A Robust Vitronectin-Derived Peptide Substrate for the Scalable Long-Term Expansion and Neuronal Differentiation of Human Pluripotent Stem Cell (hPSC)-Derived Neural Progenitor Cells (hNPCs)

by

Divya Varun

A Thesis Presented in Partial Fulfilment
of the Requirements for the Degree
Master of Science

Approved April 2016 by the
Graduate Supervisory Committee

David Brafman, Chair
Mehdi Nikkhah
Sarah Stabenfeldt

ARIZONA STATE UNIVERSITY

May 2016

ABSTRACT

Several debilitating neurological disorders, such as Alzheimer's disease, stroke, and spinal cord injury, are characterized by the damage or loss of neuronal cell types in the central nervous system (CNS). Human neural progenitor cells (hNPCs) derived from human pluripotent stem cells (hPSCs) can proliferate extensively and differentiate into the various neuronal subtypes and supporting cells that comprise the CNS. As such, hNPCs have tremendous potential for disease modeling, drug screening, and regenerative medicine applications. However, the use hNPCs for the study and treatment of neurological diseases requires the development of defined, robust, and scalable methods for their expansion and neuronal differentiation. To that end a rational design process was used to develop a vitronectin-derived peptide (VDP)-based substrate to support the growth and neuronal differentiation of hNPCs in conventional two-dimensional (2-D) culture and large-scale microcarrier (MC)-based suspension culture. Compared to hNPCs cultured on ECMP-based substrates, hNPCs grown on VDP-coated surfaces displayed similar morphologies, growth rates, and high expression levels of hNPC multipotency markers. Furthermore, VDP surfaces supported the directed differentiation of hNPCs to neurons at similar levels to cells differentiated on ECMP substrates. Here it has been demonstrated that VDP is a robust growth and differentiation matrix, as demonstrated by its ability to support the expansions and neuronal differentiation of hNPCs derived from three hESC (H9, HUES9, and HSF4) and one hiPSC (RiPSC) cell lines. Finally, it has been shown that VDP allows for the expansion or neuronal differentiation of hNPCs to quantities (>10^{10}) necessary for drug screening or regenerative medicine purposes. In the future, the use of VDP as a

defined culture substrate will significantly advance the clinical application of hNPCs and their derivatives as it will enable the large-scale expansion and neuronal differentiation of hNPCs in quantities necessary for disease modeling, drug screening, and regenerative medicine applications.

DEDICATION

For mumma, daddy and *dadu*

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my mentor, Dr. David Brafman without whom this research project would have not been this fruitful. He is an amazing person and words cannot be enough to describe how thankful I am to him. I learnt about the perspective one needs to have as a scientist while looking at one's research, solving problems, analyzing and tackling them is way more valuable than the experience in technical skills from him. Whenever encountered with unforeseen challenges or roadblocks in the experiments, his insights always indicated of the best possibilities for troubleshooting and his constant feedback motivated me to keep going ahead with the protocols until the most appropriate answers were obtained. Dr. David Brafman has been a constant support of encouragement and guided me the best way throughout my thesis project. Besides being a mentor, he has always been there to attend my personal issues and offer words of comfort and wisdom that helped me pull back together and do science. His passion for scientific research has highly motivated me to look forward into the field of research as a career option.

I would also like to acknowledge my friend Jill Tsai for teaching me tissue culture skills and my dear friend Josh Cutts for teaching me all the molecular biology skills. Josh, besides being the lab manager and a PhD student, has been a great friend who always took out time to troubleshoot my project issues and answer my queries. I express my special thanks to Sreedevi Raman for being a great moral support and keeping me optimistic whenever the experiments did not turn out well. I would like to thank Gayathri Srinivasan who will now be taking this project a step ahead and while making her familiar with it, I got to learn a

lot. Last but not the least, I would like to thank my colleagues in Brafman's lab, Francis Petty, Nick Brookhouser, Christopher Potts, Rachel Lundeen, Jake Packer, Frea Mehta, Lexi Bounds, Gavin Steeber, Ryan Magnusosum, Linxin Gu for all the technical and moral support which has contributed to the progressive completion of this project cumulatively.

I would also like to appreciate Ryan Merkley from Dr. Nick Stephanopoulos lab for extending his help for a part of my thesis project. I am grateful to Dr. Xiao Wang and Dr. Karmella Haynes and their respective labs for generously letting me use their lab resources. Their lab always kept a friendly environment around which always made me feel so comfortable and fun to work at.

I am sincerely grateful to Dr. Mehdi Nikkhah and Dr. Sarah Stabenfeldt for appreciating my thesis work and serving as members of my thesis committee.

I would like to express my love for my friends Surabhi Kabra, Aditya Reddy, Anuraag Kaparaju, Latika Wadhwani, Advaith Gvk, Anvesh Reddy, Viswanth Chadalwada, Nishant Rao, Bharat Ahuja and Anirudh Joshi for being the family away from home and giving me the love and support while I was busy working.

Last but not the least, I express my utmost love and respect to my parents, Deep Narain Varun and Bimlesh Varun, for all their love, dedication, support, wisdom, prayers, encouragement, and inspiration that has truly made me what all I am today.

TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER 1: INTRODUCTION

1.1 Motivation

With the aging population of United States, an ever increasing number of Americans are afflicted from neurodegenerative diseases. Neurodegenerative diseases are characterized by gradual and progressive loss in structure and function of neurons and other supporting cells of brain which leads to a nervous system dysfunction. Each of the neurodegenerative disease such as Alzheimer's disease (AD), Parkinson's diseases (PD) and Amyotrophic Lateral Sclerosis (ALS), involves neurodegeneration of cerebral cortex, substantia niagra, and spina cord, respectively [1]. Among all, only few occur to be as a familial condition (about or less than 10%) i.e. supporting a genetic basis like these diseases may run as an autosomal dominant condition within family, such is the case in Huntington's disease (HD). Most of the neurodegenerative diseases are diagnosed primarily to be sporadic where there is minimum genetic contribution and variety of factors are involved such as environmental factors, exposure to toxicity etc. [2]. This may lead to single or various mutations in genes that are directed to certain protein and pathways of nervous system, like mutations in the β-amyloid precursor protein causes AD; in α-synuclein causes PD; and in microtubule-associated protein tau causes frontotemporal dementia (FTD). Many of these neurodegenerative disorders often shows dichotomy between sporadic and familial forms (like AD, PD and ALS) [3].

According to National Institute of Neurological Disorders and Stroke, there are 600 different types of Neurological disorders and 50 million Americans are affected each year.

Thus Neurological disorders accounts for an unaccountable personal toll and an annual economic cost of hundreds of billions of dollars in medical expenses and lost productivity [4]. From recent statistics of 2015, 5.3 million Americans have AD from which 5.1 million individuals are 65 and older whereas the rest of the 200,000 individuals are under 65 having younger-onset Alzheimer's [5]. Nearly 44 million people worldwide have AD or other related Dementia and this is most prevalent in Europe followed by North America. The cost of caring for AD patients in U.S was estimated to be $226 billion in 2015 and the global cost for the same has been accounted to be around $605 billion [6]. Parkinson's disease (PD) affects up to 6.3 million people worldwide [7] and 1 million people in the US with cost of medical treatment around $25 billion [8].

Even though the diagnosis of the Neurodegenerative diseases have made immense progress in last four decades but the rightful cure for the same is still worked on. With these fast growing incidences of Neurodegenerative diseases and large investments in medical cost, the limitation of only a symptomatic treatment vs the actual therapies is still a big loop hole for the Neuroscience research. General protocol for the treatments that are able to ameliorate the symptoms of various neurodegenerative diseases involve: i) intake of symptomatic drugs, like in PD, the intake of Levodopa, a precursor of dopamine, gets converted into dopamine by dopa carboxylase and reduces rigidity, bradykinesia and tremors which are the most frequent symptoms of PD [9], ii) Use of battery implanted deep brain stimulations, that involves delivery of precise electrical signals to specific deep anatomical structures of the CNS and aims to alter or modulate the neural functions in order to achieve a therapeutic effect [10], iii) injecting Neurotrophic factors, that have the ability

to regulate differentiation and to support growth during development of the nervous system, is being progressed lately because they have been documented to prevent cell death and maintain cellular function of the nervous system [11], iv) Cell based therapies as a potential mainstream medicine, which involves administration of stem cells into the patients which can differentiate into the different cell types that may be damaged or dead due to the neurodegenerative disease. Human Pluripotent Stem cells (hPSCs) have the ability to extensively proliferate and differentiate into various cell types of the CNS such as, neurons, glial cells including astrocytes, oligodendrocytes etc. Thus, stem cells holds an utmost importance in the therapeutics of neurodegenerative diseases such as AD, PD, ALS, etc.

However, the other three treatments have their own limitations. The symptomatic drugs, as the name suggest, only aim to give temporary relief to the patients based on the symptoms. In AD, all the class of drugs approved for the treatment of memory loss, a symptom of AD, belongs to cholinesterase inhibitors. Cholinesterase inhibitors prevents breakdown of acetylcholine, a chemical messenger important in learning and memory function, and delays worsening of the symptoms for 6-12 months [12]. The deep brain stimulations in PD and Dystonia patients is performed by implanting a neurostimulator in collarbone which is connected to the brain via tiny electrodes that carry electrical signals. Although it might carry drawbacks such as cranial bleeding, cognitive dysfunction [13], [14]. In the neurotrophin based therapies, the family of neurotrophins like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4) is involved. Each of these factors binds to the tyrosine receptor kinase (Trk) family:

like NGF binds to TrkA, BDNF and NT4 binds to TrkB, and NT3 binds to TrkC, and theycan respond to apoptotic pathway which is initiated by binding to neurotrophin receptors. NGF and BDNF are dominant neurotrophins of the peripheral nervous system (PNS) and CNS, respectively. Although there is strong ground for using neurotrophins to degenerating neurons as potential way to restore neuronal activity in neurodegenerative diseases, delivering neurotrophins to brain is still an insignificant approach. Moreover, the diseases of thee CNS are difficult to treat due to the presence of the blood brain barrier (BBB) making it impossible for the large proteins and compounds to cross from blood into the brain. The neurotrophins are large, polar molecules that cannot cross the BBB and thus should be injected directly into the CNS making it even more difficult.

On the other hand, cell based therapies and gene transfer to the diseased and injured brain have emerged as a promising approach for the therapeutics of the neurodegenerative diseases. It has the potential to replace the diseased, dead, or lost neurons of the CNS and restore the neural functions thus acting beyond a short term symptomatic effect for neurological diseases. For successful development of the stem-cell based therapies, clinical roadmaps need to be defined. One of the major issues occurring in the basic clinical research before these therapies can be tested into humans and animal models are the type of substrate used for stem cell culture. Also, to understand the full potential of stem cells in cell based therapies, there is a need for the development of defined, robust and scalable culture conditions for their expansion and neuronal differentiation.

1.2. Neurodegeneration

Neurodegeneration is a topic that is mentioned actively everywhere and thus it is safe to assume its definition being familiar to everyone. Neurodegeneration is any pathological condition which results in damage, death, or loss of function, structure of the nerve cells or the nervous system itself. Generally, it represents pathologies which are restricted to the nervous system. Some of the most talked about neurodegenerative disorders are Alzheimer's Disease (AD), Parkinson's Diseases (PD), Huntington's Disease (HD) and Amyotrophic Lateral Sclerosis (ALS).

One of the major causes of developing a Neurodegenerative diseases is increasing age. Lately, the growth rate of the 65 aged and older population has exceeded the overpopulation as a whole which means that the proportion of the old aged will double and so will be the proportion of the people with neurodegenerative disease [15]. Thus it is of utmost importance that an effective preventive strategy is developed for treatment or else we will just have to keep expecting the values and figures of the people affected gets higher with passing time. And it cannot be ignored that nearly all the neurodegenerative disorders shorten the life span of the individuals affected besides the availability of the medicines that alleviate the symptoms. Neurodegenerative diseases are generally not attributed to single or multiple genes but are at times far more complicated involving number of known and unknown signaling pathways, protein aggregation, stress, etc.

Despite the ongoing research and neuropathological findings, sporadic neurodegenerative diseases comprises of the cases that we are still far away from understanding their molecular etiologies. To support this, we know that AD is caused bythe accumulation of

β-amyloid proteins and Tau proteins still we know only little about the sporadic form of AD. Following the disease study in the relatives or sibling of such sporadic AD patients, raises a possibility of involvement of genetic factors in this [16].

1.2.1 Alzheimer's Disease

Alzheimer's disease is associated with the progressive aggregation and accumulation of insoluble fibrous material- extracellular β amyloid protein and intraneuronal neurofibrillary tangles which are generally not found in the CNS [17]. This results in dementia that affects the memory, thinking and behavior of the individual. The β-amyloid accumulation interferes with the neuron-neuron communication and thereby hindering the synapses. The neurofibrillary tangles, caused by Tau proteins block the nutrient transport within the neuron which eventually results in cell death.

The genetical pathology of this relates to three genes: amyloid precursor protein (APP), presenelin 1 (PS1) and presenelin 2 (PS2). Based on the 'amyloid-cascade hypothesis', amyloid β is produced in normal individuals and follows the non-amyloidogenic pathway where APP undergoes a sequential cleavage by alpha and gamma-secretases, respectively, which generates water soluble and nonpoisonous peptide different from Amyloid β. But in case of AD, Amyloid β, an insoluble peptide, is formed as APP is cleaved by beta secretases followed by gamma-secretase by undergoing the amyloidogenic pathway (Figure 1A) [18], 19]. Gamma-secretase is a multiprotein complex that is made up of PS1 and PS2 proteins. Tau protein aggregation in the neurons are generated due to excessive or abnormal phosphorylation which results in the transformation of normal Tau into PHF-tau (paired helical filament) and Neurofibrillary tangles (NFTs) (Figure 1B)[20].

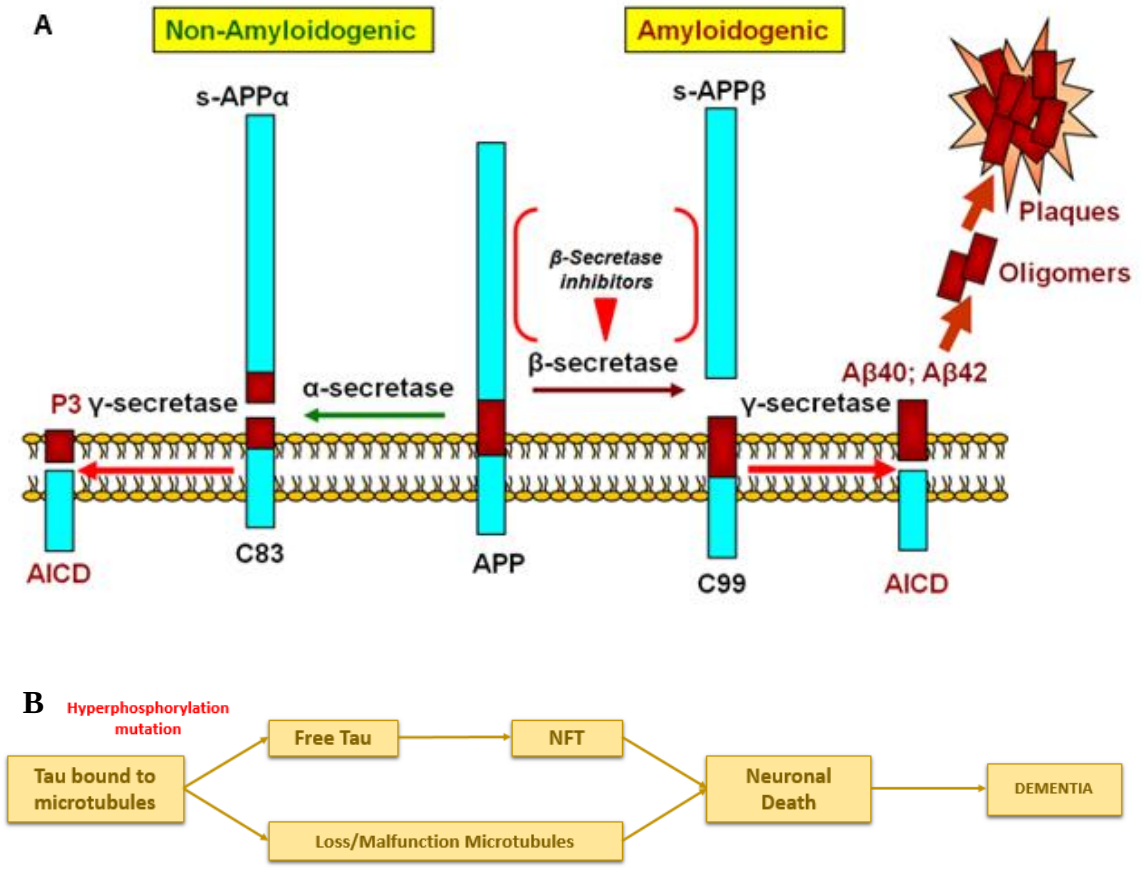


Figure 1 (A) The Amyloid-Cascade Hypothesis for Familial AD[18],[19]. (B) The Tau Hypothesis[20].

The incidence of AD has also been reported due Apolipoprotein E (ApoE) gene. There are three types of ApoE: E2, E3 and E4, of which E4 is primarily associated with AD whereas the other two are known to provide protection against AD.

Once AD develops in an individual, the cholinergic neurons and nerve synapses are affected which starts to then degenerate and gradually dies. It starts in the transentorhinal cortex and then spreads to the entorhinal cortex, the hippocampus and the cerebral cortex [21].

1.2.2 Parkinson's Disease

Parkinson's disease is the second most prevalent neurodegenerative disease in US affecting 1 million people every year [21]. It is defined by the loss of dopaminergic neurons within substantia niagra affecting ventral component of the pars compacta in particular. Dopamine is a chemical messenger responsible for transmitting signals between the substantia niagra and corpus striatum to produce movements in the body. Thus, in PD, loss of dopamine results in abnormal nerve patters thereby causing impaired movements [22]. The initial stages are generally pre-symptomatic but as the disease progresses, the substantia niagra, parts of mid brain and basal forebrain gets involved. This staging is based on the Lewy bodies and Lewy neurites which are pathological hallmark for PD. Lewy bodies are immunoreactive inclusions or deposits of α-synuclein and Lewy neurites are nerve cell processes that contain aggregates of α-synuclein and are most numerous in CA2/3 region of hippocampus and substantia niagra [23]. It was later discovered that by administration of oral drug 'Levodopa' could replenish the dopaminergic neurons and alleviates most of the symptoms associated with PD. Over time it was discovered that during its treatment over the years, individuals develop involuntary movements, dyskinesias [24].

Currently the research for PD is focused towards prevention of degeneration of dopaminergic neurons, still all the current treatments are symptomatic and none can halt this degeneration. Recent studies are focused on three cellular dysfunctions that maybe important in the pathogenesis of PD: oxidative stress, mitochondrial respiration defect and abnormal aggregation [25]. Animal models have been used to study the role of α-synuclein in PD. Eleonora Maries et al. described this by injecting neurotoxin MPTP in mice models.

MPTP effect by inhibiting the mitochondrial complex I which results in generation of reactive oxidative species, accumulation of α-synuclein thereby leads to cell death [26].

1.2.3. Huntington's Disease

Huntington's Disease (HD) is an autosomal-dominantly inherited disease which is caused due to mutation in exon 1 of huntingtin (HTT) gene on Chromosome 4 resulting in the repeats of the trinucleotide sequence CAG. The abnormal elongation of the CAG repeats expands the polyglutamine stretch of HTT protein which then becomes proportionally toxic and eventually leads to neurodegeneration [27],[28]. The proteolysis post mutation releases toxic N-terminal fragment of the HTT and this fragment forms protein aggregates in nucleus, cytoplasm and processes of neurons. Generally the exon 1 of HTT gene has 3-30 repeats in an individual but if the number exceed 35, the chances of pertinence of the disease becomes likely with chances being definitive at ≥39 CAG repeats [29]. The length of these repeats have shown to have a direct relation with the age, if the repeats are higher (50-200), the symptoms for HD will start to progress from early age, where as if the repeats are 39-50, the first signs appear at 35-40 years of age [30]-[32].

Being inherent, HD has no sporadic forms and since it occurs only in humans, to study this animal models needs to be genetically modified. M. Flint Beal et al. presented a brief overview of the transgenic mouse models for HD to understand its therapeutics better [33]. The characteristic symptoms of the disease have reflected manifestations in the motor and cognitive functions. Postmortem studies of the HD patient's brain indicates a 30% reduction in the total brain weight, neuronal loss in the caudate-putamen, collectively

known as striatum and are the major components of the basal ganglia, and the globus pallidus [34].

1.2.4 Amyotrophic Lateral Sclerosis (ALS)

ALS is a fatal neurodegenerative disease characterized by progressive degeneration of motor neurons. ‘Amyotrophic’ means muscle atrophy, fragility, vellication that represents the disease of motor system and ‘lateral sclerosis’ refers to the hardening of the lateral columns of the spinal cord which is followed by degeneration of the corticospinal tracts [35], [36].The neuropathological findings on ALS patients reveal degeneration ad loss of the large anterior horn cells of the spinal cord and lower cranial motor neurons of the brain stem. The striated muscles show denervation atrophy and the upper motor neurons in the motor cortex are also affected [37].

About 5-10% of ALS is familial following Mendelian pattern of inheritance and so far, 13 genes and loci corresponding to the defect have been identified. To name a few, SOD1, TARDBP (also known as TDP-43), FUS, ANG and OPTN cause a typical clinical phenotype. Mutations in SOD1 accounts for 20% of the familial ALS [38], [39]. The TDP-43 positive mutations have shown to overlap between ALS and fronto-temporal dementia (FTD) [40], this has given a positive approach to look at the genetic contributions of ALS.

The lack of effective therapies for all these neurological diseases are however creating a burden on the society [40]. Despite the destructive nature of the above mentioned diseases with respect to the number of people affected, billions of dollars are spent yearly for the healthcare but stunningly there is still lack of treatment with no cure available for any of these as of now. Even after decades of research and with no effective treatments available,

cell based therapies have emerged as an attractive option with applications of stem cells rapidly coming into play.

1.3 Stem Cells and Therapies

Stem cells can be defined by their ability to self –renew, i.e. to duplicate into cells without any loss in the developmental potential of the cell and the potency to develop into multiple different cell types. In many tissues they act as an internal repair system, they keep dividing into various cell types within that tissue as long as the individual lives. When a stem cell divides, each new cell generated has the potential to either remain as a stem cell or become a cell of particular type [41]-[46].

There are two types of stem cells- embryonic stem cells and non-embryonic stem cells (also known as adult stem cells). Embryonal Stem Cells (ESCs) are derived from the totipotent inner cell mass of the 8-16 cell stage of embryonic blastocyst and are capable of unlimited proliferation and differentiation into various cell types of the organism [47]. This pluripotency of the ESCs is maintained and controlled by a conserved network of transcription factors and signaling pathways. In other words, they can develop into more than 220 cell types of an adult organism when provided sufficient and necessary stimulation and nutrients for the specific cell type. ESCs can be maintained in culture systems as undifferentiated cells or can be induced to be differentiated into different lineages [48], [49].

Adult stem cells are multipotent stem cells which act as the repair system for the body in replacing the damaged or the dead cells or tissues. They have the property of self-renewal

and multipotency. They have ability to generate in to cell types of different progenies like Hematopoietic stem cells, mesenchymal stem cells, pancreatic stem cells etc. [50].

In 2006, Yamanaka and Takahashi induced pluripotency in mouse embryonic and adult fibroblasts by introducing four transcription factors, Oct3/4, Sox2, Klf4, and c-Myc and called these cells as induced pluripotent stem cells (iPSCs) [52]. His study was a major breakthrough in stem cell research as now normal cells could be induced back to pluripotency state. Current research with induced pluripotent stem cells (iPSCs) which are derived from differentiated cells have potentially expanded the resource of stem cells for therapeutics and research.

IPS cells are adult cells that are reprogrammed into the pluripotent state like ESCs. They can be differentiated into any cells types of the body. Figure 2 sums up briefly the different cell types that ESC ad iPSCs can be differentiated into [53].

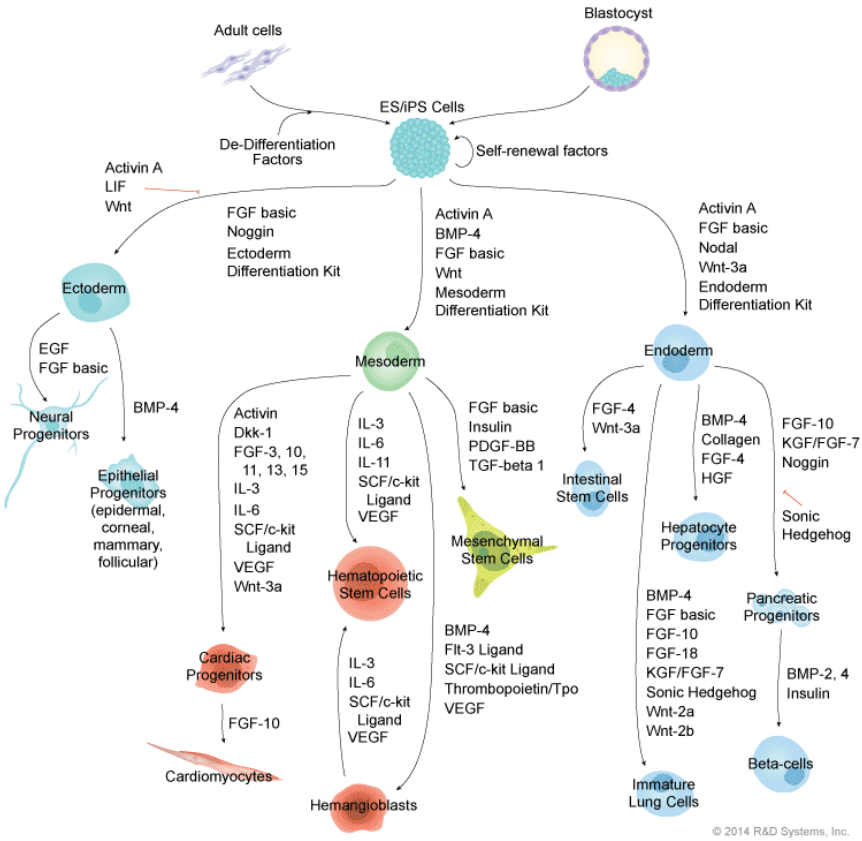


Figure 2. Stem Cell Differentiation Pathway: ESCs and iPSCs can be Differentiated into Various Cells of the Body by Inducing the Specific Pathways upon Introducing Respective Transcription Factors [54].

Stem cells are not only a promising approach for regenerative medicine and cell based therapies but also for drug discovery and development process. For diseases such as various neurodegenerative diseases, using stem cell based in vitro models have a given a new ray of hope in order to understating the disease better. Pluripotent stem cells because of their differentiation capabilities, allow researchers to follow disease progression and gain valuable insight of the pathophysiology of the disease by obtaining the relevant cell populations. Because of their capacity to self-renew, they also facilitate cell based genetic or drug screening and are considered as inexhaustible, scalable and physiologically native

material for experiments. To sum up, stem cells have been actively used in i) disease modelling, ii) drug screening, and iii) cell based therapies [55],. Figure 3 gives an overview of the potential use of iPSCs in neurodegenerative diseases with AD as a model.

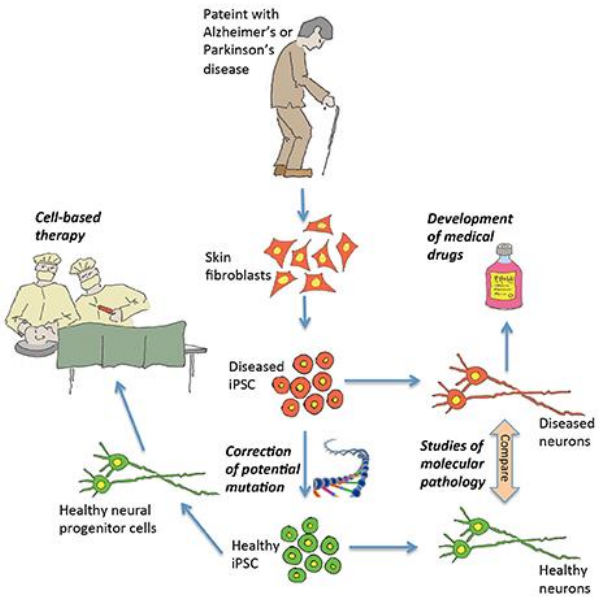


Figure 3. A Brief Overview of the use of iPSCs in Therapeutics of Neurodegenerative Diseases- AD and PD[65].

1.3.1 Stem Cells for Disease Modelling

Currently, medical research relies mostly on having appropriate model systems to study the diseases in order to develop therapies. Transgenic animal models, particularly mice models have allowed us to study many complex diseases *in vivo*. However, in vivo models hold several drawbacks such as: i) age related differences between animals and humans, ii) no help of *in vivo* models for sporadic forms of diseases iii) species differences as animals are not humans[56],[57]. Disease models serve as a platform to understand the biochemical mechanisms of normal phenotypes and process in comparison to the disease state.

Particularly in complex, multigenic diseases, molecular studies leads to a greater understanding of the disease and thus has more targeted approaches for therapies [58]-[60].

Disease modelling using pluripotent stem cells involve two parameters: their differentiation into pathologically relevant populations and their ability to sum up the key aspects of the disease [61]. Diseases in which the particular cell types are highly inaccessible, such as various neurodegenerative diseases, are of great interest of research through stem cell based disease modelling. Various disease models, using iPSCs, for neurodegenerative diseases such as PD , AD , HD and ALS have been developed [62]-[64].

The iPSC technology is very promising and has the potential to model and treat the neurodegenerative diseases. Patient derived iPSCs can be reprogrammed to affected neuronal subtypes through in vitro differentiation or using reprogrammed iPSCs using gene targeting to repair disease causing mutation. Another key potential of this is to use these affected neuronal subtypes for drug screening about which we will discuss shortly [65].

1.3.2 Stem Cells Drug Screening and Toxicity

The historical methods being followed for efficient and efficacious drug testing lack approach to understand the mechanisms of toxicity, pathogenesis of several diseases and biological and biochemical control of cellular processes in complex diseases [66]. By exposing new drugs, for drug screening and toxicity testing, into ESCs or iPSCs derived 3D cultures can reveal their ability to study efficacious or toxic changes because of drug within the structure. Several human ESCs and human iPSCs (collectively termed as hPSCs) have been reportedly commercialized and used for drug screening and drug testing. HiPSCs derived cardiomyocytes have been used for cardiotoxicity testing [67], hPSCs derived

hepatocytes have been generated and used for drug testing and screening [68]. Because these cells are derived from human source, the hPSCs will provide a valuable information about the drug safety, efficacy and any possible toxicity.

However, drug screening using hPSCs is still in its infancy. Neurodegenerative diseases provide a vast source for drug exploration due to the complexity of the diseases involved with the CNS. But now progress is being made with phenotypic evolution of iPSCs [69]. Lately, iPSCs have been developed from familial forms of ALS and necessary complementary assays and single cell longitudinal studies have been performed to understand the reduced life span of motor neurons. Through this, two compounds, tryphostin 9 and kenpaullone were identified as motor neuron protectors [70], [71].

Even though hPSCs have been used successfully for drug screening and toxicity testing, there are still many challenges that needs to be overcome, such as, cost reduction, standardizing the method of high throughput screening, improvement in cell maturation towards adult phenotypes, and standardizing the protocol for production of large cell numbers to be used in the study [72]-[76].

1.4 Stem Cell Therapies for Neurodegenerative Diseases

hPSCs have been considered as the potential treatments for various diseases to restore the tissue function either as integrated participants in the target tissue or as vehicles that deliver complex signals [77]. The objective of stem cell based cellular therapies are to utilize stem cells to treat the disease or injury. In recent years, clinical trials with stem cells have taken research into entire new direction. hESCs have begun to make their way to the Phase I

clinical trials with Oligodendrocyte precursor cells derived from hESCs for use in thoracic spinal cord injury [78].

Neurodegenerative diseases create an enormous burden on the societies as there are still no effective treatments available for them. In humans, the existence of Neural stem cells (NSCs) with multipotent properties have been reported in the brain [79]. It was evidenced in a group of cancer patients that were infused with bromodeoxyuridine for diagnostic purposes, who later died, that new neurons are continuously being generated in adult human CNS [80]. But then there is only a limited capacity of these NSCs to repair the diseased or injured CNS [81]-[83].

The cellular replacement for neurodegenerative diseases involve deriving the specific cell types that are lost, diseased or dead and grafting them into the affected area of the CNS. The transplanted neurons may then get integrated into the neural work to function normally. Also, the grafted stem cells may also act as enrichment source to the diseased cells and may support them providing them with various neurotrophic factors, removing toxic factors and creating a neural network to support the matrix around the affected matrix [84]-[87]. The appropriate cellular therapy is based on the type of neurodegenerative disease as each disease has its specific pathology. However, there are still many obstacles that need to be looked upon before clinical application of stem cell therapy in neurodegenerative diseases such as: i) it is still uncertain as to what kind of stem cells can be the ideal source of cellular grafts, ii) the mechanism by which the transplantation of stem cells can lead to the functional recover and structural reorganization of the CNS must

be understood, iii) cost effectiveness of the stem cell culture so that required amount of cells can be cultured for patients effectively.

1.5 Role of Extra Cellular Environment on Stem Cell Culture

It has been actively shown that stem cell development and differentiation require a niche, i.e. a microenvironment housing stem cells that regulates their self-renewal and fate [88] – [90]. The regulatory signals for this are provided by niche cells, soluble factors, and the extracellular matrix (ECM). Many such soluble factors such as FGFs, BMPs, Wnt have previously shown to regulate the stem cell behavior but the role of the ECM is still poorly understood. The ECM provides a scaffold for cellular support and a microenvironment that can trigger regulating signals that support stem cell proliferation, expansion, migration and differentiation [91]-[92].

The ECM is a complex mixture of various molecules like structural proteins (e.g. collagen, elastin), glycoproteins (e.g. laminin, fibronectin, and vitronectin), and proteoglycans (e.g. heparin sulfate, keratin sulfate, chondroitin sulfate). The cell adheres to the ECM and transmit signals via integrin receptors. Integrins are transmembrane cell adhesion molecules which act as matrix receptors and tie the ECM to the cell's cytoskeleton and are composed of two noncovalently associated subunits: α and β (Figure 4). A variety of human integrin heterodimers are formed from 9 types of β subunits and 24 types of α subunits [93].

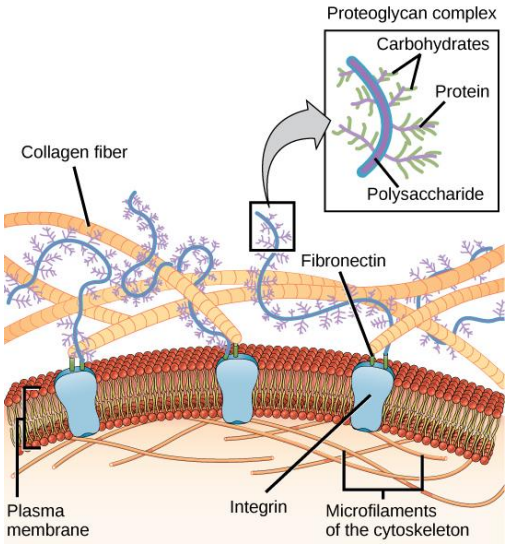


Figure 4: The Structure of the Extracellular Matrix [94]

1.5.1. ECM Interactions for Neuronal Differentiation

The ECM constitutes a basal lamina (BL) that surrounds the brain and blood vessels of the CNS [95]. It has been shown that BL is crucial for the development of the neocortex and its removal results in the detachment of the radial glial cells (RGC) fibers that eventually affects the RGC survival and lamination of the cortex. Laminins are major constituent of the BL and is also found in the ventricular zone of the developing neocortex [96]-[97]. Laminins promote the expansion, migration, and differentiation of the neural stem cells in vitro and thus NSCs can be generated and differentiated into various phenotypes if Laminin is added exogenously [98].

1.6. Natural and Synthetic Substrate for Stem Cells Culture

The hPSCs, derived from the inner cellular mass of the blastocyst hold a great interest in the clinical applications such as disease modelling, drug screening and toxicity testing, cell therapies [99], [100]. The traditional method of the hPSCs involves cell culture on mouse

fibroblast feeder layers with animal conditioned media or on Matrigel, an animal derived component that is rich in the ECM components essential for the growth [101]. Thus, these cultures not only possess an inherent risk of pathogen contamination but also the presence of the immunogenic epitopes [102].

Natural Substrates: As discussed above, hPSC expansion on feeder free cultures involve Matrigel that constitutes the basement membrane and consists of collagen, laminin, heparin sulfate, metalloproteinases, etc [103], [104]. Matrigel has been used to study the long term expansion of hESCs upto 40 passages [105] and to study the effects of bone morphogenic protein 4 (BMP4) on the differentiation of hESCs [106]. Various synthetic substrates for hPSC cultures have been considered as an alternative to Matrigel due to its unsuitability for quality control, large scale production with current good manufacturing practicesof hPSCs as it shows batch-to-batch variability and xenogenic origin. Laminin provides adequate growth of hESCs through $\alpha_6\beta_1$ integrins as these are major types of integrins present on the surface of hESCs. Various recombinant of Laminin have been derived but they show relatively less attachment and proliferation of hPSCs as these isoforms had more affinity for other integrins $\alpha_3\beta_1$, $\alpha_7\beta_1$ [107], [108]. Vitronectin binds to the $\alpha_V\beta_5$ integrins on cells via the RGD sequence. Braam et al showed that the recombinant vitronectin is as effective as the naturally purified vitronectin for hESC expansion and proliferation [109]. Heng et al examined the scale up potential of vitronectin and laminin coated polystyrene microcarriers for 3D suspension cultures to 2D culture systems on tissue culture treated polystyrene (TCPS) plates [110]. Table 1 rightly sums up the advantages and

disadvantages of these natural substrates and various other synthetic substrates that have been actively used for hPSC culture [111].

Table 1. Advantages and Disadvantages of Natural and Synthetic Substrates [111].

Substrate	Advantages	Disadvantages
Matrigel	Allows feeder-free cell culture Inexpensive Long-term hESCs culture	Xenogeneic origin Undefined components Pathogenic contamination risk Immunogenic epitope Batch-to-batch variability
Vitronectin	Long-term hESCs culture αVβ5 integrin receptor mediated cell attachment	Degradation upon sterilization Not-Scalable High production cost
Synthetic peptides	No batch-to-batch variation Immunogenicity risk avoided since chemically synthesized Scalable hPSCs culture	Sterilization difficulties Labor intensive cell passaging Limited scale-up potential of 2D platform
Synthetic Polymers	Inexpensive	Limited scale-up potential of 2D platform

Synthetic substrates: The protein based substrates like vitronectin, laminin, and fibronectin coated tissue culture plates have an advantage of being feeder free but they are not xeno-free because they are usually animal derived. Thus they pose a great chances of significant immunogenic and pathogenic responses. Due to which these substrates cannot be used for the culture of stem cells in clinical applications. Consequently, peptide and polymer based substrates can act as better alternative for the culture of hPSCs.

Heparin binding peptides have known to promote cell adhesion and interaction with cell through their cell-surface glycosaminoglycans (GAGs) which are mainly involved in cell-cell adhesion and recognition and, cell-ECM interaction. In an earlier study for determining the optimal peptides for the culture of hESC, Klim et al. showed different heparin bbinding peptide surfaces that were vitronectin derived and supported the expansion, adhesion and self-renewal of the hESCs [111].

1.7. Scale up Expansion of Stem Cells

HPSCs have two basic characteristics, first being the self-renewal i.e. they can divide indefinitely, second is pluripotency i.e. they can be differentiated into all cell types of a mature human body. It has been shown that human neural progenitor cells (hNPCs) can be generated from hPSCs by the addition of various inhibitors of bone morphogenic pathways such as Noggin and Dorsomorphin. hNPCs holds great potential in various clinical applications such as disease modelling, cell therapy, drug screening and testing [112].

These clinical applications require substantially higher number of cells. The traditional method of long term expansion and differentiation of hNPCs relies on 2D tissue culture. In this, the surface area for cell expansion is limited and multistep medium feedings, replating

and cell selection is required for differentiation. On the other hand, microcarrier suspension cultures have shown to be a great approach for culturing hNPCs in higher numbers. Microcarriers provide higher surface area to volume ratio and enable the use of stirred culture systems that result in higher folds cell expansion in comparison to 2D static plates [113].

CHAPTER 2: MATERIAL AND METHODS

2.1. Overview

In this study, we characterized the ECM and cell surface integrin profile of hNPCs to rationally design peptide-based substrates for the growth and differentiation of hNPCs. Of the peptides tested, we identified one 14 amino acid long peptide derived from the cell-binding domain of vitronectin [117] that provides for the expansion and neuronal differentiation of hNPCs. Moreover, this peptide, referred to as vitronectin-derived peptide (VDP), is easily coated onto tissue-culture treated polystyrene (TCPS) plates and microcarriers and supports the long-term propagation and directed neuronal differentiation of multiple hNPC lines in completely defined medium conditions. Overall, VDP is a completely defined and scalable substrate that support the long-term expansion and directed neuronal differentiation of hNPCs in quantities necessary for their scientific and clinical applications.

2.2. Differentiation of Neural Progenitor Cells from hPSCs in 2D Cultures.

2.2.1. HPSCs Expansion

The hPSCs (HES-3, H-9 and RiPSCs) were cultured in feeder free conditions i.e. tissue culture treated plates coated with Matrigel for a minimum of two passages. The media for the hPSC culture comprise essential 8 (E8) components: (1x DMEM F-12 (Life Technologies), $NAHCO_3$ (543 µg/mL, Invitrogen), L-Ascorbic acid-2 phosphate (64µg/ml, Sigma), Sodium selenite……). Upon 70% confluency cells, the media as aspirated out and Accutase (EMD Millipore) was added. The plates were incubated for 5 minutes to allow cell detachment. The accutase was inactivated by adding equal volume of

cell media to the cell suspension solution. The cells were pelleted down by centrifugation at 200 g for 5 minutes. The cells were resuspended in E8 media and 5 µM ROCKi, Y-267632 (EMD Millipore) and were counted using Hemocytometer. The cells were then plated at the desired densities based on the size of the culture plates for hPSC expansion or used for Embryoid body formation.

2.2.2. Embryoid Body Formation

The suspended hPSCs cells were dissociated in neural induction media [1xDMEM-12, 1% (v/v) N2 supplement (Life Technologies), 1% (v/v) B-27 supplement (Life Technologies), 50 ng/ml recombinant mouse Noggin (R&D Systems), 0.5 µM Dorsomorphin (Tocris BioScience), 1% penicillin/streptomycin (Life Technologies)] and 5 µM ROCKi, Y-267632. 1-2 x 10^6 cells were pipetted to each well of a 6-well ultra low attachment plate (Corning). The plates were then placed on an orbital shaker set at 95 rpm in a 37^OC/5% CO_2 tissue culture incubator. The next day, the cells formed spherical cultures (embryoid bodies [EBs]) and the media was changed to neural induction media without ROCKi. The EBs were cultured in suspension for 5 days in the same plate with half media change for every other day.

2.2.3. Neural Rossette Formation

After culturing in suspension for 5 days, the EBs were then transferred to a 10 cm dish (1-2 6 wells per 10 cm dish) coated with Matrigel. Most of the EBs adhere to the surface after one day of seeding. The plated EBs were cultured in neural induction media for 5-7days. Over time they form these rosettes like structures. The media was changed every other day.

The neural rosettes were dissected manually into single cells under an EVOS (Life Technologies) microscope using 1 ml pipette after incubation with Accutase for 5 minutes.

2.2.4. Neural Progenitor Cell (NPC) Culture and Expansion

The neural rosettes dissected from above were then centrifuged at 200g for 5 minutes. The cell pellet was then suspended in the NPC expansion media [NEM; 1x DMEM/F12, 1% B27 supplement, 1% N2 supplement, 1% Gluta-MAX, 1% penicillin/streptomycin]. The dissociated cells were then plated onto poly-L-ornithine (PLO; 4μg/mL; Sigma) and mouse laminin (Ln; 4 μg/mL; Life Technologies) coated dishes in NEM supplemented with 30 ng/ml mouse FGF2 and 30 ng/ml mouse EGF2 (R&D systems). For routine maintenance, the human pluripotent stem cell derived NPCs (hNPCs) were cultured onto PLO/Ln coated plates at a density of 1-5 x 10^4 cells/cm^2 and passaged upon 90% confluency.

2.2.5. Neuronal Differentiation

The hNPCs were grown to confluency and the media was changed to neuronal differentiation media [NDM; 1X DMEM-F12, 0.5% (v/v) N2 supplement (Life Technologies), 0.5% (v/v) B27 supplement (Life Technologies)] with 20 ng/ml BDNF (R&D Systems), 20 ng/ml GDNF (R&D Systems), 1 μM DAPT (Tocris Bioscience), and 0.1 mM dibutyrl-cAMP (db-cAMP)]. The hNPCs were then cultured for up to four weeks and half media was changed every day with NDM. The neuron like structures were visible from day 5-6 itself (Figure 5).

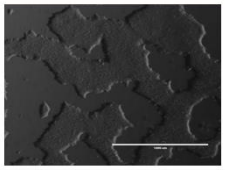
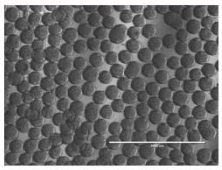
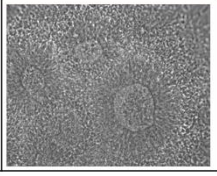
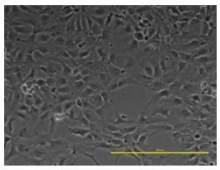
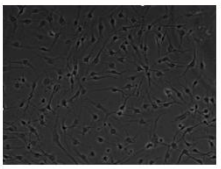
hPSCs	EB formation	Neural Rosettes	hNPCs	Neuron Differentiation
Seeded 250k cells/well of 6 well plate	Seeded 2 M hPSC/well of a 6 well cell repellent plate	Seeded EBs on Matrigel coated plates	Seeded Neural Rosettes on PLO/Ln coated plates	Changed media to NDM when hNPCs confluent
Expansion	5 days	7 days	Expansion	4 weeks
Essential 8 Media	NIM: 1x DMEM-F12, 1% N2, 1% B27, 200 ng/ml Noggin, 0.5 µM Dorsomorphin, 1% Glutamax, 1% Penicillin/Streptomycin		NEM: 1x DMEM-F12, 1% N2, 1% B27, 1% Glutamax, 1% Penicillin/Streptomycin, 30 ng/ml FGF2, 30 ng/ml EGF	NDM: 1x DMEM-F12, 1% N2, 1% B27, 1% Glutamax, 1% Penicillin/Streptomycin,
Matrigel	Suspension	Matrigel	PLO/Ln	PLO/Ln
				

Figure 5. Differentiation of hPSCs into hNPCs and Neurons

2.3. HNPC Culture and Neuronal Differentiation on Peptide Substrates

The VDP peptide was custom synthesized by AnaSpec. Peptide surfaces were prepared by reconstituting lyophilized peptide in sterile water and coating multi-well plates overnight at 37^{O}C. Peptide-coated plates were washed twice with PBS prior to culture. HNPC culture and neuronal differentiation was performed in a similar manner as described for PLO/LN-coated surfaces.

2.4. VDP Labeling with F-5-M

The coating of the VDP on TCPS was assessed by labelling the peptide with a fluorescent dye Fluorescein-5-Maleimide (F-5-M). F-5-M, at a final concentration of 1 mM was added to peptide concentrations 0.1 mM, 0.075 mM, 0.05 mM, 0.025 mM, and 0.01 mM in a multiwell plate. The positive control was 1x DPBS and 1mM F-5-M. The solutions were incubated overnight at 37^{o}C, protected from light. Later, the coated wells were washed twice with 1x DPBS. The fluorescence of the surfaces was visualized in the fluorescence microscope (Evos) at 488 nm filter.

2.5. Microcarrier Coating

Microcarriers (MC) were purchased from Corning (Corning Enhanced Attachment Microcarriers). They were weighed down into aliquots of 200 mg in sterile environment. Each aliquot was reconstituted in 5 ml of 1x DPBS to make a stock concentration of 40 mg/ml. From the stock, 20 mg of the microcarriers was aliquoted in an eppendorf and were coated with VDP (0.5mM) and PLO/Ln (0.01%/4µg/ml). The microcarriers were incubated overnight at 37^{o}C.

2.6. HNPC Expansion and Differentiation on Microcarrier Suspension Culture

The PLO/Ln and VDP coated microcarriers were washed 2x with DPBS and 1x with culture media before seeding with hNPCs. 1.5 x 10^6 cells were seeded on 4 mg microcarriers, initially dissociated at a concentration 2mg/ml in cell repellent plates. The culture plates were kept static for 12 hours to allow maximum cell attachment on MC. Later, the MC concentration was diluted to 1mg/ml and a final volume of 4 ml Nculture was used. The plates were kept on shaker at 95 rpm thereafter. Half media was changed every day and the cells were passaged upon confluency **(Figure 6).**

For neuron differentiation, hNPCs were cultured on MC as mentioned above and on Day 2 of culture, 75% media was changed from NEM supplemented with EGF and FGF to NDM. Thereafter, half media was changed with NDM for 4 weeks.

2.7. Quantitative PCR (qPCR).

RNA was isolated from cells using the NucleoSpin RNA Kit (Clontech). Reverse transcription was performed with qScript cDNA Supermix (Quanta Biosciences) or iScript RT Supermix (Bio-Rad). Quantitative PCR was carried out using TaqMan Assays or SYBR

green dye on a Bio-Rad CFX96 or CFX384 TouchTM Real-Time PCR Detection System. QPCR experiments run with TaqMan Assays was carried out using TaqMan Gene Expression Master Mix (Life Technologies). QPCR experiments run with SYBR green dye were carried out using iTaq Universal SYBR Green Supermix (Bio-Rad). For the QPCR experiments run with TaqMan® Assays a 10 min gradient to 95 °C followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s min was used. For QPCR experiments run with SYBR green dye, a 2 min gradient to 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min was used. The list of TaqMan® assays and primer sequences used is provided in **Table 3 (Appendix A)**. Gene expression was normalized to 18S rRNA levels. Delta Ct values were calculated as $C^{ttarget} - Ct^{18s}$. Relative fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method [156].

2.6. Immunofluorescence

Cultures were gently washed twice with stain buffer (BD Biosciences) prior to fixation. Cultures were then fixed for 15 min at room temperature (RT) with BD Cytofix Fixation Buffer (BD Biosciences). The cultures were then washed twice with staining buffer and permeabilized with BD Phosflow Perm Buffer II (BD Biosciences) for 30 min at $4^{O}C$. Cultures were then washed twice with stain buffer. Primary antibodies were incubated overnight at $4^{O}C$ and then washed twice with stain buffer at RT. Secondary antibodies were incubated at RT for 1 hr. Antibodies used are listed in **Table 4 (Appendix B)**. Nucleic acids were stained for DNA with Hoechst 33342 (2 μg/ml; Life Technologies) for 5 min at RT. Imaging was performed using an automated confocal microscope (Olympus Fluoview 1000 with motorized state) or EVOS microscope (Life Technologies)

2.7. Flow Cytometry

Cells were dissociated with Accutase for 5 min at 37^{O}C, triturated, and passed through a 40 μm cell strainer. Cells were then washed twice with stain buffer (BD Biosciences) and resuspended at a maximum concentration of 5 x 10^6 cells per 100 μl. For staining of extracellular membrane proteins, one test volume of antibody was added for each 100 μl cell suspension. Cells were stained for 30 min on ice, washed, and resuspended in stain buffer. For staining of intracellular proteins, cells were fixed for 10 min on ice with BD Cytofix Fixation Buffer (BD Biosciences). The cells were then washed twice with stain buffer and permeabilized with BD Phosflow Perm Buffer II (BD Biosciences) for 30 min on ice. Cells were then washed twice with stain buffer and one test volume of antibody was added for each 100 μl of cell suspension. Cells were stained for 30 min on ice, washed, and resuspended in stain buffer. Cells were analyzed on a FACSCanto (BD Biosciences) or ACCURI C6 (BD Biosciences). Antibodies and isotype negative controls are listed in **Supplementary Table 3**.

2.8. Population Doubling Time

Population doubling time of hNPCs was calculated using the following equation: PDT (h) = (T2 − T1) / (3.32 * [log(N2) − log(N1)]).

CHAPTER 3: RESULTS

3.1. Identification of Defined Peptide-Based Substrates for the Expansion of hNPCs.

We have previously developed a serum free protocol that allows for the robust generation of hNPCs from several hPSC lines [114]. Briefly, hNPCs were generated through the stepwise formation of embryoid bodies (EBs) and neuroepithelial-like rosettes. After manual dissection from EB-derived rosettes, hNPCs were replated and maintained as proliferative, multipotent cells. Expression of the neuroectoderm marker *FGF5* and neural-specific marker *SOX1* increased during hNPC generation and peaked upon hNPC expansion (**Figure 6A**). Upon continuous culture with FGF2 and EGF on laminin (LN)-coated surfaces, hNPCs proliferated extensively and maintained high levels of expression of SOX1 and NESTIN (**Figure 6B**). Subsequent differentiation of NPCs to neurons was achieved through the withdrawal of FGF2 and EGF and addition of BDNF, GDNF, dibutyrl-cAMP (db-cAMP), and the Notch inhibitor DAPT. After four weeks of treatment, cells acquired a neuronal morphology and expressed high levels of the pan-neuronal markers microtubule-associated protein 2 (MAP2) and β-Tubulin-III (B3T; **Figures 6C and 6D**).

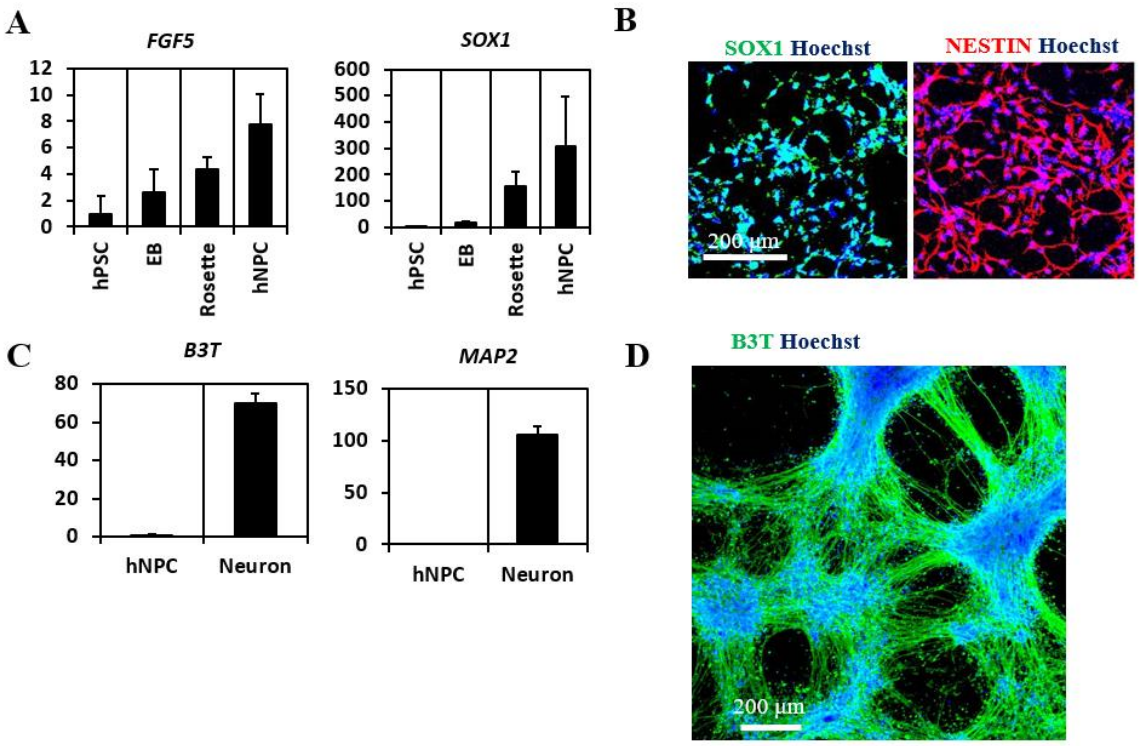


Figure 6. Differentiation of hESCs to Neural Progenitor Cells (NPCs) and Neurons. (A) Quantitative PCR analysis for expression of neuroectoderm related genes *FGF5* and *SOX1* during generated of H9-hNPCs (mean ± S.E.M). (B) SOX1 and NESTIN immunofluorescence of proliferating H9-hNPCs (scale bar = 200 µm). (C) Quantitative PCR analysis of mature neuronal markers *MAP2* and *B3T* in H9-hNPC and neuronal cultures (mean ± S.E.M). (D) B3T immunofluorescence of neurons differentiated from proliferating H9-hNPCs (scale bar = 200 µm).

The extracellular matrix (ECM) is complex network of extracellular matrix proteins (ECMPs) and proteoglycans that provides a scaffold for cell adhesion and growth. Integrins are a family of cell surface receptors that mediate binding to the ECM [115, 116]. To rationally design a set of defined peptides that could mimic the ECM and promote the adhesion as well as growth of hNPCs, we measured the expression of levels of various integrins and components of the ECM in undifferentiated hESCs, hNPCs, as well as early endoderm (EN), mesoderm (ME), and ectoderm (EC) cell populations differentiated from hESCs (**Figure 7**).

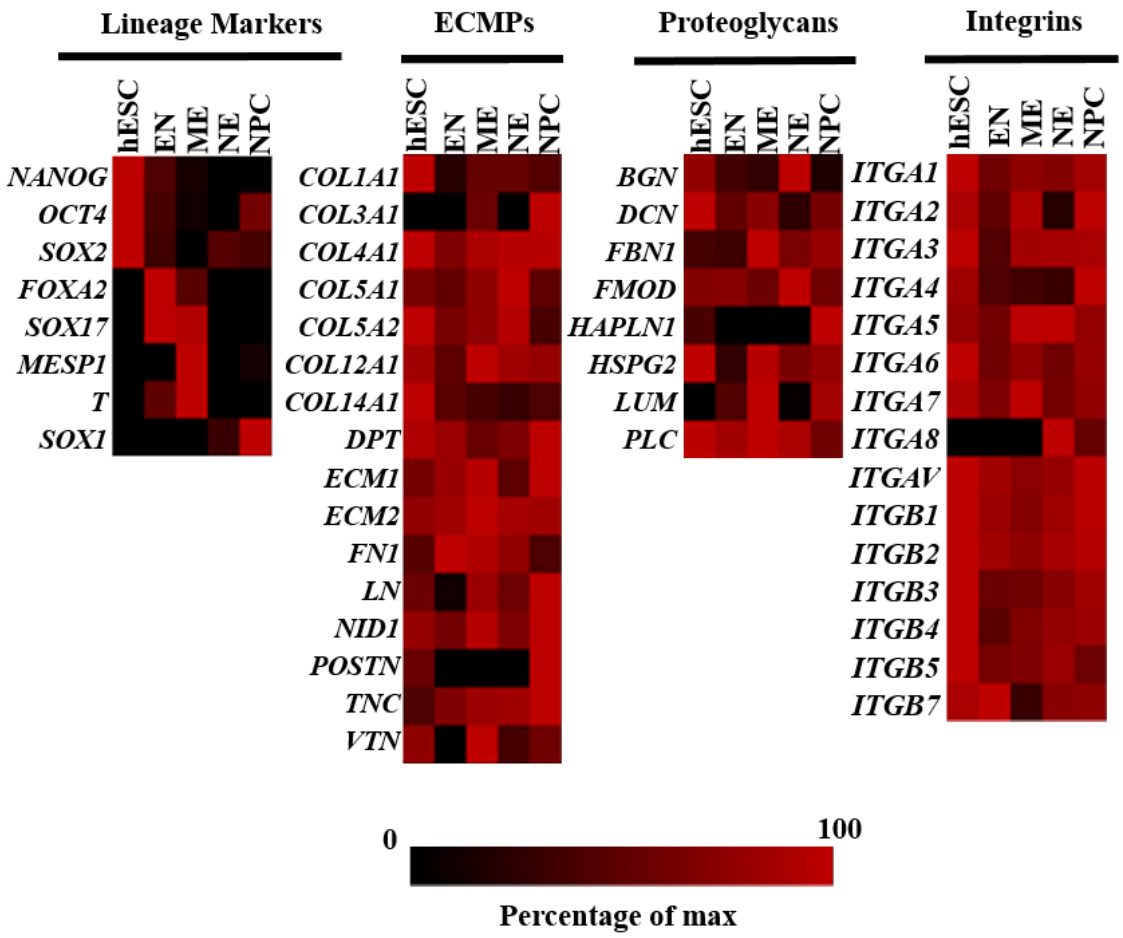


Figure 7. Analysis of Extracellular Matrix Protein (ECMP), Proteoglycan, and Integrin Expression in hPSCs, hNPCs, and hESC-Derived Endoderm (EN), Mesoderm (ME), Ectoderm (EC). Quantitative PCR analysis for expression of integrins, ECMPs, and proteoglycans in H9 hPSCs, hNPCs, and transient EC, EN, ME cell populations differentiated from H9 hPSCs. The data is displayed in a heat map where black corresponds to minimum expression levels and red corresponds to maximum levels. For each gene analyzed, the expression levels were normalized to the sample with the highest expression level.

This analysis revealed that several integrins, ECMPs, and proteogylcans were differentially expressed in hNPCs than the other cell populations examined. To confirm the expression of specific integrins in hNPCs, we used flow cytometry to measure the cell surface expression of several α- and β-integrin subunits in proliferating hNPCs (**Figure 8**). This analysis revealed that integrins α_4 (ITGA4), β_3 (ITGB3), β_4 (ITGB4), β_7 (ITGB7) were not

expressed on the cell surface of hNPCs. Integrin subunits α_1 (ITGA1), α_2 (ITGA2), α_3 (ITGA3), β_5 (ITGB5) were expressed at low levels while integrin subunits α_5 (ITGA5), α_6 (ITGA6), α_v (ITGAV), β_1 (ITGB1), β_2 (ITGB2) were highly expressed by proliferating hNPCs. Collectively, these integrin subunits can form the heterodimers $\alpha_1\beta_1$ (binds to collagen and laminin), $\alpha_2\beta_1$ (binds to collagen and laminin), $\alpha_3\beta_1$ (binds to collagen, laminin, and fibronectin), $\alpha_5\beta_1$ (binds to fibronectin), $\alpha_6\beta_1$ (binds to laminin), $\alpha_v\beta_1$ (binds to fibronectin), and $\alpha_v\beta_5$ (binds to fibronectin and vitronectin) [115].

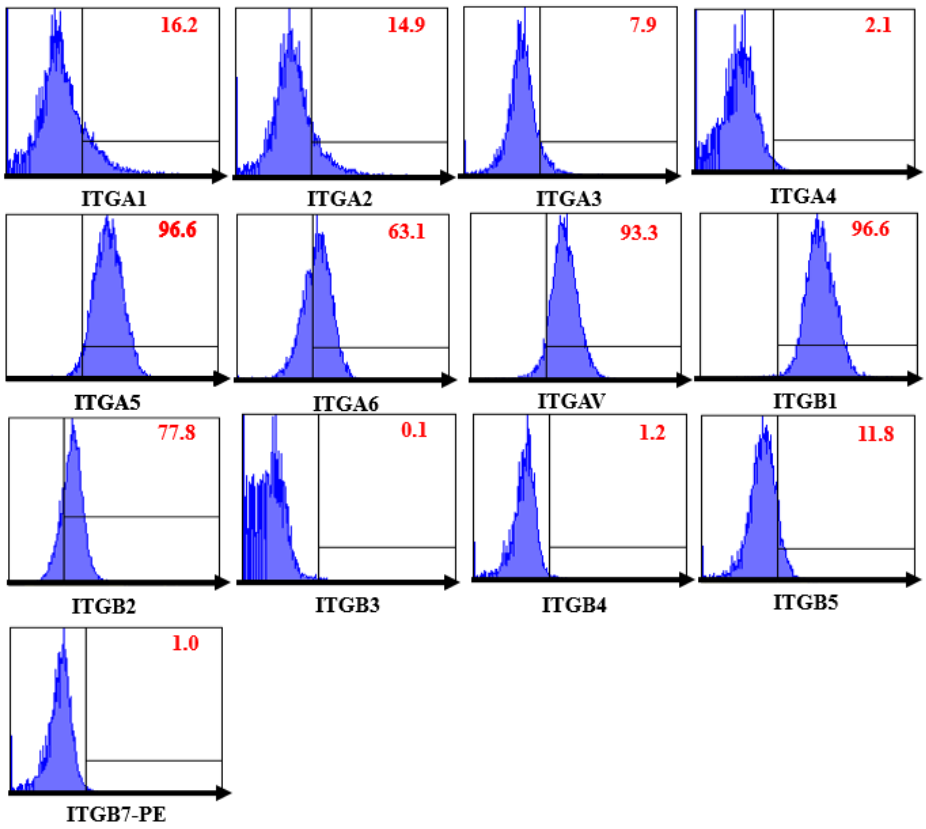


Figure 8. Expression of Integrins on hNPC. Integrin α1-6,v and β1-5,7 was measured in multipotent H9-hNPCs was analyzed by flow cytometry. Gates were determined using isotype controls.

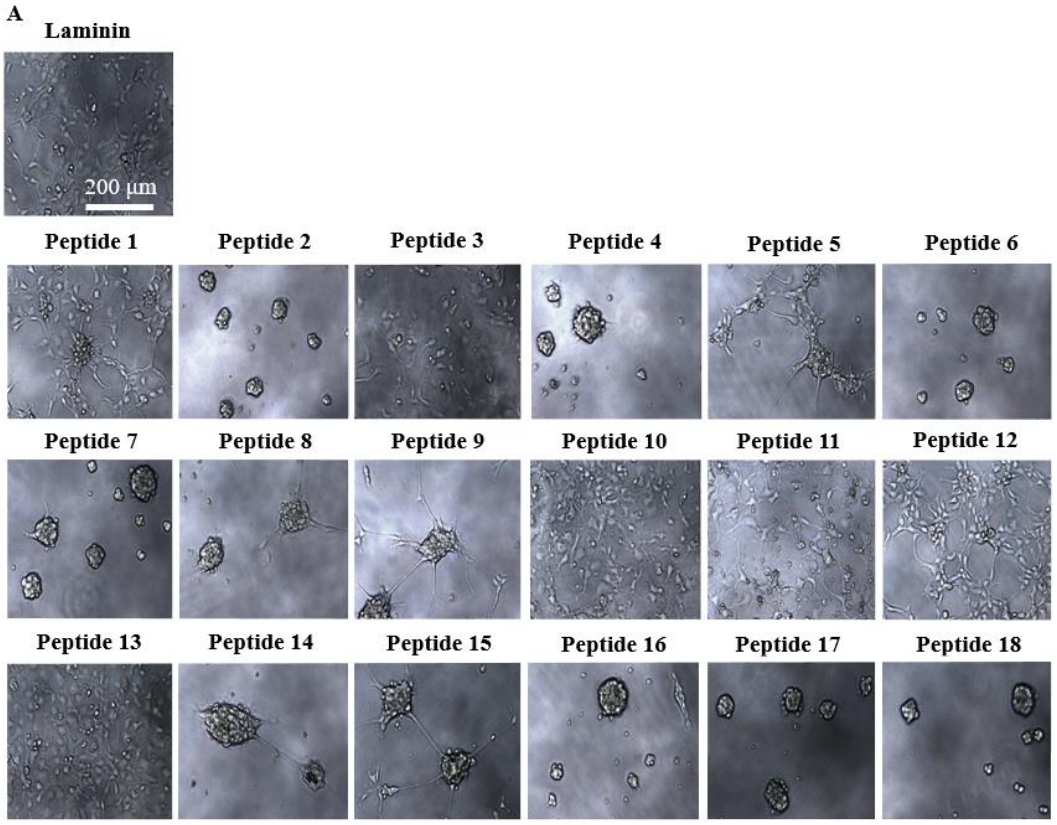
Using this information about the specific ECM components and integrins that were highly expressed in hNPCs, we designed a library of peptides with sequences that mimic these ECM components or the active domains known to interact with these integrin heterodimers [117-136] (**Table 2**).

Table 2: List of Peptides used in this Study

Peptide	Sequence	Integrins	ECMP	Reference
1	CGGTWYKIAFQRNRK	α2β1, α6β1	Laminin α1	116, 117
2	CIKLLI	α3β1	Laminin α1	118
3	CGGRKRLQVQLSIRT	α5β1	Laminin α1	116,119
4	CIKVAV	α3β1, α4β1, α6β1	Laminin α1	120-122
5	CGGNRWHSIYITRFG	α6β1	Laminin α1	116
6	CRYVVLPR	α3β1	Laminin β1	123,124
7	CRNIAEIIKDI	α6β1	Laminin β2	125
8	CDITYVRLKF	α6β1	Laminin ϒ1	126
9	CGGKAFDITYVRLKF	α5β1, αvβ3	Laminin ϒ1	127
10	CDIRVTLNRL	α6β1	Laminin ϒ1	126,128
11	CTTVKYIFR	α6β1	Laminin ϒ1	126
12	CKGGPQVTRGDVFTMP	α5β1, αvβ3, αvβ5	Vitronectin	129
13	CGKKQRFRHRNRKG	αvβ5	Vitronectin	117
14	CGWQPPRARI	α4β1	Fibronectin	130,131
15	CRGDS	α5β1, αvβ3, αvβ5	Fibronectin	132
16	CDRVRHSRNSIT	α5β1	Fibronectin	133
17	CPHSRN	α5β1	Fibronectin	134
18	CDGEA	α2β1	Collagen	135

To test if these peptides could support the growth and adhesion of hNPCs, cells were seeded into 96-well plates coated with 500 μM of each peptide. LN-coated 96-well plates were used as positive controls. Cell morphology (**Figure 9**) and cell counts (**Figure 9B**) were analyzed after 72 hrs. Of the 18 peptides tested, only four peptides allowed for

hNPCs to display a morphology and cell number similar to that of cells grown on laminin control surfaces—peptide 1 (laminin α1 derived, binds to integrins $\alpha_2\beta_1$ and $\alpha_6\beta_1$; [118, 119]), peptide 10 (laminin γ1 derived, binds to integrin $\alpha_6\beta_1$; [128, 130]), peptide 12 (vitronectin derived, binds to integrins $\alpha_5\beta_1$ and $\alpha_v\beta_5$; [131]), and peptide 13 (vitronectin derived, binds to integrins $\alpha_v\beta_5$: [117]).



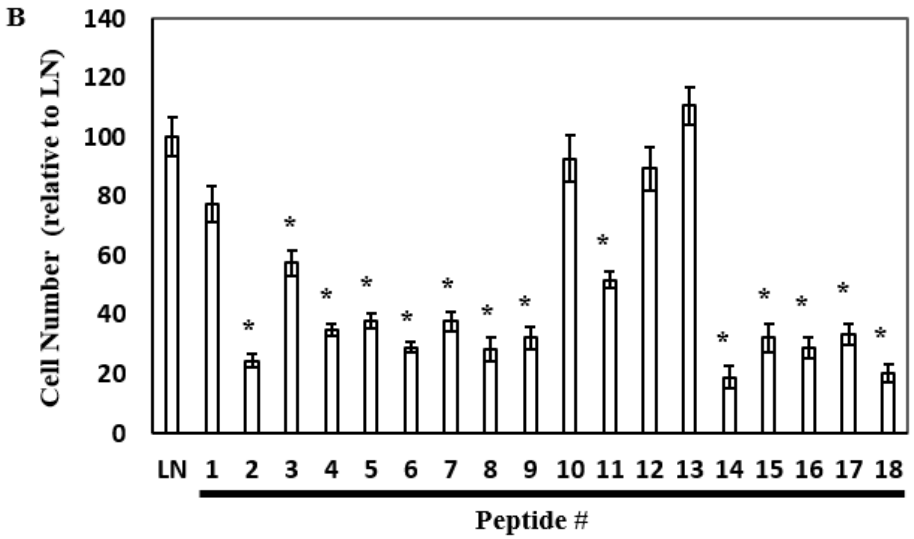


Figure 9. Identification of Peptide-Based Surfaces for hNPC Adhesion and Growth. (A) Representative phase contrast images of H9-hNPCs cultured on laminin (LN)- or peptide-coated tissue culture plates for 72 hrs. All peptides were tested at a concentration of 0.5 μM. (B) Cell count of H9-hNPCs cultured on the LN or peptide-coated tissue culture plates for 72 hrs. Quantification of images was performed by counting 9 fields at 10x magnification. Cell counts were normalized to those on LN surfaces. Image quantification of the data is presented as the average of these fields ± standard deviation (S.D.). Cell counts on peptides were compared to cell counts on LN controls using Student's t-test with Bonferroni correction ($*p < 0.05$). The sequences of the peptides tested are listed in **Table 2**.

3.2. Long-term Expansion of hNPCs on Defined Peptide Surfaces

We tested if the four 'hit' peptides that we identified to support the short-term growth of hNPCs could support hNPC proliferation and maintenance of multipotency over multiple passages. In addition, to investigate the broad utility of these 'hit' peptides, we performed this analysis with four hNPC lines derived from three independent hESC lines (H9 [137], HSF4 [138], and HES3 [139]) and one hiPSC line (RiPSC, [140]). We cultured hNPCs in 12-well plates coated with various concentrations of each 'hit' peptide. Cell detachment or differentiation, as indicated by acquisition of a neuronal morphology, was observed within the first three passages on all peptides with the exception of peptide 13 (herein referred to as vitronectin-derived peptide [VDP]). In addition, a dose-response curve revealed that 100

μM of VDP was the minimum concentration necessary to promote hNPC adhesion at levels similar to that on LN-control substrates (**Figure 10**).

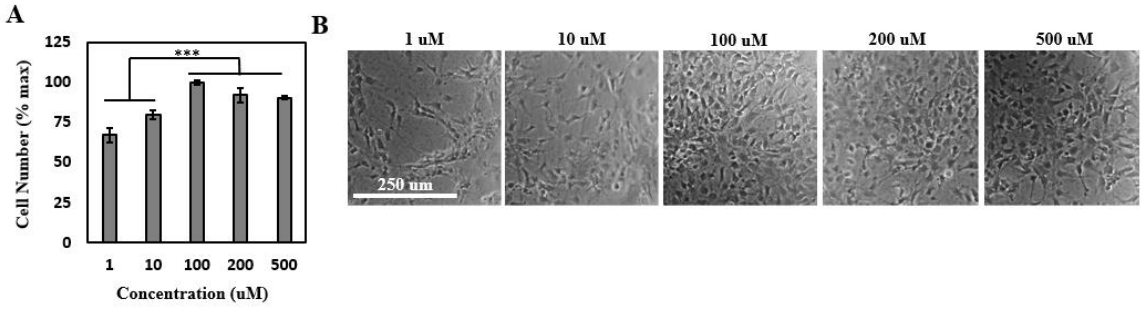


Figure 10. Effect of VDP Concentration on Growth and Adhesion of hNPCs. (A) HES3-hNPCs were grown on surfaces coated with various concentrations of VDP. Cell counts were performed after 72 hrs of culture on the VDP-coated surfaces. Quantification of images was performed by counting 4 fields at 10x magnification. Image quantification of the data is presented as the average of these fields ± standard error of the mean (S.E.M). Cell counts were compared using Student's t-test ($*p < 0.05$). (B) Representative phase contrast images of HES3-hNPCs grown on surfaces with various concentration so VDP (scale = 250 μm).

HNPCs cultured on VDP-coated substrates maintained their characteristic morphology over 10 passages (> 50 days; **Figure 11A** and **Figure 12A**). HNPCs cultured on VDP displayed a similar doubling time (**Figure 11B** and **Figure 12B**) to cells cultured on control LN substrates. In addition, the hNPC growth rate on VDP remained constant over the course **A** 0 passages (**Figure 11C** and **Figure 12C**). Cell counts taken at each passage revealed that 2 x 10^5 hNPCs could theoretically be expanded to 1 x 10^{11} over the course of 10 passages (**Figure 11C** and **Figure 12C**).

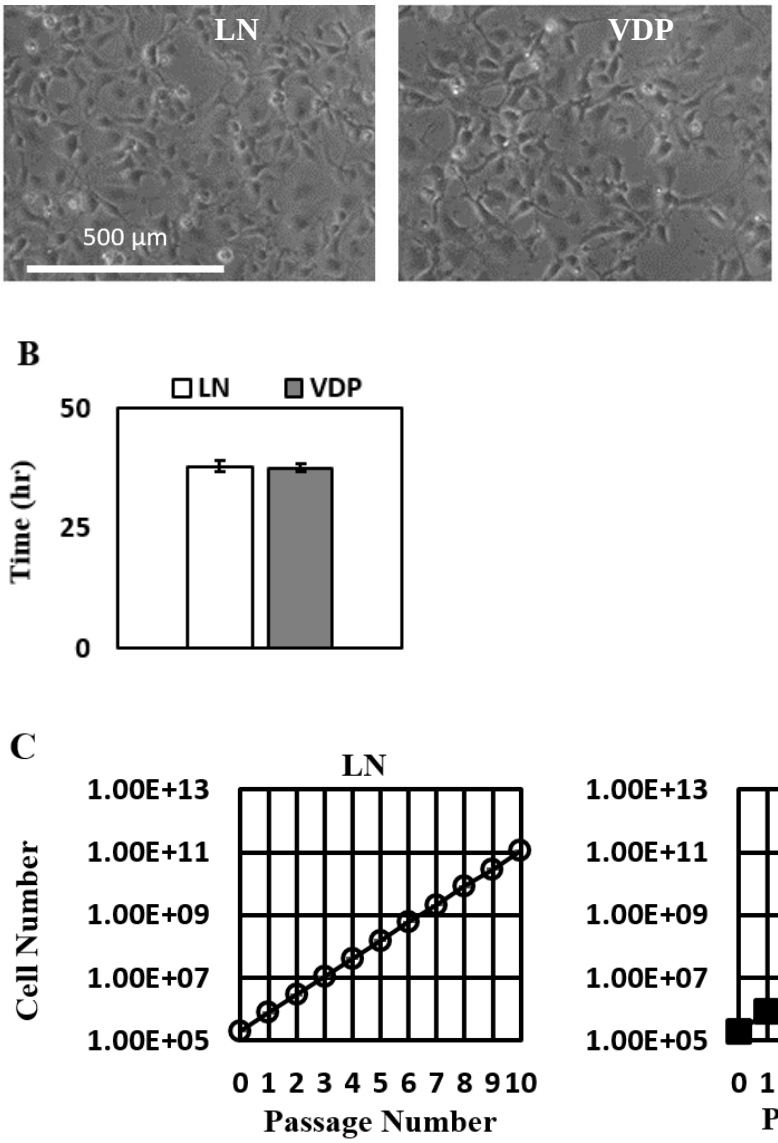


Figure 11. Long-term Expansion of hNPCs on VDP-Coated Surfaces. (A) Representative phase contrast images of H9-hNPCs cultured on LN and VDP surfaces for 10 passages (scale bar = 500 µm). (B) Doubling time of H9-hNPCs cultured on LN and VDP (mean ± S.D). (C) H9-hNPCs were cultured on LN and VDP and cell growth was analyzed by cell count at each passage (mean ± S.E.M).

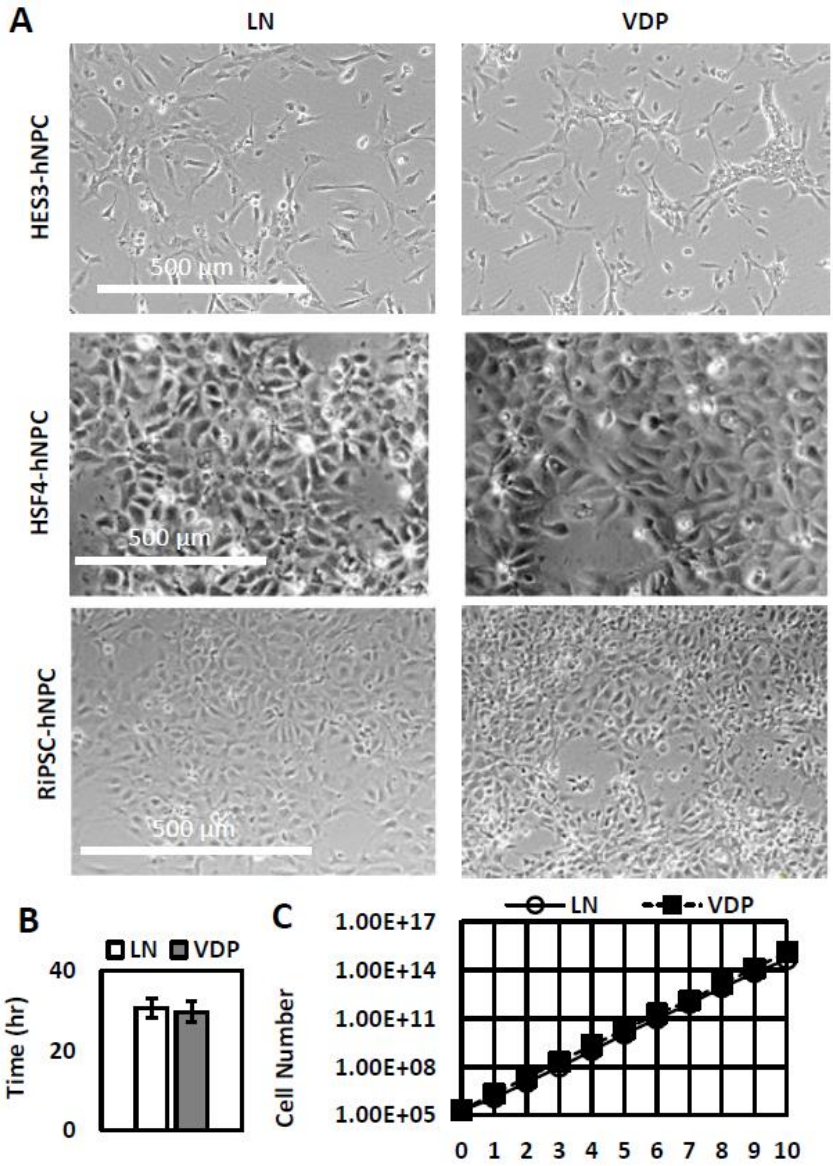


Figure 12. Long term Expansion of hNPCs Grown over 10 Passages (A) Representative phase contrast images of HES3- (top panels), HSF4- (middle panels) and RiPSC-hNPCs (bottom panels) cultured on LN and VDP surfaces (scale bar = 500 µm).(B) Doubling time of RiPSC-hNPCs cultured on LN and VDP (mean ± S.D.) (C) RiPSC hNPCs were cultured on LN and VDP and cell growth was analyzed by cell count at each passage (mean ± S.E.M.).

Quantitative RT-PCR (qPCR) showed that expression of genes associated with a hNPC phenotype (*SOX1*, *SOX2*, *NESTIN*) was similar in hNPCs grown on VDP- and LN-coated surfaces for 10 passages (**Figure 13** and **Figure 14A and 14B**).

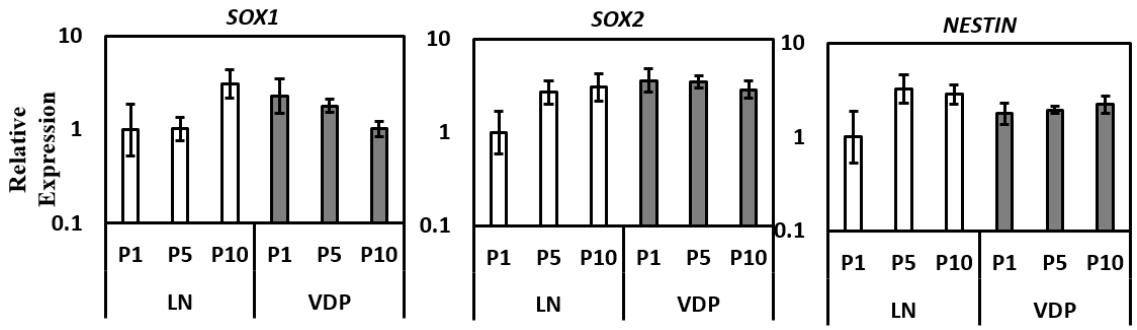


Figure 13. Gene Expression Analysis of hNPCs Maintained on VDP for 10 Passages. Quantitative PCR analysis for expression of hNPC multipotency markers *SOX1*, *SOX2*, and *NESTIN* in H9-hNPCs cultured on LN and VDP for 1, 5, and 10 passages (mean ± S.E.M). There was no statistically significant (Student's t-test, $p>0.05$) difference in expression of these genes between the hNPC populations.

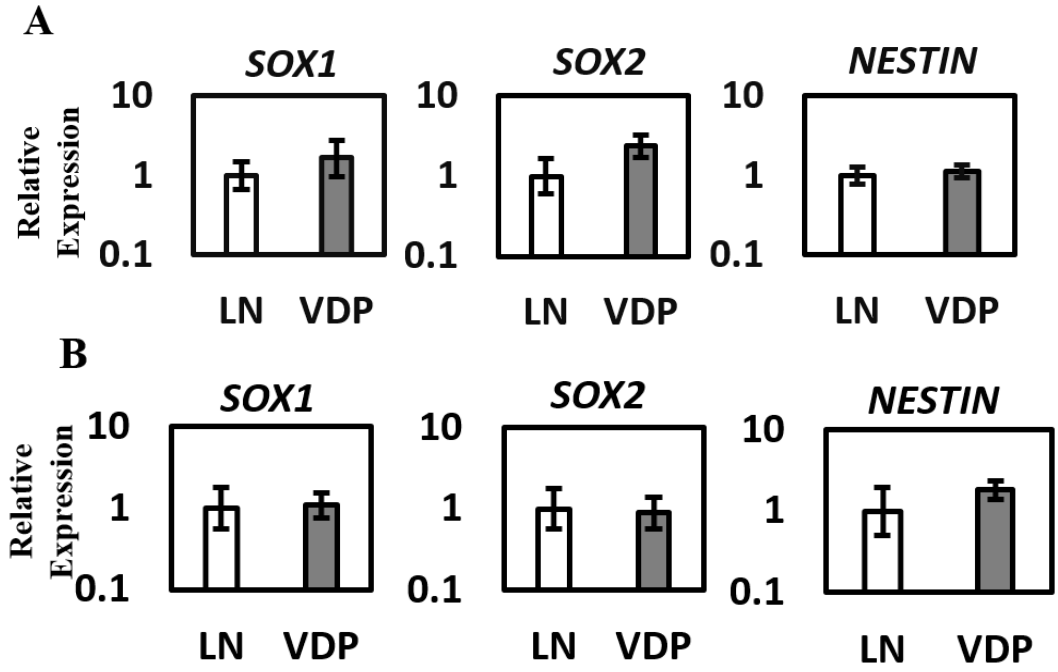


Figure 14. Quantitative PCR analysis for expression of hNPC multipotency markers *SOX1*, *SOX2*, and *NESTIN* in (A) HSF4- and (B) RiPSC-hNPCs cultured on LN and VDP for 10 passages (mean ± S.E.M). There was no statistically significant (Student's t-test, $p>0.05$) difference in expression of these genes between the hNPC populations.

Similarly, immunofluorescence (**Figure 15A** and **Figure 16A**) and flow cytometry (**Figure 15B** and **Figure 16B** and **16C**) demonstrated that a high percentage (>85%) of hNPCs cultured on VDP-coated surfaces expressed the hNPC markers SOX1, SOX2, and

NESTIN. Taken together, these results demonstrate that VDP is able to support the long-term expansion of hNPCs at a similar level to control LN substrates.

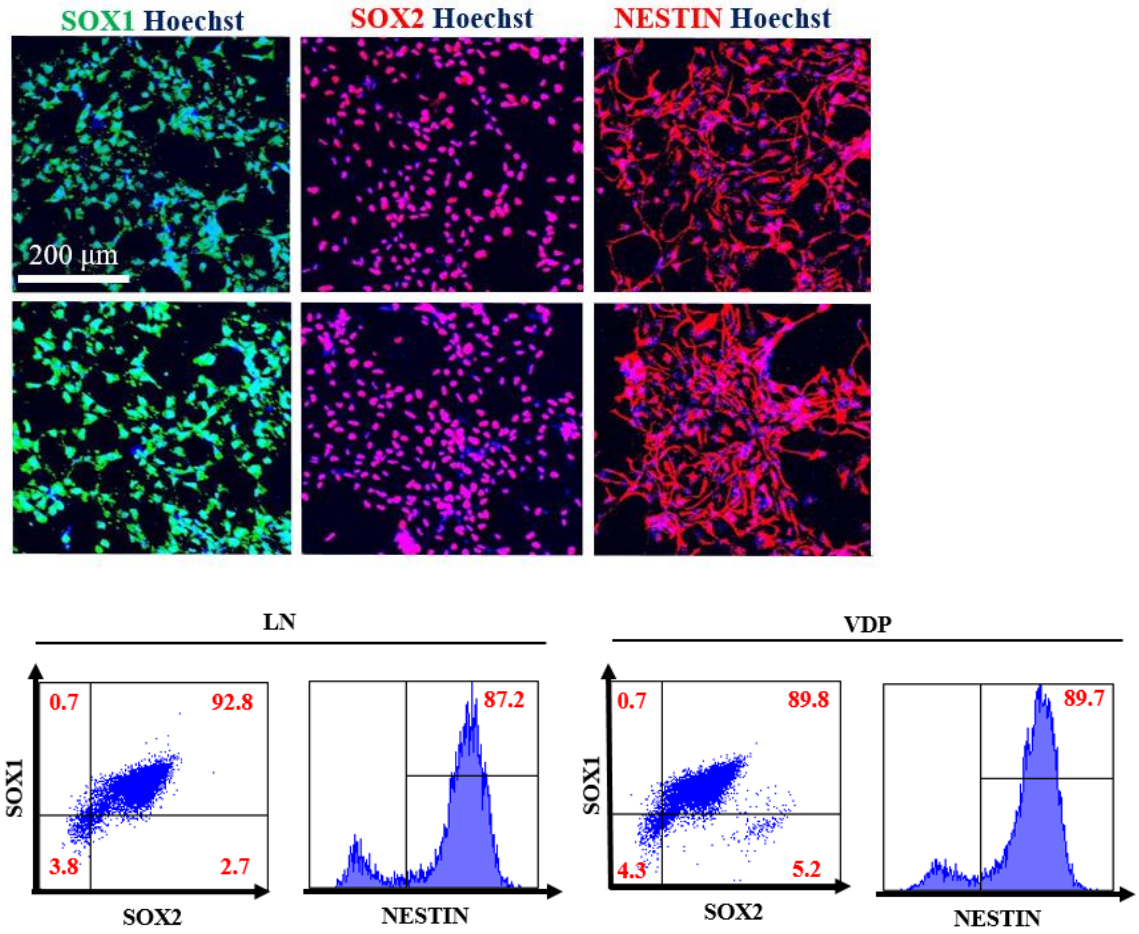
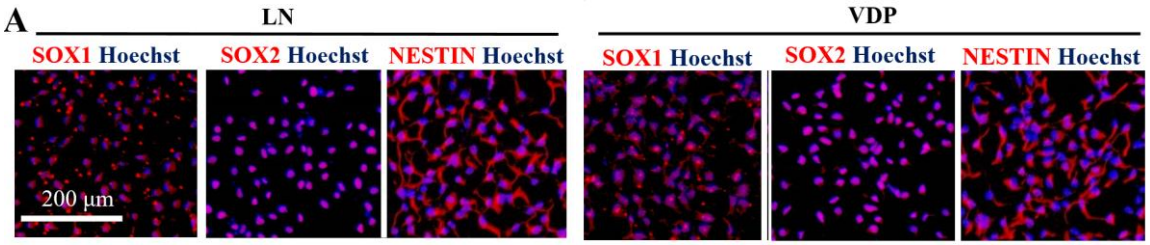


Figure 15. Protein Expression of hNPCs Maintained over 10 Passages. (A) SOX1, SOX2, and NESTIN immunofluorescence of H9-hNPCs cultured on LN and VDP for 10 passages (scale bar = 200 µm). (B) Flow cytometry analysis for SOX1, SOX2, and NESTIN expression in H9 hNPCs cultured on LN and VDP for 10 passages. Gates were determined using isotype controls. Isotype controls used are listed in **Supplementary Table 3**.



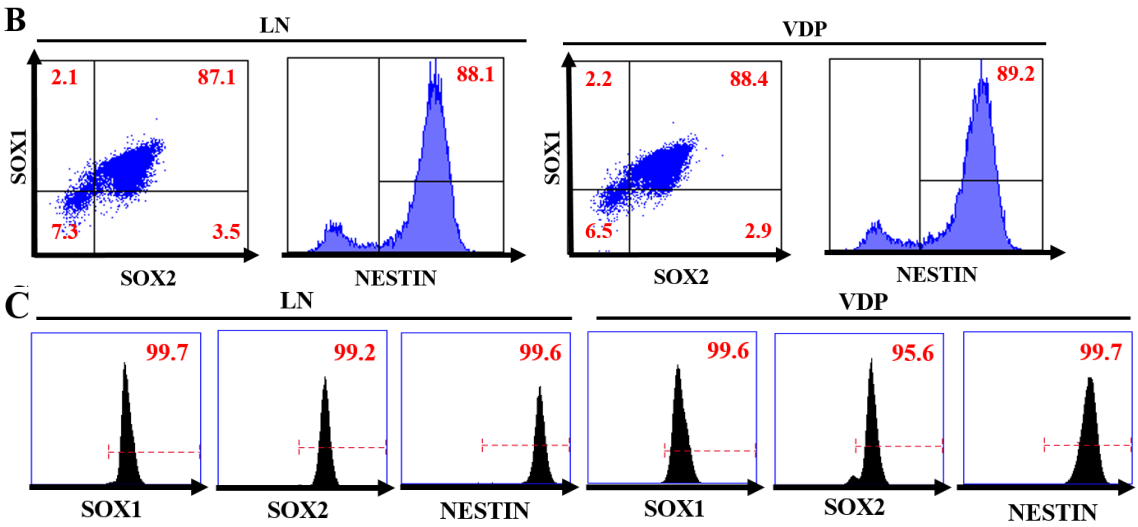


Figure 16. Protein Expression of hNPCs Maintained over 10 Passages. (A) SOX1, SOX2, and NESTIN immunofluorescence of RiPSC-hNPCs cultured on LN and VDP for 10 passages (scale bar = 200 μm). Flow cytometry analysis for SOX1, SOX2, and NESTIN expression in (B) HSF4- and (C) RiPSC-hNPCs cultured on LN and VDP for 10 passages. Gates were determined using isotype controls. Isotype controls used are listed in **Table 3**.

3.3. Characterization of hNPC Integrin Profile on VDP-Coated Surfaces

We hypothesized that long-term culture of hNPCs on VDP-coated surfaces would lead to a shift in their integrin expression profile to one of increased expression of integrins known to directly bind to VDP (i.e. integrins α_v and β5; [117]). To that end, we used qPCR to measure expression of integrins that we previously identified to be expressed by hNPCs cultured on LN substrates. Surprisingly, this analysis revealed no statistically significant differences in integrin expression between hNPCs cultured on VDP- and LN-coated surfaces (**Figure 17**). We speculate that continued expression of integrins that do bind directly to VDP may facilitate interaction with endogenous ECM components that are produced by hNPCs after their initial adhesion and subsequent growth.

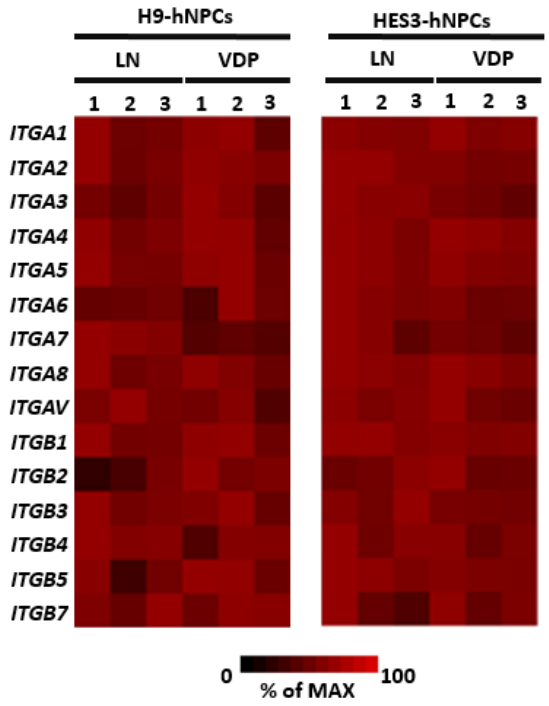


Figure 17. Analysis of Integrin of hNPCs Cultured on LN- and VDP-Coated Surfaces. H9- and HSF4-hNPCs cultured on LN and VDP have similar integrin gene expression profiles. The data is displayed for the biological triplicates in a heat map where black corresponds to minimum expression levels and red corresponds to maximum levels. For each gene analyzed, the expression levels were normalized to the sample with the highest expression level.

3.4. Neuronal Differentiation of hNPCs on VDP-Coated Surfaces

We next assessed if VDP surfaces could support the neuronal differentiation of hNPCs. To that end, hNPCs were grown on VDP and LN control surfaces until they reached confluence and neuronal induction medium was added. After 4 weeks of differentiation, cells cultured on VDP and LN substrates acquired a neuronal-like morphology. QPCR analysis revealed that neuronal cultures on both substrates expressed similarly high levels

of the pan-neuronal markers *β3T* and *MAP2* (**Figure 18** and **Figure 19**).

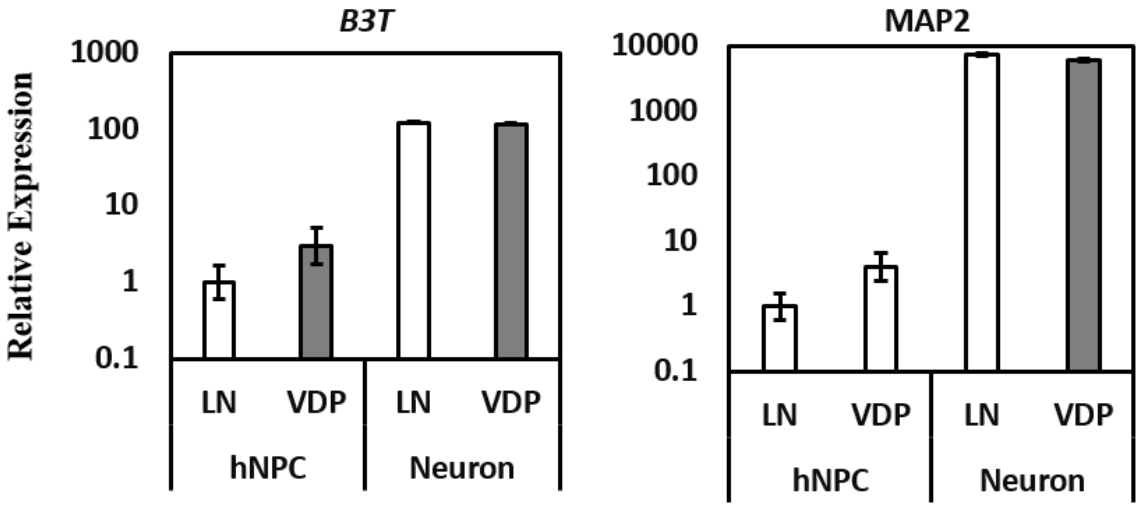


Figure 18. Gene Expression of hNPCs (HES-3) Derived Neurons on VDP-Coated Surfaces. Quantitative PCR analysis for expression of neuronal (*B3T*, MAP2). There was no statistically significant (Student's t-test, $p>0.05$) difference between expression of these genes in hNPCs differentiated on VDP and LN substrates.

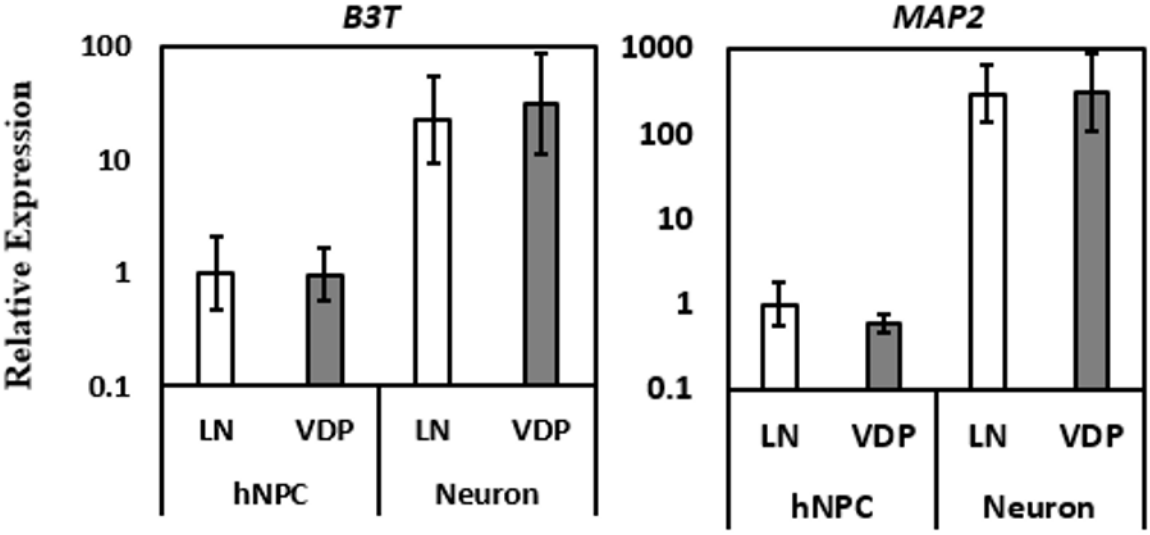
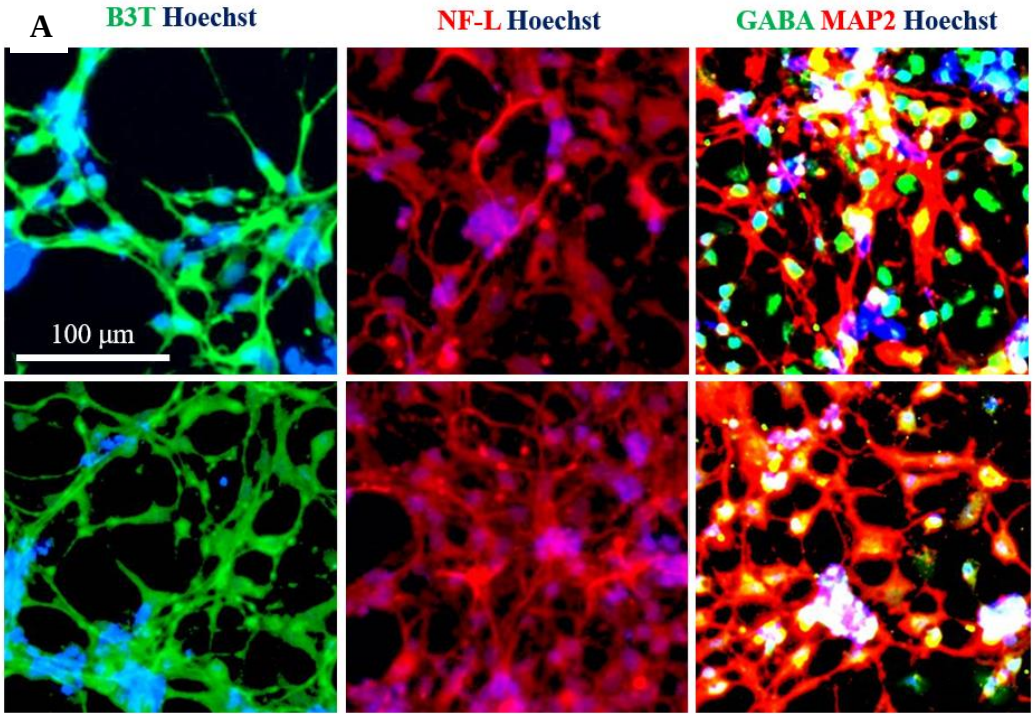


Figure 19. Gene Expression of hNPCs (RiPSC) Derived Neurons on VDP-Coated Surfaces. Quantitative PCR analysis for expression of neuronal (*B3T*, MAP2). There was no statistically significant (Student's t-test, $p>0.05$) difference between expression of these genes in hNPCs differentiated on VDP and LN substrates.

Along similar lines, immunofluorescence revealed that the percentage of cells that were positive for β3T, MAP2, neurofilament-68 (NF-L), and the neurotransmitter γ-

aminobutyric acid (GABA) was similar in neuronal cultures generated on VDP and LN substrates (**Figure 20A** and **Figure 21B**). Finally, quantification of the raw number of β3T, MAP2, and NF-L positive cells revealed that VDP was an equally efficient differentiation substrate as LN (**Figure 20B**). Overall, these results demonstrate that VDP is a highly effective neuronal differentiation matrix for hNPCs.



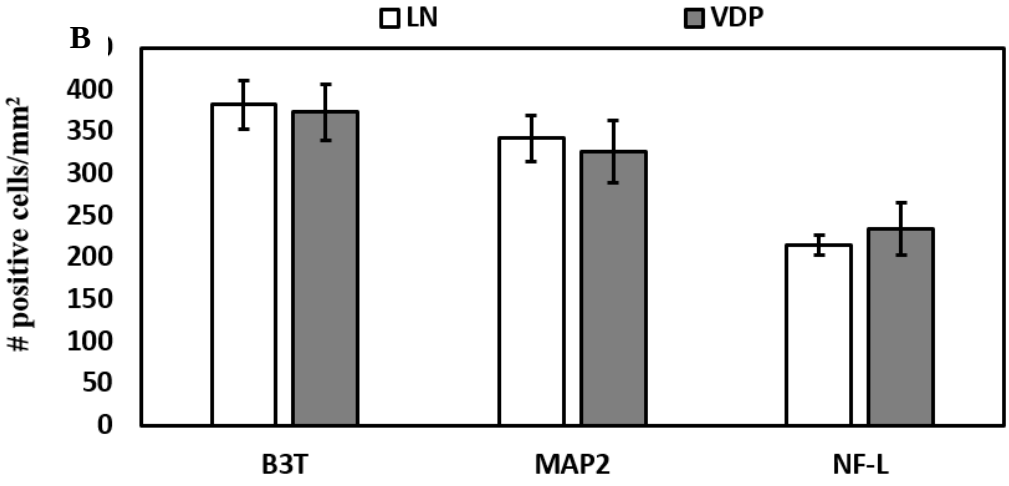


Figure 20. Immunofluoresnce Staining of Neuron Differentiation on VDP Coated Surfaces. (A) Immunofluorescence of for B3T, NF-L, MAP2, and GABA on neurons differentiated from RiPSC-hNPCs on LN and VDP substrates (scale bar = 200 μM). (B) Cell counts of the number of B3T, MAP2, and NF-L positive cells in neuronal cultures generated from RiPSC-hNPCs on LN and VDP substrates. Quantification of images was performed by counting 3 fields at 20x magnification. Image quantification of the data is presented as the average of these fields ± 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.

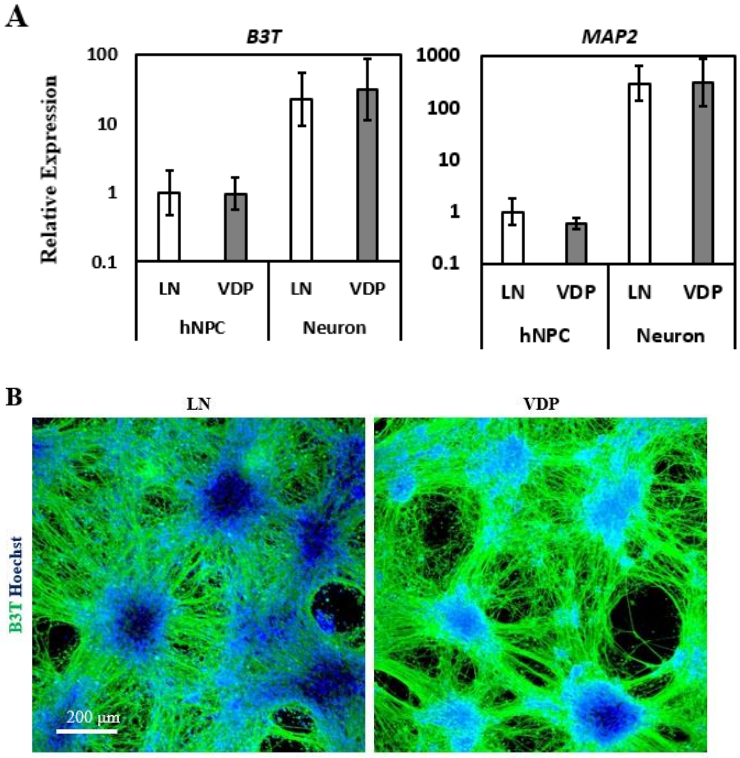


Figure 21. Neuronal Differentiation of Additional hNPCs on VDP-Coated Surfaces. (A) Quantitative PCR analysis for expression of neuronal markers *B3T* and *MAP2* of neurons differentiated from HES3-hNPCs on VDP and LN substrates (mean ± S.E.M). There was no statistically significant difference ($p>0.05$) in *B3T* and *MAP2* expression between hNPCs differentiated on VDP and LN substrates. (B) Immunofluorescence for B3T on neurons differentiated from H9-hNPCs on LN and VDP substrates (scale bar = 200 μM).

3.5. Verifying the Attachment of VDP Coating

To look at the coating chemistry, we labelled our VDP peptide with a fluorescent dye, fluorescein-5-maleimide (F-5-M). The F-5-M has a maleimide group which reacts with the Sulphur (-SH group) of the cystein which is present at the N terminal in the sequence. The maleimide forms a bond with the Cysteine through the carbon-sulphur bond thus conjugating the dye to the peptide (**Figure 22A**). To assess the labelling of the peptide, same amount of dye (1mM) was reacted with different concentrations. Maximum fluorescence was observed in the peptide concentration 1 mM followed by 0.075 mM, 0.05 mM, and least in 0.025 mM. To further prove our results, no fluorescence was observed in our positive control (**Figure 22B**).

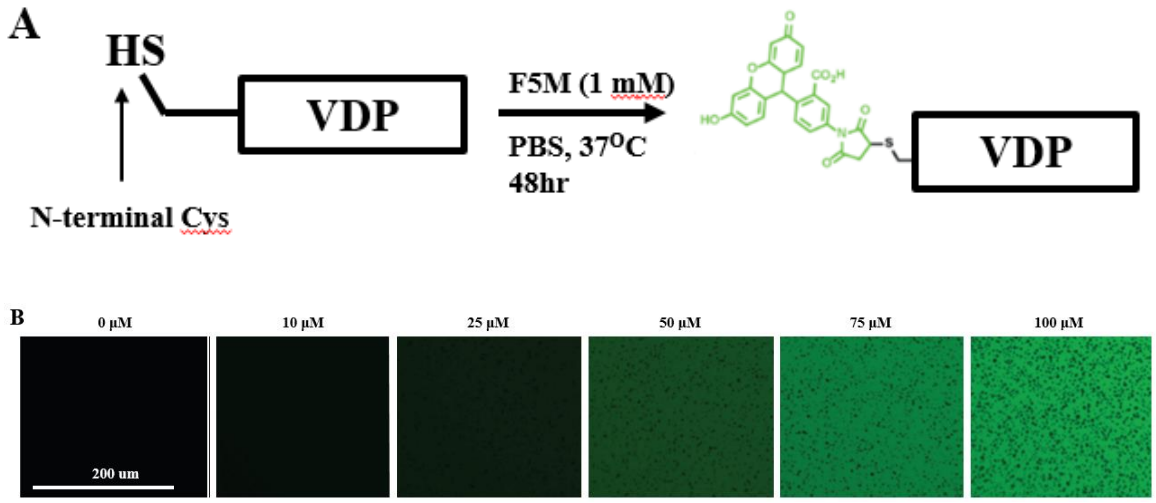


Figure 22: VDP Labelling with F-5-M. A) The thiol-maleimide chemical reaction mechanism. The fluorescently labelled maleimide reacts with the thiol group of the cysteine and couples with it thereby attaching the fluorescent dye to the cysteine of the peptide. B) The fluorescent images of TCPS coated with F-5-M labelled VDP. The same amount of dye was added to different concentrations of VDP for labeling.

3.6. Long term expansion of hNPCs on VDP coated microcarrier suspension culture

In order to create a xenofree scale up culture to meet the need of high cell numbers required for cell based therapies, drug screening and regenerative medicine, we seeded hNPCs on VDP coated microcarriers (MCs) in suspension culture. HNPCs cultured on VDP MC maintained their characteristic morphology over 10 passages (>50 days; Figure 23). HNPCs cultured on VDP displayed a similar doubling time (**Figure 24**) to cells cultured on the control LN coated MC. The hNPC growth rate on VDP MC remained constant over the course of 10 passages (Figure 16C). Cell counts at each passage revealed that $1.5x10^6$ hNPCs could theoretically be expanded to $1x10^{12}$ over the course of 10 passages (Figure 16D). Quantitative RT-PCR (qPCR) showed that expression of genes associated with hNPC phenotype (SOX1, SOX2, NESTIN) was similar in hNPCs grown on VDP and Ln coated surfaces for 10 passages. This gene expression was further compared with the hNPCs grown on VDP and Ln in 2D static cultures and we found that there was no significant difference in the expression of these markers in any of the passages in 2D static and MC suspension cultures (**Figure 25**).

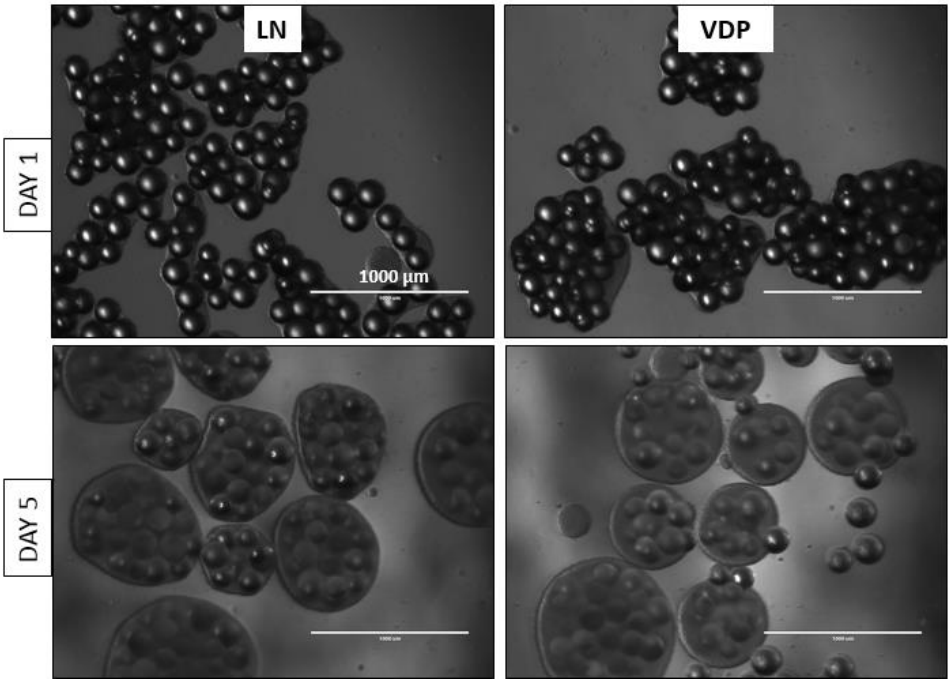
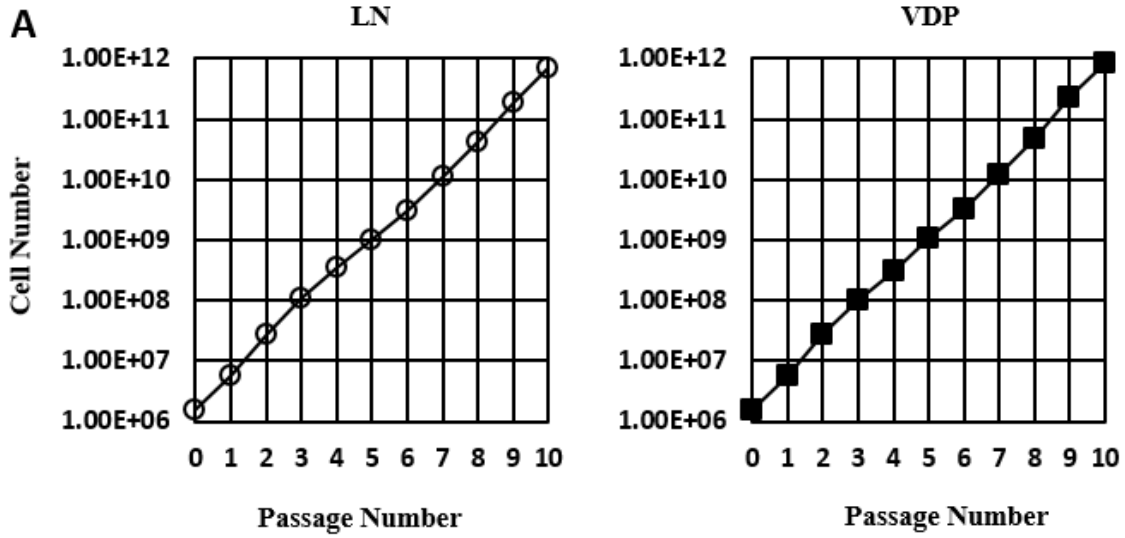


Figure 23: Phase Contrast Images of hNPC Culture on VDP Coated MC. Cell morphology of hNPCs on LN and VDP coated microcarriers. The upper row shows images of hNPCs on Day 1 and lower row shows the images of hNPCs upon confluency.



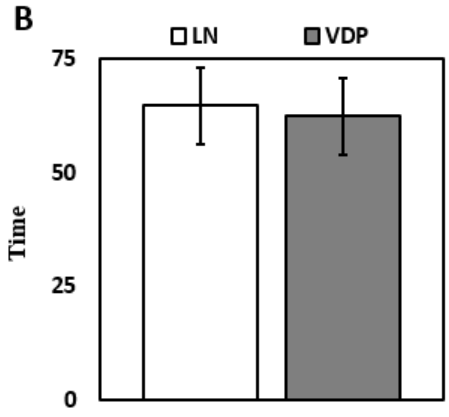


Figure 24: Growth rate Analysis of hNPCs Grown on LN and VDP Microcarrier. A) RiPSC hNPCs cultured on LN and VDP and cell growth was analyzed by cell count at each passage (mean ± S.E.M.). (B) Doubling time of RiPSC-hNPCs cultured on LN and VDP (mean ± S.D.) hNPCs grown on VDP and LN MC maintain similar growth rate over the course of 10 passages.

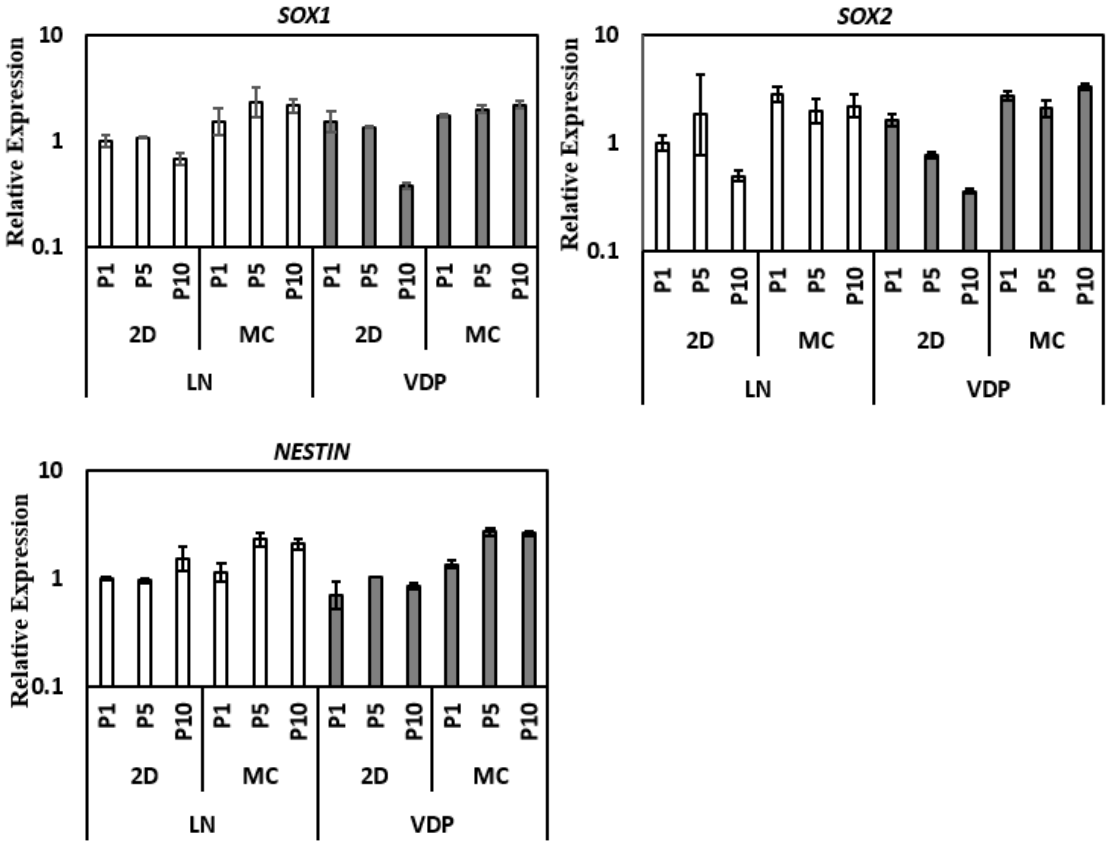
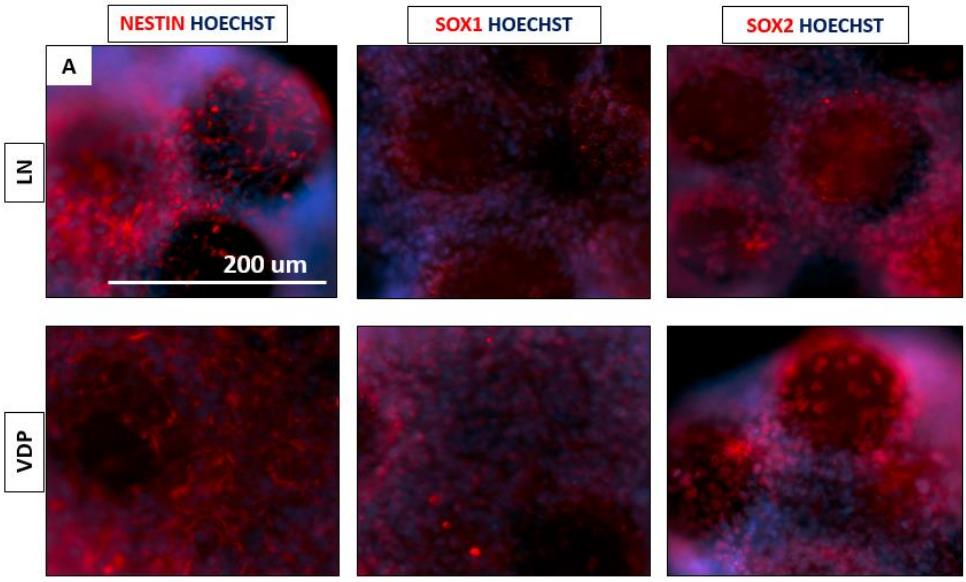


Figure 25: Gene Expression of hNPCs Derived on VDP Coated Microcarrier. Quantitative PCR analysis for expression of hNPC multipotency markers *NESTIN*, *SOX1*, and *SOX2* in RiPSC-hNPCs cultured on LN and VDP MC suspension culture for 1, 5 and 10 passages on 2D and MC suspension culture (mean ± S.E.M). There was no statistically significant (Student's t-test, $p>0.05$) difference in expression of these genes between the hNPC populations.

Similarly immunofluorescence and flow cytometry (**Figure 26**) demonstrated that a high percentage (>90%) of hNPCs cultured on VDP MC expressed hNPC markers SOX1, SOX2, and NESTIN. Thus, summing up these results demonstrate that VDP MC is able to support the long-term expansion of hNPCs at a similar level to control LN substrates.



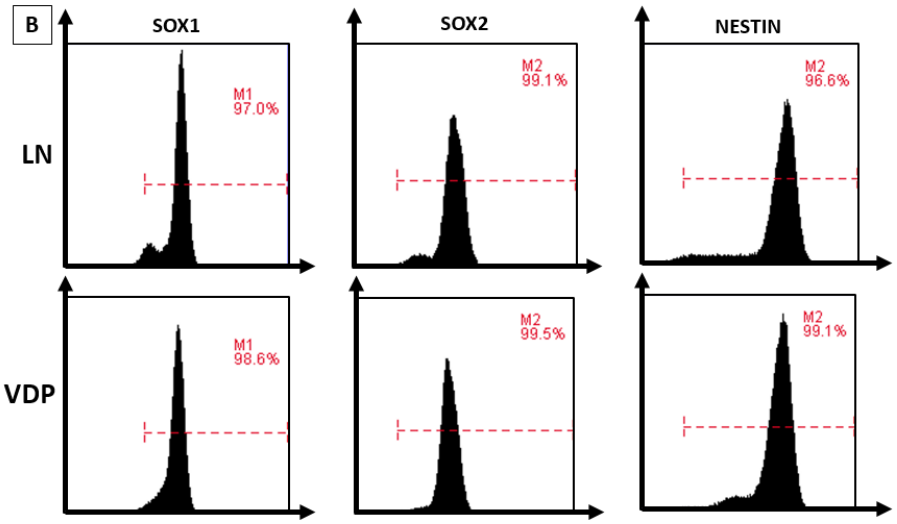


Figure 26. Protein Expression of hNPCs Maintained over 10 Passages. (A) SOX1, SOX2, and NESTIN immunofluorescence of RiPSC-hNPCs cultured on LN and VDP MC (scale bar = 200 μm). (B) Flow cytometry analysis for SOX1, SOX2, and NESTIN expression in RiPSC hNPCs cultured on LN and VDP coated MC suspension culture for 6 passages.

3.7. Neuronal Differentiation of hNPCs on VDP coated MC suspension culture

We wanted to assess if the VDP coated MC suspension culture could support the neuronal differentiation of hNPCs. So the hNPCs were cultured on VDP and LN coated MC in suspension and upon confluency, the media was switched with neuronal differentiation media (NDM). After 4 weeks of differentiation, the cells were stained positive for β3T and neurofilament-68 (NF-L) on VDP coated Mc suspension cultures (**Figure 27**). Hence, these results demonstrate that VDP is a highly effective and robust neuronal differentiation matrix for hNPCs even in MC suspension cultures

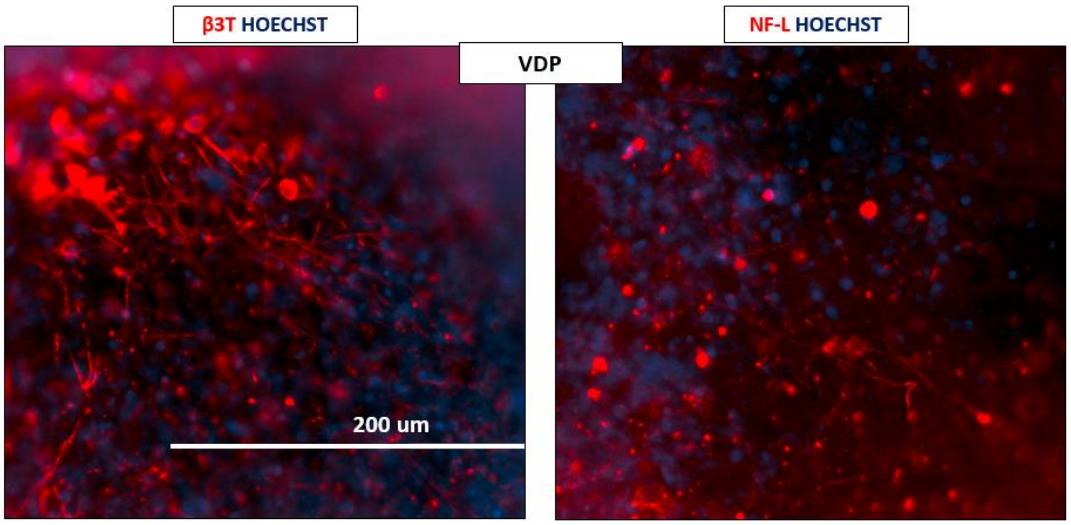


Figure 27. Protein Expression of RiPSC Neurons on VDP Coated Microcarrier Suspension Cultures. β3T and neurofilament-68 (NF-L) Immunofluorescence of RiPSC neurons differentiated on VDP coated MC suspension cultures (Scale bar = 200 um).

CHAPTER 4: DISCUSSION

The application of hNPCs for scientific and clinical purposes necessitates the engineering of completely defined and scalable substrates that support their long-term expansion and directed neuronal differentiation. In this study, we identified one peptide-based substrate, VDP, which was able to support the long-term growth of several independently derived hNPC lines over multiple passages in defined medium conditions. Compared to hNPCs cultured on ECMP-based LN substrates, hNPCs grown on VDP-coated surfaces displayed similar morphologies, growth rates, and high expression levels of hNPC multipotency markers. Furthermore, VDP surfaces supported the directed differentiation of hNPCs to neurons at similar level to cells differentiated on LN substrates.

Laminin from tissue purified or recombinant sources is the most commonly used substrate for the growth and differentiation of hNPCs. The use of LN as a substrate for hNPC culture is largely based on previously developed methods for the propagation of primary fetal and adult neural stem cells (NSCs) [141], which express high levels of the LN binding integrins α6 and β1 [142]. However, we found that only two (peptide 1 and peptide 10) of the eleven peptides derived the cell binding domains of LN or known to interact with LN-binding integrin heterodimer $\alpha_6\beta_1$ supported the attachment of hNPCs and none were able to support the long-term growth of hNPCs. Our integrin expression profiling revealed that in addition to these LN binding integrins, hNPCs also expressed high levels of integrins that bind other ECMPs such collagen (i.e. α1, α2, α3), fibronectin (i.e. α5) and vitronectin (i.e. α_v, β5). Nonetheless, peptides derived from these integrin binding domains of collagen and fibronectin did not support the attachment of hNPCs. The

two peptides (VDP and peptide 12) derived from vitronectin that we examined were able to support the attachment of hNPCs but only VDP was able to support the long-term culture of hNPCs. Previous studies have demonstrated that VDP not only mediates cell binding through interactions with integrin $\alpha_v\beta_5$ but also cell surface proteoglycans [117]. Interestingly, our analysis revealed that in addition to expressing high levels of integrins αv and β5, hNPCs also express high levels of several proteoglycans including decorin (*DCN*), fibrillin (*FBN1*), fibromodulin (*FMOD*), heparan sulfate proteoglycan (*HSGP2*), and lumican (*LUM*). Therefore, we speculate the dual ability of VDP to bind to both integrin $\alpha_v\beta_5$ as well as cell surface proteoglycans highly expressed by hNPCs allowed for VDP to support the long-term expansion of hNPCs.

Previously, peptide-based materials have been used for the expansion and differentiation of hPSCs [143,144]. Specially, a similar peptide sequence that served for the basis for VDP in this study has been used for the long-term culture of undifferentiated hPSCs [27]. More recently, surfaces displaying this same peptide sequence supported the differentiation of hPSCs to early endoderm and mesoderm cell types. Interestingly, surfaces displaying similar peptide sequences to VDP only supported the ectodermal differentiation of hPSCs when those surfaces also displayed a cyclic-RGD containing peptide. Our analysis revealed several RGD binding integrins, such as integrin α5, were expressed at significantly higher levels in early hESC-derived ectoderm cells than in proliferating hNPCs possibly explaining the need for both peptide sequences for the differentiation of hESCs to early ectoderm cell types. Another broadly used peptide-based material, Corning® Synthemax®, has been used for the long-term culture of hPSCs [24]

as well as their differentiation into retinal pigmented epithelial cells [21], mesenchymal stem cells [22], oligodendrocyte progenitor cells [23], cardiomyocytes [24], and insulin producing cells [25]. In this study, we tested the same peptide sequence (peptide 12) that serves as the basis for Corning® Synthemax®. Although this peptide was able to support the attachment and short-term expansion of hNPCs, it did provide for the expansion of hNPCs over multiple passages. Moreover, Synthemax® plates purchased directly from Corning® were unable to support the long-term growth of hNPCs (data not shown).

Several groups have reported the use of peptide-based substrates for the short-term expansion of primary NSCs [59, 60]. For example, Li et al. reported the use of laminin derived IKVAV peptide conjugated to gold-coated cover slips for the short-term growth and neuronal differentiation of immortalized human fetal NSCs [145]. Along similar lines, Little et al. identified several RGD-based peptide surfaces that allowed for the adhesion, growth and differentiation of adult rat hippocampal NSCs [146]. In this study, peptides containing IKVAV (peptide 4) and RGD (peptide 15) were unable to support the attachment and growth of hNPCs. Although NSCs derived from fetal [147-150] and adult [151-154] sources share some morphological, biochemical, and genetic similarities to hPSC-derived hNPCs, several studies have shown that the growth conditions and differentiation potential of these two cell populations are quite different. These subtle biological differences could potentially explain why peptide surfaces that have been previously used for the culture of NSCs did not support the long-term culture of hNPCs.

The development of robust, defined, and scalable substrates for hNPC culture and differentiation are necessary to realize their scientific and clinical potential. In this study,

we demonstrate that VDP is a robust growth and differentiation matrix, as demonstrated by its ability to support the expansions and neuronal differentiation of hNPCs derived from three hESC (H9, HUES9, and HSF4) and one hiPSC (RiPSC) cell lines. In addition, similar to hNPCs grown on LN cells expanded on VDP can be frozen and thawed without any detectable effects on their morphology, growth, and differentiation potential. Furthermore, similar to ECMPs, VDP can be easily coated onto TCPS plates and microcarriers, and does not require immobilization by complex chemical modification or conjugation characteristic of other peptide-based culture systems [159]. Finally, in this study we show that VDP allows for the theoretical expansion of hNPCs to quantities, $>10^{10}$ in 2D culture system and $>10^{12}$ in microcarrier suspension cultures, necessary for drug screening or regenerative medicine. In the future, VDP could potentially be used in microcarrier-based bioreactor systems [155] for the practical large-scale expansion and neuronal differentiation of hNPCs.

In the second half of this study we showed that VDP also allows for scalable long term expansion of hNPCs when cultured in microcarrier suspension culture system. The average cell count that we generated in this 4 ml suspension culture was 5.8 million cells and the cell count achieved in the 2D system of similar surface ara as the microcarrier culture well, we get 2 million cells. If we are to scale up this microcarrier suspension culture to a bioreactor system of 500 ml culture volume and 20mg/ml microcarrier density [159], we would be able to generate about 60 billion cells from each culture which is y more than the cell number required for cell based therapies (~1 billion cells).

CHAPTER 5: CONCLUSIONS

In this study, we developed a completely defined, scalable, and robust peptide-based substrate that allows for the long-term growth and directed neuronal differentiation of hNPCs. Compared to cells grown on standard LN-based substrates, hNPCs grown on VDP maintained their characteristic morphology, expressed high levels of hNPC multipotency markers, and retained their neuronal differentiation potential. In the future, the use of VDP as a defined culture substrate will significantly advance the clinical application of hNPCs and their derivatives as it will enable the large-scale expansion and neuronal differentiation of hNPCs in quantities necessary for disease modeling, drug screening, and regenerative medicine applications.

REFERENCES

1. Tsuji, S. "Genetics of Neurodegenerative Diseases: Insights from High-throughput Resequencing." *Human Molecular Genetics* 19.R1 (2010): n. pag. Web.

2. Gendelman, Howard E., and Tsuneya Ikezu. "Neurodegeneration." *Neuroimmune Pharmacology*. New York, NY: Springer, 2008. N. pag. Print.

3. Bertram, L. "The Genetic Epidemiology of Neurodegenerative Disease." *Journal of Clinical Investigation* 115.6 (2005): 1449-457. Web.

4. "NINDS Overview." *: National Institute of Neurological Disorders and Stroke (NINDS)*. N.p., n.d. Web. 15 Feb. 2016.

5. "2015 Alzheimer's Disease Facts and Figures." *Alzheimer's & Dementia* 11.3 (2015): 332-84. Web.

6. "Alzheimer's Statistics." *Alzheimers.net*. N.p., n.d. Web. 15 Feb. 2016.

7. "PD Info." *EPDA*. N.p., n.d. Web. 15 Feb. 2016.

8. "Statistics on Parkinson's." *Parkinson's Disease Foundation*. N.p., n.d. Web. 15 Feb. 2016.

9. *Parkinson's Disease: National Clinical Guideline for Diagnosis and Management in Primary and Secondary Care*. London: Royal College of Physicians, 2006. Print.

10. Oluigbo, Chima O. "Deep Brain Stimulation for Neurological Disorders." *IEEE REVIEWS IN BIOMEDICAL ENGINEERING* 5 (2012): 88-99. Web.

11. Weissmiller, April M., and Chengbiao Wu. "Current Advances in Using Neurotrophic Factors to Treat Neurodegenerative Disorders." *Transl Neurodegener Translational Neurodegeneration* 1.1 (2012): 1-14. Web.

12. "Latest Medication for Memory Loss | Alzheimer's Association." *Latest Medication for Memory Loss | Alzheimer's Association*. N.p., n.d. Web. 24 Feb. 2016.

13. Okun, Michael S., and Mustafa Saad Siddiqui. "Deep Brain Stimulation - What Has Been Learned and Where It Is Going." *Deep Brain Stimulation*. N.p., n.d. Web. 27 Feb. 2016.

14. "The Pros and Cons of Deep Brain Stimulation - Brain Health." *Brain Health*. N.p., n.d. Web. 27 Feb. 2016.

15. Przedborski, Serge, Miquel Vila, and Vernice Jackson-Lewis. "Neurodegeneration: What Is It and Where Are We?" *Journal of Clinical Investigation*. American Society for Clinical Investigation, n.d. Web. 01 Mar. 2016.

16. Tsuji, S. "Genetics of Neurodegenerative Diseases: Insights from High-throughput Resequencing." *Human Molecular Genetics* 19.R1 (2010): n. pag. Web.

17. Braak, H., and E. Braak. "Neuropathological Stageing of Alzheimer-related Changes." Acta Neuropathologica Acta Neuropathol 82.4 (1991): 239-59. Web.

18. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 1999;286:735–741.

19. Natural Compounds That Modulate BACE1-processing of Amyloid-beta Precursor Protein in Alzheimer's Disease - Can Zhang - Discovery Medicine." *Discovery Medicine*. N.p., n.d. Web. 04 Mar. 2016. <http://www.discoverymedicine.com/Can-Zhang/2012/09/25/natural-compounds-that-modulate-bace1-processing-of-amyloid-beta-precursor-protein-in-alzheimers-disease/>.

20. Mohandas, E., V. Rajmohan, and B. Raghunath. "Neurobiology of Alzheimer's Disease." Indian Journal of Psychiatry. Medknow Publications, n.d. Web. 05 Mar. 2016. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2738403/>.

21. Choi, Sung S., Sang-Rae Lee, Seung U. Kim, and Hong J. Lee. "Alzheimer's Disease and Stem Cell Therapy." Exp Neurobiol Experimental Neurobiology 23.1 (2014): 45. Web.

22. "Parkinson's Disease: Hope Through Research." Parkinson's Disease: Hope Through Research. N.p., n.d. Web. 05 Mar. 2016. <http://www.ninds.nih.gov/disorders/parkinsons_disease/detail_parkinsons_disease.htm>.

23. Davie, C. A. "A Review of Parkinson's Disease." British Medical Bulletin86.10 (2008): 109-27. Web.

24. Bekris, Lynn M. "The Genetics of Parkinson Disease." Journal of Geriatric Psychiatry and Neurology 23.4 (n.d.): 228-42. Web.

25. "Parkinson’s Disease: Review Mechanisms and Models." Neuron 39 (2003): 889-909. Web.

26. Maries, Eleonora, Biplob Dass, Timothy J. Collier, Jeffrey H. Kordower, and Kathy Steece-Collier. "The Role of α-synuclein in Parkinson's Disease: Insights from Animal Models." Nature Reviews Neuroscience Nat Rev Neurosci 4.9 (2003): 727-38. Web.

27. Bates, Gillian P. "History of Genetic Disease: The Molecular Genetics of Huntington Disease — a History." Nat Rev Genet Nature Reviews Genetics (2005): 766-73. Web.

28. Vonsattel, Jean Paul G., Christian Keller, and Etty Paola Cortes Ramirez. "Huntington's Disease – Neuropathology." Handbook of Clinical Neurology Hyperkinetic Movement Disorders (2011): 83-100. Web.

29. Estrada-Sánchez, Ana M., and George V. Rebec. "Role of Cerebral Cortex in the Neuropathology of Huntington's Disease." Frontiers in Neural Circuits Front. Neural Circuits 7 (2013): n. pag. Web.

30. Finkbeiner, Steven. "Huntington's Disease." Cold Spring Harb Perspect Biol.10 (2011): n. pag. Web.

31. Vonsattel, Jean Paul G. "Huntington Disease Models and Human Neuropathology: Similarities and Differences." Acta Neuropathologica Acta Neuropathol 115.1 (2007): 55-69. Web.

32. Vonsattel, G., and Jean Paul. "Huntington Disease." Journal of Neuropathology & Experimental Neurology 57.5 (1998): 369-84. Web.

33. Beal, M. Flint, and Robert J. Ferrante. "Experimental Therapeutics in Transgenic Mouse Models of Huntington's Disease." Nature Reviews Neuroscience Nat Rev Neurosci 5.5 (2004): 373-84. Web.

34. Cha, Jang-Ho J. "Pathology." Huntington’s Disease. N.p., n.d. Web. 06 Mar. 2016. <http://www.acnp.org/G4/GN401000151/CH.html>.

35. Rowland, Lewis P., and Neil A. Shneider. "Amyotrophic Lateral Sclerosis." N Engl J Med 344 (2001): 1688-700. Web.

36. Beleza-Meireles, Ana, and Ammar Al-Chalabi. "Genetic Studies of Amyotrophic Lateral Sclerosis: Controversies and Perspectives."Amyotrophic Lateral Sclerosis 10.1 (2009): 1-14. Web.

37. Hirano, A. "Neuropathology of ALS: An Overview." Neurology 47.Issue 4, Supplement 2 (1996): n. pag. Web.

38. DR, Rosen, Siddique T, and Patterson D. "Mutations in Cu/Zn Superoxide Dismutase Gene Are Associated with Familial Amyotrophic Lateral Sclerosis." Nature 362.6435 (1993): 59-62. Web.

39. Kiernan, Matthew C., Steve Vucic, and Benjamin C. Cheah. "Amyotrophic Lateral Sclerosis." The Lancet 377.9769 (2011): 942-55. Web.

40. Brown, Rebecca C., Alan H. Lockwood, and Babasaheb R. Sonawane. "Neurodegenerative Diseases: An Overview of Environmental Risk Factors." *Environ Health Perspect Environmental Health Perspectives* 113.9 (2005): 1250-256. Web.

41. "Stem Cell Basics." : Introduction [Stem Cell Information]. N.p., n.d. Web. 07 Mar. 2016. <http://stemcells.nih.gov/info/basics/pages/basics1.aspx>.

42. Watt, F. M. "Out of Eden: Stem Cells and Their Niches." Science 287.5457 (2000): 1427-430. Web.

43. Stachowiak, Michal K., and Emmanuel S. Tzanakakis. Stem Cells: From Mechanisms to Technologies. Singapore: World Scientific, 2012. Print.

44. Masters, J. R. W., and Bernhard Palsson. Human Adult Stem Cells. Dordrecht: Springer, 2009. Print.

45. Taupin, Philippe. Stem Cells. New York: Nova Science, 2009. Print.

46. Sugarman, Jeremy. "Ethical Issues in Stem Cell Research and Treatment."Cell Res Cell Research 18 (2008): n. pag. Web.

47. Thomson, J. A. "Embryonic Stem Cell Lines Derived from Human Blastocysts." Science 282.5391 (1998): 1145-147. Web.

48. Feng, Chunjing. "Pluripotency of Induced Pluripotent Stem Cells." Genomics Proteomics Bioinformatics (2013): 299-303. Web.

49. Evans, M. J., and M. H. Kaufman. "Establishment in Culture of Pluripotential Cells from Mouse Embryos." Nature 292.5819 (1981): 154-56. Web.

50. "What Are the Major Types of Stem Cells?" Nature.com. Nature Publishing Group, n.d. Web. 07 Mar. 2016.

51. Avasthi, S., R. N. Srivastava, and A. Singh. "Stem Cell: Past, Present and Future-A Review Article." Internet Journal of Medical Update - EJOURNAL 3.1 (2008): n. pag. Web.

52. Takahashi, Kazutoshi, and Shinya Yamanaka. "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors." Cell 126.4 (2006): 663-76. Web.

53. "Types of Stem Cells and Their Current Uses." EuroStemCell. N.p., n.d. Web. 07 Mar. 2016. <http://www.eurostemcell.org/factsheet/stem-cell-research-and-therapy-types-stem-cells-and-their-current-uses>.

54. "Embryonic and Induced Pluripotent Stem Cell Differentiation Pathways & Lineage-specific Markers." : R&D Systems. N.p., n.d. Web. 07 Mar. 2016. <https://www.rndsystems.com/pathways/embryonic-induced-pluripotent-stem-cell-differentiation-pathways-lineage-specific-markers>.

55. "New Opportunities for Stem Cells in Drug Discovery and Development."Drug Discovery & Development. N.p., n.d. Web. 08 Mar. 2016. <http://www.dddmag.com/articles/2014/01/new-opportunities-stem-cells-drug-discovery-and-development>.

56. Unternaehrer, J. J., and G. Q. Daley. "Induced Pluripotent Stem Cells for Modelling Human Diseases." Philosophical Transactions of the Royal Society B: Biological Sciences 366.1575 (2011): 2274-285. Web.

57. Srikanth, Priya, and Tracy L. Young-Pearse. "Stem Cells on the Brain: Modeling Neurodevelopmental and Neurodegenerative Diseases Using Human Induced Pluripotent Stem Cells." Journal of Neurogenetics 28.1-2 (2014): 5-29. Web.

58. Ruiz-Lozano, Pilar, and Prithi Rajan. "Stem Cells as In Vitro Models of Disease." CSCR Current Stem Cell Research & Therapy 2.4 (2007): 280-92. Web.

59. Dottori, Mirella, Mary Familari, Stefan Hansson, and Kouichi Hasegawa. "Stem Cells as In Vitro Models of Disease." Stem Cells International2012 (2012): 1-2. Web.

60. Jucker, Mathias. "The Benefits and Limitations of Animal Models for Translational Research in Neurodegenerative Diseases." Nature Medicine Nat Med 16.11 (2010): 1210-214. Web.

61. Maury, Yves, Morgane Gauthier, Marc Peschanski, and Cécile Martinat. "Human Pluripotent Stem Cells for Disease Modelling and Drug Screening." Bioessays BioEssays 34.1 (2011): 61-71. Web.

62. Nguyen, Ha Nam, Blake Byers, Branden Cord, Aleksandr Shcheglovitov, James Byrne, Prachi Gujar, Kehkooi Kee, Birgitt Schüle, Ricardo E. Dolmetsch, William Langston, Theo D. Palmer, and Renee Reijo Pera. "LRRK2 Mutant IPSC-Derived DA Neurons Demonstrate Increased Susceptibility to Oxidative Stress." Cell Stem Cell 8.3 (2011): 267-80. Web.

63. Yagi, Takuya, Daisuke Ito, Yohei Okada, Wado Akamatsu, Yoshihiro Nihei, Hideyuki Okano, and Norihiro Suzuki. "Modeling Familial Alzheimer's Disease with Induced Pluripotent Stem Cells." Rinsho Shinkeigaku52.11 (2012): 1134-136. Web.

64. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ. "Disease-specific induced pluripotent stem cells." Cell. 2008 Sep 5; 134(5):877-86.

65. Dimos, J. T., K. T. Rodolfa, K. K. Niakan, L. M. Weisenthal, H. Mitsumoto, W. Chung, G. F. Croft, G. Saphier, R. Leibel, R. Goland, H. Wichterle, C. E. Henderson, and K. Eggan. "Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons."Science 321.5893 (2008): 1218-221. Web.

66. Xie, Y. Z., and R. X. Zhang. "Neurodegenerative Diseases in a Dish: The Promise of IPSC Technology in Disease Modeling and Therapeutic Discovery." Neurological Sciences Neurol Sci 36.1 (2014): 21-27. Web.

67. "Induced Pluripotent Stem Cells (iPSC) for Modeling of Alzheimer's Disease." - Biokemisk Forening. N.p., n.d. Web. 08 Mar. 2016. <http://www.biokemi.org/biozoom/issues/537/articles/2473>.

68. Trosko, James E. "Use of Human Embryonic and Adult Stem Cells for Drug Screening and Safety Assessment." Toxicology 226.1 (2006): 31. Web.

69. Anson B, Nuwaysir E, Wang WB, Swanson B. 2011. Industrialized production of hum an iPSC-derived cardiomyocytes for use in drug discovery and toxicity testing. Biopharm Int 24 : 58–67.

70. Behbahan, Iman Saramipoor, Yuyou Duan, Alexander Lam, Shiva Khoobyari, Xiaocui Ma, Tijess P. Ahuja, and Mark A. Zern. "New Approaches in the Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells toward Hepatocytes." Stem Cell Rev and Rep Stem Cell Reviews and Reports 7.3 (2011): 748-59. Web.

71. Ito, Daisuke, Hideyuki Okano, and Norihiro Suzuki. "Accelerating Progress in Induced Pluripotent Stem Cell Research for Neurological Diseases."Annals of Neurology Ann Neurol. 72.2 (2012): 167-74. Web.

72. Egawa, N., S. Kitaoka, K. Tsukita, M. Naitoh, K. Takahashi, T. Yamamoto, F. Adachi, et al. "Drug Screening for ALS Using Patient-Specific Induced Pluripotent Stem Cells." Science Translational Medicine 4.145 (2012): n. pag. Web.

73. Yang, Yin M., Shailesh K. Gupta, Kevin J. Kim, Berit E. Powers, Antonio Cerqueira, et al. "A Small Molecule Screen in Stem-Cell-Derived Motor Neurons Identifies a Kinase Inhibitor as a Candidate Therapeutic for ALS." Cell Stem Cell 12.6 (2013): 713-26. Web.

74. Mcgivern, Jered V., and Allison D. Ebert. "Exploiting Pluripotent Stem Cell Technology for Drug Discovery, Screening, Safety, and Toxicology Assessments." Advanced Drug Delivery Reviews 69-70 (2014): 170-78. Web.

75. Müller, Benedikt, and Suzanne Kadereit. "Stem Cells in Drug Development. "Drug Discovery and Evaluation: Pharmacological Assays (2014): 1-22. Web.

76. Merkle, Florian T., and Kevin Eggan. "Modeling Human Disease with Pluripotent Stem Cells: From Genome Association to Function." Cell Stem Cell 12.6 (2013): 656-68. Web.

77. Daley, George Q., and David T. Scadden. "Prospects for Stem Cell-Based Therapy." Cell 132.4 (2008): 544-48. Web.

78. Trounson, Alan, and Courtney Mcdonald. "Stem Cell Therapies in Clinical Trials: Progress and Challenges." Cell Stem Cell 17.1 (2015): 11-22. Web.

79. Gage, F. H. "Mammalian Neural Stem Cells." Science 287.5457 (2000): 1433-438. Web.

80. Eriksson, Peter S., Ekaterina Perfilieva, Thomas Björk-Eriksson, Ann-Marie Alborn, Claes Nordborg, Daniel A. Peterson, and Fred H. Gage. "Neurogenesis in the Adult Human Hippocampus." Nature Medicine Nat Med 4.11 (1998): 1313-317. Web.

81. Christie, Brian R., and Heather A. Cameron. "Neurogenesis in the Adult Hippocampus." Hippocampus 16.3 (2006): 199-207. Web.

82. Zhang, Xinhua, and Guohua Ji. "Neurogenesis in Adult Hippocampus."Neural Stem Cells and Therapy (2012): n. pag. Web.

83. Kim, Seung U., and Jean De Vellis. "Stem Cell-based Cell Therapy in Neurological Diseases: A Review." J. Neurosci. Res. Journal of Neuroscience Research 87.10 (2009): 2183-200. Web.

84. Lunn, J. Simon, Stacey A. Sakowski, Junguk Hur, and Eva L. Feldman. "Stem Cell Technology for Neurodegenerative Diseases." Annals of Neurology Ann Neurol. 70.3 (2011): 353-61. Web.

85. Behrstock, Soshana, Allison D. Ebert, Sandra Klein, Melanie Schmitt, Jeannette M. Moore, and Clive N. Svendsen. "Lesion-Induced Increase in Survival and Migration of Human Neural Progenitor Cells Releasing GDNF." Cell Transplantation Cell Transplant 17.7 (2008): 753-62. Web.

86. Blurton-Jones, M., M. Kitazawa, H. Martinez-Coria, N. A. Castello, F.-J. Muller, J. F. Loring, T. R. Yamasaki, W. W. Poon, K. N. Green, and F. M. Laferla. "Neural Stem Cells Improve Cognition via BDNF in a Transgenic Model of Alzheimer Disease." Proceedings of the National Academy of Sciences 106.32 (2009): 13594-3599. Web.

87. Suzuki, Masatoshi, and Clive N. Svendsen. "Combining Growth Factor and Stem Cell Therapy for Amyotrophic Lateral Sclerosis." Trends in Neurosciences 31.4 (2008): 192-98. Web.

88. Spradling, A., D. Drummond-Barbosa, and T. Kai. "Stem Cells Find Their Niche." Nature 414. 6859 (2001): 98-104. Web.

89. Watt, F. M., and B. L. Hogan. "Out of Eden: Stem Cells and Their Niches."Science 287.5457 (2000): 1427-430. Web.

90. Scadden, David T. "The Stem-cell Niche as an Entity of Action." Nature441.7097 (2006): 1075-079. Web.

91. Tanentzapf, Guy, Danelle Devenport, Dorothea Godt, and Nicholas H. Brown. "Integrin-dependent Anchoring of a Stem-cell Niche." Nature Cell Biology Nat Cell Biol 9.12 (2007): 1413-418. Web.

92. Ma, Wu, Tahereh Tavakoli, Eric Derby, Yevgeniya Serebryakova, Mahandra S. Rao, and Mark P. Mattson. "Cell-Extracellular Matrix Interactions Regulate Neural Differentiation of Human Embryonic Stem Cells." BMC Developmental Biology BMC Dev Biol 8.1 (2008): 90. Web.

93. Alberts, Bruce. "Integrins." Molecular Biology of the Cell. New York: Garland Science, 2002. N. pag. Print.

94. Boundless Biology. N.p.: n.p., n.d. Boundless. 8 Jan. 2016. Web. 14 Mar. 2016. <https://www.boundless.com/biology/textbooks/boundless-biology-textbook/cell-structure-4/connections-between-cells-and-cellular-activities-63/extracellular-matrix-of-animal-cells-324-11461/>.

95. Timpl, Rupert, and Judith C. Brown. "Supramolecular Assembly of Basement Membranes." Bioessays BioEssays 18.2 (1996): 123-32. Web.

96. Radakovits, R., C. S. Barros, R. Belvindrah, B. Patton, and U. Muller. "Regulation of Radial Glial Survival by Signals from the Meninges."Journal of Neuroscience 29.24 (2009): 7694-705. Web.

97. Timpl, R., J. Engel, and G.r. Martin. "Laminin — a Multifunctional Protein of Basement Membranes." Trends in Biochemical Sciences 8.6 (1983): 207-09. Web.

98. Franco, Santos J., and Ulrich Müller. "Extracellular Matrix Functions during Neuronal Migration and Lamination in the Mammalian Central Nervous System." Devel Neurobio Developmental Neurobiology 71.11 (2011): 889-900. Web.

99. Perin, Emerson C. “Stem Cell Therapy for Cardiovascular Disease.” Texas Heart Institute Journal 33.2 (2006): 204–208. Print.

100. Thomson, J. A. "Embryonic Stem Cell Lines Derived from Human Blastocysts." Science 282.5391 (1998): 1145-147. Web.Kleinman, Hynda K., and George R. Martin. "Matrigel: Basement Membrane Matrix with Biological Activity." Seminars in Cancer Biology 15.5 (2005): 378-86. Web.

101. Hwang, Dong-Youn. "Feeder-Free Growth of Undifferentiated Human Embryonic Stem Cells." Springer Protocols Handbooks Human Embryonic and Induced Pluripotent Stem Cells (2011): 3-12. Web.

102. Martin, Maria J., Alysson Muotri, Fred Gage, and Ajit Varki. "Human Embryonic Stem Cells Express an Immunogenic Nonhuman Sialic Acid."Nature Medicine Nat Med 11.2 (2005): 228-32. Web.

103. Rosler, Elen S., Gregory J. Fisk, Ximena Ares, John Irving, Takumi Miura, Mahendra S. Rao, and Melissa K. Carpenter. "Long-term Culture of Human Embryonic Stem Cells in Feeder-free Conditions." Dev. Dyn. Developmental Dynamics 229.2 (2004): 259-74. Web.

104. Amit, Michal, and Joseph Itskovitz-Eldor. "Maintenance of Human Embryonic Stem Cells in Animal Serum- and Feeder Layer-Free Culture Conditions." Human Embryonic Stem Cell Protocols (n.d.): 105-14. Web.

105. Navarro-Alvarez, Nalú, Alejandro Soto-Gutierrez, Takesui Yuasa, Tomoki Yamatsuji, Yasuhiro Shirakawa, Takeshi Nagasaka, Sheng Sun D, Muhammad Shahid Javed, Noriaki Tanaka, and Naoya Kobayashi. "Long-Term Culture of Japanese Human Embryonic Stem Cells in Feeder-Free Conditions." Ct Cell Transplantation 17.1 (2008): 27-33. Web.

106. Amita, M., K. Adachi, A. P. Alexenko, S. Sinha, D. J. Schust, L. C. Schulz, R. M. Roberts, and T. Ezashi. "Complete and Unidirectional Conversion of Human Embryonic Stem Cells to Trophoblast by BMP4." Proceedings of the National Academy of Sciences 110.13 (2013): n. pag. Web.

107. Amit, Michal. "Feeder-Layer Free Culture System for Human Embryonic Stem Cells." Methods in Molecular Biology Stem Cell Assays (2007): 11-20. Web.

108. Miyazaki, Takamichi, Sugiko Futaki, Kouichi Hasegawa, Miwa Kawasaki, Noriko Sanzen, Maria Hayashi, Eihachiro Kawase, Kiyotoshi Sekiguchi, Norio Nakatsuji, and Hirofumi Suemori. "Recombinant Human Laminin Isoforms Can Support the Undifferentiated Growth of Human Embryonic Stem Cells." Biochemical and Biophysical Research Communications375.1 (2008): 27-32. Web.

109. Braam, Stefan R., Laura Zeinstra, Sandy Litjens, Dorien Ward-Van Oostwaard, Stieneke Van Den Brink, and Linda Van Laake. "Recombinant Vitronectin Is a Functionally Defined Substrate That Supports Human Embryonic Stem Cell Self-Renewal via αVβ5 Integrin."Stem Cells 26.9 (2008): 2257-265. Web.

110. Heng, Boon Chin, Jian Li, Allen Kuan-Liang Chen, Shaul Reuveny, Simon M. Cool, William R. Birch, and Steve Kah-Weng Oh. "Translating Human Embryonic Stem Cells from 2-Dimensional to 3-Dimensional Cultures in a Defined Medium on Laminin- and Vitronectin-Coated Surfaces." Stem Cells and Development 21.10 (2012): 1701-715. Web.

111. Enam, Sanjar, and Sha Jin. “Substrates for Clinical Applicability of Stem Cells.” World Journal of Stem Cells 7.2 (2015): 243–252. PMC. Web. 16 Mar. 2016.

112. Bardy, Jo'an, Allen K. Chen, Yu Ming Lim, Selena Wu, Shunhui Wei, Han Weiping, Ken Chan, Shaul Reuveny, and Steve K.w. Oh. "Microcarrier Suspension Cultures for High-Density Expansion and Differentiation of Human Pluripotent Stem Cells to Neural Progenitor Cells." Tissue Engineering Part C: Methods 19.2 (2013): 166-80. Web.

113. Marinho, Paulo André Nobrega, Daniel Tait Vareschini, Ismael Carlos Gomes, Bruna Da Silveira Paulsen, Daniel Rodrigues Furtado, Leda Dos Reis Castilho, and Stevens Kastrup Rehen. "Xeno-Free Production of Human Embryonic Stem Cells in Stirred Microcarrier Systems Using a Novel Animal/Human-Component-Free Medium." Tissue Engineering Part C: Methods 19.2 (2013): 146-55. Web.

114. Moya, Noel, Josh Cutts, Terry Gaasterland, Karl Willert, and David A. Brafman. "Endogenous WNT Signaling Regulates HPSC-Derived Neural Progenitor Cell Heterogeneity and Specifies Their Regional Identity."Stem Cell Reports 3.6 (2014): 1015-028. Web.

115. Humphries, J. D. "Integrin Ligands at a Glance." Journal of Cell Science119.19 (2006): 3901-903. Web.

116. Kim, S.-H., J. Turnbull, and S. Guimond. "Extracellular Matrix and Cell Signalling: The Dynamic Cooperation of Integrin, Proteoglycan and Growth Factor Receptor." Journal of Endocrinology 209.2 (2011): 139-51. Web.

117. Vogel, B. E. "A Novel Integrin Specificity Exemplified by Binding of the Alpha v Beta 5 Integrin to the Basic Domain of the HIV Tat Protein and Vitronectin." The Journal of Cell Biology 121.2 (1993): 461-68. Web.

118. Nomizu, M., W. H. Kim, K. Yamamura, A. Utani, S.-Y. Song, A. Otaka, P. P. Roller, H. K. Kleinman, and Y. Yamada. "Identification of Cell Binding Sites in the Laminin 1 Chain Carboxyl-terminal Globular Domain by Systematic Screening of Synthetic Peptides." Journal of Biological Chemistry 270.35 (1995): 20583-0590. Web.

119. Nakahara, H., M. Nomizu, S. K. Akiyama, Y. Yamada, Y. Yeh, and W.-T. Chen. "A Mechanism for Regulation of Melanoma Invasion: LIGATION OF 6 1 INTEGRIN BY LAMININ G PEPTIDES." Journal of Biological Chemistry 271.44 (1996): 27221-7224. Web.

120. Tashiro, Ken-Ichiro, Akira Monji, Ichiro Yoshida, Yoshihito Hayashi, Kazunori Matsuda, Nobutada Tashiro, and Yoshio Mitsuyama. "An IKLLI-containing Peptide Derived from the Laminin α1 Chain Mediating Heparin-binding, Cell Adhesion, Neurite Outgrowth and Proliferation, Represents a Binding Site for Integrin α3β1 and Heparan Sulphate Proteoglycan." Biochem. J. Biochemical Journal 340.1 (1999): 119-26. Web.

121. Siqueira, Adriane S., Letícia N. Gama-De-Souza, Maria Vanda C. Arnaud, João J. V. Pinheiro, and Ruy G. Jaeger. "Laminin-derived Peptide AG73 Regulates Migration, Invasion, and Protease Activity of Human Oral Squamous Cell Carcinoma Cells through Syndecan-1 and β1 Integrin."Tumor Biol. Tumor Biology 31.1 (2009): 46-58. Web.

122. Nomizu, M. "Cell Binding Sequences in Mouse Laminin Alpha 1 Chain."Journal of Biological Chemistry 273.49 (1998): 32491-2499. Web.

123. Freitas, Vanessa M., Vanessa F. Vilas-Boas, Daniel C. Pimenta, Vania Loureiro, Maria A. Juliano, Márcia R. Carvalho, João J.v. Pinheiro, Antonio C.m. Camargo, Anselmo S. Moriscot, Matthew P. Hoffman, and Ruy G. Jaeger. "SIKVAV, a Laminin α1-Derived Peptide, Interacts with Integrins and Increases Protease Activity of a Human Salivary Gland Adenoid Cystic Carcinoma Cell Line through the ERK 1/2 Signaling Pathway." The American Journal of Pathology 171.1 (2007): 124-38. Web.

124. Charonis, A. S. "A Novel Synthetic Peptide from the B1 Chain of Laminin with Heparin- Binding and Cell Adhesion-promoting Activities." The Journal of Cell Biology 107.3 (1988): 1253-260. Web.

125. Skubitz, A. P., J. B. McCarthy, Q. Zhao, and X. Y. Yi. "Definition of a Sequence, RYVVLPR, within Laminin Peptide F-9 That Mediates Metastatic Fibrosarcoma Cell Adhesion and Spreading." Cancer Res.50.23 (1990): 7612-622. Web.

126. Liesi, P., A. Narvanen, J. Soos, H. Sariola, and G. Snounou. "Identification of a Neurite Outgrowth-promoting Domain of Laminin Using Synthetic Peptides." FEBS Letters 251.1-2 (1989): 283. Web.

127. Nomizu, M., Y. Kuratomi, S.-Y. Song, M. L. Ponce, M. P. Hoffman, S. K. Powell, K. Miyoshi, A. Otaka, H. K. Kleinman, and Y. Yamada. "Identification of Cell Binding Sequences in Mouse Laminin 1 Chain by Systematic Peptide Screening." Journal of Biological Chemistry 272.51 (1997): 32198-2205. Web.

128. Ponce, M. L. "An Angiogenic Laminin Site and Its Antagonist Bind through the Alphavbeta3 and Alpha5beta1 Integrins." The FASEB Journal 15.8 (2001): 1389-397. Web.

129. Murayama, O., H. Nishida, and K. Sekiguchi. "Novel Peptide Ligands for Integrin 6 1 Selected from a Phage Display Library." Journal of Biochemistry 120.2 (1996): 445-51. Web.

130. Suzuki, S et al. “Complete Amino Acid Sequence of Human Vitronectin Deduced from cDNA. Similarity of Cell Attachment Sites in Vitronectin and Fibronectin.” The EMBO Journal 4.10 (1985): 2519–2524. Print.

131. Woods, A., J. B. Mccarthy, L. T. Furcht, and J. R. Couchman. "A Synthetic Peptide from the COOH-terminal Heparin-binding Domain of Fibronectin Promotes Focal Adhesion Formation." Molecular Biology of the Cell 4.6 (1993): 605-13. Web.

132. Sharma, A., J. Askari, M. Humphries, E.y. Jones, and D.i. Stuart. "Crystal Structure Of Heparin And Integrin Binding Segment Of Human Fibronectin." (1999): n. pag. Web.

133. Avery, R. L., and B. M. Glaser. "Inhibition of Retinal Pigment Epithelial Cell Attachment by a Synthetic Peptide Derived From the Cell-Binding Domain of Fibronectin." Archives of Ophthalmology 104.8 (1986): 1220-222. Web.

134. Wang, X., C. A. Lessman, D. B. Taylor, and T. K. Gartner. "Fibronectin Peptide DRVPHSRNSIT and Fibronectin Receptor Peptide DLYYLMDL Arrest Gastrulation OfRana Pipiens." Experientia 51.11 (1995): 1097-102. Web.

135. Danen, E. H. J., S.-I. Aota, A. A. Van Kraats, K. M. Yamada, D. J. Ruiter, and G. N. P. Van Muijen. "Requirement for the Synergy Site for Cell Adhesion to Fibronectin Depends on the Activation State of Integrin 5 1." Journal of Biological Chemistry 270.37 (1995): 21612-1618. Web.

136. Sanchez-Aparicio, P. "Activation of the Alpha 4 Beta 1 Integrin through the Beta 1 Subunit Induces Recognition of the RGDS Sequence in Fibronectin." The Journal of Cell Biology 126.1 (1994): 271-79. Web.

137. Thomson, J. A. "Embryonic Stem Cell Lines Derived from Human Blastocysts." Science 282.5391 (1998): 1145-147. Web.

138. Chavez, Shawn L., Juanito J. Meneses, Ha Nam Nguyen, Seung K. Kim, and Renee A. Reijo Pera. "Characterization of Six New Human Embryonic Stem Cell Lines (HSF7, -8, -9, -10, -12, and -13) Derived Under Minimal-Animal Component Conditions." Stem Cells and Development 17.3 (2008): 535-46. Web.

139. Sidhu, Kuldip S., and Bernard E. Tuch. "Derivation of Three Clones from Human Embryonic Stem Cell Lines by FACS Sorting and Their Characterization." Stem Cells and Development 15.1 (2006): 61-69. Web.

140. Warren, Luigi, Philip D. Manos, Tim Ahfeldt, Yuin-Han Loh, Hu Li, Frank Lau, Wataru Ebina, Pankaj K. Mandal, Zachary D. Smith, Alexander Meissner, George Q. Daley, Andrew S. Brack, James J. Collins, Chad Cowan, Thorsten M. Schlaeger, and Derrick J. Rossi. "Highly Efficient Reprogramming to Pluripotency and Directed Differentiation of Human Cells with Synthetic Modified MRNA." Cell Stem Cell 7.5 (2010): 618-30. Web.

141. Conti, Luciano, and Elena Cattaneo. "Neural Stem Cell Systems: Physiological Players or in Vitro Entities?" Nature Reviews Neuroscience Nat Rev Neurosci (2010): n. pag. Web.

142. Pan, Liuliu, Hilary A. North, Vibhu Sahni, Su Ji Jeong, Tammy L. Mcguire, Eric J. Berns, Samuel I. Stupp, and John A. Kessler. "β1-Integrin and Integrin Linked Kinase Regulate Astrocytic Differentiation of Neural Stem Cells." PLoS ONE 9.8 (2014): n. pag. Web.

143. Celiz, Adam D, et al. "Materials for Stem Cell Factories of the Future." Nature Materials 13 (2014): 570-79. Web.

144. Higuchi, Akon, Shih-Hsuan Kao, Qing-Dong Ling, Yen-Ming Chen, Hsing-Fen Li, Abdullah A. Alarfaj, Murugan A. Munusamy, Kadarkarai Murugan, Shih-Chang Chang, Hsin-Chung Lee, Shih-Tien Hsu, S. Suresh Kumar, and Akihiro Umezawa. "Long-term Xeno-free Culture of Human Pluripotent Stem Cells on Hydrogels with Optimal Elasticity." Sci. Rep. Scientific Reports 5 (2015): 18136. Web.

145. Li, X., X. Liu, B. Josey, C. J. Chou, Y. Tan, N. Zhang, and X. Wen. "Short Laminin Peptide for Improved Neural Stem Cell Growth." Stem Cells Translational Medicine 3.5 (2014): 662-70. Web.

146. Little, Lauren E., Karen Y. Dane, Patrick S. Daugherty, Kevin E. Healy, and David V. Schaffer. "Exploiting Bacterial Peptide Display Technology to Engineer Biomaterials for Neural Stem Cell Culture." Biomaterials 32.6 (2011): 1484-494. Web.

147. Ciccolini, Francesca. "Identification of Two Distinct Types of Multipotent Neural Precursors That Appear Sequentially during CNS Development."Molecular and Cellular Neuroscience 17.5 (2001): 895-907. Web.

148. Ciccolini, F., and CN. Svendsen. "Fibroblast Growth Factor 2 (FGF-2) Promotes Acquisition of Epidermal Growth Factor (EGF) Responsiveness in Mouse Striatal Precursor Cells: Identification of Neural Precursors Responding to Both EGF and FGF-2." J Neurosci.18.19 (1998): 7869-880. Web.

149. Louis, Sharon A., and Brent A. Reynolds. "Generation and Differentiation of Neurospheres From Murine Embryonic Day 14 Central Nervous System Tissue." Basic Cell Culture Protocols (n.d.): 265-80. Web.

150. Tropepe, V, et. al. "Distinct Neural Stem Cells Proliferate in Response to EGF and FGF in the Developing Mouse Telencephalon." Dev Biol. 208.1 (1999): 168-88. Web.

151. Reynolds BA, Weiss S. “Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system.” Science. 290.5052(1992): 1707-1710.

152. Doetsch Fiona, Leopoldo Petreanu, Isabelle Caille, Jose-Manuel Garcia-Verdugo, and Arturo Alvarez-Buylla. "EGF Converts Transit-Amplifying Neurogenic Precursors in the Adult Brain into Multipotent Stem Cells."Neuron 36.6 (2002): 1021-034. Web.

153. Palmer, Theo D., Jun Takahashi, and Fred H. Gage. "The Adult Rat Hippocampus Contains Primordial Neural Stem Cells." Molecular and Cellular Neuroscience 8.6 (1997): 389-404. Web.

154. Palmer, Theo D., et al."Cell Culture. Progenitor Cells from Human Brain after Death." Nature 411 (2001): 42-43. Web.

155. Chen, Allen Kuan-Liang, Shaul Reuveny, and Steve Kah Weng Oh. "Application of Human Mesenchymal and Pluripotent Stem Cell Microcarrier Cultures in Cellular Therapy: Achievements and Future Direction." Biotechnology Advances 31.7 (2013): 1032-046. Web.

156. Kumar, Nathan, Jenna Richter, Josh Cutts, Kevin T. Bush, Cleber Trujillo, Sanjay K. Nigam, Terry Gaasterland, David Brafman, and Karl Willert. "Generation of an Expandable Intermediate Mesoderm Restricted Progenitor Cell Line from Human Pluripotent Stem Cells." ELife 4 (2015): n. pag. Web.

157. Yuan, Shauna H., Jody Martin, Jeanne Elia, Jessica Flippin, Rosanto I. Paramban, Mike P. Hefferan, Jason G. Vidal., et al."Cell-Surface Marker Signatures for the Isolation of Neural Stem Cells, Glia and Neurons Derived from Human Pluripotent Stem Cells." PLoS ONE 6.3 (2011): n. pag. Web.

158. Vanguilder, Heather, Kent Vrana, and Willard Freeman. "Twenty-five Years of Quantitative PCR for Gene Expression Analysis." BioTechniques Biotech. 44 Supplement.4 (2008): 619-26. Web.

159. Rodrigues, Carlos A. V., Maria Margarida Diogo, Cláudia Lobato Da Silva, and Joaquim M. S. Cabral. "Microcarrier Expansion of Mouse Embryonic Stem Cell-derived Neural Stem Cells in Stirred Bioreactors."Biotechnology and Applied Biochemistry 58.4 (2011): 231-42. Web.

APPENDIX A

LIST OF QPCR PRIMERS USED IN THIS STUDY

Gene	ABI Assay
18s	Hs99999901_s1
MAP2	Hs00258900_m1
NES	Hs00707120_s1
SOX1	HS01057642_s1
SOX2	Hs01053049_s1
B3T	Hs00801390_s1
NANOG	Hs04399610_g1
OCT4	Hs00999632_g1
FOXA2	Hs00232764_m1
SOX17	Hs00751752_s1
MESP1	Hs01001283_g1
T	Hs00367969_m1

Gene	Forward	Reverse	Product (bp)
ITGA1	CTCACTGTTGTTCTACGCTGC	AACATGTCTTCCACCGGGC	86
ITGA2	GGTGCTCCTCGGGCAAATTA	GAGCCAATCTGGTCACCTCG	104
ITGA3	CCCACCTGGTGTGACTTCTT	CTGGTCACCCAGTGCTTCTT	137
ITGA4	TTTATGCGGAAAGATGTGCGG	ATTGGCTGAAGTGGTGGGAA	119
ITGA5	AAGACTTTCTTGCAGCGGGA	GCCACCTGACGCTCTTTTTG	122
ITGA6	GCAGCCTTCAACTTGGACAC	CACGAGCAACAGCCGCTT	129
ITGA7	TCGAACTGCTCTTCTCACGG	CCACCAGCAGCCAGCTC	147
ITGA8	ATCCTCAGGAAACTGGCAGG	CAGCAACTGAGTATCCAAGGT	85
ITGAV	AGGGTCTTTCTACCTCTGCCT	GAAGAAACATCCGGCACAACA	156
ITGB1	CCGCGCGGAAAAGATGAATTT	CCACAATTTGGCCCTGCTTG	150
ITGB2	CCAAGGAGGAGCTGAGAGGA	CCAGCATGTCCCTCGGTG	157
ITGB3	ACTGGCAAGGATGCAGTGAA	TTGGGACACTCTGGCTCTTC	125
ITGB4	CACATCCTCCACCCTCACAC	CAGTCAGGCGAGAGTCGTG	125
ITGB5	CTGCATCCAACCAGATGGACTA	ATCTCCACCGTTGTTCCAGG	151
ITGB7	TGCCGTCTCCCAGATCAGTA	GTTTCCACATAGGTGCGTGC	79
BGN	CACCGGACAGATAGACGTGC	CATGGCGGATGGACCTGGAG	96
COL1A1	GGGACACAGAGGTTTCAGTGG	CACCATCATTTCCACGAGCA	185
COL3a1	TAAAGGCGAAATGGGTCCCG	GGCACCATTCTTACCAGGCT	136
COL4A1	CCGTGGGACCTGCAATTACT	CGGCGTAGGCTTCTTGAACA	88
COL5A1	CTTGGCCCAAAGAAAACCCG	CGTCCACATAGGAGAGCAGT	72
COL5a2	GGAAGAAGACGAGGATGAAGGAT	ACACAGATCTGACAAGGGGC	108
COL12a1	AGATCTCTTCATGCCGCTGT	TGGGTCAACTTCTGCCTCAAT	118
COL14a1	CAGGACCTTCAGGGGAGAGT	ATGGGGAGCTCTCACACCATA	167
DCN	GGGAGCAGAGAAGAGGGAGA	TCACAACCAGGGAACCTTGC	117
DPT	GTGATAGTGGCCGTGAGGAG	GCACGTCTGGTACCATTCCA	156
ECM1	CTGCTCACACATTCCCCCTT	GGGGGACCCACTTCCTTTTC	117
ECM2	CAACAAAGCTGTGTGGTCGC	ATAGGAGACAGTAGCGGAGCA	157
FN1	ACAAACACTAATGTTAATTGCCCA	CGGGAATCTTCTCTGTCAGCC	74
FBN1	ACGTGAAGGAAACCAGAGCC	GCCGGCAAATGGGGACAATA	162
FMOD	AACTTGAGAGACAAAATGCAGTGG	TAGTAGGTGGACTGCTGGCT	131

HAPLN1	GAGAGCATCCGAACTCCTGG	GGGGCCATTTTCTGCTTGGA	173
HSPG2	GCAGGAGGCTTCGTTTTGC	ATGTCAGCTCTGCTCTCGACT	139
LN	CCGGTCCTCGCAGAGTTG	CGTCTTCCTTTCCGGCGAC	171
LUM	CAGTAAGGATTCAAACCATTTGCCA	ACTTGGGTAGCTTTCAGGGC	175
NID1	GTTCCTTCATGTCCCGGCTA	ACAGCGATACCTTCTGGACT	171
POSTN	CCCCGTGACTGTCTATAAGC	CCTTGGTGACCTCTTCTTGTAA	197
PLC	TCTCATCCCAGGGTGACAGT	GTTGTGGGACGAGCTCAAGG	160
TNC	CACAGCCACGACAGAGGC	AAAGGCATTCTCCGATGCCA	103
VTN	GTGCAAGCCCCAAGTGACTC	CCTCGCCATCGTCATAGACC	77

APPENDIX B

LIST OF ANTIBODIES USED IN THIS STUDY

Antibody	Vendor	Catalog #	Concentration Used
Goat anti-SOX2	Santa Cruz	SC-17320	1:50
Mouse anti-B3T	Fitzgerald	10R-T136A	1:1000
Mouse anti-NESTIN	BD	560341	1:10
Mouse anti-SOX1	BD	560749	1:10
Mouse anti-NF-L	Sigma	N5139	1:500
Rabbit anti-MAP2	Millipore	AB5622	1:500
Rabbit anti-GABA	Millipore	AB15415	1:200
Alexa-647 Mouse Anti-SOX2	BD	560294	20 µl per test
PE Mouse anti-Nestin	BD	561230	5 µl per test
PerCp-Cy5.5 Mouse anti-SOX1	BD	561549	5 µl per test
PE anit-CD49a/ITGA1	BioLegend	328303	5 µl per test
PE anti-CD49b/ITGA2	BioLegend	359307	5 µl per test
PE anti-CD49c/ITGA3	BioLegend	343803	5 µl per test
PE anti-CD49d/ITGA4	BioLegend	304303	20 µl per test
PE anti-CD49e/ITGA5	BioLegend	328009	5 µl per test
PE anti-CD49f/ITGA6	BioLegend	313611	20 µl per test
PE anti-CD51/ITGAV	BioLegend	327909	5 µl per test
PE anti-CD29/ITGB1	BioLegend	303003	5 µl per test
Alexa Fluor 647 anti-CD18/ITGB2	BioLegend	101414	5 µl per test
PE anti-CD61/ITGB3	BioLegend	359307	5 µl per test
PE anti-CD104/ITGB4	BioLegend	327807	5 µl per test
PE anti-ITGB5	BioLegend	345203	5 µl per test
PE anti-ITGB7	BioLegend	121005	5 µl per test
Alexa-647 Mouse IgG2a Isotype Control	BD	558053	20 µl per test
PE Mouse IgG1 Isotype Control	BioLegend	400113	5 µl per test
PE Mouse IgG2a	BioLegend	400213	5 µl per test
PE Mouse IgG2b Isotype Control	BioLegend	400313	5 µl per test
PE Rat IgG2a Isotype Control	BioLegend	400507	5 µl per test
PercCp-Cy5.5 MouseIgG1 Isotype Control	BD	550795	5 µl per test
Alexa 647 Rat IgG2a Isotype Control	BioLegend	400526	5 µl per test
Alexa 647 Donkey anti-Goat	Life Technologies	A-21447	1:200
Alexa 647 Donkey anti-Mouse	Life Technologies	A-31571	1:200
Alexa 647 donkey anti-Rabbit	Life Technologies	A-31573	1:200
Alexa 488 Donkey Anti-Goat	Life Technologies	A-11055	1:200

Alexa 488 Donkey Anti-Mouse	Life Technologies	A-21202	1:200
Alexa 488 donkey anti-Rabbit	Life Technologies	A-21206	1:200