Large Scale Expansion and Differentiation of Human Pluripotent Stem Cell-Derived Neural Progenitor Cells (hNPCs)

by

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ABSTRACT

Neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease and Amyotrophic Lateral Sclerosis are marked by the loss of different types of neurons and glial cells in the central nervous system (CNS). Human Pluripotent Stem Cell (hPSC)-derived Neural Progenitor Cells (hNPCs) have the ability to self-renew indefinitely and to differentiate into various cell types of the CNS. HNPCs can be used in cell based therapies and have the potential to reverse or arrest neurodegeneration and to replace lost neurons and glial cells. However, the lack of completely defined, scalable systems to culture these cells, limits their therapeutic and clinical applications. In a previous study, a completely defined, robust, synthetic peptide- a Vitronectin Derived Peptide (VDP) that supports the long term expansion and differentiation of various embryonic and induced pluripotent stem cell (hESC/hIPSC) derived hNPC lines on two dimensional (2D) tissue culture plates was identified. In this study, the culture of hNPCs was scaled up using VDP coated microcarriers (MC). VDP MC were able to support the long term expansion of hESC and hiPSC derived hNPCs over multiple passages and supported higher fold changes in cell densities, compared to VDP coated 2D surfaces. VDP MC also showed the ability to support the neuronal differentiation of hNPCs, and produced mature neurons expressing several neuronal, neurotransmitter and cortical markers. Additionally, alzheimer's disease (AD) relevant phenotypes were studied in patient hIPSC derived hNPCs cultured on laminin MC to assess if the MC culture system could be used for disease modelling and drug screening. Finally, a microcarrier based bioreactor system was developed for the large scale expansion of hNPCs, exhibiting more than a five-fold change in cell density and supporting more than 100 million hNPCs in culture. Thus, the development of a xeno-free, scalable system allows hNPC

culture under standard and reproducible conditions in quantities required for therapeutic and clinical applications.

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LIST OF ABBREVIATIONS

2-D	Two Dimensional
AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
Aβ	Amyloid Beta
BDNF	Brain Derived Neurotrophic Factor
CNS	Central Nervous System
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
FGF	Fibroblast Growth Factor
GDNF	Glial Derived Neurotropic Factor
HESC	Human Embryonic Stem Cell
HIPSC	Human Induced Pluripotent Stem Cell
HNPC	Human Neural Progenitor Cell
HPSC	Human Pluripotent Stem Cell
HRP	Horseradish Peroxidase
Ln	Laminin
MC	Microcarriers
NDC	Non Demented Control
PI	Propidium Iodide
PLO	Poly-L-Ornithine
PMT	Photo Multiplier Tube
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 Diseases and Stem Cells

Neurodegenerative diseases are one of the leading causes of death in the United States and are caused by the loss of different types of neurons and glial cells in the central nervous system. For instance, Parkinson's Disease is caused by the loss of mid-brain dopaminergic neurons, while Alzheimer's Disease is marked by the loss of forebrain cholinergic neurons and Amyotrophic Lateral Sclerosis is caused by the degeneration of motor neurons in the spinal cord and in the cerebral cortex (Lindvall & Kokaia, 2006). Additionally, conditions such as stroke, cause vascular damage resulting in ischaemia, thereby leading to neuronal and glial cell death. Current treatment methods for such diseases are only palliative and in case of diseases such as Amyotrophic Lateral Sclerosis, no effective treatment exists. Moreover, the mechanisms of these diseases are poorly understood due to the lack of models that faithfully recapitulate their pathologies. Generally animal models, such as the triple transgenic model for AD or post-mortem tissue are used to probe these diseases. However, animal models do not exhibit all the pathologies of the disease and post-mortem studies depict a terminal stage picture of the disease rather than its progression. Stem cell based therapies are a promising method of treatment for neurodegenerative disorders due to the ability of stem cells to self-renew indefinitely and to differentiate into the required cell types to replenish the lost neurons or glia.

Neural Progenitor Cells and potential applications

Stem cells with the ability to differentiate into various cell types of all three germ layers are called pluripotent stem cells and the stem cells whose fate is rather restricted, and can therefore, differentiate into only certain specific cell types are called adult stem cells.

Pluripotent stem cells derived from the inner cell mass of blastocysts are termed Embryonic Stem Cells (ESC). The ethical concerns surrounding ESCs limits their use on a wide scale. However, the ability to derive pluripotent stem cells (termed human induced pluripotent stem cells or hiPSCs) by reprogramming somatic cells by overexpressing certain transcription factors - cMyc, Oct4, Sox2, Klf4 (Takahashi et al., 2007) has introduced the possibility of using hPSCs and their derivatives for clinical applications. Moreover, patient specific hiPSCs could lend valuable insights into the mechanisms and progression of sporadic forms of certain diseases, not easily modelled by any other means. However, the ability of hPSCs to differentiate into cells of all three germ layers poses a risk of tumorigenicity, which is why in this study, we focused on expanding a multipotent progenitor population, called neural progenitor cells (hNPCs), capable of further differentiating into various cell types of the central nervous system, such as neurons, astrocytes and oligodendrocytes making them better suited for clinical applications related to neurodegenerative diseases.

Neural Progenitor Cells are derived from pluripotent stem cells by forcing ESCs/iPSCs down the neuroectodermal differentiation pathway. First, hPSCs are forced to form aggregates known as Embryoid Bodies in low adhesion plates and are cultured in neural induction media for 5 days, after which they are dissociated and plated on Matrigel™ to form neural rosettes. The rosettes are cultured in neural induction media for 7 days, after which they are dissected and plated on PLO/Ln coated tissue culture plates to form hNPCs. These hNPCs are cultured in neural expansion media with EGF and FGF and express markers such as SOX1, SOX2 and Nestin. HNPCs can be further differentiated into neurons by switching to neural differentiation media with BDNF and GDNF (Brafman, 2015).

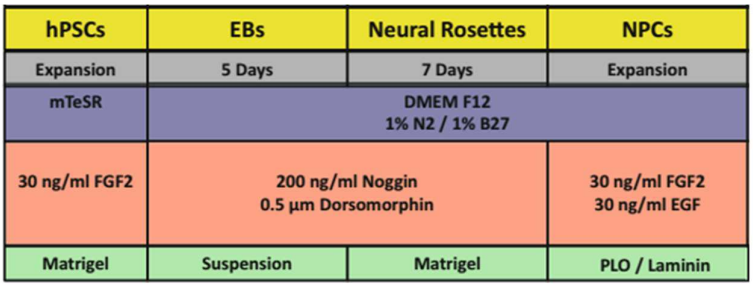

Figure 1-1: Protocol for differentiating hPSCs into hNPCs. (Brafman, 2015)

Studies suggest that hNPCs can be used in cell based therapies and have the potential to reverse or arrest neurodegeneration and to replace lost neurons and glial cells (Schwartz et al., 2012; Lindvall & Kokaia, 2006). For instance, grafting hNPCs in rats with T3 spinal cord injury showed long distance axonal growth across the injury site (Lu et al., 2012). Patient-specific iPSC derived hNPCs have also been used to model neurodegenerative diseases such as AD (Israel et al., 2012) and to facilitate drug discovery for diseases such as ALS (Egawa et al., 2012).

1.2 Bottlenecks in Cell Based Therapies

While stem cell derived hNPCs can potentially be used for therapeutic purposes, their clinical application is limited by a number of factors, including scalability and lack of xeno-free, defined culture conditions. HNPCs are typically cultured on 2-D tissue culture plates coated with laminin (Ln) or Matrigel™, which are animal derived ECMP based substrates. These substrates are expensive, subject to lot-to-lot variations and contain chemically undefined and potentially harmful xenogeneic components, thereby making hNPCs cultured on them unsuitable for clinical applications.

The cell numbers required for clinical applications far exceed the numbers typically obtained by conventional 2-D culture methods, where the surface area available for cell growth is limited and the culture cannot be easily scaled up. Moreover, monolayer culture does not resemble the cell's *in vivo* microenvironment.

1.3 Synthetic substrates

To replace the use of animal derived ECMP substrates, several chemically defined synthetic substrates have been developed for culturing cells. These can be broadly classified as synthetic polymers and synthetic peptides. Several polymers have been shown to support the growth of hPSCs (Celiz et al., 2014) and hNPCs (Tsai et al., 2015). These substrates are economical and allow cell growth under reproducible conditions. However, the preparation of such polymer coated surfaces requires complex fabrication methods which limits their use on a wider scale. Synthetic peptides such as Vitronectin Derived Peptide used in this study, on the other hand, are short amino acid sequences that can often be simply coated on tissue culture treated plates, making them easier to use.

1.3.1 Identification of Vitronectin Derived Peptide as a synthetic peptide-based substrate for hNPC culture

Cells are surrounded by a complex network of proteins and polysaccharides known as the extracellular matrix. Extracellular matrix proteins such as laminin, vitronectin and fibronectin, not only facilitate cell adhesion, but also control cell fate by influencing signalling pathways through a network of cell surface receptors known as integrins. Integrins are transmembrane proteins composed of α and β sub-units that bind to ECMPs on the extracellular domain and to

cytoskeletal elements on the intracellular domain. There are 18 α sub units and 8 β sub units and these form dimers in specific combinations according to the ECMPs in the cell's microenvironment. For instance, $\alpha_6\beta_1$ binds to laminin, $\alpha_5\beta_1$ binds to fibronectin and $\alpha_v\beta_5$ binds to fibronectin and vitronectin. Although ECMPs are complex and consist of numerous receptor binding domains, integrins can interact with certain cell binding domains of ECMPs to facilitate cell adhesion and growth. Thus, synthetic peptides consisting of short sequences, derived from the cell binding domains of ECMPS such as laminin, fibronectin and vitronectin can be used to culture cells instead of undefined ECMP substrates.

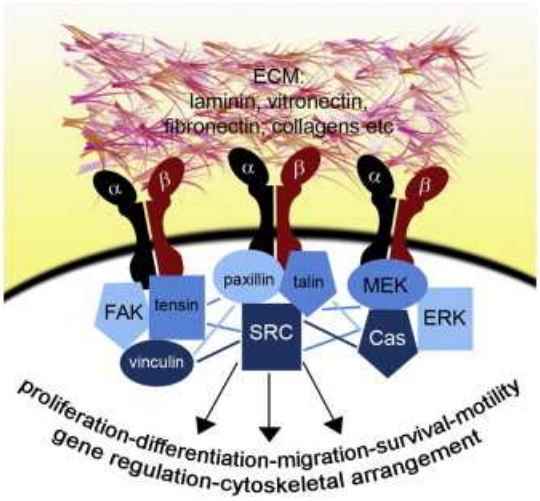

Figure 1-2: Integrin- ECMP interaction regulates stem cell fate. Integrins are transmembrane proteins that have an extracellular domain that interacts with ECMPs such as laminin, vitronectin, fibronectin etc. and a cytoplasmic domain that regulates cytoskeletal elements and affects signalling based on their interaction with ECMPs to decide cell fate. (Prowse, Chong, Gray & Munro, 2011)

To identify such a synthetic peptide-based substrate for hNPC culture, the ECMP and integrin expression of hPSC, hNPC and hPSC derived endoderm, mesoderm and ectoderm was assessed by performing qPCR and flow cytometry. Flow cytometry analysis revealed higher levels of expression of integrin subunits α_5, α_6, α_v, β_1 and β_2, lower levels of expression of α_1, α_2, α_3 and β_5 and no expression

of α_4, β_3, β_4 and β_7 in hNPCs (Figure 1-3). Using the knowledge of possible combinations of integrin subunits that form dimers and the ECMPS they bind to, a library of 18 peptides with sequences mimicking ECMPS or those that interact with these integrins was made. The adhesion and short term growth of hNPCs on these peptides was assessed by seeding hNPCs on 96 well plates coated with 500µm of these peptides, with hNPCs cultured on laminin as control. Of these peptides, four were identified to support cell adhesion and short term growth (Figure 1-4).

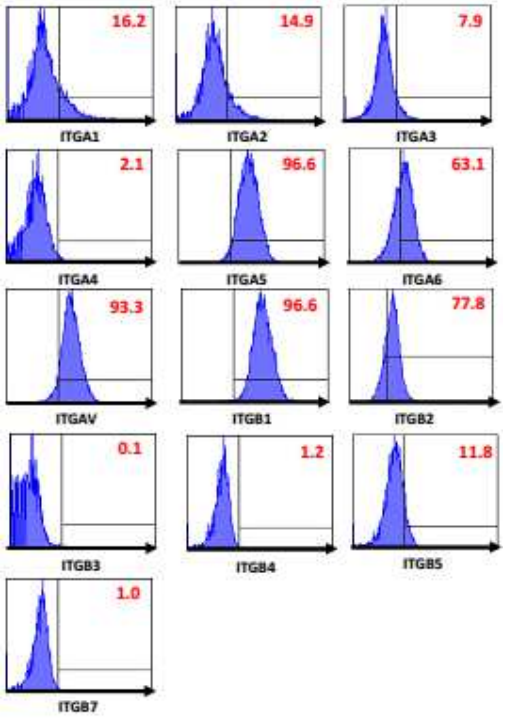

Figure 1-3: Integrin expression in hNPCs Flow cytometry analysis of integrin α1-6,v and β1-5,7 expression in hNPCs. (Varun et al., 2017)

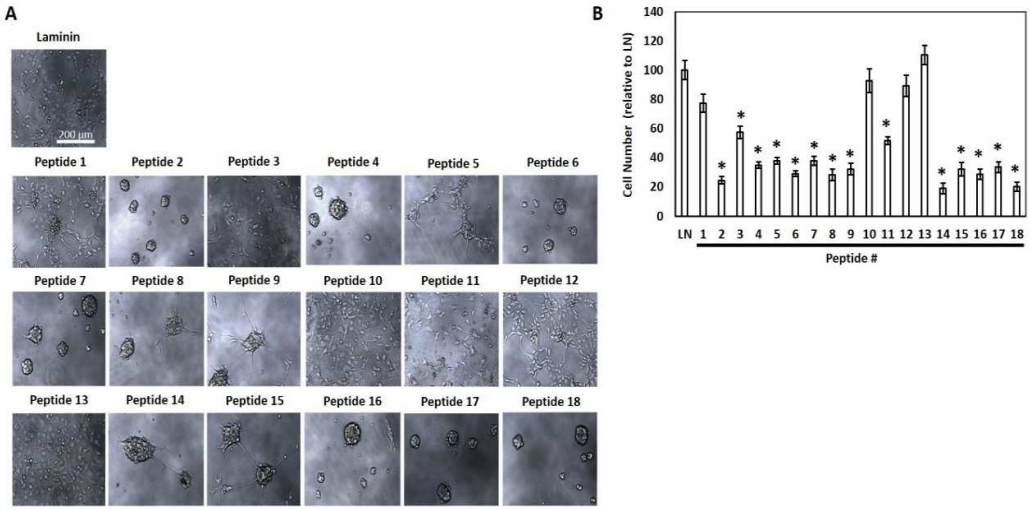

Figure 1-4: Identification of peptide-based substrate for hNPC culture. (A) Representative phase contrast images on H9 hNPCs cultured on laminin or on peptide coated tissue culture surfaces for 72 hours. All peptides were used at a concentration of 500µm. (B) Cell counts of H9 hNPCs cultured on laminin or on peptide coated tissue culture surfaces for 72 hours. Cell counts were normalized to those on laminin surfaces (mean ± s.e.m). Cell counts on peptides were compared to those on laminin using Student's t-test with Bonferroni correction (*$p<0.05$). (Varun et al., 2017)

Next, the ability of these four peptides to support long term expansion of hNPCs was assessed by culturing these cells on the peptides for multiple passages. Within 3 to 4 passages, hNPCs started acquiring a differentiated, neuronal morphology on 3 of the peptides and only one peptide (peptide 13) was identified as a substrate allowing long term expansion of hNPCs (Figure 1-4). This peptide, derived from the cell binding domain of Vitronectin (Vogel et al., 1993), and therefore, referred to as Vitronectin-Derived Peptide was subsequently used for culturing various ESC and iPSC derived hNPC lines such as HES-3, H9, HSF4 and NDC. VDP coated tissue culture plates (2D) supported the long term expansion of these hNPCs at levels similar to that on laminin (used as control) for more than 10 passages (Figure 1-5 A and B). HNPCs cultured on VDP showed similar doubling times as those cultured on laminin and showed no significant difference in the expression of genes such as SOX1, SOX2 and Nestin across multiple

passages when cultured on VDP and on laminin (Figure 1-5 D). Flow cytometry and immunofluorescence on cells cultured for more than 10 passages on VDP and laminin also revealed the presence of a high percentage (>85%) of SOX1, SOX2 and Nestin positive cells (Figure 1-5 E and F) indicating that VDP could support the expansion of hNPCs on levels similar to conventional extracellular matrix protein based substrates.

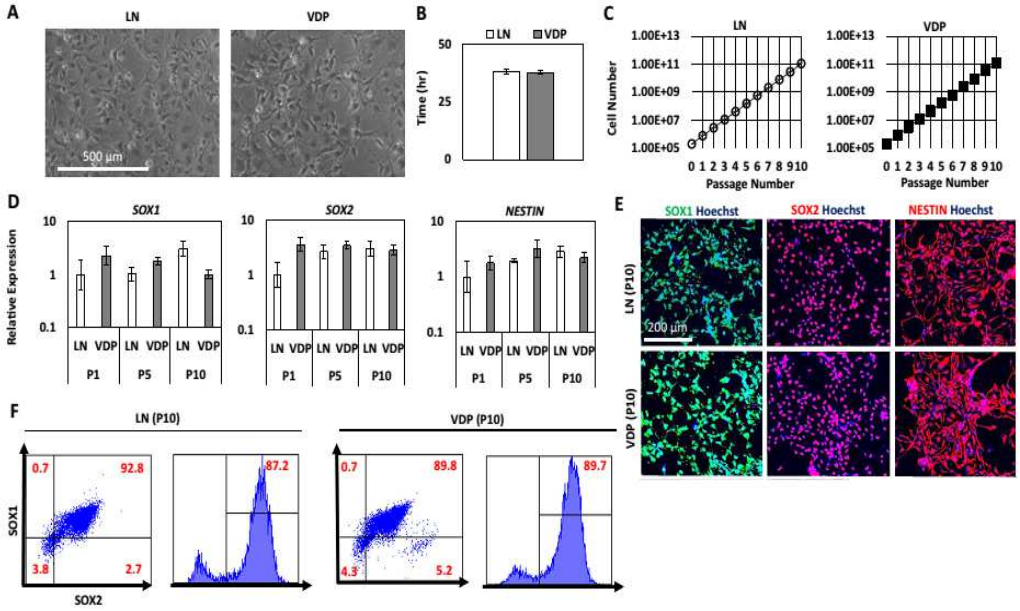

Figure 1-5: Long-term expansion of hNPCs on VDP-coated surfaces. (A) Representative phase contrast images of H9-hNPCs cultured on LN and VDP surfaces for 10 passages (scale bar = 500 µm). (B) Doubling time of H9-hNPCs cultured on LN and VDP. Data is presented as the mean ± S.D of the doubling time over the course of 10 passages. There was no statistical difference in the doubling time of hNPCs grown on LN and VDP (Student's t-test, $p > 0.05$) (C) H9-hNPCs were cultured on LN and VDP and cell growth was analysed by cell count at each passage (mean ± S.E.M). (D) Quantitative PCR analysis for expression of hNPC multipotency markers SOX1, SOX2, and NESTIN in H9-hNPCs cultured on LN and VDP for 1, 5, and 10 passages (mean ± S.E.M). Two-way analysis of variance (ANOVA) revealed that there was no statistical difference ($p > 0.05$) in expression of these genes over the course of 10 passages in hNPCs cultured on LN- or VDP-coated surfaces. (E) SOX1, SOX2, and NESTIN immunofluorescence of H9-hNPCs cultured on LN and VDP for 10 passages (scale bar = 200 lm). (F) Flow cytometry analysis for SOX1, SOX2, and NESTIN expression in H9 hNPCs cultured on LN and VDP for 10 passages. (Varun et al., 2017)

We assessed if VDP supported the neuronal differentiation of hNPCs. HNPCs were cultured on laminin and on VDP coated tissue culture plates, expanded till 80% confluent and were differentiated by switching the culture media to neural differentiation media. QPCR analysis showed higher levels of expression of neuronal markers such as β-Tubulin-III and MAP2 in neurons cultured on Ln and on VDP when compared to hNPCs cultured on these substrates (figure 1-6 A and B). Immunofluorescence revealed the presence of cells positive for neuronal markers such as MAP2, β-Tubulin-III, GABA and neurofilament (NF-L) on both laminin and VDP cultures (Figure 1-6 C, D and E). There was no statistically significant difference in the number of MAP2, β-Tubulin-III and NF-L positive cells cultured on laminin and VDP surfaces (Figure 1-6 F). Thus, VDP also showed the ability to support the differentiation of hNPCs into neurons.

In conclusion, VDP was identified as a robust, completely defined, scalable substrate for the expansion and differentiation of hPSC derived hNPCs on 2-D tissue culture surfaces.

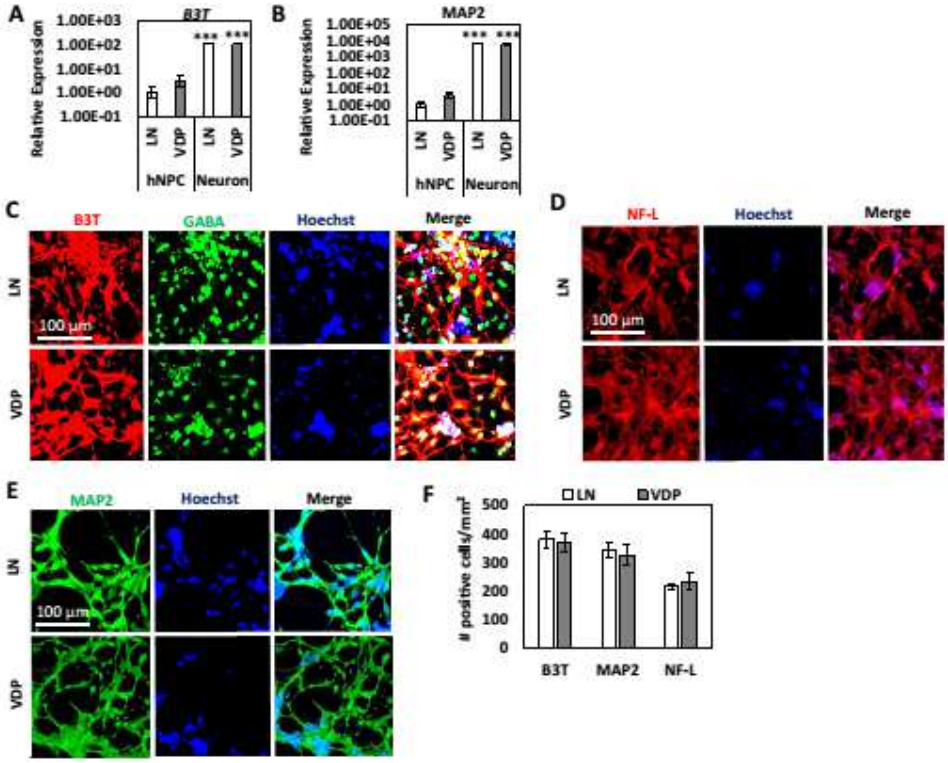

Figure 1-6: Neuronal differentiation of hNPCs of VDP-coated surfaces. Quantitative PCR analysis for expression of neuronal markers (A) B3T and (B) MAP2 of neurons differentiated from H9-hNPCs on VDP and LN substrates (mean ± S.E.M). Expression of these genes was statistically significantly higher in the neuronal cultures compared to hNPCs for cells cultured on both substrates (Student's t-test, ***p < 0.001). There was no statistically significant (Student's t-test, p > 0.05) difference between expression of these genes in neuronal cultures generated on LN- and VDP-coated surfaces. Immunofluorescence for (C) B3T and GABA (D) NF-L, and (E) MAP2 on neurons differentiated from NDC-hNPCs on LN and VDP substrates (scale bar = 100 lM). (F) Cell counts of the number of B3T, MAP2, and NF-L positive cells in neuronal cultures generated from NDC-hNPCs on LN and VDP substrates (mean ± S.E.M). There was no statistically significant (Student's t-test, p > 0.05) difference in the number of B3T, MAP2, and NF-L positive neurons generated on LN- and VDP-coated substrates. (Varun et al., 2017)

1.4 Scale up Culture of hNPCs

As mentioned earlier, cell based therapies require a large number of cells, typically of the order of 10^9 cells per patient per dose. Two dimensional culture methods have limited scalability and would therefore, not be a feasible means of culturing cells in such huge numbers. To overcome these limitations, adherent

stem cells such as hNPCs could be cultured in microcarrier based bioreactor systems.

1.4.1 Microcarriers

Microcarriers are typically spherical, bead-like structures, about 125-200 μm in diameter, used in scale up cultures due to their high surface area to volume ratio (GE Healthcare, 2005). Microcarriers were initially used to produce viral vaccines (Van Wezel, 1967) and are now being used to culture various cell types such as human Mesenchymal Stem Cells (Frauenschuh, 2007; Eibes et al., 2010), human Pluripotent Stem Cells (Oh et al., 2009; Fernandes et al., 2009; Phillips et al., 2008), and Pluripotent Stem Cell derived Cardiomyocytes (Lecina, Ting, Choo, Reuveny & Oh, 2010).

Micarocarriers can be non-porous, microporous or macroporous. Non-porous microcarriers allow cells to adhere on the surface of the beads and expose them to media, while microporous microcarriers have pores such that cells cannot enter the pores, but they create a microenvironment inside the beads and macroprous beads have larger pores (10-50 um in diameter) with cells growing inside the pores of the microcarriers. Although microporous and macroporous beads allow cell growth in high densities and macroporous beads protect the cells from shear stress, it is often difficult to harvest cells from such beads (Schaffer, Bronzino & Peterson, 2012).

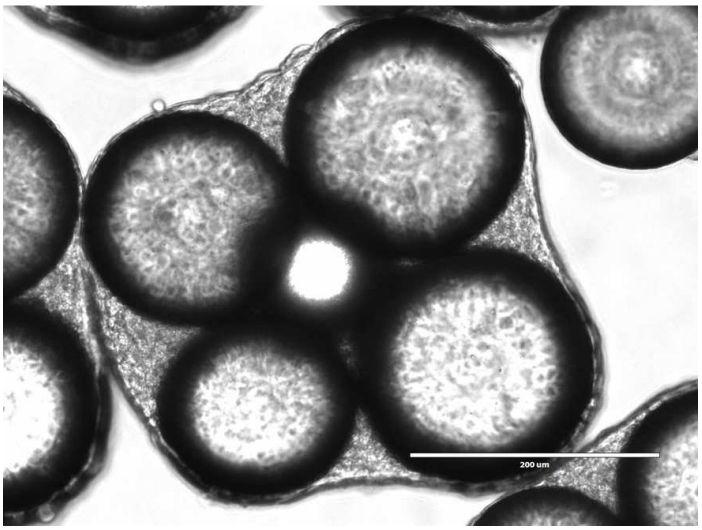

Figure 1-7: Scale up culture using microcarriers. HNPCs cultured on spherical microcarriers. The cells forming a monolayer over a cluster of spherical microcarriers can be seen above. These microcarriers have a high surface area to volume ratio that allows cells to be cultured at higher densities than on 2D tissue culture plates. Scale bar= 200 um.

Microcarriers can be surface modified to enhance attachment, for instance, Cytodex 1 microcarriers have dextran matrix and are positively charged (GE Healthcare, 2005) Cytodex 3 microcarriers consist of a thin collagen coating and are often coated with animal derived ECMP substrates such as Matrigel™ to facilitate expansion of stem cells (Nie, Bergendahl, Hei, Jones & Palecek, 2009). In this study, we used non porous, tissue culture treated polystyrene microcarriers (MC) that could be coated with the substrate of interest (Ln or VDP) to study the scalability of hNPC culture using these MC.

1.4.2 Bioreactors

Bioreactors are systems that aim at scaling up the culture of adherent and non-adherent cells. Different types of bioreactors have been used to expand stem cells, for instance, stirred suspension bioreactors have been used for the expansion of Embryonic Stem cells (Krawetz et al., 2009; Lock & Tzanakakis,

2009; Oh et al., 2009; Kehoe, Jing, Lock & Tzanakakis, 2009), spinner flasks and perfusion bioreactors for the expansion of Mesenchymal Stem Cells (Eibes et al., 2010; Zhao & Ma., 2005) and rotating wall vessel and hollow fibre bioreactors for the expansion of Haematopoietic Stem Cells (Liu, Liu, Fan, Ma & Cui, 2006; Sardonini & Wu, 1993).

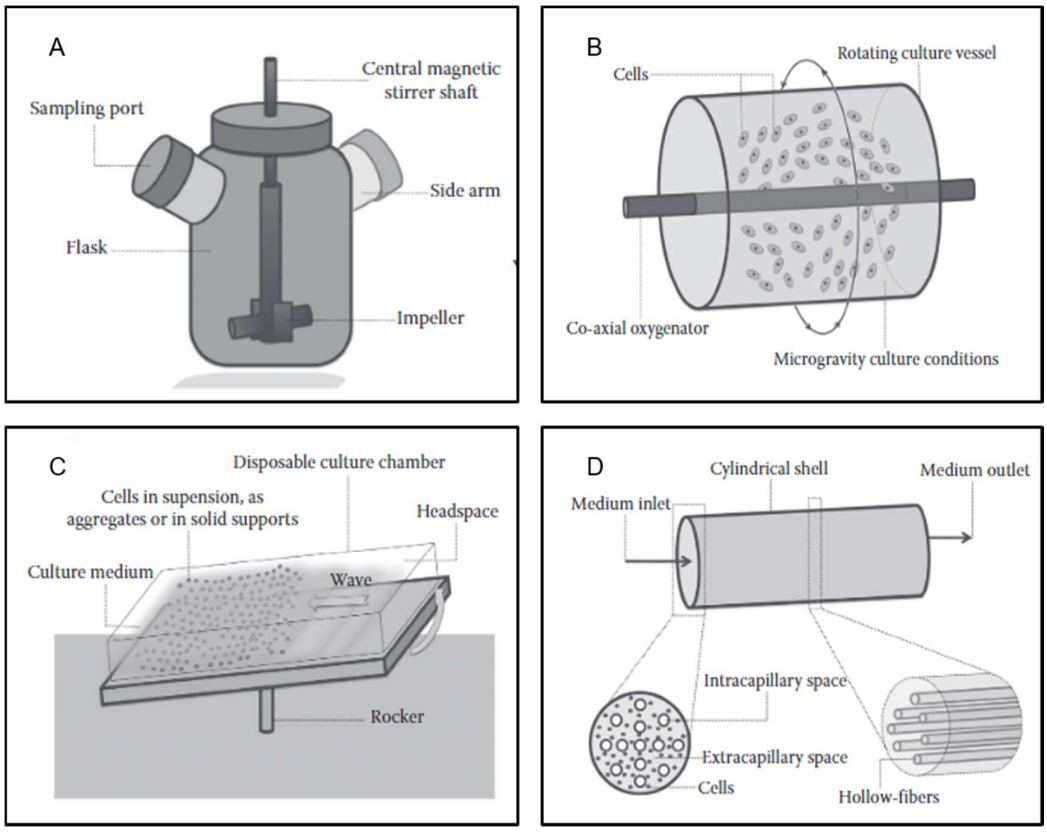

Figure 1-8: Bioreactor Configurations. (A) Spinner flask (B) Rotating wall vessel bioreactor (C) Wave bioreactor (D) Hollow fibre bioreactor (Schaffer, Bronzino & Peterson, 2012)

Bioreactors facilitate uniform distribution of nutrients, metabolites and oxygen by introducing agitation in the form of stirring or by perfusion. Spinner flasks (Figure 1-8 A) contain impellers that sparge gas bubbles throughout the medium to

ensure adequate supply of oxygen throughout the vessel. However, presence of such bubbles often creates a high shear stress environment for the cells around the bubbles and therefore, affects cell viability and growth. Additionally, the scalability of such systems is limited.

Rotating Wall Vessel bioreactors (RWV) (Figure 1-8 B) have a gas permeable silicone membrane through which oxygen exchange takes place, thereby eliminating the need for mechanical agitation to ensure oxygen distribution. These vessels can either be Slow Turning Lateral Vessels with a core oxygenator membrane and a horizontal axis of rotation or they can be High Aspect Ratio Vessels with a broad oxygenator membrane on the back of the vessel. Both configurations of RWV bioreactors simulate a microgravity environment for cells to grow in- the cells/ aggregates experience almost no shear force as their motion is limited to a circular path about the vessel's axis of rotation and their movement through the fluid medium is limited. RWVs, developed by NASA to simulate microgravity in space, are now being used to expand a variety of cells- including mesenchymal stem cells (Chen, Xu, Wan, McCaigue & Li 2006), haematopoietic stem cells (Kedong et al., 2010), rat neural stem cells (Lin, O'Shaughnessy, Kelly & Ma, 2004) etc. In addition to being a scalable system, the vessels allow cells to be cultured in conditions similar to those *in vivo*.

Other bioreactor configurations such as hollow fibre bioreactors (Figure 1-8 D) and wave bioreactors (Figure 1-8 C) are also used for stem cell culture, although not as widely as spinner flasks and RWVs. Hollow fibre bioreactor contain a bundle of hollow fibres through which cells pass through and such constant motion of cells through the fibres and of the media around the fibres ensures adequate mass transport. Wave bioreactors ensure mass transport by rocking the cells and media, placed in a disposable pouch, back and forth. However, the

presence of air pocket above the medium, subjects the cells to shear stress. Additionally, it is difficult to monitor and control the cell growth process in these bioreactors. Therefore, to culture hNPCs in a physiologically relevant shear stress environment and to facilitate their large scale expansion, we chose to use rotating wall vessel bioreactors.

In this study, we first assessed the ability of Ln and VDP MC to support the expansion and differentiation of hESC and hIPSC derived hNPC lines on a small scale and then scaled up the culture in a rotating wall vessel (RWV) bioreactor system to facilitate the large scale expansion of hNPCs.

1.5 Modelling Alzheimer's Disease

1.5.1 Alzheimer's Disease

Alzheimer's Disease (AD) is the most common form of dementia and is the sixth leading cause of death in the United States (Alzheimer's disease facts and figures, 2016). It is a neurodegenerative disease marked by cognitive decline and memory dysfuntion. Pathologically, it is characterized by senile plaques, composed of Amyloid- Beta (Aβ) deposits in the extracellular space and neurofibrillary tangles, caused by hyperphosphorylation of tau in neurons (Perl, 2010).

AD can be either familial (fAD) or sporadic (sAD); the rarer familial form is caused by mutations in the Amyloid Precursor Protein (APP), Presenilin-1 (PSEN1) or Presenilin-2 (PSEN2) genes while the more common sporadic form has been linked to various risk factors, particularly to polymorphisms in the APOE gene (Ittner & Götz, 2011). Here, we use human Neural Progenitor Cells (hNPCs) derived from iPSCs from patients with fAD, caused by the duplication of APP gene

to study AD relevant phenotypes and hNPCs derived from iPSCs from Non Demented Control individuals as control.

1.5.2 Alzheimer's Disease Pathology

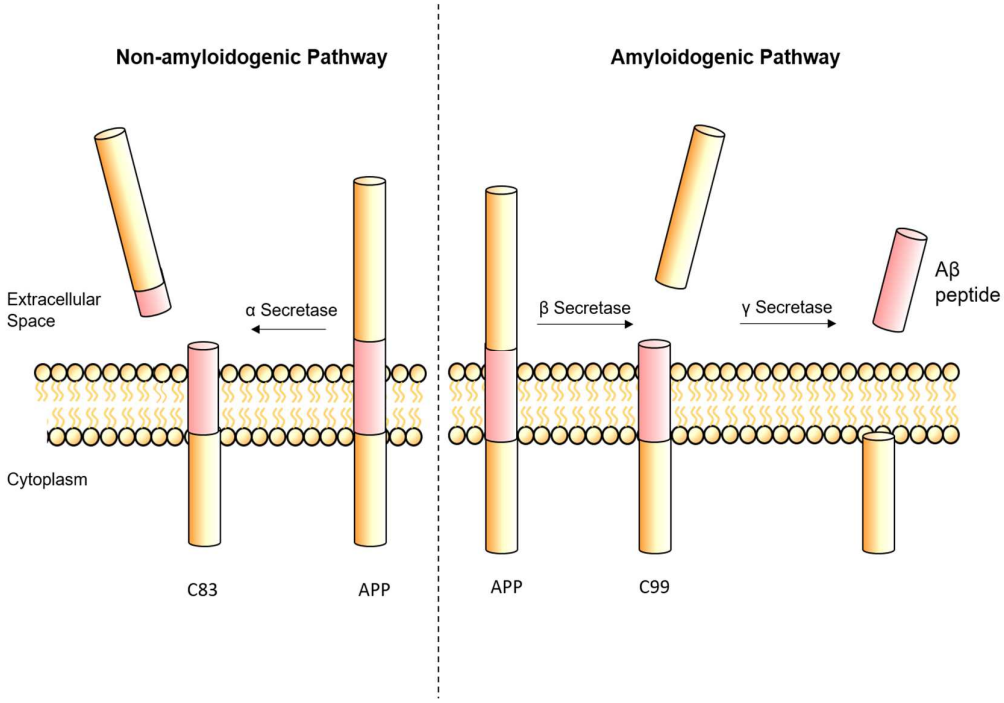

Figure 1-9: APP Proteolysis Amyloid Precursor Protein is cleaved through the non-amyloidogenic pathway by α secretase in the Aβ domain (shown in pink), thereby preventing the formation of Aβ peptide. However, in the amyloidogenic pathway, APP is cleaved by β secretase to produce a fragment with the Aβ domain intact. This fragment is further cleaved by γ secretase 38-42 amino acid residues from the extracellular N terminal (after β secretase cleavage) to generate Aβ peptides of varying lengths. $A\beta_{1-42}$ tends to aggregate and form senile plaques in the brain.

The accumulation of amyloid beta (Aβ) causing senile plaques to form, is attributed to the improper cleavage of its precursor protein APP. APP is a transmembrane protein that is processed by three enzymes- α, β and γ secretases. In the non-amyloidogenic pathway, APP is cleaved by α-secretase, secreting soluble APPα (sAPPα) into the extracellular space, leaving behind an 83

amino acid fragment in the membrane. This cleavage occurs in the Aβ peptide domain, thereby preventing the production of Aβ. However, in the amyloidogenic pathway, APP is cleaved by β secretase (BACE) 99 amino acids from the intracellular carboxy terminal of APP. This leaves behind a 99 amino acid fragment with the Aβ domain intact, in the membrane which is then cleaved by γ secretase 38-43 amino acids from the first amino acid residue in the fragment left from the β secretase cleavage. This causes the release of Aβ peptide of varying lengths into the extracellular space, with $A\beta_{1-40}$ being the most abundant to $A\beta_{1-42}$, aggregating to form plaques. Additionally, the presence of intracellular Aβ and oligomeric Aβ species has been shown to have toxic effects on neurons (LaFerla, Green & Oddo, 2007).

Tau is a microtubule associated protein that is primarily found in axons and is involved in axonal transport regulation and microtubule stabilization. Tau can undergo phosphorylation at 84 sites, at serines, threonines and tyrosines and this phosphorylation destabilizes the microtubule. Particularly, phosphorylation at Threonine 231, Serine 396 and Serine 422 causes tau to aggregate to form filaments (Gong & Iqbal, 2008). Thus, hyperphosphorylation of tau as observed in AD, destabilizes microtubules and leads to the formation of neurofibrillary tangles (Ittner & Götz, 2011).

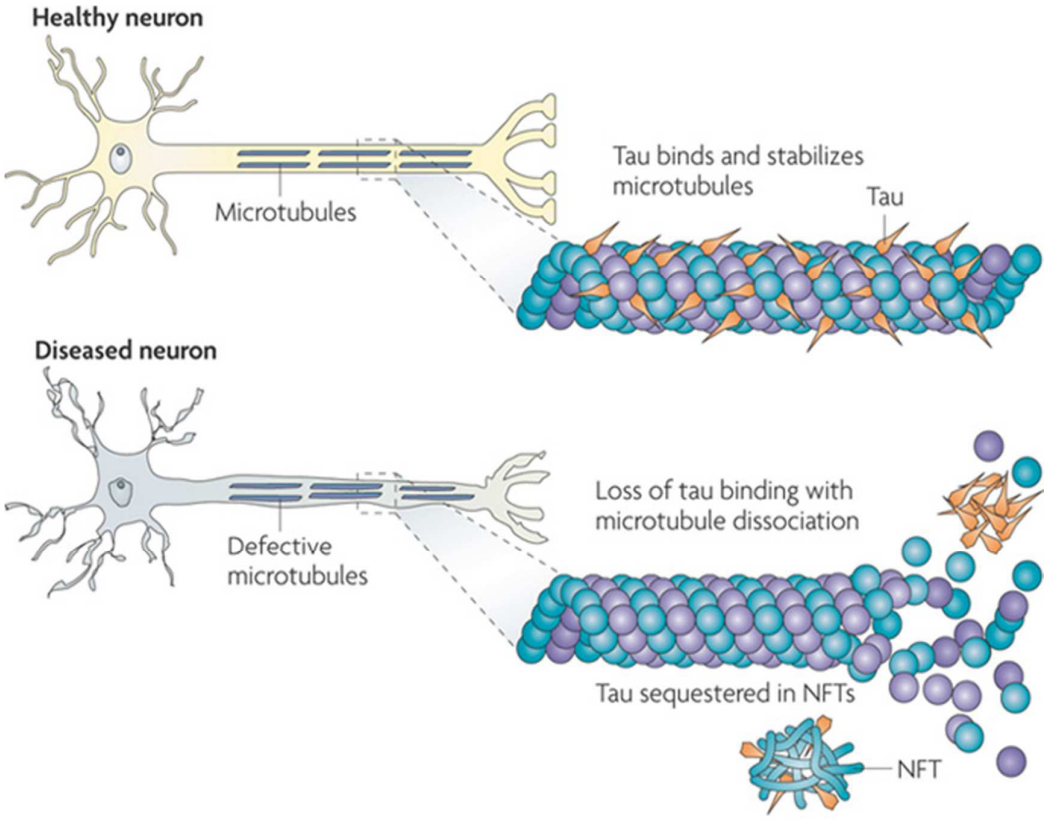

Figure 1-10. Formation of neurofibrillary tangles Tau is a microtubule associated protein that binds to microtubules and stabilizes them. Hyperphosphorylation of tau, inhibits its binding to microtubules, thereby destabilizing them. Additionally, hyperphosphorylation of tau causes it to be sequestered, leading to the formation of neurofibrillary tangles. (Brunden, Trojanowski & Lee, 2009).

The prevailing amyloid cascade hypothesis posits the deposition of amyloid beta as the central event that leads to neurodegeneration in AD. According to this hypothesis, the formation of Aβ peptide, and its subsequent oligomerization leads to abnormal phosphorylation of tau, thereby causing neurofibrillary tangles to form, which in turn leads to neuronal death and synaptic dysfunction (Hardy & Selkoe, 2002). In this study, we have assessed the levels of secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ and phosphorylated tau relative to total tau in fAD patient derived hNPCs (APP-hNPC) and in Non Demented Control hNPCs (NDC-hNPC).

2. Materials and Methods

2.1 hNPC Culture

NDC-, HES3- and APP-hNPC were cultured on PLO/Ln coated tissue culture treated 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 with 1x DPBS before use. HNPCs were plated on PLO/Ln plates at 1-2 x 10^4 cells/cm^2 and were routinely passaged upon reaching 80% confluency. Neural Expansion Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 0.5% (v/v) B27 supplement) with 30 ng/mL EGF and 30 ng/mL FGF2 was used to culture the cells.

2.2 Microcarrier Culture

Tissue cultured treated polystyrene microcarriers (Corning Enhanced Attachment microcarriers) were coated with the substrate of interest (PLO/Ln or Vitronectin Derived Peptide) as follows:

Sterile microcarriers were weighed and suspended in 1x DPBS. The required amount of microcarriers were transferred to Eppendorf tubes/ conicals and were suspended in 4 ug/mL PLO solution and incubated at 37°C overnight after which the PLO was removed using micropipettes and the PLO- coated microcarriers were washed twice with 1x DPBS. These microcarriers were then suspended in 4 ug/mL mouse laminin solution and incubated at 37°C overnight.

For coating the microcarriers with the peptide (VDP), the required amount of microcarriers were suspended in 0.5mM solution of the peptide and incubated at 37°C for 48 hours.

The microcarriers were washed once with 1x DPBS and once with NEM before use.

HNPCs cultured on PLO/Ln plates were seeded on PLO/Ln or VDP coated microcarriers in NEM containing 5uM Rho kinase inhibitor (Y-27632) to aid single cell survival and were transferred to 6 well Ultra Low Attachment plates. For the long term expansion and differentiation experiments, hNPCs were seeded at 1.5 million cells/well with 1 mg/mL of PLO/Ln or VDP microcarriers. The plates were placed in a 37°C/5% CO_2/95% humidity tissue culture incubator with half the final culture volume in each well to allow for maximum cell attachment on the microcarriers for 12 hours after which the rest of the culture media containing Rho kinase inhibitor was added and the plates were then placed on an orbital shaker with the agitation set at 95 rpm. Three fourth of the media was changed after 24 hours and half the media was changed every 24 hours after that.

Upon confluency, the cell-microcarrier aggregates were transferred to conicals, centrifuged at 200g for 3-5 minutes for the aggregates to settle and the media was aspirated. The aggregates were then resuspended in Accutase and were transferred back to Ultra Low Adhesion plates and were incubated at 37°C for 10 minutes (5 minutes with no agitation and 5 minutes on the orbital shaker). Media was added to each well to inactivate the Accutase and the aggregates were gently pipetted up and down to detach the cells from the microcarriers. This cell-microcarrier suspension was then passed through a 40 um cell strainer to obtain a single cell suspension free of the microcarriers. The cells were the centrifuged at 200g for 5 minutes, resuspended in media with rho kinase inhibitor, counted using a haemocytometer and were seeded on washed, freshly coated microcarriers.

For differentiation, hNPCs were expanded on microcarriers for 4-5 days till confluent and three fourth of the media was changed to Neural Differentiation

Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 0.5% (v/v) B27 supplement) with 20 ng/mL BDNF and 20 ng/mL GDNF. The cells were cultured in this differentiation media for 18 days after which Neural Base Media (1x DMEM-F12, 0.5% (v/v) N2 supplement, 0.5% (v/v) B27 supplement) was used for the rest of the culture duration. This protocol was followed unless otherwise specified.

For the density gradient/maximization experiments, the number of cells seeded and the microcarrier concentration were varied as mentioned in table 1. Three fourth media was changed every 24 hours.

2.3 Bioreactor Culture

Synthecon 55mL STLV was prepared according to manufacturer's instructions. Briefly, the vessel was dismantled and immersed in a mild detergent for an hour after which it was cleaned in Milli- Q water. The vessel was soaked in Milli-Q water overnight and was air dried before autoclaving at 121°C for 20 minutes. The vessel was washed in Milli-Q water and was autoclaved before beginning each experiment.

HNPCs cultured on microcarriers in 6 well Ultra Low Adhesion plates were seeded on PLO/Ln or VDP microcarriers (coated as mentioned above) and were transferred to the bioreactor vessel using its fill port. The vessel was filled with media and bubbles were removed using syringes. The vessel was rotated at 10 rpm to begin with and with the formation of clusters and increase in their size, the rpm was increased.

Samples were taken through the fill port every day for imaging, obtaining cell counts and monitoring cell viability. 30 mL media was removed everyday and the cell-microcarrier aggregates were mixed throughout the rest of the culture volume (25 mL). Two ~0.5 mL samples were taken through the fill port and

transferred to 6 well Ultra Low Adhesion plates for imaging. These cells were stained with Propidium Iodide to monitor cell viability.

When confluent, the microcarrier aggregates were transferred to 50 mL conicals and were centrifuged and the media was aspirated. Accutase was added to the conicals and the aggregates were transferred to a 6 well Ultra Low Adhesion plate with the aggregates evenly divided among all 6 wells. The plate was incubated at 37°C for 10 minutes with no agitation after which media was added to inactivate the Accutase. The cells were separated from the microcarriers by passing them through 40 um cell strainers. The cells were then centrifuged at 200g for 5 minutes and resuspended in appropriate volume of media for obtaining cell counts and for running other assays.

2.4 Quantitative PCR

RNA was isolated from cells using Nucleospin RNA kit (Macherey Nagel). cDNA was synthesised from 1ug 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 involved a 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 endogenous control and the gene expression was quantified using the ΔΔ Ct method.

2.5 Flow Cytometry

Cells cultured on microcarriers were dissociated using Accutase, triturated, passed through a 40 um cell strainer and washed once with stain buffer (BD Biosciences). The cells were fixed using fixation buffer (BD Cytofix fixation buffer) at room temperature for 30 minutes. The cells were washed twice with stain buffer and permeabilized using BD Phosflow Perm Buffer III at 4°C for 30

minutes. The cells were washed twice and stained using conjugated antibodies for SOX1, SOX2 and NESTIN as listed in Table 2 at 4°C overnight. The cells were washed twice and resuspended in stain buffer and analysed on Accuri C6 flow cytometer (BD Biosciences).

For the propidium iodide staining, the cell suspension was washed once with 1x DPBS and resuspended in stain buffer. The cells were stained with 2mg/mL propidium iodide for 1-2 minutes in dark and were analysed on Accuri C6 flow cytometer (BD Biosciences).

2.6 Enzyme Linked Immunosorbent Assay (ELISA)

Cells were cultured in media prepared using phenol-red free DMEM/F12 for 48 hours prior to media collection. The 24 hour conditioned media was collected and concentrated 10x by centrifuging at 14,000 g for 30 minutes and reverse spinning for 1.5 minutes at 4°C. The samples were stored in -80°C until use. Commercially available ELISA kits for $A\beta_{40}$ and $A\beta_{42}$ (Life Technologies catalog numbers KHB3481 and KHB3441 respectively) were used to measure the level of $A\beta_{40}$, $A\beta_{42}$ secreted by hNPCs, according to the manufacturer's protocol. The samples were incubated on wells pre-coated with monoclonal antibody specific for the $-NH_2$ terminal of Aβ, along with detection (primary) antibody specific for the –COOH terminal of Aβ and the plate was incubated for 3 hours at room temperature with shaking (250 rpm). The wells were washed four times and were incubated with HRP conjugated secondary antibody for 30 minutes at room temperature. The plate was washed four times with the wash buffer and the was incubated with the stabilized chromogen (TMB) for 30 minutes at room temperature in dark and the reaction was stopped using sulfuric acid, after which the absorbance at 450 nm was read.

For the phorphorylated tau and total tau ELISAs, the cell pellet was collected after dissociation with Accutase, trituration and passing through cell strainers. The cells were lysed in 0.1% TBST with protease inhibitors with vortexing and the lysate was centrifuged at 16000 g for 30 minutes at 4°C to collect the supernatant, which was stored in -80°C until use. A Bradford assay was done to determine total protein (absorbance read at 595 nm) and 100 uL of the lysates diluted to 50ug/mL were used for the phosphorylated & total tau ELISAs. Commercially available kits for phosphorylated tau (Life Technologies, catalog number KHB8051) and total tau (Life Technologies, catalog number KHB0041) were used for this study- briefly, the samples were incubated in wells pre-coated with monoclonal antibody specific to tau protein phosphorylated at threonine 231 for phosphorylated tau assay and antibody specific to human tau protein for the total tau assay for 2 hours. The wells were washed four times and were incubated with the detection antibody for an hour at room temperature. The wells were washed four times and the HRP labelled secondary antibody was added to wells. After an incubation period of 30 minutes at room temperature, the wells were washed four times and the stabilized chromogen (TMB) was added. The plate was incubated in dark for 30 minutes at room temperature after which the stop solution was added. The absorbance was read at 450 nm. Absorbance readings for all ELISAs were done using Synergy multi mode plate reader.

2.7 Immunofluorescence

2.7.1 Confocal Imaging

Cells cultured on microcarriers were fixed using BD Cytofix fixation buffer for 30 minutes at room temperature, after which the cell-microcarrier aggregates were was washed twice with 1x DPBS. The aggregates were permeabilized in BD Phosflow Perm Buffer III for 30 minutes at 4°C and then washed twice with 1x DPBS. The aggregates were stained using the primary antibodies listed in table 2

and incubated at 4°C overnight. Excess/unbound antibodies were washed off by rinsing with 1x DPBS twice and the aggregates were stained with the appropriate secondary antibodies (as listed in table 2) and incubated at room temperature for 1 hour. The cell-microcarrier aggregates were washed twice with 1x DPBS and were stained with Hoescht 33342 (1:5000) for 10 minutes at room temperature, followed by two washes with 1x DPBS and resuspension in enough 1x DPBS to transfer them to 6 well Ultra Low Adhesion plates where the samples were stored (at 4°C) until imaging using either EVOS fluorescence microscope or Leica TCS SP5 AOBS Spectral Confocal System. The aggregates were transferred to double cavity slides and were mounted in Vectashield mounting medium for imaging using the confocal microscope. Laser power settings are as mentioned in table 3 and the PMT gain for each image stack was set to ensure no saturation throughout the stack.

2.7.2 Plating Microcarriers

Neurons cultured on microcarriers were incubated in Accutase for 5 minutes at 37°C and were gently mechanically dissociated to break apart the clusters into smaller aggregates. Media was added to inactivate the Accutase and the aggregates were resuspended in the appropriate media with Rho kinase inhibitor (Y-27632). The aggregates were plated on Matrigel™ coated 12 well plates and the media was changed on day 1, followed by half media changes until fixation. Once the plated aggregates had spread out, the cells were fixed using BD Cytofix Fixation Buffer for 15 minutes at room temperature. The cells were washed twice with stain buffer and the permeabilized using BD Phosflow Perm Buffer III for 30 minutes at room temperature. The cells were washed twice with stain buffer and then stained with primary antibodies as listed in table 2, overnight at 4°C. The cells were washed twice and stained with the appropriate secondary antibody for one hour at room temperature and then with Hoescht 33342 for 10 minutes at

room temperature. The cells were washed twice after staining with the secondary antibodies as well as after staining with Hoescht. Samples were imaged using EVOS fluorescence microscope.

3. Results

3.1 Long term expansion of hNPCs on VDP microcarriers

We previously identified VDP as a substrate capable of supporting long term expansion and differentiation of several ESC and iPSC line derived hNPCs on tissue culture treated polystyrene plates. Next, we sought to identify if the peptide would support the culture of these hNPCs on tissue cultured treated polystyrene microcarriers. HNPCs derived from Non-Demented Control hiPSCs (NDC-hNPCs) were cultured on VDP coated microcarriers (VDP MC) and on laminin coated microcarriers as control (Ln MC) for more than 20 passages. The cells adhered to both VDP and laminin microcarriers, forming aggregates and expanding up to four fold by day 4 or 5 of culture (Figure 3-1 A and Figure 3-2 A). The doubling time of hNPCs on VDP MC was similar to that of hNPCs cultured on VDP coated 2D tissue culture plates. However, VDP MC supported 1.5±0.1 million cells/mL of culture volume when confluent as opposed to 2D VDP coated plates which supported only 0.9±0.04 million cells/mL. Also, the initial cell seeding density and microcarrier concentrations could be optimized to result in 4.5 million cells/mL of culture volume with VDP MC culture. The hNPCs cultured on VDP and Ln MC expressed multipotency markers such as SOX1 and SOX2 throughout 10 passages as shown by qPCR analysis, with no significant difference in the expression of these genes across passages (Figure 3-1.B and Figure 3-2.B). Additionally, flow cytometry analysis of hNPCs cultured on VDP and Ln MC for 20 passages revealed that >99% of the cells express these hNPC markers (Figure 3-1. E and Figure 3-2.E) and immunofluorescence on passage 13 hNPCs on VDP showed the expression of SOX2 and NESTIN (Figure 3-1. D).

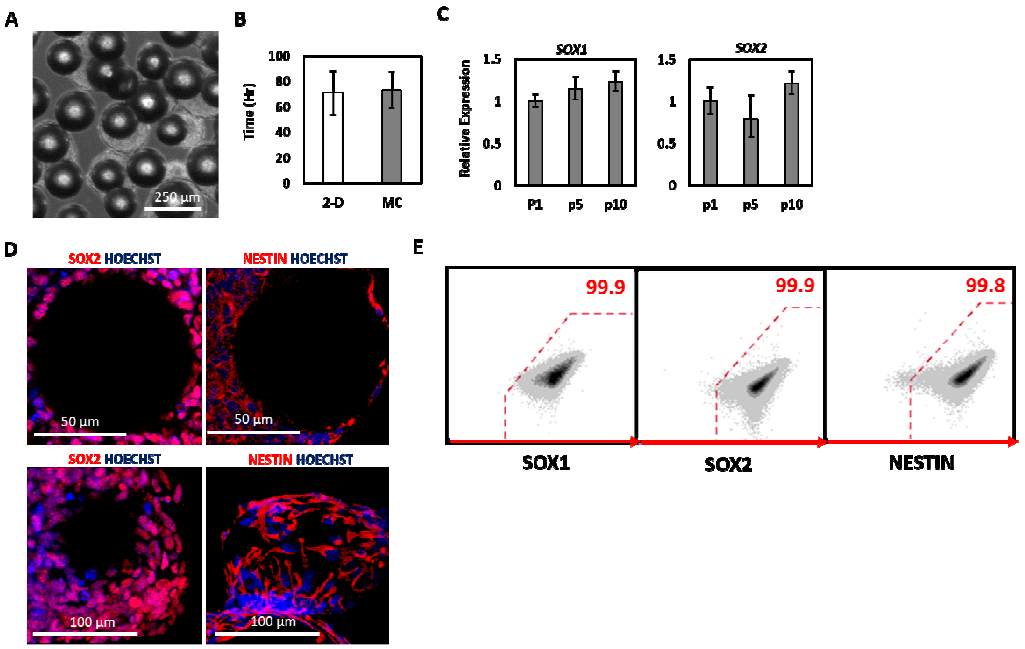

Figure 3-1: Long-term culture and expansion of NDC-hNPCs on VDP-coated microcarriers (MCs). (A) Representative phase contrast images of NDC-hNPCs cultured on VDP-coated MCs for 20 passages (scale bar = 250 µm). (B) Doubling time of NDC-hNPCs cultured on VDP-coated 2-D and MC surfaces. Data is presented as the mean ± S.D of the doubling time over the course of 20 passages. There was no statistical difference in the doubling time of hNPCs grown on MC and 2-D surfaces (Student's t-test, p = 0.626). (C) Quantitative PCR (qPCR) analysis for expression of hNPC multipotency markers SOX1 and SOX2 for in NDC-hNPCs cultured VDP-coated MCs for 1, 5, and 10 passages (mean ± S.E.M). One-way analysis of variance (ANOVA) revealed that there was no statistical difference (SOX1, p = 0.099; SOX2, p = 0.118) in expression of these genes over the course of 10 passages in hNPCs cultured on VDP-coated microcarriers. (D) SOX2 and NESTIN immunofluorescence of NDC-hNPCs cultured on VDP-coated MCs for 13 passages. Cross-section of the MC carrier cultures is shown in the upper panels (scale bar = 50 µm) and z-stack projection is shown in the lower panels (scale bar = 100 µm). (E) Flow cytometry analysis for SOX1, SOX2, and NESTIN expression in NDC-hNPCs cultured on VDP-coated microcarriers for 20 passages.

We also tested the ability of VDP MC to support the long term expansion of ESC derived hNPCs by culturing HES-3 hNPC on VDP MC for more than 10 passages. HES-3 hNPCs were also cultured on Ln MC as control and on VDP and Ln coated 2-D tissue culture plates to assess the scalability achieved using MC. The cells adhered to both Ln MC and VDP MC (Figure 3-3 A) and showed doubling times similar to Ln and VDP coated 2-D surfaces (Figure 3-3 B). VDP MC showed the

ability to support 0.83±0.04 million cells/mL of culture volume when confluent. These cells expressed hNPC markers SOX1, SOX2 and NESTIN throughout 10 passages when cultured on Ln MC and on VDP MC as shown by flow cytometry analyses (Figure 3-3.D). Additionally, immunofluorescence on passage 13 hNPCs on VDP MC revealed the expression of the NPC marker NESTIN (Figure 3-3 C).

Thus, VDP MC can support the long term expansion of both iPSC and ESC derived hNPCs on levels similar to Ln MC. Also, MC can be used to scale up of the culture of these hNPCs when compared to conventional 2-D tissue culture surfaces.

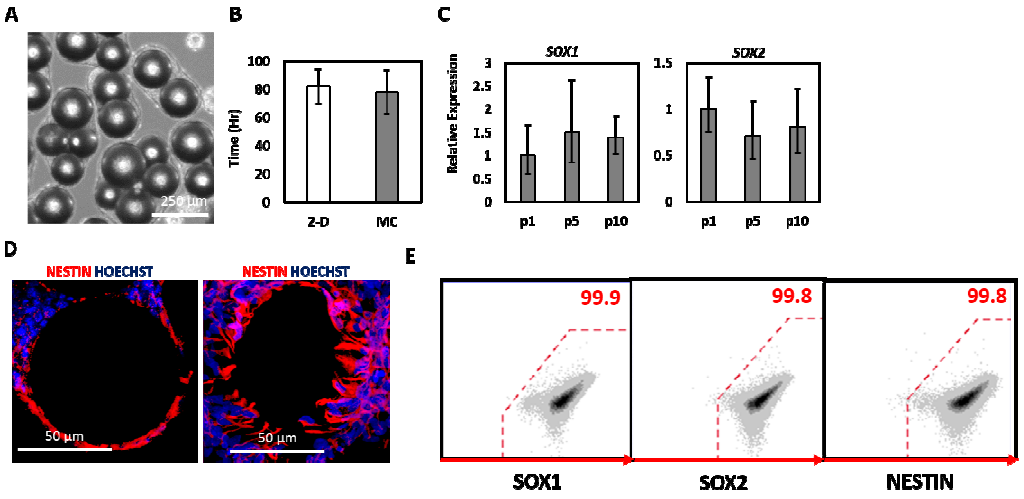

Figure 3-2: Long-term culture and expansion of NDC-hNPCs on LN-coated microcarriers (MCs). (A) Representative phase contrast images of NDC-hNPCs cultured on LN-coated MCs for 20 passages (scale bar = 250 µm). (B) Doubling time of NDC-hNPCs cultured on LN-coated 2-D and MC surfaces. Data is presented as the mean ± S.D of the doubling time. There was no statistical difference in the doubling time of hNPCs grown on MC and 2-D surfaces (Student's t-test, p = 0.358). (C) Quantitative PCR (qPCR) analysis for expression of hNPC multipotency markers SOX1 and SOX2 for in NDC-hNPCs cultured LN-coated MCs for 1, 5, and 10 passages (mean ± S.E.M). One-way analysis of variance (ANOVA) revealed that there was no statistical difference (SOX1, p = 0.565; SOX2, p = 0.570) in expression of these genes over the course of 10 passages in hNPCs cultured on LN-coated microcarriers. (D) NESTIN immunofluorescence of NDC-hNPCs cultured on LN-coated MCs for 13 passages. Cross-section of the MC carrier cultures is shown on the left panel (scale bar = 50 µm) and z-stack projection is shown on the right panels (scale bar = 100 µm). (E) Flow cytometry analysis for SOX1, SOX2, and NESTIN expression in NDC-hNPCs cultured on LN-coated microcarriers for 20 passages.

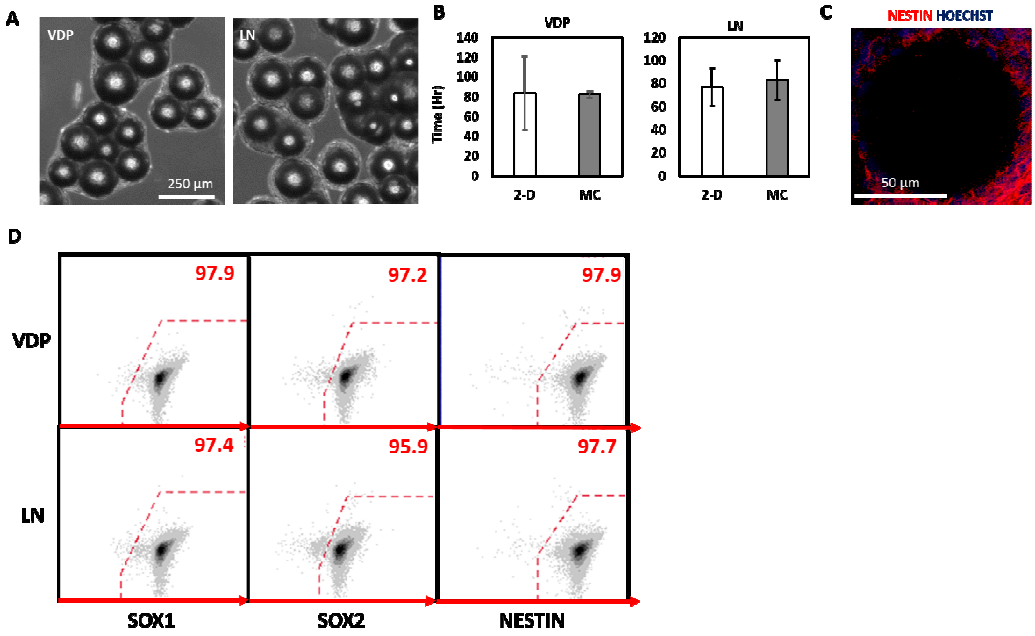

Figure 3-3: Long-term culture and expansion of HES3-hNPCs on VDP- and LN-coated microcarriers (MCs). (A) Representative phase contrast images of HES3-hNPCs cultured on VDP- (left panel) and LN-coated (right panel) for 10 passages. (B) Doubling time of HES3-hNPCs cultured on 2-D and MC surfaces coated with VDP (left panel) and LN (right panel). Data is presented as the mean ± S.D of the doubling time. There was no statistical difference in the doubling time of hNPCs grown on MC and 2-D surfaces coated with VDP (Student's t-test, p = 0.941) and LN (Student's t-test = 0.542). (C) NESTIN immunofluorescence of HES3-hNPCs cultured on VDP-coated MCs for 13 passages (scale bar = 50 µm). (D) Flow cytometry analysis for SOX1, SOX2, and NESTIN expression in HES3-hNPCs cultured on VDP- (top panels) and LN-coated microcarriers for 11 passages.

3.2 Differentiation of iPSC derived hNPC

HNPCs have the ability to differentiate into neurons, glial cells and oligodendrocytes. To assess if the hNPCs cultured on VDP MC retain their ability to differentiate, we expanded NDC-hNPCs on VDP MC for 4-5 days till confluent and started differentiating these cells by switching the media to neural differentiation media and continued to culture the cells in NDM for 30 days. QPCR analysis comparing the expression of various hNPC, neuronal, neurotransmitter, glial and cortical markers in passage 1 hNPCs and day 30 neurons cultured on

VDP MC reveal the upregulation of neuronal markers such as TUBB3, a microtubule protein and RBFOX3, a marker for neuronal nuclei and glial markers in day 30 neurons (Figure 3-4 A). On the other hand, hNPC markers such as Nestin, SOX1 and SOX2 were downregulated in day 30 neurons when compared to passage 1 hNPCs on VDP MC (Figure 3-4 A). RNA Seq analysis of day 30 neurons and passage 10 hNPCs on VDP MC also revealed a similar pattern of downregulation of genes associated with NPC phenotype (such as NES, SOX1, SOX2 and PAX6) and upregulation of genes associated with neuronal phenotype (such as DCX, TUBB3, MAP2) (Figure 3-4 B). Similarly, day 30 neurons cultured on Ln MC expressed higher levels of neuronal, neurotransmitter, cortical and astrocyte markers than neurons cultured on Ln coated 2-D plates (Figure 3-6 A). Immunofluorescence on day 31 neurons on VDP MC also show the expression of TUBB3 (Figure 3-4 D). Additionally, flow cytometry analysis of day 65 neurons on VDP MC and Ln MC indicate the presence of NEUN (neuronal nuclei) positive cells (Figure 3-4 C and Figure 3-6 B). Thus, VDP and Ln MC were shown to support neuronal differentiation in suspension cultures.

Next, we dissociated neurons cultured on VDP MC for 69 days in suspension, plated the dissociated aggregates on Matrigel™ and allowed the cells to spread out for 7 days for their morphologies to be apparent. These plated cells were positive for neuronal markers MAP2, TUBB3 and GABA and for glial marker GFAP (Figure 3-4 E). This indicates that neurons cultured on MC in suspension could be harvested for practical applications by dissociating and plating without the loss of their neuronal morphology or characteristics.

We compared the maturity of neurons generated on MC surfaces and those generated using conventional 2-D culture. RNA Seq analysis comparing the gene expression profiles of day 8 NDC neurons cultured on VDP coated 2D plates and day 30 NDC neurons cultured on VDP MC showed the upregulation of several

neuronal and neurotransmitter markers such as SYN1, DLG and NEFM in day 30 neurons on VDP MC (Figure 3-5 A). Gene ontology analysis revealed the upregulation of genes involved in the regulation of neurogenesis, neuron projection and synapse formation in day 30 neurons on VDP MC (Figure 3-5 E and F). Genes related to post mitotic neuron and synapse formation, Ca^{2+}, K^{+}, Na^{+} and Cl^{-} ion channels and secretion and release of neurotransmitters such as GABA (gamma aminobutyric acid), glutamate and acetylcholine were significantly upregulated in day 30 neurons cultured on VDP MC compared to day 8 neurons on VDP coated 2-D plates and hNPCs cultured on VDP coated 2-D and MC surfaces (Figure 3-5 B, C and D). Taken together, these results indicate that neurons cultured on MC surfaces are more mature than those obtained by differentiating hNPCs on 2-D surfaces.

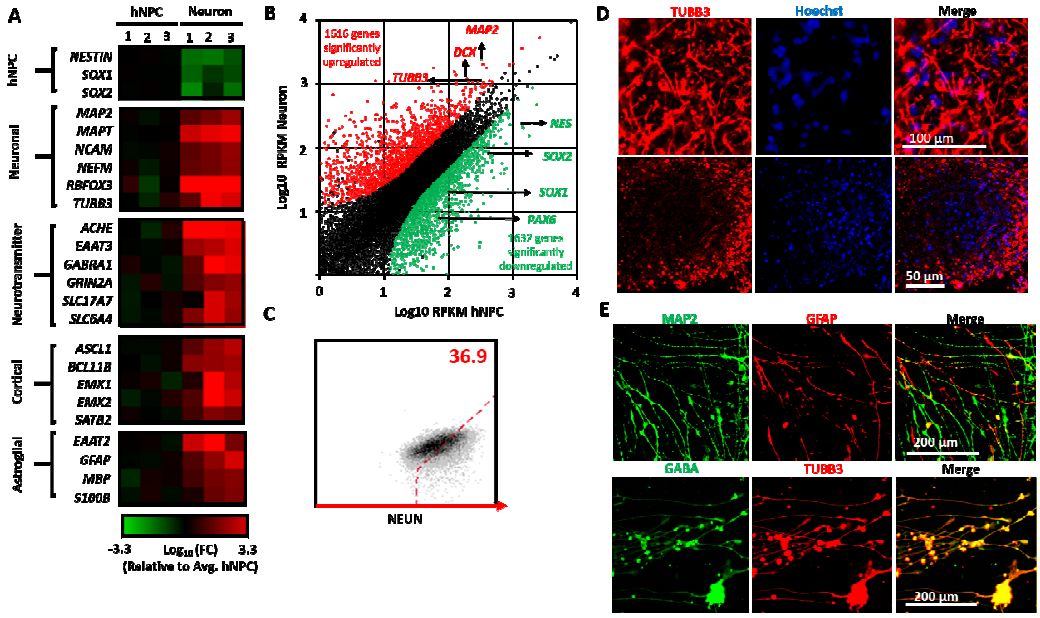

Figure 3-4: Differentiation of NDC-hNPCs on VDP-coated microcarriers (MCs). (A) Quantitative PCR (qPCR) analysis for expression of hNPC multipotency-, neuronal-, neurotransmitter-, cortical-, and astroglial-related markers in day 30 (D30) differentiated cultures. (B) RNA sequencing (RNA-seq) analysis comparing gene expression profiles of hNPC and D30 differentiated cultures. Genes with RPKM values greater than 1 were plotted. Genes showing altered expression with FDR < 0.05 and more than 2 fold changes were considered significantly upregulated (red) or downregulated (green). (C) Flow cytometry analysis for NEUN expression in D65 differentiated cultures. (D) TUBB3 immunofluorescence of D31 cultures. Cross-section of the MC carrier cultures is shown in the upper panels (scale bar = 100 µm) and z-stack projection is shown in the lower panels (scale bar = 50 µm). Immunofluorescence for (E) MAP2, GFAP, (F) GABA, and TUBB3 in differentiated MC cultures dissociated on D69, plated, and cultured for 7 days after dissociation (scale bar = 200 µm).

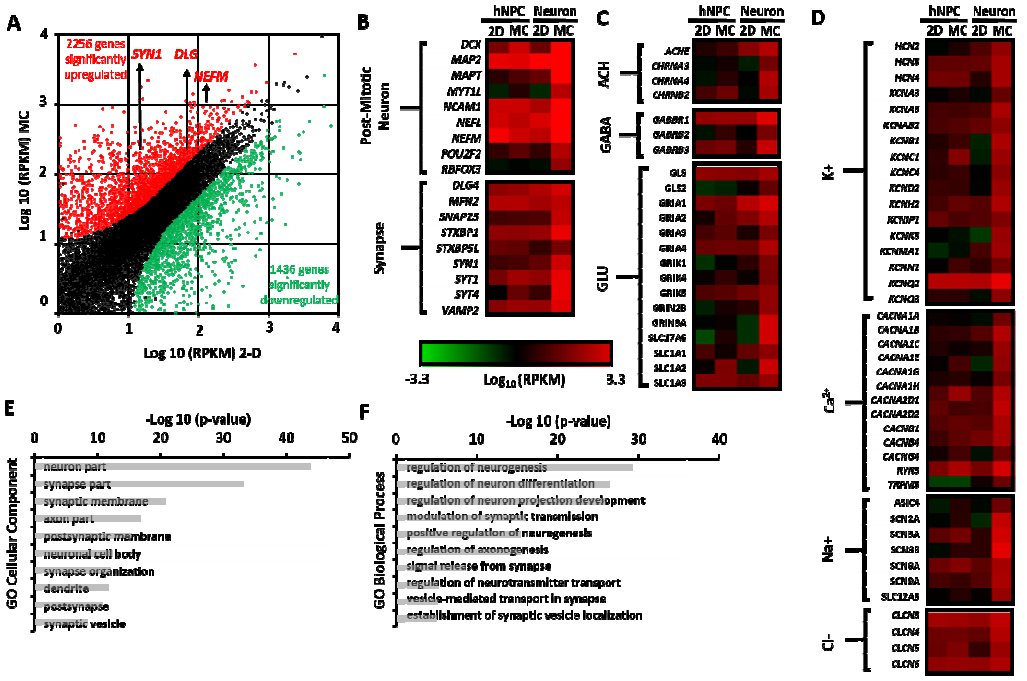

Figure 3-5: Comparison of differentiation of NDC-hNPCs on VDP-coated 2-D and microcarrier (MC) surfaces. (A) RNA sequencing (RNA-seq) analysis comparing gene expression profiles of hNPCs differentiated on 2-D and MC surfaces. Genes with RPKM values greater than 1 were plotted. Genes showing altered expression with FDR < 0.05 and more than 2 fold changes were considered significantly upregulated (red) or downregulated (green). Genes that were significantly upregulated on differentiated cultures generated on MC surfaces include those related to (B) post-mitotic neurons and synapse formation, (C) acetylcholine (ACH), γ-aminobutyric acid (GABA), and glutamate (GLU) neurotransmitter synthesis, release, and transmission, (D) potassium (K^+), calcium (Ca^{2+}), sodium (Na^+), and chloride (Cl^-) ion channels involved in action potential generation. Gene ontology (GO) analysis identified (E) cellular components and (F) biological processes related to neuron maturation, axon generation, and synapse formation were significantly enriched in differentiated cultures generated on MCs.

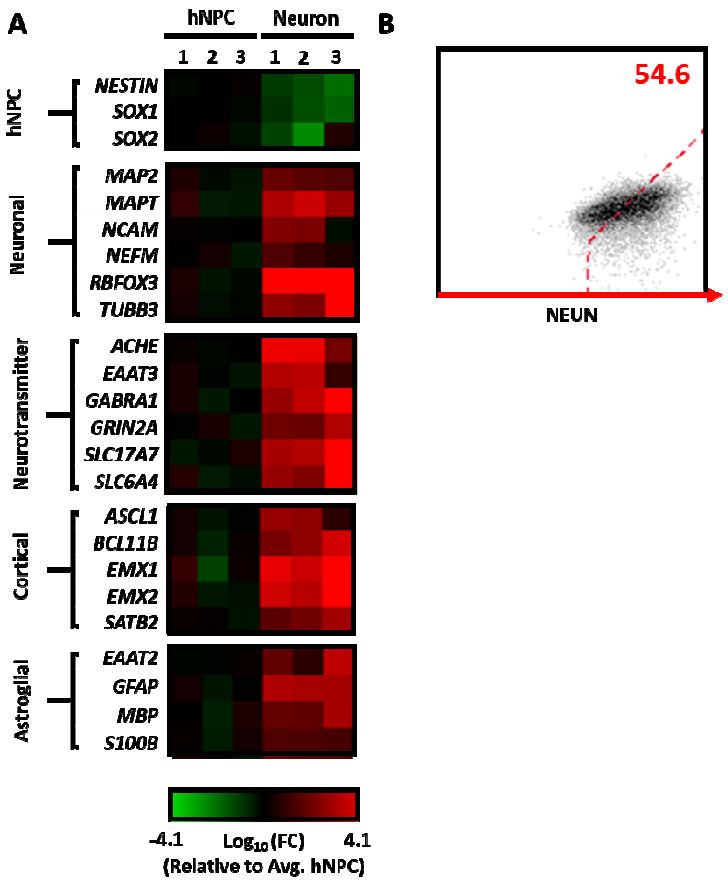

Figure 3-6: Differentiation of NDC-hNPCs on LN-coated microcarriers (MCs). (A) Quantitative PCR (qPCR) analysis for expression of hNPC multipotency-, neuronal-, neurotransmitter-, cortical-, and astroglial-related markers in day 30 (D30) differentiated cultures. (B) Flow cytometry analysis for NEUN expression in D65 differentiated cultures.

3.3 Bioreactor scale up culture

Since Ln and VDP MC could support the expansion of hNPCs over multiple passages on a small scale, in 6 well plates, we assessed if these MC could be used in a bioreactor system to further scale up the culture of hNPCs. NDC-hNPCs were expanded on Ln MC in 6 well plates for 7 days at cell and microcarrier densities optimized to maximize final hNPC counts. These hNPCs expanded on a small scale were seeded at a density of 0.36 million cells/mL with 10 mg/mL Ln MC in a rotating wall vessel (RWV) bioreactor (Figure 3-7 A) and expanded for 6

days till confluent (Figure 3-7 B). Samples were taken every day to obtain cell counts and assess cell viability (by propidium iodide- PI staining). More than a five-fold change of cell density was observed by day 4 with 2.01±0.07 million cells/mL (Figure 3-7 C). Thus, around 110 million cells were obtained by 4 days of expansion by seeding 20 million cells in the 55mL bioreactor. Flow cytometry analyses revealed the expression of NESTIN, SOX1 and SOX2 by a high percentage (>88%) of the cells cultured in the bioreactor (Figure 3-7 D).

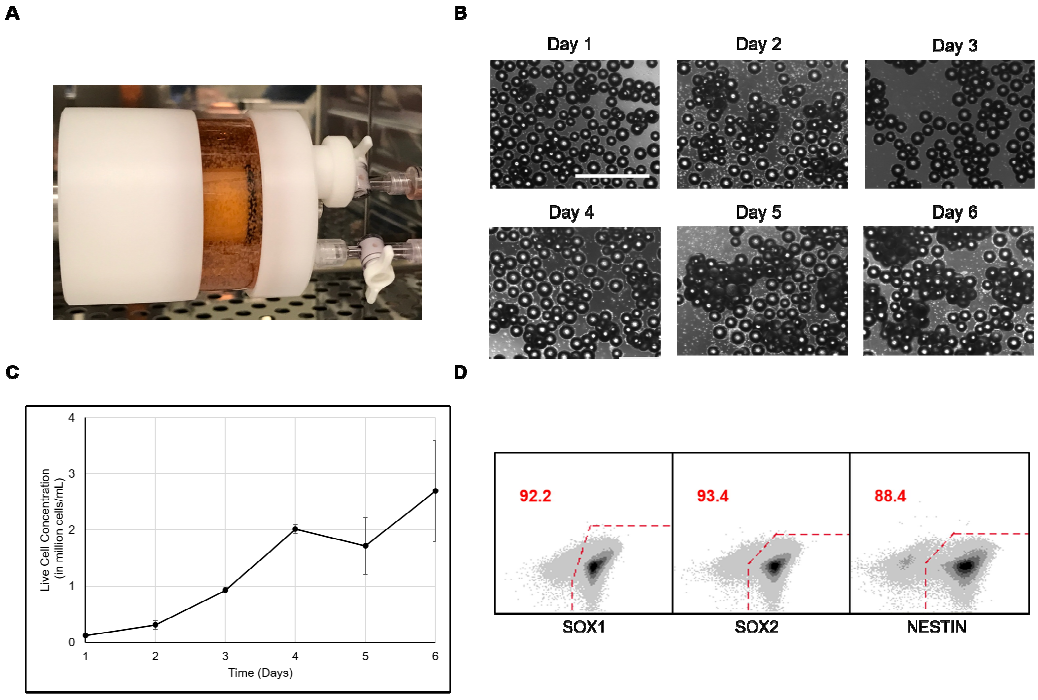

Figure 3-7: Large-scale expansion of NDC-hNPCs on Ln-coated microcarriers (MCs) in rotating wall vessel (RWV) bioreactor. (A) Image of RWV bioreactor inside tissue culture incubator. (B) Phase contrast images of samples taken from the bioreactor on days 1 through 6. Scale bar= 1000 um. (C) Live cell concentration on days 1 through 6 of bioreactor culture. Cell viability and cell counts were analysed on a flow cytometer by staining samples taken from the bioreactor with PI. The total live cell count in the bioreactor vessel was extrapolated based on this sample cell count, after accounting for the sample volumes withdrawn every day and the live cell concentration was calculated by dividing this extrapolated live cell count by the vessel volume (55 mL). (D) Flow cytometry analysis for SOX1, SOX2, and NESTIN expression in NDC-hNPCs cultured on Ln-coated MCs in the RWV bioreactor.

3.4 fAD patient derived hNPCs (APP(Dp) hNPC) cultured on microcarriers recapitulate AD

To be used for drug screening purposes, we require patient derived hNPCs to be expanded on a large scale, with the cells faithfully recapitulating disease phenotypes. Since MC surfaces could support the large scale expansion of Non-Demented Control hNPCs, we assessed if AD patient derived hNPCs expanded on MC could display disease relevant phenotypes, namely $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion and elevated levels of phosphorylated tau.

Somatic cells from an fAD patient with duplication of APP gene, were reprogrammed to form iPSCs which were further differentiated to form hNPCs, referred to as APP(Dp) hNPCs here. These APP(Dp) hNPCs and NDC-hNPCs (used as control) were expanded on Ln MC for 5 days till confluent and the conditioned media was collected to assess the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$. Cell lysates were used to assess the levels of phosphorylated and total tau. APP(Dp) hNPCs showed significantly higher concentrations of $A\beta_{1-40}$ ($p<0.001$) and $A\beta_{1-42}$ ($p<0.05$) compared to NDC-hNPCs (Figure 3-8). However, the level of phosphorylated tau relative to total tau was not significantly elevated in APP(Dp) hNPCs cultured on Ln MC when compared to NDC hNPCs (data not shown).

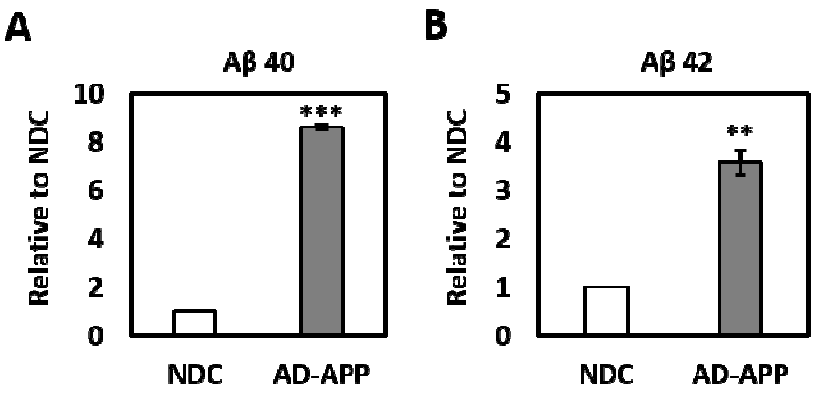

Figure 3-8: Analysis of Alzheimer's disease (AD)-related phenotypes in neural cells cultured on microcarrier (MC) surfaces. HNPCs derived from nondemented control (NDC) and AD-APP(Dp) hiPSCs were cultured on Ln MC surfaces for 5 days and analyzed for secreted (A) Aβ40 and (B) Aβ42 levels (mean ± S.E.M.). Comparisons made against NDC hNPCs ((Student's t-test, *$p<0.05$, ***$p<0.001$).

4. Discussion

The clinical and therapeutic use of hNPCs require the development of completely defined, scalable culture systems. We previously identified a synthetic peptide, VDP, derived from the cell binding domain of Vitronectin, an extracellular matrix protein (ECMP), capable of supporting the long term expansion and differentiation of various ESC and iPSC derived hNPC lines on 2-D tissue culture treated plates. In this study, we scaled up the culture of hNPCs using VDP coated microcarriers. VDP MC were able to support the long term expansion of ESC and iPSC derived hNPCs over multiple passages and supported higher fold changes in cell densities, compared to VDP coated 2-D surfaces. HNPCs cultured on VDP MC showed high levels of expression of NPC markers such as NESTIN, SOX1 and SOX2 throughout multiple passages.

VDP MC also showed the ability to support the neuronal differentiation of hNPCs, and produced mature neurons expressing several neuronal, neurotransmitter and cortical markers. Generally, the neurons differentiated from hNPCs cultured on 2D surfaces do not adhere to the surface for more than a few weeks and are often fragile, limiting their potential for long term studies. However, the neurons cultured on MC were found to be robust in their ability to adhere to the substrate and could be cultured in suspension for long periods of time (>120 days). These neurons cultured on MC were found to be more mature than the ones cultured on 2-D surfaces.

HNPCs were cultured in microcarrier based bioreactor systems and were found to expand more than five-fold, up to 110 million cells in 4 days. These cells showed high levels of expression of NPC markers, indicating that hNPCs can be expanded on a large scale in microcarrier based bioreactor system.

fAD patient derived hNPCs with duplication of APP gene, when cultured on MC displayed AD relevant phenotypes such as high levels of secreted Aβ in the culture medium. The levels of both $A\beta_{1-40}$, and the plaque forming $A\beta_{1-42}$ were found to be elevated, indicating that patient derived cells can be cultured on MC and can be used for disease modelling and drug screening.

5. Future Work

Vitronectin Derived Peptide has been identified as a completely defined, scalable substrate for culturing hNPCs. This study shows that hNPCs can be cultured on a large scale in Ln microcarrier based RWV bioreactors. Future work would involve culturing hNPCs on VDP MC in RWV bioreactor to allow the scale up of hNPC culture in standard, xeno-free and chemically defined conditions. The final cell counts obtained from the bioreactor can also be maximised by varying the initial cell seeding density and the microcarrier concentration.

Such large scale culture of hNPCs would also allow for culturing of neurons on a large scale. Characterization of such neurons differentiated in the bioreactor by examining their gene expression profiles and their electrophysiological properties would also be of interest.

This study looks at the AD relevant phenotypes shown by hNPCs derived from fAD patients. Although rare, familial form of AD has been studied widely and the mutations associated with fAD have been valuable in developing animal models of the disease. However, the mechanisms of the sporadic form of the disease are still poorly understood and animal models would not be suitable for studying the same. By developing a chemically defined, scalable system of culturing hNPCs, we can now expand sAD patient derived hNPCs on a large scale to model the disease and to allow for drug screening.

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APPENDIX A

TABLE 1: 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
ACHE	CACAGGGGATCCCAATGAGC	CGTCGAGCGTGTCGGTG
GABRA	TGCGGATTTCGTCCTGACTT	AATCTGCAGCTCTGAATTGTGC
SLC17A7	AGCTGGGATCCAGAGACTGT	CCGAAAACTCTGTTGGCTGC
SLC6A4	AGAATTTTACACGCGCCACG	GAGGTCTTGACGCCTTTCCA
EAAT3	TGGTGCTAGGCATTACCACA	CCGGATACGTTGGAATCCAGT
GFAP	AACCTGCAGATTCGAGGGGG	GGCGGCGTTCCATTTACAATC
ASCL1	TCCCCCAACTACTCCAACGA	GCGATCACCCTGCTTCCAAA
BCL11B	CTCATCACCCAGGCTGACC	GGCCACTTGGCTCCTCTATC
CUX1	TCTCAAGATGGCGGCCAATG	TTGCGCAAATCCTCTGGAGT
EMX1	GAGACGCAGGTGAAGGTGTG	CACCGGTTGATGTGATGGGA
EMX2	ATCCGTCCACCTTCTACCCC	ACCATACTTTTACCTTGGAAGCG
FOXG1	TGCCAAGTTTTACGACGGGA	AGGGTTGGAAGAAGACCCCT
LHX9	TACCACTCTAGAGGCAGCCA	GGCATGCCCGCGTCG
SATB2	CCGCACCAGAAGAAGACACC	CAGGGACTGCTCACGGTCT
TUBB3	GAGGGGCATCTCTTGAGAACAA	CCCAGAACTGTGGACGCCT
RBFOX3	TGGGACGATCGTAGAGGGAC	CTTCCAGCCGTTGGTGTAGG

APPENDIX B

TABLE 2: ANTIBODIES USED FOR FLOW CYTOMETRY AND IMMUNOFLUORESCENCE

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-GABA	Millipore	AB15415	1:200
Mouse anti-GFAP	Millipore	AB360	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	A-31571	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

APPENDIX C

TABLE 3: LEICA TCS SP5 AOBS SPECTRAL CONFOCAL SYSTEM LASER POWER AND PMT SETTINGS

Laser	Power (%)	PMT spectral range (nm)
UV	12.5	430-550
488	20	500-600
633	20	650-750