THE ISOLATION OF SYAPTIC MYONUCLEI AND SCHWANN CELL NUCLEI FOR DNA ANALYSIS FROM HUMAN SKELETAL MUSCLE

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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<table>
<thead>
<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AChR</td>
<td>Acetylcholine Receptor</td>
</tr>
<tr>
<td>BOT</td>
<td>Bruininks-Oseretsky Test</td>
</tr>
<tr>
<td>BTX</td>
<td>α-Bungarotoxin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CHD</td>
<td>Calponin Homology Domain</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>COBRE</td>
<td>Center of Biomedical Research Excellence</td>
</tr>
<tr>
<td>CP</td>
<td>Cerebral Palsy</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyography</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
</tr>
<tr>
<td>GMFCS</td>
<td>Gross Motor Function Classification System</td>
</tr>
<tr>
<td>INBRE</td>
<td>IDeA Network of Biomedical Research</td>
</tr>
<tr>
<td>KASH</td>
<td>Klarsicht, ANC-1, and Syne Homology domain</td>
</tr>
<tr>
<td>LINC</td>
<td>Linkers of the Inner Nucleoskeleton and Cytoskeleton</td>
</tr>
<tr>
<td>LMN</td>
<td>Lower Motor Neuron</td>
</tr>
<tr>
<td>LNGFR</td>
<td>Low-affinity Nerve Growth Factor Receptor</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-activated Cell Sorting</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural Cell Adhesion Molecule</td>
</tr>
<tr>
<td>Nesprin</td>
<td>Nuclear Envelope Spectrin Repeat Proteins</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofilament</td>
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</table>
NMJ ......................... Neuromuscular Junction
PNS ......................... Peripheral Nervous System
SC ............................ Schwann Cell
SR ............................. Sarcoplasmic Reticulum
Syne .......................... Spectrin Repeat Containing Nuclear Envelope
TSC ............................ Terminal Schwann Cell
UMN ........................... Upper Motor Neuron
ABSTRACT

The neuromuscular junction is a critical component of the motor system that enables transmission of impulses from the alpha-motor nerve to muscle fibers. Cerebral palsy is a neuromotor disorder that has been characterized by deficits in the central nervous system and, more recently, disruptions in the peripheral nervous system at the neuromuscular junction. The specific mechanisms accounting for neuromuscular junction disruption in cerebral palsy are not known, but the development and maintenance of the neuromuscular junctions is a complex process involving alterations in gene expression of synaptic myonuclei and terminal Schwann cells and the development of a highly-regulated synaptic structure. To understand neuromuscular junction abnormalities associated with cerebral palsy, a method for assessing the regulation of gene expression in synapse-associated nuclei is needed. This work focuses on development of a technique to isolate synaptic myonuclei and terminal Schwann cell nuclei from human skeletal muscle biopsies to enable epigenetic analyses. Using tissue dissociation and immunological separation techniques, a method to isolate synaptic myonuclei and terminal Schwann cells is accomplished using fluorescence-activated and magnetic-activated sorting technologies. Nuclear material separated from muscles of patients with different clinical diagnoses using this technique may provide a critical approach to studying the epigenetic regulation of neuromuscular junction development and maintenance in both health and disease.
Chapter 1

INTRODUCTION

1.1 Thesis Overview

The neuromotor system is responsible for the fine and gross motor functions within the human body. The neuromotor system is composed of the central nervous system (CNS), the peripheral nervous system (PNS), and muscle tissue. Together, these core components coordinate and control movement and postural positioning. Disruptions to any component of the neuromotor system can result in movement disorders. Cerebral palsy (CP) is one such disorder that occurs when injury to parts of the developing brain responsible for motor control result in deficits of muscle coordination and body movement (NINDS, 2015). Recently, abnormalities in neuromuscular junctions (NMJs) (Robinson et al., 2013) and muscle (Lucas R. Smith, Chambers, Subramaniam, & Lieber, 2012; L. R. Smith, Lee, Ward, Chambers, & Lieber, 2011; L. R. Smith et al., 2009, Mathewson, 2015) have been observed in CP. The NMJ forms a synapse between lower motor neurons (LMNs) and individual muscle fibers. The development, maintenance, and function of the NMJ is primarily attributed to the gene expression and genetic regulation of synaptic myonuclei (Merlie & Sanes, 1985; J. R. Sanes & Lichtman, 1999; Joshua R. Sanes & Lichtman, 2001) and terminal Schwann cells (TSCs) (Feng & Ko, 2008; Reddy, Koirala, Sugiura, Herrera, & Ko, 2003), and the central hypothesis guiding the work presented here is that NMJ disruption in CP is due to alterations in the epigenetic patterning of synaptic myonuclei and Schwann cells. In order to better understand how epigenetic
abnormalities in NMJs may occur in CP, a technique for isolating TSCs and synaptic myonuclei is needed. The work described in this thesis details the development of a technique for isolating both synaptic myonuclei and TSC nuclei from human skeletal muscle.

1.2 The Neuromotor System

1.2.1 Central Nervous System Control

The brain and spinal cord make up the CNS and are responsible for the integration of sensory input, motor planning, and the propagation of motor impulses. All voluntary movements are controlled by the brain. Upper Motor neurons (UMNs) originate from both the outer layer of the cerebrum, called the motor cortex, and the brain stem (Purves D, 2001). The UMNs serve as the pathway for motor signals to travel from the brain to the brain stem and down the spinal cord (figure 1.1). The axons of UMNs from the primary motor cortex form the pyramidal tracts and travel directly to the spinal cord. Once in the spinal cord, some UMNs synapse with interneurons while others synapse directly with LMNs. UMNs originating in the brainstem form the extrapyramidal tracts and generally do not directly synapse with LMNs (Purves D, 2001).

The generation of motor impulses is strictly controlled by input from multiple areas of the cerebellum and basal ganglia. The cerebellum functions by comparing the movements of the body to the desired movements and makes corrections to motor input in order to achieve the desired movement (Purves D, 2001). The cerebellum receives sensory input from the PNS system about posture, balance, muscle tone, and force. The cerebellum then utilizes this information to modulate the descending motor
commands in the pyramidal and extrapyramidal tracts in order to achieve the desired motor outcome (Purves D, 2001). The basal ganglia are interconnected with primary motor and premotor cortices and work in an inhibitory manor to prevent unwanted movement.

1.2.2 Peripheral Nervous System

The PNS consists of sensory neurons and LMNs. Sensory neurons involved in motor control include cutaneous mechanoreceptors, which are involved with sensing the external environment, mechanoreceptors specialized for proprioception of muscle length and tension, and mechanoreceptors that detect limb positioning and joint positioning (Purves D, 2001). Sensory information travels to the spinal cord and brain where adjustments to motor input can be made. Alpha, beta, and gamma LMNs in the PNS link the CNS to target muscles. LMN cell bodies reside in the spinal cord and extend axons through the ventral horn and into the periphery (Purves D, 2001) (figure 1.1 and 1.2). Descending signals from UMs are relayed to LMNs that then synapse with individual muscle fibers.

1.2.3 Neuromuscular Junction

In order for motor impulses to cause a muscle contraction, the signal must be exchanged between alpha LMN termini and the target muscle fibers. NMJs serve as the interface between the PNS and the muscle tissue.

For a signal to be propagated across the NMJ synapse to the muscle, the electrical input from the nerve is converted into a chemical signal in the form of the neurotransmitter acetylcholine (ACh) (Hughes, Kusner, & Kaminski, 2006). When an electrical impulse reaches the nerve terminus, the electrical signal initiates the fusion
of synaptic vesicles containing ACh from inside the nerve ending to the nerve terminus (figure 1.3). The fusion of the synaptic vesicles to the nerve terminus membrane then releases the contents of the synaptic vesicle into the synaptic cleft. After ACh is released into the synaptic cleft, it rapidly diffuses throughout the cleft and binds to acetylcholine receptors (AChR) on the muscle side of the synapse (Hughes et al., 2006). ACh binding leads to the depolarization of the muscle fiber and the release of calcium within the muscle fiber creating a wave of depolarization that causes contraction.

1.2.4 Muscle Physiology

1.2.4.1 Muscle Fiber Composition and contraction

Muscle fibers are large cylindrical, multinucleated cells which contain sarcomeres that function as the basic contractile unit of the fiber. Muscle fibers contain between 80 and 120 nuclei, which are aligned on the periphery of healthy, mature fibers just below the sarcolemma (J. R. Sanes & Lichtman, 1999). Additionally, fibers contain synaptic myonuclei, which are a specialized subset of myonuclei that aggregate at the site of LMN innervation on the muscle fiber (J. R. Sanes & Lichtman, 1999). The sarcomeres of fibers are arranged in a linear fashion along the axis of the cylindrical muscle fiber. The sarcomere contains a large scaffold of fibers that are encompassed by the sarcoplasmic reticulum (SR). Each sarcomere and its accompanying SR is separated by a transverse-tubule. When a nerve impulse causes depolarization at the NMJ, a wave of depolarization spreads across the fiber and into the transverse-tubules between the sarcomeres. Depolarization within the transverse-tubules causes the SR to release calcium ions from voltage-gated ion
channels which activate the contractile units of the sarcomere (Elaine N. Marieb, 2014). This process enables all sarcomeres to fire at once resulting in the contraction of sarcomeres and the shortening of the muscle fiber along its axis.

### 1.2.4.2 Muscle Organization

Muscle is composed of many individual muscle fibers which are organized by a vast system of connective tissue. The fascial system separates individual components of the muscle tissue and plays an important role in relaying the force generated by the muscle to the skeletal system (Elaine N. Marieb, 2014). The epimysium is the outer most layer of connective tissue that surrounds the entire muscle and separates the muscle from surrounding organs. The perimysium is within the muscle and separates bundles of muscle fibers into fascicles. At the lowest level or organization the endomysium separates individual muscle fibers. Each of these three layers of connective tissues is intertwined and the fascial system ultimately forms the tendon connecting the muscle to the skeleton.

### 1.2.4.3 Generation of varying levels of force

The ability to generate different levels of force is critical for environmental interaction, balance, and coordinated movement. There are two main methods for varying the force generated by muscle which are the organization of LMNs into motor units and differences in skeletal muscle fibers type.

A motor unit consists of a LMN that branches out to innervate multiple different muscle fibers (Elaine N. Marieb, 2014; J. R. Sanes & Lichtman, 1999). Although each fiber has only one innervations site, each muscle is innervated by multiple different motor units which can branch out to innervate each fiber within the
muscle. By modulating the recruitment of varying combinations of motor units, the brain can cause the contraction of a diverse range of muscle fibers. When a force is generated, the sensory apparatus of the PNS relays information about the resulting motor event back to the brain. The nervous system can then adjust the movement by recruiting more motor units to increase force or by reducing recruitment of motor units to reduce force (Shadmehr, Smith, & Krakauer, 2010). Certain muscles associated with fine motor skills have been observed to contain larger numbers of motor units enabling a greater range of control and more precise force generation (Feinstein, Lindegard, Nyman, & Wohlfart, 1955).

Skeletal muscle is composed of several types of muscle fibers. Muscle fiber type I is a slow twitch muscle fiber which generates moderate force but is capable of sustained contractions which is needed for activities such as maintaining posture and balance. Type IIA and IIX fibers are fast twitch fibers that generate larger amounts of force under anaerobic conditions (Schiaffino & Reggiani, 2011). Type II fibers are utilized for more dynamic movements.

The regulation of motor unit recruitment enables the use of different amounts of muscle fibers as well as recruitment of different fiber types. Together these two principles enable a system to generate a large range of forces that are needed in order for coordinated movement as well fine and gross motor skills.

1.2.5 Motor system development

1.2.5.1 Skeletal Muscle Myogenesis

Muscle tissue develops from the fusion of embryonic mesoderm cells called myoblasts. The fusion of myoblasts results in the formation of multinucleated cells
called myotubes that later develop sarcomeres and mature into terminally differentiated skeletal muscle fibers (Brand-Saberi, Wilting, Ebensperger, & Christ, 1996). In addition to myoblasts giving rise to muscle fibers, satellite cells which are embedded under the basal lamina of mature muscle fibers are capable of maturing into myotubes and repairing muscle fibers. Satellite cells play critical roles in muscle growth, which requires the accretion of additional myonuclei during muscle hypertrophy, and replenishing muscle tissue after injury.

1.2.5.2 Innervation of Muscle

LMNs travel a long distance in order to innervate muscle fibers. The neuronal cell body of motor neurons is embedded within the spinal cord. The axon exits the spinal cord through the ventral horn and grows toward the intended target muscle. Once the motor neuron is within the muscle, branching occurs and one motor neuron can innervate hundreds of muscle fibers. When a motor neuron’s growth cone reaches the target myotube during development or regeneration, early ACh transmission begins initiating the development of a NMJ (J. R. Sanes & Lichtman, 1999).

Prior to innervation, muscle fibers express nicotinic AChRs across a large region of the cell surface. As motor neurons become close in proximity to their target muscle fiber, synaptic activity begins to occur but synaptic transmissions are inefficient due to the wide distribution of AChRs (J. R. Sanes & Lichtman, 1999). As the NMJ matures, AChRs are localized to areas that directly appose the motor neuron terminus, and extra-junctional expression of AchRs is limited (Joshua R. Sanes & Lichtman, 2001). The shift in AChR distribution is attributed to an increase in transcriptional activation of AChR genes in synaptic myonuclei and a reduction of AChR expression in extra-synaptic nuclei (Merlie & Sanes, 1985). As this change in
myonuclear specification occurs, the morphology of the post-synaptic apparatus changes as the membrane invaginates to create post synaptic folds that contain two unique zones. The primary zone contains a high level of AChR and is located closest to the nerve terminus while the secondary zone is within the invaginations of the membrane and contains voltage-gated Na+ channels involved in muscle depolarization (J. R. Sanes & Lichtman, 1999).

In addition to alterations on the post synaptic side, the newly arrived motor axon terminus also differentiates to improve synaptic function. When the newly arrived nerve terminus meets the synaptic site, the nerve terminus is in a bulb-like shape that resembles an axonal growth cone (J. R. Sanes & Lichtman, 1999). As the synapse matures the nerve terminus spreads to create a larger area capable of synaptic transmission. Specialized regions of the nerve terminus, called active zones, are capable of synaptic transmission and are defined by the presence of synaptic vesicles containing ACh. As the NMJ stabilizes, the preterminal active zones become closely paired with AChR clusters on the apex of the muscle fiber’s synaptic folds on the post synaptic side (J. R. Sanes & Lichtman, 1999).

1.2.5.3 Aggregation of post-synaptic myonuclei and specialization of function.

During innervation a specialized subset of nuclei aggregate at the NMJ. This aggregation of synaptic nuclei has been characterized by an increase in the density of nuclei surrounding the NMJ (Kummer, Misgeld, Lichtman, & Sanes, 2004)(figure 1.4). The synaptic nuclei then begin to express AChR in large quantities which is critical to the establishment of the NMJ (Merlie & Sanes, 1985). While the mechanisms behind the migration of synaptic nuclei remains unknown, the nuclei associated with the NMJ have been shown to express nuclear envelope spectrin repeat
proteins (nesprins) (figure 1.5). Nesprin proteins are components of SUN-protein containing multivariate complexes called linkers of the inner nucleoskeleton and cytoskeleton (LINC) which form a structural link between the nucleoskeleton and actin cytoskeleton in the cells cytoplasm (Warren, Zhang, Weissberg, & Shanahan, 2005) (figure 1.6). Nesprin proteins are highly expressed in skeletal muscle and nesprin-1 isoform nesprin-1-alpha is responsible for anchoring synaptic nuclei near the NMJ. (Apel, Lewis, Grady, & Sanes, 2000; Grady, Starr, Ackerman, Sanes, & Han, 2005; Rajgor & Shanahan, 2013). Post-synaptic myonuclei have been shown to have an increased expression of nesprin-1 protein in comparison to non-synaptic myonuclei (Grady et al., 2005).

1.2.5.4 Role of Terminal Schwann Cells in NMJs

Schwann cells (SCs) are critical to neuromotor transmission. There are two functionally distinct populations of SCs associated with LMNs; extra-junctional SCs, which myelinate the nerve axon, and TSCs that cap the NMJ by surrounding the nerve terminus. During development, both SC populations follow the axon, and their growth is regulated by axonal growth (Mirsky & Jessen, 1996). When LMNs reach the target muscle fiber, the role of the TSC becomes unique from other SCs. TSCs differ from myelinating SCs by their (i) physical location, TSCs surround the synapse, (ii) function, TSCs provide support for the NMJ and do not myelinate, and (iii) differences in gene expression. TSCs have been found to exhibit elevated amounts of neural cell adhesion molecule (NCAM) and S100 in comparison to myelinating SCs (Mirsky & Jessen, 1996). Additionally, SCs express low-affinity nerve growth factor receptor (LNGFR) in high quantity.
TSCs play an important role in NMJ formation and maintenance. Signaling between TSCs and muscle fibers through neuroregulin-2 activates the transcription of AChR in synaptic myonuclei (Rimer et al., 2004). This signaling mechanism is important for initiating gene expression changes within the muscle fiber in order to establish the NMJ. SCs have been shown to be critical for the long term sustainability of function of NMJs (Reddy et al., 2003). The loss of SCs at established and functionally mature NMJs has been shown to lead to the breakdown of the synapse over time (Reddy et al., 2003).

1.3 Neuromotor Disease

1.3.1 General Information

For an organism to have a fully functional neuromotor system, the CNS, PNS, and muscular systems all need to develop and mature properly and in concert. Injury or improper development in one part of the neuromotor system can lead to abnormal function throughout. Disruptions in the CNS can impair both the ability to generate motor impulses as well as the ability to integrate sensory information needed to modify motor impulses to achieve a specific motor event. In the PNS, injuries to sensory nerves can disrupt the transmission of sensory information back to the CNS impairing the ability to modify motor events resulting in undesired movement and a lack of motor coordination. Injury to motor neurons in the PNS can cause a deficit in the transmission of motor impulses to the target muscle fibers resulting in a decrease in motor function, a loss of muscle tone, an inability to generate force, paralysis, and poor motor coordination. Finally, disease conditions affecting NMJs can have a variety of effects. Dysregulation of synaptic events could lead to excessive or
prolonged synaptic signal transmission causing muscle spasticity, poor coordination, involuntary movements and abnormal levels of force generation. Alternatively, a loss in synaptic efficiency could lead to weakness if synaptic transmission is disrupted.

1.3.2 Disruptions to the Central Nervous System

The CNS’s role in motor control and coordination is well established. Injury and disease can impair the ability of the brain to generate or transmit impulses or to integrate sensory information from the PNS. Insults to the developing brain like hypoxia / ischemia, localized infection, localized stroke, large scale hemorrhage, or global hypoxia can cause the death of neurons in the brain resulting in an encephalopathy. If the encephalopathy affects the regions of the brain associated with motor control the results of this injury can lead to deficits in motor function. Difference in the location and severity of the hypoxic injury are associated with a range of potential motor outcomes and variable degrees of recovery.

Neurodegenerative diseases also can cause motor deficits. Disease such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and spinal muscular atrophy have all been associated with neuronal degradation affecting motor control.

1.3.3 Disruptions to the Peripheral Nervous System

In the PNS both alpha motor neurons delivering motor impulses to muscle and sensory neurons relaying information back to the brain need to function properly. Physical injury to nerves can result in localized problems with muscle control and sensory perception. Physical injury can occur with nerve crush, nerve truncation, and sustained pressure on nerves, which can disrupt signal transmission.
1.3.4 Cerebral Palsy

CP is a neuromotor disorder associated with an injury to the developing brain. The specific brain injury and its cause are often not known in CP patients, but up to 45% of cases exhibit white matter brain lesions, which are often associated with perinatal conditions (Reid, Dagia, Ditchfield, Carlin, & Reddihough, 2014). In addition to evidence for CNS injury, abnormalities have also been observed in the PNS of patients with CP. In particular, NMJs were found to contain both morphologic and microanatomic disruptions of key synaptic proteins (Robinson et al., 2013; Theroux et al., 2005). Patients with CP can exhibit a wide array of movement disorders and symptoms such as abnormal muscle tone, muscle spasms, weakness, uncontrolled movement and poor coordination (NINDS, 2015).

The exact role abnormalities of NMJs play in the pathology of CP is still under investigation. However, NMJ specific pathologies with disruptions in the NMJ have been linked to a range of motor deficits. These disruptions can be caused by genetic mutations of proteins involved in the development or function of the presynaptic structure (8% of cases), synaptic basal lamina (16% of cases), and postsynaptic portions (76% of cases) of the NMJs (Engel, Ohno, Shen, & Sine, 2003). Some of these genetic defects include disruptions to the AChR (Ohno et al., 1995), rapsyn, a protein associated with AChR clustering (Milone et al., 2009), Choline acetyltransferase (Maselli et al., 2003; Shen et al., 2011), and ACh esterase (Engel, Lambert, & Gomez, 1977; Hutchinson et al., 1993). This leads to a fundamental and interesting question regarding the role gene expression and genetic regulation play in the formation of abnormal NMJs in CP.
1.3.5  Neuromyopathy Clinical Assessment and Research

In order to assess motor function both clinically and in research, a diverse range of assessments have been developed. The gross motor function classification system (GMFCS) is a system developed to classify the motor ability of a person into 5 categories. The GMFCS is reliant on an assessment of physical ability and each level of GMFCS score is reliant on the individual’s ability to perform certain motor task and their dependence of support or assistance to complete the task (R. Palisano et al., 1997; R. J. Palisano et al., 2000). Common tasks assessed to generate a GMFCS vary but can include, walking ability, ability to use stairs, ability to sit up or get out of a chair, and balancing ability. Similarly, the Bruininks-Oseretsky Test of Motor Proficiency (BOT) can be used to assess both gross and fine motor skills by examining the performance of specific tasks (Hands, Licari, & Piek, 2015). More direct testing methods can also be employed to assess motor function such as electrophysiological assessment. Electromyograms (EMGs) assess the electrical activity of muscles directly through sensory needle placed into the muscle through the skin. EMGs record the electrical activity within the muscle as it is contracted and relaxed. In addition to EMGs nerve conductivity tests can be used to examine the efficiency of electrical impulses being conducted through peripheral nerves and transmitted to muscle tissue.

In addition to the assessment of physical ability and electrophysiology, biomolecular tests can be used to look for abnormalities in metabolic function which can have profound effects on muscle due to the high energy requirements. Metabolic myopathies commonly relate to diseases involving glycogen storage, fatty acid oxidation deficits, and mitochondrial disorders (Berardo, DiMauro, & Hirano, 2010). Metabolic panels and the analysis of biomarkers in blood and urine samples are powerful tools for both clinical diagnosis and research related to metabolic disorders.
1.3.6 Limitations of existing research methods

A variety of research and clinical tools have been developed to assess motor ability and functionality of various components of the motor system; however, there remains a gap in the ability to diagnose and examine events associated with NMJ function and formation. The establishment and maintenance of NMJs involves interactions and dramatic modulation of gene expression between the motor nerve (whose nuclear material resides in the spinal cord), TSCs, and synaptic myonuclei. Together these components specialize to control NMJ form and function, processes involving sustained programs of gene expression across multiple phases of development and maturation. One possible mechanism through which genetic regulation can occur is by epigenetic modification. Due to the nature of the patients analyzed in previous studies, the abnormalities observed in NMJs in CP are not directly attributable to any genetic mutation; however, it seems possible that abnormal epigenetic regulation of NMJ associated genes could lead to the dysmorphic NMJs that have been observed.

Current clinical and research based assessments usually fail to take into consideration abnormalities in neurotransmission and the NMJ. Previous studies of whole muscle biopsies have shown that disorders affecting NMJs exhibit dysregulation of genes associated with NMJ function (L. Smith et al., 2009). The analysis of whole muscle gene expression, however, pools a large number of diverse myonuclear subtypes and ignores the functional difference between synaptic myonuclei and non-synaptic nuclei. In addition, many other cell types are found in muscle biopsies including circulating blood cells, vascular cells, stromal fibroblasts, and adipocytes as well as TSCs which have a direct and unique role in NMJ formation (Reddy et al., 2003; Rimer et al., 2004). On average about 100-120 nuclei are found in
a muscle fiber and 2-5 of those nuclei are associated with the NMJ and perform a unique role in NMJ formation and maintenance (Bruusgaard, Liestøl, Ekmark, Kollstad, & Gundersen, 2003; Joshua R. Sanes & Lichtman, 2001). Taking a whole muscle approach to examining gene expression or epigenetic regulation at NMJs may significantly distort the results as the vast majority of the analyzed DNA or mRNA will come from non-synaptic muscle regions and other cell types. Interestingly, when NMJs are disrupted and muscle fibers are being regulated abnormally, it is likely that the gene expression and epigenetic profiles of the entire muscle fiber would change due to differences in metabolic output, tensile force, and other symptoms. Such maladaptive responses of the muscle would further complicate analysis of NMJ disorders when using whole muscle biopsies.

An improved ability to assess genetic regulation of synaptic myonuclei and TSCs in NMJ disorders is needed to understand how abnormal NMJs may form in CP. One key method of genetic regulation occurs through methylation patterning, which is known to play major roles in altering gene expression throughout development and during aging (Cantone & Fisher, 2013). Given the general timing of CNS injury in CP, the Akins lab has begun testing the idea that normal development and maturation of NMJs may be disrupted with a resultant alteration in epigenetic patterning and abnormal DNA methylation. In order to assess the epigenetic regulation of gene expression in NMJ-associated cells and nuclei, a method for isolating synaptic nuclei and TSCs was tested.
1.4 Outline of Method Tested for Isolating Schwann Cells and Synaptic Myonuclei

To isolate individual cells from skeletal muscle, a tissue dissociation technique utilizing collagenase type II was used to enzymatically break muscle tissue apart. After enzymatic digestion, mononucleated cells, including SCs, and multinucleated muscle fibers were separated by density centrifugation. TSCs were isolated from other mononucleated cells using magnetic activated cell sorting (MACS) through positive selection of NCAM (CD56+) and LNGFR (CD271+) cell markers, which are highly expressed in TSCs. In order to isolate synaptic myonuclei from muscle fibers, a tissue homogenization technique was employed to further mechanically dissociate muscle fibers and release nuclei into solution. Synaptic nuclei were stained with an antibody for nesprin-1 protein, which is highly expressed on synaptic myonuclei (Apel et al., 2000; Grady et al., 2005). Synaptic myonuclei will be sorted from the homogenate using fluorescence activated cell sorting techniques (FACS). Together, these techniques were expected to enable the isolation of both TSCs and synaptic myonuclei from the same skeletal muscle sample. Furthermore, collection of nesprin-1 negative nuclei may be considered the population of non-synaptic myonuclei from skeletal muscle fibers.

1.5 Hypothesis and Aims

Hypothesis: Synaptic myonuclei and TSCs can be isolated from skeletal muscle tissue by utilizing tissue dissociation and cell sorting techniques targeting nesprin-1 rich nuclei and NCAM+/LNGFR+ Schwann cells.

Specific Aims:

1. Assess the efficacy of anti-nesprin-1 for differentially staining synaptic myonuclei.
2. Develop a tissue dissociation method for isolating mononuclear cells and multinucleated muscle fibers from a single human skeletal muscle biopsy.

3. Utilize cell sorting techniques to isolate synaptic myonuclei and TSCs.
Figure 1.1: Outline of the Human Motor System. Upper motor neurons have cell bodies situated in the brain and their axons travel through the corticospinal tract. In the spinal cord UMs may interact with interneurons before finally synapsing with LMs. LMs have cell bodies within the spinal cord and project axons into the periphery towards muscle. LMs in the brain stem exit the brain stem without entering the corticospinal tract and are involved with functions such as speech and swallowing. (Figure reused with permission from Cambridge University Press; Appendix B.1). Emily F. Goodall and Karen E. Morrison, “Amyotrophic lateral sclerosis (motor neuron disease): proposed mechanisms and pathways to treatment”, Expert Reviews in Molecular Medicine, Issue 11, page 2.
Figure 1.2: Axons of Motor Neurons Extend From the Spinal Cord to the Muscle.
This figure depicts the path of LMN leaving the spinal cord through the ventral horn and extending to the target muscle. Two motor units, shown in red and purple, consist of a LMN and all of the muscle fibers with which that LMN synapses. MARIEB, ELAINE N.; HOEHN, KATJA, HUMAN ANATOMY & PHYSIOLOGY, 10th Edition, © 2016. Printed and electronically reproduced by permission of Pearson Education, Inc., Upper Saddle River, NJ (Appendix B.2).
Figure 1.3: Representation of a Neuromuscular Junction Transmitting a Signal to Skeletal Muscle. A LMN is depicted innervating skeletal muscle. When an action potential reached the LMN terminus an increase in intracellular calcium levels occurs. The increase in calcium facilitates the fusion of ACh-containing synaptic vesicles to the nerve terminus and ACh is released into the synaptic cleft. ACh diffuses throughout the synaptic cleft and binds with AChRs on the post synaptic side causing an influx of Na$^+$ and K$^+$ ions. The ionic influx activates voltage gated ion channels resulting in a wave of depolarization to sweep across the fiber.

Figure 1.4: Molecularly Specialized Nuclei Cluster at Sites of AChR Clustering.
Cross sections of muscle (a) and longitudinal sections (b-c) were stained with antibody against nesprin-1 (a-c) and BTX (a'-c'). The merge of both nesprin-1 and BTX staining is shown (a''-c''). This figure shows an increase in nesprin-1 (Green) expression in nuclei localized in areas of AChR clustering (Red). This increase in nesprin-1 signal is associated with the anchoring of synaptic myonuclei to NMJs. Extra-synaptic nuclei are labeled “e”, while synaptic nuclei are labeled “s”. "This research was originally published in The Journal of Biological Chemistry. Apel, Lewis, Grady, & Sanes. Et al. “Syne-1, A Dystrophin- and Klarsicht-related Protein Associated with Synaptic Nuclei at the Neuromuscular Junction”. 2000; Vol 275: 31986-95. © The American Society for Biochemistry and Molecular Biology."
Figure 1.5: The Nesprin Family. Representation of known nesprin proteins 1-4. All nesprin isoforms contain a Klarsicht, ANC-1, and Syne Homology domain (KASH domain) and one or more spectrin repeats. Nesprin-1 and nesprin-2 contain 74 and 56 spectrin repeats, respectively, and both contain a calponin homology domain (CHD) enabling nesprin to bind F-actin at its N-terminus. Figure republished with permission from Cambridge University Press (Appendix B.3). Dipen Rajgor, Catherine M. Shanahan. “Nesprins: from the nuclear envelope and beyond”. Expert Reviews in Molecular Medicine, Volume 15, Page 2.
Figure 1.6: Nesprins Participate in Linker of the Nucleoskeleton and Cytoskeleton Complexes. Nesprin-1/2 function in the formation of LINC complexes in order to form a physical attachment between nuclei and the actin cytoskeleton of a cell. The nesprin KASH domain serves as the transmembrane region in the outer nuclear membrane that binds to SUN proteins in the perinuclear space. Outside of the nucleus, spectrin repeat domains form a tether between the C-terminal KASH domain and the N-terminal calponin domain. Calponin homology domains in nesprin form strong interactions with F-actin completing the connection from the nuclear lamina to cellular cytoskeleton. Figure republished with permission from Cambridge University Press (Appendix B.3). Dipen Rajgor, Catherine M. Shanahan. “Nesprins: from the nuclear envelope and beyond”. Expert Reviews in Molecular Medicine, Volume 15, Page 3.
Chapter 2
MATERIALS AND METHODS

2.1 Study Design and Tissue Acquisition.

Human skeletal muscle was obtained from surgical procedures at the Nemours/Alfred. I. duPont Hospital for Children in Wilmington, DE. Surgical candidates and their care giver(s) were consented with an Institutional Review Board approved consent process (Appendix A.1-3), and samples were stored in the Swank Neuro-Orthopedics Tissue Repository at Nemours. The demographics of the patients donating samples for use in this study are available in table 2.1.

The samples used in this study were withdrawn from the repository and de-identified; the experiments performed were considered exempt from IRB review (Appendix A.4). A full list of reagents used in this study can be found in table 2.2. Skeletal muscle samples were processed by the Histochemistry and Tissue Processing Core at Nemours. Small quantities of the muscle tissue were snap frozen in liquid nitrogen chilled isopentane. Select samples were cryosectioned at 10-50 μm thicknesses for immunologic staining. Remaining skeletal muscle samples were stored at 4°C in MACS tissue storage solution (Miltenyi Biotec, Bergisch Gladbach, Germany) for use in cell/nuclei isolation.

2.2 Histological Sample Preparation and Analysis

Slides with sectioned skeletal muscle were fixed in Dulbecco’s phosphate-buffered saline (DPBS; Corning Life Sciences, Tewksbury MA), pH 7.4, with 4%
paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 5 minutes and permeabilized with DPBS, pH 7.4, containing 0.1% Triton X-100 (Sigma-Aldrich, Saint Louis, MO), for 15 minutes. Fixed and permeabilized samples were rinsed with DPBS 3 times for 5 minutes each. Samples were blocked during primary antibody incubation in DPBS, pH 7.4, with 3% bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO). Sections were incubated in DPBS, pH 7.4, with 3% BSA overnight at 4°C with anti-nesprin-1 antibody ab24742 (Abcam Biotech Company, UK) at a dilution of 1/1000 and Anti-200kD neurofilament heavy antibody, ab4680, (NF; Abcam Biotech Company, UK) at a dilution of 1/100,000. After primary incubation, samples were rinsed in DPBS 3 times for 5 minutes each. Samples were then incubated for 1 hour at room temperature in DPBS, pH 7.4, with Hoechst 33258 (Sigma-Aldrich, Saint Louis, MO) at a dilution of 1/10,000 (0.12 μg /ml), α-Bungarotoxin conjugated with Alexa Fluor 647 (BTX; Life Technologies, Grand Island, NY) at a concentration of 1/10,000 (0.1 μg /ml), Novex goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (Life Technologies, Grand Island, NY) at a dilution of 1/500 (4 μg /ml) and goat anti-chicken IgY (H+L) secondary antibody conjugated with Alexa Fluor 555 (Life Technologies, Grand Island, NY) at a dilution of 1/500 (4 μg /ml). Samples were rinsed with DPBS, pH 7.4, 3 times for 5 minutes each and then mounted with ProLong Antifade Reagent (Life Technologies, Grand Island, NY), covered with a cover slip and sealed using nail polish. Samples were viewed using an Olympus BX-60 widefield fluorescence microscope (Olympus, Tokyo, Japan) using a 40x Oil (1.3NA) objective. Images were acquired with an Evolution QEi monochrome digital camera (Media Cybernetics, Silver Spring, MD) using Image Pro software (Media Cybernetics). For z-stack
images, z projections were made based on maximum intensity projection functions in Image J (Schindelin et al., 2012).

2.3 Tissue Dissociation and Separation of Mononucleated Cells and Muscle Fibers.

Skeletal muscle was rinsed in DPBS to remove red blood cells and MACS tissue storage solution. The sample was then placed into Dulbecco’s Modified Eagle Medium (DMEM; Mediatech-Corning, Tewksbury MA) and minced into pieces 1-3 mm³ in size (figure 2.1). During mincing, fascia, adipose tissue, and other debris (e.g., large blood vessels) were removed from the tissue. The remaining tissue was weighed and then placed into DMEM, pH 7.2, with 0.0025% Collagenase Type II (Worthington Biochemical Corporation, Lakewood, NJ) so that the total volume of enzymatic solution was 5 ml per gram of tissue. The solution and tissue were then placed into a water-jacketed spinner flask (Wheaton, Millville, NJ) that was pre-warmed using a water bath circulator at 37°C (figure 2.2). The sample was incubated at 37°C with agitation from the spinner flasks magnetic stir bar on the lowest setting. After 30 minutes, the supernatant was removed from the spinner flask and mixed with an equal volume of MACS PEB buffer (PBS, ethylenediaminetetraacetic acid, bovine serum albumin), pH 7.2, in order to attenuate the collagenase type II enzyme activity and then stored at 4°C for 30 minutes. Tissue remaining in the flask was resuspended in fresh DMEM type II collagenase solution and incubated an additional 30 minutes.

At the end of incubation, the supernatant was removed from the spinner flask following the same procedure and then combined with supernatant removed after original digestion.
Density centrifugation was performed to separate mononucleated cells from the denser multinucleated muscle fibers and extracellular debris. The density gradient consisted of 15 ml Ficoll-Paque Premium (GE Healthcare Life Sciences, Pittsburgh, PA) placed into a 50 ml conical (figure 2.3 A). The cell suspension was added to the tube and spun at 1250xg for 20 minutes at room temperature. Following centrifugation, the top fluid layer was removed through aspiration and the mononuclear layer was removed by pipetting (figure 2.3 B-D). Excess Ficoll was removed by aspiration and the pellet containing the isolated muscle fibers and debris was resuspended in DPBS.

2.4 Isolation of Schwann Cells from Mononuclear Layer

For the isolation of Schwann cells from the mononuclear cell layer, magnetic-activated cell sorting techniques were implemented. Following protocols established by Miltenyi Biotec, a double positive selection was implemented. First isolation of cells positive for cluster of differentiation 56 (CD56; or neural cell adhesion molecule / NCAM) was performed using CD56 Multisort Kits for human samples (Miltenyi Biotec). A second positive selection was performed for CD271 (low-affinity nerve growth factor receptor / LNGFR) using CD271 microbead kits (Miltenyi Biotec). Both selections were performed using miniMACS separators with MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s recommendations. Briefly, CD56 positive cells were isolated from the mononucleated cell population using a CD56 multisort kit which enabled the isolation of CD271 positive cells from the CD56 positive population. After isolation, a final cell count was determined by using trypan blue exclusion and counting cells on a hemocytometer under a light microscope.
2.5 Homogenization of Muscle Fibers for Nuclear Isolation

Cells from the multinuclear pellet were resuspended in DPBS, pH 7.4. Samples were homogenized by 5 passes of a 55mL Potter-Elvehjem tissue grinder (Wheaton, Millville, NJ), which had a total clearance of 0.96mm (0.048mm per side) at room temperature. The resulting supernatant was filtered through a 40 μm mesh cell strainer (Fisher Scientific, Pittsburgh, PA). The filtrate was then stored in DPBS, pH 7.4, at 4°C for staining and FACS.

2.6 Fluorescence-Activated Cell Sorting

Samples were centrifuged at 300xg for 10 minutes at 6°C to pellet nuclei, and the fluid layer was removed by aspiration. The pellet was resuspended in DPBS, pH 7.4, containing 3% BSA and incubated overnight at 4°C with anti-nesprin-1 antibody at a dilution of 1/1,000. The sample was centrifuged again and rinsed 3 times with PBS. The sample was then stained with goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 and Hoechst 33352 (Life Technologies, Grand Island, NY) for 1 hour at 4°C. The sample was then rinsed with DPBS 3 times for 5 minutes each and then stored on ice for approximately 1 hour until FACS was performed.

FACS was performed at the Helen F. Graham Cancer Center at Christiana Care in Newark, Delaware using a Becton-Dickinson FACS Aria II™ (BD Biosciences, San Jose, CA). The sample was split into three aliquots in DPBS, pH 7.4 at room temperature; (i) unstained, (ii) stained with secondary only (Alexa Fluor® 488), and (iii) fully stained with Hoechst 33342, anti-nesprin-1 primary antibody, and Alexa Fluor® 488 secondary antibody. Gating parameters for the FACS were determined based on fluorescence signal from the sample stained with secondary antibody only.
Nuclei were sorted into two categories; nuclei that stained positive for both Hoechst 33342 and nesprin-1 were isolated along with nuclei that were Hoechst 33342 positive but nesprin-1 negative.
Table 2.1: Demographic Information of Samples Used in Study.

<table>
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<tr>
<th>Sample ID #</th>
<th>Sex</th>
<th>Age</th>
<th>Ethnicity</th>
<th>Self-Reported Race</th>
<th>Ambulatory</th>
<th>Weight (kg)</th>
<th>Height (meters)</th>
<th>Diagnosis</th>
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<tbody>
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<td>16</td>
<td>Non-Hispanic</td>
<td>Caucasian</td>
<td>No</td>
<td>58.1</td>
<td>1.795</td>
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<tr>
<td>631</td>
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<td>3</td>
<td>Non-Hispanic</td>
<td>Caucasian</td>
<td>Yes</td>
<td>12.9</td>
<td>1</td>
<td>Idiopathic Scoliosis</td>
</tr>
<tr>
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<td>3.2</td>
<td>Unclassified CP/genetic Disorder</td>
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<tr>
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<td>Caucasian</td>
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<td>64.3</td>
<td>1.64</td>
<td>Quadruplegic CP/Spasticity</td>
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<td>615</td>
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<td>18</td>
<td>Non-Hispanic</td>
<td>African American</td>
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<td>44.9</td>
<td>1.74</td>
<td>Diabetic CP/Spasticity</td>
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<tr>
<td>606</td>
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<td>18</td>
<td>Non-Hispanic</td>
<td>Caucasian</td>
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<td>51.4</td>
<td>1.74</td>
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<td>593</td>
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<td>Non-Hispanic</td>
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<td>1.74</td>
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<tr>
<td>584</td>
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<td>Non-Hispanic</td>
<td>Other</td>
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<td>1.74</td>
<td>1.74</td>
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Table 2.2: List of Reagents.

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<th>Reagent</th>
<th>Catalog Number</th>
<th>Source</th>
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</thead>
<tbody>
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<td>Alexa Fluor® Secondary</td>
<td>A-11034</td>
<td>Life Technologies, Grand Island, NY</td>
</tr>
<tr>
<td>Antibody 488</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-200kD neurofilament</td>
<td>ab4680</td>
<td>Abcam Biotech Company, UK</td>
</tr>
<tr>
<td>heavy antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Nesprin-1 Antibody</td>
<td>ab24742</td>
<td>Abcam Biotech Company, UK</td>
</tr>
<tr>
<td>Anti-Nesprin-1 antibody</td>
<td>ab5250</td>
<td>Abcam Biotech Company, UK</td>
</tr>
<tr>
<td>BSA</td>
<td>A8577-50ML</td>
<td>Sigma-Aldrich, Saint Louis, MO</td>
</tr>
<tr>
<td>CD271 microbead kit</td>
<td>130-099-023</td>
<td>Miltenyi Biotec, Bergisch Gladbach, Germany</td>
</tr>
<tr>
<td>CD56 multisort microbead kit</td>
<td>130-055-401</td>
<td>Miltenyi Biotec, Bergisch Gladbach, Germany</td>
</tr>
<tr>
<td>Collagenase Type II</td>
<td>CLSS-2</td>
<td>Worthington Biochemical Corporation, Lakewood, NJ</td>
</tr>
<tr>
<td>DMEM</td>
<td>12800-017</td>
<td>Life Technologies, Grand Island, NY</td>
</tr>
<tr>
<td>DPBS</td>
<td>55-031-PC</td>
<td>Corning Life Sciences, Tewksbury MA.</td>
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<td>Ficoll-Paque Premium</td>
<td>17-5442-02</td>
<td>GE Healthcare Life Sciences, Pittsburgh, PA</td>
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<tr>
<td>Hoechst 33258</td>
<td>861405</td>
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<td>H3570</td>
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<td>MACS Buffer</td>
<td>130-091-221</td>
<td>Miltenyi Biotec, Bergisch Gladbach, Germany</td>
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<td>MACS Tissue storage solution</td>
<td>130-100-008</td>
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<td>ProLong Antifade Reagent</td>
<td>P36974</td>
<td>Life Technologies, Grand Island, NY</td>
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<tr>
<td>Triton X-100</td>
<td>T8787</td>
<td>Sigma-Aldrich, Saint Louis, MO</td>
</tr>
</tbody>
</table>
Figure 2.1: Preparation of Muscle Tissue. Skeletal muscle tissue was placed in a Petri dish with DPBS and minced using forceps and scissors to a final size of approximately 1-3 mm³. During mincing, adipose tissue and muscle fascia were removed along with blood vessels, and any other visible debris.
Figure 2.2: Spinner Flask Set Up for Enzymatic Digestion. Spinner flask placed on a stir plate with a hot water circulator set at 37°C to keep the flask warm. A solution of DMEM and 0.0025% collagenase type II was placed in the flask along with minced skeletal muscle. The spinner flask is activated to spin at the lowest setting available.
Figure 2.3: Separation of Mononucleated Cells.  A) After enzymatic digestion supernatant (red) added on top of 15 ml of Ficoll-Paque (clear). B) Separation of mononucleated cells after centrifugation at 1250xg for 20 minutes. The mononuclear layer can be seen as the white cloudy layer at the 15 ml mark. Multinucleated muscle fibers can be seen in the pellet below the Ficoll layer. C) Removal of excess fluid by aspiration enables easy recovery of mononuclear layer by pipetting. D) After removal of mononucleated cells with multinucleated cells remaining in the pellet.
Chapter 3

RESULTS

3.1 Testing of Nesprin-1 Antibodies

To identify a specific antibody for use in the preparation of post-synaptic myonuclei, two commercially available antibodies were screened for efficacy in the differential staining of synaptic myonuclei from non-synaptic myonuclei by nesprin-1 in human skeletal muscle. Antibody ab24742 from Abcam Biotech Company, UK produced images with low background and sufficient nuclear signal for easy detection and exhibited a specific pattern of elevated staining within myonuclei (figure 3.1). Nesprin-1 staining was observed in nuclei in close spatial relationship to NMJs, which are characterized by the clustering of AChRs labeled with fluorescent BTX. Non-synaptic myonuclei showed relatively lower staining with nesprin-1 antibody. NF staining showed the presence of axons, which were likely from the LMN that innervated the NMJ (figure 3.1).

Both synaptic and non-synaptic nuclei were present in the tissue samples. Nesprin-1 staining was predominantly localized on nuclei near NMJs with most other nuclei exhibiting low nesprin-1 levels. A third class of nuclei showed some nesprin-1 staining but were found outside of the imaged NMJ. These nuclei, however, were located in different muscle fibers than imaged NMJs and were likely associated with a NMJs that were out of the plane of the tissue section.

A second antibody generated against nesprin-1 (ab5250) was screened using the same staining procedure (figure 3.2). The resulting stain showed a large number
nesprin-1 stained nuclei that where present both at the NMJ and outside of the NMJ. In figure 3.2 there are 3 NMJs present, determined by BTX clusters, and nesprin-1 stained nuclei can be seen throughout the tissue section.

3.2 Tissue Digestion and Integrity of Isolated Nuclei

Since the ultimate goal of the method is to analyze nuclear DNA, the integrity and DNA content of isolated nuclei were assessed. Homogenates collected after enzymatic digestion and after both enzymatic digestion and homogenization were stained with Hoechst 33258 (blue) and imaged to assess the integrity of the isolated nuclei (figure 3.3). Differential interference contrast (DIC) images of the isolated material were overlaid with fluorescence images of nuclei. After enzymatic digestion individual muscle fibers were observed in solution (figure 3.3 A). The isolated fibers contained nuclei with strong staining. After both enzymatic digestion and homogenizations a population of nuclei appeared intact, exemplified by positive Hoechst staining corresponding with spherical shapes observed in DIC imaging (figure 3.3 B). A large amount of cellular debris was observed without the presence of Hoechst signal. Finally, some nuclei appeared to have abnormal shapes and lower Hoechst signal intensity indicating possible damage to the nuclear membrane and loss of DNA content (figure 3.3 B).

3.3 Fluorescence Activated Cell Sorting

Nuclei stained with both Hoechst 33342 and nesprin-1 were sorted on a BD FACS Aria II™ cell sorter at the Helen F. Graham Cancer Center in Newark, Delaware. FACS sorting was funded by a core center access award from Delaware’s IDeA Network of Biomedical Research Excellence (INBRE). Gating was determined
based upon sample stained only with Alexa Fluor® 488 secondary antibody (figure 3.4 A-B). Additionally, forward scatter and side scatter were observed during gating to monitor sample quality and aid in setting the gating parameters. Given the rarity of the target event and due to the potential for target populations to stick together, larger and more complex events were not discarded and samples were sorted for yield. Samples stained with anti-nesprin-1 antibody with Alexa Fluor® 488 and Hoechst 33358 were sorted. Events deemed positive for both Hoechst staining and nesprin-1 staining were collected and along with events deemed positive for Hoechst staining but negative for nesprin-1 staining (figure 3.4 C-D). For the samples sorted in this study the number of synaptic nuclei sorted and the percentage of synaptic to non-synaptic nuclei was calculated (table 3.1).

3.4 Isolation of Terminal Schwann Cells

The isolation of TSCs was performed with commercially available MACS microbeads to select cells positive for both NCAM (CD56) and LNGFR (CD271). The resulting populations of CD56+ / CD271+ sorted cells were counted (table 3.1). Staining with trypan blue showed CD56+ CD271+ cells remained intact and viable throughout the selection process. Cell yields were consistent across samples when cell yield was adjusted by starting tissue weight (table 3.1).
Figure 3.1: Nesprin-1 Staining At NMJs in Human Skeletal Muscle (Ab24742). A longitudinal section of skeletal muscle, 50 μm thick, stained with Hoechst33258 to show nuclei (blue), anti-nesprin-1(Ab24742) to show nesprin-1(Green), anti-neurofilament antibody to show neurons (Yellow) and BTX to show clusters of AChR indicating the presence of NMJs (Red). Image viewed is a maximum intensity projection compiled from z-stack image. Enriched staining of nesprin 1 antibody is present in nuclei associated with the NMJ. Image viewed is a maximum intensity projection compiled from z-stack image.
Figure 3.2: Nesprin-1 Staining in Human Skeletal Muscle (Ab5250). Staining of a longitudinal human skeletal muscle samples with anti-nesprin-1 antibody, Ab5250 (Green), Hoechst 33258 (Blue), and BTX (Red). Image viewed is a maximum intensity projection compiled from z-stack image.
Figure 3.3: Isolated Nuclei from Homogenized Skeletal Muscle. Nuclei stained with Hoechst33258 (blue) are shown against differential interference contrast imaging. A) An example skeletal muscle fiber from the multinucleated layer after enzymatic digestion of muscle tissue. Individual fibers were found intact containing nuclei. B) After homogenization multiple isolated nuclei appear with normal morphology with Hoechst staining. Some nuclei appear damages based on disruptions in the form of the nuclei in the DIC image. Fragments of muscle tissue remain with some nuclei aggregating on or around the debris while other nuclei appear to remain imbedded in the tissue.
Figure 3.4: Fluorescence-Activated Cell Sorting of Isolated Myonuclei. This figure shows an exemplary sample sorted by FACS. Sample 615 was split into two populations. A) Secondary only control for sample 615. Sample was stained with Alexa Fluor® 488 only. B) Quantification of events in each quadrant for secondary only stained sample. C) Sample 615 stained with Hoechst 33342 and anti-nesprin-1 antibody conjugated to Alexa Fluor® 488. Gating was set based upon secondary only sample. D) Quantification of events for fully stained sample 615. Quadrant 2 represent the target population of synaptic myonuclei showing high nesprin-1 signal and high Hoechst signal compared to the control. Quadrant 4 represents Hoechst positive nuclei that are nesprin-1 negative.
### Table 3.1: Quantities of Schwann Cells and Synaptic Myonuclei Isolated.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Tissue Weight (g)</th>
<th>Schwann Cells</th>
<th>Schwann Cells/g</th>
<th>Synaptic Myonuclei</th>
<th>Percentage of Synaptic Myonuclei to non-synaptic nuclei (Q2/Q4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>584</td>
<td>4.4</td>
<td>52,000</td>
<td>11,818</td>
<td>1,433</td>
<td>5.6%</td>
</tr>
<tr>
<td>606</td>
<td>1.2</td>
<td>28,000</td>
<td>23,333</td>
<td>789</td>
<td>N/A</td>
</tr>
<tr>
<td>615</td>
<td>2.3</td>
<td>34,000</td>
<td>14,782</td>
<td>3,953</td>
<td>5.05%</td>
</tr>
<tr>
<td>630</td>
<td>8.06</td>
<td>123,000</td>
<td>15,260</td>
<td>2,483</td>
<td>0.36%</td>
</tr>
</tbody>
</table>

Data associated with each sample used in this study is shown in this table. The starting weight of the tissue after excess debris and adipose is reported in grams. The number of SCs isolated from the sample were counted and the number of SCs per gram is shown. The quantity of synaptic nuclei was determined by the number of events double positive for nesprin-I and DNA sorted during FACS. Finally, the ratio of synaptic nuclei to non-synaptic nuclei was calculated.
Chapter 4

DISCUSSION

The NMJ is a complex synapse which is developed and maintained by the LMN ending, post-synaptic myonuclei in the innervated muscle fiber, and TSCs. Abnormalities in NMJ structures have been linked to genetic alterations in these cells (Engel et al., 2003; Shen et al., 2011) and to acute nerve or muscle injury (Kang, Tian, Mikesh, Lichtman, & Thompson, 2014; Reddy et al., 2003). Recent data indicate that NMJs in children and adolescents with CP are also disrupted, but these disruptions are not associated with genetic mutation or acute injury, and the mechanisms accounting for the sustained NMJ disruptions seen in CP are not known (Robinson et al., 2013; Theroux et al., 2005).

There are multiple methods by which protein expression control might be sustainably altered. The potential for epigenetic regulation is particularly interesting in CP due to the developmental time-frame of the presumed CNS disturbance or injury. The CNS disruption associated with CP occurs early in life while the motor system is still developing, but abnormal NMJs are found in CP patients many years after the original CNS injury (Robinson et al., 2013). Interestingly, studies in rodent models have shown that injuries to NMJs can often be recovered and that NMJs are capable of remodeling and reforming functional synapses after disruption (Kang et al., 2014). While this regenerative ability may be specific to rodents, the disruption of NMJs in CP appears to remain static. This static alteration of NMJs in CP may be due to abnormal epigenetic patterning that can persist a long time after the initial CNS
disruption occurred and long after motor maturation should have completed (Akins, unpublished).

To begin assessing the potential role of epigenetic regulation in CP, the work presented in this thesis was undertaken to develop a method for the isolation of synaptic myonuclei and TSCs from human skeletal muscle. A promising technique was accomplished using nesprin-1 as a marker for synaptic myonuclei (Apel et al., 2000; Grady et al., 2005). Stained nuclei were sorted by FACS while TSCs were isolating following MACS sorting protocols established by Miltenyi Biotec. This protocol utilized a double positive selection of NCAM (CD56 microbeads) and LNGFR (CD271) in order to isolate Schwann cell populations by magnetic activated cell sorting.

Human skeletal muscle contains high levels of nesprin-1, and synaptic myonuclei have been shown to have higher levels of nesprin-1-alpha staining when compared to non-synaptic myonuclei (Apel et al., 2000; Zhang et al., 2001). I tested anti-nesprin-1 antibodies as a biomarker to differentiate synaptic and non-synaptic nuclei within individual muscle fibers. Staining for nesprin-1 in human skeletal muscle sections supported the expected staining of synaptic myonuclei. Nuclei located near NMJs showed greatly enriched staining compared to nuclei located away from NMJs (figure 3.1). The antibody used in this study was a rabbit polyclonal antibody from Abcam (ab24742) that was generated against nesprin-1-alpha, which is one of two major isoforms found in muscle (Rajgor & Shanahan, 2013; Zhang et al., 2001). Nesprin-1-alpha and nesprin-1-beta isoforms share a large portion of their structure and it is likely this antibody stained both nesprin-1 isoforms. However, given the polyclonal nature of the antibody tested, it is possible that this antibody performed
best in differentially staining synaptic nuclei due to a stronger staining pattern to nesprin-1-alpha epitopes compared to other antibodies. This interpretation is supported by the observation that a different rabbit polyclonal anti-nesprin-1 antibody from Abcam (ab5250) which was generated against nesprin-1, and not any specific isoform, did not show the same nuclear staining preference for synaptic nuclei (figure 3.2). In figure 3.2 nesprin-1 labeled nuclei can be observed throughout the muscle tissue section as nuclei not in close proximity to the NMJ show staining that is as strong as the nuclei in close proximity to the NMJ. Alternatively, it is possible that the difference in staining patterns observed between these two antibodies was due ab2474 having a higher affinity to nesprin-1-alpha, however, given the polyclonal nature of these antibodies and the similarity between nesprin-1 isoforms it is difficult to make this statement definitively.

As shown in figure 3.3, isolation of nuclei from skeletal muscle proved to be a difficult task. The dense fibrous nature of skeletal muscle made homogenization of muscle tissue difficult and resulted in large amounts of cellular debris. Utilizing an enzymatic digestion technique with collagenase type II greatly increased the ability to homogenize tissue as minced skeletal muscle that had not been enzymatically digested often jammed the homogenizer and required a lot of force to pass tissue through the homogenizer. Muscle samples were broken down into individual muscle fibers through enzymatic digestion with collagenase type-2 while being agitated using a spinner flask (figure 2.2 and 3.3 A). Individual muscle fibers in solution required less force to homogenize than tissue that was only minced using surgical scissors. Despite these efforts to reduce the amount of mechanical force needed to homogenize the tissue, it was observed that some nuclei lost DNA content throughout the process of
isolation. This was observed both while imaging isolated nuclei after homogenization and in the spread of DNA signal seen during FACS (figure 3.3B and figure 3.4C). Typically, a population of DNA positive nuclei should be clearly separated from a population of DNA negative events. This would occur if all nuclei remained intact throughout isolation and there was no DNA lost from nuclei. In figure 3.4 C you would expect to find a population of DNA positive events in Q4 that are clearly separated from a population of DNA negative events in Q2 along the x-axis which represents the quantity of DNA signal. In these experiments a range of DNA signal intensities was seen spanning from negative events to positive events and no clearly distinct populations were seen based upon DNA content.

It is likely that the mechanical dissociation of the muscle fibers employed as part of the nuclear isolation protocol damaged some of the nuclei. The disruption of the nuclear membrane due to exposure to shear force during homogenization could have led to the partial loss of DNA. If a nuclear membrane was partly ruptured during homogenization some of the DNA within that nucleus could have leaked out before the membrane resealed. Furthermore, some released DNA may have stuck to other nuclei or cell debris. DNA that sticks to debris could cause events that should have no DNA signal to be observed with elevated DNA signal above the expected DNA negative levels. Additionally nuclei that remained intact may have come into contact with released DNA. As a result, leaked DNA sticking to the sample in addition to nuclei with reduced DNA signal from DNA loss may have caused the range of DNA signal in the events observed (figure 3.4C). Despite these drawbacks, in the method developed in this thesis, mechanical dissociation of the skeletal muscle fibers was critical to release the myonuclei into solution. Enzymatic digestion with collagenase
type II under agitating condition only dissociated muscle samples down to individual fibers. Additional improvements in the yield of isolated nuclei may be possible with further exploration of methods for enzymatic digestion and mechanical dissociation of tissue.

In order to maximize nuclear yield, a balanced approach that is aggressive enough to dissociate nuclei from their fibers but that maintains the integrity of the nuclei and the enclosed DNA may be essential. This isolation of nuclei is also complicated by the differences in nuclear anchoring between synaptic and non-synaptic nuclei as an increased level of nesprin-1, like that found in post synaptic myonuclei, would be expected to lead to stronger biomechanical interactions between synaptic nuclei and the actin cytoskeleton compared to non-synaptic nuclei. Sample variability and differences in surgical procedures may also affect sample integrity. Most notably, the use of cauterizing instruments during surgical biopsy acquisition correlated with an inability to isolate nuclei from tissue. Muscle tissue samples that had been subjected to cauterization were difficult to dissociate and antibody staining was poor likely due to heat destroying the antibodies epitope. Increasing the efficiency with which nuclei can be isolated would enable further refinements of this protocol for use on smaller tissue sample, which is important for studies involving small biopsies from surgical patients. The addition of a second enzymatic digestion after isolating muscle fibers with collagenase type II may be helpful in reducing the amount of mechanical force needed to homogenize the tissue. If the amount of mechanical force the nuclei are subjected to can be decreased the integrity of the nuclei isolated may increase.
After the isolation of nuclei, synaptic myonuclei were targeted by staining with antibody for nesprin-1. Nesprin-1 was chosen as a target for isolating synaptic nuclei for a number of reasons. The structure of nesprin-1 with its KASH domain that translocates the outer nuclear membrane and the spectrin repeat and calponin homology domains located outside the nucleus is ideal for staining isolated nuclei (figure 1.6). Antibodies targeting the spectrin repeat domains of nesprin-1 can reach and bind to its epitope without the need for fixation or permeabilization of the nuclei as nesprin-1 serves as a similar target to cell surface markers commonly used for sorting whole cells through both MACS and FACS techniques. Furthermore, nesprin-1 is likely to remain intact even after enzymatic and physical dissociation of muscle fibers due transmembrane domain spanning the outer nuclear membrane. If the nesprin-1 protein was destroyed during nuclear isolation the nucleus would likely have been ripped apart as well. Finally, nesprin-1 is also a good target for synaptic nuclei isolation as it, to some degree, avoids the issue of staining for genes that may be differentially expressed under pathological conditions. Other genes differentially expressed in synaptic myonuclei may be genes of interest in studying pathologies associated with NMJs. Screening the efficacy of nesprin-1 for the isolation of synaptic nuclei will need to be done for any pathology of interest.

Skeletal muscle nuclei isolated from their fibers were stained with nesprin-1 under conditions similar to those of methods targeting cell surface markers. Nuclei were sorted based on DNA content determined by signal strength of Hoechst 33342 and by nesprin-1 signal in comparison to gates set by secondary only controls (figure 3.4). Utilizing a double positive selection method enabled the isolation of both synaptic and non-synaptic nuclei by collecting events that were double positive for
both diploid DNA content and nesprin-1 and events positive for DNA with no nesprin-1 signal (figure 3.4 Q2 and Q4 respectively). FACS sorting was complicated due to the high amount of cellular debris that remained after homogenization of muscle fibers and sample preparation. The rarity of synaptic nuclei within the sample also made sorting difficult (figure 3.4 D). Synaptic nuclei represented 1.3% of the total events while non-synaptic nuclei represented 25.74% of the population and negative events (i.e., nesprin negative and either low or no DNA) accounted for 72.96% of total events (figure 3.4 D). Lack of sample purity is likely responsible for the high level of negative events and may have been due to the homogenization of muscle fibers, which generates a large amount of tissue debris. Cell straining techniques were used in an effort to reduce the amount of cell debris in the sample after homogenization however, large amounts of debris remained. Density-gradient centrifugation could help decrease the amount of debris, but such an additional step could also lead to further sample loss and would require further evaluation.

The ratio of synaptic nuclei to non-synaptic nuclei varied in the samples assessed during development of the isolation technique: 5.05% for sample 615, 5.6% for sample 584 but only 0.36% for sample 630 (table 3.1). The ratio of synaptic to non-synaptic nuclei is of interest because it is generally expected that 2-5 synaptic nuclei will occupy an NMJ in a muscle fiber that typically contains 100-120 nuclei (Bruusgaard et al., 2003). Based on this expectation, and since we have a relatively pure population of muscle fibers from the density separation of mononucleated cells from multinucleated cells with a Ficoll gradient, a yield of about 2-5% was expected. In the case of sample 630, the low ratio of synaptic to non-synaptic myonuclei may have been due to the insufficient quantities of nesprin-1-antibody used on a sample
that was much larger in size than other samples used in this study. Sample 630 was 8.06 grams, which was much larger than previously sorted samples (table 3.1). The staining procedure for nesprin-1 antibody was kept consistent across all samples, however in this case the amount of antibody used may not have been sufficient for the amount of nuclei isolated. Alternatively, the tissue sample may have been collected from an anatomic site with a relatively low number of innervations sites; innervation sites in some muscles are highly localized, and the biopsy may have simply come from a region of muscle with a low number of NMJs. Interestingly, the number of Schwann cells isolated for sample 630 was comparable and slightly better than other samples suggesting that the tissue dissociation procedure and sample integrity were not compromised due to sample size (table 3.1).

Some nesprin-1 positive events showed up in association with very large particles in forward scatter detection during set-up and gating. This was possibly due to nesprin-1 positive synaptic nuclei staying together through homogenization due to the high structural rigidity of the LINC complex formed. If synaptic nuclei remained clustered through staining and FACS, the clustered synaptic nuclei could show double or triple the quantity of nesprin-1 and a corresponding increase in the Hoechst signal. The clustering of myonuclei in this way would not have affected the separation, and overall, nesprin-1 staining showed strong signal over background in both FACS sorting and in the histological preps. The staining was highly-specific to synapse-associated nuclei in tissue sections, which indicated that nesprin-1 is a good target for the differential staining of synaptic myonuclei. Due to the potential for synaptic nuclei to remain clustered and the efficacy of the nesprin-1 stain, samples that appeared larger, more complex or with high signal intensity were not discarded during sorting.
Isolation of cells from skeletal muscle facilitated by MACS sorting using CD56 and CD271 microbeads proved successful (Table 3.1). The identity of the isolated cells as TSCs requires more characterization as CD56 and CD271 are present on other cell types, but the method was successful and shows potential for the collection of TSCs from muscle tissue in an efficient manner. It is likely this selection based upon CD56 and CD271 is also collected SCs and mesenchymal stem cells (Battula et al., 2009). Cell yields were consistent between most muscle samples when adjusted for tissue volume (Table 3.1); however, this isolation procedure may not be specific for TSCs. The number of TSCs in a tissue sample should be closely related to the number of synaptic nuclei (Bruusgaard et al., 2003; Reddy et al., 2003). Both synaptic nuclei and TSCs are being isolated from the same muscle biopsy, which inherently means the same number of NMJs for both isolations. The number of TSCs and synaptic nuclei per NMJ should both be around 2-5, and, therefore, under ideal circumstances the yield of synaptic nuclei and TSCs would be similar. From the data in Table 3.1 we can observe that the number of cells isolated was much larger than the number of synaptic nuclei. There are two possible explanations for this. First, during isolation, synaptic nuclei are subjected to larger amounts of mechanical stress from homogenization step that the mononuclear cells are not subjected to. This could cause a loss in the yield of nuclei and account for this discrepancy in yield. Secondly, the isolation procedure used to isolate TSCs may have isolated all SCs (or even other CD56 and CD271 positive cells present). Of these possible explanations, it seems more likely that all SCs were collected using the microbead selection procedure. It may be possible to improve TSC purity by negatively selecting SCs expressing
myelin-associated glycoprotein (MAG). Since TSCs are non-myelinating glial cells, it may be possible to segregate them from SCs by targeting this functional difference.

Abnormalities in synapses have been discovered in a variety of disorders. Research into the epigenetic and expression profiles of synaptic myonuclei and their role in diseases of the NMJ have been limited to the analysis of whole muscle tissue, which ignores both the functional differences of these distinct subsets of nuclei and the roles of TSCs at NMJs. The work described in this thesis provides initial details for methods to isolate synaptic myonuclei and TSCs from the same muscle biopsy. The ability to isolate multiple specific sample types from the same biopsies has many advantages. In addition to the SCs isolated from the mononuclear cell layer, other members of the Akins lab (for example, Stephanie Yeager, a UDel graduate student) were able to design protocols for isolating satellite cells from the same mononuclear cell population. Thus, three unique population of interest were able to be prepared from a single sample. It may be possible to isolate even more cell types, and if the multinucleated tissue layer were confirmed to be predominantly muscle fibers, it might be possible to isolate non-synaptic myonuclei in addition to the synaptic myonuclei. Another advantage to isolating multiple cell types from the same biopsy is the ability to correlate measures and limit patient-to-patient variance that might occur. This may be especially important when looking at NMJs, which in certain muscles localize to specific regions with other portions containing few if any NMJs.

The utilization of this method for isolating Schwann cells and synaptic myonuclei from skeletal muscle could provide a particularly powerful tool for understanding the functional role of epigenetic regulation at NMJs. Using nesprin-1 to isolate synaptic myonuclei should also be applicable in rodent models, however
careful screening for difference in nesprin isoform variations and developmental differences would be needed to ensure that synaptic nuclei are being targeted specifically. There is also a possibility of applying this methodology to study the role of epigenetics in NMJ formation in a developmental context with a rodent model. If the time course of nesprin isoform expression was established, it may be possible to isolate nuclei from separate developmental time points depending on when nesprin-1 becomes highly expressed in synaptic nuclei.

Overall, this thesis shows methods for tissue dissociation and the isolation of presumptive Schwann cells and synaptic myonuclei from single human skeletal muscle biopsies. This method should prove useful for epigenetic studies of NMJs. In particular, this method should allow the examination of methylation patterns in functionally-distinct subsets of muscle nuclei and could also be used for examining other nucleoproteins and non-coding RNAs found in the sorted nuclei. The ability to examine the function and regulation of synapse associated nuclei may help elucidate mechanisms of NMJ development, maturation, maintenance, and repair in both disease and in health.
REFERENCES


Appendix A

INSTITUTIONAL REVIEW BORD APPROVAL FORMS

A.1 IRB Approved Parental Permission Form – Nemours Biobank

Approved by the Nemours IRB: Valid from: 08/08/2014 through 08/07/2015
IRB# WIL: 349465

Abbreviated Study Title: Nemours Biobank

Nemours Parental Permission for Participation in an Observational / Non-Interventional Research Study
Version February 2014

You have been asked to permit your child to be in a research study. If you are a parent or legally authorized representative of a child who may take part in this study, permission from you is required. This form explains the research, your child’s rights as a research participant, and any responsibilities that you may have as a result of your child’s participation. You should understand the research study before you agree to permit your child to be in it. You will receive a copy of this form. Read this permission form carefully. You may also talk with your family or friends about it. A research team member will answer any questions you have before you make a decision.

1. WHAT IS THE TITLE OF THE STUDY?
   The title of the study is the Nemours Biobank.

2. WHO IS IN CHARGE OF THE STUDY AT NEMOURS?
   If you have a question, complaint, or problem related to the study, you can call the investigator anytime at the numbers listed below.
3. **WHO SHOULD RESEARCH PARTICIPANTS CONTACT ABOUT THEIR RIGHTS?**

If you have questions about your child’s rights as a research subject, what to do if your child is injured, if you would like to offer input or obtain information, or if you cannot reach the investigator or want to talk to someone else who is not involved with this research, you may contact the persons listed below.

Carlos Rosé, MD, CIP, Chairperson, Nemours IRB 1 at 302-651-5970  
Paul Garfinkel, MSH, CIP, Director, Nemours Office of Human Subjects Protection at 904-697-4023  
Email address: NOHSP@nemours.org

4. **WHAT IS THE PURPOSE OF THE STUDY?**

The purpose of this research study is to collect and store samples, such as tissue and blood samples, as well as information about your child so that researchers can use them to help find the causes and new treatments for conditions that affect children. Samples stored in the Biobank will be given to Nemours researchers for genetic and other testing. Genetic testing looks at pieces of your child’s DNA, called genes, that provide the instructions needed to make our bodies work.

The Nemours Biobank is a part of Nemours Department of Biomedical Research. The Biobank collects tissue and other samples for use in research and will collect samples for this project.

5. **WHO IS SPONSORING OR PAYING FOR THE STUDY?**

Nemours is the Sponsor of this study. Nemours will pay for its costs in conducting this study.

6. **WHO CAN BE IN THE STUDY?**

All children that are treated at any of the Nemours locations [Florida or Delaware] can provide samples to the Biobank. Some members (blood relatives) of the patient’s family may also provide samples.

7. **HOW MANY OTHER PEOPLE WILL BE IN THE STUDY?**

We expect samples from hundreds of people.
8. **HOW LONG WILL PARTICIPATION IN THE STUDY LAST?**
   If you have agreed to allow your child’s samples to be banked for future research, his/her samples will be stored indefinitely. This study may involve one extra visit if a blood sample was not obtained at the time your child had a procedure done for his/her medical care.
   There is no set time for destroying the information that will be collected for this study. Your permission to use and share the information and data from this study will continue until the research study ends and will not expire unless you or your child (when he/she becomes an adult) withdraws permission (see Section 12). Researchers continue to analyze data for many years and it is not possible to know when they will be completely done.

9. **WHAT ARE THE RESEARCH PROCEDURES?**
   There are several different ways that your child’s samples may be added to the study. Project staff will explain what your child’s options are from those listed below, should you choose to participate:
   - As long as your child remains enrolled in this study, if your child has a procedure (surgery or biopsy), tissue that is removed but not needed for their medical care will be collected at that time and stored for future research use. In most cases only leftover tissue that would normally be thrown away will be stored.
   - In some cases, we may ask for extra tissue to be taken specifically for the Biobank study. This sample will only be taken from tissue that is already being exposed or cut during the procedure. The tissue will be collected in a way that does not alter your child’s procedure and recovery, and will only be collected at the discretion of your child’s doctor. In these cases, the project team or your child’s doctor will discuss this with you and your family (Please see Appendix A).
   - Two (2) tubes of blood (about 2 teaspoons total) may be taken for the purpose of this study, stored and used for future research. The amount taken may be less than 2 teaspoons if your child does not weigh a lot. If blood was not collected during a procedure, a blood draw may be needed.
   - A urine sample may be collected, stored, and used for future research.
   - A saliva (spit) sample or a cheek swab (also called a buccal swab) may be taken, stored, and used for future research. The cheek swab involves taking what looks like a cotton swab and rubbing it on the inside of your child’s cheek for about 10 seconds with the same force used to brush your teeth.
   - Information about your child and your child’s health will be collected from their medical records, stored, and used for research. This will include information such as diagnosis, treatment, test results, and outcome. Information about your child will be collected when they enter the project and then periodically in the future. Information will also be collected at the time of any surgery or procedure when any samples are collected for the study.
• Relatives of children who take part in this study may also be asked for a blood sample (about 10mL, or 2 teaspoons) to store and use for future research. This blood sample is optional and not required for your child to be in the study. Relatives will be asked to sign a separate consent form for this sample. We will not be routinely collecting medical information about adult relatives.

Patient care is always a priority at Nemours. In the rare event that your doctor needs to retrieve tissue that is already in the Biobank for studies that were not foreseen at the time of banking, they could retrieve the sample or part of it for that purpose by simply requesting tissue to the Biobank staff. Unfortunately because of the processing, this possibility cannot be extended to blood, urine or bone marrow samples. If your child returns to Nemours for treatment or surgery which generates additional samples, we would like to collect and store those also.

**Future Research Use of Samples**
Any remaining portion of your child's sample(s) will be permanently stored for future research use. These samples and health information about your child might be used by other investigators for genetic research about other diseases. By participating in this study, you are also agreeing to this use of your child's sample(s) and health information.

**10. WHAT ARE POSSIBLE RISKS OF BEING IN THIS STUDY?**
Any research has some risks (things that could make your child sick, make your child feel uncomfortable, or hurt your child). The risks with the most chance of happening to someone in this study are listed below. Not all the risks can be foreseen; however, we consider this study to be of minimal risk.

The greatest risk to your child is the accidental release of information from your child's health records to someone who should not have it. Nemours will protect your child's medical records so that your child's name, address, and phone number will be kept private. Your child's participation in this research will be held strictly confidential and only a code (ID) number will be used to identify your child’s stored samples and data. The chance that this information will be given to someone else is very small.

Blood drawing may cause pain, bruising, bleeding, or infection at the site of the needle stick. Care will be taken to try to prevent this from happening. Rarely, taking blood may cause fainting or infection. If possible, the research blood sample(s) will be taken at the same time your child is having blood drawn for clinical care or through an IV catheter (small tube) if your child already has one inserted into a vein.

Possible risks related to the cheek swab could include irritation in the cheek where the swab was rubbed.
The tissue (like muscle or bone marrow) for the Nemours Biobank is only collected from routine operations and procedures, and is not needed for the doctors to make a diagnosis. The care of your child is not changed.

Genetic Research
A Federal law, called the Genetic Information Nondiscrimination Act (GINA), reduces the risk of discrimination by health insurance companies, group health plans, and most employers based on your genetic information. This law protects your child in the following ways:

- Health insurance companies and group health plans may not request your child’s genetic information we get from this research.
- Health insurance companies and group health plans may not use your child’s genetic information when making decisions regarding your child’s eligibility or premiums.
- Employers with 15 or more employees may not use your child’s genetic information that we get from this research when making a decision to hire, promote, or fire you when setting the terms of your employment.

Be aware that this new Federal law does not protect your child against genetic discrimination by companies that sell life insurance, disability insurance, or long-term care insurance.

NOTIFICATION FOR FLORIDA RESIDENTS:
By Florida law, the results of any DNA analysis, which includes DNA typing and genetic testing, are the exclusive property of the person tested, and may not be disclosed without your permission (as provided in this form). Nemours is notifying you that the DNA analysis described in this document will be performed as described, and results will be provided to those individuals and/or groups noted in the section on ‘use and disclosure of PHI’. At your request, Nemours will provide the results of DNA analysis that it receives to your child’s primary care physician. The DNA analysis is performed strictly for research purposes and will not be used in any decision regarding insurability, employment, mortgage, loan, credit or education opportunity.

11. WHAT ARE POSSIBLE BENEFITS OF BEING IN THIS STUDY?
You should not expect you or your child to benefit directly from this study. No specific research test results, findings or discoveries about your child or their health will be shared with you and your family, but it is important to understand that the care they receive will not change. It is hoped that the conclusions derived from the research using the data and samples from this study will help to better understand diseases and conditions that affect children. This may help other children in the future, and lead to improved ways of treating diseases in children.

12. IS BEING IN THE STUDY VOLUNTARY?
Being in this study is totally voluntary. Anyone who takes part in the study can stop being in it at any time. There will be no change to your child’s usual medical care if you decide not to permit your child to be in the study or decide to stop
your child’s participation in the study. No one will be angry with you or your child, or treat your child any differently than before your child was asked to be in the study.

If you decide now that your child’s tissue can be kept for research, you can change your mind later. If you do this, your child’s samples left in the Biobank will be destroyed. No additional updates will be sent to the Biobank from your child’s medical record.

To take back your authorization, you must send a letter to the doctor in charge of the project (information located on page 1 of this document). In the letter, you must say that you changed your mind and do not want us to collect any more health information about your child and you want your child’s samples to be destroyed. If you ask that we no longer collect your child’s health information for the clinical updates, your child’s participation in the project will end. It is important to know that any research that has already been done on the samples cannot be changed.

When your child turns 18 (becomes an adult) we will attempt to contact your child to tell him/her that their samples and medical information are being stored in the Nemours Biobank because you gave your consent when they were a child. We will ask for their consent to continue to store those samples and information. If they decide that they do not want their samples stored in the Biobank, we will destroy any remaining samples, and no additional updates will be sent to the Biobank from their medical record.

13. WHAT ARE THE COSTS OF BEING IN THIS STUDY?
There is no cost billed to you or your insurance company for any aspect of this study. The only cost to you and your child is a small amount of time for collecting samples.

14. WILL MY CHILD BE PAID FOR BEING IN THIS STUDY?
No arrangement exists that would allow participants to share in any profit generated from this study or future research.

15. WILL I BE TOLD OF ANY NEW INFORMATION THAT MIGHT AFFECT MY WILLINGNESS TO PERMIT MY CHILD TO STAY IN THE STUDY?
Any new information that may change your mind about allowing your child to be in this study will be given to you. A committee called the Institutional Review Board (IRB) will review this study at least once per year. If the IRB finds that there is new information that you should know about while your child is taking part in this study, it will ask the study doctor to tell you about it. You may be asked to sign a new version of this form after discussing the new information with a member of the research team.
16. WHAT INFORMATION ABOUT MY CHILD WILL BE USED OR DISCLOSED?  
(AUTHORIZATION TO USE AND / OR DISCLOSE PROTECTED HEALTH INFORMATION)

Identifiable health information about you or your child will be maintained by the
Biobank, but will not be disclosed to researchers receiving your child’s tissue
unless specific approval is obtained from the Institutional Review Board (IRB).
This is done to conduct the research project, to monitor the safety of research
participants and for auditing. Federal law requires us to tell you about, and get
your approval for research use and disclosure of health information that
includes “identifiers” that can connect the health information to you or your
child. (Names, initials, date of birth, addresses, phone numbers, and social
security numbers are examples of identifiers.) This Identifiable health
information is called Protected Health Information (PHI).

Use of Health Information by Nemours Researchers

The health information that will be used within Nemours includes all data collected for
this project. The following health information may be collected during this project:

- Demographic information, such as age or sex
- Personal medical history
- Current, past and future medications, therapies, surgeries, procedures
- Current, past and future hospitalizations
- Information from current, past and future physical examinations
- Information about the pathology (where doctors use a microscope to look at
  samples to make a diagnosis) of the blood, bone marrow, and/or tissue
  samples
- Other current, past and future medical or information from pathology,
  therapies or clinical tests that may be pertinent to research studies, including
  chromosome profiling and genetic testing

Nemours will protect your child’s health information by allowing only authorized
Nemours, Hospital and Project staff to have access to paper and electronic copies.
Study records are kept in secure offices and in password protected computer files.
The research results may be presented at meetings or published in journals to inform
other doctors and health professionals. Participants’ identities will not be disclosed in
those presentations.

To be sure information about your child is kept private; Biobank staff will assign your
child a unique identification (ID) number. This unique ID number (code) will be used
instead of your child’s name or other identifying information and the researchers will
not be able to identify your child.

Specimens, including slides, blood samples, bone marrow, tissue, urine samples or
cheek swabs will be labeled with your child’s name, the date, and type of specimen.
Once the Biobank receives these samples, the information will be recorded and
stored in a password-protected database. This database may only be accessed by
the Biobank staff with proper authority. Once the information has been transferred to
the research database, the samples will be stored with a unique identification number, which can only be identified in the password-protected database. It is crucial that the samples are originally collected with your child’s name in order to avoid errors.

The people that may view this health information may include:
- The investigators listed on the first page of this permission form and their staff
- The Nemours Institutional Review Board (IRB) (The IRB is a group of people that reviews research activities. The IRB is responsible for the safety and rights of research participants)
- Nemours internal audit staff
- U.S. and international governmental regulatory agencies involved in keeping research safe for people

By law, Nemours is required to protect your health information. The research staff will only allow access to your health information to the groups listed above. By signing this document, you are authorizing Nemours to use and/or release your child’s health information for this research. Some of the organizations listed above may not be required to protect your information under Federal privacy laws. If permitted by law, they may be allowed to share it with others without your permission.

**Disclosure of Health Information to Others**
Identifiable health information will not be disclosed outside of Nemours
17. SIGNATURES:
I am making a decision whether or not to permit my child to participate in this study. I understand that my child may also have to agree to participate in the study before he/she will be allowed to be in this study. I have read this form, or have had it read to me in a language that I understand. I have been given enough time to make this decision. I have asked questions and received answers about things I did not understand. I willingly give permission for my child to participate in this study. By signing this form, I am not giving up any rights to which I am entitled under law.

I understand that:
- I can withdraw permission for my child’s participation in this study and for the use and/or disclosure of my child’s PHI by contacting the person in charge of the study listed on the first page of this form.
- The use and/or disclosure of my child’s PHI will stop after Nemours receives the withdrawal notice. Information that is used or disclosed before the withdrawal may still be used.
- Unless I withdraw permission, the use and/or disclosure of my child’s PHI described in this form will not have an expiration date.
- My child’s PHI may be disclosed again by the person or organization (other than Nemours) that receives it. If this happens, Federal or state law may not protect the information.
- I have the right to refuse to sign this permission form.
- If I refuse to sign this permission form, my child will not be allowed to be in this research study.
- I have the right to ask Nemours to tell me who has received my child’s protected health information.
- I have the right to revoke my permission for the use and disclosure of my child’s health information at any time, which would end his/her participation in this study.
- I will receive a signed and dated copy of this form.

My signature indicates that:
- As his or her parent or legally authorized representative, I give my permission for the minor child named below to participate in the research study described in this Parental Permission Form.
- I give the researchers and Nemours permission to use and/or disclose my child’s individually identifiable health information for this research study as described in this form.

Name of Participant (Print)_________________________ Participant Date of Birth:_________________________
<table>
<thead>
<tr>
<th>Name of Parent / Legally Authorized Representative (Print)</th>
<th>Signature of Parent/ Legally Authorized Representative</th>
<th>Date</th>
</tr>
</thead>
</table>

**Check Relation to Participant:**  
- [ ] Parent  
- [ ] Legally Authorized Representative  

(Legally Authorized Representatives must have documented authority to give permission for a child’s participation in a research study according to the laws of the State in which the treatment occurs.)

I, the undersigned, certify that to the best of my knowledge the parent / legally authorized representative signing this permission had the study fully and carefully explained and that she / he understands the nature, risks and benefits of his /her child’s participation in this research study.

<table>
<thead>
<tr>
<th>Name of Person Obtaining permission (Investigator or Designee)</th>
<th>Signature of Person Obtaining permission</th>
<th>Date</th>
</tr>
</thead>
</table>

Copy of the signed form was provided to Parent/ Legally Authorized Representative on [Date] ____________

- [ ] Consent was obtained via phone and mail/fax method.
A.2 IRB Approved Patient Assent Form Ages 12-17

Approved by the Nemours IRB: Valid from: 08/08/2014 through 08/07/2015
IRB# WIL: 349465

Abbreviated Study Title: Nemours Biobank

ADOLESCENT ASSENT FORM FOR YOUTH AGES 12-17

Your parent has given permission for you to be in a project called a research study. But first, we want to tell you all about it so you can decide if you want to be in it. If you don’t understand, please ask questions. You can choose to be in the study, not be in the study or take more time to decide.

1. What is the name of the study? The name of the study is the Nemours Biobank.

2. Who is in charge of the study? The doctor in charge of the study is Dr. Diana Corao in Wilmington, Delaware.

3. What is the study about? We would like to find out why some children get sick and hopefully find new ways to help treat illnesses in children. To do this we would like to store some information about you, some of your cells, blood, urine (pee), or saliva (spit) in the Biobank.

4. Why are you asking me to be in this study? You are being asked to be in the study because you are a patient at Nemours.

5. What will happen to me in the study? If you agree to be in the study, here’s what will happen: when you are at Nemours receiving normal treatment, we will collect samples of blood, urine (pee), and saliva (spit) for lab tests for this study. If you are having a procedure (like an operation) to remove tissue, we will get a sample of that tissue. Information from your medical chart will also be collected. This study will not change the care you would normally receive at Nemours.

   There might be some things about being in this study that you won’t like. The blood tests may make you nervous and the tiny needle may hurt and bruise your arm. We are required by law to protect your health information but there is a very small chance that someone outside of Nemours might see your health records. There might be some other things about being in this study that you will like. We might find out why certain illness or diseases happen and also get information that will help to develop treatment for illness in children.

6. Will I be paid to be in this study? You will not be paid for being in this study.

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7. **Do I have to be in the study?** You don’t have to do the study if you don’t want to. If you are in the study, you can stop being in it at any time. Nobody will be upset with you if you don’t want to be in the study or if you want to stop being in the study. The doctors and nurses will take care of you as they have in the past. If you have any questions or don’t like what is happening, please tell the doctor or nurse.

You have had the study explained to you. You have been given a chance to ask questions. By writing your name below, you are saying that you want to be in the study.

________________________________________  ___________
Adolescent’s Signature                      Date

________________________________________  ___________
Name of Person Obtaining Assent (Print)     Signature of Person Obtaining Assent  Date
ASSENT FORM FOR CHILDREN AGES 7 - 11

Your parent has given permission for you to be in a project called a research study. But first, we want to tell you all about it so you can decide if you want to be in it. If you don’t understand, please ask questions.

8. What is the name of the study?
The name of this study is the Nemours Biobank.

9. Who is in charge of the study?
The doctor in charge of the study is Dr. Diana Corao in Wilmington, Delaware.

10. What is the study about?
We would like to find out why some kids get sick and help find ways to treat those kids and to help them get better.

11. What will happen to me in the study?
If you are in the study, here’s what will happen: when you are at Nemours getting care, we will get a little of your blood, pee, spit, and maybe some tissue. The doctors will keep these and use them to find out about why kids get sick and how to make them get better.

There might be some things about being in this study that you won't like. The blood tests may make you nervous and the tiny needle may hurt and bruise your arm.

There might be some other things about being in this study that you will like. We might find out things that will help other children who are sick. When you turn 18, we will contact you to see if we can still use your specimens and information.

12. Do I have to be in the study?
You don’t have to do the study if you don’t want to. If you are in the study, you can stop being in it at any time. Nobody will be upset with you. No matter what you decide, the doctors and their helpers will take care of you just like they did.
before. If you have any questions or don’t like what is happening, please tell your parent, the doctor or helper.

You have had the study explained to you. You have been given a chance to ask questions. By writing your name below, you are saying that you want to be in the study.

__________________________  _______________________
Child’s Signature                      Date

Name of Person Obtaining Assent (Print)  Signature of Person Obtaining Assent  Date

☐ The assent information was read to the child by the person obtaining assent.
☐ The child read the assent him / herself.
DATE: December 31, 2012

TO: Robert Akins, PhD
FROM: Nemours Delaware IRB
STUDY TITLE: [381494-2] Neuro-Orthopedic Tissue Repository
IRB #: 381494
SUBMISSION TYPE: Response/Follow-Up

ACTION: EXEMPT
DECISION DATE: December 30, 2012

Thank you for your submission of New Project materials for the above referenced study. Your submission received Expedited Review based upon the applicable federal regulations and meets all DHHS criteria for Exemption. The IRB determined that:

This research is exempt from further IRB review based upon the applicable federal regulations 45CFR46.101(b) under Category 4. Exempt Category 4. Research, involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens,

• If these sources are publicly available or

• If the information is recorded by the investigator in such a manner that subjects cannot be identified directly or through identifiers linked to the subjects.

• If the research does not involve prisoners as subjects.

• And, if the research is not FDA-regulated.

Although the above research in fact qualifies as exempt from further IRB review, and has been identified as such, the Principal Investigator is responsible for notifying the

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IRB, in writing of any changes that may impact on the exempt status of this protocol. Such changes in the above research cannot be initiated without prior IRB approval. If you have any questions, please contact Camille Varacchi at Nemours AI duPont Hospital for Children 1600 Rockland Road, ARB-Room 160A, Wilmington, Delaware 19803, 302-651-6807 or cvaracch@nemours.org. Please include your study title and reference number in all correspondence with this office.
Appendix B

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Daniel Barnes
University of Delaware

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Content to be included is:

- Page 291 Figure 9.8 Summary of events in the generation and propagation of an action potential in a skeletal muscle fiber

- Page 296 Figure 9.10 A motor unit consists of one motor neuron and all the muscle fibers it innervates (a) Axons of motor neurons extend from the spinal cord to the muscle

EXCEPTION: Figure 9.10(b) Branching axon terminals from neuromuscular junctions, one per muscle fiber. Illustrated Credits page lists this figure as Eric Graves/Science Source.

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