The Extracellular Environment's Effect on Cellular Processes: An In Vitro Study of Mechanical and Chemical Cues on Human Mesenchymal Stem Cells and C17.2 Neural Stem Cells

Meghan E. Casey
Lehigh University

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The Extracellular Environment’s Effect on Cellular Processes: An *In Vitro* Study of Mechanical and Chemical Cues on Human Mesenchymal Stem Cells and C17.2 Neural Stem Cells

By

Meghan E. Casey

A Thesis

Presented to the Graduate and Research Committee of Lehigh University in Candidacy for the Degree of Master of Science in Bioengineering

Lehigh University
September 2013
This thesis is accepted and approved in partial fulfillment of the requirements for the Master of Science.

Date

Thesis Advisor: Dr. Sabrina S. Jedlicka

Chairperson of Program: Dr. Anand Jagota
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Abstract

Stem cells are widely used in the area of tissue engineering. The ability of cells to interact with materials on the nano- and micro-level is important in the success of the biomaterial. It is well-known that cells respond to their micro- and nano-environments through a process termed chemo-mechanotransduction.

It is important to establish standard protocols for cellular experiments, as chemical modifications to maintenance environments can alter long-term research results. In this work, the effects of different media compositions on human mesenchymal stem cells (hMSCs) throughout normal in vitro maintenance are investigated. Changes in RNA regulation, protein expression and proliferation are studied via quantitative polymerase chain reaction (qPCR), immunocytochemistry (ICC) and cell counts, respectively. Morphological differences are also observed throughout the experiment. Results of this study illustrate the dynamic response of hMSC maintenance to differences in growth medium and passage number. These experiments highlight the effect growth medium has on in vitro experiments and the need of consistent protocols in hMSC research.

A substantial opportunity exists in neuronal research to develop a material platform that allows for both the proliferation and differentiation of stem cells into neurons and the ability to quantify the secretome of neuronal cells. Anodic aluminum oxide (AAO) membranes are fabricated in a two-step anodization procedure where voltage is varied to control the pore size and morphology of the membranes. C17.2 neural stem cells are differentiated on the membranes via serum-withdrawal. Cellular growth is characterized by scanning electron microscopy (SEM), ICC and qPCR. ImageJ software
is used to obtain phenotypic cell counts and neurite outgrowth lengths. Results indicate a highly tunable correlation between AAO nanopore sizes and differentiated cell populations. By selecting AAO membranes with specific pore size ranges, control of neuronal network density and neurite outgrowth length is achievable.

To understand differentiation marker expressions in C17.2 NSCs and how material stiffness affects differentiation, cells are cultured on substrates of varying stiffness. qPCR is used to analyze neural stem cell, neural progenitor cell, neuron-restricted progenitor and differentiated post-mitotic neuronal cell RNA expression. Results suggest a relationship between material stiffness and neuronal development in C17.2 neural stem cells.
Chapter 1

Effect of Media Formulation on Human Mesenchymal Stem Cells (hMSCs)

Maintenance In Vitro

Review of Current Literature

A stem cell’s microenvironment greatly influences cellular processes. In both in vivo and in vitro environments, chemical cues affect stem cell proliferation and differentiation. The composition of media used for in vitro experiments influences cellular proliferation, morphology and differentiation potential [1-6].

Sotiropoulou et al. investigated various components of medium formulations on human mesenchymal stem cell (hMSC) maintenance. The group found that choice of medium, glucose concentration and quality of added components affected final experiments [3].

Effects of media composition, including base media formulation, serum levels and supplements such as ascorbic acid and copper, were explored by Gong et al. Different mediums supported smooth muscle cell differentiation, while others retained hMSCs in an undifferentiated state [2].

Wagner et al. passaged hMSCs in two different media compositions, Poietics Human Mesenchymal Stem Cell Medium (PT-3001, Cambrex) and a formulation of 58% low glucose Dulbecco’s Modified Eagles Medium / 40% MCDB201 / 2% fetal calf serum with various supplements first reported by Reyes et al. [7]. The latter medium formulation was used in conjunction with fibronectin pretreatment of cell culture surfaces. The group reported differences in proliferation rate between the two mediums.
Sotiropoulou et al. studied the effects of eight available culture mediums on hMSCs. Differences in cell numbers and differentiation potential were observed between the culture mediums [3].

Serums, growth factors and additional media supplements also affect cells. Serum, while undefined, has known roles in attachment and survival of cells in culture [8]. Groups have also explored the effects of serum origination on hMSCs. Stute et al. compared proliferation and differentiation capabilities of hMSCs grown in both autologous serum and fetal calf serum. Growth of hMSCs was comparable in both serums but osteogenic differentiation was greater with autologous serum-containing growth medium [4].

Growth factors influence cell attachment, proliferation and differentiation capabilities [2,3,9]. Medium supplements (such as ascorbic acid and dexamethasone) are used to aid in both the preparation of cells prior to differentiation [10] and during differentiation [11-13]. Wang et al. reported favorable hMSC expansion in vitro with media containing low concentrations of Dex [14]. Groups also described the use of Dex ($10^{-10}$ - $10^{-7}$ M) to prevent apoptosis in cell cultures [14,15].

It is important to establish consistent protocols for cellular experiments, as chemical modifications to maintenance environments can alter long-term research results. The effects of different media compositions on human mesenchymal stem cells (hMSCs) throughout normal in vitro maintenance need to be explored. The standardization of routine hMSC cell culture is crucial for the field to advance. It will create a necessary foundation of consistency upon which future investigations can build.
Introduction

Biomedical studies involving the use of human mesenchymal stem cells (hMSCs) is a rapidly-growing research area. hMSCs, which are harvested from bone marrow, are able to differentiate into a variety of cell types including bone, muscle, cartilage, fat and tendon [11,12,16-18]. Neural differentiation potential has also been reported by groups [13,19]. Clinical applications for hMSCs have been investigated, including uses in the cardiovascular system [20,21] and with patient transplantations and grafts [22-25]. As research in this area increases, the therapeutic potential of bone marrow-derived hMSCs also grows.

hMSCs must be expanded in vitro because of the low frequency of cells isolated from bone marrow donors. Expansion of hMSCs is limited however, as cells experience decreases in population doublings [26,27] and differentiation capacity over time [1,28-30]. Telomere length decreases in hMSCs passed in vitro [29,30]. Morphological changes are also observed with cells becoming enlarged and flattened as they are passaged [1,31]. Schellenberg et al. found that cells in later passages expressed beta galactosidase, a biomarker for cellular senescence and that the frequency of fibroblastoid colony forming units decreased over passages as well [32]. DNA-methylation changes in bone marrow-derived hMSCs associated with senescence have also been reported in hMSCs cultured in vitro [32,33].

In both in vivo and in vitro environments, chemical cues affect stem cell proliferation and differentiation. The composition of media used for in vitro experiments influences cellular proliferation, morphology and differentiation potential [1-6]. Serums,
growth factors and additional media supplements alter the chemical effects of media on cells. Serum, while undefined, has known roles in attachment and survival of cells in culture [8]. Growth factors influence cell attachment, proliferation and differentiation capabilities [2,3,9]. Medium supplements (such as ascorbic acid and dexamethasone) are used to aid in both the preparation of cells prior to differentiation [10] and during differentiation [11-13].

It is important to establish standard protocols for in vitro experiments, as chemical modifications to hMSC maintenance environments can alter long-term research results including differentiation studies. In this work, the effects of different media compositions on hMSCs throughout normal in vitro maintenance are investigated. hMSCs were cultured in three different media formulations: mesenchymal stem cell growth media (MSCGM, Lonza), low glucose Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and DMEM supplemented with 10\% FBS and 10^{-7}$ M Dex. MSCGM is a commercially available MSC growth medium (Lonza, and previously under Cambrex) used in various studies [1,2,26]. DMEM + 10\% FBS is a commonly used media for the maintenance of MSCs [12,25,34-37] and the addition of 10^{-7}$ M Dex to DMEM + 10\% FBS media is reported to aid in the maintenance of MSCs [10,15].

**Materials and Methods**

**Cell Culture**

hMSCs (PT-2501, Lonza, Walkersville, MD) were routinely cultured in MSCGM supplemented with MSCGM SingleQuots (Lonza) and incubated at 37°C and 5\% CO₂
through passage 3. Media was changed three times a week and cells were passaged at 70% confluency. Initial seeding density for hMSCs was 1500 cells/cm². Cells at passages 4-10 were maintained in either MSCGM, DMEM (HyClone Laboratories, Logan, UT) supplemented with 10% hMSC-approved FBS (HyClone Laboratories) or DMEM supplemented with 10% FBS and 10⁻⁷ M Dex (Millipore, Billerica, MA). Cells were examined daily and images taken at each time point using a Zeiss AxioObserver.

Cell Counts

hMSCs, maintained in their respective media treatments, were harvested and seeded in 60 mm tissue culture-treated polystyrene dishes (Thermo Fisher Scientific, Rochester, NY), at 1500 cells/cm². Cells were maintained and routinely cultured in the respective media compositions through passage 10 and analyzed at passages 4, 5, 8 and 10. When cells reached the passage number of interest, they were harvested at days 3 and 9. Cells were removed from the plates using 0.25% trypsin-EDTA (Thermo Fisher Scientific) and counted using a hemocytometer. Each plate was counted nine times. The experiment was performed a total of three times. Averages and standard errors for each study point were calculated. A student’s t-test was used to calculate significance between media treatments and time points.

Immunocytochemistry

At passages 4, 5, 8 and 10, cells were also seeded on tissue culture-treated glass coverslips (TCTs) at a density of 2500 cells/cm². The hMSCs grew for the respective
time frame (3 or 9 days) before the cells were fixed in 10% formalin (Sigma-Aldrich Corp., St. Louis, MO). After fixation, the samples were permeabilized in 0.1% Triton X-100 (Integra Chemical Company, Kent, WA), followed by 1% bovine serum albumin (BSA) (Fisher Scientific, Fair Lawn, NJ) blocking for non-specific binding.

Protein expression was analyzed using immunocytochemistry. Proteins studied included stemness markers (Stro1, CD44), osteogenic differentiation markers (osteopontin, osteonectin) and myogenic differentiation markers (tropomyosin, sarcomeric actin).

The monoclonal antibodies MPIIIB10(1) (mouse anti-osteopontin, 1:500), developed by Michael Solursh and Ahnders Franzen, AON-1 (anti-osteonectin, 1:500), developed by John D. Termine and STRO-1 (mouse anti-stromal cell surface marker, 1:100), developed by Beverly Torok-Storb, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Sheep polyclonal anti-tropomyosin (1:500) and human monoclonal anti-CD44 FITC conjugate (1:100) were purchased from Millipore. Mouse monoclonal anti-sarcomeric actin (1:250) was purchased from Invitrogen (Carlsbad, CA). Samples incubated with solutions of Stro1/CD44, osteonectin/tropomyosin or osteopontin/sarcomeric actin at 4°C overnight. Corresponding AlexaFluor conjugated secondary antibodies (1:1000, Invitrogen) were applied at 37°C for 1 hour in two separate steps. Hoechst dye 33258 (Acros Organics) was used to counterstain nuclei.
qPCR

RNA was isolated from hMSCs at days 3 and 9 during passages 4, 5, 8 and 10 using an RNeasy Plus Micro Kit (Qiagen, Valencia, CA), per the manufacturer’s instructions. Resulting total RNA was eluted from the spin column membrane with RNase-free water.

The RT² 1st Strand cDNA Kit (Qiagen) was used with 0.5-1 μg of RNA for all samples to eliminate human genomic DNA and synthesize single-stranded cDNA. The kit was used in accordance with the manufacturer’s instructions. After conversion, the cDNA was analyzed using SABiosciences’ Human Mesenchymal Stem Cell PCR Arrays (PAHS-082A, Qiagen) using the ABI 7300 plate reader. Cycle settings were adjusted according to the manufacturer’s directions.

qPCR Analysis

Analysis of the qPCR data was completed using the ΔΔCt method, with a threshold value automatically set by the program at 20% above the baseline. Fold changes were determined through ΔΔCt comparison to the control—hMSCS at passage 3, day 3 in MSCGM.

RPL13A was chosen as the housekeeping gene, as it presented the smallest deviation across samples, when compared to the other four housekeeping genes provided in the PCR array. All data was subject to normalization to RPL13A, the housekeeping gene, corresponding to each experimental condition (passage number/day combination).
Statistical Analysis

The data was statistically analyzed by performing a student’s t-test between each experimental condition. Samples were compared between media formulations; passage number/day combinations were also compared amongst each other.

Results and Discussion

hMSCs were maintained and passaged in their respective media of study. At passages 4, 5, 8 and 10, cells were examined for changes in morphology (Figure 1.1). At passage 4, a small difference in cell shape is observable between MSCGM/DMEM medias and DMEM with the addition of Dex; the former are smaller in size. At passage 8, all media treatments produce uniformly broadened cells. By passage 10, all cells are broad, flat and large. Throughout passaging, Dex treated cells are relatively consistent, with slightly larger cells at passage 4.

Proliferation was assessed through a simple cell count. Across the study, large differences in cell numbers between passages and between mediums are observable (Figure 1.2). From day 3 to day 9, decreases in cell number were observed as early as passage 8 for MSCGM. This drop in cell number also occurred in passage 10 for hMSCs cultured in DMEM and DMEM + Dex mediums.

Further, 32,250 cells were seeded at the start of each cell count. A cell count less than this original plating number occurred at passages 8 and 10 for MSCGM and at passage 10 for DMEM and DMEM + Dex mediums. It was also observed in passage 5, day 3 cells treated with DMEM + Dex, indicating cell death.
Figure 1.1: Phase contrast images of hMSCs

Figure 1.2: Cell counts at each passage/day time point.

At the start of each cell count, 32,250 cells were seeded. P = passage, D = day
Cells were stained for stemness (Stro-1, CD44), osteogenic (osteopontin, osteonectin) and myogenic (tropomyosin, sarcomeric actin) markers. Highest expression of Stro-1, cell surface antigen expressed in stromal bone marrow, and CD44, a major adhesion molecule of the extracellular matrix, was observed in passage 5 (Figure 1.3). Loss of Stro-1 and CD44 were noted in all media treatments by passage 10. The addition of Dex into culture medium prolonged Stro-1 and CD44 expression into passage 8 cells.

![Figure 1.3: Expression of Stro-1 (red), CD44 (green) and nucleus (blue) in hMSCs.](image)

$P =$ passage, $D =$ day

In Figure 1.4, osteonectin and tropomyosin expression is shown. Osteonectin, a bone extracellular matrix (ECM) glycoprotein critical to initiation of mineralization, expression is detectable in all cells. In many cells observed, a well-defined osteonectin protein expression pattern is present. An alpha-helical coiled-coil protein found in
muscles, tropomyosin expression was observed in passages 4 and 10 for all mediums. DMEM-treated hMSCs had the highest levels of expression.

Figure 1.4: Expression of osteonectin (red), tropomyosin (green) and nucleus (blue) in hMSCs. Overlapping (red and green) expression signal appears in yellow. P = passage, D = day

Figure 1.5 shows osteopontin, a structural bone ECM protein produced by osteoblasts, and sarcomeric actin, a component of the contractile unit in cardiac muscle, expression in hMSCs. Osteopontin is observed in all mediums at passages 5 and 8, as well as passage 10 in Dex-treated mediums. The greatest expression of sarcomeric actin is observed in passage 5.

Gene expression was observed through the use of SABioscience’s qPCR arrays. The up- and down-regulation was calculated by the ΔΔCt method which compared the experimental cycle threshold to P4D3 MSCGM’s cycle threshold value. Genes CTNNB1 (cadherin-associated protein β-1), TGFB1(transforming growth factor β-1) and IGF1
(insulin-like growth factor 1) were noted for their roles in proliferation (Table 1.1).

CTNNB1 is a component of adherins junctions which regulate cell growth and adhesion between cells. It helps to anchor actin cytoskeleton and is thought to play a role in transmitting contact inhibition signals that cause cells to stop dividing. Up-regulation of CTNNB1 is observed in all mediums at passage 8, which corresponds to the drop in cell count number observed and reported in Figure 1.2.

**Figure 1.5**: Expression of osteopontin (red), sarcomeric actin (green) and nucleus (blue) in hMSCs. Overlapping (red and green) expression signal appears in yellow. P = passage, D = day

TGFB1 is a cytokine that plays a role in cellular functions such as control of cell growth, proliferation, differentiation and apoptosis. Down-regulation is observed in late passage 4 and passage 5 cells. In Figure 1.2, the largest cell increases occurred at P4D9; cell proliferation decreased after that time point.
In vitro, reduction of IGF1 signaling decelerates the degenerative aging process and therefore, it is believed that the corollary may be true, indicating that an increase in this signaling may accelerate aging. Up-regulation was observed in passages 8 and 10. This corresponds to observed decreases in cell plating (Figure 1.2) as well as large changes in cell morphology (Figure 1.1).

Table 1.1: CTNNB1, TGFβ1, IGF1 fold changes. Values were calculated with the ΔΔCt method compared to P4D3 MSCGM.

<table>
<thead>
<tr>
<th></th>
<th>CTNNB1 Fold Change</th>
<th>TGFβ1 Fold Change</th>
<th>IGF1 Fold Change</th>
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<tr>
<td></td>
<td>P4D3</td>
<td>P4D9</td>
<td>P5D3</td>
</tr>
<tr>
<td>MSCGM</td>
<td>-1.604</td>
<td>1.246</td>
<td>-1.392</td>
</tr>
<tr>
<td>DMEM</td>
<td>1.35</td>
<td>-2.821</td>
<td>-1.842</td>
</tr>
<tr>
<td>Dex</td>
<td>-1.24</td>
<td>1.339</td>
<td>-11.73</td>
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Table 1.2 shows fold regulation of IL6 (interleukin 6), MMP2 (matrix metalloproteinase-2), BMP6 (bone morphogenetic protein 6) and PPARγ (peroxisome proliferator-activated receptor gamma), genes associated with cell differentiation. IL6 and MMP2 are considered promoters of differentiation. Xiao et al. reported that passage
2 hMSCs grown in Dex for 6 days down-regulated IL6 and MMP2. This is observable across multiple passages of Dex-treated hMSCs, reported in Table 1.2.

<table>
<thead>
<tr>
<th></th>
<th>P4D3</th>
<th>P4D9</th>
<th>P5D3</th>
<th>P5D9</th>
<th>P8D3</th>
<th>P8D9</th>
<th>P10D3</th>
<th>P10D9</th>
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<td>IL6 Fold Change</td>
<td>+1.472</td>
<td>+2.716</td>
<td>-1.01</td>
<td>+2.374</td>
<td>+4.139</td>
<td>+2.369</td>
<td>+3.658</td>
<td></td>
</tr>
<tr>
<td>MMP2 Fold Change</td>
<td>-2.922</td>
<td>-1.204</td>
<td>+1.939</td>
<td>+2.575</td>
<td>+2.022</td>
<td>+2.006</td>
<td>+4.037</td>
<td>+6.908</td>
</tr>
<tr>
<td>BMP6 Fold Change</td>
<td>-4.952</td>
<td>-4.287</td>
<td>-6.989</td>
<td>-4.187</td>
<td>-10.73</td>
<td>-2.516</td>
<td>1.568</td>
<td>1.662</td>
</tr>
<tr>
<td>PPARG Fold Change</td>
<td></td>
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</table>

**Table 1.2**: IL6, MMP2, BMP6 and PPARG fold changes. Values were calculated with the ΔΔCt method compared to P4D3 MSCGM.

Further, Xiao et al. reported that the addition of Dex promotes osteogenic, chondrogenic and adipogenic differentiation. BMP6 induces bone and cartilage growth in MSCs. PPARG is a regulator of adipocyte differentiation. Both BMP6 and PPARG are up-regulated with the treatment of Dex. BMP6 up-regulation is observed over the entire
course of study presented in Table 1.2. This correlates with the observed expression of osteonectin in all media treatments at all passages. Osteopontin was also observed in passages 5 and 8, as well as in passage 10 cells treated with Dex.

**Conclusions**

Overall, the choice of media greatly affects cellular processes. The morphology of the cells changed in passage 8 for MSCGM- and DMEM-treated cells but was consistent in Dex-treated cells until passage 10. The addition of Dex to media extended proliferation capacity when compared to MSCGM and DMEM mediums. The loss of stemness markers was observed in passage 8 for MSCGM and DMEM mediums; stemness markers were expressed in cells treated with Dex until passage 10. Gene regulation of proliferation and differentiation markers correlates with observed cell counts and protein expression.

Morphology, proliferation, protein expression and RNA expression are affected by media composition. These typical growth mediums affect cellular processes during regular maintenance of hMSCs and are causing observable changes in cells before experimental conditions begin. Consistent protocols in hMSC research are needed to eliminate discrepancies between groups. By instituting standardization protocols, experiments will correlate between different research groups. These base-line changes will help to advance the field of hMSCs for use in therapeutic research.
References


Chapter 2
Anodic Aluminum Oxide (AAO) Membranes for Neurite Outgrowth

Review of Current Literature

Stem cells are widely used in the area of tissue engineering. The ability of cells to properly interact with materials on the nano- and micro- level is important in the success of the biomaterial. It is well-known that cells respond to their micro- and nano-environments through a process termed chemo-mechanotransduction.

When a cell is near a surface, nonspecific interactions such as electrostatics and van der Waals forces pull the two into close proximity with one another. Binding proteins in the on the surface bind to specific receptor integrins on the cell. This signal is rapidly transmitted to the actin filaments by the association of the focal adhesion complex proteins. Changes in the cell surface, whether they are mechanical or chemical in nature, change the integrin-focal adhesion signaling cascade. These changes result in variances in cellular processes such as differentiation, proliferation and migration.

This signaling cascade has to be properly transmitted for materials to integrate well with biological samples such as cells and tissues. Both chemical and mechanical cues can dictate how a cell responds to a surface. Unfavorable properties of a surface, both in the chemistry and the mechanics, can cause undesirable effects such as apoptosis. By controlling the properties of materials, cell response can be, to a degree, controlled.

Many groups are researching how mechanical properties affect stem cells, particularly in neural tissue engineering. As damaged neural tissue is unable to repair in vivo, the ability to generate and control neuronal growth in vitro from stem cell
precursors is a growing area of interest. Researchers are exploring various methods for therapeutic use in patients with neural tissue injuries. One such method involves integrating stem cells into the damaged tissue and allowing cells to properly differentiate via mechanical and chemical cues from the surrounding tissue. In the area of neural tissue engineering, it is important to understand how the mechanical environment alters stem cell fate.

Carbon nanotubes (CNTs) for use in neural tissue engineering are an area of research. Fabricated into both single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs), CNTs are constructed from sheets of graphine (0.4-100nm in diameter) [1]. CNTs are being explored for neural systems because they have many favorable properties including high tensile strength, electrical conductivity and nano-scale features [2-4]. Additionally, CNTs have been proven to be biocompatible [1,5,6].

Many groups have researched the applications of CNTs in vitro and in vivo. Stacked-cup carbon nanotubes (carbon nanofibers, CNFs) with both high and low energy fibers were created by McKenzie et al. [7]. Composite materials were formed by mixing polycarbonate urethane with each CNF type. Rat astrocytes were seeded onto the materials and the adhesion and proliferation rates observed. Astrocyte adhesion was the greatest on composites with the largest CNF diameter and lowest surface energy. Decreased adhesion of the astrocytes occurred with increasing weight percents of high surface energy CNFs in the composites. These results indicate CNFs’ potential in limiting glial scar tissue during neural implantation.
Webster et al. created CNF composites with polycarbonate urethane. The materials were seeded with rat astrocytes and PC-12 cells (rat pheochromocytoma). The researchers found that the composites supported neural cell function. A decrease in astrocyte adhesion density was also observed on composites with increasing CNF amounts. This study supports the idea of using CNFs in vivo due to the possibility of decreasing glial scarring after implantation.

Layer-by-layer assembled SWCNT composite films were studied in conjunction with mouse embryonic neurospheres isolated from the cortex by Jan et al. [8]. The cells were seeded on the composites at a low density of 200 neurospheres per sample to assess the interaction between the cells and the substrate. The SWCNT composites supported the differentiation of cells into neurons, astrocytes and oligodendrocytes, as shown through the positive staining of MAP-2 (neurons), glial fibrillary acidic protein (GFAP, astrocytes) and O4 (oligodendrocytes).

CNTs with modified surfaces have also been explored by researchers. Mattson et al. was the first group to report the use of CNTs in neuroscience research [9]. They grew embryonic rat-brain neurons on unmodified and 4-hydroxynonenal-modified MWCNTs; neurons grew and attached to both types of CNTs. When compared to unmodified CNTs, the CNTs that were coated with bioactive molecules had extensive growth and branching of neurites suggesting that a modified surface may be beneficial.

Neurotrophin (or nerve growth factor) covalently linked to CNTs was researched by Matsumoto et al. [10]. The group grew embryonic chick dorsal root ganglion neurons on the modified CNTs. Neuronal growth on the modified CNTs was similar to neural
growth using soluble neurotrophin in culture medium. Results indicated the ability of the CNTs to stimulate neurite outgrowths.

Chemically modified CNTs were also researched by Hu et al. [1]. The group studied the effects of changing surface charge of the CNTs on the growth of hippocampal neuronal cultures isolated from Sprague-Dawley rats. The modified substrates allowed neuronal growth, as characterized by the presence of growth cones, neurite outgrowths and branching. However, differences in neurite outgrowth lengths, number of growth cones and neurite branching were observable between the positively-charged, zwitterionic and negatively-charged CNTs.

Béduer et al. patterned SiO$_2$ cell culture surfaces with CNTs [11]. The group found that neuroblastoma mouse cells preferentially adhered to the CNT patterns. The neurite outgrowths were guided by the pattern created by the CNTs. Additionally, the researchers gave a possible reason for the preferential growth; they reported an enhanced protein adsorption on the CNTs when compared to the SiO$_2$ surface.

Groups have researched the enhanced electrical signaling of neuronal networks on CNTs. Lovat et al. reported attachment of hippocampal neurons to purified CNTs [12]. Using single-cell patch-clamp recordings, the group monitored the occurrence of spontaneous postsynaptic currents (PSCs). Neurons seeded on CNTs displayed a six-fold increase in the frequency of spontaneous PSCs over cells on glass coverslips. Average spontaneous action potential frequency of neurons on CNTs increased over neurons on glass, suggesting a significant increase in neuronal network operation.
The potential use of vertically-aligned carbon nanofibers (VACNFs) in excitable cell matrices was explored by McKnight et al. [13]. Surfaces were untreated, pretreated with poly-L-lysine (PLL) or pretreated with fibronectin prior to seeding with rat hippocampal cells or PC-12 cells. The researchers showed that the VACNF-containing electrode arrays supported the direct culture, differentiation and electroanalytical evaluation of neuronal cells. The results presented suggested a platform for studying temporal electrophysiological events in neural tissue.

Kam et al. constructed SWCNT-laminin composites through a layer-by-layer assembly [14]. When neural stem cells (NSCs) were seeded on the materials, cell adhesion and differentiation were observed. Longer neurite outgrowths were observed on SWCNT-laminin composites than laminin-coated glass surfaces. Positive staining for MAP-2 protein and GFAP indicated the ability of the SWCNT-laminin composites to induce differentiation in the NSCs. Functional neuronal networks were confirmed through the positive staining of synapsin protein. Additionally, calcium imaging showed the generation of action potentials after applying a lateral current through the composites.

The use of nanofibrous material networks are a growing area of research in neural tissue engineering. Typically created via electrospinning, these nano-featured fiber scaffolds have high surface to volume ratio. They also have a controllable 3D structure, with variable fiber size and porosity [15]. The scaffolds are also biocompatible with neural tissue [16-18].

Yang et al. (2004) created poly(L-lactic acid) (PLLA) nanofibrous scaffolds through liquid-liquid phase separation [19]. Resulting fiber diameters ranged from 50 to
350 nm and porosity ranged from 81% to 93%. After seeding the materials with C17.2 neural stem cells, some cell migration into the scaffolds was observed. Additionally, neuronal differentiation and neurite outgrowth was observed in cells seeded on the materials, indicating that the PLLA 3D, nanofibrous scaffolds have potential in neural tissue engineering.

Electrospun PLLA nanofibrous scaffolds were studied by Corey et al. [20]. Scaffolds with different levels of fiber alignment were seeded with primary rat dorsal root ganglia explants. Samples with highly aligned fibers supported highly aligned neurites; samples with random fiber alignment supported the lowest levels of neurite alignment. Further, neurite length was greatest on the PLLA scaffolds with the highest amount of fiber alignment. The change in neurite alignment and outgrowth, as a result of variance in fiber alignment, indicates that electrospun PLLA scaffolds have potential in directed tissue engineering applications.

Some groups have utilized electrospinning with bioactive additions, such as proteins, to create scaffolds for use in cell culture studies. Xu et al. studied the effects of seeding Schwann cells on electrospun silk-fibroin mats [17]. Results of the fabrication revealed that the electrospun mats were nanofibrous, with an average fiber diameter of approximately 420 nm. Additionally, the fibers were randomly oriented and interconnected which created a large, three-dimensional (3D), porous network. The average pore size created was 18 nm. When Schwann cells were seeded on the materials, they grew into the pores of the mat. The researchers also found that the electrospun mats had no significant cytotoxic affect on the cells. This study suggests the use of electrospun
materials in neural tissue engineering due to their biocompatibility and favorable extracellular matrix-like structure.

Electrospun poly-ε-caprolactone (PCL) and collagen/PCL (C/PCL) nanofibrous substrates were studied by Schnell et al. [16]. The researchers created fibers with average diameters of 559 and 541 nm for PCL and C/PCL, respectively. Chick embryonic DRG explants were placed on the materials and neurite outgrowth was observed after 24 hours; neurite outgrowth elongation was aligned with the fibers. Glial migration was also observed on both materials. The C/PCL nanofiber material was preferential for both neurite outgrowth and glial migration. Additional studies with single cells (dissociated DRG, Schwann cells and olfactory ensheathing cells) also highlighted the enhanced biological effects C/PCL materials had over the PCL substrates. The results suggest that electrospun C/PCL nanofibrous materials have potential in neural implants due to their favorable interactions with neural cells, including directed neurite outgrowth and elongation.

Ahmed et al. created polyamide nanofibrous scaffolds for use in neural cell culture [21]. After electrospinning, the nanofibers were covalently modified with neuroactive tenascin-C-derived peptides. The peptide-modified nanofibers supported more neurite generation and neuronal adhesion in cerebellar granule neurons than nanofiber surfaces without peptides. The peptide-modified, porous, nanofiber scaffolds may prove beneficial in neural studies due to their 3D, *in vivo*-like environment.

Johansson et al. studied the growth of superior cervical ganglion and DRG explants on nano-printed patterns in polymethylmethacrylate (PMMA) [22]. The
researchers created 17 different striped patterns with the width of the stripes ranging from 100 to 400 nm and the pitch ranging from 200 to 2000 nm. After the cells were seeded on the materials, it was observed that most axons aligned along the stripes imprinted by the patterning. Additionally, the axons grew on top of the stripes and not in the grooves formed between the ridges. Patterns with 100 nm widths had less alignment than surfaces with larger widths. The results suggest employing nano-patterning of polymer substrates to direct neuronal outgrowth in tissue engineering.

Surface roughness was researched by Fan et al. [23]. Silicon wafers were patterned via photolithography, producing surfaces with Ra values ranging from 2 to 810 nm. Raman spectra revealed little difference in surface chemical groups between wafers. Nigral cells, isolated from the subthalamus of prenatal E13-14 Wister rats, were cultured on the wafers. Significance of surface roughness on cell adherence and morphology was observed. The lowest surface roughness (Ra of 2.2) supported the least number of adhered cells; cells that did adhere exhibited abnormal morphologies compared to controls. Further, surfaces with Ra values greater than 70 nm also adversely affected cell growth. Due to the resulting cellular growth, the researchers identified an optimal range for surface roughness between Ra values of 20 and 50 nm. On patterned surfaces with Ra values of both 3 and 25 nm, cells migrated to areas of the wafer with optimal roughness. Surface roughness, a feature on the nano-scale, was shown to be influential in cell growth and should be considered when designing neural growth surfaces.

Many groups have shown the ability of the mechanical environment to influence neural processes such as differentiation and outgrowth extension. However, functional
neurons are required for implantable, therapeutics in neural tissue engineering. The ability of the cells to transmit signals across the synaptic cleft via neurotransmitters indicates functionality of the cells [24]. Neurotransmitter release and subsequent uptake is difficult for researchers to quantify \textit{in vitro}, as secretion levels are low.

**Introduction**

The ability to generate and control neuronal growth \textit{in vitro} from stem cell precursors is a growing area of interest in biomedicine. While primary neurons are functional, they are difficult to manipulate and incorporate into existing tissue. Neural stem cells have the ability to differentiate into a variety of neural cells and integrate into the existing tissue during development and maturation.

Neuronal development occurs in various stages from immature precursor cells to fully integrated and functionally mature neurons [25]. These developmental steps are classified into two categories: activity independent and activity dependent. Independent landmarks are thought to be genetically determined and include neuronal differentiation, migration and axon guidance [26]. Activity dependent stages of neuronal growth are heavily regulated by secreted molecules such as hormones and neurotransmitters [26]. The overall effects of the secreted molecules \textit{in vivo} are well researched; however, the effects on \textit{in vitro} differentiation are not fully understood. Researchers are unable to identify and measure the small molecules \textit{in vitro}, as the secreted hormones are absorbed by neighboring cells. Therefore, a substantial opportunity exists in neuronal interface research to develop a material platform that allows for both the proliferation and
differentiation of stem cells into neurons and the ability to quantify the secretome of neuronal cells.

C17.2 neural stem cells (NSCs) are an immortalized and multipotent cell line established by Snyder et al. [27,28]. Derived from the external germinal layer of neonatal mouse cerebellum, C17.2 neural precursors have been show to successfully implant into mouse germinal zones [28]. The NSCs integrate into the implanted tissue and contribute to cerebellum development [28]. Because C17.2 cells are functional in vivo, they are a relevant cell model for studying neuronal platforms.

AAO membranes are biocompatible and composed of highly-ordered nanopores that penetrate the entire material [29-31]. The inert properties of AAO membranes support the growth of neuronal cells and the nanopores may allow for selective concentration of secreted molecules. Nanopore sizing, surface functionalization and morphologies are controllable based upon experimental parameters and allow for precise segregation and selection of secreted molecules [32].

The use of AAO membranes in biology-related applications is a growing field of study. Hoess et al. used AAO membranes as a substrate for HepG2 (heptoma cell line) cell growth [33], while Walpole et al. investigated MG63 (osteoblast cell line) growth and differentiation on the membranes [31]. Adhesion and proliferation of fibroblasts and epidermal cells (NIH3T3 and HaCaT cells, respectively), were also assessed by groups [34,35]. In this work, neuronal differentiation of C17.2 neural stem cells on AAO membranes is examined as a means for developing an artificial cell/material synapse system.
Materials and Methods

Anodic Aluminum Oxide Membrane Fabrication

The fabrication procedure for the AAO membranes used in this experiment was based on a two-step mild anodization procedure originally proposed by Masuda and Fukuda in 1995 [36]. 99.99% pure aluminum was electropolished at a current of 6.5 A to remove any surface scratches. The electrolyte used for both anodization steps was 2.7% oxalic acid by weight mixed with ethanol in a ratio of 5:1. The temperature was held constant at 0°C during anodization with a recirculating chiller and air stirring.

The first anodization step was conducted for a total of 2 hours to form ordered pore nucleation sites. The oxide layer was then etched away at 65°C for 1 hour in a 1:1 mixture of 8% by volume orthophosphoric acid and 4% by weight chromic acid. The second anodization step was carried out for approximately 35 hours. The second anodization has a much more stable growth pattern because of the already established nucleation sites leading to an ordered pore structure, but there is no significant benefit to further etch-anodization cycles in terms of pore structure [37]. The voltage was varied during the first and second anodization steps in order to control the pore size and morphology of the AAO membranes as proposed by Bai et al. [30]. An etch cycle in a 1:1 mixture of 10% hydrochloric acid by volume and 0.1 M CuCl removed the remaining aluminum on the back of the foil leaving only the AAO membrane. The final etching step in 0.1 M orthophosphoric acid at 30°C for 75 minutes removed the barrier layer resulting in membranes with straight channel through-pores for cell growth. Resulting pore structures were quantified using SEM and ImageJ software.
Polycrystalline Alumina Controls Fabrication

Polycrystalline Al₂O₃ (PXA) was chosen as a control for cellular response studies. The alumina control samples have similar chemistry to the AAO but lack the porous and topographic features. AKP-HP Al₂O₃ powder from the Sumitomo Company with a mean particle size of 0.45 µm was spark plasma sintered to form the PXA samples. The powder was placed in a graphite die and held at 10 MPa and 700 °C for 30 min to burn off any organic impurities. Following a ramp up to 1300 °C and 60MPa, the samples were held for 25 min to sinter the particles and create alumina cylinders that were approximately 20 mm diameter by 8 mm long. The PXA was then sectioned using a high-speed diamond blade into thin sheets for cell culture.

Materials Sterilization and Preparation

The AAO membranes and PXA samples were UV sterilized overnight. The samples were washed with sterile 1X Phosphate Buffered Saline (PBS), followed by a 30 minute wash with growth medium (GM) [high glucose Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 5% Horse Serum (HS), 1% L-glutamine]. The membranes were incubated in GM overnight at 37°C and 5% CO₂.

Cell Culture

C17.2 NSCs were routinely maintained in GM. The NSCs were seeded onto the AAO membranes at 10,000 cells/cm² and incubated at 37°C and 5% CO₂. Controls
(tissue culture-treated glass [TCT], polystyrene [PS] and PXA) were also seeded with 10,000 cells/cm² and incubated at 37°C and 5% CO₂ after sterilization. The cells grew on the samples for 2 days before starting the serum-withdrawal protocol to differentiate the NSCs. Half of the total volume of media was removed and replaced with serum-free (DMEM high glucose with 1% L-glutamine) media every 2 days. The cells grew on the membranes for 14 days after the serum concentration had dropped below 1%.

Undifferentiated cells were also cultured for comparison to differentiated cells. The cells were grown on the samples for a comparable time frame of 21 days without undergoing the serum-withdrawal process; cells were maintained in regular GM for the experimental time frame.

**Immunocytochemistry (ICC)**

Following the 21 day growth period, the cells were formalin fixed and analyzed for neuronal and astrocytic differentiation using β-tubulin III (1:1000, Covance #A488-435L, neuronal) and glial fibrillary acidic protein (GFAP) (1:500, Sigma #C9205, astrocytic) antibodies, respectively.

Additional samples were analyzed with nestin (1:100, Developmental Studies Hybridoma Bank #Rat-401, NSCs) and neurofilament H/M (1:100, Covance #SMI-33R, neuronal) antibodies. Nuclei were counter-stained with Hoechst 33258.

**SEM Preparation**

After the cells grew for the 21 day time period, the samples were prepared for
SEM. The samples were fixed in 5% glutaraldehyde. Following fixation, the samples underwent dehydration via ethanol and hexamethyldisilazane incubations. After air drying overnight, the samples were sputter coated with iridium to provide a conductive surface for SEM observation.

qPCR

qPCR was performed on undifferentiated and differentiated cells. RNA was isolated from cells using an RNeasy Plus Micro Kit (Qiagen, Valencia, CA), per the manufacturer’s instructions. Resulting total RNA was eluted from the spin column membrane with RNase-free water. RNA was converted to cDNA via Qiagen’s Omniscript RT Kit. Gene expression was assessed through use of Qiagen’s SYBR Green qPCR kit. Primers were optimized for melting temperature and cycle number. Primer sequences are shown in Table 2.1

qPCR Analysis

Analysis of the qPCR data was completed using the ΔΔCt method, with a threshold value of 2 above the baseline. Fold changes were determined through ΔΔCt comparison to the control—undifferentiated C17.2 NSCs grown for 21 days on polystyrene. β-actin was chosen as the housekeeping gene, as it presented the smallest deviation across samples, when compared to the other possible housekeeping gene, Hsp90ab1. All data was subject to normalization to β-actin. The ΔΔCt was calculated by comparing the threshold cycle of the housekeeping gene (HKG) and the gene of interest.
Results and Discussion

SEM images show that AAO membrane fabrication resulted in regular pore geometry (Figure 2.1a-c). The pores penetrated the entire membrane; consistent pore sizes on the top and bottom of the membrane were calculated through ImageJ. The
fabrication of the PXA samples resulted in an irregular surface roughness, with features on both the macro- and nano-scale (Figure 2.1d).

After material fabrication, the cells were seeded onto the surfaces. The NSCs that underwent the 21 day growth period were examined using ICC and SEM. All differentiated samples (AAO, PXA, PS, TCT) were positive for β-tubulin III, nestin and neurofilament H/M, indicating a mixed phenotype population (Figure 2.2); GFAP did not result in positive staining, indicating no astrocytic differentiation.

Undifferentiated cells on TCT were positive for neurofilament H/M and nestin, but negative for β-tubulin III and GFAP expression (Figure 2.3a). Undifferentiated cells on PXA and 67 nm AAO samples were positive for neurofilament H/M, nestin and β-
tubulin III expression, but negative for GFAP expression (Figure 2.3b-d). The observed expression of β-tubulin III in undifferentiated (did not undergo serum-withdrawal) cells on PXA and AAO membranes may indicate that the materials serve as means of mechanically stimulating differentiation.

**Figure 2.2:** Differentiated C17.2s on AAO membranes stained for expression of nuclei (blue). Expression of (a) neurofilament H/M and (b) nestin are shown in red.

The β-tubulin III stained samples were used for neurite measurements in NeuronJ (Figure 2.4), as neurite outgrowth is often correlated to increased neuronal differentiation [37]. Compared to the tissue culture treated glass controls, all AAO membranes supported enhanced neurite outgrowth. The samples with the longest measured outgrowths were within the 60-69 nm and 70-79 nm ranges, suggesting the possibility of an optimal range of pore sizes (Figure 2.5). The PXA samples supported neurite lengths similar to neurons grown on 60-69 nm and 70-79 nm AAO samples. As shown in Figure 2.1d, the PXA surfaces exhibit surface roughness on the nano-scale. The preceding, combined with similar chemistries between PXA and AAO samples, may explain the observed neuronal similarities between PXA and 60-69 nm and 70-79 nm AAO samples.
Samples were stained for a,b) β-tubulin III (green) and NF (red), c) β-tubulin III (green) and GFAP (red) and d) β-tubulin III (green) and nestin (red). Nuclei are shown in blue.

Samples were also analyzed for neuronal population percentages (Figure 2.6). Tissue culture treated glass controls resulted in the highest neuronal population. Additionally, AAO membranes with smaller pore sizes (30-39 nm) supported larger neuronal populations than the other ranges of pores (60-69, 70-79 nm). These results suggest a relationship between pore size and neuronal population dynamics.

Cell layer morphology was examined via SEM. Figures 2.7a and 2.7b illustrate dense, tissue-like cell growth on AAO membranes. The tissue layer was mixed in
phenotype, as observable through morphological differences in the images. Neurons are outstretched and interacting with both the underlying cell layer and the AAO membrane (Figures 2.7c-e).

**Figure 2.4:** C17.2s differentiated on AAO membranes and stained for β-tubulin III (green) and nuclei (blue). AAO membrane pore sizes are (a) 38 nm, (b) 64 nm and (c) 78 nm. Tissue cultured treated glass control is shown in (d).

**Figure 2.5:** Neurite outgrowth measurements on AAO, PXA and TCT.
Cell layer morphology was examined via SEM. Figures 2.7a and 2.7b illustrate dense, tissue-like cell growth on AAO membranes. The tissue layer was mixed in phenotype, as observable through morphological differences in the images. Neurons are outstretched and interacting with both the underlying cell layer and the AAO membrane (Figures 2.7c-e).

Figure 2.6: Neuronal population percentage of C17.2 cells differentiated on AAO, PXA and TCT.

Results for qPCR are shown in Table 2.1. Fold changes were calculated with the \( \Delta \Delta Ct \) method, by comparing the experimental cycle threshold to the cycle threshold calculated for undifferentiated C17.2s grown on tissue cultured-treated polystyrene dishes. Neural stem cell differentiation markers studied include: neural stem cell (ABCG2, NeuroD1), neural progenitor cell (BLBP, TBR2), neuron-restricted progenitors (MAP2, \( \beta \)-tubulin III) and differentiated post-mitotic neuronal cells (Synaptophysin 1, Homer).
ABCG2 is a neural stem cell marker. Zhou et al. reported high levels of ABCG2 mRNA in primitive murine hematopoietic stem cells, with sharp down-regulation observed after differentiation [38]. For most samples, ABCG2 is down-regulated, with the largest down-regulation occurring on PXA samples. This may indicate a mature phenotype of neurons present on PXA samples.

Figure 2.7: SEM images of differentiated C17.2 cells grown on AAO membranes. Images illustrate (a, b) dense cellular growth (c) with neuronal interactions on underlying cell layers and (d, e) AAO membranes.

NeuroD1 is essential for the development of the central nervous system, particularly for the generation of granule cells [39-41]. Over-expression of NeuroD1 has
been shown to result in neuronal differentiation in adult hippocampal neural progenitors; deletion of NeuroD1 results in decreased survival and maturation of new adult neurons [42,43]. In Table 2.1, NeuroD1 is up-regulated in all samples that have undergone differentiation, while it is down-regulated in undifferentiated samples. NeuroD1 fold-change is not as up-regulated on AAO and PXA samples when compared to PS and TCT; this may indicate a more mature phenotype for cells grown on AAO and PXA samples.

BLBP is a neural progenitor cell marker for radial glial cells. In situ hybridization and ICC show that BLBP is transiently expressed in radial glial, and Feng et al. reported that BLBP is required for the establishment of the radial glial fiber system [44]. BLBP is down-regulated with most of the differentiated samples, with the exception of 50-59 nm AAO. Undifferentiated PXA samples have a large up-regulation of BLBP comparatively. The up-regulation may indicate an inherent radial glial phenotype for the preceding samples.

Sessa et al. found that TBR2 is critical for the development of intermediate basal progenitor cells [45], while Englund et al. reported a drop in TBR2 expression levels to undetectable levels in post-mitotic projection neurons [46]. TBR2 is down-regulated in differentiated samples on PXA and 70-79nm and 80-89nm. The down-regulation of TBR2 indicates a mature phenotype of neurons present. These results mirror neurite outgrowth measurements reported in Figure 2.4, where longer neurite extensions were measured for PXA and AAO membranes above 60 nm.

β-tubulin III is expressed in early, post-mitotic neurons and is one of the earliest neuronal cytoskeletal proteins in the development of the central nervous system [47,48].
All samples resulted in up-regulation of β-tubulin III, with largest up-regulation observed on PXA and 70-79nm AAO samples. These results correlate with the down-regulation of TBR2 observed, as well as the longest neurite extensions measured in Figure 2.4.

MAP2 has been suggested to be essential for dendritic growth by maintaining the neuronal morphology. It stabilizes microtubules by serving as cross-bridges between tubulin in dendrites through binding domains at the COOH-terminal [49,50]. MAP2 is up-regulated in differentiated PS and TCT samples, but down-regulated for differentiated PXA, 50-59 nm and 70-79 nm AAO samples. The increased expression observed in PS and TCT may indicate an immature phenotype, as the cells are still preparing for β-tubulin III expression. The observed down-regulation in samples suggests that MAP2 production is no longer necessary, as the β-tubulin III-MAP2 cross-linking structures are well-established.

**Table 2.2:** qPCR fold change

Values were calculated with the ΔΔCt method compared to undifferentiated C17.2s grown on polystyrene for 21 days.

<table>
<thead>
<tr>
<th>Gene</th>
<th>uTCT</th>
<th>uPXA</th>
<th>dPS</th>
<th>dTCT</th>
<th>dPXA</th>
<th>d50-59nm AAO</th>
<th>d70-79nm AAO</th>
<th>d80-89nm AAO</th>
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<tr>
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<td>-1.47</td>
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<td>-25.4</td>
<td>-1.17</td>
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<td>-1.85</td>
<td>-1.17</td>
<td>-4</td>
<td>4.32</td>
<td>-4</td>
<td>-1.47</td>
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<tr>
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<td>2.38</td>
<td>2.38</td>
<td>-6.73</td>
<td>5.99</td>
<td>-2.83</td>
<td>6</td>
<td>-3.56</td>
<td>1.88</td>
</tr>
<tr>
<td>Homer2</td>
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<td>69.09</td>
<td>4.32</td>
<td>17.27</td>
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<td>6.85</td>
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<td>-8</td>
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<td>-8</td>
<td>-1.59</td>
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<td>10.08</td>
<td>127.97</td>
<td>-6.35</td>
<td>-2.52</td>
<td>-3.17</td>
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<td>9.51</td>
<td>15.11</td>
<td>3.56</td>
<td>7.55</td>
<td>4.49</td>
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<tr>
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<td>17.27</td>
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<td>21.76</td>
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<tr>
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Up-regulation of synaptic proteins Synaptophysin and Homer (1,3) by PS and TCT indicate that the cells are maturing into functional cells. The synaptic proteins are required for the cells to transmit signals across the synaptic cleft. Down-regulation of synaptic proteins Synaptophysin and Homer (1, 3) by PXA and 70-79 nm AAO suggests a matured neuronal phenotype, as the cells are not preparing to form synapses, but may have already formed the proteins necessary for functional synapses.

Conclusions

The resulting ICC images indicate that neurite outgrowths were the greatest on AAO membranes with pore sizes of 60-69 and 70-79 nm. Outgrowths on 30-39 nm were greater than those measured on tissue culture treated glass. Neuronal percentage populations also changed based upon the membrane pore size, with TCT and 30-39 AAO membranes resulting in the largest percentage of neurons. As illustrated by ICC and SEM images, the resulting cell layer is dense, with a mixed phenotype population. qPCR fold changes observed in neural differentiation markers correlate with observed neurite measurements, indicating that differentiated cells on PXA and 70-79nm AAO samples may exhibit the most mature phenotype.

The resulting data suggest that AAO membranes support greater neurite outgrowth than traditional cell culture surfaces such as tissue culture treated glass. The presence of the dense, mixed phenotype population suggests the possibility of tissue growth on the membranes. The combinatorial results indicate the AAO membrane pore size can directly affect the differentiated cell population. Data suggests a highly tunable
correlation between AAO nanopore sizes and differentiated cell populations. By selecting AAO membranes with specific nanopore size ranges, control of neuronal network density and neurite outgrowth length was achieved.
References


37. Jaworski, J. et al. (2005). Control of dendritic arborization by the phosphoinositide-


Chapter 3

Effect of Substrate Stiffness on Neural Stem Cell Differentiation Markers

Review of Current Literature

Mechanical cues from the extracellular environment have been shown to effect stem cell fate [1-3]. The cytoskeleton of the cell is important in cellular processing of microenvironments. Comprised of dynamic polymer fibers (microtubules, actin filaments and intermediate filaments), the cell’s main mechanical supports aid in the interaction with neighboring cells. Actin fibers, concentrated at the membrane, help with mobility [4] and cell-to-cell and cell-to-substrate connections via integrins [5].

Attached to actin filaments are integrins, which transmit extracellular cues intracellularly. Approximately 10 nm in diameter, integrin receptors on cells are able to transmit surface cues into signals for various cellular processes [6-8]. The clustering of integrins and the formation of focal adhesions, both necessary processes in the signaling cascade, are on the order of nanometers to microns [9-10].

Additional cellular proteins contained in focal adhesion complexes are α-actinin, cinculin, paxillin, tallin and vinculin [5,11]. In particular, the protein paxillin interacts with both the integrin and the actin cytoskeleton [12]. The arrangement of proteins in the focal adhesion complex between the membrane-spanning integrin and the corresponding intracellular actin fibers help to transmit signals from the surrounding environment into the cell.

When a cell is near a surface, nonspecific interactions such as electrostatics and van der Waals forces pull the two into close proximity with one another. Binding proteins
on the surface bind to specific receptor integrins on the cell. This signal is rapidly transmitted to the actin filaments by the association of the focal adhesion complex proteins. Changes in the cell surface, whether they are mechanical or chemical in nature, change the integrin-focal adhesion signaling cascade. These changes result in variances in cellular processes such as differentiation, proliferation and migration. By controlling the properties of materials, cell response can be, to a degree, controlled.

The process by which a cell internalizes extracellular mechanical cues into biochemical signals is termed mechanotransduction. Mechanical properties influence numerous cellular processes. Cell shape has been shown to influence differentiation [13,14]. The elastic modulus of the surface upon which a cell sits influences cell fate as well. Migration [15,16], differentiation [17], apoptosis [18] and proliferation [19] have been affected by the elastic modulus of the culture surface. The topography of a surface also alters cell fate. Focal adhesion number and alignment, actin stress fiber alignment and cell body alignment as a whole are influenced by topography [20-22]. Differences in surfaces include molecular conformation and surface roughness. Shear stress on cells can alter cellular processes. Further, osmotic pressure; cyclic strain and compression; and generated electric fields affect cells [23-26].

Fine-tuning these surface mechanical properties may result in highly-refinable tissues, particularly in the area of neural tissue engineering. As damaged neural tissue is unable to repair in vivo, the ability to generate and control neuronal growth in vitro from stem cell precursors is a growing area of interest in biomedicine. Researchers are exploring various methods for therapeutic use in patients with neural tissue injuries. One
such method involves integrating stem cells into the damaged tissue and allowing cells to properly differentiate via mechanical and chemical cues from the surrounding tissue. While primary neurons are functional, they are difficult to manipulate and incorporate into existing tissue. Neural stem cells have the ability to differentiate into a variety of neural cells and integrate into the existing tissue during development and maturation.

Neuronal development occurs in various stages from immature precursor cells to fully integrated and functionally mature neurons [27]. These developmental steps are classified into two categories: activity independent and activity dependent. Independent landmarks are thought to be genetically determined and include neuronal differentiation, migration and axon guidance [28]. Activity dependent stages of neuronal growth are heavily regulated by secreted molecules such as hormones and neurotransmitters [28].

Differentiation of neural stem cells into functional neurons requires various precursor divisions. A cell begins as a neural stem cell and transitions into a neural progenitor cell. Neural progenitors can either be basal progenitors or radial glial progenitors, with the former differentiating into neuron-restricted progenitor cells and the latter becoming glial-restricted progenitors. Neuron-restricted progenitors, once fully differentiated and functional, are classified as differentiated post-mitotic neuronal cells.

Neural stem cell markers include ABCG2/ (BCRP1) (ATP-binding cassette subfamily G member 2) and NeuroD1/(BETA2). ABCG2 is a membrane-associated protein that is thought to be a molecular determinant of side population phenotypes [29,30]. ABCG2 is conserved in stem cells from a wide variety of sources, including murine bone marrow, skeletal muscle and cultured embryonic stem cells [30]. Zhou et al. reported
high levels of ABCG2 mRNA in primitive murine hematopoietic stem cells, with sharp
down-regulation observed after differentiation [30].

A member of the proneural helix-loop-helix class of transcription factors,
NeuroD1 is essential for the development of the central nervous system, particularly for
the generation of granule cells [41-43]. Over-expression of NeuroD1 has been shown to
result in neuronal differentiation in adult hippocampal neural progenitors; deletion of
NeuroD1 results in decreased survival and maturation of new adult neurons [44,45].

Neural progenitor cell markers include radial glial cell marker BLBP/(FABP7)
(brain lipid-binding protein) and basal progenitor cell marker TBR2/(EDMES) (T-brain
gene-2). BLBP, present in both the nucleus and the cytoplasm, carries small hydrophobic
signaling molecules between compartments. In situ hybridization and ICC show that
BLBP is transiently expressed in radial glial, and Feng et al. reported that BLBP is
required for the establishment of the radial glial fiber system [46].

TBR2 is a T-domain transcription factor present in the developing brain. TBR2 is
expressed in high levels in neuronal progenitors in the subventricular and ventricular
zones [47]. Sessa et al. found that TBR2 is critical for the development of intermediate
basal progenitor cells [48], while Englund et al. reported a drop in TBR2 expression
levels to undetectable levels in post-mitotic projection neurons [47].

Neuron-restricted progenitor cell markers include β-tubulin III and MAP2
(microtubule-associated protein 2). β-tubulin III is a highly-conserved, neuronal
vertebrate isotype of tubulin, the structural unit of microtubules. β-tubulin III is expressed
in early, post-mitotic neurons and is one of the earliest neuronal cytoskeletal proteins in the development of the central nervous system [49,50].

MAP2 has been suggested to be essential for dendritic growth by maintaining the neuronal morphology. It stabilizes microtubules by serving as cross-bridges between tubulin in dendrites through binding domains at the COOH-terminal [51,52].

Even though a neuron has become post-mitotic, functional neurons are required for implantable, therapeutics in neural tissue engineering. The ability of the cells to transmit signals across the synaptic cleft via neurotransmitters indicates functionality of the cells [53].

Neurons propagate signals through synapses. Synapses consist of pre-synaptic cells, which transmit signals, and post-synaptic cells, which receive signals. The space between the cells is termed the synaptic cleft. Chemical synapses rely on the release of neurotransmitters into the synaptic cleft and subsequent uptake of the molecules to transmit signals [54,55].

The pre-synaptic protein synaptophysin consists of four membrane-spanning domains and can form homo-oligomers [56]. It is a major component of synaptic vesicles contained in the pre-synaptic cell [57,58]. Because it binds calcium, synaptophysin is thought to be involved in calcium-dependent exocytosis of neurotransmitters from synaptic vesicles into the synaptic cleft [59]. However, the exact function of synaptophysin is still unknown.

Synaptophysin interacts with vesicle-associated membrane protein 2 (VAMP2), a v-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)
involved in synapse vesicle fusion with the outer cell membrane of the pre-synaptic cell [60-63]. Edelmann et al. reported that the binding of synaptophysin to VAMP2 seems to be mutually exclusive with the binding of VAMP2 to the SNARE complex [51]. Further, Pennuto et al. found that when synaptophysin forms homo-oligomers, it binds to VAMP2 and synaptic vesicle fusion with the plasma membrane is inhibited [54]. The group also observed vesicle fusion with the outer membrane when synaptophysin was not oligomerized and therefore, unbound to VAMP2. As a result, it is proposed that synaptophysin may act as a regulator of SNARE complex assembly in pre-synaptic cells [51,54].

Homer is a post-synaptic molecular scaffold protein that clusters specific synaptic proteins [55-57]. It is thought to provide organization to regulate post-synaptic signaling cascades propagated by the cell’s uptake of released neurotransmitters [58]. Homer proteins contain a C-terminal coiled-coil domain which allows for homo-dimerization [59,60]. The dimerization of Homer in post-synaptic cells is crucial in creating the scaffolding signal transduction pathway essential for propagating signals [61].

The development of a neuron from a neural stem cell occurs over several distinct divisions. Protein expression at each step in the differentiation process is also unique to that stage in development. Studying the gene expression of particular neural markers over the course of differentiation may provide the ability to better understand how mechanical properties of a surface influence cellular differentiation events. In turn, fine-tuning of neuronal properties through the use of mechanical materials platforms may be possible.
Introduction

Recent work highlights the ability of the mechanical environment to influence stem cell fate. Termed mechanotransduction, the process by which cells convert extracellular mechanical cues into internal biochemical cues is a growing area of research. Cell migration, proliferation, differentiation and apoptosis have been shown to be affected by a cell’s microenvironment [1-3, 19-26]. Substrate elasticity is a main focus for researchers in the field of stem cell mechanobiology. A material’s resistance to deformation, the property of elasticity has been shown to influence cellular processes such as migration, proliferation and differentiation [1-3, 62, 63]. Engler et al. showed that a material elastic modulus similar to a cell’s native tissue can cause stem cells to differentiate into that cell-specific lineage [2]. The researchers used polyacrylamide (PA) gels of varying stiffness to direct human mesenchymal stem cells into specific fates.

The effects of substrate elasticity on neuronal cell processes have been researched by a variety of groups, with neurite extension lengths as the most widely studied neuronal process [64,65]. Most research into neural mechanotransduction utilizes primary neurons [64-68], with a few studies exploring neural stem cells [69-71]. While primary neurons are functional, they are difficult to manipulate and incorporate into existing tissue. Neural stem cells have the ability to differentiate into a variety of neural cells and integrate into the existing tissue during development and maturation.

C17.2 neural stem cells (NSCs) are an immortalized and multipotent cell line established by Snyder et al. [72,73]. Derived from the external germinal layer of neonatal mouse cerebellum, C17.2 neural precursors successfully implant into mouse germinal
zones [73]. The NSCs integrate into the implanted tissue and contribute to cerebellum development [73]. Because C17.2 cells are functional in vivo, they are a relevant cell model for studying neuronal platforms. However, characterization work of the C17.2 cells in vitro is minimal. Recently, the cells were shown to form synapses in vitro through the co-localization staining of pre-synaptic and post-synaptic vesicles. This finding is important in the study of neural stem cells and the development of the differentiated neuronal cells needs to be further investigated and characterized.

To understand how material stiffness affects differentiation in C17.2 neural stem cells were cultured PA substrates of varying stiffness. qPCR was used to analyze neural stem cell (ABCG2, NeuroD1), neural progenitor cell (BLBP, TBR2), neuron-restricted progenitors (MAP2, β-tubulin III) and differentiated post-mitotic neuronal cells (Synaptophysin 1, Homer) RNA expression. Results suggest a relationship between material stiffness and neuronal development in C17.2 neural stem cells.

**Materials and Methods**

**Polyacrylamide (PA) Gel Fabrication**

PA gels were fabricated on cover glass (VWR) through a modified process first described by Pelham and Wang [16,74,75]. To activate the glass substrates, the cover slips were flamed, coated with 0.1 N NaOH and allowed to dry. Three-aminopropyltrimethoxysilane (Acros Organics) was evenly spread on glass before air drying for 5-10 minutes. The cover slips were washed with ddH₂O. Seventy percent
glutaraldehyde (Alfa Aesar) was incubated with the cover slips for 30 minutes, followed by washing with ddH$_2$O. Activated glass was allowed to air dry.

PA gels were fabricated on the activated glass. Low stiffness (140 Pa) and high stiffness (60,000 Pa) PA gels were achieved by varying the ratios of polyacrylamide and bis-acrylamide in gel solution, as reported by Johnson et al. [75]. Briefly, the gel solutions were degassed and pipetted onto the activated glass cover slips. Rain-X-coated glass cover slips were added on top of the PA gel solutions. The PA gels were allowed to polymerize for 30-60 minutes; after polymerization, the Rain-X-coated glass was removed from the gel. Gels were then functionalized with 0.2 mg/mL collagen (BD) through Sulfo-SANPAH (Thermo Scientific) cross-linking. PA gels were UVed overnight to sterilize.

**Collagen-Coated Glass Fabrication**

As a control, collagen-coated glass was fabricated for cell culture. Briefly, glass cover slips (VWR) were incubated with 0.2 mg/mL collagen (BD) solution overnight. Glass was sterilized via UV light treatment.

**Cell Culture**

C17.2 NSCs were routinely maintained in GM. The NSCs were seeded onto the PA gels and collagen-coated glass at 10,000 cells/cm$^2$ and incubated at 37°C and 5% CO$_2$. The cells grew on the substrates for 2 days before starting the serum-withdrawal protocol to differentiate the NSCs. Half of the total volume of media was removed and
replaced with serum-free (DMEM high glucose with 1% L-glutamine) media every 2 days. The cells grew on the membranes for up to 14 days after the serum concentration had dropped below 1%.

Subconfluent, undifferentiated C17.2s were also cultured for comparison. C17.2s were seeded onto polystyrene culture dishes and allowed to grow to 70% confluency.

qPCR

qPCR was performed on cells at various time points throughout the differentiation process. RNA was collected 2 days after medium serum percentages reached: 15%, 7.5%, 1.88% and 0.94%, as well as 2 and 3 weeks after the start of serum-withdrawal. RNA was isolated from cells using an RNeasy Plus Micro Kit (Qiagen, Valencia, CA), per the manufacturer’s instructions. Resulting total RNA was eluted from the spin column membrane with RNase-free water. RNA was converted to cDNA via Qiagen’s Omniscript RT Kit. Gene expression was assessed through use of Qiagen’s SYBR Green qPCR kit. Primer melting temperature and cycling times were optimized. Primer sequences are shown in Table 3.1

qPCR Analysis

Analysis of the qPCR data was completed using the ΔΔCt method, with a threshold value of 2 set above the baseline. Fold changes were determined through ΔΔCt comparison to the control—subconfluent C17.2 NSCs grown to 70% confluency on polystyrene. β-actin was chosen as the housekeeping gene, as it presented the smallest
deviation across samples, when compared to the other possible housekeeping gene, Hsp90ab1. All data was subject to normalization to β-actin. The ΔΔCt was calculated by comparing the threshold cycle of the housekeeping gene (HKG) and the gene of interest (GOI) for both the studied time point (PA gel or collagen-coated glass) and the control (undifferentiated C17.2s on polystyrene).

**Table 3.1: C17.2 qPCR primers**

<table>
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<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Tm</th>
<th>Accession</th>
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<tbody>
<tr>
<td>ABCG2</td>
<td>F: 5'- TGGACTCAAGCACAGCGAAT -3’&lt;br&gt;R: 5'- ATCCGCAGGTTGGTAGTGG -3’</td>
<td>F: 59.96&lt;br&gt;R: 60.04</td>
<td>NM_011920.3</td>
</tr>
<tr>
<td>NeuroD1</td>
<td>F: 5'- AATCATACAGCGAGAGCGG -3’&lt;br&gt;R: 5'- TGAGTCTGGGAGTAGAAGG -3’</td>
<td>F: 59.97&lt;br&gt;R: 59.02</td>
<td>NM_010894.2</td>
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<td>BLBP</td>
<td>F: 5'- TGATCCGGACACAATGCAA -3’&lt;br&gt;R: 5'- TCCATCAAACCACACAG -3’</td>
<td>F: 59.96&lt;br&gt;R: 59.96</td>
<td>NM_021272.3</td>
</tr>
<tr>
<td>TBR2</td>
<td>F: 5'- ATCTCCACGGATCCCCTA -3’&lt;br&gt;R: 5'- GCTTTGTTGGAGTAGCGGT -3’</td>
<td>F: 59.43</td>
<td>NM_010136.3</td>
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<tr>
<td>β-tubulin III</td>
<td>F: 5'- GGGCGCATGTCTATGAAGGA -3’&lt;br&gt;R: 5'- GCTTCCGATTCCTCGTCATCA -3’</td>
<td>F: 59.89&lt;br&gt;R: 60.2</td>
<td>NM_023279.2</td>
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<tr>
<td>MAP2</td>
<td>F: 5'- CATCAAAACATTGCTGGGG -3’&lt;br&gt;R: 5'- GCTTCCGATTCCCTCGATCA -3’</td>
<td>F: 59.52&lt;br&gt;R: 60.04</td>
<td>NM_001039934.1</td>
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<td>Synaptophysin</td>
<td>F: 5'- TGCCAACAAGACGGAGAG -3’&lt;br&gt;R: 5'- TAGTCGCCCTTAAAGCAG -3’</td>
<td>F: 60.25&lt;br&gt;R: 60.04</td>
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<td>F: 59.31&lt;br&gt;R: 60.04</td>
<td>NM_011982.2</td>
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<tr>
<td>Homer 2</td>
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<td>F: 60.09&lt;br&gt;R: 59.96</td>
<td>NM_001164086.1</td>
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<td>Homer 3</td>
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<td>F: 59.64&lt;br&gt;R: 59.83</td>
<td>NM_001146153</td>
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<td>β-actin (HKG)</td>
<td>F: 5'- AGTGTCGTTGGAGCATCCGT -3’&lt;br&gt;R: 5'- AGCTCAACAGGCAGCTTCCCTA -3’</td>
<td>F: 59.61&lt;br&gt;R: 60.62</td>
<td>NM_007393.3</td>
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Results and Discussion

The C17.2 NSCs were seeded onto the low and high stiffness PA gels, as well as the collagen-coated glass controls. RNA was collected at six time points throughout the 21-day differentiation process, as highlighted in Table 3.2.

**Table 3.2**: Serum percentages corresponding to days in culture.

*Asterisk indicates sampling day.

<table>
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<th>Day</th>
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<tr>
<td>1*</td>
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</tr>
<tr>
<td>3*</td>
<td>7.5</td>
</tr>
<tr>
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</tr>
<tr>
<td>9*</td>
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</tr>
<tr>
<td>11</td>
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<td>17</td>
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<tr>
<td>21*</td>
<td>0.01465</td>
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</table>

Results for qPCR experiments are shown in Tables 3.3 and 3.4. Fold changes are reported as up- or down-regulation of expression levels compared to subconfluent, undifferentiated C17.2 NSCs grown on polystyrene for 21 days.

ABCG2 is a neural stem cell marker. Zhou et al. reported high levels of ABCG2 mRNA in primitive murine hematopoietic stem cells, with sharp down-regulation observed after differentiation [30]. Overall, the regulation of ABCG2 remained consistent at low levels, with a larger down-regulation observed at 2 weeks. This down-regulation suggests the presence of maturing neurons.
NeuroD1 is essential for the development of the central nervous system, particularly for the generation of granule cells [31-33]. Over-expression of NeuroD1 has been shown to result in neuronal differentiation in adult hippocampal neural progenitors; deletion of NeuroD1 results in decreased survival and maturation of new adult neurons [34,35]. In Tables 3.3 and 3.4, NeuroD1 is up-regulated throughout the differentiation process for all sample types. An increase in NeuroD1 expression is observed for cells at 15% serum, when compared to expression levels in subconfluent, undifferentiated C17.2 cells on polystyrene. This may indicate the material’s inherent ability to influence neuronal differentiation. Additionally, NeuroD1 is highly up-regulated for samples at 0.94% serum and 2 weeks, with a decrease in up-regulation observed at 3 weeks. The preceding may indicate that mature neurons are forming and NeuroD1 production is declining.

BLBP is a neural progenitor cell marker for radial glial cells. In situ hybridization and ICC show that BLBP is transiently expressed in radial glial, and Feng et al. reported that BLBP is required for the establishment of the radial glial fiber system [36]. BLBP is up-regulated throughout the differentiation process until less than 1% serum is reached for cells grown on low and high PA gels. The largest up-regulation of BLBP is observed at 7.5% serum, which suggests the neural stem cells have begun the process into neural progenitor cells. Up-regulation of BLBP is still reported in cells grown on collagen-coated glass until 2 weeks, which may indicate a slower differentiation process compared to PA gel samples that have already down-regulated BLBP. This may also suggest that the NSCs are differentiating into radial glial cells on the collagen-coated glass substrates.
Down-regulation of BLBP occurs at 3 weeks for collagen-coated glass, while levels on PA gel samples are similar to control levels.

Sessa et al. found that TBR2 is critical for the development of intermediate basal progenitor cells [38], while Englund et al. reported a drop in TBR2 expression levels to undetectable levels in post-mitotic projection neurons [37]. TBR2 is up-regulated throughout the experiment, with samples at 3 weeks having levels similar to the control. For high PA gels, the highest up-regulation occurs at 7.5%, indicating that cells on these materials have become neural progenitors; on low PA gels, TBR2 levels are the highest at 0.94% serum. For collagen-coated glass, up-regulation is greatest at 2 weeks, suggesting that the cells undergoing differentiation have progressed more slowly, when compared to cells on PA gels.

β-tubulin III is expressed in early, post-mitotic neurons and is one of the earliest neuronal cytoskeletal proteins in the development of the central nervous system [39,40]. Most samples resulted in up-regulation of β-tubulin III, with largest up-regulation observed at 7.5%/1.88% serum for high PA gels and 1.88% serum for low PA gels. The largest observed up-regulation of β-tubulin III for collagen-coated glass occurs later at 0.94% serum. The preceding may indicate a longer differentiation period for C17.2 NSCs grown on glass, when compared to PA gel cell growth. This expression pattern is mirrored with TBR2 neural progenitor levels.

MAP2 has been suggested to be essential for dendritic growth by maintaining the neuronal morphology. It stabilizes microtubules by serving as cross-bridges between tubulin in dendrites through binding domains at the COOH-terminal [41,42]. MAP2 is
generally down-regulated for all three materials. The observed large down-regulation in levels between 1.88% serum and 2 weeks suggests that MAP2 production is no longer necessary, as the β-tubulin III-MAP2 cross-linking structures are well-established.

Table 3.3: Fold changes for 15%, 7.5% and 1.88% serum.

<table>
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<tr>
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<th>15%</th>
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<th>7.50%</th>
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<td></td>
<td>Glass</td>
<td>Low</td>
</tr>
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<tr>
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<td>1.41</td>
<td></td>
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<td>-1.58</td>
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Table 3.4: Fold changes for 0.94% serum, 2 weeks and 3 weeks.

*Indicates data not available at time of publishing

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<th>0.94%</th>
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<th>3 weeks</th>
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Up-regulation of synaptic proteins Synaptophysin and Homer 2 generally occurs across all samples. The synaptic proteins are required for the cells to transmit signals across the synaptic cleft. Homer 2 is highly up-regulated at 2 and 3 weeks in cells grown on collagen-coated glass, whereas expression levels in low and high PA gel samples are no longer highly up-regulated at those time points. This suggests that production of Homer 2 occurs earlier in cells differentiated on PA gels than in cells grown on glass, indicating that the differentiation process is slowed for collagen-coated glass samples.

**Conclusions**

Expression levels of neuronal differentiation markers were studied throughout the differentiation process. Fold changes were reported for cells grown on collagen-coated glass, low stiffness PA gel and high stiffness PA gel samples. Generally, cells progressed through the differentiation process, with key landmarks occurring at different time points for the varying materials. Overall, it appears as though the PA gels (both low and high elastic moduli) supported a faster differentiation of NSCs over collagen-coated glass. This statement is corroborated by the up-regulation expression levels of BLBP and TBR2 (neural progenitor cell markers), β-tubulin III (neuron-restricted progenitor cell marker) and Homer 2 (differentiated post-mitotic neuronal cell marker) that occurred later in glass samples than in PA gel samples.

Understanding expression patterns of neuronal differentiation markers may lend itself to a better comprehension of mechanobiology in neural tissue engineering. PA gels, with elasticities closer to native brain tissue, supported a faster and more robust
differentiation progression over collagen-coated glass. From neural progenitors to fully differentiated, mature neurons, each step in the process occurred earlier in cells grown on PA gels than on glass. These findings indicate an ability to fine-tune neuronal differentiation through mechanical properties of substrates. The capacity to alter differentiation periods of neural stem cells will lead to better neural platforms for implantation and overall improvements in the field of neural tissue engineering.
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replacement of neurons undergoing targeted apoptotic degeneration in adult mouse 
Vita

Meghan Casey was born on July 31, 1989 in Mountaintop, Pennsylvania to Joseph Casey and Diane Grochowski. She has a younger sister, Jillian and an older brother Joseph. Meghan graduated from Lehigh University in 2011 with a Bachelor of Science in Bioengineering and a minor in Political Science. During her undergraduate time, she spent a semester abroad in Townsville, Australia. Meghan also worked for Dr. Jedlicka as an undergraduate researcher, which allowed for a smooth transition as a Graduate Research Assistant in the Jedlicka lab. She graduated from Lehigh University in 2013 with a Master of Science in Bioengineering and entered the biotechnology industry.