An Integrated Biomanufacturing Platform for the Large-Scale Expansion and Differentiation of Neural Progenitor Cells

by

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ABSTRACT

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, or amyotrophic lateral sclerosis are defined by the loss of several types of neurons and glial cells within the central nervous system (CNS). Combatting these diseases requires a robust population of relevant cell types that can be employed in cell therapies, drug screening, or patient specific disease modeling. Human induced pluripotent stem cells (hiPSC)-derived neural progenitor cells (hNPCs) have the ability to self-renew indefinitely and differentiate into the various neuronal and glial cell types of the CNS. In order to realize the potential of hNPCs, it is necessary to develop a xeno-free scalable platform for effective expansion and differentiation. Previous work in the Brafman lab led to the engineering of a chemically defined substrate—vitronectin derived peptide (VDP), which allows for the long-term expansion and differentiation of hNPCs. In this work, we use this substrate as the basis for a microcarrier (MC)-based suspension culture system. Several independently derived hNPC lines were cultured on MCs for multiple passages as well as efficiently differentiated to neurons. Finally, this MC-based system was used in conjunction with a low shear rotating wall vessel (RWV) bioreactor for the integrated, large-scale expansion and neuronal differentiation of hNPCs. Finally, VDP was shown to support the differentiation of hNPCs into functional astrocytes. Overall, this fully defined and scalable biomanufacturing system will facilitate the generation of hNPCs and their derivatives in quantities necessary for basic and translational applications.

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LIST OF ABBREVIATIONS

2D	Two Dimensional
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
BDNF	Brain Derived Neurotrophic Factor
BMGP4	Bone Morphogenic Protein 4
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate Buffered Saline
ECMP	Extra Cellular Matrix Protein
EGF	Epidermal Growth Factor
FAD	Familial Alzheimer's Disease
FALS	Familial Amyotrophic Lateral Sclerosis
FGF	Fibroblast Growth Factor
GDNF	Glial Derived Neurotropic Factor
HIPSC	Human Induced Pluripotent Stem Cell
HNPC	Human Neural Progenitor Cell
HPSC	Human Pluripotent Stem Cell
HRP	Horseradish Peroxidase
LN	Laminin
LPS	Lipopolysaccharide
MC	Microcarriers
NDC	Non-Demented Control
NRG-1	Neuregulin 1-β1
PI	Propidium Iodide
PLO	Poly-L-Ornithine

RWV	Rotating Wall Vessel
SAD	Sporadic Alzheimer’s Disease
STLV	Slow Turning Lateral Vessel
TMB	3,3',5,5'-tetramethylbenzidine
VDP	Vitronectin Derived Peptide

1. INTRODUCTION

1.1 Neurodegenerative Disease and Stem Cells

Neurodegenerative diseases represent a leading cause of death within the United States and are characterized by a loss of neurons and glial cells within the central nervous system. The effected region of the brain or nervous system is closely tied to the associated condition. Alzheimer's disease (AD) is typically associated with a loss of neurons within the cerebral cortex. Whereas Amyotrophic Lateral Sclerosis (ALS) is associated with a loss or dysregulation of upper and lower motor neurons. Without effective treatment methodologies these diseases are a significant challenge to clinicians. Additionally, the precise mechanisms of these diseases have eluded researchers due to a lack of reliable models which can recapitulate all aspects of the associated disease. Historically, animal models have been utilized to study aspects of these diseases, one such example is the triple transgenic mouse model of AD. Additionally, researchers have relied on post-mortem tissue samples. However, animal models fail to completely mimic human systems of disease and post-mortem tissue only provides an end-point of disease. Stem cell-based therapies may provide a new avenue of treatment for these typically robust neurodegenerative diseases. Stem cells have the ability to self-renew indefinitely as well as the ability to differentiate into all the cell types of the CNS.

Stem cells can be more specifically categorized by their differentiation potential. Pluripotent stem cells having the ability to differentiate into cell types characteristic of all three germ layers. Comparatively, adult stem cells are fate-restricted and are limited to

specific cell types. It has been shown that the overexpression of four transcription factors, cMyc, Oct4, Sox2, and Klf4 can reprogram somatic cells into what has been termed human induced pluripotent stem cells (hiPSCs) (Takahashi and Yamanaka). HiPSCs provide a robust source of pluripotent cells useful for research and clinical applications without the ethical concerns associated with the isolation of human embryonic stem cells (hESCs). Furthermore, it is possible to generate patient specific hiPSCs that may provide additional insight into various disease mechanisms. Although hiPSCs offer significant potential, their ability to differentiate into all three germ layers poses risks of tumorgenicity. A multipotent progenitor such as human neural progenitor cells (hNPCs) capable of differentiating into various cell types such as neurons, astrocytes, and oligodendrocytes may provide a better clinical solution.

Neural progenitor cells are generated by directing hiPSCs down a neuroectodermal differentiation pathway. HNPCs express markers such as Sox1, Sox2, and Nestin and have already demonstrated their usefulness in previous studies. For instance, neural progenitor/stem cells have demonstrated some success as a means to deliver small molecules in a gene therapy for glioblastomas (Benedetti et al.). HNPCs have also demonstrated use in cell therapies to increase neurogenesis and formation of glial cells (Lindvall et al.). It has also been preliminary demonstrated that autologous hNPC-based cell therapies are less likely to be immunogenic (Huang et al.).

1.1.2 Alzheimer's Disease

Alzheimer's disease (AD) represents the most common form of neurodegenerative disease, an estimated 5.7 million Americans are currently living with AD ("2018

Alzheimer's Disease Facts and Figures"). Additionally, this number is expected to rise dramatically as the baby boom population increases in age. The pathology of AD consists of two main pathological hallmarks: extracellular amyloid beta plaques and intracellular neurofibrillary tangles. There are two forms of AD, generally distinguished by the time of onset and associated risk factors, familial Alzheimer's disease (fAD) and sporadic Alzheimer's disease (sAD). FAD is a rarer form of the disease resulting in early onset as a result on mutations in Amyloid Precursor Protein (APP), Presenilin-1 (PSEN1) or Presenilin-2 (PSEN2) genes. SAD is the more common form of the disease and has been linked to many genetic risk factors including polymorphisms in the Apolipoprotein E (APOE) gene (Giri et al.). In this study, hiPSCs generated from patients who were homozygous for the APOE ε4 allele were made into to hNPCs and subsequently differentiated into neurons and astrocytes.

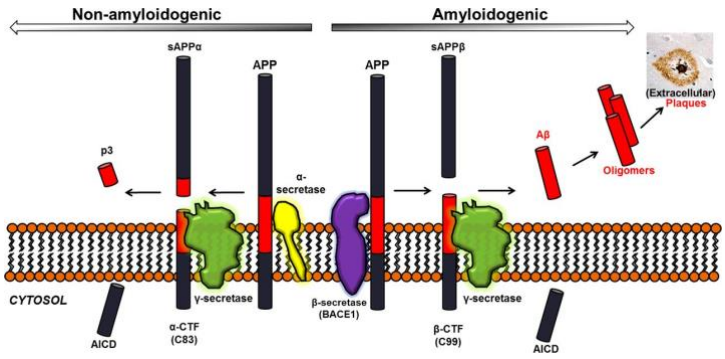

Figure 1-1: APP Processing. Amyloid Precursor Protein is cleaved through a non-amyloidogenic pathway by α secretase within the Aβ domain (shown in red). In the amyloidogenic pathway, APP is cleaved by β-secretase which will in turn allow for a peptide fragment with the Aβ domain intact. This fragment is additionally cleaved by γ-secretase between 38-42 amino acid residues from the by β-secretase cleavage site (Toh and Gleeson).

The amyloid beta plaques that have formed within the extracellular space are likely a result of improper processing of its precursor, APP. This is a transmembrane protein that is processed by three separate enzymes α-, β- and γ-secretase. With two different

pathways, a non-amyloidogenic and amyloidogenic one. In the non-amyloidogenic, APP is cleaved by α-secretase, generating soluble amyloid precursor protein alpha (sAPPα). This cleavage occurs within the Aβ domain of the precursor, preventing in its oligomerization. In the amyloidogenic pathway, APP is first cleaved by β-secretase, 99 amino acids from the intracellular carboxy terminal of APP, generating a 99 amino acid fragment. This fragment containing the intact Aβ domain, is cleaved by γ-secretase generating Aβ peptides at varying lengths. With $A\beta_{1-40}$, being most abundant as well as $A\beta_{1-42}$, which leads to the primary aggregation of amyloid plaques. It is likely due to hydrophobicity, that $A\beta_{1-42}$ it is more prone to fibril formation (Toh and Gleeson) .

In addition to the presentation of amyloid plaques, an AD patient's pathology will also include neurofibrillary tangles. An abnormally hyperphosphorylated tau protein, due to various pathways, will lead to destabilized microtubules and formation of toxic neurofibrillary tangles (Gong and Iqbal). The prevailing underlying pathology for the cause of AD could be explained through the amyloid cascade hypothesis. This hypothesis suggests that increased $A\beta_{1-42}$ production, possibly through missense mutations in PSEN1, PSEN2, and APP, leads to increased Aβ oligomerization and plaques. This in turn could be causing activation of microglial and astrocytes or directly impacting neuronal survivability. An altered neuronal ionic homeostasis; and oxidative injury resulting from synaptic and neuritic injury. This may in turn lead to altered kinase/phosphatase activity causing downstream formation of neurofibrillary tangles (NFTs). This widespread neuronal dysfunction along with cell death and disruption of transmitter efficacy ultimately resulting in dementia (Hardy and Selkoe). HiPSC-based

models of AD may allow for the discovery of better therapeutic targets as to date there are no effective treatment options.

1.1.3 Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease which primarily effects motor neurons. It is estimated to have a prevalence of approximately 5 in every 100,000 people (Julien). Similar to AD, ALS is typically split between familial and sporadic, with familial only accounting for 10% of cases. There have been several genes identified in familial forms of ALS including C9ORF72, TARDBP, and SOD1, among others. TDP-43, a DNA/RNA binding protein coded for by TARDBP is a common phenotype within 97% of patients. TDP-43 is a protein typically found within the nucleus and is responsible for many important RNA processing mechanisms. TDP-43 plays a role in the splicing of many mRNA transcripts as well as the generation of microRNAs. Additionally, TDP-43 has shown the ability to stabilize its own mRNA, suggesting a mechanism of autoregulation. TDP-43 is of particular importance in cellular survival as it is involved in the formation of cytoplasmic stress granules under conditions of cellular stress (Scotter et al.).

Studies have examined the role of TDP-43 as either a gain or loss of function in disease. The overexpression of TDP-43 recapitulates disease phenotypes in transgenic mice models, suggesting a gain of toxic function in disease (Wils et al.). Initial investigations into a loss of function in mice showed embryonic lethality, indicating the importance of TDP-43 in early development (Wu, Cheng, Hou, et al.). Conditional and partial knockout models did successfully demonstrate that a loss of TDP-43 function, can lead to motor

neuron defects, typical of TDP-43 proteinopathy (Wu, Cheng, and Shen). It is likely that the disease mechanisms underlying TDP-43 are both a loss and gain of function. Stem cell models of ALS will prove useful in better understanding disease mechanisms.

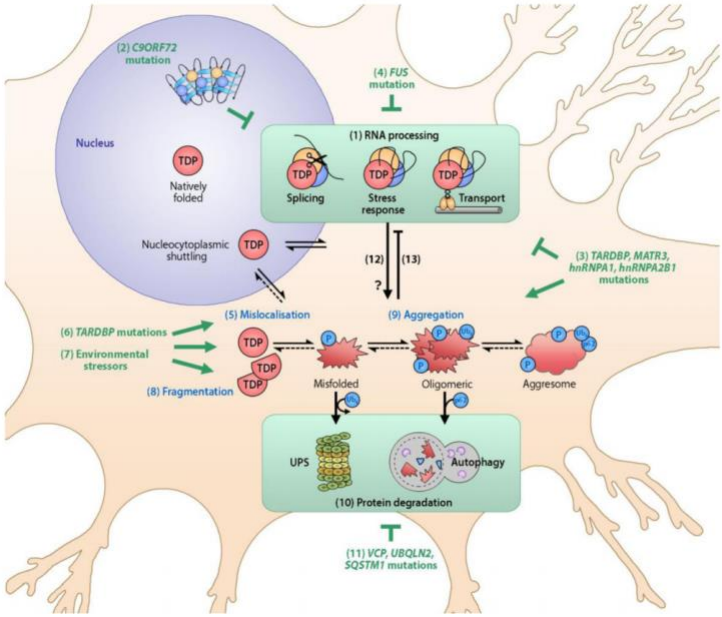

Figure 1-2: TDP-43 Proteinopathy and ALS. TDP-43 is a DNA/RNA binding protein responsible for important RNA processing mechanisms. Mislocalization of TDP-43 in ALS results in misfolding, oligomerization, and ultimately aggregation leading to cytotoxicity within cells. Repeat sequences within C9ORF72 lead to sequestration of important RNA processing proteins. (Scotter et al.)

1.2 Limitations Associated with Cell Based Therapies, Disease Modeling and Drug Screening

Although hNPCs have the potential to provide significant benefits in therapeutic and research applications there are a number of limiting factors associated with their use. Particularly, as it relates to scalability and defined culture conditions. HNPCs are historically cultured in 2D plates coated in either Laminin or Matrigel. These substrates are both comprised of extracellular matrix proteins derived from animals, typically mice.

Animal-derived substrates are expensive, chemically undefined, and subject to significant variation between lots. This variation in substrates or culture reagents can contribute to differences in experimental outcomes. Additionally, these substrates contain xenogeneic components which may be harmful for cells and ultimately make them unsuitable for clinical applications.

Also, it is prohibitively expensive as well as labor intensive to scale up these cultures. Recent clinical trials have demanded upwards of 6 billion cells per dose well beyond what 2D cultures could reasonable accommodate (Chen et al.). Additionally, 2D monolayer cultures are unable to fully recapitulate disease mechanisms found within the complex *in vivo* microenvironment. Current biomanufacturing processes are unable to produce the number of cells that will be necessary in clinical therapy development and disease modeling. Therefore, in this study a previously engineered, chemically defined peptide is utilized within a microcarrier-based bioreactor to produce a number of neurons that could more reasonably be used in drug development and disease modeling.

1.3 Synthetic Substrates

In order to replace ECMP-based animal derived substrates researchers have developed synthetic polymers as well as synthetic peptides. Polymer based substrates have been shown to allow for the expansion and differentiation of hNPCs (Tsai et al.). While these polymers have been shown to be effective they have limited ease-of-use on a larger scale. Synthetic peptides such as vitronectin derived peptide (VDP), a chemically defined substrate engineered within the Brafman Lab, are short amino acid sequences which are simple to use in order to coat the surfaces of plates to be used in adherent cell culture.

1.3.1 Identification of Vitronectin Derived Peptide as a Scalable Substrate for hNPC Expansion and Differentiation

Surrounding cells is a diverse organization of proteins and polysaccharides otherwise referred to as the extracellular matrix. These matrix proteins, such as laminin, vitronectin, and fibronectin increase cell surface adhesion as well as influence cell fate through signaling pathways via surface receptors known as integrins (Prowse et al.). It is possible to use this interaction to engineer short peptides which bind these various integrin domains as a substitute for undefined ECMP substrates.

Previously in the lab, a synthetic peptide was rationally designed from the cell binding domain of vitronectin, and subsequently referred to as vitronectin derived peptide (VDP) (Varun et al.). A library of peptides was designed using the experimentally determined integrin expression of hNPCs and screened for their ability to support cell adhesion and growth of hNPCs. This led to the identification of four peptides which were assessed on whether they could support the long term expansion of hNPCs. Only one of these four peptides was shown to maintain the multipotency of hNPCs and not result in presentation of neuronal-like morphologies. This peptide was able to demonstrate across multiple cell lines the ability to expand hNPCs for at least ten passages comparable to laminin. Cells cultured on VDP showed similar morphology and doubling time as compared to laminin. HNPCs cultured on VDP also showed similar expression of the three multipotency markers SOX1, SOX2, and Nestin, as assessed by qPCR, immunofluorescence, and flow cytometry.

VDP-coated surfaces were also shown to support the neuronal differentiation of hNPCs. Cells were differentiated by removal of EFG and FGF from culture media and addition of BDNF and GDNF. QPCR showed no statistically different expression of B3T and MAP2 between neurons differentiated on laminin versus VDP. Immunofluorescence revealed the expression of B3T, γ- aminobutyric acid (GABA), neurofilamnet-68 (NF-L), and MAP2. This overall demonstrates the ability of VDP to support the long term expansion and differentiation of hNPCs.

1.4 Large Scale Culture of hNPCs

As previously stated, the future clinical and research applications of hNPCs will require a significant number of cells. Generating $>10^9$ cells is not feasible on 2D tissue culture plates, both logistically and financially. It is possible to scale up the culture of hNPCS within a microcarrier based bioreactor system.

1.4.1 Microcarriers

Microcarriers are small spherical polystyrene beads, ranging in diameter (100-400 um), that are capable of supporting the culture of adherent cells. There are several factors that influence the efficiency of microcarrier cell attachment including, chemical composition, surface topography, degree of porosity and charge density. Several different microcarrier compositions have been produced, including dextran matrix, plastic coated with collagen, polystyrene, or cellulose (Malda and Frondoza). In a previous study, polystyrene

microcarriers were coated in VDP to encourage cell adhesion and subsequent aggregate formation (Srinivasan et al.).

HNPCs can be enzymatically dissociated from 2D tissue culture plates and seeded on Microcarriers coated with VDP in Ultra Low Attachment plates. Cells attached to microcarriers to form an initial monolayer around the surface as well as form aggregates consisting of multiple MCs. A higher surface area-to-volume ratio allowed for a greater expansion of hNPCs than in 2D wells of comparable size. Varying initial microcarrier and cell densities allowed for up to 8-fold expansion of RiPSC-hNPCs. HNPCs cultured on VDP-MC also continued to highly express the multipotency markers SOX1, SOX2, and Nestin as shown through immunofluorescence and flow cytometry.

Following the successful expansion of hNPCs on VDP-MC, it was investigated whether this 3D culture system could be used for the differentiation of hNPCs into neurons. Cells were differentiated by removal of EGF and FGF2 and addition of BDNF and GDNF for 18 days and then media containing no factors was used for the remainder of the culture duration. RNA sequencing and qPCR analysis of these neuronal cultures revealed downregulation of hNPC markers SOX1, SOX2, Nestin and PAX6. This analysis also showed high expression of MAP2, MAPT, RBFOX3, and TUBB3, markers of mature neurons, as well as increased expression of markers such as DLG4, SNAP25, and SYN1, which are associated with formation of functional synapses. Additionally, flow cytometry revealed >80% expression of TUBB3 and >50% expression of NEUN. These neurons could be enzymatically dissociated and replated on 2D surfaces with high survivability. Immunofluorescent staining showed neurons were positive for the pan-neuronal markers MAP2, TUBB3, and NEUN as well as the forebrain marker FOXG1.

1.4.2 Bioreactors

While microcarriers do provide an improved scalable tissue culture model they are still insufficient in producing the number of cells needed for clinical and research applications. This will require the robust and reproducible production of an order of magnitude higher number of cells. Microcarrier cultures integrated within a bioreactor system have the potential to provide this solution. There has been the development of many different bioreactor systems for use in mammalian cell culture. Four of these bioreactors types are represented in figure 1-7 below.

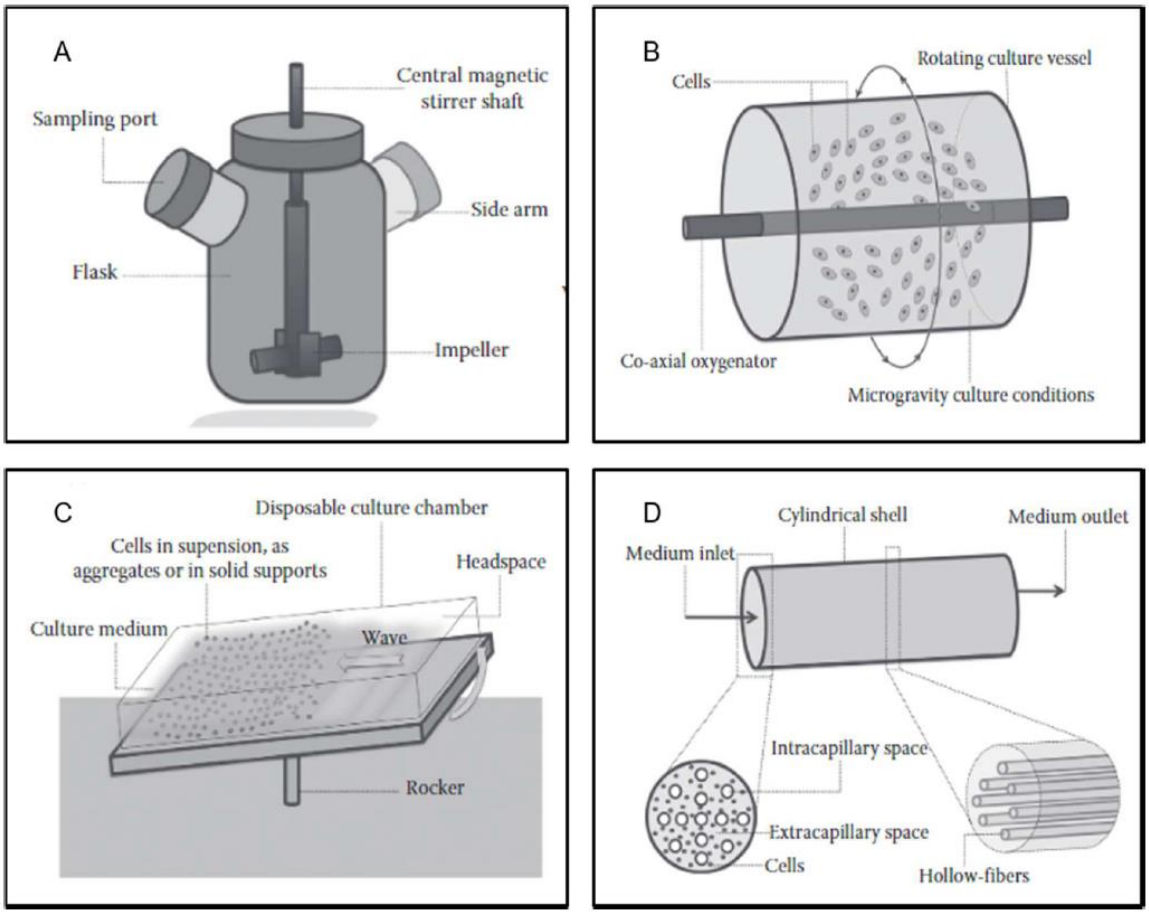

Figure 1-3: Possible Bioreactor Configurations. (A) Spinner flask (B) Rotating wall vessel bioreactor (C) Wave bioreactor (D) Hollow fiber bioreactor (Schaffer et al.)

Spinner flasks (Figure 1-7A) are one of the more common bioreactors used in suspension based cultures. They rely on an impeller which imparts rotation into cell suspension and allows for better diffusion of nutrients, however this can lead to high levels of shear stress within the reactor. Rotating wall vessels (RWV) (Figure1-7B) rely on a semipermeable membrane for oxygenation and were designed to simulate microgravity, resulting in a system with relatively low shear by comparison. Spinner flasks have been shown to support the generation of 3D stem cell derived β-cells (Millman et al.). RWV bioreactors have been used in the culture of various cell types ranging from extravillous trophoblast cells (Zwezdaryk et al.), vaginal epithelial cells (Hjelm et al.), and also retinal organoids (DiStefano et al.).

In this study, a RWV bioreactor was chosen to be integrated with polystyrene microcarriers coated in VDP and was investigated for its ability to support the large scale expansion and differentiation of hNPCs. This system is able to provide a physiologically relevant low shear environment for the 3D culture of hNPCs.

2. MATERIALS AND METHODS

2.1 HiPSC Culture

2.1.1 HiPSC Maintenance

All hiPSCs were cultured on Matrigel (Corning) coated plates. SAD- and NDC-hiPSC were cultured in Essential 8 Medium (E8) (1x DMEM-F12, 1% (v/v) Penicillin/Streptomycin, 543 ug/mL $NaHCO_3$, 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 TFGB). While, fALS-hiPSCs were cultured in either mTeSR1 (Stemcell Tech) or a solution of 50% mTeSR1 and 50% E8. SAD- and NDC-hiPSC were routinely passaged in Accutase, resuspended in E8 with 5 uM Rho Kinase inhibitor, and seeded on Matrigel coated 6-well plates. FALS-hiPSCs were initially cultured in a medium of only mTeSR1 and were gradually transitioned to 50% E8. FALS-hiPSCs were routinely passaged in Accutase, resuspended in either of the pluripotency medias previously described with 5 um Rho Kinase inhibitor, and seeded on Matrigel coated 6-well plates.

2.1.2 Neural Induction of hiPSCs

To induce neural fate, hiPSCs were expanded to 80% confluency. Media was aspirated from plate and 1.5 mL of ReLeSR (Stemcell Tech) was added to each well. Cells were incubated with ReLeSR for 30 seconds at room temperature. ReLeSR was gently aspirated and plate was incubated at 37°C for 5 minutes. Appropriate culture media was added to each well to lift small colonies off of plate. HiPSC colonies were resuspended in pluripotency media with 5 uM Rho Kinase inhibitor and placed in a 6-well Ultra Low

Adhesion plate on an orbital shaker with agitation (95 rpm). Cells were allowed to aggregate for 48 hours and form embryoid bodies. After 48 hours, three fourths of media was changed to Neural Induction Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 1% (v/v) B27 supplement, 1% (v/v) GlutaMAX, 1% (v/v) Penicillin/Streptomycin) with 0.2 ug/mL Noggin and 6.25 uM Dorsomorphin.

Embryoid bodies were cultured in NIM for an additional 6 days with half media changes each day. On day 6, the media and Embryoid bodies were transferred to a 50 mL conical and suspended in more NIM containing 5 uM Rho Kinase inhibitor. Embryoid bodies and media were transferred to a Matrigel coated 6-well tissue culture treated plate.

After 48 hours, half media changes were performed each day. After an additional 5 days, media was aspirated and cells were incubated in Accutase for 5 minutes at 37 °C. Cells were gently removed from plate using p1000 micropipette and transferred to a 15 mL conical. Neural Expansion Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 1% (v/v) B27 supplement, 1% (v/v) GlutaMAX, 1% (v/v) Penicillin/Streptomycin) with 30 ng/mL EGF and 30 ng/mL FGF2 was added to conical to deactivate Accutase. Cell suspension was centrifuged at 200xg for 5 minutes and cell pellet was resuspended in NEM with 5 uM Rho Kinase inhibitor. Cells were seeded on a PLO/Ln coated plates. The plates were first coated with poly-L-ornithine (PLO) (4 ug/mL) solution and incubated at 37 °C overnight and washed twice with 1x DPBS. The plates were then coated with mouse laminin (Ln) (4 ug/mL) and incubated at 37°C overnight and were washed twice at with 1x DPBS before use.

2.2 HNPC Culture

hNPCs were cultured on PLO/Ln coated tissue culture treated plates coated as previously described. HNPCs were plated on PLO/Ln plates at 1-1.5 x 10^4 cells/cm^2 in Neural Expansion Media with Rho kinase inhibitor (Y-27632) to aid single cell survival. Cells were routinely passaged upon reaching 80% confluency. Cells were incubated in Accutase for 5 minutes at 37°C. After which NEM was added to plate to deactivate Accutase. Suspension was transferred to 15 mL conical and centrifuged at 200xg for 5 minutes. Cells were resuspended in NEM with 5 uM Rho Kinase inhibitor to aid single cell survival. Cells were plated as described above.

2.3 Astrocyte Culture

HNPCs were seeded on either PLO/Ln or VDP coated plates. These cells were cultured in Neural Expansion Media for 24 hours and then media was changed to Astrocyte Medium (ScienCell) with 10 ng/mL CNTF, 10 ng/ml BMP4, and 10 ng/mL NRG-1. Cells were routinely passaged using Accutase similar to methods described above upon reaching 80% confluency. After 50 days within differentiation media cells were expanded and used for astrocyte marker expression analysis and functional characterization assays.

2.4 Microcarrier Culture

2.4.1 Microcarrier Coating

Tissue culture treated polystyrene Enhanced Attachment Microcarriers (Corning) were coated with either PLO/Ln or Vitronectin Derived Peptide (VDP). To coat in PLO/Ln microcarriers were suspended in a solution of PLO (4 ug/mL) and incubated at 37°C overnight. Microcarriers were gently washed twice with 1xDPBS and the resuspended in

a mouse laminin solution (4 ug/mL) and incubated at 37°C overnight. Prior to use in culture microcarriers were washed once with 1xDPBS and once with appropriate cell culture media. To coat with VDP microcarriers were suspended in a 0.5 mM solution of the synthetic peptide and incubated at 37°C for 48 hours. Prior to use in culture microcarriers were washed once with 1xDPBS and once with appropriate cell culture media.

2.4.2 HNPC Culture on Microcarriers

HNPCs cultured on PLO/Ln tissue culture treated plates as described above were dissociated into a single cell suspension and resuspended in NEM containing 5 uM Rho kinase inhibitor. Cells were seeded at 1.5 $x10^6$ cells/well with 1mg/mL of PLO/Ln or VDP microcarriers. Plates were incubated for 12 hours with half final culture volume in 6 well Ultra Low Attachment plates to encourage optimal attachment. Following 12 hours, additional media containing Rho kinase inhibitor was added to wells and plate was placed on an orbital shaker with agitation set to 95 rpm. After 24 hours following initial seeding of cells on microcarriers, three fourths of media was changed and half of media was changed each day following.

Upon reaching confluency, microcarrier-aggregates and cell media were transferred to a 15 mL conical and centrifuged at 200xg for 3 minutes. Without disturbing aggregates, media was removed and the aggregates resuspended in Accutase. Then this solution was transferred to 6-well Ultra Low Adhesion plates and placed within incubator at 37°C for 3 minutes on static and an additional 5 minutes on an orbital shaker with agitation set to 95 rpm. Following agitation NEM was added to wells to deactivate the Accutase and

gently pipetted up and down to allow cell detachment from microcarriers. This cell microcarrier suspension was passed through a 40 um cell strainer to remove microcarriers from cell suspension. Cells were centrifuged at 200xg for 5 minutes and resuspended in NEM with 5 uM Rho kinase inhibitor. Cells were seeded on freshly coated microcarriers as described above.

2.4.3 Neuronal Differentiation on MCs

HNPCs were expanded on microcarriers for 3-4 days as described above and then three fourths of media was changed to Neural Differentiation Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 1% (v/v) B27 supplement, 1% (v/v) GlutaMAX, 1% (v/v) Penicillin/Streptomycin) with 20 ng/mL BDNF and 20 ng/mL GDNF. Cells were cultured in this media, with half media changes each day. After 18 days, media was switched to Neural Base Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 1% (v/v) B27 supplement, 1% (v/v) GlutaMAX, 1% (v/v) Penicillin/Streptomycin) for the remainder of culture.

2.5 Bioreactor Culture

2.5.1 HNPC Expansion in RWV Bioreactor

Synthecon 55 mL STLVs were prepared according to manufacturer's instructions. Prior to initial use the vessel was dismantled and immersed in a mild detergent for one hour and then rinsed using Milli-Q water. The vessel was soaked in Milli-Q water overnight and allowed to dry before being autoclaved at 121°C for 20 minutes. Prior to each experimental use the vessel was rinsed clean with Milli-Q water and then autoclaved as described above.

HNPCs were cultured on microcarriers for a single passage prior to use in bioreactor. A single cell suspension was obtained from 6-well Ultra Low Adhesion plates as previously described. HNPCs were seeded within the bioreactor at 2 x 10^7 cells per vessel with 10 mg/mL of enhanced attachment microcarriers coated in either PLO/Ln or VDP as described previously. Vessel was filled completely with media and all bubbles were removed using syringe ports. Vessels were initially spun at a rate of 8-10 rpm and along with formation and enlargement of aggregates, the rpm was increased.

Samples were taken from the fill port each day of expansion for phase contrast imaging and in order to obtain cell counts. To take samples, 30 mL of media was removed and the cell-microcarrier aggregates were gently mixed until thoroughly dispersed within remaining 25 mL media volume. Two samples of approximately 5 mL were taken through the fill port and transferred to a 6 well Ultra Low Adhesion plate for imaging. Cells were then stained with Propidium Iodide to assess cell viability.

Upon reaching confluency, aggregates and media were transferred to 50 mL conicals. Following centrifugation and aspiration of the media cells were resuspended in Accutase. Aggregates were transferred to a 6 well Ultra Low Adhesion plate and incubated at 37°C for 10 minutes with no agitation. Following incubation media was added to the plate to inactivate Accutase. Cells were separated from microcarriers using 40 μm cell strainers. Cells were then centrifuged at 200xg for 5 minutes and suspended in appropriate volume of media for either cell counting or use in other assays.

2.5.2 Neuronal Differentiation in RWV Bioreactor

For neuronal differentiations in bioreactor, hNPCs were seeded on microcarriers and expanded for 4 days in Neural Expansion Media. Following expansion 45 mL of media was replaced with Neural Differentiation Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 1% (v/v) B27 supplement, 1% (v/v) GlutaMAX, 1% (v/v) Penicillin/Streptomycin) with 20 ng/mL BDNF and 20 ng/mL GDNF. Cells were cultured within this media for 18 days after which Neural Base Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 1% (v/v) B27 supplement, 1% (v/v) GlutaMAX, 1% (v/v) Penicillin/Streptomycin) was used for the remaining duration of culture. Cells were allowed to differentiate for at least 30 days before being used in assays. Cells were either dissociated from microcarriers as previously described or were plated for use in immunofluorescence imaging.

2.6 QPCR

RNA was isolated from cells using a Nucleospin RNA kit (Macherey Nagel). cDNA was synthesized from 1 ug of the isolated RNA using iScript Reverse Transcription Supermix (Bio-Rad). QPCR was performed using iTaq™ Universal SYBR© Green Supermix (Bio-Rad) and the primers listed in Table 1 on a CFX384 Touch™ Real-Time PCR Detection System. The reaction was performed with an initial 2 minute elevation to 95°C, followed by 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds. 18s rRNA levels were used as an endogenous control and the gene expression was quantified by using a ΔΔCt methodology.

2.7 Flow Cytometry

2.7.1 Fixed Cell Staining

Cells were dissociated from either plates or microcarriers as previously described. The cells were then washed once with stain buffer (BD Biosciences). The cells were first fixed using BD Cytofix fixation buffer at room temperature for 30 minutes. Cells were then washed twice with stain buffer and permeabilized using BD Phosflow Perm Buffer III at 4°C for 30 minutes. Following permeabilization, cells were washed twice with stain buffer and stained using conjugated antibodies as listed in table 3 at 4°C overnight. Cells were washed twice and resuspended in stain buffer and analyzed with Accuri C6 Flow Cytometer (BD Biosciences).

2.7.2 Live Cell Staining

For propidium iodide staining, live cell suspension was washed once with 1xDPBS and resuspended in stain buffer. Cells were stained with 2 mg/mL propidium iodide for 1-2 minutes at room temperature protected from light and were analyzed on Accuri C6 Flow Cytometer (BD Biosciences).

For surface marker staining, cells were washed once and resuspended in 1xDPBS. The cells were stained for 1 hour at 4°C protected from light. Cells were next washed twice and resuspended in 1xDPBS and analyzed with Accuri C6 Flow Cytometer (BD Biosciences).

2.8 Immunofluorescence

2.8.1 2D Cultured Cell Staining

Cells were seeded on PLO/Ln or Matrigel coated 12 well plates and incubated for at least 24 hours not allowing the cells to become more than 50% confluent. Culture media was aspirated and cells were fixed using BD Cytofix fixation buffer at room temperature for 30 minutes. Wells were then washed twice with 1xDPBS. Cells were then permeabilized using BD Phosflow Perm Buffer III at 4°C for 30 minutes. After permeabilization, cells were stained with primary antibodies as described in table 3 at 4°C overnight. Cells were then washed twice with 1xDPBS and stained with secondary antibodies as described in table 3 at room temperature for 2 hours. Cells were washed twice with 1xDPBS and stained with Hoescht 33342 at a dilution of 1:5000 for 10 minutes. Cells were then washed twice with 1xDPBS and imaged using fluorescent microscope.

2.8.2 Plating Microcarriers

Neuron-microcarrier aggregates were carefully transferred from suspension culture to 15 mL conical using serological pipette. Aggregates were allowed to settle at bottom of conical and media was carefully removed. Accutase was added to conical and incubated at 37°C. Aggregates were mechanically dissociated into smaller aggregates by gently pipetting up and down. Neural Base Media was added to deactivate Accutase and conical was centrifuged at 200xg for 5 minutes. Smaller aggregates were resuspended in NBM and plated on either Matrigel coated plates or Matrigel coated plates with astrocytes. Microcarrier aggregates remained in culture for approximately 7 days to allow for

neurons to spread from aggregate. At which point, the cells were fixed, permeabilized and stained as described previously.

2.9 ELISAs

2.9.1 IL-6

Cells were seeded on a 24 well plate at 100k cells per well and allowed to attach and expand for 24 hours. The media was then changed to either NEM or Astrocyte Media containing either 25 ug/mL or 50 ug/mL of lipopolysaccharide (LPS) (eBiosciences). Additionally, a saline control was used for comparison. After treatment for 24 hours the conditioned media was collected and stored at -20°C until use. The commercially available IL-6 Human Instant ELISA Kit (Invitrogen) was used to measure inflammatory response due to LPS according to the manufacturer's protocols. Samples were incubated in wells pre-coated with monoclonal antibody for Human IL-6, conjugated antibody to IL-6 and Streptavidin-HRP for 3 hours at room temperature with shaking (400 rpm). The wells were then washed four times and incubated with TMB for 10 minutes at room temperature protected from light. A stop solution was added and the absorbance immediately read at 450 nm.

2.9.2 ApoE

Cells were seeded on a 12 well plate at 100k cells per well and allowed to attach and expand for 48 hours. The cell culture media was changed once at 24 hours. After an additional 24 hours the media was collected and concentrated 8-10X. Concentrated media samples were stored at -80°C until use. Cells were lysed in 0.1% TBST with protease inhibitors and the lysate was collected and stored at -80°C until use. A Bradford assay

was used to determine the total protein. The commercially available Apolipoprotein E Human ELISA Kit (Thermo Fisher) was used to assess level of ApoE in the concentrated media. Samples were incubated in wells pre-coated with antibody specific to human ApoE for 2.5 hours at room temperature with shaking (200 rpm). The wells were washed four times and then incubated with a biotinylated antibody for 1 hour with shaking (200 rpm). The wells were then washed four times and incubated with Streptavidin-HRP reagent for 45 minutes at room temperature with shaking. The wells were again washed four times and then TMB substrate was added to each well and the plate was developed at room temperature protected from light. A stop solution was added and absorbance was measured at 450 nm.

2.10 Glutamate Uptake Assay

To assess glutamate uptake cells were seeded in a 12 well plate at 100k cells per well. After 24 hours cells were treated with glutamate in Hank's Buffered Salt Solution (HBSS) with varying concentrations. Samples were taken from the wells at three time points: 0, 1, and 2 hours. A commercially available Glutamate Assay Kit (Sigma) was used, according to manufacturer's protocols, to determine the levels of glutamate within the supernatant. Samples were incubated with a reaction mix comprised of an assay buffer, developer, and enzyme mix for 30 minutes at 37°C protected from light. Following incubation, the absorbance was then measured at 450 nm.

3. RESULTS

3.1 Generation and Characterization of hNPCs.

Previous work has shown a robust and efficient means of differentiating human induced pluripotent stem cells into neural progenitor cells (hNPCs) by modulation of TGF-β signaling (Brafman). This protocol has been used within the lab to generate stable hNPC lines from a number of healthy and disease patient samples. In particular, this protocol was adapted to generate hNPCs from two different familial amyotrophic lateral sclerosis (fALS) patient lines: 034 and A4V. Three fALS-hiPSC lines were received from the Sattler lab at Barrow Neurological Institute. These lines have three different mutations which have been identified as genetic risk factors in fALS. The related gene mutations associated with each line can found in table 1. 034- and A4V-hNPCs were characterized by their expression of the markers SOX1, SOX2, and Nestin. As compared to their respective hiPSCs, 034- and A4V-hNPCs showed upregulation of the markers SOX1 and Nestin by RT-qPCR (Figure 3-1B). Additionally, flow cytometry analysis revealed greater than 99% expression of these three markers in fALS-hNPCs (Figure 3-1C). In addition, immunofluorescent staining confirmed the expression of relevant hNPC markers (Figure 3-1D).

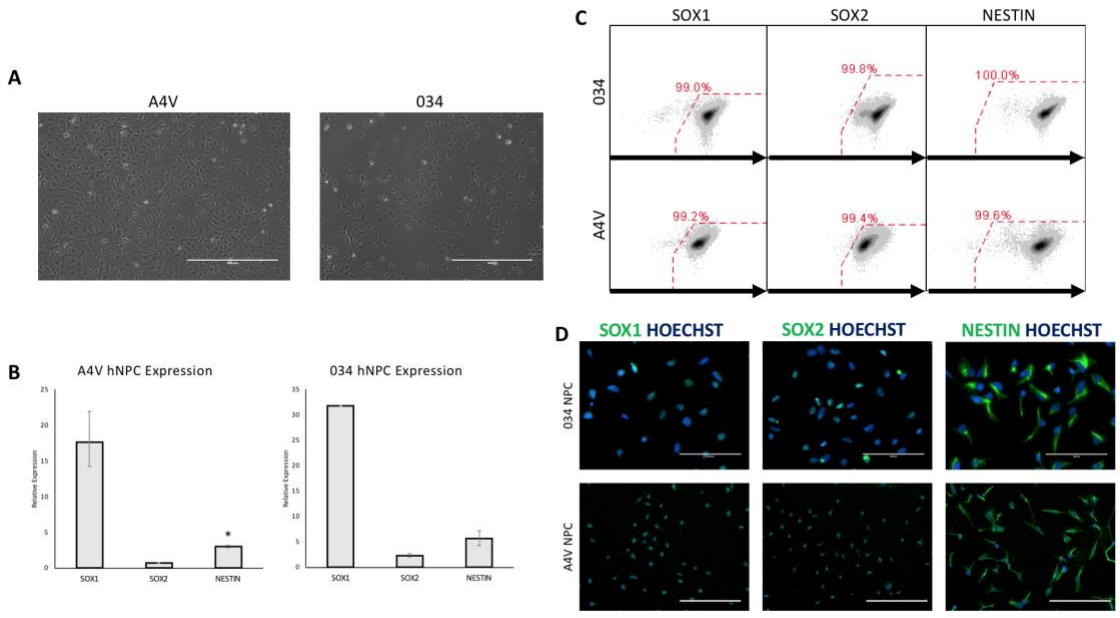

Figure 3-1: Generation of hNPCs from patient derived fALS-hiPSCs. (A) Representative phase contrast images of A4V- and 034-hiPSCs that have been passaged using Accutase (scale bar =1000 um) (B) Quantitative PCR analysis for expression of hNPC markers SOX1, SOX2, and Nestin as compared to avg. hiPSC expression (mean ± S.E.M) (Student's t-test, * $p<0.01$) (c) Flow cytometry analysis for expression of SOX1, SOX2 and Nestin in hNPC lines. (D) SOX1, SOX2, and Nestin immunofluorescence in 034- and A4V-hNPCs (034, scale bar = 100 um; A4V, scale bar = 200 um).

More broadly the lab has generated numerous neural progenitor cell lines from different patient samples. These cell lines will be useful for the study of relevant disease mechanisms including familial Alzheimer's disease, sporadic AD, as well as familial amyotrophic lateral sclerosis. Information pertaining to these lines is summarized within table 1 below.

Cell Line	Patient's Diagnosis	Mutation
160-sc1	Sporadic AD	APOE E4/E4
384-sc4	NDC	APOE E4/E4
414-sc8	NDC	APOE E4/E4
188-sc18	Sporadic AD	APOE E4/E4
TGEN 10_08	NDC	N/A
HVRD-001A	Familial AD	APPV717I
CS40iFAD-n1	Familial AD	PSEN1A246E
UCSD239iAPP2-1	Familial AD	APP^{dp}
JH034	Familial ALS	C9orf72
SODA4V	Familial ALS	SOD1

Table 1: Summary of Patient Derived hNPCs

3.2 Expansion of hNPCs on VDP-MC in RWV Bioreactor

Previously, VDP was identified as a substrate capable of supporting the long-term expansion and maintenance of hNPCs derived from pluripotent stem cells. Additionally, it has been shown that polystyrene enhanced attachment microcarriers coated in VDP (VDP-MC) can support the long-term culture of hNPC. It was investigated whether this could be successfully integrated within a RWV bioreactor to increase the scale of the culture further. RiPSC-hNPCs were seeded on VDP-MC and cultured within the bioreactors for at least 6 days. This culture system was able to support the expansion of RiPSC-hNPCs on VDP-MC to over 200 million live cells ($215.94 \pm 52.60 \times 10^6$ cells; n=4) (Figure 3-2D). In addition, flow cytometry and immunofluorescence demonstrated high levels of expression for the NPC markers, SOX1, SOX2 and Nestin (Figure 3-2C and E).

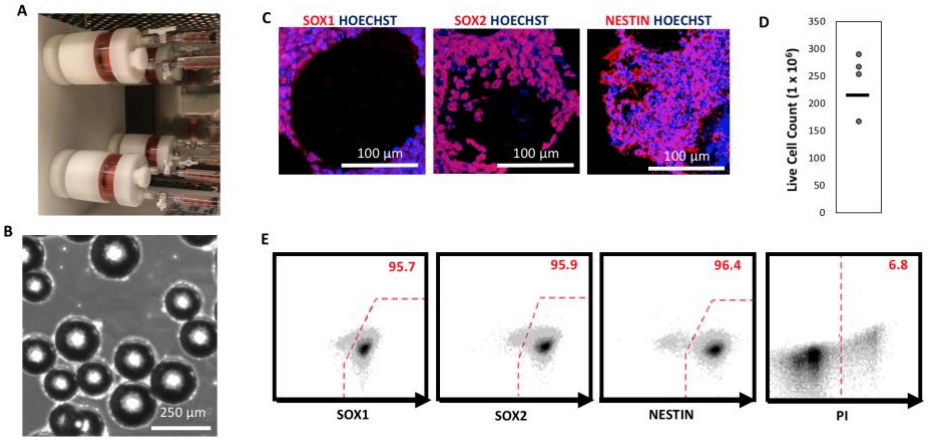

Figure 3-2: Expansion of RiPSC-hNPCs on VDP-MC in RWV Bioreactor. (A) Image of four vessel bioreactor configuration within tissue culture incubator. (B) Representative phase contrast image of NDC-hNPCs cultured on VDP-coated microcarriers in RWV. (C) Immunofluorescent images of SOX1, SOX2, and Nestin in hNPCs cultured on VDP-MC in RWV. (D) Live cell count of NDC-hNPCs expanded in bioreactors (n=4). (E) Flow cytometry analysis of SOX1, SOX2, Nestin and propidium iodide staining in NDC-hNPCs. (adapted from Srinivasan et al.)

3.3 Neuronal Differentiation of hNPCs in RWV Bioreactor

Following the confirmation of a reproducible hNPC expansion within the bioreactors. Previously developed cortical neuronal differentiations protocols were adapted for use in the bioreactor as described within the methods. RiPSC-hNPCs were seeded on VDP-MC and expanded for 3-4 days within the bioreactor, then three fourths of the media was changed to Neural Differentiation Media (NDM). Aggregates were cultured within the bioreactor in this media for 18 days, at which the media was replaced with NBM containing no growth factors. After at least 30 days of differentiation, these cells were assessed for their expression of mature neuronal markers. Differentiation of RiPSC-hNPCs within the bioreactors allowed for the generation of over 130 million neurons ($133.25\pm 47.98 \times 10^6$ neurons; n=3) (Figure 3-4A). Further analysis of these cultures by

RNA-seq revealed high levels of gene expression associated with mature neurons such as MAP2, MAPT, RBFOX3, TUBB3. Additionally, RNA-seq showed the expression of markers typical of cortical/forebrain neurons (e.g. ASCL1, CTIP2, EMX1/2, LHX9, TBR1) as well as, genes associated with functional synapse formation (e.g. SNAP25, SYN1, SYP) (Figure 3-4D). It was also confirmed using flow cytometry that cells were expressing TUBB3 and NEUN (Figure 3-4B). Finally, aggregates that were plated on 2D plates coated in Matrigel were confirmed to be expressing the markers MAP2, TUBB3 and FOXG1 through immunofluorescent staining (Figure 3-4E).

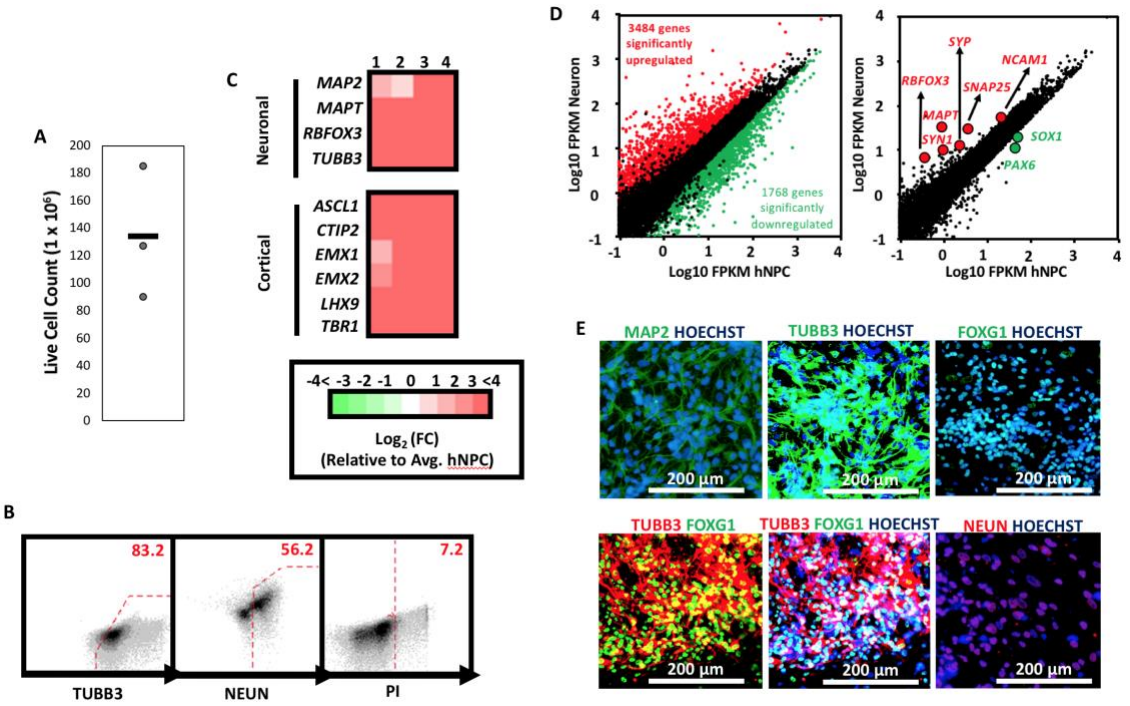

Figure 3-3: Cortical Neuronal Differentiation of NDC-hNPCS on VDP-MC in RWV Bioreactors. (A) Live cell count of NDC-NPC derived cortical neurons obtained through PI staining (n=3). (B) Flow cytometry analysis for TUBB3, NEUN and PI in differentiated cells. (C) Quantitative PCR for several neuronal and cortical markers in NDC neurons on VDP-MC relative to expression in hNPCs. (D) RNA sequencing was performed comparing the expression profiles of hNPCs and cortical neurons. Average values of FPKM from four independent samples are shown. Genes that had an altered expression with FDR <0.001 and more than 2-fold changes were considered significantly upregulated (in red) or downregulated (in green). Genes that are related to hNPC identity, post mitotic neurons and synapse formation are highlighted. (E) Immunofluorescence staining of MAP2, TUBB3, FOXG1, and NEUN in plated neuron-MC aggregates (scale bar = 200 um). (adapted from Srinivasan et al.)

3.4 Astrocytic Differentiation of hNPCs on VDP and LN

HNPCs have the capability of differentiating into all cell types of the CNS including astrocytes. As such, AD- and NDC-hNPCs were differentiated into astrocytes using the commercially available Astrocyte Medium (ScienCell) with the three growth factors CNTF, BMP4, and NRG-1. Initially, these cells were differentiated on laminin coated plates for 50 days and assessed for their expression of astrocyte markers and functional characteristics. After at least 50 days, AD- and NDC-hNPC expressed the astrocyte markers GFAP and S100B as assessed through immunofluorescence (Figure 3-7B). Flow cytometry showed both AD- and NDC-astrocytes expressed S100B as well as the surface cell marker CD44 (Figure 3-7C). A characteristic of functionally mature astrocytes is their propensity to generate an inflammatory response within the CNS. To assess this, astrocytes were treated with lipopolysaccharide (LPS) for 24 hours with a saline control and media was collected. The level of IL-6, one of many possibly secreted cytokines, was measured using a Human IL-6 ELISA kit (Invitrogen). AD- and NDC-astrocytes both showed statistically significant higher levels of IL-6 secretion than hNPCs (Figure 3-7D). Additionally, astrocytes secrete Apolipoprotein E (ApoE), which has been identified as an important genetic risk factor in sporadic Alzheimer's disease. To determine if astrocytes were producing ApoE, cells were cultured in serum-free media for 24 hours and supernatant was collected and subsequently concentrated. Amount of ApoE was determined using a Human ApoE ELISA kit (Thermo Fisher) and normalized to total protein as measured using a Bradford assay. Astrocytes produced approximately 110 ng/mg total protein (Figure 3-7E). Glutamate, an important neurotransmitter, can lead to neuron cytotoxicity in high concentrations. Astrocytes mediate the levels of glutamate

within the extracellular. Astrocytes differentiated on laminin were incubated in HBSS containing glutamate and samples were taken at 1 and 2 hours. The level of glutamate within the supernatant was used to determine if cells had taken up glutamate as expected. Astrocytes decreased the glutamate within the supernatant after 1 hour, however the level of glutamate within the supernatant increased after another hour albeit still below the initial concentration (Figure 3-7F).

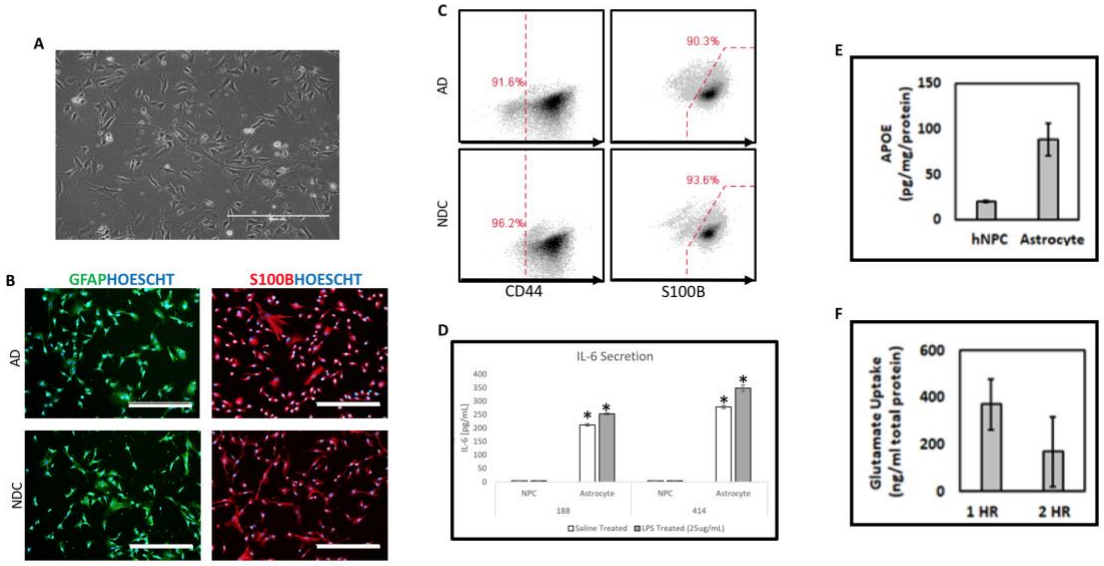

Figure 3-4: AD- and NDC-hNPC Derived Astrocytes on Laminin Coated Plates. (A) Representative phase contrast image of D50+ Astrocytes differentiated on laminin. (scale bar = 400 µm) (B) Immunofluorescence for GFAP and S100B expression in NDC and AD-Astrocytes. (scale bar = 400 µm) (C) Flow cytometry analysis for CD44 and S100B expression in NDC and AD-Astrocytes. (mean ± S.E.M.) (D) IL-6 secretion following treatment with LPS in both NDC and AD-Astrocytes and hNPCs. (mean ± S.E.M.) (Student's T-test, *$p<1x10^{-5}$) (E) ApoE secretion normalized to total protein in Astrocytes and hNPCs. (mean ± S.E.M.) (F) Glutamate uptake in astrocytes measured at 1 and 2 hrs normalized to total protein. (mean± S.E.M.)

Next AD(188)- and NDC(414)-hNPCs were differentiated into Astrocytes as described above on both VDP and laminin coated plates. AD- and NDC-astrocytes on both laminin and VDP appeared to have an immature morphology as there was an absence of star-like

projections from the cell bodies (Figure 3-8A). Immunofluorescent staining however did reveal expression of GFAP and S100B in astrocytes differentiated on both VDP and laminin (Figure 3-8C). AD- and NDC-hNPC derived astrocytes also showed expression of the markers CD44 and S100B through flow cytometry (Figure 3-8B). Additionally, these astrocytes were functionally characterized as described above for secretion of ApoE and IL-6. AD-astrocytes differentiated on laminin showed high levels of ApoE secretion. However, NDC-astrocytes on both substrates and AD-astrocytes on VDP showed lower amounts of ApoE within the supernatant (Figure 3-8D).

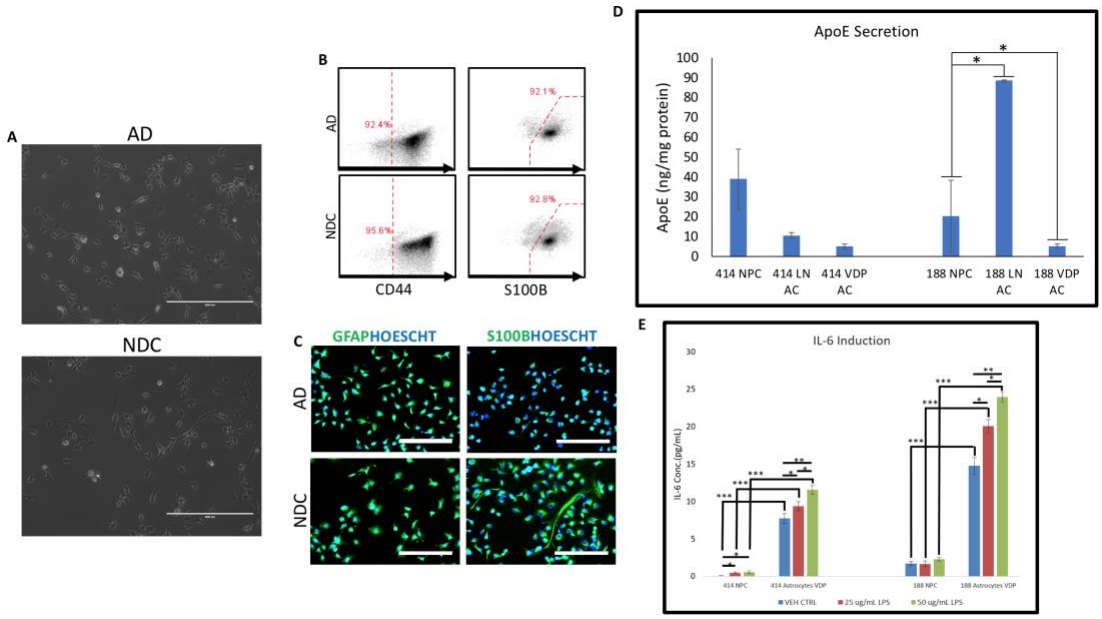

Figure 3-5: AD- and NDC-hNPC Derived Astrocytes on VDP Coated Plates. (A) Representative phase contrast images of D90+ AD- and NDC-Astrocytes differentiated on VDP coated surface. (scale bar = 400 um) (B) Flow cytometry analysis for CD44 and S100B expression in NDC and AD-Astrocytes. (mean ± S.E.M.) (C) Immunofluorescence for GFAP and S100B expression in NDC and AD-Astrocytes. (scale bar = 400 µm) (D) ApoE secretion normalized to total protein in Astrocytes and hNPCs. (mean ± S.E.M.) (Student's T-test, *p<0.05) (E) IL-6 secretion following treatment with LPS in both NDC and AD-Astrocytes differentiated on laminin and VDP and hNPCs. (mean ± S.E.M.) (Two-way ANOVA, *p<0.05, **p<0.001, ***p<0.00001)

Lastly, astrocytes on VDP and Ln were treated with LPS for 24 hours at varying concentrations, and inflammatory response through IL-6 secretion was measured. Both AD- and NDC-astrocytes had statistically significant higher levels of IL-6 as compared to hNPCs, however it appears that only in astrocytes differentiated on VDP was there a dose response. AD- and NDC-astrocytes differentiated on both substrates did not demonstrate an uptake of glutamate (data not shown).

Finally 160-hNPCs, another AD patient-derived cell line, was differentiated into astrocytes on both laminin and VDP. After 80 days within a commercial differentiation media these cells showed a morphology expected of astrocytes with fibrous star-like projections (Figure 3-6A). Additionally, flow cytometry analysis demonstrated expression of the surface marker CD44 as well as the mature astrocyte marker, S100B (Figure 3-6B). Immunofluorescent microscopy also demonstrated expression of S100B in addition GFAP was shown to be expressed in astrocytes on both laminin and VDP (Figure 3-6C). Next, it was investigated whether these cells performed functions physiologically typical of astrocytes. Firstly, it was confirmed that astrocytes secreted the protein ApoE through an ELISA kit (Figure3-6D). To determine if 160-astrocytes would simulate an inflammatory response cells were treated with LPS as previously described. 160-astrocytes showed increased levels of IL-6 secretion in the presence of LPS. Finally, cells differentiated from 160-hNPCs were shown to uptake glutamate after both 1 and 2 hours, however the result was not statistically significant for laminin or VDP.

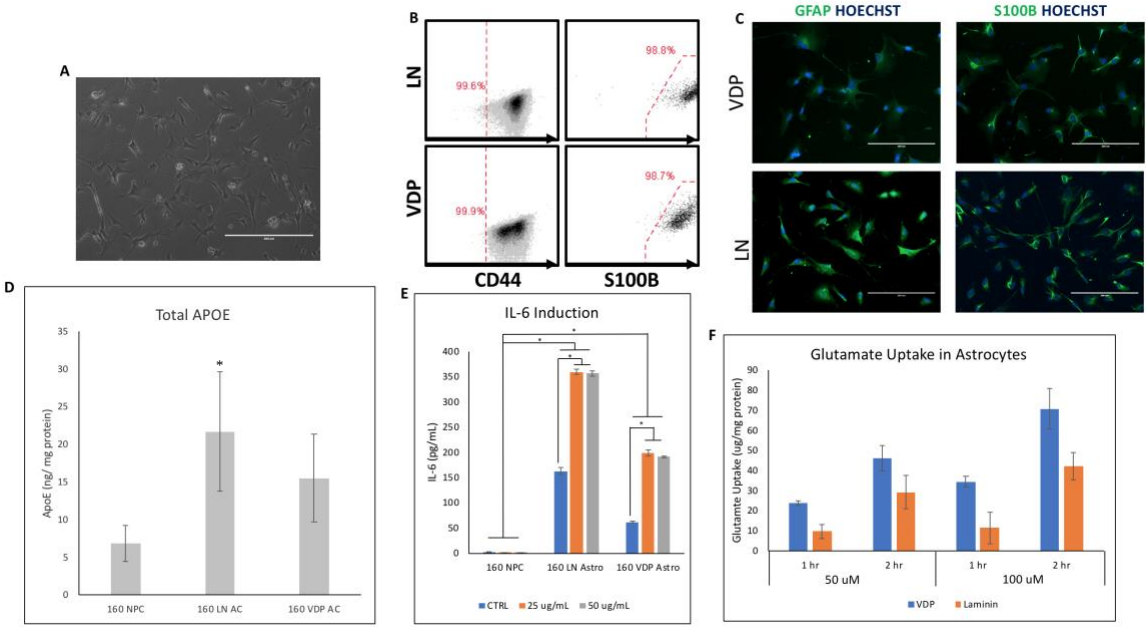

Figure 3-6: 160-hNPC Derived Astrocytes on VDP Coated Plates. (A) Representative phase contrast images of D80+ 160-Astrocytes differentiated on a VDP coated surface. (scale bar = 400 um) (B) Flow cytometry analysis for CD44 and S100B expression in 160-Astrocytes. (C) Immunofluorescence for GFAP and S100B expression in 160-Astrocytes. (scale bar = 200 µm) (D) ApoE secretion normalized to total protein in Astrocytes and hNPCs. (mean ± S.E.M.) (Student's T-test, *p<0.05) (E) IL-6 secretion following treatment with LPS in 160-Astrocytes differentiated on laminin and VDP and hNPCs. (mean ± S.E.M.) (Two-way ANOVA, *p<0.05) (F) Glutamate uptake in 160-Astrocytes at two starting concentrations normalized to total protein. (mean ± S.E.M.)

4. DISCUSSION

The widespread implementation of hNPCs in both translational and clinical applications requires the development of a completely defined and scalable biomanufacturing platform. Previously, a synthetic peptide was engineered from the cell binding domain of Vitronectin, an extracellular matrix protein (ECMP), and shown to be capable of supporting the long-term expansion and differentiation of hNPCs on 2D tissue culture treated plates. Additionally, microcarriers, small polystyrene beads, coated in this synthetic peptide were able to scale up the culture of hNPCs and allow for their long-term expansion in greater numbers than on 2D surfaces. While also, allowing for the differentiation of hNPCs into neurons expressing several cortical and neurotransmitter markers.

VDP coated MC were integrated into a rotating wall vessel bioreactor culture system and allowed for a 10-fold increase in the number of viable hNPCs following 6-8 days of expansion. HNPCs cultured within the bioreactor continued to highly express the markers SOX1, SOX2, and Nestin. Utilizing all four 55 mL vessels, would allow for the generation of nearly 1 billion viable hNPCs for use in drug screening and disease modeling.

This bioreactor culture system was also shown to support the differentiation of hNPCs into mature neurons expressing several neuronal, cortical, and neurotransmitter markers. This system could produce neurons on a much larger scale than typical 2D culture methods. Additionally, these neurons were able to remain as aggregates on VDP-MC for greater than 30 days. This platform will be useful to the further implementation of hiPSC-

derived neurons in the development of drugs and new treatment strategies for neurodegenerative disease such as Alzheimer's and ALS.

VDP was able to support the differentiation of hNPCs into cells expressing markers typical of astrocytes. Also, astrocytes differentiated on VDP demonstrated a release of inflammatory cytokines, secretion of apolipoprotein E, as well as glutamate uptake. While initial differentiations may have shown immature phenotypes this was consistent across substrates and cell lines. Further work will be needed to optimize the differentiation of hNPCs into astrocytes on VDP.

Current biomanufacturing systems fail to produce physiologically relevant cells for use in disease modeling, cell-based therapies and clinical drug development. The culture system described here addresses the primary issues facing development of such a process. This CNS-specific cell culture system eliminates the use of reagents such as laminin which are subject to significant batch-to-batch variations. Additionally, it produces cells of a neuronal phenotype in numbers greater than demonstrated previously. It is also possible that this 3D suspension culture could be adapted to other adherent cell types. This system will be of particular value to both clinicians and researchers investigating neurodegenerative disease.

5. FUTURE WORK

The integrated biomanufacturing platform developed within this study will allow for the chemically defined large scale expansion and differentiation of hNPCs. This technology could support the development of cell therapies, and importantly provide a better platform for modeling and studying diseases such as Alzheimer's Disease or amyotrophic lateral sclerosis. Currently, this platform has successfully demonstrated mature neurons. It will be of interest to expand this protocol to allow for the differentiation of hNPCs into more specific neuronal subtypes within the central nervous system. As well as, use this platform for the generation of functionally mature neurons derived from patients who suffer from neurodegenerative disease. This will be necessary to develop small molecule therapies or understand the mechanisms of the various diseases impacting the CNS.

Vitronectin derived peptide, a chemically defined substrate, has been shown to support the differentiation of hNPCs into Astrocytes. However, given the inconsistent phenotype of these cells it will be necessary to optimize their differentiation on VDP. It will also be of interest to integrate astrocytes within the biomanufacturing platform described herein for their large scale expansion and differentiation. Many neurodegenerative diseases such as ALS, exhibit non-autonomous cell toxicity and require more complex models of the disease capable of incorporating these features.

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APPENDIX A

FORWARD AND REVERSE PRIMERS USED FOR RT-QPCR

Gene	Forward	Reverse
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
NESTIN	CGCACCTCAAGATGTCCCTC	CAGCTTGGGGTCCTGAAAGC
SOX2	TTTGTCGGAGACGGAGAAGC	CCCGCTCGCCATGCTATT
MAPT	AGGGGGCTGATGGTAAAACG	TTGCTTAGTCGCAGAGCTGG
NCAM	GATGCGACCATCCACCTCAA	CCAGAGTCTTTTCTTCGCTGC
RBFOX3	TGGGACGATCGTAGAGGGAC	CTTCCAGCCGTTGGTGTAGG
TUBB3	GAGGGGCATCTCTTGAGAACAA	CCCAGAACTGTGGACGCCT
LHX9	TACCACTCTAGAGGCAGCCA	GGCATGCCCGCGTCG
EMX1	GAGACGCAGGTGAAGGTGTG	CACCGGTTGATGTGATGGGA
EMX2	ATCCGTCCACCTTCTACCCC	ACCATACTTTTACCTTGGAAGCG
ASCL1	TCCCCCAACTACTCCAACGA	GCGATCACCCTGCTTCCAAA
GFAP	AACCTGCAGATTCGAGGGGG	GGCGGCGTTCCATTTACAATC

Table 2: Forward and Reverse Primers Used for RT-qPCR

APPENDIX B

ANTIBODIES USED FOR IMMUNOFLUORESCENCE AND FLOW CYTOMETRY

Antibody	Vendor	Catalog Number	Concentration Used
Goat anti-SOX2	Santa Cruz	SC-17320	1:50
Mouse anti-SOX1	BD	BD 56749	1:50
Mouse anti-Nestin	BD	BD 560341	1:50
Mouse anti-B3T	Fitzgerald	10R-T136A	1:1000
Rabbit anti-GFAP	Millipore	AB360	1:500
Rabbit anti-FOXG1	Abcam	AB18259	1:500
Mouse anti-S100B	Sigma	S2532	1:500
Rabbit anti-MAP2	Millipore	AB5622	1:500
Alexa-647 Mouse anti-SOX2	BD	BD 560294	1:10
Alexa-647 Mouse anti-Nestin	BD	BD 560341	1:10
PerCP-Cy5.5 Mouse anti-SOX1	BD	BD 561549	1:10
Alexa-647 Donkey anti-Mouse	Life Technologies	A31571	1:200
Alexa-647 Donkey anti-Goat	Life Technologies	A-21447	1:200
Alexa-488 Donkey anti-Mouse	Life Technologies	A-21202	1:200
Alexa-488 Donkey anti-Rabbit	Life Technologies	A-21206	1:200

Table 3: Antibodies Used for IF and Flow Cytometry