Tissue Engineered Myelination And The Stretch Reflex Arc Sensory Circuit: Defined Medium Formulation, Interface Design And Microfabrication

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University of Central Florida

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TISSUE ENGINEERED MYELINATION AND THE STRETCH REFLEX ARC SENSORY CIRCUIT: DEFINED MEDIUM FORMULATION, INTERFACE DESIGN AND MICROFABRICATION

by

JOHN WAYNE RUMSEY
B.S. University of Florida, 2001
M.S. University of Central Florida, 2004

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Burnett School of Biomedical Sciences in the College of Medicine at the University of Central Florida Orlando, Florida

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Major Professor: James J. Hickman
ABSTRACT

The overall focus of this research project was to develop an in vitro tissue-engineered system that accurately reproduced the physiology of the sensory elements of the stretch reflex arc as well as engineer the myelination of neurons in the systems. In order to achieve this goal we hypothesized that myelinating culture systems, intrafusal muscle fibers and the sensory circuit of the stretch reflex arc could be bioengineered using serum-free medium formulations, growth substrate interface design and microfabrication technology.

The monosynaptic stretch reflex arc is formed by a direct synapse between motoneurons and sensory neurons and is one of the fundamental circuits involved in motor control. The circuit serves as a proprioceptive feedback system, relaying information about muscle length and stretch to the central nervous system (CNS). It is composed of four elements, which are split into two circuits. The efferent or motor circuit is composed of an $\alpha$-motoneuron and the extrafusal skeletal muscle fibers it innervates, while the afferent or sensory circuit is composed of a la sensory neuron and a muscle spindle. Structurally, the two muscular units are aligned in parallel, which plays a critical role modulating the system’s performance. Functionally, the circuit acts to maintain appropriate muscle length during activities as diverse as eye movement, respiration, locomotion, fine motor control and posture maintenance. Myelination of the axons of the neuronal system is a vertebrate adaptation that enables rapid conduction of action potentials without a commensurate increase in axon diameter. In vitro neuronal systems that reproduce these effects would provide a unique modality to study
factors influencing sensory neuronal deficits, neuropathic pain, myelination and diseases associated with myelination.

In this dissertation, results for defined \emph{in vitro} culture conditions resulting in myelination of motoneurons by Schwann cells, pattern controlled myelination of sensory neurons, intrafusal fiber formation, patterned assembly of the mechanosensory complex and integration of the complex on bio-MEMS cantilever devices. Using these systems the stretch sensitive sodium channel BNaC1 and the structural protein PICK1 localized at the sensory neuron terminals associated with the intrafusal fibers was identified as well as the Ca$^{2+}$ waves associated with sensory neuron electrical activity upon intrafusal fiber stretch on MEMS cantilevers. The knowledge gained through these multi-disciplinary approaches could lead to insights for spasticity inducing diseases like Parkinson’s, demyelinating diseases and spinal cord injury repair. These engineered systems also have application in high-throughput drug discovery. Furthermore, the use of biomechanical systems could lead to improved fine motor control for tissue-engineered prosthetic devices.
“It is not the critic who counts; not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood, who strives valiantly; who errs and comes short again and again; because there is not effort without error and shortcomings; but who does actually strive to do the deed; who knows the great enthusiasm, the great devotion, who spends himself in a worthy cause, who at the best knows in the end the triumph of high achievement and who at the worst, if he fails, at least he fails while daring greatly. So that his place shall never be with those cold and timid souls who know neither victory nor defeat.” ~ Theodore Roosevelt
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CHAPTER 1: GENERAL INTRODUCTION

Tissue engineering is an interdisciplinary field that applies the concepts and methodologies of the engineering field in an effort to study problems in the life sciences [1]. The intent of these studies are to understand the physical processes and engineering aspects of biological performance under both normal and abnormal conditions in an effort to design and develop artificial devices meant to measure, improve, protect or replace life functions [2]. More specifically, Eugene Bell described the following goals for the field of tissue engineering in 1992:

1. Provide cellular prostheses or replacement parts for the human body
2. Provide formed non-cellular replacement parts capable of inducing regeneration
3. Provide tissue or organ-like model systems populated with cells for basic research and for applied uses such as the study of diseased states using aberrant cells
4. Provide vehicles for delivering engineered cells to an organism
5. Surface non-biological devices to create hybrid devices and systems [3].

Tissue engineering typically, but not exclusively, focuses on solving human problems. Consequently, the field integrates knowledge acquired from a diverse range of biological and engineering sciences including: stem cell and developmental biology, reconstructive and transplantation surgery, anatomy and physiology, veterinary medicine, biophysics, biomechanics, biomaterials and nanotechnology [4]. Meeting the challenges and goals of tissue engineering depends largely on how one answers the
philosophical question “can nature be imitated?” There are several ways this question can be approached:

1. It can mean making an exact biological replica exhibiting all of the properties of the original tissue or organ at the time of implantation

2. It can mean making a closely approximate biological replica exhibiting some of the properties of the original tissue or organ at the time of implantation

3. It can mean providing an undeveloped precursor of the original tissue or organ at the time of implantation with the expectation that it will develop into a fully functional replacement

4. It can mean combining biological and non-biological components exhibiting some, all or enhancement of (to) the properties of the original tissue or organ at the time of implantation

5. It can mean using a non-biological replacement.

The first four approaches aim to remedy a deficit and restore or improve native functionality in the form of a fully integrated living/hybrid tissue or organ. Examples include joint replacement procedures (arthroplasty), tendon and ligament engineering and more ambitious project like heart and lung engineering [5-8]. The last approach aims to achieve the same goals using non-biological replacements to mimic the functionality of native tissues or organs as closely as possible. Successful examples of this type of tissue engineering include external replacements such as artificial limbs, hearing aids and dentures and internal replacements such as pacemakers, heart valves
and joint or skeleton components in the form of plastic or metal which are grafted into the body [9].

In vivo, biological tissues and organs are composed of three main components: cells, extracellular matrix (ECM) (consisting of a variety of glycosaminoglycans (GAGs), proteins and proteoglycans) and signaling systems (paracrine, autocrine or endocrine). In a delicately balanced system, progenitor cells of various types produce and interact with the ECM and signaling systems in order to differentiate into functional tissues and organs [10]. In tissue-engineered systems, the components can be biological, modified biological or non-biological in origin. Consequently, successful tissue engineering depends on the synergistic interaction of the biological and / or non-biological components of the engineered system in order to achieve a replacement tissue, organ or prosthetic device. Figure one illustrates a tissue-engineering paradigm and lists some examples for each component in a typical prosthesis.

The goal of tissue engineering is to develop functional tissues and organs using both biological and non-biological components. Each component of the tissue-engineering paradigm plays a vital role in successful prosthesis development. Therefore, techniques, materials and methodologies must be designed and implemented to address each component of the paradigm both separately and together and ensure ease of device integration with the host when necessary. Several areas must be addressed for each project and their reproducibility ensured:

1. Development of cell isolation, preparation and culture methodologies

2. Development of defined medium formulations containing specific signaling molecules
3. Development of growth substrates and environments

4. Development of data collection instruments and analysis metrics

5. Development of host integration and monitoring methodologies.

This dissertation research focuses on the development of tissue-engineered systems for repair and recovery of the peripheral nervous system (PNS), bridging the gap between in vivo and in vitro systems. Specifically, the work focuses on the development of an in vitro tissue engineered system that mimics the functionality of the monosynaptic stretch reflex arc and to develop a model of peripheral motoneuron myelination. To that end, this work has been divided into three interrelated topics: skeletal muscle differentiation and development, sensory neuron regeneration and reinnervation of intrafusal myotubes and myelination of motoneurons and sensory neurons. These topics will be addressed using the following tissue engineering parameters: microfabrication of micro-electro-mechanical systems (MEMS), cellular interface / substrate design and defined medium formulation development. Therefore, the overall hypothesis for this dissertation project is that tissue engineered constructs of the sensory circuit of the monosynaptic stretch reflex arc and peripheral myelination can be developed using microfabrication, interface design and defined medium formulation.

The monosynaptic stretch reflex arc is formed by a direct synapse between motoneurons and sensory neurons and can be thought of as a simple circuit (Fig.1). This circuit serves as a proprioceptive feedback system, relaying information about muscle length and stretch to the central nervous system (CNS) [11]. It is composed of four elements, which are split into two circuits. The efferent or motor circuit is composed of an α-motoneuron and the extrafusal skeletal muscle fibers it innervates,
while the afferent or sensory circuit is composed of a Ia sensory neuron and a muscle spindle. Structurally, the two muscular units are aligned in parallel, which plays a critical role modulating the system’s functionality [12]. Furthermore, the monosynaptic nature of the sensory neuron to motoneuron connection provides a rapid, involuntary response to stimuli. Functionally, the circuit acts to maintain appropriate muscle length during activities as diverse as eye movement, respiration, locomotion, fine motor control and posture maintenance [13, 14]. This is achieved through the action of afferent impulses, generated in response to muscle stretch, signaling motoneuron excitation and compensatory contraction of extrafusal muscle fibers in opposition to the initial stretch.

A more detailed investigation of the structure of the sensory circuit reveals that the muscle spindle consists of two to twelve specialized muscle fibers called intrafusal fibers that are distinct from the extrafusal fibers in both structure and function (Fig.2). These unique fibers can be categorized morphologically as nuclear bag1, nuclear bag2 and nuclear chain fibers based on the location of their nuclei [15, 16]. In nuclear bag fibers, the nuclei are clustered in an enlarged central region, while in nuclear chain fibers, the nuclei are arranged in a single row localized in the equatorial region [17, 18]. The spindle fibers are also unique in that morphological characteristics such as striation and myofibril density vary proportionately with distance from the center [19]. In fact, they are heavily striated at their polar regions indicating the presence of contractile sarcomeric units, a feature that decreases moving equatorially until it is nearly absent. This feature plays an important role in the sensitivity to stretch seen in fibers and consequently the nerve terminals that innervate them.
The sensory innervation of intrafusal fibers is of two types: the primary ending present in all spindles, supplied by a single group Ia afferent fiber, is an annulospiral wrapping of the nerve terminal around the fibers at the equatorial region; the secondary ending, not present in all spindles, is provided by group II afferents and occur at the equatorial region of the spindle with endings characterized as flower-spray [20]. As their names imply, annulospiral endings wrap around the intrafusal fibers and flower-spray endings terminate in a flower-like appearance (Fig.3). These specialized nerve terminations form the mechanically sensitive receptors necessary to monitor stretch of the muscle, or mechanoreceptors [21]. Essentially, as the surrounding extrafusal fibers stretch under tension, the muscle spindle stretches in parallel distorting the equatorial region of the intrafusal fibers and the associated nerve endings. This mechanical distortion results in graded action potential transmission along the afferent neuron and is caused by mechanically gated ion channels present in the membrane of the annulospiral wrapping [9]. Sodium ion influx into the nerve terminal generates action potentials that relay the proprioceptive information to the spinal cord α-motoneurons [21, 22]. The frequency and amplitude of these impulses reflects the strength of the distortion being transmitted through the spindle fibers [22]. This causes excitatory depolarization of the α-motoneuron and consequent contraction of the extrafusal muscle fibers where the stretch was first detected.

The physiological properties of the muscle spindle are directly related to their structure [23, 24]. These specialized proprioceptors transmit information about muscle length and speed of stretch to the group Ia sensory neurons via mechanoreceptors. Each fiber’s morphology can be related to a distinct pattern of myosin heavy chain
(MHC) expression that has been documented using immunohistochemical techniques [25-29]. The corresponding MHC expression reflects specialized properties for each fiber in terms of its contractile ability, or its ability to distort under stretch [30]. For example, the presence of contractile units of myosin heavy chains localized at the polar regions of the spindle fibers reflects this region's ability to contract under stimulation by γ-motor neurons, whereas the absence of such units at the equatorial regions reflect the presence of sensory nerve terminals [31-33]. This dichotomy is so great that in adulthood it has been shown that intrafusal fibers synthesize exclusively embryonic, neonatal, slow-development and α-cardiac-like isoforms of MHC [31, 34-36].

Interestingly, the site where γ-motor neurons form neuromuscular junctions with intrafusal fibers, the MHC expression pattern is more similar to extrafusal fibers [37]. Taken together this information reflects the precise function of the muscle spindle as part of the larger musculoskeletal system. While the skeletal muscles are under relaxed conditions the spindles remain taut and are able to relay stretch information. However, as the skeletal muscles contract, the parallel running muscle spindles begin to unload rendering them unable to accurately sense the length and stretch of the surrounding muscle fibers. Signaling by γ-motor neurons synapsed at the polar regions of the intrafusal fibers cause contraction of the spindle and a return to tautness in the equatorial region and sensitivity to the spindle. Therefore, the functional significance of site-specific MHC expression in muscle spindles is dual in nature. The equatorial region expressing myosin heavy chains reflects its role in proprioception, while the polar regions expressing MHC proteins are more adept at contraction due to innervation by γ-motor neurons [36, 38].
The efferent neurons of the reflex circuit are responsible for interpreting signals from the sensory neurons terminating there, and in turn, generating action potentials that reflect the strength of the incoming impulse. These neurons fall into two categories: α-motor neurons innervating the same muscle where the stimulated spindle is located as well as surrounding synergistic muscles; and interneurons that synapse on α-motoneurons terminating on antagonistic muscles. Therefore, the responsibility of the sensory circuit is to provide accurate information both in strength and duration of stimulus to the spinal cord neurons in order to maintain proper muscle tension and coordinate movement by amplifying necessary contractions while inhibiting unnecessary ones.

The synapse formed between the α-motoneuron and extrafusal muscle fibers is called a neuromuscular junction (NMJ). The formation of an NMJ requires specialized differentiation of both the pre-synaptic and post-synaptic terminals. This is premeditated by the presence of synaptic vesicles in the pre-synaptic terminal containing proteins including synaptophysin and the neurotransmitter acetylcholine (ACh), which in conjunction with acetylcholine receptors (AChRs) on the post-synaptic muscle membrane serves to direct NMJ formation [39]. Additional maturation of the NMJ is characterized by AChR class switching and development of numerous folds in the post-synaptic membrane containing clusters of AChRs [40, 41]. Like other vertebrate synapses, glial cells are closely associated with the pre- and post-synaptic structures of the NMJ [42, 43], and peri-synaptic Schwann cells play an important role both short-term NMJ plasticity as well as long-term maintenance of the structural integrity of the NMJ [44-47].
An additional role for glial cells in the functioning of the stretch reflex arc is the formation of myelin sheaths surrounding the axons of both motoneurons and Ia sensory neurons which functions as an insulator for electrical impulses generated by cells [48, 49]. These myelinating Schwann cells synthesize and deposit specialized myelin proteins in their membranes that in turn wrap around axons multiple times forming compact myelin [50, 51]. The proteins deposited include myelin basic protein (MBP), myelin associated glycoprotein (MAG) and P₀ protein (PLP) [52]. During the myelination process, organization modification of several proteins occurs in the neuronal axon. Specifically, the voltage-gated Na⁺ and K⁺ channels cluster into distinct compartments between myelin segments forming a node of Ranvier [53]. This structural remodeling results in the rapid and essentially lossless transmission of electrical current through the axon in a process called saltatory conduction [53]. Saltatory conduction is the rapid transmission of electrical impulses through axons at speeds more than 10 – 100 times that of unmyelinated axons [54, 55].

While much has been elucidated about the development and physiology of the components of the stretch reflex arc, the majority of studies have been conducted in vivo and little attempt has been made to tissue engineer the circuit in vitro [14, 18, 56-61]. The overall goal of this dissertation research was to tissue engineer the sensory circuit of the stretch reflex arc, bridging the gap between the in vivo and the in vitro systems. An innovative approach to the problem, including serum-free medium formulations and non-biological substrates, was employed. Furthermore, an array of flexible cantilever micro-electrical mechanical systems (MEMS) device was developed and used as a growth surface for the stretch reflex arc components (Fig.4-5). This
microcantilever device has been used to successfully monitor the contractile force generated by myotubes responding to electrical field stimulation [62, 63]. Here, the microcantilever system will be used to help discern how mechanical stretch information is coded electrically by la sensory neurons and how electrical information from motoneurons is converted to mechanical contractile force (Fig.4-5).

Overall, the monosynaptic stretch reflex arc is a combination of two relatively simple circuit units. The synergy of these two circuits provides for the senses of proprioception and kinesthesia and perturbations in either circuit unit can lead to diseases like infantile spinal muscular atrophy type I (Werdnig-Hoffman disease) and spasticity inducing diseases like Parkinson’s, muscular dystrophy and myasthenia gravis [64-69]. Furthermore, the development of a biomimetic microcantilever-based model of the stretch reflex arc will provide insights into how mechanical sensory input is coded electrically and lead to the development of prosthetic devices with greater fine motor control. Consequently, further examination of the circuit elements in isolation and combination will lead to a greater understanding of their development and function, potentially providing insights into the diseases related to their dysfunction.
Figure 1.1. A tissue-engineering paradigm.
Figure 1.2. The mammalian stretch reflex arc.
Figure 1.3. The mammalian muscle spindle.
Figure 1.4. Sensory circuit of the stretch reflex arc on a bio-MEMS cantilever.
Figure 1.5. Complete stretch reflex arc circuit on a bio-MEMS cantilever.
References


53. Rasband MN, J.S. T. Developmental clustering of ion channels at and near the node of Ranvier. Developmental Biology 2001;236:5-16.


CHAPTER 2: SUBSTRATE DIRECTED MYELINATION AND NODE OF RANVIER FORMATION ON SENSORY NEURONS IN VITRO

Introduction

The rapid conduction of action potentials in both the central nervous system (CNS) and peripheral nervous system (PNS) depends on the formation of a myelin sheath around neuronal axons. In the PNS, myelination initiation requires an interaction between Schwann cells and an individual axon, a process known as axonal sorting [1]. During myelination, Schwann cells form an insulating, multi-lamellar sheath around associated axonal segments, resulting in the formation of four specialized domains: the internode, the juxtaparanode, the paranodal region and the node of Ranvier. In the internode, axons are ensheathed by compact myelin consisting of the Schwann cell membrane and expressing myelin basic protein (MBP). The juxtaparanodal region sits adjacent to the paranode and contains localized clusters of voltage-gated potassium channels (vgpc’s) in the axon. In the paranodal region, the axon and myelin sheath form axo-glial junctions where Schwann cell expressed neurofascin 155 forms heterodimers with the axonal protein contactin-associated protein (CASPR) [2]. At nodes of Ranvier, specialized regions of unmyelinated axon between two myelin segments, the presence of clusters of voltage-gated sodium channels (vgsc’s) facilitate the saltatory conduction of action potentials [3].

Myelination of neurons by Schwann cells has been extensively studied using dorsal root ganglia (DRG) cultures in a variety of serum containing and serum-free in vitro systems, with most systems relying on the use of biological substrates for cell culture [4]. Among the important findings from previous work with in vitro myelinating
systems are the requirement of ascorbic acid in basal lamina modification to facilitate myelination by Schwann cells, the key role played by the extracellular matrix protein collagen type IV, the importance of β-1 integrin activation by laminin, the role of cyclic AMP in activation of myelin genes and the importance of factors such as insulin-like growth factor I (IGF-I) and neuregulin-1 in enhancing myelination [5-11]. However, in these studies axonal outgrowth and Schwann cell myelination was non-uniform in its organization.

Previously, we developed a DRG culture system utilizing the non-biological substrate N-1[3 (trimethoxysilyl) propyl] diethylenetriamine (DETA) [12]. The utility of this substrate comes from the ability to form a self-assembled monolayer (SAM) on any hydroxylated surface and its non-degradability by cells. Additionally, photolithographic patterning of SAMs has been used to control the directional outgrowth of axons [13, 14]. These features make DETA a useful substrate for bioengineering applications, a major goal in hybrid electronic systems, tissue engineering and cell-based biosensor development.

Laser ablation photolithography of SAMs by deep ultraviolet (UV) radiation has been shown to modify organosilanes by photocleavage, rendering the surface amenable to additional SAM modification [15]. By rederivatizing with an organosilane resistant to protein adsorption and consequently cell adhesion, a patterned surface can be generated with regions that support cell adhesion and regions that do not support cell adhesion. Poly(ethylene glycol) (PEG) SAMs prevent the adsorption of proteins on glass surfaces by the entropy/hydrated surface hypothesis [16-18]. Therefore, a
surface composed of alternating regions of PEG-DETA would facilitate the patterned adhesion of cells.

In this study, we demonstrate myelination and node of Ranvier formation on sensory neurons in a chemically defined, serum-free medium on the biomimetic, non-biological substrate DETA. Additionally, this directional outgrowth of the axons and their subsequent myelination by Schwann cells was controlled using photolithographic patterned substrate PEG-DETA. These findings fill a major void in the area of myelinating in vitro tissue engineered systems and could play an important role in the development of cell-based biosensors and bio-hybrid devices where cell location and directional control of process outgrowth are critical to functionality.
Materials and Methods

PEG surface modification

Glass coverslips (VWR 48366067, 22×22 mm2 No. 1) were first cleaned using 1:1 HCl-methanol followed by a concentrated H2SO4 soak for 2 hours. The coverslips were then coated with a PEG-terminated silane by a modified protocol from Papra et al. Dry toluene was prepared by distillation over metallic sodium to remove any water or other contaminants. The alkylsilane 2-[Methoxypol(ethyleneoxy)propyl]trimethoxysilane (Gelest, Tullytown, PA), was added to the toluene to a final concentration of 0.1% by volume in an MBraun glove box (MBraun, Stratham, NH). Concentrated HCl was added to a final volume of 0.08 % (0.8 ml HCl/L) to the PEG-toluene solution and the solution briefly stirred. The coverslips were then incubated in the PEG-toluene solution for 1 hour at room temperature. After 1 hour the samples were removed and rinsed in serial washes of toluene (1x), ethanol (2x), and diH2O (1x). The washed samples were blown dry under a stream of ultrapure nitrogen and were used immediately or stored in a desiccator until needed.

Deep-UV photolithography of PEG-silane monolayers

PEG-silane modified silica substrates were patterned using deep ultraviolet (DUV) photolithography. The samples were patterned in a photolithography system of our own design, which was based on a mask aligner, 193 nm ArF excimer laser (Lambda Physik, Santa Clara, CA) with an in-line beam homogenizer. Samples were placed on the stage of the mask aligner under a 5x5 inch chrome plated photomask,
which contained the pattern, to be ablated. The masks were written in dark-field polarity such that the areas corresponding to the ablated pattern were transparent and the remaining areas were opaque. When necessary the substrate was precision aligned using the aligner stage to ensure micrometer precision placement of the pattern. The substrate was then brought into contact with the mask and a vacuum applied between the stage and mask to ensure a hard contact. A hard contact was used to minimize the gap between the substrate and mask to ensure a high contrast pattern with minimal edge effects due to refraction of the laser light. The substrates were then exposed to 193 nm ultraviolet laser light for 30 seconds with a pulse intensity of 200 mJ/pulse and a frequency of 10Hz. After ablation the samples were removed from the aligner stage and stored for subsequent processing.

Back-fill of patterned PEG-silane monolayers with DETA-silane

After ablation the patterned PEG-silane substrates were backfilled with the alkylsilane (3-Trimethoxysilyl propyl) diethylenetriamine (DETA). Fresh distilled dry toluene was prepared as discussed before. DETA was added to the toluene to a final concentration of 0.1% (vol:vol) inside the glove box. The DETA-toluene solution was removed from the glove box and transferred to a beaker and the samples were immersed in the solution. To drive the reaction forward the solution was gently heated to no more than 65°C for 30 minutes. After reaction with DETA the samples were allowed to cool to room temperature, washed 3 time with dry toluene and heated to 65°C for 30 more minutes. Surfaces were characterized by static water contact angle measurements using a Rame-Hart Model 250 goniometer, and by X-ray photoelectron
spectroscopy (XPS) using an Escalab 200i spectrometer (VG Scientific) by monitoring the N 1s peak [14, 19, 20]. The values are reported as the mean ± SEM.

**Animals**

Dated pregnant Sprague-Dawley rats were housed in an animal facility at the University of Central Florida. All research was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Central Florida and conformed to NIH guidelines. Pregnant rats were anesthetized and sacrificed at embryonic day 15, embryos were removed by caesarean section and fetuses dissected under a stereomicroscope (Carl Zeiss, Stemi, 2000).

**Dorsal Root Ganglion Culture and Myelination of Sensory Neurons**

Embryonic rats day 15 (E15) were euthanized inhalation of an excess of CO$_2$. The procedure was in agreement with the IACUC of the University of Central Florida. The DRGs were collected in cold Hibernate E + B27 + Glutamax + antibiotic / antimycotic. Two mL 0.05% trypsin-EDTA is added to the tissue and then put in the water bath for 10 minutes at 37°C. After 10 minutes, the trypsin was removed and the reaction stopped by addition of 5mL of Hibernate E + 10% FBS. The DRG tissue was manually triturated using a 1000µL pipette until a homogenate was obtained and then centrifuged for 5 min at 200*g. Next, the medium was removed and 1mL of DRG plating media (Table 1) was added, and the cells gently suspended. A cell count was conducted and the cells were plated on 22×22mm$^2$ DETA coated coverslips at a density
of 200 cells/mm². DRG neurons were grown for two weeks in vitro prior to myelination induction. After two weeks, the medium was supplemented with 50μg/mL ascorbic acid to induce endogenous Schwann cell myelination of axons. The cultures were grown an additional two weeks to facilitate myelination and node of Ranvier formation.

**Immunocytochemistry & Laser Scanning Confocal Microscopy**

The co-cultures were fixed in fresh 4% paraformaldehyde in PBS for 5 minutes and then rinsed twice with PBS. Next, cells were permeabilized with a solution of 0.5% Triton-X 100 in PBS + 5% bovine serum albumin (BSA) for 5 minutes, rinsed once with PBS and then blocked with permeabilization solution + 5% donkey serum. The cells were then incubated with primary antibody solutions in blocking buffer overnight at 4°C. The following primary antibodies were obtained commercially from Chemicon: anti-neurofilament heavy chain (1:12,000) (AB5539), anti-voltage-gated sodium channel pan (1:200) (AB5210), and anti-MBP (1:40) (MAB382). The anti-CASPR antibody (1:300) (sc-14340) was obtained from Santa Cruz Biotechnology, Inc. The next day primary antibody solutions were aspirated and the cells rinsed three times with PBS. Then, AlexaFluor® 488nm, 594nm and 647nm secondary antibodies diluted 1:200 in blocking solution were added to the cells and incubated for 2 hours at room temperature in the dark. The secondary antibody solution was then aspirated and the coverslips rinsed three times in PBS and allowed to dry. Finally, coverslips were mounted on glass slides using VectaShield mounting medium with DAPI (Vector Labs, H-1200) and fixed using clear nail polish.
Results

**PEG-DETA Line Pattern Surface Preparation and Cellular Pattern Conformity**

The DETA foreground surrounded by PEG background provides a pattern supporting differential cell adhesion to the surface (Fig1A). Two control coverslips were used in order to test the quality of the PEG-DETA patterns: (1) one PEG coverslip was ablated without the photomask and rederivatized with DETA, and (2) a second PEG coverslip was rederivatized with DETA only. LASER irradiation and DETA rederivatization were done in the same conditions as for the PEG-DETA patterns.

The XPS measurements of the control coverslips show that PEG forms a self-assembling monolayer (SAM) on glass coverslips (Fig 1A). Additionally, DETA forms a SAM on ablated PEG, but it is not incorporated (or only incorporated in traces amounts) in the unexposed PEG regions (Fig 1B-C). Further, static water contact angle measurements of 92±2 validated the hydrophilicity of the LASER exposed PEG after DETA rederivatization. However, the non-ablated PEG monolayer is not affected by the reaction with DETA, as is also revealed by the contact angle values of 45±3 of unexposed PEG control coverslip, values that are close to the ones for pure PEG.

The pattern uniformity was verified by palladium catalyzed copper reduction metallization (Fig. 1D). These results were stable throughout the study indicating the reproducibility of the lines pattern. The cellular conformity to the patterns was also analyzed throughout the study. As shown in table one, pattern conformity was stable throughout the time in culture. During the early days, pattern conformity was with 49.03±1.57 out of 60 boxes (100%) showing cells only on the lines (Fig. 2). As the
cultures aged, conformity to the pattern remained stable with 4.11±0.25 lines out of 60 (98%) showing cells conforming to the pattern (Fig. 2).

Myelination Promoting Medium Formulation

The defined medium formulation described in Table 1 supports the growth and development of sensory neurons and Schwann cells. In this medium, sensory neurons develop a singular axonal process and a branching dendritic field (Fig. 2A). Schwann cells grown in this medium exhibit a spindle-like morphology characteristic of this cell-type (Fig. 2B). Cultured together, motoneurons and Schwann cells exhibit similar morphologies (Fig. 2C). Furthermore, with the temporal supplementation of ascorbic acid, Schwann cells form myelin sheaths and cause the subsequent clustering of nodal proteins (Fig. 3-4).

Immunocytochemical Evaluation and Quantification of Myelination

As compact myelin forms around neuronal axons, Schwann cells express MBP as a component of the myelin sheath. Using immunocytochemistry we evaluated MBP expression as a standard for compact myelin formation in our culture systems at day 25 to day 30. We imaged the neuronal processes using anti-neurofilament-H (NF-H) antibodies and then determined the fluorescence co-localization of the two antibodies. Myelin segments were observed in DRG cultures and imaged using Z-stack confocal microscopy (Figure 3) (Video 1). After staining, myelin segments were quantified in order to determine the efficiency of Schwann cells myelination in the culture system. As
shown in Table 2, we identified 192.17±2.74 myelinated segments in the DRG culture. Additionally, myelination results in the rearrangement and clustering of voltage-gated sodium channels (vgsc’s) and voltage-gated potassium channels (vgpc’s) in the axonal segment. This clustering resulted in the formation of physiologically functional nodes of Ranvier.

**Node of Ranvier Formation**

In order to visualize nodal development in this system, we used immunocytochemistry to stain vgsc’s and CASPR localized at the nodes of Ranvier. As shown in Figure 4, vgsc’s were found clustered between two myelinated segments of a sensory neuron axon, verifying node of Ranvier formation (Fig. 4A-E). Additionally, clusters of CASPR (Fig. 4C) were also seen in this culture system. The presence of these nodal proteins indicates maturation of the nodes of Ranvier in this patterned culture system.
Discussion

The development of an in vitro system guiding axonal outgrowth and their subsequent myelination using photolithography represents a significant technological development. These experiments indicate that a PEG-DETA surface prepared by laser ablation photolithography is sufficient to drive organization of axonal outgrowth and subsequent myelination and node of Ranvier formation in dissociated DRG cultures. This patterned myelination system has important implications for the development of cell-based biosensors and other bio-hybrid devices where the direction of cell growth and development plays a key role in device functionality.

Concurrently, we developed a system resulting in myelination and node of Ranvier formation on motoneurons using neonatal Schwann cells [21]. In that study, the number of myelinated segments outnumbered the number of nodes of Ranvier formed to a greater degree than in this study. While many factors influence Schwann cell myelination of axons, it is likely that patterned organization of the axons led to an increased ratio of nodes of Ranvier formed relative to myelinated segments [3, 22-24].

In these experiments, pattern conformity over time in culture was monitored to determine the effectiveness of PEG as a negative, cell repulsive surface. As indicated by Table 1, pattern conformity remained stable throughout the time in culture. In contrast, a previous study conducted using DETA-13F patterns showed a significant decrease in pattern conformity over time in culture (still unpublished). This data indicates that PEG is an effective cell repulsive surface both during initial plating and during maturation of the culture. This observation is likely due to the fact that PEG
resists protein adsorption while in culture, rendering cell motility onto the PEG region unlikely.

DETA’s utility from a bioengineering standpoint stems from its defined and reproducible nature as well as its ease of patterning with photolithography. Its role here, as a patterned biomimetic, hydrophilic growth substrate, is especially useful because it cannot be degraded by the cells plated on it and because it easily facilitates the study of deposited extracellular matrix molecules on the growth surface by the cells. DETA can be coated onto any hydroxylated surface or material. Consequently, PEG-DETA coated micro-electro-mechanical systems (MEMS) devices like multi-electrode arrays (MEAs) can provide a high throughput system for evaluating the electrical differences between myelinated and non-myelinated neurons. As previous studies have indicated, the deposition of a basal lamina and the subsequent modification of that layer are required for the formation of Schwann cell myelin [25-27]. The use of the non-biological substrate DETA for these experiments supports the findings that the neurons and / or the Schwann cells are secreting sufficient extracellular matrix (ECM) components necessary for the formation of the myelin sheath.

In this study, cellular adhesion, axonal outgrowth and myelination were spatially controlled using a patterned substrate of PEG-DETA. This work represents the first spatially directed development of myelination using tissue-engineering principles. The system provides a unique platform to study the substrate cues resulting in cellular adhesion and the development of myelinated axons as well as soluble molecules influencing the myelination process. Furthermore, this substrate directed developmental system could be used in tissue engineering applications where the
direction axonal outgrowth and myelination were important such as peripheral nerve regeneration models and an in vitro model of the stretch reflex arc.
Table 2-1. Culture conformity to PEG-DETA lines pattern

<table>
<thead>
<tr>
<th>Culture Day</th>
<th>Number of lines</th>
<th>Conforming Patterns</th>
<th>% Conformity</th>
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</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>60.00</td>
<td>60.00±0.00</td>
<td>100.00</td>
</tr>
<tr>
<td>Day 10</td>
<td>60.00</td>
<td>59.83±0.11</td>
<td>99.72</td>
</tr>
<tr>
<td>Day 20</td>
<td>60.00</td>
<td>59.58±0.19</td>
<td>99.30</td>
</tr>
</tbody>
</table>

Conformity was quantified by counting the number of lines with cells attached to only the DETA surface. Data is the average±SEM of three experiments performed on six coverslips / experiment at each time point.
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount / Concentration</th>
<th>Company</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurobasal</td>
<td>500ml</td>
<td>Gibco</td>
<td>10888</td>
</tr>
<tr>
<td>B27</td>
<td>50μl/ml</td>
<td>Gibco</td>
<td>17504-044</td>
</tr>
<tr>
<td>Glutamax</td>
<td>10μl/ml</td>
<td>Invitrogen</td>
<td>35050-061</td>
</tr>
<tr>
<td>Antibiotic / Antimycotic</td>
<td>10μl/ml</td>
<td>Invitrogen</td>
<td>15240-062</td>
</tr>
<tr>
<td>aFGF</td>
<td>20ng/ml</td>
<td>Invitrogen</td>
<td>13241-013</td>
</tr>
<tr>
<td>VEGF 165</td>
<td>20ng/ml</td>
<td>Invitrogen</td>
<td>P2654</td>
</tr>
<tr>
<td>h BDNF</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRB 600B</td>
</tr>
<tr>
<td>h GDNF</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRG 400B</td>
</tr>
<tr>
<td>r CNTF</td>
<td>50ng/ml</td>
<td>Cell Sciences</td>
<td>CRC 401B</td>
</tr>
<tr>
<td>h CT-1</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRC 700B</td>
</tr>
<tr>
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<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRN 500B</td>
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<tr>
<td>Heparan sulfate</td>
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<td>Sigma</td>
<td>D9809</td>
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<tr>
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<td>100ng/ml</td>
<td>Sigma</td>
<td>V0132</td>
</tr>
<tr>
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<td>10ng/ml</td>
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<td>256-GF</td>
</tr>
<tr>
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<td>11905-031</td>
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<tr>
<td>L-ascorbic acid</td>
<td>50μg/ml</td>
<td>Sigma-Aldrich</td>
<td>396-HB</td>
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</table>

1 Supplemental component added only at indicated medium changes
Table 2-3. Quantification of myelin segments and nodes of Ranvier

<table>
<thead>
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<th></th>
<th>Culture 1</th>
<th>Culture 2</th>
<th>Culture 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelinated segments</td>
<td>191.00±3.93</td>
<td>189.67±1.20</td>
<td>193.67±2.08</td>
</tr>
<tr>
<td>Nodes of Ranvier</td>
<td>66.33±6.94</td>
<td>70.33±5.36</td>
<td>69.00±4.73</td>
</tr>
</tbody>
</table>

The data shown is a mean of three coverslips evaluated per culture. The values are the mean ± the standard error of the mean (SEM).
Figure 2.1. XPS analysis and metallization reaction for PEG-DETA patterns. A) XPS survey spectrum of PEG-coated glass coverslip (insert shows high resolution C1s spectrum), (B) XPS survey spectrum of DETA on PEG-coated glass coverslip (insert shows high resolution N1s spectrum), (C) XPS survey spectrum of DETA on ablated PEG-coated glass coverslip, (D) image of the lines pattern visualized using palladium catalyzed copper reduction metallization (dark lines indicate the DETA regions). Scale bar = 90μm.
Figure 2.2. Dissociated DRG cells in culture on PEG-DETA line patterns.

(A-C) 100x phase contrast images of DRG cells on the different line widths, (D-F) 200x phase contrast images of DRG cells growing on the different line widths, (G) 300x phase contrast image of DRG cells growing on line pattern, (H-I) 400x phase contrast images of DRG cells growing on different line widths. Scale bars = 50μm.
Figure 2.3. Confocal image Z-stack of a myelinated sensory neuron axon.

(A) phase contrast image, (B) bottom image of the stack, (C) middle image of the stack, (D) top image of the stack. (NF-H = green, MBP = red, colocalization = yellow) Scale bar = 60μm.
Figure 2.4. Node of Ranvier formation on a sensory neuron axon on PEG-DETA patterned coverslips.

(A) phase contrast image, (B) MBP immunocytochemistry, (C) CASPR immunocytochemistry, (D) Nav cluster immunocytochemistry, (E) color composite image. Scale bar = 60µm.
References


CHAPTER 3: NODE OF RANVIER FORMATION ON MOTONEURONS IN VITRO

Introduction

The rapid conduction of action potentials in both the central nervous system (CNS) and peripheral nervous system (PNS) depends on the formation of a myelin sheath around neuronal axons. In the PNS, myelination initiation requires an interaction between Schwann cells and an individual axon, a process known as radial sorting [1]. During myelination, Schwann cells form an insulating, multi-lamellar sheath around associated axonal segments, resulting in the formation of four specialized domains: the internode, the juxtaparanode, the paranodal region and the node of Ranvier. In the internode, axons are ensheathed by compact myelin consisting of the Schwann cell membrane and expressed myelin basic protein (MBP). The juxtaparanodal region sits adjacent to the paranode and contains localized clusters of voltage-gated potassium channels (vgpc's) in the axon. In the paranodal region, the axon and myelin sheath form axo-glial junctions where Schwann cell express neurofascin 155 form heterodimers with the axonal protein contactin-associated protein (CASPR) [2]. At the Nodes of Ranvier, which are specialized regions of unmyelinated axon between two myelin segments, the presence of clusters of voltage-gated sodium channels (vgsc's) facilitate the saltatory conduction of action potentials [3].

Model systems that can represent the myelination of motoneurons by glial cells have previously proven difficult to develop. Myelination of neurons by Schwann cells has been extensively studied using dorsal root ganglia (DRG) cultures in a variety of serum containing and serum-free in vitro systems [4]. However, while many groups
have reported the successful co-culture of primary motoneurons and Schwann cells, the success of myelinating sensory neuron systems has not been translated to motoneuron systems [5-10]. The development of a functional myelinating motoneuron/Schwann cell system is a necessary first step in describing the molecular events surrounding the interactions between these cells that have myelination as the end result. Additionally, such a system would benefit scientists’ ability to study both central and peripheral demyelinating neuropathies such as multiple sclerosis, Guillain-Barré Syndrome, diabetes associated peripheral neuropathies and progressive muscular atrophy, under controlled conditions. Previous studies have described methods to created defined systems to understand hippocampal function [11] and motoneuron regeneration [12]. The adaptation of these culture systems to motoneurons/Schwann cell co-culture would be an ideal solution to this problem.

In this study, we demonstrate the myelination of motoneurons in a chemically defined, serum-free medium on the biomimetic, non-biological substrate N-1[3(trimethoxysilyl) propyl] diethylenetriamine (DETA). The utility of this substrate comes from its ability to form a self-assembled monolayer on any hydroxylated surface [13], the ease of photolithographic patterning [14] and the postulation that cells do not degrade this surface modification due to its non-biological origins and covalent attachment to the surface [11, 15]. In the defined medium we have identified the minimum combination of growth factors required for neuronal growth, as well as Schwann cell survival, proliferation and myelination of motoneuron axons that results in complete Node of Ranvier formation. System maturation was determined by analysis of the clustering of voltage gated sodium (vgsc’s) and potassium channels (vgpc’s) at the nodes as well as
from the presence of contacting-associated protein (CASPR). This defined system provides a reproducible model for studying Schwann cell interactions with motoneurons as well as the myelination process, and most importantly, remyelination.
Materials and Methods

DETA Surface Preparation and Characterization

Glass coverslips (VWR 48366067, 22×22 mm2 No. 1) were first cleaned using 1:1 HC l-methanol followed by a concentrated H2SO4 soak for 2 hours. The DETA (United Chemical Technologies Inc. T2910-KG) film was formed by the reaction of the cleaned surfaces with 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (VWR BDH1151). The cleaned surfaces were heated to about 100 °C in the organosilane mixture, rinsed with toluene, reheated to about 100 °C in toluene, and then dried in the oven overnight (100 °C). Surfaces were characterized by static water contact angle measurements using a Rame-Hart Model 250 goniometer, and by X-ray photoelectron spectroscopy (XPS) using an Escalab 200i spectrometer (VG Scientific) by monitoring the N 1s peak [15-17]. The values are reported as the mean ± SEM.

Animals

Dated pregnant Sprague-Dawley rats were housed in an animal facility at the University of Central Florida. All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines. Pregnant rats were anesthetized and sacrificed at embryonic day 15, embryos were removed by caesarean section and fetuses dissected under a stereomicroscope (Carl Zeiss, Stemi 2000).

Purified Embryonic Motoneuron Culture
Rat spinal cord motoneurons were purified from the ventral horn cords from embryonic day 15 (E15) embryos as described by Henderson et al. [18]. Briefly, pregnant rats were anaesthetized and killed by inhalation of excess CO2. Spinal cords were removed from the E15 pups and the ventral horn tissue was dissected out and digested in 0.05% trypsin-EDTA for 15 minutes in a 37°C water bath (Gibco 25300-120). Following incubation, the trypsin-EDTA was aspirated and the cells suspended in dissection media + 10% FBS and the tissue gently triturated. The dissociated cell suspension was then centrifuged at 500g for 10 minutes at 4°C to pellet the cells. Next, the tissue was layered on a density gradient of Opti-prep (Sigma D1556) solution and centrifuged at 500g for 15 minutes at 4°C. After centrifugation, the resulting top two bands were collected in a 15 ml tube and the pellet discarded. The ventral horn cells were then applied to an immuno-panning dish coated with goat affinity purified antibody to rat IgG and the low affinity nerve growth factor receptor p75 (Chemicon MAB365) in dissection medium for 45 minutes. This positive selection process provides attachment for the motor neurons while the other cells remain in suspension. After immuno-panning the non-adherent cells were aspirated and the adherent motor neurons were removed from the dish in dissection medium to a 15mL tube. Lastly, the neurons were pelleted by centrifugation at 500g for 10 minutes and then resuspended in culture medium and plated at 100 cells / mm2 (Table 1).

Neonatal Schwann Cell Culture

Primary rat Schwann cells (SC) were cultured from neonatal rat sciatic nerves as described originally by Brockes et al. [19]. Briefly, sciatic nerves from newly born
Sprague-Dawley (Charles River; Raleigh, NC) rat pups were dissected from the hind limb and then digested with 0.3% collagenase in Dulbecco’s modified Eagle’s medium (DMEM) + 10% FBS, forskolin and pituitary extract on poly-L-lysine coated 100 mm tissue culture dishes. After two days in culture, fibroblasts were eliminated using an anti-mitotic treatment followed by Thy1.1 antibody/complement mediated lysis (Chemicon MAB1406). Purified SC cultures were passaged no more than three times before plating with the embryonic motoneurons for the myelination experiments.

Immunocytochemistry and Laser Scanning Confocal Microscopy

The co-cultures were fixed in fresh 4% paraformaldehyde in PBS for 5 minutes and then rinsed twice with PBS. Next, cells were permeabilized with a solution of 0.5% Triton-X 100 in PBS + 5% bovine serum albumin (BSA) for 5 minutes, rinsed once with PBS and then blocked with permeabilization solution + 5% donkey serum. The cells were then incubated with primary antibody solutions in blocking buffer overnight at 4°C. The following primary antibodies were obtained commercially from Chemicon: anti-neurofilament heavy chain (1:12,000) (AB5539), anti-voltage-gated sodium channel pan (1:200) (AB5210), anti-voltage gated potassium channel (1:200) (AB5483) and MBP (1:40) (MAB382). The anti-CASPR antibody (1:500) (sc-14340) was obtained from Santa Cruz Biotechnology, Inc. The next day primary antibody solutions were aspirated and the cells rinsed three times with PBS. Then, AlexaFluor® 488 nm, 594 nm and 647 nm secondary antibodies diluted 1:200 in blocking solution were added to the cells and incubated for 2 hours at room temperature in the dark. The secondary antibody solution was then aspirated and the coverslips rinsed three times in PBS and allowed to dry.
Finally, coverslips were mounted on glass slides using VectaShield mounting medium with DAPI (Vector Labs, H-1200) and fixed using clear nail polish.
Results

DETA Surface Modification

The aminosilane, trimethoxy-silylpropyl-diethylenetriamine (DETA), functions efficiently as a non-biological substrate due to its self-assembling monolayer properties and the multiple amines contained in the terminal group. This group confers hydrophilic properties to the surface, and that combined with the partial positive change on the amines at physiological pH make it an ideal surface for neuronal cellular attachment and survival. The system is similar to poly-D-lysine, but has been found to be more robust and consistent [11]. XPS measurements of the DETA coated coverslips indicated a complete monolayer formed during the self-assembly process (Fig.1). The normalized area values of N1s (401 and 399 eV) to the Si 2p3/2 peaks were stable throughout the study at 1500 ± 200 and were similar to previously published results (Fig.1A-C) [11, 14, 15, 17, 20]. Static contact angle measurements of 45.6 ± 2° validated the hydrophilicity of the DETA surfaces (Fig.1D). Stable XPS readings and contact angles across coverslips throughout the study indicate uniformity and reproducibility of the self-assembly of the DETA monolayer.

Myelination Promoting Medium Formulation

As previously reported, embryonic and adult motoneurons, grown in serum-free medium on DETA recovered morphologically and electrically, firing repetitive action potentials under patch clamp conditions [12]. In this study, rat motoneurons and Schwann cells were isolated and grown in serum-free medium on DETA substrates.
The defined medium formulation described in Table 1 supported the growth and development of motoneurons and Schwann cells as shown in Figure 2. Rat motoneurons and Schwann cells were first individually isolated and grown separately as controls to ensure suitable morphology. In the individual cultures these motoneurons developed a singular axonal process and branching dendritic field (Fig. 2A). Schwann cells exhibited a spindle-like morphology characteristic of this cell-type (Fig. 2B). Cultured together, motoneurons and Schwann cells exhibited similar morphologies to the individual cultures (Fig. 2C). Furthermore, with the temporal supplementation of ascorbic acid, Schwann cells formed myelin sheaths and this also caused the subsequent clustering of the nodal proteins (Fig. 3-4).

**Immunocytochemical Evaluation and Quantification of Myelination**

As compact myelin forms around neuronal axons, Schwann cells express MBP as a component of the myelin sheath. Using immunocytochemistry MBP expression was evaluated as a standard for compact myelin formation in the culture system for day 25 to day 30. The neuronal processes were imaged using anti-neurofilament-H (NF-H) antibodies and then the fluorescence co-localization was determined using the two antibodies. Myelin segments were observed in motoneuron+Schwann cell co-cultures (Figure 3). After staining, myelin segments were quantified in order to determine the efficiency of Schwann cells myelination in the co-culture system. As shown in Table 2, 63.11±1.70 myelinated segments per coverslip were identified in the motoneuron+Schwann cell co-culture. Additionally, myelination resulted in the rearrangement and clustering of voltage-gated sodium channels (vgs's) and voltage-
gated potassium channels (vgpc’s) in the axonal segment. This clustering resulted in the formation of physiologically correct Nodes of Ranvier as defined below.

**Node of Ranvier Formation**

In order to visualize nodal development in this system, immunocytochemistry was used to stain for vgsc’s, vgpc’s and CASPR localized at the nodes. As shown in Figure 4, vgsc’s were found clustered between two myelinated segments of a motoneuron axon, verifying node of Ranvier formation (Fig. 4A,B). Additionally, clusters of CASPR (Fig. 4C) and vgpc’s (Fig. 4D) were also seen in this culture system. The presence of these nodal proteins indicates maturation of the nodes into the physiologically correct morphologies. After staining, the number of nodes was quantified in order to determine the efficiency of Schwann cell myelination and node formation in the co-culture system. As shown in Table 2, the formation of 20.67±0.61 Nodes of Ranvier were indentified per coverslip.
Discussion

The development of an in vitro system defining the minimum requirements for the survival, maturation and myelination of a motoneuron + Schwann cell co-culture represents a significant scientific and technological breakthrough. These experiments indicate that this medium formulation is sufficient to not only recover cellular functionality, but also to provide an environment conducive to further cell-cell interactions and relevant physiological development that results in physiologically correct Node of Ranvier formation. Using this basic serum-free medium formulation we have also shown the ability to grow dorsal root ganglia sensory neurons and both intrafusal and extrafusal muscle fibers [21-23]. The ability of the same basic serum-free medium formulation to sustain growth and facilitate myelination of a variety of interacting cell types facilitates future studies where all cells could be combined (Table 1). For example, studying motoneuron/sensory neuron electrical connectivity or recreating the stretch reflex arc in vitro will require all of these cell types to be in close proximity and will be more easily achieved using one basic medium formulation. This also is an essential requirement for drug discovery applications. Furthermore, the reported importance of culturing motoneurons, sensory neurons and Schwann cells together with muscle to form a significant number of neuromuscular junctions in vitro makes this basic medium even more critical [24, 25].

Schwann cell interaction with axons in the periphery is critical for efficient myelin sheath formation. Here we have shown both myelin sheath formation and subsequent development of nodes of Ranvier using this defined in vitro system (Fig3-4). The quantity of myelinated segments relative to nodes of Ranvier indicate that not all
myelinated segments formed in such a fashion as to result in the clustering of nodal proteins. While the processing of nodal proteins is influenced by the presence of myelinating Schwann cells opposing the initial segment, what regulates the Schwann cell “decision” to elongate an initial myelin segment or begin the process of forming a new segment? The likely candidate is interactions between the motoneuron and the extra-nodal proteins of the myelinating Schwann cell [26]. Due to the significant level of physiological development, the system also provides a model for further investigation into the potential molecular differences between Schwann cell interaction with motoneurons and sensory neurons. For example, it could be useful in the evaluation of additional factors that could play a role in enhancing motoneuron myelination and node formation relative to sensory neurons. This is especially true for evaluating factors that are normally abundant in serum infused medium formulations typically used to facilitate Schwann cell myelination of sensory neurons.

DETA’s utility from a bioengineering standpoint stems from its defined and reproducible nature. Its role here, as a biomimetic, hydrophilic growth substrate, is especially useful because we believe it is not degraded by the cells plated on it and because it easily facilitates the study of deposited extracellular matrix molecules on the growth surface by the cells. DETA can be coated onto any hydroxylated surface or material. All of these features make DETA a useful substrate for bioengineering applications, a major goal in hybrid electronic systems, tissue engineering and cell-based biosensors. Consequently, DETA coated micro-electro-mechanical systems (MEMS) devices like multi-electrode arrays (MEAs) can provide a high throughput system for evaluating the electrical differences between myelinated and non-myelinated
neurons. As previous studies have indicated, the deposition of a basal lamina and the subsequent modification of that layer are required for the formation of Schwann cell myelin [6, 7, 27]. Therefore, the use of DETA as the growth substrate for these experiments suggests that the neurons and/or the Schwann cells are secreting sufficient extracellular matrix (ECM) components necessary for the formation of the myelin sheath. This raises the questions of which cells generate the basal lamina, which cells secrete what ECM proteins, and how the ECM deposition influences cell-cell interaction between neurons and Schwann cells. These questions are currently under investigation in our laboratory.

We have used a completed defined in vitro system to demonstrate Node of Ranvier formation by Schwann cells on motoneurons with concurrent K channel clustering and (CASPR) formation. The development of this system, one where motoneurons are myelinated by Schwann cells, is a critical breakthrough in understanding the interactions between these two cell types and represents significant progress towards culturing a stretch reflex arc in vitro [24, 28, 29]. Additionally, it provides a novel system to evaluate the utility of a variety of factors not easily analyzed using an in vivo model. Such a system could provide enhancement to or recovery of myelin segments for patients suffering from demyelinating neuropathies. This defined system provides a reproducible model for studying Schwann cell interactions with motoneurons as well as the myelination process, and most importantly, remyelination.
Table 3-1. Serum-free medium composition for growth and myelination of motoneurons by Schwann cells

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount / Concentration</th>
<th>Company</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurobasal</td>
<td>500ml</td>
<td>Gibco</td>
<td>10888</td>
</tr>
<tr>
<td>B27</td>
<td>50μl/ml</td>
<td>Gibco</td>
<td>17504-044</td>
</tr>
<tr>
<td>Glutamax</td>
<td>10μl/ml</td>
<td>Invitrogen</td>
<td>35050-061</td>
</tr>
<tr>
<td>Antibiotic / Antimycotic</td>
<td>10μl/ml</td>
<td>Invitrogen</td>
<td>15240-062</td>
</tr>
<tr>
<td>aFGF</td>
<td>20ng/ml</td>
<td>Invitrogen</td>
<td>13241-013</td>
</tr>
<tr>
<td>VEGF 165</td>
<td>20ng/ml</td>
<td>Invitrogen</td>
<td>P2654</td>
</tr>
<tr>
<td>h BDNF</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRB 600B</td>
</tr>
<tr>
<td>h GDNF</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRG 400B</td>
</tr>
<tr>
<td>r CNTF</td>
<td>50ng/ml</td>
<td>Cell Sciences</td>
<td>CRC 401B</td>
</tr>
<tr>
<td>h CT-1</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRC 700B</td>
</tr>
<tr>
<td>NT-3</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRN 500B</td>
</tr>
<tr>
<td>NT-4</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRN 501B</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>80ng/ml</td>
<td>Sigma</td>
<td>D9809</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>100ng/ml</td>
<td>Sigma</td>
<td>V0132</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>50ng/ml</td>
<td>Sigma-Aldrich</td>
<td>396-HB</td>
</tr>
</tbody>
</table>

1 Supplemental component added only at indicated medium changes
Table 3-2. Quantification of myelin segments and nodes of Ranvier

<table>
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<th></th>
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<th>Culture 2</th>
<th>Culture 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelinated segments</td>
<td>61.67±3.71</td>
<td>63.67±4.91</td>
<td>64.00±2.65</td>
</tr>
<tr>
<td>Nodes of Ranvier</td>
<td>20.33±0.88</td>
<td>20.00±1.73</td>
<td>21.67±1.20</td>
</tr>
</tbody>
</table>

The data shown is a mean of four coverslips evaluated per culture. The values are the mean ± the standard error of the mean (SEM).
Figure 3.1 XPS and contact angle analysis of DETA monolayer on glass coverslips. (A) XPS survey spectra analysis of DETA coverslip, (B) XPS high resolution spectrum of N1s peak on DETA coverslip, (C) XPS high resolution spectrum of Si2p peak on DETA coverslip, (D) contact angle image of water on DETA coverslip.
Figure 3.2: Phase contrast images of motoneuron+Schwann cell co-cultures. (A) EMN culture image at day seven, (B) pure neonatal Schwann cell culture at day 14 (C) EMN+SC co-culture at day 7 (arrow indicating MN). Scale bars = 60µm.
Figure 3.3: Immunocytochemical evaluation of the myelination of motoneurons by Schwann cells. (A-D) embryonic MN+SC co-culture images at day 29, (A) phase contrast image of the MN+SC co-culture, (B) NF-H antibody staining neuronal processes throughout the culture, (C) MBP antibody staining showing a segment of compact myelin and the outline of the Schwann cell, (D) merge image showing the co-localization of the NF-H and MBP antibody staining, (E-H) embryonic MN+SC culture images at day 27, (E) phase contrast image of the MN+SC co-culture, (F) NF-H antibody staining showing neuronal process, (G) MBP antibody staining revealing a segment of compact myelin in the culture, (H) merge image showing co-localization of the NF-H and MBP antibody staining. Scale bars = 50µm.
Figure 3.4: Immunocytochemical characterization of node of Ranvier formation on motoneurons. (A) phase contrast image of day 29 MN+SC co-culture showing an axonal segment and multiple Schwann cell bodies, (B) MBP and vgsc staining indicating node formation, (C) CASPR staining indicating paranode formation, (D) vgpc staining indicating juxtaparanode formation. Scale bar = 50µm.
References


CHAPTER 4: DEVELOPING A NOVEL SERUM FREE CELL CULTURE MODEL OF
SKELETAL MUSCLE DIFFERENTIATION BY SYSTEMATICALLY STUDYING THE
ROLE OF DIFFERENT GROWTH FACTORS IN MYOTUBE FORMATION

Introduction

The goal of this study was to develop a serum-free cell culture model to study and understand skeletal muscle differentiation and to better understand the process by which individual skeletal muscle cells migrate and align in order to form functional, contractile, multinucleated myotubes [1-11]. The serum-free cell culture model required the development of a medium, which was supplemented with different growth factors and utilized a synthetic, biocompatible silane substrate for cell growth. We formulated the serum-free medium by systematically studying the effects of individual growth factors on myotube formation in vitro.

In vivo and in vitro studies carried out in the previous two decades have indicated that skeletal muscle differentiation involves the specific interaction of multiple growth factors with both the myocytes and, subsequently, the developing myotubes. The different growth factors implicated in skeletal muscle differentiation include vitronectin, bFGF, CT1, GDNF, BDNF, NT3, and NT4 [1-10, 12]. However, no systematic in vitro study had been carried out that combined these different growth factors in order to develop a defined medium. In this study, the effects of bFGF, CT1, GDNF, BDNF, NT3, NT4, and vitronectin on myocyte fusion and skeletal muscle differentiation were analyzed. Through this analysis, a simple chemical and growth factor based medium formulation was developed and a novel technique, which promoted the formation of robust, functional, contractile, multinucleated myotubes in culture was proposed.
Immunocytochemical characterization was performed on the different myotube morphologies using embryonic myosin heavy chain (MHC) antibody (F1.652). Preliminary reports documented the enhanced myotube formation as well as their integration on silicon microstructures but not the mechanism of myotube formation[13, 14]. This technique, which promoted robust myotube formation, was also observed to support nerve-muscle co-culture[15]. Immunocytochemical evidence of the muscle-nerve co-culture was also established. We believe that this chemically defined formulation and the proposed mechanistic development model will be a useful tool in studying myocyte biocompatibility, muscle differentiation, myopathies, muscle tissue engineering and neuromuscular junction formation.
Materials and Methods

Surface Modification

Glass coverslips (Thomas Scientific 6661F52, 22 x 22mm No.1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 minutes at 100 mTorr. The DETA (United Chemical Technologies Inc. T2910KG) films were formed by the reaction of the cleaned surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated to just below the boiling point of the toluene, rinsed with toluene, reheated to just below the boiling temperature, and then oven dried [14, 16]

Surface Characterization

Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and X-ray photoelectron spectroscopy (XPS) (Kratos Axis 165). XPS survey scans, as well as high-resolution N1s and C1s scans, utilizing monochromatic Al Kα excitation, were obtained[13, 17].

Skeletal Muscle Culture and Serum-free Medium

The skeletal muscle was dissected from the thighs of the hind limbs of fetal rats (17-18 days old). The tissue was collected in a sterile 15 ml centrifuge tube containing 1 ml of phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075). The tissue was enzymatically dissociated using 1 ml of 0.05% of trypsin-EDTA (Gibco 25300054) solution for 30 minutes in a 37°C water bath (100 rpm). After 30 minutes the trypsin solution was removed and 2 ml of L15 + 10% fetal calf serum (Gibco 16000044) was added to terminate the trypsin action. The tissue was then mechanically triturated.
The supernatant was then transferred to a 15 ml centrifuge tube. The same process was repeated two more times by adding 2 ml of L15 + 10% FBS each time. The 6 ml cell suspension obtained after mechanical trituration was suspended on a 2 ml, 4% BSA (Sigma A3059) (prepared in L15 medium) cushion and centrifuged at 300g for 10 minutes at 4°C. The pellet obtained was washed 5 times with L15 medium, then resuspended in 10 ml of L15 and plated on 100 mm uncoated dishes for 30 min. The non-attached cells were removed, centrifuged on a 4% BSA cushion, and plated on the coverslips. The cells were plated at a density of 700-1000 cells/mm2. The cells attached to the substrate in 1 h. The serum-free medium (containing different formulations of growth factors) was added to the culture dish after 1 h and the cells were maintained in a 5% CO2 incubator (relative humidity 85%). Half of the medium was changed every 4 days [14].

*Three Cell Types Co-cultured in the Serum-free Medium*

For co-culturing of the skeletal muscle, DRG (sensory neurons) and spinal cord neurons, a simple co-culture technique was developed. The dissociated muscle cells were mixed with dissociated sensory neurons (DRG) and the spinal cord cells. The DRG isolation protocol was described in an earlier paper [18]. In brief, DRG’s were isolated from embryonic, day 14 (E14) rat embryos and dissociated using trypsin. The resulting single cell suspension was then mixed with the dissociated muscle cells in the serum-free medium. Similarly, the whole spinal cord was removed from E14 embryos and dissociated in a trypsin solution. Subsequently, the single cell suspension of the spinal cord cells were combined with the dissociated muscle/DRG cell suspension mixture. The combined three cell suspension was then plated on the coverslips at a
density of 800 cells/mm². After 30 minutes, the wells containing coverslips were filled with serum-free medium (Figure 1). The first medium change was done at day 4 as described in Figure 1 and the cultures were maintained for 3 weeks.

**Immunocytochemistry of Skeletal Muscle**

Coverslips were prepared for immunocytochemical analysis as previously described [14]. Briefly, coverslips were rinsed with PBS, fixed in -20°C methanol for 5-7 min, washed in PBS, incubated in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution) for 10 minutes, and blocked for 2h with 10% goat serum and 1% BSA. Cells were incubated overnight with primary antibodies against embryonic myosin heavy chain (F1.652) (Developmental Studies Hybridoma Bank) diluted (1:5) in the blocking solution. Cells were washed with PBS and incubated with the secondary antibody (Cy3 conjugated anti-mouse, Jackson Labs., 1:200 dilution in PBS) for 2 hours. After 2 hours the coverslips were rinsed with PBS and mounted on glass slides and observed under a confocal microscope.

**Immunocytochemistry of Co-cultures Double Stained with Neurofilament 150 and Embryonic MHC Antibodies**

Co-cultures were processed for immunocytochemistry as described above. Next, cells were incubated overnight at 4°C with rabbit anti-neurofilament M polyclonal antibody, 150 kD (AB1981, diluted 1:2000; Millipore/Chemicon, Temecula, CA, USA) and fetal MHC (F1.652, IgG, Developmental Studies Hybridoma Bank, diluted 1:5). After
incubating overnight, the coverslips were rinsed three times with PBS and incubated again with the appropriate secondary antibodies for 2 h. After rinsing three times in PBS, the coverslips were mounted with Vectashield DAPI mounting medium onto the glass slides. The coverslips were visualized and images collected using a confocal microscope (UltraVIEW™ LCI, PerkinElmer). Controls without primary antibody were negative.

**AChR Labeling of Myotubes**

AChRs were labeled as described previously [19] by incubating cultures with 5x10^{-8} M of α-bungarotoxin, Alexa Fluor® 488 conjugate (B-13422; Invitrogen/Molecular Probes, Carlsbad, CA, USA) for 1.5 h at 37°C before observation. Following incubation in α-bungarotoxin, the cultures were fixed as mentioned above for further staining with embryonic myosin heavy chain (F1.652) antibodies.
**Results**

*DETA Surface Modification and Characterization*

Static contact angle and XPS analysis was used for validation of the surface modifications and for monitoring the quality of the surfaces. Stable contact angles (40.64° ± 2.9 /mean ± SD) throughout the study indicated a high reproducibility and quality of the DETA coatings and were similar to previously published results[13, 16, 17, 20-23]. Based on the ratio of the N (401 and 399 eV) and the Si 2p3/2 peaks, XPS measurements indicated that a complete monolayer of DETA was formed on the coverslips.

*Development of Serum-free Medium Formulation*

The results have been summarized in Table 1. Below the effects of the different growth factors present in the nine different formulations are discussed. Unless otherwise stated, half of the medium was changed every three to four days.

Formulation I (F I) was the basal medium. It consisted of Leibovitz’s medium and M199 in a 3:1 ratio (v/v). The basal medium did not promote myotube formation and the cells died after 4 days in culture.

In Formulation II (F II) vitronectin was added to the basal medium. Myotube formation was not observed and cells did not survive in the culture for more than 4 days.

In Formulation III (F III) B27 supplement was added to Formulation II (F II). The cells survive for 8-10 days, but there was no myotube formation.
In Formulation IV (F IV) bFGF was added to Formulation III (F III). The addition of bFGF led to the formation of robust myotubes. Myotubes started appearing by day 2 in culture. Contracting myotubes were observed by day 4. Myotubes covered 50% of the total area of the coverslip and they survived for 10-12 days in culture. Many of the myotubes popped out of the coverslip due to extensive contractions. Extensive proliferation of fibroblasts in culture was also observed.

In Formulation V (F V) CT1 was added to Formulation III (F III). The addition of CT1 led to the formation of small myotubes with 4-6 nuclei. Myotubes started appearing by day 2 in culture and by day 4 most of these small myotubes showed mild contractions. Contractile myotubes covered 10% of the total area of the coverslips and the myotubes survived for 6-7 days in culture. The Formulation V had no bFGF in it. Small myotube formation was observed even without the presence of bFGF.

In Formulation VI (F VI) both bFGF and CT1 were added to the Formulation III (F III). Robust myotube formation with a reduction in fibroblast proliferation was observed. Fibroblast proliferation was less compared to Formulation IV (F IV) where only bFGF was added. Contractile myotubes covered 60% of the total surface area of the coverslips. Most of the myotubes began contracting by day 4 and exhibited extensive contractions as well as surviving for 10-12 days in culture.

In Formulation VII (F VII) GDNF and BDNF were added to Formulation VI (F VI). Robust myotube formation as well as a significant reduction in fibroblast proliferation was observed. The presence of GDNF and BDNF significantly reduced fibroblast proliferation and increased the total surface area covered with myotubes. Almost 65% of
the total surface area of the coverslip was covered with contractile myotubes. Most of the myotubes began to show contractions by day 4.

In Formulation VIII (F VIII) NT3 and NT4 were added to formulation VII (F VII). No significant qualitative difference from Formulation VII (F VII) was observed. Functionally, the myotubes started contracting by day 2. Additionally, a significantly reduced fibroblast proliferation was observed compared to Formulation III (F III).

In Formulation IX (F IX) no additional growth factors were added; instead two changes were made in the growth factor application. First, instead of replacing half of the medium during the first change, the entire medium was replaced. Second, in the process of changing the whole medium, bFGF was withdrawn, which resulted in the following formulation: Neurobasal/ vitronectin/ B27/ CT1/ GDNF/ BDNF/ NT3/ NT4. These two changes brought about a significant increase in the total number of myotubes formed. The final medium formulation has been enumerated in Table 2 and the medium change protocol is represented in Figure 1. By day 6, 90% of the total surface area of the coverslip was covered with robust, contractile myotubes. Additionally, there was minimal fibroblast contamination. Contractions began by day 2. The myotubes survived in culture for 16-20 days. All the different morphologies of myotubes observed in the culture were stained with embryonic myosin heavy chain antibodies (Figure 2 A-D). Chain like (Fig 2 A), branched (Fig 2 B), spindle shaped (Fig 2 C) and cylinder like (Fig 2 D) morphologies of myotubes were observed in the culture. The clustering of acetylcholine receptors on the membrane surface of the different morphologies of myotubes are shown in Figure 1 E-H. All of the myotubes showed clustering of acetylcholine receptors on their membrane surface.
Medium Formulation IX (F IX) and the subsequent change at day 4, further supported the nerve-muscle co-culture (Figure 3 A-F). In Fig 3 A, the sensory neurons along with the myotubes in the cultures were observed. The sensory neurons were seen as large cell bodies. In Fig 3 B, the neuron processes run parallel to the myotubes. In Fig 3 C, multi-polar cell morphologies of motoneurons, along with the large sensory neurons were detected. In Fig 3 D, branched, striated myotubes, and neuronal process crossing over a myotube was noted. In Fig 3 E-F, the wrapping of a neuronal process on a myotube is represented in three dimensions.
Discussion

In this study, a step-by-step process was described to develop of a novel, serum-free, in vitro cell culture system which results in the formation of robust, contracting, multinucleate myotubes from dissociated skeletal muscle cells that were obtained from the hind limbs of fetal rats. The step-by step development consisted of experimentally evaluating the effect of individual growth factors on myocyte survival and subsequent myotube formation. At the conclusion of the study, the most optimal formulation i.e. Formulation IX (F IX), was shown to support muscle as well as nerve-muscle co-culture growth. This serum-free medium supporting the survival, proliferation and fusion of fetal rat myoblasts into contractile myotubes. The rational for selecting these growth factors (vitronectin, B27, bFGF, CT1, GDNF, BDNF, NT3 and NT4) was solely based upon the distribution of their receptors within the developing myotubes in the rat fetus.

Role of Vitronectin

Vitronectin was added to the culture medium because its receptors promote cell adhesion and provide an anchoring function during the skeletal muscle differentiation process in vitro [24, 25]. The addition of vitronectin by itself did not promote myotube formation.

Role of B27

As previously documented, B27 supplement had supported cardiomyocyte growth [26]. In this culture medium B27 supplement was added to the medium as a
serum-replacement. Although the addition of B27 supplement promoted cell survival, it did not promote myotube formation.

Role of bFGF and the Controversy Concerning the Role of bFGF in Differentiation

Basic fibroblast growth factor (bFGF-2) is a 17-kDa member of the heparin binding growth factors. Basic FGF (bFGF) plays a complex, yet poorly understood, role in skeletal muscle differentiation. Several studies have indicated that bFGF promoted limb development, and some in vitro studies indicate that bFGF promotes the division of skeletal muscle cells, but inhibits the differentiation process. It had been documented that the terminal differentiation of the skeletal muscle occurs in the G1 phase but is repressed by fibroblast growth factor [27-36]. Interestingly, in this study, even in the presence of bFGF, most myoblasts fused and differentiated to form functional myotubes. These in vitro results support the hypothesis that there are at least two different pools of myoblasts present in the developing limb bud. In one population, bFGF promotes differentiation and in the other it inhibits muscle differentiation.

Role of CT-1

Cardiotrophin-1 (CT-1) is a cytokine belonging to the IL-6 family. It is expressed at high levels in the embryonic limb bud development and secreted by differentiated myotubes. CT1 promotes cardiac myocyte survival, regeneration of extraocular muscle, exhibits increased immunoreactivity in regenerating muscle and promotes motoneuron survival [37-45]. In this study, the addition of CT1 to basal medium/ vitronectin/ B27 was
observed to promote the formation of small myotubes. This indicated that CT1 by itself has the potential to initiate myotube formation, but it needs support from other growth factors.

Role of GDNF and BDNF

The glial cell line derived neurotrophic factor (GDNF) is a glycosylated, disulfide-bonded homodimer that is a distantly related member of the transforming growth factor-beta superfamily. GDNF plays a role in the differentiation and survival of central and peripheral neurons as well as in kidney organogenesis. GDNF is widely expressed in the development of skeletal muscle and is involved in regulating the distribution of acetylcholine receptors in mouse, primary skeletal muscle cells [46-50].

Brain-derived neurotrophic factor (BDNF) is a ligand for the low-affinity NGF receptor, p75, and for the high affinity neurotrophin receptor, trkB. It is expressed in the developing skeletal muscle, promotes motoneuron survival and also plays a vital role in the formation of the neuromuscular junction. BDNF rescues myosin, heavy chain, IIB muscle fibers after neonatal nerve injury [51-53] and along with NT4 promotes phenotypic recovery of both fast and slow twitch fibers. We believe that the above mentioned effects of GDNF and BDNF promote enhanced myotube formation in culture.

Role of NT3 and NT4

Neurotrophin-3, or NT3, is a neurotrophic factor in the NGF (Nerve Growth Factor)-family of neurotrophins. It is one of five neurotrophin growth factors which shape
the development of the nervous system by regulating neuronal survival and differentiation [54]. Recent studies have indicated that NT3 has an essential non-neuronal function. It plays a key role in cardiac development [55]. NT4 [51, 52, 56] promotes the normal development of slow muscle fiber phenotypes and phenotypic recovery of fast and slow twitch fibers. Previously, studies in this lab indicated that the addition of NT3 and NT4 results in early contractions in the myotubes[13]. Speculation is that the NT3 and NT4 have a synergistic effect in muscle differentiation.

**Co-culture studies**

One interesting feature of the differential application of growth factors in Formulation IX (F IX) was that it supported the survival and growth of skeletal muscle, sensory neurons and spinal cord motoneurons in a three cell co-culture model. While this seems intuitive, it was a crucial finding for easily building functional muscle-motoneuron, muscle-sensory neuron and muscle-motoneuron-sensory neuron constructs and for ultimately reconstructing the stretch reflex arc in vitro.

This work documents the development of a medium formulation that resulted in robust myotube formation and provided an analysis of the role of the individual factors and their mechanism of action in that process. Furthermore, the myotubes developed a MHC profile, which resulted in functional contractile properties. The final medium formulation was determined to support the growth of motoneurons and sensory neurons as well as their co-culture with myotubes. Consequently, this medium will be a powerful tool in nerve-muscle tissue engineering, discovering the molecules which triggers the switching between different myosin heavy chain proteins during muscle development,
dissecting the molecules involved in synapse formation at the neuromuscular junction and for applications in diseases such as ALS, muscular dystrophy, other diseases and injuries of spinal cord.
Table 4-1. Development of the chemically defined serum-free medium by systematically adding individual growth factors in the culture

<table>
<thead>
<tr>
<th>Components of the Medium Formulation</th>
<th>Myotube Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>L15:M199 3:1 Vt B27 bFGF CT1 BDNF GDNF NT3 NT4</td>
<td>(- - -)</td>
</tr>
<tr>
<td>I + Φ Φ Φ Φ Φ Φ Φ</td>
<td>(---)</td>
</tr>
<tr>
<td>II + + Φ Φ Φ Φ Φ Φ</td>
<td>(---)</td>
</tr>
<tr>
<td>III + + + Φ Φ Φ Φ Φ</td>
<td>(-+)</td>
</tr>
<tr>
<td>IV + + + + Φ Φ Φ Φ Φ</td>
<td>(+++)</td>
</tr>
<tr>
<td>V + + + Φ + Φ Φ Φ Φ</td>
<td>(+)</td>
</tr>
<tr>
<td>VI + + + + + Φ Φ Φ Φ</td>
<td>(+++)</td>
</tr>
<tr>
<td>VII + + + + + + Φ Φ</td>
<td>(++++)</td>
</tr>
<tr>
<td>VIII + + + + + + + + +</td>
<td>(++++)</td>
</tr>
<tr>
<td>IX + + + + + + + + +</td>
<td>(++++)</td>
</tr>
</tbody>
</table>

Abbreviations and signs used in Table 1.

F: Formulation number indicating the components added in the medium. If it indicates “+” sign, that means the factor had been added. If it indicates “Φ”, that means the component had been omitted.

L15: Leibovitz's medium, Vt: Vitronectin, B27: B27 supplement, bFGF: Basic fibroblast growth factor, CT1: Cardiotrophin 1, BDNF: Brain derived neurotrophic factor, GDNF: Glial derived neurotrophic factor, NT3: Neurotrophin 3, NT4: Neurotrophin 4

(---): No myotube formation  (- +): No myotube formation but cells survive for 8-10 days  (+): Small myotubes consisting of 4-6 nuclei  (+++): Robust myotube formation (10-30
nuclei), but with many fibroblasts that proliferated in the culture (+++): Robust myotube formation (10-30 nuclei) with a reduction in fibroblast proliferation (++++): Robust myotube formation (10-30 nuclei) and a significant reduction in fibroblast proliferation (+++++): Robust myotube formation (10-30 nuclei), minimal fibroblast proliferation and further supports nerve-muscle co-culture
### Table 4-2. Composition of serum-free medium in 500mL

<table>
<thead>
<tr>
<th>Component</th>
<th>Company</th>
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<th>Amount</th>
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<tbody>
<tr>
<td>L15</td>
<td>Invitrogen</td>
<td>11415064</td>
<td>375mL</td>
</tr>
<tr>
<td>M199</td>
<td>Invitrogen</td>
<td>11150059</td>
<td>125mL</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>Sigma</td>
<td>V0132</td>
<td>100ng/mL</td>
</tr>
<tr>
<td>B27</td>
<td>Invitrogen</td>
<td>17504044</td>
<td>10mL</td>
</tr>
<tr>
<td>Basic FGF</td>
<td>Invitrogen</td>
<td>13256029</td>
<td>10ng/mL</td>
</tr>
<tr>
<td>CT-1</td>
<td>Cell Sciences</td>
<td>CRC700B</td>
<td>50ng/mL</td>
</tr>
<tr>
<td>GDNF</td>
<td>Invitrogen</td>
<td>10907012</td>
<td>20ng/mL</td>
</tr>
<tr>
<td>BDNF</td>
<td>Invitrogen</td>
<td>10908019</td>
<td>20ng/mL</td>
</tr>
<tr>
<td>NT3</td>
<td>Cell Sciences</td>
<td>CRN 500B</td>
<td>20ng/mL</td>
</tr>
<tr>
<td>NT4</td>
<td>Cell Sciences</td>
<td>CRN 501B</td>
<td>20ng/mL</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Fisher</td>
<td>5233500</td>
<td>140μg/mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Osmolarity</th>
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<tbody>
<tr>
<td></td>
<td>320-325 mOsm</td>
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</table>

<table>
<thead>
<tr>
<th>pH</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>7.3</td>
<td></td>
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</table>
Figure 4.1. Flow chart showing the technique utilized to grow robust myotubes and muscle-nerve co-cultures. The Formulation IX and the medium change protocol, which is the most optimal for myotube and co-culture growth.
Figure 4.2. Different morphologies of myotubes stained with embryonic myosin heavy chain antibodies and clustering of acetylcholine receptors on the surface of myotubes. (A) Chain-like morphology of the myotubes, (B) Branched morphology of the myotubes, (C) Spindle shaped morphology of a single myotube, (D) Cylinder shaped morphology of a single myotube, (E-H) Different morphologies of myotubes showing the clustering of acetylcholine receptors (green) on the membrane surface of different myotubes. Scale bar = 50µm.
Figure 4.3. Co-culture of skeletal muscle + sensory neurons (DRG) / spinal cord neurons. All neurons were stained with the antibody against neurofilament-M (150 KD) (red) and the myotubes were stained with the antibody against embryonic myosin heavy chain (F 1.652) (green). (A) The large sensory neurons (DRG) were stained with NF 150 (red). (white arrows). The myotubes stained with F1.652 (green) were seen in the same field, (B) The neuron process in red are noted running parallel to the myotubes (green), (C) The multi-polar motoneuron was noted in close proximity with the myotubes. In the frame multiple large sensory neurons were observed (white arrows), (D) A bundle of processes of neurons crossed the striated, branched myotube, (E) A single multi-polar motoneuron was observed to wrap around the myotube, (F) Neuron processes wrapping on the myotubes. Scale bar = 75μm.
References


Introduction

Investigations into primary myocyte growth and differentiation have significant clinical applications ranging from functional prosthetic device development to muscular dystrophy. While much research has focused on the existence of extrafusal muscle fibers, much less effort has been applied to understanding spindle development in vitro and the intrafusal fibers that comprise the spindle. Research on spindle development would not only have interest from a basic research prospective, but identification of the factors regulating muscle spindle development would also enhance scientists’ understanding of proprioception/kinesthesia. Interspersed in most muscles, muscle spindles act as mechanoreceptors, providing proprioceptive sensory information to the brain and form the feedback circuit of the stretch reflex arc [1, 2]. Structurally, each muscle spindle contains several intrafusal myotubes; identified morphologically as either nuclear bag fibers with nuclei clustered at the center of the myotube or nuclear chain fibers with nuclei aligned to form a chain-like appearance [3, 4]. Furthermore, individual intrafusal fibers, either nuclear bag or nuclear chain, are also characterized by distinct patterns of myosin heavy chain expression profiles [5]. A key unanswered question in developmental muscle biology is the cellular origin of the intrafusal fibers that compose the muscle spindle, as well as the factors responsible for their differentiation.
Currently, two theories on the origin of intrafusal fibers dominate the field: one suggests a single bi-potential population of myocytes that develop into both fiber types while the other advocates the presence of two distinct population of cells, one for extrafusal fibers and the other for intrafusal fibers [6, 7]. To resolve this debate, investigations focused on identifying proteins and cell types necessary for intrafusal fiber differentiation are ongoing. For example, the Ia sensory neuron’s impact on spindle morphogenesis through ErbB2 signaling has been clearly established, *in vivo* [8, 9]. Additionally, the transcription factor Egr3 has been shown to play a role in protein expression profiles necessary for muscle spindle development [10]. Finally, the extracellular matrix molecule type IV collagen, observed using scanning electron microscopy, has been proposed as a molecule important in intrafusal fiber differentiation [11, 12]. While significant advances have been made using *in vivo* techniques, a clear definition of all the factors required for intrafusal fiber differentiation has not been documented [13-17]. Therefore, one approach to gain additional insight into intrafusal fiber myogenesis is to utilize a defined *in vitro* [18]. Such a system would permit the analysis of specific factors, in a defined environment, on the differentiation of skeletal muscle without variable interference from undefined sources such as serum or adsorbed biological substrates.

*In vitro* primary myocyte culture has attracted the interest of multi-disciplinary scientific endeavors such as cell patterning and tissue engineering for the development of biosensors and bio-robotic systems [19-21]. Also, much effort has been towards identifying growth factors, hormones and culture conditions that yield robust primary myocyte growth, as well as subsequent fusion into contractile myotubes *in vitro* [22-24].
For example, *in vitro* studies have identified neuregulin as a growth factor that induces expression of the transcription factor Egr3 which is critical for intrafusal fiber development [25-27]. These studies have provided some insight into the conditions required for myocyte growth and differentiation. However, variability of adsorbed biological growth substrates, and the undefined factors present in serum containing media formulations introduce unknown variability into these *in vitro* systems.

Definition of the minimal components required to create a functional system and subsequent control of those conditions are paramount for truly answering mechanistic questions using an *in vitro* system. We have developed a serum-free medium composition facilitating myocyte growth and fusion into functional myotubes on the non-biological substrate N-1[3-(trimethoxysilyl)propyl]-diethylenetriamine (DETA) [28, 29]. The nonbiological substrate, DETA, is ideally suited for *in vitro* studies due to its ability to form self-assembling monolayers on glass coverslips, effectively eliminating inconsistencies in growth surfaces [29]. Furthermore, the use of a defined, serum-free media formulation eliminates the unwanted variability of serum containing media. These two modifications facilitate direct correlation of the tested variable to the observed outcome. In this study we have utilized such a system to understand the role of neuregulin 1-β-1 on the development of nuclear bag fibers.

Our results indicate that by utilizing a specific *in vivo* marker for intrafusal fibers, the anti-alpha cardiac myosin heavy chain (MHC), and by morphological analysis that the addition of neuregulin 1-β-1 EGF (Nrg1-β-1) into a defined muscle culture medium drives the *in vitro* differentiation of primary rat myocytes towards intrafusal fiber formation. These findings clearly illustrate the utility of Nrg1-β-1 to enhance nuclear bag
fiber formation *in vitro*. Additionally, we have used electrophysiology to characterize the electrical properties of the proposed nuclear bag fibers and this represents the first *in vitro* analysis of the electrical properties of isolated nuclear bag fibers. Finally, no difference was found in the development of intrafusal fibers utilizing DETA as the culture substrate when compared to a routine biological surface composed of collagen.
Materials and Methods

DETA Surface Preparation and Characterization

Glass coverslips (Thomas Scientific 6661F52) were cleaned using an O\textsubscript{2} plasma cleaner (Harrick PDC-32G) for 20 minutes at 100 mTorr. The N-1\[3 (trimethoxysilyl) propyl] diethylenetriamine (DETA) (United Chemical Technologies Inc. T2910KG) self assembling monolayer film was formed by reacting the cleaned coverslip with a 0.1\% (v/v) mixture of the DETA organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were heated in toluene to just below boiling for 30 minutes, rinsed with fresh toluene, reheated to just below boiling again for 30 minutes and then oven dried overnight. The DETA surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by N 1s peak monitoring using XPS (Kratos Axis 165) as previously described [29-32]. The values are reported as the mean ± SEM.

Collagen Surface Preparation

The collagen surfaces were prepared by adsorbing type IV collagen [100µg/mL] on to previously prepared DETA surfaces for one hour at room temperature. These surfaces will be evaluated for their ability to influence intrafusal fiber formation relative to the DETA surfaces.

Animals
Dated pregnant Sprague-Dawley rats were housed in an animal facility at the University of Central Florida. All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines. Pregnant rats were anesthetized and sacrificed at embryonic day 18, embryos were removed by caesarean section and fetuses dissected under a stereomicroscope (Carl Zeiss, Stemi 2000).

*Primary Culture of Rattus norvegicus Myocytes*

Skeletal muscle was removed from the hind limb of E18 rat fetuses, collected in a 15ml tube in cold Hibernate E + GlutaMAX™ + antibiotic-antimycotic + B27 (dissection media) and incubated in 0.05% trypsin-EDTA for 1 hour in a 37°C water bath. Following incubation, the trypsin-EDTA was removed and the cells suspended in dissection media + 10% FBS and the tissue gently triturated. The dissociated cell suspension was then centrifuged at 800g for 10 minutes at 4°C to pellet the cells. Following centrifugation, the supernatant was aspirated and the cells resuspended in dissection media + 10% FBS. Fibroblasts were removed by panning the cell suspension in a 100 mm cell culture dish containing dissection media + 10% FBS for 20 min [33]. After panning, the myocytes were aspirated off the panning dish and then pelleted by centrifugation at 800g for 10 minutes at 4°C [34]. Finally, the supernatant was removed and the myocytes were suspended in the serum-free culture medium and a cell count was conducted using the trypan blue method. Myocytes were then plated on DETA coverslips at a density of 600-700 cells/mm².
Treatment with Neuregulin 1-β-1 EGF Domain

Myocytes were initially grown for 3 days in the serum-free culture medium. On the third day the medium was changed and Nrg1-β-1 EGF was added to the culture wells at a concentration of 100 ng/ml. At each subsequent medium change Nrg1-β-1 EGF was supplemented at the same concentration as the original treatment.

Myotube Fusion Index and Bag Fiber Differentiation Index

The myotube fusion index was calculated as the number of nuclei incorporated into myotubes relative to the total number of nuclei per unit area [35]. For each culture, a minimum of 10 random fields were counted and the fusion index expressed as the mean fused myocytes: total myocytes ± SEM. The intrafusal fiber differentiation index (bag fibers: total myotubes) of myocytes on untreated control coverslips and on Nrg1-β-1 EGF treated coverslips was assessed at day 12. The differentiation index was determined as the ratio of bag fiber myotubes to the total number of myotubes (greater than 4 nuclei). The index was calculated as the mean from three coverslips ± SEM for each experiment (treated and controls) and the experiments were conducted over 10 different cultures. A differentiation index was also calculated for experiments conducted on collagen adsorbed surfaces in order to address its potential enhancement of intrafusal fiber formation.

Preparation BA-G5 alpha Cardiac Myosin Heavy Chain
The mouse B lymphocyte hybridoma cell line HB-276 was cultured according to ATCC guidelines [36]. Briefly, cells were grown in DMEM (Gibco 10313-021) + 10% FBS (Gibco 16000-077) or Hybridoma-SFM (Gibco 12045-076) in 75 mm² tissue culture flasks and placed in an incubator at 37°C and 5% CO₂ at a concentration of between 1x10⁵ and 1x10⁶ cells/ml. The medium was changed twice weekly. The BA-G5 antibody is constantly secreted by the cells in culture and was harvested by removing 12ml of conditioned culture medium, transferring it to a 15 ml tube, followed by centrifugation at 4000g for 15 minutes at 4°C. The antibody containing supernatant was then removed and the concentration quantified using the microBCA method (Pierce 23235). The antibody concentration ranged from 9-12 µg/ml (data not shown) and was used 4 to 1 in blocking solution (see below).

Phenotypic Assessment of Myotubes by Immunocytochemistry & Laser Scanning Confocal Microscopy

The myocyte cultures were fixed in fresh 4% paraformaldehyde in PBS for 5 minutes and then rinsed twice with phosphate buffered saline (PBS). Next, cells were permeabilized with a solution of 0.05% saponin in PBS + 5% bovine serum albumin (BSA) for 5 minutes, rinsed once with PBS and then blocked with permeabilization solution + 5% donkey serum [37]. The cells were then incubated with primary antibodies (ATCC: HB-276) diluted in blocking solution (4:1) overnight at 4°C. The primary antibodies used include: mouse anti-alpha cardiac-like myosin heavy chain (see above), mouse anti-slow myosin heavy chain (A4.840 Developmental Studies Hybridoma Bank), rabbit anti-ErbB2 (06-562 Upstate), rabbit anti-phospho ErbB2 (06-
229 Upstate), rabbit anti-Egr3 (sc-22801 Santa Cruz Biotechnology, Inc). The next day primary antibody solutions were aspirated and the cells rinsed three times with PBS. Then, AlexaFluor® 488nm secondary antibodies (Molecular Probes A21200) diluted 1:200 in blocking solution were added to the cells and incubated for 2 hours at room temperature in the dark. The secondary antibody solution was then aspirated and the coverslips rinsed three times in PBS. Finally, coverslips were mounted on glass slides using VectaShield mounting medium with DAPI (Vector Labs, H-1200) and fixed using clear nail polish.

**Electrophysiology**

Whole-cell patch clamp recordings were performed in a recording chamber located on the stage of a Zeiss AxioScope 2FS Plus upright microscope. The chamber was continuously perfused (2 ml/min) with the extracellular solution (culture medium @ 35 °C). Patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller and filled with intracellular solution (in mM: K-gluconate 140, EGTA 1, MgCl₂ 2, Na₂ATP 2, Phosphocreatine 5, Phosphocreatine kinase 2.4, Hepes 10; pH = 7.2). The resistance of the electrode was 6–8 MΩ. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A amplifier (Axon, Union City, CA). Signals were filtered at 2 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis were performed with pClamp 9.2 software (Axon). Membrane potentials were corrected by subtraction of a 15 mV tip potential, which was calculated using Axon's pClamp 9.2 program. Membrane resistance and capacitance were calculated using 50 ms voltage steps from −85 to
−95 mV without any whole-cell or series resistance compensation. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a −85 mV holding potential. Action potentials were evoked with 1 s depolarizing current injections from a −85 mV holding potential [28, 30, 38]. For these experiments, five Nrg1-β-1 EGF treated myotubes meeting the morphological requirements of nuclear bag fibers were selected and patched from three different experiments (n=15).
Results

DETA Surface Modification

The aminosilane, trimethoxy-silylpropyl-diethylenetriamine (DETA), functions efficiently as a non-biological substrate due to its self-assembling monolayer properties and the multiple amines contained in the terminal group. This group confers hydrophilic properties to the surface, and that combined with the partial positive charge on the amines at physiological pH make it an ideal surface for cellular attachment and survival. The system is similar to poly-D-lysine, but has been found to be more robust and consistent [29]. X-ray photon spectroscopic (XPS) measurements of the DETA coated coverslips indicated a complete monolayer formed during the self-assembly process. The normalized area values of N 1s (401 and 399 eV) to the Si 2p_{3/2} peaks were stable throughout the study at 1500 ± 200 and were similar to previously published results [39, 40]. Static contact angle measurements of 40.13 ± 1.35° validated the hydrophilicity of the DETA surfaces. Stable XPS readings and contact angles across coverslips throughout the study indicate uniformity and reproducibility of the self-assembly of the DETA monolayer.

Serum-free Medium and Defined System Formulation

The basic serum-free medium composition developed in our laboratory for the co-culture of embryonic motoneurons and myocytes was used as a starting point for these experiments [41]. The additional critical component added to the previous formulations was nerve growth factor (NGF) at a concentration of 10 ng/ml (Table 1).
Using media supplemented with NGF, myotube formation progressed at the usual rate, but the longevity of the culture increased. NGF was added to the medium to assist in myocyte recovery and to more accurately represent the plausible environment that exists inside a muscle spindle in vivo due to secretion of the factor by schwann cells and myocytes and to also serve as a starting point for future sensory neuron / muscle co-culture experiments [42, 43] (Table 1).

**Immunocytochemical Evaluation of Nuclear Bag and Chain Fibers**

Initially, myotube cultures were grown on DETA and fusion indexes were determined to be 0.59 ± 0.02 for untreated cultures. While evaluating these cultures, we observed a distinct morphology that appeared to be nuclear bag fibers (Figure 1). The monoclonal antibody BA-G5 was used to evaluate alpha cardiac-like myosin heavy chain (MHC) (Figure 1A,B) expression in the morphologically apparent nuclear bag fibers. This antibody has been previously shown to be specific for intrafusal fibers in vivo, and was therefore the choice for evaluating their presence in vitro [7, 16, 44]. The presence of myotubes expressing intrafusal fiber specific, alpha cardiac-like MHC, was clearly observed in all cultures (Figure 2A, B). All of the myotubes stained displayed medially clustered nuclei characteristic of nuclear bag fibers in vivo. This immunocytochemical data shown in Figure 2 confirmed the morphological data illustrated in Figure 1, and verified the formation of nuclear bag fibers. Although their presence was not quantified, myotubes that were consistent with the morphology of nuclear chain fibers were also positive for BA-G5 MHC antibody staining in all cultures (Figure 2C, D), while other fibers exhibiting similar morphologies, assumed to be
extrafusal fibers, did not. In certain cases, myocytes in proximity to bag fibers were positive for alpha cardiac MHC (Table 3) (Figure 2D). Additionally, all myotubes were evaluated for their expression of slow MHC. In both treated and untreated cultures, myotubes as well as some myocytes were positive for slow myosin heavy chain expression (Figure 2 E, F). This expression pattern is consistent with in vivo data where slow myosin heavy chain expression is present in both intrafusal and extrafusal fiber subtypes. In our system, this likely represents an incomplete development of extrafusal fibers due to lack of the necessary cell-cell interactions or trophic factors required for their complete differentiation and is an area of further investigation in our laboratory.

**Nrg 1-β-1 EGF Treatment Enhances Nuclear Bag Fiber Differentiation**

The ability of Nrg 1-β-1 to influence skeletal myocyte differentiation was evaluated in a dose and time dependent manner (Table 2). Nrg1-β-1 EGF treatment of the myocytes using 10 ng/ml and 20 ng/ml at the time of plating, resulted in significant cell death by day 3 (images not shown). Next, treatments utilizing 10ng/ml and 20ng/ml concentrations were administered at day 3 while the myocytes were further along in the fusion process. This resulted in a culture of myotubes comparable to controls; there was no significant increase in intrafusal fiber formation or a substantially different number of total myotubes present (Table 2). Having established that day 3 application of Nrg 1-β-1 permitted cell growth and myocyte fusion, the treatment concentration was increased to 100ng/ml. Morphologically, this treatment led to the formation of significantly greater numbers of nuclear bag fibers in culture (Figure 1, Table 2). These cultures were then stained using the anti-alpha cardiac-like antibody to confirm the
development of intrafusal fibers due to Nrg 1-\(\beta\)-1 treatment. At this point, we quantified the myotube fusion index of treated cultures and determined it to be 0.60 ± 0.02. This value was found not to be significantly different from untreated culture fusion indexes. We then quantified the average number of bag fiber myotubes in treated cultures. The mean number of nuclear bag fibers was 30.90 ± 0.69 compared to 6.28 ± 0.32 in untreated cultures (Table 2). This difference was shown to be significant using a Student’s T-test (P < 0.001). Sporadically, tandem double bag fibers were observed in treated cultures and are noteworthy due to their observed presence in vivo (Figure 1D) [45].

After establishing that Nrg 1-\(\beta\)-1 EGF induced the morphological changes in nuclear bag fiber formation described above, other neuregulin isoforms were evaluated for their ability to induce similar changes. We tested Nrg 1-SMDF, Nrg 1-\(\alpha\)-EGF and Nrg 1-\(\beta\)-ECD with the same sequence of applications as we used for the Nrg 1-\(\beta\)-1 EGF and they showed no phenotypic changes (data not shown).

Additionally, as collagen fibers have been identified inside muscle spindles and in contact with intrafusal fibers, the potential role of collagen adsorbed to culture surfaces was also evaluated to determine if it further influenced intrafusal fiber differentiation [12, 23]. On the collagen surface the average number of bag fibers on treated coverslips was 32.67 ± 1.53 compared to 6.13 ± 0.53 on untreated coverslips (Table 2). These data sets evaluated together show no significant difference between the collagen and DETA surfaces for bag fiber differentiation using a Student’s T-test (P < 0.001).
Immunocytochemical evaluation of expression of the neuregulin receptor ErbB2 and its activation after Nrg1-β-1 treatment

In order to further evaluate the role of Nrg 1-β-1 signaling on the development of nuclear bag fibers, we tested the expression of the ErbB2 signaling receptor on the surface of treated and untreated myotubes in vitro. Furthermore, after Nrg 1-β-1 treatment, we evaluated the activation of the ErbB2 receptors using the phosphorylated [activated] ErbB2 receptor using the phospho-ErbB2 antibody (Figure 3). In both treated and untreated cultures, the presence of the inactivated form of the ErbB2 receptor was seen in all myotubes [(Table 3) (Figure 2E, K)] making this receptor a poor determinant of intrafusal fiber formation. Therefore, we determined the activation of the ErbB2 receptor in both treated and untreated cultures. In the untreated cultures, no activation of the ErbB2 receptor was seen (Figure 3 G, H, I). Conversely, in Nrg 1-β-1 treated cultures, the activated ErbB2 receptor was present in all treated myotubes exhibiting the nuclear bag phenotype (Figure 3 A, B, C). Concurrently, we evaluated the expression of BA-G5 MHC in these myotubes. We showed that the BA-G5 MHC was present in treated myotubes expressing the active phosphorylated ErbB2 receptor, but not present on any untreated myotubes. This makes the phosphorylated ErbB2 receptor a better determinant of intrafusal fiber formation and supports the evidence from Figure 2 (Figure 3 F, I, L). The summary of these expression experiments was quantified in Table 3.

Immunocytochemical Evaluation of the Activation of Intrafusal Fiber Specific Transcription Factor Egr3 after Nrg1-β-1 Treatment
In order to further verify our finding that Nrg 1-β-1 induced nuclear bag fiber formation, we also evaluated the expression of the intrafusal fiber specific transcription factor Egr3 in treated and untreated myotube cultures. We concurrently analyzed the myotubes co-expression of either intrafusal specific BA-G5 MHC or the nonspecific slow MHC (Figure 4). In Nrg 1-β-1 treated intrafusal fibers, the co-expression of BA-G5 MHC and the Egr3 transcription factor was present in all myotubes evaluated over three experiments [(Table 3) (Figure 4A, B, C)]. In untreated cultures, myotubes did not express either the Egr3 transcription factor or the BA-G5 MHC (Table 3). Consequently, these myotubes were visualized using the slow MHC (Figure 4 D, E, F).

In summary, while expression of the ErbB2 receptor was not sufficient to distinguish intrafusal from extrafusal fibers, the phosphorylation of ErbB2 after Nrg 1-β-1 treatment and the expression of the transcription factor Egr3 conclusively distinguishes the formation of nuclear bag fibers from other non-bag fibers on the nonbiological substrate DETA.

*Electrophysiological Evaluation of Nrg 1-β-1 Treated Myotubes*

Electrophysiology was used to characterize the Nrg 1-β-1 treated myotubes capacity for inward Na+ currents and outward K+ currents. These experiments represent the first in vitro evaluation of the electrophysiological properties associated with the individual intrafusal fibers rather than examination of the entire muscle spindle. The resting membrane potential of the nuclear bag fibers was -50.6 ± 1.6mV (n = 5). The current traces of the treated cultures that showed characteristics consistent with nuclear bag fibers exhibited maximal inward Na+ currents of -5900pA and outward K+ currents.
currents of 1800pA (Figure 5A). Additionally, the Nrg 1-β-1 treated bag fiber myotubes’ ability to fire action potentials was also investigated. All myotubes consistent with the nuclear bag fiber morphology demonstrated single action potential firing (Figure 5C).
Discussion

Neuregulin 1-β-1 treatment in a minimalistic \textit{in vitro} system of developing myotubes resulted in a significant increase in nuclear bag fiber formation. This demonstrates that Nrg 1-β-1 EGF plays a positive role in intrafusal fiber development. This finding supports \textit{in vivo} and \textit{in vitro} experiments indicating that Nrg 1-β-1 activates transcription factors highly expressed in muscle spindle fibers and is required for their development. Although the incomplete differentiation of all myotubes into nuclear bag or chain fibers in culture would suggest a role for other factors in this process as well [25-27]. Additionally, the incomplete differentiation could represent that certain myotubes are refractory to Nrg 1-β-1 treatment. This suggests the possibility of two distinct populations of cells, one that can become intrafusal fibers, and one that will become extrafusal fibers. This idea is supported by the finding that while all myotubes were positive for ErbB2 receptor expression, only a subset of the myotubes was positive for the activated form of the receptor after Nrg 1-β-1 treatment.

The additional experiments using the ErbB2 signaling and the activation of the transcription factor Egr3 by the Nrg 1-β-1 growth factor further suggest that these fibers are in fact intrafusal fibers of the nuclear bag phenotype. These experiments show that the combination of BA-G5 MHC expression, activated ErbB2 signaling and expression of the transcription factor Egr3 are more precise indicators of nuclear bag fiber formation than BA-G5 MHC expression alone. Furthermore, the expression of the inactivated ErbB2 receptor in seemingly non-intrafusal fibers could indicate that these myotubes were unable to complete the morphogenesis to the intrafusal fiber phenotype, an area of development needing further research. In contrast, it is possible that by
embryonic day 18 certain fate specification processes have occurred that limit the ability of every myotube to become an intrafusal fiber.

The similarity in bag fiber formation on the DETA surface compared to the collagen surface supports previous work illustrating the utility of DETA as a growth promoting surface for neurons and muscle [18, 28-30, 38, 40]. While a collagen adsorbed surface did not significantly affect bag fiber formation, other extracellular matrix components are good candidates for further influencing muscle spindle morphogenesis through integrin signaling [46]. Specifically, the close proximity of tenascin-c, s-laminin and chondroitin sulfate proteoglycan to the intrafusal fibers invites speculation about the role these ECM components play in muscle spindle mechanoreceptor development and physiology [47, 48]. This defined in vitro system provides an ideal environment to further evaluate the role of additional factors in intrafusal fiber development.

The necessity and utility of each growth factor present in the media formulation was derived from previous experiments from our groups and others [28, 31, 38]. The addition of nerve growth factor (NGF) to the formulation came as a result of experiments done by Murphy et al. showing an active form of NGF was secreted by rat skeletal muscle cells and a myogenic cell line [42]. They postulated the molecule acted in a support role for associated nerves. Here we hypothesized the molecule could also play an autogenic role in the support of muscle cells.

Using electrophysiology, we have begun to describe the electrical properties of nuclear bag fibers in this defined system. These findings are significant because previous experiments on the electrical properties of muscle spindle fibers had to be
conducted *in vivo* on the whole muscle spindle [49, 50]. Here we were able to being evaluating the electrical properties of isolated nuclear bag fibers *in vitro*. Compared to recording properties of non-bag fiber myotubes, we found no significant difference in the properties of nuclear bag fibers [28]. This data set raises questions regarding the cell-cell signaling events that might modulate ion channel expression in the intrafusal fibers. In future studies, it will be interesting to compare motor neuron innervated myotubes to sensory neuron innervated myotubes. Furthermore, defining ion channel expression in intrafusal fibers is essential in understanding the functional behavior of the mammalian mechanoreceptor system, a still poorly understood structure, and is something that can be readily studied using this *in vitro* system.

The important role of sensory innervation by Ia afferent proprioceptive neurons for the development and maintenance of muscle spindles, as well as the retrograde support provided by NT-3 from innervated intrafusal fibers, is well documented [22, 51, 52]. Additionally, the role that various neurotrophic factors play in the interaction between the Ia afferents and the motor neurons for the maintenance of the monosynaptic stretch reflex arc have been elucidated [1, 53, 54]. Although ion channel expression has been related to these findings, a clear picture of the trophic factors required for expression of appropriate ion channels necessary for functioning of the reflex arc has also not been clearly defined either and could also benefit from studies with this system.

In conclusion, we used an *in vitro* serum-free system to further explore the role of neuregulin 1-β-1 on intrafusal fiber development. We characterized the dose and time dependent nature of the neuregulin 1-β-1 in the formation of nuclear bag fibers on the
nonbiological substrate DETA. We also showed the permissive nature of DETA in supporting the formation of nuclear bag fibers. Finally, we began to characterize the electrical properties of isolated intrafusal fibers in vitro, something that has not been done until now. The high reproducibility of this system will facilitate further investigation into intrafusal fiber and muscle spindle morphogenesis. The complete development of such a system will permit the systematic evaluation of mechanoreceptor formation, maintenance and function, as well as its integration into an in vitro model of the stretch reflex arc.
Table 5-1. Serum-free medium composition in Neurobasal used for myocyte growth and intrafusal fiber differentiation

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Company</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>B27</td>
<td>20µl/ml</td>
<td>Gibco</td>
<td>17504-044</td>
</tr>
<tr>
<td>Glutamax</td>
<td>10µl/ml</td>
<td>Invitrogen</td>
<td>35050-061</td>
</tr>
<tr>
<td>Antibiotic/Antimycotic</td>
<td>10µl/ml</td>
<td>Invitrogen</td>
<td>15240-062</td>
</tr>
<tr>
<td>aFGF</td>
<td>20ng/ml</td>
<td>Invitrogen</td>
<td>13241-013</td>
</tr>
<tr>
<td>VEGF 165</td>
<td>20ng/ml</td>
<td>Invitrogen</td>
<td>P2654</td>
</tr>
<tr>
<td>G5 (100x)</td>
<td>2µl/ml</td>
<td>Invitrogen</td>
<td>17503-012</td>
</tr>
<tr>
<td>h BDNF</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRB 600B</td>
</tr>
<tr>
<td>h GDNF</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRG 400B</td>
</tr>
<tr>
<td>r CNTF</td>
<td>50ng/ml</td>
<td>Cell Sciences</td>
<td>CRC 401B</td>
</tr>
<tr>
<td>h CT-1</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRC 700B</td>
</tr>
<tr>
<td>NT-3</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRN 500B</td>
</tr>
<tr>
<td>NT-4</td>
<td>20ng/ml</td>
<td>Cell Sciences</td>
<td>CRN 501B</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>80ng/ml</td>
<td>Sigma</td>
<td>D9809</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>100ng/ml</td>
<td>Sigma</td>
<td>V0132</td>
</tr>
<tr>
<td>h β-NGF</td>
<td>10ng/ml</td>
<td>R&amp;D Systems</td>
<td>256-GF</td>
</tr>
<tr>
<td>h Nrg 1-β-1*</td>
<td>100ng/ml</td>
<td>R&amp;D Systems</td>
<td>396-HB</td>
</tr>
</tbody>
</table>

*Supplemental component added only as indicated.
<table>
<thead>
<tr>
<th>Surface</th>
<th>Nrg1-β-1 (ng/mL)</th>
<th># of Bag Fibers</th>
<th># of Myotubes</th>
<th>Intrafusal fiber Different. Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>DETA</td>
<td>0.00</td>
<td>6.00±0.32</td>
<td>306.17</td>
<td>0.0196</td>
</tr>
<tr>
<td>DETA</td>
<td>10.00</td>
<td>6.00±44</td>
<td>333.33</td>
<td>0.0209</td>
</tr>
<tr>
<td>DETA</td>
<td>20.00</td>
<td>7.66±0.38</td>
<td>330.33</td>
<td>0.0233</td>
</tr>
<tr>
<td>DETA</td>
<td>100.00</td>
<td>30.83±0.69</td>
<td>310.17</td>
<td>0.1002</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.00</td>
<td>6.13±0.53</td>
<td>325.00</td>
<td>0.0189</td>
</tr>
<tr>
<td>Collagen</td>
<td>100.00</td>
<td>32.67±1.53</td>
<td>325.00</td>
<td>0.1005</td>
</tr>
</tbody>
</table>

The number of bag fibers and the number of myotubes are averages of ten experiments all performed in triplicate. Data is reported as the mean ± the standard error of the mean. The differentiation index is the ratio of observed bag fibers to total myotubes with greater than four nuclei.
Table 5-3. Immunocytochemical determination of nuclear bag fiber morphogenesis after neuregulin 1-B-1 EGF treatment

<table>
<thead>
<tr>
<th></th>
<th>ErbB2 +</th>
<th>P-ErbB2 +</th>
<th>Egr3 +</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA-G5 (+) nuclear bag fibers</td>
<td>30.67±1.14</td>
<td>30.00±0.65</td>
<td>30.89±0.68</td>
</tr>
<tr>
<td>BA-G5 (-) myotubes</td>
<td>279.78±4.05</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Total myotubes</td>
<td>310.44±4.85</td>
<td>30.00±0.65</td>
<td>30.89±0.68</td>
</tr>
</tbody>
</table>

Values are reported as the mean ± SEM. Experiments were performed three different times in triplicate. Differentiation index = 0.60 ± 0.02.
Figure 5.1. Nrg 1-B-1 induces the formation of nuclear bag fiber in vitro. (A-C) Representative images used to tabulate the number of bag fibers present in treated and untreated cultures and total myotubes (red arrows indicate counted bag fibers). (D) Continuous double bag fibers were observed sporadically in treated cultures (red arrows) and represent a physiologically relevant phenomenon. Scale bars = 100µm.
Figure 5.2. Phase contrast and immunocytochemical characterization of nuclear bag and chain fibers using the intrafusal fiber MHC antibody BA-G5. (A) phase contrast image of characteristic nuclear bag fibers after Nrg 1-β-1 treatment, (B) alpha cardiac-like MHC expression using BA-G5 antibody immunocytochemistry, (C) phase contrast image illustrating characteristic nuclear chain fibers in culture after Nrg 1-β-1 treatment, (D) representative alpha cardiac-like MHC immunocytochemical expression in nuclear chain fibers using the BA-G5 antibody. (E) phase contrast image of nuclear bag fibers after Nrg 1-β-1 treatment, (F) slow-developmental MHC immunocytochemical expression in nuclear bag fiber. Scale bars = 60µm.
Figure 5.3. Expression of the phosphorylated and unphosphorylated forms of the ErbB2 receptor in Nrg1-B-1 treated and untreated cultures. (A-C) Nrg 1-β-1 treated myotube, (A) phase contrast image of a bag fiber, (B) phospho-ErbB2 immunostaining shows an activated receptor cluster, (C) BA-G5 + phosphor-ErbB2 merge image, (D-F) Nrg 1-β-1 treated myotube, (D) phase contrast image, (E) unphosphorylated ErbB2 staining, (F) BA-G5 + unphosphorylated ErbB2 merge image, (G-I) myotube from untreated culture, (G) phase contrast image, (H) phosphor-ErbB2 staining shows no receptor activation, (I) BA-G5 staining shows no immunoreactivity, (J-L) untreated myotubes, (J) phase contrast, (K) unphosphorylated ErbB2 immunostaining shows reactivity in untreated myotube cultures, (L) BA-G5 staining is absent in non-intrafusal myotubes. Scale bars = 40μm.
Figure 5.4. Activation of the transcription factor Egr3 in Nrg1-B-1 intrafusal fibers. (A-C) Nrg 1-β-1 treated myotube, (A) phase contrast image of the treated bag fiber, (B) positive Egr3 transcription factor immunoreactivity localized in the nuclei, (C) BA-G5 + Egr3 immunoreactivity merge image (D-F) untreated myotube, (D) phase contrast image of an untreated two untreated myotubes, (E) absent Egr3 transcription factor immunoreactivity, (F) Slow MHC immunoreactivity of the untreated myotube. Scale bars = 60µm.
Figure 5.5. Electrophysiological properties of Nrg1-B-1 treated nuclear bag fiber myotubes on DETA. (A) voltage clamped, current traces of Nrg 1-β-1 treated myotube showing maximal inward Na⁺ currents of -5900pA and outward K⁺ currents of 1800pA, (B) current clamped, single action potentials of a Nrg 1-β-1 treated myotube, (C) phase contrast image of a patched nuclear bag fiber.
References


CHAPTER 6: SKELETAL MUSCLE TISSUE ENGINEERING: A MATURATION MODEL PROMOTING LONG TERM SURVIVAL OF MYOTUBES, DEVELOPMENT OF THE EXCITATION CONTRACTION COUPLING APPARATUS AND EXPRESSION OF NEONATAL MYOSIN HEAVY CHAIN EXPRESSION

Introduction

Skeletal muscle differentiation and maturation is a complex process involving the synergy of different growth factors and hormones interacting over a broad time period [1-4]. The differentiation process is further complicated by neuronal innervation, where neuron to muscle cell signaling can regulate myosin heavy chain (MHC) gene expression and acetylcholine receptor clustering [5-7]. Consequently, understanding the role of growth factors, hormones and cellular interactions in skeletal muscle differentiation would be a key step in generating physiologically relevant tissue engineered constructs, developing advanced strategies for regenerative medicine and integrating functional skeletal muscle with bio-hybrid MEMS devices for non-invasive interrogation in high-throughput screening technologies.

In order for skeletal muscle myotubes developed in vitro to be useful in tissue engineering applications, they must exhibit as many of the functional characteristics of in vivo skeletal muscle fibers as possible. During muscle fiber development in vivo, several critical structural changes occur that indicate functional maturation of the extrafusal myotubes. These changes include sarcomere organization, clustering and colocalization of ryanodine (RyR) and dihydropyridine (DHPR) receptors and MHC class switching [8, 9]. Each of these structural changes reflects the physiological maturation of the skeletal muscle and is critical for consistent muscular contraction. For example,
organization of the contractile proteins myosin and actin into sarcomeric units gives skeletal muscle myotubes organized and structured contraction, a property lacking in smooth muscle. The organization of sarcomeres in skeletal muscle gives rise to anisotropic and isotropic bands of proteins (A and I bands) and gives skeletal muscle a striated appearance. The clustering and colocalization of RyR and DHPR is indicative of transverse tubule (T-tubule) biogenesis and excitation contraction coupling. This developmental step structurally links electrical excitation to the internal contractile system by providing close apposition of DHPR located in the T-tubule and RyR located in the sarcoplasmic reticulum. Finally, a properly functioning skeletal muscle must express the appropriate MHC proteins required for the task it must perform. For example, different muscle fibers express different MHC proteins depending on the rate of contraction and force generation required by the work to be done. Without these modifications, an in vitro model of skeletal muscle maturation cannot achieve full physiological relevance.

One approach for identifying the role of specific growth factors and hormones in muscle differentiation is to develop an in vitro model system consisting of a serum-free medium supplemented with the factors of interest [10]. Such a model provides the opportunity to evaluate the role of each factor individually or in combination with others known or believed to be important in skeletal muscle development. It also has the benefit of removing factors that may be present in serum that would inhibit maturation. Employing a non-biological growth substrate such as trimethoxy-silylpropyl-diethylenetriamine (DETA) provides an additional measure of control. DETA is a silane molecule that forms a covalently bonded monolayer on glass coverslips, resulting in a
uniform, non-hydrophilic surface for cell growth and may partially mimic the three dimensional features of an extracellular matrix, which may be responsible for the robust growth found for different cell types on this synthetic substrate [11-15]. Previously, studies have demonstrated the usefulness of the DETA silane substrate for in vitro culture systems [10, 16, 17]. The use of DETA surfaces is advantageous from a tissue engineering perspective because it can be covalently linked to virtually any hydroxolated surface, it is amenable to patterning using standard photolithography [17] and it promotes long-term cell survival because it is non-digestible by matrix metalloproteinases secreted by the cells [15, 18].

A defined system that promoted differentiation of different skeletal muscle phenotypes was developed earlier and resulted in the formation of contractile myotubes, but with only short-term survival [12, 15]. In a technological advance we have also developed a novel bio-hybrid system to integrate functional myotubes with cantilever based bio-MEMS devices for the study of muscle physiology, neuromuscular junction formation, bio-robotics applications and for use in a model of the stretch reflex arc [19]. More recently, using our defined model system, we have achieved a significant breakthrough by creating mechanosensitive intrafusal myotubes in vitro [20]. Intrafusal fibers are the myotubes present in the muscle spindle which functions as the sensory receptor of the stretch reflex circuit [7] and combined with extrafusal fibers represent the primary component necessary to reproduce functional muscle activity in vitro.

This system has been utilized as a model for different developmental and functional applications; however, further improvements were necessary to enhance the physiological relevance of the skeletal muscle myotubes [19, 20]. Specifically, in order
to create a working model of the stretch reflex arc, myotubes are needed that more accurately represent extrafusal fibers \textit{in vivo}. In this study, we have now demonstrated sarcomere assembly, the development of the excitation-contraction coupling apparatus and myosin heavy chain (MHC) class switching. These results suggest we have discovered a group of biomolecules that act as a molecular switch promoting the transition from embryonic to neonatal MHC expression as well as other structural adaptations resulting in the maturation of skeletal muscle \textit{in vitro}. The discovery of these biomolecular switches will be a powerful tool in regenerative medicine and tissue engineering applications such as skeletal muscle tissue grafts. It should also be useful in the creation of higher content high-throughput screening technologies.
Materials and Methods

Surface Modification and Characterization

Glass coverslips (Thomas Scientific 6661F52, 22 x 22mm No.1) were cleaned using an O₂ plasma cleaner (Harrick PDC-32G) for 20 minutes at 100 mTorr. The DETA (United Chemical Technologies Inc. T2910KG) films were formed by the reaction of the cleaned glass surface with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (Fisher T2904). The DETA coated coverslips were then heated to approximately 100°C, rinsed with toluene, reheated to approximately 100°C, and then oven dried [12]. Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by X-ray photoelectron spectroscopy (XPS) (Kratos Axis 165). XPS survey scans, as well as high-resolution N1s and C1s scans utilizing monochromatic Al Kα excitation were obtained [12].

Skeletal Muscle Culture and Serum-free Medium

Skeletal muscle was dissected from the thighs of the hind limbs of fetal rat (17-18 days old). The tissue was collected in a sterile 15 mL centrifuge tube containing 1 mL of phosphate-buffered saline (calcium- and magnesium-free) (Gibco 14200075). The tissue was enzymatically dissociated using 2 mL of 0.05% of trypsin-EDTA (Gibco 25300054) solution for 30 minutes in a 37°C water bath at 50 rpm. After 30 minutes the trypsin solution was removed and 4 mL of Hibernate E + 10% fetal bovine serum (Gibco 16000044) was added to terminate the trypsin reaction. The tissue was then
mechanically tritutrated with the supernatant being transferred to a 15 mL centrifuge tube. The same process was repeated two times by adding 2 mL of L15 + 10% FBS each time. The 6 mL cell suspension obtained after mechanical trituration was suspended on a 2 mL, 4% BSA (Sigma A3059) (prepared in L15 medium) cushion and centrifuged at 300g for 10 minutes at 4°C. The pellet obtained was washed 5 times with L15 medium then resuspended in 10 mL of L15 and plated in 100 mm uncoated dishes for 30 minutes. The non-attached cells were removed and then centrifuged on a 4% BSA cushion [12]. The pellet was resuspended in serum-free medium according to the protocol illustrated in Figure 1 and plated on the coverslips at a density of 700-1000 cells/mm². The serum-free medium containing different growth factors and hormones was added to the culture dish after one hour. The final medium was prepared by mixing medium one (Table 1) and medium two (Table 2) in a 1:1 v/v ratio. Figure 1 indicates the flowchart of the culture protocol. Tables 1 and 2 list the growth factor and hormone supplement compositions of medium one and medium two. The cells were maintained in a 5% CO₂ incubator (relative humidity 85%). The full medium was replaced after four days with NBactiv4 medium according to the protocol in Figure 1 [21]. Thereafter three-fourths of the medium was changed every three days with NBactiv4.

Immunocytochemistry of Skeletal Muscle Myotubes

Coverslips were prepared for immunocytochemical analysis as previously described. Briefly, coverslips were rinsed with PBS, fixed in -20°C methanol for 5-7 min, washed in PBS, incubated in PBS supplemented with 1% BSA and 0.05% saponin (permeabilization solution) for 10 minutes, and blocked for 2h with 10% goat serum and
1% BSA. Cells were incubated overnight with primary antibodies against embryonic myosin heavy chain (F1.652) (dilution>1:5), neonatal myosin heavy chain (N3.36) (1:5) (Developmental Studies Hybridoma Bank), ryanodine receptor (AB9078, Millipore) (1:500) and dihydropyridine binding complex (α1-Subunit) (MAB 4270, Millipore) (1:500) diluted in the blocking solution. Cells were washed with PBS and incubated with the appropriate secondary antibodies for two hours in PBS. After two hours the coverslips were rinsed with PBS and mounted on glass slides and evaluated using confocal microscopy [14].

AChR Labeling of Myotubes

AChRs were labeled as described previously by incubating cultures with 5 x 10⁻⁸ M of α-bungarotoxin, Alexa Fluor® 488 conjugate (B-13422; Invitrogen) for 1.5 h at 37°C [5, 14]. Following incubation in α-bungarotoxin, the cultures were fixed as above for subsequent staining with embryonic myosin heavy chain (F1.652) antibodies.

Patch-clamp Electrophysiology of Myotubes

Whole-cell patch clamp recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope as described previously [15, 20]. The chamber was continuously perfused (2 ml/min) with the extracellular solution (Leibovitz medium, 35°C). Patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller and filled with intracellular solution (K-gluconate 140 mM, EGTA 1 mM, MgCl₂ 2 mM,
Na₂ATP 2 mM, phosphocreatine 5mM, phosphocreatine kinase 2.4 mM, Hepes 10 mM; pH=7.2). The resistance of the electrodes was 6–8MΩ. Voltage clamp and current clamp experiments were performed with a Multi-clamp 700A amplifier (Axon Laboratories, Union City, CA). Signals were filtered at 2 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis were done with pClamp 8 software (Axon Laboratories). Membrane potentials were corrected by subtraction of a 15 mV tip potential, which was calculated using Axon’s pClamp 8 program. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a -85 mV holding potential. Action potentials were evoked with 1 second depolarizing current injections from a -85 mV holding potential [12, 15].
Results

*DETA Surface Modification and Characterization*

Static contact angle and XPS analysis was used for the validation of the surface modifications and for monitoring the quality of the surfaces. Stable contact angles (40.64° ± 2.9 /mean ± SD) throughout the study indicated high reproducibility and quality of the DETA surfaces and these characteristics were similar to previously published results [14, 15]. Based on the ratio of the N (401 and 399 eV) and the Si 2p\textsubscript{3/2} peaks, XPS measurements indicated that a reaction-site limited monolayer of DETA was formed on the coverslips [22].

*Development of the Serum-free Medium Formulation and Culture Timeline for Long-term Survival and Maturation of Myotubes*

The serum free medium composition was developed semi-empirically. The final medium is derived from two different medium compositions described in Tables 1 and 2. Table 1 constitutes the same medium composition used previously for a motoneuron-muscle co-culture and adult spinal cord neurons culture [11, 14]. Table 2 is composed of twelve additional factors that had been shown to promote skeletal muscle maturation and neuromuscular junction formation individually. The final medium was prepared by mixing these two mediums in a 1:1 v/v ratio. After first 4 days of culture the whole medium was replaced with NBactiv4 medium [21]. Thereafter, every three days three-fourth medium was changed with NBactiv4. The culture technique has been illustrated in the flowchart in Figure 1.
Using this new medium formulation and timeline, myotubes were successfully cultured for more than 90 days. Figure 2 indicates 50 days old myotubes in culture. As the myotubes aged and grew they began to form the characteristic anisotropic (A band) and isotropic (I band) banding pattern observed with mature in vivo muscle fibers [9, 23]. This banding pattern is caused by differential light diffraction due to the organization of myofibril proteins forming sarcomeres within the myotubes [9, 23]. The arrowheads in the images (Figure 2 A-D) indicate myotubes where sarcomeric organization has occurred and is visualized by the appearance of the A and I bands.

*Myotube Expression of Fetal MHC*

The myotubes formed were evaluated for the expression of fetal MHC to establish a baseline as comparison to our previous results [12]. In Figure 3, the different myotube phenotypes formed at approximately day 50 in vitro are shown. The myotubes ranged from having clustered nuclei (Figure 3 A-D) to having diffuse nuclear organization (Figure 3 E-H). The arrowheads in the images indicate the characteristic striations.

*Differential Expression of Neonatal MHC in the Myotubes*

In order to determine if the myotubes were maturing in a physiologically relevant way as they aged in vitro, the expression of neonatal MHC protein was evaluated. After approximately 50 days in vitro, 25% of the myotubes expressed neonatal MHC (Figure 4 A-M). Additionally, the myotubes were stained to detect the clustering of acetylcholine
receptors (AChR) using alpha bungarotoxin (Figure 5 B,F). This clustering of the AChR receptors, which are induced by the motoneuron protein agrin in vivo, are locations on the myotube where neuromuscular junction formation occurs and another indication of functional myotube formation.

Formation of the Excitation – Contraction Coupling Apparatus

The presence of the ryanodine (RyR) receptor and dihydropyridine (DHPR) receptor clusters, as well as their colocalization in vivo, represents the development of excitation-contraction coupling apparatus in skeletal muscle myotubes [8, 9, 24]. The clustering of both RyR and DHPR receptors was observed on the myotubes after 30 days in culture (Figure 5 A-D). The clustering and colocalization of the RyR + DHPR clusters was observed with a number of different myotube morphologies (Figure 5 E-L). This functional adaptation illustrated that the medium formulation facilitated not only the structural maturation but also the functional maturation of myotubes in this in vitro system. The clustering of the RyR + DHPR receptors was also observed in 90 day old myotubes, indicating that the older myotubes maintained their functional integrity (Figure 6 A-F).

Myotube Electrophysiology

The myotubes contracted spontaneously in the culture and the contractions began generally by day four and continued throughout the life of the culture. Most of the myotubes expressed functional voltage gated sodium, potassium and calcium ion
channels as reported previously [12]. The voltage clamp electrophysiology of the myotubes indicated the inward and outward current that demonstrates functional sodium and potassium channels (Figure 7 A). Figure 7B shows the single action potential fired by the myotubes in current clamp mode. The electrical properties of the myotubes were also consistent with the formation of mature, functional myotubes.
Discussion

In this report we have documented the development of a system for long-term, functional, skeletal muscle culture. This system was in response for the need to develop more physiologically relevant skeletal muscle myotubes for functional in vitro systems. For our specific research, they are a component needed for the creation of a realistic model of the stretch reflex arc as well as their integration with bio-MEMS cantilevers for screening applications. The results indicate that we have achieved three significant structural modifications within the myotubes, causing both the developmental profile and functionality of the fibers to better mimic in vivo physiology. This skeletal muscle maturation resulted from modifications to the cell culture technique, a new medium formulation and the use of NBactiv4 as the maintenance medium.

We developed this serum-free medium, supplemented with growth factors, that supported the survival, proliferation and fusion of fetal rat myoblasts into contractile myotubes in a semi-empirical fashion. The rational for selecting the growth factors was based on the distribution of their cognate receptors in the developing myotubes in rat fetus [3, 4, 25]. Tables 1 and 2 reference the literature where these individual growth factors, hormones and neurotransmitters were observed to support muscle and neuromuscular junction development. The composition in Table 1 is the formulation used for a previously published medium used for motoneuron-muscle co-culture and adult spinal cord neuron culture [11, 14]. Table 2 lists the twelve additional factors we have identified in muscle development and neuromuscular junction formation. The use of NBactiv4 for the maintenance of the cells significantly improved the survival of the skeletal muscle derived myotubes despite the original development of NBactiv4 for the
long-term maintenance and synaptic connectivity of fetal hippocampal neurons *in vitro* [21].

We observed a ratio of 25% neonatal to 75% embryonic MHC expression of the myotubes after 50 days, which contrasts with the previous study in which MHC expression was strictly embryonic. We believe that the myotubes matured in this culture system because the long-term survival provided adequate time for the myotubes to respond to the additional growth factors, which activated the necessary signaling pathways to achieve MHC class switching [26]. This suggests that a different growth factor profile could be utilized to activate alternative signaling pathways and drive myotube differentiation down other pathways. For example, the effects of adding steroid hormones like testosterone to the system could be critically examined.

The colocalization of RyR and DHPR clusters in the myotubes indicated the formation of the excitation-contraction coupling apparatus and was another indication of functional maturation in the fibers. Excitation-contraction coupling is the signaling process in muscle by which membrane depolarization causes a rapid elevation of the cytosolic Ca^{2+} generating contractile force [27]. The close proximity of the DHPR and RyR complexes occurs at specialized junctions established between the transverse tubule and sarcoplasmic reticulum (SR) membranes in skeletal muscle myotubes [28]. At these junctions, T-tubule depolarization is coupled to Ca^{2+} release from the SR resulting in muscle contraction [29, 30]. This structural adaptation represents a significant functional change due to the fact that excitation-contraction coupling is required for successful extrafusal muscle fiber development as well as neuromuscular junction formation [8, 9, 23]. This improved model provides the potential to study
excitation-contraction coupling in a defined system as well as for myotonic and myasthenic diseases.

The development of sarcomeric structures, the excitation-contraction coupling apparatus and MHC class switching in the skeletal muscle myotubes is a result of the improvements to the model system documented in this research. This improved system, supports the goal of creating physiologically relevant tissue engineered muscle constructs and puts within reach the goal of developing functional skeletal muscle grafts. This defined model can also be used to map the developmental pathway regulating the formation of the excitation-contraction coupling apparatus as well as MHC class switching. Furthermore, we believe this serum-free culture system will be a powerful tool in developing advanced strategies for regenerative medicine in muscular dystrophies, stretch reflex arc development and integrating skeletal muscle with bio-hybrid prosthetic devices. Due to the use of a serum-free defined culture system this also has applications for new high-throughput screening systems for use in drug discovery research and toxicology investigations.
Table 6-1. Composition of medium 1

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Figure 6.1. Schematic diagram of the culture protocol and timeline.
Figure 6.2. Phase contrast images of 50 day old myotubes in culture. (A-D). Red arrows indicate characteristic striations present in most of the myotubes. Scale bars = 75 μm.
Figure 6.3. Myotubes stained with antibodies against the embryonic myosin heavy chain (F1.652) antibody at day 50. (A) Panel showing phase + fluorescent image of the myotubes, (B) Myotubes image only in fluorescent light, (C) Panel showing phase + fluorescent image of the myotubes, (D) Myotubes image only in fluorescent light, (E)
Panel showing phase + fluorescent micrograph of the myotubes, (F) Myotube image only in fluorescent light, (G) Panel showing phase + fluorescent picture of the myotubes, (H) Panel G observed only in fluorescent light (white arrowheads indicate the striations). Scale bar = 75μm.

Figure 6.4. Myotubes immunostained with neonatal myosin heavy chain antibody (N3.36) and alpha-bungarotoxin at day 50. (A) Phase contrast image of 2 myotubes indicated by white arrows, (B) Both the myotubes shown in phase (panel A) have acetylcholine receptor clustering indicated by alpha-bungarotoxin staining, (C) Only one myotube out of the two seen in panel A stained for N3.36, (D) Double stained image of panel A with alpha-bungarotoxin and N3.36, (E) Phase image of 6 myotubes indicated by white arrows, (F) All the myotubes shown in phase (panel E) have acetylcholine receptor clustering shown by alpha-bungarotoxin staining, (G) None of the myotubes in panel E stained for N3.36, (H, I, J) Differential staining of the myotubes with N3.36, (K, L, M) Differential staining of the myotubes with N3.36.
Figure 6.5. Ryanodine receptor and DHPR clustering in a 30 days old myotube culture. (A) phase and fluorescent-labeled micrograph of the myotubes, (B) merged fluorescent picture of the ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes shown in panel A, (C) ryanodine receptors (green) on the myotubes shown in panel A, (D) DHPR receptors (red) on the myotubes shown in panel A, (E) phase and fluorescent-labeled micrograph of the myotubes, (F) merged fluorescent picture of the ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes (panel E), (G) ryanodine receptor (green) on the myotubes (panel E), (H) DHPR receptors (red) on the myotubes (panel E), (I) phase and fluorescent-labeled micrograph of the myotubes, (J, K, L) merged fluorescent micrograph of the ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes (panel I) at three different planes (white arrows indicate the striations and the receptor clustering). Scale bar = 75 μm.
Figure 6.6. Ryanodine receptor and DHPR clustering in a 90 days old myotube culture. 
(A) phase and fluorescent-labeled micrograph of the myotubes, (B) merged fluorescent micrograph of the ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes (panel A), (C) ryanodine receptor (green) on the myotubes (panel A), (D) DHPR receptors on the myotubes (panel A), (E, F) same panels at a different plane indicating the merged fluorescent picture of the ryanodine receptor (green) and DHPR receptor (red) clustering on the myotubes. Scale bars = 75 μm.
Figure 6.7. Patch-clamp electrophysiology of the myotubes. (A) representative voltage clamp trace obtained after patching a 48 days old myotube in culture, (B) representative current clamp trace of the same myotube for which voltage clamp trace had been obtained (insets show the phase contrast image of the patched myotubes).
References


Introduction

The enormous complexity of natural neuronal networks makes it a significant challenge to study their development, activity and dynamics in vivo. A promising approach to solve this problem is to create simplified neural network models in vitro on silicon-based devices. The stretch reflex arc is the simplest functional reflex circuit in the nervous system [1, 2]. This neuronal circuit sends afferent signals through DRG Ia sensory neurons and efferent signals through spinal cord motoneurons. We are attempting to recreate this circuit on a micro-cantilever based bio-MEMS device. In vitro neuronal network models have multiple potential applications in advanced medical diagnostic, cell-based biosensors, neurological implants, and high throughput drug/toxin screening as well as fundamental neurobiological studies.

In order to create the in vitro stretch reflex arc, the first step is to culture the components of the reflex arc system in a defined environment. We have selected a synthetic silane substrate, N-1[3-(trimethoxysilyl) propyl] diethylenetriamine (DETA), to grow and pattern the cells. The rational for using DETA is that it forms a self-assembled monolayer on a variety of silicon substrates, MEMS devices and on glass. Furthermore, it can be easily patterned using photolithography [3-11]. We have experimentally established a bio-MEMS system where all the major culture parameters are known and quantifiable, which includes a reproducible dissociated culture methodology, serum-free media, and a surface composed of self-assembled monolayers (SAMs). In addition, we
have characterized the embryonic motoneurons in a serum free system and have successfully co-cultured embryonic motoneurons with embryonic skeletal muscle [3-5, 12, 13]. We have also successfully differentiated fetal skeletal muscle into intrafusal muscle spindle fibers in a defined system, [14] making DRG characterization and subsequent co-culture with intrafusal muscle fibers one of the final steps in a defined system creating an *in vitro* model of the stretch reflex.

The enormous complexity of natural neuronal networks controlling our movements makes studying their development, activity and dynamics *in vivo* difficult. A promising approach to solve this problem is to create a simplified neural network circuit and integrate such circuits into bio-MEMS devices. Some of the potential applications of such tissue-engineered constructs are in advanced medical diagnostics, cell-based biosensors, neurological implants, and high throughput drug/toxin screening [11, 15, 16].

In this study, sensory neurons were cultured in serum-free medium on the non-biological surface, N-1[3-(trimethoxysilyl) propyl] diethylenetriamine (DETA) and on a biological control surface ornithine/laminin (O/L). The sensory neurons were characterized using immunocytochemistry and whole cell electrophysiology. Furthermore, the electrical properties of the sensory neurons grown on DETA were compared with cells grown on the O/L surface. This study is the first step in integrating the sensory neurons in our already well established bio-MEMS device, in order to recreate the sensory circuit of the reflex arc *in vitro*. Furthermore, this system could be used as a high-throughout method to screen compounds for ectopic discharges or stimulated responses in DRG neurons.
Materials and Method

DETA Surface Preparation

Glass coverslips (Thomas Scientific, Swedesboro, NJ) were cleaned using HCl/methanol (1:1), soaked in concentrated H₂SO₄ for 30 min then rinsed in double distilled H₂O. Coverslips were boiled in deionized water, rinsed with acetone then oven dried. The DETA monolayer was formed by reaction of the cleaned surface with 0.1% (v/v) mixture of DETA organosilane in distilled toluene (Fisher T2904). The DETA coverslips were heated just below the boiling point of toluene for 30 minutes, rinsed with toluene, reheated just below the boiling temperature for 30 minutes, and then oven dried.

Ornithine/Laminin Surface Preparation

Coverslips were cleaned by acid treatment, incubated with polyornithine (Aldrich, 1: 1000 in DI water) overnight at room temperature, and then dried in a biological safety cabinet. Laminin (Natural Mouse, Invitrogen) was dissolved in the culture medium at 1:500 and the coverslips were covered with laminin containing solution and incubated at 37°C for 30min.

Surface Characterization

Surfaces were characterized by contact angle measurements using an optical contact angle goniometer (KSV Instruments, Cam 200) and by XPS (Fisions 220i). XPS survey scans, as well as high-resolution N 1s and C 1s scans, utilizing monochromatic
Al Kα excitation, were obtained according to our previously published procedures [3-5, 13, 14].

*Rat Embryonic DRG Neuron Culture*

Embryonic rats day 14 (E14) were anaesthetized and killed by inhalation of an excess of CO₂. The procedure was in agreement with the Animal Research Council of Clemson University and the University of Central Florida. The DRG tissue was collected in PBS. 1 mL 0.05% trypsin-EDTA is added to the tissue and then put in the water bath for 15 minutes. After 15 minutes, the trypsin was removed and 4 mL of L15 medium was added containing 4% BSA (Invitrogen 15561-020) to quench the trypsin reaction and 100 µl of Dnase I (Invitrogen, 18047-019). The DRG tissue was manually triturated using a 1000µL pipette until a single cell homogenate was obtained and centrifuged for 10 min at 300 g on a 4% FBS cushion. Next, the medium was removed and 1 mL of DRG plating media was added, and the cells gently suspended. A cell count was taken and the cells were plated in 22×22 mm² ornithine-laminin (Sigma) coated and DETA coated coverslips at a density of 1000 cells/mm². The culture medium was: Neurobasal (Gibco-BRL) supplemented with B27 (2% v/v; Invitrogen), L-glutamine (0.5mM). Three growth promoting factors were added: glial cell line-derived neurotrophic factor (GDNF) (100 pg/ml; Invitrogen), brain derived neurotrophic factor (BDNF) (100 pg/ml; Invitrogen), and ciliary neuropathic factor (CTNF) (1ng/ml; Cell Sciences). The culture medium was changed every 4 days and L-glutamate (25 µM) was added to the culture medium during the first 4 days of growth. DRG neurons were maintained *in vitro* for at least two weeks.
Characterization of DRG Neurons and Live/Dead Assay

DRG neurons were identified and characterized by immunostaining. The primary antibodies used in this study were: 1) mouse Anti-MAP2a&b monoclonal antibody (Chemicon, MAB378), 2) chicken anti-neurofilament 150kDa (Chemicon, AB1981), 3) mouse anti-parvalbumin (Chemicon MAB1572) 4) rabbit anti-trkA (Chemicon AB1577), 5) rabbit anti-trkB (Upstate 07-225), and 6) rabbit anti-trkC (Chemicon AB5918). The secondary antibodies were donkey anti-mouse IgG Alexa Fluor 594nm, donkey anti-chicken Alexa Fluor 647nm and donkey anti-rabbit Alexa Fluor 488nm (Molecular Probes A-21203, A-21449, A-21206). DRG neurons were fixed in 4% paraformaldehyde in calcium magnesium free PBS plus 0.1% glutaraldehyde and 4% sucrose for 10 min then permeabilized by lysine 50 mM and 0.1% triton X-100 for 15 min at room temperature. Nonspecific staining was blocked using 2% BSA and 5% goat serum in PBS. Primary antibody (1:200) was added to the coverslips in blocking buffer overnight at 4°C. After washing with PBS, the coverslips were incubated with secondary antibody (1:200) for 2 hours. Coverslips were then fixed on glass slides with clear nail polish and viewed using a fluorescence microscope. Molecular Probe’s L-3324 Live/Dead Assay kit was used for the live/dead assays as indicated by the manufacturer’s instructions.

Morphological Analysis

Phase-contrast pictures were taken with a commercial Nikon Coolpix 990 camera using the 40× objective of a Zeiss Axiovert S100 microscope. Pictures were analyzed
using Scion Image Software (Scion Corp., Maryland). The size of the soma was measured.

**Electrophysiological Analysis**

Whole-cell patch clamp recordings were performed using a Zeiss Axiovert S100. Electrodes (3~6 MΩ) which were pulled from borosilicate glass (BF150-75-10; Sutter, Novato, CA) with Sutter P97 pipette puller and then were filled with intracellular solution (in mM): 5.2 NaCl, 120 KCl, 2 MgCl₂, 10 HEPES, 10 EGTA, 2 ATP-tris, 0.4 GTP-tris; pH was adjusted to 7.2 with NaOH (1M). Osmolarity was 275. The extracellular solution includes (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 10 Dextrose, 2 CaCl₂; pH was adjusted to 7.4 using NaOH (1M). All experiments were done at a room temperature of 23±2°C. Neurons with somal diameters ranging from 28-33 µm were used, representing the range of Ia sensory neurons seen in the cultures (Loth et al., 1991). After formation of a tight seal (>2GΩ) and compensation of pipette capacitance with amplifier circuitry, whole cell access was established. Multiclamp 700A was used to perform voltage and current clamp experiments. PClamp 8 software (Axon) was used to acquire and analyze the recordings. Under the voltage clamp mode, sodium and potassium channel currents were obtained by holding the cells at the potential of –60 mV. The membrane resistance and capacitance were calculated using a 50 ms voltage step from -60 to -70 mV without any whole-cell or series resistance compensation. Whole cell capacitance and series resistance was compensated using a p/6 protocol. Only cells with access resistance less than 22 MΩ were analyzed. Electrophysiological parameters were recorded and analyzed. The input resistance (Rin, MΩ) was calculated from the slope of a steady-
state I-V curve. Duration of action potential, amplitude of action potential was recorded in the current clamp mode [4, 14, 17]

**Statistical Analysis**

Means and standard errors of the means (SEM) were calculated, and results were expressed as means±SEM. A two-sample *t* test was performed for the statistical analysis of morphological and electrophysiological data. Parameters obtained from DETA plated neurons were compared with the ornithine/laminin as control. When necessary, p-values were obtained by the student *t*-test.
Results

Surface Modification

The surface modification controls were tested by contact angle and X-ray photoelectron spectroscopy (XPS). The contact angle for DETA modified coverslips was 39.43 ± 1.67° (mean ± SD). This value represents a hydrophilic surface which has been shown to support the growth various cell types including neurons (Das et al., 2003, Stenger et al. 1993). XPS data indicated the glass surface was modified by a complete monolayer of DETA (characterized by the 399 eV N peak).

Survival of DRG Cells in the Defined Environment

Rat embryonic sensory neurons, grown either on DETA or ornithine/laminin surfaces (data not shown) (Fig. 1), survived at least 2 weeks in serum-free neurobasal culture medium supplemented with growth factors at plating. The initial number of live neurons was higher on ornithine/laminin than on DETA, and the numbers remained constant throughout the time period of the study, indicating that both surfaces supported the survival of DRG neurons (Table 1). We also analyzed the size of the neurons at day 7 in both culture systems. These measurements were made at day 7 to allow time for the cells to mature in size and no significant size differences were observed after day 7. We identified slightly higher numbers of smaller neurons (1111±29) compared to larger (896±17) neurons. We also found slightly more neurons on the O/L surface (Table 1).

Immunocytochemical Characterization of Sensory Neurons on DETA
The efficacy of the medium formulation to support the survival and growth of all three sensory neuron subtypes was evaluated immunocytochemically. We observed trkA positive sensory neurons in culture (Fig.2 a-d). These neurons are significant in culture for studying nociception. We also observed trkB positive sensory neurons in our cultures (Fig.2 e-h). These neurons represent another distinct population of sensory neurons found in the dorsal root ganglion. Finally, we observed trkC positive sensory neurons in culture (Fig.2 i-l). These large somal size and large axon diameter neurons are also positive for parvalbumin, a calcium binding protein present in these cells, and represent the pool of proprioceptive neurons significant for future studies of the reflex arc.

Effect of DETA Surface on the Morphology and Electrophysiology of DRG Neurons

The morphology of sensory neurons on DETA (Fig.1) and on ornithine/laminin (data not shown) was not significantly different. Also, there was no significant difference in the size of the cells on the two surfaces. The somal diameter of the cells increased as the cells matured in the culture system (Table 1). The resting membrane potential of the DRG neurons became more positive with time (Table 2). The amplitudes of the voltage-dependent sodium and potassium currents do not change with time. However, the ability of sensory neurons to fire repetitively decreased after 5 days culture. At day 2 on the DETA surface, 27 percent of the neurons patched fired repetitively while by day 7 the percentage decreased to 10 percent. These values were not significantly different from the neurons plated on the ornithine/laminin surface (Table 2). There was no
significant difference between the passive or active membrane properties of DRG neurons plated on DETA or ornithine/laminin surfaces.
Discussion

In a completely serum-free defined environment, rat embryonic sensory neurons were cultured on two different surfaces, the non-biological surface DETA, and a standard biological surface, ornithine/laminin as the control. The sensory neurons survived and matured during a 14-day study period on both surfaces. No differences were found regarding the morphology and electrophysiological properties of DRG neurons on DETA and ornithine/laminin surfaces.

According to the live/dead assay, for the day 2 culture, the amount of live neurons on ornithine/laminin is higher than DETA surface. However, for day 5, the surviving neurons on two surfaces were similar (Fig. 2). The different adhesive strength of the surfaces, and the different initial environment provided by the two surfaces may contribute to this phenomenon (Haper et al., 1985; Georger et al., 1992; Stenger et al., 1992). However, since survival of neurons on both surfaces was stable, this variation was not significant for this study.

Whole cell patch clamp technique was used to study the electrophysiological properties of sensory neurons on two different surfaces. The resting membrane potential became more positive and the repetitive firing ability of the cells became lower as the neurons aged. This finding could be a result of changing ion channel expression profiles as the cells developed and aged in culture. Different sizes of the DRG neurons may express different ion channels and thus process different electrophysiological characteristics (Spargo et al., 1994). Many factors including neuron size, growth environment and target innervation can affect sensory neuron ion channel expression (Haper et al., 1985; Itoh et al., 1991).
This is the first study showing the long-term survival of sensory neurons on a synthetic silane surface, DETA, in a serum-free system. Morphological and electrophysiological properties of sensory neurons were not different on the DETA surface compared to the biological surface, ornithine/laminin. Based on this and combined with our earlier results with motoneurons, skeletal muscle and muscle spindle fibers we will be able to integrate the sensory neurons in our cantilever based bio-MEMS device and recreate the stretch reflex circuit.
Table 7-1. Neuron somal size and survivability on DETA and O/L surfaces

<table>
<thead>
<tr>
<th>Surface</th>
<th>Day 2</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DETA</td>
<td>O/L</td>
<td>DETA</td>
</tr>
<tr>
<td>Smaller cells$^1$</td>
<td>--</td>
<td>--</td>
<td>1111±29</td>
</tr>
<tr>
<td>Larger cells$^1$</td>
<td>--</td>
<td>--</td>
<td>896±17</td>
</tr>
<tr>
<td>Survivability$^2$</td>
<td>1906±37</td>
<td>2024±5</td>
<td>2162±49</td>
</tr>
</tbody>
</table>

1 somal size diameter values are the average of 3 experiments performed in triplicate ± standard error of the mean (SEM).

2 survivability values are the average number of live cells from 3 experiments performed in duplicate ± SEM.
Table 7-2. Electrophysiology characteristics of DRG neurons on DETA and O/L surfaces

<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DETA</td>
<td>O/L</td>
<td>DETA</td>
</tr>
<tr>
<td>Vm (mV)</td>
<td>-51±3.4</td>
<td>-52±5.7</td>
<td>-49±4.9</td>
</tr>
<tr>
<td>Cm (pF)</td>
<td>12.5±2.1</td>
<td>10.2±2.9</td>
<td>11.4±1.6</td>
</tr>
<tr>
<td>Rin (MΩ)</td>
<td>75.8±6.3</td>
<td>68.5±4.9</td>
<td>67.5±5.2</td>
</tr>
<tr>
<td>Rm (MΩ)</td>
<td>576±49.8</td>
<td>439±36.8</td>
<td>553±74.9</td>
</tr>
<tr>
<td>APD (ms)</td>
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<td>6.2±1.2</td>
<td>6.4±0.8</td>
</tr>
<tr>
<td>APA (mV)</td>
<td>86.4±3.1*</td>
<td>94.5±2.2</td>
<td>78.6±4.2*</td>
</tr>
<tr>
<td>AHPA (mV)</td>
<td>13.0±2.4</td>
<td>15.6±4.3</td>
<td>16.2±3.6</td>
</tr>
<tr>
<td>N</td>
<td>16</td>
<td>20</td>
<td>22</td>
</tr>
</tbody>
</table>

Values are expressed as the mean±SEM.

Vm: resting membrane potential  Cm: membrane capacitance  Rin: input resistance  APD: action potential duration,  APA: amplitude of action potential (AP)  AHPA: amplitude of after hyperpolarization  N: number of cells

Student t-test was used for statistical analyses.
Figure 7.1. Morphological results of sensory neurons plated on a DETA surface. (A, B) sensory neurons at day 2 (C, D) sensory neurons at day 7. Scale bar = 100μm.
Figure 7.2. Immunocytochemical characterization of sensory neuron subtypes grown on the non-biological substrate DETA. (A) phase contrast image of trkA positive neurons (B) parvalbumin negative staining channel (C) trkA staining channel (D) merge of panels b and c + NF-M green (E) phase contrast image of trkB positive neurons (F) parvalbumin positive staining channel (G) trkB staining channel (H) merge of panels f and g + NF-M green (I) phase contrast image of trkC positive neurons (J) parvalbumin positive staining channel (K) trkC staining channel (L) merge of panels j and k + NF-M green. Scale bar = 50μm.
Figure 7.3. Electrophysiological characterization of rat embryonic sensory neurons grown on a DETA surface. (A) Representative recording of whole cell currents obtained from a day 7 neuron on the DETA surface (-60 mV holding potential, 10 mV steps) (B) Repetitive firing of day 7 sensory neuron on DETA surface. (C) Phase contrast image of a patched large diameter sensory neuron.
References


Introduction

The ability of multi-cellular organisms to function in cohesive synergy depends on the integration and communication of sensory information throughout the body. One principle type of sensory information is mechanical in nature. Organisms from bacteria and worms to humans have developed mechanisms to detect mechanical force dynamics in the environment and play a role in activities as diverse as development, immunity and fracture repair to balance and hearing [1-6]. Consequently, the vertebrate body has developed several conserved mechanisms for the transduction of mechanical forces to sensory neural impulses, also called mechanotransduction [7]. These include groups of mechanoreceptors, mainly cutaneous in nature, that respond to touch, pressure and vibration as well as proprioceptors sensitive to changes in muscle length, stretch and tension [8-10]. Both mechanoreceptors and proprioceptors are composed of a specialized receptors innervated by a specific type of sensory neuron [8, 9, 11]. Physical deformation of the receptor results in depolarization of the sensory neuron and action potential generation.

In the case of muscle spindles, a major type proprioceptor, the sensory receptor is composed of an encapsulated group of specialized muscle fibers called intrafusal fibers. Within the spindle, the intrafusal fibers are of two main types: 1) nuclear bag fibers, so named because their nuclei cluster in a bulging equatorial region, and 2) nuclear chain fibers, so named because their nuclei align next to each other. Several
different neuronal types innervate these fibers. The sensory innervation is accomplished by type Ia and type II neurons located in dorsal root ganglia. These sensory neurons form specialized synapses on intrafusal muscle fibers called annulospiral wrappings (ASWs) (type Ia) and flower-spray endings (FSEs) (type II) [12]. Muscle spindles also receive motor innervation from γ-motoneurons and β-motoneurons. This motor innervation occurs at the polar regions of the intrafusal fibers and functions to maintain the sensitivity of the spindle during extrafusal fiber contraction[13, 14].

Our basic knowledge of these mechanosensory systems comes from years of physiological analysis using vertebrates from frogs and cats to humans [11, 15-17]. However, the molecular components and processes of the vertebrate mechanosensory transduction system are poorly understood due to difficulty in directly observing the sensory structures in vivo. This is due to their sparse and random distribution in the body. Additionally, their encapsulated structure spatially limits electrical and biochemical examination. Therefore, some of the most interesting information gathered regarding the molecular components of the system have come from experiments done in the worm Caenorhabditis elegans [18]. Ultimately, the work resulted in the identification of a mechanoreceptive complex, including a mechanically sensitive ion channel called MEC-4/10 [19-21]. Using a genetic screen, homologous vertebrate sequences were identified and determined to be members of the degenerin / epithelial sodium channel (DEG/ENaC) superfamily [22-24]. These proteins, brain sodium channels 1 and 2 (BNaC1 & BNaC2), form an additional branch of the superfamily and have been shown to localize at mechanosensory terminals of dorsal root ganglion.
neurons [25-27]. Specifically, BNaC1 is expressed in large diameter sensory neurons and BNaC2 in medium and small diameter sensory neurons [27]. Furthermore, the channels were found localized at specialized mechanosensory terminals like Meissner corpuscles and Merkel discs [25]. Also, BNaC1 has been shown to interact with protein interacting with PRKCA 1 (PICK1) and both localize to mechanosensory terminals of type Ia sensory neurons [25, 28]. However, further progress in identifying additional functional or structural proteins present in the vertebrate stretch receptor complex has not been successful.

The development of an in vitro system capable of supporting the growth and differentiation of both intrafusal fibers and sensory neurons would provide a critical building block for studying the vertebrate mechanosensory complex. Furthermore, systems using serum-free defined medium formulations and nonbiological, uniform growth substrates provide an environment ideal for manipulation of specific factors in order to generate a specific cell phenotype without concern for confounding variability associated with systems based on biological substrates and serum containing medium. This system will also provide the added benefit of being readily modified using photolithography to generate patterned sites for cell attachment.

In our previous work, we showed the ability to grow embryonic skeletal muscle on trimethoxy-silylpropyl-diethylenetriamine (DETA) surfaces and direct myotube differentiation towards intrafusal fiber formation [29]. Also, we have shown that the same culture conditions will support the growth and phenotypic development of DRG neurons [30]. The next critical segue for the design and engineering of neural and myoskeletal tissue based devices is the formulation a medium that provides an
environment conducive to the growth and differentiation of both cell types simultaneously. This work documents the development of an in vitro system supporting the growth and differentiation of both intrafusal muscle fibers and sensory neurons as well as the development of the specialized mechanosensory endings seen in vivo.

Engineering an in vitro system to study the mechanical interactions between intrafusal muscle fibers and la sensory neurons and the subsequent generation of electrical activity is an integral step in building a functional stretch reflex arc in vitro. Such a system would capacitate studies of nerve-muscle and nerve-nerve communication, aiding in the enhanced functioning of prosthetic devices and providing insight into the pathologies of neuromuscular diseases such as infantile spinal muscular atrophy type I (Werdnig-Hoffman disease) and spasticity inducing diseases like Parkinson’s, muscular dystrophy and myasthenia gravis [31-36].
Materials & Methods

DETA Surface Modification

Glass coverslips (VWR 4836067, 22×22 mm² No. 1) were first cleaned using 1:1 HC l-methanol followed by a concentrated H₂SO₄ soak for 2 hours. The DETA (United Chemical Technologies Inc. T2910-KG) film was formed by the reaction of the cleaned surfaces with 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (VWR BDH1151). The cleaned surfaces were heated to about 100 °C in the organosilane mixture, rinsed with toluene, reheated to about 100 °C in toluene, and then dried in the oven overnight (100 °C). Surfaces were characterized by static water contact angle measurements using a Rame-Hart Model 250 goniometer, and by X-ray photoelectron spectroscopy (XPS) using an Escalab 200i spectrometer (VG Scientific) by monitoring the N 1s peak [37-39]. The values are reported as the mean ± SEM.

Boxed Line Pattern Surface Preparation

Boxed line patterned quartz photomasks were designed using the CleWin layout editor (WieWeb, Hengelo, The Netherlands) and fabricated through a commercial vendor (Bandwidth Foundry Pty Ltd., Australia). The surface of the DETA coated coverslips not covered by the photomask were ablated using a 193 nm Ar/F LPX200i laser beam (Lambda Physik, Ft. Lauderdale, FL) combined with a beam homogenizer (Microlas, Ft. Lauderdale, FL, Energy density: 50 mJ/cm²) to create the patterns. The ablated regions of the DETA coverslips were then rederivatized with the fluorinated silane tridecafluoro-1,1,2,2-tetrahydroctyl-1-dimethylchlorosilane (13F) as previously described [38, 40]. Patterned surfaces were characterized by XPS.
Animals

Dated pregnant Sprague-Dawley rats were housed in an animal facility at the University of Central Florida. All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines. Pregnant rats were anesthetized and sacrificed at embryonic day 14 or 18 (E14 / E18). The embryos were removed by caesarean section and fetuses dissected under a stereomicroscope (Carl Zeiss, Stemi 2000).

Primary Culture of Rattus norvegicus myocytes

Skeletal muscle was removed from the hind limb of E18 rat fetuses, collected in a 15ml tube in cold Hibernate E + GlutaMAX™ + antibiotic-antimycotic + B27 (dissection medium) and incubated in 0.05% trypsin-EDTA for 45 minutes in a 37°C water bath. Following incubation, the trypsin-EDTA was removed and the cells suspended in dissection media + 10% FBS and the tissue gently triturated. The dissociated cell suspension was then centrifuged at 500g for 10 minutes at 4°C to pellet the cells. Following centrifugation, the supernatant was aspirated and the cells resuspended in dissection media + 10% FBS. Fibroblasts were removed by panning the cell suspension in a 100 mm cell culture dish containing dissection media + 10% FBS for 20 min [41]. After panning, the myocytes were aspirated off the panning dish and then pelleted by centrifugation at 500g for 10 minutes at 4°C [42]. Finally, the supernatant was removed and the myocytes were suspended in the serum-free culture medium and...
a cell count was conducted using the trypan blue method. Myocytes were then plated on DETA coverslips at a density of 600-700 cells/mm².

*Primary culture of Rattus norvegicus Sensory Neurons from DRG*

Dorsal root ganglia (DRGs) were removed from E14 rats and collected in dissection medium. Following dissection, the DRGs were incubated in 0.05% trypsin-EDTA for 15 minutes in a 37°C water bath. Following incubation, the trypsin-EDTA was removed and the cells suspended in dissection media + 10% FBS and the tissue gently triturated. The dissociated cell suspension was then centrifuged at 500g for 10 minutes at 4°C to pellet the cells. Following centrifugation, the supernatant was aspirated and the cells resuspended in the serum-free culture medium and a cell count was conducted using the trypan blue method. DRG cells were then plated on DETA coverslips at a density of 100 cells/mm².

*Preparation BA-G5 alpha Cardiac MHC*

The mouse B lymphocyte hybridoma cell line HB-276 was cultured according to ATCC guidelines [43]. Briefly, cells were grown in DMEM (Gibco 10313-021) + 10% FBS (Gibco 16000-077) or Hybridoma-SFM (Gibco 12045-076) in 75 mm² tissue culture flasks and placed in an incubator at 37°C and 5% CO₂ at a concentration of between 1×10⁵ and 1×10⁶ cells/ml. The medium was changed twice weekly. The BA-G5 antibody is constantly secreted by the cells in culture and was harvested by removing 12ml of conditioned culture medium, transferring it to a 15 ml tube, followed by
centrifugation at 4000g for 15 minutes at 4°C. The antibody containing supernatant was then removed and the concentration quantified using the microBCA method (23235, Pierce). The antibody concentration ranged from 9-12 µg/ml (data not shown) and was used 4 to 1 in blocking solution (see below).

**Co-culture assessment: Immunocytochemistry and Laser Scanning Confocal Microscopy**

The co-cultures were fixed in fresh 4% paraformaldehyde in PBS for 10 minutes and then rinsed twice with phosphate buffered saline (PBS). Next, cells were permeabilized with a solution of 0.05% triton-X 100 in PBS + 5% bovine serum albumin (BSA) for 5 minutes, rinsed once with PBS and then blocked with permeabilization solution + 5% donkey serum [44]. The cells were then incubated with primary antibodies diluted in blocking solution overnight at 4°C. The primary antibodies used include: mouse anti-alpha cardiac-like myosin heavy chain (HB-276, ATCC), anti-neurofilament heavy chain (AB5539, Millipore), rabbit anti-ACCN1 (ab65699, Abcam). The next day primary antibody solutions were aspirated and the cells rinsed three times with PBS. Then, donkey AlexaFluor® secondary antibodies (Molecular Probes) diluted 1:200 in blocking solution were added to the cells and incubated for 2 hours at room temperature in the dark. The secondary antibody solution was then aspirated and the coverslips rinsed three times in PBS. Finally, coverslips were mounted on glass slides using VectaShield mounting medium with DAPI (H-1200, Vector Labs) and fixed using clear nail polish.
Results

DETA Surface Modification

The aminosilane, trimethoxy-silylpropyl-diethylenetriamine (DETA), functions efficiently as a non-biological substrate due to its self-assembling monolayer properties and the multiple amines contained in the terminal group. This group confers hydrophilic properties to the surface, and that combined with the partial positive change on the amines at physiological pH make it an ideal surface for cellular attachment and survival. The system is similar to poly-D-lysine, but has been found to be more robust and consistent [45]. XPS measurements of the DETA coated coverslips indicated a complete monolayer formed during the self-assembly process. The normalized area values of N 1s (401 and 399 eV) to the Si 2p_{3/2} peaks were stable throughout the study at 1500 ± 200 and were similar to previously published results [38, 39, 45-47]. Static contact angle measurements of 44 ± 2° validated the hydrophilicity of the DETA surfaces. Stable XPS readings and contact angles across coverslips throughout the study indicate uniformity and reproducibility of the self-assembly of the DETA monolayer.

Box Line Pattern Surface Preparation and Cellular Pattern Conformity

The DETA foreground surrounded by 13F background provides a pattern supporting differential cell adhesion to the surface (Fig1A). Two control coverslips were used in order to test the quality of the DETA/13F patterns: (1) one DETA coverslip was ablated without the photomask and rederivatized with 13F, and (2) a second DETA
The XPS measurements of the control coverslips show that 13F is incorporated in the LASER exposed DETA control coverslip (Fig 1B), but it is not incorporated (or only incorporated in traces amounts) in the unexposed DETA control coverslip (Fig 1C). Further, static water contact angle measurements of 92±2 validated the hydrophobicity of the LASER exposed DETA control coverslip after 13F rederivatization. However, the non-ablated DETA monolayer is not affected by the reaction with 13F, as is also revealed by the contact angle values of 45±3 of unexposed DETA control coverslip, values which are close to the ones for pure DETA.

The pattern uniformity was verified by palladium catalyzed copper reduction metallization (Fig. 1D). These results were stable throughout the study indicating the reproducibility of the box line patterns. The cellular conformity to the patterns was also analyzed throughout the study. As shown in table one, pattern conformity decreased throughout the time in culture. During the early days, pattern conformity was at its highest, with 49.03±1.57 out of 56 boxes (88%) showing cells only on the lines (Fig. 2). As the cultures aged, conformity to the pattern decreased with only 4.11±0.25 boxes out of 56 (8%) showing cells conforming to the pattern (Fig. 2).

**Serum-free Medium and Defined System Formulation**

The basic serum-free medium composition developed in our laboratory for the co-culture of embryonic motoneurons and myocytes was used as a starting point for the
development of a medium that enhanced synaptic connectivity between neurons and myotubes in vitro [48-51]. The addition of 10 new growth factors known to play a role in neuromuscular junction formation and synaptic maintenance led to increased recovery and development of neurons and myocytes as well as increased development of synaptic structures in the system (Table 2) (Figure 2-4).

**Immunocytochemical Evaluation of Ia Sensory Neuron Endings on Intrafusal Fibers**

The synapses formed between Ia sensory neurons and intrafusal fibers are morphologically distinct (unique) structures [13]. Annulosprial wrappings (ASWs) and flower-spray endings (FSEs) terminate on intrafusal fibers in vivo and form a mechanosensitive complex capable of relaying muscle stretch information to spinal cord motoneurons. In order to determine if the co-culture of sensory neurons and skeletal muscle would result in the formation of ASWs and/or FSEs, cultures were evaluated using immunocytochemistry (Figure 3). Additionally, in order to evaluate the rapidity of Ia sensory neuron innervation in this system, the cultures were evaluated at three different time points (Table 3). As shown in figure 3, ASWs are evident on nuclear chain fibers as shown by sensory neuron axon wrapping around the intrafusal fiber (Fig.3A-D). Flower-spray endings were also observed in the co-culture as shown by axonal “flower-spray” termination upon contact with the nuclear chain fiber (Fig.3E-H). Annulospiral wrappings were also observed on nuclear bag fibers as shown by the Z-stack slices of an axon wrapping around an intrafusal fiber and terminating near the equatorial region (Fig.3I-L). As shown in table three, a small number of ASWs and FSEs were observed at six days in culture, seven and five percent respectively. As the
cultured aged, the number of endings increased, reaching their maximal values at day 23 with 18% ASWs and 15% FSEs.

*Immunocytochemical Evaluation of the Mechanosensory Complex*

In order for the action potential generation to occur in the mechanosensitive terminals, stretch sensitive ion channels located in Ia sensory neurons must open, thereby depolarizing the axolemma. Due to the localization of BNaC1 to the nerve terminals of mechanosensitive skin structures as well as the channels sensitivity to amiloride and the attenuation of stretch activated spindle firing by amiloride, the ion channel’s presence at ASWs and FSEs was investigated in vitro [52]. Using immunocytochemistry, clusters of BNaC1 ion channels were shown to localize at the terminals of ASWs and FSEs (Fig.4A-J). We also evaluated the localization of PICK1 with BNaC1 at the proposed mechanosensory ASWs of the type Ia sensory neurons. As shown in Figure 4, the annulospiral wrappings associated with intrafusal fibers contain BNaC1 and PICK1 colocalized in the axonal terminals (Fig.4A-E). Additionally, BNaC1 and PICK1 colocalized with flower-spray endings terminating on intrafusal myotubes (Fig.4F-J).
Discussion

The formulation of a set of conditions resulting in the development of annulosprial wrappings and flower-spray endings on intrafusal muscle fibers in vitro is a critical step in tissue engineering the stretch reflex arc. In this work, we showed that temporal application of growth factors in NBActiv4 medium resulted in the development of annulosprial wrappings and flower-spray endings using morphological and immunocytochemical evaluation criteria. Furthermore, we were able to identify BNaC1 and PICK1 localized to the terminals of type Ia sensory neuron ASWs and FSEs, both components of the proposed mammalian mechanosensory complex.

The utility of each growth factor present in the medium formulation was derived from previous experiments done by our group and others and was developed for the purpose of enhancing neuromuscular junction (NMJ) development. Due to the similarities between traditional NMJs and the terminations made by type Ia sensory neurons on intrafusal muscle fibers (ASWs and FSEs), we hypothesized the same growth factor cocktail would support their development [53]. Additionally, similarities in myoblast origin, myosin heavy chain isoforms expression and encapsulation of the fibers further supported the idea that similar developmental cues would facilitate the formation of ASWs and FSEs [54-58]. Essentially, both ASWs and FSEs are alternative types of neuromuscular junctions; in this case, mechanosensory junctions formed between sensory neurons and intrafusal muscle fibers.

The use of photolithography and surface chemistry modification to pattern cytophilic and cytophobic regions on glass coverslips resulted in distinct cell adhesion areas and helped direct cell-cell interactions. Pattern conformity decreased over time in
culture, primarily due to myotube overgrowth from the DETA regions of the pattern area onto the 13F regions. Regardless, the initial patterning enhanced localized interaction between sensory neurons and intrafusal muscle fibers, resulting in easily quantified ASWs and FSEs. One advantage of surface chemistry modification is the potential to use a variety of silanes for cell repulsion at plating. One silane in particular, polyethylene glycol (PEG), has been shown to minimize protein adhesion while maintaining biocompatibility and work done in our lab suggests it might enhance cellular pattern conformity [59, 60].

The observation that BNaC1 and PICK1 colocalize at the sensory neuron terminals associated with intrafusal myotubes demonstrates the utility of this system to investigate basic developmental questions regarding the formation of the mammalian mechanosensory complex. Consequently, this in vitro system will be a useful tool for identifying additional components of the mechanosensory complex. This is due to the ease of access to both individual intrafusal fibers and their associated axonal endings. For example, while the presence of proteins linking the stretch activity of the intrafusal fibers to the mechanically sensitive ion channels in the sensory neurons is postulated, these proteins have yet to be identified. It also provides for an efficient way to analyze electrical impulses generated in the sensory neurons caused by stretch deformation of the intrafusal fiber. Also, by integrating this system with a cantilever micro-electrical mechanical systems (MEMS) device, it will be possible to determine how intrafusal fiber stretching due to measurable cantilever deflection is converted to electrical impulses in the type Ia sensory neurons.
In this report we document the development of a tissue-engineered system resulting in physiologically relevant interactions between Ia sensory neurons and intrafusal muscle fibers. Using photolithographic patterning, the formation of annulospiral wrapping and flower-spray endings were documented and quantified. Furthermore, two components of the proposed mechanosensory system, the stretch sensitive ion channel BNaC1 and the membrane support protein PICK1, were also shown to be localized at the Ia sensory neuron terminals interacting with the intrafusal fibers. This engineered system provides a platform to investigate the mechanosensory activity of muscle spindles in a controlled and reproducible environment. This system is an integral component of an in vitro model of the stretch reflex arc, which has applications in functional prosthetic device design and the study of spasticity inducing diseases such as Parkinson's, muscular dystrophy and myasthenia gravis.
<table>
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<tbody>
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</tr>
<tr>
<td>Day 8</td>
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<td>69</td>
</tr>
<tr>
<td>Day 16</td>
<td>56</td>
<td>4.11±0.25</td>
<td>8</td>
</tr>
</tbody>
</table>

Quantified by counting myotube and Ia sensory neuron conformity to box line pattern using phase contrast microscopy. Data is the average±SEM of at least five experiments performed on six coverslips / experiment at each time point.
Table 8-2. Serum-free medium for enhanced synaptic connectivity in NB4A (500mL)

<table>
<thead>
<tr>
<th>Component</th>
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<th>Company</th>
<th>Catalog Number</th>
</tr>
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<td>AnaSpec</td>
<td>61170</td>
</tr>
<tr>
<td>h BDNF¹</td>
<td>20ng/mL</td>
<td>Cell Sciences</td>
<td>CRB 600B</td>
</tr>
<tr>
<td>h GDNF¹</td>
<td>20ng/mL</td>
<td>Cell Sciences</td>
<td>CRG 400B</td>
</tr>
<tr>
<td>r CNTF¹</td>
<td>50ng/mL</td>
<td>Cell Sciences</td>
<td>CRC 401B</td>
</tr>
<tr>
<td>h CT-1¹</td>
<td>20ng/mL</td>
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Quantified by counting the number of respective ending morphologies on box line patterned coverslips using immunocytochemistry and laser scanning confocal microscopy. Data is the mean±SEM of at least three experiments performed on four coverslips / experiment at each time point.
Figure 8.1. Photomask image and chemical analysis of box line patterns. (A) CleWin drawing of the box line pattern, (B) XPS survey of an ablated DETA monolayer exposed to 13F solution, (C) XPS survey of a non-ablated DETA monolayer exposed to 13F solution, (D) image of a box line pattern visualized using palladium catalyzed copper reduction metallization (dark bars and lines indicate DETA regions). Scale bar = 90µm
Figure 8.2. Intrafusal myotubes and DRG sensory neurons in co-culture on box line patterns. (A) Day 1 image illustrating myocytes conforming to DETA patterns, (B) Day 7 image illustrating developing myotubes conforming to the box line pattern, (C-D) Day 16 image showing developed intrafusal myotubes (red arrows) on box line patterned DETA coverslips. Scale bars = 60µm.
Figure 8.3. Ia sensory neuron annulospiral and flower-spray endings on intrafusal myotubes. (A) phase contrast image of nuclear chain fiber, (B) neurofilament-H (NF-H) illustrating classic annulospiral wrapping morphology (C) M-cadherin (green) and alpha cardiac-like MHC (blue) staining verifying nuclear chain fiber phenotype, (D) composite image, (E) phase contrast image of a nuclear bag fiber, (F) NF-H displaying classic flower-spray morphology (G) alpha cardiac-like MHC staining verifying nuclear bag fiber phenotype, (H) composite image, (I) phase contrast image of nuclear bag fiber, (J-L) rising slice confocal stack showing a Ia sensory neuron axon (NF-H – green) wrapping around a nuclear bag fiber (alpha cardiac-like MHC – red) to form an annulospiral wrapping. Scale bars = 50µm.
Figure 8.4. BNaC1 and PICK1 localize to la sensory neuron annulospiral and flower-spray endings. (A) phase contrast image, (B) NF-H illustrating annulospiral wrapping, (C) PICK1 localized at the superior surface wrapping, (D) BNaC1 localized at the superior surface wrappings, (E) color composite image, (F-I) localization at a flower-spray ending, (F) phase contrast image, (G) NF-H illustrating flower-spray ending, (H) PICK1 localized at the flower-spray terminal, (I) BNaC1 localized at the flower-spray terminal, (J) color composite image. Scale bars = 50µm.
References


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Introduction

The monosynaptic stretch reflex arc functions as a negative feedback loop to maintain appropriate muscle fiber length [1]. From an engineering perspective, the arc can be thought of as two circuits working in concert to maintain muscle length specified by descending motor pathways. The sensory circuit is composed of muscle spindles acting as stretch sensors and Ia sensory neurons relaying that stretch information to spinal cord motoneurons via action potentials generated by mechanically sensitive ion channels. The motor circuit, composed of motoneurons and contractile extrafusal fibers, responds to signals sent from the type Ia sensory neurons thereby maintaining appropriate muscle length [1].

In muscle spindles, a major type proprioceptor, the sensory receptor is composed of an encapsulated group of specialized muscle fibers called intrafusal fibers [2]. Within the spindle, the intrafusal fibers are of two main types: 1) nuclear bag fibers, so named because their nuclei cluster in a bulging equatorial region, and 2) nuclear chain fibers, so named because their nuclei align next to each other. Several different neuronal types innervate these fibers. The sensory innervation is accomplished by type Ia and type II neurons located in dorsal root ganglia [3]. These sensory neurons form specialized synapses on intrafusal muscle fibers called annulospiral wrappings (ASWs) (type Ia) and flower-spray endings (FSEs) (type II) [4]. The synapses formed are mechanosensitive in nature, relying on a mechanically gated ion channel for initiation of
signaling in the type Ia sensory neurons [5-7]. The proposed mechanically sensitive ion channel, BNaC1 has been localized at specialized mechanosensory terminals like Meissner corpuscles and Merkel discs [5]. These proteins, brain sodium channels 1 and 2 (BNaC1 & BNaC2), form an additional branch of the degenerin / epithelial sodium channel (DEG/ENaC) superfamily and have been shown to localize at mechanosensory terminals of dorsal root ganglion neurons [6-8]. Muscle spindles also receive motor innervation from γ-motoneurons and β-motoneurons. This motor innervation occurs at the polar regions of the intrafusal fibers and functions to maintain the sensitivity of the spindle during extrafusal fiber contraction [3, 9].

The development of a tissue engineered in vitro bio-MEMS system capable of supporting the growth and differentiation of both intrafusal fibers and sensory neurons would provide a critical transition in the study of vertebrate mechanosensory complex. Furthermore, systems using serum-free defined medium formulations and nonbiological, uniform growth substrates provide an environment ideal for manipulation of specific factors in order to generate a specific cell phenotype. These systems also provide the added benefit of being readily modified using photolithography to generate patterned adhesive sites for cells. These systems provide the added ease of evaluating the manipulated factor’s effect without concern for confounding variability associated with systems based on biological substrates and serum containing medium.

In our previous work, we showed the ability to grow embryonic skeletal muscle on trimethoxy-silylpropyl-diethylenetriamine (DETA) surfaces and direct myotube differentiation towards intrafusal fiber formation using serum-free conditions [10]. Also, we have shown that the same culture conditions will support the growth and phenotypic
development of DRG neurons [11]. In a co-culture system on patterned DETA surfaces, we showed that sensory neurons form annulospiral wrapping and flower-spray endings on intrafusal myotubes. Furthermore, we identified the presence of two components of the purposed mammalian mechanosensory complex, BNaC1 and PICK1, at the sites of innervation (). While these discoveries shed light on the structural elements composing the sensory circuit of the reflex arc, in order to fully investigate the physiology of the circuit the components need to be integrated with a micro-electro-mechanical systems (MEMS) device.

MEMS devices are mechanical technologies made up of components ranging in size from 1 and 1000 micrometers. These devices are an integration of mechanical and electronic elements on a substrate built using microfabrication technologies. Cantilevers, a type of MEMS device fabricated from silicon wafers, are diving board-like structures organized in an array, which can be used for biosensing or bending detection [12]. For bending detection, the deflection of an optical beam aimed at the free end of the cantilever is monitored by a detector [13]. In this case, the bending caused by myotube contraction, monitored by beam deflection, can be used to calculate myotube contractile force [14].

Engineering an in vitro cantilever system to study the mechanical interactions between intrafusal muscle fibers and Ia sensory neurons and the subsequent generation of electrical activity is an integral step in building a functional stretch reflex arc in vitro. This work documents the development of a functional cantilever based system supporting the growth and differentiation of both intrafusal muscle fibers and sensory neurons. This system can be directly used to study nerve-muscle
communication and describe how mechanical stretch is converted to electrical impulses in sensory neurons. This information could directly aid in the enhanced functioning of prosthetic devices and provide insight into the pathologies of neuromuscular diseases such as infantile spinal muscular atrophy type I (Werdnig-Hoffman disease) and spasticity inducing diseases like Parkinson’s, muscular dystrophy and myasthenia gravis [15-20].
Materials and Methods

Cantilever Fabrication

Bio-MEMS cantilevers were fabricated as previously described [14]. Briefly, a four-inch double-sided polished SOI wafer containing a 5μm thick top layer of crystalline silicon bonded onto a 500μm thick silicon dioxide layer was photoresist spun and developed in a Suss ACS200 wafer coater/developer. First, the 5μm layer was primed with 100Å layer of hexamethyldisilazaine (HMDS). Then the was placed silicon side up on the resist spinner and the AZ5214e photoresist was applied and spun at 1,900 rpm to a final thickness of 2.1μm. Next, the resist was baked at 110˚C. Then the wafer was exposed on the Suss MA6 aligner to the photomask containing the cantilever pattern and the resist developed in a 1:1 mixture of AZ320 and dH2O. Next, deep reactive ion etching (DRIE) was performed on the wafer in a PlasmaTherm 770 DRIE at a rate of 4μm min-1. The 5μm device layer was etched through to the silicon dioxide layer and the photoresist was removed using a Metroline M4L plasma resist etcher. Next, the wafer was inverted and the silicon dioxide layer was primed with a 100Å layer of HMDS then coated with AZ9245 photoresist to a final thickness of 10μm (1,000rpm) and soft baked at 100˚C for 2min. The coated wafer was placed in a Suss MA6 aligner and exposed with the mask containing the backside pattern. After exposure, the wafer was immersed in AX400K developer to develop the patterned resist followed by a hard bake at 120˚C for 30min. Etch until approximately 50μm etch depth is left. Mount the wafer on a dummy wafer using Nitto Model 3195V thermal release tape and complete the DRIE. Demount the wafer by heating to 170˚C on a hotplate. Remove the photoresist
by plasma etching in a Metroline M4L plasma resist etcher. Perform a final etch in 49% hydrofluoric acid for 10 min to remove the buried oxide layer.

**PEG Surface Modification**

Glass coverslips (VWR 48366067, 22×22 mm2 No. 1) were first cleaned using 1:1 HCl-methanol followed by a concentrated H2SO4 soak for 2 hours. The cantilevers were then coated with a PEG-terminated silane by a modified protocol from Papra et al. Dry toluene was prepared by distillation over metallic sodium to remove any water or other contaminants. The alkylsilane 2-[Methoxypoly (ethyleneoxy)propyl] trimethoxysilane (Gelest, Tullytown, PA), was added to the toluene to a final concentration of 0.1% by volume in an MBraun glove box (MBraun, Stratham, NH). Concentrated HCl was added to a final volume of 0.08% (0.8 ml HCl/L) to the PEG-toluene solution and the solution briefly stirred. The cantilevers were then incubated in the PEG-toluene solution for 1 hour at room temperature. After 1 hour the samples were removed and rinsed in serial washes of toluene (1x), ethanol (2x), and diH2O (1x). The washed samples were blown dry under a stream of ultrapure nitrogen and were used immediately or stored in a desiccator until needed.

**Deep-UV Photolithography of PEG-silane Monolayers**

PEG-silane modified cantilevers were patterned using deep ultraviolet (DUV) photolithography. The samples were patterned in a photolithography system of our own design, which was based on a mask aligner, 193 nm ArF excimer laser (Lambda
Physik, Santa Clara, CA) with an in-line beam homogenizer. Samples were placed on the stage of the mask aligner under a 5x5 inch chrome plated photomask, which contained the pattern, to be ablated. The masks were written in dark-field polarity such that the areas corresponding to the ablated pattern were transparent and the remaining areas were opaque. When necessary the substrate was precision aligned using the aligner stage to ensure micrometer precision placement of the pattern. The substrate was then brought into contact with the mask to ensure a hard contact. A hard contact was used to minimize the gap between the substrate and mask to ensure a high contrast pattern with minimal edge effects due to refraction of the laser light. The substrates were then exposed to 193 nm ultraviolet laser light for 45 seconds with a pulse intensity of 200 mJ/pulse and a frequency of 10Hz. After ablation the samples were removed from the aligner stage and stored for subsequent processing.

*Back-fill of Patterned PEG-silane Monolayers with DETA Silane*

After ablation the patterned PEG-silane substrates were backfilled with the alkylsilane (3-Trimethoxysilyl propyl) diethylenetriamine (DETA). Fresh distilled dry toluene was prepared as discussed previously. DETA was added to the toluene to a final concentration of 0.1% (vol:vol) inside the glove box. The DETA-toluene solution was removed from the glove box and transferred to a beaker and the samples were immersed in the solution. To drive the reaction forward the solution was gently heated to no more than 65°C for 30 minutes. After reaction with DETA the samples were allowed to cool to room temperature, washed 3 time with dry toluene and heated to 65°C for 30 more minutes. Surfaces were characterized by static water contact angle
measurements using a Rame-Hart Model 250 goniometer, and by X-ray photoelectron spectroscopy (XPS) using an Escalab 200i spectrometer (VG Scientific) by monitoring the N 1s peak [21-23]. The values are reported as the mean ± SEM.

**Animals**

Dated pregnant Sprague-Dawley rats were housed in an animal facility at the University of Central Florida. All research was approved by the Institutional Animal Care and Use Committee at the University of Central Florida and conformed to NIH guidelines. Pregnant rats were anesthetized and sacrificed at embryonic day 14 or 18 (E14 / E18). The embryos were removed by caesarean section and fetuses dissected under a stereomicroscope (Carl Zeiss, Stemi 2000).

**Primary Culture of Rattus norvegicus Myocytes**

Skeletal muscle was removed from the hind limb of E18 rat fetuses, collected in a 15ml tube in cold Hibernate E + GlutaMAX™ + antibiotic-antimycotic + B27 (dissection medium) and incubated in 0.05% trypsin-EDTA for 45 minutes in a 37°C water bath. Following incubation, the trypsin-EDTA was removed and the cells suspended in dissection media + 10% FBS and the tissue gently triturated. The dissociated cell suspension was then centrifuged at 500g for 10 minutes at 4°C to pellet the cells. Following centrifugation, the supernatant was aspirated and the cells resuspended in dissection media + 10% FBS. Fibroblasts were removed by panning the cell suspension in a 100 mm cell culture dish containing dissection media + 10% FBS for 20
min [24]. After panning, the myocytes were aspirated off the panning dish and then pelleted by centrifugation at 500g for 10 minutes at 4°C [25]. Finally, the supernatant was removed and the myocytes were suspended in the serum-free culture medium (Table 1) and a cell count was conducted using the trypan blue method. Myocytes were then plated on cantilevers at a density of 200 cells/mm².

**Primary Culture of Rattus norvegicus Sensory Neurons from DRGs**

Dorsal root ganglia (DRGs) were removed from E14 rats and collected in dissection medium. Following dissection, the DRGs were incubated in 0.05% trypsin-EDTA for 15 minutes in a 37°C water bath. Following incubation, the trypsin-EDTA was removed and the cells suspended in dissection media + 10% FBS and the tissue gently triturated. The dissociated cell suspension was then centrifuged at 500g for 10 minutes at 4°C to pellet the cells. Following centrifugation, the supernatant was aspirated and the cells resuspended in the serum-free culture medium (Table 1) and a cell count was conducted using the trypan blue method. DRG cells were then plated on cantilevers at a density of 200 cells/mm².

**Preparation BA-G5 alpha Cardiac MHC**

The mouse B lymphocyte hybridoma cell line HB-276 was cultured according to ATCC guidelines [26]. Briefly, cells were grown in DMEM (Gibco 10313-021) + 10% FBS (Gibco 16000-077) or Hybridoma-SFM (Gibco 12045-076) in 75 mm² tissue culture flasks and placed in an incubator at 37°C and 5% CO2 at a concentration of
between 1x10^5 and 1x10^6 cells/ml. The medium was changed twice weekly. The BA-G5 antibody is constantly secreted by the cells in culture and was harvested by removing 12ml of conditioned culture medium, transferring it to a 15 ml tube, followed by centrifugation at 4000g for 15 minutes at 4°C. The antibody containing supernatant was then removed and the concentration quantified using the microBCA method (23235, Pierce). The antibody concentration ranged from 9-12 µg/ml (data not shown) and was used 4 to 1 in blocking solution (see below).

Co-culture assessment: Immunocytochemistry and Laser Scanning Confocal Microscopy

The co-cultures were fixed in fresh 4% paraformaldehyde in PBS for 10 minutes and then rinsed twice with phosphate buffered saline (PBS). Next, cells were permeabilized with a solution of 0.05% triton-X 100 in PBS + 5% bovine serum albumin (BSA) for 5 minutes, rinsed once with PBS and then blocked with permeabilization solution + 5% donkey serum [27]. The cells were then incubated with primary antibodies diluted in blocking solution overnight at 4°C. The primary antibodies used include: mouse anti-alpha cardiac-like myosin heavy chain (HB-276, ATCC) and anti-neurofilament heavy chain (AB5539, Millipore). The next day primary antibody solutions were aspirated and the cells rinsed three times with PBS. Then, donkey AlexaFluor® secondary antibodies (Molecular Probes) diluted 1:200 in blocking solution were added to the cells and incubated for 2 hours at room temperature in the dark. The secondary antibody solution was then aspirated and the coverslips rinsed three times in PBS.
Finally, coverslips were mounted on glass slides using VectaShield mounting medium with DAPI (H-1200, Vector Labs) and fixed using clear nail polish.

Calcium Imaging of type Ia Sensory Neuron Currents in Co-culture

Co-cultures were grown for 10-14 days in serum-free medium (Table 1). Due to the fact that Ca2+ flux has been shown to be important for muscle spindle afferent activity, we investigated Ca2+ dynamics in this model system [28]. The calcium sensitive dye, Fluo-4 AM (F14201, Invitrogen), was prepared as described by the manufacturer. Briefly, a 5mM dye stock was prepared in DMSO, and then mixed 1:1 with DMSO+20% pluronic F-127 to prepare a 2.5mM working solution. Next, the working solution was added to the co-cultures for a final concentration of 10μM and incubated for 30 minutes. The co-cultures were then washed twice with fresh medium and placed in the field stimulation chamber in fresh medium. The field stimulator was run using the following parameters to specifically stimulate myotube contraction: 1 Hz, 40 millisecond pulse width and 2 volts peak-to-peak. In DRG only control experiments, field stimulation did not result in neuronal electrical activity. During field stimulation of co-cultures, the confocal microscope was set to record video files of Fluo-4 AM conformational change due to spatio-temporal Ca2+ binding.

Amiloride Blockage of type Ia Sensory Neuron Currents in Co-culture

Amiloride hydrochloride is a known blocker of mechanotransduction and has been shown to block the BNaC1, the proposed mechanosensitive ion channel [6, 7]. Co-
cultures were grown and Ca2+ flux was monitored as previously described. After imaging the Ca2+ flux generated in type Ia sensory neurons in co-culture, amiloride hydrochloride (A7410, Sigma-Aldrich) was added to the bath solution at a final concentration of 500μM with the field stimulation of intrafusal muscle fibers still in progress.
Results

Serum-free Medium and Defined System Formulation

The basic serum-free medium composition developed in our laboratory for the co-culture of embryonic motoneurons and myocytes was used as a starting point for the development of a medium that enhanced synaptic connectivity between neurons and myotubes in vitro [29-32]. The addition of 10 new growth factors known to play a role in neuromuscular junction formation and synaptic maintenance led to increased recovery and development of neurons and myocytes as well as increased development of sensory synaptic structures in the system (Table 1) (Fig.3).

Cantilevers and PEG-DETA Pattern Analysis

The quality of cantilever fabrication was verified using electron microscopy (Fig.1). After verification of cantilever fabrication, the devices were exposed to a solution of PEG for SAM formation. The aminosilane, trimethoxy-silylpropyl-diethylenetriamine (DETA), functions efficiently as a non-biological substrate due to its self-assembling monolayer properties and the multiple amines contained in the terminal group. This group confers hydrophilic properties to the surface, and that combined with the partial positive change on the amines at physiological pH make it an ideal surface for cellular attachment and survival. The system is similar to poly-D-lysine, but has been found to be more robust and consistent [33]. As shown in figure 2, PEG forms a SAM on cantilever devices (Fig.2A). The PEG was then laser ablated and DETA was back-filled in the ablated region forming a cell adhesive region on the cantilevers.
(Fig.2C). No DETA incorporation into PEG coated regions of the cantilever could be detected (Fig.2B).

*Immunocytochemical Evaluation of type Ia Sensory Neuron Endings on Intrafusal Fibers*

The synapses formed between Ia sensory neurons and intrafusal fibers are morphologically unique structures [3]. Annulospiral wrappings (ASWs) and flower-spray endings (FSEs) terminate on intrafusal fibers in vivo and form a mechanosensitive complex capable of relaying muscle stretch information to spinal cord motoneurons. In order to determine if the co-culture of sensory neurons and skeletal muscle would result in the formation of ASWs and/or FSEs, cantilevers were evaluated using immunocytochemistry (Fig.3). As shown in figure 3, ASWs are evident on nuclear bag fibers as shown by sensory neuron axon wrapping around the intrafusal fiber (Fig.3A-D). The intrafusal myotubes were verified using the $\alpha$ cardiac-like myosin heavy chain antibody and axons were visualized using neurofilament-H antibody. Flower-spray endings were also observed in the co-culture as shown by axonal “flower-spray” termination upon contact with the nuclear chain fiber (Fig.3E-H). Annulospiral wrappings were also observed on nuclear bag fibers as shown by the Z-stack slices of an axon wrapping around an intrafusal fiber and terminating near the equatorial region (Fig.3I-L).

*Calcium Imaging of type Ia Sensory Neuron Currents in Co-culture on Cantilevers*
Action potentials generated in the sensory neuron axon results in spatio-temporal calcium flux. Dynamic calcium flux measurement is an indicator of electrical activity in neurons. Therefore, in order to evaluate the functionality of the neurons in co-culture, a Ca\(^{2+}\) flux imaging technique was performed using Fluo-4 AM dye. Fluo-4 AM reversibly binds to intracellular Ca\(^{2+}\) resulting in a conformational change that with an excitation emission spectra of 488nm / 516nm. We hypothesized that a change in length of the intrafusal fibers due to contraction would result in electrical activity by the innervating type Ia sensory neuron axon that can be visualized as spatio-temporal Ca\(^{2+}\) flux. Using the Fluo-4 AM dye, we visualized electrical activity in real time, and in this case, the type Ia sensory neuron currents generated as a result of intrafusal fiber innervation. Using field stimulation, intrafusal muscle fibers contracted and we visualized the subsequent Ca\(^{2+}\) flux moving along a type Ia sensory neuron axon resulting from innervation (Fig.4) (Vid.1). Conversely, in control experiments conducted with only DRG neurons no Ca\(^{2+}\) flux could be observed, suggesting that field stimulation in the absence of myotubes does not result in any form of electrical activity on the type Ia sensory neurons.

Amiloride Attenuation of Ca\(^{2+}\) flux Imaging in type Ia Sensory Neurons

After establishing that field stimulation resulted in intrafusal myotube activity and Ca\(^{2+}\) currents in type Ia sensory neurons, we hypothesized the activity was generated by mechanically gated ion channels present where sensory neurons innervate intrafusal myotubes. Amiloride hydrochloride is a potent inhibitor of members of the degenerin / epithelial sodium channel (DEG/ENaC) superfamily. The mechanosensitive ion channel
BNaC1, a member of the DEG/ENaC has been shown to be sensitive to amiloride. After amiloride injection into the system, Ca^{2+} electrical activity in the sensory neuron ceased even while field stimulation of the intrafusal fibers continued, suggesting the functional connection between the two elements had been blocked (Vid.2).
**Discussion**

This report documents the development of a bio-MEMS device facilitating functional analysis of the sensory circuit of the stretch reflex arc in vitro. Specifically, a co-culture of DRG cells and intrafusal myotubes were grown on cantilevers coated with patterned nonbiological substrates PEG and DETA and cultured in a serum-free medium. Structurally, we identified sensory neuron annulospiral wrappings and flower-spray endings on intrafusal myotubes on the cantilevers. Physiologically, we used Ca$^{2+}$ imaging to visualize electrical currents in the sensory neurons after intrafusal myotube deformation by field stimulation. Subsequently, we blocked stretch sensitive ion channels with amiloride and observed an attenuation of the Ca$^{2+}$ flux suggesting a mechanical connection between the sensory neurons and the intrafusal myotubes.

The utility of each growth factor present in the medium formulation was derived from previous experiments done by our group and others and was developed for the purpose of enhancing neuromuscular junction (NMJ) development. Due to the similarities between traditional NMJs and the terminations made by type Ia sensory neurons on intrafusal muscle fibers (ASWs and FSEs), we hypothesized the same growth factor cocktail would support their development [34]. Additionally, similarities in myoblast origin, myosin heavy chain isoforms expression and encapsulation of the fibers further supported the idea that similar developmental cues would facilitate the formation of ASWs and FSEs [35-39]. Essentially, both ASWs and FSEs are alternative types of neuromuscular junctions; in this case, mechanosensory junctions formed between sensory neurons and intrafusal muscle fibers.
The use of photolithography and surface chemistry modification to pattern cytophilic and cytophobic regions on the cantilevers resulted in distinct cell adhesion areas and helped direct cell-cell interactions. The aminosilane, trimethoxy-silylpropyldiethylenetriamine (DETA), functions efficiently as a non-biological substrate due to its self-assembling monolayer properties and the multiple amines contained in the terminal group. This group confers hydrophilic properties to the surface, and that combined with the partial positive change on the amines at physiological pH make it an ideal surface for cellular attachment and survival. The use of polyethylene glycol (PEG) as the cell repulsive substrate worked particularly well as it has been shown to resist protein adsorption over time in culture [40, 41].

Building on our DRG+ESM model grown on patterned coverslips, the integration of the sensory circuit of the stretch reflex arc on bio-MEMS cantilevers represents a significant step towards understanding the physiology of this most basic of neuromuscular circuits. The Ca2+ flux imaging of sensory neuron electrical activity after field stimulation of intrafusal fibers and flux attenuation after amiloride treatment suggests a functional innervation between the neuron and myotube. When combined with the immunocytochemistry data, these results suggest the development of the sensory circuit of the stretch reflex arc on a MEMS cantilever device. This is significant because it provides for an efficient way to analyze electrical impulses generated in the sensory neurons caused by stretch deformation of the intrafusal fiber. Also, by integrating this system with a cantilever micro-electrical mechanical systems (MEMS) device, it is possible to quantify how intrafusal fiber stretch due to measurable cantilever deflection is converted to electrical impulses in the type Ia sensory neurons.
In this report we document the development of a tissue-engineered bio-MEMS resulting in physiologically relevant interactions between Ia sensory neurons and intrafusal muscle fibers. Using field stimulation of the intrafusal fibers, calcium flux resulting from sensory neuron action potential generation was observed. This engineered system provides a platform to investigate the mechanosensory activity of muscle spindles in a controlled and reproducible environment. This device can be used for high-throughput drug screening applications. The cantilever system is an integral component of an in vitro model of the stretch reflex arc, which has applications in functional prosthetic device design and the study of spasticity inducing diseases such as Parkinson’s, muscular dystrophy and myasthenia gravis.
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<sup>1</sup> component of original medium 1

<sup>2</sup> component of original medium 2
Figure 9.1. Electron micrograph images of the bio-MEMS cantilevers. (A) overhead view, (B) angled view showing the depth of the undercut area, (C) overhead view. Scale bars = 50μm.
Figure 9.2. XPS analysis of PEG-DETA self-assembling monolayer patterns on cantilevers. (A) XPS survey spectrum of PEG-coated cantilever (insert shows high resolution C1s spectrum), (B) XPS survey spectrum of DETA on PEG-coated cantilever (insert shows high resolution N1s spectrum), (C) XPS survey spectrum of DETA on ablated PEG-coated cantilever.
Figure 9.3. Ia sensory neuron annulospiral and flower-spray endings on intrafusal myotubes on cantilevers. (A) α cardiac-like MHC image of nuclear bag fiber, (B) bottom slice composite of a flower-spray ending, (C) middle slice composite of a flower-spray ending (D) top slice composite of a flower-spray ending, (E) α cardiac-like MHC image of nuclear bag fiber, (F) bottom slice composite of an annulospiral ending (G) middle slice composite of an annulospiral ending, (H) top slice composite of an annulospiral ending. Scale bars = 50µm.
Figure 9.4. Ca2+ flux in a type Ia sensory neuron generated after field stimulation of intrafusal myotubes on cantilevers. (A-D) time course images in sequence from beginning to end the Ca2+ wave traveling through a type Ia sensory neuron. (red arrowheads indicate location of the soma) (white arrowheads indicate location of the axon) Scale bars = 50µm.
Vid.1 Ca2+ flux in a type Ia sensory neuron generated after field stimulation of intrafusal myotubes on cantilevers.

Vid.2 Ca2+ flux attenuation after amiloride blockage of mechanically sensitive ion channels in DRG+ESM co-cultures on cantilevers.
References


27. Daniels MP. Localization of actin, beta-spectrin, 43 x 10(3) Mr and 58 x 10(3) Mr proteins to receptor-enriched domains of newly formed acetylcholine receptor aggregates in isolated myotube membranes. Journal Of Cell Science 1990;97 (Pt 4):615-626.


CHAPTER 10: GENERAL DISCUSSION

Tissue engineering and regenerative medicine represent two branches in the emerging field of translational research. Translational research can be described as the rapid conversion of new knowledge from basic science into new drugs, devices and treatment options for clinical patients [1]. This process requires the integration of basic scientific discoveries and clinical observations about the effectiveness of the various treatment options. One reason tissue engineering and regenerative medical approaches fit so well in this arena is they are by nature integrative, frequently combining scientists from varied disciplines to answer a research question. Specifically, these in vitro systems, designed and implemented using multiple scientific resources, can be translated into growth factor therapeutics for patients.

One of the key challenges involved in translational research is applying the knowledge gained from basic research, often relying on poorly defined in vitro or animal models, to human patients in the clinic. This lack of clearly defined model systems to study clinical phenomenon arises from the following discrepancies and limitations:

1. Lack of uniform cell isolation and culture methodologies
2. Reliance on medium formulations supplemented with animal serum
3. Limited understanding of cellular growth factor requirements
4. Limited understanding of the importance of cell-cell interactions in vivo
5. Limited understanding of the importance of cell-substrate (ECM) interactions

The approach taken in this dissertation research was to develop in vitro systems, which removed or minimized these translational limitations while simultaneously answering
basic biological questions about the systems under development. This work sought to integrate cell biology techniques and growth factor biology with substrate chemistry and engineering and microfabrication technology. Specifically, easily reproducible cell culture methodologies were adopted and then modified with serum-free medium formulations resulting in elimination of the confounding variability introduced by serum containing media. The surface engineering tools facilitated the development of unique self-assembling monolayers (SAMS) capable of cell adhesion and the development of patterned substrates capable of selective cell adhesion. Microfabrication of bio-hybrid MEMS cantilever devices provided the platform to investigate myotube functionality in a unique way, allowing access to the cell’s physiology in a controlled environment. In the future, these devices will be useful not only in translational research but also addressing basic biological and medical questions as well as for developing functional prosthetic devices. These culture models were also developed with the intention of providing scientists with systems to perform high throughput screening of drugs and other small molecules aimed at disease treatment, thereby reducing the limitations inherent in translation research.

In this dissertation project various tissue-engineering strategies and technologies have been employed to develop functional in vitro systems mimicking myelination and the mechanosensory circuit of the stretch reflex arc. The generation of these complex physiological systems was multi-step, relying on the integration of individually developed components reflected in the three parts of the dissertation research. In part one, a serum-free system resulting in Schwann cell myelination of peripheral sensory and motoneuron axons was developed [2]. The experiments conducted in part two of
the project resulted in a functional skeletal muscle tissue construct [3]. Finally, part three integrated type Ia sensory neurons with specially differentiated skeletal muscle fibers from part two resulting in the creation of an *in vitro* model of the stretch reflex arc mechanosensory circuit. In order to minimize integration related pitfalls, the strategies and technologies chosen had to be compatible with the wide range of anticipated cells types and functional applications. Consequently, the DETA substrate, serum-free medium formulations and bio-MEMS cantilevers were chosen or designed to support the growth and differentiation of neurons, glia and myocytes both separately and in co-culture.

Myelination is a widely studied neurological event due to its importance in nervous system functionality and maturation and dysfunctions of this process result in a variety of disease states [4, 5]. It is also interesting from a cell biology perspective as cell-cell interactions dictate many of the structural and functional changes seen during the process [6-8]. Myelination of neurons by Schwann cells has been extensively studied using dorsal root ganglia (DRG) cultures in a variety of serum containing and serum-free *in vitro* systems, with most systems relying on the use of biological substrates for cell culture [9]. Among the important findings from previous work with *in vitro* myelinating systems are the requirement of ascorbic acid in basal lamina modification to facilitate myelination by Schwann cells, the key role played by the extracellular matrix protein collagen type IV, the importance of β-1 integrin activation by laminin, the role of cyclic AMP in activation of myelin genes and the importance of factors such as insulin-like growth factor I (IGF-I) and neuregulin-1 in enhancing myelination [10-16]. However, in these studies, axonal outgrowth and Schwann cell
myelination was random/variant/non-uniform in its organization. In chapter two,
experiments documented the development of a patterned substrate supporting the
directed outgrowth and myelination of sensory neuron axons. These findings fill a major
void in the area of myelinating \textit{in vitro} tissue engineered systems and could play an
important role in the development of cell-based biosensors and bio-hybrid devices
where cell location and directional control of process outgrowth are critical to
functionality.

\textit{In vitro} myelinating cultures of Schwann cells and sensory neurons have been
studied for many years [9]. However, no basic \textit{in vitro} system composed of
motoneurons and Schwann cells has been shown to myelinate. This lack of a system
represented a major gap in the basic molecular understanding of motoneuron+Schwann
cell interactions. In this dissertation research, the development of a functional
myelinating motoneuron+Schwann cell system was the first step in describing the
molecular events surrounding the interactions between these cells that end in
myelination. In chapter three, a system resulting in the myelination of motoneurons in a
chemically defined, serum-free medium on the biomimetic, non-biological substrate
DETA was developed. The work also identified the minimum combination of growth
factors required for neuronal growth, as well as Schwann cell survival, proliferation and
myelination of motoneuron axons. These findings filled a major void in the area of
myelinating \textit{in vitro} tissue engineered systems [2].

\textit{In vitro} skeletal muscle systems are a commonly used platform to study basic
cellular events such as cell fusion, differentiation and changes in gene expression (\textit{).}
They also represent an important research area for tissue engineers due to their
potential for generating functional prosthetic devices and other bio-hybrid systems [17-19]. However, there has been limited success in these bioengineering areas because no serum-free, defined culture system was available and no investigations into the substrate requirements of skeletal muscle had been conducted. Serum-free systems are critical when attempting to integrate an engineered construct into living host tissue for immunological reasons. Non-biological substrates represent an important component of bio-hybrid and bio-MEMS devices, therefore, identification of one permissive to skeletal muscle growth was important to further tissue engineering research. These gaps in the basic knowledge of skeletal muscle biology were revisited from a tissue engineering perspective in part two this dissertation project. The goals for this part of the dissertation were:

1. Develop a reproducible cell culture methodology for fetal skeletal muscle
2. Define the substrate requirements of the skeletal muscle myoblasts
3. Investigate and define the growth factor requirements necessary for myoblast fusion and myotube formation and maturation under serum-free conditions
4. Differentiate myotubes into intrafusal muscle fibers using growth factors
5. Differentiate embryonic myotubes into extrafusal muscle fibers

The experiments designed to test these goals and their results were documented in chapters 4-6. The major findings from chapter four were the development of a reproducible cell culture methodology and identification of a growth permitting silane surface (DETA) capable of supporting cell adhesion, growth and differentiation. These results documented for the first time, a group of growth factors and their role in the
development of functional myotubes [20]. This basic system was used as the foundation for the differentiation experiments that came in chapters five and six.

The goal for the experiments conducted in chapter five was to differentiate forming myotubes into the intrafusal phenotype. Building on the system designed in chapter four, the role of a molecule known to be important in the development intrafusal fibers in vivo was investigated. Neuregulin 1-β-1 EGF (Nrg1-β-1) was examined in a dose and time dependent manner for its ability to drive intrafusal fiber differentiation. After Nrg1-β-1 treatment, the intrafusal fiber specific transcription factor Egr3 was only expressed in fibers exhibiting the intrafusal fiber phenotype. This finding supported in vivo and in vitro experiments indicating that Nrg1-β-1 activates transcription factors highly expressed in muscle spindle fibers and is required for their development. The discovery that Nrg1-β-1 is sufficient to drive intrafusal fiber differentiation in both a dose and time specific manner shed significant light on muscle spindle development. The high reproducibility of this system will facilitate further investigation into intrafusal fiber and muscle spindle morphogenesis. The complete development of such a system will permit the systematic evaluation of mechanoreceptor formation, maintenance and function, as well as its integration into an in vitro model of the stretch reflex arc.

In chapter six, a modified culture system and medium formulation for the culture embryonic myoblasts was developed and characterized. These experiments documented the maturation of embryonic myotubes into physiologically mature extrafusal fibers. Specifically, immature myotubes were induced to develop several aspects of mature muscle including: sarcomere assembly, development of the excitation-contraction coupling apparatus and myosin heavy chain (MHC) class
switching from the embryonic to the neonatal type. The maturation was facilitated by the addition a new growth factor containing medium formulation. These results suggest we have identified a group of biomolecules that act as a molecular switch promoting the transition from embryonic to neonatal MHC expression as well as other structural adaptations resulting in the maturation of skeletal muscle \textit{in vitro}. This defined model system for skeletal muscle maturation supports the goal of developing physiologically relevant muscle constructs for use in tissue engineering and regenerative medicine as well as for high-throughput screening applications.

The development of an \textit{in vitro} model system mimicking the mechanosensory circuit of the stretch reflex arc will provide a highly accessible system for investigations into the structure and function of the mechanosensory complex. The integration of the sensory circuit element of the stretch reflex arc on microcantilever MEMS devices will increase the understanding of how mechanical stretch signals are converted to electrical signals via Ia sensory neurons. This system will be invaluable for developing enhanced fine motor control in bioengineered prostheses and would capacitate studies of nerve-muscle and nerve-nerve communication. The system can also be used to study the pathology of neuromuscular diseases such as infantile spinal muscular atrophy type I (Werdnig-Hoffman disease) and spasticity inducing diseases like Parkinson’s, muscular dystrophy and myasthenia gravis [21-26]. In part three of this dissertation project, experiments were conducted to develop and characterize the mechanosensory circuit of the stretch reflex arc.

In chapter seven, dorsal root ganglia (DRG) cultures were grown in serum-free medium on the non-biological substrate DETA and then characterized both
immunocytochemically and electrically [27]. In these experiments, we developed a serum-free medium formulation supporting the growth of trkA, trkB and trkC positive sensory neurons on DETA substrates. The type Ia sensory neurons were examined using electrophysiology and found to generate repetitive firing action potentials after seven days in culture. This defined sensory neuron system could have applications in drug discovery for neuropathic pain [28]. Furthermore, the development and characterization of this basic system was critical for tissue engineering the more complex co-culture systems that followed.

In chapter eight, DRG+ESM co-cultures were grown on patterned DETA/13F coverslips. The major findings from this group of experiments were the utility of the box-line pattern to enhance the number of observed annulospiral and flower-spray endings in vitro as well as the localization of the stretch sensitive ion channel, BNaC1, at the axonal terminals of Ia sensory fibers interacting with intrafusal myotubes. Consequently, this in vitro system could be used to identify additional components of the mechanosensory complex. This is due to the ease of access to both individual intrafusal fibers and their associated axonal endings. For example, while the presence of proteins linking the stretch activity of the intrafusal fibers to the mechanically sensitive ion channels in the sensory neurons is postulated, these proteins have yet to be identified. It also provides for an efficient way to analyze electrical impulses generated in the sensory neurons caused by stretch deformation of the intrafusal muscle fiber. Also, by integrating this system with a cantilever MEMS device, it will be possible to determine how intrafusal fiber stretching due to measurable cantilever deflection is converted to electrical impulses in the type Ia sensory neurons.
In chapter nine, DRG+ESM co-cultures were integrated with bio-MEMS cantilever devices. The significant findings from these experiments were the visualization of annulospiral wrappings and flower-spray endings on myotubes grown on cantilevers and the verification of functional connectivity between the intrafusal myotubes and type Ia sensory neurons using Ca$^{2+}$ imaging. In the future, experiments can be conducted using this system to determine the way mechanical stretch is converted to electrical impulses. Furthermore, these findings illustrate the utility of bio-hybrid devices.

Growth factor manipulation within the medium formulations was a technique used throughout this project to direct the development of many of the tissue-engineered systems. In chapters two and three, the addition of ascorbate to the medium during changes after day 14, led to type IV collagen synthesis and subsequent basal lamina formation facilitating myelination by Schwann cells. In chapters three, seven, eight and nine the supplementation of the base medium with nerve growth factor (NGF) and the presence of neurotrophin-3 (NT-3) was critical for the survival of the Ia proprioceptive sensory neurons necessary for development of those systems. NGF and NT-3 act in concert to activate the trkC receptor and p75 neurotrophin receptor (p75NTR) thereby promoting cell survival and the differentiation of Ia proprioceptors. The experiments documented in chapter four described a role for neuregulin 1-β-1 in the increased differentiation of intrafusal muscle fibers through the activation of ErbB2 signaling and downstream Egr3 transcription factor activation. In order to direct skeletal muscle myotube maturation as described in chapter six, we developed a new medium formulation, which included insulin-like growth factor-1 shown to down regulate
embryonic MHC expression and agrin shown to play a role in the maturation of the excitation-contraction coupling apparatus. These examples illustrate the wide range of effects that exogenous growth factor application can have on the development of in vitro cultures.

The development of a biomimetic microcantilever-based model of the sensory circuit of the stretch reflex arc will provide insights into how mechanical input is coded electrically and lead to the development of prosthetic devices with greater fine motor control. Consequently, further examination of the circuit elements in isolation and combination will lead to a greater understanding of their development and function, potentially providing insights into the diseases related to their dysfunction.

Through the use of interface design, microfabrication technology and defined medium formulation we hope to further the understanding of the reflex arc circuit elements and Schwann cell myelination. The knowledge gained through these multi-disciplinary approaches could lead to insights for disorders like spasticity and spinal cord injury. Furthermore, the use of biomechanical systems could lead to improved fine motor control for bioengineered prosthetic devices.
References


