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ABSTRACT

NEURAL DIFFERENTIATION OF PLURIPOTENT STEM CELLS AND APPLICATIONS TO MICROPATTERNED AGAROSE HYDROGEL FOR NERVE GUIDANCE

by
Jamila S. Gittens

Many central nervous system (CNS) disorders are attributable to damaged oligodendrocytes, the cells that form the myelin sheath of axons. An effective source of human oligodendrocytes is crucial, making pluripotent stem cells, such as embryonic stem (ES) cells, an attractive source for nerve tissue repair and regeneration due to their unlimited capacity for self-renewal and their ability to differentiate into all major cell lineages. Bioengineered 3-D hydrogel culture systems have the potential to elucidate mechanisms required for regeneration of CNS cells. The goals of this study are: (1) to investigate the effects of retinoic acid (RA), sonic hedgehog (shh) agonist, and glucose levels on neural differentiation of mouse embryonic stem cells (mESCs), and (2) to employ micropatterned agarose hydrogels for nerve guidance. A protocol utilizing embryoid body (EB) formation, under both high and low glucose conditions, was devised for RA-induced neural lineage differentiation and RA/shh-induced oligodendrocyte differentiation. EBs exposed to lower glucose concentrations with agonists exemplified a more rapid differentiation. After 8 days in culture, there was a higher expression level of oligodendrocyte precursor cell markers in low glucose conditions. These results illustrate that physiologic glucose concentrations are more conducive to the development of EBs and favor the differentiation of mESCs into neural lineages. In order to fabricate 3-D microchannel hydrogel structures for nerve guidance, agarose solution was poured onto the plastic mold master with gridded microchannels and peeled off after hydrogel formation. An extracellular matrix (ECM) coating, such as collagen, was used to promote cell adhesion and neurite outgrowth limited to the channels. Exploitation of various cell types, including fibroblasts and PC12 cells, to optimize the hydrogel conditions, exhibited good cell and ECM patterns on the microchannels. Further studies using dorsal root ganglion (DRG)

neurons, Schwann cells, and differentiating ES cells demonstrated good cell growth and nerve guidance through the micropatterned channels. Micropatterned hydrogels can be used as an *in vitro* model to replicate complex tissue organization, and to investigate nerve signal propagation and myelination.

NEURAL DIFFERENTIATION OF PLURIPOTENT STEM CELLS AND APPLICATIONS TO MICROPATTERNED AGAROSE HYDROGEL FOR NERVE GUIDANCE

by
Jamila S. Gittens

A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Biomedical Engineering

Department of Biomedical Engineering

January 2012

APPROVAL PAGE

NEURAL DIFFERENTIATION OF PLURIPOTENT STEM CELLS AND APPLICATIONS TO MICROPATTERNED AGAROSE HYDROGEL FOR NERVE GUIDANCE

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Gittens, J.S., The Quantification of Collagen using Histological Staining and Polarized Microscopy, Center for Biophotonics Science and Technology Annual Scientific Meeting, Sacramento, CA, (2008).

This thesis is dedicated to my family who has always supported me throughout my academic career. I would not have accomplished this without their love and support.

ACKNOWLEDGEMENT

First and foremost, I would like to thank Dr. Cheul H. Cho for allowing me to work in his lab and for all of the guidance he has provided during this past year. I am very thankful not only for Dr. Cho's inspiring leadership, but also his exceptional accessibility to me and the other students.

I would like to express my gratitude to Dr. Bryan Pfister and Dr. Max Roman for providing notable expertise to my thesis committee.

I am also grateful for all of the members, past and present, of Dr. Cho's lab with special appreciation for Ali Hussain, Derek Yip, Nolan Skop, Divya Rajendran, Ishnoor Sinhu, Margaret Christian, Amad Saulut and Josette Pierre.

I would like to thank Dr. Haesun Kim's lab (Rutgers-Newark) for providing DRG neurons and Schwann cells.

Finally, I would like to thank my family and friends for their support.

This study was partially supported by Wallace H. Coulter Foundation and NJIT Start-Up Fund.

TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF FIGURES
(Continued)

Figure **Page**

CHAPTER 1

INTRODUCTION

1.1 Objective

There are many disorders of the central nervous system, such as multiple sclerosis, traumatic brain injury, and Alzheimer's disease, which are attributable to the loss of neurons and oligodendrocytes. The diseases that cause the most burdens on society are due to the lack of critical cell populations that the body is unable to naturally replace. The basic element of tissue repair and regeneration is to generate a clinically relevant number of the cell populations [1].

Neurons are the cells that transmit information in the nervous system through electrical and chemical signals. Neurons are composed of three components: dendrites, which are short branching projections that receive information; the cell body, inclusive of the nucleus and mitochondria; and the axon, a long fiber-like extension that relays ongoing information to the next neuron at junctions called synapses. Oligodendrocytes, cells supportive of the neuron, have thin extensions from their cell body, which wrap around the axons of neurons creating an insulating sheath of myelin.

Since there are no cures for these neurological disorders, research is being conducted on the use of pluripotent stem cells for cell replacement therapy and injectable hydrogels for repair and regeneration of neuronal tissue. For cell replacement therapy, effective sources of neurons and oligodendrocytes are crucial. Pluripotent stem cells, such as embryonic stem cells (ESCs), possess an unlimited ability to replicate themselves and to differentiate or transform into all cell types, under the correct conditions, making

them the most attractive option. The correct conditions include the physical environment of the cells, which plays a vital role in tissue repair. Hydrogels, 3D networks of polymer chains that retain water, can be used to provide physical cues and have mechanical properties similar to neuronal tissues. *In vitro* hydrogel culture systems aid in the elucidation of mechanisms required for the regeneration of the CNS.

The 1st objective is to investigate the effects of small molecules: retinoic acid (RA) and sonic hedgehog (shh) agonist and the effects of glucose levels on neural differentiation of mouse ESCs. RA is regularly used to differentiate stem cells into the neural lineage. RA with the addition of Shh is known to differentiate stem cells into oligodendrocytes at a similar rate observed in embryonic development. The rational for why I am investigating glucose levels is that it has not been established whether high glucose (HG) conditions or low glucose (LG) conditions are more efficient for the differentiation of stem cells.

The 2nd objective is to employ micropatterned agarose hydrogels for nerve guidance. Agarose, a natural polymer extracted from marine algae, is a solid at room and body temperatures, and a liquid at high temperatures (185°F or 85°C). This thermo-induced gelation ability makes agarose hydrogels easy to manipulate without the requirement of any chemicals that may be harmful to the cells. Cells do not attach to agarose so the addition of more cell friendly polymer and/or proteins are required to promote cell attachment. Patterning of cells allows the complex order of cells that exist *in vivo*.

1.2 Physiology and Anatomy of the Nervous System

The CNS is made of the brain and the spinal cord, which integrate and synchronize the processing of sensory data and transmission of motor commands. The CNS is also responsible for a person's memory, intelligence, and emotions [2].

Neural tissue contains two different types of cells: neurons and neuroglia. There is a variety of neuroglia cells which all create a supportive environment for neurons. Neurons communicate through electrical signals. Neurons are the longest cells in our body and can reach a length of thirty-nine inches. Neurons cannot undergo division because of their lack of centrioles, which are involved in the movement of chromosomes during mitosis. Although they are not capable of mitosis, neurons can regenerate a severed axon or "sprout new processes" in certain conditions [3].

Neuroglia cells are much smaller than neurons, but are comparatively much higher in numbers and possess the ability to divide. The cell body or the soma which contains a larger nucleus; dendrites, which project from the cell body, and one axon composed of nerve fibers are the three main parts of the neuron [2,3]. The dendrites are responsible for the retrieval of information while the nerve fibers transmit the signal to other cells through synaptic terminals. There are there structurally different neurons: multipolar, unipolar and bipolar. Multipolar forms are the most common neuron found in the CNS [2].

There are four types of neuroglia located in the CNS, which are: astrocytes, oligodendrocytes, microglia, and ependymal (Figure 1.1). Astrocytes are the most prevalent and are responsible for preserving the blood-brain-barrier, which keeps the CNS secluded from the rest of the circulation system, by releasing vital chemicals. In

addition to managing the blood-brain-barrier, astrocytes establish the structural environment for neurons and are involved in the regeneration of damaged neural tissues [2].

Microglia are the smallest and the least abundant glial cells in the CNS. They are phagocytic and are responsible for engulfing cellular waste and pathogens. Ependymal cells make up the lining of the central canal of the spinal cord and the cavities of the brain that are filled with cerebrospinal fluid [2].

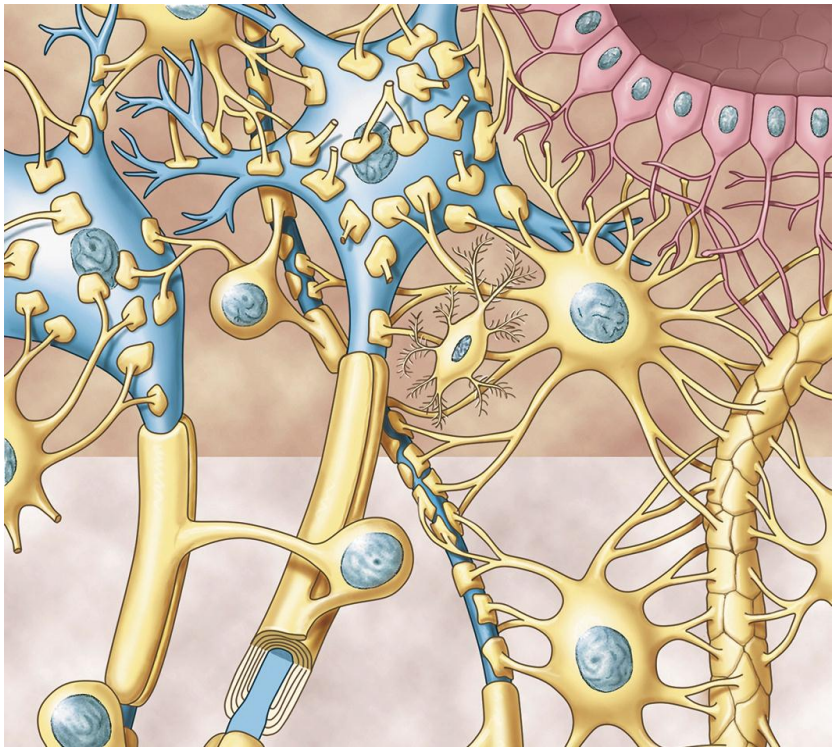


Figure 1.1 The cells of the Central Nervous System
Source: [4]

Oligodendrocytes have fewer and thinner extensions from their cell body compared to astrocytes, which wrap around the axons of neurons creating an insulating sheath of myelin. Each oligodendrocyte covers short sections of the axon, creating a space between each sheath, called a node. These sheaths allow an increase in the speed at which the action potential travels through the axon by minimizing the escape of ions and

capacitance of the axonal membranes. The propagation speed of the signal is significantly important for axons that extend to a length of one meter [3,5].

Oligodendrocytes are different from astrocytes in several ways including: smaller in size, denser cytoplasm and nuclei, lack of intermediate filaments and glycogen, and a larger number of microtubules. Oligodendrocytes have several processes that form sheaths of myelin around axons. These processes vary in number depending on the location in the CNS. In early development, oligodendrocyte progenitors differentiate into mature oligodendrocytes and begin the production of myelin and the wrapping of the sheath around axons, after receiving the necessary signal from the axons. These signals have yet to be fully understood. One oligodendrocyte forms myelin around more than one axon causing adjacent myelin segments to belong to different oligodendrocytes. Myelin consists of lipids and has low water content, which allows the electrical insulation of axons. There are four different types of myelinating oligodendrocytes that are categorized based on the length and diameter of the axon around which they form the myelin sheath [6,7].

1.3 Nerve Injury and Neurodegenerative Diseases

Neurodegenerative diseases like multiple sclerosis and Alzheimer's disease lead to often debilitating neurological dysfunction due to the diffuse loss of cells. Traumatic injuries and diseases such as Parkinson's disease affect only a certain type of cell or area. As a result of these disease processes, secondary injuries occur including anoxia, ischemia or hemorrhaging, free-radical formation and excitotoxicity [8].

Injury to the central nervous system through a physical injury or a neurodegenerative disease causes the loss of neuronal cell bodies, axons and supportive

glial cells. Death of these cells due to a lack of blood supply can also be caused by ischemia, a hemorrhage or trauma. The infiltration of macrophages, which aids in the removal of inhibitory myelin, occurs slowly in the CNS. Macrophage recruitment is limited because of weakening of the blood-spine barrier and the lack of up-regulation of cell adhesion molecules at the injury site [3]. Once the CNS has been damaged it leads to an inflammatory response, which causes microglial infiltration and proliferation of astrocytes on the site. This process is called glial scarring and prevents the regeneration of neurons. Not only does this pose as an obstacle, but the cells themselves do not have the capacity to proliferate, leading to permanent damage. Once this is overcome, the cells then require cues for cell migration, acquisition of the correct neuronal phenotype, and axon guidance to the relevant target. These signals are present in a developing brain, but are not present in an adult brain. In other organs there is a balance of suppression and proliferation maintained by supporting cells and basement membranes. The supporting cells in the CNS are oligodendrocytes and astrocytes which suppress proliferation and neurite outgrowth.

1.3.1 Axon Growth and Glial Scarring

The tip of a neurite is the growth cone, which has many filopodia. The filopodia are supported by microfilaments and are the part of the neurite that react to cues of their environment. Growth-inducing chemicals signal the axon toward the correct target. These chemicals are “gradients of diffusible trophic factors,” and also may be attached to the surrounding cell surfaces or extra cellular matrices. Adhesion molecules are responsible for regulating the adhesion of the growth cone and their micro-spikes to their surrounding surfaces by maintaining neurite outgrowth in the appropriate direction

through cell to cell adhesion. These two types of chemical cues work in cohesion as guidance molecules for the growing axon.[9].

Adhesion factors also play important roles in axon growth. The growth cone interacts with the substrate surface and the more strongly the F-actin filaments of the growth cone attach to the surface, the faster the neurite grows. "Differential adhesion can therefore direct growth and this selective adhesion can occur through cell-cell interactions via cell adhesion molecules (CAMs), or through cell-substrate interaction via (SAMs)." Laminin and fibronectin allow cell adhesion and neurite outgrowth [9].

Mechanical cues also activate neurite outgrowth. These mechanical cues which effect neurite outgrowth and focal-adhesion include hydrogel pore size and the elastic properties of the substrate, respectively. For stem cells, the cytoskeleton mediates their differentiation and proliferation [9]. When the GC reaches its destination cell, the filopodia of the GC penetrate the cell, which is prohibited by glial scars when an injury occurs in the CNS [9]. The glial scar consists of fibrous astrocytes with interwoven processes and basement membrane proteins, like collagen type IV and chondroitin sulfate proteoglycans [9].

1.4 Present Methods of Nerve Regeneration

Currently, there is no therapeutic intervention that restores nerve function in the CNS; the only viable options are most often taking the necessary precautions to prevent secondary injury. These interventions include surgery if there are any bone fragments near the site of injury, decompressive craniectomy and anti-inflammatory drugs. When the swelling from the injury subsides, the patient undergoes a long period of rehabilitation which forces the remaining nerves to compensate for the damaged ones [3]. There has been

progress with regeneration of fibers in the CNS with the support of embryonic spinal cord and peripheral nerve tissue grafts. Unfortunately, these grafts were unable to get the fibers to grow across the PNS-CNS transition zone. Utilization of grafts only prevents neurons from dying and serves as a support for sprout growth. This lead to the realization of the need for physical or mechanical guidance cues, cellular components and biomedical signals to be included in the device [3].

There are several key steps in the process of regeneration of nervous in the central nervous system. The first is to regenerate the lost cell bodies and axons of the neurons. Second is to re-establish the synaptic connections between these neurons. Third, the supporting glial cells, blood supply and extracellular scaffolds must be generated. And lastly, anatomical layers and overall microenvironment must be stabilized [8]. Table 1.1 lists the obstacles of nerve regeneration and the possible strategies for each hurtle.

Table 1.1 Regeneration Obstacles and Strategies for Repair

Regeneration Obstacles	Strategies for Repair

• **Cell Body Response**
  ○ **Retrograde Cell Death**
  ○ **Low Expression of Regeneration-associated genes**
• **Glial Scar Formation**
• **Inhibitory Molecules**
  ○ **Myelin-associated glycoprotein**
  ○ **Nogo**
  ○ **Chondroitin Sulfate Proteogylcans**
  ○ **Semaphorins, ephrins, netrins**
  ○ **Tenascin**
	• Guidance Therapies
	  ○ Graphs
	  ○ Support Matrices
	• Biomolecular Therapies
	  ○ Neurotrophic Factors
	  ○ Regeneration-associated and Antiapoptosis Genes
	  ○ Blocking Inhibitory biomolecules
	• Cellular Therapies
	  ○ Stem Cells
	  ○ Schwann Cells
	  ○ Macrophages
	  ○ Genetically Modified Cells

Source: [3]

1.5 Mouse Embryonic Stem Cells (mESCs)

During early stages of development in an embryo, the CNS is created by the neuroectoderm and is predominately composed of neuroepithelium. These neuroepitheliumal cells are columnar in shape, proliferate rapidly; and subsequently differentiate and mature into neurons, astrocytes and oligodendrocytes [10]. *In vitro* differentiation of stem cells into neuroectoderm lineage cells will aid in the understanding of the molecular events that occur in the early developmental stages of the CNS *in vivo* [10]. Pluripotent stem cells, such as mouse embryonic stem cells (mESCs), are taken from the inner cell mass of developing blastocysts. Under the correct *in vitro* conditions, these cells have the ability to proliferate and generate several types of cells from the three primitive germ layers of an embryo.

Stem cells give scientists the ability to study the processes that guide *in vivo* differentiation in the embryo and the promise of potential new cell replacement therapies for human neurological disease[10,11,12,13]. Pluripotent stem cells are the most attractive option due to their intrinsic capabilities. In addition to their pluripotency and ability to expand, they are relatively easy to isolate. Neural stem cells are a less reliable and effective source of myelinating cells than ESCs, although the issue of immune rejection in patients is still an obstacle that needs to be overcome by scientists [13].

1.5.1 Differentiation of mESCs

The proliferation and differentiation of stem cells can be manipulated by a set of distinct influences [12]. Their pluripotency or their ability to self-renew is maintained by exposure to leukemia inhibitory blastocyst (LIF) or with a feeder layer of mouse embryonic fibroblast (MEFs) [14]. The addition of other factors can be used to facilitate

the differentiation of cells into specific lineages, for example RA and shh agonist is used to differentiate cells into the neuroectoderm lineage, like neurons and oligodendrocytes [1].

Along with these chemical cues, physical stimuli are required for the differentiation of ESCs. There are three different approaches to generating somatic cells from mouse embryonic stem cells: embryoid body formation, monolayer cultures on extracellular matrix proteins, and culturing ESCs directly on supportive stromal layers in serum-free or undefined medium [1,11].

EBs are used because they imitate features of cell differentiation during early mammalian embryogenesis [15]. EBs allow cell-to-cell and 3D interacts, similar to the environment in an embryo during natural development, and develop gap function formation. They are used instead of embryos and whole animals [15]. There are two phases of early formation and differentiation of EBs. The first stage is the formation of simple EBs, which forms from day 2 – 4. These simple EBs are composed of an endoderm on the surface of the EBs. On day 4, the EBs begin to form a central cavity and a columnar epithelium with a basal lamina. If allowed, culturing EBs will allow the differentiation of cells indicative of the ectoderm, mesoderm, and endoderm germ lineages. The specificity of the cell types can be altered by changes in the culturing environment and extended culturing of the EBs. [16].

There are several different ways to create these cell aggregates, methods include: cell suspension in bacterial-grade dishes, culture in methylcellulose semisolid media, hang-drop (HD) formation, and the use of round bottom plates or conical tube. The utilization of spinner flasks and bioreactors are other methods more recently discovered

to be useful. The employment of a particular method for ES differentiation depends on the scientist's end goal. Each method has its own unique aspects, disadvantages and advantages [14]. Some methods create more morphologically uniform EBs than others.

Methylcellulose cultures are being used for hematopoietic differentiation, but are difficult to use because of the semisolid solution. The use of spinner flask or bioreactors allows a scalable production of EBs and easy control of process strategies, but requires two steps in order to prevent agglomeration. The employment of round-bottom 96-well plates also produces controlled sizes of EBs, enables to EBs to be observed by microscope and allows change of the medium. Hang-drop culture produces more controlled sizes of EBs, is usually used for mESCs and requires the use of bacterial grade dishes due to the limited liquid volume in each drop. The cell suspension method is the most basic and creates EBs with a large variety of morphologies. [14].

1.5.2 Differentiation of ESCs into the Neural Lineage

The first step in the differentiation of ESCs is the development of epiblasts of the mouse embryo. If pathways to the formation of the primitive streak are not induced, the epiblasts will form into cells on the ectoderm lineage (neural or epidermal). Therefore, differentiation into the neuroectoderm is referred to as the "default" pathway, even though it has been show that differentiation into this lineage is dependent on Fibroblast Growth Factor (FGF).

When using EB formation, exposure to high concentrations of retinoic acid has been shown to be required to promote the generation of neural cells. These EBs must then be placed in a serum-free environment after the EB formation is complete [17].

The most commonly used method, which was developed by Bain in 1994 to differentiate ESCs into neural cells, is the 4-/4+ protocol. This protocol consists of forming EBs for 4 days without RA and exposure to RA the subsequent 4 days [18].

1.5.2.1 Oligodendrocyte Differentiation. There are five key developmental stages that an embryonic stem cell goes through before becoming a terminally mature, myelin producing oligodendrocyte. There are several markers used to classify and to confirm these stages of differentiation during experimentation. During the first stage or the pre-progenitor stage, the morphology of the cells is round and they positively express nestin, PSA-NCAM, Group B1, Sox, and Olig2. Next is the oligodendroblast stage during which these early bipolar progenitors express A2B5 and NG2 positively. These cells are also called oligodendrocyte-type-2 astrocytes. The cells become pre-oligodendrocytes and are morphologically multipolar or arborizing during the third stage. These cells are positive for the expression of O4, which identifies the sulfatide glycolipids in the immature oligodendrocytes. Immature oligodendrocytes are established next and start to express GalC and O1 markers. Finally, as mature oligodendrocytes, the cells begin to express positively for myelin basic protein (MBP) and CNPase (Figure 1.2) [19].

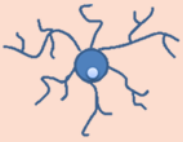
Embryonic Stem cell	Neural Stem Cell	OL Progenitor	Pre-OL	Immature OL	Mature OL
					
Oct 4	Nestin	Olig 1 Olig 2 NG2 A2B5	Olig 1 Olig 2 O4	O1 O4 CNP GPDH	O1 O4 CNP GPDH MBP

Figure 1.2 Differentiation stages of oligodendrocytes from embryonic stem cells.

Fraichard and colleagues worked on differentiating ESCs into not only functional neurons, but also glial cells. They formed EBs by cell suspension in medium containing RA for 2 days and then plated these aggregates. On day 3, the majority of the cells were positive for the expression of nestin, signifying the presence of neuro-glia precursor cells. The O4 positive cells continued to be a minority in the cell population of about 1 percent. The GFAP positive cells increased to about 75% on day 9. On day 5, MAP2 positive cells were first observed and increased in expression from day 9 at 25% to 90% on day 20. They concluded that differentiation of ESCs into the neural lineage does not require complex conditions compared to differentiation into oligodendrocytes to a detectable level [20].

Tokumoto and colleagues were able to differentiate ESCs into oligodendrocytes with more than a 30% efficiency rate by using ITS/fibronectin medium for the early stages of differentiation and N2 plus/bFGF medium for more mature differentiation after the EBs were dissociated [13]. Studies performed by Su Lie et al. and Jiang et al. produced very efficient protocols for oligodendrocyte differentiation. Su Liu et al.

increased the percent yield of oligodendrocyte cells and were successful in implanting these cells to determine their ability to myelinate neurons in a damaged CNS. Their protocol was unique in that it required the isolation of oligodendrocytes from the rest of the cell population by taking advantage of the oligodendrocyte's inability to adhere firmly to flasks in which they were cultured. With this additional step, they were able to produce a cell culture primarily of oligodendrocytes or 92 ± 7%, with few neurons or astrocytes present [21]. Jiang and colleagues used shh agonist purmorphamine to generate 80.3% GFP positive cells of which 96.4% were NG2 positive [22].

1.5.3 Retinoic Acid Concentration Effects

All-trans retinoic acid (ATRA) is the biologically active Vitamin A and plays a key role during embryogenesis and cellular differentiation [11,23]. It specifically impacts "neural development in the early stage of CNS development and is required to establish patterned territories of cell groups" [11]. ATRA regulates gene expression through the retinoic acid receptor (RAR) [23]. Therefore, RA has been determined to be the most efficient extrinsic inductive signal to differentiate mouse ESCs *in vitro* [11].

The aggregation of cells in serum containing medium and without the influence of RA promotes the differentiation of mESCs into non-neural lineages, like cardiac cells. This can easily be altered with the addition of 0.5 to 1μM RA to yield results with high numbers of neurons and astrocytes [24]. It has been found that RA up-regulates markers of the neuroectoderm, pancreatic beta cells, hepatocytes and cardiomyocytes, while other differentiation pathways seem to be repressed [23]. It has been shown that ESCs cultured in medium with serum are in an active retinoid signaling state and RARs are present on

these ESCs, while the enzymes involved in the processing of RA are not present. Therefore, it can only be concluded that there is RA in serum and is used by the cells. With the combination of the removal of LIF and beta-mercapto-ethanol and the formation of EBs, the existing amount of RA in the serum is enough for the differentiation of cells, but a spontaneous or non-specific differentiation. During this transition state, the RAR mediated signaling is blunted, allowing a wider range of lineages to develop. If the cells are exposed to a higher level of RA there is subsequently an alteration in the present spontaneous differentiating program and regression to a previous differentiation state. A restricted stem cell program and a newly initiated retinoid driven differentiation program remain active in the EBs.

They also proved that the time at which RA is added to the culture affects the differentiation of the ESCs. If the RA was induced during the first 4 days, the gene expression for the endoderm was present in high levels. Whereas, if the RA was induced after the fourth day there was a significant increase in the neuroectoderm markers [23].

A study by Okada and colleagues revealed that the amount of RA on mouse embryonic stem cells is directly related to the neutralization and the positional specificity of mouse embryonic stem cells during differentiation[11]. Nestin positive cells were more prevalent in EBs exposed to low concentrations of RA. GFAP and β-III tubulin positive cells increased their expression directly with the increase in RA concentration. But the CNPase, an oligodendrocyte marker, was not strongly detected for all differentiation conditions and its expression was not determined by the change in RA concentration. Immunocytochemistry also supported these findings except the Olig2 marker was increased 9.1-fold in the low RA concentration in contrast to the high RA

concentration. They concluded that higher concentrations of RA induce embryonic stem cells to differentiate into post-mitotic glial cells and neurons and low concentrations of RA generate undifferentiated neural progenitor cells [11].

Prior to the Okada study, it was widely accepted that RA increases the rate of differentiation of ESCs and that the concentration of RA determined what kind of cells where generated. Lower concentrations of RA induce the differentiation of mesodermal cells, while high RA concentrations promote the differentiation of ESCs into the neural lineage. This study shows that RA concentration affects the terminal differentiation of neural cells. The results revealed that the concentration level of RA was directly proportional to the expression levels of differentiated and undifferentiated neural cell markers. Levels of differentiated neural cell markers were more prevalent in EBs exposed to higher levels of RA [11].

1.5.4 Sonic Hedgehog Agonist Effects

Sonic hedgehog (shh) agonist is one of the three proteins of the hedgehog-family that are responsible for activating the signaling pathway that regulates cell fate during development and during adulthood [25,26].

It has been proven that this signaling pathway is hedgehog (Hh) ligand concentration sensitive and can direct the differentiation of cells into specific neuronal cell types. It has also been shown that Hh signaling persuades the proliferation of granule neuron precursors that patterns the cerebellum [26]. Shh signaling pathway patterns the ventral neural tube by promoting the expression of transcription factors such as Olig1 and Olig2, two markers associated with early differentiation stages of oligodendrocytes [27]. Differentiation into different cell types requires different concentrations of Shh. The

addition of Shh to embryoid bodies promotes their differentiation into interneurons and motorneurons. It has also been concluded that Hh signaling aides in the proliferation of neural stem cells or a more specialized neural cell type. Maye and colleagues used mutant cells for components of the Hh signaling cascade. They found that these cells were not capable of generating EBs with a neuroectoderm, were unable to respond to the induction of RA and, therefore, were unable to differentiate into mature neurons and glia [28].

Directing progenitor cells along specific pathways of the neural lineage is difficult; because the mechanisms that lead to the variety of CNS cells have not been well defined [29]. Shh is found in high concentrations in the notochord and floor plate where oligodendrocytes and motorneurons are located in the CNS [30].

1.5.5 Glucose Concentration Effects

Although it has been established that it is most efficient to maintain embryonic stem cells in high glucose medium, it has not been extensively investigated if the high glucose conditions are best for the formation of embryoid body formation and differentiation of these stem cells. It has been assumed that high glucose is required to form aggregates in a high density cell culture [15]. High glucose medium contains 25mM of glucose in contrast to low glucose medium which contains only 5.5mM of glucose. High glucose concentration medium has been used for the maintenance and proliferation of embryonic stem cells, although "It has been hypothesized that the physiological glucose concentration in the final differentiation stage was instrumental in developing improved cell function" [16]. Studies have supported such hypotheses with the findings of loss of differentiation and insulin secretion in adult pancreatic beta cells that were cultured in

high glucose conditions. The lack of glucose exposure has been shown to enhance the development of human and mouse embryos with no glucose exposure [16]. There is also evidence that diabetic levels of glucose have a negative impact on the developing embryo *in vivo* [31,32].

Mammalian pluripotent stem cells rely on glycolysis as their major source of energy to proliferate due to their immature mitochondria. When embryonic stem cells differentiate, their mitochondria become more mature and metabolic activity shifts to oxidative phosphorylation (OXPHOS) [15,33].

High glucose concentration medium is also used for the studying of ESC differentiation. The high concentration of glucose is similar to hyperglycemic conditions, while normal physiological conditions are 5.5mM of glucose. Previous studies show that there were increased levels of intracellular reactive oxygen species (ROS) and inhibition of 2-deoxyglucosee uptake in cells exposed to high glucose medium. The inhibition of 2-deoxyglucose uptake then causes oxidative stress on mouse ESCs. It has also been shown that the differentiation human and mouse embryonic stem cells is improved in glucose deficient media.

Mochizuki et al. used a variety of glucose concentrations to determine the differences in the EBs physical features, gene expression lineages and the efficiency of induction for mESCs. The EBs in the high glucose did not grow more rapidly than the EBs exposed to the LG medium and the medium with the super high level of glucose. There was no significantly different decreased expression of Oct3/4 and Rex1 in the HG and LG medium. The EBs of LG medium produced more β-III tubulin than the EBs exposed to HG concentrations. LG EBs only expressed genes for nestin and microtubule-

associated protein (MAP), while HG EBs outgrowth also expressed positively for myosin heavy chain (α-MHC) and transthyretin (TTR). The glucose consumption by the EBs in the LG cultures was inhibited even though there was more glucose left to consume. It was concluded that pyruvate was consumed instead of glucose. This amount of pyruvate uptake is similar to that consumed in fertilized mouse ova, making the LG condition more feasible for EB formation and differentiation of mESCs [15].

Khoo et al. performed a study based on the investigation of the morphology and gene expression of embryoid bodies over a period of 104 days. They used human embryonic stem cells and exposed the EBs to various concentration of glucose and Fibroblast Growth Factor 2 (FGF-2). They concluded that EBs exposed to lower glucose concentrations had a more rapid growth rate than the EBs exposed to 25mM glucose concentrations. The gene expression in both concentrations was similar, leading to the conclusion that the lower glucose concentration provides the necessary conditions for differentiation without the possible impairment in cell function. They also found that the use of FGF-2 supplement enhances the gene expression of neural endocrine markers in EBs formed by cell suspension [16].

1.6 Hydrogels

There are two types of hydrogels, synthetic and natural. Natural hydrogels are more likely to be biocompatible and degraded by natural existent enzymes. Also, they are likely to be capable of appropriate cell signaling without the requirement of the addition of growth factors [9].

Hydrogels may be preferred over scaffolds because they contain low interfacial tensions and are permeable to nutrients and oxygen due to their macroporosity. Low

interfacial tension does not provide much of a barrier to cells in neighboring areas from migrating into the hydrogel. The mechanical properties of hydrogels are similar to their natural supporting tissue and their elasticity can be changed by controlling the crosslink density of the material. Hydrogels can also conform to unique defect shapes and can "include [neurotrophins] NTs for control of neuronal cell adhesion, proliferation and axonal extension." The hydrogel must also be able to remain intact the entire duration of the regeneration process *in vivo* and solidify without chemical manipulation because of the easily disrupted nature of the nervous system [9,34].

The charge and the material properties of the hydrogel determine the polymer's ability to promote cell adhesion and extension. Hydrogels with a positive charge enable cell adhesion due to a cell's net negative charge. It has also been discovered that softer polymers or polymers closer to the mechanical structure of natural brain matter better facilitate cell adhesion and proliferation [35].

1.6.1 Agarose

Agarose is a linear galactan hydrocolloid purified from marine algae [34]. Structurally, agarose consists of repeating disaccharide units (Figure 1.3). This natural polymer solidifies at 8 - 17 °C and melts at approximately 37°C. The physical structure of agarose can vary depending on the concentration of agarose in the gel. The variation in concentration alters the pore sizes of the gel, inevitably changing the chemical structure of the gel.

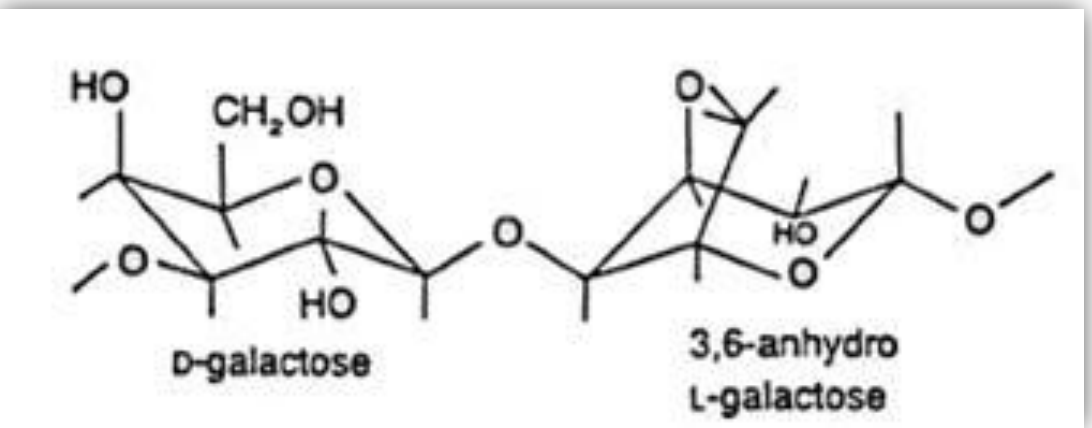


Figure 1.3 Chemical structure of agarose. This figure illustrates that agarose consists of a repeated chain of D-Galactose and 3,6-Anhydro-L-Galactose subunits.
Source: [36]

Agarose forms thermally reversible hydrogels which are widely used as three dimensional scaffolding for neuronal engineering and cartilage tissue engineering [37]. Agarose has also been considered for the use of drug and living cell delivery. Although not biodegradable, agarose is biocompatible and porous, which provides the appropriate environment for the spreading and proliferation of cells. In contrast to its friendly environment for cell proliferation and spreading, it is not cell adhesive, restricting its application. It order to enable agarose to promote cell adhesion, chemical functionalization and blending with other materials (natural polymers) has been under investigation. While hydrogel blends alter the mechanical and thermal properties of agarose, chemical functionalization is costly and time-consuming [38].

1.6.2 Micro-patterned Cell Cultures

There are several techniques, including photolithography, micro-contact printing, microfluidic patterning using microchannels, laminar flow patterning, and stencil patterning, used today in order to reconstruct the structural environment that cells grow in

naturally. These approaches aim to control cell growth, proliferation and differentiation on scaffolds. Cells interact with external stimuli, physical and chemical cues, provided by their environment, which include topography and composition of the extracellular matrix. These techniques have been used to help scientists and engineers gain knowledge about interactions between cells, the surface on which they exist and the medium from which they get nutrients [39,40].

Photolithography is a well-developed technique and has been used in various ways to pattern cells. The photolithography technique requires the use of a light, photoresist and a mask to create the desired pattern of cell adhesion material. Often polylysine, fibronectin, collagen, and Matrigel are used as the cell adhesion material. The cell suspension is then incubated on the fabricated surface creating cell adhesion in the desired pattern. Although this technique is well developed, there are many disadvantages including the need to fabricate the substrate in a clean room, a requirement for expensive equipment, and the need for the use of toxic chemicals.

Micro-contact printing uses a technique called soft lithography to pattern cells on a substrate with a stamp, made of what is usually PDMS (polydimethylsiloxane). The PDMS transfers the material of interest, using alkanethiols, onto a gold and silver surface. The disadvantage of this technique is that the precision of feature size is limited.

An alternative technique is microfluidic patterning using microchannels formed by a PDMS structure on a substrate. The patterned PDMS structure seals itself to the substrate due to its elastic nature and hydrophobicity, restricting the flow of the cell adhesive material and allowing the cells to only attach to the desired areas. Microfluidic patterning techniques have been used to create a substrate with thin lines of ligands and

then coated with specific peptides to study the extension of neurite outgrowth and guidance of Schwann cells.

Stencil Patterning is another technique in which a metallic or PDMS stencil is sealed to the substrate; and the cell suspension is then added to the substrate. The cell suspension does not come in contact with the areas of the substrate covered by the stencil. Once the cells attach to the substrate, the stencil is removed and the desired pattern of cells is established [40].

1.6.3 The Use of Hydrogels for Nerve Regeneration Applications

Although other approaches to guiding nerve growth have been investigated, such as electrospun nanofibers, hydrogels show the most promise for the reasons listed above. [39]. A variety of different hydrogels are used for nerve regeneration including agarose, alginate, chitosan, Matrigel, polyethylene glycol (PEG), methylcellulose, and hyaluronan. All of these hydrogels have different chemical and mechanical properties and require different fabrication techniques in order to establish a substrate appropriate for cell cultures.

Since agarose is naturally not cell adhesive, the use of ultraviolet light or irradiation treatments is utilized to change the chemical properties of the agarose films, creating a surface that allows biomaterials attachment. This technique allows for the establishment of patterns by blocking part of the light with a mask. Mercey et al. coated agarose fills, which were covalently bonded to glass coverslips, with an ECM component like fibronectin to allow the culture of cells, after they were exposed to ultra violet lighting through a chromium mask [41].

Immobilization of biomolecules by light activation was used by Luo and colleagues for the attachment of embryonic chick dorsal root ganglia neurons to agarose hydrogels. Hydroxyl groups, activated by carbonyldiimidazole (CDI), are present in many natural polysaccharides and synthetic polymers, allowing photoliable S-NBC to be introduced. The addition of the photoliable S-NBC enables the hydrogel to photoreact because of the exposure of sulfhydrl groups. The irradiation doses determined the amount of peptides or proteins that adhered to the hydrogel [42].

Sakai et al. also used CDI to create a cell adhesive agarose, but used it to covalently cross-link gelatin to the agarose hydrogel. The mixture of hydrogels is another approach to changing the natural properties of hydrogels to the desired environment for cells. The gelatin conjugation allowed for the attachment of cells to the hydrogel, but did not decrease the mechanical stability. They also determined that a simple mixture of gelatin and agarose did not produce positive results and that the covalent cross-linking was necessary for the promotion of cell adhesion [37].

Chitosan is another commonly used hydrogel and was mixed with agarose at an optimum concentration for neuronal adhesion and growth by Zheng Cao and colleagues. A simple mixture of hydrogels avoids the use of chemical that may be harmful to the cells. They took advantage of the characteristics of both gels to create a hydrogel with the appropriate properties for the culturing of cells. The addition of chitosan did decrease the mechanical stiffness of agarose; it remained very close to the complex modulus of that of brain tissue [38].

After *in vitro* hydrogel culture systems aid in the investigation of the mechanisms required for nerve regeneration, hydrogels can be used *in vivo* for nerve repair.

Hydrogels are most appealing for this indication because they are capable of conforming to the shape of the size of the injury. The objective to produce an injectable hydrogel, which quickly gels at physiological temperatures and fills irregular injury geometries, has also been investigated by Zuidema et al. They utilized a previously well-developed agarose and methylcellulose hydrogel with the addition of other polysaccharides, including dextran and chitosan. These substances were only blended and not covalently coupled, eliminating the complex fabrication processes similar to the ones previously mentioned. Varying concentrations of agarose, methylcellulose, chitosan and dextran mixed to create an overall softer and more positive surface will improve cell adhesion and allow neurite outgrowth [35].

Martin et al. created a novel agarose and methylcellulose hydrogel blend for nerve regeneration applications. Since agarose has a low gelation temperature and injecting it at a higher temperature can cause further damage, the gelation properties need to be changed in order to make it more feasible as an injectable hydrogel for nerve regeneration. In this study, Martin and his colleagues used methylcellulose to serve this purpose. Methylcellulose is a thickening agent commonly used in food and has properties opposite of agarose. The hydrogel was added to a dorsal root ganglion (DRG) culture and did not affect the morphology or the viability of the neuron. [34].

1.6.4 Collagen and Laminin

For neural cells the substrate must not only be suitable for the attachment of the cell body, but also for the growth of the neurite. The direction and the rate of growth of these processes are determined by the substrate. Even though the surface may be cell adhesive, it may not be favorable for the growth of an axon. This makes the choice of a substrate

for neural cells more selective. Collagen and laminin are both proteins of the extracellular matrix which are thought to be important parts of neural differentiation [43,44]. It has been established that neural cells like PC12 cells do not adhere well to fibronectin, which is readily used for cell attachment with non-neural cells [44].

Collagen is an insoluble fibrous protein present in the extracellular matrix (ECM) and in connective tissues. It is the single most abundant protein and has at least 16 different types. The body contains collagen I, II and III [45].

Laminin is an intricate, cross shaped molecule that is naturally present in the nervous system. Laminin possesses many domains that are responsible for its functional properties (Figure 1.4). It has been established that the heparin binding domain is specifically responsible for the positive effects laminin has on the neurite growth and survival of neurons [43].

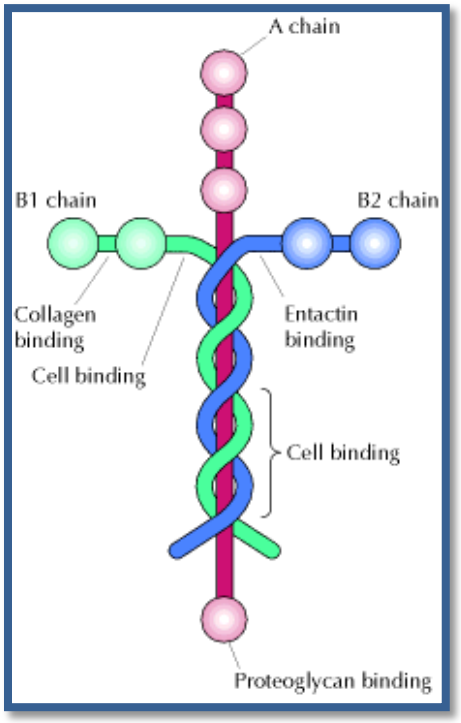


Figure 1.4 The binding sites of laminin

CHAPTER 2

MATERIALS & METHODS

2.1 Neuronal Cell Differentiation

2.1.1 ES Cell Culture

Mouse ES cell line D3 (ES-D3, ATCC) cells were maintained on 0.1% gelatin-coated p60 dish in mESC undifferentiating medium. mESC undifferentiating medium consisted of Dulbecco's modified Eagle's medium (DMEM [High Glucose]) supplemented with 20% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 0.1 mM β-mercaptoethanol (β-ME), 1% nonessential amino acids (NEAA), 1% penicillin/streptomycin(p/s) and 1,000U/ml leukemia inhibitory factor (LIF).

2.1.2 Oligodendrocyte Differentiation

When cells were confluent, stem cell colonies were trypsinized and single cells were suspended in Knockout Serum (KS) medium in petri-dishes, allowing embryoid bodies (EBs) to form. KS medium consisted of DMEM supplemented with 20% KS, 1mM sodium pyruvate, 1% NEAA, and 0.1 mM β-ME.

On days 4 through 7, RA (.5uM) was added. On days 5 through 7, shh agonist (1uM) was added. On days 6 through 8, the EBs were suspended in N2 medium. N2 medium consisted of DMEM supplemented with 1X N2 Supplement, 1mM sodium pyruvate, 1% NEAA, and 0.1 mM β-ME. The medium was changed every day.

On day 8, the EBs were disaggregated using accutase (12min, 37°C) and plated on 0.01% polyorthine/Fibronectin(FN)-coated p35 dishes in Oligodendrocyte Precursor

Cell (OPC) medium. OPC medium consisted of N2 medium supplemented with 20ng/ml of fibroblast growth factor-2(FGF-2) and 2% FBS. Medium was changed every two days. The cells were trysinized and re-plated and/or fixed approximately every 4 days for FACs analysis, immunofluorescent staining or further differentiation.

2.1.3 Effects of RA and Glucose Concentrations on EB Size and Differentiation

The murine Oct4-GFP ES cell line R1 (provided by Dr. Andras Nagy, Mount Sinai Hospital, Toronto, ON, Canada) were trypsinized (5min at 37°C) when confluent and suspended in LG-KS medium or HG-KS medium. 30µl drops of cell suspension were placed on p100 tissue culture dish lids (1,000cells/30ul drop). The dish lid with cell suspension drops were then inverted and placed on the dish bottom filled with 5ml of sterile dH_2O and incubated at 37°C.

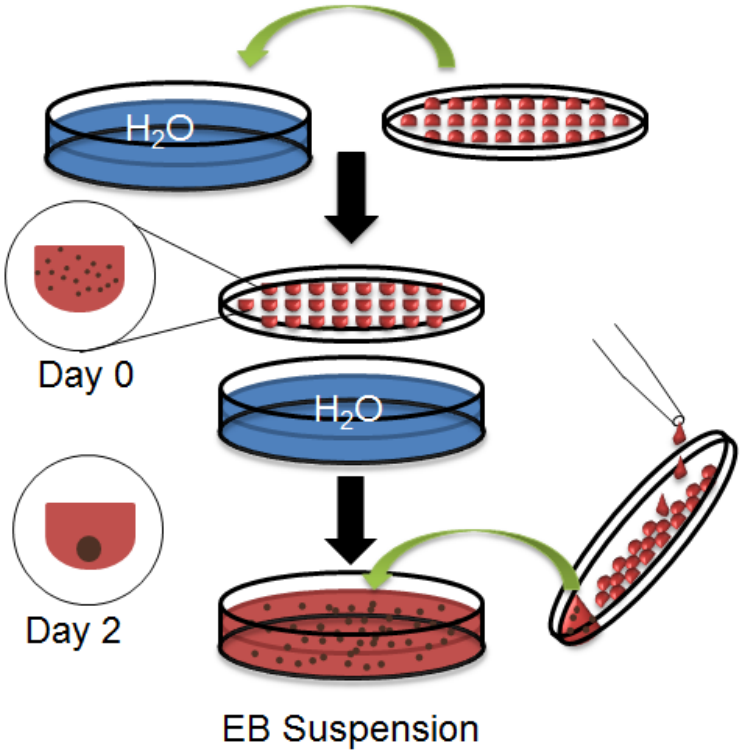


Figure 2.1 Embryoid body formation – hangdrop method.

After 2 days of incubation, the formed EBs were transferred to non-coated p60 in suspension of fresh medium for further differentiation. On day4, the EBs were exposed to RA (.5uM) for four days. The medium was changed every 2 days, from day 4 through day 8. Images were taken on day 2, 4, 6, and 8 with a Nikon Eclipse microscope with 2x, 4x, 10x and 20x objectives and a Nikon DS-U2 digital camera. On day 8, the EBs were disaggregated for FACS analysis.

2.1.4 EB Size and Differentiation Rate Analysis

Analysis was performed on the HD EB images using Sigma Scan Pro software to regulate the changes in the size of EBs and rate of differentiation. The size of the EB was determined by measuring the mean diameter. The differentiation rate was determined by the expression levels of Oct4/GFP, measured by the average green intensity changes over the period of 8 days.

2.1.5 Immunofluorescent Staining

Differentiated ES cells were washed with PBS at room temperature (RT). The cells were then incubated in 4% PFA in PBS (1ml/p35) at RT for 20 min. The cells were washed three times with PBS at RT and permeabilized with 0.2% Triton X-100 for 10 min. The cells were washed two times with PBS and blocked with PBS/10% FBS/1%BSA (1ml/p35) to block non-specific antibody binding. The cells were incubated in this blocking buffer for 30min at RT. Primary antibodies in blocking buffer were then added and incubated 1 hr at RT in blocking buffer (or incubated overnight at 4°C). The primary antibodies used were rabbit anti-Olig2 (1:1000), chicken anti-beta III tubulin (1:500), and mouse anti-NG2 (1:100), actin phalloidin (1:1000). The cells were washed 3 times with

PBS, each time, and incubated for 5 min. The secondary antibodies conjugated with Alexa fluor 488 (1:500) or Alexa fluor 594 (1:500), in blocking buffer were then added and incubated for 1 hr at RT. The cells were washed 3 times with PBS (each time, incubated for 5 min). DAPI (1:1000) was added and incubated for 5 min. The cells were washed 1 time with PBS (1ml/p35).

For live staining of fibroblasts 1μl/p35 dish of Calcein-AM and Ethidium homodimer III (EH) was added to the cell media and incubated for 5 min at 37°C. The media was then removed and the cells were washed two times with PBS and fresh medium was added. Images of the samples were acquired with a Nikon Eclipse microscope with 40x objective and a Nikon DS-U2 digital camera.

2.1.6 Flow Cytometry Analysis

For day 8 HD EBs and day 8 ES-D3 EBs, the cell suspension was transferred from the petri dish to a 15ml centrifuge tube. 10mL of PBS was added and centrifuged at 700 rpms for 5 min. The supernatant was removed and the cells were re-suspended in 0.5 ml of accutase. The cells were incubated for 10 minutes at 37°C and then dissociated by pipetting up and down. PBS was added to make a total of 10 ml. The cell suspension was then centrifuged at 1000 rpm for 5 minutes and re-suspended in 0.3 ml of medium. The cell suspension was transferred to a 15ml tube (or FACS tube) for fixation. 1ml of 4% PFA in PBS was added and incubated for 20 minutes at RT. 10 ml of PBS was added and centrifuged at 1000 rpms for 5 minutes. The cells were re-suspended in 1 ml of PBS and then stained or stored at 4°C.

Following trypsinization, differentiated ES-D3 cells on day 7, 8, 12, 13, and 19, were collected in a 15 ml tube and 5 ml of PBS was added. The cell suspension was

centrifuged and the supernatant was removed. The cells were re-suspended in 0.5 ml of 4% PFA in PBS and incubated for 20 min at RT. The cells were washed by adding 5 ml of PBS, centrifuged and the supernatant was removed. 0.5 ml of blocking buffer was added and the cells incubated for 30 min at RT. The primary antibody, mouse anti-NG2 (1:100), was added and incubated for 1 hr or overnight. 5 ml of PBS was added, centrifuged and the supernatant was removed. 0.5 ml of blocking buffer and secondary antibody Alexa fluor 488(1:2,500) was added and incubated for 1 hr. The cells were washed by adding 5 ml of PBS centrifuged and the supernatant was removed. 1 ml of PBS was added and the solution was transferred into a BD brand FACS tube. Flow cytometry analysis was performed with the BD Biosciences FACSCaliburs and the data was analyzed with WinMDI software.

2.1.7 Statistical Analysis

The means and standard error of mean (SEM) were calculated and statistically significant differences were determined by the two-tailed student's t-test. The statistical difference was determined at $p<0.05$ and the sample size is referred to by n.

2.2 Micropatterned Agarose Hydrogel

2.2.1 4% Agarose Synthesis

Agarose powder (2.0 g) was suspended in 50 ml of sterile dH_2O. The suspension was stirred and heated at 140°C for 20 min. The solution was then cooled to RT and stored at 4°C.

2.2.2 Micropatterned Agarose Fabrication

4% agarose solution was heated to 140°C and 1 ml of the solution was evenly distributed on top of the plastic mold master (positive). After 5 min or until the solution solidified, the agarose gel was flipped over and placed in a p35 dish.

The agarose gel was quickly washed with deionized water (dH_2O) and 12 µl of collagen solution (1.2 mg/ml) was dropped in the center of the micropatterned agarose gel. The collagen solution was allowed to travel through the microchannels via capillary action providing a thin layer of collagen limited to the channels.

After the solution from the first drop coated all of the channels and diffused, another drop of collagen was added and the coating was allowed to dry for 30 min. The collagen was then neutralized with NH_4OH for 10 min and washed three times for 5 min with dH_2O. 10 µl of 1 mg/ml of laminin was then dropped into the center of the hydrogel and allowed to dry for 30 min. The hydrogel was soaked in DMEM supplemented with 10% FBS medium for several minutes before cells were seeded.

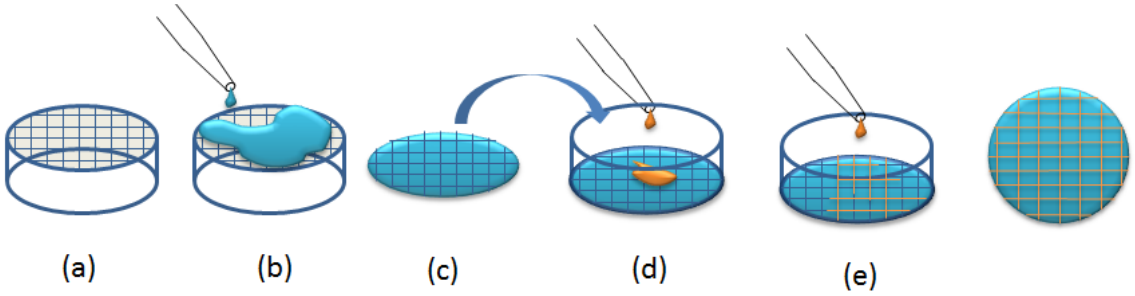


Figure 2.2 Micropatterned agarose hydrogel fabrication.

2.2.3 Characterization of Hydrogel

To determine the true structure and 3D features of the master mold, 3D images were captured and created by a VHX-600 digital microscope (Keyence Corp, NJ).

After the channels of the micropatterned agarose hydrogel were coated and neutralized, the channels were viewed by phase contrast microscopy to confirm the coating of the channels. Images were taken with a Nikon Eclipse microscope with 10x and 20x objectives and a Nikon DS-U2 digital camera.

Also, after channel coating, the hydrogels were covered with 100 µl of dye and then washed with water. Trypan blue (TB) is negatively charged and only bonds to areas of the hydrogel coated with 0.5% chitosan, which is positively charged. Safranin O is positively charged and only stains areas of the hydrogel coated with 2% alginate, which is negatively charged. Images were then taken with a Nikon Eclipse microscope with 2x, 4x, 10x and 20x objectives and a Nikon DS-U2 digital camera.

2.2.4 Fibroblast, PC12, Dorsal Root Ganglion (DRG) and Oct4/GFP EB Cultures

Fibroblast were maintained in DMEM medium supplemented with 10% FBS. Fibroblasts were trypsinized and plated on the sterile agarose hydrogels at a density of .2M/p35.

PC12 cells were maintained and cultured in PC12 medium, which consists of DMEM medium supplemented with 5% FBS and 5% horse serum (HS) and 1% p/s. Cells were trypsinized and plated at .2Mcells/p35 on sterile hydrogels coated with collagen and laminin. At the time of seeding, the PC12 cells were induced with 50 ng/ml of nerve growth factor (NGF) to induce neural differentiation.

Fetal rat dorsal root ganglions (DRG) obtained from Dr. Haesun Kim's laboratory, (Rutgers University-Newark) were isolated from E 14.5 rat embryos. The DRG's were dissociated using trypsin-EDTA treatment and cultured in DMEM/F-12 with 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine, 20% glucose, and 50 ng/ml Nerve Growth Factor (NGF).

The Schwann cells isolated from the neonatal rat sciatic nerves were obtained from Dr. Haesun Kim's laboratory (Rutgers University-Newark, Biology Department). The Schwann cells were cultured on 0.05% poly-L-lysine coated tissue culture dishes. The Schwann cell medium consists of DMEM, 10 % Fetal Bovine Serum, 2mM L-glutamine, 10 ng/ml recombinant human neuregulin-β-1EGF domain, 2.5 μM forskolin, and 1% penicillin streptomycin. The cells were maintained at 37^{o}C and 10 % CO_2 in a humid incubator. The medium was changed every 3 days.

Oct4/GFP EBs were formed as previously described and on day 8 were seeded on the collagen and laminin coated micrpopatterned hydrogel in DMEM medium supplemented with 10% FBS and 1% p/s.

CHAPTER 3

RESULTS

3.1 Neural Cell Differentiation

3.1.1 Effects of Glucose Levels and RA on EB Size and Differentiation

Oct4/GFP labeled stem cells were used for general neural studies to investigate the effect of glucose levels and RA on EB size and differentiation rate. EBs were formed via the hang drop method, which allowed more uniform EB sizes. Images of the EBs were taken on days 2, 4, 6, and 8, (Figure 3.1). Image analysis was performed to determine the size or the diameter of the EBs and the GFP/Oct4 expression by determining the average green expression of the EBs, (Figure 3.2). The average green intensity gradually decreases in all EBs but at different rates. EBs exposed to LG levels had a faster rate of GFP/Oct4 expression. The average diameter of the EBs in all conditions increased over the 8 days in culture, which confirms that cell proliferation and differentiation, did occur. The LG EBs with and without RA were smaller on day 2, but on day 8 they were much larger than the HG EBs. Overall, the lack of RA exposure led to a slower rate of differentiation, but larger EB sizes on day 8.

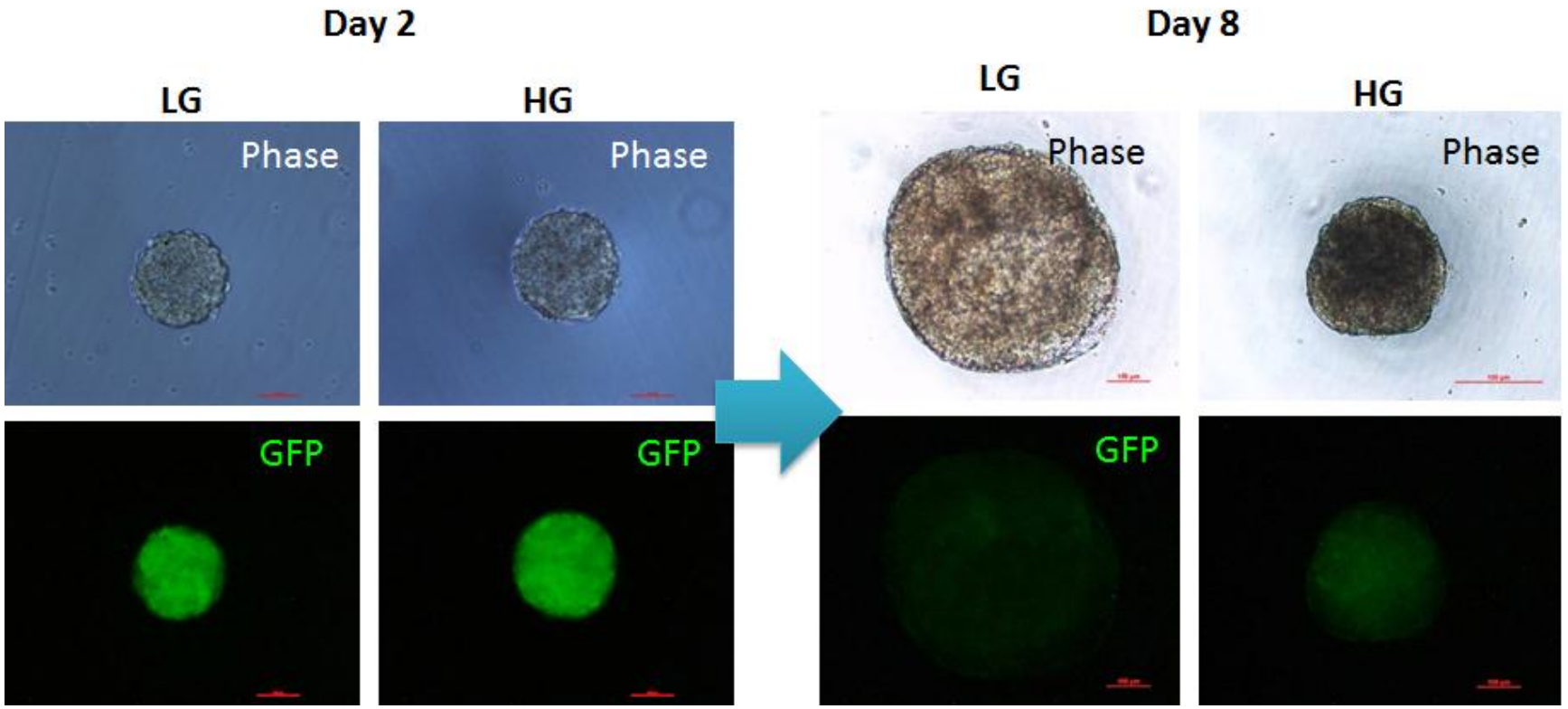


Figure 3.1 EB morphology and Oct4/GFP expression in different glucose conditions (These images are all captured at 10x magnification).

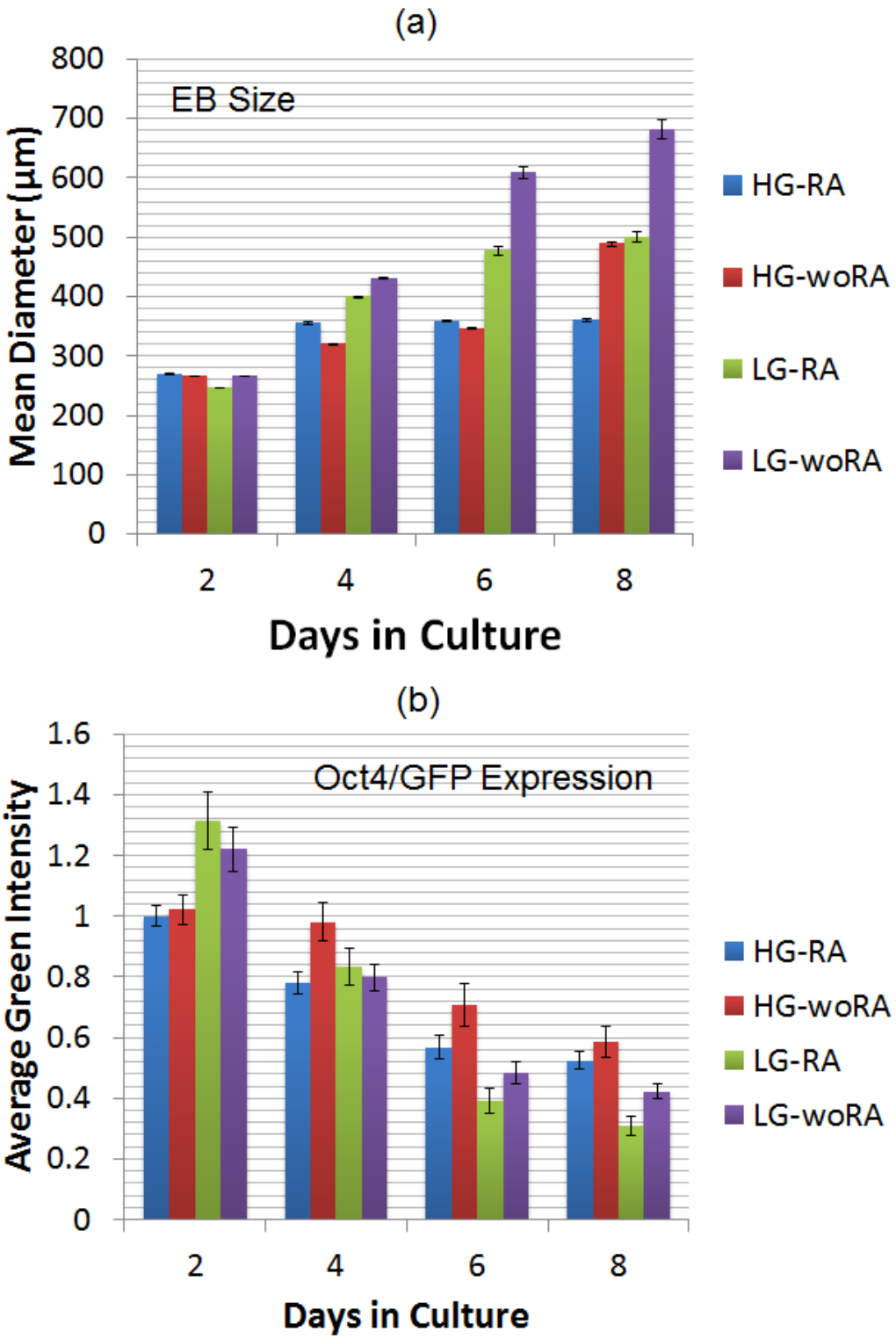


Figure 3.2 Neural differentiation in low and high glucose conditions, with and without RA (a) The mean ± SEM diameter was used to determine the EB size. (b) The average green intensity represents the expression levels of Oct4/GFP.

After 8 days in culture, the EBs were dissociated and fixed for flow cytometry analysis, (Figure 3.2). Undifferentiated Oct4/GFP stem cells were used as a control of which 97.2% of cells were positive for Oct4, a stem cell marker. The FACs analysis determined that 23.94% of the cells exposed to LG levels without RA were Oct4 positive. 10.90% of the cells exposed to LG levels with RA were Oct4 positive. 68.88% of the cells exposed to HG levels without RA were Oct4 positive. 27.61% of the cells exposed to HG levels with RA were Oct4 positive, (Figure 3.4). The FACs analysis revealed that more of the cells in the EBs in LG conditions were committed to differentiation than the EBs in the HG conditions. The addition of RA in both high and low glucose conditions decreased the number of Oct4 positive cells.

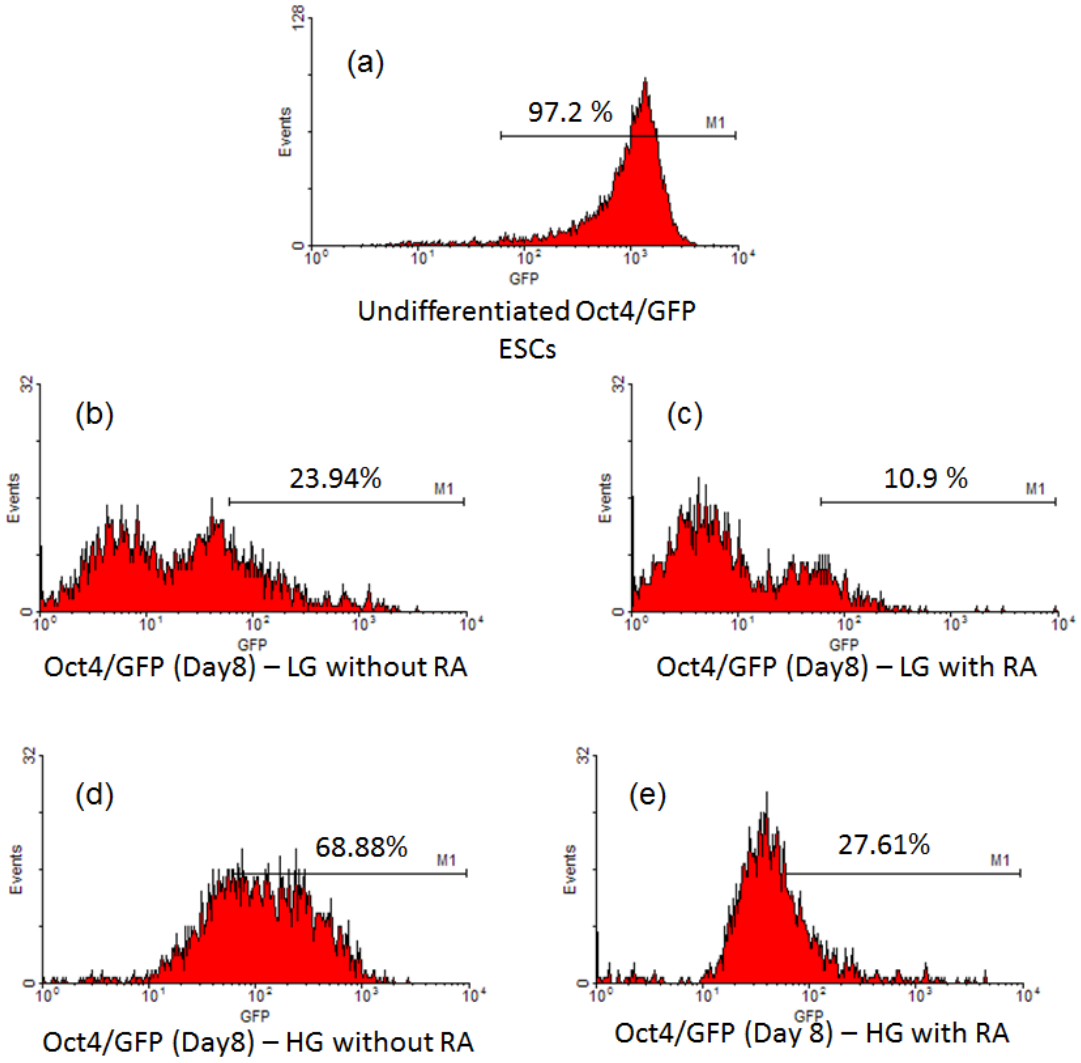


Figure 3.3 Flow cytometry analysis on dissociated Oct4/GFP HD EBs (Day8) (a) Undifferentiated Oct4/GFP cells for a control, (b) Cells exposed to LG levels without RA, (c) Cells exposed to LG levels with RA , (d) Cells exposed to HG levels without RA , (e) Cells exposed to HG levels with RA.

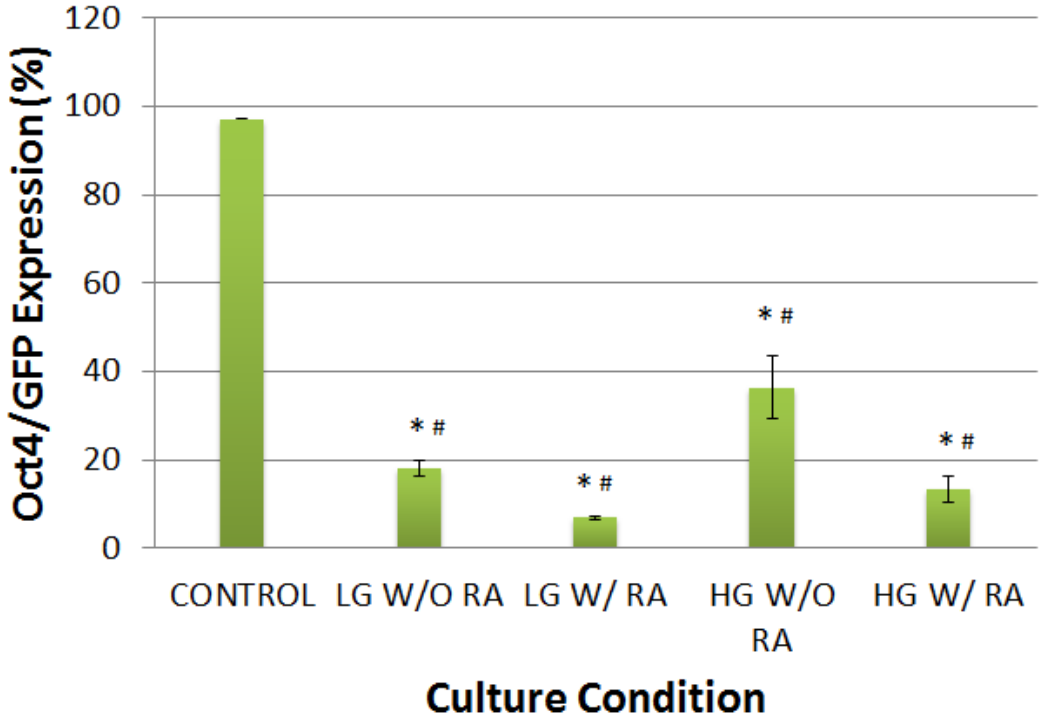


Figure 3.4 Percent of Oct4 positive cells in different conditions LG with and without RA and HG with and without RA. (n = 3, mean ± SEM, (*), $p < 0.05$, LG without RA vs. LG with RA, and HG without RA vs. HG with RA. (#), $p < 0.05$, LG without RA vs. LG with RA and HG without RA vs. HG with RA).

3.1.2 Effects of Glucose and RA on Oligodendrocyte Differentiation

ES-D3 cells were differentiated into oligodendrocytes by forming EBs and exposing them to RA and shh agonist, under high and low glucose conditions. On day 7-8 in culture, EBs were dissociated and then single cells were fixed for FACs analysis or replated for immunofluorescent staining and further differentiation. Undifferentiated stem cells were used as a control.

Flow cytometry analysis results show that 0.39% of undifferentiated stem cells were positive for NG2 (an oligodendrocyte progenitor marker). A low percentage of cells should be positive for NG2 because they were not differentiated and have not committed to a specific cell lineage. The flow cytometry analysis also revealed that

13.69% of the cells exposed to HG levels were oligodendrocyte progenitors, and 26.97% of the cells exposed to LG conditions were oligodendrocyte progenitors, (Figure 3.5 and 3.6). Differentiation of cells in LG conditions was more efficient than in HG conditions.

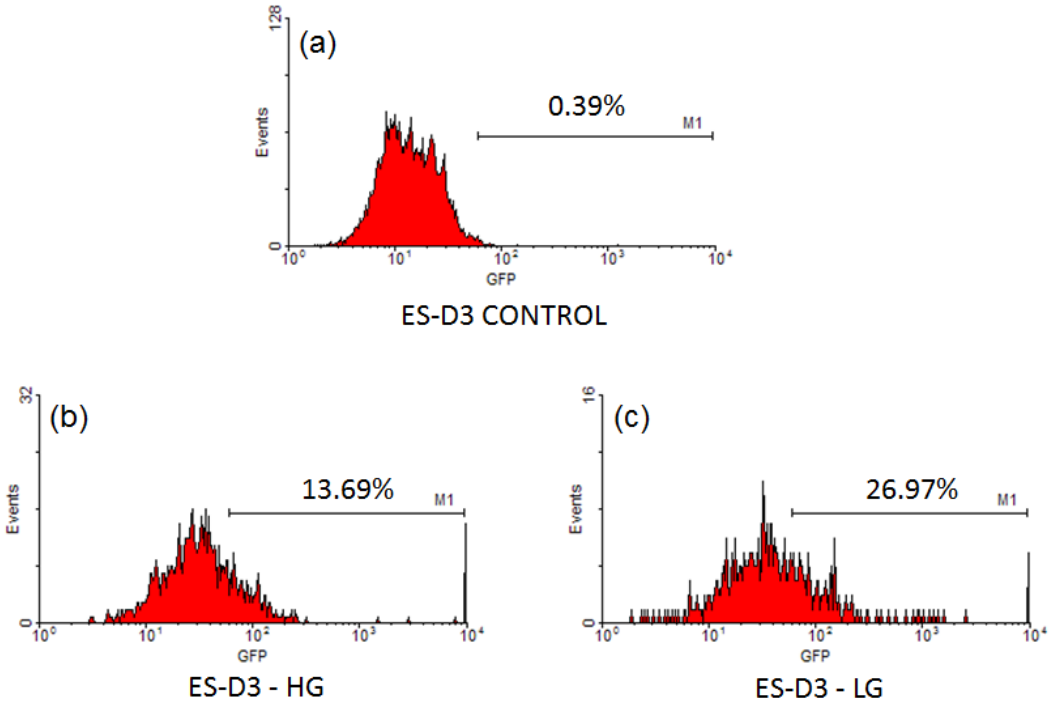


Figure 3.5 FACs analysis of oligodendrocyte precursors on Day 7 – 8 of Differentiation Antibodies against NG2 (oligodendrocyte progenitor marker) in different glucose conditions (a) Percent of undifferentiated ES-D3 cells, control, (b) Percent of the cells exposed to HG levels, (c) Percent of the cells exposed to LG levels.

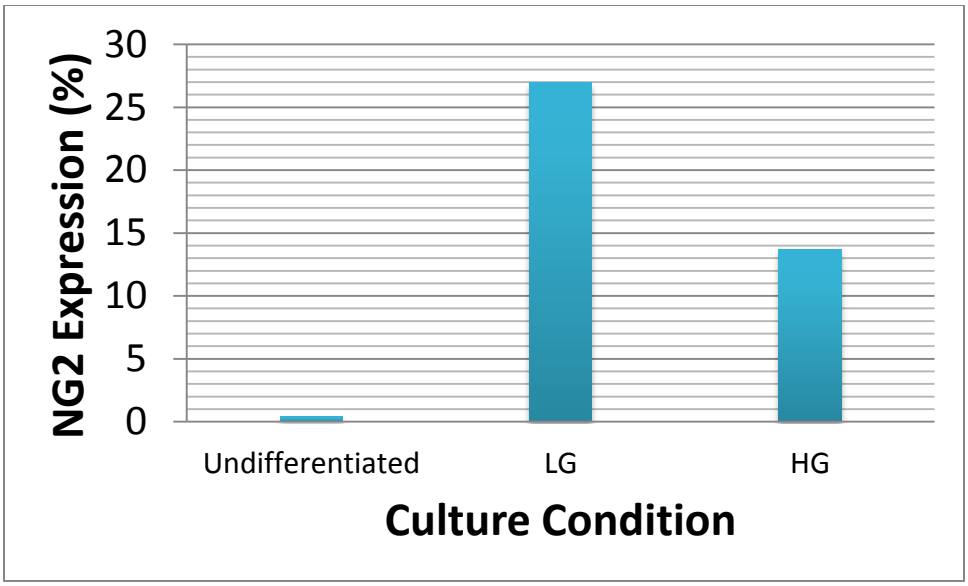


Figure 3.6 Bar graph of FACs analysis data of oligodendrocyte differentiation Day 7-8 On Day 8 there were more cells that expressed the oligodendrocyte progenitor marker in the LG conditions than the HG conditions. Undifferentiated ES-D3 cells were used as a control.

On day 13 in culture, cells were stained with O4 and DAPI. The cells showed bipolar and multipolar morphology similar to oligodendrocyte progenitor cells and pre-oligodendrocytes, (Figure 3.7). None of the cells were found positive for β-III tubulin (data not shown).

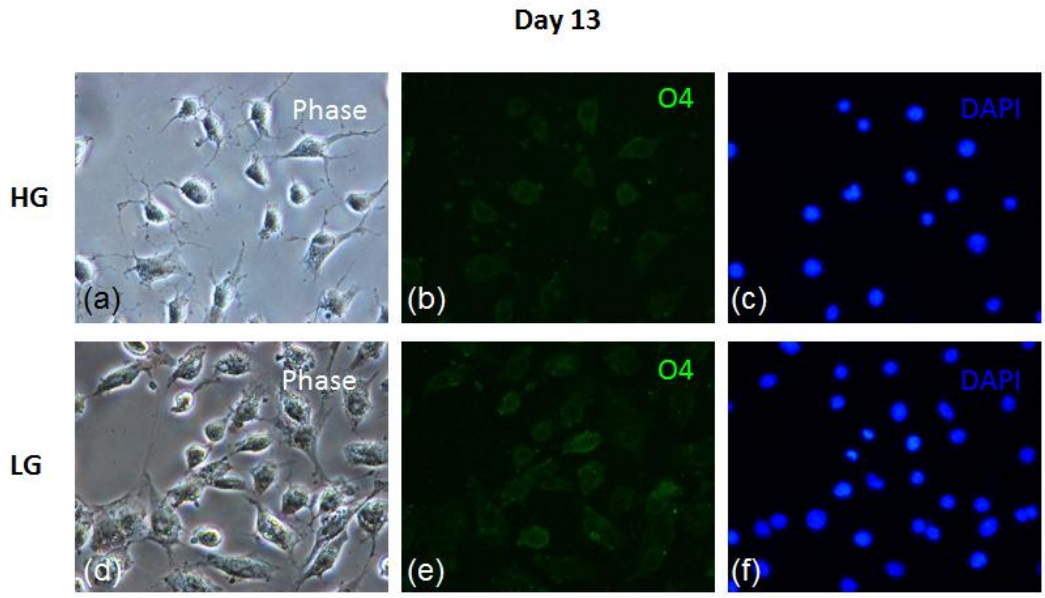


Figure 3.7 Immunofluorescent staining for oligodendrocyte differentiation (Day 13) (a) HG–Phase contrast (40x), (b) HG –Immunofluorescence for O4 (Pre-Oligodendrocyte Marker), (green)(40x), (c) HG –DAPI (blue), which stains the cell nucleus (40x) (d) LG – Phase contrast (40x), (E) LG –Immunofluorescence for O4 (Pre-Oligodendrocyte Marker), (green)(40x), (F) LG –DAPI (blue), which stains the cell nucleus (40x).

3.2 Micropatterned Agarose

Phase contrast images were taken of the master mold and image analysis was performed to determine the width of the channel (Figure 3.8). The image analysis was determined to be approximately 250µm wide. The 3D images of the master mold shows that the depth of the master mold is 250µm wide and 150µm deep (Figure 3.9). After the micropatterned hydrogels were coated with collagen they were observed by microscopy. The fibers of the collagen coated were clearly visible under phase contrast (Figure 3.10).

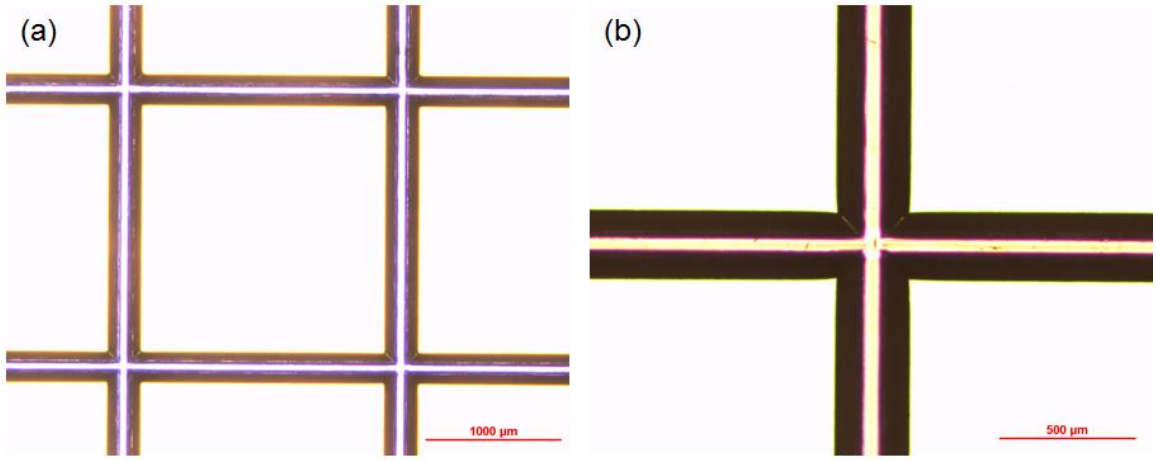


Figure 3.8 Phase contrast images of the master mold.

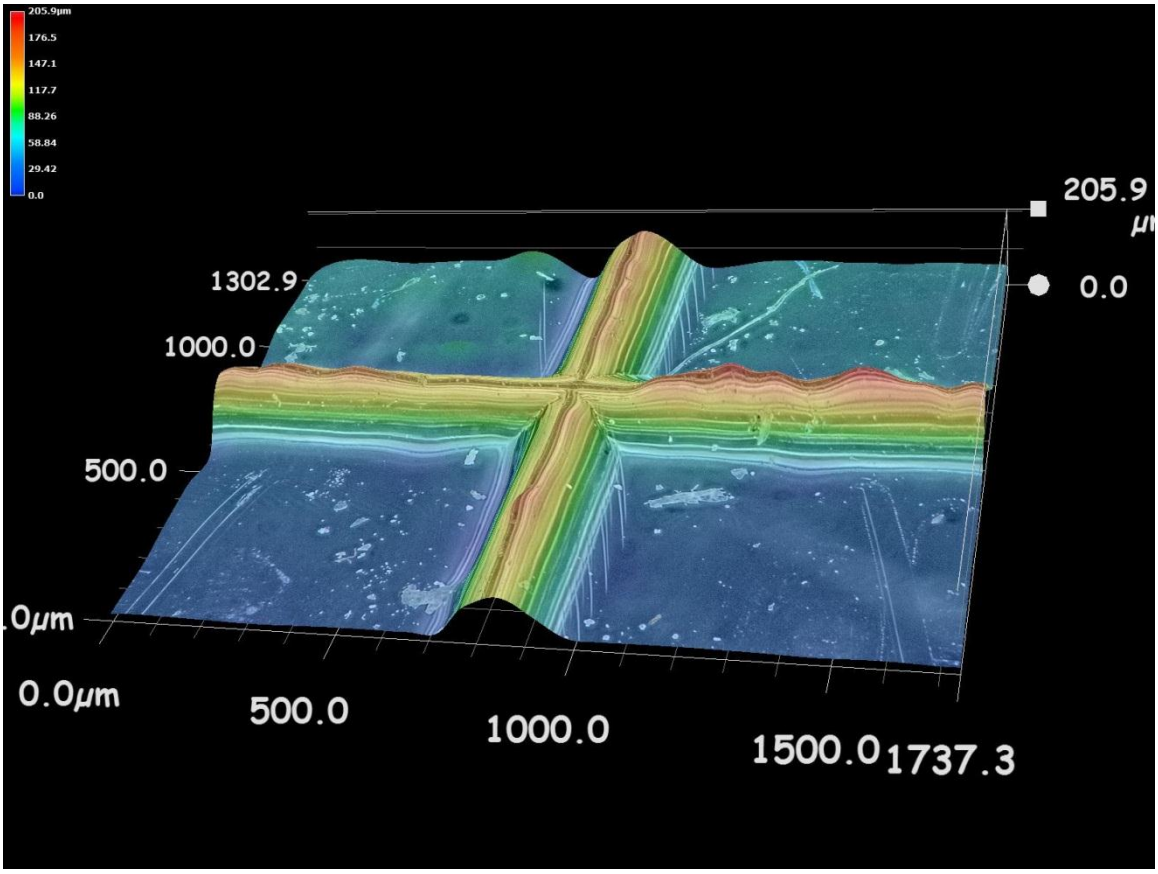


Figure 3.9 3D image of plastic master mold with grid.

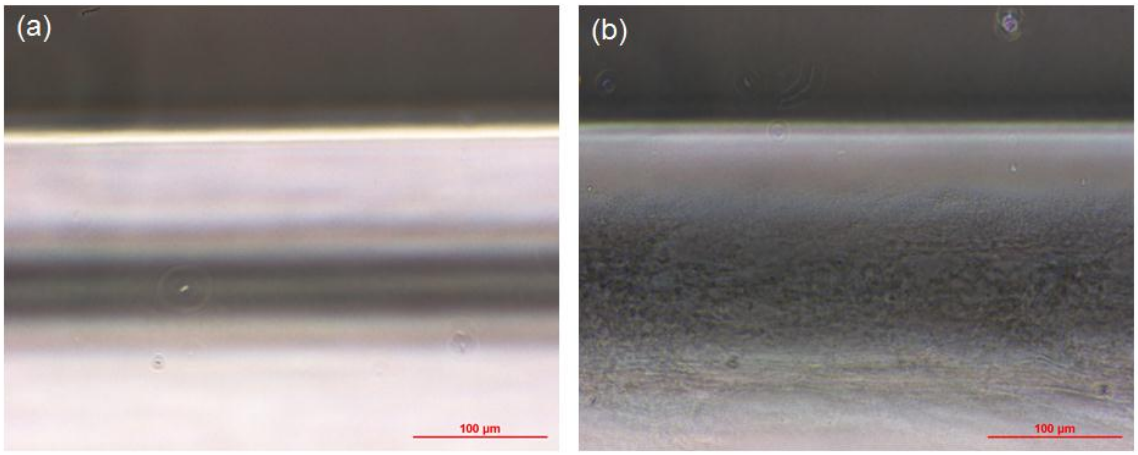


Figure 3.10 Characterization of micropatterned agarose hydrogel (a) Channel of micropatterned agarose hydrogel without collagen coating (b) Channel of micropatterned agarose hydrogel with collagen coating (Images were taken at 20x magnification).

In order to confirm that the 0.5% chitosan and 2.0% alginate coatings were covering the channels only, positive and negatively charged dyes were used in order to take advantage of the charges of the coatings. TB was used to stain the chitosan coating and was limited to the channel areas of the micropatterned hydrogel. Safranin O was used to stain alginate and only stained the channel areas of the agarose hydrogel, confirming that the method of coating the channels via capillary action only is efficient in limiting cell adhesive areas on the agarose hydrogel (Figure 3.11).

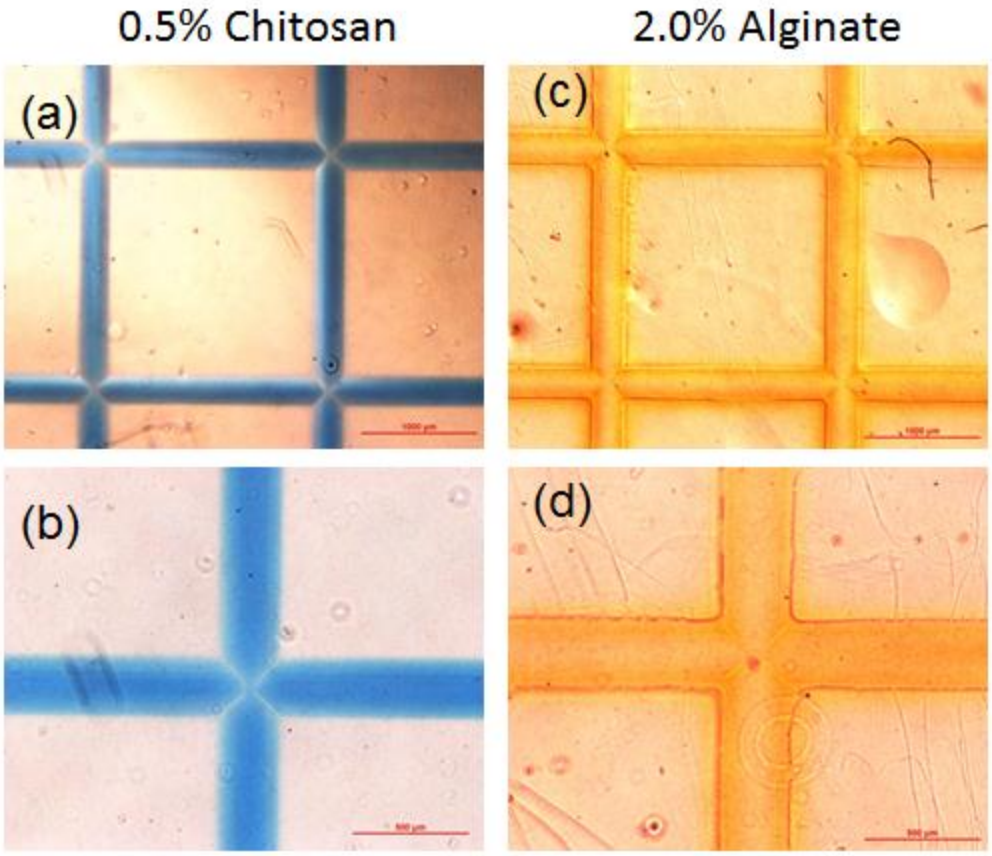


Figure 3.11 Characterization of agarose microchannels coated with chitosan and alginate (a) TB (blue) was used to determine the location of the chitosan coated areas (2x), (b) TB stained chitosan coated micropatterned agarose channels,(4x). (c), Safranin O (red) was used to determine the location of the alginate coated areas (2x), (d), Safranin O stained alginate coated micropatterned agarose (4x).

A variety of cell types were used to optimize the agarose conditions for stem cell derived neurons. After the micropatterned hydrogel was coated with collagen, fibroblasts were seeded on the hydrogel. They attached and spread well in the channel areas of the gel and grew within these boundaries. The cells were stained alive to determine the viability of the cells after seeding. A majority of the cells expressed calcein and few cells expressed EH, confirming the viability of the cells. The cells were then fixed and stained with Actin and DAPI to stain the cytoskeleton and nuclei of the fibroblasts (Figure 3.12).

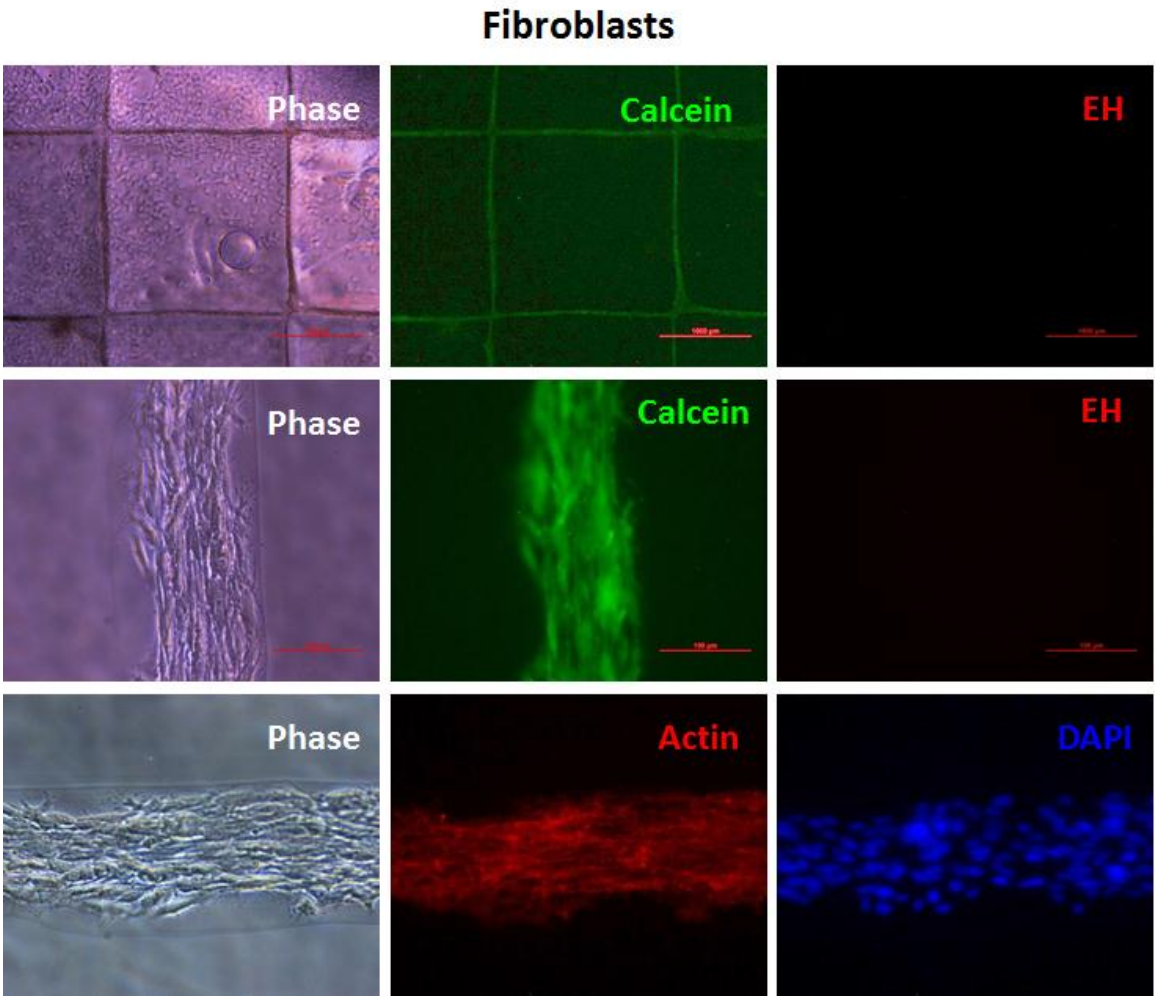


Figure 3.12 Immunofluorescent staining of micropatterned fibroblasts (a) Phase contrast (2x), (b) Calcein (stains live cells) (2x), (c) EH (stains dead cells) (2x), (d) Phase contrast (20x), (e) Calcein (20x), (f) EH (20x), (g) Phase contrast (20x), (h) Actin(stains cytoskeleton of live cells) (20x), DAPI (stains cell nuclei) (20x).

PC12 cells, induced with NGF, were seeded on the micropatterned agarose and also adhered well to the channel areas. DRG neurons and Schwann cells were seeded on the micropatterned agarose hydrogel, and subsequently showed attachment and neurite outgrowth (Figure 3.13). Oct4/GFP EBs were also seeded on the agarose hydrogel and subsequently showed attachment and spread into the channels. The positive expression of actin and DAPI from the patterned EBs confirmed the viability of the cells. Neurite

outgrowth from the EBs was guided along the micropatterned agarose hydrogel, as shown in Figure 3.14.

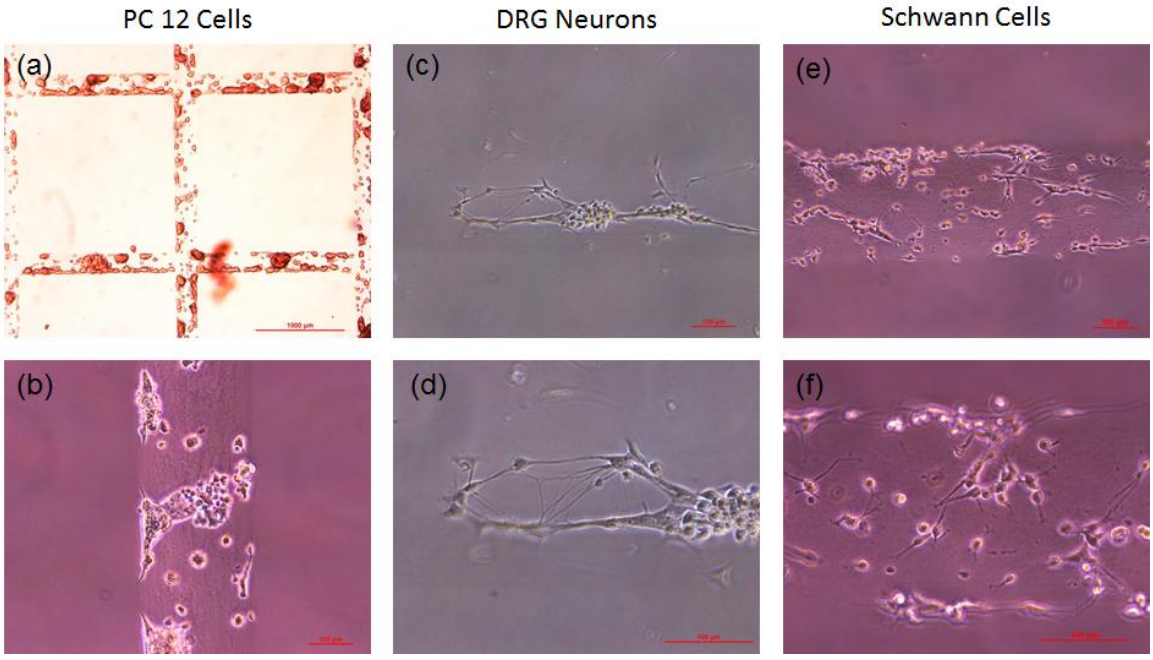


Figure 3.13 Cells micropatterned on agarose coated with collagen and laminin (a) Patterned PC12 cells induced with NGF (2x), (b) Patterned PC12 cells induced with NGF (10x), (c) Patterned DRG neurons (10x), (d) Patterned DRG neurons(20x), (e) Patterned Schwann Cells (10x), (f) Patterned Schwann Cells (20x).

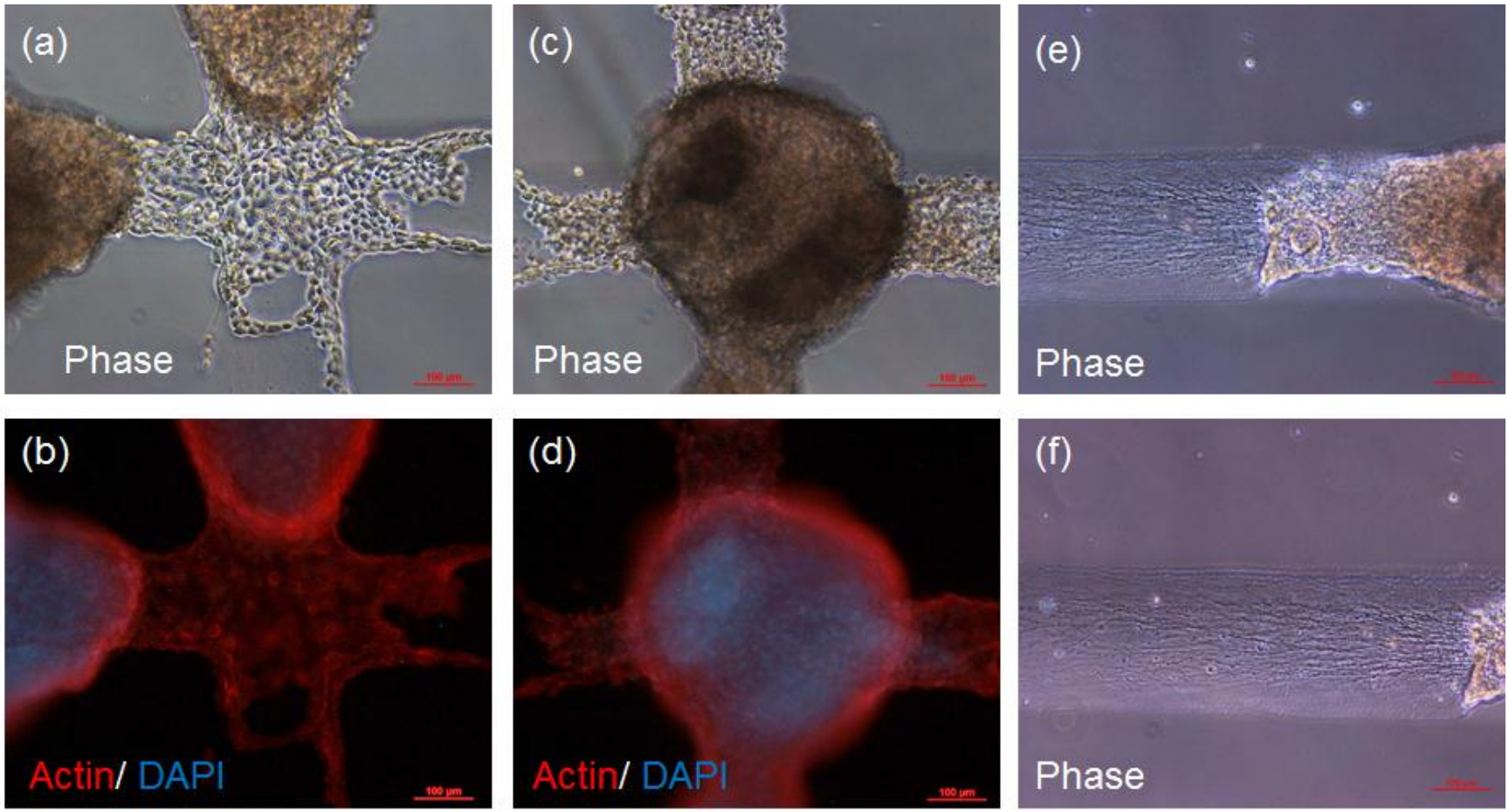


Figure 3.14 Patterned embryoid bodies on micropatterned agarose hydrogel (a) Phase contrast image of patterned cells from differentiated EBs (b) Actin and DAPI staining of patterned cells from differentiated EBs (c) Phase contrast image of patterned cells from differentiated EBs (d)) Actin and DAPI staining of patterned cells from differentiated EBs (e) Nerve guidance of neurite outgrowth from EB (f) Nerve guidance of neurite outgrowth from EB (All of these images were taken at 10x magnification).

CHAPTER 4

DISCUSSION

The first objective of this study was to determine the effects of small molecules RA and shh agonist and the effects of glucose levels on the differentiation of embryonic stem cells into cells of the neural lineage. RA is a known to induce the differentiation of embryonic stem cells into neurons; and RA with the addition of shh agonist has been used to promote the differentiation of stem cells into oligodendrocytes.

Although it has been well established that high glucose medium is the best environment for the maintenance of ESCs, it has not been extensively investigated whether high glucose is the most efficient medium to use for the differentiation of these cells. Also, it was originally thought that high glucose was required in order to form EBs in suspension. This study proves that EBs can be formed efficiently in both high and low glucose conditions. The protocol to differentiate stem cells into a mixed population of neurons, astrocytes and oligodendrocytes is less complex than generating a predominately oligodendrocyte population.

The second objective of this study was to take advantage of the properties of agarose hydrogels to guide neurite outgrowth of RA induced EBs. Agarose, a naturally occurring polymer, is regularly used for investigations involved in nerve regeneration and repair. By creating this 3D scaffold with similar mechanical properties to neural tissue *in vivo*, scientists can investigate the possible mechanisms required for the regeneration of neural tissue.

This investigation shows that EBs formed by the hang-drop method grew and differentiated faster in low glucose conditions compared to high glucose conditions. On

day 2, the EBs in the HG conditions were not significantly larger than the EBs exposed to LG media. Both HG and LG conditions were capable of forming viable EBs and, therefore, it is not necessary to have HG to form EBs in high cell density.

The highest rate of growth and fastest rate of decreased expression of Oct4/GFP was seen in the EBs exposed to LG conditions. In both high and low glucose conditions the addition of RA decreased the growth rate and increased the differentiation rate. After 8 days in culture the LG EBs not exposed to RA were significantly larger than the EBs in the other three conditions. This must be due to the differentiation of these cells to cell types that are of a larger size. The results show that the Oct4/GFP expression of EBs exposed to LG was initially high and then quickly decreased after the eight days in culture. The diameter of these LG EBs also quickly increased over the 8 days in culture. The LG Oct4/GFP expression and diameter increase was faster than the EBs in the HG conditions. The lack of growth and slow rate of decreased expression of Oct4/GFP implies that the cell in the EBs exposed to HG differentiated more slowly, if at all, compared to the HG EB cells.

The EBs that are exposed to RA in both high and low glucose conditions both exemplify that RA does favor the differentiation of ESCs by their expression of Oct4/GFP, which was less than their counterparts not exposed to RA. Mochizuki et al. found that there was no difference between the mouse EB growth rate between EBs formed in HG and LG media [15]. They did discover a positive difference in LG and HG EBs in their respective attachment cultures. The results of this study are more similar to the results found by Khoo and colleagues, which show that the human EB growth rate is faster in LG conditions [16]. The discrepancy can be explained by the cell line used.

Mochizuki et al. also observed that HG conditions produced beating cardiac muscle more efficiently than LG medium. Therefore, the significant differences in diameter and Oct4/GFP expression can be due to the certain conditions being favorable to different differentiation lineages.

It was also demonstrated that in low glucose conditions cells that were differentiated into oligodendrocytes expressed a higher level of oligodendrocyte markers than cells exposed to high glucose conditions. EBs were formed and induced with shh and RA, after 8 days in culture they were dissociated and re-plated or fixed for FACs analysis. The single cells were stained with an oligodendrocyte precursor marker, NG2. The results of the FACs analysis showed that there was a significant difference in the number of cells that differentiated into oligodendrocytes in the LG conditions compared to the HG conditions. Re-plated single cells were stained with β-III tubulin, a marker for neurons, and there were no cells that expressed positive for this marker. This confirms that the use of RA and shh induction did aid in the differentiation into oligodendrocytes and not neurons.

This study also sought to employ micropatterned agarose hydrogels for the guidance of neurites from RA induced EBs. Agarose is a natural occurring polymer isolated from marine algae. The unique property of agarose to be a liquid at high temperatures and to solidify at room and physiological temperatures was used to establish a micropatterned hydrogel. The fabrication of the micropatterned agarose hydrogel proved to be inexpensive, quick and capable of being used for a variety of materials. A variety of cell types were used to optimize the agarose conditions. By taking advantage of the non-cell-adhesive properties of agarose, cell adhesive materials were used to

pattern cells in the micropatterned channels only. Collagen was used due to its known use with neural cells. Collagen is a part of the extracellular matrix which is involved in cell adhesion through the integrin protein family in the cell membrane [46]. Laminin is also used for the attachment of neural cells. When cells are plated on laminin coated dishes, the neurite outgrowth and length is significantly longer than on collagen [47]. The seeded cells including fibroblasts, Schwann cells, PC12 cells and DRG neurons attached and grew well on the micropatterned agarose.

CHAPTER 5

CONCLUSIONS

Due to the results related to the Oct4/GFP EBs of this study, it can be concluded that a glucose level closer to physiological glucose concentrations is more efficient than high glucose levels for the differentiation of stem cells to cells of the neural lineage. Further studies should include the determination of the types and size of the cells in the Oct4/GFP EBs to confirm that the reason why the EBs exposed to low glucose levels but not induced with RA are the largest in diameter or size. Also the percentage of neural cells in the EBs in all four groups (LG with RA, LG without RA, HG with RA and HG without RA) should be compared to confirm that RA is not just inducing a spontaneous differentiation and that it has a vital role in neural differentiation.

The differentiation of mESCs into oligodendrocytes in high and low glucose has also lead to the conclusion that low glucose is more beneficial for the differentiation of pluripotent stem cells into a lineage committed cell type. Further studies should be performed to determine the expression of other markers for oligodendrocytes. These differentiated oligodendrocytes can be tested to determine whether they are capable of myelinating neurons. This can be conducted in an *in vitro* culture system such as the one developed in this study or in an *in vivo* model. PCR can also be used to measure the expression of oligodendrocyte lineage specific genes to compare the efficiency of both high and low glucose conditions.

The micropatterned agarose hydrogel has proven to be a viable *in vitro* agarose hydrogel culture system. This study utilized the well-defined use of an agarose hydrogel to fabricate a three dimensional environment that promotes the cell adhesion and neurite

outgrowth proliferation of a variety of cell types. Further studies should include cell patterning of cells outside of the neural lineage and quantitative analysis of the proliferation of the cells on the micropatterned agarose gel compared to the two dimensional culture dishes. This *in vitro* model system can not only be used for cell patterning to replicate complex tissue organization, but can also be used to investigate nerve signal propagation as well as myelination.

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