUJ-1. MSCs stained negative for TH and TUJ-1. Images were captured at 40x magnification.

Cell Viability

Cell viability assays were performed in triplicate. DA neuron progenitors were 94.7% viable after undergoing differentiation. 5.3% took in the trypan blue stain and were determined nonviable. These results demonstrate that UC-MSCs are a viable option for treating PD. This also demonstrates that the mixture of cytokines and growth factors serves as a successful method for producing functional cells, generating a clinically significant amount of cells, and increasing survivability of cells upon transplantation.

Discussion

Parkinson's disease is characterized by the progressive, localized degeneration of DA neurons in the SNpc. Cell therapy serves as the ultimate treatment option by replenishing degenerated DA neurons with functional neurons. UC-MSCs are the most desirable source for cell therapies because UC is an inexpensive and ethical method of obtaining stem cells, and also

provides a large reservoir of cells. Notably, UC-MSCs are isolated in a more primal state compared to BM-MSCs. This property of UC-MSCs confers greater proliferative and differentiation potential⁸. In addition, UC-MSCs are a better choice for cell therapy because they are non-tumorigenic and have immunomodulatory properties, meaning they are not likely to illicit an immune response upon transplantation and will not cause graft versus host disease, unlike ESCs which have been known to cause teratomas and require immunosuppression in order to be transplanted.

Cell therapy serves as a method of curing or reversing the etiology of PD. Current pharmaceuticals function to only provide symptomatic relief and begin to lose effectiveness overtime. A surgical intervention called deep brain stimulation is another option for those with PD. However, this is an invasive neurosurgical procedure, only those with advanced PD qualify, and this procedure also only provides symptomatic relief. Cell therapy using UC-MSCs presents as an innovative way to solve current issues providing therapeutic relief to individuals with debilitating diseases where medications only provide symptomatic relief. Currently, stem cells are clinically used to be transplanted into the knee, spine, and bone marrow.

The results from this study are important in laying the foundation for future methods of developing stem cell therapies. This study used UC-MSCs to generate DA neuron progenitors in the absence of cytotoxic chemicals, such as retinoic acid, and in the absence of animal products, such as fetal bovine serum, which is commonly used as a supplement in stem cell culture to support proliferation. The absence of animal products in this differentiation protocol is clinically significant because these DA neuron progenitors can be transplanted into a human host without the potential of causing a xenogenic reaction. Since these cells were cultured in the absence of cytotoxic chemicals, functionality and survivability was significantly increased. Cytotoxic

chemicals in cell culture causes the cells to undergo apoptosis, deeming them nonviable for cell therapy.

Conclusion and Future Directions

The results of this study demonstrate that UC-MSCs can be differentiated into DA neuron progenitor cells. The data obtained demonstrates that the differentiation procedure developed yields viable DA derivatives that have the differentiation potential to be used for cell therapy to treat and/or cure Parkinson's disease. This is a significant finding because this is one of the first studies to cultivate DA neuron progenitors *in vitro* from UC-MSCs in the absence of animal products and cytotoxic chemicals.

Further studies are needed to determine the safety and efficacy of using these DA derivatives in an animal model of Parkinson's disease. Using an animal model will allow further understanding of the mechanism of DA neuron regeneration, to learn more about the regenerative properties of UC-MSCs and DA neuron progenitors, and to reverse and/or halt the progression of PD. There are currently no registered clinical trials using human UC-MSCs to treat PD. The results obtained from this study hold the potential of contributing to human clinical trials using UC-MSCs as a form of cell therapy to treat PD, and also impacting the lives of millions of people.

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Abbreviations

6-OHDA 6-hydroxydopamine

BDNF Brain derived neurotropic factor

bFGF Basic fibroblast growth factor

BM Bone marrow

DA Dopaminergic

ELISA Enzyme-linked immunosorbent assay

GDNF Glial derived neurotrophic factor

IP intraperitoneal

MSCs Mesenchymal stem cells

PBS Phosphate buffered saline

PD Parkinson's disease

qRT-PCR Quantitative reverse transcriptase- polymerase chain reaction

SHH Sonic hedgehog

SNpc Substantia nigra pars compacta

TGF β -3 Transforming growth factor beta-3

UC Umbilical cord

UCB Umbilical cord blood

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