UNIVERSITY OF CALIFORNIA, SAN DIEGO

Engineering of Synthetic Peptide Substrates for the Expansion and Differentiation of Human Pluripotent Stem Cell-Derived Neural Progenitor Cells

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by

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The thesis of Hyun-Je Kim is approved, and

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Dedication

To My Father God in Heaven,

Jesus Christ

Epigraph

“Love the Lord your God with all your heart and with all your soul and with all your mind and with all your strength.’ The second is this: ‘Love your neighbor as yourself.’ There is no commandment greater than these.” (Mark 12:30-31)

小醫治病, 中醫治人, 大醫治國 (Unanimous)

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ABSTRACT OF THE THESIS

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Professor Shu Chien, Chair

Neurodegenerative diseases, characterized by traumatic or progressive loss of neurons in the brain and spinal, present significant medical and economic challenges to society with fast growing patient populations and yet unavailability of permanent cure-giving therapies. Current drug-based treatments are highly limited in a sense that they merely alleviate symptoms, delay the onset or slow down the progression of the disease at best—even so, with low efficacy and wide

variability in therapeutic outcome at the cost of many side effects. With prospect to induce permanent recovery in affected neural functions at molecular level, stem cell therapies based on neural progenitor cell (NPC) transplantation have been extensively performed in animal models of neurodegeneration. However, laminin (LN) substrates commonly used to culture these NPCs may contain animal-derived pathogens and are relatively expensive to produce. In this project, we developed optimal combinations of synthetic peptides that mimick functional sequences of extracellular matrix proteins, on which human embryonic stem cell (hESC) derived NPCs were expanded for 10 passages and differentiated into neurons after 10^{th} passage followed by molecular profiling. Cells cultured on the optimized peptide substrates proliferated at a rate similar to those on the LN substrate, also expressing multipotent NPC markers such as NESTIN, SOX1, and SOX2 at a comparable degree to LN-based culture as verified by gene expression analysis, immunofluorescence staining and flow cytometry. Cells differentiated on the synthetic peptides expressed neuronal markers, MAP2 and B3T, at a similar level to those on LN. Therefore, our synthetic peptide combinations support the long-term expansion and neuronal differentiation of NPCs, providing cheaper and xenogenic contaminant-free alternatives for the LN substrate, and holding promise as a feasible substrate for the large-scale production of NPCs and neurons for cell-based therapies.

Chapter 1: Introduction

1.1: Motivation

Neurodegenerative diseases are characterized with the structural and functional loss of neurons and their supporting cells in the brain and spinal cord, where the cells have no or highly limited capacities to self-renew in response to traumatic injuries or become subjected to progressive deterioration associated with genetic mutations or epigenetic modifications related to environmental factors. Sporadic forms of the disease whose genetic contributions are usually multiple and unclear account for the majority cases, as opposed to familial forms whose etiology bears a clear connection to mutation in a particular gene.

In 2010, almost 7 million people were diagnosed with some kind of neurodegenerative disease or related disorders, including 5 million Americans suffering from Alzheimer's disease (AD), 1 million from Parkinson's disease (PD), 400,000 from multiple sclerosis (MS), 30,000 from Huntington's disease (HD), 30,000 from amyotrophic lateral sclerosis (ALS), entailing the annual medical expenditure of more than $100 billion in the U.S. [1]. It is estimated that more than 12 million Americans will be struck with these neurodegenerative diseases by 2030, provided that susceptibility to neurodegenerative diseases increases with age.

The Alzheimer’s Association, in its 2012 annual statistics report, estimated that as many as 11 to 16 million people in the U.S. will fall victim to AD by the year 2050, with one new case appearing every 33 seconds [2]. The same report showed that the medical expense associated with AD treatment is approximately $200 billion per year and will increase to $1.1 trillion per year by 2050, suggesting a huge economic burden. In addition, Alzheimer’s Disease International reported that approximately 35.6 million people had AD worldwide as of 2010 and that the number will soar up to 115.4 million people by 2050 [3]. It also estimated that health care costs for AD-related dementia were $604 billion, which corresponds to about 1% of world gross

domestic product. In a 2006 NIH-released report, PD affected up to an estimated 1.5 million Americans with approximately 50,000 new cases diagnosed every year, for which medical costs exceeded $6 billion per year [4]. The estimated costs for 500,000 affected patients were estimated as $11 billion in 2009 alone.

Despite the fast growing incidence of the neurodegenerative diseases and overwhelming medical costs, currently available methods of treatment provide a temporary symptomatic relief at best rather than permanent cure. General treatment protocols against the neurodegenerative diseases encompass 1) the administration of anti-inflammatory drugs (AIDs) and anti-oxidants, known to delay the onset or slow down the progression of the diseases, 2) the injection of neutrophic factors, growth factors capable of ameliorating disease symptoms by promoting cellular growth and differentiation in a lesion site and 3) deep brain stimulation (DBS), involving high-frequency electrode stimulation of discrete brain areas producing functional and reversible inhibition of the lesion site.

However, drug-based treatments utilizing AIDs and neutrophic factors have demonstrated low efficacy in animal and clinical trials with many pharmacological side-effects, necessitating the development of a cell-based therapy with prospects to realize more permanent, fundamental recovery from neurodegeneration. Since the delivery of large, polar neutrophic factors across the blood brain barrier (BBB) selectively targeting only affected neurons proves extremely difficult, invasive and surgical methods of delivery are inevitable for neutrophic factor injection. But these particular approaches are prone to entailing toxic side-effects, including hyperinnervation of cerebral blood vessels, hypophagia, and Schwann cell hyperplasia with sprouting of sensory and sympathetic neurons, and neutrophic pain [5]. Significant concerns persist with regards to the occurrence of psychiatric side effects following DBS, as well as intracranial hemorrhage, stimulation-induced dyskinesia, and cognitive dysfunction. Pallidotomy and thalatomy, invasive procedures designed to induce an improvement in motor function and provide moderate anti-

parkinsonian benefits, necessitate making a destruct lesion in the pars interna of the globus pallidus and subthalamic nucleus, which induce cognitive deficits and neuropsychiatric problems [6].

Cell-based therapies can surmount these drawbacks associated with drug administration and invasive surgical procedures for combating neurodegeneration, bearing potentials to target the disease pathology at molecular level and induce permanent recovery beyond a short-term symptomatic improvement with little concerns for undesired side-effects. Pluripotent stem cells and multi-potent neural progenitors supply excellent raw materials for the functional replacement of damaged neurons in a lesioned brain or spinal cord, but also produce neuroprotective effects and stimulate endogenous repair mechanisms via trophic factor secretion. Indeed, cell transplantation has yielded promising results in numerous animal models representing different types of neurodegeneration, reinnervating sites of central nervous system (CNS) injury with grafted cells differentiating into neurons, repopulating and integrating with the host local environment, restoring previously impaired electrophysiological functions, and even correcting behavior deficits.

In order to facilitate the translation of stem cell-based therapies into a feasible method to treat neurodegenerative humans and animal models, an optimal culture substrate should be designed that supports the long-term expansion and differentiation of the stem cells *in vitro* up to a sufficient number for transplantation to be effective. For example, current substrates used for embryonic stem cell-derived neural progenitors self-renewal and differentiation, such as laminin, prove difficult to isolate, vary between lots, and contain xenogenic components which limits their use for clinical applications. The heterogeneous composition of these currently available matrices often results in variable cell expansion rates, non-homogenous expansion, and inability to respond to differentiation signals. These limitations are a significant bottleneck in clinical application of these cells for neurodegenerative diseases, where large quantities of homogenous neural

progenitors and neuronal populations are required. In contrast, synthetic materials that are inexpensive and easily fabricated represent a reliable alternative for the expansion and differentiation of neural progenitor cells. Therefore, identifying optimal synthetic material substrates and culture conditions to 1) yield the quantity of neural progenitor cells sufficient for transplantation, 2) maintain their cellular phenotypes for a long-term during *in vitro* expansion, and 3) instruct their differentiation into mature neuronal cell types are critical to enhancing the efficacy of cell-based therapies in treating the neurodegenerative disease.

1.2: Non Cell-Based Treatment Options for Neurodegenerative Diseases

Not only characterized with amyloid beta (Aβ) peptide deposits and neurofibrillary tangle formation, AD has been also associated with chronic inflammation and microglia activation, its pathogenesis involving the activation and secretion of inflammatory factors in AD brains, including cytokines, growth factors, complement proteins, proteases, and reactive oxygen intermediates [9-13]. Epidemiological studies have shown that prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) decreases the risk of developing AD and delays the onset of the disease. In animal studies, NSAIDs, such as indomethacin and flurbiprofen, reduced microglia and astrocytes activation by suppressing the levels of inflammatory mediators, prostaglandin E2 (PGE2) and IL-1 [12]. Ibuprofen and sulindac could lower the level of Aβ peptides in both AD transgenic mice and cell cultures of peripheral, glial, and neuronal origin, shifting the βAPP metabolism toward shorter and less fibrillogenic forms of Aβ peptides by interfering with β- and γ-secretase activities [12-13]. However, low-efficacy, gastro-intestinal side effects, and susceptibility to increased risk for cardiovascular events have been reported for the NSAIDs use. Another form of AD treatment centers on regulating neurotransmitter activity, with the purpose of enhancing cholinergic function. Administering cholinesterase inhibitors were shown to delay the degradation of acetylcholine and improve behavioral and cognitive defects [11]. Neutrophins also

proved effective for mitigating AD symptoms, implicated in promoting neurogenesis and protecting neuronal function. But their relatively large size and polarity hamper their penetration across BBB into the CNS, necessitating their direct administration or injection based on invasive surgical procedures with adverse side-effects [5].

In the case of PD, the loss of dopamine-producing midbrain neurons in the substantia nigra leads to a decreased level of dopamine in the striatum and dysfunctional motor complications, warranting the development of dopamine replacement drugs, such as levodopa, dopamine agonists, and drugs that slow the catabolism of dopamine in the brain [6, 14-17]. Though levodopa is recognized as the most effective, gold standard of treatment for PD, its therapeutic efficacy significantly diminishes over time and its long-term administration often complicated by patients' increased motor fluctuations between "on" (good mobility period) and "off" (impaired mobility) periods, as well as increased dyskinesias [6]. Dopamine agonists also fail to address neurodegeneration detected in other parts of the brain such as the locus of coeruleus and cerebral cortex, and prove ineffective for a number of autonomic symptoms in PD patients [16]. A Monoaminooxidase B inhibitor known to stabilize dopamine levels in the striatal synaptic cleft by inhibiting dopamine catabolism, rasagiline demonstrated disease-modifying effects in PD patients as evidenced by an increase in behavioral scores compared to placebo groups [15]. However, it bears risk for blood pressure elevation and an undesired side-effect like increased liver enzyme production. Using an electrical impulse to stimulate the subthalamic nucleus and pars interna of the globus pallidus could improve parkinsonian motor features, such as bradykinesia, tremor, rigidity, and drug-related motor complications in clinical trials [17]. There remained, however, a significant concern with regards to the occurrence of psychiatric side effects, depression, intracranial hemorrhage, stimulation-induced dyskinesia, and cognitive dysfunction following the deep brain stimulation.

Treatment efforts to improve ALS symptoms focused on preventing motor neuronal death by blocking glutamate-related toxicity, which causes harmful Ca^{2+} influx, oxidative stress, and neural degeneration when released in large amounts [18]. In SOD1-mutated transgenic mouse models embedding a defective glutamate transporter and other ALS phenotypes, glutamate receptor antagonists riluzole and gabapentin hampered disease progression and prolonged mouse survival, whereas an antioxidant vitamin E delayed the onset of symptoms [19]. However, the efficacy of these drugs was poorly reproducible in later experiments involving murine ALS models, and the drugs failed to significantly benefit ALS patients during clinical trials [20, 21]. They tended to prolong the early stage of ALS development but failed to affect long-term disease progression and survival in the human ALS patients [22]. In addition to glutamate-induced oxidative stress and neural toxicity, microglial activation and proliferation have also earned spotlights as pathological hallmarks of ALS. The hyper-expression of endogenous tumor necrosis factor (TNF) and prostaglandin-E2 (PGE_2) by astrocytes and microglia in ALS mouse models, as well as increased level of TNF and inflammatory cytokines in human ALS cerebrospinal fluid or blood serum, have initiated many preclinical and clinical trials to identify drugs with capabilities to inhibit TNF production and microglia activation itself [18]. For example, thalidomide derivatives could successfully block TNF message translation in SOD-mutant ALS mouse models [23, 24], but human trials involving the drug thalidomide for ALS proved unsuccessful owing to patient tolerance and excessive occurrence of sinus bradycardia [25,26]. Doses of thalidomide already tolerated by the ALS patients were insufficient to modulate patient cytokine profiles any significantly [25]. Other drugs include cyclooxygenase (COX) and lipoxygenase inhibitors, which are standard NSAIDS also used to treat AD symptoms. These anti-inflammatory drugs have not proven efficacious in the human clinic, generally failing to prevent or slow down the progression of sporadic ALS. The COX-II inhibitor, which hampered murine ALS progression by lowering its level, did not prove efficacious for human trials [27]. A clinical trial involving minocycline, an

antibiotics acting on the MAPK pathway to inhibit microglial activation, resulted in the onset of neurological and gastrointestinal side-effects without significant symptomatic improvement [28].

1.3: Stem Cell-Based Approach to Treat and Model Neurodegenerative Diseases

1.3.1: Neural Progenitor and Stem Cells for Transplantation

In order to surmount the low pharmacological efficacy problems and various side-effects associated with current drug-based treatments against the neurodegenerative diseases, neural progenitor cells (NPCs) and neural stem cells (NSCs) have been extensively derived, characterized, and transplanted into animal models and their therapeutic potentials evaluated. Despite persisting trends in literatures that the two terms are used somewhat interchangeably, there is a general distinction agreed upon by most sources. NSCs specifically refer to endogenous adult stems cells located in the hippocampus, cortex, or subventricular zone of lateral ventricles, often limited in their differentiation potentials. Typically, these cells are derived straight from primary mouse or human brain or spinal cord tissues. On the other hand, NPCs are progenitor cell populations derived and expanded from pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), retaining capacities to proliferate indefinitely and differentiate into all neural, glial, and supporting cell types that comprise the central nervous system.

Neural progenitor or stem cell-based therapies are generally intended to induce recovery based on three mechanisms: 1) cell replacement, in which cell transplants directly replace lost or damaged cells in the lesion site to reconstruct functional neural circuitry, 2) trophic support, in which the cell transplants secrete neuroprotective factors to promote the survival of affected neurons and endogenous repair of the diseased brain areas, and 3) modulation of inflammation, in which the cell grafts down-regulate the production and release of cytokines in the local

environment of lesion in order to suppress inflammatory responses often underlying the disease pathology [7-8].

1.3.2: NPC & NSC Transplantation for Alzheimer's Disease

Ibotenic is often used to induce AD pathologies in animal models, by creating a lesion in the nucleus basalis of Meynert (nbM) from which cholinergic fibers project to the cerebral cortex. This procedures lead to nbM degeneration, a decrease in the amount of choline acetyltransferase (ChAT) released in the cortex, and eventually disorder in memory and learning.

Wang et al. transplanted mouse ESC-derived neurospheres (or NPCs) into the prefrontal and parietal cortices of AD mice having lesion in the nbM, which significantly ameliorated cholinergic deficits and recent memory disruption in the animal models [29]. The grafted cells primarily differentiated into cholinergic neurons in and around the transplantation site with a high level expression for ChAT markers, migrating into and integrating with the surrounding cortex, and ultimately rescuing cholinergic impairment and working memory deterioration in the NBM lesioned mice. Indeed, cholinergic neurons of nbM projecting to the hippocampus directly pertain to cognitive performance such as attention, learning, and memory. Provided that AD pathology is characterized with an irreversible decline of cognitive functions due to cell deterioration in the forebrain cholinergic projection system, particularly in NBM, these results hold a great promise for treating AD via stem cell transplantation. Xuan *et al.* found that hippocampus-derived NSC grafted into the transected basal forebrain of mice increased the number of cholinergic neurons, enhancing memory and learning abilities in AD-model rats [30]. Kern *et al.* reported that murine NSC transplantation into the hippocampus of Down syndrome mice lowered the number of tau clusters and neurofibrillary tangles (which are also pathological hallmarks of AD), suggesting that soluble growth factors secreted by NSCs may regulate tau phosphorylation [31]. Blurton-Jones et al. demonstrated that the bilateral engraftment of murine NSCs into transgenic AD mice

expressing mutant APP and PS proteins rescued cognitive deficits without altering the level of AB and tau protein levels, promoting hippocampal synaptic density and axonal outgrowth via increased BDNF secretion that mediates synaptogenesis and neuronal networking [32]. Tang produced AD rat models by intra-dentate Aβ (1-40) peptide injections which resulted in a significant neuronal loss of granule cells in the denate gyrus and subsequent memory loss, followed by mouse ESC-derived NPC transplantation which significantly improved spatial learning abilities [33].

In addition to NPCs and NSCs, other studies have utilized mesenchymal stem cells (MSCs) as raw materials for transplantation studies since they also bear potentials for differentiation into multiple lineages including a neural cell type. Lee et al. transplanted murine bone marrow-derived MSCs into mouse models with lesion-induced hippocampus, where AB deposition was cleared after 7 days of transplantation possibly due to elevated microglial activation and phagocytic activity [34]. In another study, they grafted human umbilical cord blood-derived MSCs into AD mice, which decreased glial activation, oxidative stress, and apoptosis with the rescue of learning and memory function [35]. Similarly, Nivet et al. demonstrated that human olfactory MSC transplantation could stimulate endogenous neurogenesis in the hippocampus lesion model while restoring synaptic transmission and long-term potentiation, confirmed by improvement in learning abilities and memory retention [36]. Babaei et al. showed that rat MSC transplantation could significantly improve learning and memory functions in AD rat models, which had memory impairment either naturally induced by aging or purposefully inflicted upon by excitotoxic lesion of NBM following infusion of Ibo that decreases cholinergic activities in the hippocampus and frontal cortex [37].

1.3.3: NPC & NSC Transplantation for Parkinson's Disease

Prior to the development of well-established culture protocols to generate a homogeneous population of dopaminergic neurons in a standardized and quality-controlled fashion, clinical studies involving PD patients in the 1980s almost exclusively relied on fetal mesencephalic tissues as raw materials for cell transplantation. The mesencephalic tissues, rich in dopamine neurons, were grafted into the striatum of PD individuals and assessed for their capabilities to survive, produce dopamine, exhibit normal electrical firing patterns, integrate functionally with host neurons, and improve motor functions. Indeed, such grafted dopaminergic neurons could often re-innervate the denervated striatum, restore regulated dopamine release and movement-related frontal cortical activation, and promote significant symptomatic relief [38]. In the most successful cases, patients have been able to withdraw levodopa treatment after transplantation and continue on with an independent life. While this approach suggested a new paradigm and proof-of-concept for cell replacement therapies in treating PD, there have been inherent limitations associated with the use of fetal tissue: the limited supplies of primary donor tissues, variability in functional outcome, and onset of dyskinesias as a side-effect in a larger number of patients following transplantation. In order to fundamentally overcome these limitations, stem cell-based therapies have been actively implemented not only to produce a virtually unlimited source of dopaminergic neurons for cell transplantation and improve reproducibility in clinical recovery outcome. 6-hydroxydopamine (6-OHDA), a highly specific neurotoxin which targets catecholamine neurones via the dopamine active transporter, has been used to mimick PD pathologies in animal models. When injected stereotaxically into the brain, either into the median forebrain bundle or into the neostriatum, it causes extensive, irreversible loss of dopamine neurons in the ventral midbrain.

Anderson et al. showed that human NPCs transplanted into the subthalamic nucleus survived up to 5 months and promoted functional recovery in the unilateral 6-OHDA lesioned PD

rat model, spontaneously producing VEGF with potentials to induce vascularization and thus establish the host environment more permissive for recovery [39]. Around 90% of the grafted cells expressed an early neural marker nestin and were found throughout the striatum, internal capsule and corpus callosum, which indicated their multi-potent nature and migration potentials. Svendsen et al. demonstrated that NSCs derived from a human fetus and expanded in culture using a combination of EGF and FGF-2 growth factors could differentiate into neurons and glia upon intracerebral transplantation into the PD rat models [40]. Not only did they show a long-term survival up to 20 weeks, but the grafted cells could also migrate extensively into the host striatum while undergoing differentiation and partially improved lesion-induced behavior dysfunction in the PD animals. Ben-Hur showed that hESCs-derived NPCs could survive for at least 12 weeks when directly transplanted into the striatum of PD rats, undergoing differentiation into DA neurons *in vivo* [41]. These rats displayed a significant partial correction of D-amphetamine and apomorphine-induced rotational behavior, along with a remarkable reversal in behavioral deficits, suggesting the therapeutic potentials of NPCs. Daadi et al. used glial conditioned media and growth factors, FGFs, and LIFs, to synergistically induce the differentiation of hESC-derived midbrain-specific neural precursors into a DA-specific neuronal population *in vitro*, which maintained their neuronal phenotype, extended neurite outgrowths and showed synaptophysin-expressing terminals when grafted into a monkey model of PD [42]. Kriks et al. showed that midbrain DA neurons differentiated from hESC-derived neural precursors led to complete restoration of amphetamine-induced rotation behavior and improvement in forelimb use and akinesia, during a long-term transplantation study in PD mice and rats [43]. However, the same DA neurons survived but lacked neural outgrowths and failed to rescue the impaired motor functions in PD monkey models. Chung et al showed that when transplanted respectively into the striatum of Pitx3-deficient *aphakia* PD mice and 6-OHDA–lesioned PD rats, hESC-derived dopaminergic NPCs successfully re-innervated the lesion site by differentiating into midbrain

specific dopaminergic neurons *in vivo,* demonstrating an excellent migratory capacity throughout the host striatum reaching more than 3.3 mm in length, well integrating with the local environment and producing a significant improvement in motor functions including amphetamine-induced rotation and paw movement [44]. Rather than grafting progenitors, Cho et al. instead grafted pre-differentiated hESC-derived DA neurons into 6-OHDA lesioned PD rats, which led to substantial increase in amphetamine- and apomorphine-induced rotation and forepaw locomotion throughout the 12-week period post-transplantation [45].

1.3.4: NPC & NSC Transplantation for Stroke

Stroke is an acute cerebrovascular disorder resulting from lack of oxygen supplies to the brain parenchyma, causing the loss of neural and endothelial cells and leading to memory deficits, motor complications, and impairment in sensory and vibratory sensations. Aside from thrombolytic medications which are only effective when administered to a patient during the first hours of the disease onset [46], no efficacious therapy exists yet to improve functional recovery in the post-ischemic phase. NPCs hold potentials for restoring functional deficits in stroke patients, by replacing and reconstructing neural circuitry, stimulating endogenous repair by brain resident neural stem cells, and modulating inflammatory or neuroprotective mechanisms.

Daadi *et al.* showed that hESC-NPCs migrated toward ischemia-injured adult brain parenchyma in stroke rat models and induced the recovery of sensorimotor function [47]. Enriched NPCs derived from mouse and monkey ESCs produced behavioral improvements when transplanted into stroke mice models with transient cerebral ischemia and hemiplegic mice models with cryogenic brain injury, respectively [48,49].

Lee et al. reported that human NSCs transplantation mounted anti-inflammatory responses in hemorrhagic stroke rats to induce functional improvement [50]. Bacigaluppi et al. showed that intravenous injection of mouse NSCs hampered inflammation and glial scar

formation in stroke mice, while inducing delayed neuroprotection and functional recovery in 18 days from the injury [51]. Horie et al. found that human NSCs implanted into a cortical lesion of stroke rats suppressed inflammation and neovascularization in the peri-infarct region by secreting VEGFs, which led to improved forelimb locomotion within the first week of lesion [52]. The transplantation of iPSC-derived NPCs (neuroepithelial-like stem cells) into stroke-damaged mouse striatum and rat cortex induced forepaw movement recovery within the first week of transplantation, accompanied with an increased VEGF level [53].

The transplantation of human ESC-derived NPCs into the cortical infarct from middle cerebral artery occlusion (MCAO) of stroke rats promoted neurogenesis based on the increased number of cells expressing a neuronal migration protein, doublecortin, in SVZ [54] and that of human fetal NSCs stimulated endogenous cell proliferation in SVZ up to at least 15 days post-stroke and enhanced angiogenesis in peri-infarct regions [55]. Mine et al. showed that the implantation of fetal striatum-derived human NSCs in the striatum of stroke rats following MCAO promoted the generation and recruitment of new endogenous neuroblasts, reduced inflammation in the injury site, stimulated stroke-induced cell proliferation in SVZ, and finally induced improvement in behavioral deficits [56].

1.3.5: NPC & NSC Transplantation for Spinal Cord Injury

Spinal cord injury is an acute form of neurodegenerative disease caused by traumatic insults to the spinal cord resulting in either a temporary or permanent modification in the motor, sensory, or autonomic function of the spinal cord which incurs devastating neurologic deficits and disability.

Hatami et al. showed that the implantation of collagen scaffold-embedded hESC-derived NPCs induced the recovery of hindlimb locomotion and sensory responses in the hemisection-damaged spinal cord of rats. The transplanted cells underwent differentiation *in vivo* into neuronal and glial

lineages, demonstrating migratory capacity throughout the spinal cord [57]. In another study, oligodendrocyte progenitors derived from hESCs enhanced the remyelination of surviving axons and motor function recovery when transplanted into the contusion-induced spinal cord injury site of rats [58]. Lu et al. embedded hESC-derived NPCs into fibrin matrices containing a growth factor cocktail including EGF and bFGF, transplanting them into the T3 vertebra transected spinal cord of rats. The implanted NPCs differentiated into mature neural cells in vivo, extended large numbers of axons to form synaptic connections with the host spinal cord over long distances, filled the lesion cavity establishing functional relay formation, and improve the hindlimb locomotion of the animal models [59]. Essentially the same studies were conducted using human iPSCs rather than hESCs as a source material for differentiation into NPCs, which further differentiated *in vivo* into neurons and glia, extended numerous axons from the lesion site over white matter, gray matter, and virtually entire length of the animal CNS, and functionally integrated with host neurons when transplanted into the C5 lateral hemisected spinal cord of rats [60].

1.3.6: NPC & NSC Transplantation for Huntington's Disease

HD pathologies have been mimicked in animal models by intrastriatal injection of an excitotoxin such as quinolinic acid (QA), a tryptophan metabolite that causes the degeneration of spiny GABAergic neurons with a relative preservation of interneurons in the striatum [61].

Song et al. showed that NPCs derived from hESCs by co-culturing with PA6 stromal cells resulted in a behavioral recovery in the apomorphone-induced rotation 3 weeks post-transplantation, showing evidences of neuronal differentiation and migration into the cortex when transplanted into the striatum of quinolinic acid (QA)-induced HD rodents [62]. Lee at al. showed that immortalized NSCs derived from human embryonic cerebrum migrated to the damaged striatum in HD rat brains after intravenous transplantation, decreasing excitatory asymmetry and

progressive atrophy in the animal models 2 weeks post-transplantation, which was manifested in improvement in apomorphine-induced rotation score [63]. Signs of neuronal differentiation were observed. Visnyei et al. showed that the intrastriatal injection of embryonic rat neocortex-derived NSCs resulted in improved metabolic function and sparing of host neurons in the striatum and overlying cortex of the QA-lesioned HD rats, because of trophic action by the engrafted cells on the host neurons rather than restoration of complex circuitry [64]. The transplanted progenitors showed migratory and differentiation capabilities into neurons and glia. NSCs isolated from human fetal cortex and expanded in the presence of ciliary-derived neutrophic factor (CNTF) demonstrated robust survival, migration over long-distance, and differentiation into neurons and astrocytes in vivo when grafted into QA-lesioned HD rat striatum [65]. Significant improvement in forelimb and forepaw movement was observed in transplanted HD rats during the 8-week experiment period.

1.3.7: NPC & NSC Transplantation for Amyotrophic Lateral Sclerosis

Pathological features of ALS are often mimicked by creating transgenic animal models over-expressing the mutated form of superoxide dismutase 1 gene, whose impairment underlies the development of ALS and its associated symptoms.

Klein et al. showed that NPCs isolated from human fetal cortex and transfected with lentivirus to secrete GDNF showed survival up to 11 weeks after transplantation into spinal cord of ALS rats expressing SOD1G93A mutation, integrating into both gray and white matter. GDNF delivered by NPCs promoted motor neuronal survival in the transplanted site and upregulated cholinergic activities with an increased expression in ChAT [66]. Xu et al. showed that human NSCs derived from a fetal cervical-upper thoracic cord underwent extensive neuronal differentiation forming synaptic connections with host neurons, releasing GDNF and BDNF for neuroprotective effects on degenerating motor neurons, delaying the onset and progression of

ALS in transplanted rats while prolonging their lifespan by more than 10 days [67]. Hwang et al. showed that immortalized human NSCs derived from fetal telencephalon culture and genetically modified to overexpress the human *VEGF* gene were capable of replacing lost motor neurons and secreting vascular endothelial growth factor (VEGF), which provided neuroprotective effect by concomitant upregulation of antiapoptotic proteins and downregulation of proapoptotic proteins, when intrathecally transplanted into the spinal cord of ALS model mice [68]. The grafted cells integrated into the spinal cord anterior horn, migrating into the gray matter within 4 weeks of transplantation, differentiating into motor neurons, delaying the disease onset and prolonging the survival of ALS SOD1G93A mouse model.

1.4: Disease Modeling & Drug Screening

Cell reprogramming technology has provided opportunities to model neurodegenerative diseases and recapitulate their early-stage developmental and pathological features in live neurons under controlled environments, surmounting limitations with post-mortem animal and human tissues that only represent late-stage disease phenotypes. By isolating somatic cells from a patient with a certain neurodegenerative disease, reprogramming them into induced-pluripotent stem cells (iPSCs), and differentiating the cells into NPCs and eventually a mature neuronal cell type implicated in the pathology of the disease, it is feasible to obtain a cellular model capturing the patient-specific disease genome embedding mutations in previously-identified genes and any epigenetic modifiers whose causative roles in disease onset are yet to be elucidated (Figure 1). Then, this cell-based disease model can be analyzed to unravel disease-specific molecular pathways and pathological mechanisms of action, before the onset and during the progression of the disease. This would potentially enable early intervention therapies to either prevent disease occurrence or slow down its progression, as symptoms of the neurodegenerative diseases are often not manifested until later stages of development and it may be too late for any treatment

measures to be effective by then. Once the neuropathological phenotype is fully characterized in the model, it can be finally used as a drug screening platform to identify chemical compounds with therapeutic potentials to ameliorate or rescue the disease phenotype. These drugs can be administered to general patient populations sharing the same type of neurodegenerative disease or used for a personalized medicine approach, to induce symptomatic recovery in the tested subjects. As such, patient-specific iPSC-derived neuronal models have been extensively characterized and used to investigate the development and progression of neurodegeneration, with hope for discovering early diagnostics and therapeutic drugs.

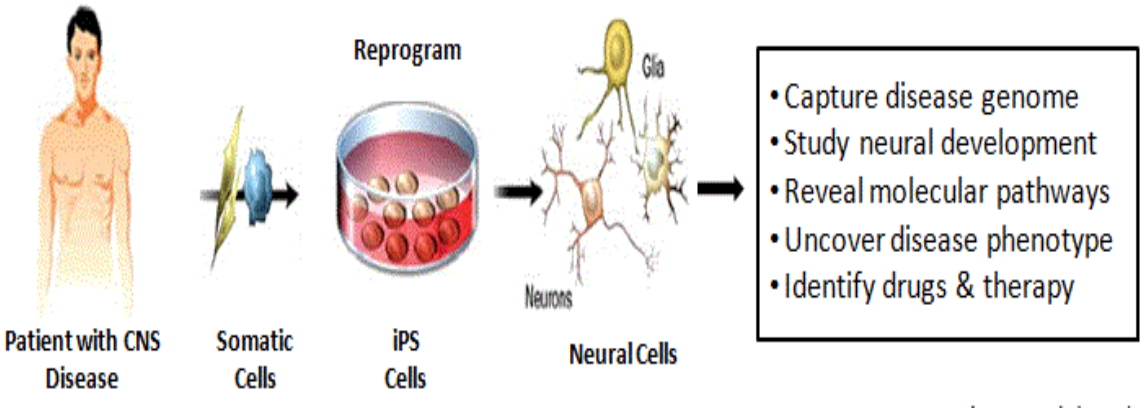

Figure 1. Cell Reprogramming Technology to Model Neurodegenerative Disease [69]

1.4.1: Parkinson's Disease Model

Parkinson's disease (PD) is characterized by the progressive loss of midbrain dopamine neurons in the substantia nigra, which results in decreased level of dopamine in the striatum and consequently dysfunctional movement symptoms including bradykinesia, rigidity, and tremor. Genetic mutations have been consistently associated with the onset of PD, and several mechanisms by which the mutations result in disease phenotypes have been identified. [69] Some mutations appear to impair the cell machinery responsible for degrading undesired protein

aggregates in dopaminergic neurons, such that the accumulated proteins trigger cell apoptosis via microglial activation and subsequent oxidative stress response, the exact molecular pathways of which still await elucidation [71]. These intraneuronal protein deposits serve as a major pathological hallmark of PD and are called Lewy bodies, whose major components are α-synuclein proteins that are ubiquitously distributed throughout the brain and seem to bear associations with membranes and vesicular structures for a supporting role in exocytotic vesicle trafficking [73]. Other kinds of mutation may disrupt the function of mitochondria, which leads to the overproduction of reactive oxygen species and/or reduced endogenous anti-oxidant capacities of affected cells to effectively counteract toxic effects caused by the free oxygen radicals [72, 73]. Thus generated oxidative stress eventually leads to cell death. Studies have shown that mutations in the *LRRK2* (encoding leucine-rich repeat kinase 2) gene and *PINK1* gene (PTEN-induced putative kinase) can increase their kinase activity and promote neurotoxicity via oxidative stress pathways, making cells more vulnerable to peroxide-induced cell death [74].

In order to model PD pathogenesis involving mitochondrial dysfunction and increased cell susceptibility to oxidative stress, Cooper et al. produced a cellular model of the disease by deriving fibroblasts from familial PD patients and at-risk individuals carrying mutations in the PINK1 and LRRK2 genes, reprogramming the cells into iPSCs, and ultimately differentiating them into neural cells embedding the PD-associated mutations. When treated with chemical stressors and toxins disrupting mitochondrial function or protein degradation, such as valinomycin, MPP+, concanamycin A, and hydrogen peroxide, these iPSC-derived neural cells demonstrated increased cell vulnerabilities and mROS concentration, decreased GSH level and basal oxygen consumption rate, and altered mitochondrial dynamics and morphology. Cellular vulnerability associated with mitochondrial dysfunction could be rescued with coenzyme Q10, rapamycin, or the LRRK2 kinase inhibitor GW5074 [74]. Along lines of concepts, Nguyen et al. also generated iPSC carrying the mutation in the LRRK2 gene from a PD patient and

differentiated them into dopaminergic neurons, which expressed upregulation of major oxidative stress-response genes and increased accumulation of a-synuclein protein. The mutant DA neurons were more susceptible to caspase-3 activation and cell death when exposed to stress agents, such as hydrogen peroxide, MG-132, and 6-OHDA than control DA neurons. The ROCK inhibitor Y-27632, known to inhibit the kinase activity of LRRK2, did not protect the PD patient-derived DA neurons against neurotoxins but rather exacerbated cell death [75].

1.4.2: Alzheimer's Disease Model

Alzheimer's Disease (AD) pathology develops typically in the entohinal cortex and hippocampus, but spreads to the cerebral cortex and other regions of the brain with disease progression over time [76, 77]. Caused by mutations or a duplication of the *APP* gene, which encodes the amyloid precursor protein (APP), or mutations in the Presenilin genes, which encode proteolytic enzymes that cleave APP into Aβ peptides, AD is characterized with amyloid plaques (extracellular deposits of Aβ peptides) and neurofibrillary tangles (intraneuronal aggregations of microtubule-associated hyperphosphorylated tau protein) [76]. Cell transplantation as a therapeutic option against AD has yielded some promising results.

Israel et al. modeled both familial and sporadic forms of AD by creating diseased neurons differentiated from iPSCs derived from AD patients, where the familial case of the disease was specifically due to a duplication of the *APP* gene. The mutant neurons demonstrated significantly elevated levels of AD pathological markers such as Aβ40 peptide, phosphorylated tau (p-tau) proteins, and active form of glycogen synthase kinase-3β (GSK-3β), and large RAB51 early endosomes, where the kinase GSK-3β is known to phosphorylate tau at Thr231 epitope and co-localize with neurofibrillary tangles in AD post-mortem neurons and RAB. Treating the neurons with β-secretase inhibitors, but not γ -secretase inhibitors, significantly reduced active GSK-3β and p-tau levels in the AD neurons, though both inhibitor types could reduce Aβ40 [78]. This

suggested the strong causative role of APP proteolytic processing in tau-phosphorylation and GSK-3β activation. Yahata et al. also developed a disease model of AD by reprogramming patient-derived iPSCs into NPCs and subsequently forebrain neurons, which expressed APP, β-secretase cleavaged products such as sAPPβ, APP-CTFβ and BACE1, and γ-secretase component. These forebrain neurons could produce two major pathological hallmarks of AD, Aβ40 and Aβ42 peptides, as evidenced by their increased concentrations in the cell conditioned media. The NSAID sulindac sulfide, as well as β- and γ-secretase inhibitors, could partially or fully inhibit Aβ production in a drug-dose and cell-differentiation-stage dependent manner [79].

1.4.3: Huntington's Disease Model

Huntington's disease (HD) is dominantly inherited, caused by a mutation in the *HTT* gene which in turn leads to a polyglutamine expansion in the N-terminus of the huntingtin protein [80-85]. The mutated *HTT* gene usually carries the CAG trinucleotide sequences repeated 36 to more than 120 times, as opposed to the normal CAG segment repeated between 10 to 35 times. Whereas those with 36-39 CAG repeats may or may not develop the signs and symptoms of the disease, people having 40 or more repeat lengths are almost certain to develop the disease [80-82]. Such an abnormal length CAG segment produces an abnormally long version of the huntingtin protein, which is then cleaved into smaller fragments that bind together and accumulate in neurons. These toxic protein aggregates cause the massive loss of medium spiny neurons in the striatum and loss of neurons in the cortex, by disrupting normal intracellular functions via proteolysis to generate toxic N-terminal fragments, alterations in vesicular trafficking, mitochondrial function and transcriptional dysregulation [84].

Zhang et al. generated a human HD cell model carrying a CAG expansion mutation in the endogenous huntingtin gene, by differentiating patient-derived iPSCs into neural precursors and striatal neurons carrying the same number of CAG repeats as the parent cells. The HD neural

precursors exhibited an increase in caspase-3/7 activity upon growth factor withdrawal and a decrease in ERK activation in response to bFGF, both recapitulating well-characterized disease phenotypes of HD in rodent and human models [84]. Especially, provided that caspase-mediated cleavage of huntingtin is implicated in generating toxic fragments responsible for cell deaths, this iPSC-derived HD model would provide a feasible platform for drug screening. The same group corrected these HD-iPSCs by direct substitution of the expanded CAG repeat with a normal length repeat using homologous recombination, which resulted in the normalization of pathogenic HD signaling pathways involving cadherin, TGF-β, BDNF, and caspase-3/7 activation, the rescue of disease phenotypes such as vulnerability to cell death, and improvement of mitochondrial bioenergetics in NPCs differentiated from the corrected HD-iPSCs [85]. The corrected HD iPSC-derived NPCs could populate the striatum of HD mice model brains, undergoing differentiation in vivo to DARPP-32-expressing striatal neurons.

1.4.4: ALS Model

More generally known as Lou Gehrig's disease, Amyotrophic Lateral Sclerosis (ALS) is characterized by progressive muscular paralysis reflecting the degeneration of motor neurons in the primary motor cortex, brainstem and spinal cord [86]. Typical symptoms include different forms of motor weakness such as difficulty buttoning shirts, lifting weight, chewing, slowing, and slurring of speech. Patients ultimately die of respiratory distress from the failure of diaphragms, as the disease progresses further towards a complete loss of the brain's ability to voluntarily initiate and control muscle movement. Mutations in various genes have been identified as underlying familiar ALS and it pathological features, including SOD1 gene, whose enzymatic activity is crucial for antioxidant defense, and FUS/TLS, TDP43, and c9ORF genes which regulate RNA metabolism and potentially gene expression [78]. Astrocyte dysfunctionality is often related to ALS, due to its implicated role in supporting motor neuronal health [78, 86, 87].

Egawa et al. created motor neurons with abilities to recapitulate ALS phenotypes using iPSCs derived from familial ALS patients, which carried mutations in the gene encoding Tar DNA binding protein-43 (TDP-43). TDP-43 is associated with multiple aspects of RNA metabolism including the transcription, splicing, and transport of mRNA [89-91]. The ALS patient-specific iPSC-derived motor neurons exhibited the formation of cytosolic aggregates of TDP-43 proteins in a detergent-insoluble form, shortened neurite lengths, up-regulation of genes involved in RNA metabolism and down-regulation of genes coding for cytoskeletal intermediate filaments, and increased vulnerability to cell death in response to arsenite, an oxidative stress-inducing agent. Four compounds reported to modulate transcription through histone modification or RNA splicing, trichostatin A, spliceostatin A, anacardic acid, and garcinol, were screened to assess if they could reverse perturbations in RNA metabolism and rescue the disease phenotypes in ALS neurons. In fact, only anacardic acid exerted neuroprotective effects against the arsenite-induced death of ALS motor neurons, lowering TDP-43 mRNA expression along with the insoluble fraction of TDP-43 proteins in cytosols, and increasing the expression of cytoskeletal protein-encoding genes consistent with an increase in the neurite lengths [88].

1.5: Extracellular Matrix and Stem Cell Culture

1.5.1: The Role of Extracellular Matrix in Neurogenesis

The extracellular matrix (ECM) is a mixture of various glycoproteins, fibronectins, collagens, laminins and proteoglycans that assemble into fibrils or other complex marcomoleculuar arrays, providing a scaffold for cell adhesion and migration, as well as chemical and biomechanical cues which can be transduced into intracellular signals regulating cell fates and decisions [93]. The ECM constitutes a basal lamina (BL) surrounding the brain and blood vessels throughout the CNS, where BL is located under the pia matter and found in the ventricular zone (VZ) of an embryonic neocortex during early neural development. The role of

BL is directly implicated in neocortical development, as it provides an attachment site for radial glial cells which are precursors of NPCs maintained in the adult brain and also regulates cortical lamination. Laminin is a major composing unit of BL and present in the VZ of the developing neocortex, promoting the expansion, migration, and differentiation of NSCs both *in vivo* and *in vitro* [92]. Based on the identified structural and functional roles of ECM in regulating neurogenesis, numerous substrate materials have been developed, characterized, and tested to culture NPCs and NSCs *in vitro* for the ultimate goal of fully recapitulating the *in vivo* microenvironments of neural tissue development.

1.5.2: Natural and Synthetic Material Substrates for Stem Cell Culture

In order to expand and maintain stem cells over a long-term, the properties of substrate materials on which the cells are cultured are extremely important as they mediate 1) cell adhesion based on integrin-ligand coupling, 2) activate intracellular signaling pathways and gene expressions through mechanotransduction of extracellular signals, and 3) provide biochemical and physical cues for cell growth, migration and differentiation. Substrates can be nature-derived like extracellular matrix proteins from animal tissues or artificially synthesized like synthetic peptides or polymers, either approach having its own pros and cons.

Extracellular matrix proteins (ECMPs) are usually poorly defined with heterogeneous compositions, therefore showing batch-to-batch variation dependent upon sources of isolation. Therefore, any experimental results and clinical outcomes involving cells expanded on these proteins may be potentially inconsistent and even inaccurate. Since ECMPs are derived from animals, there are inevitable issues with pathogen transmission and immunogenic responses in host animals or humans if cells cultured on these surfaces are to be used for transplantation purposes and other clinical applications. Despite their off-the-shelf availability, ECMPs are relatively expensive to derive and produce in large quantities.

Synthetic peptides, on the other hand, have well-characterized properties and homogeneous compositions that ensure batch-to-batch consistency and reproducibility in terms of experimental outcomes. Custom-designed to mimick certain active components of ECMPs such as specific integrin binding domains, synthetic peptides adequately support the proliferation and self-renewal of stem cells at a level similar to the whole proteins from which they are derived. Since these peptide derivatives are much shorter in sequence than the whole proteins of their origin, one can elucidate which specific peptide sequence is engaged with specific cell surface integrins and obtain a better insight into the mechanism of cell adhesion. They are also relatively inexpensive to produce and be cost-effective for expanding stem cells up to a number sufficient for cell-based therapies. There are no concerns for xenogenic contaminants, due to the synthetic nature of the peptides not carrying any biologically active components from animal tissues. In this way, synthetic peptides can provide excellent alternative substrates that overcome some of inherent drawbacks in ECMP-based culture. Another well-established synthetic alternatives, polymer materials, share most characteristics and advantages with the synthetic peptides except for difficulties in predicting biological activities and mechanisms underlying cell attachment.

1.5.3: Application of Extracellular Matrix Proteins for NPC Culture

Reubinoff et al. showed that neurospheres (or NPCs) derived from human embryonic bodies and expanded on laminin-coated dishes formed a monolayer displaying the characteristic morphologies and structural markers, representative of both immature and mature neurons, such as β-III tubulin, NF-M, MAP-2ab, and synaptophysin [94]. When plated on fibronectin-coated dishes and cultured with the growth factor combination of EGF, bFGF, and PDGF-AA, however, these same neurospheres differentiated primarily into astrocytes expressing GFAP and oligodendrocytes expressing the RNA transcripts of MBP and the *plp* gene. Consistent with these findings, Wu et al. demonstrated that laminin and laminin-rich Matrigel substrates could support

the expansion, differentiation, and neural outgrowths of hESC-derived NPCs more optimally than fibronectin-, laminin- and type I collagen-coated substrates [93]. Raghavan et al. obtained similar results by showing that laminin-, collage I-, collagen IV-, and heparan sulfate-immobilized substrates regulate the balance of neuronal and glial cell differentiation and that the substrate containing a higher content of laminin and heparin sulfate induced more neuronal differentiation. When expanded on various combinations of laminin, collagen I, collagen IV, and heparan sulfate that enteric neurons and glia come in contact with *in vivo* in the adult myenteric plexus, rabbit intestine-isolated adult enteric NPCs differentiated into neuronal and glial cells to varying extents. The composite substrates of collagen I and IV with laminin and/or heparan sulfate addition enhanced neuronal differentiation of NPCs, with significantly increased neurite lengths and more extensive branching and initiation of neuronal network formation. However, in the absence of laminin and heparan sulfate, the NPCs tended to differentiate more towards a glial lineage. In addition, substrates with a combination of laminin and collagen IV induced neuronal differentiation more effectively than a substrate based on collagen I only, as evidenced by the greater numbers and lengths of neurites for neurons differentiated on the combination substrate [95].

1.5.4: Application of Synthetic Peptides for hESC, NPC, and Neuronal Cell Culture

Many studies have been published that utilize and identify synthetic peptides as feasible culture substrates for the long-term culture of various cell types, where the peptides mimick certain functional components of ECM proteins to generate biochemical effects comparable to the whole proteins.

Research efforts to develop synthetic peptide alternatives have been largely directed towards the long-term culture of hESCs more than any other cell types, mainly because of their pluripotent nature and relatively wide applicability. Klim et al. employed a high-throughput

platform in which synthetic peptides were screened individually, in different combinations, and at varying densities on gold surface conjugated via alkanethiol linkage, and the vitronectin-derived heparin binding peptide was identified as a hit molecule supporting the long-term expansion and self-renewability of hESCs by engaging with glycosaminoglycans on cell-surface at the lowest concentration of all tested compounds. The peptide also stimulated the differentiation of expanded cell culture into a heterogeneous population, which expressed lineage-specific genetic markers of ectoderm, mesoderm, and endoderm at a level and timescale comparable to Matrigel culture. Cells propagated on streptavidin-coated surfaces presenting a combination of heparin binding and KGRGDS peptides maintained high expressions of pluripotency genes for more than three passages, growing in compact colonies and at a rate similar to Matrigel culture [96]. Along similar line of concept, Melkoumian et al. showed that bone sialoprotein and vitronectin peptides conjugated to acrylate surface, BSP-PAS and VN-PAS, induced the tightly-packed colony formation of hESCs and proliferation in cell number with doubling time comparable to culture on Matrigel for more than ten passages in xeno-free, chemically defined media. The long-term maintenance of pluripotency for the cells cultured on either peptide was demonstrated by teratoma formation in immunodeficient mice, containing tissues from all three germ layers represented by secretory epithelium, cartilage, and neuroepithelium. Both peptides promoted the differentiation of hESCs into spontaneously beating cardiomyocytes with action potential firing properties of ventricular and atrial cell types, which also expressed cardiomyocyte-specific markers Nkx2.5 and α-actinin. Cells cultured in T75 flasks coated with either BSP-PAS or VN-PAS peptide exhibited the uniform distribution of colonies with characteristic hESC morphology and expressed pluripotency genes, demonstrating the scalability of the synthetic peptides for long-term hESC culture [97].

Kolhar et al. synthesized a cyclic RGD peptide that supported long-term culture of hESCs, where the peptide was conjugated to tissue culture plates via a bi-functional linker reacting with

amine on the culture substrate and thiol on the peptide. The hESC lines expanded on this peptide surface exhibited the rate of proliferation similar to Matrigel control, along with hESC-like colony formation and characteristic morphology with tightly packed cells and large nucleus to cytoplasm ratio. The CRGDC peptide surfaces supported the self-renewability and pluripotency of the hESC colonies up to 10 passages, as well as their differentiation into three germ layers after 10^{th} passage. The peptide also supported hESC maintenance in serum-free, defined mTESR1 culture media for 5 days [98]. Meng et al. developed a combination of synthetic peptides that supported hESC adhesion, expansion, and maintenance by associating with the same set of integrins exploited by hESCs for adhesion to the Matrigel-coated surface, where the peptides were designed based on the cell adhesion motifs of a basement membrane matrix, laminin-111: AG-10 peptide for engagement with A6B1 integrin, C-16 for AVB3 integrin, and AG-73 for syndecan-1, a heparan sulfate proteoglycans. Whereas cells expanded on the individual peptides showed low adhesion, deterioration in colony morphology and loss of pluripotency at later passages, those cultured on the triple-peptide combination displayed characteristic hESC morphology and strong adhesion while maintaining self-renewability up to three passages. The combinatorial peptide also exhibited capabilities to promote the multi-lineage differentiation of hESCs into ectoderm and mesoderm at level similar to Matrigel, thus faithfully recapitulating the pattern of hESC integrin engagement on Matrigel [99].

In addition to hESC lines, other studies tested a wide variety of cell types including neural cell lines to evaluate the potentials for synthetic peptides to be used as an effective substrate material for cell culture. Silva et al. reported that murine NPCs differentiated into mature neuronal and glial cells expressing β-III tubulin and GFAP markers, respectively, when encapsulated within a nanofibrous scaffold incorporating the laminin-derived peptide IKVAV (Ile-Lys-Val-Ala-Val) on its surface, which is known to promote neurite sprouting and direct neurite growth [100]. Tashiro et al. reported that a 19-amino acid synthetic peptide, designated as

PA-22 and containing the IKVAV motif from the alpha chain of laminin, stimulated cell attachment, spreading, migration and neurite extension in a dose-dependent fashion to the similar extent of whole laminin. Among smaller truncated fragments containing partial sequences of the PA-22 peptide, only those including the IKVAV sequence generated comparable biochemical effects to the whole peptide for rat adreneal medulla-derived PC12 cells. The loss of cell adhesion and spreading induced by the replacement of isoleucine by glycine confirmed the role of isoleucine in the IKVAV as an active component regulating cellular behavior. Aside from PC12 rat pheochromocytoma cells, the PA-22 peptide supported the adhesion of various cell types including human embryonal rhabdomyosarcoma, mouse melanoma, murine neural-glial hybrid, and calf pulmonary artery endothelial cells. The neurite outgrowth was also observed for cerebellar neurons cultured on the synthetic peptide [101]. Kiryushuko et al. designed a synthetic ligand, the C3d peptide, of neural cell adhesion molecule (NCAM) which plays a crucial role in morphogenesis of the nervous system and in remodeling of neuronal connections accompanying regenerative and cognitive processes. The immobilized C3d peptide strongly promoted neurite outgrowth in a dose-dependent manner and functional synapse formation in primary cultures of rat hippocampal neurons, demonstrating a significantly faster synaptogenesis than the control poly-L-lysine substrate. Also, the C3d peptide modulated the presynaptic function in the long-term neuronal cultures, primarily via the FGF receptor activation-mediated signaling pathways and in a concentration-dependent manner [102]. Cooke et al. designed oligonucleotides to encode desired motifs of ECM proteins, ligating them into the gene coding for an engineered domain of the Escherichia coli outer membrane protein (OmpA) in a plasmid construct, and purifying the desired ECM protein-mimicking peptides from the plasmid culture. Thus synthesized peptide sequences presented on the OmpA surfaces, each representing collagen I, collagen IV, fibronectin and laminin, supported the adhesion of a neuron-like rat pheocytochroma cell line, PC12, at a

level similar to that of their respective whole proteins. The fibronectin motif, PHSRN, showed the greatest level of cell attachment [103].

1.5.5: Application of Polymers and Synthetic Proteins for NPC culture

In addition to these natural ECMPs and synthetic peptides, polymer materials and synthetic proteins such as chitosan and poly-l-acids have been demonstrated to induce the survival, migration and differentiation of NPCs into mature neuronal cell types, as well as promote the maturation and myelination of oligodendrocytes by triggering the activation of Schwann cell functions post-spinal cord injury [92-93]. Binal et al. used a co-electrospun poly-L-lactic acid and gelatin scaffold to direct the differentiation of NPCs into motor neurons, as demonstrated by positive-staining for relevant markers such as β-III tubulin, HB-9, and Islet-1 [104]. Leipzig et al. reported that the chitosan-based hydrogel scaffold functionalized with free amine groups, designated as photocrosslinkable methacrylamide chitosan (MAC), could instruct the differentiation of NPCs into either neurons, oligodendrocytes, or astrocytes based on its Young's elastic modulus, which could be fine-tuned by varying the respective concentrations of the photoinitiator and solvent used for polymerization. NPCs grew robustly on the scaffold with elastic modulus at 10 kPa with maximal proliferation at 3.5 kPa. Neuronal differentiation was favored on the softer scaffold having elastic modulus less than 1 kPa, whereas oligodendrocyte differentiation was favored on the stiffer scaffolds with modulus greater than 7 kPa. The chitosan scaffold also promoted astrocyte differentiation to a minor extent for its elastic modulus less than 1 kPa and at 3.5 kPa, around the ranges where NPC proliferation was most optimal [105]. These findings suggest a direct correlation between the mechanical properties of a culture substrate and differentiation pathway of NPCs. Johnson et al. demonstrated that mESC-derived NPCs encapsulated in a fibrin scaffold, embedded with growth factors such as neurotrophin-3 (NT-3) and platelet derived growth factor (PDGF-AA), enhanced the survival of the cells and promoted

their differentiation in vivo when transplanted into spinal cord-injured rats. The NPC transplanted rats showed an improvement in behavioral function 4 weeks after transplantation [106]. Lu et al. showed that chitin-alginate 3D microfibrous scaffolds promoted the self-renewal, neuronal differentiation, and maturation of encapsulated hPSCs. The transplantation of these terminally differentiated neurons into the kidney capsule of immunodeficient mice resulted in grafts expressing a mature neuronal marker, MAP2, without teratoma formation, showing a good integration of the cells with the kidney [107]. Mahairaki et al designed polycaprolactone-based nanofiber matrices with aligned mesh orientations and poly-L-ornithine/laminin coating on surface, which optimally supported the adhesion and differentiation of NPCs into neural cells where the differentiating cells adopted polarized morphology with axonal processes stretching along the fiber axis [108]. Outinen et al. identified the effectiveness of a 3D synthetic hyrodgel matrix, PuraMatrix, in supporting the growth, maturation, and migration of hESC-derived neural cells, with neuronal cells forming electrically active firing networks [109]. Kuo et al. showed that neuron growth factor (NGF)-grafted poly(ε-caprolactone) (PCL)-poly(b-hydroxybutyrate) (PHB) scaffolds with surface heparin guided the differentiation of iPSCs into neurons, while inhibiting other differentiations into non-neural lineages [110].

Chapter 2: Materials and Methods

2.1: Neural Progenitor Derivation from Human Embryonic Stem Cells

Neural progenitor cells, with potentials to proliferate indefinitely and differentiate into all neural cell types such as neurons, astrocytes, and oligodendrocytes, have been derived from human embryonic stem cells via embryonic body and neural rosette formation using different media compositions, soluble factor combinations, and culture conditions at each stage of differentiation.

2.1.1: Human Embryonic Stem Cell Expansion

Human embryonic stem cells (H9 cell line) were cultured on tissue culture plates coated with Matrigel (1:25 in KnockOut DMEM, BD Biosciences) in serum-free mTeSR™ media (STEMCELL Technologies) [400 ml basal medium with 100 ml 5x supplement containing Bovine Serum Albumin, rh bFGF, rh TGF-β, Lithium Chloride, Pipecolic acid, and GABA], supplemented with 5 μM ROCK Inhibitor Y-267632 (Stemgent). The cells were passaged at ~70% confluency by rinsing the plate with PBS twice following media aspiration, incubating the cells with Accutase (Millipore) for 5 minutes, centrifuging them for 5 minutes at 200x RCF, resuspending the cells in fresh mTeSR media and replating them at various densities from 20,000 to 80,000 cells/ml. The media were changed every day. All reagents were purchased from Life Technology unless indicated otherwise.

2.1.2: Embryonic Body Formation

In order to generate embryonic bodies, H9 cells previously maintained on Matrigel-coated plates in mTeSR™ media were dissociated using the steps described above by exposure to Accutase and resuspending them in NPC EB Media [1x DMEM/F12, 1% B27 supplement, 1% N2 supplement, 1% Gluta-MAX, 1% penicillin/streptomycin, 50 ng/ml recombinant mouse

Noggin (R&D Systems), 0.5 μM Dorsomorphin (Tocris Bioscience)], supplemented with 5 μM ROCK Inhibitor Y-267632. Approximately 1 million cells were transferred onto each well of a 6-well ultra low cluster plate (Corning), which was then placed on an orbital shaker at 95 RPM in a 37℃/ 5% CO2 tissue culture incubator. After incubation for 24 hours, the H9 cells aggregated to form embryonic bodies (EBs). The EBs were cultured in suspension for 5 days in the same plate with media change for every two days.

2.1.3: Neural Rosette Derivation

After 5 days of culture, the EBs were transferred onto a Matrigel-coated 6-well plate for adhesion. Most EBs adhered to the plate after the first day of seeding and spread out over the next 6 days to from neuroepithelial cell-like rosettes. The EBs were cultured in NPC EB media for total 7 days with media change every other day. The neural rosettes within the EB culture were manually dissected into single cells using a 1 mL pipet following incubation with Accutase for 5 minutes, which was then centrifuged at 200x RCF and resuspended in NPC expansion media [1x DMEM/F12, 1% B27 supplement, 1% N2 supplement, 1% Gluta-MAX, 1% penicillin/streptomycin]. The dissociated cells were then plated at a density of 300,000 cells/ml onto “double-coated” tissue culture plates, which had been prepared by overnight incubation with 100 μg/mL poly-L-ornithine (PLO; Sigma; 1:8) and subsequently with 0.5 mg/mL mouse laminin (LN; Sigma; 1:125) on top of the PLO coating. Thus plated cells were cultured in NPC expansion media supplemented with 5 μM ROCK Inhibitor Y-267632, 30 ng/ml EGF, and 30 ng/ml bFGF.

2.1.4: Neural Progenitor Cell Formation and Expansion

Neural progenitor cells formed one day after plating of the neural rosettes and were maintained on PLO/LN-coated tissue culture plates in NPC expansion media containing 30 ng/ml EGF and 30 ng/ml bFGF. The media were filtered with a 0.22um stericup (Millipore) filter prior to use. The cells were passaged when about 70% confluency by exposure to Accutase for 5

minutes in an incubator, followed by mechanical detachment of the cells using a sterile plastic cell scraper, centrifugation for 5 minutes at 200x RCF, and resuspension in NPC expansion media with 5 µM ROCK Inhibitor Y-267632. The resuspended cells were then seeded on a new PLO/LN coated plate at the density of 300,000 cells/ml for the next passage (Figure 2).

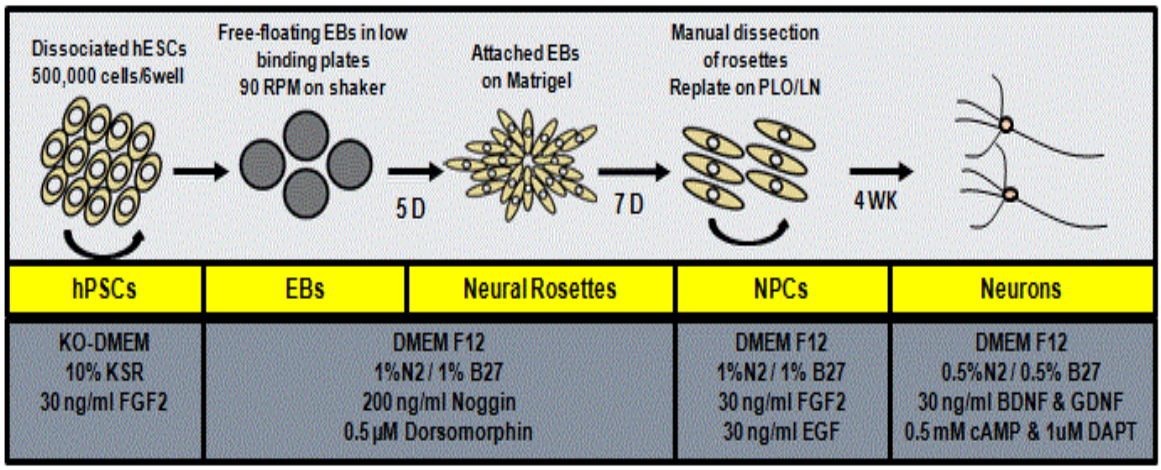

Figure 2. Protocol for NPC Derivation and Expansion from hESCs and Neuronal Differentiation from NPCs

2.2: Screening Optimal Peptide Substrates for NPC Morphology and Proliferation

In order to identify an optimal synthetic substrate with ability to support NPC morphology and expansion, 18 custom synthesized peptides that mimick different ECM components, such as laminin, vitronectin, collagen, fibronectin, cRGDs and various integrin binding domains, were screened individually for primary screening, in combinations for secondary screening , and at various doses for tertiary screening. For primary screening, tissue culture plates were coated with the peptides via thermal adhesion. NPCs were seeded and cultured on the plates for 3 days, followed by morphology assessment and cell counts for each substrate condition for comparison with the LN control (Figure 3).

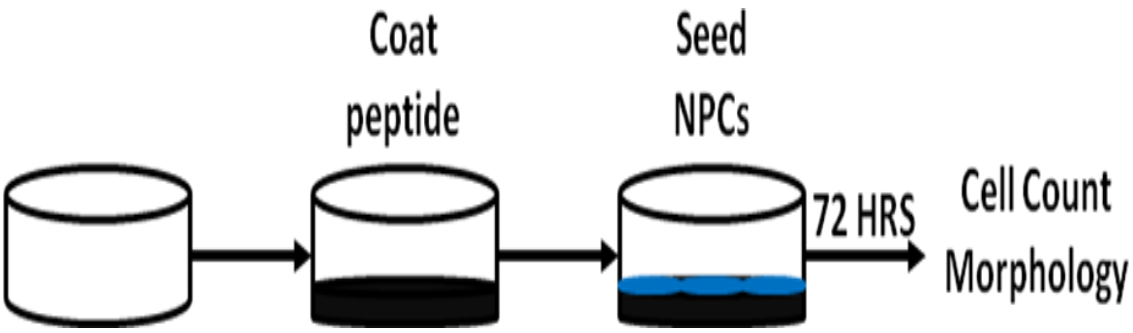

Figure 3. Synthetic Peptide Screening Procedure

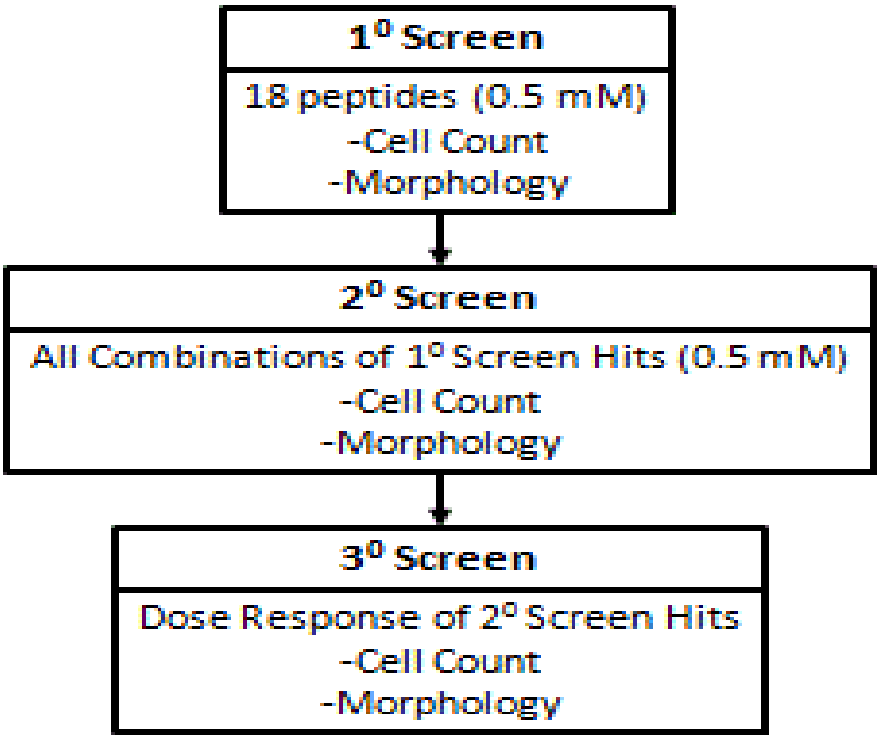

Figure 4. Synthetic Peptide Screening Paradigm

For secondary screening, these individual hit peptides from primary screening were made into multi-factor combinations and assessed for cell count and morphology based on the same steps described above. For tertiary screening, combinatorial hits from the previous screening were tested at a set of lower constituent peptide concentrations to identify the minimum concentration

required to optimally support cell growth and morphology at a level similar to LN control substrate (Figure 4).

2.2.1: Primary Screening for Individual Peptide Hits

The eighteen custom synthesized peptides mimicking ECM subunits and integrin binding sites were purchased from Anaspecs, carrying the following amino acid sequences tabulated. The peptides were numbered for convenience in future referencing (Figure 5).

Name	Sequence	Peptide
Peptide 1	CRGDS	Common integrin binding site
Peptide 2	CCGGKAFDITYVRLKF	αVβ3 integrin binding
Peptide 3	CDITYVRLKF	Laminin α1 chain
Peptide 4	CDIRVTLNRL	Laminin α1 chain
Peptide 5	CTTVKYIFR	Laminin α1 chain
Peptide 6	CRNIAEIIKDI	Laminin β2 chain
Peptide 7	CGWQPPRARI	Fibronectin
Peptide 8	CKGGPQVTRGDVFTMP	Vitronectin
Peptide 9	CDRVRHSRNSIT	Fibronectin
Peptide 10	CGGNRWHSIYITRFG	α6β1 integrin binding
Peptide 11	CGGKAFDITYVRLKF	αVβ3 integrin binding
Peptide 12	CGGRKRLQVQLSIRT	α5β1 integrin binding
Peptide 13	CGGTWYKIAFQRNRK	α6β1 integrin binding
Peptide 14	CPHSRN	Fibronectin
Peptide 15	CIKVAV	laminin α1 chain
Peptide 16	CIKLLI	laminin α1 chain
Peptide 17	CDGEA	collagen I
Peptide 18	CRYVVLPR	laminin b1 chain

Figure 5. Library of 18 Custom-Synthesized Peptides

Each synthetic peptide originally in powder form was reconstituted in sterile PBS to make a stock solution of 2 mM or 5 mM dependent upon its molar mass, which was subsequently diluted to the final concentration of 0.5 mM. A 96-well tissue culture plate (Corning) was coated with the eighteen synthetic peptides individually at 0.5 mM concentration and laminin at 0.004 mg/mL concentration, followed by 24-hour incubation. The peptide-coated plate was then washed twice

with PBS to remove any non-adherent peptide residues and UV sterilized three times for 10 minutes each with in-between washing steps. NPCs were seeded at a density of 62,500 cells/ cm^2 per each substrate condition in the sterilized plate and cultured for 3 days in NPC expansion media supplemented with 30 ng/ml EGF, 30 ng/ml bFGF, and 5 μM ROCK Inhibitor Y-267632. Cell morphology was monitored using a brightfield microscope (Zeiss) and cells were counted on a hemocytometer after three days of culture. Results were compared with those of LN substrate culture.

2.2.2: Secondary Screening for Combinatorial Peptide Hits

The individual hit peptides identified from primary screening were made into a combination of two, three, four, and five factors to generate distinct composite substrate conditions for assessment on supporting cell proliferation. A 96-well tissue culture plate was coated with the distinct peptide combinations each at the final concentration of 0.5 mM concentration, and laminin at 0.004 mg/mL concentration was used as a control. After overnight incubation, PBS washing, and UV sterilization of the peptide-coated plate as described earlier, NPCs were seeded at a density of 62,500 cells/ cm^2 per each substrate condition in the sterilized plate and cultured for 3 days in NPC expansion media supplemented with 30 ng/ml EGF, 30 ng/ml bFGF, and 5 μM ROCK Inhibitor Y-267632. Cell morphology and proliferation were assessed using the Zeiss bright field microscope and hemocytometer, respectively. Results were compared to those of LN control.

2.2.3: Tertiary Screening for Dose Response of Combinatorial Peptide Hits

With two peptide combinations identified as “hits”—namely, combination #1 (peptide 8+13) and combination #2 (peptide 4+8+13)—we lowered and varied the concentrations of individual constituent peptides within the two combinatorial hits from 0.03 to 0.003 mM for dose response test. For peptide combination #1, the concentrations of its constituent peptides #8 and

#13 were both varied from 0.02 mM, 0.01 mM, down to 0.002 mM and mixed to form 9 distinct substrate conditions still representing the same compositional make-up, but with a different set of individual peptide concentrations. Similarly, for peptide combination #2, the concentrations of its constituent peptides #4, 8, and 13 were varied from 0.03 mM, 0.015 mM, down to 0.003 mM, respectively, and combined to generate 27 unique substrate conditions all representing the same molecular composition, but with a different set of individual peptide concentrations leading to a different final composite concentration. A 96-well plate was coated with these 36 distinct substrate conditions and incubated for thermal adhesion, followed by PBS washing and UV sterilization. NPCs were seeded at a density of 62,500 cells/ cm^2 per each substrate condition (which corresponds to 17,000 cells/well) in the sterilized plate and cultured for 3 days in NPC expansion media supplemented with 30 ng/ml EGF, 30 ng/ml bFGF, and 5 μM ROCK Inhibitor Y-267632. Cell morphology and proliferation were assessed using the Zeiss bright field microscope and hemocytometer, respectively, for comparison with readouts for the LN control.

2.3: NPC Expansion and Differentiation Scale-Up on Synthetic Peptide Combinations

In order to determine the feasibility of the synthetic peptides in supporting the long-term self-renewal of NPCs as raw materials to be used for cell transplantation therapies, the scalability of NPC culture on the optimized combinatorial peptide substrates was assessed by 1) expanding NPCs up to 10 passages, 2) differentiating the expanded NPCs into a neuronal lineage after 10^{th} passage, and 3) characterizing the cell phenotype after expansion and differentiation using molecular biology techniques.

2.3.1: Cell Morphology and Growth Rate Assessment during NPC Expansion

A 12-well tissue culture plate (Corning) was coated with hit peptide combinations identified from tertiary screening, combination #1 [peptides 8 (0.02mM) +13 (0.02mM)] and combination #2 [peptides 4 (0.03mM) + 8 (0.03mM) + 13 (0.015mM)], each condition having

triplicate wells to ensure reproducibility. After overnight incubation, PBS washing, and UV sterilization of the plate, about 0.25 million NPCs were seeded in each well and expanded for 3-4 days until ~70% confluency in NPC expansion media containing 30 ng/ml EGF, 30 ng/ml bFGF, and 5 μM ROCK Inhibitor Y-267632. Cell morphology was monitored every day under the Zeiss bright field microscope. The cells were passaged using the same procedures as described above. After complete detachment and dissociation of cell clumps after exposure to accutase for 5 minutes in the incubator, the total number of cells from each well of the plate was counted manually using a hemocytometer at the end of each passage. Cell counts from triplicate wells of each substrate condition were added and averaged for growth rate assessment, without the merging of the cell populations from the three wells. With about 0.25 million cells per well set aside for next passaging, the remaining cells were pelleted and stored at -80'C for gene expression analysis later. The NPC culture was maintained up to 10 passages with media change every other day. Cell counts and morphology from each peptide combination substrate were compared to those of LN control.

2.3.2: Neuronal Differentiation of NPCs on Synthetic Peptide Combinations

In order to evaluate the synthetic peptides' potentials as instructive materials for neuronal differentiation, NPCs expanded on the synthetic peptide combinations for 10 passages were subjected to a neuronal differentiation protocol upon reaching ~90% confluency. The NPC expansion media, previously supplemented with bFGFs and FGFs, were now treated with 30 ng/ml BDNF and GDNF (R&D Systems), 0.5 mM cAMP (Sigma Aldrich), and 1 uM DAPT (R&D Systems) to induce the neuronal differentiation of NPCs. The cells were maintained and differentiated on the combination peptide substrates for three weeks, with half-amount media change every day.

2.3.3: RNA Isolation and Gene Expression Analysis Using qPCR

Total RNA was isolated from the cell pellets harvested from each passage using the NucleoSpin RNA Kit (Macherey-Nagel). Approximately, 200-1000 ng of RNA was reverse-transcribed into cDNA using qScript cDNA SuperMix (Quanta Biosciences). qPCR was performed on a 7900HT Real Time PCR machine (Life Technologies) using TaqManFast Universal PCR Master Mix (Life Technologies), TaqMan probes (Life Technologies), and lineage-specific gene primers (Life Technologies). The cycling parameters were pre-programmed, with 95 ℃ for 2 minutes for initial denaturation followed by 40 cycles of 95 ℃ for 15 seconds for denaturation, and 60 ℃ for 1 min for primer annealing and extension. Relative gene expression levels were calculated based on the ΔΔCt method, with the error bars representing the propagated errors determined from the standard deviation of triplicate reactions. The gene-of-interest threshold cycle (Ct) values were all normalized to Ct values for the housekeeping gene *18S.* Three technical replicates were created per biological condition to ensure accuracy in fold amplification level. Data are presented as the average of the biological replicates ± standard error of the mean.

2.3.4: Immunofluorescence Staining

Immunofluorescence images were obtained using an Olympus Fluoview 1000 confocal microscope. After aspirating culture media and gently washing once with FACS buffer (PBS, 10 mM EDTA, and 2% FBS), the cells in each well were fixed with PBS containing 4% paraformaldeyde (w/v) for 10 minutes at room temperature. Then the cells were permeabilized with PERM buffer (B&D Biosciences) and incubated at 4’C for 30 minutes, followed by blocking with 1% BSA FACS buffer and incubation at room temperature for another 30 minutes. After washing with FACS buffer, the cells were incubated with primary antibodies diluted in the blocking buffer overnight at 4 ‘C with the plate wrapped in aluminum foil. On the following day,

the cells were washed twice with FACS buffer and incubated with secondary antibodies diluted in the blocking buffer for 1 hour at room temperature. Following FACS buffer wash, the cells were incubated with Hoechst 33342 (2 μg/ml; Life Technologies; 1:1000) for 10 minutes at room temperature for DNA staining in the cell nucleus. The stained cells were washed with FACS buffer twice, after which some volume of FACS buffer were left in each well for imaging. Primary antibodies used were mouse anti Sox-1 (BD Biosciences, 1:100), goat anti SOX-2 (Santa Cruz Biotechnology, 1:100), mouse anti NESTIN (Santa Cruz Biotechnology, 1:100), and mouse anti β-III tubulin (R&D Systems, 1:3,000). Secondary antibodies used were Alexa Fluor® 488 donkey anti-mouse (2 mg/ml; Life Technology, 1:200) and Alexa Fluor® 647 donkey anti-goat (2 mg/ml; Life Technology, 1:200).

2.3.5: Flow Cytometry

Cells were incubated with Accutase for 5 min at 37℃ for dissociation, triturated, and passed through a 40 μm cell strainer. After washed twice with FACS buffer (PBS, 10 mM EDTA, and 2% FBS), they were resuspended at a maximum concentration of 5 x 10^6 cells per 100 μl. Then, the cells were stained with antibodies-of-interest for 30 minutes on ice, washed, and resuspended in stain buffer. Cells were analyzed and sorted using a FACSCanto (BD Biosciences). The resulting FACS data were analyzed with FACSDiva software (BD Biosciences)

Chapter 3: Results

3.1: Screening Optimal Peptide Substrates for NPC Morphology and Proliferation

During three rounds of peptide screening, cell morphology assessment and cell counts at the end of each passage were established as preliminary guidelines for identifying synthetic peptide candidates with potentials to induce the expansion of NPCs. Cell morphology provided a qualitative insight into whether NPCs can properly adhere and spread out over culture surfaces for biophysical interactions with the substrate material, where as cell counts at the end of each passage during expansion culture could be interpreted and analyzed as an index of proliferation. These two evaluation criteria proved to be both quick and reliable forms of assessment, as evidenced by reproducibility in data for 10 passages and promising results from scale-up culture based on preliminary hits identified from the screening paradigm employing those criteria.

3.1.2: Primary Screening for Individual Peptide Hits

For primary screening, NPCs were seeded and expanded on a 96-well tissue culture plate coated with the 18 peptides individually, and cell morphology along with total number of expanded cells were evaluated after 3 days of culture for each peptide condition. Substrates yielding cell counts at least 90% of LN control substrate along with characteristic NPC morphology were identified as initial "hits." Based on analysis of the collected data, synthetic peptides #4, 5, 8, 12, and 13 were selected as primary hits that each yielded the number of cells and NPC morphology similar to those of LN substrate at the end of 3-day culture period (Figure 6 and 7). These results suggested the functional roles of these individual peptides in supporting NPC proliferation and morphology. Though peptides #1 and #10 also showed cell counts similar to those of LN, they were excluded from further analysis due to poor morphology demonstrated not characteristic of NPCs.

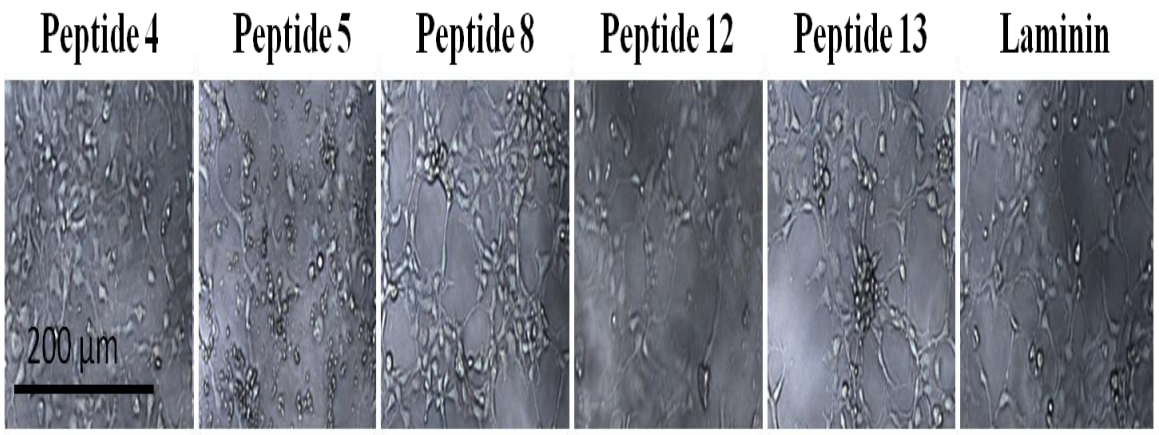

Figure 6. NPC Morphology on Individual Peptide Hits from Primary Screening

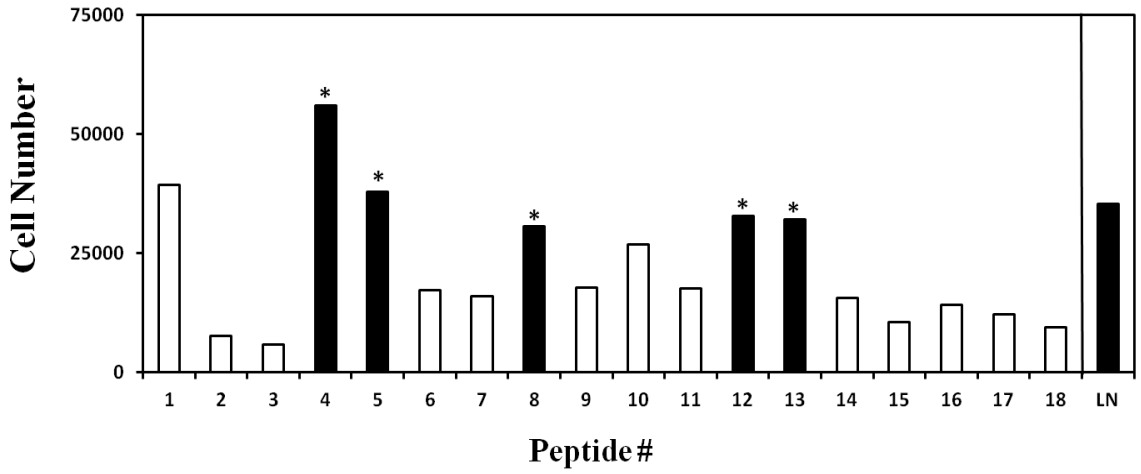

Figure 7. NPC Counts on All Individual Peptides from Primary Screening

3.1.3: Secondary Screening for Combinatorial Peptide Hits

Provided the heterogeneous composition of ECM and complicated nature of its microenvironment where multiple different ligands are engaged in a concerted fashion with cell surface integrins for cell adhesion to the matrix, it is reasonable to hypothesize that synthetic peptides representing distinct components of ECMPs may generate more optimal results for cell adhesion and proliferation when combined as a whole rather than as individuals. In fact, a high-throughput screening of ECMPs using a multi-factorial array technology showed that the

combination of collagen I, collagen IV, fibronectin, and laminin promoted much higher proliferation and maintenance of pluripotency for hESC culture than these proteins individually and a combination missing any one of these four components [111]. Similarly, the composite substrates of collagen I and IV with laminin and heparin sulfate addition promoted the neuronal differentiation of rabbit intestine derived-NSCs more significantly those without laminin and heparin sulfate addition [95].

As a way to validate and measure potential advantages with synthetic peptide composites over their individual constituents in supporting the expansion of NPCs, the individual "hit" peptides identified from primary screening—namely, peptide #4 (CDIRVTLNRL), peptide #5 (CTTVKYIFR), peptide #8 (CKGGPQVTRGDVFTMP), peptide #12 (CGGRKRLQVQLSIRT), and peptide #13 (CGGTWYKIAFQRNRK)—were made into a combination of two, three, four, and five factors to generate 31 distinct substrate conditions in a 96-well tissue culture plate. NPCs were seeded on each combination substrate and cultured for 3 days, followed by cell morphology and proliferation assessment using cell counts. Based on the total number of cells counted and their morphology observed in each combinatorial substrate, a two-factor combination of peptides #4 and #8 (combination #1) and a three-factor combination of peptides #4, 8, and 13 (combination #2) were identified as "hits" that supported the growth and characteristic morphology of NPCs for the secondary screening at an extent similar to the LN control substrate and higher than the individual peptides that constitute each of these combinations (Figure 8 and 9). There were quite a few number of other peptide combinations, such as Peptides #4+8, #4+13, #5+12+13, and #4+5+8+13, that yielded cell counts similar to the LN substrate, but were excluded from future characterizations due to poor morphology not representative of NPCs.

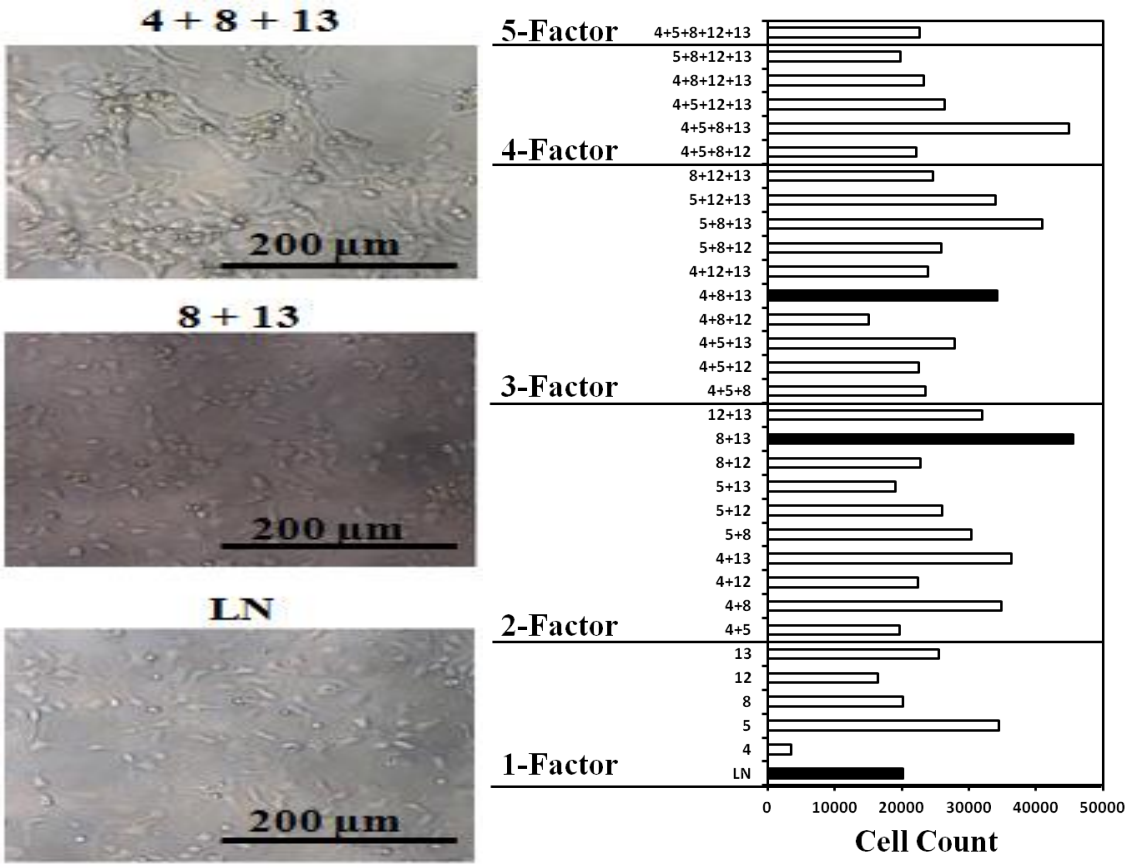

Figure 8. NPC Morphology on the Two Combinatorial Hits from Secondary Screening (Left)

Figure 9. NPC Counts on All Possible Peptide Combinations from Secondary Screening (Right)

3.1.4: Tertiary Screening for Dose Response of Combinatorial Peptide Hits

Synthetic peptide-based culture offers cheaper alternatives to the use of natural ECMPs in expanding stem cells *in vitro*, which are relatively expensive to purify and produce in large quantities despite their off-the-shelf availability. The cost required to synthesize or engineer a substrate material is one of the primary design criteria, by which the feasibility of the substrate for long-term and large-scale cell culture is determined. In attempt to develop a cost-effective, optimal synthetic peptide substrate to potentially replace the more expensive, widely used LN substrate for the long-term expansion of NPCs, the two combinatorial peptide hits identified from

previous screening were subjected to the tertiary screening in which to identify the minimal dose of these peptides required to support cell expansion at a similar level to LN substrate. For peptide combination #1, its constituent peptides #8 and #13 were both diluted to the final concentrations of 0.02 mM, 0.01 mM, and 0.002 mM and mixed to generate 9 distinct combinatorial substrate conditions, still representing the same molecular composition but with a different set of individual peptide concentrations and therefore different final composite concentrations. For peptide combination #2, its constituent peptides #4, 8, and 13 were made into concentrations of 0.03 mM, 0.015 mM, and 0.003 mM, respectively, and combined to generate 27 unique culture surfaces. NPCs were cultured for 3 days on a 96-well plate coated with these 36 distinct substrate conditions, followed by cell morphology evaluation and cell counts.

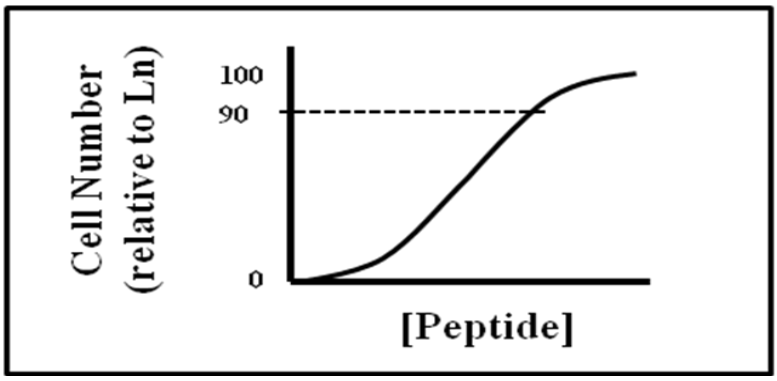

Figure 10. Quantitative Readout for Tertiary Screening

Based on the dose response from each substrate condition, combination #1 with both of its peptides at 0.02 mM concentrations and combination #2 with peptides #4 at 0.015 mM and peptides #8 and 13 at 0.03 mM concentrations were identified as tertiary "hits" that yielded cell counts at least 90% of the LN substrate along with characteristic NPC morphology maintained throughout the culture (Figure 10 and 11). These sets of concentrations respectively for each of the two combinatorial peptides were the bare minimum thresholds, below which NPCs either failed to adhere on the tested substrate for a correct morphology demonstration or showed a low

proliferation level. These outcomes represent a significant reduction in materials needs, compared to the original concentrations of the two combinatorial peptides at 0.5 mM, implying potentially huge savings in cost for scale-up

culture.

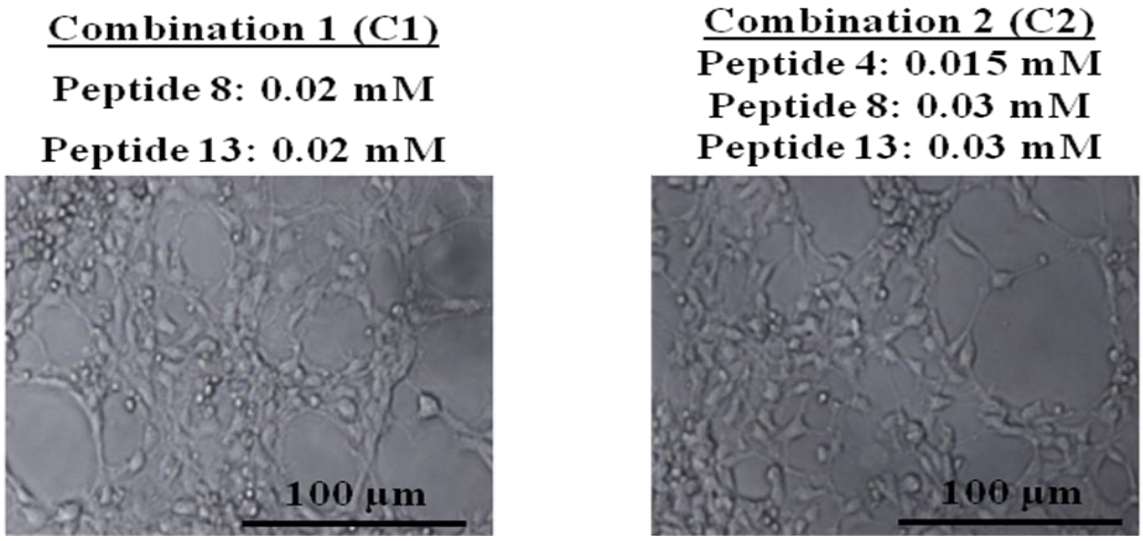

Figure 11. NPC Morphology on Combinatorial Hit Peptides for Minimal Optimal Doses Identified from Tertiary Screening

3.2: NPC Expansion and Differentiation Scale-Up on Synthetic Peptide Combinations

Stem cell-based therapies generally require 5 to 10 billion cells dependent upon the size and severity of a lesion, in order for the transplantation procedure to exert any measurable functional recovery in animal models or patients. Therefore, it is absolutely critical to design a culture substrate with abilities to support the long-term expansion and differentiation of cells up to a quantity sufficient for transplantation, without any undesired compromise in cellular phenotypes or alteration in molecular make-up. In this project, the scalability of the combinatorial hit peptides in supporting the NPC growth, maintenance, and differentiation was assessed by 1) expanding NPCs up to 10 passages, 2) differentiating the expanded NPCs into a neuronal lineage after 10^{th} passage, and 3) characterizing the cellular phenotype after expansion and differentiation

using molecular biology techniques, such as qPCR (quantitative polymerase chain reaction), immunofluorescence staining, and flow cytometry (Figure 12). The two evaluation criteria adopted during initial peptide screening, cell morphology and cell counts, only provided an approximate measure of cell expansion which lacks quantitative information on the molecular identities of cultured cells. By integrating additional evaluation parameters such as gene expression level, immunofluorescence intensity of target molecules, and the fraction of cells expressing markers of a desired phenotype, the synthetic peptides can be more accurately assessed for their potentials in supporting the expansion and differentiation of NPCs.

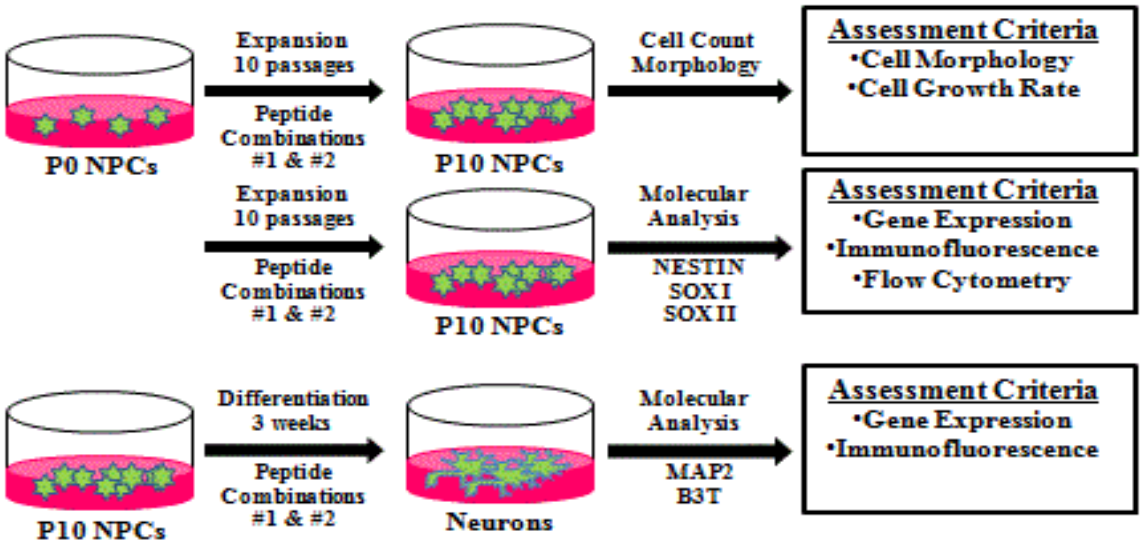

Figure 12. Scale-Up Paradigm for Long-Term Expansion and Differentiation of NPCs

3.2.1: Cell Morphology and Growth Rate Assessment during NPC Expansion

Two different NPC lines, derived from human embryonic stem cells H9 and HSF respectively, were expanded on the synthetic peptide combinations #1 and 2 over 10 passages. The H9- and HSF-derived NPCs on both peptides substrates clearly demonstrate characteristic NPC morphology, indistinct from that on the LN substrate (Figure 13).

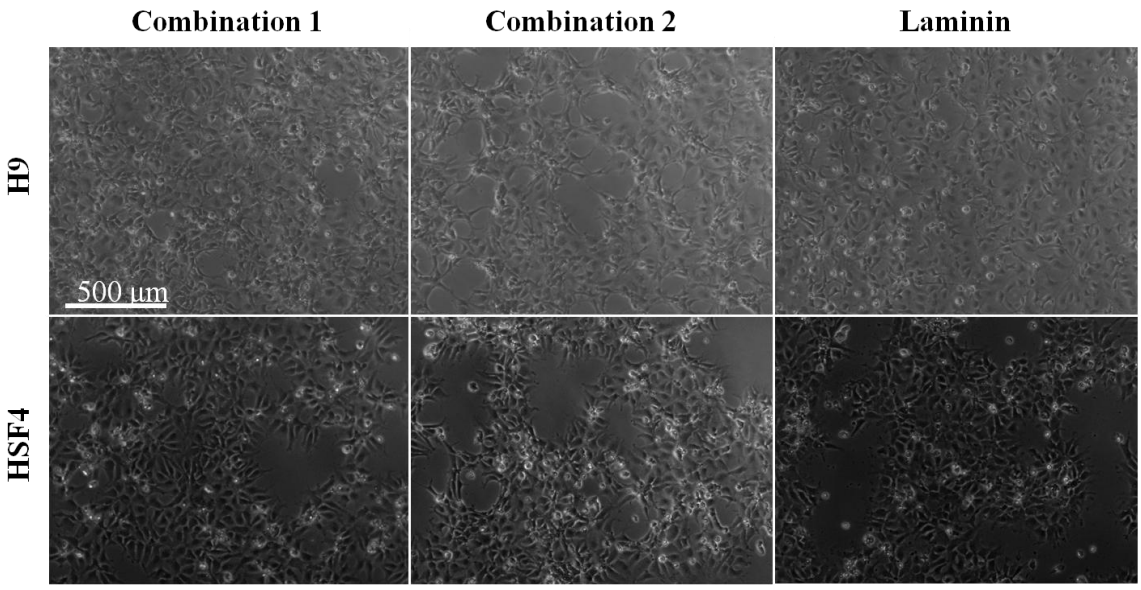

Figure 13. Morphology of H9- and HSF4-Derived NPCs on Combinatorial Peptide Substrates

For an assessment on proliferative capabilities of NPCs on each of these substrate conditions, cell growth rates were compared between the two peptide combination and LN substrates by counting the number of cells after every passage and extrapolating it on a logarithmic scale as if the cells would continue to be propagated up to 10 passages without setting aside any portions of the cells in- between passages for molecular characterizations. The rate of cell growth was amazingly steady across all three conditions, with cell doubling times (T_D) on the peptide combinations almost equivalent to each other and that of the LN substrate at ~50 hours, which corresponds to about two days (Figure 14). Indeed, the numbers of cells obtained at the end of each passage were highly similar for all three substrates and remained consistently so until the 10^{th} passage. These results verify that the combinatorial synthetic peptides support both the characteristic morphology and proliferation of NPCs at a level similar to the LN substrate, confirming the previous observations and data collected from the preliminary peptide screening.

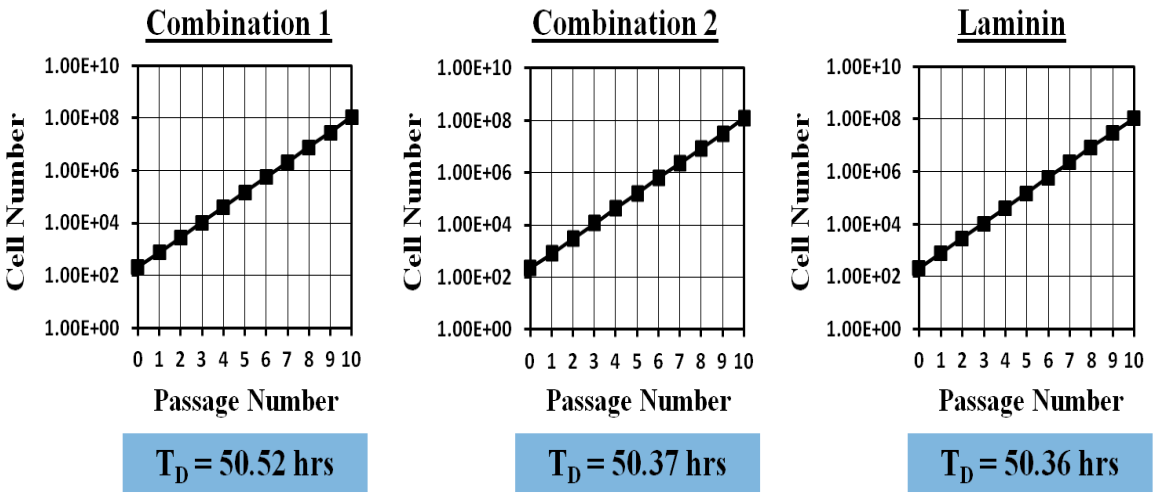

Figure 14. Rate of NPC Growth on Synthetic Peptide Combinations and Laminin for 10 Passages

3.2.2: Gene Expression Analysis of NPCs Maintained on Peptide Combinations

In order to next verify whether the synthetic peptide combinations support the multipotent characteristics of NPCs over a long-term culture, NPCs expanded on the peptide substrates were collected at the end of every passage up to 10th passage and analyzed for the expression of NPC markers, such as NESTIN, SOX 1, and SOX 2. All gene expression levels were normalized to that of LN substrate culture at passage 0. Cells maintained on the synthetic peptide substrates for 10 passages showed expressions of NETIN, SOX1 and SOX2 at a level similar to those cultured in parallel on the LN substrate up to 10th passage (Figure 15). Standard error of the mean calculated from the biological triplicates was not significant across all substrate conditions, ensuring validity in the analysis. These results confirm that the multipotency of NPCs is maintained on both synthetic peptide combination substrates.

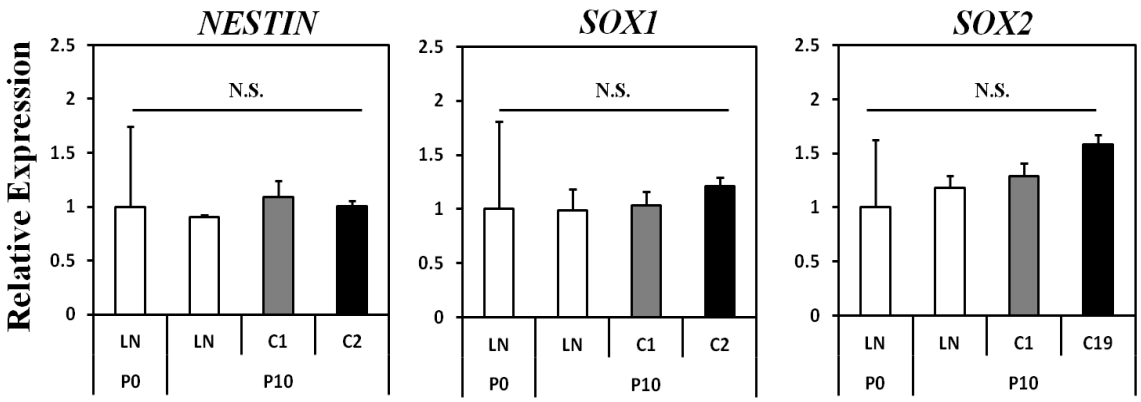

Figure 15. Gene Expression Analysis of NPCs Maintained on Peptide Combinations and Laminin for 10 Passages

3.2.3: Immunofluorescence Staining of NPC Markers on Peptide Combinations

The molecular identity of cells expanded on the synthetic peptide combinations was further characterized using immunofluorescence staining for NPC multipotency markers. NPCs on the peptide substrates were stained with antibodies specific to NESTIN, SOX1, and SOX2 markers after 10^{th} passage, and visualized on an automated confocal microscopy for immunofluorescence intensity. Cells maintained for 10 passages on peptide combinations #1 and #2 robustly express all three NPC markers at a level similar to that of the LN substrate, consistent with the qPCR findings that NPC multipotency remains intact on the peptide substrates (Figure 16).

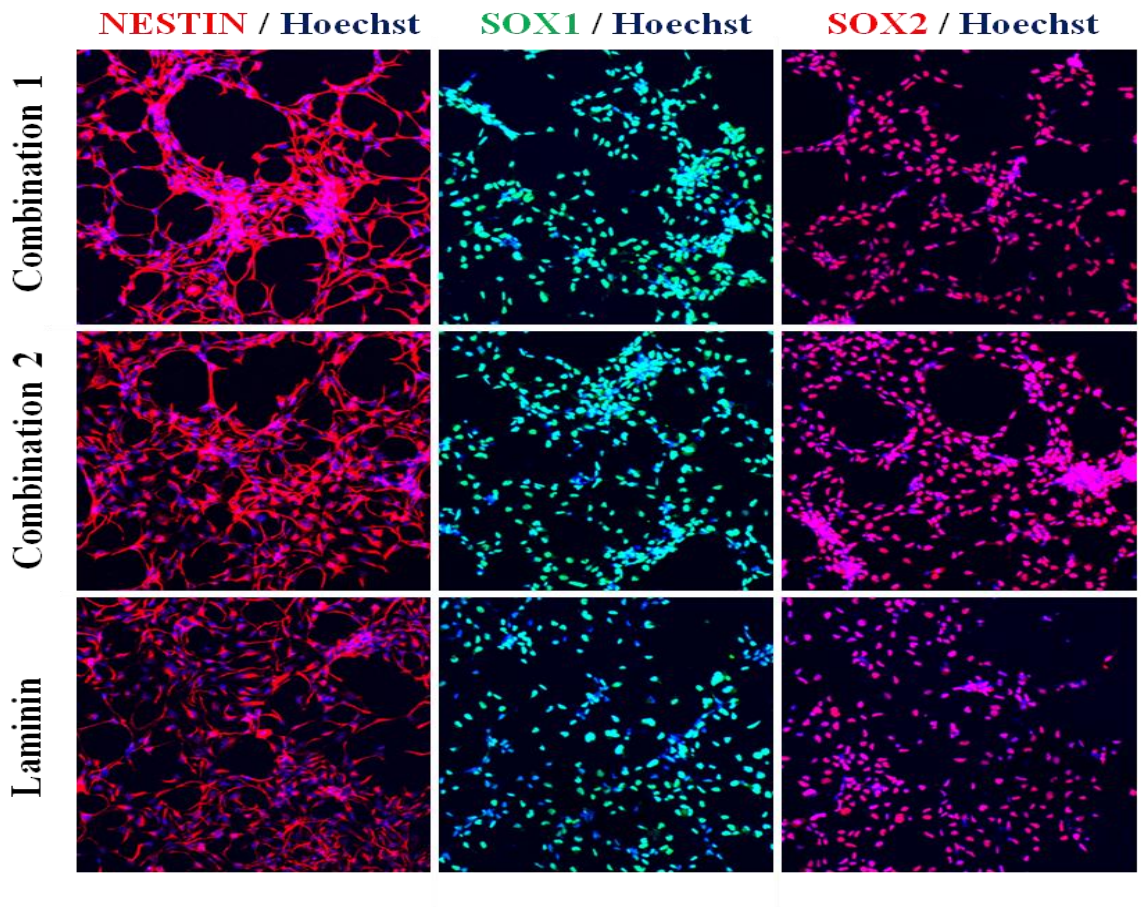

Figure 16. Immunofluorescence Staining of NPCs Maintained on Peptide Combinations and Laminin for 10 Passages

3.2.4: Quantification of Cell Fraction Expressing NPC Markers on Peptide Substrates

Data from immunofluorescence staining are only qualitative in a sense that they do not provide information on how much fraction of the cell population actually expresses NPC markers for each substrate condition. In other words, immunofluorescence intensity detected for a target molecule may not necessarily correlate well with the amount of cells positive for the expression of that molecule. For a quantitative assessment of NPC marker expression, flow cytometry was performed to determine % fraction of cell populations expressing NPC markers on the synthetic peptide substrates. NPCs cultured for 10 passages on the combinatorial peptides were dissociated with Accutase, triturated and filtered through a cell strainer for aggregate removal. The cells were

then stained with antibodies specific to NPC markers, NESTIN, SOX1, and SOX2, for 30 minute incubation on ice. After washed and resuspended in stain buffer, the stained NPCs were analyzed and sorted. Based on this fluorescence-activated cell sorting (FACS) procedure, plots were generated to show percentage of cells expressing the NPC markers from the synthetic peptide- and LN substrate-base culture. The FACS plots demonstrated that more than 90% of the cell populations expressed both SOX1 and SOX2 and 87% expressed Nestin for peptide combinations #1 and #2, which were results comparable to those representing the LN substrate where more than 90% of cells expressed all three markers (Figure 17). These results indicate that the majority of cells maintained on the synthetic peptide substrates for 10 passages are indeed NPCs and have not lost their original phenotype.

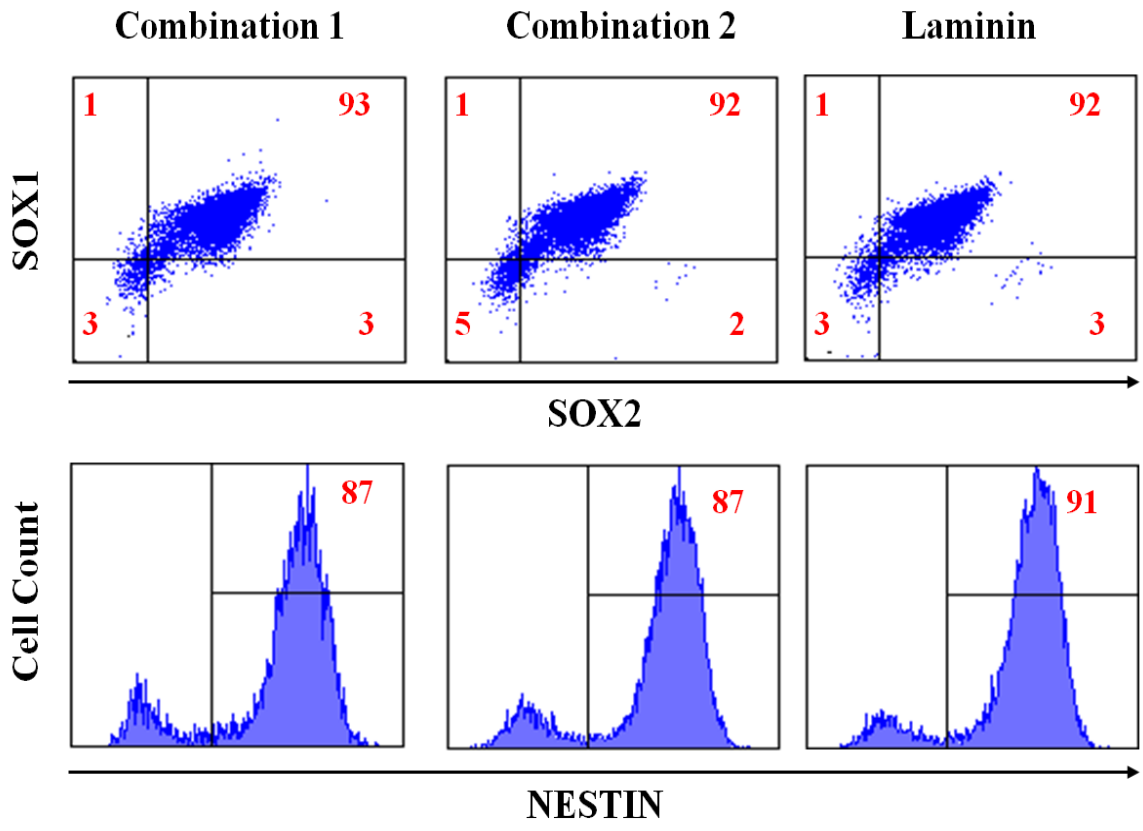

Figure 17. FACS Analysis of NPCs for NESTIN, SOX1, and SOX2 Expressions on Peptide Combinations and Laminin after 10th passage

3.3: Neuronal Differentiation of NPCs on Synthetic Peptide Combinations

Despite the multipotent characteristics of hESC-derived NPCs to differentiate into all neural cell types in response to site-specific molecular cues of a host environment for cell transplantation and their versatile applicability to the various contexts of neurodegenerative disease, it remains a matter of concern whether the transplantation of these cells may potentially lead to teratoma formation because of their pluripotent ESC origin. This concern is certainly not to be overlooked, considering that most tumors observed following experimental transplantation of hESC-derived progenitors are caused by a minor population or even single still pluripotent cells contaminating the grafts [112].

In order to minimize even sheer possibilities for teratoma formation, many studies have used fully differentiated neuronal cell types rather than immature NPCs as raw materials for transplantation in neurodegenerative animal models. Daadi et al. differentiated hESC-derived midbrain-specific NPCs into dopaminergic neurons *in vitro*, which effectively integrated into the host environment via functional synapse formation and extended neurite outgrowths in a PD monkey model [42]. In a similar study, Kriks et al. also used dopaminergic neurons differentiated from hESC-derived NPCs for cell-based therapies, which induced complete restoration of amphetamine-induced rotational behavior and significant improvement in motor functions in PD mice and rats [43]. Since the pathology of a specific neuronal subtype is usually implicated in the chronic form of neurodegenerative diseases, transplanting pre-differentiated neurons relevant to the neurodegenerative disease-of-interest rather than unspecified NPCs may be a reasonable choice. In addition to their newly identified role in supporting both proliferation and multipotency of NPCs, the combinatorial synthetic peptide matrices were tested for their potentials in instructing NPC differentiation into a neuronal lineage by subjecting the NPC culture at 10^{th} passage to an established neuronal differentiation protocol. Induction of differentiation was initiated when the cells expanded on the synthetic peptide combinations for 10 passages reached

~90 % confluency, by supplementation of differentiation-inducing factors such as BDNF, GDNF, cAMP, and DAPT to the culture media and withdrawal of growth factors, bFGF and EGF. Cells were differentiated on the peptide substrates for 3 weeks, followed by molecular characterizations.

3.3.1: Molecular Characterization of NPCs on Synthetic Peptide Combinations

After 3 weeks from the initiation of differentiation culture, neurons were harvested from all substrate conditions for molecular profiling based on gene expression analysis and immunofluorescence staining of mature neuronal markers, such as B3T and MAP2. All gene expression levels were normalized to that of LN substrate culture at passage 0 prior to differentiation induction. Cells differentiated on the synthetic peptide combinations #1 and #2 after 10^{th} passage expressed B3T and MAP2 markers at a level similar to those differentiated in parallel on the LN substrate (Figure 18). Standard error of the mean propagated from the biological triplicates was not significant across all substrate conditions, conferring validity to the analysis. Neurons on both peptide substrates showed ~120 and ~7000 fold amplifications in B3T and MAP2 genes expression, compare to NPCs expanded on the peptides at passage 0 prior to differentiation.

The phenotype of differentiated cells on the synthetic peptide combinations was further characterized using immunofluorescence staining for a mature neuronal marker, B3T. Neurons on the peptide substrates were stained with antibodies specific to B3T at the end of 3-week-culture period, followed by visualization on an automated confocal microscopy for immunofluorescence intensity. Cells differentiated on peptide combinations #1 and #2 robustly expressed B3T marker at a level comparable to those of the LN substrate, consistent with qPCR findings (Figure 19). These results overall confirm the neuronal identity of cells differentiated on the synthetic peptides.

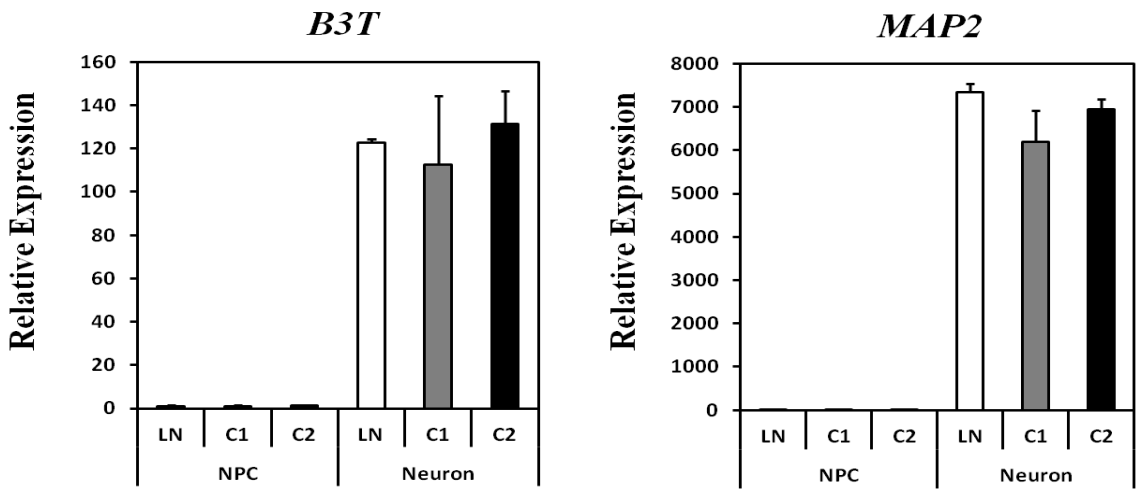

Figure 18. Gene Expression Analysis of Neurons Differentiated on Peptide Combinations and Laminin after 3 Weeks from Differentiation Induction

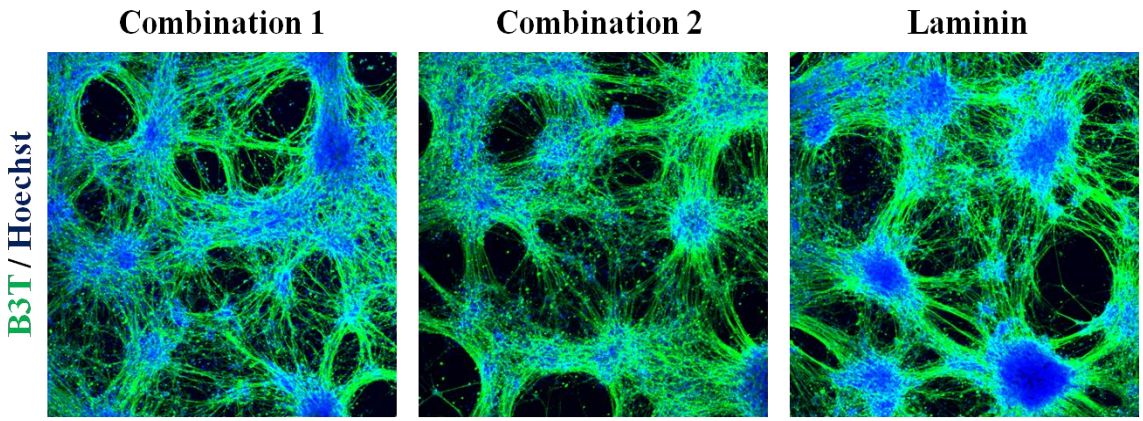

Figure 19. Immunofluorescence Staining of Neurons on Peptide Combinations and Laminin

3.4: Cost Analysis of Synthetic Peptide Combinations in Comparison to LN

As a way to evaluate the relative cost-effectiveness of our synthetic peptide substrates compared to a more generally used LN substrate for long-term NPC culture, costs required to coat a single well of each of different-sized tissue culture plates were calculated. Concluded from this analysis is that it costs about 5 times less for a single well coating with either of these synthetic peptide combinations than with human laminin (Figure 20). This 5-factor difference can lead to an extreme difference in production cost, if NPC culture is to be scaled-up to yield billions of

cells for cell-based therapies. So, the combinatorial synthetic peptides provide much cheaper and more feasible alternatives to LN substrate for the mass production of NPCs towards potential clinical applications.

Culture Size (single well coating)	Human Laminin	Peptide Combination 1	Peptide Combination 2
24 well	$2.68	$0.63	$0.50
12 well	$5.36	$1.26	$1.00
6 well	$12.06	$2.84	$2.25
60 mm	$28.14	$6.62	$5.25
100 mm	$73.70	$17.33	$13.75

Figure 20. Cost Analysis for Single Well Coating of Tissue Culture Plates of Various Sizes Using Peptide Combinations and Laminin

Chapter 4: Discussion

Current methods of NPC culture that require laminin or other kinds of ECMPs purified from natural sources pose significant limitations, because they contain animal-derived factors with potentials to trigger both pathological and immunogenic responses, if cells that are cultured on these substrates are to be used for transplantation-based therapies. Though many studies have been published that employ synthetic materials for the long-term culture of stem cells, synthetic peptides are yet to be developed that promote NPC growth and maintenance specifically. Provided that biological effects elicited by key components of the ECM are attributable to certain short peptide motifs within the whole molecule [103,113], total 18 synthetic peptides mimicking known sequences of various ECMPs and integrin binding domains were initially screened for abilities to support NPC proliferation and maintenance for a short-term. Since multiple different ECM ligands are engaged in a combinatorial manner with their corresponding integrins present on cell surface to modulate cell adhesion, this synergistic effect was modeled and reproduced by making a multi-factor combination of promising "hits" from individual screening of the 18 peptide candidates. Among all possible peptide combinations assessed for morphology and proliferation, ones that supported H9-derived NPC expansion at a comparable level to the LN control were identified as secondary "hits," which were subjected to dose-dependency test. By varying the individual concentrations of constituent peptides within the combinatorial "hits" towards lower values than originally tested concentration, multiple combinatorial substrate conditions were generated with the same molecular composition but different sets of individual peptide concentrations that constitute their respective whole. The combinatorial substrates with set of lowest individual peptide concentrations that could still yield cell counts at least 90% of the LN substrate with comparable NPC morphology were identified as final "hits" for the following

scale-up studies, which were combination #1 [peptides 8 (0.02mM) +13 (0.02mM)] and combination #2 [peptides 4 (0.03mM) + 8 (0.03mM) + 13 (0.015mM)].

With research aims to demonstrate the scalability of cell culture on the synthetic peptide combinations, H9-derived NPCs were expanded on both peptide substrates in parallel with the LN substrate for 10 serial passages and subjected to molecular characterizations for evaluation parameters being cell morphology and growth rate, gene expression level, immunofluorescence staining and flow cytometry of multipotent NPC markers, such as NESTIN, SOX1, and SOX2. Subsequently, these same NPCs maintained on the synthetic peptides were subjected to a neuronal differentiation protocol after 10^{th} passage and cultured for 3 weeks, following which gene expression analysis and immunofluorescence staining of mature neuronal markers, B3T and MAP2, were performed. Based on collective analysis of qualitative and quantitative results obtained for the assessment criteria, it was concluded that synthetic peptide combinations #1 and #2 could indeed support the long-term expansion and differentiation of H9-derived NPCs to a level similar to the well-established LN substrate. There still remain several ways to expand on these current findings, for a more complete understanding of cell-peptide interactions and additional assessment on the larger scalability of the synthetic peptide-based NPC culture for cell-based therapies.

4.1: Elucidation of Cell-Peptide Binding Mechanism

Despite the certain existence of biophysical interactions between the synthetic peptides and NPCs, binding mechanisms underlying cell anchorage on to the peptide substrates require a further investigation. As a way to expand on the current findings, one can elucidate which specific integrins present on NPC surface are engaged with the synthetic peptide combinations in mediating cell adhesion to the substrate. The first step is to profile types of integrins expressed on NPCs, and then either use shRNA to knockdown genes encoding those profiled integrins or

instead use integrin blocking antibodies to disrupt specific integrin-peptide interactions. By examining changes in cell attachment level and morphology as result of this integrin knockdown or blocking assay, one can determine which sets of integrins bind the synthetic peptide substrates and modulate cell adhesion.

We have recently profiled types of integrins expressed on NPCs to probe their potential engagement with our combinatorial synthetic peptides using flow cytometry, and the FACS plots generated from this analysis illustrate that 96.6% of the cell population expresses the laminin-binding ITGA6; 94.7% expresses the vitronectin-binding ITGAV; and 16.3% expressing the fibronectin-binding ITGA5 (Figure 21). These results make sense in the context of our previous findings because the two highly expressed integrins types, ITGA6 and ITGAV, serve as binding sites for laminin and vitronectin, respectively, from which the sequences of our synthetic peptide hits are derived. Since both peptides #4 (CDIRVTLNRL) and #13 (CGGTWYKIAFQRNRK) represent laminin-derived sequences, respectively mimicking a laminin alpha-1 chain and a particular laminin domain interacting with the very ITGA6, it is highly likely that they are associated with the lamining-binding ITGA6 for cell adhesion. Peptide #8 (CKGGPQVTRGDVFTMP) represents a vitronectin-derived sequence, which may engage with the vitronectin-binding ITGAV for cell-substrate binding. The low expression of fibronectin-binding ITGA5 by NPCs indicates that synthetic peptides containing functional sequences of fibronectin may not interact well these cells, consistent with our previous observations that fibronectin-derived peptides # 7, 9, 12, and 14 indeed failed to support cell adhesion and appropriate NPC morphology.

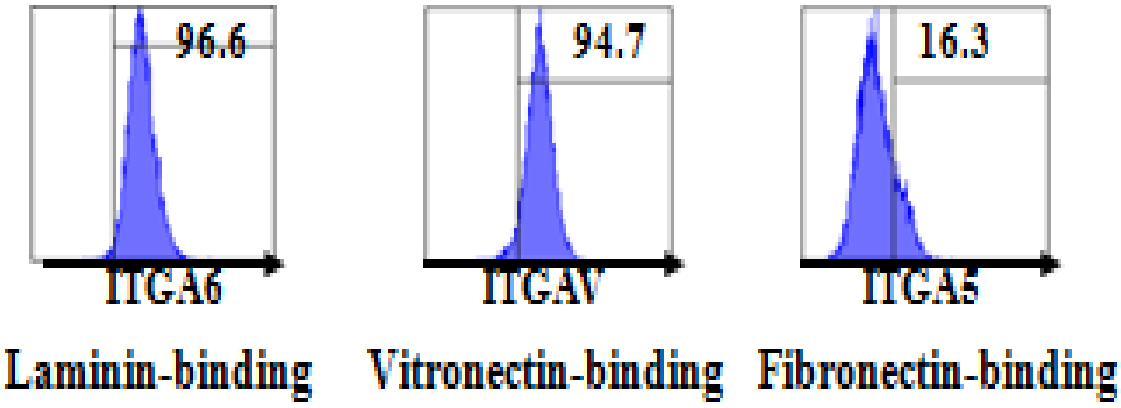

Figure 21. Profiling of Integrins Expressed on H9-Derived NPCs Using Flow Cytometry

The next course of action would be to verify whether the blocking of these integrins or any other ones expressed on NPCs actually leads to cell detachment from the peptide substrates, for which luminescence assay can be employed to quantify % cell binding to the substrate along with qualitative assessment of morphological changes. Once it is identified which integrins specifically engage with our synthetic peptide substrates, a full-scale molecular analysis on how this integrin-peptide coupling is transduced into biochemical signals for regulating gene expressions and intracellular machineries can be performed.

We have recently tested a synthetic heparin binding peptide (HBP) singularly for its potentials to support H9-derived NPC expansion and differentiation, by following the same screening paradigm and scale-up framework utilized for our initial library of 18 custom synthesized peptides. Gene expression analysis, immunofluorescence staining, and flow cytometry analysis indeed confirmed the long-term self-renewal and differentiation of NPCs on the optimized HBP substrates (data not shown). We anticipate that surfaces exhibiting this heparin-binding peptide promote NPC adhesion and spreading by engaging with cell-surface glycosaminoglycans, large unbranched polysaccharides containing repeating units of alternating uronic acids and amino sugars with identified roles in receptor-signaling complexes, cell-cell

recognition, cell-cell adhesion and cell-matrix interactions [96, 115, 116]. For future directions, we can verify the possible involvement of glycosaminoglycans in regulating cell adhesion to the heparin-binding peptide substrate, by exposing NPC culture to the enzyme chondroitinase ABC which catalyzes the hydrolysis of a subset of glycosaminoglycans and subsequently assaying whether cells exhibit any decreased binding to the synthetic surfaces presenting this heparin-binding peptide. The greater the cell detachment becomes with the enzyme addition, the greater the engagement of glycosaminoglycans in cell adhesion proves to be. Alternatively, we can expose NPC culture to soluble heparin and examine if this would inhibit cell interactions to the synthetic heparin-binding peptide substrate. As soluble heparin should directly compete with cell-surface glycosaminoglycans, the greater the cell attachment level is negatively impacted with soluble heparin addition the greater the possibility becomes that glycosaminoglycans are directly involved in cell adhesion to the heparin-binding peptide surface.

4.2: Testing the Feasibility of Synthetic Peptides for Expanding Multiple Cell Lines

Lastly, our synthetic combinatorial and heparin-binding peptides can be tested for the long-term expansion and differentiation of NPCs derived from other human embryonic stem cell lines and NSCs derived from primary tissues, to ensure the consistency of these outcomes across multiple different cell lines and therefore a wider applicability of these peptides for cell-based therapies.

4.3: Large-Scale NPC Expansion on Peptide-Coated Microcarriers in Bioreactors

Major drawbacks regarding conventional 2D static tissue culture include the limited available surface area for cell propagation, time consuming and labor-intensive passaging and harvesting protocols, and lack of continuous monitoring and tight control of the culture microenvironment, which may lead to spontaneous stem cell differentiation, culture heterogeneity, and consequently the serious impairment in their therapeutically applicability [114]. In the

context of our project, stem cell-based therapies for neurodegenerative diseases usually require the quantity of cells ranging from 5 to 10 billion, but expansion and differentiation of NPCs up to these numbers is not feasible with current 2D culture systems. Provided that 100 mm tissue culture plates used to expand NPCs in this project become 70% confluent per about 4 million cells, about 1200 to 2400 of these plates are necessary to obtain cells in order of billions in theory. A feasible alternative to this monolayer culture for the larger-scale propagation of cells would be 3D microcarrier-based culture in a bioreactor system, where the microcarriers provide a high surface area-to- volume ratio and thus facilitates high density cell expansion and scale-up in a timely-effective manner (Figure 22). In the meantime, bioreactors can provide mechanical cues by "stirring action" to better mimick the *in vivo* cell growth environment and further optimize NPC expansion and differentiation protocols which are not available in 2D platforms. Growing expectations for cell replacement therapies in neurodegenerative diseases, coupled with severe shortage of donor tissues and many limitations of current 2D culture system for large-scale cell cultivation, warrant the implementation of 3D culture system for NPC production up to clinically relevant quantities.

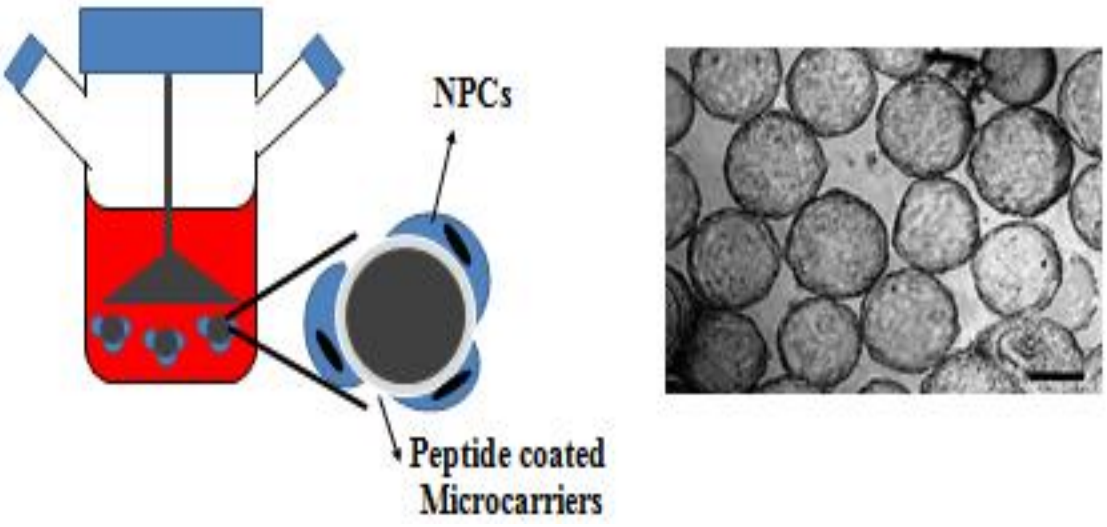

Figure 22. 3D Microcarrier-Based Culture of NPCs in Bioreactor System

As an extension of our scale-up studies based on monolayer culture, we can see NPCs on 3D microcarriers coated with our synthetic combinatorial peptides and expand them in a bioreactor for over 10 passages, to assess the feasibility of this system for producing sufficient number of cells for clinical applications, followed by molecular characterizations of the cultured cells. The same procedures can be applied for differentiation culture.

4.4: Conclusive Remarks

In this project, we demonstrated that combinatorial synthetic peptides mimicking functional sequences of laminin and vitronectin supported the long-term expansion and differentiation of H9-derived NPCs at a level comparable to the animal-derived laminin substrate. These findings are particularly noteworthy and valuable, as the synthetic peptides can provide highly cost-effective and pathogen-free alternatives for more commonly used ECMP-based substrates, with respect to the large scale production of NPCs for stem cell transplantation therapies.

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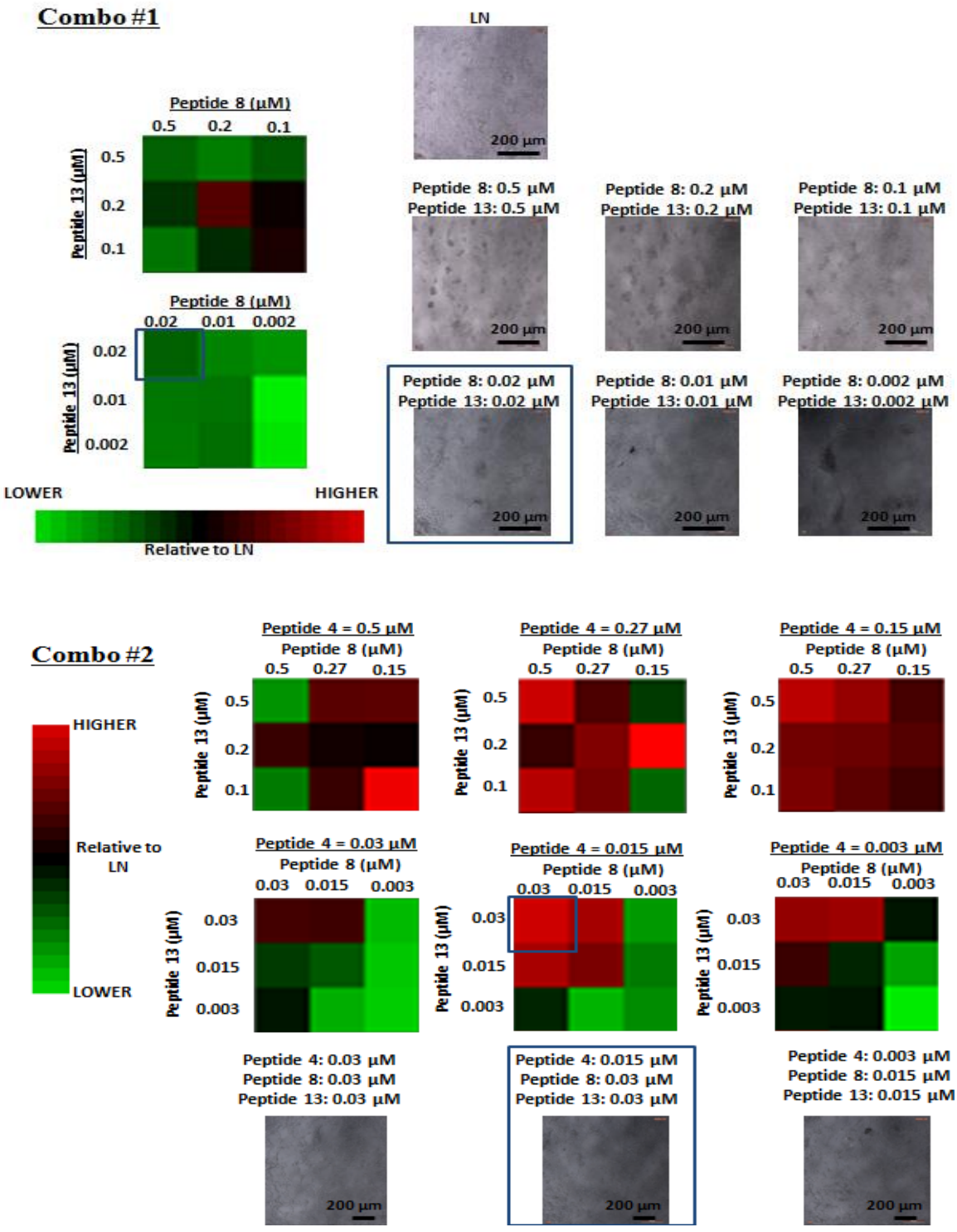
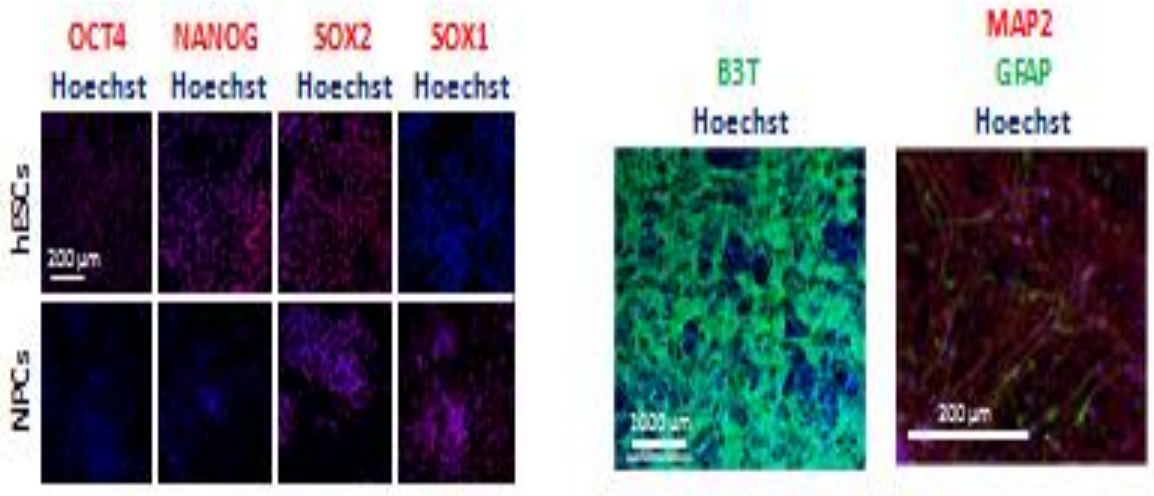
Chapter 6: Supplementary Data



Supplementary Figure 1. Heatmap Analysis of Cell Counts for Tertiary Screening



Supplementary Figure 2. Immunofluorescence Staining of hESCs (Left, Upper Panels), hESC-Derived NPCs (Left, Lower Panels), and Neurons Differentiated from NPCs (Right)