UNIVERSITY OF CALIFORNIA, SAN DIEGO

Development of Synthetic Materials for Long-term Expansion and Differentiation of Neuron Progenitor Cells

A Thesis submitted in partial satisfaction of the requirements
for the degree of Master of Science

in

Materials Science and Engineering

by

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2014

The Thesis of Yi-Huan Tsai is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2014

DEDICATION

To my wonderful family, for supporting me while studying abroad in order to experience a new life and expand my horizons.

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LIST OF ABBREVIATIONS

hNPC	**Human neural progenitor cell**
hESC	**Human embryonic stem cell**
iPSC	**Induced pluripotent stem cell**
FGF	**Fibroblast growth factor**
EGF	**Epidermal growth factor**
PBS	**Phosphate buffered saline**
ECM	**Extracellular matrix**
ECMP	**Extracellular matrix protein**
FACS	**Fluorescence activated cell sorting**
qPCR	**Quantitative polymerase chain reaction**

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ACKNOWLEDGEMENTS

In my studies here at UC San Diego, I have had the honor and privilege of working with so many experienced people on one of the most fascinating topics in bioengineering today. I would first like to recognize Dr. David Brafman. His support, patience, and belief in me during my time here have given me the confidence to push forward towards new discoveries in this field. He has been both a great mentor and friend since the beginning, and his passion for the subject continues to inspire me.

I would like to thank Prof. Shu Chien for introducing me to such a great opportunity, allowing me to enter and grow in knowledge within the field of bioengineering.

I would like to give thanks to Prof. Karl Willert, who has always provided an inviting environment for research and for giving me priceless feedback on my presentations. My lab team members Stephanie Grainger, Nathan Kumar, Ian Huggins, Tomas Bos, Jenna Richter, Josh Cutts, Wayne Kim, Daniel McCoy, Anne Conway, John Hur, Erin Pang, and Sam Badie have always been there to give me advice and technical support whenever I needed it. Special thanks to Noel Moya for training me from the beginning and sharing his lab experience, his encouragement has always made the lab an inviting place. Everyone in the team has left me great memories that I will cherish wherever I go.

I would like to thank Charlotte Lauve for all the advice, paperwork, and

reminders that she has help me with during my time here. She has been easy to work with even under any circumstances.

I would like to thank my friends at UC San Diego who have accompanied and supported me in this experience like family: Blair Kao, Jack Lin, Alex Liu, Jason Chuang and Jimmy Chuang. Together we have shared great memories through ups and downs while studying abroad.

Finally, I would like to show my deepest love for my family: Chao-Pao Tsai, Jaxon Tsai, Kai-Ling Tsai, Pei-Jung Tsai, Li-Wei Tsai, Wen-Suan Tsai and Hui-Jun Ma. Thank you all for always being there for me and giving me the support I need to study outside of the country.

ABSTRACT OF THE THESIS

Development of Synthetic Materials for Long-term Expansion and Differentiation of Neuron Progenitor Cells

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University of California, San Diego, 2014
Professor Bandaru Prabhakar Rao, Chair

This thesis focuses on creating an alternative substrate to replace current exogenous material, which has limited clinical use for cell applications. The subjects of study were on neurodegenerative diseases, particularly those that resulted in the damage or loss of a patient's neurons and supporting cells. Of the various sources of transplants for the cell therapy of these diseases (like embryonic, fetal, adult, and induced pluripotent

stem cells), we chose to focus our research on NPCs derived from human pluripotent stem cells, which have the ability to rapidly proliferate and differentiate into all neuron lineages. It was also important to have a system for these cells to survive and multiply, while maintaining functionality. To quickly screen a library of polymers, we used a high throughput approach on a polymer array to find the best material suited for expansion. As a result, we determined that P4VF was the best substrate polymer for long-term support of NPC self-renewal and growth. In addition, this polymer also supported neuron differentiation.

CHAPTER 1: Introduction

1.1 Motivation

Neurodegenerative diseases, which result in the damage or loss of neurons in brain or spinal cord, create an enormous burden on society with over 7 million people in the United States diagnosed with diseases like Alzheimer's, Parkinson's and Sclerosis. Treatments on Alzheimer's (which is currently considered as the most common neurodegenerative disorder) alone in year 2010 totaled over $100 billion in health care costs in the US. Lunn, et al. [1] Stem cell therapies have thus attracted more and more attention as it is widely regarded as a potentially effective treatment for neurodegenerative disease. The need to find effective treatment is urgent, especially with an increasingly aging population, the burden from Alzheimer's disease (AD) is anticipated to rise. The second most common neurodegenerative disorder, Parkinson's disease (PD), affects up to an estimated 1.5 million people in the USA, with around 50,000 new cases diagnosed each year. In 2009, estimated costs of PD treatment stood at $11 billion per 500,000 affected Americans. [1-3]

Amyotrophic lateral sclerosis (ALS) affects another 30,000 Americans, with death typically resulting from respiratory failure within 2 to 5 years of diagnosis[1]. The mechanisms underlying this disease pathology remain unclear and there are currently no effective treatment options. Without full comprehension of the causes of ALS, it is difficult to design treatments[1, 4-7].

Currently there is a lack of cellular therapy options which utilize cell or tissue grafts to treat diseases or injury. As scientists grasp a greater understanding of the capacity of stem cell technologies, there is a growing public hope that stem cell therapies will continue to evolve into realistic and efficacious treatments for these and other neurodegenerative diseases. [1]

1.2 Cell Therapy for Neurodegenerative Diseases

Neurodegenerative diseases can be categorized into acute and chronic injuries, each with its own characteristic biomechanical responses. When responding to acute injury, such as ischemic stroke or spinal cord injury, distinct types of neuronal cells rapidly die over a short time period within affected areas of the nervous system. In chronic disorders selected regions of the brain are affected by the slow degeneration of neuronal cells over a period of years, such as motor neurons in amyotrophic lateral sclerosis, dopaminergic neurons in Parkinson's disease, or the widespread loss of many types of neurons that occurs in Alzheimer's disease [5, 8-11]. As Alzheimer’s disease is characterized by memory deterioration and graduated cognitive function decline [12], current treatment options for AD focus on regulating neurotransmitter activity. For instance, it has been reported that after neural stem cell transplantation on AD-related aged triple transgenic mice, cognitive function improved and hippocampal synaptic density increased through brain-derived neurotrophic factor (BDNF) mediation [13].

Parkinson's disease (PD) is characterized by the loss of dopaminergic (DA)

neurons in the substantia nigra area of the midbrain. Recent statistics show that 2 percent of aged people over 65 experience some impairment in movement, balance and some cognitive abilities [14, 15], which are typical PD symptoms.

Deep brain stimulation or therapies that aim to increase dopamine levels are two of the many treatment options for Parkinson's disease [16, 17]. Animal study on PD demonstrated that deep brain stimulation (DBS) of the subthalamic nucleus at early stage PD promoted survival rate of dopaminergic neurons[16, 18].

However, current therapies (including oral administration) are only effective in symptoms control but not cure Parkinson's disease by reconstituting cells back to their normal states. Long-term negative side effects have also been reported of these medicines. Moreover, the effectiveness of treatments will decrease as degeneration progresses over time. As such, stem cell therapies have garnered more attention, as studies have indicated that they provide a more promising approach such as replacement of lost DA neurons for Parkinson's diseases [3, 15, 17, 19].

More importantly, it has been reported that induced pluripotent stem cells generated from patients with Parkinson's disease (PD) can be differentiated into dopaminergic (DA) neurons, which survive in large amounts after being transplanted onto the adult rodent striatum. In addition, functional deficits improvement and cell integration in the host tissue were also observed after transplantation. These observations provided motivation for further research and development on iPSCs for neurodegenerative diseases [20, 21].

1.3 Application of stem cell for Drug screening

Recent publications have heralded the use of iPSCs derived from patients with spinal muscular atrophy and amyotrophic lateral sclerosis for drug screening and disease study. Studies have shown a disease-linked in vitro phenotype in iPSC derived cells and utilizing cells extracted from patients with Spinal Muscular Atrophy (SMA) for drug tests [22]. This possibly provides a platform to study pathogenesis of the disease, when progressive degradation of motor neurons is observed. The mutation in gene coding for motor neuron 1 (SMN1) protein survival (conditions of SMA) results in progressive degradation of lower alpha-motor neurons. It is thus crucial that in vitro modeling of SMA is reliable, as SMA is genetically unique to humans (models from rodents are not relevant because SMA for humans comprises of SMN1 and SMN2 while rodents only possess a single version of SMN) [5].

As such, iPSC derived motor neurons with mutation linked to SMA can be directly used for tests. Ebert et al utilized fibroblasts that were i) derived both from a healthy participant and SMA type 1 patient , ii) differentiated into motor neurons in vitro and iii) reprogrammed into iPSCs with excess expression of Nanog, Lin28, Oct4 and Sox2 genes. Comparative research was conducted to address molecular features and growth of SMA neurons relative to healthy cells, which illustrated factors associated with the development of diseases (For example, drug treatment with valproic acid and tobramycin can reverse low level condition of SMN proteins).

Lee et al. studied patients with familial dysautonomia (FD), which was a disease attributed to tissue-specific splicing defect in processing of mRNA for IKB Kinase complex associated protein (IKBKAP). The mutation depletes autonomic and sensory neurons in the neutral tissue. As of now, there exists no optimal model for familial dysautonomia, so the iPSC based method may be advantageous for the research of new treatment methods and disease pathogenesis. iPSCs derived from familial dysautonomia patients were differentiated into peripheral neural crest precursors and neurons that mutated. They were also used for drug tests with 3 distinct drugs shown to be affecting splicing with the following candidates for familial dysautonomia treatment: epigallocatechin gallate (ECGC), kinetin and tocotrienol. Results showed that mutant IKBKAP splice form was decreased with the treatment of IPSC neutal crest precursors. On the other hand, there was no observerable change in IKBKAP splicing after treatment with ECGC or tocorienol [23].

Therefore, research on iPSCs derived from SMA and familial dysautonomia (FD) patients show the effectiveness of drug screening and modeling for serious neurodegenerative diseases. In retrospect, they present new opportunities for building iPSC based models for Parkinson's disease and lateral sclerosis.

1.4 Neuron Progenitor Cells

In the adult brain, neurogenesis occurs in the subgranular zone (SGZ) and subventricular zone (SVZ). Neural progenitor cells (NPC) can also be isolated from the

adult cerebral cortex, cerebellum, and spinal cord. However, the central neuron system has limited ability to self-renew, giving these cells a restricted healing capability. During injury, the brain will stimulate more cells towards repair but because of these limited capabilities, scientists believe that there is more opportunity in the ability to implant stem cells for the purpose of healing. The source of transplants can come from embryonic, fetal, adult, and induced pluripotent stem cells. However it is difficult to isolate adult NPCs, due to the inability to harvest the cells noninvasively. Therefore our focus is on NPCs derived from embryonic stem cells.

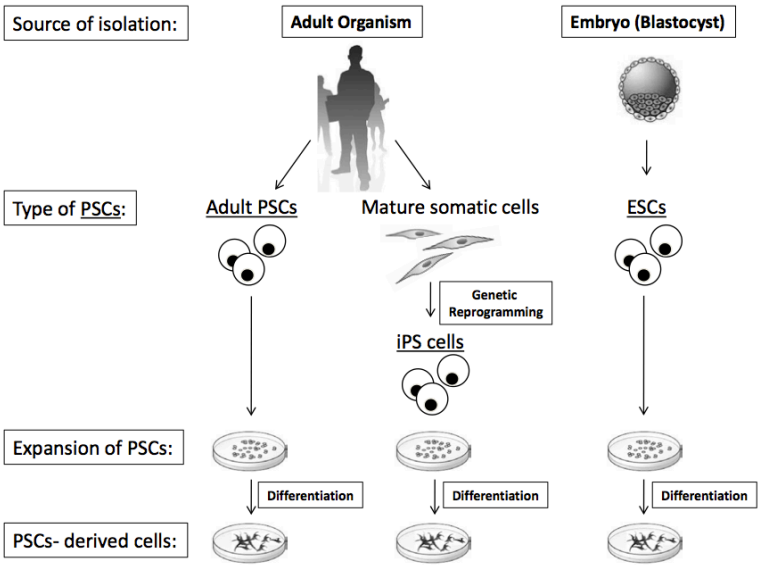


Figure 1: cell source[5]

1.5 Extracellular Matrix

Before using these cells for clinical applications, there are still several problems that first need to be resolved. When cells are cultured in vitro, it is difficult to do a large cell expansion. Animal modeling is problematic because there are still differences

between humans and other animals (like mice), so biomechanics between the two might show differences. Advantages of human pluripotent stem cells (hPSC) have the ability to rapidly proliferate and differentiate into all neuron lineages. NPCs are a good cell source for toxicity tests and drug screening for specific neural diseases, with the ability to be customized. Cells from induced pluripotent stem cells (iPSC) can be specifically customized from any individual patient for treatments. However if we need those cells for cell therapy, we need to develop an efficient way to produce enough of them while avoiding any potential obstacles. The exogenous products derived from animals such as the extracellular matrix protein, unclear mechanisms for binding, and survivability of the cell on the matrices. The environment of the cell when it is grafted still contains many unknown factors. Therefore we are developing a culture system for these cells to survive and multiply, while maintaining functionality. This would enhance the viability and its ability to differentiate into any other future cell lines post-implant. Nowadays people use laminin substrates for capture, but this method limits the use for clinical applications. Research shows that when using laminin substrates there are variable hPSC expansion rates, non-homogenous hPSC expansions, and an inability of hPSCs to respond to differentiation signals. Clinical use requires large quantities for homogenous hPSC populations. On the other hand, synthetic-based polymer strands are inexpensive and easy to fabricate with defined physical and chemical properties. Polymeric materials have already been developed to support hPSC for in vitro expansion. However a matrix to support the growth and differentiate of hNPCs have not yet been developed. In order to

quickly screen a library of polymers, we use a high throughput approach on a polymer array as described below to find the best material suited for expansion.

1.6 Summary of aims

1. Neural progenitor cells generation from human embryonic stem cells.
2. Screening promising synthetic polymers out by high-throughput method for further investigation.
3. Development of scalable synthetic polymer-based hydrogel culture system for NPC proliferation.
4. Optimization of culture system and condition for large scale NPC long-term expansion.
5. Validation of NPC multipotency after multiple passages on P4VP-coated hydrogel by IF, FACS, qPCR.
6. Growth rate measurement of NPCs on P4VP and compare with laminin-contained substrate.
7. Optimization of culture condition for NPC differentiation.
8. Validation of neuron differentiation ability after 2 weeks culture on P4VP-coated hydrogel.

1.7 Summary of results

1. Obtaining high purity of neuron progenitor cells from human embryonic stem cells by EB formation method.

2. Identification of one promising polymer, which is poly(4-vinyl phenol) [P4VP] by using polymer microarray technology.
3. Poly(4-vinyl phenol) support large scale self-renewal and expansion of NPC for more than 10 passages and at the same time maintain their multupotency.
4. Poly(4-vinyl phenol) coated hydrogel supports neuron differentiation and the derived neurons express neuron markers after 2 weeks culture.

CHAPTER 2: Materials and Methods

2.1: Tissue culture

2.1.1: hPSC culture

HUES9 human embryonic stem cell line was cultured in mouse embryonic fibroblast conditioned media (MEF-CM). The preparation of MEF condition media is to seed mouse embryonic fibroblasts on gelatin coated tissue culture plate with MEF media [1x DMEM with glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin]. After 24 hours, we aspirated previous media and added HUES media [1x Knockout DMEM, 10% Knockout serum replacement, 10% human Plasmanate (Talecris Biotherapeutics), 1% non-essential amino acids, 1% penicillin/streptomycin, 1% Gluta-MAX, 55mM 2-mercaptoethanol (sigma), 30 ng/ml bFGF]. All media components were purchased from Life Technoloies unless indicated otherwise. After 24 hours of incubation, we collected mouse embryonic fibroblast conditioned media and re-added HUES media. Then, we collected MEF-CM after another 24 hours. This step was repeated for six consecutive days.

H9 human embryonic stem cell line was cultured in mTeSR™ media [400 ml basal medium with 100 ml 5x supplement contain Bovine Serum Albumin, rh bFGF, rh TGFβ, Lithium Chloride, Pipecolic acid, and GABA] purchased from STEMCELL Technologies and supplemented with 5 μM ROCK Inhibitor Y-267632 (Stemgent).

HUES9 and H9 hESC lines were maintained on Matrigel (BD Biosciences) coated tissue culture plate and were routinely passaged by exposure to Accutase (Millipore) for 5 minutes and centrifuged for 5 minutes at 200 RCF after a one time rinse. Then we resuspended cells and plated them at various densities from 20,000 to 80,000 cells/ml. The media was routinely changed every day.

2.1.2: hNPC culture

H9 and HUES9 NPC derived from human poluripotent stem cell were cultured in NPC expansion media [1x DMEM/F12, 1% B27 supplement, 1% N2 supplement, 1% Gluta-MAX, 1% penicillin/streptomycin] supplemented with 30ng/ml EGF, 30ng/ml FGF and 5 μM ROCK Inhibitor Y-267632 (Stemgent). All mediums were filtered with a 0.22um stericup (Millipore) filter prior to use.

hNPC lines were cultured on poly-L-ornithine/ Laminin coated tissue culture plate [PLO diluted with PBS at concentration of 0.004mg/mL and Laminin at concentration of 0.004mg/mL] and were regularly passaged as single cells when it was 90% confluent by exposure to Accutase for five minutes. We utilized sterile plastic cell scrapper to detach cells, followed by one rinse and centrifugation for five minutes at 200 RCF. Then we resuspended cells and seeded cells at a density of 100,000 cells/ml on tissue culture plate and seeded cells onto quadriPERM culture dish at various densities from 160,000 to 200,000 cells/ml. The media was routinely changed every other day.

2.2: Generation of NPC from hPSC

Human neural progenitor cells derived from human pluripotent stem cells have the ability to proliferate and differentiate into neurons, astrocytes and oligodendrocytes. Induced cellular differentiation of human pluripotent stem cells undergoes three steps towards neural progenitor cells, namely embryonic body formation, neural rosette formation and neural progenitor cells formation.

2.2.1 Embryonic Body Formation

To induce neuron differentiation, H9 and HUES9 were cultured on Matrigel (BD Biosciences) with mTeSR™ and MEF-CM respectively and supplemented with 30 ng/ml FGF2. When cells became confluent, hESCs were exposed to Accutase for 5 minutes and rinsed once, then centrifuged for 5 minutes at 200 RCF. We gently resuspended cells 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)] and supplemented them with 5 μM ROCK Inhibitor Y-267632 (Stemgent). We then transferred 1 million cells onto one well of a 6-well ultra low cluster plate (Corning) and placed it on an orbital shaker at 95 RPM in a 37°C/ 5% CO2 tissue culture incubator. After 24 hours, the hESCs aggregated to form embryonic body (EB). The media was subsequently changed every other day in a length of 5 days, with the plate kept on an orbital shaker in an incubator during the period.

2.2.2 Neural Rosette Formation

After 5 days in suspension culture, EBs were transferred to a Matrigel (1:25 in KnockOut DMEM; BD Biosciences) coated 6-well plate for hESCs attachment. The plate was cultured in NPC EB media for additional 7 days with media change every other day. After one day, EB adhered to the plate and in following 6 days the EB spread out and neural rosettes formed.

2.2.3 Neural Progenitor Cell formation

The neural rosettes were manually dissected by exposure to Accutase for 5 minutes and resuspended in NPC expansion media with a 1 mL pipet to triturate to single cells. The cells were then plated onto poly-L-ornithine (PLO; 10 μg/mL; Sigma) and mouse laminin (Ln; 5μg/mL; Sigma) coated tissue culture plate at a density of 100,000 cells/ml in NPC expansion media supplemented with 30ng/ml EGF, 30ng/ml FGF and 5 μM ROCK Inhibitor Y-267632 (Stemgent). The neural progenitor cells formed after one day.

For routine maintenance, NPC was cultured on PLO/Laminin coated plate with NPC expansion media supplemented with 30ng/ml EGF, 30ng/ml FGF and the media was changed every other day.

2.3: Polymer array Screening

2.3.1: Fabrication of polyacrylamide gel

Glass slides (75mm x 25mm x 1mm) were washed five times with Millipore water prior to being washed with 100% acetone and 100% methanol respectively for 30 minutes on an orbital shaker in order to remove residual debris and oils.

The slides were etched in 0.05 N NaOH solution on the orbital shaker for one hour, followed by five times rinsing with Millipore water. They were then slightly blow-dried with filtered compressed air in order to remove the residual water and then baked in a vacuum oven (70°C, 20 psi) for 1 hr.

The slides were then silanized in a 2% solution of 3-(trimethoxysilyl)propyl methacrylate in anhydrous toluene for 30 minutes, rinsed in fresh toluene twice, dried with compressed air, and baked for 15 minutes in a vacuum oven (70°C, 20 psi).

After Glutaraldehyde solution (0.5% (vol/vol)) was prepared, the slides were then Glutaraldehyde-activated for 15 minutes, rinsed three times with Millipore water, dried with compressed air, and baked for 15 minutes in a vacuum oven (70°C, 20 psi).

A stock solution of 10% (w/v) acrylamide, 0.55% (w/v) bis-acrylamide and 10% (w/v) photoiniator I2959 (200 μg/ml in 100% methanol; Igacure 2959, Ciba Specialty Chemicals) was prepared and 120 μl of this stock solution was then placed on a silanized slide and covered with a 75mm x 25 mm coverslip (Bellco Glass). The slide was then

placed in Stratalinker for 7 minutes under exposure to 1.5 mW/cm2 365-nm ultraviolet A light and after that immersed in Millipore water for 10 minutes.

The coverslip was removed with a razor blade, leaving a thin polyacrlyamide gel layer on the glass slides. The polyacrlyamide slides were then soaked in Millipore water and placed on the orbital shaker for 48 hour to remove residual unpolymerized acrylamide and photoinitiator, and then dehydrated by placing the slide face-up on the slide warmer for 10 minutes[24, 25].

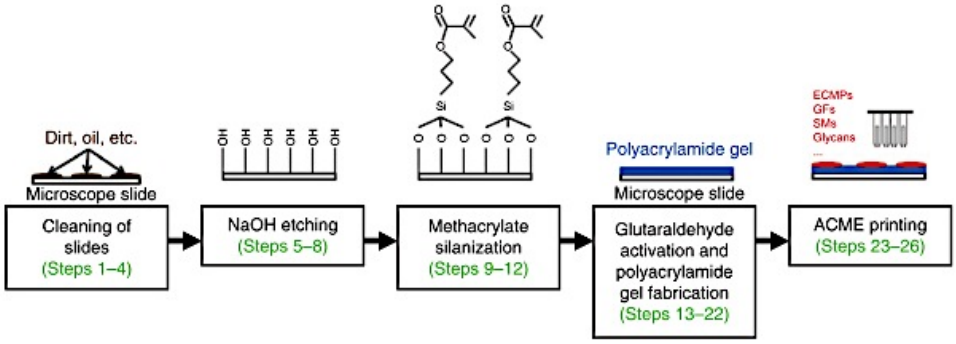


Figure 2: Arrayed microenvironment (ACME) slide fabrication[25]

2.3.2: Preparation of polymer solution

The synthetic polymer candidates were dissolved in appropriate solvent (either PBS or in *N,N*-Dimethylformamide (DMF, Sigma Aldrich)) with various concentration.

All of the polymers in the form of dry powder were purchased from Sigma. The polymers after weighted were added into solvent and vortexed vigorously until all

materials were completely dissolved. Store the solutions in dry and clean location at room temperature.

2.3.3: Synthetic polymer printing

We used a SpotArray24 spotter to print polymers. The spots were printed on range of 16x4 subarray. Each subarray was a designed matrix of 10x10 spots. Therefore, there were 6400 spots on a single slide; since we spotted each polymer in replicates of five for reducing experimental variability, each slide carried 1280 distinct polymers. Each spot had a diameter round 150 to 200 μm and the center-to-center distance between spots was 450 μm.

Before printing, the synthetic polymers were arranged within the 384-well plate to the final desired layout of the printed microarray. Each 384-well plate was covered with a plate sealer and the reagent plate centrifuged for 5 minutes at 1000 RPM. We then spotted arrays onto acrylamide-coated glass slides, and after printing we let the slides dry overnight at room temperature. Slides were stored in a dry, dark location at 4 °C before use

2.3.4: Cell seeding on polymer arrays

After polymer arrays printed slides were dry, each slide was placed face-up onto one well of quadriPERM culture dish, and each well gently washed twice with 5 ml of cell culture media. We harvested NPCs from tissue culture plate and resuspended in NPC expansion media supplemented with 30ng/ml EGF, 30ng/ml FGF and 5 μM ROCK

Inhibitor Y-267632 (Stemgent). After that, we seeded NPCs at an amount of 1 millions onto each well of quadriPERM culture dish and allowed them to grow for 7 days.

2.3.5: Identification of candidate substrates

We used "FACS-like" analysis with high-content imaging to identify potential hits. After NPCs settled on the feasible printed spots for 7 days, the cells were fixed and stained for NPC markers SOX1 and Nestin. The NPCs were then imaged with high-content automated microscopy by detecting and analyzing the intensity of fluorescence; the promising hits could then be identified.

2.4: Synthetic polymer scale-up

In order to apply stem cell therapies for neurodegenerative disease, it has been reported that 5 to 10 million cells are required per patient.[26, 27] Therefore, generating sufficient hNPCs for clinical research is imperative. According to the results from screening, one hit stood out from the others; therefore I took the promising hit into the next step, scaled up.

2.4.1: Synthetic polymer coated slides fabrication

I took the prepared polymer solutions (the preparation of polymer solution was described in section 2.3.2) and polyacrylamide gel coated glass slides into a sterile flow hood; at the same time expose FAST frame slide holders (Sigma Aldrich) to UV-light before use. Polymer solution was pipetted onto the top of polyacrylamide gel coated glass slide and solution was covered with coverslip.

The covered slides were carefully slid into FAST frame slide holders and incubated in 37°C/5% CO2 tissue culture incubator.

There were three parameters that control the quality and ability of the polymer coated polyacrylamide gel slides as follows:

1. The concentration of polymer solution
2. The pipetted volume of polymer solution
3. The incubation time

2.5: Characterization of hNPC proliferation on synthetic polymer

2.5.1: Multiple Passages

The hNPCs were passaged onto other polymer coated polyacrylamide gel slides when they were at 80 percent conflueny. I used the same method as I cultured those hNPCs on 10 cm tissue culture plate. However, after those cells were exposed to Accutase for 5 minutes, they were harvested without utilizing sterile plastic cell scrapper and replaced by twice washing with culture media. After centrifuging for 5 minutes at 200 RCF seeded 1 million cells to another slide, I saved the rest cells for further analysis.

2.5.2: Growth rate measurement

The purpose of this growth rate measurement was to quantify the growth rate of hNPC on P4VP and also compare the result to the growth rate of hNPC on laminin-coated slide. hNPC typically reaches 90 percent confluency after being cultured

on P4VP coated slide for 5 days. Therefore, we designed a five-day observation period for the test.

On day zero, we prepared 18 sample slides by coating 110 ul of P4VP onto 9 slides and 130 ul of laminin onto another 9 slides. This was then followed by 3 hours of incubation. In general, the 18 slides all were fabricated under the same experimental conditions. Lastly, hNPCs were seeded onto 18 coated slides at a density of 160,000 cells/ml per well of quadriPERM culture dish at the same time. On days one, three and five, three wells of cells on one P4VP coated slide and three wells of cells on one laminin-coated slide were accutased and counted. The media were replaced subsequently at days one and three for all remaining wells.

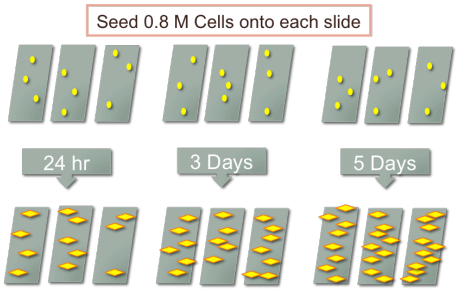


Figure 3: Schematic of growth rate experiment design

2.6: Characterization of hNPC differentiation on synthetic polymer

For characterization of hNPC differentiation on synthetic polymer, the hNPCs were seeded onto the polymers and cultured in NPC expansion media until the hNPCs reached approximately 80 percent confluency. At that time, I added 30 ng/ml BDNF and GDNF (R&D Systems), 0.5 mM cAMP (Sigma Aldrich), and 1 uM DAPT (R&D

Systems) onto NPC expansion media to induce neuron differentiation. I also changed half of the media everyday for two weeks and then collected neuron cells for further analysis.

2.7: RNA isolation and q-PCR

Cell were dissociated and collected by using Accutase, then stored at -80°C after being pelleted. RNA was isolated using the NucleoSpin RNA Kit and cDNA was synthesized using the qScript cDNA SuperMix (Quanta Biosciences). Quantitative real-time PCR (qPCR) was performed using TaqMan probes (Life Technologies) and TaqManFast Universal PCR Master Mix (Life Technologies) on a 7900HT Real Time PCR machine (Life Technologies), with a 10 min gradient to 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Taqman gene expression assay primers (Life Technologies) were also used. Gene expression was normalized to 18S RNA levels. All experiments were performed with two or three technical replicates. Data are presented as the average of the biological replicates ± standard error of the mean.

2.8: Flow Cytometry

Cells were dissociated and collected by using Accutase, collected and spun down. Cells were washed twice with FACS buffer (PBS, 10 mM EDTA, and 2% FBS) and stained with antibodies for 30 minutes on ice. The cells were then washed twice with FACS stain buffer and analyzed with FACSCanto.

2.9: Immunofluorescence Staining

For cell fixation, I aspirated the cell culture media and gently washed the array slides with FACS buffer (PBS, 10 mM EDTA, and 2% FBS). Then, I aspirated the FACS buffer and added 4 ml fixation buffer, fresh paraformaldeyde (4% (w/v)), into each well. This was then incubated for 10 minutes at room temperature and gently washed twice with 5 ml FACS buffer. Then the cells were permeablized with 5 ml PERM buffer (B&D Biosciences) for 30 minutes at room temperature. After being gently washed twice with 5 ml FACS buffer, the slides were stained with Primary antibodies and incubated at 4°C overnight covered with foil. On the next day the slides were gently washed twice with 5 ml FACS buffer and the Secondary antibodies were incubated at room temperature for an hour. Nucleic acids were stained for DNA with Hoechst 33342 (2 μg/ml; Life Technologies) for 10 minutes at room temperature covered with foil. The slides were than washed twice with FACS buffer and left 5 ml of FACS buffer in each well.

Fluorescence images were obtained using an automated confocal microscope (Olympus Fluoview 1000 with motorized stage and incubation chamber).

CHAPTER 3: Results

3.1: Polymer array Screening

Previously, high-throughput microarray technology was used in the lab to screen a library of hundreds of polymers in order to identify two synthetic matrices, which support the long-term expansion of hPSCs[24, 28]. Since the culture system built on synthetic materials for hNPC is yet to be developed, we used this polymer array technology to identify candidate polymers which support hNPC self-renewal and proliferation, and at the same time support neuronal differentiation. We screened 25 polymers at five levels of concentrations (15, 7.5, 5, 1, and 0.5 μM), which have been identified to support hPSC adhesion and growth.

After screening, those polymers were dissolved into appropriate solvent (PBS or DMF) and deposited onto polyacrylamide coated glass slides with microarray spotting instrument (Figure 4a). The polymers interpenetrated with the polyacrylamide gel and anchored on the polyacrylamide gel while the solvents evaporated (Figure 4d). After this, hNPC was seeded onto the polymer printed slides, cultured with NPC expansion media and supplemented with EGF and FGF for 7 days (Figure 5a). Since polyacrylamide gel inhibits cell growth, seeded cells could only be found on the polymer printed spots (Figure 4b). Of each slide, there were 6,400 spots. Also, each polymer was deposited with 5 replicates so a single slide contained 1,280 different polymers (Figure 4c).

We then fixed and stained each slide with NPC marker, SOX1, and NESTIN after those hNPC adhered and proliferated on printed slide for 7 days (Figure 5b). Finally, we analyzed the result by using high-content automated microscopy (Cellomics Array ScanVti) to measure Nestin and SOX1 fluorescence intensities at a single-cell level and plotted these intensities as a dot plot where each dot represents the intensity from a single cell (Figure 5c). We also compared the results to Laminin printed spots, which was the positive control we chose. As a result, we have successfully found several polymers so far, which are poly(4- vinylphenol) [P4VP], wpoly(azelaic anhydride) [PAzA], poly(styrene-co-allyl alcohol) [PSco-AA] and poly(styrene-maleic acid) [PSMA]). The polymers displayed similar characteristics of cell adhesion and growth as Laminin printed spots (Figure 6).

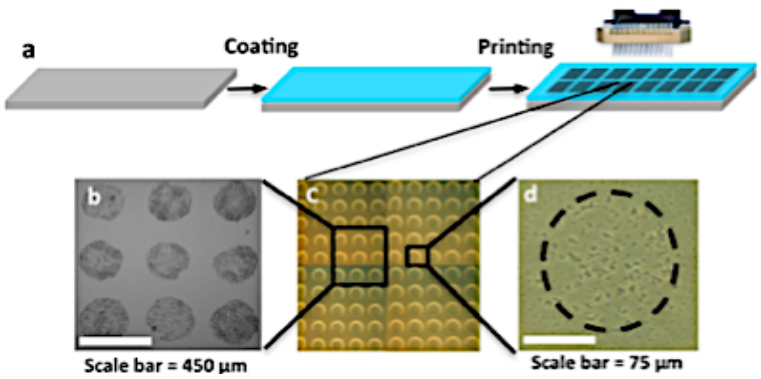


Figure 4: High-throughput polymer array technology

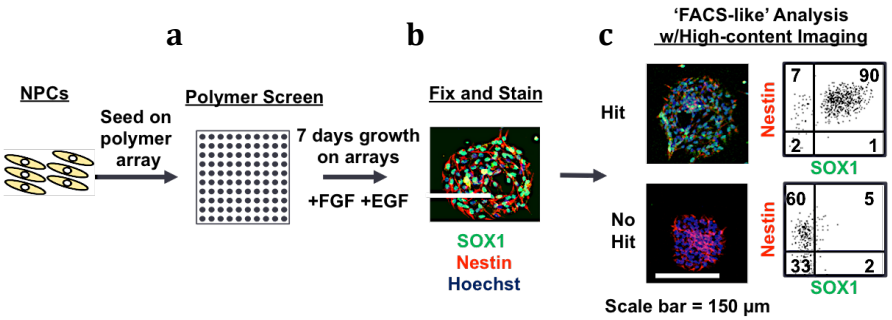


Figure 5: Schematic of polymer array screen to identify synthetic substrates

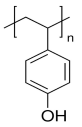
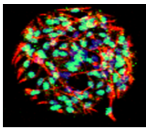
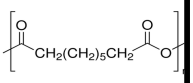
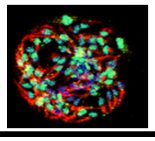
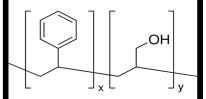
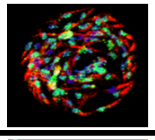
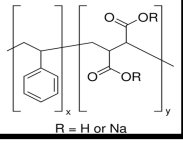
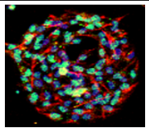
Polymer	Structure	SOX1 Nestin Hoechst
Poly(4-vinyl phenol) [P4VP]		
Poly(azelaic anhydride) [PAzA]		
Poly(styrene-co-allyl alcohol) [PS-co-AA]		
Poly(styrene-maleic acid) [PSMA]		

Figure 6: The four identified polymers that support hNPC growth

3.2: Optimization of large scale coating

After screening out the promising hits, the next step was to scale-up the culture area to the glass slide (of size 75 mm × 25 mm × 1 mm).

We tried several methods to coat polymers. One of the methods was to do it by printing where we deposited condensed polymer spots onto polyacrylamide coated glass slides. However, a drawback of this method was that it took 40 hours to print 5 slides, which was highly inefficient. Another alternative was to pipette polymer solutions with a 16-well chamber gasket; however, it was difficult to coat flat and thin layers. The third option was to pipette polymers solutions onto polyacrylamide coated glass slides by slowly placing coverslip along the edge of glass slide and incubating the slide with coverslip covered (Figure 5). So far we have found this method to be most feasible and successful in coating polymers. In addition, we also coated Laminin in a larger scale since we used Laminin (0.25 ng/ml, Invitrogen) as positive control. As the third method did not work for Laminin, I resolved the issue by pipetting Laminin solution onto polyacrylamide coated glass slides, using the coverslip's edge to wipe even the solution and incubate the slide without any coverslip.

As a result, we have developed a consistent system of polymer coated slide fabrication and have also seeded hNPC on those four promising hits. Additionally, we experimented several polymer combinations with different ratios but poly(4- vinylphenol) [P4VP] presented better results compared to the others. Hence, we decided to focus on this polymer for subsequent experiments.

As mentioned earlier in section 2.4.1, three variables influence coating conditions, namely the concentration of polymer solution**,** volume of polymer solution and incubation time. After several trials, we successfully optimized parameters for the coating processes. Concentration for P4VP was determined as 4% and proper pipetting volume as 110 ul and 130 ul for P4VP and Laminin respectively. Optimum incubation time was 3 hours and 7 hours for for P4VP and Laminin respectively.

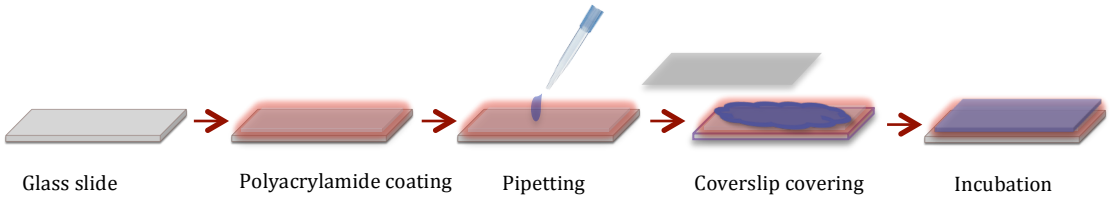


Figure 7: method of large-scale coating

3.3: Characterization of hNPC Proliferation on P4VP

3.3.1: Cell Adhesion

Prior to the process of seeding onto polymer coated slides, hNPCs were treated with Accutase and re-suspended into single cells. The polymer-coated slides were placed face-up into a quadriPERM culture dish and washed twice with culture media.

H9 and HSF NPCs were then seeded onto P4VP coated slides with 1×10^6 cells per well and then washed off the non-adhering cells and polymer debris after 24 hours of incubation. With this amount of cells seeded, the polymer coated slide provided sufficient

area for cells to attach and proliferate. Examination of the cells under microscope revealed that most NPCs were able to successfully adhere to P4VP coated slides (in both H9 and HSF lines). Cell survival rate was approximately 90 percent.

Subsequent observation after several days showed that P4VP demonstrated the ability to support growth of H9 NPCs. In fact, H9 NPCs could proliferate on the polymer-coated slides for 5 to 7 days to reach 80 percent confluency. On the other hand, P4VP supported limited growth for HSF as HSF NPCs began curling up and died gradually after the 72 hours culture (Figure 9) period.

We also verified the cells multipotency by flow cytometry after 5 days of culture on P4VP. FACS plots showed high percentage of NPCs-expressed NPC marker, SOX1, SOX2 and Nestin, which indicated that P4VP has the ability to maintain multipotency of hNPC (Figure 8).

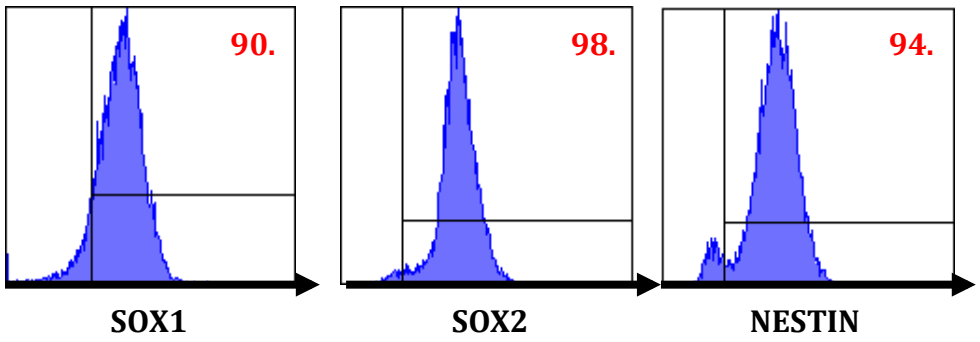


Figure 8: Flow Cytometry for H9 NPCs markers

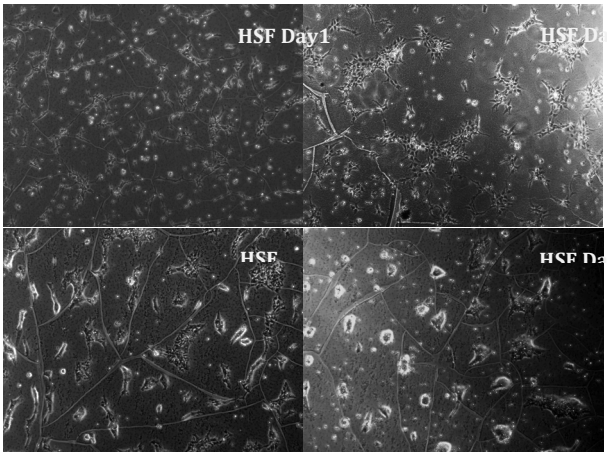


Figure 9: HSF cultured on P4VP for 4 days

3.3.2: Multiple Passages

Our discovery that P4VP has the ability to support NPCs adhesion prompted us to investigate if this polymer also has the ability to support long-term expansion of NPCs and at the same time maintain their multipotency.

When H9 NPCs were at approximately 80 percent confluency, the cells were passaged to the next P4VP coated slide. So far, P4VP supported H9 NPCs expansion for more than ten passages. Furthermore, the hNPCs maintained their morphology and expressed NPC markers of multipotency. Maintenance of multipotency was verified by the immunostaining (Figure 12) and quantitative RT-PCR (Figure 10) for neural progenitor cells markers SOX1, SOX2, and NESTIN. The expression level of the multipotency of NPC cultured on P4VP was comparable to the level expressed by NPCs cultured on Laminin. Hence, our results suggest that P4VP is able to support long-term

culture of hNPC. In addition, the phase images (Figure 11) show that hNPCs maintained their morphology even after 10 passages.

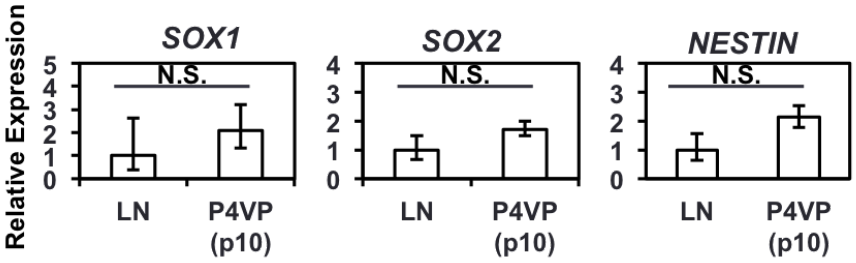


Figure 10: qPCR result from H9 NPC after cultured on P4VP for 10 passages

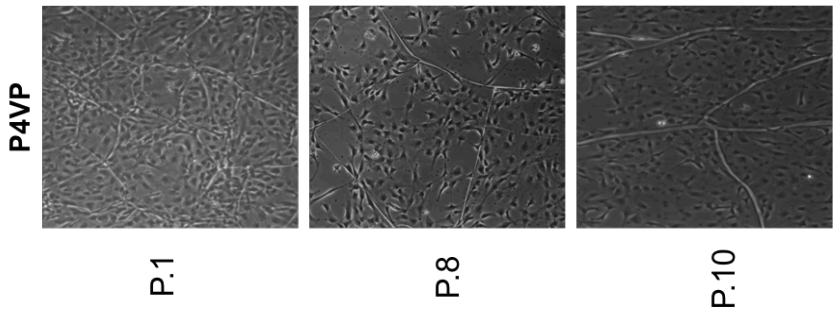


Figure 11: H9 morphology after 10 passages on P4VP

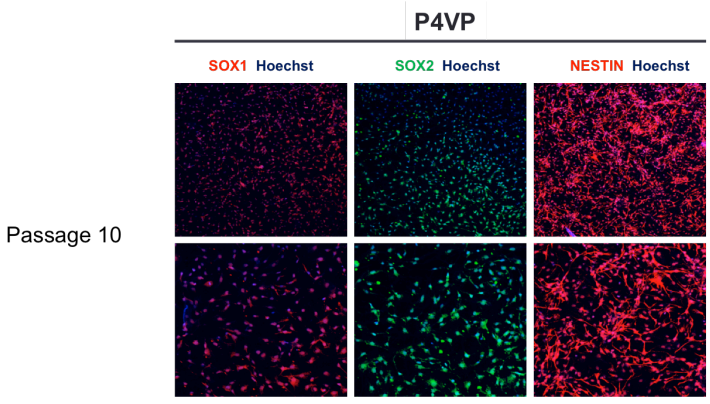


Figure 12: IF staining of NPC markers, SOX1, SOX2, and NESTIN

3.3.3: Growth Rate Measurement

After we counted cells at each point of time, we plotted the average cell number at each point of time against time and obtained a steadily increasing curve (Figure 13). This result verified that existence of cell proliferation on P4VP-coated slide.

The population doubling time (PDT) of cells on treatment and control group were also determined based on the curves.

Here is the slope intercept form of P4VP and laminin samples.

P4VP: $Y = 0.4461\,X\ + 0.3426$

LN: $Y = \ 0.4032\,X\ + 0.2966$

Hence, the doubling time of hNPCs were 53.8 and 59.5 hours on P4VP-coated slides and laminin-coated slides respectively.

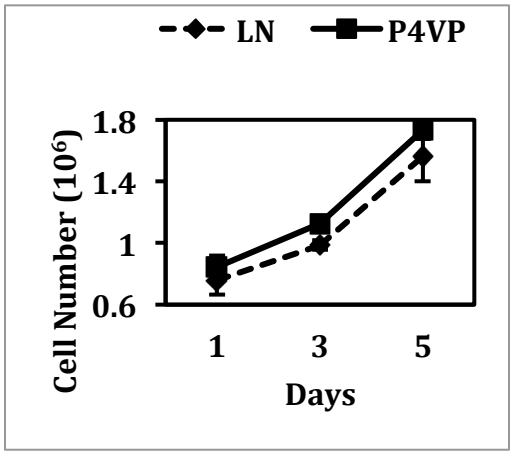


Figure 13: The growth curve of H9 NPC on P4VP and on laminin, respectively

3.4: Characterization of hNPC Differentiation on P4VP

To qualify for consideration as a confidential substrate for medical treatment, P4VP should possess the ability to support long-term self-renewal of NPC at the same time to induce NPC differentiation. Hence, after H9 NPCs cultured on P4VP reached 80 percent confluency, I added 30 ng/ml BDNF and GDNF (R&D Systems), 0.5 mM cAMP (Sigma Aldrich), and 1 uM DAPT (R&D Systems) into NPC expansion media. Then I aspirated and refilled half of fresh media amount everyday for two weeks. Usually, the P4VP coated slides with 3 hours incubation cannot be soaked in culture media for more than 7 days. The slides would crease and peel off. For this reason, I tried experimenting with longer incubation times and observed that 8 hours incubation periods exhibited better result. The slides with 8 hours incubation could last for 12 days without creasing and coming off the slide.

After being cultured for 2 weeks, some of the neuron samples were treated with Accutase and centrifuged at 200 RCF for 5 minutes; the collected cells were then used for quantitative RT-PCR (Figure 14). Some of the neuron samples were also fixed and stained for neuron marker, beta3 Tubulin (B3T) and MAP2 (Figure 15). The results of quantitative RT-PCR and immunostaining confirmed that P4VP coated glass slides were able to support induced differentiation of H9 NPC.

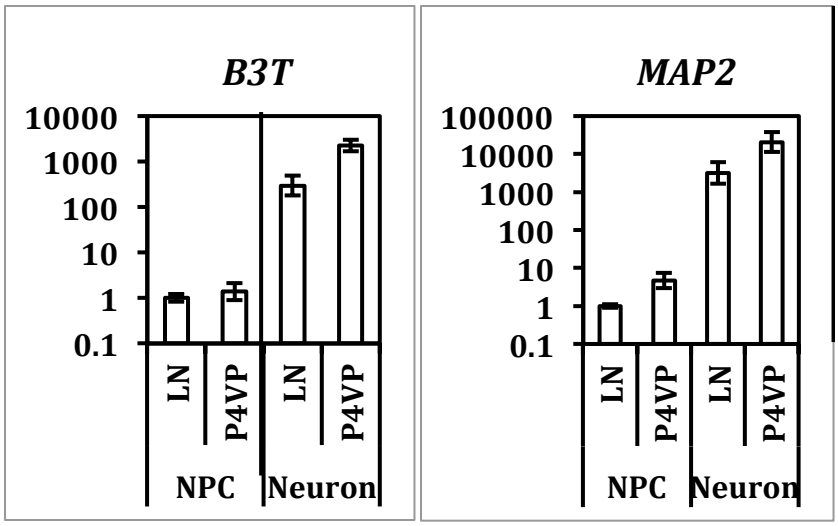


Figure 14: Expression of neuron marker, MAP2 and B3T in neurons after differentiated on P4VP for 2 weeks

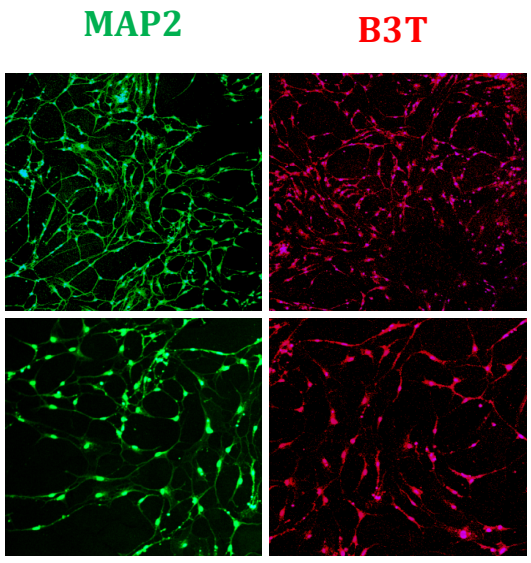


Figure 15: Neuron differentiated on P4VP expressed neuron markers, MAP3 and B3T in immunofluorescence analysis

13 Days Differentiation on P4VP

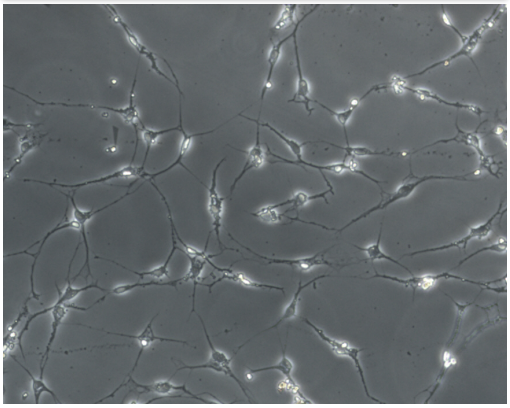


Figure 16: Phase contrast imaging of developing neurons

CHAPTER 4: Discussion and Future Perspective

4.1 Mechanism of cellular attachment

After obtaining positive results for our tests, our next objective is to understand the mechanism of cellular adhesion. Usually, cells bind to the surface through integrin and it had been also reported that positive charge surface highly affect cell adhesion which increased protein adsorption [15, 29].

There are other possible mechanisms: (1) Integrin dependence, (2) positive charge of polymer surface. We will investigate cellular adhesion based on these three aspects, starting with integrin dependence.

CHAPTER 5: Conclusion

In this research, we presented an alternative substrate for xenogeneic product replacement for hNPC long-term expansion and differentiation.

In order to be a feasible substrate, the polymer should be able to support NPC long-term *in vitro* self-renewal and at the same time maintain their multipotency and genomic integrity.

By utilizing high-efficiency printing technology, we obtained several potential candidates efficiently from the first screening. Followed by large-scale cell culture, the most feasible synthetic polymer stood out, which is P4VP.

P4VP possess the ability to support NPC proliferation and differentiation, since the NPC expressed NPC markers (SOX1, SOX2, and NESTIN) after multiple passages and also expressed neuron markers (MAP2 and B3T) after 2 weeks differentiation on the P4VP coated hydrogel.

However, the polymer-coated hydrogel cannot soak in culture media for more than 2 weeks as usually the hydrogel will crease and peeled off gradually. Therefore, in future we will modify our gel fabrication slightly in order to provide a better culture condition for neuron differentiation.

Appendix A – Polymers for screening

Number	Polymer Name
1.	Poly(ethylene glycol-co-acryloyl caproic acid)
2.	Poly(acryloyl-6-amino caproic acid)
3.	Poly(acryloyl-2-acrylamido glycolic acid)
4.	Poly(2-hydroxyethyl methacrylate)
5.	Poly(N-isopropylacrylamide)
6.	Poly(trimethylene carbonate)
7.	Poly(acryloyl- 4-aminobenzoic acid)
8.	Poly(acrylamido-methyl-propane sulfonate)
9.	Poly([3-(Methacryloylamino)propyl]dimethyl(3-sulfopropyl)ammonium hydroxide)
10.	Poly([3-(Methacryloylamino)propyl]trimethylammonium chloride)
11.	Poly(ethylene-co-acrylic acid)
12.	Poly(acrylic acid)
13.	Poly(L-lactide)
14.	Poly(D-lactide)
15.	Poly (DL-lactide-co-glycolide) 85:15
16.	Poly (DL-lactide-co-glycolide) 75:25
17.	Poly (DL-lactide-co-glycolide) 65:35
18.	Poly (DL-lactide-co-caprolactone) 86:14
19.	Poly (DL-lactide-co-caprolactone) 40:60
20.	Poly(caprolactone)
21.	Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid)
22.	Poly(3-hydroxybutyric acid)
23.	Poly(propylene carbonate)
24.	Poly(methyl vinyl ether-alt-maleic anhydride), hydrophillic
25.	Poly(sodium 4-styrenesulfonate)
26.	Poly-L-argine hydrochloride)
27.	Poly-D-lysine hydrobromide
28.	Poly-L-glutamic acid sodium salt
29.	Poly-L-ornithine hydrobromide
30.	Poly(2-ethyl-2-oxazoline)
31.	Poly(oligoethylene glycol methyl ethyl methacrylate
32.	Poly(butyl methacrylate)
33.	Poly(ethyl methacrylate)
34.	Poly(styrene-co-methacrylic acid)
35.	Poly-L-argnine hydrocloride
36.	Poly(ethylene gylcol) methacrylate
37.	Poly(styrene-alt-maleic acid)
38.	Poly(styrene)
39.	Poly(ethylene-alt-maleic anhydride)
40.	Poly(4-styrenesulfonic acid-co-maleic acid)
41.	Poly(methyl vinyl ether-alt-maleic acid)
42.	Poly(methyl vinyl ether)
43.	Poly(styrene-co-maleic anhydride)
44.	Poly(maleic anhydride-alt-1-octadecene)
45.	Poly(styrene-alt-maleic anhydride), methyl ester
46.	Poly(ter-butyl methacrylate)
47.	Poly(2-hydroxyethyl methacrylate)
48.	Poly(benzyl methacrylate)
49.	Poly(2-(dimethylamino)ethyl methacrylate)
50.	Poly(4-vinylphenol-co-methyl methacrylate)

51.	Poly(ethylene-co-gylcidyl-methacrylate)
52.	Poly(cyclohexyl methacrylate)
53.	Poly(tert-butyl acrylate-co-ethyl acrylate-co-methacrylic acid)
54.	Poly(ethylene-co-methyl acrylate-co-glycidyl methacrylate)
55.	Poly(ethylene-co-acrylic acid)
56.	Poly(ethylene-co acrylic acid)
57.	Poly(vinyl alcohol)
58.	Poly(vinylphosphonic acid)
59.	Poly(vinyl sulfate) potassium salt
60.	Poly(4-vinylpyridine hydrocholride)
61.	Poly(4-vinylphenol)
62.	Poly(4-vinylpyridine) crosslinked
63.	Poly(vinyl -co-ethylene)
64.	Poly(vinyl butyral-co-vinyl alcohol-co-vinyl acetate)
65.	Poly(ethylene-co-vinyl acetatate-co-carbon monoxide)
66.	Poly(allylamine hydrochloride)
67.	Poly(anetholesulfonic acid)
68.	Poly(epoxysuccinic acid)
69.	Poly(1,4-butylene terepthalate)
70.	Poly(styrene-co-4-bromostyrene-co-divinylbenzene)
71.	Poly(1,6-hexanediol/neopentyl glycol-alt-adipic acid)
72.	Poly(acrylonitrile)
73.	Poly(styrene-co-allyl alcohol)
74.	Poly(N,N'-(1,3-phenylene)-isophthalamide)
75.	Poly(trimellitic anhydridie chloride-co-4,4'-methylene-dianiline)
76.	Poly(Bispenol A carbonate)
77.	Poly(azelaic anhydride)
78.	Poly[trimethylolpronae/di(propylene gylcol)-alt-adipic acid/phtyhalic anhydride)
79.	Poly[di(ethylene glycol adipate]
80.	Poly(allyamine)
81.	Poly(diallyl dimethyl ammonium)
82.	Poly(diallyl methyamine hydrochloride)
83.	Poly(1-glyceryl monomethacrylate)
84.	Poly(3-chrolro-2-hydroxypropyl-2-methacroxyethydimethylammonium chloride)
85.	Poly(butadienne maleic acid)
86.	Poly(vinyl pyrroliodone)
87.	Poly(n-vinylpyrrolidone-vinylacetate)
88.	Poly(ethylenimine)
89.	Chitosan
90.	Poly(1-glyceryl monomethacrylate)

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