Using Bioengineering Approaches to Generate a Three-Dimensional Human Induced Pluripotent Stem- Cell Based Model of Alzheimer's Disease

by

Rachel Elizabeth Lundeen

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Graduate Supervisory Committee:

David Brafman, Chair
Samira Kiani
Mohammad Ebrahimkhani

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ABSTRACT

The pathophysiology of Alzheimer's disease (AD) remains difficult to precisely ascertain in part because animal models fail to fully recapitulate many aspects of the disease and postmortem studies do not allow for the study of the pathophysiology. *In vitro* models of AD generated with patient derived human induced pluripotent stem cells (hiPSCs) could provide new insight into disease mechanisms. Although many protocols exist to differentiate hiPSCs to neurons, standard practice relies on two-dimensional (2-D) systems, which do not accurately mimic the complexity and architecture of the *in vivo* brain microenvironment. This research aims to create three-dimensional (3-D) models of AD using hiPSCs, which would enhance the understanding of AD pathophysiology thereby, enabling the generation of effective therapeutics.

ACKNOWLEDGMENTS

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CHAPTER 1: Introduction

1.1.1 Motivation

Alzheimer's disease (AD) is the sixth leading cause of death in the United States, with a 71% increase in deaths between the years 2000 and 2013[1]. In the United States alone there are an estimated 5.4 million people that suffer from Alzheimer's disease, with a new case arising every thirty three seconds and an expected 2.5 fold increase in cases by mid-century[2]. The complex pathophysiology of Alzheimer's disease leads to a difficulty in finding a cure or even an effective treatment method. For this reason, it is imperative to develop a model of Alzheimer's that fully recapitulates all of the complexities of the disease.

1.1.2 Alzheimer's Disease

Alzheimer's disease is the most common form of dementia, which can be defined as the loss of cognitive functioning and behavioral abilities to the point where it interferes with a person's daily life[3]. This loss of cognitive functioning and behavioral abilities is irreversible, again highlighting the need for a closer study at Alzheimer's disease. The disease is progressive, leading to in increase in dementia symptoms over time. The progression is grouped into three main stages based on the severity of the impairment[4]. The "early stage" refers to mild Alzheimer's disease during which individuals have mild impairment but can still function independently [5]. The "middle stage" is the typically the longest stage of the disease and refers to moderate Alzheimer's during which the symptoms will be more noticeable and the individual typically begins to require assistance in performing daily tasks [5]. The final stage is "late stage" or severe Alzheimer's during which the individual loses the ability to respond to their environment

and communicate, leading them to require to full time care[4]. This progression of the disease is due to the development of extracellular amyloid beta plaques and intracellular neurofibrillary tangles in different regions of the brain (Fig1-1). These plaques and tangles are the two pathological hallmarks of Alzheimer's disease[6]. The insoluble plaques are formed from beta amyloid fragments aggregating together, causing a neurotoxic environment. The plaques block ion channels, disrupt calcium homeostasis, cause mitochondrial oxidative stress, impair energy metabolism, and cause abnormal glucose regulation, which all lead to neuronal cell death[7]. The tangles are formed from hyperphosphorylation of tau, a microtubule associated protein. The hyperphosphorylation of tau results in microtubule destabilization and ultimately apoptosis of neurons[7]. The excess accumulation of amyloid beta plaques are hypothesized to lead to neurofibrillary tangles, and together these contribute to Alzheimer's disease[8] (Fig 1-2).

1.1.3 Familial Versus Sporadic Alzheimer's Disease

Alzheimer's disease is divided into two forms: familial (FAD) and sporadic (SAD). While the familial form is due to mutations in three major genes (amyloid precursor protein (APP) gene, presenelin1 (PSEN1) gene, and presenilin2 (PSEN2) gene), the sporadic form is thought to be due to a combination of genetic and environmental factors, including Apolipoprotein E (ApoE)[9].

Sporadic Alzheimer's disease accounts for 95% of all AD cases and has an estimated genetic component of 50%-70%, half of which is due to ApoE allele, specifically ApoE ε4[10]. ApoE releases cholesterol that is used to support synaptogenesis and the maintenance of synaptic connections[11]. There are numerous studies that are attempting to uncover the complex mechanisms behind SAD. Although the exact pathophysiology is

still unknown, it is thought that ApoE ε4 either increase toxic function or decrease neuroprotective function, thus leading to cognitive decline[12]. Specifically, it has been hypothesized that ApoE is involved in deposition or clearance of amyloid beta by direct protein-to-protein interaction[13].

Familial Alzheimer's disease accounts for only 5% of all AD cases. FAD is due to mutations in the APP, PSEN1, or PSEN2 genes, which cause an increased production of amyloid plaques. These three gene mutations affect the cleavage of the amyloid precursor protein (APP) and lead to the formation of amyloid beta plaques, the first hallmark of Alzheimer's disease which then gives rise to the second hallmark, neurofibrillary tangles. APP is either cleaved by α-secretase, which is normal cleavage, or by β- and γ- secretase, which is abnormal cleavage. When α-secretase cleaves APP, the cleavage occurs in the middle of the Aβ domain and is not amyloidogenic, but when β- and γ- secretase cleave APP, it does not occur in the middle of the Aβ domain and results in the release of neurotoxic Aβ peptides, which accumulate together into oligomer aggregates[9]. A mutation in the APP gene enables preferential cleavage by β-secretase by inhibiting α-secretase cleavage. PSEN1 and PSEN2 are both components of the γ-secretase complex. As such, a mutation in either PSEN1 or PSEN2 results in an increase in γ-secretase cleavage (Fig 1-3). The mutations in APP, PSEN1, and PSEN2 and resulting increase in amyloidogenic cleavage, lead to an increase in Aβ peptide production. The excess Aβ can impair synaptic function between neurons and trigger inflammatory response. This leads to neuronal death and the Aβ plaques[14].

1.2 Models to Study Alzheimer's Disease

Alzheimer's disease remains an elusive disease, as the mechanisms driving it are not yet understood. In order to gain the knowledge necessary for effective treatment and eventually a cure, it is imperative to study the disease in depth. There are three main techniques used to study AD. The first is animal models, the second is human cadaveric studies, and the third is the use of stem cells. The use of these various models helps in the understanding of AD.

1.2.1 Animal Models

Animal models provide researchers with the unique opportunity to study Alzheimer's disease as it is developing, thus allowing the study of the pathophysiology and manifestation of the disease. Animal models may also be used to study the effect of various drugs on the disease. As previously mentioned, the specific causes of SAD are not known, while FAD is associated with various gene mutations. Due to this, animal models have been limited to recapitulating the disease in only the familial form[15].

The standard mouse model used to study AD contains one mutation, but there has been advancement in the field that led to a triple transgenic mouse model. First, a tau transgenic mouse, which has been shown to reproduce aggregation and neurofibrillary tangle formation, was crossed with an APP transgenic mouse, which has been shown to reproduce β-amyloid plaque formation and memory impairment. Then, by combining the expression of APP and tau on a PSEN1 background, a triple transgenic mouse model was created[16] (Fig 1-4). This model allows for a deeper understanding of AD, as it more closely recapitulates the human pathology, through the tangle and plaque formation.

The use of transgenic mouse models has exponentially expanded the understanding of disease manifestation, but there are limitations when it comes to animal studies. For example, the disease may manifest differently in a mouse compared to how it manifests in humans. Although the new triple transgenic mouse model more closely recapitulates the human form of the disease, it is necessary for the mouse to carry multiple mutations. This is not how the disease presents itself in a human; a human with FAD either has an APP, PSEN1, or PSEN2 mutation.

1.2.2 Postmortem Studies

A second model used to study Alzheimer's disease is postmortem studies. The need for postmortem studies arises from the limitations of animal models. Namely, animal models do not fully recapitulate human disease complexities due to genetic and environmental factors[17]. Postmortem studies allow researchers to look at the disease as it is presented in humans. With this, we are able to further understand the affects that Alzheimer's have on the brain. However, these studies show just a snap shot of the disease and do not help in the understanding of the pathophysiology. There is also a limited amount of these samples available to study.

1.2.3 Stem Cells

The previous two models of Alzheimer's disease, animal models and postmortem studies, are helpful in certain aspects of the disease, but are lacking key features necessary to fully understanding its complexities. For animal models, the key features missing are that the disease is not manifested in the same way within the mouse as it is in humans, and the sporadic case of AD is very hard to recapitulate since the pathophysiology is not fully understood. For postmortem studies, the key feature missing

is that there is only a snapshot of the disease and it is not possible to see how the disease came to be. Stem cell models address these various limitations of the animal models and postmortem studies. There are two types of stem cells, embryonic stem cells (ESCs) and human induced pluripotent stem cells (hiPSCs). ESCs are derived from the inner cell mass of blastocysts and can indefinitely self-renew or be differentiated into different cell types[18]. This gives rise to the ability to understand disease pathophysiology through disease modeling, screening drugs, and cell replacement therapy. The limitation of ES cells is the difficulty of generating disease-specific ES cell lines. For this reason, human induced pluripotent stem cells (hiPSCs) and neural progenitor cells (NPCs) are used throughout the research being presented.

1.2.4 Human Induced Pluripotent Stem Cells

Human induced pluripotent stem cells are derived from reprogramming somatic cells through the overexpression of the transcription factors Oct3/4, Sox2, c-Myc, and Klf4[19]. With this, the ability to study the exact mechanisms behind the disease for both familial and sporadic cases of Alzheimer's disease has become a possibility. The ability to reprogram somatic cells from familial AD, sporadic AD, and healthy individuals and then differentiate these cells into neurons allows for the study of the disease in both its forms and helps to reveal the mechanisms behind it (Fig 1-4). As with all models, these models still have limitations. Particularly, the *in vitro* culturing may not be able to mimic the *in vivo* system quite right and lead to some difficulties in revealing vital information[20].

1.2.5 Neural Progenitor Cells

In order to use stem cells to investigate mechanisms of neurological disorders and develop more physiologically relevant disease models, methods for generation of disease

specific neural progenitor cells are needed. NPCs are multipotent stem cells that are able to differentiate into the various cell types of the central nervous system[21]. NPCs can be generated from disease specific hiPSCs and provide invaluable insights into AD mechanisms that cannot be recapitulated in animal models and cannot be seen in the snap shot from postmortem studies. The benefit of using NPCs to model Alzheimer's disease rather than hiPSCs comes from the fact that the NPCs are a population of cells closer to the final differentiated neurons and are biased to a forebrain fate.

1.2.5.1 Materials and Methods

1.2.5.1.1 Medias

Various medias were used for hiPSC maintenance and NPC formation. Essential 8 (DMEM/F12 supplemented with 543 ug/mL NaHCO3, 64 ug/mL L-Ascorbic acid-2-phosphate, 140 ng/mL sodium selenite, 10.7 ug/mL Transferrin, 20 ug/mL insulin, 100 ng/mL FGF2, and 2 ng/mL TGFB) is a xeno-free, defined maintenance media used for long-term expansion of hESCs and hiPSCs. Several neuronal differentiation medias were used to drive cell fate of hiPSCs into cells of the central nervous system. Neural base media [NBM (DMEM/F12 supplemented with 0.5% N2, 0.5% B27, and 1% GlutaMAX)] is supplemented with small molecules and growth factors to make the neural induction media [NIM (NBM supplemented with 200 ng/mL Noggin and 0.5 uM Dorsomorphin)], neural expansion media [NEM (NBM supplemented with 20 ng/mL basic fibroblast growth factor and 20g/mL epidermal growth factor)], and neural differentiation media [NDM (20 ng/mL brain-derived neurotrophic factor and 20 ng/mL glial-derived neurotrophic factor)].

1.2.5.1.2 Methods

HiPSCs were grown on Matrigel™ coated polystyrene tissue culture plates with E8 medium. Upon 80% confluency, cells were dissociated down to single cell using Accutase®. Cells were then plated at $2x10^6$ cells per well on an ultra-low attachment plate in 3 mL NIM with 3 uL 5mM ROCK inhibitor (ROCKi) and placed on an orbital shaker at 95 RPM inside the CO_2 incubator. Embryoid bodies (EBs) should aggregate in the wells. The first day, the total volume was brought up to 4 mL per well, and then each day afterward half of the medium was changed. After five days, EBs were plated on Matrigel™ coated polystyrene tissue culture plates to form neural rosettes. A full media change was performed each day. After seven days of neural rosette formation, rosettes were dissociated with Accutase® and replated onto poly-L-ornthine/Laminin (PLO/LN) coated plates in 3mL NEM and 3uL 5mM ROCKi per well. A full media change was done on days 1 and 3. Upon 90% confluency, cells were passaged. After several passages, only NPCs will be adhered to the PLO/LN plates. The NPCs can then be expanded indefinitely or differentiated into cells of the CNS. To differentiate the NPCs into neurons, NDM was exchanged in place of NEM and changed daily for four weeks[21] (Fig1-6 A-B).

1.2.5.1.3 Immunofluorescence Analysis

Immunofluorescence was performed on the differentiated hiPSCs to evaluate the protein expression to confirm NPC and neuronal identity. NPCs and differentiated neurons were dissociated into single cell suspension by using Accutase® and plated onto Matrigel™ coated plates in NEM/ROCKi. After 24 hours, the wells were washed twice with PBS. The cells were fixed by adding fixation buffer to each well, followed by a 10-

minute incubation at room temperature. The wells were then again washed twice with PBS. The cells were permeablized by adding perm buffer to each well, followed by a 30-minute incubation at room temperature. Primary antibodies were added to the wells and incubated overnight at 4°C. The wells were then washed twice with PBS and secondary antibodies were added to each well. After an hour incubation at room temperature in the dark, the wells were washed twice with PBS. Hoescht was added to each well to counterstain nuclei. The plate was incubated for 10 minutes at room temperature in the dark, followed by washing the wells twice with PBS. The cells were then imaged using a fluorescent microscope.

1.2.5.1.4 Flow Cytometry Analysis

Flow cytometry was used to determine the percent of hiPSCs that were differentiated into NPCs by staining the cells with SOX1, SOX2, and Nestin antibodies. To fix the NPCs, the cells were spun down at 5000 rpm for three minutes at 4°C and 500 uL of fixation buffer was added to the cell pellet after removing the supernatant. The cells were then incubated at room temperature in foil for thirty minutes, followed by two FACs washes. The cells were then permeabilized. The cells were centrifuged at 5000 rpm for three minutes at 4°C and 500 ul of perm buffer was added. The cells were then incubated at 4°C on ice for thirty minutes in foil. The cells were then washed twice with FACs buffer. The cells were then stained with conjugated antibodies Nestin, SOX1, and SOX2 and allowed to sit for an hour, followed by two FACs washes. The cell pellets were then resuspended in 100 ul of PBS and ran on the flow cytometer. Gates were set using an unstained control.

1.2.5.2 Results

In order to confirm that the hiPSCs were differentiated into NPCs and then into neurons, two assays were performed: immunofluorescence and flow. Immunofluorescence to confirm NPC identity used SOX1 and SOX2 antibodies, both of which are NPC markers. The cells were positively stained for both of these markers, indicating that the cells were NPCs (Fig 1-6 C). Flow cytometry was run using SOX1, SOX2, and Nestin to confirm NPC identity. Over 90% of the cells were positively stained for SOX1, SOX2, and Nestin, indicating that the hiPSCs were differentiated into NPCs (Fig 1-6 D). Immunofluorescence to confirm neuronal identity used MAP2 and B3T antibodies, which are dendrite and mature neuronal markers. The cells were stained for both MAP2 and B3T, indicating that the cells were differentiated into neurons (Fig 1-6 E).

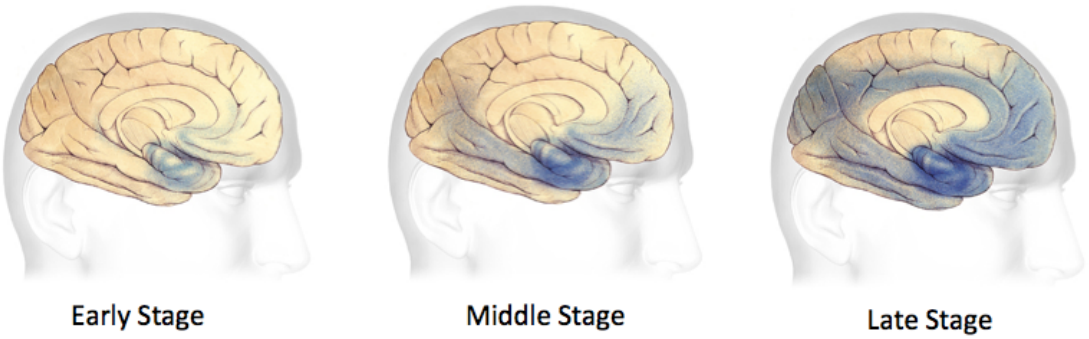

Figure 1-1: Progression of Alzheimer's Disease
Amyloid beta plaques and neurofibrillary tangles accumulate in different regions of the brain during the three stages of Alzheimer's disease, leading to the cognitive decline[4].

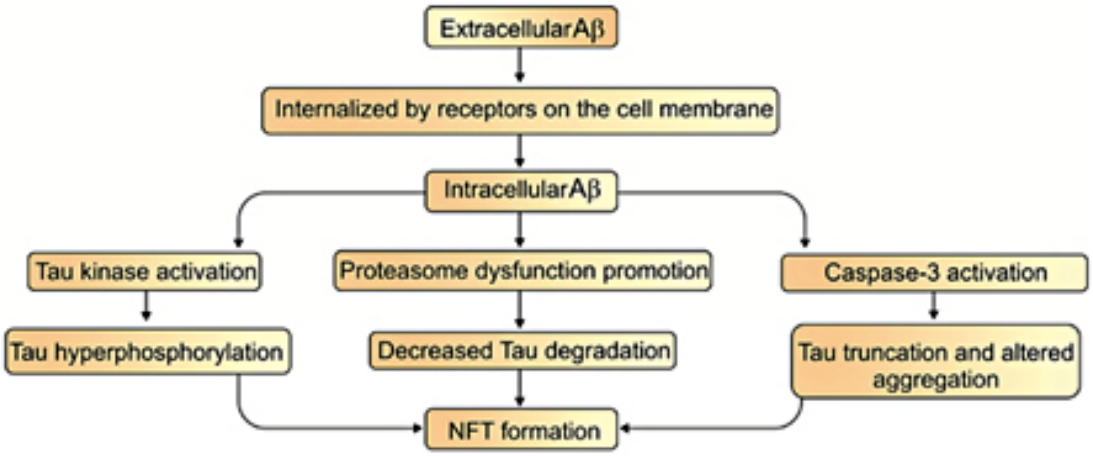

Figure 1-2: Interrelationship Between Amyloid Beta Plaques and Neurofibrillary Tangle Formation
Schematic depicting how extracellular amyloid beta is internalized by receptors on the cell membrane, then the intracellular amyloid beta leads to neurofibrillary tangle formation through tau kinase activation, proteasome dysfunction promotion, or caspase-3 activation[8].

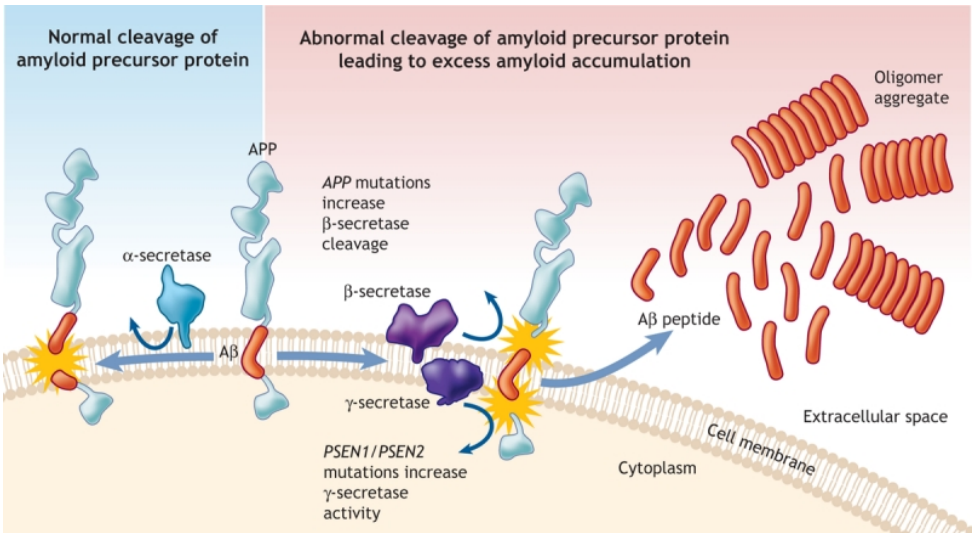

Figure 1-3: Cleavage of Amyloid Precursor Protein

Figure depicts the non-amyloidogenic pathway versus the amyloidogenic pathway. Alpha-secretase cleavage of APP prevents formation of a-beta through the non-amyloidogenic pathway, while beta- and gamma-secretase cleavage of APP forms a-beta through amyloidogenic pathway[14].

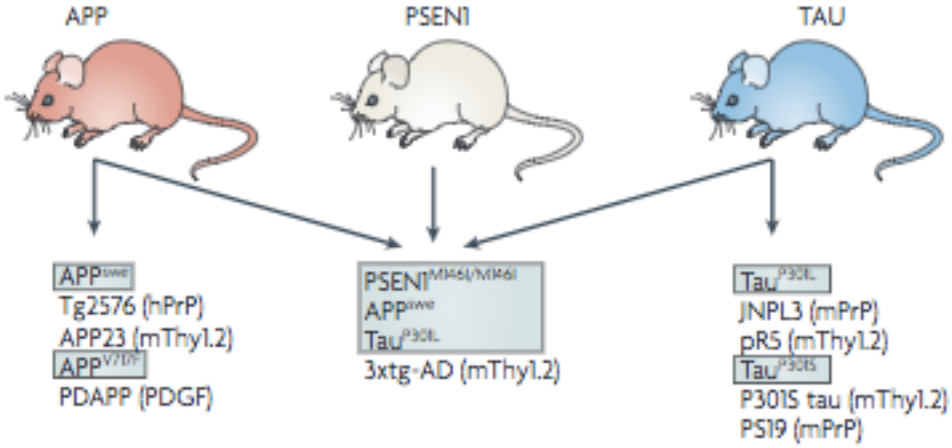

Figure 1-4: Triple Transgenic Mouse Model
The triple transgenic mouse model was created by crossing APP, PSEN1, and TAU transgenic mice together. This triple transgenic mouse model more closely recapitulates the human form of AD[16].

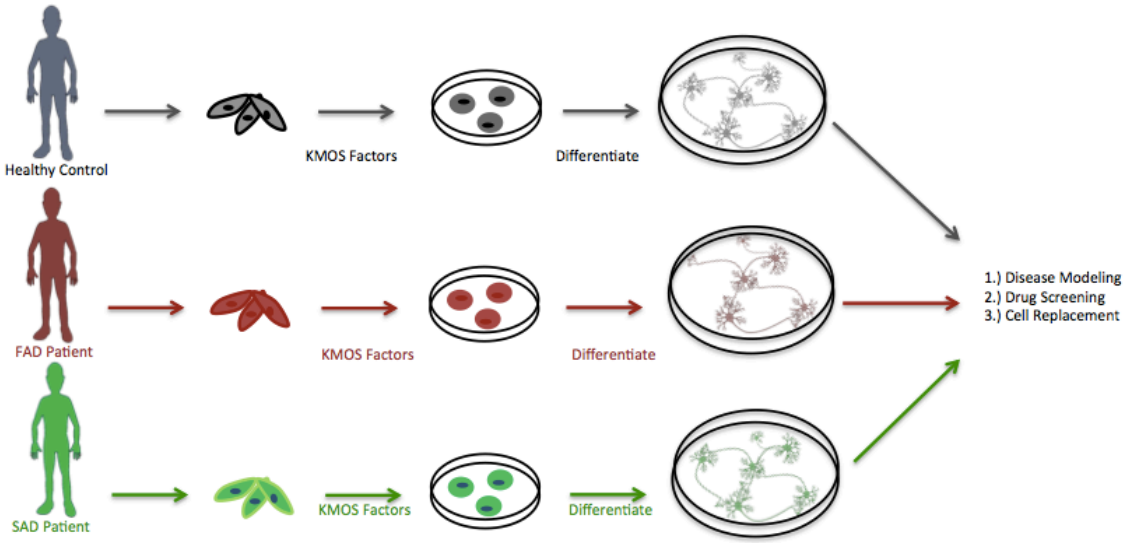

Figure 1-5: HiPSC Reprogramming for Stem Cell Modeling of Alzheimer's Disease
Somatic cells from a healthy control, a FAD patient, and a SAD patient can be reprogrammed and differentiated into neurons to model the disease, screen drugs, and for cell replacement therapy.

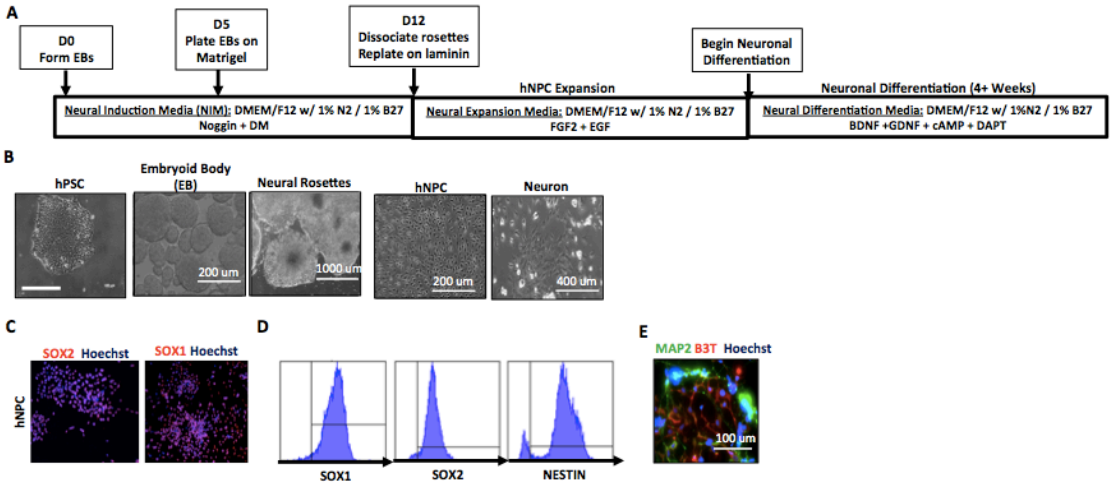

Figure 1-6 Generation and Neuronal Differentiation of Human Pluripotent Stem Cell (hPSC)-Derived Neural Progenitor Cells (hNPCs). (A) Schematic of protocol for the generation and neuronal differentiation of hPSC-derived hNPCs. (B) Phase contrast images of hPSCs, embryoid bodies (EBs), neural roesttes, hNPCs, and neurons. (C) Immunofluorescent and (D) phase contrast images of hNPCs for multipotent markers SOX1, SOX2, and NESTIN. (E) Immunofluorescent analysis of mature neuronal markers MAP2 and B3T from neuronal cultures generated from hNPCs.

CHAPTER 2: Two Dimensional Stem Cell Models of Alzheimer's Disease

2.1 Introduction

The use of hiPSCs has provided a unique opportunity to study Alzheimer's disease in a way not possible before. The most common method of hiPSC modeling is to use a two-dimensional (2-D) system. Primary fibroblasts can be reprogrammed from individuals with FAD, SAD, or a healthy individual into hiPSCs. These hiPSCs can then be differentiated to study relevant phenotypes of Alzheimer's disease[22].

2.2 Materials and Methods

2.2.1 Two- Dimensional Differentiation of hiPSCs to Neuronal Lineage

HiPSCs were grown on Matrigel™ coated polystyrene tissue culture plates with E8 medium. Upon 80% confluency, cells were dissociated down to single cell using Accutase®. Cells were then plated at $2x10^6$ cells per well on an ultra-low attachment plate in 3 mL NIM with 3 uL 5mM ROCK inhibitor (ROCKi) and placed on an orbital shaker at 95 RPM inside the CO_2 incubator. Embryoid bodies (EBs) should aggregate in the wells. The first day, the total volume was brought up to 4 mL per well, and then each day afterward half of the medium was changed. After five days, EBs were plated on Matrigel™ coated polystyrene tissue culture plates to form neural rosettes. A full media change was performed each day. After seven days of neural rosette formation, rosettes were dissociated with Accutase® and replated onto poly-L-ornthine/Laminin (PLO/LN) coated plates in 3mL NEM and 3uL 5mM ROCKi per well. A full media change was done on days 1 and 3. Upon 90% confluency, cells were passaged. To differentiate the NPCs into neurons, NDM was exchanged in place of NEM and changed daily for four weeks[21] (Fig 2-1).

2.3 Results

Various assays to were performed to determine if the differentiated hiPSCs were able to reproduce AD phenotypes. These 2-D derived neurons generated from FAD patients showed increased levels of secreted Aβ, increased levels of phospho-tau, and sensitivity to glutamate excitotoxicity (Fig 2-2 A-C). However, phenotypes that are often associated with late onset such as synaptic loss or pathological hallmarks such as Aβ plaques or neurofibrillary tangles are absent. Moreover, when SAD patient derived hiPSCs are differentiated, no disease related phenotypes are observed.

2.4 Discussions and Conclusions

Although these 2-D systems recapitulate Alzheimer's disease to some degree, they fail to show robust AD hallmarks[23]. The crucial limitation of 2-D culture systems is the simplicity of them; the brain is a complex three-dimensional system and is not recapitulated. In 2-D culture systems, the amyloid beta that is secreted by the hiPCSs is released into the media, which is subsequently switched out everyday[24] (Fig 2-3). It is imperative that the model being used is able to fully recapitulate all of the aspects of the disease, including hallmarks. It is hypothesized that a three-dimensional model would better provide a native brain-like environment and therefore better model AD[24]. It is also hypothesized that having a pure population of neurons of forebrain identity would help to recapitulate the disease and its phenotypes. Therefore, this three-dimensional model would need to be optimized to produce only neurons of forebrain identity.

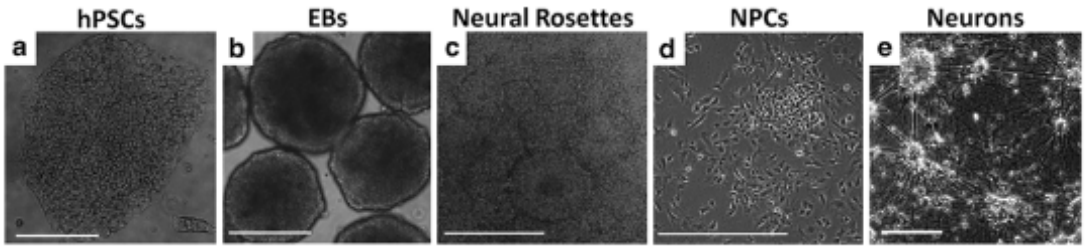

Figure 2-1: Morphologies of Cell Types at Each Stage of the Protocol.
Phase contrast images of (**a**) hPSCs (scale bar = 200 um), (**b**) day 5 EBs (scale bar = 200 um), (**c**) day 7 neural rosettes (scale bar = 500 um), 9**d**) passage 3 NPCs (scale bar = 100 um), and (**e**) week 4 neurons (scale bar = 200 um).

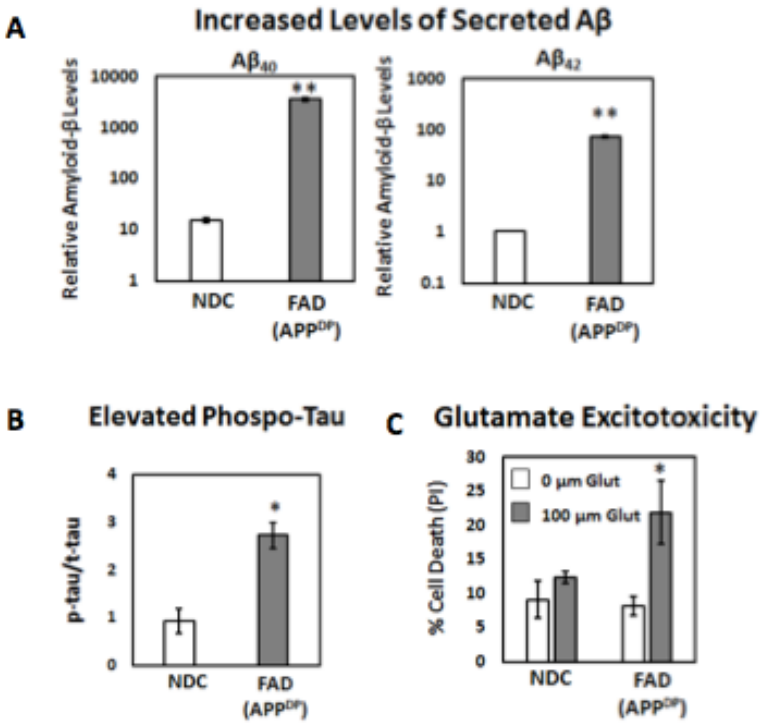

Figure 2-2: Alzheimer's Disease Phenotype Analysis. (A) Levels of secreted Aβ in control and FAD patient derived hiPSCs, (B) Levels of phosph-tau in control and FAD patient derived hiPSCs, and (C) Glutamate excitotoxicity of control and FAD patient derived hiPSCs. Asterisks represent statistical significance.

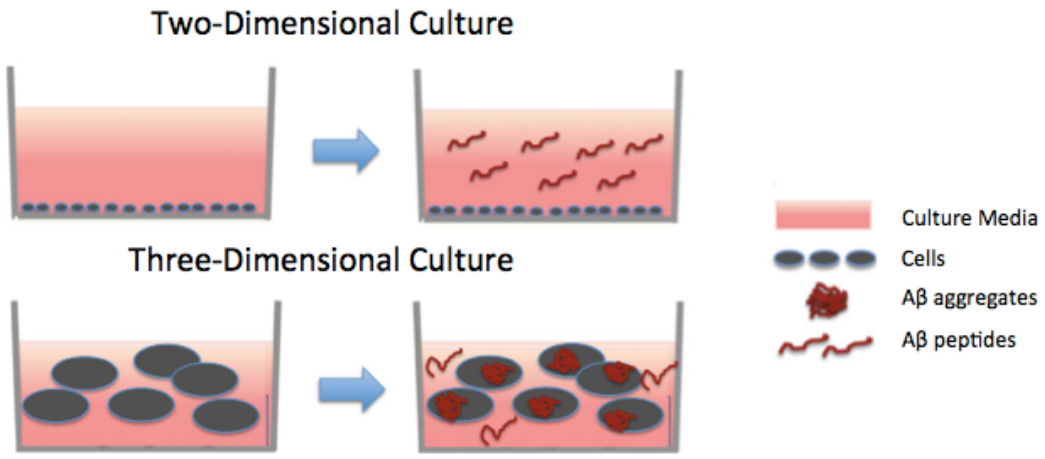

Figure 2-3: 2-D Versus 3-D Cell Culture Systems.
Due to media changes, two-dimensional cell culture systems do not allow for the aggregation of amyloid beta, one of the pathological hallmarks. Three-dimensional cell culture systems could solve this problem by keeping the Aβ peptides out of the media changes [24].

CHAPTER 3: Development of a Three- Dimensional Model of Alzheimer's Using hiPSCs

3.1 Introduction

In order to model Alzheimer's disease as accurately as possible, a model that recapitulates the complex microenvironment of the brain as well as the disease itself is required. There are two main categories for three-dimensional hiPSC modeling. The first is 3-D hydrogels and the second is organoids. Typically, Matrigel™ is used as the 3-D matrix in hydrogels, as it contains high levels of brain extracellular matrix proteins[20]. Because three-dimensional hydrogels lack the vascular systems that support oxygenation, nutrition, and waste removal present in *in vivo* tissue, these occur through diffusion. Therefore, the thickness of the 3-D models is very important in allowing proper diffusion. The hydrogels contain cells in various depths and therefore varying amounts of nutrients are being supplied to the cells[25]. Organoids are not in a matrix, allowing for even diffusion of nutrients to all of the cells. Organoids are capable of self-organization and are structurally similar to native tissue[26].

3.1.2 Development of a Basic hiPSC-Based Organoid System

Here, a three-dimensional organoid hiPSC model is developed to model AD. Through this research the gap in knowledge left by 2-D cultures and *in vivo* models is bridged, due to the fact that organoids are very close to native physiology and are much easier to manipulate in terms of signaling pathways and genome editing[27]. The development of this 3-D organoid system uses non-specific differentiation of hiPSCs, thus containing neurons of the forebrain, midbrain, and hindbrain regions.

3.1.3 Development of Regionally Patterned hiPSC Organoid System

Alzheimer's disease affects the cholinergic neurons within the basal forebrain[28]. The non-specific differentiation of the hiPSCs in the 3-D model leads to differentiation of neurons of all regions of the brain. In order to more closely study Alzheimer's disease, the ability to generate a homogenous culture of forebrain neurons is imperative[29]. Therefore, the 3-D hiPSC model was adapted to incorporate regional patterning. Through research it has been found that endogenous WNT signaling gives rise to the heterogeneity that is seen in hiPSC differentiation[30]. Exogenous modulation of Wnt signaling has been shown to influence the regional identity of NPCs and reduces the heterogeneity of neuronal differentiation[30] (Fig 3-1). The manipulation of Wnt signaling levels is done through the addition of IWP2 or CHIR. IWP2 is used to inhibit the Wnt pathway[31], whereas CHIR is used to stimulate Wnt signaling[32]. Using these small molecules to manipulate the level of Wnt signaling allows for a homogenous population of neuronal cells of the forebrain region, which can then be used to study Alzheimer's in a more specific way.

3.2 Materials and Methods

3.2.1 Non-Specific Three-Dimensional Neuronal Differentiation Using hiPSCs

HiPSCs were maintained on Matrigel™ coated plates until the cells reached 80% confluency. The hiPSCs were then dissociated into single cell using Accutase ® and plated at 2.0 x 10^6 cells per well on ultra-low attachment plates and placed on an orbital shaker at 95 RPM in a 37°C, 5% CO_2 incubator. Media volume was 3 mL of NIM and 3 uL 5mM ROCKi per well. The cells begin to form into embryoid bodies (EBs) on the first day. The EB media is exchanged with 2 mL fresh NIM everyday during the first six

days to force the cells down the neural ectodermal pathway using dual SMAD inhibition. Through this, the hiPSCs become NPCs. On the seventh day, the media is changed from NIM to NEM to expand the EBs. NEM is used for 18 days, exchanging half of the media daily. The NPCs are proliferating during this time and growing in size. After the expansion phase, on day 25, the media is changed from NEM to NDM to begin to differentiate the NPCs into neurons and neuronal subtypes. Half of the media was exchanged daily. After 18 days of differentiating, on day 43, the media was switched from NDM to NBM. Half of the media was exchanged daily[33] (Fig 3-2 A).

3.2.2 Regionally Patterned Three-Dimensional Neuronal Differentiation Using hiPSCs

HiPSCs were maintained on Matrigel™ coated plates until the cells reached 80% confluency. The hiPSCs were then dissociated into single cell using Accutase ® and plated at 2.0 x 10^6 cells per well on ultra-low attachment plates and placed on an orbital shaker at 95 RPM in a 37°C, 5% CO_2 incubator to form EBs. Media volume was 3 mL of NIM and 3 uL 5mM ROCKi per well. To pattern the EBs, three different conditions were maintained. The first condition was forebrain inducing, where 0.2 uM IWP2 was added to the NIM media to inhibit Wnt signaling. The second condition was midbrain inducing, where 0.1 uM CHIR was added to the NIM media to introduce low levels of Wnt. The third condition was hindbrain inducing, where 0.75 uM CHIR was added to the NIM media to introduce high level of Wnt. These various medias are exchanged with 2 mL of fresh conditioned NIM everyday for the first six days. After these first days, the NPCs are formed with determined lineages to forebrain, midbrain, or hindbrain regions. The

remaining protocol to differentiate these NPCs follows that of the non-specific 3-D differentiation (Fig 3-2 A).

3.2.3 Real Time Quantitative Polymerase Chain Reaction Analysis

Real time quantitative polymerase chain reaction (RT-qPCR) was performed at specific time points in order to confirm the identity of the hiPSCs throughout the differentiation process. For both the non-specific and the regionally patterned embryoid bodies, EBs were collected directly from the well and spun down in 1 mL epitubes. RNA isolation was performed using the NucleoSpin™ RNA kit (Macherey-Nagel) and cDNA was synthesized using iScript RT Supermix (BioRad). RT-qPCR was done with iTaq Universal SYBER Green SMX 500 (BioRad) to form the master mix. The BioRad CFX384 real-time PCR detection was used to run the RT-qPCR. Results were analyzed from the cycle threshold (CT) values using delta-delta CT method, with the fold change normalized to D0 samples. Results were compiled into heatmaps using the log base 10 of the fold change.

3.3 Results

Real time quantitative polymerase chain reaction was performed on the both the non-specific and the regionally pattened 3-D samples at various time points in order to confirm that the hiPSCs were differentiating from hiPCSs into neurons and neuronal subtypes. First, the non-specific differentiated hiPSCs were analyzed. Samples were taken on D0 for normalization and three samples were taken past D50, so the hiPSCs would be fully differentiated into neurons and neuronal subtypes. There were three panels of primers that the samples were run through (Fig 3-2 C). The first panel was post-mitotic neuron markers. These primers include MAPT, NCAM, RBFOX3, and TUBB3. It can be

seen that the cells are not expressing any of these markers on day zero. However, the samples from day 50 onward express these markers highly, specifically the cells express MAPT and NCAM (Fig 2-3 C). The second panel was neurotransmitter markers, which included ACHE, EAAT3, GABRA, GRIN2A, SLC17A7, and SLC6A4. Again, the samples taken on day 0 did not express these markers, but the day 50 onward samples showed some expression. Specifically, ACHE, GABRA, and SLC17A7 were relatively highly expressed in these samples, whereas EAAT3, GRIN2A, and SLC6A4 were not (Fig 2-3 C). The final panel was astroglial markers, which included GFAP, MBP, and S100B. The samples taken on day 0 showed no expression of these markers, while the day 50 onward samples showed some expression (Fig 2-3 C).

Next, the 3-D regionally patterned hiPSC differentiation was analyzed using RT-qPCR. Samples were taken on D0 for normalization and two samples from day 50 onward were taken from each condition, so that the hiPSCs would be fully differentiated. The samples from all three conditions were run through various panels of primers for early anterior, posterior, and CNS markers (Fig 3-2 D) and then the forebrain patterned samples were run through a panel of mature cortical markers (Fig 3-2 E). The 200 nM IWP2 treated samples, which are forebrain patterned, showed high expression of forebrain markers, including DLX2, FOXG1, GSX2, GS2, LHX2, and SIX3, and little to no expression of midbrain or hindbrain markers (Fig 3-2 D). These forebrain-patterned samples also showed some expression of two of the mature cortical markers ASCL1 and EMX2 (Fig 3-2 E). The 100 nM CHIR treated samples, which are midbrain patterned, showed little to no expression of the forebrain or hindbrain markers, while showing expression of midbrain markers OTX2, EN2, and WNT1A (Fig 3-2 E). The 750 nM

CHIR treated samples showed expression of all three panels of markers- forebrain, midbrain, and hindbrain, though the expression of the hindbrain markers is the highest (Fig 3-2 E).

3.4 Discussions

Alzheimer's disease has a complex pathophysiology that is yet to be fully understood. Various models have been used to attempt to uncover these mechanisms, but due to limitations of each, the disease remains elusive. HiPSCs are becoming a popular way to study diseases *in vitro*, but heavily rely on 2-D substrates that do not reflect the 3-D complexity of native brain tissue and are therefore unable to replicate all aspects of AD. Therefore, the aim of this research was to develop a 3-D hiPSC model that can recapitulate the complexity of the brain and the disease.

3.4.1 Three- Dimensional hiPSC Differentiation

A 3-D organoid - based method, which provides a similar architecture to *in vivo* neural tissue, was developed as a way to model Alzheimer's disease. Through RT-qPCR analysis it was found that the hiPSCs could differentiate into post-mitotic neurons, astroglial cells, and neurotransmitters. It is important that the hiPSCs were able to differentiate into all three cell types due to the fact that many AD – related neuronal phenotypes are mediated by their interactions and signals from astrocytes, so a model lacking the presence of these cells would not be able to recapitulate the disease accurately[34]. The hiPSCs were successfully differentiated into neural cells, therefore overcoming the two-dimensional limitations. The second hypothesis in recapitulating the disease *in vitro* was that there is a necessity for a homogenous population of neurons of

forebrain identity. Therefore, this protocol was adapted to create homogenous populations of the three regions of the brain.

3.4.2 Regionally Patterned Three-Dimensional hiPSC Differentiation

Because Alzheimer's disease is directly related to the loss of synapses and neurons in the forebrain, it is critical to be able to generate a homogenous neuronal culture of forebrain identity in order to better model the disease and understand the mechanisms behind it. Previous methods based on modulating the amount of Wnt signaling in two-dimensions in order to differentiate hiPSCs into homogenous neural populations of specific regions were applied to the 3-D model that was developed[29]. RT-qPCR analysis revealed that manipulating Wnt levels during the neural induction phase of differentiation leads to more homogenous samples of the three brain regions. IWP2 treatment led to an increase in forebrain markers, low levels of CHIR treatment led to an increase in expression of midbrain markers, and high levels of CHIR treatment led to an increase in expression of hindbrain markers. The low and high levels of CHIR treatment led to a more heterogeneous population containing both midbrain and hindbrain markers present in both. This is likely due to the fact that the levels of CHIR used during treatment were not optimal in modulating the levels of Wnt signaling. In the future, these levels would need to be adjusted. Importantly, the 0.2 uM IWP2 treated samples produced an almost entirely homogeneous population of anterior patterned neurons. This produced a model in which the specific cells affected by AD can be studied.

3.5 Conclusion

Through this research, a three - dimensional hiPSC - derived organoid model has been developed that has laid the groundwork to begin to uncover the complex

pathophysiology behind Alzheimer's disease. This model has the ability to be adapted using AD patient - derived hiPSCs, providing the unique opportunity to see the disease manifest itself in both sporadic and familial cases in an environment very similar to that of *in vivo* native tissue. Because AD is manifested within the cortex, it is imperative to use a population of neurons within this region to truly understand the disease. For this reason, the original model was adapted to determine if homogenous populations of forebrain, midbrain, and hindbrain neuronal cells could be differentiated in three - dimensions, in order to further the understanding of AD. Homogeneous neuronal populations were created using the 3-D differentiation, allowing for a closer look into disease mechanisms.

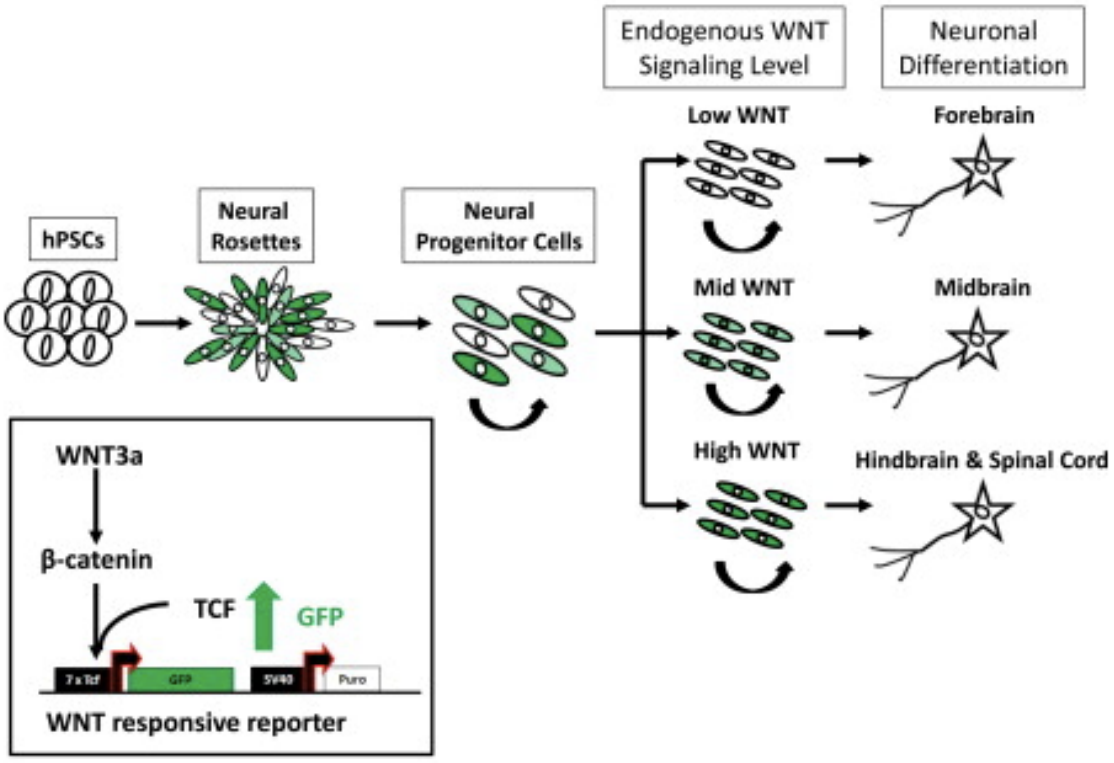

Figure 3-1: Summary of Endogenous WNT Signaling Using a GFP Reporter.
Manipulating endogenous WNT signaling levels give rise to the differentiation of forebrain, midbrain, and hindbrain neuronal subtypes. The presence of low WNT gives rise to forebrain, the presence of middle levels of WNT gives rise to midbrain neurons, and the presence of high levels of WNT gives rise to hindbrain and spinal cord neurons[30].

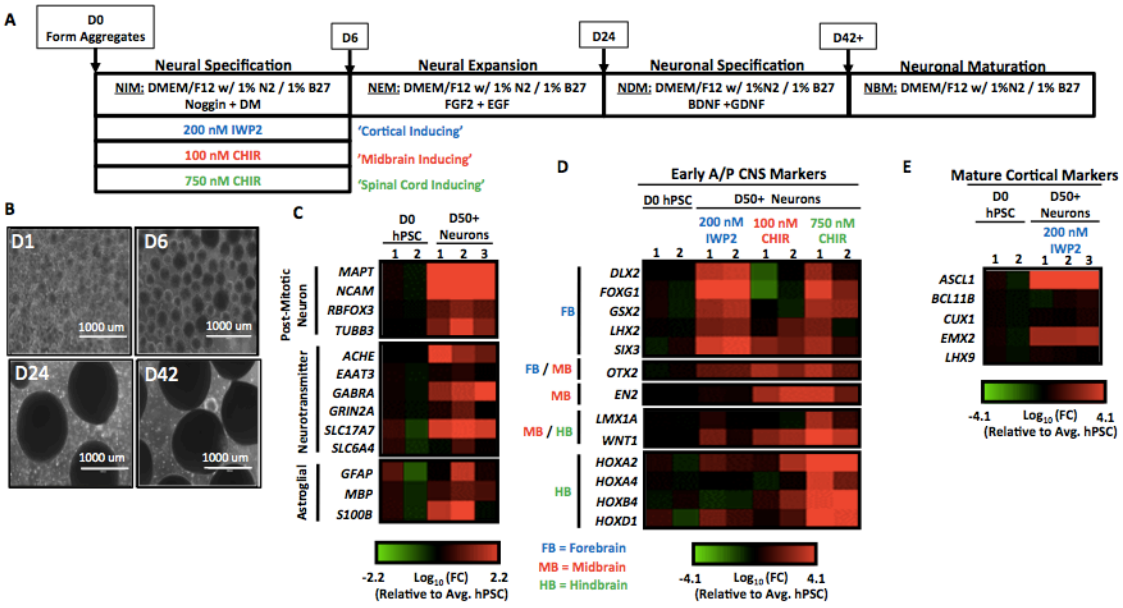

Figure 3-2. Generation of 3-D Neuronal Cultures from hPSCs. (A) Schematic of protocol for the generation of 3-D neuronal cultures from hPSCs. (B) Phase contrast images of 3-D aggregates at D1, D6, D24, and D42. (C) Quantitative PCR (qPCR) analysis for expression of neuronal-, neurotransmitter-,and astroglial-related markers in day 50+ (D50) differentiated cultures. (D) QPCR analysis of D50+ neuronal cultures generated using-specific subtype-differentiation protocols. (E) QPCR analysis of D50+ neuronal culture generated using cortical-specific differentiation condition.

CHAPTER 4: Development of a Three-Dimensional Model Using Neural Progenitor Cells

4.1 Introduction

While the results from the 3-D hiPSC model showed some promise, the differentiation was not as efficient as expected. With that, it was hypothesized that starting with a population of cells closer to the endpoint would help to improve the process. Here, a 3-D model was developed using non-demented control neural progenitor cells. This NPC line provide the unique opportunity to model AD in three-dimensions and provide new insights into the disease. The relative gene expression and protein expression were analyzed to determine if the NPCs were differentiating into neuronal cell types.

4.2 Materials and Methods

4.2.1 Three-Dimensional Differentiation Using Non-Demented Control NPCs

NPCs were maintained on PLO/LN coated plates until the cells reached 90% confluency. The NPCs were then dissociated into single cell using Accutase ® and plated at 1.0 x 10^6 cells per well on ultra-low attachment plates and placed on an orbital shaker at 95 RPM in a 37°C, 5% CO_2 incubator. Media volume was 3 mL of NEM and 3 uL 5mM ROCKi per well. The cells begin to form into embryoid bodies (EBs) on the first day. The EB media is exchanged with 2 mL fresh NEM everyday during the first seven days to support NPC proliferation and expansion. On the eighth day, the media is changed from NEM to NDM to expand the EBs. NDM is used for 18 days, exchanging half of the media daily. After the NPCS have differentiated for 18 days, the media is

changed from NDM to NBM. Half of the media was exchanged daily (ISSN 1548-7091) (Fig 4-1 A-B)

4.2.2 RT-qPCR Analysis

The RT-qPCR analysis followed the same protocol as previously described for the hiPSC 3-D models.

4.2.3 Cryopreservation

To maintain the 3-D structure of the differentiated EBs, cryopreservation was used. First, the EBs were fixed in 4% paraformaldehyde overnight. They were then washed with PBS and transferred to a 30% sucrose solution and kept in 4°C for 48-72 hours. The EBs were then transferred into embedding molds with OCT compound (Tissue-Tek), which were then frozen using methylbutane over dry ice. A cryostat was used to section EBs into 10 um-thick sections.

4.2.4 Immunohistochemistry Analysis

Immunohistochemistry was performed on NDC NPCs in their differentiated aggregate form to determine the protein expression and structure in three dimensions. Slides containing the aggregate sections were washed with PBS in a Coplin jar. Slides were then treated with a permeabilization solution for 15 minutes at room temperature in a Coplin jar, followed by a rinse with PBS. Excess fluid was removed and the sections were enclosed in a circle using a PAP pen. Blocking serum was added to the slides for one hour at room temperature. Slides were tapped on a paper towel to remove the excess blocking serum. Primary antibodies were added to the slides and incubated in a staining box overnight at 4°C. The slides were then rinsed with PBS and washed in PBS three times for 15 minutes each in a Coplin jar. Slides were tapped on a paper towel to remove

excess PBS. The secondary antibody was added and the slides were incubated in the dark at room temperature for one hour. Slides were rinsed with PBS and washed two times with PBS for 15 minutes each in a Coplin jar. DAPI was added for 5 minutes and the sections were washed PBS. Mounting solution was added and a coverslip was applied and sealed with clear nail polish. Slides were allowed to dry for one hour at room temperature in the dark. Slides could then be imaged using a fluorescent microscope.

4.3 Results

Real time quantitative polymerase chain reaction was performed on the NDC NPC 3-D samples on day 0 and two samples from day 50 onward in order to confirm that the NPCs were differentiating from NPCs into neurons and neuronal subtypes. Immunohistochemistry was also performed on the NDC NPC samples on day 50 to confirm the protein expression of these samples while still in the 3-D structure. First, the NDC NPC samples were analyzed using RT-qPCR and run through five panels of primers: post-mitotic neuron, neurotransmitter, astroglial, early forebrain, and mature cortical markers (Fig 4-1 C, E). The samples taken on day 0 show no expression of post-mitotic neurons, neurotransmitter, astroglial markers, early forebrain, or mature cortical markers. The samples taken on day 50 onwards show mid to high expression post-mitotic neuron markers, specifically RBFOX3 and TUBB3 (Fig 4-1 C). The day 50 onward samples also show mid to high levels of expression of neurotransmitter markers, specifically GABRA, SLC17A7, and SLC6A4. The day 50 onward samples also show mid levels of expression of astroglial markers EAAT2, GFAP, and MBP. Importantly, the NDC NPCs were able to produce neurons that express early forebrain and mature cortical markers (Fig 4-1 E) without needing to pattern them. The NDC NPC 3-D samples were

then analyzed using immunohistochemistry in order to determine protein expression. The NDC NPC 3-D aggregates were stained for post-mitotic neuronal (NESTIN, RBFOX3, TUBB3) and astroglial markers (GFAP) on day 50 cultures (Fig 4-1 D). It can be seen that the samples are stained positively for all four of these markers. The protein expression of a forebrain marker, FOXG1, was also analyzed on day 50 and it can be seen that the sample is expressing this (Fig 4-1 F).

4.4 Discussion

Through hiPSC modeling of Alzheimer's disease, the pathophysiology begins to be understood, but the techniques have not provided an optimal system for modeling the disease. The hiPSC 2-D models do not allow for the development of AD hallmarks and the hiPSC 3-D models did not efficiently differentiate hiPSCs into neuronal cell types. Thus, AD remains elusive in its development and a better model needed to be developed. Thus, a 3-D NPC organoid model was created, as NPCs are closer to the final lineage and are biased to a forebrain fate. A control neural progenitor cell line, NDC NPC, was used in the development of this 3-D NPC organoid model to determine if the three-dimensional organoid protocol could be used with NPCs and optimized to produce a higher efficiency of cells being differentiated into neurons, astroglia, and neurotransmitters. It was found through RT-qPCR as well as immunohistochemistry that the NDC NPC EBs expressed high levels of post-mitotic neuronal, astroglial, and neurotransmitter markers. It was also found that the NDC NPCs differentiated into a high population of neurons with anterior identity. It was found that beginning with a population of pure NPCs leads to a higher efficiency in differentiation.

4.5 Conclusions

Here, a three-dimensional model of Alzheimer's disease using hiPSCs was developed and then adapted for the use of NPCs. This NPC model provides the ability to use patient-derived hiPSCs that are then differentiated into a pure population of NPCs. Once these NPC lines are created, the 3-D differentiation can be used to study the pathogenesis of AD. The differentiation protocol for the diseased cell lines needs to be optimized further in order to uncover information about AD.

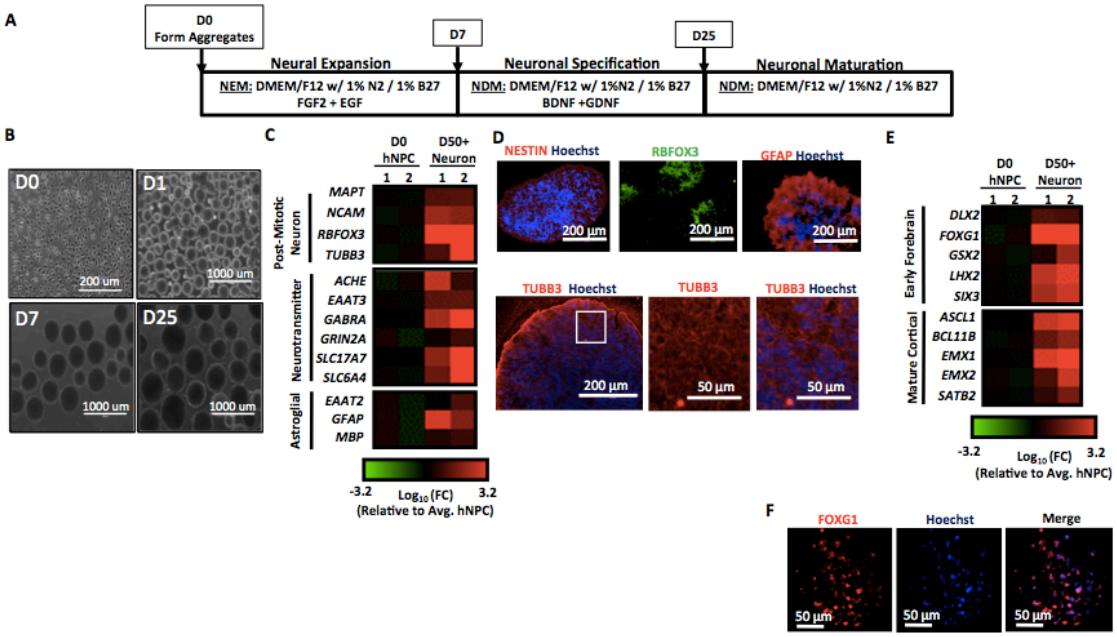

Figure 4-1. Generation of 3-D Neuronal Cultures from hNPCs. (A) Schematic of protocol for generation of 3-D neuronal cultures from hNPCs. (B) Phase contrast images of hNPCs as well as D1, D7, and D25 3-D aggregates. (C) QPCR analysis for expression of neuronal-, neurotransmitter-, and astroglial-related markers in day 50+ (D50) differentiated cultures. (D) Immunofluorescence for post-mitotic neuronal (NESTIN, RBOX3, TUBB3) and astroglial markers (GFAP) in D50+ neuronal cultures. (E) QPCR analysis for expression of early forebrain and mature cortical markers in D50+ neuronal cultures. (F) Immunofluorescence analysis for FOXG1 in D50+ neuronal cultures.

CHAPTER 5: Conclusions and Future Work

5.1 Conclusions

The overall goal of this research was to develop a robust and adaptable platform in which to model Alzheimer's disease. A three-dimensional organoid - based system was chosen over other 3-D platforms, such as hydrogels, due to the fact that organoids are self-organizing and better recapitulate the *in vivo* native tissue in which AD is developed. First, a 3-D organoid model was developed using hiPSCs. After it was determined that the hiPSCs could differentiate in this 3-D platform, the aim was to more closely mimic the *in vivo* environment in which AD occurs. In order to accomplish this, the Wnt pathway was modulated to generate a homogeneous population of hiPSCs of anterior identity, where AD is developed. This gives a closer look at the region in which the brain is affected by AD. The hiPSC protocol was then adapted for use with NPCs due to the fact that NPCs are closer developmentally to neurons and are biased towards a forebrain fate. In doing so, a control line, NDC NPC, was differentiated in 3-D. The NDC NPC line showed much higher expression of neuronal, astroglial, and neurotransmitter markers than did the hiPSC line, and also expressed numerous anterior neuronal markers. These results show that the 3-D protocol using NPCs is successful in efficiently differentiating cells into the neuronal cell types as well as in the region of interest. The 3-D NPC protocol allows for a higher efficiency of differentiation as well as a shorter amount of time to do so. For these reasons, the focus of the research has been shifted to using various NPC lines. Through the research that was conducted during my thesis, a three-dimensional model of Alzheimer's disease was developed and then optimized for highly efficient differentiation of NPCs into neuronal subtypes. This research provides an *in*

vitro model that recapitulates the complexities of AD and can be used to study disease mechanisms and develop therapies.

5.2 Future Work

In order to better characterize the cells that are being differentiated with the various 3-D models presented within this research, various assays need to be performed. The first assay to characterize the neurons is electrophysiology. There have been attempts to patch clamp the differentiated hiPSCs and NPCs in order to determine if they are firing action potentials, but they have remained unsuccessful. Differentiated neurons have been taken from their 3-D state and replated onto 2-D cultures and then patch clamped. It is hypothesized that during this replating process, the neuronal synaptic network may be disrupted, causing no action potentials to be detected. Different methods are being explored on measuring these action potentials, including MEA. The next goal going forward is to adapt this protocol for use with diseased cell lines. This will allow the study of the pathophysiology of both the sporadic and familial cases of Alzheimer's disease. Once these diseased lines are in culture, assays that characterize the extent of AD hallmarks will need to be performed.

BIBLIOGRAPHY

1. 2016 Alzheimer's disease facts and figures. *Alzheimer's & Dementia* **12,** 459–509 (2016).

2. Alzheimer's Association. 2016 Alzheimer's disease facts and figures. *Alzheimers. Dement.* **12,** 459–509 (2016).

3. Alzheimer's Disease Fact Sheet | National Institute on Aging. (2016). Available at: https://www.nia.nih.gov/alzheimers/publication/alzheimers-disease-fact-sheet. (Accessed: 28th February 2017)

4. Alzheimer's Disease & Dementia | Alzheimer's Association. Available at: http://www.alz.org/alzheimers_disease_what_is_alzheimers.asp. (Accessed: 28th February 2017)

5. Progression.

6. Ballard, C. *et al.* Alzheimer's disease. *Lancet* **377,** 1019–1031 (2011).

7. Phiel, C. & Juret, Je. Alzheimer's Disease Amyloid Plaque Signaling Pathway | CST. (2009). Available at: https://www.cellsignal.com/contents/science-pathway-research-neuroscience/amyloid-plaque-formation-in-alzheimer-s-disease-signaling-pathway/pathways-alz. (Accessed: 28th February 2017)

8. Sadigh-Eteghad, S. *et al.* Amyloid-beta: a crucial factor in Alzheimer's disease. *Med. Princ. Pract.* **24,** 1–10 (2015).

9. Piaceri, I., Nacmias, B. & Sorbi, S. Genetics of familial and sporadic Alzheimer's disease. *Front. Biosci. (Elite Ed).* **5,** 167–77 (2013).

10. O'Brien, R. J. & Wong, P. C. Amyloid precursor protein processing and Alzheimer's disease. *Annu. Rev. Neurosci.* **34,** 185–204 (2011).

11. Pfrieger, F. W. Cholesterol homeostasis and function in neurons of the central nervous system. *Cell. Mol. Life Sci.* **60,** 1158–1171 (2003).

12. Kim, J., Basak, J. M. & Holtzman, D. M. The role of apolipoprotein E in Alzheimer's disease. *Neuron* **63,** 287–303 (2009).

13. Carter, D. B. The interaction of amyloid-beta with ApoE. *Subcell. Biochem.* **38,** 255–72 (2005).

14. Patterson, C. *et al.* Diagnosis and treatment of dementia: 1. Risk assessment and primary prevention of Alzheimer disease. *CMAJ* **178,** 548–56 (2008).

15. LaFerla, F. M. & Green, K. N. Animal models of Alzheimer disease. *Cold Spring Harb. Perspect. Med.* **2,** (2012).

16. Götz, J. & Ittner, L. M. Nucleus basalis of Meynert Animal models of Alzheimer's disease and frontotemporal dementia. doi:10.1038/nrn2420

17. Gomez-Nicola, D. & Boche, D. Post-mortem analysis of neuroinflammatory changes in human Alzheimer's disease. *Alzheimers. Res. Ther.* **7,** 42 (2015).

18. Vazin, T. & Freed, W. J. Human embryonic stem cells: derivation, culture, and differentiation: a review. *Restor. Neurol. Neurosci.* **28,** 589–603 (2010).

19. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131,** 861–872 (2007).

20. Yang, J., Li, S., He, X.-B., Cheng, C. & Le, W. Induced pluripotent stem cells in Alzheimer's disease: applications for disease modeling and cell-replacement therapy. *Mol. Neurodegener.* **11,** 39 (2016).

21. Brafman, D. A. in 87–102 (2014). doi:10.1007/7651_2014_90

22. Israel, M. A. *et al.* Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. *Nature* **482,** 216 (2012).

23. Choi, S. H., Kim, Y. H., Quinti, L., Tanzi, R. E. & Kim, D. Y. 3D culture models of Alzheimer's disease: a road map to a "cure-in-a-dish". *Mol. Neurodegener.* **11,** 75 (2016).

24. D'Avanzo, C. *et al.* Alzheimer's in 3D culture: challenges and perspectives. *Bioessays* **37,** 1139–48 (2015).

25. Yamada, K. M. & Cukierman, E. Modeling Tissue Morphogenesis and Cancer in 3D. *Cell* **130,** 601–610 (2007).

26. Turner, D. A., Baillie-Johnson, P. & Martinez Arias, A. Organoids and the genetically encoded self-assembly of embryonic stem cells. *BioEssays* **38,** 181–191 (2016).

27. Fatehullah, A., Tan, S. H. & Barker, N. Organoids as an in vitro model of human development and disease. *Nat. Cell Biol.* **18,** 246–254 (2016).

28. Mufson, E. J., Counts, S. E., Perez, S. E. & Ginsberg, S. D. Cholinergic system during the progression of Alzheimer's disease: therapeutic implications. *Expert Rev. Neurother.* **8,** 1703–18 (2008).

29. Cutts, J., Brookhouser, N. & Brafman, D. A. in 121–144 (2016). doi:10.1007/7651_2016_357

30. Moya, N., Cutts, J., Gaasterland, T., Willert, K. & Brafman, D. A. Endogenous WNT Signaling Regulates hPSC-Derived Neural Progenitor Cell Heterogeneity and Specifies Their Regional Identity. *Stem Cell Reports* **3,** 1015–1028 (2014).

31. Chen, B. *et al.* Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat. Chem. Biol.* **5,** 100–7 (2009).

32. Blauwkamp, T. A., Nigam, S., Ardehali, R., Weissman, I. L. & Nusse, R. Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. *Nat. Commun.* **3,** 1070 (2012).

33. Paşca, A. M. *et al.* Functional cortical neurons and astrocytes from human pluripotent stem cells in 3D culture. *Nat. Methods* **12,** 671–678 (2015).

34. Verkhratsky, A., Olabarria, M., Noristani, H. N., Yeh, C.-Y. & Rodriguez, J. J. Astrocytes in Alzheimer's disease. *Neurotherapeutics* **7,** 399–412 (2010).