UNIVERSITY OF CALIFORNIA, SAN DIEGO

SKELETAL MUSCLE ADAPTATIONS IN HAMSTRING CONTRACTURES OF CHILDREN WITH CEREBRAL PALSY

A dissertation submitted in partial satisfaction of the

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by

Lucas R. Smith

Committee in charge:

Professor Richard L. Lieber, Chair
Professor Shankar Subramaniam, Co-Chair
Professor Henry G. Chambers
Professor Koichi Masuda
Professor Robert L. Sah
Professor Samuel R. Ward

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The dissertation of Lucas R. Smith is approved, and it is acceptable in quality and form for publication on journal of physiology:

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Co-Chair

_____
Chair

University of California, San Diego

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VITA

2003	B.S., Bioengineering University of Washington, Seattle, Washington
2006-2011	Graduate Student Researcher Muscle Physiology Labratory University of California, San Diego, La Jolla, California
2011	Ph.D., Bioengineering University of California, San Diego, La Jolla, California

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ABSTRACT OF THE DISSERTATION

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Professor Shankar Subramaniam, Co-Chair

Cerebral palsy is an upper motor neuron lesion to the developing brain that results in motor impairments. Despite best clinical practices muscle often secondarily develops the pathologic state of contracture, where muscle stiffness limits the functional range of motion. This work aims to elucidate the mechanism of contracture in children with cerebral palsy.

Chapter 2 presents the first transcriptional study of muscle in cerebral palsy performed on both effected wrist flexors and their antagonist extensors. Results show that muscle in contracture is fundamentally different than control muscle, or other muscle pathologies. Many transcripts related to calcium handling were altered in cerebral palsy, the first such evidence of disruption within contracture. Immature

transcripts were present in the muscle suggesting regeneration. This was accompanied by large increases in extracellular matrix transcription. Muscle signaling was confounding with signals of muscle growth and growth inhibition..

To better understand the relationship between gene products Chapter 3 provides a network of proteins specifically related to skeletal muscle function. The functions described include: neuromuscular junction, excitation contraction coupling, muscle contraction, cytoskeleton, extracellular matrix, energy metabolism, inflammation, and muscle hypertrophy and atrophy. These functional networks were created to provide networks specific to muscle for high throughput analysis.

These networks are applied in Chapter 4 to a more comprehensive microarray study on muscle contracture in hamstrings of patients with cerebral palsy. Again muscle in cerebral palsy was distinct from controls and there were many signs of immature muscle. However the fiber type shift was from fast-to-slow, opposite of that seen in chapter 2. A critical consistency was the large increase in extracellular matrix transcripts further implicating fibrosis.

To study the effects of this fibrosis the mechanics of single fibers were compared to that of fiber bundles and their constituent extracellular matrix. Although fiber stiffness was not changed in cerebral palsy an increase in bundle stiffness points to a functional consequence of increased extracellular matrix. This tissue stiffening is exacerbated by evidence of increased in vivo strain imposed on muscle in cerebral palsy. These studies provide the basis for future research into muscle contracture and targets for novel therapeutics.

CHAPTER 1

INTRODUCTION

1.1 General Introduction to the Dissertation

Skeletal muscle is the motor responsible for producing force and voluntary joint movement under control of the somatic nervous system. Voluntary motor signals travel through central nervous system with upper motor neurons and then extend through the peripheral nervous system to muscle via lower motor neurons. Lower motor neurons connect to the skeletal muscle at the neuromuscular junction and release neurotransmitter during an action potential. The neurotransmitter induces an action potential within the muscle that elicits the release of calcium with in the muscle cell. Calcium is the trigger within the sarcomere that allows the interaction of actin and myosin to create force within the muscle. This force is extended through the cell within the cytoskeleton and out to the periphery via the extracellular matrix. This is a highly energetic process that requires tightly integrated metabolic system. The skeletal muscle is very responsive to the neurologically induced signal and undergoes hypertrophy or atrophy accordingly.

Cerebral palsy is a motor disorder that consists of a lesion of the upper motor neurons of the developing brain. This disruption of upper motor neurons, which are often responsible for inhibition of lower motor neurons leads to the hallmark of

spasticity associated with cerebral palsy. Spasticity is a hyper reflexia caused by a lack of inhibition of the reflex system and non-voluntary activation of the lower motor neurons. Cerebral Palsy is a spectrum disorder with various types, of which spasticity is the most common and the focus of this dissertation. Cerebral palsy is also characterized by the region of the body affected and based on the severity. These neurological changes have many secondary effects on skeletal muscle due to aberrant control of the inherent plasticity. These muscular adaptations are where much of the impairement is observed in cerebral palsy with muscle contractures, which restrict the range of motion around a joint, severely limiting motor capability. This dissertation examines the biochemical and biomechanical alterations present in contracture of muscle in cerebral palsy using biopsies from human subjects.

Chapter 2, which has been published in Biomedical Central: Medical Genomics, is a descriptive study examining the transcriptional alterations that in contractured muscle of the upper extremity. The biopsies used for this study include a wrist flexor and wrist extensor extracted during surgery. The study revealed that the transcriptional profile of muscle in cerebral palsy is distinct from controls and that in other muscle disease. It also highlighted many muscle functions in which proteins were altered in cerebral palsy.

Chapter 3, which has been accepted for publication in Wiley Interdisciplinary Reviews: Systems Biology and Medicine, is a review of genes vital for skeletal muscle function. The review was written to facilitate advanced analysis of high-throughput technologies that allow the simultaneous investigation of many genes. The genes and gene products are broken down into important functional networks specific the skeletal muscle.

Chapter 4, which has been submitted for publication in Human Molecular Genetics, is a more extensive study of the transcriptional alterations that occur in hamstring muscle contractures of children with cerebral palsy. The study further establishes unique adaptations of muscle in contracture. Using advanced analytic techniques the importance of functional networks such as the extracellular matrix are established.

Chapter 5, which has been published in the Journal of Physiology, is an investigation of the passive mechanical properties of muscle from hamstring biopsies of children with cerebral palsy. The results show individual muscle fibers are not stiffer in cerebral palsy, but when the extracellular matrix is included the tissue is more stiff. The study also demonstrates muscle architectural changes that have a direct effect and compounding effect of muscle tissue stiffness in cerebral palsy.

Chapter 6, summarizes the major findings and significance of this research. Potential further areas of research are also explored.

1.2 Basic Principles of Muscle Physiology

The mechanical force required for voluntary motor activity is generated within skeletal muscles. The signal begins in the motor cortex is part of the cerebral cortex involved in planning, control, and execution of voluntary motor function. The corticospinal tract contains the upper motor neurons as they extend from the cell bodies in the motor cortex past the ventricle system and into the spinal cord. The spinal cord provides a conduit for the upper motor neurons as they extend to the appropriate spinal level and interact with lower motor neurons. The lower motor neurons extend axons from the spinal cord out to the skeletal muscle at the

neuromuscular junction. Activation of lower motor neurons is complex, tightly regulated, and depends on many pre-synaptic inputs. Much of the signal from the upper motor neurons is inhibitory. The motor neurons responsible for initiating muscle contraction are termed α motor neurons and a single α motor neuron along with the muscle fibers it innervates is referred to as a motor unit.

When a motor unit fires, the neurotransmitter acetylcholine is released from the motor neuron into the synaptic cleft of the neuromuscular junction. Activated acetylcholine receptors on the muscle trigger an action potential to travel across and importantly through muscle cells via the T-tubule system [1]. Voltage gated calcium channels permit calcium to enter the cell and induce a calcium triggered calcium release from the sarcoplasmic reticulum. Calcium binds to the regulatory machinery on skeletal muscle inducing a translocation of the troponin/tropomyosin complex to reveal the myosin binding site on actin [2], collectively termed the thin filament. Force is generated when myosin from the thick filament binds the thin filament and under goes a power stroke to displace the filaments relative to each other, thus the sliding filament theory [3, 4]. A set of thick and thin filaments that interdigitate is referred to as a sarcomere and the basic functional unit of muscle contraction. The sarcomere is bordered by Z-discs, connecting thin filaments from adjacent sarcomeres to form a series of sarcomeres into a myofibril. Myosin in an ATPase, which requires ATP to release the thin filament and repeat the force generating cross-bridge cycle [5]. When calcium is pumped back into the sarcoplasmic reticulum the tropomyosin resumes its inhibitor position on the thin filament and muscle relaxation is initiated.

The nerve has two important methods of controlling the amount of force a muscle produces. Temporal summation achieved when action potentials are generated sequentially in less time than is required for the muscle to relax. The faster the

frequency the greater the calcium concentration within the cell and the more force generating myosin crossbridges that will be formed [6]. Alternatively spatial summation is the simultaneous firing of multiple motor neurons, which in turn activate more muscle fibers. The more fibers activated to promote crossbridge cycling the greater the magnitude of force.

The sliding filament theory leads to important implications based on the length of a sarcomere, referred to as the length-tension curve [7]. Typically divided up into three regions, the length tension curve is based on the fact that the more overlap between thick and thin filaments that exist, the more force a sarcomere will generate. Thus there is an optimal length region when thin filaments directly overlap the thick filaments to produce maximal force, termed the force plateau. As the sarcomere is stretched less overlap exists between filaments and thus less capacity for force generation in a region denoted the descending limb. If sarcomeres are shortened from plateau, opposing thin filaments disrupt crossbridge formation and any shortening beyond the length of thick filament is opposed mechanically. This region is referred to as the ascending limb.

These lengths are all based on a static, or isometric, sarcomere length. Another force determining property is the force-velocity curve. When a muscle undergoes a shortening, or concentric, contraction the force produced is inversely proportional to the velocity of shortening [8, 9]. This is derived from the crossbridge cycling rates, where if filaments are sliding past each other the crossbridge has less chance to form. Alternatively if muscle is activated while being lengthened (eccentric contraction), the force generated by muscle exceeds that produced isometrically. The large force generated in an eccentric contraction is largely independent of lengthening velocity: a property that is not fully understood [10].

Spatial summation indicates that the size of a muscle is a critical component of muscle activity. Further the arrangement of muscle fibers, termed muscle architecture, is fundamental to determining function. The ability to produce force is proportional to the number and size of fibers in a muscle: the muscle cross-sectional area [11]. Many muscles fibers do not run the length of a muscle from origin to insertion parallel to the action of the muscle, but are instead at an angle, termed the pennation angle. The pennation angle is used to correct the muscle cross-sectional area into physiological cross-sectional area, which is a strong predictor of force [12]. The other important architectural component is the fiber length. The fiber length is related to the muscle excursion, the range of lengths in which the muscle can produce force [13]. As described above, the length of fibers and sarcomeres changes as a muscle contracts so it is important when comparing muscles to normalize the fiber lengths. This can be done by using a reference sarcomere length. Muscle architectural measurements are done in order to estimate the number of sarcomeres in parallel, physiological cross-sectional area, and number of sarcomeres in series, normalized fiber length. Various muscles have specialized architectural properties based functional requirements for either high force capacity or high excursion ability [14].

1.3 Basic principles of passive mechanical properties of muscle

The section above describes how skeletal muscle works to produce force and perform work when activated by the nervous system. However, muscles are also capable of generating considerable passive tension, without crossbridge cycling, in response to stretch. Thus a passive length tension curve may be added to the active length tension curve described above. The origin of this passive tension has been

controversial and is not the borne out equally in each muscle. A seminal study in the field showed that in frog muscle much of the passive tension was within a single myofibril [15]. This demonstrates that the impact of intermediate filaments and cytoskeletal structures all the way to the membrane do not play a large role in passive tension. It also mitigates the impact of extracellular matrix components in contributing to passive tension of whole muscle.

It was originally unknown what could be bearing the passive load in the sarcomere in the absence of crossbridges. Eventually the giant protein titin, the largest protein found to date, was discovered and shown to span half of the sarcomere from the Z-disc to the middle of the thick filament and provide a physical link between filaments [16]. This made it a prime candidate for being responsible for the passive tension generated within the myofibril. Indeed experiments where titin is enzymatically removed result in fibers that are very compliant [17]. Furthermore it was demonstrated that titin had many splice variants with much of the variation in the length of the extensible PEVK region. Studies showed that the size and corresponding length of a titin molecular was related to the stiffness, with shorter isoforms being stiffer [18, 19]. This presented strong evidence for titin bearing considerable muscle passive tension.

Later experiments showed that titin did not bear as much of the passive load in other muscle tissue [20]. In this case the primary components responsible for load bearing aside from titin were found to be extracellular. The extracellular matrix is a scaffold linking together muscle fibers on multiple levels. The endomysium surrounds individual fibers, the perimysium surrounds groups of fibers called fascicles, and the epimysium is a layer of connective tissue surrounding whole muscle. The extracellular matrix consists of many components including collagens and

proteoglycans. Collagen is however the primary component of extracellular matrix in muscle thought to be responsible for much of the extracellular matrix stiffness [21, 22].

The passive tension in skeletal muscle is not purely a function of stretch. Indeed a stretched muscle will immediately resist stretch with a high force, followed by a period of relaxing stress to a lower steady state. This stress-relaxation principle makes muscle a viscoelastic material where the speed and time course of stretch are important components in determining passive tension [23, 24]. These additional parameters have complicated models of passive tension in skeletal muscle [25, 26]. It is important to remember that muscle passive tension in not static in vivo.

1.4 Basic principles of plasticity in muscle

Skeletal muscle is a very adaptive tissue and is subject to remodeling the properties discussed previously based on the usage pattern. Muscles that are used in producing high forces respond by undergoing muscle hypertrophy. Studies have shown that eccentric contractions, which produce the highest stresses, elicit the largest hypertrophy signal [27, 28]. After development muscle hypertrophy is accomplished primarily by fiber hypertrophy rather than the addition of new muscle fibers [29]. However, muscles that undergo repeated high stress contractions are subject to damage and injury. Repair of injured muscle often results in fibrotic response.

Conversely, muscles that are not subject to use undergo atrophy, as in the cases of limb immobilization, bed rest, or even space flight [30]. Muscle atrophy is an active process in which proteins are targeted for degradation in order to conserve the

high-energy demand required for muscle upkeep. Furthermore, muscle undergoing atrophy due to immobilization is also subject to a fibrotic response.

Muscle plasticity is often referenced in relation to a change in fiber types. Muscle fiber types have important functional consequences for the muscle. Human muscle has primarily three fiber types, which are based largely on the myosin heavy chain isoform expressed. Type I, or slow, fibers have slower crossbridge cycling rates and produce less force than other fiber types. However type I fibers are highly oxidative and capable of repeated contractions with very little fatigue. Type IIa, fibers have faster crossbridge cycling rates and are capable of producing larger forces than type I fibers. They still maintain oxidative metabolism and are relatively fatigue resistant. Type IIx, are the fastest and most powerful fibers in human muscle. However they have very little oxidative capacity and rely upon glycolytic metabolism for energy demands and are thus very susceptible to fatigue. Each fiber from a motor unit is of the time fiber type so that slow fibers dominate repetitive low force contractions and then fast fibers can be recruited for periodic high force contractions [31, 32]. The proportion of fast to slow fibers varies across muscles depending on their function. With altered usage pattern it is possible to shift the proportion of fiber types within a muscle to a slower phenotype with over-activity, or to a faster phenotype with decreased use. The potential magnitude of these changes is controversial.

Muscle is not only capable of adding sarcomeres in parallel to increase force, but also of adding sarcomeres in series to create longer fibers and greater excursions. Much of the research in the area was done in cats, showing that when muscles are immobilized in a shortened position they reduce the number of sarcomeres in series and when they are placed at longer lengths they add sarcomeres in order to stay at the

same point on the sarcomere length tension curve [33]. This property of muscle is important during skeletal development as limbs extend the length of muscles and also during surgical operations in which muscle length is altered [34]. However, pathologic muscles may not demonstrate the ability to natively adapt the number of sarcomeres in order to maintain optimal sarcomere length.

1.5 Etiology of cerebral palsy

Skeletal muscle is also known undergo plastic changes in response to disease states. The purpose of this dissertation is to examine the response of skeletal muscle in patients with cerebral palsy. Cerebral palsy describes a spectrum of movement disorders caused by injury to the immature brain. It is the most common childhood mobility disorder with a prevalence of 3.6 cases per 1,000 live births in the United States [35]. The prevalence has not decreased with improved medical practices, possibly due to the increase in premature births that have a higher risk of cerebral palsy [36].

The upper motor neuron lesion that initiates the disease process occurs in the developing brain, often prenatally, but up to 3 years of age [37]. The exact etiology is varied, but can be caused by asphyxia, hypoxia, infection, or by trauma. The result is most commonly periventricular leukomalacia, a white matter lesion near the ventricles that has a high concentration of upper motor neuron axons [38]. The loss of connections to lower motor neurons results in the negative features of upper motor neuron syndrome. However, much of the upper motor neuron signal is inhibitory and the loss of inhibition results in positive features of upper motor neuron syndrome.

Few abnormalities are found in the function of lower motor neurons in cerebral palsy aside of their altered input [39].

Although the upper motor neuron lesions in cerebral palsy are non-progressive the secondary pathology often is progressive. The negative features of cerebral palsy result in muscle weakness and increased fatigability due to decreased drive to the lower motor neurons. Poor balance and other sensory deficits are also common negative features of cerebral palsy. The positive features of the upper motor neuron syndrome include hyper-reflexia, clonus, and co-contractions of antagonist muscles. The most researched negative feature is spasticity, defined as a velocity dependent resistance to stretch [40]. The unique mix of positive and negative features often result in muscle contractures, however the mechanism of this not known [41]. Muscle contractures result from a passive inextensibility of muscle that limits range of motion around a joint. Contractures represent a major form of disability in patients with cerebral palsy. Additionally joint contractures have further downstream effects creating bony torsion, joint instability, and often degenerative arthritis.

These secondary effects are often progressive in cerebral palsy. Many patients follow typical timelines of motor function, distinct timelines are described by the Gross Motor Function Classification System (GMFCS) [42]. GMFCS is the most common severity measure used to describe patients who are completely independent for their mobility needs to those who are fully dependent for functional mobility. Cerebral palsy is often classified by the limbs effected as well. Hemiplegia occurs if the brain lesion occurs unilaterally, as patients maintain function on one side of the body. Diplegia is a limited function of the lower extremity and is observed commonly as the lower extremity motor axons travel closest to the ventricles, being most susceptible to damage and malformation. Quadriplegia or total body cerebral palsy

effects all limbs and may involve also involve trunk muscles. Spasticity in muscle groups is often classified by the Ashworth Score, or a modification thereof. However, recent studies call into question the functional significance of spasticity as there is little correlation to functional measures [43]. Contractures in muscle groups are determined by measuring the passive range of motion about a joint.

There are a variety of treatments available for patients with cerebral palsy, primarily focused on limiting the activity of lower motor neurons and the positive features of upper motor neuron disease. Baclofen is a GABA agonist promotes inhibition of lower motor neurons. Baclofen is often given orally, but intrathecal pumps may also be surgically implanted to deliver the drug intrathecally to regulate dosage and limit side effects [44, 45]. Botulinum toxin is a potent inhibitor of acetylcholine release in the neuromuscular junction. Botulinum toxin is delivered by injection and can be used to target muscles individually for a period of 4-6 months [46]. Phenol and ethanol are also used to destroy the neuromuscular junction with longer lasting effects [47]. Neurosurgical interventions include selective dorsal rhizotomy, in which the sensory nerve rootlets are individually identified and tested to determine those that illicit the largest response in spastic muscles. This permanent procedure involves cutting those rootlets in order to reduce spasticity [48]. Physical therapy is almost universally recommended in cases with cerebral palsy in an effort to increase or prevent loss of range of motion [49]. Despite best clinical practices patients often end up with muscle contracture and associated deformities requiring orthopedic surgery. Muscle “lengthening” procedures generally involve tendonotomy, tendon lengthening, or aponeurotic lengthening [50]. All of these treatments for cerebral palsy aim to limit the activity of the muscle and thus weaken an already weak

muscle. This demonstrates the need to further research in order to develop new therapies to treat muscle contracture and preserve or enhance muscle function.

1.6 Histological Properties of Muscle in Cerebral Palsy

A complicating factor in any cerebral palsy research is that there is no commonly accepted animal model available the replicates the disease pathology [51]. This necessitates the use of human muscle biopsies in order to study the adaptations. The most basic form of analysis of muscle tissue it to section for histology. Typically skeletal muscle has tightly packed polygonal fibers and this contractile material represents the vast majority of muscle area. However muscle from patients with spasticity undergoes many histological changes including, increased variability in fiber size, increased number of abnormally shaped fibers, and increased extracellular matrix space [52-56].

Another aspect of muscle that can be examined using a histological staining method is the distribution of fiber type. Although fiber type distributions can also be determined based on myosin heavy chain content [57]. The negative features of upper motor neuron syndrome would predict a shift to a decreased use faster phenotype, while the positive features such as spasticity would predict an increased use shift to slower phenotype. Several studies have reported an increased percentage of slow fibers [52, 55, 58] in patients with upper motor neuron syndrome, while others have reported a shift to a faster distribution of fibers ([59-61]). Still more studies have shown no change in fiber type [54, 56], demonstrating there is no consistent model of activity that encapsulates cerebral palsy muscle.

Additional studies have made use of immunohistochemical techniques to study specific proteins within cerebral palsy muscle. Knowing the etiology is neurological studies investigated the neuromuscular junction. Typically the neuromuscular junction is defined by acetylcholine esterase, a prominent protein involved in breaking down acetylcholine to terminate synaptic transmission and allow reuptake by the presynaptic nerve. In biopsies from cerebral palsy muscle acetylcholine receptors were regularly found outside of the neuromuscular junction [62]. This indicates a disorganization of the neuromuscular junction in cerebral palsy. Another protein investigated with this method was collagen type I [53]. The study showed that overall collagen content as measured by hydroxyproline increases with severity. The histological evidence supported this result and identified the endomysium as the primary area of collagen accumulation. This data supports the claim that muscle in patients with cerebral palsy is fibrotic.

1.7 Architectural Properties of Muscle in Cerebral Palsy

The impact of architectural adaptations in cerebral palsy has also been investigated. There is a general belief within the clinical community that muscle contractures are the result of shortened muscle. Indirect studies performed on both passive and active joint mechanics present inconsistent evidence on fiber lengths [63-65]. Recently techniques using ultrasound have been employed to directly measure fiber lengths in muscle from children with cerebral palsy. Again the results have been inconsistent with some indicating normal fiber lengths [66], and other showing shortened fiber lengths [67]. These discrepancies could be partially resolved based on the use of normalization procedures, including joint position and bone lengths. It is

important to remember that fiber shortening could be the result of shortening the sarcomeres within a muscle or a reduction in the number of sarcomeres in series. Measuring equivalent fiber lengths relative to bone length and joint angle would be possible with much different normalized fiber lengths have important functional consequences. One study has directly measured sarcomere lengths in patients with cerebral palsy, finding dramatically increased sarcomere lengths [68]. This suggests a decrease in normalized fiber length, however since fiber length was not concurrently measured there is still no study directly determining normalized fiber length in cerebral palsy.

Fiber lengths relation to excursion is important in cerebral palsy, but so is cross sectional area and the associated strength of muscle in cerebral palsy. The results of studies into cross sectional area are less controversial with a near universal finding of decreased area in cerebral palsy muscle. MRI studies on hemiplegic patients allow for well-controlled comparisons of muscle area [69]. This study also finds that the decreased area did not fully account for the weakness in cerebral palsy subjects indicating a decrease in functional quality of muscle as well. Further studies have examined muscle thickness using ultrasound as a correlate to muscle area in supporting the findings of decreased area [70, 71]. This decreased thickness has also been shown to correlate with severity in some muscle, emphasizing the functional importance of muscle strength.

1.8 Biomechanical Properties of Muscle in Cerebral Palsy

Spasticity is defined as a velocity dependent resistance to stretch, with which there is an active response of muscle to resist stretch. Previous studies have been

conducted to delineate the active and passive increase in stiffness of spastic muscle through the use of dynamometry, electromyography, and nerve stimulation. While an increase in reflex mediated stiffness may be observed, both studies also implicate the intrinsic passive muscle stiffness as well [72, 73]. This indicates that the passive biomechanical properties of the muscle are being altered in cerebral palsy to increase joint stiffness. Particularly in the case of fixed contracture where there is no reflex contribution, but joint motion is negatively limited by increase in muscle stiffness.

The increased stiffness of muscle in cerebral palsy could be due to alterations within muscle fibers themselves. A study looking directly at the stiffness of isolated and demembranated single fibers from surgical muscle biopsies. By measuring sarcomere lengths and passive force the investigators were able to determine that fibers in cerebral palsy were approximately twice as stiff as controls [74]. This also corresponded to a lower resting sarcomere length in cerebral palsy muscle. The molecules responsible for this altered stiffness were not investigated, but titin is the most likely candidate. Believed to be the major molecule responsible for fiber stiffness, shorter titin molecules in cerebral palsy would suggest both the stiffness increase and decrease in resting sarcomere length [74]. Further studies in stroke victims also found increased fiber stiffness in spastic patients, but only within type IIx fibers [75]. These data suggest intrinsic changes to the fibers themselves contribute to muscle contracture.

Outside of the muscle fibers the extracellular matrix may also contribute to passive stiffness. One method of examining the extracellular matrix is to mechanically test bundles of fibers that include the constituent extracellular matrix and subtract the stiffness tested from the same biopsy. This method surprisingly revealed that bundles from cerebral palsy biopsies were much more compliant than controls,

even though bundles in both cases were stiffer than fibers. These same bundles did show a greater area faction of extracellular matrix as observed in previous studies. This confounding result suggests that even with stiffer fibers and more extracellular matrix present, the extracellular is organized in a way that confers much less mechanical stiffness to the tissue [76]. It also opposes the hypothesis that stiffness is due to an intrinsic change of muscle properties.

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CHAPTER 2

NOVEL TRANSCRIPTIONAL PROFILE IN WRIST MUSCLES FROM CEREBRAL PALSY PATIENTS

2.1 Abstract

Cerebral palsy (CP) is an upper motor neuron disease that results in a progressive movement disorder. Secondary to the neurological insult, muscles from CP patients often become spastic. Spastic muscle is characterized by an increased resistance to stretch, but often develops further complications such as contractures which represents a prominent disability in children with CP. This study's purpose is to characterize alterations of spastic muscle on the transcriptional level. Increased knowledge of spastic muscle may lead to novel therapies to improve the quality of life for children with CP.

The transcriptional profile of spastic muscles were defined in children with cerebral palsy and compared to control patients using Affymetrix U133A chips. Expression data were verified using quantitative-PCR (QPCR) and validated with SDS-PAGE for select genes. Significant genes were determined using a 2x2 ANOVA and results required congruence between 3 preprocessing algorithms.

CP patients clustered independently and 205 genes were significantly altered, covering a range of cellular processes. Placing gene expression in the context of physiological pathways, the results demonstrated that spastic muscle in CP adapts transcriptionally by altering extracellular matrix, fiber type, and myogenic potential. Extracellular matrix adaptations occur primarily in the basal lamina although there is increase in fibrillar collagen components. Fiber type is predominately fast compared to normal muscle as evidenced by contractile gene isoforms and decrease in oxidative metabolic gene transcription, despite a paradoxical increased transcription of slow fiber pathway genes. We also found competing pathways of fiber hypertrophy with an increase in the anabolic IGF1 gene in parallel with a paradoxical increase in myostatin, a gene responsible for stopping muscle grown. We found evidence that excitation-contraction coupling genes are altered in muscles from patients with CP and may be a significant component of disease.

This is the first transcriptional profile performed on spastic muscle of CP patients and these adaptations were not characteristic of those observed in other disease states such as Duchenne muscular dystrophy and immobilization-induced muscle atrophy. Further research is required to understand the mechanism of muscle adaptation to this upper motor neuron lesion that could lead to the development of innovative therapies.

2.2 Introduction

Cerebral palsy (CP) is a disorder in which children experience a non-progressive brain lesion that results in permanent and progressive secondary postural and movement disorders [1]. CP has an incidence of 2.0-2.5 occurrences per 1000 live

births in developed nations, making it the most common cause of physical disability in children [2]. There is a spectrum of disease states in CP that affect upper and lower limbs to varying degrees. Since the primary lesion in CP is in the central nervous system, most CP research has been focused on the neurological disorder [3-5]. However, since the secondary effects of CP disrupt posture and movement, most conservative and surgical treatments address the musculoskeletal system [6].

It is clear that skeletal muscles from CP patients are altered secondary to the neurological lesion. There are many neurological symptoms secondary to the brain lesion including dystonia, ataxia, athetosis and particularly spasticity [7, 8]. Loss of upper motor neuron (UMN) inhibition on the lower motor neurons (LMN) results in spasticity, altered muscle tone and increased/impaired motor unit firing. Loss of UMN excitation of LMNs leads to negative features of UMN syndrome that include weakness, fatigability, poor balance, and occasionally, sensory deficits. Although the mechanism is unknown, spastic muscle often shortens to create muscle contractures, which is a primary disability of CP that leads to further complications [6]. There are many clinical approaches to managing spasticity to increase function, potentially decrease muscle contractures and most importantly improve quality of life. Oral medications, physical therapy techniques, chemical neurectomies with phenol or alcohol, chemodenervation using neurotoxins (BTX), and surgical neurectomies have all been utilized to decrease spasticity in children with CP [9]. Unfortunately, while there has been some success in this management, many children ultimately require orthopedic surgery to lengthen the tendons of contracted muscle so that arm or leg

function can be increased. If the adaptation of the muscle tissue were more completely understood, it might lead to novel medical treatments of contractures.

Skeletal muscle from children with CP has been characterized at a variety of levels, with most studies reporting muscle tissue and muscle fiber atrophy, decreased muscle cross-sectional area, muscle shortening, and decreased specific tension [10, 11]. All of these changes implicate physiological mechanisms of growth being involved in the pathology of muscle from CP patients. Interestingly, recent intraoperative studies of human muscles revealed abnormally long muscle sarcomere length in vivo [12] that were associated with muscle tissue of altered properties. Specifically, muscle fiber sarcomere length under no load (i.e., slack sarcomere length) was significantly decreased and the muscle tissue itself contained a hypertrophic extracellular matrix of poor material quality [11, 13]. These changes implicate the mechanical force generating system of the muscle cell as well its extracellular matrix tissue. Muscle has been shown to adapt its mechanical function to neurological input [14], however the biochemical process of how an UMN lesion could lead to alterations in muscle myogenesis, force generation, force transmission and extracellular matrix properties is unknown. While there is evidence that neurotrophic factors dramatically affect muscle properties [15, 16], there is neither mechanistic understanding as to how such factors might alter tissue properties, nor information as to which specific biosynthetic pathways might lead to these changes.

To develop an understanding of the physiological processes altered in spastic muscle secondary to CP, we exploited the fact that muscle tissue from a previous study, in which the clinical severity of the spasticity was clearly established, was

available for transcriptional profiling [17]. We used GeneChip technology to contrast the transcriptome from CP patients with age-matched control patients whose muscles were completely normal. We performed a variety of analyses to identify a robust set of genes that were significantly altered in CP and interpreted these genes in their biological context to explain previously defined muscle changes. We also compared our transcriptional data to two other disease states to determine whether spasticity secondary to CP results in a unique muscle disorder at the gene expression level.

2.3 Methods

Muscle Sampling

Children were recruited for this study because they were receiving tendon transfers of the flexor carpi ulnaris (FCU) muscle into the extensor carpi radialis brevis (ECRB), the extensor carpi radialis longus muscle, or the extensor digitorum communis muscles [18]. All patients had CP and developed a contracture indicating surgery, despite receiving conservative treatment that included splinting and occupational therapy. Parental consent and patient assent was obtained in accordance with our institutional review boards. From the original sample size of 23 [18],a subset (n= 6 children, average age 12.8 ± 1.5 years) was selected to cover a range of clinical severities determined from the House [19], Ashworth [20], and Zancolli [21] classification systems as well as characteristics of sarcomere length and range of motion. Control tissue was obtained incidentally (n=2 children, average age 8.5 ± 2.1 years) from the FCU and ECRB muscles in children with no previous history of any

neural injury who were already undergoing surgery. None of the surgeries injured the control muscles in any way. We suggest that these samples are true controls for the following reasons: 1) the surgeon verified that the muscles from which biopsies were taken were in pristine condition and showed no signs of damage, 2) surgery was emergent in these children, and therefore, control samples were obtained within 24 hours of fracture, 3) controls showed no significant effect for many of the transcripts associated with trauma or immobilization and were, in fact, often altered in the opposite direction (data not shown) (21, 60). Just prior to harvesting of the spastic muscle biopsies, sarcomere length of the FCU was measured by laser diffraction *in vivo*. While the wrist was held in neutral, a small fiber bundle was transilluminated with a HeNe light. The sarcomere length could be calculated from the diffraction pattern obtained [18]. CP biopsies were snap frozen in isopentane chilled by liquid nitrogen (-159°C), and stored at -80 °C until analyzed (Table 2.3). No patients had undergone serial casting prior to surgery, two patients (AN and BF) had BTX injections into the FCU in months prior to surgery, and one patient (AQ) had a prior BTX injection in the biceps.

RNA preparation and gene expression profiling

RNA was extracted using a combination of standard Trizol (Invitrogen, Carlsbad, CA) and RNeasy (Qiagen, Valencia, CA) protocols. Briefly, 30 mg of frozen muscle was homogenized in a rotor-stator homogenizer on ice in 0.5 ml of Trizol; 0.1 ml of chloroform was added to the solution, which was then vigorously vortexed for 15 s followed by centrifugation at 4°C for 15 min. The upper aqueous layer was removed and mixed with an equal volume of 70% ethanol before being

added to the RNeasy spin column. After the column was washed, it was incubated with RNAse-free DNAse (Qiagen) for 15 min and then washed again three more times before being eluted as described in the manufacturer's protocol. RNA concentration was determined by the absorbance at 260 nm, and the 260 nm-to-280 nm absorbance ratio was calculated to define RNA purity.

Microarray data analysis

Affymetrix microarrays ("GeneChip" HG-U133A; Affymetrix, Santa Clara, CA) were used for each muscle biopsy (n=16 chips; 2 muscles x 8 patients) and the data are available (Gene Expression Omnibus (GEO) accession number: GSE11686). RNA processing for the GeneChip, including stringent quality control measures, was performed by the Gene Chip Core at the Department of Veterans Affairs San Diego Health Care System, (San Diego, CA). GeneSpring software (version 7.3; SilconGenetics, Redwood City, CA) was used to identify those genes that were significantly altered in CP. Initially, a 12.5% (2/16 chips) present call on MAS5 (Affymetrix) was used to filter out poorly performing probe sets in the analyses. Three independent probe set algorithms were used for signal generation and normalization: MAS5, RMA, and GCRMA. Recent reports support requiring concordance among different probe set algorithms as an approach to reduce false positives in data sets [22-24]. Each feature was normalized per chip (to the median of all features on each chip) and per gene (to the median of that feature on all chips). Normalized gene values were subjected to a 2 x 2 Welch ANOVA of muscle type (FCU vs. ECRB) and disease state (CP vs. CTRL) with a required statistical significance ($P < 0.05$) with a Benjamini and Hochberg False Discovery Rate (FDR)

multiple testing correction for present features. Thus 5% of the genes deemed significant for an individual preprocessing algorithm are suspected to be false positives. Features that passed in all three preprocessing algorithms were deemed significantly altered in CP.

The condition tree was created using a Pearson Correlation similarity score and average linkage clustering algorithm for all samples on present features. For severity analysis, a Welch ANOVA for each severity parameter was run on the MAS5 data of the features deemed significantly altered in CP without the control patients and a required statistical significance of ($P < 0.05$) also with an FDR multiple testing correction.

Promoter sequence analysis was conducted using GeneSpring on the list of genes altered in CP. The upstream sequence from -10 to -1000 base pairs was analyzed for a nucleotide sequence of from 6 to 10 nucleotides long and containing at most 2 N values in the middle. Significance was determined based on the number of times the given sequence appeared in the upstream sequence of all other genes and was corrected for multiple testing. The analysis was performed on the whole list of genes altered in CP and the sub lists of up- or down-regulated.

Quantitative real-time PCR

QPCR was performed to validate expression levels of selected genes to the GeneChip data and to provide mRNA expression data for genes not contained on the HG-U133A chip. After RNA was extracted from the muscle as described previously and diluted 1:5 with DNase/RNase free water (Invitrogen), 1 μl of each sample was reverse transcribed using standard protocols (Superscript III; Invitrogen). cDNA was

amplified with the Cepheid SmartCycler (Sunnyvale, CA) with primers specific to the genes of interest (Table 2.4). All primers were tested for cross-reactivity with other transcripts using nBLAST and Oligo (version 6.6; Molecular Biology Insights, Cascade, CO). All samples were run at least in triplicate, along with a standard curve. The PCR reaction vessel (25 μl) contained 1x PCR buffer, 2 mM $MgCl_2$ (Invitrogen), 0.2 mM sense and antisense primers, 0.2 mM dNTP, 0.2x SYBRgreen, and 1 U of platinum Taq polymerase (Invitrogen). Amplification conditions were as follows: An initial hold at 95°C for 2 min was followed by 40 cycles of denaturing at 95°C for 15 s, followed by annealing/extension at 68°C for 40 s. The success of each reaction was deduced based on the observation of a single reaction product on an agarose gel and a single peak on the DNA melting temperature curve determined at the end of the reaction. To express QPCR results, we used the standard curve method with the "cycles to threshold" value representing the number of PCR cycles at which the SYBRgreen signal was increased above the threshold. Each sample's value was measured in triplicate, normalized to the housekeeping gene GAPDH, and then averaged. QPCR data were normalized to the median value of the gene to permit comparison to the GeneChip data.

Myosin protein content biochemistry

Myosin heavy chain protein content was measured (Table 2.3) for comparison to the GeneChip data as previously described [25]. Three bands were identified corresponding to MyHC I, MyHC IIa/ fetal and MyHC IIx/embryonic. (Using this methodology, embryonic MyHC cannot be separated from MyHC IIx and fetal MyHC cannot be separated from MyHC IIa.) The gels were scanned in a soft laser

densitometer (Molecular Dynamics Sunnyvale, CA, USA). The relative proportion of each MyHC isoform was determined by using a densitometric system (ImageQuant TL Software v 2003.01, Amersham Biosciences, Uppsala, Sweden).

Gene Ontology analyses

Gene ontology analysis provides a means of converting a list of differentially expressed genes into a hierarchical list of gene ontologies that are significantly altered. We used the web-based software GOTree (http://bioinfo.vanderbilt.edu/gotm/; [26]) to compare the list of features altered in CP to the list of features present on the HG-U133A chip. In this analysis, a P-value is generated for each ontology based on hyperbolic comparison of the number of genes present in that list to the number of genes expected to be present based on the size of the list. The analysis was performed on the entire list of genes altered in CP, with a required $P < 0.01$.

Biological pathway analyses

To gain understanding into the biological context of transcript changes, we investigated the way in which genes were involved in various muscle pathways. We analyzed pathways from databases including: Ingenuity Pathway Analysis (IPA; http://www.ingenuity.com), Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg/pathway.html), and Gene Map and Pathway Profiler (GenMAPP; http://www.genmapp.org/). These pathways permit establishment of pathways specific to muscle involving critical muscle functions such as: neuromuscular junction function, excitation-contraction coupling, muscle contraction, extracellular matrix formation, muscle hypertrophy/atrophy, myogenesis, and fiber type switching. Based on the pathway databases and relying heavily our own

literature review, we created pathways specific to muscle with particular emphasis on genes altered in CP. To quantify gene expression for pathway analysis, MAS5 data were normalized to the averaged control data within each muscle type and across CP samples. This value is termed the expression ratio.

Finally, to compare the CP transcriptome to other conditions we examined the pathways specific to muscle against transcriptome deposited for three other disease states, Duchene Muscular Dystrophy (DMD; GSE465; [27]), immobilization (IMB; GSE8872; [28]), and hereditary spastic paraplegia (HSP; GSE1300; [29]). The DMD experiment used muscle from patients age 6-9 years and further details are described in the reference [27]), but we compared data only from those U95A chipset. The IMB experiment used medial gastrocniemus muscle from adult patients and further details are described in the reference [28], but we compared only data from voluntary controls and ankle facture patients immobilized for 4-9 days. The HSP experiment used vastus lateralis muscle from adult patients and further details are described in the reference [29], but we used the U133A chipset and controls (1-10) from GSE3307. As these data sets are from subjects of different ages and muscles, and are acute (in the case of 4-9 days of immobilization), direct comparison to our CP dataset is somewhat problematic; however, we are able to investigate whether similar transcriptional trends are present for these muscle conditions. The expression ratio for each feature was taken as the MAS5 ratio of the average disease state:average control state of the particular study so disease values are normalized to their own controls. The genes expression ratio of a pathway for each disease (CP, DMD, IMB, HSP) is log averaged across the pathway with inverse expression value used for inhibitors. We similarly

investigated a list of genes involved in satellite cell states of quiescence and activation [30-32].

2.4 Results

Of the 22,283 probe sets on the HG-U133A GeneChip, 11,312 met the criteria of being "present" on 2/16 GeneChips and were therefore considered for further analysis. The number of genes that were significant for CP ($P < 0.05$) on the 2 x 2 Welch ANOVA of disease state and muscle (CP vs. CTRL; flexor carpi ulnaris (FCU) vs. extensor carpi radialis brevis (ECRB)) with FDR among the three preprocessing algorithms were: 495 for Microarray Suite Version 5.0 (MAS5), 1,141 for Robust Multiarray Analysis (RMA), and 1,207 for GCRMA. The overlap of these 3 preprocessing algorithms produced a final list of 205 genes (319 features) that were considered significantly altered secondary to CP (Sup. Table 2.1). Of these, more were up-regulated (143 genes, 220 features) than down-regulated (62 genes, 99 features). Table 2.1 reports the 72 genes subset of these 205 genes that were identified as relevant to specific muscle functions. Genes in the Tables (Table 2.1; Sup. Table 2.1) are reported with the P-value for each preprocessing algorithm as well as the expression ratio. The 2 x 2 ANOVA yielded no genes significant ($P < 0.05$) for muscle type and only one gene with a significant interaction, MYH1. This was due to control ECRB tissue having a very low MYH1 mRNA content. This important result supports our previous contention that, even though these children present with wrist

flexion contracture, the FCU and ECRB are equally affected and the wrist flexion simply results from the large size of the FCU [18].

Promoter sequence analysis performed on each gene altered in CP did not reveal any 6-10 base pair sequences that were overrepresented 10-1000 base pairs upstream of the gene. Of course, regulation can occur farther upstream than 1000 base pairs and regulation sequences can be outside of the 6-10 base pair range. Thus, further sequence analysis may reveal significant promoter or enhancer sequences, but none were identified using these criteria.

Condition tree correlates with clinical severity scores and treatment

The condition tree resulted in the control patients being clustered together separate from CP patients (Fig. 2.1). Figure 2.1 illustrates the condition tree based on all present genes. The tree shows that patients are grouped together in most cases rather than by muscle type suggesting more between-patient than between-muscle variability.

We had hoped that clinical severity [1, 19-21] would allow us to define transcriptionally, the severity of CP or differences between flexor and extensor muscles. In this way, clinical parameters would be seen as representative of the state of the muscle tissue. These parameters were applied to an analysis of all of the 319 features altered in CP (Sup. Table 2.1) but only sarcomere length and active wrist extension with fingers flexed had features that were significant, containing the same two genes, *RBM9* and *RHOBTB1*. Heat plot of these data reveals that even these genes undergo a much larger change in expression from the control sarcomere length (3.37 μm) to CP sarcomere lengths than they do in CP progression (data not shown).

Thus it appears that our study is underpowered to reveal transcriptional correlation with clinical severity scores.

Treatment with BTX was investigated by comparing injected muscles vs. non-injected CP muscles, muscle from patients receiving injection of any muscle vs. non-injected patient muscle, and injected FCU muscles vs. non-injected FCU muscles. None of these analyses yielded any genes that met our requirement for statistical significance, and thus we show no significant transcriptional effect of BTX injection.

RT-PCR results compared to chip results

As a quality control measure, correlation of data between the GeneChip and QPCR was highly significant ($P < 0.001$) indicating internal consistency. To validate the GeneChip data, 10 genes covering a variety of cellular processes and expression levels were compared directly to transcript levels determined by QPCR on the same cDNA samples (Fig. 2.2). For 9/10 genes studied, the direction of the transcript change (i.e., up- or down-regulation) was confirmed, and there was a good correlation between methodologies in terms of the magnitude of the effect. For two genes, (*PVALB*, *GDF8*) expression levels were evaluated relative to *GAPDH* transcript levels. These genes were selected based on their significant differences on the chip and their relevance to the disease state. Significant positive correlations were observed for both genes (*PVALB*, $r^2 = 0.924$, $P < 0.001$; *GDF8*, $r^2 = 0.864$, $P < 0.001$). The QPCR data were also subjected to 2 x 2 ANOVA (CP vs. CTRL; FCU vs. ECRB) and both *PVALB* and *GDF8* were confirmed as significantly up-regulated in CP (Figs. 2.3C and 2.3D).

Two genes of particular interest that are related to muscle atrophy, MAFbx (*FBXO32*) and MURF1 (*TRIM63*) were not represented on the chip [28, 33]. Their expression was determined in the same manner as the genes described above (Figs. 2.3E and 3F). Both of these genes were down-regulated in CP, but neither reached statistical significance.

Myosin heavy chain protein-mRNA comparison

The GeneChip and QPCR provide only transcriptional data and we wished to determine whether the transcriptional changes resulted in translational changes in the case of myosin heavy chain (MHC) for these samples [25, 34]. All of the muscles were of a mixed fiber type, however the control ECRB tissue showed no evidence of type 2X MHC. The spastic muscles had a higher proportion of fast fibers than the controls of the corresponding muscle, with most of the increase in type 2X MHC. Comparison between protein and mRNA was confounded by the fact that MHC expression was normalized as percent of total myosin while mRNA was normalized to the median of that individual transcript across subjects. In spite of this difference, we still expected to see the same trend across samples, which was the case for type 1 MHC (gene *MYH8*) and type 2X MHC (gene *MYH1*) where protein and mRNA levels were significantly correlated (Figs. 2.4A and 2.4C; $P < 0.05$) while type 2A MHC (gene *MYH4*) did not quite reach significance (Fig. 2.4B; $P = 0.065$). Taken as a whole, these results suggest that, in the case of the MHC, protein levels reflected transcript levels.

Gene ontology analysis

Thirty-eight different ontologies were overrepresented based on the 143 up-regulated genes (Sup. Table 2.2; Sup. Fig. 2.1). The biological processes that stood out as most relevant to the disease state included striated muscle contraction, muscle development, cytoskeletal anchoring, negative regulation of metabolism, protein ubiquitination, and RNA processing. The cellular components of these genes were generally grouped into muscle components and ECM components, particularly the basement membrane. Twenty-eight different ontogenies were overrepresented based on the 99 down-regulated genes (Sup. Table 2.3; Sup. Fig. 2.2). The two major functions of the down-regulated biological processes were fatty acid metabolism and transport. This corresponded with the molecular function ontologies involved in fatty acid/acyl CoA binding and also contained cadmium and copper ion binding. Cellular component categories were almost exclusively related to the mitochondria, however it was interesting that sheet forming collagen type IV of the basement membrane also was over represented using this analytical approach.

Gene pathways related to muscle function

To understand muscle tissue adaptation to CP from a physiological perspective, we analyzed gene expression ratio patterns within muscle-specific pathways of gene products that interact in a given muscle function.

Because CP is a neurological disorder, a pathway describing the neuromuscular junction (NMJ) was created (NMJ; Fig. 2.5A). No postsynaptic genes were significantly altered in CP including subunits of the nACHR receptor. Collagen type IV subunits (*COL4A3*; 2.89 and *COL4A4*; 3.26) and laminin (*LAMB2*; 1.74) of

the synaptic basal lamina were significantly up-regulated. A Ca^{2+}-activated K^{+} channel (*KCNN3*; 12.98), was dramatically up-regulated in CP.

The process of converting the action potential into muscle contraction is referred to as excitation-contraction coupling (ECC; Fig. 2.5B). The β1 regulatory subunit of the L-type voltage gated Ca^{2+} channel was significantly up-regulated in CP (*CACNB1*; 1.59). Although the ryanodine receptor responsible for releasing Ca^{2+} from the sarcoplasmic reticulum (SR) was not altered, the genes *FKBP1A* (0.56) and *PDE4D* (0.68) that prevent channel leaking, were significantly down-regulated [35]. Myomegalin (*PDE4DIP*; 2.10) was altered significantly in CP and is thought to anchor *PDE4D* near the SR [36]. Calmodulin (*CALM1*; 1.70) was significantly up-regulated. The most drastic change with CP on gene expression was in the up-regulation of muscle-relaxing protein, parvalbumin (*PVALB*; 62.6). The process of pumping Ca^{2+} back into the SR is assisted by triadin (*TRDN*; 2.39), which was significantly up-regulated.

Muscle contraction obviously requires the myosin heavy chain motor and a cytoskeletal framework (MC; Fig. 2.5C). *MYH1* (type 2X MHC; 8.57) was significantly up-regulated in CP along with *MYH4* (type 2B MHC; 4.33) a gene not normally expressed in humans [37]. The developmental MHCs, *MYH3* (embryonic MHC; 15.74) and *MYH8* (perinatal MHC; 7.74) showed large up-regulation. *MYH7* (type 1 MHC; 0.72) was unchanged. The *MYBPC2* (2.16) is a fast isoform of myosin binding protein and was up-regulated in CP. Several sarcomeric structural components were also up-regulated as well: dystrophin (*DMD*; 2.11), nebulin (*NEB*; 2.54), and muscle LIM domain binding protein 3 (*LDB3*; 1.93).

We suspected that ECM transcription would be altered based on previous biomechanical results ([11, 13, 38]; ECM; Fig. 2.5D). Fibrillar collagens all increased modestly. Interestingly, basal laminar collagen IV was altered with *COL4A1* (0.51) and *COL4A2* (0.43) decreasing significantly while *COL4A3* (2.89) and *COL4A4* (3.26) increased significantly. Basigin, (*BSG*; 0.48) was significantly down-regulated. Various other ECM components were also up-regulated in CP: *ECM2* (1.86), *KAL1* (2.57), *MATN2* (2.24), *MFAP5* (3.64), *CILP* (2.09) and *SMC3* (1.88).

Myogenesis describes the pathway that produces muscle growth (MYG; Fig. 2.5E). *IGF1* (2.63) was up-regulated along with *IGFBP5* (2.48) (IGF1; Fig. 2.5F). Myostatin (*GDF8*; 3.65), an inhibitor of myogenesis, was also significantly up-regulated. Other significantly up-regulated genes implicated in myogenesis are *NEO1* (1.44, [39]), *PLCB1* (2.34, [40]), *PBX1* (1.86, [41]), and *HMGB1* (1.65, [42]), *MBNL1* (1.74, [43]), and MAPK6 (1.77, [44]). However, the muscle regulatory factors (*MYOD1* 1.22; *MYF6* 0.87; *MYF5* 1.06; *MYOG* 1.75) did not show a significant transcriptional change. Mitogen activated protein kinases (MAPK) have been proposed as a major pathway in muscle hypertrophy [45], however our study showed minimal transcriptional affects on this signaling family. Another segment of myogenesis relates to satellite cell activation, proliferation and incorporation into adult muscle fibers. None of the markers for quiescent or activated satellite cells (quiescence: *PAX7*, *FOXK1*, *MET*, *CDH15*, *NCAM1*, *VCAM1*, *SDC3*, *SDC4*; activation: *MYF5*, *MYOD1*, *MYOG*, *MYF6*, *PCNA*, *CDKN1A*, *MYH3*, *MYH8*) were significantly altered in CP suggesting minimal involvement of satellite cells in the

disease. Additional cell cycle transcripts were investigated, but did not show a significant change.

Although slow fiber creation is related to myogenesis, there is also a specific pathway for slow oxidative muscle fiber type determination (FT; Fig. 2.5F). Sensing and signaling factors, *CALM1* (1.70) and calcineurin (*PPP3CA*; 1.95) respectively, had significantly increased transcription along with transcription factor *MEF2A* (1.53), but NFATs and other MEF2 expressions levels were unchanged.

Cerebral palsy compared to other muscle pathologies

To determine whether the CP transcriptome was unique or simply a secondary adaptation of decreased activity in these children (as might be observed with immobilization (IMB)), or whether the response was a generic muscle pathology (Duchene Muscular Dystrophy (DMD) being the most-commonly studied), or was similar to spastic muscle in an alternative more developed muscle (Hereditary Spastic Paraplegia (HSP) being a spastic condition with adult subjects) we compared our GeneChip data to these three muscle pathologies for which GeneChip data were available [27-29]. To make these comparisons, the expression ratio values for the pathways were compared amongst the three conditions (Table 2.2). While averaging over an entire pathway may be misleading (similar scores may result from different gene expression patterns), different scores do emphasize pathways that are unique among disease states. This analysis revealed significant satellite cell activation, as expected, in DMD [27] as well as increased NMJ components (primarily nicotinic acetylcholine receptor subunits) and loss of contractile material as expected in IMB [28]. HSP represents muscle adaptation to altered neuronal input, although there was

a negative correlation in most pathways, ECC seemed to be handled in a similar manner. CP was unique relative to the other two pathologies based on the IGF1 pathway increase, slow fiber activation, and increased expression of ECC activators and inhibitors. Thus, the correlation data support the assertion that CP is unique relative to other disease states.

2.5 Discussion

The purpose of this study was to define the muscle transcriptional adaptations in children with cerebral palsy (CP) to gain insights into the cellular mechanisms that might explain muscular adaptation in this neurological condition. We show that the transcriptional profile of CP muscle is fundamentally different compared to normal controls (Fig. 2.1). Previous CP muscle studies of intraoperative sarcomere length [12], in vitro tissue biomechanics [38, 46], and immunohistochemical and biochemical assays suggested adaptation of extracellular matrix regulation [11, 13], myogenenic pathways [10, 38], and fiber type determination pathways [25] in this condition. Our transcriptional analyses provide potential explanations of the cellular bases for these adaptations. Based on a general understanding of muscle physiology and biology, we placed the gene expression patterns into the context of six major muscle physiological systems—the neuromuscular junction (Fig. 2.5A), excitation-contraction coupling (Fig. 2.5B), muscle contraction (Fig. 2.5C), extracellular matrix regulation (Fig. 2.5D), myogenesis (Fig. 2.5E) and fiber type determination (Fig. 2.5F). As will be seen, one

feature of CP is that conflicting tendencies occur within and between these various systems.

The initial insult in CP is located in the central nervous system, but this primary insult leads to a secondary effect on the skeletal muscle system. Thus the NMJ, as the nerve-muscle interface, may play a role in CP. Studies have shown disrupted NMJ in that acetylcholine receptors appear outside the NMJ area more often in CP, although they were unable to find any change in transcriptional regulation [47, 48]. However, *KCNN3* was the 2nd most up-regulated gene on the entire chip and this gene plays a role in causing after-hyperpolarizations which may be a cellular attempt to limit the excessive motor unit firing that has been reported in spastic muscle [49]. Interestingly, *KCNN3* is usually expressed in immature muscle and inhibited after innervation, which may indicate a sort of "immature state" of this muscle [50]. Although their localization in these samples is unknown the standard collagenous component of the synaptic basal lamina (*COL4A3*; *COL4A4*) was transcriptionally increased, the opposite activity of primary muscle basal lamina collagens (*COL4A1*; *COL4A2*) [51]. If these synaptic collagen IV subunits occurred outside the NMJ it would suggest a further degree of NMJ disorganization, alternatively they could be another indicator of muscle in an "immature state."

We also uncovered significant evidence of altered calcium handling secondary to CP. Our data appear to reflect chronically increased intracellular calcium since the L-type voltage gated Ca^{2+} channel (*CACNB1*) was up-regulated (leading to activation of the ryanodine receptor) and leakage through the ryanodine receptor would be increased by down-regulation of two genes that prevent leakage (*FKBP1A*; *PDE4D*).

Another "attempt" by the muscle to re-regulate $[Ca]_i$ can be inferred by the up-regulation of *TRDN*, which reclaims Ca^{2+} to the SR by localizing calsequestrin within the SR [52]. Chronically altered calcium levels and subsequent activation of the intramuscular calcium-activated proteases (Calpains) would cause dramatic muscle lesions, although they are not transcriptionally regulated in CP. Indeed, a relatively new class of Calpain-mediated myopathies has recently been described [53, 54]. Perhaps in response to this chronic change in $[Ca]_i$ a huge 63-fold increase in *PVALB*, a Ca^{2+} binding protein was induced in order to force muscle relaxation [55]. This dramatic adaptation could have significant effects on the $[Ca]_I$ and may even lower it below control levels.

Of the proteins involved in calcium induced force generation, MHC isoforms are the most responsive to CP. They are primarily responsible for determining muscle fibers type [56] and undergo a transformation in the direction of a slow-to-fast phenotype. This shift included immature myosins, which saw large increases, although they were only significant in 2/3 algorithms, and lends further evidence to muscle in an "immature state." The many oxidative metabolic genes that are down-regulated in CP (Table 2.1) support this slow-to-fast transition. The ontology analysis revealed the loss of metabolic and mitochondrial related transcripts represented the majority of down regulated ontologies (Sup. Table 2.3). Although previous research is mixed on whether spastic muscles become more fast or slow, our data is in concordance with recent research that fast fibers dominate spastic muscle in CP [25, 34]. Paradoxically, this transformation occurs despite an overall increase in gene transcription related to the determination of the slow fiber phenotype, particularly

calmodulin (*CALM1*) and calcineurin (*PPP3CA*) [57]. A potential explanation may be that the dramatic PVALB expression actually leads to a decrease in intracellular calcium, thus turning off the initiation of the slow gene program. The validity of the calcineurin/NFAT pathway for transcription of a slow muscle fiber program has also come under question [58, 59].

The slow fiber program represents only one segment of myogenesis that is controlled by many other genes. While the majority of the pathway elements (receptors, second messengers, signaling molecules) involved in myogenesis were not changed, two of the most important initial factors were both up-regulated—insulin-like growth factor (*IGF1*) and myostatin (*GDF8*). Interestingly they produce opposing effects on myogenesis with IGF1 leading to hypertrophy and myostatin opposing growth [60, 61]. What this means for the net level of myogenesis is unclear. Satellite cells are an important contributor to muscle growth, but their role in CP is difficult to discern as neither quiescent nor activated satellite cell markers were altered transcriptionally.

Muscle development was indicated in the ontology analysis (Sup. Table 2.2) and some genes related to myogenesis were up-regulated (Table 2.1). While the muscle regulatory factors were not significantly altered, apparently fewer "growth" proteins must be activated since muscle growth in children with CP is decreased [10]. The reduction of parallel growth would lead to decreased muscle strength in CP patients. Reduced longitudinal growth would limit range of motion, and this has been suggested as the cause for extraordinarily long in vivo sarcomere lengths in children with wrist flexion contractures [12]. The increase in *GDF8* could be responsible for

this lack of growth in spastic CP muscle and thus represent a potential therapeutic target. Other evidence pointing toward muscle degradation is in the expression ontology of protein ubiquitination being increased, based on the up-regulation of 4 related genes (*FBXO3*, *PCNP*, *RBBP6*, and *UBE2V2*) and supported by an up-regulation of *CACYBP*, a gene involved in calcium dependent ubiquitination. The opposing actions of IGF1 to increase muscle mass are also controlled by a number of IGF binding proteins and we revealed *IGFBP5* was significantly up-regulated in CP, however the effects of *IGFBP5* in muscle have been questioned [62, 63]. These results make the activation of the IGF1 pathway is difficult to decipher on a transcriptional level. Furthermore the hypertrophic effect of IGF1 is primarily from an increase in translation efficiency, which could have broad effects that would be unobservable in our study.

One of IGF1's broad anabolic effects could be a contribution to the increased ECM in muscle from CP patients [64, 65]. While the ECM is altered transcriptionally, it is unclear which components are most affected. The fibrillar components of collagen in muscle are primarily collagen types I and III and each alpha chain of these collagen types were slightly up-regulated. The most dramatic changes were in the collagens of the basal lamina discussed in reference to the NMJ. Overall the basal lamina has been demonstrated as an area of excessive growth, and thus may be important in understanding muscle pathology [13]. Gene ontology analysis revealed a set of genes associated with the ECM that were all significantly up-regulated (Sup. Table 2.2). This supports the hypothesis of a prolific ECM in spastic muscle of CP patients. The decreased transcription of basigin (*BSG*; 0.48) could also lead to

extensive ECM through the reduced activation of MMPs [66]. Basigin may also implicate a disorganized ECM lacking full functionality as MMP activity is usually increased along with increases in ECM production. However TIMP's are the primary MMP inhibitors and did not show a corresponding transcriptional increase [67].

It is important to note the distinct pathology of CP, as spastic muscle does not fit neatly into any of the other "altered use" muscle models [68]. The transcriptional control of muscle in CP was qualitatively different compared to DMD, IMB, or HSP (Table 2.2). DMD actually showed the most similarity to CP, particularly among contractile genes and satellite cell markers. DMD is known to have activated satellite cells and regenerating muscle and this correlation implicates the same in CP, although no satellite cell activation markers were significantly up-regulated in UP. IMB and HSP had an opposing effect on satellite cells, which shows this is not consistent with all disease states. IMB has been shown to result in muscle atrophy, fibrosis, and a shift from slow to fast muscle fibers. Although we were not able to compare our results to a human overactivity microarray study we clearly did not show the increase in slow fibers and mitochondrial transcripts expected. In fact HSP, which may be expected to the most similar to CP, resulted in little correlation ($R < 0.1$) with CP in all pathways except ECC. This suggests that ECC alterations may be a defining characteristic of spastic muscle. It is also interesting to note that, in the other cases, *IGF1* and *GDF8* acted alternatively—IGF1 increased while GDF8 decreased in DMD and conversely for IMB and HSP. This highlights the unique adaptation of CP, where myogenesis is turned on and off simultaneously.

While we are able to demonstrate the transcriptional effects of CP we also investigated this effect on two separate muscles and at different levels of clinical severity. Tendon transfer surgery is relatively common procedure for CP patients and is implicated when there is a muscular imbalance around a joint. It involves transferring the distal tendon of a muscle on the side of a joint considered to have a contracture or relative over activity to a tendon on the opposing side of the joint. Transfer of FCU to ECRB to correct wrist position is one of the common tendon transfer surgeries. Thus FCU is considered the more pathologic muscle and we might have expected a different transcriptional profile. However, we were unable to show any transcriptional difference between the muscles, indicating that both wrist flexors and extensors have a similar adaptation to CP. While the FCU is known to exhibit contractures in CP, we conclude that the contracture is developed due to its architecture, not a fundamental difference is secondary adaptations to the altered neuronal input of CP. The FCU is a larger muscle than the ECRB and the larger wrist flexor muscles may dominate the disease state based on their size. We were also unable to show significant transcriptional differences among various clinical severity scores in CP patients. This may be because CP transcriptional profiles are either on or off. More likely our study was unable to resolve a severity effect as the study is biased towards the most severe cases (patients recruited based on corrective surgery) or the study is simply underpowered. We would likely need more patients across the range of clinical severity scores to define the genes most closely correlated with severity. However the low power of the severity analysis is increased in our comparisons of CP vs. control muscle. Further, a discussion of statistical power does

not apply to significant differences detected in CP vs. control muscle. We do acknowledge, however, that we are clearly not detecting all transcripts that are altered in CP.

Our study has some inherent limitations, one of which is the small sample size noted above, especially in the case of control patients. As with any human study there is a high degree of heterogeneity among the samples. These patients have been treated in a variety of ways, and it is important to note that our transcriptional profile is not solely based on CP, but include conservative treatment. We must also point out that this muscle is in a chronic disease state, making it difficult to discern the primary effects of CP from compensatory mechanisms that have taken place. As with any GeneChip study, we discuss only transcriptional control and any observation is subject to post-transcriptional modification.

Despite these inherent limitations we have been able to highlight areas where future work on spastic CP muscle may lead to innovative therapies. Our altered calcium handling data points to chronically elevated calcium levels which are highly dangerous since they may activate endogenous proteases. Fortunately a variety of calcium channel blockers have been developed and tested which could be of use in treating CP. Another potential application of current techniques could come from anti fibrotic therapy to combat the increase in ECM components, which is suggested by the transcriptomes. Of the most promising may be myostatin inhibiters, currently under investigation, since growth is inhibited in muscle from CP patients and myostatin, a major inhibiter of muscle growth is significantly up-regulated. This transcriptional

study helps point the way to these and other areas of protein modifications, cell signaling, and biomechanics where future investigations should be focused.

Dramatic transcriptional alterations occur in muscle secondary to CP. These transcriptional changes ultimately lead to derangement of the ECM components of spastic muscle along with alteration of transcripts involved in myogenesis. A number of genes alter their expression in order to create a slow-to-fast transition of MHC isoforms and metabolic profile. GeneChip analysis has also allowed us to demonstrate the many changes in Ca^{2+} handling occur in CP that was not suggested previously. Together we are able to postulate the mechanisms known to affect muscle function in CP and predict new ones. This will aid future research into CP muscle and therapies to treat CP patients.

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Table 2.1: Table of genes within function groups related to skeletal muscle. Groups defined by ratio is the expression ratio of CP:CTRL. P-values are listed for the three separate preprocessing algorithms used (MAS5, RMA, GCRMA).

		P-Values					P-Values		
GENE	**Ratio**	**MAS5**	**RMA**	**GCRMA**	**GENE**	**Ratio**	**MAS5**	**RMA**	**GCRMA**
Neuromuscular Junction					*Muscle Contraction and Structure*				
KCNN3	12.98	0.013	0.011	0.018	MYH1	8.57	0.001	0.001	0.000
COL4A 3	2.89	0.043	0.014	0.019	MYH4	4.33	0.001	0.002	0.015
LAMB2	1.74	0.042	0.018	0.015	NEB	2.54	0.015	0.008	0.013
					MYBPC 2	2.16	0.027	0.004	0.004
Excitation Contraction Coupling					DMD	2.11	0.023	0.020	0.027
PVALB	62.60	0.001	0.004	0.001	LDB3	1.93	0.030	0.015	0.015
ATP2B2	2.60	0.033	0.023	0.021	*Metabolism/Mitochondria Related*				
TRDN	2.38	0.028	0.019	0.017	WARS	0.64	0.044	0.040	0.019
ATP2C1	2.30	0.046	0.047	0.041	CAV1	0.64	0.037	0.013	0.017
PDE4DIP	2.10	0.005	0.003	0.004	CERK	0.63	0.033	0.006	0.014
CALM1	1.70	0.005	0.004	0.008	A2M	0.62	0.015	0.016	0.010
CACNB1	1.51	0.045	0.014	0.017	MDH1	0.60	0.050	0.017	0.011
FKBP1A	0.56	0.039	0.016	0.011	PECI	0.58	0.029	0.024	0.012
Myogenesis/Fiber type pathways					MRPL3 5	0.57	0.040	0.043	0.037
					MRPS1 8B	0.56	0.009	0.002	0.001
GDF8	3.65	0.025	0.032	0.007	SLC25 A20	0.55	0.044	0.049	0.022
IGF1	2.63	0.013	0.010	0.008	CPT2	0.54	0.044	0.022	0.013
IGFBP5	2.48	0.002	0.000	0.000	MRPS1 2	0.53	0.044	0.008	0.010
PLCB1	2.34	0.028	0.014	0.007	UCP2	0.51	0.030	0.023	0.015
RASA4	2.26	0.037	0.027	0.029	MLYCD	0.48	0.017	0.004	0.001
PPP3CA	1.95	0.012	0.003	0.005	PPIF	0.48	0.014	0.011	0.010
PBX1	1.86	0.028	0.010	0.007	ADM	0.47	0.019	0.012	0.018
CALM1	1.70	0.005	0.004	0.008	UCP3	0.47	0.014	0.010	0.007
MBNL1	1.55	0.033	0.015	0.024	ALDH6 A1	0.46	0.023	0.013	0.007
MEF2A	1.53	0.028	0.006	0.007	ACSL1	0.43	0.014	0.008	0.004
NEO1	1.44	0.015	0.005	0.001	GOT1	0.42	0.039	0.027	0.021
HMGB1	1.34	0.015	0.013	0.015	TST	0.41	0.015	0.002	0.001
Extracellular Matrix					MT1G	0.37	0.015	0.009	0.011
MFAP5	3.64	0.012	0.004	0.014	LPL	0.35	0.013	0.010	0.007
COL4A3	2.89	0.043	0.014	0.019	RETSA T	0.35	0.002	0.006	0.005
COL21A1	2.86	0.021	0.022	0.015	MT1M	0.34	0.015	0.006	0.004
KAL1	2.57	0.002	0.003	0.003	MT1X	0.32	0.014	0.012	0.010
MATN2	2.24	0.013	0.011	0.007	GLUL	0.31	0.018	0.017	0.013
CILP	2.09	0.035	0.043	0.034	MT2A	0.29	0.015	0.013	0.011
SMC3	1.88	0.021	0.026	0.019	MT1H	0.29	0.015	0.015	0.012
ECM2	1.86	0.037	0.035	0.021	MT1F	0.27	0.019	0.012	0.009
LAMB2	1.74	0.042	0.018	0.015	MT1E	0.26	0.012	0.006	0.005
COL4A1	0.51	0.048	0.032	0.015	LIPE	0.22	0.028	0.001	0.002
BSG	0.48	0.033	0.026	0.030					
COL4A2	0.43	0.012	0.010	0.006					

Table 2.2: Quantification of gene pathways in various disease states (CP, DMD, IMB, HSP). Quantification represents MEAN (geometric mean of expression ratios in specific muscle pathways defined in Figure 2.5 using inverse values for pathway inhibitors) and CORR (correlation of CP with the other disease states, reported with an R value). Satellite cells markers are separated into genes expressed in the quiescent (SCQ) and activated states (SCA).

	CP	**DMD**		**IMB**		**HSP**	
	MEAN	MEAN	CORR	MEAN	CORR	MEAN	CORR
NMJ	2.39	0.98	-0.24	1.19	-0.05	0.97	-0.02
ECC	0.66	0.90	0.12	1.26	0.14	1.39	0.62
MC	2.91	2.18	0.91	0.65	-0.74	1.07	-0.48
ECM	1.63	1.73	-0.23	0.69	0.44	0.66	0.02
FT	1.35	0.70	-0.21	0.92	0.37	0.91	-0.79
IGF1	1.19	1.58	N/A	1.04	N/A	0.76	N/A
MYG	1.14	1.11	-0.16	1.01	-0.24	1.01	-0.09
SCQ	1.19	1.18	-0.67	1.11	0.66	1.23	0.07
SCA	2.17	3.73	0.91	0.86	-0.81	0.94	-0.54

Table 2.3: Table of the eight subjects used in this study with 2 muscles (FCU and ECRB) per patient with sex and age recorded. Measures of severity were taken to include: intraoperative measurement of sarcomere length (SL) on the FCU, House clinical assessment of activity, Severity grouped by House (Severe Severe (SS), Severe Moderate (SM), Mild (M), and Control (C)), Ashworth clinical assessment, Zancolli classification based on finger extension, passive extension with flexed fingers (PEFF) passive extension with straight fingers (PESF) and active extension with flexed fingers (AEFF). Samples that were available for MyHC SDS-PAGE gels are noted in the final column

Subject	Sex	Age	Muscle	SL (μm)	House	Severity	Ashworth	Zancolli	PEFF (°)	PESF (°)	AEFF (°)	MyHC gel
AI	M	13	FCU ECRB	6.36	1	SS	4	3	-5	-90	-70	x x
AN	F	12	FCU ECRB	4.56	3	SS	3	2B	50	45	-90	
AO	F	11	FCU ECRB	4.01	5	SM	2	2B	65	60	-50	x x
AQ	M	15	FCU ECRB	4.01	7	M	2	2B	80	80	-50	
AT	M	14	FCU ECRB	3.57	0	SS	3	3	45	40		
BF	F	12	FCU ECRB	5.31	6	SM	3	2A	85	85	55	x x
BP	M	10	FCU ECRB	-	-	C	1	0	90	90	90	x
AZ	M	7	FCU ECRB	-	-	C	1	0	90	90	90	x x

Table 2.4: Table of primers used for QPCR analysis. Genbank accession number and PCR product length given in parentheses after each transcript name

COL1A2 transcript (NM_000089, 225 bp):
Sense primer: 5′-TCC AAA GGA GAG AGC GGT AA-3′
Antisense primer: 5′-GCC ACT TGC ACC ACG ACT A-3′
COL4A2 transcript (NM_001846, 269 bp):
Sense primer: 5′-CTG GGT GGC GGA GTT TGT G-3′
Antisense primer: 5′-GCT GAT GTG TGT GCG GAT GAG3′
COL4A3 transcript (NM_000091, 157 bp):
Sense primer: 5′-CAC CAG CTC TGA TGC CAA TG-3′
Antisense primer: 5′-AGA GAA ATC CAG CCG TGA GG-3′
DMD transcript (NM_004019, 151 bp):
Sense primer: 5′-GAC CAG CAC AAC CTC AAG CAA-3′
Antisense primer: 5′-TCA GCA GCC AGT TCA GAC ACA-3′
FBXO32 (NM_058229, 376 bp):
Sense primer: 5′-GTC CAA AGA GTC GGC AAG T-3′
Antisense primer: 5′-TTG GGT AAC ATC GGA CAA GT-3′
GAPDH (NM_002046, 172 bp):
Sense primer: 5′-TCT GAC TTC AAC AGC GAC AC-3′
Antisense primer: 5′-TGG TCC AGG GGT CTT ACT C-3′
IGF1 transcript (NM_000618, 355 bp):
Sense primer: 5′-AGC AGT CTT CCA ACC CAA TTA-3′
Antisense primer: 5′-CAC GGA CAG AGC GAG CTG-3′
IGFBP5 transcript (NM_000599, 339 bp):
Sense primer: 5′-CCA AAC ACA CCC GCA TCT3′
Antisense primer: 5′-CAG CTT CAT CCC GTA CTT GTC-3′
GDF8 (NM_005259, 167 bp):
Sense primer: 5′-TAT CAC GCT ACA ACG GAA AC-3′
Antisense primer: 5′-GGA GTC TCG ACG GGT CTC-3′
MYH1 (NM_005963, 132 bp):
Sense primer: 5′-AAG AGC AGG GAG GTT CAC AC-3′
Antisense primer: 5′-TTA TCT CCA AAA GTC ATA AGT ACA-3′
NEB (NM_004543, 131 bp):
Sense primer: 5′-CCG TGC CAT GTA TGA CTA TAT-3′
Antisense primer: 5′-CGG TCC TGC CAG TCC TCT G-3′
PVALB (NM_002854, 329 bp):
Sense primer: 5′-GAT GAC AGA CTT GCT GAA CGC-3′
Antisense primer: 5′-CTT AGC TTT CAG CCA CCA GAG-3′
TRIM63 (NM_032588, 393 bp):
Sense primer: 5′-GAG GAT TCC CGT CGA GTG AC-3′
Antisense primer: 5′-AAT GGC TCT CAG GGC GTC T-3′

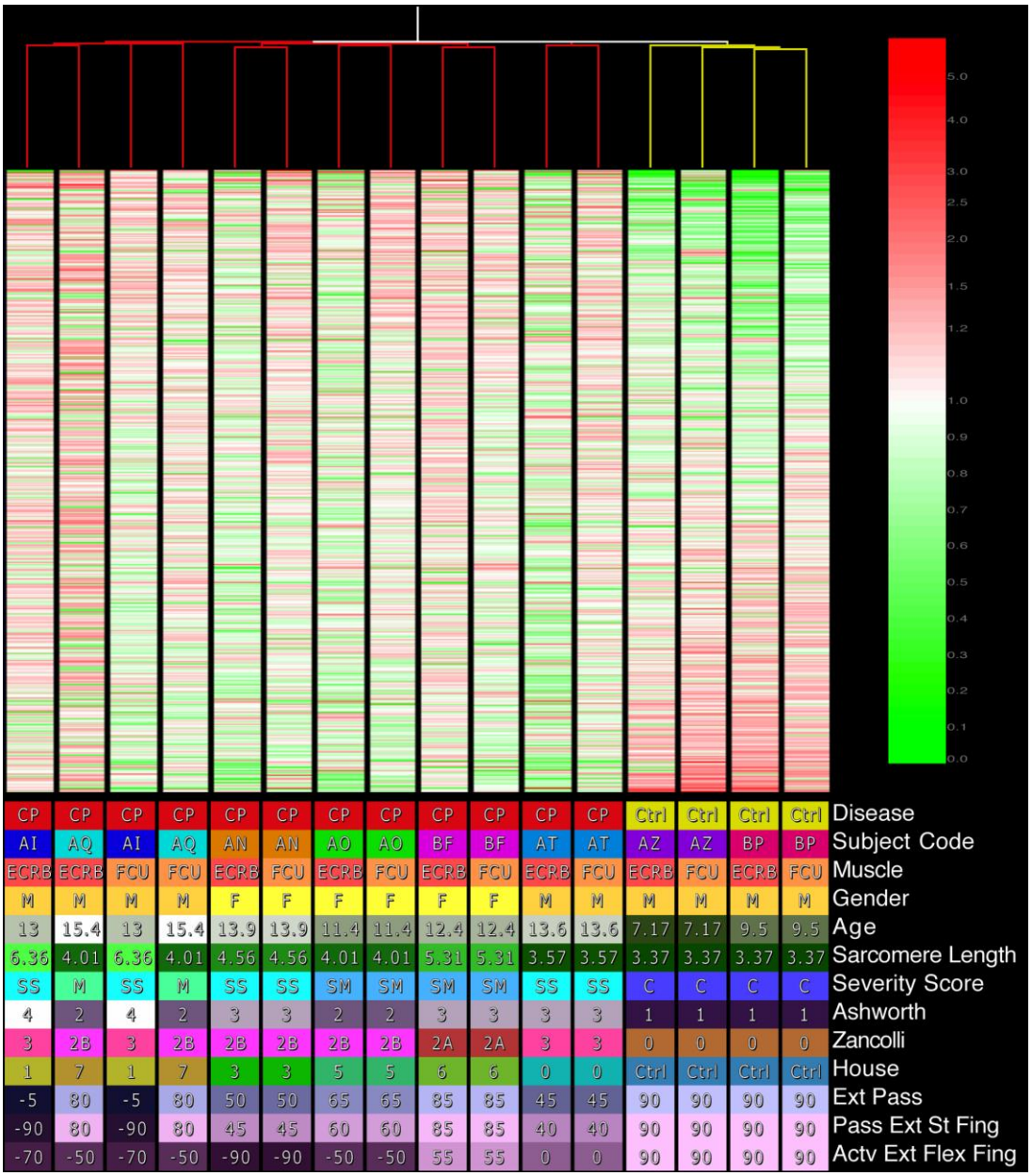

Figure 2.1: (**Top**) Condition tree created using Pearson Correlation for the similarity score and an average linkage clustering algorithm. The tree was created based on all present features. MAS5 data were used with expression values normalized to each features median. Features are ordered from highest expression ratio to lowest. **(Bottom)** Clinical conditions color-coded with values for each sample.

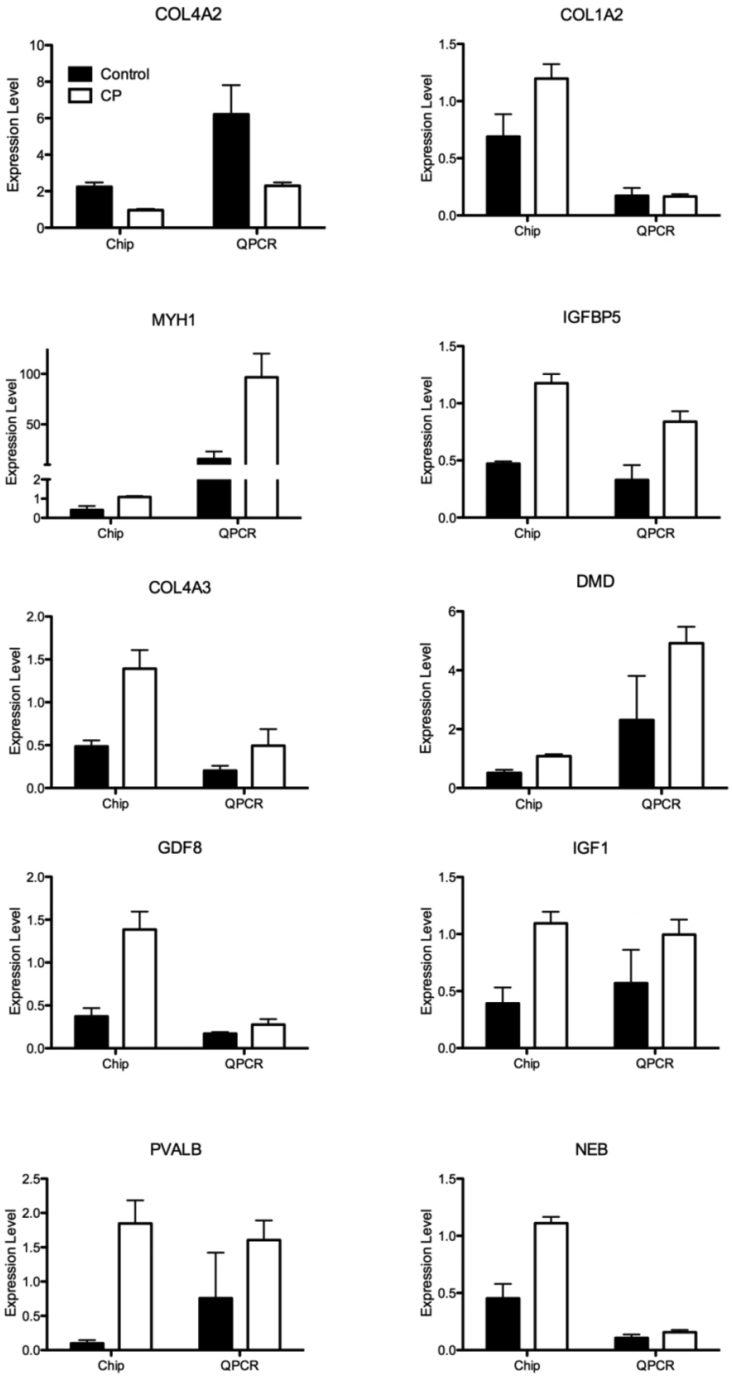

Figure 2.2: QPCR results compared to GeneChip results for several individual genes shown from CP (open bars) and CTRL (filled bars) patients. Error bars represent SEM. QPCR data are from dilute (1:100) samples to test multiple genes and represent transcript level relative to total RNA (fg/μg). GeneChip data are normalized to the median value for each gene and averaged across CP or CTRL samples.

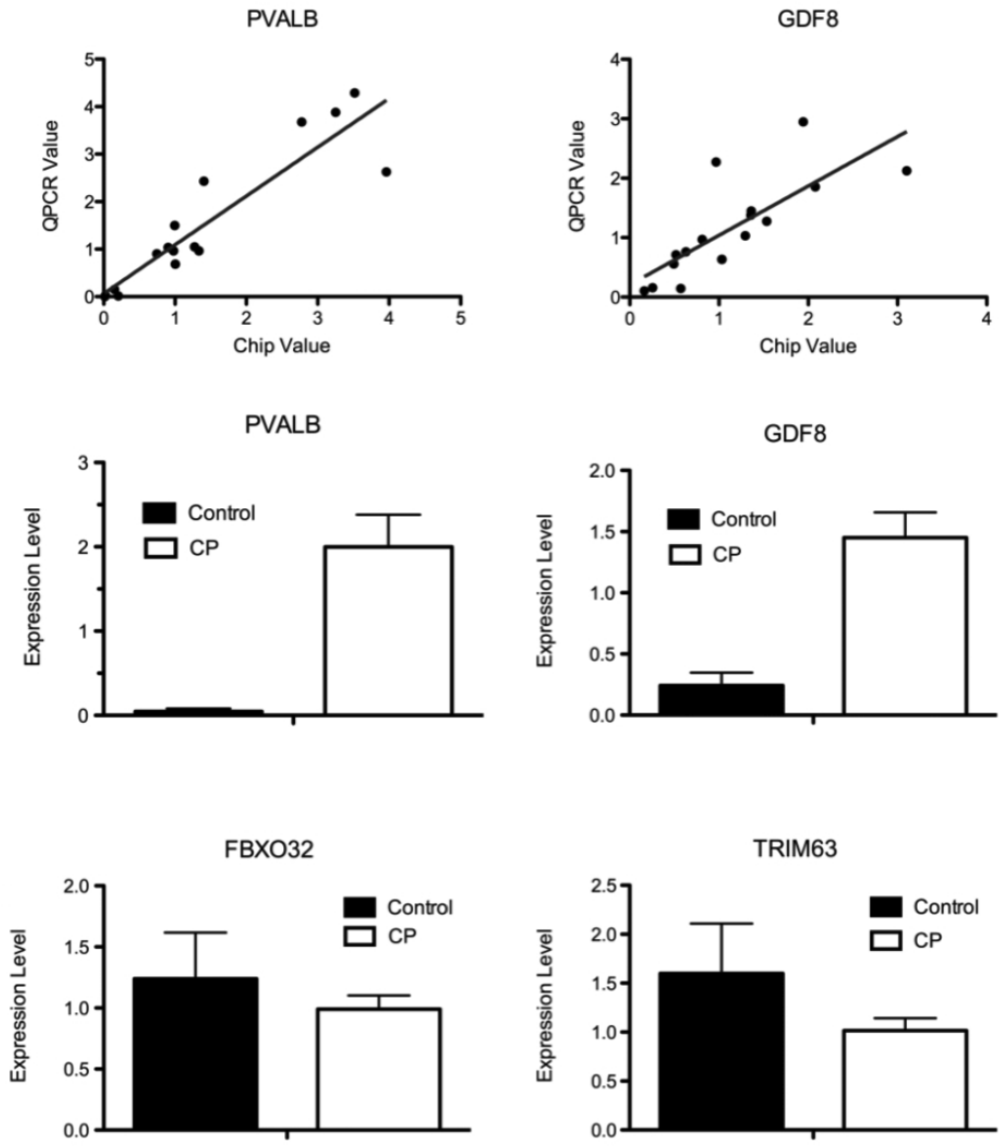

Figure 2.3: (**A/B**) Sample-to-sample correlation between the QPCR and GeneChip results. (**A**) PVALB, parvalbumin; (**B**) GDF8, myostatin. The solid line is a best fit regression line. (**C-F**) QPCR results showing the difference between CP and CTRL patients for specific genes, (**C**) PVALB, (**D**) GDF8, (**E**) FBXO32/MaFBX/Atrogin, (**F**) TRIM63/MuRF. Values are expressed determined relative to GAPDH and normalized to the median value for each individual gene. (*) represents significant difference ($P < 0.05$). Error bars represent SEM.

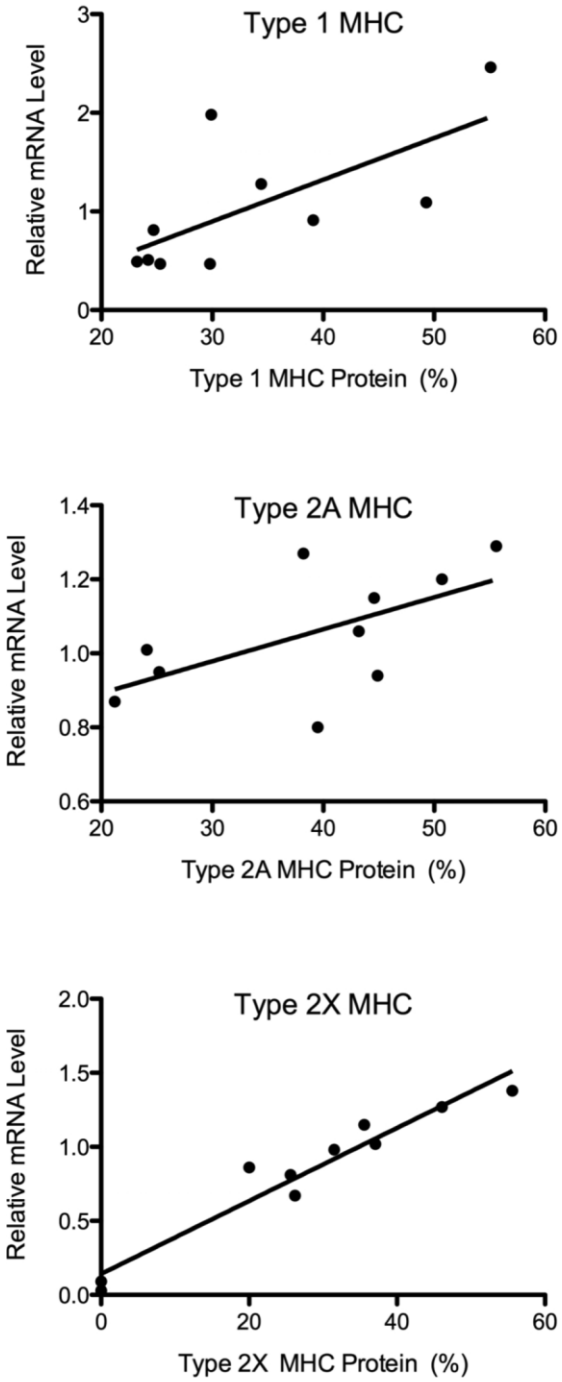

Figure 2.4: Comparison of the GeneChip mRNA data to protein SDS-PAGE gel data for three myosin heavy chains commonly expressed in human skeletal muscle, (**A**) Type 1 MHC (MYH6), (**B**) Type 2A MHC (MYH2), (**C**) Type 2X MHC (MYH1). mRNA data are normalized to the median value for each gene on the chip using MAS5 preprocessing and protein data are normalized to total MyHC content.

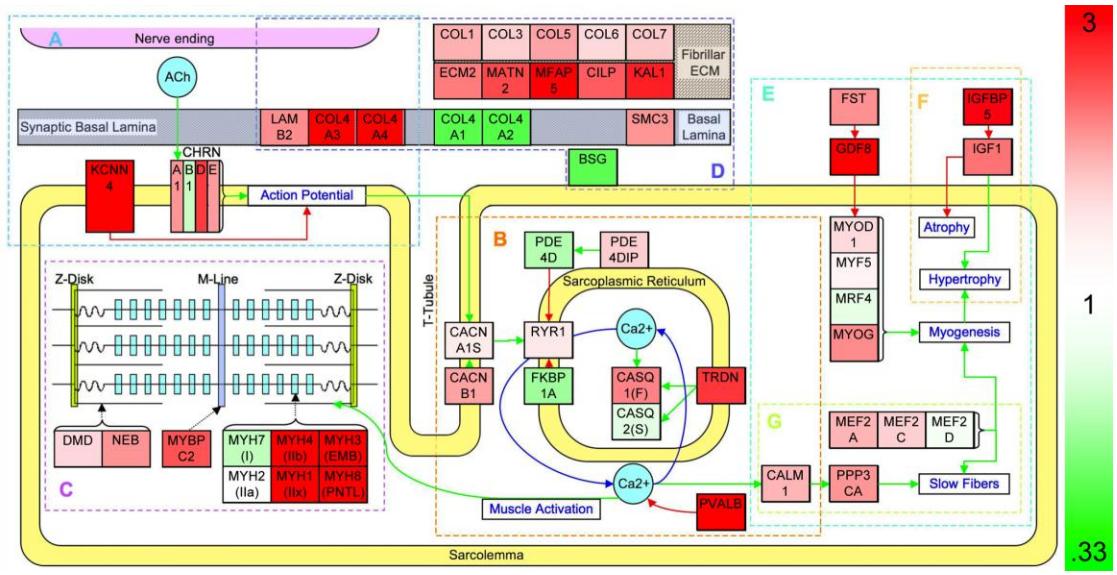

Figure 2.5: Pathways specific to muscle analysis of transcription in CP muscle. Pathways A-G involved in muscle function. Color is determined by the expression ratio. Up-regulated genes are red and down-regulated genes are green. Green connectors represent activation and red connectors represent inhibition in the direction of the arrow. Bolded genes represent those that are significantly altered in all three preprocessing algorithms. Italic genes (RAS, MAPK) are sets of genes involved in muscle MAPK pathway in muscle, but are not colored by expression because many individual genes are applicable and not altered in CP. Pathways represented are (**A**) NMJ, (**B**) ECC, (**C**) MC, (**D**) ECM, (**E**) MYG, (**F**) FT, and (**G**) IGF1.

CHAPTER 3

SYSTEMS ANALYSIS OF GENES RELATED TO SKELETAL MUSCLE FUNCTION

3.1 Abstract

Current technology allows researchers to probe the genome to investigate tissue biology. Skeletal muscle is dependent proteins encoded by a subset of genes in order to function efficiently. We have defined 9 gene networks whose coordination is critical to muscle function in order to facilitate objective analysis of muscle systems. We begin with genes involved in the neuromuscular junction to create an action potential. That action potential is transmitted to proteins encoded by genes involved in excitation-contraction coupling for Ca^{2+} release. Ca^{2+} then activates contractile proteins supporting actin and myosin crossbridge cycling. The force generated in the sarcomere is transmitted through proteins that produce cytoskeletal proteins through the sarcolemma and out to critical genes that support the extracellular matrix of skeletal muscle. Muscle contraction is fueled through many proteins that regulate energy metabolism within muscle. Inflammation is a common response that can regulate multiple genetic pathways within muscle. Muscle also has multiple

physiological pathways that regulate muscle size through atrophy or hypertrophy. Finally, the transcripts that are associated with fast muscle fibers and their corresponding isoforms in slow muscle fibers are delineated. While these pathways are not comprehensive, they represent important genes that have been established to function in skeletal muscle specifically through a review of the literature. Combining high throughput systems analysis with advanced networking software allows researchers to use these networks to objectively study skeletal muscle systems.

3.2 Introduction

Skeletal muscle's primary function is to generate force and produce movement. This requires coordination among many physiological pathways, and obviously their associated genes. Loss of skeletal muscle function due to disease results from a variety of biological factors and altered transcriptional pathways. Thus, understanding genes that regulate muscle function is a prerequisite to understanding the mechanisms of muscle pathology. This review highlight the genes that are most critical to muscle function and places them in a context of muscle physiology as a whole using current reviews in muscle physiology and muscle gene onotology [1]. We do not provide an exhaustive list of genes that regulate muscle function, but rather explore how various pathways are distorted in a variety of muscle pathologies and the downstream consequences of altered gene expression. The networks created here will provide a foundation from which to build more detailed and specific networks. The networks have been create in a Cytoscape (Cytoscape Consotrium)[2] for use in the

interpretation of current high throughput and system level technologies such as microarrays [3] and protein arrays [4]. This work will also be useful to provide a general reference for studying the interaction between genes and muscle physiology.

As stated, proper muscle function requires the coordination of many integrated gene networks. Muscle contraction is initiated at the specialized neuromuscular junction (NMJ) where acetylcholine is released from the nerve ending, diffuses to muscle receptors and initiates an action potential. The action potential propagates along the sarcolemma, down the transverse tubules and, in a process known as excitation contraction-coupling (ECC) calcium is released from the sarcoplasmic reticulum. Calcium binding to the regulatory units of the thin filament trigger the myosin cross-bridge cycle that creates muscle contraction (MC). Muscle contraction force is transmitted through specialized networks of proteins within the cell to the costameres and out to the extracellular matrix (ECM). Myosin cross bridge cycling requires ATP, and thus skeletal muscle function also requires metabolism (MET) and storage of carbohydrates and fatty acids. When a muscle undergoes damage, particularly in eccentric contractions, there is an inflammatory (INF) system response that occurs within the muscle. As a very plastic tissue, muscle responds to injury by increasing muscle size dependent upon resident muscle progenitors, satellite cells. With altered use, muscle adapts by coordinating a change in muscle mass via synchronized muscle hypertrophy or atrophy (HA). Many aspects of muscle function required distinct protein isoforms specific to various fiber types. Slow muscles are designed more for chronic use and high metabolic demand while fast muscle is designed more for rapid force production that requires tight calcium handling. Thus, it

is not surprising that isoforms related to muscle contraction and gene regulatory networks related to fiber type determination would be present.

Understanding muscle diseases requires knowledge of the protein used for force production. Duchenne Muscular Dystrophy (DMD) is the most frequently studied muscle disease and, although it results from the loss of a single gene product, dystrophin, many muscle functions are compromised[5]. Dystrophin is part of the costamere complex that links MC to the ECM and, when disrupted, allows mechanically-induced membrane damage [6]. This allows calcium influx that contributes ECC alterations and muscle degradation and damage [7] and is associated with a large INF response [8, 9] as well as cycles of regeneration [10]. As the HA pathway is exhausted, the muscle undergoes an increase in ECM fibrosis[11]. This illustrates the interconnectivity of these muscle functions and demonstrates how understanding a single protein's role is critical to understanding muscle pathology. This work will provide a framework for those investigating muscle disease and adaptation to efficiently inspect muscle function as a system of related proteins, especially to take advantage of the high-throughput technologies currently available.

3.3 Neuromuscular Junction

The NMJ function requires coordination of many transcripts that are expressed primarily or exclusively local to the NMJ (Fig. 3.1). The action is initiated by acetylcholine (ACh) release from the motor neuron, which crosses the synaptic basal lamina to bind to the nicotinic acetylcholine receptor (*CHRN*), which consists of 5

subunits [12]. The gamma subunit (*CHRNG*) is expressed in immature muscle and replaced by the epsilon subunit upon maturity [13]. *CHRN* is a ligand gated channel that allows sodium influx upon binding to create an endplate potential. This potential activates the voltage gated sodium channels in muscle *SCN4A* to transform the end plate potential into an action potential which is propagated throughout the muscle and initiates ECC [14].

To maintain proper clustering in the postsynaptic muscle, motor neurons also release agrin (*AGRN*). *AGRN* binds to a receptor on the muscle transmembrane receptor *MUSK* along with its extracellular co-receptor *LRP4 [12]*. *MUSK* binding eventually results in the activation of *RAPSN*, which acts as an intracellular scaffold for *CHRN*. MUSK also interacts with 14-3-3γ (*YWHAG*), a signal transduction protein involved in synaptic gene expression. Synaptic gene expression is also mediated through neuregulin (*NRG1*) a glycoprotein, which binds to a family epidermal growth factor receptor (*ERBB*). *ERBB* bind to *NRG1* results in activation of *GABP* transcription factors for synaptic genes [13].

The synaptic basal lamina plays an important role in organizing the NMJ and includes a unique subset of genes that are expressed preferentially in the region. It is primarily made up of *COL4* with the α3-6 subunits, instead of the α1-2 subunits found around the rest of muscle, and laminin subunits *LAMA5* and *LAMB2 [12]*. The collagen and laminin networks are linked by glycoprotein NID1. Perlecan (*HSPG2*) is the major proteoglycan present. The synaptic basal lamina also includes *COLQ*, which along with *HSPG2* binds and anchors acetylcholine esterase (*ACHE*) to the

neuromuscular junction. The function of *ACHE* is to hydrolyze acetylcholine which inactivates it and terminates activation [14].

3.4 Excitation Contraction Coupling

The action potential generated at the neuromuscular junction travels throughout the muscle and to the interior of the muscle via the transverse-tubule (T-tubules) system (Fig. 3.2). The action potential activates the voltage gated Ca^{2+} channel *DHPR,* which consists of 5 subunits. The close proximity of DHPR and the ryanodine receptor *RYR1* on the sarcoplasmic reticulum (SR) ensures that Ca^{2+} entering the cell triggers opening of *RYR1* to release Ca^{2+} from the SR store [15, 16]. A unique ryanodine receptor (*RYR3*) is expressed in immature muscle and is replaced by *RYR1* during development [17]. Ca^{2+} serves as the intracellular trigger for muscle contraction. RYR1 requires multiple proteins to modulate the open probability for Ca^{2+} release. *FKBP1A* is an SR gene that directly interacts with *RYR1* and is required for full conductance [15]; *S100A1* also binds to *RYR1* to increase open probability [18], and *SYLP2* acts from the T-tubules to increase open probability without increasing current amplitude [17].

For muscle relaxation to occur, Ca^{2+} is pumped back into the SR via the ATP dependent SERCA pumps (*ATP2A*), which have different isoforms for fast and slow muscle (Figure 2) [17]. *PVALB* can act as an intracellular Ca^{2+} store by binding free Ca^{2+} [19]. Conversely SR proteins *SLN* and *PLN*, when dephosphorylated, slow relaxation by inhibiting *ATP2A* reuptake of Ca^{2+} [17, 20]. Maintaining the high

concentration of Ca^{2+} within the SR requires Ca^{2+} binding to *CASQ*, which also has different isoforms in fast and slow muscle (Figure 9). *CASQ* is held within the SR and near *RYR1* by luminal proteins triadin (*TRDN*) and junctin (*ASPH*). The SR is held in close approximation to the T-tubule system physically by the linking protein junctophilin (*JPH1*) [21].

Aside from initiated contraction, Ca^{2+} also has other signalling mechanisms in muscle. Ca^{2+} activates *CAPN*, a family of proteolytic enzymes important in muscle [22]. Through the activation of calmodulin (*CALM1*) Ca^{2+} can also activate many growth and metabolic responses that are discussed in subsequent sections through *CAMK2* or calcineurin (*PPP3CA*) activation [19]. Sarcolemmal channels such as *TRPC*'s that allow Ca^{2+} into the cell also contribute to activation of these Ca^{2+} pathways [23].

3.5 Sarcomere Contraction

Sarcomere contraction is based on the sliding of interacting thick and thin filaments within the sarcomere [24, 25] (Fig. 3.3). The thick filament is made up primarily of type 2 myosin, which contains two myosin heavy chain (*MYH*) and four myosin light chain (MYL) subunits [26]. The myosin head interacts with the thin filament by binding to actin (*ACTA*) in a force generating mechanism is termed the cross-bridge cycle. *TPM* wraps around the thin filament and obscures the MYH binding pocket of ACTA filaments. *TPM* is regulated by the troponin (*TN*) complex, which consists of three subunits, each with different isoforms according to muscle

type [27]. Regulation is mediated by Ca^{2+} binding to *TNNC,* which removes the inhibitory subunit *TNNI* from its position with *TPM* and both subunits are anchored to the thin filament by the third *TNNT* [28]. *MYH* is an ATPase that requires ATP for the release phase of the cross-bridge cycle. ATP levels are maintained in skeletal muscle by *CKM,* which uses phospho-creatine to transfer a phosphate group to ATP [29]. Myosin binding proteins (*MYBPC*) are important for thick filament formation and maintenance. *MYH* is the major determinant of fiber type and both *MYL* and *MYBPC* have different isoforms corresponding to fiber type. *MYH* and *MYL* also have particular isoforms (*MYH3*, *MYH8*) and (*MYL4*, *MYL5*) respectively that are expressed in immature muscle [26].

The sarcomere structure is maintained by a variety of proteins. The largest protein in the body is *TTN*, which spans a half sarcomere from myosin in the middle of the sarcomere near the m-line and interacts with myomesin (*MYOM*) at the Z disc end of the sarcomere. The Z disc is made up primarily of *ACTN* with many interacting proteins. One of those is *TCAP*, which localizes titin to the Z disc. It also interacts with *CAPZ*, which caps the barbed end of the actin thin filament. The large protein *NEB*, which extends the full length of the thin filament and contains many repeated actin binding sites, maintains the thin filament structure [26]. On the pointed end, *NEB* interacts with the actin capping protein *TMOD*, which has different isoforms corresponding to muscle type [30]. *NEB* is anchored to the Z disc by *MYPN* and *MYOT* also plays a role in thin filament stability [26].

3.6 Cytoskeleton

The force generated within the sarcomere is transmitted throughout the cell to the surrounding tissue through various cytoskeleton proteins (Fig. 3.4). The most studied in skeletal muscle is dystrophin (*DMD*), which mechanically links the cytoskeleton to the ECM [31]. Utrophin (*UTRN*) provides a similar role as *DMD*, but functions in the NMJ and may be able to partially compensate in absence of *DMD*. *DMD* is associated with many proteins that interact to form the dystrophin-associated glycoprotein (DAG) complex. Dystroglycan (*DAG1*) serves as a link from the DAG to the ECM through its laminin binding properties. *DAG1* is glycosolated by membrane proteins *LARGE* and *FCMD*. Also associated with the DMD are sarcoglycans (*SGC*), transmembrane proteins that help stabilize the sarcolemma and also link to the cytoskeleton through interactions with filamen γ (*FLNC*). *FLNC* binds *SGC* at the sarcolemma and also filamen associated protein myozenin (*MYOZ2*) in the Z-disc [26]. Membrane stability is also maintained by dysferlin (*DYSF*) with its function in membrane fusion [32]. Dystrobrevins (*DTN*, two isoforms) bind *DMD* and syntrophins (*SNT*), which localize nitric oxide synthase 1 (*NOS1*) near the sarcolemma. *DTN* also connects the DAG complex to intermediate filaments syncoilin (*SYNC*) and synemin (*SYNM*).

These intermediate filaments connect to desmin (*DES*) the primary intermediate in skeletal muscle. However, *VIM* predominates expression during muscle development, but is then lost at maturity. *DES* links both mitochondria and the

nucleus within the cell. The nuclear membrane anchorage to the cytoskeleton is mediated by emerin (*EMD*), which also binds nuclear lamin (*LMNA*) with its role in nuclear stability and gene expression. *DES* binds these organelles to the muscle structure at the Z-disc. The Z-disc itself consists of the overlapping barbed end actin filaments from adjacent sarcomeres with its principle component *ACTN* connecting actin filaments. The Z-disc also contains *ACTN* binding protein cypher (*LDB3*), for the linkage of filamens through *MYOZ2,* and muscle LIM protein (*CSRP3*) which links to the ankryn (*ANK*) and spectrin (*SPT*) network within the muscle. *ANK* and *SPT* interact with actin and support membrane stability. They also interact with *OBSCN*, which plays a role in localizing the SR through interactions with both the SR and *TTN* in the sarcomere [26].

Integrins also provide a physical link between the cell and the ECM. Integrins bind the actin cytoskeleton and also directly to laminins in the ECM. Integrins are dimers formed by α and β subunits, of which *ITGA7* and *ITGB1* are the most common forms in muscle [26].

3.7 Extracellular Matrix

The cytoskeleton provides a network to transmit force out to the ECM (Fig. 3.5). This is initially transmitted to the basal lamina, a mesh like network consisting primarily of *COL4* (*COL4A1* and *COL4A2*) and laminin (*LAM*; most commonly *LAMA2*; *LAMB1*/*LAMB2*, *LAMC1* in muscle) [33]. *HSPG2* is a proteoglycan in the basal lamina that binds both *COL4* and *LAM*. Other basal laminar proteoglycans

include syndecans (*SDC*), which play an important role in satellite cell differentiation and biglycan (*BGN*) an important binding partner [31, 34]. The glycoprotein fibronectin (*FN1*) is a multimeric protein that serves as a link to many proteins within the basal lamina [35].

As opposed to the basal lamina, the fibrillar ECM has strong load bearing capabilities and is made up primarily of collagen I (*COL1*) and collagen III (*COL3A1*). Collagen VI (*COL6*) serves as an important link between the fibrillar and laminar ECM [33]. Decorin (*DCN*) is the major fibrillar proteoglycan in muscle [34]. *FN1* also plays an important link in fibrillar ECM. It binds to glycoprotein tenascin C (*TNC*) and provides strength and elasticity to the ECM and is highly expressed in regenerating fibers [35]. ECM strength is also determined by the amount of collagen crosslinking and lysyl oxidase (*LOX*) is a copper enzyme primarily responsible for crosslinking [36].

The ECM also has a program for degradation through zinc dependent matrix metalloproteinases (MMPs). *MMP2* and *MMP9* are abundant in muscle and breakdown *COL4* found in the basal lamina. *MMP2* is activated by membrane type *MMP14* at the sarcolemma. *MMP1* functions as a protease in the fibrillar ECM breaking down both *COL1* and *COL3A1*. MMP activity is controlled by tissue inhibitors of metalloproteinases (TIMPs) and are represented by *TIMP1* and *TIMP2* and skeletal muscle [37].

Production of ECM proteins is controlled by specific growth factors. The most well studied inducer of ECM in skeletal muscle for its role in fibrosis is transforming growth factor, beta 1 (*TGFB1*) [38]. *TGFB1* binds *BGN* in skeletal muscle and its

activity is also inhibited through binding to *LTBP4* [39, 40]. Connective tissue growth factor (*CTGF*) is another critical component in ECM signalling that leads to expression of collagen genes and fibrosis [38]. Following expression, collagen must be processed before becoming functional. Prolyl 4-hydroxylase (*P4*) catalyzes the formation of hydroxyproline and *PLOD3* catalyzes posttranslational modification and both proteins reflect the rate of collagen biosynthesis [36].

3.8 Energy Metabolism

Muscle is a very energetically active tissue, requiring ATP to power the crossbridge cycle and also for calcium transport in relaxation (Fig. 3.6). Glucose is a primary substrate for energy metabolism and is transported across the sarcolemma by GLUT4 (*SLC2A4*). *SLC2A4* translocation to the sarcolemma is controlled by *AMPK*. *AMPK* is an energy sensing enzyme that becomes activated in response to low energy levels [41]. Intracellular glucose can be stored in the form of glycogen via the enzyme glycogen synthase 1 (*GYS1*), which can then be broken down back into glucose via the enzyme glycogen phosphorylase (*PYGM*) when necessitated [42]. Glucose is broken down further into pyruvate through glycolysis, which nets ATP. Glucose is prepared for glycolysis by the phosphorylating enzyme hexokinase (*HK1*), glycolysis is maintained by lactate dehydrogenase A (*LDHA*), and the rate-limiting step of glycolysis is controlled by the enzyme phosphofructokinase (*PFKM*) [43].

Pyruvate conversation to Acetyl-CoA by pyruvate dehydrogenase (*PDH*) allows for progression through the TCA cycle and subsequent oxidative

phosphorylation [43]. Acetyl-CoA can also be produced from fatty acids in skeletal muscle. Fatty acid uptake into the cell is also through an *AMPK* mediated transporter *CD36* [41]. Shuttling proteins fatty acid binding protein 3 (*FABP*) and lipoprotein lipase (*LPL*) also mediate fatty acid transport [44]. Intracellular fatty acids may be stored as triglycerides for which the enzyme *GPAM* catalyzes the initial and committing step [41]. Hormone sensitive lipase (*LIPE*) is responsible for triglyceride breakdown to free fatty acids in muscle along with ATGL (*PNPLA2*) for the initial step and also assisted by *LPL* for triglyceride hydrolysis [44]. To be used in energy metabolism fatty acids must be transported into the mitochondria via *CPT1B* [43]. Here fatty acids can undergo beta-oxidation to produce Acetyl-CoA and NADH, which is catalysed by enzymes *MYLCD* and *HADH* [44].

Acetyl-CoA enters the TCA cycle to produce NADH. Citrate synthase (*CS*) catalyses the rate limiting step within the TCA cycle, which also requires the enzyme succinate dehydrogenase (*SDH*) [45]. NADH is then used as an electron carrier in oxidative phosphorylation that also uses oxygen as an oxidising agent [46]. Oxygen is provided to the muscle through the vasculature, which is stimulated by *VEGF*, and transported within the muscle by *MB* [47, 48]. Oxidative phosphorylation is catalysed by a set of 4 complexes in series (*NDUF*, *SDH*, *CYC*, *COX*). The energy from the electron gradient produced is then converted to ATP by ATP synthase (*ATP5*) [46].

The production of metabolic transcripts is largely controlled PGC-1α (*PPARGC1*) within skeletal muscle in conjunction with many other transcription factors [49]. *AMPK* activates *PPARGC1A* when energy levels are low [41]. Ca^{2+} also plays a role through the activation of *PPP3CA*, *CAMK4*, and *CAMK2*. *PPP3CA*

activates the transcription factor *NFATC1* that produces muscle metabolic genes including myoblogin (*MB*) [50]. *PPP3CA* and *CAMK4* both activate *CREB1*, a transcription factor that is integral to *PPARGC1A* mediated expression [45]. *CAMK2* activates p38 (*MAPK14*) that activates transcription factors *MEF2* and *ATF2*, which participate in metabolic transcription through *PPARGC1A* [45, 46]. NRF1 together with PPARGC1A plays a large role in mitochondrial expression through transcription factor A, mitocontrial (*TFAM*). *TFAM* works in concert with transcription factor B1 and B2, mitochondrial (*TFB*) and nuclear respiratory factor 2 (*NFE2L2*) in the mitochondrial transcription complex which is also maintained by *TP53* [51].

3.9 Inflammation

Injury to skeletal muscle initiates a coordinated and precisely controlled inflammatory response that is localized to the damage site (Fig. 3.7). This is a critical step in the process of muscle repair and if not properly regulated can lead to deterioration and fibrosis [38, 52, 53]. In the early stages of the inflammatory process, pro-inflammatory cytokines such as interleukin 8 (*IL8*), interferon gamma, (*IFNG*), and COX-2 (*PTGS2*) are released at the injury site attracting circulating neutrophils and classically activated macrophages, which then act to clear myofiber debris and promote myoblast proliferation [52, 53]. These monocytes secrete other pro-inflammatory cytokines such as tumor necrosis factor-alpha (*TNF*) and interleukin-1 beta (*IL1B*), which stimulate phagocytosis [38, 54].

The primary pathway for inflammation mediated protein degradation is the nuclear factor kappa B (*NFKB*) dependent pathway. Activation of *NFKB* is controlled by the Iκβ kinase (*IKBKE*) complex, which phosphorylates Iκβ targeting it for degradation and enabling the translocation of NFKB to the nucleus [55, 56]. *NFKB* affects protein turnover by increasing the expression of the ubiquitin ligase MuRF1 (*TRIM63*) and by binding to and activating interleukin-6 (*IL6*). *IL6* is thought to act in a hormone-like manner to regulate glucose homeostasis possibly via *AMPK* and can also be produced [56, 57]by the muscle itself [58, 59]. *IL6* may also be activated by heat shock factors 1 and 2 and by calcium via its activation of *NFKB* [52, 59]. *NFKB* is also activated by reactive oxygen and nitrogen species as well as *SOCS3* [60, 61], however, inside the nucleus reactive species act to inhibit *NFKB* activity [60]. Once activated, *NFKB* and *IL6* act to inhibit muscle regeneration. Evidence suggests that the mitogen-activated protein kinase p38 (*MAPK14*) is also activated in response to *TNF* and *IL1B*. It then acts to upregulate atrogin-1 (*FBXO32*), a gene involved in muscle atrophy. Additionally, p38 has been shown to activate *IL6* [58]. *TNF* is also shown to increase circulating levels of interferon-γ (*IFNG*), which activates the JAK-STAT pathway and inhibits cell growth and proliferation [62]. *TNF* and *IL1B* inhibit the expression of IGF1 a key muscle growth factor [63].

After the initial invasion of neutrophils and classically activated macrophages, a second population of macrophages secrete cytokines such as interleukin 10 (*IL10*) and *TGFB1* and act to reduce the inflammatory response [38]. *IL10* and *TGFB1* negatively regulate *IFNG* production and *IL10* inhibits the proteolytic effects of *IL1B* [52, 62]. *TGFB1* plays a role in both the initiation of fibrosis in skeletal muscle by

stimulating fibroblast proliferation and inducing myogenic cells to differentiate into myofibroblastic cells. *TGFB1* has been shown to inhibit regeneration via activation of Smad proteins [38].

3.10 Muscle Hypertrophy and Atrophy

Skeletal muscle hypertrophy and atrophy are critical processes required to maintain skeletal muscle mass, recover from injury and adapt to exercise. These processes can be triggered at the cellular level by a variety of cues including growth factors, nutritional signals and mechanical stress [56, 57] (Fig. 3.8). Insulin and insulin-like growth factor 1 (*IGF1*) have been shown to be potent inducers of hypertrophy via *IGF1* receptor (*IGF1R*) and the PI3K/Akt pathway [57]. Activated phosphatidylinositol 3 kinase (PI3KR) creates a lipid binding site on the cell membrane for *AKT1*, a serine/threonine kinase. *AKT1* then results in the activation of the mammalian target of rapamycin (*MTOR*). Activation of *MTOR* then in turn activates *RPS6KB1*, which activates genes responsible for protein synthesis. In addition, PDK1 has also been shown to phosphorylate *RPS6KB1* directly [57]. *MTOR* also participates in the growth process by phosphorylating *EIF4EBP1*, which results in the dissociation of the *EIF4EBP1*/*EIF4E* complex allowing *EIF4E* to initiate protein translation. A regulatory associated protein of *MTOR* (*RPTOR*) facilitates the phosphorylation of *RPS6KB1* and *4EBP1* by *MTOR* and has been shown to bind both proteins. *AKT1* may also influence translation by inhibiting the activity of glycogen synthase kinase 3 beta (*GSK3B*) as *GSK3B* inhibits *EIF2B*, thereby blocking its

promotion of protein translation [56, 57]. *AKT1* also prevents the forkhead box proteins (*FOXO*) from entering the nucleus via phosphorylation [56, 58]. *FOXO* transcription factors are known to promote transcription of *TRIM63* and *FBXO32*, which are ubiquitin ligases involved in muscle degradation [64].

Transcriptional regulation also plays a key role in muscle hypertrophy and control is centred around the four muscle regulatory transcription factors: muscle differentiation factor (*MYOD1*), myogenic factor 5 (*MYF5*), muscle regulatory factor 4 (*MYF6*), and myogenin (*MYOG*). *MYOD1* and *MYF5* are early myogenic factors important in differentiation [65]. *MYOD1* activates p21 (*CDKN1A*) to arrest the cell cycle and promote differentiation of muscle progenitors [66]. *MYOD1* and *MYF5* lead to the expression of *MYOG* and *MYF6* that directly to control the transcription of many muscle specific genes [65]. These transcription factors work in concert with *NFATC1* and muscle enhancement factor 2 (*MEF2*) for muscle transcription [67].

The family of mitogen-activated protein kinases (MAPK) have also been implicated in growth and hypertrophy in response to exercise [55]. The MAPK family of proteins includes extracellular signal-related kinases 1 and 2 (*MAPK1/3*), *MAPK14* and c-Jun NH2-terminal kinases (*MAPK8*), which are thought to couple cellular stress with an adaptive transcriptional response and are activated by *MAP2K* [67]. *MAPK1/3* acts to activate downstream target (*EIF4E*) to initiate protein translation and also to activate *MYOD1* [67, 68]. *MAPK1/3* can also be activated downstream of growth factors *HGF* and *FGF2* [67]. *MAPK14* has also been shown to activate *MYOD1* as well as *MYF5* and *MEF2*. The activation of *MAPK8* is correlated with an increase in

transcription of the early response genes c-jun and c-fos, which may enhance muscle regeneration [55].

Ca^{2+} can also signal muscle hypertrophy through activation of *CALM1* and *PPP3CA*. These proteins prevent the translocation of *NFATC1* to the nucleus where it acts as part of the muscle transcription machinery [50]. Calcium/calmodulin-dependent protein kinase II (*CAMK2*) is also activated by *CALM1* and prevents the binding of histone deacetylase complexes (*HDAC*). *HDAC* blocks the binding of important muscle transcription factors such as *MEF2* [69].

Skeletal muscle also contains a key autocrine signal to limit muscle growth. Myostatin (*MSTN*), a member of the transforming growth factor β pathway, has been shown to play a significant role in muscle growth as an inhibitor of hypertrophy. *MSTN* signalling is mediated through its receptor activin IIB (*ACVR2B*), which conducts a signal to the nucleus through the SMAD pathway. Expression of follistatin (*FSTN*) has been shown to increase muscle mass through action as a *MSTN* antagonist [38, 56].

3.11 Muscle Fiber Type

Skeletal muscles have different fiber types that play a role in muscle function (Fig. 3.9), with myosin heavy chain as the major determinant of fiber type in skeletal muscle [70]. Slow fibers are characterized by myosin heavy chain I (*MYH7*) and are more oxidative and used for repetitive contractions. Fast fibers are generally larger, more glycolytic, and required for brief high force contractions [70]. Fast fibers have

two myosin heavy chain isoforms expressed in mature muscle, myosin heavy chain IIa (*MYH2*), from fast fibers with oxidative capacity, and myosin heavy chain IIx (*MYH1*), from the fastest most glycolytic fibers. Many transcripts related to muscle function may be differentially regulated according to fiber type. A full description of the proteins related to muscle fiber type is beyond the scope of this review. Including *MYH*, however, some of the gene complexes listed in the sections above have distinct genes that are activated in specifically in either fast or slow muscles fibers. Figure 9 was created to specify these individual genes within complexes and provide a useful analysis of muscle fiber type beyond *MYH*.

3.12 Conclusions

For skeletal muscle to function properly, coordination among these multiple gene networks is required. Each of these networks has critical genes responsible for the function of that network, which have been established within the literature. We have delineated the critical components of these and their relationship to muscle function, however there are additional genes that may play an important role. These gene networks include: the NMJ signal transduced through ECC to trigger MC, genes critical for the transmission of force through the CYSK out to the ECM, genes involved in MET, gene signals for INF, genes regulating muscle size through MHA, and finally which gene isoforms are present in different FT. This work will allow research on muscle using high throughput technologies with advanced software packages for data analysis designed to investigate muscle tissue specifically. This will

also allow objective research onto which networks are altered in a variety of muscle adaptations or pathologies and highlight the primary targets for future therapies.

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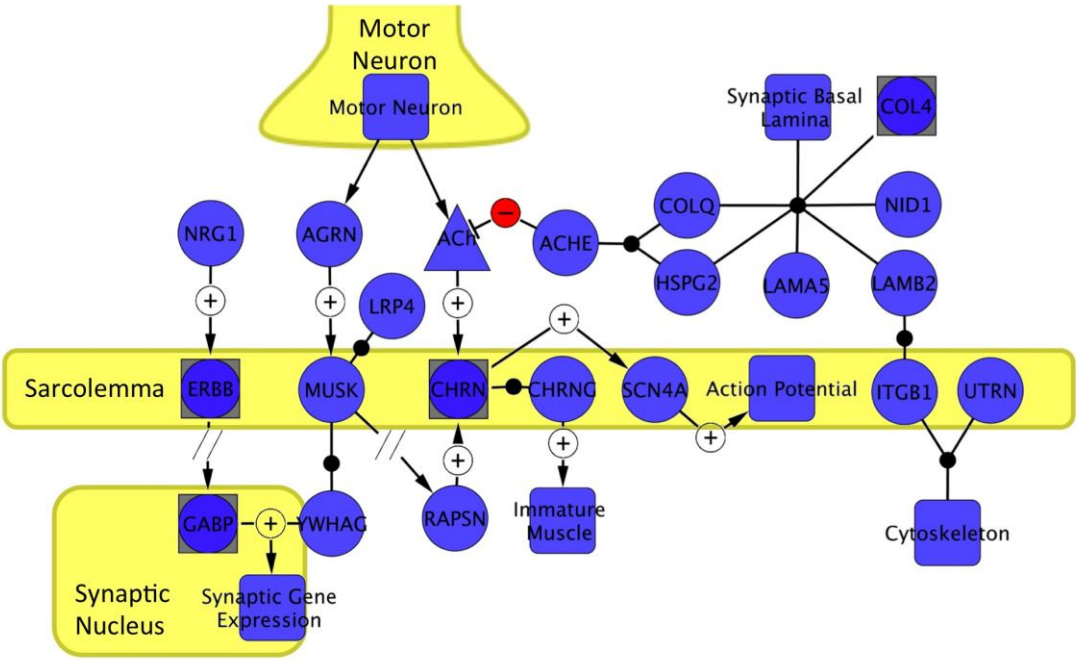

Figure 3.1: Neuromuscular Junction. Motor neurons release the neurotransmitter acetylcholine, which trigger an action potential in the muscle. NMJ formation is also induced by motor neuron factors that signal muscle proteins. For all figures there are the following nodes: (●) entrez gene symbols, (■) complexes, (▲) non-protein molecules, (■) modules or functions. There are the following interactions: (⊕) positive, (●) negative, (●) binding, (//) intermediate and (?) unknown. There are the following lines (–) basic, (→) A proceeds to B, (⊣) A does not proceed to B, and (↝) translocation of A. The genes within complexes are listed in the Supplementary Table 3.1. Some complexes are interacting proteins, however, others are multiple isoforms of a protein that have the same function.

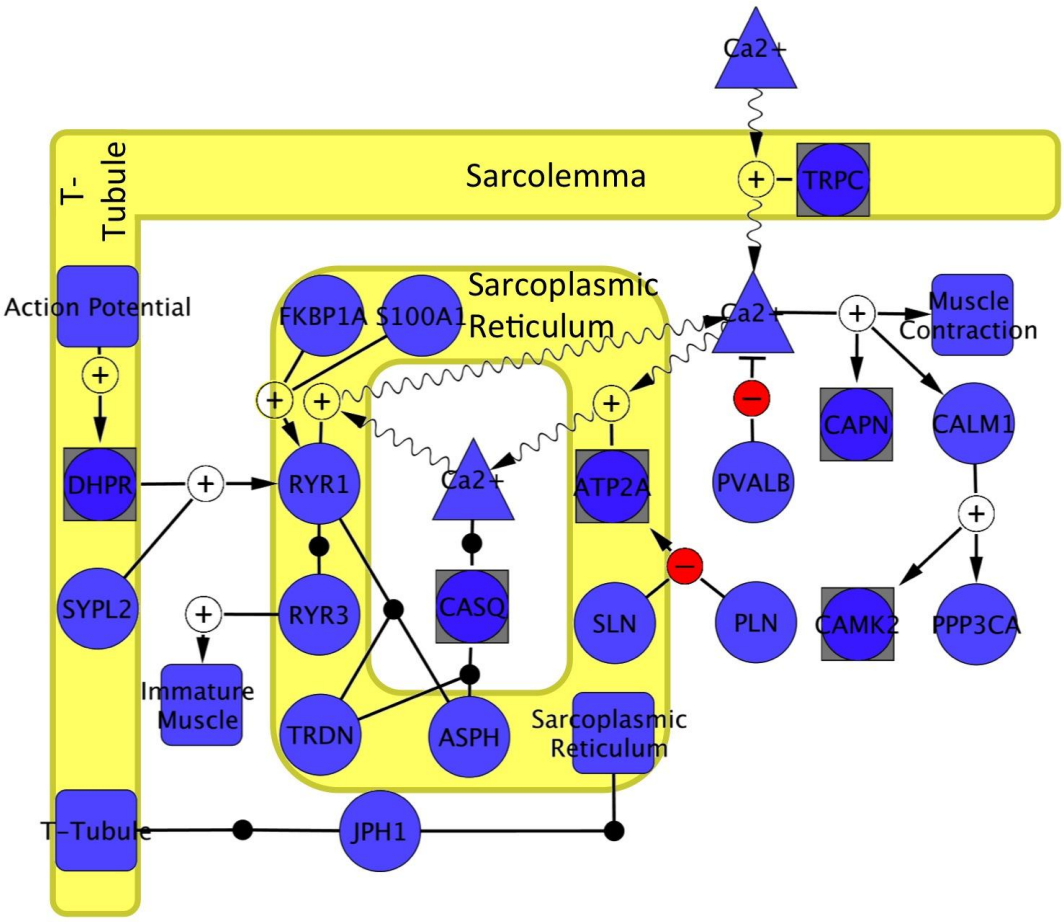

Figure 3.2: Excitation Contraction Coupling. Action potentials travel through the T-tubule system and induce Ca2+ release from the SR through the ryanodine receptor. Intracellular Ca2+ triggers muscle contraction and is then pumped back into the SR. Ca2+ plays a role in many intracellular signaling pathways.

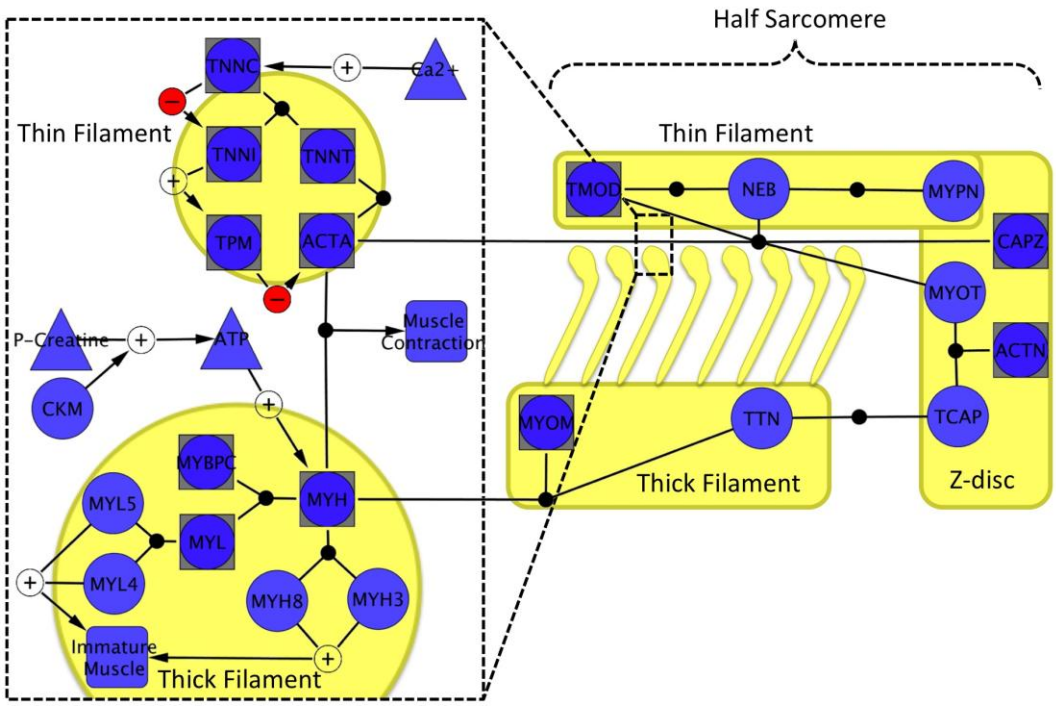

Figure 3.3: Muscle Contraction. Myosin binds to actin and undergoes cross-bridge cycling to produce contractile force. Myosin (thick) and actin (thin) filaments slide past each other during contraction. Sarcomeres are separated by Z-discs.

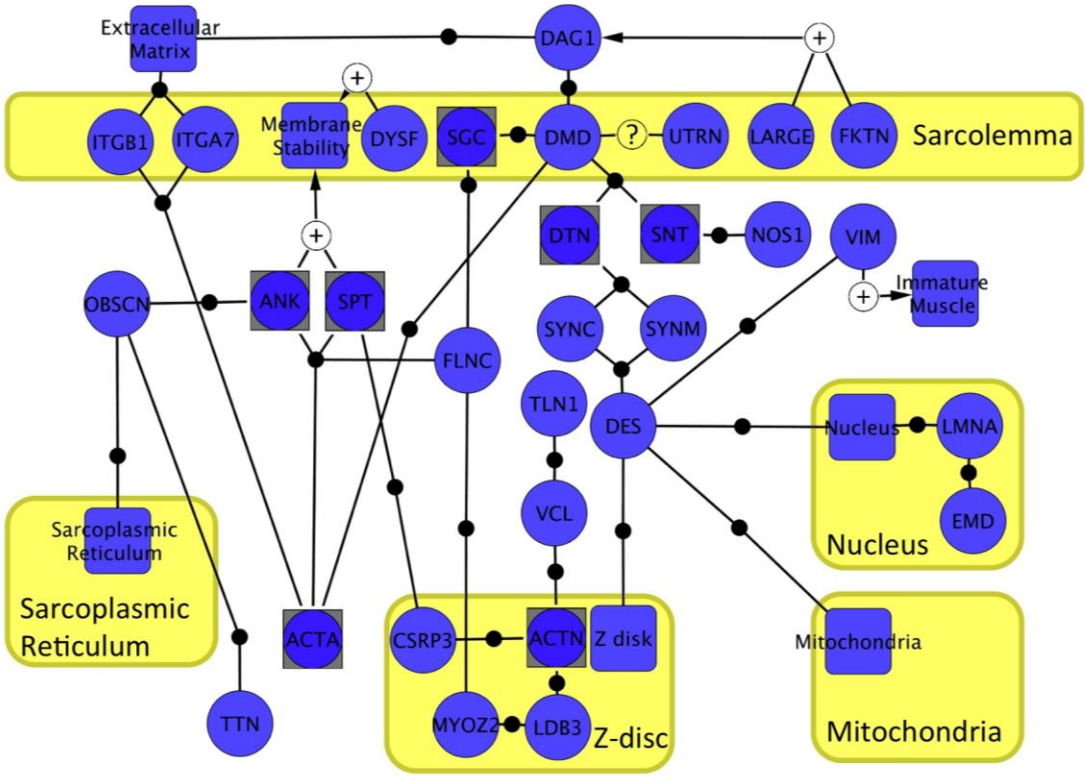

Figure 3.4: Cytoskeleton. Muscle force generated in the sarcomere is transmitted from myofibrils to the sarcolemma through the dystroglycan complex or integrins. Loads are transmitted to intracellular organelles through the intermediate filament network.

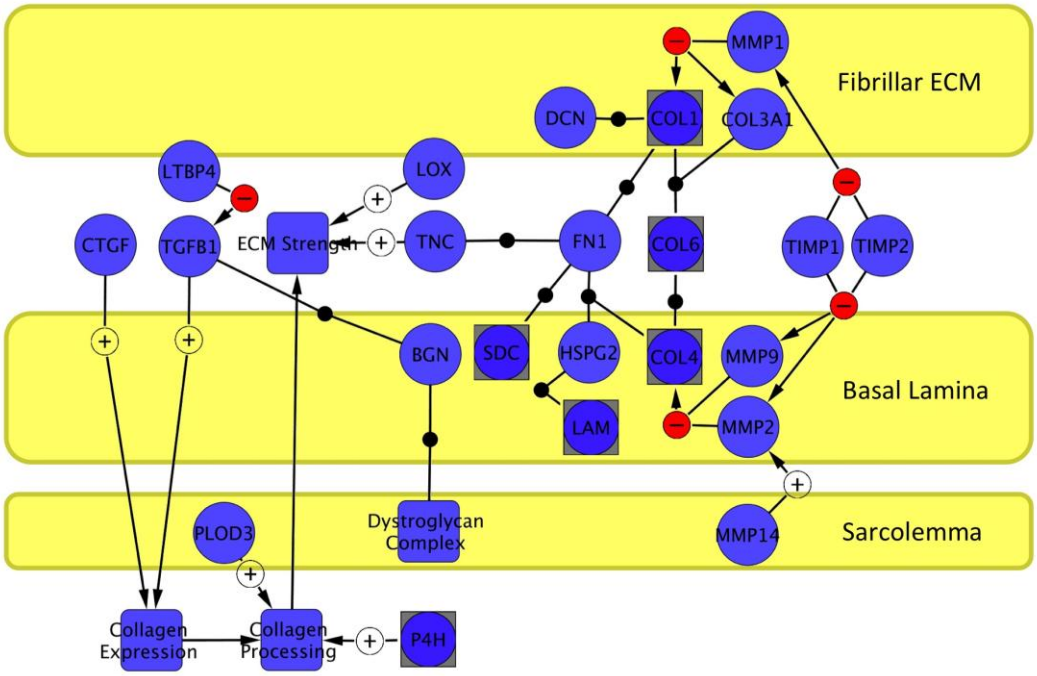

Figure 3.5: Extracellular Matrix. Provides network for intracellular loads to be transmitted extracellularly. The basal lamina is a mesh like network, while the fibrillar ECM is made up of larger collagen fibrils and associated proteins. Important growth factors are involved in ECM formation.

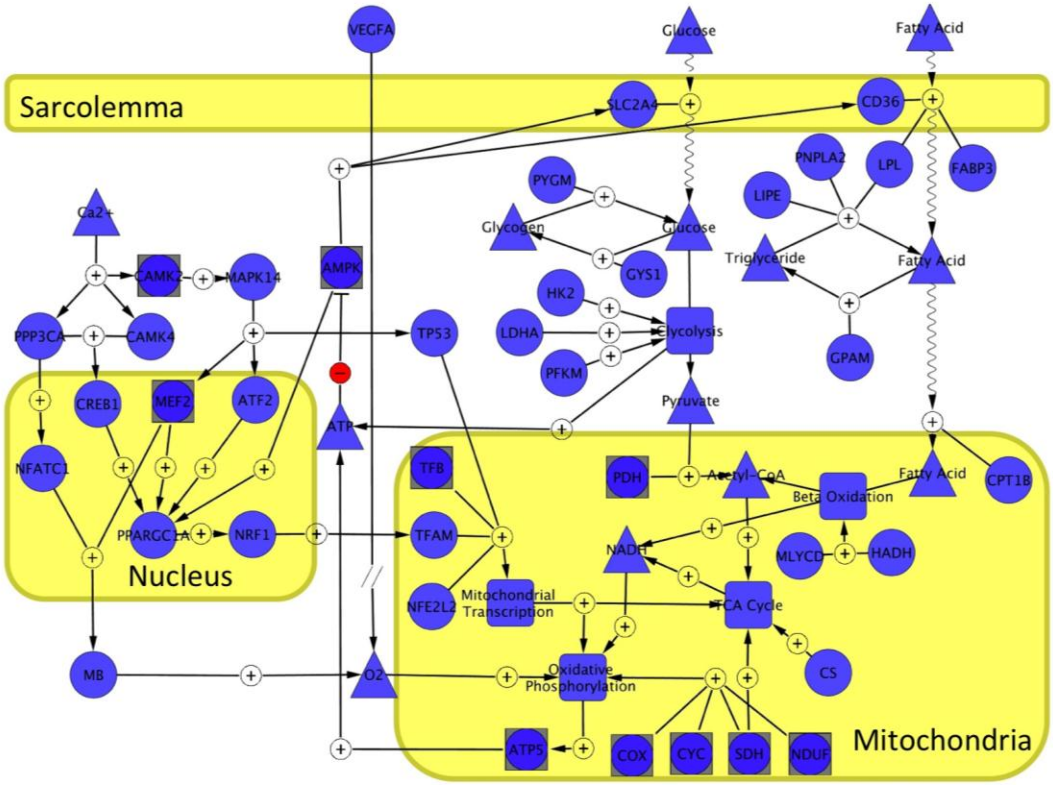

Figure 3.6: Energy Metabolism. Muscles use ATP as the energy source for contraction. ATP is generated both glycolytically and oxidatively in the muscle from glucose or fatty acids. Muscle has energy sensing mechanisms to adapt transcriptionally to demand changes.

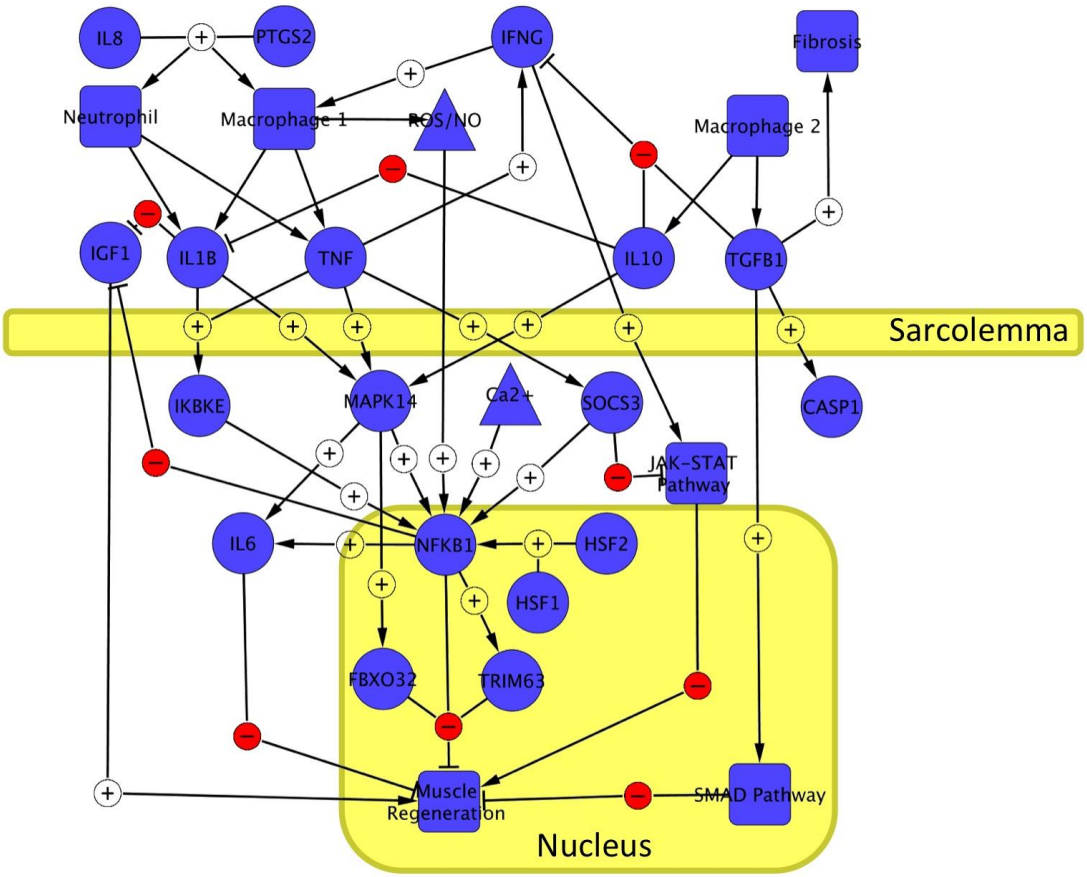

Figure 3.7: Inflammation. Early macrophages and neutrophils enter damaged muscle to clear debris and produce an inflammatory signal. If sustained inflammation can lead to degradation. Secondary macrophages enter to limit the inflammatory signals and repair muscle.

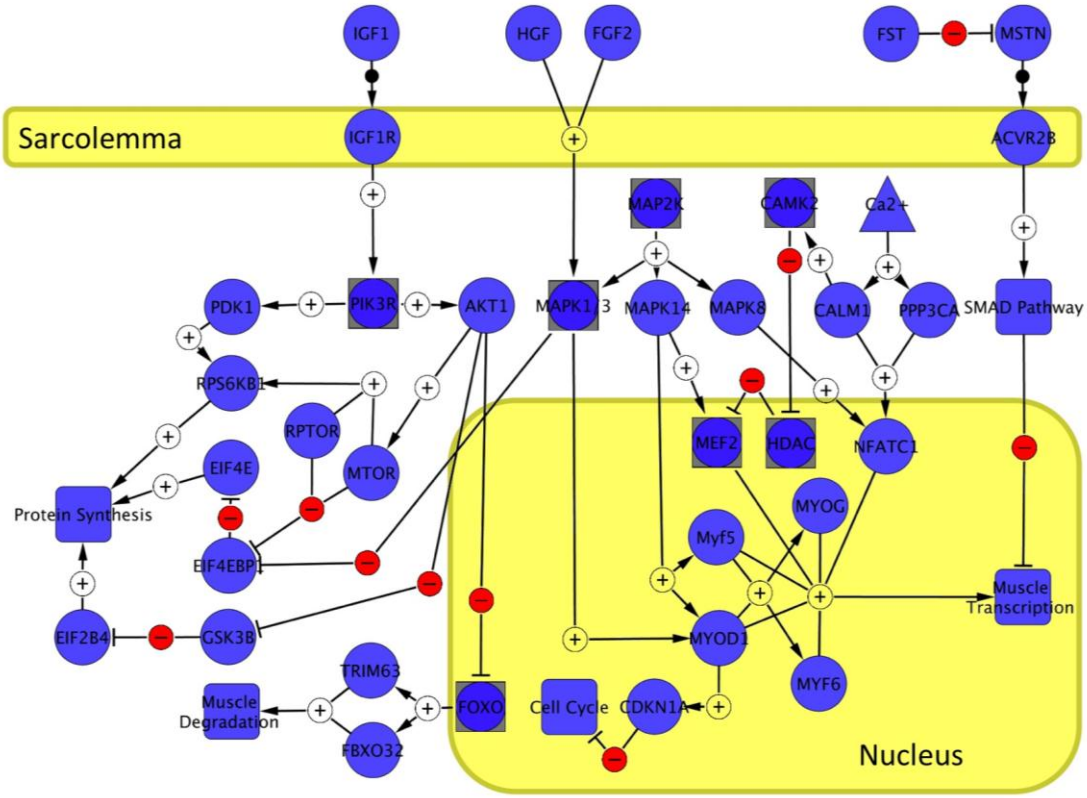

Figure 3.8: Muscle Hypertrophy and Atrophy. Multiple pathways determine muscle size. IGF1 signals fiber growth and increased protein production. MAPK's can elicit muscle myogenic factors in response to stresses. Autocrine factor MSTN limits muscle growth through the SMAD pathway.

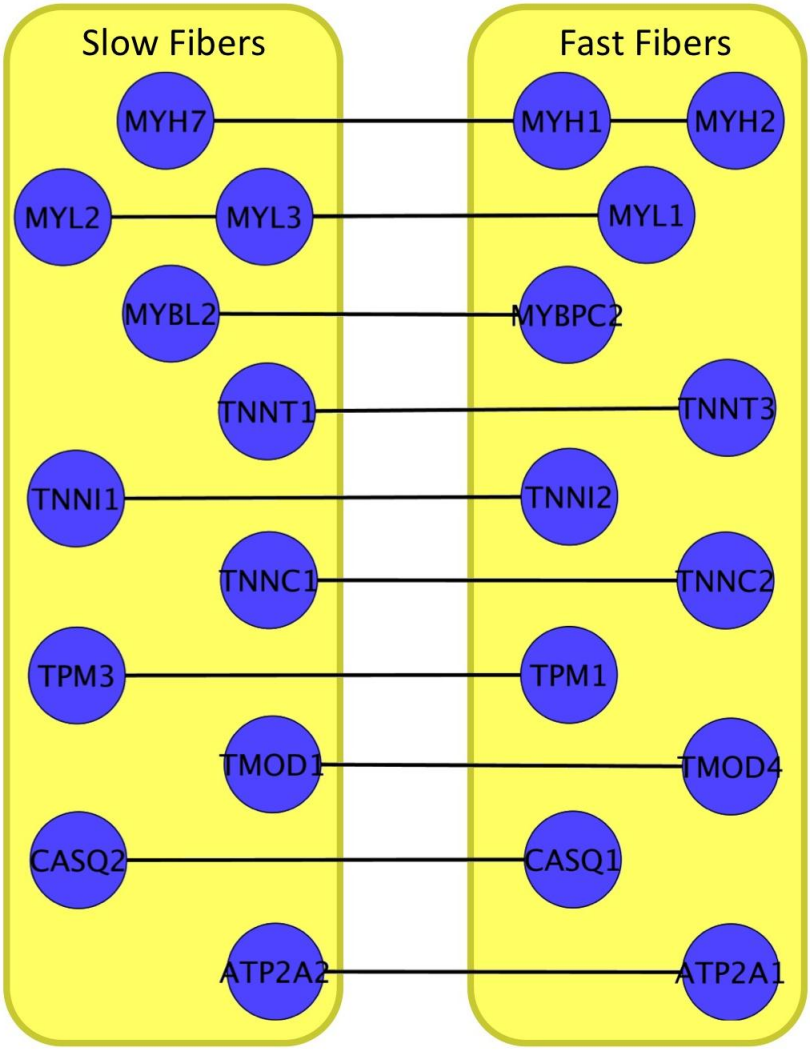

Figure 3.9: Muscle Fiber Type. Genes that have similar function, but are expressed specifically in either a fast or slow muscle fiber type. MYH is the major determinant of fiber type

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CHAPTER 4

TRANSCRIPTIONAL ALTERATIONS OF HAMSTRING CONTRACTURES IN CHILDREN WITH CEREBRAL PALSY

4.1 Abstract

Cerebral palsy is primarily an upper motor neuron disease that results in a spectrum of progressive movement disorders. Secondary to the neurological lesion, muscles from patients with cerebral palsy are often spastic and form debilitating contractures that limit range of motion and joint function. With no genetic component, the pathology of skeletal muscle in cerebral palsy is a response to aberrant neurological input in ways that are not fully understood. This study was designed to gain further understanding of the skeletal muscle response to cerebral palsy using microarrays and correlating the transcriptional data with functional measures. Hamstring biopsies from gracilis and semitendinosus muscles were obtained from a cohort of patients with cerebral palsy (n=10) and typically developing patients (n=10) undergoing surgery. Affymetrix HG-U133A 2.0 chips (n=40) were used and

expression data was verified for 6 transcripts using quantitative real-time PCR, as well as for two genes not on the microarray. Chips were clustered based on their expression and those from patients with cerebral palsy clustered separately. Significant genes were determined conservatively based on the overlap of three summarization algorithms (n=1,398). Significantly altered genes were analyzed for over-representation among gene ontologies, transcription factors, pathways, microRNA and muscle specific networks. These results centered on an increase in extracellular matrix expression in cerebral palsy as well as a decrease in metabolism and ubiquitin ligase activity. The increase in extracellular matrix products was correlated with mechanical measures demonstrating the importance in disability. These data lay a framework for further studies and novel therapies.

4.2 Introduction

Cerebral palsy (CP) is a movement disorder caused by an upper motoneuron (UMN) lesion in the developing brain [1]. CP covers a spectrum of severities and is the most common childhood movement disorder with a prevalence of 3.6 cases per 1000 in the US [2]. While the UMN lesion that initiates CP is non-progressive, many secondary changes occur within the musculoskeletal system that are progressive [3]. Among the hallmarks of CP is muscle spasticity, in which the muscle contracts in a velocity dependent resistance to stretch that results, in part, from reduced inhibition of the stretch reflex [4]. While the disability that results from spasticity is debatable [5, 6] patients with spastic CP also develop muscle contractures secondary to the lesion.

Muscle contractures represent a unique muscle adaptation in which muscle stiffness reduces the range of motion around a joint. Thus muscle contractures can severely limit mobility, may be painful, and represent a major disability among those affected by CP or anyone with an UMN lesion [7].

There are a variety of treatments designed to inhibit muscle activity in CP and prevent contracture formation. Physical therapy techniques, oral and intrathecal muscle relaxants, chemical neurectomies with phenol or alcohol, chemodenervation using neurotoxins, and surgical neurectomies have all been employed to decrease spasticity in children with CP [8]. However, despite best clinical practices, contractures still develop and often require surgery to correct [9]. It should also be noted that all of these therapies reduce muscle strength in a condition in which strength is already compromised, clearly current therapies are not ideal.

There are no known genetic defects in patients with CP and their muscles, as it is a direct consequence of the UMN lesion [10]. Although skeletal muscle is known to be highly adaptive in response to neurological input, muscle contractures that develop are part of an adaptive mechanism that is not fully understood. Contracture does not develop in animal models of increased muscle use, which could be present from decreased motoneuron inhibition, or even decreased muscle use, which could result in decreased functionality [3]. Indeed UMN contractures are not readily reproducible in animal models, thus necessitating research on human subjects [11].

Previous research demonstrated that muscle stiffness in contracture is independent of active muscle contraction [12, 13]. Recent data in hamstring muscle indicates that the increased muscle stiffness is due to alterations in ECM rather than

the stiffness of muscle fibers themselves [14]. Multiple studies have also shown an increase in sarcomere length of muscle in contracture, demonstrating contractured muscle experiences high intrinsic strain [15] due to dramatic, but unknown structural alterations. This suggests a decrease in the serial sarcomere number despite conflicting evidence as to whether muscle fascicle length decreases [16-19]. Studies have shown that muscle and muscle fiber cross sectional area are reduced, which decreases force production, and even that the remaining muscle has decreased force generating capacity [20-22]. These mechanical and architectural changes in muscle implicate a disruption of the biological components involved in of myogenesis, force generation, force transmission, extracellular matrix maintenance, and perhaps additional pathways. Recent microarray data from the upper extremity supports the assertion that CP muscle is altered transcriptionally and that in addition to the pathways listed above, neuromuscular junction, excitation-contraction coupling, and energy metabolism are also deranged [23].

As a purely adaptive muscle disorder, contractures are believed to have an altered transcriptional profile. The current study has taken advantage of a large surgical population of both children with CP and typically developing children to conduct a robust microarray analysis. Our previous study was limited by a very small control subject population which was not age matched, N=2) [23], and microarray studies in humans subjects generally require larger sample sizes to identify differences due to the higher variability present in human tissues compared to most inbred animal strains. Additionally the same biopsies reported here were collected as part of an additional study and thus, we have matched mechanical data, myosin heavy chain

content, and collagen content to which we can compare transcriptional data [14]. We will also take advantage of recent additions of muscle specific gene ontologies and muscle specific gene networks to probe the muscle and compare the pathology to microarrays from other published muscle conditions ([24]; Smith et al. in press). A mechanistic understanding of muscle adaptation to contracture may provide possible therapeutic targets that can delay or even reverse the debilitating effects of CP or other UMN lesions.

4.3 Results

Condition tree clustering

The condition tree was conducted based on all present genes on the microarray and resulted in a unique clustering of cerebral plays subject biopsies from typically developing subject biopsies, with the exception of a mild cerebral palsy subject (Fig. 4.1). The condition tree also shows that biopsies form the same patient cluster closely together. This result indicates that the two hamstring muscles, gracilis and semitendinosus, have less variability than that between subjects of the same condition. With a large sample size there was an expectation that biopsies from patients with similar clinical severity scores would cluster together. This trend was not obviously observed in the data.

Quantitative Real Time PCR verification

As a quality control measure, qRT-PCR was performed on a subset of 8 significant genes and genes of interest. The qRT-PCR data was normalized to the median value and correlated to genechip data also normalized to the median value. Comparison of the two datasets revealed a significant correlation ($p < 0.05$) in all 6 genes examined (Fig. 4.2A-F). For the genes that were deemed significant in microarray analysis a 2x2 ANOVA was also conducted on qRT-PCR data. In the case of *MYH1* and *MSTN* the significant main effect of cerebral palsy on the microarray was also significant ($p < 0.05$) with qPCR. Both *COL1A2* and *COL4A2* were significantly different on the microarray and did not reach significance with qPCR, but were regulated in the same direction with similar expression ratios. qRT-PCR was also conducted on two genes of interest that were not included on the microarray with expression ratios of (0.48) *TRIM63* and (0.79) *FBXO32*. *TRIM63* expression did have a significant main effect of cerebral palsy ($p < 0.01$) (Fig. 4.2G-H).

Myosin Heavy Chain Content

The microarray is limited to transcriptional data so in order to determine if transcriptional quantities were reflected in translated protein quantities we used myosin heavy chain content. It is not a direct comparison as protein content is measured as a percent of total myosin heavy chain and microarray quantities are normalized based on the chip. The protein gel also only includes analysis of bands for myosin heavy chains: type I, type IIa, and type IIx. There was a positive correlation for each isoform measured (Supplemental Fig. 4.1), however the relationship was only significant for myosin heavy chain IIa.

As the major determinant of muscle fiber type we also wished to determine if there was a change in patients with cerebral palsy. A 2-way ANOVA on disease and muscle for each isoform revealed a significant difference only for myosin heavy chain I ($p < 0.05$). This shows that regardless of transcript levels there are proportionally more slow fibers in the hamstrings of patients with cerebral palsy.

Significantly altered genes

Among the 22,283 probesets existing on the HG-U133A 2.0 GeneChip (Affymetrix), 13,787 were considered present for further analysis based on a 25% "present" call on the affymetrix chips. The number of probesets that were significantly different for CP based on a Welch 2x2 ANOVA (GeneSpring 11.5; CP vs. typically developing and gracilis vs. semitendinosus; false discovery rate of 5%) for each summarization algorithm was: 2,836 for Microarray Suite Version 5.0 (MAS5), 3,954 for Robust Multiarray Analysis (RMA), and 4,009 for GC-RMA. The probesets were reduced to genes for each summarization algorithm genes significant in the 1,398 genes identified by overlap of all 3 algorithms were deemed significantly altered in CP (Supplementary Table 4.1). Of these genes 533 had expression increased in CP while 865 genes had expression decreased. The 2x2 ANOVA yielded only 3 genes (*MAB21L1*, *SIM1*, and *EN1*) with unknown roles in muscle as significantly different between muscles, demonstrating both muscles to have similar expression profiles. There were also no genes that produced a significant interaction between muscle type and disease state, indicating that both muscles undergo similar changes in CP.

Categorical analysis

Secondary analysis was performed using predefined gene sets for Gene Ontology, pathways, microRNA, and transcription factors (WebGestalt). There were 87 gene ontologies over-represented from the list of significantly genes up-regulated in CP (Supplementary Table 4.2: Table 4.1). Of note are a large portion of extracellular matrix ontologies as well as those for calcium ion binding and cytoskeleton ontologies. Categories discussed are presented in Table 4.1. Among genes that were down-regulated in CP 85 ontologies were significantly over-represented (Supplementary Table 4.3: Table 1). These ontologies primarily fell into the categories of metabolic processes and ubiquitin related pathways and also interestingly included skeletal muscle contraction.

Established pathway databases (KEGG, Wiki Pathways, and Pathway Commons) were also examined using the significant gene set for over-representation (Table 4.2). Of the 5 KEGG pathways up-regulated in CP; 3 were related to cell adhesion (ECM-receptor interaction, Focal Adhesion, and Tight Junction) while the other 2 suggest an inflammatory response (Leukocyte transendothelial migration and Pathogenic E. Coli Infection). In Wiki Pathways only Focal Adhesion was significant. Pathway commons had 8 pathways up-regulated, primarily related to platelet activation. There were no KEGG pathways that met the $p < 0.01$ threshold, but the 4 pathways with $p<0.05$ overrepresented in down-regulated genes in CP include: Insulin signaling pathway, ubiquitin mediated proteolysis, TCA cycle, and Systemic lupus erythematosus. This corresponds with the decrease in metabolic activity and ubiquitination from Gene Ontologies as well as a role for insulin signaling and inflammation. Only type II interferon signaling (IFNG) was overrepresented for

among wiki pathways, further implicating inflammation. From the Pathway Commons database 0 pathways met even the ($p < 0.05$) criteria, yet the top pathways were primarily related to metabolism and insulin signaling.

Transcription factors with known target genes were also investigated to determine which may be active in producing the significantly altered gene sets in CP (Table 4.2). Zero transcription factors met the ($p < 0.01$) threshold for significance, and the level was raised to $p < 0.05$. This yielded 7 transcription factors that could play a role in increasing expression of genes in CP. Two were related to AP-1 transcription factor related to c-Jun a known player in mechanosensation of skeletal muscle [25]. Other transcription factors are related to activity (CREB, [26]), development (PAX4, [27]), MEF2C enhancer (TEF-1, [28]), skeletal muscle growth (SRF, [29]), and Maf recognition element repressor (BACH-1, [30]). Only 2 transcription factors met the $p < 0.05$ threshold for down-regulated genes, one of which is unknown. The other, SP3 has can be involved in positive or negative regulation of expression, but has been shown to negatively regulate slow myosin heavy chain [31].

MicroRNAs (MIR) have also been shown to play an important role in regulating muscle function. Genes up-regulated in CP were over-represented for sequences related to 2 microRNAs (Table 4.2). MIR-133A/B has been demonstrated to decrease during skeletal muscle hypertrophy [32], while MIR-518C has little described function in muscle. The only MIR related to down-regulated genes in CP was MIR-302c. The action of MIR-302c may be related to the estrogen receptor in skeletal muscle [33].

Network analysis

Recent work on creating networks of genes related to their function in muscle permits a more detailed investigation of individual gene expression. A heatmap, using expression ratios, is created with genes listed in their respective functions with genes in those determined as significantly different in cerebral palsy according to previous analysis (Fig. 4.3). Some genes appear in multiple functions and some genes listed within gene complexes (Supplementary Table 3.1). For complexes the geometric mean of expression ratios is reported and significance is denoted if at least one gene in the complex was significantly different.

The neuromuscular junction network demonstrates that important genes are significantly altered in cerebral palsy. *CHRN* is the acetylcholine receptor complex, which has decreased expression in cerebral palsy. Yet only the *CHRNB1* subunit had expression that was denoted as present and is thus the only subunit in which a difference is observed. The differentially regulated genes were primarily found in the extracellular matrix region of the neuromuscular junction. *COLQ* and *HSPG2* are up-regulated and help bind acetylcholine esterase [34]. *NID1* is a basement membrane glycoprotein that is also up-regulated. *COL4* is a complex that within the neuromuscular junction is made up from collagen IV alpha chains 3-6 [35], and this decrease is driven primarily by *COL4A4* expressed at a ratio of 0.42 in cerebral palsy muscle. *UTRN* is the dystrophin analogue in the neuromuscular junction and also down-regulated in cerebral palsy [36]. These changes represent some inconsistent change in the extracellular matrix of the neuromuscular junction and the link to the cells through *UTRN*.

The only transcript increased among those described in the excitation contraction coupling network is *RYR3*. *RYR3* is a ryanodine receptor expressed in immature muscle [37]. The ryanodine receptor is activated by *DHPR*, however the transcript for *DHPR* is down-regulated [38]. This is also the case for calsequestrin binding protein *ASPH* [39]. *CALM1* is an important calcium binding signaling protein that is down-regulated in cerebral palsy [40]. The calcium/calmodulin dependent protein kinase type II (*CAMK2*) does not have an overall expression change; however the beta subunit is significantly increased. Also down regulated was *SLN*, an inhibitor of the SERCA pumps (*ATP2A*) which pump calcium back into the sarcoplasmic reticulum [41]. There are separate *ATP2A* genes that are present in either fast or slow muscle and related to fiber types that are described later.

Transcripts directly involved in muscle contraction are also altered in cerebral palsy. Many of the proteins have isoforms coded by separate genes for fast and slow muscle. Those that are differentially regulated in cerebral palsy include: *MYH*, *MYL*, *MYBPC*, *TNNT*, and *TNNI*, which are described in relation to fiber types below. Two transcripts that are involved with capping filaments at the Z-disc of skeletal muscle are down regulated in cerebral palsy, *TCAP* and *CAPZ* [42]. Among the most increased transcripts in the muscle from children with cerebral palsy is that of embryonic myosin heavy chain (*MYH3*). This also corresponds with a significant increase in the embryonic myosin light chain transcript (*MYL4*).

The contractile elements transmit force through the cytoskeleton, in which further elements are affected in cerebral palsy. Many of the actin linking transcripts are up-regulated including: *SPT*, *FLNC*, and *ANK*. The linkages extend out to the cell

membrane and the dystroglycan complex. Some important members of the complex are down-regulated in cerebral palsy, namely *SGCB* and *SNTB1*. The cytoskeleton also links the nucleus with *EMD* and *LMNA* critical points that have associated genetic muscle diseases [42]. In cerebral palsy interestingly, *EMD* is significantly down-regulated while *LMNA* is down-regulated. *DES* is the primary intermediate filament within muscle, but in immature muscle *VIM* is more prevalent and is up-regulated similar to other immature muscle markers [42].

Force is then transmitted through the cytoskeletal to the extracellular matrix, the most uniformly altered network in cerebral palsy. Many transcripts are significantly up-regulated in cerebral palsy including fibrillar collagens (*COL1*, *COL3*, *COL6*), proteoglycans (*SDC*, *HSPG2*), laminins (*LAM*) and key binding proteins (*LTBP4*, *TNC*, *FN1*). These increases coexist with an increase in *MMP2* expression, which breaks down extracellular matrix, as well as *TIMP1*, which inhibits MMP activity. Upstream of the increase in extracellular matrix transcripts is an increase in *CTGF*. Interestingly *COL4* expression overall was not changed, however previously we discovered a decrease in *COL4* isoforms around the neuromuscular junction, but *COL4A1* and *COL4A2* were significantly up-regulated.

Muscle is a very metabolically active tissue in which some of the key transcripts are altered in cerebral palsy. Each member of the oxidative phosphorylation pathway complexes has at least 1 subunit with decreased expression (*NDUF*, *SDH*, *CYC*, *COX*, *ATP5*). *AMPK* is an important switch that is activated by phosphorylation, but also has significantly down-regulated expression in cerebral palsy, along with another signaling molecule *MAPK14*. Important transcription

factors also have decreased expression including nuclear (*CREB1*) and mitochondrial (*TFAM*). Interestingly, while an important glycolysis enzyme in muscle is down-regulated (*PFKM*) the only up-regulated transcript (*LPL*) is involved in fatty acid usage.

The disruption of the inflammation network of genes can be a hallmark of diseased or damage muscle. Unfortunately only 7 out of 19 transcripts were both on the chip and at expression levels high enough to be present. Surprisingly two of the markers were down-regulated. One heat shock protein transcript was down-regulated (HSP2). *MAPK14*, an important player in the inflammation signaling cascade was also down-regulated [43].

Many of the signaling molecules have been discussed in regards to previous functions, but some of the key signals for muscle growth are also altered. *MSTN* is a vital muscle growth inhibitor and both it and its receptor are down-regulated in cerebral palsy, while a natural inhibitor of *MSTN* is up-regulated (*FST*) [44]. The *FOXO3* transcription factor responsible for transcribing the atrogenes is also down-regulated [45]. *FGF2* plays a role in activating muscle differentiation and highly up-regulated in cerebral palsy [46]. All of these signaling molecules point towards a muscle that is growing, however there are also gene profiles that predict inhibited growth. *RPS6KB1* is an involved in protein synthesis and downstream of *IGF1* signaling pathways, but is down-regulated. *MAPK8* is a down-regulated signaling molecule known to play a role in mechanotransduction in skeletal muscle [25].

The program for fast versus slow muscle turns on many separate genes as discussed above. None of those described for slow fibers have any significant change

in transcription. However 5 (MYH1, MYBPC2, TNNT3, TNNI2, ATP2A1) out of the 11 transcripts described for fast fibers were significantly down-regulated in cerebral palsy. This suggests a decrease in fast fiber transcription and a relative shift to slower muscle.

Transcriptional correlations

A unique aspect of this study was the corresponding mechanical measurements from the same muscle biopsies. Individual fibers were isolated and mechanically tested to determine a stiffness value [14]. Fiber bundles, consisting of a group of approximately 20 fibers and their constituent extracellular matrix, were testing in the same way. Mechanical stiffness values for fiber and fiber bundles were correlated with each of the significant genes in CP. 27 genes had a significant ($p < 0.05$) positive correlation with fiber stiffness and 50 genes had a significant negative correlation (Table 4.3 A/B; Fig. 4.4). These genes lists were used for categorical gene ontology analysis with the total significantly altered in CP gene list as the reference set to determine which categories were over-represented. From the genes positively correlated with fiber stiffness there were no categories significantly up-regulated. However among the negatively correlated genes 29 ontologies were significantly ($p < 0.05$) over-represented, with most ontologies related to ubiquitin ligase activity. This suggests that when genes in the ubiquitin protease system are most active muscle fibers lose mechanical stiffness. When fiber bundles are considered 141 genes had a positive and 95 genes a negative significant correlations with stiffness values (Table 4.3 C/D). There were 36 ontologies significantly over-represented with a positive correlation to bundle stiffness. These consisted almost exclusively of ontologies

related to the extracellular matrix, indicating the matrix plays an important role in the stiffness of fiber bundles. Only 2 ontologies were significantly over-represented among genes negatively correlated with bundle stiffness. Those related to mitochondrial structure, suggesting a relationship between muscle stiffness and energy production.

Comparison analysis

To determine if the results of this study follow a similar pattern to previous research the data was compared to the only other dataset available of microarrays from children with cerebral palsy [23]. The comparison dataset comes from patients with undergoing wrist surgery, with biopsies taken from flexor carpi ulnaris and extensor carpi radialis brevis. Using the significantly altered genes published from the data set, it was surprising that only 13 transcripts were up-regulated and only 6 transcripts down-regulated in both studies (Table 4.4). When gene ontology analysis is performed on these genes the only over-represented ontologies are all directly related to extracellular matrix for transcripts increased with cerebral palsy, while no ontologies were over-represented based on the down-regulated transcripts.

4.4 Discussion

The objective of this study was to sufficiently describe the transcriptional adaptations that occur in skeletal muscle of patients with cerebral palsy and incorporate functional data to determine the cellular mechanisms that are driving the

muscle pathology secondary to the upper motor neuron lesion. This research has seen little prior work as there is no commonly accepted animal model necessitating the challenges of direct human research [11]. Without any genetic defect in the muscle, it is clear that the pathology has a large transcriptional component across many genes (Fig. 4.1). The large number of altered transcripts fall into a variety of gene ontologies and biological pathways, as well as important functional networks within skeletal muscle. Some of these vital muscle systems such as the extracellular matrix have a correlation between transcript levels and tissue stiffness. These new insights are put into the context of the literature as well as a direct comparison to a previous study performed on the upper extremity of cerebral palsy muscle transcription that shows little constancy.

The condition tree clustering demonstrates the difference between the transcriptional profile of muscles from patients with cerebral palsy or typically developing (Fig. 4.1). This separation was not 100% as a single cerebral palsy patient clustered more closely with the controls. It should be noted that this was one of the more mild cases of cerebral palsy with a GMFCS score of II and the highest popliteal angle of the group (120^{o}). The clustering algorithm did not however group the cerebral palsy muscle by the clinical severity scores, age, or even which muscle. The patients themselves had both biopsies clustered together consistently indicated that the variation from subject to subject is higher than that between muscles of the same subject. This finding was described in a previous study using multiple muscles from a single subject [23].

Microarray verification

To ensure that the microarray data was reliable we used quantitative real time PCR to independently assess the transcript levels. We used 2 genes that were up-regulated, 2 with no significant change, and 2 down-regulated in cerebral palsy to assess the validity. In each case there was a significant correlation between the microarray and PCR results giving us high confidence in the data from the chip that was used for the rest of the analysis.

Using transcript levels allow us to investigate the muscle in a very broadly using microarray technology to assess thousands of genes in parallel. However, these transcript levels are not necessarily reflected in protein quantities due to a multitude of factors post-transcriptional factors. To establish relevance of transcript levels on a small subset of protein quantity we analyzed myosin heavy chain content [47]. The results indeed showed a positive correlation in each isoform to support the importance of transcript levels in this case, although the correlation was only significant in one instance. This demonstrates the exploratory nature of this experiment in that changes observed in the transcriptional study will need to undergo further study to assess downstream mechanisms. The broad nature of the experiment also allows the unbiased investigation of many systems

Categorical analysis

To determine the various functions that are altered in cerebral palsy we first established a list of genes with altered transcription. We sought to narrow our results by using a more stringent analysis that uses the congruence of three summarization algorithms as well as a restrictive false discovery rate of 1%. This method still produced a list of 1,398 genes altered. Our large sample size allowed us to have

increased power to detect smaller differences, but we choose not to implement a fold change cutoff as this assumes an arbitrary level of change is required to be functionally important [48]. The list of genes is too large to describe individually in the scope of this study and was analyzed to delineate groups of genes that are over represented among the gene list. Prior to this the list was separated in to up and down-regulated genes so that within a group the alteration was consistent.

Among the up-regulated transcripts there were a total of 87 gene ontologies over-represented, but they generally fell into a few different categories. The most extensive were those related to extracellular matrix with large ontologies and more focused ontologies such as collagen binding. The increase in extracellular matrix transcripts has been well documented in muscle from cerebral palsy [23, 49, 50]. However the structure of the extracellular matrix increases is not well known. This study shows how extensive the increase in extracellular matrix is with categories ranging from fibrillar collagen, basal lamina and even collagen metabolic process. Increased structural categories continue through the cell with ontologies including integrin binding, cytoskeletal binding protein, and cytoskeleton. This could have an effect on individual fiber stiffness, but the stiffness of fibers in cerebral palsy is inconsistent [14, 51]. There were ontologies important in muscle function that had over-representation as well with calcium ion binding. A disruption of calcium handling had been suggested previously, where *PARV* was drastically altered in cerebral palsy muscle [23], however here *PARV* was unchanged. The impact of growth factor binding is also crucial in skeletal muscle, with many of the transcripts being IGF binding proteins known to be important in skeletal muscle.

Contrastingly there were 62 ontologies that were over-represented in down-regulated genes. The most extensive of these were related to metabolic processes and ubiquitin ligases. Skeletal muscle is a very metabolically active tissue and these results suggest that despite spasticity the muscle has less metabolic machinery. This decrease was also seen in the previous transcriptional study in cerebral palsy, however this was accompanied by a shift to faster fiber types which was not the case here [23]. The ubiquitin ligase role is surprising here as it is generally accepted that muscle in cerebral palsy has decreased mass [20, 52]. This result suggests that decreased muscle mass is not coming from active muscle degradation. The obvious importance of the skeletal muscle contraction ontology is also represented. Together these results imply that muscle protein turnover is decreased in cerebral palsy.

The analysis of pathways was conducted in a similar manner as gene ontologies, with some similar results. The extracellular matrix was also represented in up-regulated genes, but some important other pathways were also over-represented. Pathways such as pathogenic Escherichia Coli infection and Leukocyte transendothelial migration may not have obvious implications in muscle, but indicate a role for an inflammatory which could have an important impact in muscle [53]. Focal adhesions come up in multiple pathways as well and integrate the extracellular matrix and cytoskeletal changes discussed previously and have important function in muscle [54]. Down regulated pathways also follow gene ontologies with change in metabolism and ubiquitination. The down regulation of the IGF1 pathway is surprising as it is opposite of previous studies [23].

Many of the significantly altered genes have known transcription factor binding sites in the promoter region and allow similar analysis over-representation analysis. None of the transcription factors are muscle specific or dominates the gene list as none met the initial threshold ($p<0.01$). Instead the transcription factors identified illustrate the complexity of the adaptation as they work in concert with other transcription factors in processes including mechanosensation, development, and muscle growth. Interestingly as well the only known transcription factor related to down regulated genes, SP3, is known to negatively regulate slow myosin heavy chain and could play an important role in fiber type shifts in muscle from children with cerebral palsy.

Similar to transcription factors, microRNAs also play a role in regulating transcript availability, particularly in skeletal muscle [55]. Only three were identified in the categorical analysis, but MIR-133A/B has the most connection to muscle. A disproportionally large number of the up-regulated genes interact with MIR-133A/B, and as microRNA generally lower transcript level [56] it is possible that MIR-133A/B is decreased in cerebral palsy. A decrease in MIR-133A/B is also seen in muscle hypertrophy and is sign that the muscle does have growth signals, yet has impaired muscle growth [32].

Network analysis

Categorical analysis provides an avenue to analyze vast gene lists into manageable pieces. However, we also sought to investigate more deeply into networks of genes critical to muscle function using a recently established gene networks (In press). As cerebral palsy is neurological in origin the neuromuscular

junction could be altered and has indeed been shown to have a disorganized nature in cerebral palsy [57]. The results did not show overwhelming changes in the neuromuscular junction, the acetylcholine receptor (CHRN) was down-regulated. CHRN is dramatically up-regulated when muscle is denervated [58], which our results clearly show is not the case in cerebral palsy muscle. Most of the changes were associated with extracellular matrix proteins localized to the neuromuscular junction. However, unlike the majority of extracellular matrix transcripts COL4 was down-regulated as was UTRN an important link to the extracellular matrix of the neuromuscular junction. It is unclear how these adaptations change the function of the neuromuscular junction, but it does support evidence for disorganization [57].

Excitation contraction coupling has been largely unexplored in cerebral palsy, but has been shown to be altered and is also the target of therapeutic intervention [23, 59]. The transcripts altered in this study were unique however, and primarily down-regulated in cerebral palsy. Genes such as *ASPH* and *DHPR* which have a role in activating the -ryanodine receptor are down-regulated. As are mechanisms for pumping calcium back in to the sarcoplasmic reticulum with *ATP2A* and its regulator *SLN*. Further a decrease in transcripts of calcium binding proteins of *CALM1* and downstream *CAMK2* suggest that there is less calcium cycling in cerebral palsy muscle. The only up-regulated transcript is *RYR3*, which is a ryanodine receptor expressed in immature muscle, and a theme that is common for immature transcripts.

That theme is also observed in the contractile transcripts of skeletal muscle. The only up-regulated genes are immature isoforms embryonic myosin heavy chain (*MYH3*) and embryonic myosin light chain (*MYL4*). Many of the contractile elements

have well defined isoforms that have genes expressed in either fast or slow muscle [60]. Many of the down-regulated transcripts are isoforms of the fast isoforms, which will be discussed in regard to fiber type. Interestingly both thin filament Z-disc capping protein *CAPZ* and titin filament Z-disc capping protein *TCAP* are down-regulated in cerebral palsy. This could lead to Z-disc disorganization and *TCAP* itself is associated with destabilization in limb girdle muscular dystrophy 2G [61].

The force generated in the sarcomere is transmitted through the cytoskeleton to the cell periphery, but the effects in cerebral palsy have been largely unexplored. Our results are somewhat difficult to interpret with many genes both up and down-regulated. Of those associated with the dystroglycan complex, sarcoglycans (*SGC*) and snytrophins (*SNT*) as well as *UTRN* are down-regulated. This is while many crosslinking transcripts of the cytoskeleton are up-regulated (ankyrin (*ANK*), sprectrin (*SPT*), filamin (*FLNC*)) along with an important connector to the Z-disc (*MYOZ2*). What role these increased cytoskeletal filament connections may play is unknown, but they did not lead to increased fiber stiffness [14]. The cytoskeletal connection to the nucleus is interesting as *EMD* and *LMNA*, which both lead to Dreifuss-Emery muscular dystrophy when absent are not co-regulated with *EMD* decreased and *LMNA* increased [62, 63]. One aspect that is consistent is that *VIM* is up-regulated, which is the primary immature muscle intermediate filament that is replaced with desmin (*DES*) during development.

The force generated in the cell is ultimately transferred to the extracellular matrix, which is significantly altered in various analyses here as well as numerous other studies of cerebral palsy [23, 49, 50]. This alteration is nearly uniformly up-

regulated, with the exception of the *COL4* isoforms in the neuromuscular junction previously described. It is important to note that the increase includes many categories, fibrillar collagens, laminar collagens, proteoglycans, matrix metalloproteinases, matrix metalloproteinase inhibitors, and extracellular matrix growth factors. This uniform increase does not permit speculation on how the extracellular matrix may be prolific, yet disorganized as speculated in cerebral palsy muscle [50]. With all the increase in extracellular matrix components it would be expected that *TGFB1*, an important fibrosis signal in muscle, is increased [64]. Although it did have higher expression in cerebral palsy it was not significant and shows that increases can occur independent of a large *TGFB1* autocrine signal.

Another network with broad regulation was metabolic transcripts, which were down-regulated in cerebral palsy. This was observed in gene ontologies, pathways, as well as previous studies [23]. The fold change values on many of the transcripts were relatively low. It should be noted that this change occurred despite a decrease in fast muscle isoforms that have fewer mitochondria present. The only increased transcript was *LPL* associated with fat metabolism that is more prevalent in slow muscle. Despite an increase in non-voluntary muscle contractions associated with spasticity [65], the muscle is not producing more metabolic machinery. This could be a result of the muscle being in the state of contracture and thus have decreased functionality which contributes to disuse [66].

The state of muscle contracture inducing damage is not well known, but an inflammatory response is common in damaged muscle [53]. Wound healing and inflammatory pathways were up-regulated in categorical analysis, but unfortunately

few inflammatory transcripts were able to be analyzed in this study. Of those that were altered were p38 (*MAPK14*) and a heat-shock transcription factor (*HSF2*). The role of muscle damage in pathologic cerebral palsy muscle is not discernable from this data.

The critical aspect of muscle growth is a complicated system of many genes and viewed the lack of muscle growth is considered the primary cause of contracture [67-69]. However there are many transcripts altered to induce muscle growth including down-regulation of myostatin (*MSTN*) a critical muscle growth inhibitor, its receptor (*ACVR2B*), and up-regulation of natural inhibitor (*FST*). *FGF2* also plays a role in stimulating muscle growth and differentiation [46]. The muscle atrogene program is also decreased with a drop in MURF1 (*TRIM63*) and its transcription factors *FOXO* [70]. This *MSTN* signal is in contrast to the previous study in cerebral palsy muscle and is a candidate for being responsible for blocking the growth signal in these muscles. The question of what limits muscle growth in cerebral palsy is not readily apparent from these results.

The role of fiber type has been discussed in regard to multiple networks. The results here show clearly that while slow isoform levels remain unchanged many fast isoforms have significant decreases in transcription. The role of fiber type in upper motor neuron lesions has been inconsistent [71, 72], but these results imply a proportional shift to slower muscle. This can be important functionally, but is also very important in terms of a transcriptional study. Many of the changes observed could be the result in transcriptional changes from fast to slow muscle, such as the

decrease in glycolytic transcripts. However it is clear the pathology of muscle in cerebral palsy is not purely from a shift in fiber type.

Correlations to mechanics

Having mechanical stiffness measurements for both the muscle fibers and the muscle fiber bundles with their extracellular matrix was a unique aspect of this study. There is literature to support the role of the giant protein titin (*TTN*) in being the major contributor to passive tension of individual fibers [73]. When considering the muscle bundle much of the passive stiffness is believed to arise from collagen, especially at larger sarcomere lengths [74]. Certainly many transcriptional factors could contribute to the production of these important proteins or other proteins that have a direct impact on the passive mechanical stiffness. Indeed 77 genes were correlated with fiber stiffness and 236 with bundles stiffness. To determine the fundamental nature of these transcripts we again used categorical analysis. It should be noted that since only significantly altered genes were considered that was the gene set used as a reference set, meaning correlated genes had to be enriched beyond the significant gene list be over-represented.

For individual fiber there were actually no ontologies that had a significant positive correlation with stiffness. However many ontologies were associated with decreasing fiber stiffness, the vast majority of which were related to ubiquitin ligases. This supports the idea that as ubiquitin ligases become active and begin degrading the muscle filaments, particularly titin, causing a loss of passive stiffness [75].

When bundle stiffness is considered there are many more ontologies over-represented (Table 4.3). Fittingly the most prevalent ontologies are related to

extracellular matrix, however there are many more transcripts than fibrillar collagens thought to provide the passive stiffness to bundles [76]. For example one of the most highly correlated genes was *COL21A1* which is a fibril associated collagen that is found with collagen I and serves to maintain the integrity of the extracellular matrix. This illustrates the complex nature and the many factors that lead to tissue stiffness and that could be contributing to fibrosis in cerebral palsy. Additional ontologies were over-represented including immune system process. This is an indicator of damage in bundles that are stiffer and supports the claim that muscle tissue damage leads to fibrosis in cerebral palsy. Conversely there were only two ontologies negatively correlated to bundle stiffness, related to mitochondria. It is difficult to speculate in too much detail from this small data set, but this suggests that increased mitochondria and thus more metabolically active tissue is more compliant.

Comparison to upper extremity

This study is similar to one conducted in the upper extremity of muscle from patients with cerebral palsy [23]. There are some important differences in that the current study has a much larger sample size, especially in terms of controls (10 in this study compared to 2 in the previous). Additionally the typically developing control subjects in the current study did not have recent acute trauma as an indication for surgery as a confounding factor. Regardless we expected to see many similar results between the two studies. However this was not the case with only 19 genes significantly altered in the same direction between the two studies (Table 4.4). A possible explanation for this is the change in fiber type, which was opposing between the studies, was driving many of the transcriptional changes. For example in the

previous study many calcium binding genes were up-regulated, such as *CALM1*, where as in the current study *CALM1* and other calcium binding transcripts were down-regulated. Fast fibers have a more extensive calcium cycling apparatus thus offering an explanation other than a disruption of typical calcium handling in cerebral palsy [23]. It is still important to look at the similarities between the studies. The obvious similarity is an increase in extracellular matrix in both upper and lower extremity contracture, shown with ontology analysis of genes altered in both studies. This is important in placing fibrosis as a consistent property of muscle contractures in cerebral palsy. It should also be noted that both studies had an increase in genes related to immature muscle, another possible hallmark of muscle in a contractured state.

Study Limitations

Some of the limitations of this study have been discussed in relation to previous topics. As with any microarray study only transcript levels are investigated with the relationship to actual protein quantity having other important factors. Without an animal model of cerebral palsy this study was conducted in humans, which leads to a much greater variability between subjects. Particularly in the cerebral palsy patient population, as it is a spectrum disorder with a range of severities that are non-homogenous in our subjects [77]. Many of the patients with cerebral palsy are also undergoing treatments prior to surgery, and although each is treated conservatively in this study it is a confounding factor. This also brings up the difficulty in discerning which changes are primary is causing pathology in the muscle and which alterations

may be a compensatory response. In contrast the human subjects do make the study more relevant to the clinical condition.

This study is valuable in highlighting many functions of skeletal muscle that are disrupted in contractures caused by cerebral palsy and detailing the gene transcripts involved. It does not directly answer which programs are inducing contracture or contributing to the lack of muscle growth. However the most dramatic changes were seen in the drastic increases in extracellular matrix, which could blunt the intracellular growth signals observed. Indeed it has been shown that the extracellular stiffness can modulate skeletal muscle satellite cells proliferation and differentiation [78, 79]. Further matrix digestion has been shown to have a positive effect on muscle growth through satellite cell activation [80]. Combining previous knowledge with this study targets the extracellular matrix as a novel treatment for cerebral palsy.

Summary

Skeletal muscle undergoes significant transcriptional alterations secondary to upper motor neuron lesion in cerebral palsy. These fall into several categories with increases extracellular matrix components and muscle growth signals along with decreases in metabolic, and muscle degradation systems. These are overlaid on a decrease in fast muscle isoform transcripts with an increase in immature muscle states. The increases in extracellular matrix were seen to be associated with an increase in the passive stiffness of the muscle tissue and one of the few components consistent with previous transcriptional studies into cerebral palsy muscle. This work will assist

future research into cerebral palsy muscle and aid the design of novel therapies for these patients.

4.5 Materials and Methods

Muscle Biopsy Collection

Ethical approval for this study conformed to the standards of the Declaration of Helsinki and was approved by the Institutional Review Board at the University of California, San Diego Human Research Protection Program. Age appropriate assent from the patient as well as consent of the parent or guardian was obtained. After obtaining consent, subjects with spastic CP (n=10) were recruited into the study based on undergoing distal hamstring lengthening surgery involving both the gracilis and semitendinosus muscles such that 2 muscle biopsies could be acquired per subject. "Control" subjects (n=10) were pediatric patients undergoing ACL reconstructive surgery with hamstring autograft using gracilis and semitendinosus tendons that were excised along with a distal portion of the muscle that was obtained prior to trimming of the tendon. Control patients did not have any neuromuscular disorders and were ambulatory prior to surgery suggesting no damage to the hamstring muscle from the injury. However, because they were having surgery to repair a torn ligament we acknowledge that these are not purely normal muscles. However, given the ethical constraints associated with taking muscle biopsies, we believe that this is the best possible comparison group that can be envisioned. Patients with CP had developed a fixed contracture requiring surgery and were classified based on the clinical measures

of Gross Motor Function Classification System [81], popliteal angle, and limb(s) affected. Patients had not received any neurotoxin injection or previous surgical lengthening within the 2 years prior to surgery. All muscle biopsies (n=40) were snap frozen in liquid nitrogen (-159 ^{o}C) within ~1 minute of excision and stored at -80 ^{o}C.

RNA extraction

RNA was extracted using a combination of standard Trizol (Invitrogen, Carlsbad, CA) and RNeasy (Qiagen, Valencia, CA) protocols. Briefly, approximately 30 mg of frozen muscle tissue was homogenized using approximately 50 mg of RNase free 0.5 mm zirconium oxide beads (Next Advance, Averill Park, NY) in 0.5 ml Trizol using a Bullet Blender (Next Advance, Averill Park, NY). 0.1 ml of chloroform was added to the solution, then vigorously vortexed for 15 seconds, then centrifuged at 4^{o}C for 15 min. The upper aqueous layer was removed and mixed with an equal volume of 70% ethanol before being added to the RNeasy spin column. The column was washed and then incubated with RNAse-free DNAse (Qiagen) for 15 minutes and then washed again three times prior being eluted as described in the manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm, and the 260 nm-to-280 nm absorbance ratios were calculated to define RNA purity.

Microarray processing

Affymetrix microarrays (HG-U133A 2.0; Affymetrix, Santa Clara, CA) were used for each individual muscle biopsy (n=40 chips). RNA processing including cDNA synthesis, cDNA labeling, microarray hybridization, microarray scanning, and stringent quality control measures were performed by the Gene Chip Core at the Department of Veterans Affairs San Diego Health Care System, (San Diego, CA).

The raw data are available (.cel files) at the Gene Expression Omnibus (GEO) under accession number GSE31243.

Quantitative real time PCR

Quantitative real-time PCR (qPCR) was conducted to validate the expression levels of select genes (*DMD*, *COL1A2*, *MSTN*, *IGF1*, *COL4A2*, and *MYH1*) and also to provide expression values for two transcripts not present on the microarray (*FBXO32* and *TRIM63*). The primers used for each gene are listed in Supplementary Table 4.1. The same RNA extracted for microarray analysis was used for qPCR. Isolated RNA was diluted 1:5 with DNase/RNase free water (Invitrogen) and 1 µl of each sample was reverse transcribed using standard protocols (Superscript III; Invitrogen). cDNA was amplified with the Cepheid SmartCycle (Sunnyvale, CA) with primers designed specific to each gene of interest (Supplementary Table 4.6) using nBLAST and Oligo (version 6.6; Molecular Biology Insights, Cascade, CO). Each sample was run in triplicate along a standard curve. The PCR reaction tube contained 1 x PCR buffer, 2 mM $MgCl_2$ (Invitrogen), 0.2 mM sense and antisense primers, 0.2 mM dNTP, 0.2 x SYBR green, and 1 U of platinum Taq polymerase (Invitrogen). Amplification conditions included an initial hold at 95^{o}C for 2 minutes with 40 cycles of denaturing at 95^{o}C for 15 seconds, followed by annealing and extension phases adjusted for each transcript. Success of each the reaction was determined based on observation of a single reaction product on an agarose gel and a single peak on the DNA melting temperature curve determined after the 40 cycles. The results of qPCR were expressed using a standard curve method with the "cycles to threshold" values represented the number of PCR cycles at which the SYBR green

signal was increased above threshold. The triplicate measures were normalized to the housekeeping gene GAPDH and then averaged. QPCR data were normalized to the median value of the gene to facilitate comparisons to microarray data.

Microarray analysis

Data files were loaded into GeneSpring Software (version 11.5.1; Agilent Software, Santa Clara, CA) for determination of significantly altered genes and clustering analysis. Present genes were determined from the MAS5 (Affymetrix) probe set algorithm based on a 12.5% (5/40) present call. Each sample was clustered based on MAS5 for present genes based on Pearson Correlation similarity score and average linkage clustering algorithm.

In order to provide a conservative choice of significantly altered genes in CP, three independent probe set algorithms were used: MAS5, RMA, and GCRMA. Requiring concordance among different probe set algorithms has recently been used as an approach to reduce false positives in data sets specific to any individual algorithm. Each probe set was normalized to the median of the microarray and then to the median of that probe set on all chips. The probe set data were then condensed into gene level data in GeneSpring by calculating the median value of all probe sets belonging to a single gene. Gene values were analyzed by 2 x 2 Welch ANOVA based on pathology (CP vs. Control) and muscle (gracilis vs. semitendinosus) with a Benjamini and Hochberg False Discovery Rate for multiple testing correction setting the required statistical significance to ($p < 0.01$). Accordingly, 1% of the genes deemed significant for an individual probeset algorithm are suspected to be false positives and genes that

passed in all three algorithms were designated as significantly different for further analysis.

Microarray categorical analysis

After the significantly altered genes were defined, categorical analysis was used to provide information on over represented subsets of genes. Enrichment analysis was performed on up-regulated and down-regulated genes independently with WebGausalt (http://bioinfo.vanderbilt.edu/webgestalt/) on Gene Ontology, KEGG Pathways, transcription factor targets, and microRNA targets [82, 83]. The hypergeometric statistical method was used with a Benjiman-Hochberg multiple testing correction with a significance level of ($p < 0.01$) requiring a minimum of 3 genes per category.

Network analysis was also conducted to determine the proportion of significantly altered genes present in a recently-published muscle gene network (L. Smith, G. Meyer, and R. Lieber, submitted). As these networks are specific to skeletal muscle function, each gene in the network was also investigated in order to avoid arbitrary cutoffs. The networks were visualized using Cytoscape (Version 2.8.1; Cytoscape Consortium) [84] and the nodes colored based on expression level defined as the average expression in CP:average expression of controls.

Microarray correlation analysis

The same biopsies used for microarray analysis also underwent experiments from another study that allowed for matching of microarray data to physical properties where they are described in detail. Briefly, all biopsies underwent passive mechanical testing of muscle fibers and muscle fiber bundles. Fibers and bundles were isolated

and then stretched with the result of the mechanical tests yielding a tangent stiffness (kPa/μm sarcomere length). For fibers this represents the stiffness of components within the cell and for fiber bundles includes cellular components as well as extracellular components.

Correlations analysis was performed using MATLAB software (Mathworks; Natick, MA). Each gene that was considered significantly altered in children with CP from the analysis above was correlated with each of the physical parameters measured. Correlations below $p<0.05$ with a Benjiman-Hochberg multiple testing correction were considered significantly correlated.

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Table 4.1: Highlighted gene ontologies significantly over-represented in genes significantly altered in cerebral palsy. (Category) is the number of genes on the reference list or in this case on the microarray that fall under the ontology. (Observed) is the number of genes on either the significantly up or down-regulated in cerebral palsy list. (Expected) is the number of genes expected to be in the significantly altered list based on the relative sizes of lists.

Gene Ontology	GO#	Category	Observed	Expected	p-value
Biological Process		*Increased in Cerebral Palsy*			
extracellular matrix organization	0030198	86	20	3.57	1.55e-07
collagen metabolic process	0032963	38	8	1.58	0.0044
actin cytoskeleton organization	0030036	213	22	8.85	0.0044
Molecular Function					
calcium ion binding	0005509	680	64	28.22	3.91e-08
collagen binding	0005518	32	8	1.33	0.0005
growth factor binding	0019838	97	14	4.02	0.0006
Cellular Component					
collagen	0005581	31	12	1.31	4.60e-08
cytoskeleton	0005856	1008	75	42.73	1.58e-05
basal lamina	0005605	15	6	0.64	0.0003
Biological Process		*Decreased in Cerebral Palsy*			
ubiquitin-dependent protein catabolic process	0006511	190	33	12.18	9.76e-06
glucose metabolic process	0006006	116	19	7.43	0.0041
skeletal muscle contraction	0003009	15	6	0.96	0.0073
Molecular Function					
ligase activity	0016874	281	43	18.12	6.48e-06
ubiquitin-protein ligase activity	0004842	107	23	6.90	1.39e-05
zinc ion binding	0008270	1447	128	93.32	0.0028
Cellular Component					
proteasome complex	0000502	54	11	3.35	0.0047
cis-Golgi network	0005801	12	5	0.74	0.0056
nuclear lumen	0031981	1203	101	74.65	0.0081

Table 4.2: Significantly over-represented pathways from public databases (KEGG, WikiPathway, and Pathway Commons) along with transcription factors and microRNA. (Category) is the number of genes on the reference list or in this case on the microarray that fall into the listed category. (Observed) is the number of genes on either the significantly up or down-regulated in cerebral palsy list in the category. (Expected) is the number of genes expected to be in the significantly altered list based on the relative sizes of lists.

Increased with Cerebral Palsy	**Observed**	**Expected**	**p-value**
KEGG			
ECM-receptor interaction	20	3.44	8.10e-09
Focal adhesion	30	8.08	1.60e-08
Pathogenic E. coli infection	14	2.25	6.90e-07
Tight junction	15	5.02	0.0015
Leukocyte transendothelial migration	14	4.46	0.0015
Wiki Pathway			
Focal Adhesion	28	7.40	5.19e-08
Pathway Commons			
Hemostasis	27	6.97	2.66e-07
Integrin cell surface interactions	13	2.64	0.0002
Formation of Platelet plug	15	3.66	0.0002
Cell surface interactions at the vascular wall	12	2.72	0.0007
Platelet Activation	12	3.06	0.0016
Signaling in Immune system	21	7.91	0.0016
Collagen-mediated activation cascade	4	0.30	0.0030
Integrins in angiogenesis	10	2.59	0.0053
Transcription Factors			
AP1 Q6	20	7.14	0.0123
TAXCREB	6	0.72	0.0123
PAX4	58	35.3	0.0129
TEF1	17	5.95	0.0129
SRF	17	6.38	0.0172
BACH1	18	7.06	0.0172
AP1 C	49	29.8	0.0295
microRNA			
GGGACCA, MIR-133A	16	5.61	0.0070
TCCAGAG, MIR-518C	14	4.21	0.0070

Table 4.2: Continued

Decreased with Cerebral Palsy	Observed	Expected	p-value
KEGG			
Systemic lupus erythematosus	18	7.56	0.0201
Citrate cycle (TCA cycle)	8	2.06	0.0201
Insulin signaling pathway	20	8.80	0.0201
Ubiquitin mediated proteolysis	17	7.91	0.0473
Wiki Pathways (P<.01)			
Type II interferon signaling	13	3.37	0.0012
Pathway Commons			
Integration of energy metabolism	26	15.5	0.0766
APC/C:Cdc20 mediated degradation of mitotic proteins	10	3.99	0.0766
eNOS acylation cycle	3	0.48	0.0766
APC/C:Cdh1 mediated degradation of Cdc20 in late mitosis/early G1	9	3.57	0.0766
IGF1 pathway	6	1.99	0.0766
Glucose Regulation of Insulin Secretion	23	13.5	0.0766
Activation of APC/C mediated degradation of mitotic proteins	10	4.12	0.0766
Glucose + ATP => glucose-6-phosphate + ADP	23	13.4	0.0766
APC/C-mediated degradation of cell cycle proteins	10	4.47	0.0766
Regulation of nuclear SMAD2/3 signaling	28	16.84	0.0766
Transcription Factors			
WCAANNNYCAG UNKNOWN	23	9.69	0.0270
SP3 Q3	24	10.4	0.0270
microRNA			
ATGTTAA, MIR-302C	28	11.3	0.0012

Table 4.3: Gene ontologies that were over-represented among genes that had a significant correlation ($p < 0.05$) with either fiber or fiber bundle passive stiffness measurements. (Category) is the number of genes on the reference list or in this case significantly altered in cerebral palsy. (Observed) is the number of genes significantly correlated with mechanical stiffness that fall into the category. (Expected) is the number of genes expected to be in the significantly altered list based on the relative sizes of lists.

Gene Ontology	**Observed**	**Expected**	**p-value**
Biological Process	*Pos Correlation with Bundle Stiffness*		
extracellular matrix organization	13	2.25	5.54E-06
extracellular structure organization	13	2.46	1.29E-05
collagen fibril organization	8	1.07	7.53E-05
collagen biosynthetic process	5	0.54	1.40E-03
cell adhesion	23	9.95	3.00E-03
biological adhesion	23	9.95	3.00E-03
skin development	5	0.64	4.40E-03
collagen metabolic process	5	0.86	2.91E-02
response to external stimulus	19	9.10	2.91E-02
blood vessel development	10	3.42	2.91E-02
regulation of response to external stimulus	6	1.39	2.91E-02
immune system process	17	7.60	2.91E-02
myeloid leukocyte mediated immunity	3	0.32	2.91E-02
response to chemical stimulus	25	13.59	2.91E-02
response to wounding	15	6.42	2.91E-02
bone morphogenesis	3	0.32	2.91E-02
multicellular organismal marco metabolic proccess	5	0.96	2.91E-02
multicellular organismal metabolic process	5	0.96	2.91E-02
vasculature development	10	3.53	3.44E-02
cell-substrate adhesion	7	2.03	4.78E-02
cell activation	8	2.57	4.78E-02
Molecular Function			
extracellular matrix structural constituent	12	2.25	2.58E-05
structural molecule activity	22	7.17	3.41E-05
platelet-derived growth factor binding	5	0.64	2.70E-03
growth factor binding	8	2.25	2.56E-02
proteoglycan binding	3	0.32	2.60E-02
Cellular Component			
extracellular matrix	23	5.99	1.65E-07
proteinaceous extracellular matrix	22	5.46	1.65E-07
extracellular region part	28	9.31	1.17E-06
extracellular region	35	14.23	3.46E-06
collagen	10	1.50	3.46E-06
extracellular matrix part	14	3.00	4.50E-06

Table 4.3: Continued

Gene Ontology	Observed	Expected	p-value
fibrillar collagen	6	0.75	2.00E-04
basement membrane	8	1.93	4.70E-03
myosin complex	5	1.18	4.23E-02
cytoskeleton	26	14.98	4.26E-02
Cellular Component	*Neg Bundle Stiffness Correlation*		
mitochondrial intermembrane space	4	0.36	6.70E-03
organelle envelope lumen	4	0.43	1.00E-02
Biological Process	*Neg Fiber Stiffness Correlation*		
ubiquitin-dependent protein catabolic process	7	1.34	2.54E-02
modification-dependent protein catabolic process	9	2.72	3.18E-02
cellular protein catabolic process	9	2.89	3.18E-02
proteolysis involved in cellular protein catabolic process	9	2.89	3.18E-02
modification-dependent macromolecule catabolic process	9	2.72	3.18E-02
mitotic cell cycle	6	1.31	3.18E-02
proteasomal protein catabolic process	4	0.60	3.81E-02
proteasomal ubiquitin-dependent protein catabolic process	4	0.60	3.81E-02
regulation of ubiquitin-protein ligase activity during mitotic cell cycle	3	0.40	3.93E-02
negative regulation of ligase activity	3	0.40	3.93E-02
regulation of ubiquitin-protein ligase activity	3	0.40	3.93E-02
positive regulation of ubiquitin-protein ligase activity	3	0.37	3.93E-02
positive regulation of ubiquitin-protein ligase activity during mitotic cell cycle	3	0.37	3.93E-02
cellular macromolecule catabolic process	10	3.86	3.93E-02
negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle	3	0.40	3.93E-02
DNA metabolic process	7	2.05	3.93E-02
anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	3	0.40	3.93E-02
negative regulation of ubiquitin-protein ligase activity	3	0.40	3.93E-02
cellular response to DNA damage stimulus	5	1.24	3.93E-02
positive regulation of ligase activity	3	0.37	3.93E-02
Cellular Component			
nucleus	28	16.61	1.44E-02
proteasome complex	4	0.48	2.16E-02
intracellular membrane-bounded organelle	35	25.73	2.76E-02
membrane-bounded organelle	35	25.73	2.76E-02
chromosome	6	1.59	3.84E-02
intracellular part	41	34.47	4.26E-02
organelle	37	29.32	4.26E-02
intracellular organelle	37	29.28	4.26E-02

Table 4.4: The list of genes that are significantly regulated in the same direction with cerebral palsy between the current study in hamstring muscle and a previous study in wrist muscle. Only 13 genes were up-regulated in both and only 6 down regulated in both. The 13 up-regulated genes were significantly over-represented in the 4 extracellular matrix gene ontologies listed.

Increased in Cerebral Palsy		**Decreased in Cerebral Palsy**	
Entrez Gene	*Gene Symbol*	*Entrez Gene*	*Gene Symbol*
22872	SEC31A	5209	PFKFB3
120	ADD3	27095	TRAPPC3
6421	SFPQ	1052	CEBPD
4147	MATN2	4501	MT1X
5157	PDGFRL	682	BSG
10234	LRRC17	645745	MT1M/E/H/P2
1842	ECM2		
8483	CILP	**Gene Ontologies from Increased in Cerebral Palsy**	
65110	UPF3A		
81578	COL21A1	Extracellular Matrix	
8076	MFAP5	Proteinaceous Extracellular Matrix	
91851	CHRDL1	Extracellular Region	
6934	TCF7L2	Extracellular Region Part	

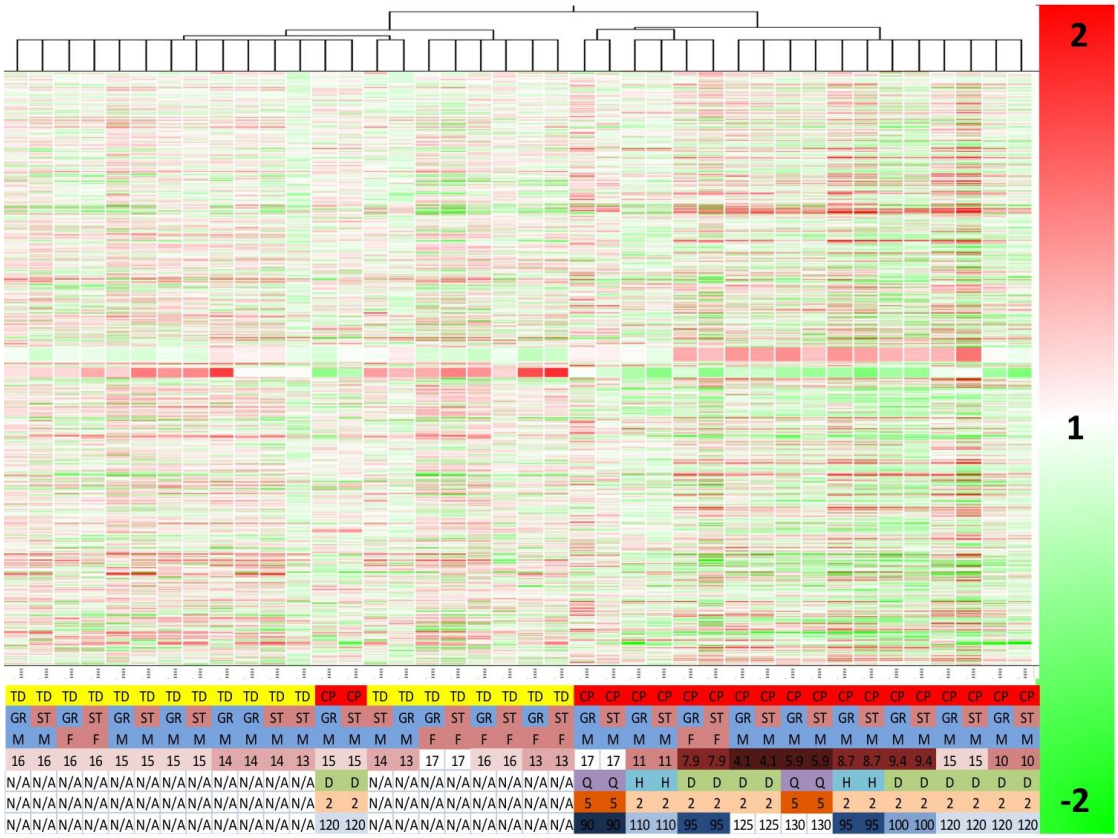

Figure 4.1: Condition tree based on a Pearson correlation clustering algorithm. Individual genes are colored according to expression ratio. Cerebral palsy samples cluster separately from typically developing controls, except for one subject. Table contains subject data. Row 1: typically developing (TD) or cerebral palsy (CP), Row 2: gracilis (GR) or semitendinosus (ST), Row 3: sex, Row 4: age, Row 5: diplegia (D), hemiplegia (H), or quadriplegia (Q), Row 6: gross motor function classification system score, Row 7: popliteal angle.

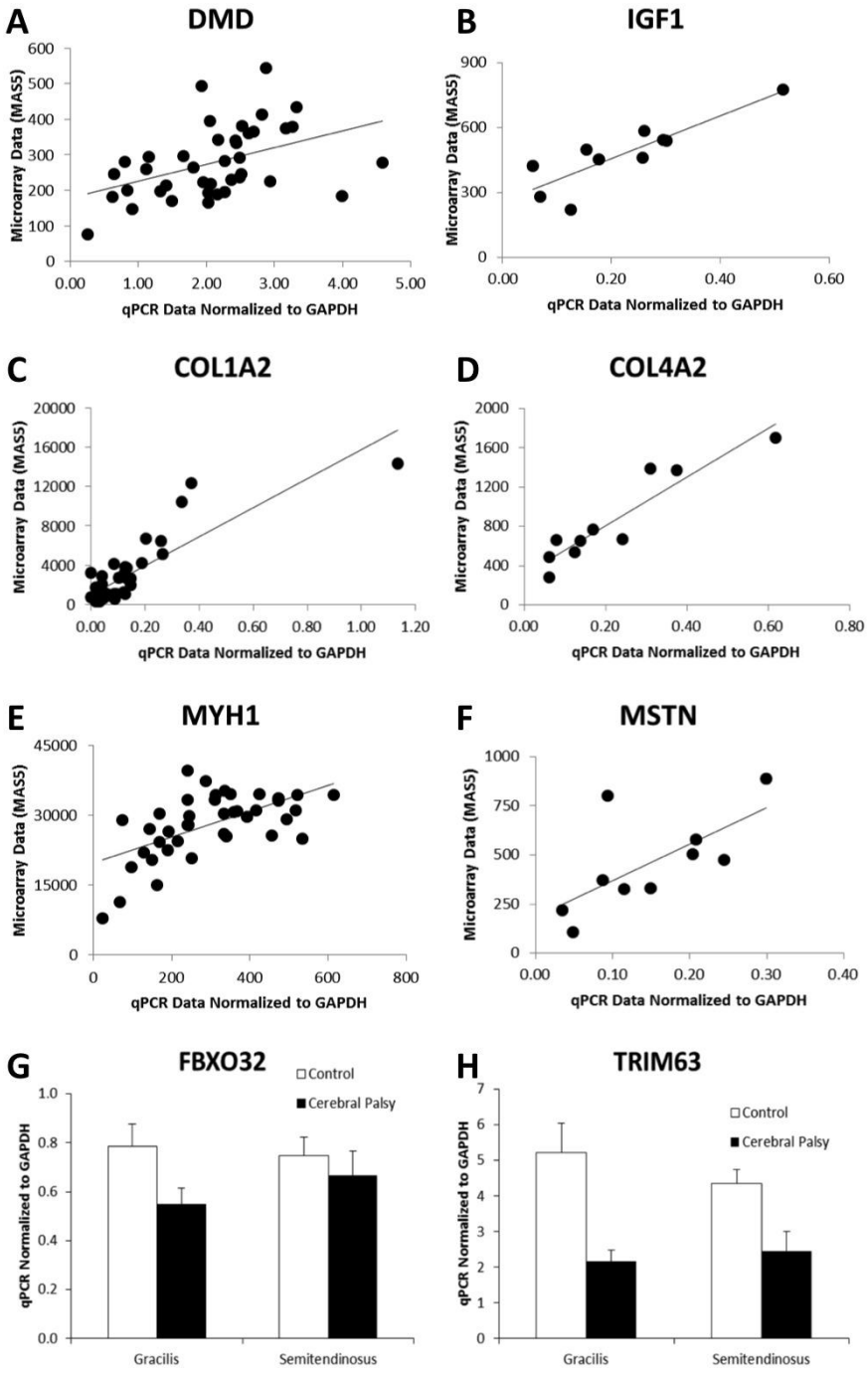

Figure 4.2: Comparison of quantitative real-time PCR (qPCR) data to microarray data based on MAS5 summarization algorithm. Each gene in (**A-F**) has a significant correlation ($p < 0.05$). (**G**) atrogin-1 (FBXO32) and (**H**) MURF-1 (TRIM63) are quantified based on disease state and muscle as they are not present on the microarray.

Neuromuscular Junction		Calcium Handling		Muscle Contraction		Cytoskeleton		Extracellular Matrix	
Gene	*FC*	*Gene*	*FC*	*Gene*	*FC*	*Gene*	*FC*	*Gene*	*FC*
UTRN	**0.52**	**ASPH**	**0.70**	**TCAP**	**0.76**	**UTRN**	**0.52**	MMP9	0.85
CHRN	**0.70**	**DHPR**	**0.78**	**MYH**	**0.80**	**EMD**	**0.59**	**COL4**	**1.02**
MUSK	0.70	**CALM1**	**0.80**	**TNNT**	**0.84**	NOS1	0.73	P4H	1.04
COL4	**0.76**	**SLN**	**0.84**	**MYBPC**	**0.84**	VCL	0.85	PLOD3	1.11
GABP	0.81	PLN	0.85	TMOD	0.85	**SGC**	**0.87**	DCN	1.15
SCN4A	0.89	**ATP2A**	**0.87**	**CAPZ**	**0.86**	DMD	0.88	TGFB1	1.22
ERBB	0.89	TRDN	0.87	CKM	0.89	LDB3	0.89	**LTBP4**	**1.31**
RAPSN	0.93	PVALB	0.89	**TNNI**	**0.89**	SYNM	0.89	**SDC**	**1.39**
AGRN	0.94	PPP3CA	0.90	TTN	0.90	DTN	0.90	BGN	1.42
ITGB1	1.00	S100A1	0.92	TPM	0.96	**SNT**	**0.90**	**LAM**	**1.43**
LRP4	1.08	**CAMK2**	**0.98**	MYOT	0.96	SYNC	0.90	**HSPG2**	**1.43**
LAMA5	1.10	FKBP1A	1.01	NEB	0.96	LARGE	0.95	**COL6**	**1.57**
ACHE	1.12	RYR1	1.02	TNNC	0.97	DES	0.96	LOX	1.57
LAMB2	1.17	CAPN	1.06	**MYL**	**1.00**	TLN1	0.99	**FN1**	**1.77**
HSPG2	**1.43**	CASQ	1.14	MYOM	1.09	ITGB1	1.00	**MMP2**	**1.90**
NID1	**1.56**	TRPC	1.22	ACT	1.27	ITGA7	1.00	**CTGF**	**2.09**
COLQ	**1.76**	**RYR3**	**2.37**	ACTN	1.29	OBSCN	1.01	**TIMP1**	**2.26**
CHRNG	#N/A	SYPL2	#N/A	MYH8	1.49	DYSF	1.03	**TNC**	**2.32**
YWHAG	#N/A	JPH1	#N/A	**MYH3**	**6.14**	DAG1	1.05	**COL3A1**	**2.74**
NRG1	#N/A			MYPN	#N/A	**ANK**	**1.08**	**COL1**	**2.79**
						CSRP3	1.09	MMP14	#N/A
						LMNA	**1.18**	TIMP2	#N/A
						FLNC	**1.31**	MMP1	#N/A
						SPT	**1.34**		
						VIM	**1.37**		
						MYOZ2	**1.79**		
						FKRP	#N/A		

Metabolism		Inflammation		Muscle Signaling	
Gene	*FC*	*Gene*	*FC*	*Gene*	*FC*
AMPK	**0.55**	**HSF2**	**0.61**	**MAPK8**	**0.45**
TFAM	**0.73**	HSF1	0.79	**MSTN**	**0.50**
LIPE	0.77	**MAPK14**	**0.87**	**ACVR2B**	**0.59**
SDH	**0.80**	NFKB1	1.08	**RPS6KB1**	**0.66**
PFKM	**0.81**	CASP1	1.22	**FOXO3**	**0.73**
LDHA	0.82	TGFB1	1.22	MYOG	0.73
CREB1	**0.83**	IGF1	1.37	IGF1R	0.77
ATF2	0.85	IL1B	#N/A	**CALM1**	**0.80**
VEGFA	0.86	TNF	#N/A	EIF4E	0.82
MAPK14	**0.87**	IL6	#N/A	**MAPK14**	**0.87**
PPARGC1A	0.87	IL10	#N/A	GSK3B	0.89
TFB	0.88	IL1RN	#N/A	PPP3CA	0.90
PDH	0.89	SOCS3	#N/A	MEF2	0.91
CS	0.89	IFNG	#N/A	MYF6	0.93
MB	0.89	IL8	#N/A	PIK3R	0.93
CYC	**0.90**	PTGS2	#N/A	MYOD1	0.94
COX	**0.91**	IKBKE	#N/A	MYF5	0.96
NDUF	**0.91**	TRIM63	#N/A	FRAP1	0.96
PYGM	0.92	FBXO32	#N/A	**CAMK2**	**0.98**
ATP5	**0.94**			MAPK1	1.01
PNPLA2	0.94	NFATC1	1.07	HDAC	1.03
MEF2	0.95	HADH	1.10	EIF2B4	1.03
FABP3	0.95	TP53	1.12	MAP2K1	1.04
CAMK2	**0.98**	GYS1	1.12	NFATC1	1.07
NRF1	0.99	CPT1B	1.24	EIF4EBP1	1.10
NFE2L2	1.00	HK2	1.47	AKT1	1.18
MLYCD	1.03	**LPL**	**1.87**	IGF1	1.37
CD36	1.05	CAMK4	#N/A	**FST**	**1.62**
SLC2A4	1.06	GPAM	#N/A	**FGF2**	**2.12**

Fiber Type Isoforms			
Slow Isoform		*Fast Isoform*	
Gene	*FC*	*Gene*	*FC*
MYH7	0.97	**MYH1**	**0.70**
		MYH2	0.89
MYL2	0.94	MYL1	0.87
MYL3	1.09		
MYBPC1	0.92	**MYBPC2**	**0.77**
TNNT1	0.95	**TNNT3**	**0.73**
TNNI1	0.99	**TNNI2**	**0.81**
TNNC1	0.96	TNNC2	0.90
TPM3	1.08	TPM1	0.87
TMOD1	0.85	TMOD4	#N/A
CASQ2	1.41	CASQ1	0.92
ATP2A2	0.94	**ATP2A1**	**0.80**
PVALB	0.89		

RPTOR	#N/A
PDK1	#N/A
CDKN1A	#N/A
HGF	#N/A

Figure 4.3: Heatmap based on expression ratio and separated by functional muscle gene networks. Entrez gene symbols are used for individual entities with the exception of gene families found in supplementary table 3.1 in which case geometric means of multiple genes determine expression ratio. Gene symbols found to be significantly different in cerebral palsy are colored based on direction of regulation. Gene families are colored if any individual gene in the family is significantly altered in cerebral palsy. Genes with N/A were either not present on the chip or did not have expression to qualify as present in analysis.

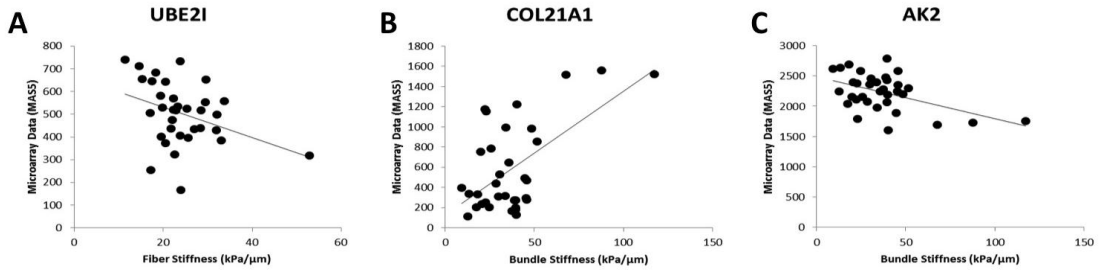

Figure 4.4: Examples of significant correlation ($p < 0.05$) between mRNA expression levels and passive mechanical stiffness measurements. (**A**) Ubiquitin-conjugating enzyme E2I (UBE2I) has a negative correlation with fiber stiffness. (**B**) Collagen XXI alpha I (COL21A1) has a positive correlation with fiber bundle stiffness. (**C**) adenylate kinase 2 (AK2) and a mitochondrial intermembrane transcript has a negative correlation with bundle stiffness.

CHAPTER 5

HAMSTRING CONTRACTURES IN CHILDREN WITH SPASTIC CEREBRAL PALSY RESULT FROM A STIFFER ECM AND INCREASED IN VIVO SARCOMERE LENGTH

5.1 Abstract

Cerebral Palsy (CP) results from an upper motor neuron (UMN) lesion in the developing brain. Secondary to the UMN lesion, which causes spasticity, is a pathologic response by muscle—namely, contracture. However, the elements within muscle that increase passive mechanical stiffness, and therefore result in contracture, are unknown. Using hamstring muscle biopsies from pediatric patients with CP (n=33) and control (n=19) patients we investigated passive mechanical properties at the protein, cellular, tissue, and architectural levels to identify the elements responsible for contracture. Titin isoform, the major load bearing protein within muscle cells, was unaltered in CP. Correspondingly, the passive mechanics of individual muscle fibers were not altered. However, CP muscle bundles, which include fibers in their constituent ECM, were stiffer than control bundles. This

corresponded to an increase in collagen content of CP muscles measured by hydroxyproline assay and observed using immunohistochemistry. In-vivo sarcomere length of CP muscle measured during surgery was significantly longer than that predicted for control muscle. The combination of increased tissue stiffness and increased sarcomere length interact to increase stiffness greatly of the contracture tissue in-vivo. These findings provide evidence that contracture formation is not the result of stiffening at the cellular level, but stiffening of the ECM with increased collagen and an increase of in vivo sarcomere length leading to higher passive stresses.

5.2 Introduction

Cerebral Palsy (CP) describes a spectrum of movement disorders caused by upper motor neuron (UMN) lesions that occur in the developing brain [1]. CP is the most common childhood movement disorder with a prevalence of 3.6 cases per 1,000 in the US [2]. Although the primary UMN insult is not progressive, the resulting muscle pathology does progress [3]. Pathologic muscle in CP is described as spastic, which is a velocity-dependent resistance to stretch due to reduced inhibition of the stretch reflexes [4]. Despite best clinical practices, children with CP often develop contractures that limit their range of motion, decrease their mobility and may be painful. While muscle spasticity and hyper-activity are commonly seen in cerebral palsy, contracture represents a unique muscle adaptation in which the muscle increases passive stiffness such that range of motion around a joint is limited without active

force production of the muscle. Thus muscle contractures represent a major disability to those affected by CP in particular and those with UMN lesions in general [5]

The skeletal muscle mechanism by which spasticity results in contracture is not known. Transcriptional data suggest many physiological pathways are altered in contracture [6]. One consistent finding is that spastic muscles from children with CP are weaker than those of typically developing control children due to a combination of decreased neuronal drive, decreased muscle size, and decreased specific tension [7-9]. Previous studies also demonstrated that increased resistance to stretch in spastic muscle has both an active and passive component [10-12]. However the passive elements responsible for this increased stiffness have not been identified and these presumeably represent the therapeutic targets of physical therapy [13], surgery [14] and neurotoxin injection [15]. To date, these treatments do not prevent contracture formation [16].

It should be noted that the term “contracture” is typically referred to in the muscle physiology literature as an increase in tension of isolated muscles or fibers in response to external activation by caffeine or potassium [17]. Caffeine induces calcium release from the sarcoplasmic reticulum and potassium depolarizes the muscle as methods to activate the cross bridge cycle that produces muscle active tension [18, 19]. However, the common clinical use of the term “contracture” does not refer to such activation. Rather, a clinical “contracture” represents a condition where a muscle becomes extremely stiff, limiting range of motion, perhaps causing pain, and deforming joints. These contractures often result from upper motor neuron lesions such as those that occur after stroke, head injury, or cerebral palsy and represent

tremendous challenges to treat [20, 21]. Often, clinical contractures result from chronic activation of a muscle, referred to as "spasticity" and the net result is a stiff muscle that limits the range of motion around a joint in the absence of any active component of cross bridge cycling [22].

As muscle architecture is the most important determinant of muscle force generating capacity and excursion, previous studies have sought to describe the macroscopic structural adaptation of muscle in CP. It has been suggested that contracture results from shortened muscles and thus multiple studies have used ultrasound technology to measure fascicle length in contractured muscle and, while these experiments confirm reduced CP muscle volume, evidence for shortened fascicles is inconclusive [23-26]. A major drawback of ultrasound studies is that there is no normalization of fascicle length to sarcomere length so it is conceivable that a CP muscle and control muscle could have exactly the same fascicle lengths, yet have different numbers sarcomeres in series and correspondingly different functional mechanical properties. This would be invisible to the ultrasound method. Direct measurement of intraoperative sarcomere length revealed that sarcomere lengths are indeed longer in CP muscle, suggesting increased passive stiffness [27, 28].

Another proposed mechanism for increased passive stiffness in contractured muscle involves alteration of the tissue itself. Previous studies demonstrated that individual fibers from contractured muscles are stiffer than controls indicating an alteration within the muscle cell [29]. This increased stiffness from within the fiber was hypothesized to arise from titin, considered the major passive load bearing protein within the muscle fiber [30]. Further studies confounded this result showing that

bundles of fibers, which includes extracellular matrix (ECM), from contractured muscles were more compliant compared to controls, and thus unable to explain the increased stiffness on the whole muscle scale [31]. A drawback of our previous mechanical studies is that they studied a variety of human muscles, and we have since shown that healthy human muscles have different passive mechanical properties [32] as was shown for rabbit muscle [30].

To avoid complications that arise when making comparisons across different muscles, we have taken advantage of the fact that children who are undergoing anterior cruciate ligament (ACL) reconstruction with a hamstring autograft have muscle trimmed from the tendon graft that can be harvested and directly compared to the same hamstring muscles from children with CP undergoing surgery. We hypothesized that the passive mechanical properties of spastic muscle are altered in CP and that this could arise across the levels of: protein (titin), cellular (fiber), tissue (fiber bundle including ECM), and/or architecture (sarcomere length). This work will provide further insight into the debilitating mechanism of muscle contracture and drive research on targeted therapies to treat contractures.

5.3 Methods

Muscle Biopsy Collection

Ethical approval for this study conformed to the standards of the Declaration of Helsinki and was approved by the Institutional Review Board of the University of California, San Diego Human Research Protection Program. After obtaining consent

from parents and age-appropriate assent from children, subjects with CP (n=17) were recruited for this study because they were undergoing hamstring lengthening surgery that involved gracilis and semitendinosus muucles. Control children (n=14) with no history of neurological disorder were recruited because they were undergoing ACL reconstructive surgery with a hamstring autograft using gracilis and semitendinosus tendons that were excised along with a portion of muscle that could be obtained as it was trimmed from the tendon. All patients with CP had developed a contracture requiring surgery, despite receiving conservative treatment. Patients were classified based on clinical measures of Gross Motor Function Classification System [33], popliteal angle, limbs affected and treatment measures of previous surgical lengthening or botulinum toxin injection (Table 5.1). Muscle biopsies were obtained and either snap frozen in isopentane chilled by liquid nitrogen (-159°C), and stored at -80°C or placed in glycerinated muscle relaxing solution and stored at -20^{o}C.

In Vivo Sarcomere Lengths

Custom muscle biopsy clamps, modified for pediatric use with 0.5 cm jaw spacing were used to determine *in vivo* sarcomere length (Fig 5.1). We previously validated this method against intraoperative laser diffraction [34]. After skin incision and prior to lengthening, gracilis and semitendinosus were identified. A small segment of each muscle was atraumatically isolated by blunt dissection. The custom clamp was then slipped over the bundle, with care to prevent undue tension on the muscle. The child's leg was positioned with 90^{o} of hip flexion and 90^{o} of knee flexion, and neutral hip abduction-adduction, the clamp was engaged, and the section of muscle within the jaws of the clamp was resected and immediately placed in Formalin to fix the biopsy

specimen in its in vivo configuration. After 2-days of fixation, muscle bundles were isolated on glass slides and sarcomere length was measured by laser diffraction (see below). For control patients receiving ACL reconstruction, hamstring muscles are not accessible in their *in vivo* position, which precludes the use of the biopsy clamps to obtain control values. Thus, for estimation of control sarcomere lengths, we extracted these values from our previous musculoskeletal model [35].

Muscle Mechanical Testing

Biopsies for mechanics were stored in a glycerinated relaxing solution overnight, composed of (mM): KPropionate (170.0), K_3EGTA (5.0), $MgCl_2$ (5.3), imidazole (10.0), Na_2ATP (21.2), NaN_3 (1.0), glutathione (2.5), 50 μM leupeptin, and 50% (v/v) glycerol. For dissection of fiber or fiber bundle samples, muscles were removed from storage solution and transferred to a relaxing solution at pCa 8.0 and pH 7.1 consisting of (mM): imidazole (59.4), KCH_4O_3S (86.0), $Ca(KCH_4O_3S)_2$ (0.13), $Mg(KCH_4O_3S)_2$ (10.8), K_3EGTA (5.5), KH_2PO_4 (1.0), Na_2ATP (5.1), and 50.0 μM leupeptin. Single fiber segments (1.5-3 mm in length) were carefully dissected and mounted in a chamber in a custom apparatus at room temperature (20°C). Fibers were secured using 10-0 monofilament nylon suture on one end to a force transducer (Model 405A, sensitivity 10 V/g, Aurora Scientific, Ontario, Canada) and on the other end to a titanium wire rigidly attached to a rotational bearing (Newport MT-RS; Irvine, CA; Sup. Fig. 5.1). Segments displaying obvious abnormalities or discoloration were not used. The sample was transillumated by a 7-mW He–Ne laser to permit sarcomere length measurement by laser diffraction (Lieber et al., 1984). Resolution of this method is approximately 5 nm [36]. The system was calibrated with

a 2.50-μm plastic blazed diffraction grating prior to experimentation (Diffraction Gratings, Inc., Nashville, TN).

The fiber was brought to slack length, defined when passive tension was just measurable above the noise level of the force transducer. Sample dimensions were measured optically with a cross-hair reticule mounted on a dissecting microscope and micromanipulators on an x-y mobile stage. The fiber was then loaded with strains of approximately 0.25 μm/sarcomere at 100 fiber lengths/sec. Each stretch was held for 2 or 3 minutes during which stress relaxation was measured, before a sequential stretch was made. Fibers were stretched in total to approximately 100% strain and were saved for titin analysis after mechanical testing. Force data were converted to stress by dividing force by the baseline cross-sectional area value determined assuming a cylindrical sample with an average diameter determined from 3 separate points along the fiber. Samples were discarded if they did not produce a clear diffraction pattern, if any irregularities appeared along their length during testing, or if they were severed or slipped at either suture attachment point during the test. Muscle bundles were mechanically tested in the same manner as fibers and consisted of approximately 20 fibers and their constitutive ECM.

Mechanical Data Analysis

All analysis was performed using Matlab (Mathworks Inc., Natick, MA). Relaxed stress after 2 or 3 minutes was used to fit a relaxed stress vs. sarcomere length curve. This curve was fit with a line for fibers, but with a quadratic for bundles, as there was notable non-linearity in bundle data. For sample fits, sarcomere lengths below slack length are assigned a stress of 0. This produces a “toe region” due to

averaging of the fits across the range of slack sarcomere lengths tested in fibers, generally below 2.5 μm sarcomere length . Only the data beyond the toe region in which a most fibers are generating tension is depicted (Fig. 7.2A/C). Tangent modulus was calculated at given sarcomere length by taking the derivative of the relaxed stress vs. sarcomere length fit at that length. Comparisons of tangent stiffness were conducted with a 3-way ANOVA for pathology (CP vs. control), muscle (gracilis vs. semitendinosus; repeated measure), and scale (Fiber vs. Bundle; repeated measure) with results considered significant at $p < 0.05$. All data are presented in the text as mean±SEM unless otherwise noted.

Protein Gels

Titin isoform and MyHC content were analyzed on gels from both single fibers after mechanical experiments and from sections of biopsies. Single fibers were stored at -80˚C until analyzed and boiled for 2 minutes in 10uL SDS-VAGE sample buffer and. SDS-VAGE sample buffer was comprised of 8M urea, 2M thiourea, 3% SDS w/v, 75mM DTT, 0.03% bromophenol blue, and 0.05M Tris–Cl, pH 6.8 [37]. For biopsies, a myofibril-rich fraction (~10mg wet weight) of individual biopsies (n=24 biopsies from 12 patients for titin and MyHC) was homogenized in sample buffer using the Bullet Blender (Next Advance, Inc., Averill Park, NY).

To quantify titin isoforms, the molecular mass of titin in muscle samples was determined using sodium dodecyl sulfate-vertical agarose gel electrophoresis (SDS-VAGE). An acrylamide plug was placed at the bottom of the gel to hold the agarose in place. The final composition of this plug was 12.8% acrylamide, 10% v/v glycerol, 0.5M Tris–Cl, 2.34% N,N0-diallyltartardiamide, 0.028% ammonium persulfate, and

0.152% TEMED. The composition of the agarose gel was 1% w/v SeaKem Gold agarose (Lonza, Basel, Switzerland), 30% v/v glycerol, 50mM Tris–base, 0.384M glycine, and 0.1% w/v SDS. Titin standards were obtained from human cadaver soleus (3700 kDa) and rat cardiac muscle (2992 kDa). The standard titin molecular weights are based on sequence analysis of the 300 kb titin gene with a coding sequence contained within 363 exons (Labeit and Kolmerer, 1995; Freiburg et al., 2000). These tissues were also homogenized and stored at -80˚C until analysis. Before loading onto the gel, a titin standard "cocktail" was created with the following ratio: 1 unit of human soleus standard:3 units rat cardiac standard:6 units sample buffer. Sample wells were then loaded with both biopsy and rat cardiac homogenate. Human soleus and rat cardiac titin homogenates were loaded into standard lanes. This enabled titin quantification on each gel as previously described [37]. Gels were run at 4˚C for 5 h at 15 mA constant current.

To quantify MyHC isoform distribution homogenized protein solution was resuspended to 0.125 ug/uL protein (BCA protein assay, Pierce, Rockford, IL) in a sample buffer consisting of dithiothreitol (DTT, 100 mmol/L), sodium dodecyl sulfate (SDS, 2%), Tris-base (80 mmol/L) pH 6.8, glycerol (10%), and Bromophenol blue (0.01% w/v). Samples were boiled (2 minutes) and stored at -80°C. Before loading onto the gel, protein was further diluted 1:15 (0.008 ug/uL) in the same sample buffer to account for the approximately 50-fold greater sensitivity of the silver stain. 10 uL of each sample were loaded in each lane. Total acrylamide concentration was 4% and 8% in the stacking and resolving gels, respectively (bisacrylamide, 1:50). Gels (16 22 cm, 0.75 mm thick) were run at a constant current of 10 mA for 1 hour, and thereafter

at constant voltage of 275 V for 22 hours at 4°C to 6°C. Gels were silver stained (BioRad, Hercules, CA). MyHC bands were identified and quantified with densitometry (GS-800, BioRad). The progression of the band was compared and identified based on its relative molecular weight to that of a human protein standard prepared (as described above) from a normal semitendinosus biopsy that showed all three human MHC bands (IIa, IIx, I).

Hydroxyproline Content

Collagen percentage was determined using a colorimetric analysis of hydroxyproline content. Briefly, muscle samples were hydrolyzed in 6N HCl for 18h, neutralized, and samples were treated with a chloramine T solution for 20 min at room temperature followed by a solution of p-diaminobenzaldehyde for 30min at 60°C. Sample absorbance was read at 550nm in triplicate and compared to a standard curve to determine the hydroxyproline content. Hydroxyproline content was converted to collagen using a constant (7.46) that defines the number of hydroxyproline residues in a molecule of collagen.

Immunohistochemistry

Biopsies previously snap frozen in isopentane were used for immunohistochemistry. Cross-sections (10 μm thick) taken from the midportion of the tissue block were cut on a cryostat at −25°C (Microm HM500, Walldorf, Germany). Serial sections were stained with hematoxylin–eosin to observe general tissue morphology. To investigate ECM components sections were labeled with primary antibodies to fibrillar collagen type I (rabbit polyclonal, Rockland, Gilbertsville, PA) and laminin (rabbit polyclonal, Sigma, Saint Louis, MI). The

secondary antibody used for visualization was an Alexa Fluor 594 goat anti-rabbit immunoglobulin G (Invitrogen, Carlsbad, CA).

Fiber cross sectional areas were measured from laminin stained slides using a custom-written macro in ImageJ (NIH, Bethesda, MD). Filtering criteria were applied to insure measurement of actual muscle fibers. These criteria rejected regions with areas below 50 μm^2 or above 5,600 μm^2 to eliminate neurovascular structures and "optically fused" fibers, respectively. Fibers touching the edge of the field were excluded as they were assumed to be incomplete. Regions with circularity below 0.30 or above 1.0 were excluded to prevent inclusion of fibers that were obliquely sectioned.

5.4 Results

Passive Mechanics

Passive mechanical properties were determined for 3 fibers per muscle and 2 muscles per subject, for both control children (n=14) and children with CP (n=17). Fiber diameter was smaller for CP (66.3 ± 2.6 μm) than for control (80.2 ± 2.6 μm; $p < 0.001$) as previously described [29]. Slack sarcomere length for control (2.31 ± 0.04 μm) fibers was not significantly different from CP (2.31 ± 0.04 μm SD; $p > 0.9$) fibers. The stiffness of CP fibers was not significantly different from control for gracilis (control 21.5 ± 1.8 kPa/μm; CP 21.5 ± 2.4 kPa/μm; Fig 5.2A/5.3A) or semitendinosus (control 22.4 ± 1.8 kPa/μm; CP 23.6 ± 1.7 kPa/μm; Fig 5.2B/5.3A), nor was it significantly different between muscles.

Passive mechanical properties were determined for 3 bundles on the same biopsy as for fibers. Fiber bundle diameters were not significantly different between control (366.1 ± 15.0 μm) and CP (354.9 ± 15.2 μm; $p > 0.4$) bundles, nor were slack sarcomere lengths (control: 2.27 ± 0.03 μm; CP 2.29 ± 0.03 μm; $p > 0.5$). CP bundles had higher stresses at longer sarcomere lengths for both gracilis and semitendinosus muscle. When comparing the tangent stiffness at 4.0 um the stiffness of CP bundles was significantly greater than control ($p<0.05$) for both gracilis (control 36.1 ± 3.9 kPa/μm; CP 60.4 ± 11.8 kPa/μm; Fig 5.2C/5.3B) and semitendinosus (control 25.2 ± 2.9 kPa/μm; CP 40.7 ± 4.9 kPa/μm; Fig 5.2D/5.3B).

The 3-way ANOVA with muscle (semitendinosus/gracilis; repeated measure), scale (fiber/bundle; repeated measure), and condition (CP/control) on tangent stiffness revealed a main effect of all three independent measures ($p<0.05$ for condition and muscle; $p<0.001$ scale). The results also showed a significant interaction between muscle and scale ($p<0.05$), with post-hoc tests revealing gracilis bundles are stiffer than semitendinosus, and a significant interaction of condition and scale, with post-hoc tests revealing an effect of CP only at the bundle level for semitendinosus ($p<0.05$).

To determine whether the mechanical changes were related to the clinical observations made on the patients, stiffness was correlated with clinical severity score. There was no significant correlation between stiffness and either Gross Motor Function Classification System or popliteal angle at either the fiber or bundle level (Sup. Fig. 5.2A). To determine consistency within patients, a correlation was run between gracilis and semitendinosus stiffness within the same patient or fiber stiffness to bundle stiffness within the same biopsy, but again there were no significant

correlations (Sup. Figs. 5.2B and 5.2C). There was also a concern that there might be an age effect since the control subjects were slightly older than CP subjects (Table 5.1) but no significant correlation was found, validating the comparison of control to CP subjects with different ages (Sup. Fig. 5.2D). Further the mechanics were compared from patients who underwent a previous hamstring lengthening surgery or botulinum toxin injection prior biopsy as these may affect stiffness. No significant difference was observed for prior botulinum toxin injection for bundles ($p>0.3$) or fibers ($p>0.8$). Only one patient with mechanics measured had undergone a previous lengthening surgery, which was not an outlier among any mechanical measure.

In-vivo Sarcomere Lengths

In-vivo sarcomere length from cerebral palsy patients of contractured hamstring muscles ($n=22$) was $3.54 \pm .14$ μm for gracilis and $3.62 \pm .13$ μm for semitendinosus at 90^o of hip and knee flexion. For control comparison, model results were used as described in Methods [35]. Both gracilis and semitendinosus had significantly longer sarcomere lengths at 90^o of hip and knee flexion than predicted by the model by about 0.5 μm (Fig 5.4A, $p<0.05$ for semitendinosus and gracilis). Combining the sarcomere length values with passive mechanical properties demonstrates that CP muscle tissue at these joint angles bears a higher passive load compared to control muscle (Fig 5.3C).

To determine whether the sarcomere length was associated with functional changes, clinical measures were correlated to sarcomere length. There was a significant correlation between in vivo sarcomere length and both Gross Motor Function Classification System ($p<0.05$) and sarcomere length ($p<0.05$) indicating that

more severely involved patients had longer *in vivo* sarcomere lengths (Fig 5.4B). There was also a significant negative correlation between popliteal angle and *in vivo* sarcomere length indicating that longer sarcomere lengths were present in joints with more severe contractures (Fig 5.4C). Together these correlations provide further evidence that in-vivo sarcomere lengths are elevated in CP,

To compare predicted in-vivo stiffness of CP muscles compared to control we also evaluated the tangent stiffness at the average in-vivo sarcomere length of 90^{o} of hip and knee flexion for each muscle and condition from the data above. Combining the in-vivo sarcomere lengths with the mechanical data shows that in-vivo stiffness is predicted to be much larger for CP muscle (Fig 5.3C). As fibers had linear stress strain relationships, the tangent stiffness does not vary with sarcomere length and is thus the same result as the tangent stiffness at 4.0 μm.

Titin isoforms

To determine if titin size was related to overall muscle stiffness we measured titin molecular weight from a biopsy. The results of a two-way ANOVA showed no significant difference ($p>0.05$) for CP with mean values of gracilis (control 3588 ± 18 kDa; CP 3667 ± 22 kDa) and semitendinosus (control 3625 ± 19 kDa; CP 3658 ± 26 kDa; Fig 5.5) among the samples measured (n=24, 6 per muscle condition). The mass of CP titin was actually larger than that of control suggesting a more compliant isoform and unable to account for any increased passive stiffness of the muscle as a whole.

The effect of titin isoform was also investigated on single fibers, which had previously undergone passive mechanical testing. A two-way ANOVA showed no

significant difference ($p>0.1$) between titin isoform sizes for gracilis (control 3758 ± 24 kDa; CP 3772 ± 36 kDa) or semitendinosus (control 3729 ± 40 kDa; CP 3797 ± 40 kDa) among the single fibers measured (n=55). CP fibers' having equivalent titin isoform size to control fibers is consistent with the fact that CP and control fibers have equivalent stiffness. The effect of titin isoform size on the variability in mechanical stiffness of fibers was also investigated, but there was not a significant correlation between titin size and fiber stiffness within single fibers (Sup Fig 5.3). The molecular weights are larger for single fibers than for the whole biopsies, possibly due to modified preparation methods.

Collagen Content

Collagen content of the biopsies was measured (n = 40, 10 per muscle per condition) as collagen is thought to be the primary load bearing structure of the ECM within muscle [38]. CP muscles had significantly higher collagen concentrations in both gracilis (control 8.0 ± 1.6 μg/mg wet weight; CP 11.2 ± 2.6 μg/mg wet weight) and semitendinosus (control 4.0 ± 0.3 μg/mg wet weight; CP 8.8 ± 0.8 μg/mg wet weight) as determined by a 2 way ANOVA on muscle and condition (Fig 5.6, $p<0.05$). Collagen content was elevated in both gracilis and semitendinosus, although post-hoc tests revealed a significant difference only in semitendinosus ($p<0.001$). Gracilis also tended to have higher collagen concentrations corresponding to the relationship seen in passive bundle stiffness. The collagen content was not significantly different for patients who underwent a previous lengthening surgery ($p>0.5$), or botulinum toxin injections ($p>0.8$) prior biopsy.

Collagen was also visualized by immunohistochemistry. Qualitative results show an increase in fibrillar collagen type I (Fig 5.7 A-D) in muscle from children with CP, corresponding the hydroxyproline results. There was no apparent mislocalization of collagen, however, an increased frequency of large collagen deposits was observed. Laminin, a component of the basal lamina, also showed marked increase in CP muscle (Fig 5.7 E-H). These results demonstrate an increase of ECM material that includes, but is not limited to collagen. While histological evidence shows an increase in ECM material in muscle from children with cerebral palsy, there is also a corresponding decrease in fiber cross-sectional area from (3141 ± 375 μm^2) for controls to (1255 ± 226 μm^2; $p<0.001$) for cerebral palsy as has been previously reported [29].

Myosin Heavy Chain

To determine whether any of the single mechanical fiber data might be confounded by systematic differences in muscle fiber type between patient populations, myosin heavy chain isoform content was measured (n = 35 fibers). One-way ANOVA comparing tangent stiffness of different fiber types did not produce a significant result for either CP or control fibers ($p > 0.05$; Sup Fig 5.4).

To determine the distribution of different fiber types for hamstring muscles in CP myosin heavy chain content was measured from a sample of biopsies (n = 24, 6 per muscle per condition). CP muscles had increased slow myosin heavy chain expression (gracilis – control 29.3 +/- 1.9% to CP 40.0 +/- 2.5%; semitendinosus – control 29.7 +/- 1.7% to CP 41.0 +/- 3.3%; $p<0.001$; Fig 5.8), but there was no significant change in either of the fast isoforms measured IIa or IIx. Myosin heavy

chain is the primary determinant of fiber type, so these results demonstrate a shift to slower fibers in CP muscle. There was no significant difference between gracilis and semitendinosus muscles.

5.5 Discussion

The most significant finding of this study is that muscle tissue from children with CP is significantly stiffer compared to typically developing children. This increased passive stiffness is accompanied by an increase in collagen content and is made even more functionally significant in that in-vivo sarcomere length of CP hamstring muscles is significantly longer compared to predictions for control children. Taken together, these data provide a mechanistic explanation for the increased joint and muscle stiffness observed in these contrature patients. While fiber bundles were different between CP and control muscle, we found no significant difference in mechanical properties at the single fiber level of muscle and no change in titin isoform size. Thus, we conclude that, for human hamstring muscles, increased passive tension in contracture is due to a change in ECM stiffness and increased in-vivo functional sarcomere length rather than any intracellular alteration.

Bundle Mechanics

While fibers contribute to passive tension of muscle, muscle ECM plays an important role in passive mechanics, especially at longer sarcomere lengths. The results show a significant increase in the tangent stiffness of fiber bundles from CP patients. While fibers were fit well with a linear stress-sarcomere length relationship,

fiber bundles required a non-linear quadratic fit. This nonlinearity results in similar tissue stiffness at small strains, but significantly increased stiffness at long sarcomere lengths of the CP muscle tissue. Muscle contractures often limit joint range of motion suggesting that there are large in-vivo strains on the muscle. We thus believe that, in vivo, the ECM bears a large portion of the passive muscle load.

Collagen is considered the primary load bearing structure within muscle ECM [38]. We hypothesized that an increase in collagen content of CP muscle could lead to the increased passive stiffness seen in bundles. Using a hydroxyproline assay to test the hypothesis, the results demonstrate a significant increase in collagen within CP muscle. Although these data are presented in μg collagen/mg of muscle wet weight, they is within similar to previous data presenting collagen as a percent of dry weight [39], using the assumption muscle is approximately 80% water [40]. Increased collagen was also observed by immunohistochemistry along with another ECM component, lamanin, a critical component for cellular attachment to the basal lamina. These results are in agreement with a previous study showing increased collagen content within CP muscle [41]. Many additional factors may be playing a role in the increased ECM stiffness. The organization of collagen, the distribution of collagen types, or the proteoglycan content all could be altered in CP to create a stiffer ECM and represent areas of further investigation. Immunohistochemistry of other muscle proteins (□-actinin, desmin, dystrophin) revealed no obvious differences between patient groups.

One previous study investigated the mechanics of fiber bundles from contractured muscle tissue [31]. Despite finding stiffer fibers we found more

compliant bundles in CP muscle. This result is difficult to reconcile with the increased passive stiffness of the whole muscle that has been reported. The previous study was conducted on biopsies taken from various muscles that were not matched between populations which could account for some these differences and all muscles were from upper extremities which may respond differently to spasticity. In addition, the ECM from upper extremity muscles was highly deranged in the contractured muscles (see Fig. 2 of Lieber, *et al.* 2003) making area fraction measurements from these specimens difficult. It is possible that the area fraction of ECM was overestimated, resulting in artificially low values for bundle modulus. Finally, the nonlinear behavior of upper extremity muscles was quantified by only fitting data to the linear portion of the sarcomere length-stress curve. The current method represents a more accurate method for handling analysis of the nonlinear relationships.

Sarcomere Organization

Sarcomere length operating ranges of semitendinosus and gracilis muscles are unknown, although muscles are typically believed to operate on the plateau of the length-tension curve—2.5-2.7 μm for human skeletal muscle [42]. A previous study demonstrated that spastic muscle operates at longer sarcomere lengths than control [27], which would lead to a larger observed passive stiffness such as that seen in contracture. We measured in-vivo sarcomere lengths of patients with CP at a defined joint angle and compared these values to sarcomere lengths of control subjects that were calculated based on in-vivo sarcomere lengths, moment arms, and muscle-tendon lengths[35]. The results showed that the CP sarcomere lengths are significantly longer than those predicted from the model. The lengths measured were also much longer

than optimal sarcomere length lending further evidence to the idea that they are overly stretched in contracture. With CP subjects operating at longer lengths of the passive length tension relationship, this means that the muscle is experiencing higher stresses not only due to material property changes, but also due to this shift along the passive length-tension curve. This difference becomes more pronounced as the knee extends and the hip flexes and may limit range of motion for children with contractures.

It is often stated that muscle adds or subtracts serial sarcomeres to optimal sarcomere length in-vivo [43]. Long in-vivo sarcomeres suggest an inability of the muscle to add sarcomeres in series, which would be exacerbated during growth spurts, which have been associated with the onset of muscle contractures [44]. The very long sarcomere lengths observed in vivo clearly imply that muscles from children with CP are under high stress. The source of the force that creates or opposes this stress is not known. However, we have speculated, based on analysis of the transcriptome, that muscles from children with CP are unable to grow serially in response to the stretch imposed by osteogenesis [6]. It is also possible that muscles would decrease their serial sarcomere number, which would provide a resistive force since the changes could be slow and accompanied by reinforcement of the muscle fiber by the ECM. Muscle contracture is often described as a "shortened" muscle; our finding of increased in-vivo sarcomere length corresponds with that notion that muscle shortening is derived from fewer series sarcomeres, not shortened sarcomeres. Longer in-vivo sarcomere lengths are an important factor for both passive and active force production of skeletal muscle. Previous research has demonstrated that muscles from children with CP are smaller than that of control children, yet muscle force production

is reduced to an even greater extent indicating a dysfunction of active muscle force production in CP [9, 45]. A consequence of having longer in-vivo sarcomere lengths for children with CP is the muscle will be working at different portions along its active length tension curve [46] compared to control subjects. Based on measured human filament lengths [42] and the increase in ~0.5 μm sarcomere length, the decrease in force from a typically developing child on the plateau of the length tension curve to a child with CP on the descending limb would be 33%. It is interesting to note that this is on the same scale as the reduction in force that is not accounted for by decreased muscle size in these patients [9, 45]. Thus, perhaps altered in vivo sarcomere length operating range represents a significant functional alteration in muscles from children CP and demonstrate that these muscles are not simply changing sarcomere number to "re-optimize" the muscle after injury.

Potential mechanisms of contracture formation

It is possible that the changes in ECM and in vivo sarcomere length take place simultaneously and independently, or that one precedes and directly affects the other. If these two alterations of CP muscle are not causal, they could be a consequence of the same factors within spastic muscle. Previous research showed that myostatin, a negative regulator of muscle growth, also stimulates proliferation of muscle fibroblasts and the release of ECM proteins [47]. Myostatin mRNA has also been shown as significantly increased in CP muscle of the upper extremity [6]. Alternatively, transforming growth factor-beta1 has been shown to induce a shift in satellite cells from a myogenic lineage to fibrobasts [48]. This process also has the potential to limit growth through satellite cell depletion and increase the ECM secreting cell population.

Longer in-vivo sarcomeres of CP muscles demonstrate that there is increased sarcomere strain, which has been shown to directly induce skeletal muscle injury [49]. Repeated strain induced injuries have been shown to drastically increase collagen content and fibrosis in skeletal muscle [50]. The effects of chronic strain injuries persist for months or even years and could be responsible for the effects of muscle in contracture. Repeated strain induced injury also results in lower force producing capacity of muscle which may provide another explanation for the reduced specific tension of CP muscle [51]. The increased fibrosis and stiffness of muscle contracture could also be a compensatory mechanism to limit further strain-induced injury.

There is also potential for a fibrosis induced from spasticity to lead directly to a limitation of longitudinal growth. Satellite cells responsible for muscle growth rely on migration across the basement membrane during activation with the release of matrix metalloproteases [52]. Skeletal muscle fibrosis could impede muscle regeneration by forming a mechanical barrier to this process [52]. Stem cell differentiation is also sensitive to the elasticity of the matrix in which it is embedded [53]. Our study demonstrated an altered stiffness of the ECM in contracture tissue that could lead to an inhibition of satellite cell activation or proliferation [54, 55] and perhaps even predispose muscle stem cells to differentiate toward the fibroblast lineage. It is also possible that fibrosis and lack of growth create a vicious cycle that leads to muscle contracture.

Titin isoforms

Since titin isoform size is related to muscle passive tension [30], we hypothesized that shorter titin isoforms would be present in muscle contracture leading

to increased stiffness. However our results show no difference in titin size between CP and control muscles. Thus, we conclude that titin is not altered in CP to cause contracture, at least not in a manner that alters size. Titin isoform changes have been reported in cardiac disease [56], but literature on titin isoform changes in skeletal muscle is sparse. A previous study investigating titin isoform in spastic muscles of stroke patients also found no change in titin isoform size [57].

Fiber Mechanics

While titin isoform size contributes to single fiber mechanics, other proteins or organization of fiber material could be responsible for an increased passive tension at the cellular level. Two previous studies did demonstrate stiffer muscle fibers for spastic patients. These studies each had important differences however. In one the muscle fibers tested were from a range of muscles which was not the same in the spastic and control groups [29]. This is a confounding issue because it is known that different muscles have different passive mechanical properties [30]. Another showed increased stiffness only in fast fibers with an increase in the proportion of fast fibers [57]. We did not see a corresponding shift to fast fibers of our muscles, and in fact showed a significant increase in type I myosin heavy chain of CP muscles indicated a shift to slower fibers (Fig 5.8). Overall, previous studies have shown disagreement on whether spastic muscles gain a faster or slower phenotype, which could be muscle specific [58-60]. However, the most straightforward interpretation of our data is that over activity resulting from spasticity drives a shift to slower fibers. It is also important to note that the previous study was performed with vastus lateralis muscle biopsies [57], which show much less spasticity and contracture development compared

to the medial hamstrings studied here [61, 62]. Future studies across a wider range of muscles are required to ultimately resolve these ambiguities.

Study Limitations

One important limitation of this study is the subject heterogeneity. CP is a spectrum disorder and here we have primarily examined only the commonly shared parameters of this range of subjects with spastic CP. A more detailed analysis could be attempted using patient stratification by clinical parameters, Gross Motor Function Classification System, popliteal angle, limbs affected, age, and treatment regimens. However, due to limitations of the sample size and the high variability of parameters in working with human subjects, this was not possible. We were able to demonstrate a significant correlation between severity measures and sarcomere length, which helped to mitigate the fact that we are relaying on model data for our comparison with in-vivo sarcomere lengths control children.

The source of controls for this study is not ideal since the patients have sustained an ACL tear. However, these patients are several months removed from the injury and have normal mobility at the time of surgery. Out approach represents the best available source of normal hamstring muscle from a pediatric population. These subject groups were not perfectly age matched, although they all came from a pediatric population, as ACL surgeries do not occur prior to the teenage years whereas hamstring lengthening surgeries often occur much earlier. However, our passive mechanical data did not correlate with age, suggesting that these small age discrepancies did not affect our outcomes. The subjects also underwent varied previous treatment, of which previous hamstring lengthening surgery and botulinum

toxin injections into the hamstrings was tracked. These variables were analyzed in relation to mechanical measures and collagen content, but no relationship was found. These treatment effects are further complicated by highly variable times since treatment.

Summary

It is known that muscle contractures result from the UMN lesion in CP. Here, using a larger and more controlled study then previous studies, we showed increased passive stiffness of fiber bundles and increased sarcomere length *in vivo*. Together, these properties create a muscle in CP that undergoes much higher stresses with increasing muscle length and clearly contributes to the development of muscle contractures. Future studies are required to understand the mechanistic basis for the sarcomere length change and increased ECM content in CP as these clearly represent targets for therapy.

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Table 5.1: Patient Parameters. Patient parameters for the control (CTRL) and CP groups, control patients do no have Gross Motor Function Classification System (GMFCS) or popliteal angle measurements. The right columns are the number of subjects whose biopsies were used in the various analysis, many biopsies were used for multiple analysis: passive mechanics (Pass Mech), in-vivo sarcomere length (SL), hydroxyproline (OH-pro), myosin heavy chain biopsy analysis (MyHC), titin biopsy analysis (Titin).

Group	N	Age	Sex	GMFCS	Popliteal Angle	Pass Mech	SL	OH-Pro	MyHC	Titin
Control	19	15.8 ±1.8	8 M 11 F	N/A	N/A	14	N/A	12	6	6
CP	33	9.6 ±4.2	23 M 10 F	I(2), II(13), III(2), IV(6), V(10)	114 ±15	17	11	12	6	6

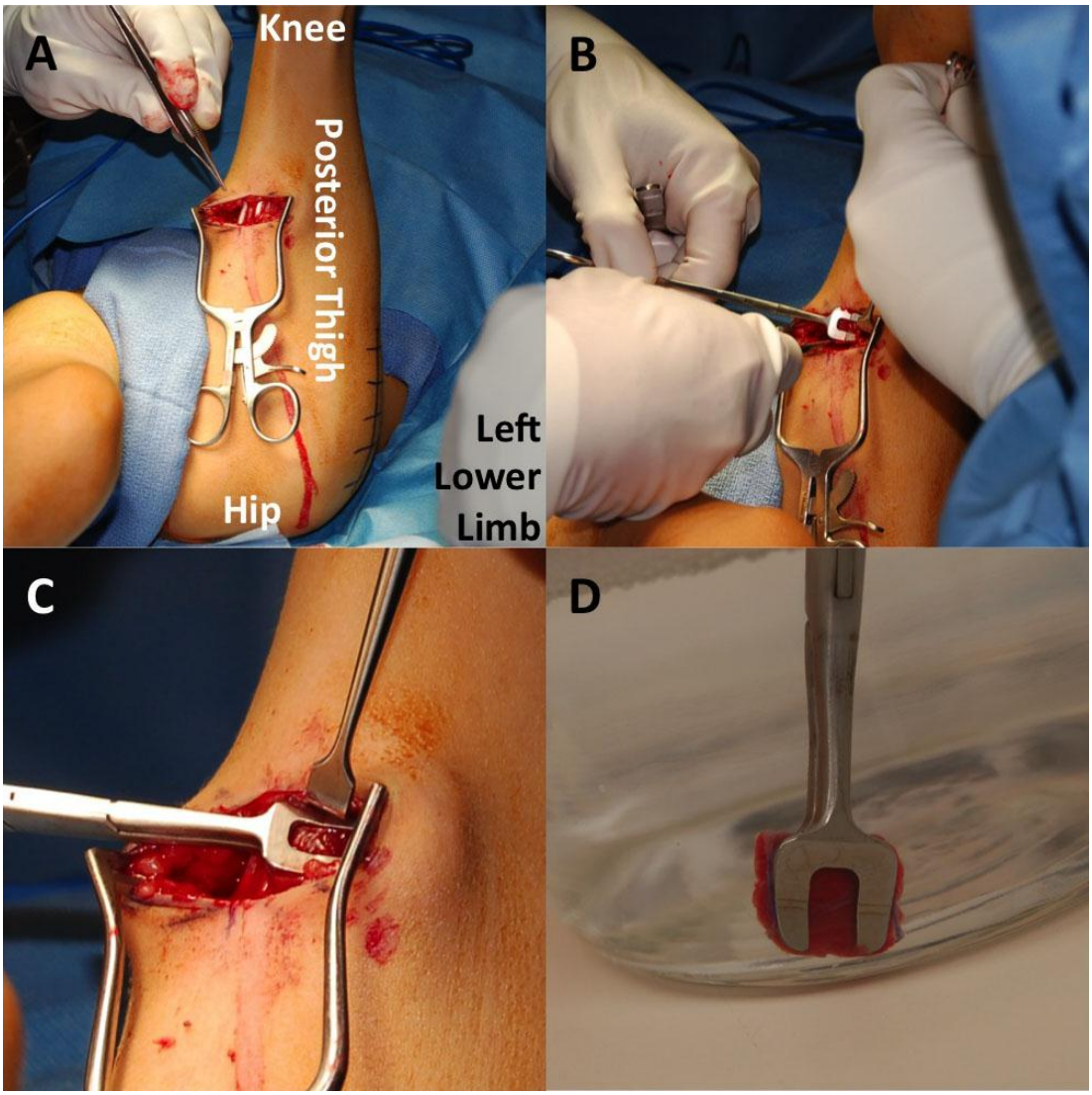

Figure 5.1: Images of biopsy collection method using clamps for in vivo sarcomere length determination. (**A**), hamstring muscle is exposed. (**B**) Clamp is secured around gracilis muscle with joint position at 90^{o} of hip and knee flexion. (**C**) Close-up view of muscle clamps around the biopsy tissue. (**D**) Biopsy is dissected from muscle while clamped and fixed in formalin for subsequent sarcomere length detection.

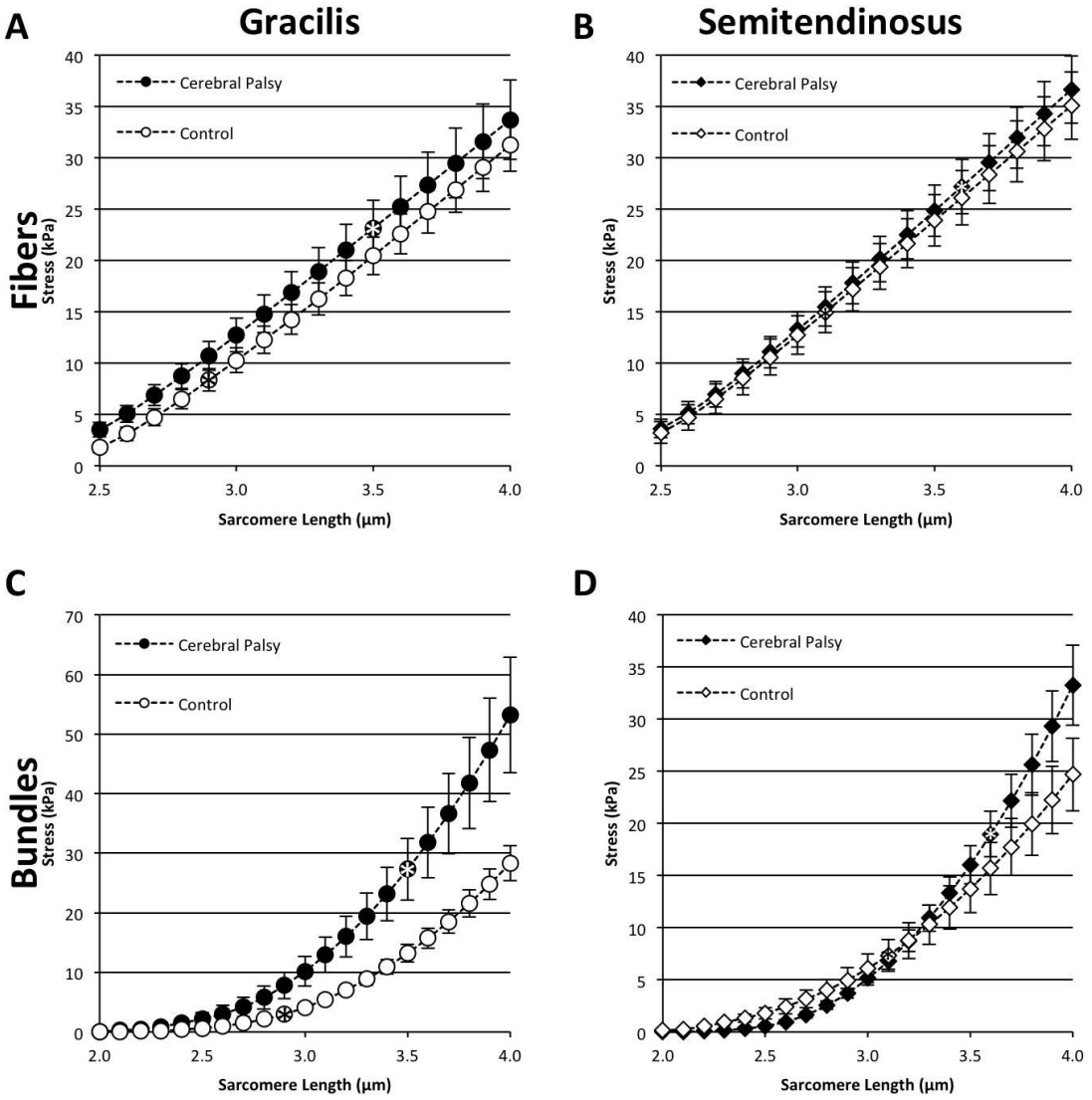

Figure 5.2: Passive tension as a function of sarcomere length for fibers and bundles, after stress relaxation. Plots represent the average of the fits from each individual sample ± SEM. The stress vs. sarcomere length fit was linear for fibers with a R2 value of 0.962 ± 0.003 (**A&B**) and quadratic for bundles with a R2 value of 0.985 ± 0.002 (**C&D**). (**A**) gracilis fibers show no difference between CP and control. (**B**) semitendinosus fibers show no difference between CP and control. (**C**) CP gracilis bundles show a significant increase in stress at high sarcomere lengths compared to control. (**D**) CP semitendinosus bundles show a significant increase in stress at high sarcomere lengths compared to control. (*) In symbol designates the approximate sarcomere length at 90o of hip and knee flexion.

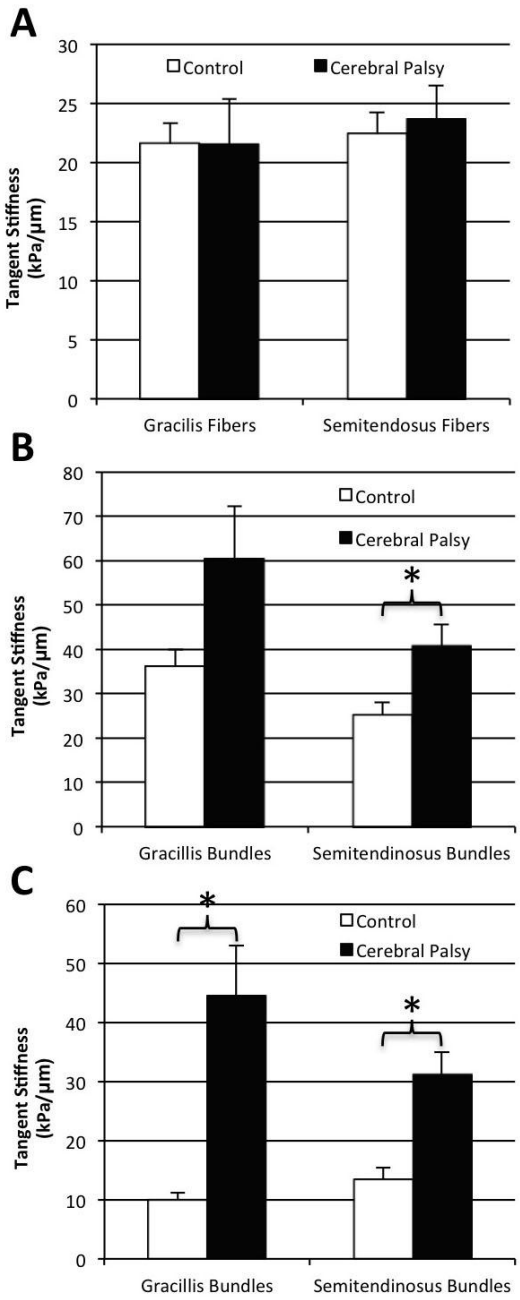

Figure 5.3: Tangent stiffness of fibers and bundles, samples are represented with either a linear fit for fibers or a quadratic fit for bundles. (**A**) tangent stiffness values at 4.0 µm for single fibers are not changed with CP for either gracilis or semitendinosus muscles. (**B**) tangent stiffness values at 4.0 µm for fiber bundles are significantly greater in CP compared to control bundles in both gracilis and semitendinosus ($p<0.05$). (**C**) tangent stiffness values at measured average in vivo sarcomere length for CP bundles or the predicted in vivo sarcomere length for control bundles show highly elevated values in CP for a joint configuration of 90^{o} hip and knee flexion.

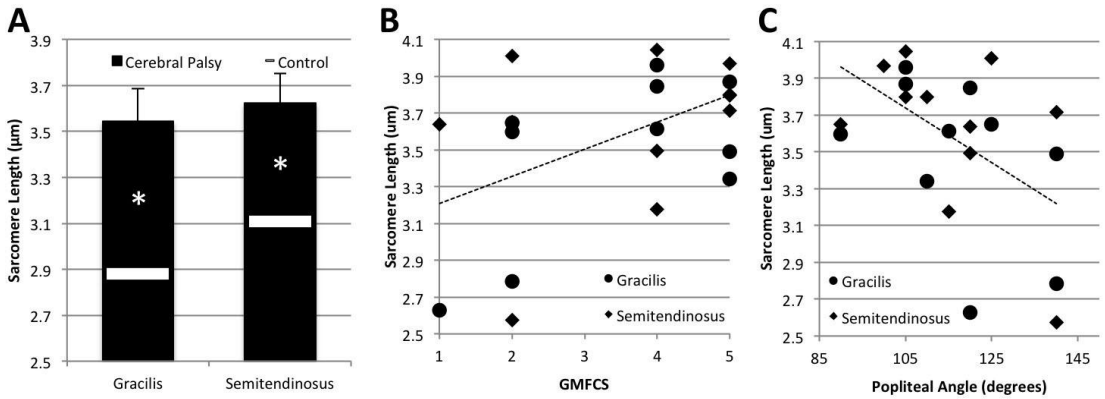

Figure 5.4: In-vivo sarcomere length of gracilis and semitendinosus (**A**) Measured in-vivo sarcomere length with 90^{o} of hip and knee flexion ± SEM for CP subjects in gracilis and semitindenosus, ($p<0.05$). Dotted line represents predicted sarcomere length for control children. (**B**) Correlation between in-vivo sarcomere length measured for CP subjects and their Gross Motor Function Classification System shows a positive significant correlation ($p<0.05$), meaning subjects with longer in-vivo sarcomeres are more severely involved patients. (**C**) Correlation between in-vivo sarcomere length and popliteal angle is a negative and significant ($p<0.05$), meaning subjects with less knee extension have longer sarcomere lengths.

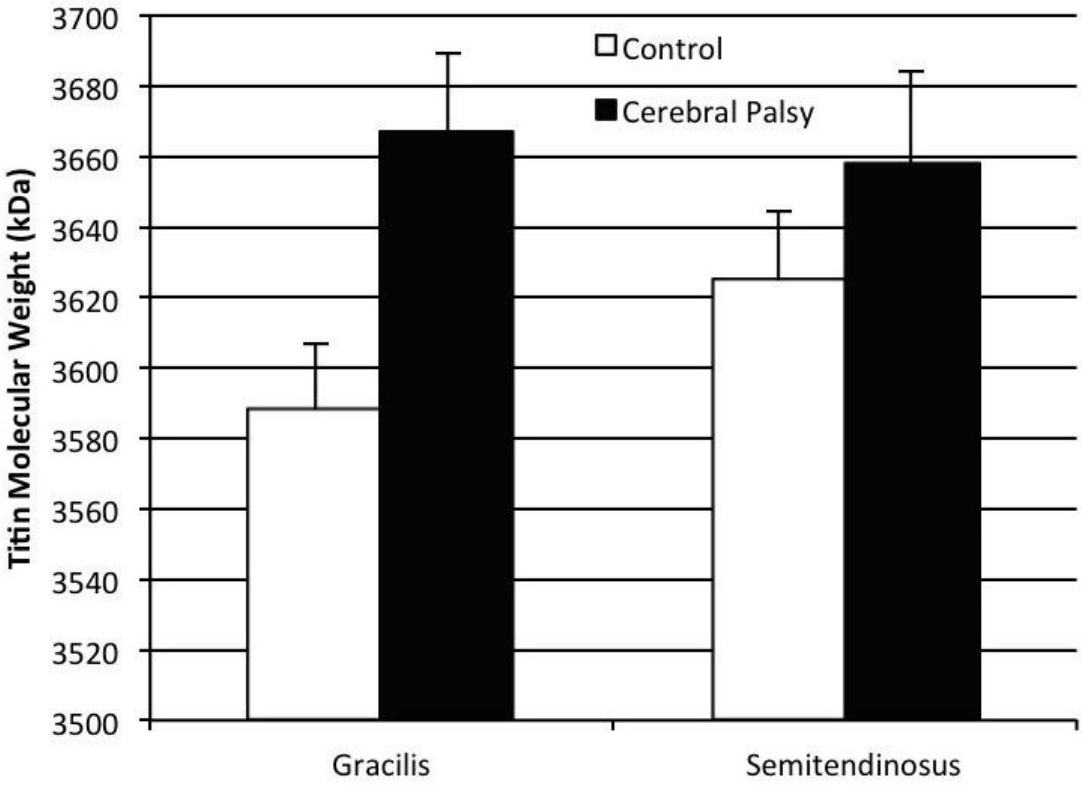

Figure 5.5: Titin isoforms molecular weight of CP and control subjects in gracilis and semitendinosus muscles. Two-way ANOVA shows no significant effect of pathology on molecular weight ($p>0.05$). Although not significant, the trend for molecular weight of titin in CP muscles is larger than control suggesting, if anything, more compliant fibers due to titin alterations.

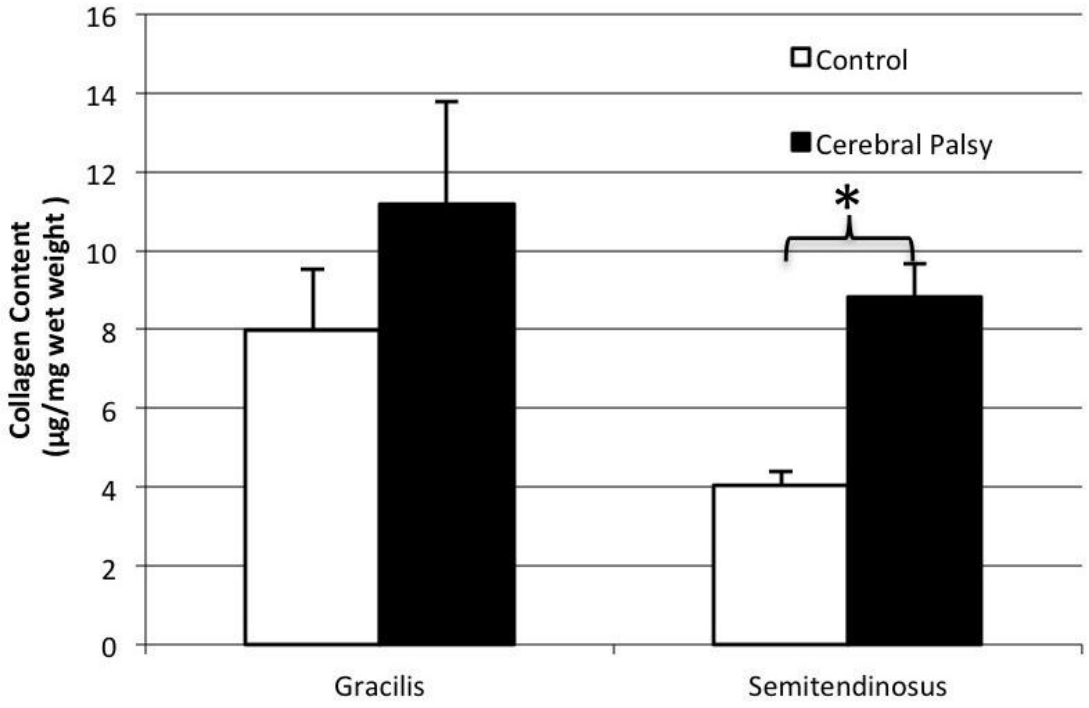

Figure 5.6: Collagen content of muscle biopsies shows significantly higher collagen content in CP biopsies. The results of this assay are consistent with the increased stiffness observed in fiber bundles (Fig. 5.4).

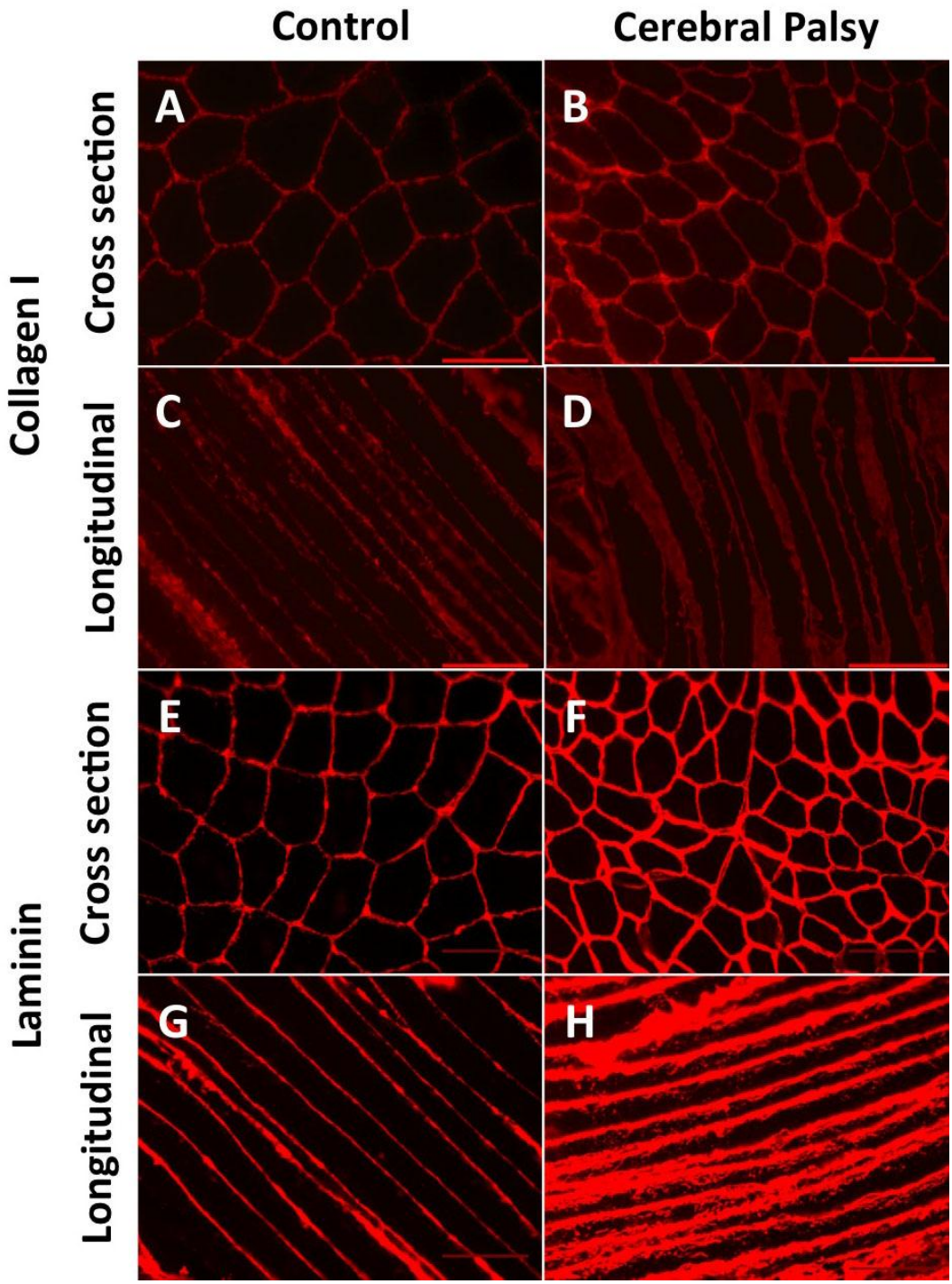

Figure 5.7: Immunohistochemistry of muscle biopsies show qualitatively increased levels of ECM in CP (**B,D, F, and H**) compared to control (**A,C, E, and G**) children. Representative images with primary antibody to collagen type I, fibrillar, in cross section (**A/B**) and longitudinal section (**C/D**). Representative images with primary antibody to laminin, basal lamina, in cross section (**E/F**) and longitudinal section (**G/H**). Scale bar represents 100 μm.

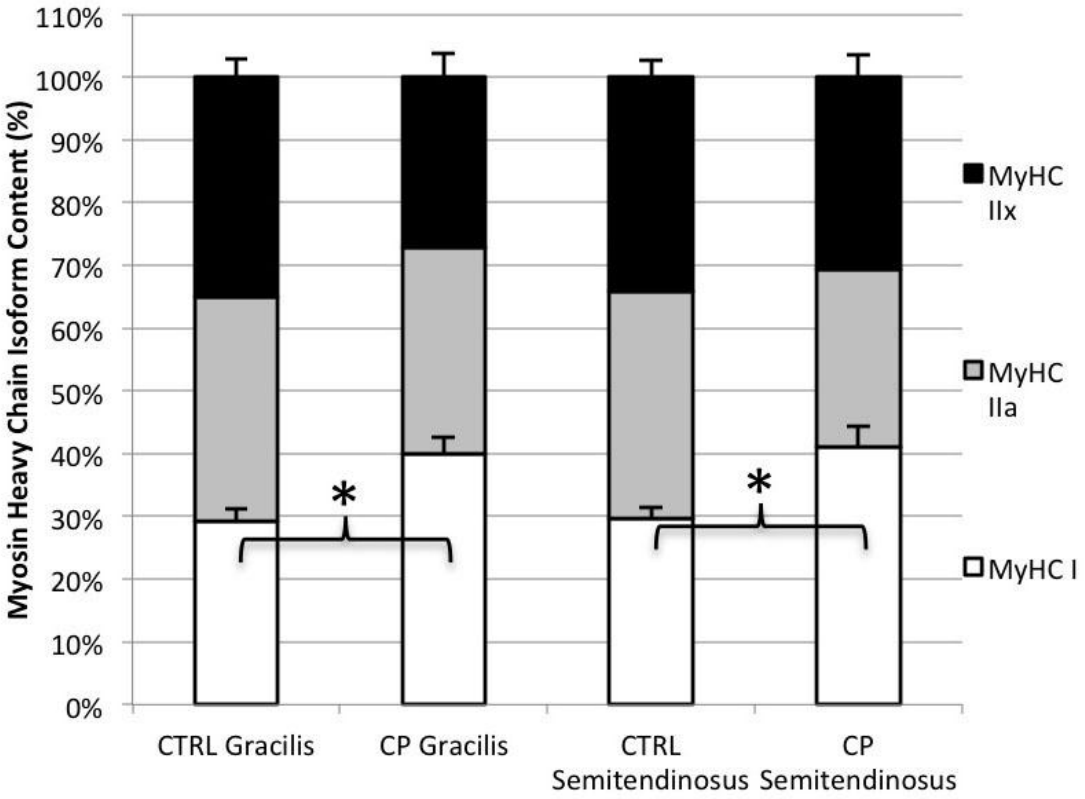

Figure 5.8: Myosin heavy chain isoforms. There was a significant increase in myosin heavy chain I in CP muscles compared to control suggesting contractured fibers have a slower phenotype. There was no significant difference between muscles.

CHAPTER 6

SUMMARY AND SIGNIFICANCE

6.1 Summary of Findings

This work has sought to deepen the understanding of muscle contractures in cerebral palsy from a variety of perspectives. Without an animal model that recapitulates the disease phenol type the study relied upon surgical muscle biopsies from children with cerebral palsy and typically developing pediatric patients. This increases the variability of the experiment relative to genetically identical animal studies and also causes concern over proper control tissue. However it also presents the distinct advantage of being directly pertinent to clinical cases of cerebral palsy.

In Chapter 2 we sought to investigate the muscle as a whole tissue without preconceived notions of which proteins were important. Using microarray technology we were able to examine the expression changes across nearly the entire genome using wrist flexors which undergo contracture and wrist extensors that do not present clinically with contracture. The results showed that muscle from children with cerebral palsy was objectively different than controls as well as being a distinct from other altered muscle states. Using conservative approach to identify significantly

altered genes we were able to identify important gene ontologies that were significantly altered, including extracellular matrix, calcium binding, metabolism, and others. We also placed select genes that were altered into their functional context within muscle to examine the resultant alterations. Few changes were observed in the neuromuscular junction, but many calcium handling genes were up-regulated, a novel discovery in cerebral palsy muscle. Further we found a shift to fast muscle fiber types along with sharp increase in immature muscle myosins present to go along with other immature muscle isoforms. Extracellular matrix components were consistently increased, while metabolic genes were consistently decreased. We found a confounding result showing increases in both muscle hypertrophy and muscle growth inhibition signals, potentially indicating a mechanism by which muscle contractures are induced. This was an exploratory experiment that had too few controls and nonobjectively defined genes in muscle function.

Chapter 3 was done in order to provide an objective list of proteins involved in muscle function. The proteins were broken down into a number of functional categories including: neuromuscular junction, excitation contraction coupling, muscle contraction, cytoskeleton, extracellular matrix, metabolism, muscle hypertrophy and atrophy, and muscle fiber type. Defining how these genes interact provides a more in depth method to investigate high-throughput experiment, including microarrays. The gene networks were created in Cytoscape (Cytoscape Consortium) in order to provide a generic media to distribute the work to outside investigators.

The follow-up microarray experiment presented in Chapter 4 was conducted on muscle hamstring biopsies. This was a more robust study using 40 microarrays and

a well-controlled patient population. Quantitative Real-Time PCR was performed and confirmed the transcriptional results of the microarray on a select set of six genes. The list of significantly altered genes was determined in a similar process as Chapter 2, but yielding many more significantly altered genes and again showing that cerebral palsy muscle was distinct from controls. The ontology analysis revealed several categories of genes that were altered in cerebral palsy, and was expanded to also investigate pathways, transcription factors, and microRNA. This enabled identification of a role for of serum response factor, a muscle growth transcription factor, and microRNA 133A, also critical for muscle growth. In order to investigate the functional consequence of these transcriptional alterations we used the gene networks established in Chapter 3. This identified further markers of muscle immaturity as up-regulated in cerebral palsy. It also displayed the vast increase in extracellular matrix transcripts and growth factors. Metabolic transcripts were generally depressed as in the previous experiment. However this was in spite of a fast-to-slow fiber type transition, opposite of that found in the wrist. In fact calcium handling transcripts were also down-regulated as well growth inhibition signals. In fact there was very little overlap between the upper and lower extremity changes, which were relegated to consistently increasing extracellular matrix. These extracellular matrix changes also were significantly correlated with the results found further in Chapter 5.

Chapter 5 was designed to expound on previous mechanical measurements of muscle tissue in cerebral palsy using the same muscle for comparison and increasing sample size. Contrary to previously published data [1, 2] we showed that muscle fiber

in cerebral palsy were not stiffer than controls. This did correspond with titin molecular weight data showing no difference as well. However, when the extracellular matrix was included with bundles of fibers cerebral palsy muscle tissue did become stiffer. This followed an increase in collagen content in muscle from cerebral palsy as well. The stiffness of muscle tissue depends on the strain it experiences in vivo as well. We used novel muscle biopsy clamps [3] and computer models [4] to show that muscle in cerebral palsy was indeed under increased strain with markedly longer sarcomeres. Combining the increased strain and the stiffer tissue predicts that cerebral palsy experiences much higher passive in vivo.

6.2 Significance of Findings

These results shed light onto the unknown mechanism of muscle contracture in children with cerebral palsy. One notable result of Chapter 2 is that there was no difference between pathologic flexors and extensors. This demonstrates that opposing muscles can be effected similarly, but that the larger muscle group dominates the contracture formation. It should be noted that although the wrist flexion contractures limit the function of the wrist muscles, the results were distinct from immobilized muscle.

The results of Chapter 5 were very different than previous data collected on the mechanics of muscle fibers and bundles of fibers [1, 5]. Previous worked displayed an increase in fiber stiffness while this research shows no change. The earlier study also showed a decrease stiffness of bundles in cerebral palsy while the current research

shows the opposite. An important factor to recognize is that in earlier studies biopsies were taken from multiple muscles made accessible during surgery, whereas we have directly compared two individual muscles. This emphasizes the need to compare the same muscle group when analyzing results in human muscle studies, especially when various muscle have unique responses to disease.

Research on cerebral palsy muscle has been confounded by variable and inconsistent results. However one aspect that was consistent throughout this thesis is the increase in extracellular matrix components in cerebral palsy muscle. Particularly among muscle among the full breadth of the microarray experiments an increase in extracellular matrix was the only consistently regulated gene ontology. This has been shown to have important mechanical considerations in Chapter 5, but could also have further implications in muscle growth. The inhibitors to muscle growth seen active in Chapter 2 were not substantiated in Chapter 4, suggest another muscle inhibition factor. It is conceivable that the increase in extracellular matrix itself is responsible for limiting muscle growth. This could be related to the increased stiffness as studies have shown that satellite cells fail to differentiate into muscle precursors on rigid surfaces [6, 7]. Alternatively the increased extracellular matrix could present a physical barrier for satellite cell incorporation into mature fibers. Indeed satellite cells express proteins to breakdown the extracellular matrix and migrate to the appropriate position for integration. Manipulating expression to increase production of these factors has led to increased muscle regeneration capacity [8]. This presents a possible therapeutic target of introducing anti-fibrotic agents into cerebral palsy muscle in order to prevent or even reverse contracture and facilitate muscle growth.

6.3 Future Directions

Whole Muscle Mechanics

While we have shown that muscle tissue stiffness can contribute to contracture at the levels of muscle bundles, showing this relationship at the whole muscle level would be ideal. The advantage of testing whole muscle is that skeletal muscle is not homogenous above the level of fiber bundles. Connective tissue structures including the perimysium and epimysium are not accounted for when testing fiber bundles alone, but could play an important role in whole muscle stiffness [9]. Isolating whole muscles for mechanical testing is obviously not feasible in a human study; however, there are tools that could make such a study possible.

Previous methods have been established to identify sarcomere length in vivo that are applicable to the surgical setting [3, 10] so that sarcomere strain can be determined. Buckle transducers can be placed around the tendon of the muscle of interest, which can measure linear muscle force by the displacement of the buckle [11]. Furthermore ultrasound imaging of the muscle is currently used to determine fascicle lengths as well as muscle thickness [12, 13]. Measuring these components would be required at multiple specific joint angles. Combining these three protocols in a single patient would provide stress vs. sarcomere strain as well as normalized fiber lengths.

This method of future study is promising, but there are considerations that need to be made. In order to measure sarcomere lengths the surgery requires direct access

to the muscle of interest. Our biopsies have been from gracilis and semitendinosus muscles that are directly accessed in common surgeries for cerebral palsy, but not commonly in typically developing children. Model based predictions of sarcomere length changes may need to be included. The buckle transducers are able to reliably measure force, however when in-vivo considerations need to be made to isolate the force directly applied from the tendon from other tissues in the body that could apply a transverse force during knee motion. Finally ultrasound in skeletal muscle has been used frequently, but is more difficult in non-superficial muscles. MRI may be an alternative and would provide 3-dimentional images and more contrast. However this would be much more expansive, time intensive, and difficult with spastic children at a fixed knee orientation.

Decellularization

Each of the experiments within this thesis implicates a role of the extracellular matrix in muscle contracture. In Chapter 5 a subtraction technique is used to estimate the contribution of the extracellular matrix. Being able to directly test the mechanical properties and composition of the extracellular matrix would provide more information on the alterations taking place. Recently decellularization protocols have been developed to remove cells from tissue while leaving the extracellular matrix intact as a scaffold for implanting cells [14, 15]. One protocol in particular has been developed with the purpose of leaving the mechanical properties of muscle extracellular matrix intact [16]. This method was performed in whole mouse muscle,

but it is possible to obtain surgical muscle biopsies of similar size to mouse tibilias anterior muscle.

Unfortunately there are also drawbacks to this analysis method. During the cell lyses and removal procedure it is unknown if the shape of the extracellular matrix remains constant, especially in biopsies without perimysium or epimysium. Further in response to stretch it is unknown if the extracellular matrix would deform in the same manner as when fibers are present. If the extracellular matrix is collapses together without fibers present it would likely alter the mechanical properties. In fact a recent study has shown that extracellular matrix exerts a transverse stress to muscle fibers under stretch. The non-continuous nature of the extracellular matrix would area calculations for determining stress values more challenging as well, however they may be overcome by careful histology techniques [5]. An experiment to confirm the extracellular matrix mechanical properties may also be possible in reverse, selectively removing the extracellular matrix. Changing from the mouse whole muscle to the human biopsies may require adaptations to the protocol as well. In fact we have attempted decellularization of human muscle biopsies, but have been unable to completely remove cellular material. One study has used collagenase to digest the extracellular matrix and tested the mechanical properties as an alternative method [17].

Flow Cytometry

Knowing the changes in cell populations present in muscle contracture would enable deeper understanding of results we have observed. Flow cytometry has recently been employed in muscle tissue enabling analysis of small mono-nuclear cells

[18]. Despite being unable to record muscle fibers, these methods are useful in identifying progenitor cell populations including satellite cells and a recently discovered population that has the potential to differentiate into fibroblasts or adipocytes [19, 20]. Mononuclear cells within muscle including fibroblasts, macrophages, endothelial cells, and other may also be recorded with proper labeling. Having knowledge of the cells present in the tissue would provide deeper understanding of how the tissue develops into the state of contracture, if the increased fibrosis the result of more fibroblasts?

Beyond the question of determining cell populations, cell sorting is also possible to gather more information on the various cell populations. Quantitative real-time PCR could be employed to determine the activity of the cell, such as if native fibroblasts are producing more collagen per cell. Knowing the expression of satellite cells based on transcripts for *PAX7*, *MYOD*, and *MYOG* would provide information on whether the cells are replicating or what stage of differentiation they are in [21]. Furthermore the cells may be cultured in different media to determine if they gain or lose potential due to the cerebral palsy environment. These experiments can provide useful knowledge of the mechanism of contracture and also possibly form the basis for a cell therapy.

We were able to collect some preliminary data on flow cytometry experiments performed on cells isolated from hamstring muscle contracture biopsies and well as controls. The antibodies used were a combination of NCAM and PAX7 for satellite cell determination [22], CD45 for hematopoietic cells [23], CD34 for hematopoietic plus endothelial cells [24], ER-TR7 for fibroblast determination [25], and PDGFRα

for fibro/adipogenic progenitor cells [20]. Initial results showed that NCAM and PAX7 co-localized, enabling the use of NCAM only in further experiments. Initial results have also demonstrated an approximately 2-fold decrease in the satellite cell population in biopsies from patients with cerebral palsy, however this has not met the significant threshold ($p < 0.06$). Further comparisons are inclusive at this time.

Flow cytometry experiments rely upon well efficient antibody labeling of isolated cell suspensions. Unfortunately, many muscle antibodies in human have not been well tested for flow cytometery. This has led to poor labeling for some of our antibodies, particularly ER-TR7 and PDGFRα. The availability of human biopsies is also inconsistent which has led to a necessity to fix cells after isolation and cell surface labeling. This does provide the ability to permeablize the cells and investigate intracellular markers. However this process eliminates the ability to sort living cells. Protocols would have to be adapted in order to maintain a living cell population for experiments after sorting in order to culture or quantify transcript levels. However we believe this preliminary work will pave the way to more robust experiments and a further knowledge of muscle contracture.

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