The Impact of Stretch, Exercise and Drug Treatments on Structure, Function and Satellite Cell Activation in Aging Muscle

by

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ABSTRACT

Age-related muscle atrophy and the importance of satellite cells in muscle maintenance, growth and repair led us to examine the effects of mechanical stretch, nitric oxide (NO), and age on satellite cell (SC) activation and gene expression in normal young and old mice. Baseline variables (body mass, muscle mass, fiber cross-sectional area (CSA), muscle strength, SC population, stretch activation and gene expression) were obtained from normal C57BL/6 mice at 3-, 8-, 12- and 18-months-of-age. Activation was assayed by 3H-thymidine incorporation into extensor digitorum longus (EDL) muscles isolated for culture. In a second experiment, muscle from 8- and 18-month-old mice was treated with one or more of: stretch; NO-donors (L-Arginine (LA), isosorbide dinitrate (ISDN)) and; N^{ω}-nitro-L-Arginine methyl ester (LN). EDL muscles from 6-month-old mice required a greater stretch stimulus (20% vs. 10% length increase) than EDL from younger mice to increase SC activation. Stretch did not increase SC activation in mice older than 6 months-of-age. NO supplementation from an exogenous source (ISDN) increased SC activation by stretch in 8- but not 18-mo-old EDLs. In a third experiment, 8- and 18-month-old mice were subjected to 3 weeks of voluntary wheel running, or not. The EDL, tibialis anterior (TA), gastrocnemius (GAST) and quadriceps (QUAD) muscles were selected for analysis following sacrifice. The QUAD muscle from 8-month-old mice was the only muscle that demonstrated an exercise-induced increase in SC activation, elevated expression of neuronal nitric oxide synthase (NOS-I) and downregulation of myostatin, a gene that inhibits muscle growth. These results suggest mechanical stimulation of satellite cells and regulation of gene expression that controls muscle growth in voluntary contractile tissue is muscle-specific and age-dependent.

Perturbed sensitivity to mechanical stimulation and NO in muscle from 18-mo-old mice, in culture and in vivo, may partly explain loss of muscle mass, fiber CSA, relative grip strength and SC pool size with age. Similar to dystrophic muscle, a disrupted dystrophin-glycoprotein complex and subsequent alteration in NO availability may affect the ability of native satellite cells to maintain or effectively regenerate aged muscle. Therefore, NO treatment and exercise have the potential to stimulate satellite cell activation and increase muscle growth.

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CHAPTER 1. INTRODUCTION

In the postnatal period, skeletal muscle is a post-mitotic, voluntary contractile tissue responsible for movements of the skeleton [1]. Skeletal muscle also plays a role in: protection of bones, organs, vessels and nerves; metabolism; oxygen consumption; and regulation of body temperature. Muscle is a dynamic tissue that is constantly undergoing maintenance, growth and repair through the processes of protein synthesis and degradation. A balance of these two processes results in maintenance of muscle size and shape, whereas increased levels of protein synthesis or degradation lead to muscle growth (hypertrophy) or muscle loss (atrophy), respectively.

Since skeletal muscle is post-mitotic, nuclei located within individual muscle fibers do not have the ability to maintain, increase or repair fiber size and shape. Fortunately, located between the sarcolemma and basement membrane of the fiber are muscle precursor cells called satellite cells. Satellite cells were named by Mauro in 1961, more for their location than function [2]. Muscle precursor cells surround the muscle fiber much like a satellite orbits the earth; sensitive to signals sent or received by the dominant mass. In 2000, Anderson discovered that the chemical messenger, nitric oxide (NO), governs the activation state of satellite cells [3]. In normal adult muscle, a pulsatile release of NO maintains the quiescent state of the cells whereas a bolus release of NO results in the activation, proliferation and differentiation of satellite cells. Unlike the nuclei of the muscle fiber, satellite cells are stem cells and have the ability to divide into a large number of daughter cells, identical to itself (self-renewal), or into precursor cells that eventually become mature muscle cells [4]. Therefore, muscle satellite cells are responsible for the maintenance, growth and repair of muscle tissue.

Satellite cells must be activated in order to contribute to the processes of muscle maintenance, growth and repair [5]. Activation of satellite cells can occur through stretch, exercise, trauma, denervation [6] and the addition of growth factors, specifically hepatocyte growth factor (HGF) [7-10]. For the purposes of this research, the two models used to study satellite cell activation are stretch in vitro and exercise in vivo. In both of these models, the mechanical stimulus of stretch or exercise is utilized to produce a bolus release of NO from the muscle fiber to activate satellite cells. Although stretch- and exercise-induced satellite cell activation have been well documented in young muscle of both humans [11-14] and animals [7, 15-21], the effects of mechanical stimulation on satellite cell activation in aged muscle has yet to be realized.

In young muscle, satellite cells typically respond to stimuli by becoming activated within 10 minutes, however, in aged muscle there is a 24 hour lag between the application of a stimulus and activation of muscle precursor cells [22-24]. However, the age-related delay in satellite cell activation has been demonstrated using in vivo models of severe muscle trauma or in vitro studies incorporating growth factors. Therefore, the current study is unique in the fact that in vitro stretch and in vivo exercise are adopted to activate satellite cells from aged muscle. Since muscle atrophy and decreased muscle function is associated with aging, it is important to determine how typical life-style activities, such as stretch and voluntary exercise, can be utilized to combat the negative consequences of age-related muscle atrophy.

Age-related changes in animal and human muscle include decreased mass and volume; reduced fiber CSA; diminished strength and power; impaired contractile function; increased cell death (apoptosis); and a decreased ability to respond to

hypertrophic stimuli [25, 26]. A diminished response to hypertrophic stimuli may be the cause of age-related changes in skeletal muscle with reduced mass, volume, CSA, strength, power and contractile function being the effects. Central to the lack of response to activating stimuli is the satellite cell. If satellite cells cannot be activated to maintain, enlarge or repair muscle, atrophy will ensue. Since muscle precursor cells are the main contributor to these processes, intuitively, defunct recruitment of satellite cells would result in the structural and functional deficits associated with aging.

To date, NO [7, 16, 27-31] and HGF [8, 17, 23] are the only two chemical messengers proven to activate satellite cells . As mentioned previously, NO production (in young muscle) increases in response to mechanical stimulation. In addition, NO has been implicated in the formation of neuromuscular junctions, glucose uptake, muscle contraction and vasodilatation [32]. NO is produced via the muscle specific isoform of neuronal nitric oxide (NOS-Iμ) [32, 33]. Since the topic of this thesis is skeletal muscle, the abbreviation NOS-I will refer to the muscle specific isoform of neuronal NOS. NOS-I is anchored in the dystrophin-glycoprotein complex (DGC) that connects the sarcomere (contractile component of the muscle fiber) to the basement membrane [34]. In normal muscle, NOS-I is located in an optimal position to communicate with satellite cells. As NOS-I converts L-Arginine (internal source of NO) to L-citrulline and NO, diffusion of NO across the sarcolemma of the muscle fiber either maintains satellite cell quiescence, or activates satellite cells depending on the amount of NO produced [6, 31].

Blake and colleagues demonstrated that the lack of response to mechanical stimuli in the EDL and soleus muscles of aged rats can be attributed, in part, to the alterations in mitogen-activated protein kinase phosphorylation [35]. Although the mechanisms

responsible for these changes are poorly understood, data confirm that aged muscle does not respond to an applied load to the same extent as young muscle [36]. Interestingly, in genetic diseases such as muscular dystrophies, similar deficits in load-induced signaling of skeletal muscle are present [37]. Dystrophic muscle is characterized by varying degrees of DGC defects (depending on the type of dystrophy) which may also be responsible for deficits in mechanically-induced satellite cell activation since the DGC plays a role in sensing mechanical forces imposed on the sarcolemma of muscle fibers [38]. Similar to dystrophic muscle, contractile tissue from the aged muscle of rats has demonstrated an absence or discontinuous distribution of dystrophin along the sarcolemma of muscle fibers [39]. In addition, suspension-induced atrophy models in young normal mice have resulted in a dislocation of NOS-I from the sub-sarcolemmal position to the cytoplasm of muscle fibers. Dislocation of NOS-I from the DGC not only disrupts the structure of muscle fibers but actually increases atrophic signaling within the fiber[40]. These results suggest that two prominent changes in aged muscle, increased susceptibility to contraction-induced injury and the lack of response to hypertrophic signaling, may be a result of disruption to the DGC and dislocation of NOS-I into the cytoplasm of fibers. Both of these changes would ultimately affect the ability of aged muscle to activate satellite cells for the purposes of muscle maintenance, growth and repair.

If the DGC is disrupted, and NOS-I is dislocated into the cytoplasm of the muscle fiber, stretch and exercise may not be able to activate satellite cells via NO production since this messenger molecule has a very short half-life and is only able to travel 150-300 μm during this time [41]. Although muscle fibers typically range in diameter from 10-

100 μm [1], the quantity of NO that reaches a satellite cell when NOS-I is dislocated may not be sufficient to activate satellite cells. In addition, the sensitivity of satellite cells to NO may be altered with age. Thus, it is important to determine if exogenous sources of NO that do not have to be converted to NO via NOS-I can rescue muscle from age-related atrophy. If satellite cell sensitivity is not changed with age, an exogenous source of NO would have the ability to activate satellite cells since the displacement of the enzyme would be irrelevant.

Despite the fact that age-related changes in skeletal muscle have been well documented, the progression and sequence of these changes are not well understood. To develop effective, non-invasive treatments through exercise and NO supplementation, a preventative approach may be more effective than reactive intervention. In order to prevent sarcopenia (age-related loss of muscle mass), it is important to identify when structural and functional changes in skeletal muscle begin to occur. Therefore, a component of this research has been dedicated to re-investigating the effects of age on muscle structure and function in normal female mice from a young age to a period when the animals are considered senescent.

Evidence suggests that the age-related structural and functional changes that occur in muscle may be a result of a diminished ability to respond to hypertrophic stimuli [42]. Absent or discontinuous DGC and dislocated NOS-I may be responsible for the loss of mechanically-induced satellite cell activation in aged muscle. Therefore, the purpose of this research is: (1) to determine the progression of age-related changes in muscle structure and function, (2) to investigate the effects of stretch and NO supplementation/inhibition on satellite cell activation in aged muscle that demonstrates a

reduced sensitivity to mechanical stimuli and, (3) to determine if voluntary exercise can rescue different muscles from the structural and functional changes that occur with age via increased satellite cell activation.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Skeletal Muscle

Skeletal muscle is a vital and dynamic tissue both morphologically and functionally. The "heart" of any striated muscle is the muscle cell which is a multinucleated syncitium called a fiber. Morphologically, muscle tissue is constantly undergoing processes responsible for growth, degeneration, maintenance and repair, depending on the imposed requirements of the tissue and the state of the environment in which it is exists. Functionally, skeletal muscle is responsible for movement of the body by producing tension on bones to move, stabilize or neutralize the articulations of osseous tissue that forms the framework of the skeleton. In addition, muscle helps to regulate body temperature and provides protection for other systems of the body (i.e. skeletal, digestive system).

2.1a Structure

Here, structure will refer to gross organization, then it will be discussed from "outside – in" or from the extracellular matrix and connective tissue to the complex and intricate features of the structurally dynamic contractile component of muscle known as the sarcomere. The architecture of skeletal muscle, which was well reviewed by MacIntosh and colleagues, is responsible not only for the appearance, at a macro- and microscopic level, but also the dynamic function of the contractile tissue [43].

Superficially, the entire muscle is covered by a dense connective tissue known as the epimysium. The epimysium not only compartmentalizes individual muscles but also pierces the muscle belly to further section the contractile tissue by surrounding bundles of

muscle fibers with connective tissue known as the perimysium. The perimysium is thinner than the epimysium but is still a strong and dense connective tissue since it serves as a pathway for vessels and nerves to penetrate the central aspect of the muscle belly. Arterioles, venules, and intramuscular nerve branches are located underneath the perimysium in a mesh-like network of collagen fibrils, some of which connect to the delicate connective tissue surrounding individual muscle fibers known as endomysium. The endomysium is a delicate and dense layer of connective tissue that connects, in some capacity, to the basement membrane of the muscle fiber.

The basement membrane is an extracellular component of muscle tissue surrounding each individual muscle fiber. Within the basement membrane are two distinct structures: the basal lamina that links the basement membrane to the plasmalemma (also known as sarcolemma); and the reticular lamina, superficial to the basal lamina but deep to the endomysium [44]. The basal lamina is composed of two layers that can be identified with electron microscopy and are known as the (a) lamina densa and (b) lamina lucida. The lamina lucida lies underneath the lamina densa, but is superficial to the plasmalemma of the myofiber that envelopes the cell and ensures the chemical composition within this membrane is different from the extracellular environment [43]. These membranes are not only important for the structure of contractile tissue but also form a microenvironment or "niche" that contains muscle stem cells [45]. Muscle stem cells or satellite cells (SCs) were first identified by Mauro in 1961 and lie between the plasmalemma and basal lamina of the muscle fiber [2].

Myogenic satellite cells, which will be discussed later in more detail, are essential for normal muscle growth and repair from injury or disease [5]. Depending on animal

group, age, and/or muscle type, satellite cells only comprise 3-6% of all muscle nuclei whereas myonuclei make up the largest percentage [45]. Myonuclei lie within the syncitium of muscle, internal to the plasmalemma, and do not have the ability to divide. Conversely, satellite cells are external to the fiber membrane, are contained within in a specific cell membrane, and have the ability to activate, proliferate and differentiate given the appropriate stimuli.

Near the tendon of a muscle, the collagen fibrils of the reticular lamina connect to the collagen fibers within the muscle tendon. Collectively, these collagen fibrils transmit force from the contractile component of the muscle fiber to the tendon and, subsequently, the articulating bone [43]. Several proteins such as acetylcholinesterase, collagen, fibronectin, tenascin and agrin lie within, or are bound by the basement membrane layer, and are pivotal to achieving normal structure, contractile function and innervation of the muscle fiber. Collagen, or fibronectin and tenascin connect the basement membrane and the plasmalemma, respectively, to the collagen fibers of the endomysium. This ensures a mechanical link between the contractile component of the muscle cell and the connective tissue, and forms the ultimate link to the skeleton.

Deep to the basement membrane is the plasmalemma, and it is between these two layers that Mauro (1961) made the novel discovery of muscle satellite cells. As in all cells, the plasmalemma of the muscle cell is a lipid bilayer that surrounds the entire muscle fiber and separates the intracellular- from the extracellular-environment. The dynamic properties of the plasmalemma govern the transport of sugars, lipids, amino acids and ions across the membrane via several pumps, channels, and transporter proteins (e.g. for glucose) embedded within the lipid bilayer. Proteins that form ion channels

either cross one (intrinsic) or both (extrinsic) layers of the plasmalemma. Proteins pivotal for structure, and ultimately for mechanical force transfer from the cytoskeletal structures and plasmalemma to the basement membrane and endomysium are called integrins. In addition to integrins, two laminin receptors and the dystrophin/glycoprotein complex assist in linking the sarcomere to the basement membrane. The importance of the dystrophin-glycoprotein complex to the stability and effectiveness of force transfer in muscle is no more evident than in individuals with Duchenne Muscular Dystrophy (DMD). In DMD, the dystrophin-associated glycoprotein complex is deficient which leaves the muscle susceptible to extensive damage even when the simplest of contractions are performed (e.g. walking). The integrin proteins are bound to laminin receptors which are a component of a greater structure known as the costomere [46]. Costomeres link the Z-disk of the sarcomere, via γ-actin, desmin and vimentin, with the basal lamina through the integrin proteins [47].

Up to this point, the extracellular components of muscle tissue and proteins that traverse the plasmalemma of the muscle cell have been described. Several references have been made to proteins that form the link between the muscle cell cytoskeleton and the connective tissue that ultimately transfers force to the bones of a joint. The contractile component of the muscle fiber, or sarcomere, is the mechanical component of the muscle fiber and enables the tissue to shorten with tremendous force and/or velocity. Each muscle fiber is comprised of several to several thousand myofibrils that give the muscle fiber its striated appearance. Striations are a result of alternating dark (anistropic) and light (isotropic) filaments that correspond to myosin and actin, respectively.

Individual myofibrils contain many sarcomeres depending on the length, function and pennation of the muscle and fiber to which it belongs. Sarcomeres are composed of actin and myosin filaments. Actin and myosin filaments run longitudinally in a myofiber, and are bordered on each end of the sarcomere unit by a Z-disk that is orientated perpendicular to the filaments. Myosin filaments are located within the A-band of the sarcomere and are bisected by the M-region that maintains the appropriate 3-dimensional spacing between the thick filaments.

The longitudinal orientation of the myosin filaments is achieved by an extremely large protein called titin. Titin wraps around the myosin filament and passes through the I-band to connect to the Z-disk. Lateral to the A-band, in a longitudinal section, is the I-band comprised of actin filaments. Similar to the A-band, proteins midway along the I-band maintain the position and stability of the actin filaments. Nebulin, which has a similar role to that of titin with myosin filaments, contributes to the position and stability of actin filaments along with the intermediate filaments desmin, vimentin, symenin, and α-actin. The intermediate filaments connect the Z-disk to the filamentous cytoskeleton proteins actin, dystrophin, and spectrin, completing the mechanical link between the endomysium and basement membrane to the sarcomere. The H-zone of the sarcomere is the central region of the A-band and contains only myosin and the M-region. Immediately lateral to the H-zone is the section of the A-band where actin and myosin overlap, giving this region a darker appearance. Each myosin filament is surrounded by 6 actin filaments in a hexagonal arrangement. This hexagonal arrangement allows for the formation of cross-bridges between actin and myosin filaments. Each actin filament is surrounded by a rope-like molecule known as tropomyosin. Tropomyosin is a binding

site for troponin which contains three subunits (TnT, TnC, and TnL) responsible for the attraction of calcium ions and the promotion and/or inhibition of actin-myosin interaction [1].

Each myofibril is encased by a membrane meshwork called the sarcomplasmic reticulum (SR) that plays an important role in the calcium release-mediated contraction of skeletal muscle. At the junction of A- and I-bands, the sarcoplasmic reticulum abuts on both sides of the transverse tubule system to form a triad consisting of 2 terminal cisternae (expansion of SR) and a T-tubule.

The structure of skeletal muscle cannot be discussed without reference to the neuromuscular junction since voluntary contraction would not be possible without the connection between the nervous system and striated muscle. Myelinated motor nerves branch and lose the insulating myelin sheath at the terminal end of the axons as the axon approaches the muscle fibers and forms the neuromuscular junction (NMJ). The branched fiber at the terminal end of the axon forms the neuronal bouton that contains numerous mitochondria and acetylcholine (ACh) vesicles. Opposite the axon terminal and across the intervening cleft of the neuromuscular junction, the plasmalemma of the muscle fiber invaginates to form junctional folds of the motor end plate region that contain many ACh receptors and sodium (NA^{+}) channels. Excitation of the motoneuron is communicated to the muscle fiber at the NMJ via the release of ACh that travels across the NMJ cleft to bind to ACh receptors, depolarize the T-tubules and stimulate $Ca2^{+}$ release from the SR. This $Ca2^{+}$ induces myosin-actin interaction in a process that is described as excitation-contraction coupling (described below).

2.1b Function

Skeletal muscle has four distinct functions that include contractility, excitability, extensibility and elasticity [48]. Contraction is the most evident functional characteristic of muscle as it causes movement, stabilization and neutralization of the skeletal system. For example, when performing a bicep curl the biceps brachii muscle produces "movement" by pulling the forearm closer to the upper arm; the rhomboid muscles contract to "stabilize" the scapula against the body wall; and the pronator teres is activated to prevent supination of the forearm by "neutralizing" the position of the radius. The excitability of skeletal muscle allows for the voluntary control of muscle contraction via the nervous system. Passive structures that form the framework and maintain the architecture of sarcomeres contribute to the extensibility of muscle tissue along with the connective tissue that invests and invades the muscle belly. The series and parallel elastic components are extensile features of muscle derived from its tendinous and membranous structures, respectively [49]. Elasticity, also derived from the series and elastic components, is the property of muscle that returns the tissue to original length after being extended beyond resting length. Titin is the third most abundant protein in skeletal muscle, after actin and myosin, and is an integral part of the series elastic component, contributing to the extensible and elastic characteristics of muscle [50].

The chemical and mechanical processes of muscle contraction will be described from the axon of the motoneuron to the tendinous attachment on bone. An action potential travels down the axon from the cell body to the motor end plate causing release of the acetylcholine-filled vesicles from the boutons and into the synaptic cleft. Acetylcholine binds to the acetylcholine receptors, located in the plasmalemma of the

junctional folds, which increases the permeability of the membrane and leads to an influx of Na^+ ions. The depolarization travels deep into the muscle via the transverse tubular system. The depolarization signal is passed to the sarcoplasmic reticulum at the triad that causes a release of $Ca2^+$ from the sarcoplasmic reticulum and into the sarcoplasm of the muscle fiber. $Ca2^+$ binds to troponin on the actin filaments and exposes the active binding site for myosin. The myosin head binds to the actin filament via troponin, forming a cross bridge and causing a release of phosphate from the head of the myosin molecule. Energy stored in the head of the myosin molecule causes bending of the myosin head that pulls the cross bridge closer to the M-region of the sarcomere. Consequently, the Z-disks move closer together and narrow the diameter of the H-zone and the I-band since actin filaments slide towards the central portion of the contractile unit. As the Z-disks move closer together, the costameres, which attach to the Z-disk, place tension on the plasmalemma and basement membrane via the integrin molecules [47]. The basement membrane is subsequently attached to the collagen fibers of the endomysium, via reticular fibers, and extends to the perimysium and outwards to the epimysium [1]. Through the epi-, peri- and endomysium connective tissue layers, mechanical force is transferred from the sarcomere to the muscle tendon and ultimately, the bone targeted for intended movement.

The ability of muscle to cause movement is determined by the resultant vector from the sum of all muscle fiber actions and total resistance forces in 3 dimensions, including inertia. Torque is the product of force and the perpendicular distance from the axis of rotation to the line of action of the force (i.e. muscle or resistance). The musculoskeletal system in mammals is actually inefficient in utilizing the force produced

by a muscle; however the design maximizes the range of motion based on the mechanical advantage of the muscle-bone system [49]. For example, the elbow joint is classified as a 3rd class lever because the mechanical advantage (moment arm of the force divided by the moment arm of the resistance) is less than one, since the length of the force arm (from the axis of rotation to the insertion of the muscle) is shorter than the length of the moment arm of the resistance (from the axis of rotation to center of gravity of the forearm and hand). Therefore, the force of the muscle must be much greater than the force of the resistance (greater than the magnitude of the difference between the lengths of the moment arm) in order to achieve movement. The smaller the discrepancy in the length of the moment arms, the less force is required to overcome the resistance to movement. Conversely, the musculoskeletal system is designed to maximize the range of motion at a joint since minimal linear displacement of the insertion of the muscle results in a large range of motion at the distal aspect of the limb. The magnitude of the difference between the length of the insertion of the muscle to the axis of rotation, and the length of the point of interest to the axis of rotation, is the multiplier for the difference in linear displacement of the two points.

As mentioned above, the torque of a muscle is dependent on the length of the moment arm of the force and the magnitude of the muscle force. Several variables influence the amount of force or tension that a muscle can produce including, but not limited to, the size and cross-sectional area of muscle; muscle architecture; fiber type; length of muscle; type of contraction; velocity of contraction; muscle endurance, and the number of motor units recruited. The general features of muscle fiber type and type of muscle contraction, and how each contributes to muscle torque will be discussed briefly

here. A more detailed review of fiber type and type of contraction will be presented later since these two variables are particularly important in relation to age and muscle damage, respectively.

The size (length) and cross-sectional area (diameter) of a muscle are related to the anatomical location and proposed function of that muscle. Smaller muscles used for fine motor skills and tasks requiring less strength, such as the first lumbrical muscle, contain only about 10 muscle fibers [51]. By contrast, the human medial gastrocnemius muscle has, on average, over 1,000,000 fibers [52]. Considering all muscles, as the number of fibers in a muscle increases the ratio of muscle fiber per motor unit increases. In other words, contractile tissue that has hundreds of thousands of muscle fibers is intended for large powerful movements and one motor unit can supply over a thousand fibers. By contrast, the external rectus muscle of the eye may contain only nine fibers for every motor unit. [52].

There is a general consensus in the literature that the cross-sectional area (CSA) of a muscle fiber is positively correlated with peak force (P0) production [53-55]. In fact, a review by Malisoux illustrated that the correlation is so strong that training programs that are designed to promote muscle hypertrophy do not demonstrate a change in CSA/P0 since both variables increase proportionately [56-61]. Only two studies demonstrated a disproportionate increase and decrease in the ratio of fiber CSA to peak force, but these studies included bodybuilders on steroids and recreational runners training for a marathon, respectively. Steroid supplementation accounted for the disproportionate increase in fiber size whereas marathon training lead to a decrease in fiber CSA in relation to P0 [58, 62].

The orientation of muscle fibers in relation to the tendon establishes the architecture of the contractile tissue and determines the amount of force each sarcomere contributes to the tension placed on the bone via the tendon. This orientation of muscle fibers constitutes the pennation of the muscle. Two general categories of pennation exist; pennate- and parallel-fiber arrangement [63].

Fibers within parallel-fiber arrangement muscles are aligned parallel to the tendon; therefore, the force vector of each fiber is directed along the tendon and the majority of force produced is transferred to the tendon and ultimately, the bone. The parallel-fiber structure allows large proportional shortening of the muscle and, therefore, a very large range of motion.

Muscles with a pennate-fiber arrangement have the fibers aligned at an angle to the tendon that results in forces transmitted along the longitudinal and transverse axis of the tendon. Using vector analysis one can observe that the greater the angle of pennation the less effectively force is transferred to the tendon-bone structure [49]. In a positive manner, a pennate-fiber arrangement allows for a greater number of fibers per unit volume of muscle compared to parallel-fiber arrangement. This increases force production and stability at the expense of range of motion. For example, the resultant force of a muscle fiber pulling at an angle of 45° to the tendon is comprised of a vertical (along the length of the tendon) and horizontal force vector (perpendicular to the tendon). Because the angle of pull is 45°, the two force vectors have equal magnitude and approximately 70% of the resultant force is directed in both the vertical- and horizontal-direction (figure 2.1). Similarly, about 30% of the change in length of the muscle fiber will not contribute to the vertical displacement of the tendon due to the angle of muscle

pennation. The pennate-fiber arrangement of a muscle, therefore, allows for greater force production by that muscle since more fibers connect on a tendon, albeit with a compromise in the resultant force produced by each fiber. The compromise, however, means there is less range of motion since the proportional shortening is lower than in muscles with a parallel fiber arrangement where there are fewer fibers per volume of muscle.

Muscle fiber type determines the force, fatigability and twitch speed of a muscle fiber. Various muscle fiber-type classifications will be described later in greater detail. A type I fiber has slow contraction velocity, produces small forces, and is resistant to fatigue. Type IIA fibers have a faster contraction speed, intermediate force production, and relatively low fatigability. Type IIB fibers are characterized by a fast contraction speed but produce more force and have high fatigability compared to type IIA fibers. Johnson and colleagues demonstrated that the majority of muscles in humans are heterogeneous and are composed of type I- and type II-fibers [64]. Two exceptions to this generalization are the soleus- and obicularis oris-muscle that consist mainly of type I and type II fibers, respectively. Conversely, fiber type percentages in wild type mice are more homogeneous than humans and have a much lower percentage of type I fibers [65].

The next variable that affects the force of a muscle contraction is the type of contraction that occurs. Concentric contraction is activation of a muscle that causes the shortening of muscle fibers and ultimately the entire muscle. Hill was the first to propose the force-velocity relationship of concentric contraction which suggested there is a negative correlation between the force of a concentric contraction and the velocity of the shortening of the muscle. When the maximum amount of force a muscle can produce

equals the force of the resistance, the system is in equilibrium and no movement occurs. This type of contraction is an isometric contraction and defines the transition point between concentric and eccentric contraction. An eccentric contraction is lengthening of a muscle during contraction and occurs when the torque of the resistance at a joint is greater than the torque of the muscle contraction acting at the same joint. As the magnitude of the eccentric contraction increases, the velocity at which the muscle contracts also increases. The combination of increasing muscle force and increasing velocity often leads to muscle damage since fewer cross-bridges are engaged at any one time and cause sarcomeres to become weaker and more easily disrupted [66].

Velocity of Contraction – The velocity of a contraction can either be correlated with the type of contraction or can be independent. For instance, concentric contractions with a low resistance can be performed at a high velocity; they can also be executed slowly. Similarly across all types of muscle (i.e. cardiac, smooth and skeletal), in a concentric contraction the greatest force can be produced at a velocity near zero and force declines as the velocity of the movement increases. However, the opposite holds true for eccentric contractions as the faster the movement, the greater the force produced. If the velocity of a contraction reaches zero, the contraction is considered isometric (iso = equal; uniform) since there is no change in muscle length.

Length-tension relationship – There are major differences between the force-tension relationship of a whole muscle and that of single-fiber units. Single fibers isolated from whole muscle can produce peak force at resting length. However, in whole muscle, maximum force is produced beyond resting length as the connective tissue components (i.e. series elastic component, parallel elastic component) become engaged in

producing maximum tension. For example, analogous to pulling a brick along a platform with an elastic, the extensibility of the tissue or elastic material must be maximized to eliminate the "slack" or laxity in the system evident at resting length. Once the laxity has been removed from the system, the majority of the applied force will be transferred from the elastic- to the resistance-components.

The characteristics of muscle described above not only affect the strength of the muscle, but also the power of the contraction. If strength is deemed as the amount of force produced, then power is defined as the rapidity by which the force can be generated [49, 67-69]. Peak power is produced at about 33% of maximum force and approximately one-third of peak velocity of the muscle [49]. Power is a requirement for elite athletic performance in most sports (hockey, football, sprinting, etc.). However power is also essential for activities of daily living (ADL). Although strength has been studied extensively in the literature, the subject of power training in older adults has received a lot of attention recently. Hazell and colleagues cited several studies that demonstrate a much stronger relationship between muscle power and ability to perform ADL compared to muscle strength and proficiency in executing ADL [69]. One of the major problems with strength training is that it does not necessarily result in an increased ability to perform ADL; whereas, power training increases the proficiency at which ADL can be performed. Resistance training may provide a false sense of security since individuals may be under the misconception that increased strength will reduce the risk of falls when in fact, power is the most critical variable when trying to right oneself from an unbalanced position. Increased disability in executing ADL is strongly and consistently correlated with decreased muscle power [70-72]. Fortunately, the benefits of power

training are two-fold since the majority of power training studies report an increase in strength and an improvement in most functional tests [69].

Although research continues to emerge on the subject of power training in older adults, additional studies with a high level of evidence are required since little is known about the most effective training protocol. Porter [73] reviewed literature and stated the overall benefits of power training occur at a high intensity (80% of one-repetition maximum); however, higher intensity training may also lead to a greater incidence of adverse events as reported by de Vos and colleagues [73, 74]. The American College of Sports Medicine (ACSM) recommends that the intensity of power training be executed at 40%-60% of one repetition maximum (1 RM) [75]. Two other factors need to be considered before determining the appropriate method of power training for older adults which include the following: muscle groups to be trained, and the effectiveness of the program at improving the ability to perform ADL (transfer validity). The transfer validity of the training program highlights the importance of the specificity of exercises not only for training but testing as well. Bean and colleagues had subjects wear a weighted vest (2% of BW) and execute exercises including, but not limited to, chair stands, toe raises, and seated triceps dips [76]. After 12 weeks of training subjects increased muscle strength, power, and most importantly, functional performance in ADL-specific tests [67]. It is important to understand that power training programs for older adults refer to high velocity movements during the concentric phase of an exercise and not the eccentric component of the activity. Although this type of power training has demonstrated significant improvement in ADL, results should not be extrapolated and

applied to reducing the risk of falls since the mechanism of maintaining balance often requires a powerful eccentric contraction to "right" oneself.

Additionally, power training has not only demonstrated an increase in balance, but an increase in muscle strength and endurance after 8- to 12-week programs. Muscle endurance and strength were positively correlated with intensity of training (80% 1RM), but this high intensity training did not improve balance. The greatest improvements in balance occurred in the low intensity group (20% 1RM) which suggested that velocity of training may be more closely associated with balance since the lowest intensity group had the highest movement velocity [77]. Caserotti and colleagues also used a power training protocol (75-80% 1RM) with two groups of older women aged to 60- and 80-years-of-age. After 12 weeks of training, 2 days per week, both age groups demonstrated significant improvements in maximal isometric strength, isometric explosive force characteristics and muscle power. Even at 80 years-of-age, a substantial positive response to heavy-resistance training eliminated baseline differences between age groups in explosive force characteristics and maximum voluntary contraction indices [78]. This was the first study to investigate the effects of explosive, heavy-resistance training on 80-year-old subjects and the results were very promising. Further investigation into optimal training protocols is warranted, but evidence suggests explosive, heavy-resistance training at 75-80% 1RM provides the greatest benefit in older individuals. Power training has the potential to restore the power required to perform ADL and, ultimately, reduce the risk of falls.

2.1c Types of Contraction

The nature of muscle contraction influences the amount of force that is produced, the velocity of the contraction, the range of motion that is supported, and the susceptibility of muscle to damage. Classically, three types of contractions have been described: concentric, isometric and eccentric. Although these three terms have been used extensively in the literature and text books, Faulkner states that the terminology is incorrect (by over-simplification) and leads to misinterpretations [79]. All muscle contractions involve metabolic activation of the muscle and development of force, but not all contractions cause shortening of the muscle as stated in Taber's medical dictionary [79, 80]. Therefore, for the purposes of this paper and to adhere to the recommendations of Faulkner, the terms shortening and lengthening of muscle will replace the descriptions of concentric and eccentric contraction, respectively. The term isometric will continue to be used [79].

Shortening contraction describes the activation of muscle which causes the ends of a muscle to be brought closer together. For example, a shortening contraction of the rectus femoris muscle will rotate the tibial tuberosity closer to the anterior superior iliac spine and extend the knee joint while flexing the hip joint. Isometric contraction refers to activation of the muscle and generation of force without any change in length of the contractile tissue. If the quadriceps muscles are contracting against resistance and the net torque about the knee joint is zero, the length of the muscle will not change and the contraction is isometric. Lastly, a lengthening contraction involves the activation of a muscle and generation of force as the two ends of the muscle move further apart. For instance, when landing from a jump the quadriceps muscle contracts to decelerate the

center of gravity of the body and control the velocity of knee flexion. As the quadriceps muscle contracts, the knee flexes under load or torque and the tibial tuberosity rotates away from the anterior inferior iliac spine of the pelvis; this causes the quadriceps muscle to lengthen. Nearly all muscle strains and ruptures are a result of lengthening contractions that occur at a high rate of acceleration (positive or negative). The hamstrings muscle group has the highest incidence of strain and rupture in the human body [49]. The majority of hamstrings muscle strains occur during high-velocity movements such as kicking a soccer ball. During the force producing phase of the soccer kick the hip is being flexed and the knee extended at a tremendous angular velocity. After ball contact (i.e. critical instant), the hamstrings muscle group contracts with a significant amount of force to decelerate hip flexion and especially knee extension. As the muscles contract, the angular velocity of the lower leg causes the hamstrings to lengthen until knee extension is eventually terminated. Highly-skilled athletes are able to prevent co-contraction of the hamstrings muscle group until after critical instant which means the hamstrings muscle group is not recruited until the lower leg is rotating at an extremely high velocity. Thus, the risk of muscle strain or tear of the semitendinosus, semimembranosus, and biceps femoris muscles is increased. A similar mechanism of injury occurs in the gastrocnemius muscle during running or sprinting when the forefoot contacts the ground during foot strike. A lengthening contraction of the gastrocnemius muscle occurs to decelerate dorsiflexion of the ankle since the centre of mass of the body travels over the foot and forces the ankle into dorsiflexion.

2.1d Fiber Types

The dynamic, functional diversity of skeletal muscle is due, in part, to the various fiber type patterns in muscles of the body, including both homogeneous and heterogeneous fiber type distributions. Fiber type classification using many different methods has been discussed over the years, yet only a few methods have been generally accepted [81, 82]. The most established research method is to group fiber types according to myosin heavy chain (MHC) isoform content [81]. This method has only been accepted since the development of our knowledge of immune-based detection methods (since 1987), even though isoforms were well described prior to that time, from biochemical studies. For example, early immunostaining studies were corroborated by enzyme histochemistry [83]. MHC isoforms determine the velocity of contraction by influencing the rate at which cross bridges are formed and released [43, 84, 85].

Regardless of the methodology selected, it has been discovered that whole muscle contains either homogenous or heterogeneous fiber types. Staron and Pette have shown that there are at least four different MHC isoforms in adult mammalian muscle which include: slow type I (MHC Iβ), fast type IIA (MHCIIa), fast type IID (MHCIId), and fast type IIB (MHCIIb) [82]. It is important to note that fast type IID in humans does not contain MHCIId and should therefore be referred to as fast type IIX [43, 86]. In addition to these four homogenous types of fibers, Pette and Staron list several combinations of isoform co-expression that have been identified. These include type I/IIA or IC (MHCIβ>MHCIIa); type IIA/I or IIC (MHCIIa>MHCIβ); type IIAD (MHCIIa>MHCIId); type IIDA (MHCIId>MHCIIa); TypeIIDB (MHCIId>MHCIIb) and type IIBD (MHCIIb>MHCIId).

Several studies have found a strong correlation between MHC-based fiber type and contraction velocity. From slowest to fastest, these are: Type IA, Type IIA, Type IID and Type IIB [81]. The contractile velocity of a type of fiber is also strongly associated with the ratio of adenosine triphosphatase activity (ATPase), an enzyme that dissociates the phosphate bond in ATP, and with isometric tension generating capacity. ATPase activity is the greatest and fastest in Type IIB fibers and progressively declines in activity and kinetics in the same order of fiber type as in contraction velocity [81, 87]. This is consistent with the finding that the rate of ATP breakdown determines the velocity at which actin can slide past the cross-bridges formed by myosin heads [88].

As with the interaction of several other mammalian systems, the muscular system is closely associated with the nervous system. A number of studies have demonstrated the influence of the nervous system on the phenotype of muscle fibers. For example, both denervation and cross-reinnervation have the ability to change the original contractile phenotype [89-91]. When the innervation of a muscle was compromised, slow-twitch fibers become faster and fast-twitch fibers become slower. This relates to the concentration of MHCIa and MHCIIb which decreases in relation to the increase in concentration of MHCIIa and MHCIId myosins [92, 93]. Muscle phenotype can be altered by changing the innervation of a muscle from fast to slow or by applying an impulse pattern different from that of the native nerve. In either case, the change in innervation induces a systematic and sequential transformation of phenotype [94]. The nervous system is only one of many other systemic factors that can influence muscle phenotype; others include mechanical loading and unloading; hormones; disease and aging. It is important to recognize that these other systemic factors not only affect

skeletal muscle tissue independently, but also interact with one another to influence muscle phenotype.

Mechanical loading and unloading, as well as the type of training (resistance vs. endurance), has been shown to cause muscle fiber type transformation. Adams and colleagues demonstrated a decrease in type IIB (X) fibers and an increase in Type IIA fibers with 19 weeks of resistance training [95]. This data was in agreement with a study that investigated the effects of a 3-month sprint training program on fiber-type transformation in the human vastus lateralis muscle [96]. However, opposite findings were demonstrated in male elite soccer players exposed to a 3-month resistance training program in which the percentage of type IIA fibers decreased with strength training [97]. Although the effects of resistance training to transform fiber type proportions has demonstrated conflicting results, fiber type transformation associated with endurance training is much more consistent. Endurance training causes a decrease in the percentage of type IIB(X) fibers and a subsequent increase in either type IIA or type I fibers in both humans and rodents [98-102]. Therefore, evidence suggests that type IIB(X) fibers are driven toward a type IIA phenotype with endurance training and to a lesser extent, with resistance training. Muscle fiber phenotype is plastic and can be altered by training, but is also susceptible to transformation with detraining or unloading.

A study by Rusko (1992) demonstrated a decrease in the percentage of type I muscle fibers in retired skiers after the skiers had decreased the volume and intensity of training [103]. Similarly, the percentage of type I fibers significantly declined in endurance-trained athletes after a period of limb immobilization following knee surgery [104]. However, the percentage of type II fibers decreased in the atrophied vastus

medialis muscle of patients with end-stage knee osteoarthritis which may be a reflection of pain-induced limb immobilization. Evidence suggests fiber-type transformation does occur in human muscle with detraining, denervation, limb immobilization and space flight, but it is important not to generalize these findings since the direction of fiber-type transformation is dependent on several factors including: activity level, training status, age of the subject, muscle of interest and model of unloading.

Previous research has documented an age-related decrease in the size and number of motoneurons in cervical nerve roots and the lumbosacral cord [105, 106]. More important than the absolute number of motoneurons is the change in function of these nerves with age. Fortunately, up until the age of 60 years, the number of functional motor units remains relatively the same but a drastic decrease (up to 50%) occurs in the next decade and beyond [107]. To compensate for a decreased number of functional motor neurons, some motor neurons will develop additional axons to innervate denervated muscle fibers. When a motor neuron sprouts to innervate denervated fibers, those fibers adopt the contractile properties of the new nerve. This process leads to fiber type "grouping", and with age favors the adaptation and grouping of type II fibers to type I fibers. The theory of fiber type grouping with age is supported by the increase in the size of action potentials in older subjects. Larger action potentials suggest the diameter of the functioning motoneuron axons has also enlarged. The loss of motoneurons with age appears to parallel similar patterns in other neurons. In 1972, Johnson and Erner demonstrated a significant loss of neurons in the brains of very old mice [108]. Regardless of the location of motoneuron loss with age, the negative impact of this loss on the functional capacity of skeletal muscle is further exacerbated by the slowing of

nerve pulses within nerves of older subjects. Nerve impulses of older adults are transmitted more slowly than in young individuals due to segmental demyelination, remyelination, decreased internodal length and loss of the motoneurons with the largest diameter [108].

Although the mechanism of age-related motoneuron loss is not fully understood, ciliary neurotrophic factor (CNTF) appears to play an important role in maintaining motoneuron function. In rats, age-related strength decrements are associated with decreased CNTF expression but resolved with the administration of CNTF [109].

Franssen and colleagues reviewed numerous studies that had investigated changes in structure and function associated with age-related muscle atrophy (See section 2.5e), as well as several other pathologies. Almost unanimously, data indicate type II fibers atrophy to a greater extent than type I fibers with increasing age [110]. Nine out of twelve studies (75%) that analyzed biopsies from the vastus lateralis muscle of humans showed atrophy in type II fibers whereas only 3 investigators indicated a reduction in the size of Type I fibers. In all three of these studies, atrophy of type I fibers was less than type II fibers. It is also interesting to note that very few of the studies (33%) that investigated the gastrocnemius and biceps brachii muscles demonstrated atrophy of either fiber type. However, the changes in fiber type distribution associated with aging are much less convincing. Some studies demonstrate an increase in the percentage of type I fibers and a decrease in the proportion of type II fibers. Other investigators did not demonstrate any difference in distribution between type I and type II fibers in age-related atrophy [110]. It is important to note that different muscles are affected to a greater extent with aging. For example, lower limb or weight-bearing muscles in older humans

demonstrate a greater decrease in muscle CSA and strength than upper limb muscles. This may account, to some extent, for conflicting evidence regarding alterations in fiber type distribution associated with sarcopenia. In addition to the effects of age-associated motoneuron changes on muscle, hormones significantly influence the structure, function, and phenotype of voluntary contractile tissue.

Several hormones have been implicated in shaping the growth, function and characteristics of skeletal muscle including, but not limited to, testosterone, insulin, human growth hormone, and mechano growth hormone. Testosterone has received significant attention over the last several years because of its muscle building and strengthening effects, especially in the illegal synthetic form. Despite all the research on testosterone, the mechanism by which testosterone stimulates muscle growth is not fully understood. Evidence supports the theory that this anabolic hormone promotes protein synthesis in muscle [111], since increased protein synthesis has been demonstrated in both young hypogonadal men [112] and older men after treatment with testosterone [113, 114].

Testosterone increases protein synthesis and muscle size in both type I and type II muscle fibers. Increased muscle size is due to an increase in the diameter of individual fibers (hypertrophy) and not in the number of fibers (hyperplasia). In 2002, Sinha-Hikim and colleagues studied human males from which biopsies of the vastus lateralis muscle were obtained. Results demonstrated that fiber cross-sectional area increased incrementally according to the dose of testosterone supplementation [115]. Increased fiber cross-sectional area was associated with a greater number of myonuclei and not an increase in the absolute number of type I and type II fibers. These data suggest hypertrophy was a

result of satellite cell activation and the fusion of satellite cells to existing myofibers. The same study showed there was no change in the proportion of type I and type II fibers after testosterone supplementation; this observation supports the hypothesis that testosterone has a growth-stimulating effect on both types of muscle fibers [111]. However, testosterone levels decrease with age and depending on the magnitude of the decrease, the quantity of this anabolic hormone may not be sufficient to support muscle growth.

Although there was previously a controversy as to whether testosterone levels decreased with age, there is now significant evidence from longitudinal and cross-sectional studies to conclude that testosterone does decrease with aging, regardless of confounding factors such as exercise levels, illnesses, diet, etc [116]. The Massachusetts Male Aging Study reported testosterone deficiency in at least 4% of men 40- to 70-years-old. Insufficient androgen hormone levels were also associated with elevated luteinizing hormone levels which have been correlated with testicular dysfunction. Testosterone levels in males are not only lower in older men but decline progressively beginning in the third decade of life. Research indicates 40-90% of men beyond 80 years-of-age have serum testosterone levels below normal values for young males [111]. Perry and colleagues demonstrated that below normal anabolic hormone levels resulted in decreased muscle mass and quadriceps strength in African-American males between 70 and 102 years-of-age [117].

2.1e Fiber type transformation with loading and unloading

The plasticity of skeletal muscle and its ability to respond to the functional demands imposed on it has been well documented [118]. Muscle tissue not only

hypertrophies and atrophies in response to loading and unloading, respectively, but has also demonstrated transformation of fiber type proportions depending on the muscle and the functional demands placed on it. Sugiura and colleagues reported a 36% decrease in soleus muscle mass of young Wistar rats. Subsequent reloading of the hindlimb for 10 days, after a period of unloading for 10 days, resulted in a return of muscle mass to pre-unloading values [119]. Reloading also resulted in a decrease of type IId/x MHC isoforms, which is consistent with previous findings that unloading of the soleus muscle results in a shift in phenotype from type I to type II fibers [120-123]. Decreased muscle volume (32%) and percentage of type I fibers (19%) were demonstrated in humans after 84 days of bed rest; this was accompanied by an increase in MHC IIa/IIx and the total number of hybrid muscle fibers (expressing two MHC isoforms together) [124]. An increased percentage of hybrid fibers provide strong evidence of fiber transformation. Conversely, the group of subjects exposed to bed rest plus exercise did not show a transformation of fiber type proportions despite decrements in strength and muscle volume. These data suggest that the threshold for preventing fiber type transformation is less than the intensity and volume of exercise required to counteract the deficits in strength and volume documented in predominately slow-twitch muscles. On the other hand, biopsies of the vastus lateralis muscle from the same experiment revealed that exercise maintained muscle size and strength but did not ameliorate fiber type transformation to a more hybrid phenotype [59]. A transformation in fiber type was also evident in the vastus lateralis of human subjects exposed to only 11 days of space flight since the decrease in the percentage of type I fibers was 2-8% compared to pre-flight [125].

Reloading hindlimb muscles in Wistar rats after 14 days of hindlimb unloading reversed fiber type transformation. Initially, a decrease in the percentage of type I fibers (~22%) and an increase in type II fibers (~21%) were evident in the soleus muscle after 2 weeks of unloading. Interestingly, the percentage of type I fibers increased to control levels after only 1 week of reloading whereas it took an additional 2 weeks of reloading for the percentage of type II fibers to return to pre-unloading levels [126]. Therefore, restoration of fiber type back to the original proportion of type I and type II fibers can occur after reloading, but evidence suggests that the percentage of type I fibers is restored at a greater rate than type II fibers. It is also important to note that this immunostaining study did not specifically investigate hybrid fibers and previous studies suggest that the proportion of hybrid fibers would have also fluctuated in response to loading conditions. It is most likely that the shift in percentage of type I- and type II-fibers was a transformation to a hybrid fiber expressing both phenotypes rather than a shift from one homogenous fiber type to the other.

Skeletal muscle responses to loading and unloading in humans and rodents include alterations in muscle size, mass and volume; decreases or increases in muscle strength, function and fiber cross sectional area; as well as, fiber type transformation. The magnitude of change in fiber type is a reflection of: the type of unloading (space flight versus hind limb unloading), the size, function and location of the muscle (soleus versus vastus lateralis); and the original fiber type distribution of the muscle. Evidence suggests that exercise protocols designed to restore the structure, function and fiber type distribution must meet a specific threshold before change occurs, and that this threshold is muscle- and fiber type-specific.

2.1f Muscle Damage and Necrosis

Muscle damage can originate from either internal or external events. Internal causes of myofibril damage result in strain or tearing of the muscle belly or myotendinous junction following a forceful stretch or lengthening contraction. More specifically, damage to muscle fibers caused by unaccustomed lengthening contractions is known as delayed onset muscle soreness (DOMS) and is characterized by pain that peaks at 24- to 48-hours after exercise [43, 81, 108]. External causes of muscle damage include mechanical trauma from lacerations and high- or low-velocity blows, as well as, thermal trauma from excessive heat or cold. External traumatic events can cause muscle necrosis (or cell death) if the blood and/or nerve supply is compromised during the injury. Several diseases are also characterized by muscle necrosis which include, but are not limited to, alcoholism, polymyositis, dermatomyositis and muscular dystrophy [43]. Medical procedures such as the injection of a vasoconstricting anaesthetic (i.e. bupivacaine) [127] or sustained application of a tourniquet have also been shown to initiate necrosis in skeletal muscle [128].

Several models of muscle damage have been utilized to analyze the sequence of events which take place during muscle repair. Murine models of muscle damage include, but are not limited to, crush injury [129, 130]; denervation and devascularization [131]; toxin injection [132]; barium chloride injection [133]; lengthening contractions in situ [134], in vitro [135], and in vivo [136]. Damaged muscle is characterized by disruption of cytoskeleton proteins, sarcomeres and/or the plasmalemma [137]. Depending on the mechanism and severity of injury, damage can involve sarcomeres in parallel- and/or series-arrangement. Damage can be segmental (i.e. a few sarcomeres) or disrupt the

whole fiber. If all sarcomeres in a fiber are damaged the fiber becomes necrotic [138]. Carlson and Faulkner stated that regardless of the mode of injury, the mechanism of degeneration and regeneration appears to follow a similar mechanism [139]. However, each model shows distinct, subtle differences in the stages of degeneration and repair related especially to immune responses, timing, extent of damage, and proportion of repair versus scar formation. Recently, Parise and colleagues suggested that the type of cells recruited for muscle repair (satellite cells vs. $CD45^+$:$Sca\text{-}1^+$ cells) is dependent upon the model of muscle damage [140]. For instance, muscle damage via cardiotoxin injection completely obliterates the satellite cell population of the damaged muscle; therefore, $CD45^+$:$Sca\text{-}1^+$ cells are recruited for repair since satellite cells are absent. Conversely, exercise-induced muscle damage in muscle that had a viable satellite cell population utilized satellite cells for the repair process and not $CD45^+$:$Sca\text{-}1^+$ cells. These results suggest that muscle satellite cells are the preferred population of cells for muscle repair unless there is a requirement to use an alternate population of cells due to the absence of satellite cells.

2.1g Muscle Tissue Repair

One of the fascinating characteristics of skeletal muscle is the ability to regenerate, regardless of the mechanism of degeneration. Regeneration of striated muscle has been a topic of interest for many years [141]. Muscle cells are multinucleated with the nuclei positioned at the periphery of the muscle fiber. However, myonuclei (inside fibers) are post-mitotic and do not have the ability to divide and contribute to muscle regeneration. Therefore, muscle satellite cells are pivotal for repair and regeneration.

Satellite cells are sensitive to signals emanating from exercise and trauma which initiate proliferation, self-renewal and differentiation into myoblasts [142]. Similar to embryological myogenesis, myoblasts fuse with one another and begin to form new myotubes (nascent fibers). Myotubes synthesize proteins necessary for the construction of contractile components of muscle cells. The cells then begin to enlarge and mature, and take on the appearance of a muscle fiber [139].

Friden and Lieber characterized muscle damage as a process including disruption of the sarcolemma, fiber swelling, damage to the sarcotubular system, distortion of contractile proteins, damage to the cytoskeleton and abnormalities in the extracellular matrix [137]. Immediately following damage, fluid and then neutrophils enter the cell and recruit macrophages. These in turn produce free radicals and cytokines (Interleukin-1, Interleukin-6, and tumor necrosis factor) [143]. In cardiotoxin (snake venom) induced injury of the adult murine gastrocnemius muscle, proliferating satellite cells and inflammatory cells are evident within 2 days of injury. Only 3 days later, characteristic central-nucleated, regenerated fibers appear and contribute to striated muscle repair that is nearly complete ten days post-injury [144, 145]. Although the role of macrophages in the repair process is not completely understood, dissolution of damaged proteins is mandatory for successful regeneration [139]. As young myotubes begin to hypertrophy, their ends extend towards the damaged segments of the fiber [130], connect, fuse, and unite to form a regenerated muscle cell that is continuous with the remnant part of the fiber left after injury [43]. MacIntosh and colleagues state that the repair process is much more effective if the original endomysium around fibers is still intact. Disruption of the endomysium results in migration of satellite cells outside the old muscle cell location

which form new muscle cells in the suburb of the original fiber. Myogenic precursor cells (satellite cells) have the ability to repair damaged muscle fibers or to form new muscle fibers, depending on the extent of insult to the striated muscle.

Despite the ability of striated muscle to regenerate after internal or external damage, repetitive repair of muscle cells results in structural deformation and functional deficits. Repaired fibers appear as branched or split fibers and are more susceptible to subsequent damage due to concentrations of stress at the forked segment of the striated cell. Branched fibers have been observed in *mdx* muscle that has been denervated or devascularized [146], exposed to lengthening contractions [147], isolated from aged mice 26- to 84-weeks-of-age [148], or after injury in a muscle with low nitric oxide concentration [28]. Interestingly, branched or split fibers are not specific to *mdx* muscle and have been observed in normal muscle exposed to repeated trauma via crush injury, lengthening contractions and bupivacaine hydrochloride injection [149]. Of these three processes, Tamaki and Akatsuka demonstrated that bupivacaine hydrochloride injection resulted in the greatest increase in the number of branched fibers whereas lengthening contractions produced the least [149]. In addition to the morphological alterations with repeated trauma or disease, branched fibers are more susceptible to damage [150], and demonstrate a force deficit that is positively correlated with the prevalence of branched fibers [147].

2.1h Mechanisms of muscle hypertrophy

Muscle hypertrophy is defined as an increase in muscle mass, cross-sectional area (CSA) and quantity of protein within the muscle cell [151]. An increase in the CSA of an entire muscle is a result of individual fiber expansion and increased fiber CSA. Striated

contractile tissue is sensitive to the stresses placed upon it and may correspondingly hypertrophy, atrophy and maintain size by regulating protein synthesis and degradation. If protein synthesis and degradation are equal, the size of the muscle is maintained. However, if protein synthesis is greater than degradation, or vice versa, the muscle hypertrophies or atrophies, respectively. Individual muscle fibers contain hundreds of myonuclei, each of which is responsible for governing a certain volume of cytoplasm within the cell. The cytoplasmic volume of governance is known as the myonuclear domain. The myonuclear domain is the ratio of myonuclei/cytoplasm, a ratio that remains relatively constant despite the dynamic regulation of muscle size in adults [152-154]. However, during growth the myonuclear domain can lengthen [155]. As mentioned previously, voluntary contractile tissue is post–mitotic and the myonuclei located in the sub-sarcolemmal region of the muscle fiber do not have the ability to divide. In order to maintain the myonuclear domain ratio during fiber hypertrophy, activation of satellite cells is required to ensure that new nuclei are contributed to the native number of nuclei.

Activation of satellite cells via hypertrophic stimuli occurs in fast- and slow-twitch muscles of rodents, humans and birds [151]. Several researchers have demonstrated satellite cell activation in response to hypertrophic stimuli and it has been suggested that the muscle fiber has to reach a certain size before satellite cells are recruited [115, 156, 157]. Conversely, many investigators refute the suggestion that satellite cell activation is absolutely required for muscle hypertrophy to occur [158-162]. Bodine stated “while the role of satellite cells in muscle regeneration is well established and accepted, the role of satellite cells in hypertrophy of adult skeletal muscle is less clear

and widely debated…" [158]. To date, it has not been determined if satellite cell activation is mandatory for skeletal muscle hypertrophy and several issues need to be addressed before a definitive answer can be given. O'Connor and colleagues. listed the following issues: "the type of growth stimulus (i.e. hormonal vs. mechanical), the magnitude of the growth response, the age of the animal, potentially whether the animal is still in a more active growth phase, the species studied, and the elapsed time from a stimulus to sampling. [163].

One growth factor, in particular, responds to mechanical stimulus and plays an integral role in muscle growth. Mechano growth factor (MGF) is a muscle-specific splice-variant of insulin-like growth factor (IGF-I). In addition to naming the growth factor, Goldspink demonstrated that MGF increased satellite cell proliferation in response to mechanical stress, such as contraction-induced injury, in young and middle aged mice. Evidence also suggested that adequate circulating levels of insulin-like growth factor are required to support increased expression of MGF in response to a mechanical stimulus [164]. The effects of MGF on satellite cell proliferation in response to exercise highlight the complexity of the mechanisms necessary for muscle hypertrophy and the interplay of the various systems of the body. Therefore, several models of hypertrophy have been developed to study specific aspects of skeletal muscle growth and have been used in humans, rodents and birds. Hypertrophic stimuli used to activate satellite cells are synergistic ablation [165-167], testosterone [115], clenbuterol [168, 169], stretch overload [19, 152, 170], and exercise [169]. Several other models and mechanisms of muscle hypertrophy exist including growth factors, IGFs, anabolic steroids, hormones and the immune system [153].

2.1i Atrophy and models of muscle atrophy

Atrophy 'stems' from the Latin word atrophia which means "a wasting away" and is a descriptive generalization of what happens to skeletal muscle when atrophy occurs. Opposite to muscle hypertrophy, at least in the context of the measures traditionally used to quantify muscle loss or growth (i.e. CSA, muscle mass), atrophy is a loss of muscle mass and muscle fiber CSA [153]. Muscle atrophy is correlated with decreased function and can severely affect quality of life since loss of strength and power is more extensive in the weight-bearing limbs of humans. Adequate leg strength is required for several activities of daily living including walking, stair climbing and rising from a chair. Although muscle atrophy has been defined in simplistic terms and studied extensively, the mechanisms responsible for the loss of muscle tissue are poorly understood. In addition, the various models used to investigate muscle atrophy may have dissimilar characteristics and mechanisms of soft tissue demise. Several structural and functional changes in muscle are consequences of muscle atrophy, but effective treatments to restore muscle mass and quality are not relevant to every model of atrophy or every application to improve human performance.

On a macroscopic level, atrophy of contractile tissue is associated with a decrease in muscle mass and volume [171, 172], a decrease in muscle fiber CSA [173-176], an increase in interstitial fluid volume [177], and an accumulation of connective tissue [178]. A reduction in the number of muscle fibers is reported as possible [179] although most research does not support this latter finding [180-182]. Atrophy is muscle specific and affects each individual muscle to a varying degree depending upon the location, action, function, environment, fiber type proportion, innervation and/or pathology of the

muscle, and systemic influences. For example, in an immobilization model, atrophy affects type I fibers more severely than type II fibers [183] and has been associated with a shift in fiber type from type I- to type II-fibers [179, 184]. Conversely, detraining in power athletes is associated with a significant decline in the CSA of type II fibers [185]. Atrophy related to muscle unloading is usually greater in slow extensor muscles compared to fast extensors, but generally is more extensive in the most dominant fiber type of the specific muscle under investigation [186]. Therefore, the model of atrophy and the muscle(s) under study must be taken into consideration before conclusions can be drawn with regards to atrophy-induced changes in muscle size and fiber type transformation.

Microscopically, apart from changes in fiber type distribution and fiber CSA, several differences have been noted in atrophied muscle compared to normal muscle tissue including decreased capillary density, loss of sarcomeres , endothelial degradation, decrease in the number of mitochondria, and loss of myonuclei [153, 174, 178]. Until recently, it was generally accepted that muscle atrophy is associated with increased cell death (i.e. apoptosis) [187-190]. However, a recent study by Brusgaard and Gunderson used in-vivo time lapse microscopy and did not detect apoptotic loss of myonuclei, but rather death of both stromal and satellite cells[190]. Stromal cell nuclei were defined as being located outside the laminin ring. Based on these recent findings, it is possible that the death of stromal and satellite cells, as indicated by DNA fragmentation, was mistaken for apoptosis of myonuclei. Brusgaard and Gunderson suggest atrophy is due to an imbalance between protein synthesis and degradation rather than a process of programmed cell death[190]. This theory has been supported by previous research that

implicated reduced protein synthesis [153, 191], or increased protein degradation [188, 192] as a cause of decreased muscle mass. Regardless of whether morphological changes are identified macroscopically or microscopically, muscle atrophy results in decreased function.

Various models of atrophy have been used in animals to replicate muscle wasting in humans caused by aging, space flight, limb immobilization, denervation, injury, obesity, detraining, lack of hormone production, neuromuscular conditions and disease (i.e. HIV, alcoholism, cachexia, etc.). Another less practical, yet effective model of atrophy is hindlimb suspension [193, 194] in which rats are anesthetized and suspended from the side of the cage by both forelimbs and one hindlimb [195]. A few problems with the hindlimb suspension model are as follows: the animal being anesthetized, possible nerve damage to the suspended leg as recently demonstrated in a human hip arthroscopy study [196], and the fact that this model does not replicate any ethical human situation. In fact, some animal ethics boards may not approve this model of muscle atrophy. Therefore, caution must be taken when extrapolating such data to human conditions of muscle wasting. It is also important to realize that aging is only one condition of muscle atrophy and that the mechanisms responsible for age-related atrophy cannot be applied to every model of muscle wasting. However, it is clear that to reverse muscle atrophy or to minimize the progression of muscle wasting, the mechanisms responsible for the negative changes in muscle, both structurally and functionally, must be explored.

Loss of muscle mass is often due to a combination of factors described previously in addition to the intimate involvement of the nervous, skeletal, digestive, urinary,

endocrine, cardiovascular, respiratory, and immune systems. When studying the mechanisms, progression and treatment of muscle atrophy, it is not possible to investigate the implications of every system on this condition but it is imperative to understand the environment in which muscle exists has a significant impact on the events of muscle wasting and/or treatment.

2.2 Satellite cells

Stem cells have been the 'nucleus' of much research and controversy in the 21st century and the recent discovery in 2007 that pluripotent cells can be produced from human adult fibroblasts may prompt stem cell ethics to be revisited [197, 198]. Political and moral issues aside, stem cell therapies have been the focus of many investigators and funding agencies in all sub-disciplines of medicine. Of importance is the type of stem cell being investigated for potential as a treatment intervention, since not all stem cells are surrounded by controversy. Stem cells are characterized as totipotent, pluripotent, multipotent, or unipotent, depending on the differentiation potential of the cell [199]. Totipotent cells are capable of producing any type of cell whereas pluripotent stem cells have the capability of producing nearly, but not all types of cells. Finally, multipotent cells can only divide into a more restricted set of cell types similar to the cell of origin [199]. Unipotent cells can only divide into one cell type. Two criteria must be met in order for a cell to be classified as a stem cell: the cell must be capable of producing a large number of daughter cells, identical to itself, with stem cell character (self-renewal) [200] and can divide into precursors of mature cells that differentiate into structurally and functionally distinct cells [201]. According to these criteria, muscle satellite cells are stem cells. In addition, muscle satellite cells have demonstrated the capacity to

alternatively proliferate into non-myogenic mesenchymal cells and are considered multipotent [202]. Therefore, these multipotent stem cells require warrant further discussion here [203].

2.2a Location

Muscle stem cells were first discovered by Mauro in 1961, via electron microscopy, and were named 'satellite cell' based on the peripheral location of the cell between the sarcolemma and external lamina of the skeletal muscle fiber [2]. The near abutment of the satellite cell to the sarcolemma of the muscle fiber provides an optimal location for the myogenic stem cell to respond to mechanical and/or chemical stimuli elicited by the muscle fiber. In normal adult muscle, satellite cells are metabolically and mitotically quiescent on the periphery of the muscle fiber. However, exposed to the appropriate stimuli, satellite cells become activated and enter the cell cycle to contribute to the growth, repair and regeneration of skeletal muscle fibers [5]. Satellite cells are not equally distributed around the longitudinal axis of a fiber since the concentration of cells is greater around myonuclei, motoneurons, and capillaries [204]. De novo muscle formation has also been identified in the interstitial space of post-natal muscle fibers; however, satellite cells, as described here, do not contribute to this process [205]. Interstitial muscle fiber formation originates from myo-endothelial progenitor cells located outside the external lamina of the muscle fiber. To date, the relationship between satellite cells and myo-endothelial cells has not been determined [203]. Additional cell types have been implicated in the processes of muscle growth, maintenance and repair, but have not demonstrated a significant and/or consistent contribution to these processes

compared to muscle satellite cells. Therefore, for the purposes of this paper, muscle satellite cells will refer to those cells originally described by Mauro.

2.2b Satellite cell activation

Skeletal muscle satellite cell activation is pivotal for normal muscle growth and the repair of muscle damaged by injury or disease [5]. The term activation refers to entry of satellite cells into the cell cycle and mobilization from a quiescent state [28, 206]. Quiescent satellite cells are spindle-shaped, with the nucleus occupying a vast majority of the volume of the cell. Inactivated cells have very little cytoplasm, few organelles, and are void of myofilaments [206, 207]. Upon activation, the size of the nuclei and organelles increase, heterochromatin disperses and the volume of the satellite cell expands causing a change in size and shape of the cell [167, 206, 207]. Several markers have been utilized to quantify satellite cell activation including incorporation of tritiated thymidine or BrdU into new DNA [206, 208]; expression of proliferating cell nuclear antigen (PCNA) [209]; co-localization of c-met with hepatocyte growth factor (HGF) [23, 206]; and the expression of immediate early genes, c-fos and c-jun, within 3- to 6-hours after injury [210]. Our lab has demonstrated that nitric oxide (NO) not only activates satellite cells, but is also responsible for the maintenance of the quiescent state of these cells [5, 6]. A previous investigation by Tatsumi and colleagues demonstrated a rapid co-localization of HGF to c-met receptors (located on satellite cells) immediately following injury in vivo [23]. HGF is produced by both muscle and non-muscle tissues, and is stored in the extracellular matrix (ECM) around muscle fibers. There is strong evidence to suggest that the release of HGF from the ECM is dependent on NO. Recently, an intermediate step between nitric oxide release and mobilization of HGF was

identified both in culture and in vivo. Yamada and colleagues demonstrated that NO synthesis activates matrix metalloproteinase-2 (MMP-2) and the activated form of MMP-2 liberates HGF from the extracellular matrix [7, 21]. These results corroborate previous evidence that nitric oxide (NO) regulates the normal activation of satellite cells. A consistent pulsatile release of NO maintains satellite cell quiescence whereas a bolus release of NO, caused by stretching of individual muscle fibers, activates satellite cells from quiescence and initiates the downstream events necessary for myotube formation [20]. Original work by Bischoff identified that an extract of crushed muscle could activate satellite cells [211]. Crushed muscle extract was later identified by Tatsumi and colleagues to contain HGF and bFGF (basic fibroblast growth factor), although bFGF does not activate satellite cells from quiescence [23]. In addition to NO, HGF is the only growth factor that has demonstrated the ability to activate satellite cells in culture and in vivo [7].

2.2c Proliferation

Following activation of myogenic precursor cells by mechanical and chemical stimuli, satellite cells enter the proliferation stage [212, 213]. Two muscle specific regulatory genes, MyoD and myogenin, are expressed as early as 6 hrs after the onset of a stimulus and remain highly expressed up to 42 hours later [214]. Muscle differentiation can occur within 8 hrs, based on expression of myogenin, but most precursor cells do not divide until just prior to 24 hours after application of a stimulus [215, 216]. MyoD and myogenin are muscle specific proteins translated from the myogenic regulatory gene family (MRF) that regulates muscle differentiation. Although MRFs act in sequence there is significant overlap in the peaks of their individual expression during proliferation

and differentiation. Satellite cell derived myoblasts can express both MyoD and myogenin from cell division until formation of a myofiber, although co-expression is greatest during differentiation and fusion. As mentioned earlier, in order to be classified as a stem cell, a cell must be capable of self-renewal by dividing into two identical daughter cells. However, if all cells divided and differentiated the satellite cell pool size would become depleted after repeated bouts of injury, trauma, exercise, and/or stretch. Moss and Leblond proposed that satellite cells replenish the satellite cell population by stochastic or asymmetrical cell division that results in one daughter cell committed to differentiation while the other either continues to proliferate or returns to a quiescent state [217]. Several recent studies have demonstrated asymmetrical division of SCs and the avoidance of cell differentiation by proliferation or a return to quiescence [22, 218-224]. The capacity for self-renewal of satellite cells grants skeletal muscle tissue the ability to continuously regenerate after the presentation of repeated stimuli known to activate satellite cells. During muscle maintenance, growth or repair, satellite cell proliferation can be intensified with the addition of growth factors such as insulin-like growth factor 1 (IGF-1) [152, 225], mechano growth factor (MGF) [164], and fibroblast growth factor (FGF) [8, 226].

2.2d Differentiation.

As discussed previously, after the initial proliferative phase, daughter cells can proceed to differentiation or delay the process by continuing to proliferate or return to quiescence. Myoblast differentiation has been characterized by the expression of myogenin and MRF4 in both cell- and single-fiber-cultures [227-229]. However, myogenin is also expressed on the proliferation side of the proliferation-differentiation

phase in vivo and should not be used solely as a marker of differentiation [230]. Cells that differentiate were previously activated and withdrew from the GO phase of the cell cycle and entered the G1 phase to become myoblasts [208]. Myoblasts then either fuse to existing damaged fibers to elicit repair, become post-mitotic myocytes, or fuse to one another to form new myofibers. A primary fiber must be present for both events to occur [231]. Lagord and colleagues. demonstrated that satellite cells from various rat muscles (fast- vs. slow-twitch) have dissimilar or differential proliferation and activation patterns [232]. Satellite cells from the soleus muscle (slow-twitch) exhibited higher rates of satellite cell proliferation but less efficient myotube fusion compared to the EDL muscle (fast-twitch). The asynchronous or heterogeneous population of satellite cells considered, with respect to proliferation and differentiation, may be influenced by muscle function since the EDL muscle is an anterior, fast-twitch muscle of the lower leg whereas the slow-twitch soleus muscle is on the distal, posterior aspect of the lower leg. Similar to bone, muscle adapts and remodels to the loads placed upon it. This theoretical version of "Wolff's law" may be evident in the satellite cells of specific muscles and would explain why some satellite cells are more or less sensitive to external stimuli than others. MyoD and myogenin expression indicate that differentiation of muscle precursor cells is in progress and the muscle is regenerating or hypertrophying [214, 233-235]. MyoD, myogenin and another MRF, Myf5, are critical for myotube formation following the differentiation of muscle precursor cells and the assembly of myotubes [228, 236], as are other proteins such as N-CAM which are not specific to muscle. Similar to MyoD, Myf5 is expressed during satellite cell proliferation and overlaps with the period of myogenin expression during differentiation and muscle fusion [237, 238]. Grounds and McGeachie

discovered that satellite cell division in vivo peaks at 72 hours post-injury and declines to control levels 6 days after the original insult [212]. Due to the excessive overlap of MRFs from activation until myotube formation, and the expression of these genes over an extended period of time (6–72 hrs), it is likely that proliferation and differentiation occur simultaneously within a regenerating muscle [129]. Only examination of muscle on a cell by cell basis or fiber by fiber basis, using particular markers, can separate or identify the individual patterns of expression that govern the detailed pattern of differentiation.

2.2e Markers

The extensive amount of research committed to skeletal muscle satellite cells is no more evident than in the search for specific satellite cell markers. A wealth of research articles and reviews has been published on the subject and the list of satellite cell markers continues to expand. Satellite cell markers have been reviewed by several authors in the last couple of years, yet very few articles provide a comprehensive list of all the cell surface markers and transcription factors that can be used to identify satellite cells [4-6, 239-242]. The large quantity of satellite cell markers available for research and its interpretation is further complicated by evidence that indicates specific markers are expressed or repressed depending on the state of the satellite cell at the particular time, condition and model of the study. In addition, gene expression often extends beyond discrete stages of the cell cycle and is co-expressed with other genes at various stages of activation, proliferation, differentiation and self-renewal. Satellite cell markers can be divided into two categories, cell surface markers and transcription factors. Stages of the cell cycle are typically segregated into quiescence (G0), activation (G0 $\rightarrow$ G1) and proliferation (G1 $\rightarrow$ S $\rightarrow$ M). Due to the large number of markers and the three

stages of cycling, many different combinations of markers are used within the literature, which makes comparison of results difficult.

Cell surface markers that are expressed through all 3 cell cycles include: Pax7, c-met, Caveolin-1, CD34, CXCR4/SDF-1b, Integrin α7 and β1, M-cadherin, NCAM, Syndecans 3 and 4, and VCAM-1/VLA-4d. Transcription factors present during quiescence, activation and proliferation are: Foxk1, HoxC10, Myf5, MyoD, as well as the growth and differentiation factor, myostatin, from the family of transforming growth factors beta (TGF-β). Of these markers, the most commonly used are CD34, M-cadherin, Pax7, syndecan-3, syndecan-4 and c-met [240]. Pax7 may be the most universal marker since it is expressed in nearly all satellite cells and the antibody works well in the majority of laboratories [4, 238, 240, 242, 243]. However, Pax7 is downregulated during differentiation and thus, should be used in combination with other transcription factors, such as MyoD and Myf5 that are expressed in satellite cells during proliferation and differentiation.

C-met (also called mesenchymal epithelial transition factor) is a proto-oncogene protein on the cell surface of satellite cells and is a receptor for hepatocyte growth factor (HGF). When MMP-2 liberates HGF from its tethering in the ECM, HGF binds to the c-met receptor which activates satellite cells to enter the proliferative stage. C-met is expressed in both quiescent and activated satellite cells within the basement membrane and in the extracellular space [16, 23, 27, 236]. Expression of c-met is expressed in single cell myotubes and myocytes but is downregulated when they fuse into myotubes or fibers [9]. Wozniak stated that the inconsistent results associated with tracking c-met expression in skeletal muscle tissue are due to the breadth of protocols to which it has

been applied including: single-fiber cultures, dispersed-cell cultures, muscle sections, enzymatic digestion, migration assays, muscle injury and exercise, to name a few. Moreover, these protocols have included rodents with varying genetic backgrounds including wild type, transgenic and diseased strains [30].

Although several satellite cell markers have been discovered in the mouse model, very few have been identified in human muscle. Fortunately, some of the more elaborately used transcription factors (i.e. Pax7, Myf5, and MyoD) and cell surface markers (i.e. c-met, M-cadherin) have transferred well into research with muscle from human subjects.

Muscle maintenance, growth, repair and regeneration are multi-step, well orchestrated processes characterized by the expression/repression and binding/unbinding of transcription factors and cell surface markers, respectively. Prior to the selection of satellite cell markers for a study protocol, several factors must be considered to ensure the research is reliable and valid. These factors consist of, but are not limited to, animal model; isolation technique; experimental design, assay procedure, and ultimately, the hypothesis. For example, if the objective of the research is to determine the total number of satellite cells present on a single muscle fiber from an *mdx* mouse, the satellite cell marker can be less specific as opposed to a study designed to investigate the activation state of muscle satellite cells in humans. These markers are interesting from a historical perspective as only 30 years ago satellite cells were "presumptive" satellite cells. These cells were only stained for high RNA content activated in regenerating muscle due to the lack of knowledge about gene expression and undeveloped tools such as immunostaining.

The first quiescent satellite cell stained with antibodies was reported by Cornelison and Wold in 1997 [236].

2.2f Activation Stimuli

In 2005, Wozniak stated that satellite cells can be activated by injury, exercise, loading of skeletal segments of the body, stretch and denervation [6]. In addition to mechanical stimuli, chemical stimuli such as nitric oxide and hepatocyte growth factor (also in crushed muscle extract) have been implicated as powerful triggers for satellite cell activation. Satellite cells, therefore, can be activated by chemical and/or mechanical stimuli. However, chemical stimuli can activate muscle precursor cells without the presence of a mechanical stimulus, but satellite cell activation that results from mechanical stimulation is dependent on an intermediary step that involves chemical messengers.

Mechanical activation of satellite cells has been investigated extensively with several models of injury, stretch, mechanical overloading, exercise and denervation being used. Muscle injury protocols include muscle crush injury [28], neotoxin or cardiotoxin venom injection [140, 244]; and cryodamage [245]. Investigation of activation via stretch includes the use of satellite cell-derived muscle precursor cells [16, 18, 21], isolated single fibers [31], whole-muscle cultures (Anderson, unpublished), and in vivo studies [195]. Stretch protocols include passive stretch [31], and stretch during electrical stimulation which is designed to replicate lengthening (eccentric) contractions [246]. Overloading skeletal segments of the body to induce satellite cell activation can be achieved by chronic stretch [247], hindlimb- or tail-suspension [17, 40], elimination of agonist musculature [248, 249], or tenotomy [250, 251]. Several exercise protocols have

been introduced to induce satellite cell activation in rodents and humans. Rodent models consist of voluntary wheel running [252], level treadmill running [253], uphill treadmill running [254], and downhill treadmill running [140]. Investigation of satellite cell activation in humans has incorporated short- and long-term resistance training [12, 255, 256], acute (single) bouts of eccentric exercise [11], voluntary eccentric contractions, involuntary eccentric contractions with electrical stimulation, voluntary concentric contractions [11, 257], and endurance exercise [258, 259]. Denervation models proposed to increase satellite cell activation include excision [260-262], or crush of the sciatic nerve [146, 263, 264].

Protocols that have been designed to invoke mechanical activation of satellite cells in both rodents and humans have had varying degrees of success. Although all models purport to activate satellite cells, the mechanism of activation may differ. For instance, pathways of satellite cell recruitment for hypertrophy and repair of muscle following mechanical overload or crush injury, respectively, may follow a different process than non-physiological models of muscle damage (i.e. cardiotoxin- and neotoxin-injection) since venom injections completely obliterate muscle fibers and reduce satellite cell populations [140, 265].

Regardless of the mechanical stresses inflicted on voluntary contractile tissue, chemical stimuli (or messengers) act as transducers that respond to mechanical stimuli and in turn, activate satellite cells. If a mechanical stimulus is not significant enough to cause a chemical messenger to be released, satellite cells will not be activated. However, chemical signals can activate satellite cells without a mechanical stimulus. To date, the

only two chemical signals proven to activate satellite cells are nitric oxide and hepatocyte growth factor.

Mechanical stimuli, such as shear forces, stretch, exercise, loading and injury cause a bolus release of nitric oxide [28, 31, 80, 246, 253, 266-269]. Nitric oxide release, downstream from mechanical stimulation, has been confirmed in several models including satellite cell derived myoblast cultures, single fiber preparations, and in vivo, later followed by corroborative studies [28, 31]. Release of nitric oxide by skeletal muscle fibers, in response to crush injury or by mechanically stretched muscle satellite cells, was postulated to be the first chemical signal involved in the activation of satellite cells [16, 27]. Several investigators have demonstrated that immediately following stretch and release of nitric oxide, HGF is liberated from its attachment in the ECM [16, 18]. HGF then binds to its receptor, c-met, which is a proto-oncogene found on the surface of quiescent and activated satellite cells [8, 23, 236]. In addition, this sequence of events has also been demonstrated in vivo with the use of the hindlimb suspension model in rats [15, 17]. Very recently, Yamada and colleagues proposed an intermediary step between the release of nitric oxide and HGF. There is strong evidence to suggest that nitric oxide-activated MMP-2 liberates HGF from its tethering in the ECM which enables or allows the subsequent binding of this scatter factor to c-met [7, 21].

To date, HGF is the only growth factor with a demonstrable, integral role in the activation of satellite cells from quiescence in culture and in vivo [7-10, 80]. Despite the evidence that HGF has a positive effect on the activation and proliferation of satellite cells [8, 42], administration of HGF in culture inhibits myoblast differentiation by suppressing muscle specific protein expression [10]. Preventing or delaying the

differentiation of satellite cell-derived daughter cells amplifies the number of cells that can proliferate and enhances the ability of satellite cells to hypertrophy or repair muscle fibers.

2.2g Activation Pathways

A few pathways have been confirmed for satellite cell activation whereas alternative potential pathways have been proposed based on evidence from other tissues (See Section 2.2b).

The most well-studied and confirmed activation pathway for skeletal muscle satellite cells is through the binding of HGF to c-met [236, 270]. HGF is released from the extracellular matrix in response to a bolus release of nitric oxide from muscle fibers as a result of mechanical strain [16, 28]. The relationship between nitric oxide and HGF release was confirmed by experiments that blocked muscle-specific nitric oxide synthase (NOS-Iμ) function with N^{ω}-nitro-L-Arginine methyl ester (L-NAME), a nonspecific NOS inhibitor. When the production of nitric oxide was reduced with L-NAME treatment, satellite cell activation and the binding of HGF to c-met was prevented [28]. Our lab demonstrated a significant increase in satellite cell activation in whole muscle cultures of C57BL/6 EDL muscles from 8-month-old mice when a mechanical stimulus (passive stretch) was combined with isosorbide dinitrate (ISDN), an exogenous source of nitric oxide (Leiter and Anderson, unpublished data). Until recently, the mechanism by which nitric oxide stimulated the release of HGF from the extracellular matrix was unknown. Yamada and colleagues have implicated MMP-2 as a possible bridge between nitric oxide and HGF release [7]. Results of immunocytochemistry experiments of satellite cells in culture indicated less than a 1% difference between the percentage of cells

staining positive for Pax7 and MMP-2 (97.3 ± 1.12% and 98.0 ± 0.71%), respectively. Therefore, MMP-2 is definitely present in nearly every single satellite cell identified by Pax7 gene expression. Satellite cells were then cultured and stretched which resulted in a conversion of MMP-2 to its activated form. However, when L-NAME was added to the culture, MMP-2 was not converted to its active form. This NO-dependent conversion in response to stretch was confirmed by treating cells with an NO donor (NOC-7), which amplified the expression of MMP-2 and caused a release of HGF from its tethering in the extracellular matrix [15]. Several studies have demonstrated that the binding of liberated HGF to its c-met receptor on satellite cells results in satellite cell activation [16-18, 21]. Therefore, to date, the sequence of events for mechanical activation of satellite cells is as follows: mechanical passive stretch results in nitric oxide release, nitric oxide release converts MMP-2 to its activated form, the activated form of MMP-2 causes the release of HGF from its association to the extracellular matrix, HGF binds to its c-met receptor on the surface of the satellite cell. Binding of HGF triggers the rapid autophosporylation of c-met which initiates the transcription of genes specific for hypertrophy and proliferation of cells [271] by stimulating the mitogen-activated protein kinase (MAPK) and phosphatidyl inositol-3 kinase pathways [272]. Although HGF activates satellite cells, it is also inhibits myoblast differentiation by suppressing muscle specific protein expression [10]. Of the four MAPK families identified, the p38 MAPKs appear to be the most likely candidate to activate satellite cells in response to the binding of HGF to c-met. Jones and colleagues have demonstrated that p38χ/β becomes activated (pp38χ/β) and is localized to the nucleus of satellite cells prior to differentiation and MyoD induction. If p38χ/β is inhibited during the G1-phase, not G0-phase, of the cell cycle, proliferation and

differentiation of satellite cells does not occur. Interestingly, p38χ/β appears to be involved in the activation of satellite cells as pp38χ/β inhibition prevents the activation of satellite cells via external stimuli [273].

The sequence of events from the application of chemical and/or mechanical stimulus to satellite cell differentiation is a sensitive and complex process. To further complicate matters, NOS-I$^{-/-}$ knockout mice and *mdx* mice demonstrate muscle repair even in the absence of NOS-I, which suggests satellite cell activation has occurred [28]. Although activation of satellite cells is delayed in these mouse strains, the fact that satellite cells are activated at all, strongly suggests an alternative pathway as discussed by Wozniak and Anderson [6].

Once satellite cells are activated and enter the G1-phase of the cell cycle, signals are required to govern the progression from G1-to S-phase of the cycle. Notch signaling is an extremely important mediator of progenitor cell progression in skeletal muscle and ensures an efficient transition from G1- to S- phase [274, 275]. In young regenerating murine muscle, the Notch ligand Delta is expressed in both satellite cells and at the myofiber membrane near the region of muscle injury. However, when old muscle was damaged via freeze injury there was no upregulation of Delta in satellite cells and myofibers [274]. Consequently, the regeneration capacity of old muscle was decreased compared to young muscle, but was restored when forced expression of Notch was achieved [274]

2.2h Changes with aging

Skeletal muscle tissue, which is post-mitotic in adult mammals and humans, has the amazing ability to completely regenerate following injury. However, as age increases

the regenerative capacity of muscle declines. To date, it is not universally accepted that the number of satellite cells decreases with age. As discussed previously, the structural characteristics and functional requirements of different muscles within the same individual influence the type and extent of change associated with the aging process. For example, two studies performed on aged mice demonstrated a greater decline in the number of satellite cells associated with the extensor digitorum longus muscle as compared to the soleus muscle [22, 204]. On the other hand, in both studies, the number of satellite cells from the tibialis anterior muscle either increased with age, or showed very little or no difference; corroborating evidence from rat experiments [207]. There is also no evidence of an age-related decrease in satellite cell number of the rat levator ani muscle. These results suggest that the satellite cell number associated with muscles that maintain the same function and intensity of action throughout a lifetime (i.e. postural control, support of pelvic viscera), is preserved. However, growth and atrophy of the levator ani muscle is sensitive to gonadal hormone levels and must be considered when satellite cells are studied in this tissue [276]. Conversely, fast-twitch muscles in which the functional demand fluctuates to a greater extent than slow-twitch muscles throughout the life of an individual or species demonstrate a more pronounced and rapid decrease in satellite cell number with age [22, 204, 277]. This was confirmed by data from very old mice (senile) since EDL muscles demonstrated a major decline in satellite cell number by 12 months-of-age whereas the soleus muscle did not demonstrate a significant decline in satellite cell pool size until 28-33 months-of-age [22]. Unfortunately the study by Shefer and colleagues was from isolated fiber studies versus electron microscopy and satellite cells were activated during isolation. This study also did not include functional data

which would have identified the association between activity level and the rate of loss in the satellite cell pool size of slow-and fast-twitch muscles. The lack of agreement between studies is partly due to the very large variation in techniques and markers used to identify satellite cells, as well as the discrepancy in muscles selected for study among the different reports. For example, Shefer and colleagues supplemented basal growth medium with fibroblast growth factor 2 (FGF2) to determine the proliferation potential of satellite cells on fibers from the flexor digitorum brevis muscle of senile (28 to 30 months-of-age) mice [22]. Increased proliferation of satellite cells from aged muscle fibers in this report suggested that satellite cells were activated during the isolation procedure as only nitric oxide and hepatocyte growth factor are chemical signals proven to activate muscle precursor cells. In addition, the implications of a decreased satellite cell pool size are a subject of controversy. Brack and Rando suggested that even if the number of satellite cells decreases with age, the change in the quantity of precursor muscle cells would not be significant enough to deter muscle regeneration [203]. Conversely, Shefer and colleagues stated the decreased ability of a muscle fiber to activate satellite cells and the decline in the number of muscle precursor cells may contribute to the deficiency in muscle maintenance and repair with age [22]. Therefore, decline in satellite cell pool size and the effects of this change (if evident) warrant further investigation.

Our study is designed to address deficiencies in previous studies by investigating the effects of age on satellite cell activation in whole muscle cultures as well as in vivo. Proliferation of satellite cells in aged muscle is irrelevant if the preceding step of satellite cell activation cannot be achieved.

Collins and colleagues reported a significant decrease in the number of satellite cells per fiber in mice aged up to 30 months-of-age (2.4 ± 0.6%) compared to 1 – 2-month-old mice (4.6 ± 0.3%) [277]. In addition, almost 10% of aged muscle fibers did not have any $Pax7^+$ cells within the satellite cell position whereas every young muscle fiber had at least one satellite cell associated with it. Despite the decline in the absolute number of satellite cells, after 96 hours in culture, the maximum number of satellite cells surrounding fibers from old mice did not differ from young cultured fibers. The significant difference in the mean number of satellite cells reflected the absence of precursor cells on some fibers rather than a deficiency in the proliferative capacity of aged cells. These data are consistent with the finding from Shefer and colleagues that there was no difference in progeny clone size between young, adult, old and senile age groups, but the frequency of myofibers with no associated satellite cells was greater in the old and senile groups [22]. Further evidence to support the regenerative capacity of aged muscle was reported in an experiment by the same group in which aged single fibers from EDL muscle were transplanted into irradiated TA muscles from *mdx* nude mice. Even though the aged muscle fibers had fewer satellite cells per fiber, the extent and quality of muscle restoration was similar to results using young fibers [218]. These findings suggest that in addition to the possible decrease in satellite cell number and the delayed proliferative capacity of aged satellite cells, external cues from the environment play an integral role in satellite cell activation, proliferation and differentiation. This was demonstrated by earlier cross-transplantation studies, as discussed below.

Numerous studies demonstrate a 24-hour lag in the activation of satellite cells from aged muscle compared to young muscle [22-24], but this delayed response is likely

a result of the environment rather than intrinsic dysfunction of satellite cells. Shefer and colleagues demonstrated that FDB myofibers, from aged mice up to 33 months-of-age, in culture with fibroblast growth factor 2 (FGF2) proliferated and differentiated at the same magnitude and rate as young myofibers [22]. Environmental influences are also evident in heterochronic transplantation studies in rodents in which old muscles or myofibers are transplanted into a young host. When exposed to a youthful environment, transplanted tissue from an old host regenerated to a greater extent than if left in the old environment. Often the results could not be discerned from regeneration resulting from young fibers [80, 277, 278]. Further evidence to support the positive effects of a young environment on aged satellite cells are reflected in heterochronic parabiosis experiments, where the circulatory systems of one younger and one older mouse are united to form a single blood supply to a pair of mice connected side by side. Satellite cells resident in old mice are able to activate, proliferate and regenerate in a similar manner to young muscle, whereas young muscle precursor cells exposed to an aged environment demonstrate a decline in function [278]. Similarly, enhanced Wnt signaling, which leads to a fibrotic fate in aged satellite cells, is evident in young muscle, but decreased in aged muscle after an anastomosis of the parabiotic pair (between the young and old circulatory systems). Therefore, strong evidence exists to implicate changing environmental conditions as a major factor in the decline of satellite cell function with age. This refers to the environment of the muscle tissue including connective tissue, innervation, blood supply, and cellular and a-cellular components of the body that affect muscle.

Extensive changes take place with age in both the macro- (body systems) and micro- (niche) environment in which satellite cells exist. Age-associated changes in the

endocrine system include a decrease in mechno growth factor (MGF), the muscle-specific splice variant of insulin-like growth factor 1 (IGF-1) that is involved in the activation of satellite cells, in response to a mechanical stimulus [164]. In addition, circulating growth hormone levels in older humans are only one-third to that of young adults but a combination of growth hormone (GH) supplementation and exercise have increased MGF levels [164, 279]. Testosterone is a major growth-promoting stimulus in muscle of males and there is significant evidence to confirm its positive effect on protein synthesis, although the mechanisms responsible for increased protein synthesis are not fully understood [111]. In 2002, an experiment involving human males, demonstrated that fiber cross-sectional area increased incrementally with the dose of testosterone supplementation and hypertrophy was evident in both type-I and type-II muscle fibers [115]. Unfortunately, evidence from longitudinal and cross-sectional studies confirm a decrease in testosterone with age, regardless of exercise levels, illnesses, diet, etc. [111, 280]. Below-normal anabolic hormone levels lead to decreased fat-free (FFM) and strength [117]. Morphological investigation of aged muscle demonstrated an increase in fibrotic tissue surrounding muscle fibers [281]. This was a result of a positive correlation between age, Wnt signaling, and conversion of myogenic precursor cells to fibrotic cells [203]. An increase in the deposition of fibrotic cells surrounding muscle fibers may negatively influence the satellite cell niche and affect the communication between cells and motility of factors necessary for satellite cell activation [203].

The muscle satellite cell is central to the processes of muscle maintenance, hypertrophy and regeneration. This myogenic precursor cell is also at the mercy of the macro- and micro-environment in which it is housed. Several changes in both

environments have been proposed to occur in addition to inherent differences in satellite cells from young and old animals. Taken together, it is apparent that sarcopenia is a multifactoral process and in order to treat or prevent this condition, an extensive knowledge and understanding of all internal and external influences on the muscle satellite cell are required. Fortunately, specific stimulating factors, such as nitric oxide, provide strong potential for solving a piece of this very complex puzzle.

2.3 Nitric Oxide

Nitric Oxide (NO) is a colourless, poisonous gas and a diatomic free radical with one unpaired electron [282]. Despite its classification as a poisonous gas and an atmospheric pollutant, nitric oxide is produced by almost every organism and is present in exhaled breath of humans and other animals [80, 283-289]. Nitric oxide has numerous functions in many systems and tissues of the body including, but not limited to, regulation of vascular tone, mediation of thrombosis, pro-inflammatory role in response to pain stimulus, immune response, maintenance of gastrointestinal mucosa function, neuroendocrine and autoimmune homeostasis, and most relevant to the purposes of this paper, cellular proliferation [282, 289-294]. As a mediator and regulator of physiological homeostasis, the sensitivity of tissues and cells to nitric oxide is extremely high. For example, under physiological conditions, nitric oxide protects the bronchi of the lungs whereas excessive levels of nitric oxide induce inflammation and have been implicated in diseases such as asthma and chronic obstructive pulmonary disorder (COPD) [289]. Similar inflammatory effects resulting in disease have been identified from studies involving muscles of animal colitis models that suggest non-physiological levels of nitric oxide may play a role in the human condition of inflammatory bowel disease [292].

Although the mechanisms by which nitric oxide regulates several functions in the body are not yet fully understood, it is universally accepted that physiological levels of nitric oxide have a positive effect on tissues and too much or too little of the poisonous gas has deleterious effects. The problem is that normal or physiological levels of nitric oxide have not been identified for each tissue, which makes treatment with NO donors and inhibitors difficult.

2.3a Nitric Oxide Synthase

Nitric oxide synthases (NOSs) were first identified in 1989 and because its isoforms are found extensively throughout mammalian tissues, the amount of research on the topic is overwhelming. The nitric oxide synthase enzyme functions to convert L-Arginine (LA) to citrulline, nitric oxide (NO), and S-nitrothiols and peroxynitrite [32, 33]. Three different isoforms of NOS have been recognized and are very different in both structure and their impact on function. The three isoforms are neuronal NOS (also known as nNOS, Type I, NOS-I, NOS-1), inducible NOS (or iNOS, NOS-II, NOS-2) and endothelial NOS (synonymously Type III, NOS-III, NOS-3) [33]. Neuronal NOS is generally found in nerve tissue, endothelial NOS is prominent in vasculature whereas as inducible NOS is less specific and is prominent in a vast selection of cells following an immunological response [33, 34]. Although several authors have speculated that NOS-III; and NOS-I and II; from satellite cells and mdx fibers, respectively, may play a role in maintaining satellite cell quiescence, nitric oxide synthase-μ, a splice variant of NOS-I, is the main source of NO in skeletal muscle and will be the focus here [17, 266, 295, 296]. Previous reports in rats indicate NOS-Iμ is present in all skeletal muscles of mammals but is more highly expressed in muscle comprised primarily of type-II-

compared to type-I-fibers. However, a recent study on human muscles indicated there is no difference in NOS-Iμ protein expression between types I-, IIa- and IIx-fibers [297]. The age-related decrease in type II fiber CSA is greater than type I fibers, therefore, it is possible the greater expression of NOS-Iμ in type-II fibers, and its subsequent decrease in aged muscle, contributes to the more extensive decline of type II muscle fiber CSA. Neuronal nitric oxide synthase-μ (NOS-Iμ) is specifically found in skeletal muscle with only trivial levels identified in the heart of rodents [34]. In skeletal muscle, NOS-Iμ is associated with the sarcolemmal dystrophin-associated glycoprotein complex and functions as a regulator of muscle contractility since the formation of nitric oxide is consistent with depolarization of the muscle fiber during contraction [34]. Abnormal expression or dislocation of NOSIμ, evident in humans with Duchenne Muscular Dystrophy or in *mdx* mice, results in perturbed production of NO. Research has indicated that deficient levels of NO in skeletal muscle impairs structure, function and the ability to successfully regenerate [295, 298, 299]. In 2000, Anderson reported that repair of skeletal muscle tissue is initiated by a bolus release of NO to activate satellite cells and begin the regeneration process [28]. The intrinsic need for NO by muscle tissue to begin the repair process was recently confirmed in a study by Chen and colleagues in which the expression of NOS-Iu, in a sciatic nerve crush model, was demonstrated in both satellite cells and new myotubes [296]. Additionally, expression of eNOS and iNOS was evident in satellite cells and new myotubes, and may indicate NO is being produced by alternate sources to ensure satellite cells are activated [296]. Tatsumi and colleague also demonstrated increased expression of NOS-Iμ in satellite cells from 9-month-old rats in response to a stretch stimulus [16]. Despite the fact that all three isoforms of nitric oxide

synthases have been identified in skeletal muscle, NOS-Iμ is the main source of nitric oxide production and alteration in its expression may result in hypo- or hyperactivation of satellite cells as a consequence of non-physiological levels of NO.

2.3b NOS and stretch

In 2000, Anderson discovered that the activation and quiescence of satellite cells is regulated by NO [28]. Since the original discovery, Anderson and several other groups demonstrated that the release of NO is triggered by mechanical stimuli, such as stretch in cultured myoblasts, single myofibers, whole-muscle cultures, and in vivo [7, 17, 21, 27, 300, 301]. As previously explained, NO production is dependent on the expression of the nitric oxide synthases, in particular, NOS-Iμ. Therefore, if stretch results in an increase in NO production, NOS-Iμ is also implicated in the process.

Over 10 years ago, Reiser and colleagues reported that chronic electrical stimulation of rabbit TA and EDL muscles increased NOS-Iμ expression which supports evidence from the EDL, soleus and diaphragm muscles of rats [253, 302, 303]. In addition to electrical stimulation, other models of stretch have increased NOS-Iμ expression including passive stretch of whole muscle [268], single fibers (20% length increase) [270], satellite cells (25% stretch) [7, 16, 21]; cyclic loading (6.7% mean deformation) of cultured myotubes [268, 269]; and passive stretch in vivo using hindlimb suspension [17]. Regardless of the mechanism used to stretch or electrically stimulate striated muscle, muscle fibers, myotube or satellite cells, NOS-Iμ expression increases in response to mechanical stress. Therefore, evidence suggests that the bolus release of NO that results from mechanical stimuli is due, in part, to a greater rate of NO production from increased expression of NOS-Iμ. This also discounts the original theory that NOS-

Iμ was a constitutive enzyme. The role of NOS-Iμ is further confirmed by stretch experiments of single fibers, from the FDB muscles of C57BL/6 mice, in which inhibition of NOS with N^{ω}-nitro-L-Arginine methyl esther (L-NAME) significantly decreased satellite cell activation [31]. Perturbed expression of NOS-Iμ in aged muscle may decrease nitric oxide production in response to mechanical stimuli and contribute to decreased satellite cell activation. This could possibly be a factor in delayed satellite cell activation in aged muscle.

2.3c NOS and exercise

The effects of exercise and NOS-I μ expression have been investigated in both rodents and humans. Two studies involving rats demonstrated an increase in NOS-Iμ expression with exercise despite a vast difference in exercise protocols. Balon and Nadir implemented an intense treadmill program for a total of 8-weeks to induce increased expression of the muscle specific isoform of NOS-I [253]. After 2 days of treadmill acclimatization, Roberts and colleagues used an acute bout of exhaustive treadmill exercise to investigate the effects of exercise on NOS-Iμ expression [304]. Results from both the long term and acute exercise protocols demonstrated a significant increase of NOS-Iμ expression in the soleus and gastrocnemius muscles, respectively [253, 304].

Currently, a limited number of studies have investigated the effects of exercise on NOS-Iμ protein content in healthy human subjects [297, 305, 306]. Comparison between studies is difficult since the protocols differ extensively, but two out of the three studies mentioned above report an increase in NOS-Iμ activity with exercise. For instance, McConell and colleagues demonstrated greater NOS-Iμ protein levels in the vastus lateralis muscle of elite cyclists and triathletes versus sedentary controls. The study

reported similar differences after only 10 days of an intense cycling program, but levels of NOS-Iμ expression did not differ between fiber types before or after the exercise intervention [297]. In 2004, Rudnick and colleauges studied the effects of 90 days of bed rest (simulating space flight) on NOS-I protein content in the vastus lateralis and soleus muscles of healthy male subjects [305]. The participants were separated into a control and an exercise group; results from the exercise group were in agreement with the McConell laboratory for the vastus lateralis muscle but no difference in NOS-Iμ protein content was detected in the soleus muscle [297]. Conversely, 4 weeks of cycling did not increase NOS-Iμ protein content in the vastus lateralis muscle of healthy subjects or individuals with type 2 diabetes or prediabetes [306]. Although VO_2 peak and insulin sensitivity improved in both groups, the home exercise cycling program performed at 60% of VO_2 peak may not have been a sufficient enough resistance load to stimulate increases in NOS-Iμ protein content. The absence of change in NOS-Iμ protein content may indicate the cycling program did not surpass the theoretical threshold for satellite cell activation. It would have been of great interest to compare satellite cell activation in both groups, before and after exercise, to determine if the threshold for satellite cell activation had been exceeded. Although few studies have investigated the effects of training on NOS-Iμ expression, there is evidence from both humans and animals to indicate that intense exercise training results in greater levels of NOS-Iμ protein. It would be important in relation to understanding age-related sarcopenia to determine if well-designed activity protocols could be used to help prevent or combat age-related muscle atrophy.

2.4 Pharmacological Manipulation of Nitric Oxide

As mentioned previously, nitric oxide is a powerful activator of quiescent satellite cells and initiates the entrance of these precursor cells into the G1 phase of the cell cycle in response to exercise, stretch, trauma and denervation. Due to its role in the activation of muscle precursor cells and the potential therapeutic benefits of stimulating muscle growth and regeneration, the effects of pharmacological enhancement of nitric oxide availability in normal mice have been the focus of numerous investigations [252, 307-310]. Several studies have also inhibited the production of nitric oxide by blocking nitric oxide synthase activity to determine the significance of nitric oxide in satellite cell activation [7, 17, 27, 28, 299, 311], vasodilation [312], and mitochondrial respiration [313]. Although the importance of nitric oxide has been implicated in several processes of the body, the effects of nitric oxide on satellite cell function will be discussed in greater detail here.

2.4a Nitric Oxide Donors

Nitric oxide donors, relative to satellite cell activation, can come from either an endogenous or exogenous source. An endogenous source of nitric oxide indicates that internal production of nitric oxide must occur in order for the levels of nitric oxide to be increased. For example, L-Arginine (LA) is a nitric oxide synthase substrate and must be converted by nitric oxide synthase to produce citrulline and nitric oxide. Therefore, in order for LA treatments to be effective, NOS-Iμ or another NOS must be present. Conversely, nitric oxide donors that do not have to be converted to nitric oxide by NOS-Iμ, such as isosorbide dinitrate (ISDN), are known as exogenous sources of nitric oxide.

The structural and functional changes in skeletal muscle of mice resulting from LA administration have been investigated extensively. LA treatments have been used in single fiber cultures and in vivo experiments which have included C57BL/6, *mdx* and $NOS^{-/-}$ mouse strains [27, 252, 295, 314]. Several methods have been adopted to administer the NOS substrate in vivo including intraperitoneal (IP) injection [28, 314], osmotic pumps implanted under the skin [314], and oral treatment via drinking water [252]. The dose and method of administration is not a trivial matter, since results from single fiber experiments demonstrate a dose-dependent increase in satellite cell activation [28]. LA dispensed in a high dose through osmotic pumps decreased force deficits in single fibers to a greater extent than IP injection [314]. Overall, administration of LA has resulted in both structural and functional improvements since studies have demonstrated that the NOS substrate increased satellite cell activation [27], increased utrophin and γ sarcoglycan protein content [314], decreased eccentric contraction induced damage [314], enhanced neuronal NOS activity [295], improved muscle regeneration following crush injury [28], decreased exercise-induced muscle damage in pathological muscle tissue and increased distance travelled by *mdx* mice on a rodent wheel [252].

In addition to LA, other NO donors (exogenous donors) that do not have to be converted to nitric oxide by NOS-Iμ have been utilized to study the effects of NO on muscle. Most recently, Betters and colleagues used diethylenetriamine NONOate (DETA-NO) to ameliorate the detrimental consequences of glucocorticoid (GC) treatment by activating satellite cells on single fibers [307]. Additional NO donors which include S-Nitroso-N-acetylpenicillamine (SNAP) and sodium nitroprusside (SNP) have increased satellite cell activation [21], liberated HGF [21], and increased mitochondrial

content in culture [315]. IP injection of ISDN, often used to treat coronary heart disease, demonstrated increased satellite cell activation in muscle from *mdx* mice damaged with myotoxin injection [309].

Regardless of the NO donor, endogenous and exogenous sources of nitric oxide have provided excellent structural and functional benefits to muscles studied in culture and in vivo. In muscle where NOS-Iμ expression is perturbed, an exogenous source of nitric oxide would be the preferred treatment modality, since it does not require NOS-Iμ activity to supply nitric oxide to the muscle. The challenge that lies ahead is to determine physiological levels of nitric oxide that would provide therapeutic benefits to muscle tissue without negatively affecting other tissues and organs. Nitric oxide is involved in both the activation and quiescence of satellite cells and could potentially be used to effectively counteract muscle wasting related to age, disease, or disuse.

2.4b Nitric Oxide Synthase Inhibitors

The critical role nitric oxide plays in satellite cell activation and quiescence, as well as several other essential cellular processes has been further emphasized in studies investigating the effects of NOS inhibition. N^{ω}-nitro-L-Arginine methyl ester (L-NAME) has been used to determine the implications of preventing endogenous production of nitric oxide via the NOS-Iμ enzyme [4, 7, 17, 27, 28, 31, 311, 316, 317]. L-NAME is a non-isoform-specific inhibitor of NOS and has produced side effects including altered hemodynamics, contractility of muscle tissue, and expression of genes in tissues other than muscle [316]. An alternate, and more specific inhibitor of neuronal NOS is 1-(2-trifluoromethyl-phenyl)-imidazole (TRIM) and to date, no side-effects have been reported [318]. However, L-NAME has been used more extensively and has been

studied in C57BL/6, *mdx*, and $NOS^{-/-}$ mouse strains, as well as, Sprague-Dawley rats. L-NAME has been incorporated into experiments that have involved the use of satellite cells [7, 317] and single fibers in culture [27, 31], as well as in vivo models including crush injury [28], hindlimb suspension (passive stretch) [15, 17] and overload experiments [311, 316]. The main consequences of L-NAME administration are decreased nitric oxide activity [17, 316, 317], reduced NOS-Iμ, decreased satellite cell activation [15, 17, 27, 28, 31], reduction of myonuclei addition [28, 311] and inhibition of the conversion of MMP-2 to it activated form [7]. However, one study that involved the daily administration of L-NAME via drinking water did not prevent satellite cell activation and proliferation in an overload model of the plantaris muscle in rats [311]. Despite this conflicting result, there is strong evidence to support the inhibitory role of L-NAME as it relates to satellite cell activation via the NOS-Iμ pathway, and the use of L-NAME or NOS inhibition is fundamental to examining the role of nitric oxide. L-NAME treatment has also been used in combination with NO donors and has demonstrated that the effects of endogenous sources of NO (i.e. LA, sodium nitroprusside) are inhibited by blocking the NOS-Iμ enzyme [7, 27, 252, 295].

Pharmacological manipulation, either to increase or decrease the availability of nitric oxide in skeletal muscle, has emphasized the importance of nitric oxide in muscle maintenance, growth and repair. Administration of nitric oxide is an attractive potential treatment for several pathological conditions of skeletal muscle including muscular dystrophies, cachexia and sarcopenia. Further investigations of nitric oxide donors must be performed in other animals and humans to determine side effects, most effective method for administration, and physiologically-effective dose.

2.5 Functional Measures

Several measurements of functional performance in humans allow for assessment of strength, power, endurance and fatigue relating to activities of daily living or more sport-specific tasks. Functional measurements in rodents are more difficult since the animal must be trained or administered stimuli to perform a task, rather than receive verbal or visual instruction. A plethora of physical functional tests are available for rodents and include, but are not limited to the following: voluntary wheel running [252, 319] [320]; involuntary level- [321], uphill- [322, 323] and downhill-treadmill running [324]; swimming [325]; grip strength [319]; balance beam [326]; squats [327]; and rota-rod [326]. For the purposes of this paper, voluntary wheel running and grip strength will be discussed in further detail.

Mice are frequently used as an animal model to investigate the effects of exercise on various disease- or age-related processes [320]; however, the voluntary exercise model has been used more frequently in rats than in mice and results between the two are not always synonymous [65]. In fact, the lack of change in skeletal muscle fiber type with endurance exercise in mice suggests the contractile tissue of mice is less sensitive and adaptive than rat muscle [65, 328, 329]. From the other perspective, it suggests that mouse muscle has greater metabolic plasticity, in response to challenges, than rats. The mechanisms responsible for enhanced metabolic plasticity in mouse muscle, when compared to the contractile tissue of rats, have yet to be determined.

Functionally, mice and rats have very similar endurance capacity, in terms of distance, when exposed to voluntary wheel running or involuntary treadmill exercise [65]. Both voluntary wheel running and involuntary treadmill exercise are utilized to

increase the endurance capacity of mice but the characteristic results of each type of exercise are quite different and comparisons should be made with caution since few studies compare changes in murine skeletal muscle with treadmill versus voluntary wheel running [323]. Interestingly, effects of voluntary wheel running are more synonymous with interval training and treadmill exercise is more closely associated with marathon-like activities in humans [323, 330, 331]. Voluntary wheel running with resistance caused muscle growth and the extent of muscle hypertrophy may be enhanced by increasing intensity and volume of training [330]. Several studies have demonstrated the positive effects of both treadmill exercise and voluntary wheel running on satellite cell activation in rats and mice, often combining exercise with treatments such as estrogen, prednisilone, and L-Arginine [224, 252, 324, 332-334]. Although some mouse studies have investigated the association between nNOS levels in skeletal muscle and exercise, no such studies have been performed on aged mice [335-337].

2.5a Voluntary wheel running

Although treadmill running is commonly used in exercise protocols for rodents; due to the considerations of repeatability and control over training volume and intensity, involuntary treadmill running can produce a physiological stress response. Moraska and colleagues reported adrenal hypertrophy, thymic involution, decreased serum CBG, increased levels of lymphocyte nitrite and other physiological changes indicative of stress after invoked treadmill running [322]. Conversely, voluntary wheel running is a 'natural' activity for captive rodents and consists of short spurts of high velocity running [330, 331]. Rodents run at a greater velocity (42 meters/minute vs. 16 meters/minute) and travel a further distance (8.8 kilometres vs. 1.9 kilometres) when running voluntarily on a

wheel compared to involuntary treadmill exercise [323]. Long-term voluntary wheel running protocols in rats have resulted in increased soleus muscle mass up to 27 months-of-age and an increase in the CSA of the plantaris muscle at 10 months-of-age [338, 339]. A 24-hour voluntary wheel running intervention in *mdx* mice resulted in extensive fiber damage in the quadriceps-, gastrocnemius-, tibialis anterior- and diaphragm-muscles [252]. Voluntary wheel running was also used as a tool to measure functional performance in a study that demonstrated halofuginone treatment decreased muscle fiber damage and time to functional recovery following exercise by resolving fibrosis [319]. Several studies in mice have incorporated this more 'natural' running method to show earlier satellite cell activation following muscle transplantation [340], increased quantity of apoptotic nuclei [333], and elevated NOS-Iμ activity in skeletal muscle [336]. Voluntary wheel exercise has been used as both a functional performance measurement tool and a training apparatus in rodents. A 'natural' activity was one of the training protocols selected for the current study since the effects of aging and exercise on NOS-Iμ have not been investigated and it is important to eliminate as many confounding variables as possible in studying aged animals, more specifically, physiological stress responses. In addition, exercise on a voluntary wheel apparatus has demonstrated muscle damage (especially in *mdx* mice), increased satellite cell activation, and elevated NOS-Iμ activity, all of which are essential for this research.

2.5b Treadmill Running

The benefit to using treadmill running protocols for rodent training and functional testing is that the volume and intensity are under the control of the investigator. As mentioned above, treadmill training does elicit a physiological stress response but this

can be minimized with gradual increases in the volume and intensity of exercise [322]. An added benefit to treadmill exercise is the ability to change intensity by altering the angle of incline/decline of the running surface. It has been well established in both humans and rodents that downhill treadmill running induces extensive muscle fiber damage [80, 136, 341-343]. Most recently, Enns and Tidus adopted a downhill treadmill running protocol to induce muscle damage and confirm the positive influence of estrogen supplementation on the recruitment of satellite cells for muscle repair in ovariectomized female rats [324]. Similar eccentric downhill treadmill exercise protocols have been used by other groups to investigate satellite cell activation after acute muscle injury caused by lengthening contractions in mice and rats [334, 343-345]. To date, only one mouse study has used a downhill treadmill running protocol and the purpose of the study was to determine if exercise-induced NOS-Iμ levels in skeletal muscle are associated with mitochondrial biogenesis; the association of NOS-I and satellite cell activation was not assessed [335]. Downhill treadmill exercise protocols in rodents have been effective for inducing muscle damage and provide a useful method to investigate repair of muscle tissue in vivo, caused by a health enhancing activity such as exercise. Although muscle damage caused by exercise may not be as extensive as tissue disruption inflicted by cardiotoxin injection or crush injury, it is the most prominent type of muscle damage. Therefore, the more information acquired about this process, the greater the opportunity for resolving muscle wasting by the use of exercise protocols.

2.5c Grip Strength

Grip strength measurements in rodents are a popular and effective functional assessment tool to determine the significance of nerve lesions, sedative drugs, age,

steroid administration, halofuginone treatment, NO-donor treatment and neuromuscular disease on function, and more specifically forelimb muscle force production [3, 252, 319, 346-348]. Several variations of grip strength testing equipment exist which include the following: mesh grid attached to a balance [349, 350]; lateralized grip strength meter that allows for simultaneous force assessment of each limb [348]; and a trapeze bar attached to a strain gauge [3, 252, 319]. Despite the variation in equipment used, the protocol for testing is generally similar. Rodents are gently grabbed by the tail, as close to the body as possible, and lowered toward either the mesh grid, ring or trapeze bar. As the animal grabs the trapeze bar the investigator gently pulls the rodent in the opposite direction until grip can no longer be maintained; peak force or the greatest amount of weight that was supported by the rodent is recorded. Grip strength testing is quick, non-invasive, well-documented in the literature, and can be repeated as often as necessary. However, in addition to several benefits of grip strength testing, the motivation of the animal can be an issue and contribute to large variation between repetitions [349, 351]. The magnitude of force produced and the motivation of the animal are also dependent on how hard and how quickly the investigator pulls on the tail of the rodent. Therefore, it is recommended that a single tester perform all the repetitions and be as consistent as possible to minimize measurement error [349]. Ingram and Reynolds reported that grip strength was a positive predictor of lifespan at 22 months-of-age; unfortunately, at 27 months-of-age testing was too inconsistent, possibly confounded by learning variations [351]. Even though equipment and methods differ slightly, our lab has extensive experience with grip strength testing using the trapeze bar attachment; thus, it was utilized for this research [3, 252, 319].

2.6 Changes in Skeletal Muscle with Aging

Taber's cyclopedic medical dictionary defines aging as "getting older" and states "there is no precise method for determining the rate or degree of aging" [80]. A recent article by Haylfick indicated that there is still no universal definition of aging which results in communication breakdowns and misinterpretation of research performed in the field of aging [352]. Further confounding the problem of aging in relation to skeletal muscle are the 12 other systems in the body which include the integumentory system, skeletal system, central nervous system, peripheral nervous system, autonomic nervous system, endocrine system, cardiovascular system, lymphatic system, respiratory system, digestive system, urinary system and reproductive system. The interaction between the muscular system and the other systems of the body is a bidirectional process in which each system has an influence on, and provides feedback, to the other. For example, muscle is the most abundant insulin-sensitive tissue in the body and accounts for up to 50% of body weight. At rest, it is responsible for 20 to 30% of oxygen consumption and 75 to 90% of glucose absorption [353]. The point is that it would be impossible to study the implication of every system on muscle tissue, but in the same context, the age-related changes that occur in other tissues of the body must be recognized when investigating skeletal muscle. A very brief summary of age-related alterations in the nervous, endocrine, skeletal, cardiovascular, urinary (table only) and immune system are described below to highlight the intricate and complex interplay between various tissues and systems of the body (table 2-1).

With age, several changes occur in the endocrine system that ultimately affects skeletal muscle. These age-related alterations include decreased levels of growth

hormone (GH) and mechano growth factor (MGF); reduced resting metabolic rate; increased insulin resistance and hyperglycemia; and decreased levels of testosterone. Lower levels of MGF and GH lead to a deficiency in the proliferation and fusion of satellite cells [164]. Increased insulin sensitivity and hyperglycemia disrupt the equilibrium between protein synthesis and degradation [354, 355] and may account, in part, for the 40% reduction in myosin heavy chain protein synthesis in aged individuals.

The extensive effects of testosterone in mammalian tissue have been summarized by Herbst and Bhasin and there is now substantial evidence from longitudinal and cross-sectional studies to conclude that testosterone production in males decreases with age [111, 356]. Although the mechanism by which testosterone stimulates muscle growth is not fully understood, age-associated decline in this anabolic hormone is associated with decreased muscle mass and strength [117]. Decreased satellite cell activation and fusion, and an imbalance in protein synthesis contribute to the lack of exercise-induced skeletal muscle repair and growth evident in older subjects. Goldspink has demonstrated that the combination of growth hormone supplementation and exercise resulted in increased MGF levels and could be one avenue by which treatment could stimulate satellite cell activation and muscle growth in aged individuals [164]. A review by Bhasin and colleagues documented the positive effects of testosterone supplementation on young hypogonadal men including increased fat-free mass (FFM) and increased fiber CSA of type I- and type II-fibers. However, testosterone supplementation in older men is a controversial issue since there is extensive public concern, although unproven, that this treatment could increase the incidence of prostate cancer and cardiovascular disease.

In 1917 D'arcy Thompson stated "Muscle and bone, for instance, are inseparably associated and connected, they are molded one with the another; they come into being together…" and over 80 years later a similar statement was made "Muscle and bone grow in proportion to one another, presumably because bone is biomechanically linked to muscle and bones adapt to muscle forces during development" [357]. In fact, it has been demonstrated that up to 80% of the variance in total bone mineral content can be explained by lean body mass and that muscle mass is the greatest predictor of bone mineral content [358]. With the exception of trauma, muscle produces the greatest strain on bone as it must overcome resistances to produce body movement [359], and thus contributes to the remodeling of bone [360, 361]. Similarly, muscle contraction performed to produce movement against the resistance of bone is required to maintain and increase muscle mass. Walsh demonstrated an increasing association between osteopenia, osteoporosis and muscle wasting with advancing age [362]. It is also interesting to note that the incidence of sarcopenia increases by almost 20% between the ages of 40- and 60-years-old and precedes the increasingly prevalent conditions of osteopenia and osteoporosis; this illustrates the interplay and feedback between the two systems and the need to maintain muscle mass and strength.

The influence of the cardiovascular system on muscle growth and development is extensive. In addition to the delivery of growth-stimulating hormones mentioned above, the cardiovascular system supplies the gases and nutrients required for muscle growth, development and function, as well as, removing the waste products of muscle activity such as hydrogen, potassium and lactate [108]. Blood flow is also a major determinant of protein turnover, as it regulates amino acid concentrations and influences the metabolism

and function of muscle. Anabolic hormones are influenced by blood flow as insulin, GH/IGF-I and protein turnover may be mediated by an increase in peripheral blood flow and nitric oxide production [363]. Blood flow in contracting muscle is reduced with age in both humans and animals [364]. Decreased blood flow may be a result of structural and functional changes that take place within the cardiovascular- and muscle-systems with age. Russel cites studies that indicate there is a decrease in capillary density and capillary-to-fiber ratio in aged muscle which is accompanied by a decrease in mitochondrial volume and oxidative function. Haidet documented similar findings in a study of beagles which demonstrated a decrease in muscle capillary density and capillary-to-fiber ratio with age [365]. Although change in blood flow distribution compensates, to some extent, for age-related changes in the cardiovascular system, complete restoration of blood flow is not restored in skeletal muscle. Deficits in muscle vascularization may contribute to the morphological changes and functional deficits evident in muscles affected by sarcopenia.

Within the immune system, cytokines are small nonstructural proteins that transport inter-cellular chemical signals. Although cytokines are an integral part of immune responses, recent research suggests these chemical messengers are involved in cell growth, differentiation, repair and regeneration, as well as, aging [366]. More specifically, in muscle, cytokines play a role in anabolic and catabolic processes; muscle contraction; injury and contraction-induced repair; and many muscle disease processes [367].

Cytokines obviously play a significant role in the inflammatory response associated with muscle damage, but these chemical messengers can also be produced by

muscle fibers. Muscle fibers are capable of directing the inflammatory response, expressing cytokine receptors, adhesion molecules and co-stimulatory molecules [368]. It is important to understand, depending on the chemical messengers and stimuli involved, that cytokines can act in an endocrine, paracrine or autocrine fashion [367]. In other words, chemical signals can be transported in the blood to target cells (endocrine), produced and metabolized locally (paracrine) or transduced by autocrine signaling in which the chemical messenger acts on the same cell that produced it [1].

Muscle tissue has the ability to produce cytokines internally in response to a variety of stimuli. Proinflammatory cytokines can stimulate the secretion of interleukin-1 beta (IL-1β), IL-16, IL-8 and transforming growth factor. [367]. Although the role of individual cytokines in muscle catabolism is controversial, Zoico cites several studies that demonstrate increased protein degradation with elevated levels of TNF-α [367]. The role of IL-1β in muscle degradation is not convincing to date. Basically, despite extensive research in this area, little is known about the individual contribution of various cytokines to muscle atrophy. It is evident, however, that several cytokines such as tumor necrosis factor-alpha (TNF-α), IL- β, IL-6, ciliary neutrophic factor (CNTF), interferons and growth/differentiation factor-8 form an intricate network of signals which ultimately lead to muscle catabolism.

Age-related sarcopenic changes in striated muscle are due to an imbalance between protein synthesis and degradation. The majority of research suggests decreased muscle mass is a result of increased muscle catabolism rather than decreased anabolism. Several diseases including osteoarthritis (OA), rheumatoid arthritis (RA), congestive renal failure (CRF) and congestive heart failure (CHF) have been associated with

inflammation and increased cytokine production. Roth suggested age-related muscle loss is also associated with increased cytokine levels and decreased growth factors [369]. Therefore, with a decrease in anabolic factors and an associated increase in catabolic factors, the scale is heavily tipped to increased muscle loss with aging. More specifically, Roubenoff and colleagues demonstrated a strong association between increased IL-6 and TNF-α levels and mortality in older adults. Elevated levels of TNF-α have also been associated with decreased quadriceps strength and increased activation of skeletal muscle apoptic pathways. In muscle, apoptosis leads to a loss of myonuclei and death of muscle cells (myofibers) [370, 371]. It is also interesting to note that some of these structural changes are associated with functional limitation and compromised quality of life in older adults. The mechanisms by which growth and inflammatory factors interact are still unknown but may provide further insight into the loss of homeostasis between anabolism and catabolism in aged skeletal muscle. There are several studies that look at the association between lean body mass and fat mass. It has been well documented that decreased muscle mass is associated with aging, but recently the term 'sarcopenic obesity' has been used to describe a coincident increase in fat mass and a decrease in lean body mass. Interestingly, obesity is also related to inflammation as adipose tissue releases TNF-[372]α, as well as, IL-6. In both older men and women, elevated levels of inflammatory cytokines have been associated with an increase in fat mass and a decrease in fat free mass [373, 374]. This may not be a chicken before the egg argument as research suggests all the inflammation in sarcopenic obesity is a result of fat mass, therefore, obesity precedes sarcopenia.

2.6a Structural Changes

The intent of this section is to collate age-related changes in the structure of muscle together, and add some evidence from previous sections of the literature review. Aging is associated with changes in both structure (morphology) and function of muscle tissue and the relationship between the two is intimate and inseparable. Alterations in the structure of muscle tissue as a result of aging, injury, disease, etc. ultimately affect the ability of sarcomeres, individual muscles fibers, bundles of muscle fibers, and the entire muscle to function to their respective full capacity. Functional deficits include, but are not limited to, loss of force production, increased fatigue, lack of endurance, altered angle of peak torque, and decreased contraction velocity. Conversely, functional deficits feedback to muscle morphology and may result in loss of sarcomeres in series or in parallel, decreased fiber CSA, loss of motor units, decline in the number of muscle fibers, fiber type transformation, and change in fiber arrangement (pennation). The sequence in which changes in muscle structure and function occur is dependent on the mechanism(s) responsible for the alterations.

Sarcopenia is an age-related loss of muscle cross-sectional area [375, 376] and although changes in muscle structure are well documented, the mechanisms responsible for these changes are still under intense investigation. Decreased muscle cross-sectional area is a secondary process due to motor unit loss and atrophy of individual muscle fibers. Several studies have reported a significant decrease in muscle fiber cross-sectional area in both humans and rodents [295, 375-380]. In addition to a loss of muscle fiber CSA, the number of muscle fibers in aged muscle is less than in young contractile tissue [372, 381] such that the vastus lateralis muscle of an 80 year old human has 1/3 the

number of fibers compared to an individual 50 years younger [376]. The CSA of the human vastus lateralis muscle is more an indication of the total number of fibers rather than the atrophy or loss of type II fibers [372, 382] .

Type II fibers are adversely affected by age to a greater extent than type I fibers in the vastus lateralis [380, 383, 384], tibialis anterior, gastrocnemius and biceps brachii muscles [385]. Atrophy of individual fibers throughout the muscle is inconsistent due to the heterogeneity of fiber types. Aged, atrophied muscle is characterized by clusters of fiber types, rather than a random distribution of fibers evident in young muscle, which indicates fiber type grouping [372, 386]. Fiber type grouping usually occurs in the eighth decade of life and is described as the result of a process of partial denervation and reinnervation of muscle fibers, which may change fiber type proportion. Axonal branches near a denervated fiber will reinnervate it. [101, 385, 387-390]. This also results in an increased size of the motor unit or greater number of fibers per motoneuron [386].

Andersen noted a change in fiber shape with age since muscle fibers from the vastus lateralis of an aged section of muscle demonstrated a ‘flattened’, ‘crushed’, or ‘banana shaped’ appearance as opposed to the angular cobble stone shape of younger fibers [386]. Change in muscle fiber shape may be due to the initiation of apoptosis (programmed cell death). The most prevalent morphological changes in aged muscle occur in type II fibers and include decreased fiber CSA, loss of muscle fibers and fiber type grouping [385]. Therefore, intervention strategies intended to combat sarcopenia should be designed to effectively target type II fibers to maintain or increase muscle CSA.

Several researchers have investigated age-related changes in the proportion of type I and type II muscle fibers but there is still discrepancy between the results of various studies. While research has demonstrated that there is no change in the proportion of muscle fiber types with age, others have provided evidence to support a decrease in the number of type II fibers [153, 385]. Biopsy technique (depth of tissue sample), age of subject (difference, before and after the age of 76-years-old), muscle sampled, study design (cross-sectional vs. longitudinal), and the change and/or co-expression of histochemical characteristics contribute to the discrepancy of the results between studies.

Regardless of the lack of agreement in the age-associated change of muscle fiber type percentagewithin an individual muscle, there is strong evidence to indicate muscle atrophy during aging affects type II muscle fibers to a greater extent than type I fibers. In addition to a decrease in the CSA of individual fibers, a change in shape and distribution of fibers occurs as aged muscle exhibits some flattened fibers and fiber type grouping. These structural changes cause fibers to be more susceptible to contraction-induced injury and ultimately affect muscle function. In turn, these changes make a significant impact on the ability to perform activities of daily living and function independently.

2.6b Functional Changes

The implications to function of age-related structural changes in skeletal muscle have yet to be fully realized. One of the most detrimental functional deficits in older muscle is increased susceptibility to contraction-induced injury since the regenerative capacity of aged muscle is impaired [204, 263, 380, 391-393]. Type II fibers are at greater risk of contraction-induced injury than type I fibers which may be a result of their

more extensive atrophy than type I fibers. There is substantial evidence from both cross-sectional-and longitudinal-studies in humans to confirm a loss of strength with age in muscles that flex and extend the elbow-, knee- and ankle-joints [394-397]. These studies indicate that after the age of 50-years-old, strength decreases at a rate of about 12–15% per decade, but in general, strength deficits associated with changes in muscle structure tend to be underestimated due to differences in study design (longitudinal versus cross-sectional) and sampling techniques (diagnostic imaging versus muscle biopsies) [394, 397].

Lynch and colleagues stated that proximal lower limbs demonstrate the greatest reduction in strength although later reports do not confirm these results [393-395]. It is important to realize that the percentage of change in strength reported in the literature is dependent on the velocity at which testing was performed and the type of contraction that was assessed (concentric versus isometric versus eccentric). Eccentric strength is maintained to a greater extent with aging than both isometric and concentric strength [398-401], although the underlying mechanisms for the maintenance of eccentric strength are not fully understood. It is quite possible that, due to the uncomfortable and unnatural movement pattern, isokinetic strength testing is not the most accurate and valid measure of eccentric strength in older adults and may be reflected in the lack of recorded changes in strength with age. Another confounding variable that could account for a lack of change in eccentric strength with age is muscle co-contraction. Evidence suggests that substantial antagonist contraction of the hamstring muscle group occurs during agonist contraction of the quadriceps muscles and vice versa [402, 403]. Also, co-contraction of the quadriceps muscle group was velocity dependent and increased at greater velocities

[402]. If velocity of contraction decreases with age, co-contraction torques would also decrease which could result in greater recordings of agonist force production, thus, underestimating deficits in eccentric strength of the older- compared to younger-subjects.

Several studies of aging indicate muscle power is affected to a greater extent than muscle strength [385, 397, 404, 405]. This again illustrates the importance of maintaining the size and force production of type II fibers since these fibers are responsible for muscle power. Muscle power has demonstrated a closer association to function than strength [73] and decreases at a greater rate with age [73, 404]. Bassey and colleagues demonstrated the significance of power in performing activities of daily living such as stair climbing, rising from a chair and walking [406].

In addition to decreased strength and power, aged muscle demonstrates a decreased rate of contraction, increased time-to-peak tension [407], and a longer rate of relaxation compared to younger muscle [408]. Narici and colleagues stated "Slowing of relaxation and type II atrophy may also explain why older people demonstrated more resistance to isometric fatigue than younger individuals" [407]. Collectively, these changes in contraction rate and relaxation time demonstrate significant deficits in skeletal muscle function with age that culminate as a decreased ability to perform activities of daily living, and an increased rate of injury resulting from decreased response time and an inability to right oneself when balance is compromised.

As discussed previously, insufficient power in older individuals compromises function to a greater extent than strength deficits. Fortunately, there is a wealth of literature that identifies the benefits of power training in aged subjects greater than 64 years-of-age, after training for only 2 to 6 months [73]. Although the study protocols

vary considerably, the majority of studies demonstrate an increase in power, strength and CSA after power training [74, 409-417]. More importantly, power training resulted in decreased disability and an increase in functional measures such as walking speed [411, 413, 418], balance [418]; chair stands [143, 413, 418], and stair climbing [414, 418].

With the appropriate training protocols and possible treatments to maximize the production of nitric oxide to stimulate satellite cell activation and growth of type II muscle fibers, the extensive improvements demonstrated with power training alone may be further enhanced. If that enhancement were possible, it would improve the ability of older subjects to perform activities of daily living and to live independently with a reduced risk of falls.

2.6c Neuromuscular system

As mentioned previously, one cause of decreased muscle fiber CSA is motor unit loss [375, 376]. A motor unit is described as a single nerve fiber and all the muscle fibers it innervates [1]. Although the number of motor units was originally determined by counting ventral root fibers [43, 106, 419], this method gives no indication of the number of functioning motor neurons. Counting the anatomical presence of an axon during the loss of function will underestimate the decrease of motor units with age and does not provide insight into the implications of motor unit loss on muscle function. A classic experiment by Campell and colleagues used electrophysiological studies to investigate the decline in the number of functioning motor units of the extensor digitorum brevis (EBD) muscle of healthy human subjects aged 7-months-old to 97-years-old [107]. This study incorporated a physiological approved model that stimulated a single motor nerve axon to fatigue and used muscle histology (stained for glycogen) such that fibers depleted

in glycogen (no stain) were part of the motor unit stimulated in vivo. Up until the age of 60-years-old, the number of motor units varied considerably but there was a significant drop in number beyond the start of the 7th decade. In addition, research has demonstrated that seventy-year-old subjects lose as much as 50% of functional motor units [107]. The denervation and reinnervation process of muscle fibers causes fiber type grouping and loss of random fiber type distribution within the muscle which may cause functional deficits in muscle performance. Although the implications of fiber type changes on strength, power and functional status have yet to be fully characterized, data suggest the neuropathic process contributes to loss of muscle fibers and muscle atrophy [378, 420]. Fiber type grouping doesn't necessarily result in a change in the overall proportion of type I and type II fibers but deductive reasoning suggests the denervation and reinnervation process contributes to a decline in the CSA of type II fibers. Booth and colleagues stated that studies which report motor unit loss and a decrease in the number of spinal motor nerves are primarily cross-sectional in design, therefore, longitudinal studies are warranted to accurately quantify changes in the neuromuscular system with age [381].

There is significant evidence that suggests motor unit loss plays an essential role in sarcopenia, especially after 60 years-of-age. Structural changes that occur in muscle with age ultimately affect muscle function and impede ability. However, the feedback between structure and function, and the order in which these events occur during atrophy and aging is still under intense investigation. Well-designed longitudinal studies with reliable techniques investigating several upper and lower muscles of agonistic- and

antagonistic-function are required to provide further insight into the epidemiology of sarcopenia.

2.6d Cellular Changes

Genes provide the blueprint for cellular structure and function that are responsible for the assembly of proteins and biomechanical processes that take place in both satellite cells and mature muscle fibers. Even though Hayflick provides a convincing argument that aging is not a purposeful program driven by genes, there is no doubt that age-related changes in gene expression alter the structure and function of cells, regardless of whether these alterations are preprogrammed or random, accidental events [352]. The purpose of this section is not to debate the evidence presented by Haflick, but to identify changes in gene expression and protein levels that do occur with aging, regardless of the cause.

Neuronal NOS, and more specifically the spliced form NOS-Iμ, which presents only in skeletal muscle and the myocardium, has been implicated in the processes regulating contractile force and for the purposes of this research, satellite cell activation. Satellite cell activation is delayed and decreased with age (see Section 2.2f), therefore because NOS-Iμ is involved in satellite cell activation, it is hypothesized that NOS-Iμ protein levels decrease with age. The results from a study by Capanni and colleagues did not agree with this hypothesis as comparison between neuronal NOS protein levels of the gracilis muscle from 6-and 24-month-old Wistar Rats demonstrated an increase in neuronal NOS levels with age [421]. Conversely, Richmonds and colleagues found decreased levels of NOS activity in the extensor digitorum longus- and soleus-muscle of 24-month-old Fischer 344 rats [422]. Two possible reasons for this discrepancy are the different muscles being investigated and, more likely, the methods used to assay NOS-Iμ

protein levels. Capanni and colleagues determined protein levels whereas Richmonds and colleagues investigated NOS-Iμ activity by analyzing the conversion of 3H arginine to 3H citrulline. Increased NOS-Iμ levels demonstrated in the study by Capanni and colleagues may have been a compensatory mechanism to counteract deficient NOS activity illustrated in the study by Richmonds and colleagues. To date, no murine studies have investigated the effects of age on the changes in expression or activity of NOS-Iμ in skeletal muscle. In addition, the potential benefits of NO donor supplementation, stretch, and exercise to enhance NO production and, subsequently, increase satellite cell activation to combat muscle atrophy have yet to be realized.

Myogenic regulatory factors (MRFs) which include Myf5, MyoD, MRF4 and myogenin play a significant role in muscle growth, maintenance and repair, and are active in response to loading and unloading of limbs in young rodents [423-425]. However, the plantaris and soleus muscles of rats aged up to 39 months-of-age were reported to have decreased levels of MyoD and myogenin protein and did not respond to unloading by increasing protein levels as identified in young rats [426]. Contrary to these results, Edstrom and Ulfhake found an upregulation of all four MRF genes in the gastrocnemius muscle of 30-month-old Sprague Dawley rats [427]. Similar to neuronal NOS expression, elevated levels of MRFs with aging may be an intrinsic mechanism that is unsuccessful in counteracting atrophy of muscle fibers.

Analogous to myogenic regulatory factors, myostatin (GDF-8), a member of the transforming growth factor-β superfamily, inhibits muscle growth [428]. Knockout mice and naturally mutated 'double-muscle' cattle have increased muscle mass (< 20-30%) compared to animals with normal expression of myostatin [428, 429]. Therefore, it is

hypothesized that age-related muscle atrophy may be associated with increased myostatin expression and a more stringent negative control of muscle growth. An earlier study investigating the changes in myostatin expression with age in C57BL/6 mice did not demonstrate a difference in myostatin expression in the gastrocnemius- and plantaris-muscles from 11- to 92-weeks-of-age [430]. The only change in myostatin expression occurred during the reloading phase of gastrocnemius and plantaris muscle from 11-week-old mice subjected to hindlimb suspension for 14 days. However, muscles from animals aged to 11-weeks also demonstrated decreased myostatin expression during growth which suggests myostatin expression may be more responsive at a younger age [430]. Data from human studies have revealed that genetic variations of the myostatin gene, in particular R153, may be associated with greater muscle weakness than the non-mutated form of myostatin [431, 432]. The prevalence of the R135 mutation in the human population is extremely low and it should not be assumed that decreased myostatin levels (specifically R135) cause age-induced weakness in the general population.

To date, there is no convincing evidence to suggest that age-associated alterations in NOS-Iμ, MRFs and myostatin play a significant role in sarcopenia. That being stated, similar to motor units, the expression of a gene or presence of protein in aged muscle does not necessarily reflect effective function or an important functional change. Further research is needed in this area to determine if gene expression and protein levels can be altered in aged muscle, with treatments and/or exercise, to restore the structural and functional characteristics evident in young muscle.

2.6e Age-related Sarcopenia

Sarcopenia means “flesh loss” in Greek and was originally proposed by Rosenberg in 1987 to describe the age-associated loss of muscle mass and function [433]. In old age, skeletal muscles atrophy, evidenced as a significant loss of muscle cross-sectional area, a process that is a secondary to motor unit loss and atrophy of individual muscle fibers [375, 376]. The decline in lean body mass is associated with decreased strength that results in decreased function due to the positive correlation between muscle mass and strength [248, 385]. Although many researchers have studied sarcopenia since the first Sarcopenia Workshop held by the National Institute on Aging in 1994, the mechanisms responsible for the deterioration of voluntary contractile tissue have yet to be elucidated. The importance of making progress on this question is highlighted by the change in demographics of our society.

The loss of muscle mass is accompanied by strength deficits and impaired function [434], resulting in increased disability amongst older individuals . In fact, Morley and colleagues suggested that clinically significant sarcopenia affects 8.8% of the older female population and up to 17.5% of older males [435]. The rates of clinically significant sarcopenia alone have a significant impact on the health care system. Further, when the subsequent loss of bone mass and demographics of our population are taken into account, muscle atrophy becomes a serious problem. The proportion of the North American population over the age of 50-years-old continues to increase along with life expectancy. Therefore, the number of individuals experiencing sarcopenia continues to increase, placing a greater strain on health care dollars. The fact that over 50% of our

workforce could retire in the next 10 years gives a vision of the impact of the current demographic shift.

In addition, bone mineralization and strength are stimulated by the pull or tension placed on the bone during muscle contraction. Stronger muscles have the ability to produce more force and place greater tension on bone, subsequently stimulating bone calcification. Decreased muscle mass and strength reduce the effects of muscle pull on bone and allow osteoporosis to develop from misuse.[361].

Forty-percent of muscle mass is lost by 80 years-of-age, with 30 % of the reduction occurring after the fifth decade of life. Age-related muscle atrophy is accompanied by a decrease in muscle-force output and limitation of function compared to young, healthy muscle. Age-related muscle loss has a greater affect on the weight-bearing capacity of lower limb muscles, as up to 33% of the decline in CSA of the quadriceps muscle occurs between the third and eighth decade of life [436].

Currently, the most effective way to treat age-related muscle atrophy is resistance training. A review by Johnshon hilighted the positive effects of resistance training in older adults which included an increase in muscle strength, muscle mass, type II fiber CSA, proportion of type II fibers, and function, as well as, decreased morbidity and mortality [437]. Tornopolsky and Safdar discussed additional benefits of resistance training in older adults such as an increased rate of muscle protein synthesis, and increased fiber CSA of type I and IIx fibers. Resistance training has also improved functional measures (i.e. muscle strength, gait velocity, stairclimbing power, and spontaneous physical activity) in older adults up to 98 years-of-age.[438]. The increase in muscle size with resistance training in an older population may be due, in part, to

increased satellite cell activation since 3 months of resistance training in older men and women (70-82 years-of-age) increased the number of satellite cells per fiber in the vastus lateralis muscle[439]. Although resistance training has been shown to enhance muscle structure and function in older adults, the mechnisms responsible for these positive changes are largely unknown. If the mechanisms responsible for the positive benefits of resistance training can be identified, the potential to combat sarcopenia with strength training could be enhanced even further. Unfortunately, to date, the prevalence of sarcopenia continues to rise and negatively affect older adults and society as a whole.

As the average life-expectancy rises in Canada, the financial impact of sarcopenia on the health care system increases. Age-related muscle atrophy directly affects quality of life by increasing the risk of osteoarthritis, decreasing physical function and, concomitantly, increasing the risk of falls. Muscle weakness makes it more difficult for older adults to quickly correct posture when balance is lost, which results in falls [281]. Older individuals are more likely to be affected by reduced bone mass, in fact, the prevalence of osteoporosis in Canadian women aged 50 year of greater is almost 16% [440]. Osteoporosis currently affects approximately 1 in 4 women and 1 in 8 men in Canada and is a significant health care problem since about 20% of women and 40% of men die within of year of sustaining a fracture of the hip [440]. Muscle is also used an emergency protein store during times of stress, such as major surgery, and atrophy therefore reduces survival rates of affected individuals from other health and rehabilitation conditions [281].

There is a wealth of evidence to confirm that age-associated structural and functional changes in muscle have a significant impact on the older population, as well

as, the community as a whole, in terms of health, economy and prosperity. Aging research has increased exponentially over the last decade, yet there are still several important questions that need to be answered if effective treatments are to be realized. Sarcopenia is a multifactoral process that involves the interaction of several complex systems within the body and comorbidities in aged humans and animals further complicate this condition. Fortunately, positive results have been associated with exercise, drug treatments and nutritional supplementation, but further progress in research is necessary to find ways to restore function in the older population. More than likely, a combination of exercise, drug treatments and nutritional supplementation will be required to fully elucidate the effects of aging on skeletal muscle tissue and eliminate disability resulting from sarcopenia.

2.7. Significance

Over the last decade, age-related muscle atrophy has received a lot of attention and has been the focus of many research studies. Sarcopenia is characterized by loss of whole muscle mass, decreased fiber CSA, motor unit loss, reduced strength, and an impaired ability to perform activities of daily living. Despite what is known about the structural changes in muscle and the functional deficits that occur with aging, the mechanisms responsible for alterations in aged contractile tissue are still not fully understood.

Muscle satellite cells are the main source of myonuclei during muscle maintenance, hypertrophy and regeneration. Adult muscle tissue is post-mitotic, therefore, without satellite cells, maintenance, growth and repair of muscle would not be possible. Satellite cells from aged muscle demonstrate decreased functional capacity and

a delayed response to activation stimuli. Although growth factors have been used to increase the proliferation of satellite cells from aged muscle, the initial activation of muscle precursor cells is a prerequisite for the multiplication of satellite cells.

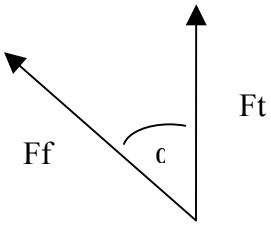
To date, only nitric oxide and hepatocyte growth factor have been proven to activate satellite cells and initiate the exit of these precursor cells from GO to G1 of the cell cycle. Nitric oxide concentration in muscle increases in response to stretch, exercise, denervation and trauma. Stretch and exercise are the only two mechanical stimuli that would receive ethics approval to be used on human subjects. Hence, although several models of denervation and trauma have been used to study satellite cell activation in young-and old-animals, stretch and exercise are the most realistic and promising intervention to increase nitric oxide production in humans with sarcopenia. However, it is not known if stretch and exercise can increase nitric oxide concentration to physiological levels required to stimulate satellite cell activation in aged muscle. For this reason, endogenous and exogenous sources of nitric oxide will be investigated to determine if inadequate levels of nitric oxide exist in aged muscle and if so, which source of nitric oxide is most therapeutic.

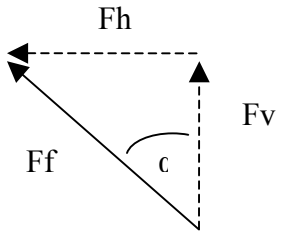
Re-investigating the progression of the structural and functional changes that occur in muscle with age may provide clues to the mechanisms responsible for these age-related alterations in skeletal muscle. Circumstantial evidence exists to implicate muscle satellite cells in the process of muscle wasting, but further research is required to determine if satellite cell pool size or declined regenerative capacity of satellite cells is responsible for atrophy. The work presented in this thesis was designed to determine if age-related changes in the structure and function of muscle are related to a decreased

satellite cell pool size and/or the inability of satellite cells to activate to the same extent as in young muscle. Both mechanical and chemical stimuli were incorporated in these studies to investigate the effects of nitric oxide on satellite cell activation in aged muscle. Results of this research will determine the role of nitric oxide in age-related muscle atrophy and possibly provide evidence to develop mechanical stimulation protocols and drug therapies that have the potential to combat sarcopenia by increasing satellite cell activation.

2.8. Tables and Figures

Figure 2-1. Vector resolution for muscle fiber pennation
The force applied to the tendon of a multi-pennate muscle was determined using the principles of vector geometry. Tension applied to the tendon during contraction of a muscle fiber was divided into these two components: vertical tension and horizontal tension. Vertical tension can also be described as the effective force since the force acting along the tendon is pulling on the bones in an attempt to cause motion. The greater the angle of pennation the less vertical tension applied to the tendon. Muscle fibers oriented at 45º to the tendon result in equal magnitudes of horizontal and vertical tension when fibers contract.





Ft (tension in the tendon) = ?
Ff (tension in the fiber) = 100 N
Fh (horizontal tension on tendon) = ?
Fv (vertical force on tendon) = ?
α (angle of pennation) = 45°

$Fh = (Ff)(\sin\alpha)$
$Fh = 70.7$ N

$Fv = (Ff)(\cos\alpha)$
$Fv = 70.7$ N

Table 2-1. The effects of systemic changes with age on muscle growth.

Aging causes alterations in every system of the body and these changes ultimately impact the structure and function of skeletal muscle. Age-related changes in the nervous system (NS), endocrine system (ES), skeletal system (SS), cardiovascular system (CS), urinary system (US) and the immune system (IS) are identified and summarized, along with the impact of these changes on skeletal muscle.

System	Changes with age	Effect on muscle
NS	↓ motoneuron size, ↓ # motoneurons, ↓ functional motoneurons, fiber type grouping	↓ fiber CSA, ↓ strength, ↓ muscle mass, ↓ power, ↓ balance, ↓ heterogenity of fiber types
ES	↓ MGF, ↓ GH, resting metabolic rate, ↑ insulin resistance, ↑ hyperglycemia, ↓ testosterone	↓ proliferation of SCs, ↓ fusion of SCs, ↓ protein synthesis
SS	↓ bone density, ↑ rates of osteoporosis	↓ resistance → atrophy/weakness
CS	↓ blood flow, ↓ capillary density, ↓ capillary-to-fiber ratio, ↓ capillary #	↓ mitochondrial volume, ↓ muscle function, ↓ amino acid transport
US	↑ angiotensin II associated with adipose tissue, hypertension	↓ protein synthesis, ↓ mitochondial volume, ↓ muscle function, ↓ amino acid transport
IS	↑ cytokine levels, ↑ inflammation due to age-related diseases, obesity	↓ muscle mass, ↓ strength, ↑ activation of apoptotic pathways

CHAPTER 3. HYPOTHESIS AND AIMS

3.1. Hypothesis

The overall hypothesis of this thesis is that the age-related structural and functional changes in skeletal muscle of normal mice can partly be attributed to decreased satellite cell pool size and perturbed activation due to deficits in NO signals. It is also hypothesized that increasing NO production through stretch, exercise and drug treatments will restore the structure and function of aged muscle by enhancing satellite cell activation.

3.2 Specific Aims:

Determine the effects of stretch and drug treatments on satellite cell activation and gene expression in normal aged muscle (Chapter 4).

Determine the progression of morphological changes in skeletal muscle of normal mice that occur with age (Chapter 5).

Establish the age-related functional changes that take place in muscle of normal mice up to 18 months-of-age (Chapter 5).

Determine the alterations in satellite cell pool size and satellite cell activation that occur with age in normal muscle (Chapter 5).

Characterize the gene expression profile of normal muscle from young, adult and senescent mice (Chapter 5).

Characterize the effects of exercise on muscle structure, function, satellite cell activation and gene expression in normal mice aged to 18 months (Chapter 6).

Determine if there is an age- and muscle-specific response to exercise in terms of satellite cell activation and gene expression. (Chapter 6)

The experiments described here include whole-muscle cultures and in vivo models of exercise. Stretch and drug treatments were applied to whole-muscle cultures to increase NO production whereas exercise was used to enhance the production of NO in vivo.

CHAPTER 4. THE EFFECTS OF AGE AND DRUG TREATMENTS ON SATELLITE CELL ACTIVATION IN CULTURED EDL MUSCLES OF NORMAL FEMALE MICE

4.1 Introduction

Skeletal muscle satellite cell activation is essential for normal muscle growth, as well as for the repair of muscle damaged by injury or disease [5]. In normal adult muscle, satellite cells are quiescent and become activated in response to exercise, trauma, denervation, stretch and exercise. However, satellite cells from older muscle demonstrate a delayed response to activation stimuli, up to 24 hours [22-24], and are not able to repair or regenerate muscle to the same extent as satellite cells from younger animals [218, 278, 441]. The implications of these deficiencies on the aged population are extensive. In both animals and humans, aging is associated with a significant decrease in muscle fiber cross sectional area [295, 375-380]. In addition to a loss of muscle fiber CSA, the number of muscle fibers in aged muscle is less than in young contractile tissue [372], such that the vastus lateralis muscle of an 80 year old human has 1/3 the number of fibers compared to an individual 50 years younger [376]. The implications to function of age-related structural changes in skeletal muscle have yet to be fully realized. One of the most detrimental functional deficits in older muscle is increased susceptibility to contraction-induced injury since the regenerative capacity of aged muscle is impaired [204, 263, 380, 391-393].

Activation of satellite cells has been demonstrated extensively according to studies of proliferation which use incorporation of BrdU or [^{3}H]–thymidine incorporation into DNA [8, 16, 18, 23, 27, 28, 31, 211, 270]. Our lab has previously demonstrated that passive mechanical stretch of isolated single muscle fibers in culture from the flexor

digitorum brevis (FDB) muscle of 5- to 8-week-old normal mice increased satellite cell activation [31]. That study also indicated that satellite cell activation is dependant on nitric oxide, since inhibition of nitric oxide production with the NOS inhibitor, L-NAME, prevented mechanically-induced satellite cell activation in vitro. However, the effects of stretch and NO manipulation on satellite cell activation in muscle from aged mice have yet to be investigated. Stretch studies in rats have demonstrated that EDL and soleus muscle from aged rats do not respond to mechanical stimuli to the same extent as muscle from young animals. Alterations in mitogen-activation protein kinase (MAPK) phosphorylation suggest that mechanical stimulation of aged muscle is disrupted [36]. One possible explanation for a deficient response to mechanical stimuli may be disruption of the dystrophin-glycoprotein complex (DGC) and subsequent displacement of neuronal nitric oxide synthase (NOS-I).

The DGC is responsible for securing the muscle fiber to the basal lamina; transmitting tension from the fiber to the muscle tendon and ultimately bone; sensing mechanical forces placed on the sarcolemma; and suspending the muscle-specific isoform of neuronal nitric oxide (NOS-I) between the sarcolemma and basal lamina [38]. Rice and colleagues demonstrated that the DGC in EDL muscles of 36-month-old rats were disrupted compared to the DGC in the same muscle from 6-month-old mice. Dystrophin was either completely absent or discontinuous along the sarcolemma in aged mice whereas a uniform distribution of dystrophin was evident in younger muscle [39]. In young mice, a suspension-induced muscle atrophy model demonstrated that NOS-I is displaced into the cytoplasm of muscle fibers rather than being anchored in the DGC between the sarcolemma and basement membrane[40]. NOS-I responds to mechanical

stimuli by releasing a bolus of nitric oxide that activates satellite cells, as previously described. It is possible that the disruption of the DGC in aged muscle is associated with the dislocation of NOS-I into the cytoplasm of the muscle fiber since NOS-I is anchored to the sarcolemma via α1-syntrophin. The implications of these proposed changes are two-fold. First, disruption of the DGC would lead to increased susceptibility to contraction-induced injury, similar to dystrophic muscle. Secondly, displaced NOS-I into the cytoplasm would prevent or limit NO availability for satellite cells when a mechanical-stimulus triggers a bolus release of NO which would lead to decreased satellite cell activation. In fact, Suzuki and colleagues have suggested that the release of NO within the cytoplasm triggers atrophic signaling in young muscle [40]. Therefore, the purpose of this set of experiments was to determine if passive stretch activates satellite cells from aged muscle in culture. In addition, to determine if NO manipulation can enhance in vitro satellite cell activation in EDL muscles from 8- and 18-month-old mice.

4.2 Methods

4.2a Experimental animals

Normal female C57BL/6 mice aged to 6 weeks, 6-, 8-, 10- and 18-months-of-age were used to study the effects of stretch and age on satellite cell activation in whole-muscle cultures of extensor digitorum longus (EDL) muscles. Muscles from mice aged to 8- and 18-months-of-age were selected for tests of drug treatments, in addition to stretch, to investigate the influence of age, nitric oxide (NO) supplementation, NOS inhibition or both on muscle precursor cell activation. These age groups were chosen for further investigation since an increase in satellite cell activation with stretch was not

demonstrated beyond 6 months-of-age. Additionally, muscles from mice aged to 6-months-of-age required a greater magnitude of stretch (10%- vs. 20%-increase in length) to increase satellite cell activation compared to unstretched control muscles from 6-weel-old mice (Leiter and Anderson, unpublished). To successfully treat muscle atrophy caused by impaired satellite cell activation, it is important to attempt to rescue stretch-induced satellite cell activation nearest the age when mechanically-stimulated activation ceases to occur. Therefore, 8-month-old mice were considered to be in the initial stages of satellite cell latency with respect to stretch-induced stimulation of muscle precursor cells. Six week, 6-, 8- and 10-month-old mice were bred and housed in the Central Animal Care Facility at the University of Manitoba Bannatyne Campus. Mice aged to 18 months-of-age were bred at The Jackson Laboratory (Bar Harbor, Maine, USA), transported to the University of Manitoba at 8 months-of-age and housed for an additional 10 months until the animals were 18-months-old. Ethics approval was obtained prior to commencement of any research activities and the study protocol adhered to the guidelines set forth by the Canadian Council on Animal Care.

4.2b Muscle isolation and pinning

Mice were anaesthetized with PRAErrane isoflourane USP (Baxter Corporation, Mississauga, ON) and then immediately sacrificed by cervical dislocation. The extensor digitorum longus (EDL) muscles were isolated (figure 4-1) with precise dissection to avoid muscle damage and activation of satellite cells. EDL muscles were then placed in proliferative medium (PM) (PM; 1X Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum, 2% chick embryo extract, 1% antibiotic/antimytotic and 0.1% gentamycin) for ½- to 1-hour. The connective tissue of EDL muscles was removed under

a dissecting microscope (50X magnification) and muscles were placed in an individual well of a FlexCell^{TM} plate (FlexCell International Corporation, Hillsborough, North Carolina). Each well contained 2 ml of basal growth medium (BGM), which uses serum replacement instead of high concentrations of fetal bovine serum to limit satellite cell activation under control conditions in culture (BGM; 1X DMEM with 2% controlled replacement serum-2 (Sigma Aldrich, St. Louis, MO) diluted in 18% 5X DMEM, 1% fetal bovine serum, 1% antibiotic/antimycotic and 0.1% gentamycin), as previously reported for muscle-fiber cultures [270]. Under moderate magnification (200X), muscle tendons were secured with insect pins to ensure muscles were at resting length (figure 4-2). Muscles were randomly assigned to either the unstretched (US) group or stretched (S) group. Additional muscles from the 8- and 18-month-old age groups were randomly assigned to the stretch plus drug treatment groups. Muscles from the US group were pinned in FlexCell^{TM} plates with a rigid bottom, whereas muscles from the S groups (including S plus drug treatment) were placed in FlexCell^{TM} plates with a silastic flexible base that allowed the EDL muscles to be stretched when a vacuum was applied [270].

4.2c Drug treatments

As mentioned previously, muscles were randomly assigned to the US group, S group, or S plus drug treatment groups. The stretch plus drug treatment groups included: stretch plus L-Arginine (LA), stretch plus isosorbide dinitrate (ISDN), stretch plus N^{ω}-nitro-L-Arginine methyl ester (LN), stretch plus LA and LN (LALN), or stretch plus ISDN and LN (ISDNLN). All drug treatments were administered to basal growth medium, pipetted onto muscles after pinning in the wells, and before stretch was applied. It should be noted that in the 18 month–old-group, LN was re-administered after 24 hours

in culture since LN dissociates after 24 hours. Muscles placed in the LA group were treated with L-Arginine (LA, Sigma Chemical Co., 105 μg/ml). The S + LN group was administered the non-specific NOS inhibitor, Nω-nitro-L-Arginine methyl ester (L-NAME, Sigma Chemical Co., 0.5 μg/ml). Whole-muscle cultures assigned to the ISDN group had isosorbide dinitrate (Sigma Chemical Co., 0.046 μg/ml) added to the basal growth medium (BGM). Two groups of muscles received a combination of the above treatments which included the following: LA (105 μg/ml) and LN (0.5 μg/ml); and ISDN (0.046 μg/ml) and LN (0.5 μg/ml). Following the administration of treatments, plates were placed in an incubator set to 37ºC and 5% CO_2.

4.2d Stretching of EDL muscles

After EDL muscles were isolated and pinned in FlexCell^{TM} wells, plates were secured in the vacuum mat of the FlexCell^{TM} system. Muscles that were assigned to the US group were also loaded into the FlexCell^{TM} system, but since the plate had a rigid bottom, no mechanical stresses were applied to the muscle. The EDL muscles were subjected to mechanical stretching at 4 cycles/minute (8 seconds on, 7 seconds off), for a total of 30 minutes, or 120 cycles. Preliminary results indicated that activation was a function of %length change or %stretch, with a 20% stretch inducing greater satellite cell activation than a 10% stretch (97% vs. 0% increase) in 6-month-old mice (figure 4-3). Although a 10% stretch was sufficient lengthening to increase activation in individual fibers isolated from 6-week-old mice, whole-muscle cultures from 6-month-old mice required twice the length increase to activate satellite cells. A vacuum of 50 kPa was cyclically applied to the system, and increased the length of the muscle by approximately

20%. This %lengthening is within the physiological range of muscle stretch in vivo [442].

Following stretch, whole-muscle cultures were immediately treated with 4 µl of [^{3}H]-thymidine (4 µCi) to label DNA synthesis, and placed in an incubator set at 37° C and 5% CO_2, for 22- or 44-hours. Following 22 hours of incubation for 8-month- old mice or 44 hours of incubation for 18-month-old mice, the tendons of EDL muscles were removed, washed in 1X cold PBS and weighed.

4.2e Tissue homogenization and DNA assay

EDLs were immediately placed on ice, minced with a razor blade and homogenized. Minced tissue was placed in a 12 ml polystyrene conical tube (Falcon, Lincoln Park, New Jersey), 1 ml of DNA extraction solution was added and then tissue was homogenized on ice. Homogenate was placed in a water bath (37°C) for 10 minutes. Samples were removed from the tissue bath, shaken and placed in a 1 ml eppendorf® tube (Eppendorf, Westbury, New York). Homogenate was neutralized with 600 µl of acetic acid and centrifuged at 4°C for 30 minutes at 2500 revolutions per minute (RPM). Samples were removed from the centrifuge and placed on ice. The supernatant was extracted and placed in a new eppendorf® tube. Three hundred microlitres of the supernatant was placed in 5 ml of Scinti-Safe™ scintillation fluid (Fisher Scientific, Ottawa, ON) for scintillation counting. To quantify DNA, 5 µl of the supernatant was placed in a 96 well plate to which 200 µl of Hoechst dye (Sigma Aldrich, St. Louis, MO, 1 µg/ml) was added. Samples and 12 incremental standards (5 ng/ul–119 ng/µl of calf thymus DNA) were exposed for 1 second in a VICTOR® microplate reader (PerkinElmer, Waltham, MA) to determine concentration of DNA. Following these

processes, dissociations per minute (DPM) were normalized to µg of DNA in the same sample. DPM/µg DNA was used as a measure of satellite cell activation, as previously reported [5].

4.2e. Statistical analysis

Statistical significance ($p<.05$) was determined by performing an analysis of variance (ANOVA). Data were entered into NCSS 2004 and PASS Trial (Number Cruncher Statistical Systems, Kaysville, Utah). Least Significant Difference (LSD) tests were used for pair-wise comparisons to identify differences between groups. Data are expressed as mean and standard error (SEM) values.

4.3 Results

4.3a Effects of age on satellite cell activation

Analysis of the US EDL muscles in each age group indicated that satellite cell activation was greater at 8 months-of-age than for any other age group. There was no difference in satellite cell activation between muscles from the 6 week-, 6- , 10- or 18-month-old age groups (figure 4-4).

4.3b Stretch-activation of satellite cells

EDL muscles from 6-week- and 6-month-old mice were the only age groups to demonstrate an increase in satellite cell activation with passive stretch (10%- and 20%-length increase, respectively). Muscles from mice 6 weeks-of-age showed a 134% (± 0.31%) increase in satellite cell activation whereas satellite cell activation increased 97% (± 0.32%) in EDL muscles from 6-month-old mice. In contrast, EDL muscles from 8- (-

11% ± 0.01%), 10- (-38% ± 0.07%) and 18- (-7% ± 0.009%) month-old-mice demonstrated a trend towards a decrease in satellite cell activation with age (figure 4-5).

4.3c Drug Treatments

All drug treatments were combined with mechanical stretch to determine if a mechanical stimulus and nitric oxide manipulation could rejuvenate satellite cell activation in the EDL muscles of 8-and 18-month-old mice. Although 8-month-old mice did not respond to mechanical stretch alone, mechanical stretch combined with an exogenous source of nitric oxide (ISDN) resulted in an increase in satellite cell activation ($p<.01$). In fact, the only two treatment groups to demonstrate an increase in satellite cell activation in 8-month-old mice were the groups treated with ISDN (ISDN group) or with ISDN and LN (ISDNLN group) (table 4-1a). ISDN is an external source of nitric oxide and does not have to be converted to NO by the enzyme nitric oxide synthase as does LA. Therefore, when ISDN was combined with LN (NO inhibitor), which blocks the NOS-I enzyme, satellite cell activation was not attenuated compared to the ISDN group. Figure 4-6 illustrates the effects of treating whole-muscle cultures with LN; satellite cell activation did not decrease after LN treatment but when LN was combined with LA, satellite cell activation decreased significantly, to levels below activation in the control US group ($p<.01$).

Although the combination of stretch plus ISDN rejuvenated satellite cell activation in 8-month-old mice, stretch and drug treatments did not have a significant effect on muscle precursor cell activation in 18-month-old mice. These data suggest that NOS-I and the availability of nitric oxide in aged muscle is even more perturbed than in younger, 8-month-old mice. Administration of LA to cultured muscles of younger mice

demonstrated a trend toward increased satellite cell activation. This suggests that the NOS-I enzyme is still functional, and able to convert LA to nitric oxide and activate satellite cells. However, in aged muscle, manipulation of NOS-I with LA or LN did not have any measurable effect on satellite cell activation as measured by DNA synthesis (table 4-1b).

4.4 Discussion

Activation of satellite cells via mechanical stretch was previously demonstrated using both single-fiber cultures and whole-muscle cultures [31, 301]. However, the effects of age on stretch-induced satellite cell activation of EDL whole-muscle cultures in normal female mice have yet to be investigated. Age-related muscle atrophy in both humans and rodents has been well established [295, 375-380], but the changes in mechanically-induced activation of muscle precursor cells is unknown. To our knowledge, this is the first report to investigate the differences in satellite cell activation via mechanical stretch in whole-muscle cultures from mice aged from 6 weeks- to 18 months-of-age. This study is also unique in the fact that EDL muscles from 8- and 18-month-old mice were subjected to stretch and drug treatments to determine the role of nitric oxide in satellite cell activation during the aging process.

The effect of age on satellite cell activation was investigated using normal female mice aged to 6 weeks-, 6-, 8-, 10- and 18-months-of-age. In the absence of stretch, results indicated that satellite cell activation in the EDL muscles from normal mice peaked at 8 months-of-age and then showed a continuous decrease up to 18 months-of-age. Satellite cell activation in whole-muscle cultures from 8-month-old mice was greater than all other age groups. However, when stretch was introduced, satellite cell

activation in the same age group did not increase in response to the mechanical stimulus. Muscle from mice aged to 6 weeks- and 6 months-of-age demonstrated a significant increase in satellite cell activation when subjected to 30 minutes of cyclical stretch. These data are in agreement with previous studies of stretch activation in cell- [16, 18] and single-fiber cultures [27], and in vivo [17], however, muscle from 6-month-old mice required twice the length increase as 6-week-old mice to increase satellite cell activation.

Conversely, it is important to note that the passive-stretch stimulus did not increase satellite cell activation in muscle from older mice (8-, 10- and 18-months-of-age). This finding is consistent with a study by Mylabathula and colleagues in which load-induced alterations in the EDL muscle of aged rats were perturbed compared to those from younger rats [36]. Interestingly, similar alterations in response to mechanical stimuli are evident in muscular dystrophies and knockout mice lacking the NOS-I protein [31, 35, 376]. Rice and colleagues also demonstrated that the dystrophin-glycoprotein complex is disrupted in aged EDL- and soleus-muscles of rats, which may account not only for age-related dysfunction in muscle but also the diminished ability of the satellite cells to respond to a mechanical stimulus at an older age in the present experiments.

In skeletal muscle, the nitric oxide that signals to activate, or maintain the quiescent state of satellite cells is very largely produced by NOS-I which is anchored to the sarcolemma via the DGC. Due to its position in muscle, NOS-I is a mechanical transducer and responds to mechanical stimuli such as shear forces, stretch, exercise, loading and trauma, by increasing expression and producing a bolus release of nitric oxide that activates satellite cells [28, 31, 253, 266-269, 304]. Alterations in NOS-I, evident in $NOS^{-/-}$ mice and muscular dystrophies prevent the activation of satellite cells

after a half-hour of cyclical passive stretch because the disrupted DGC and lack of NOS-I activity cannot produce the bolus release of nitric oxide. In the current study, aged muscle demonstrated a similar diminished or absent response to mechanical stimulation. Alternatively, if NOS-I is dislocated to the cytoplasm of the fiber, as demonstrated in a tail suspension model of young wild type mice [40], the availability of nitric oxide to satellite cells may be inadequate to surpass the threshold of NO concentration required to activate satellite cells. As stated by Rice and colleagues, an age-related disruption of DGC may contribute to the attenuated response of muscle from aged animals to passive stretch [39]. Drug treatments that supplement or inhibit nitric oxide were incorporated into this research to determine if NOS-I expression and/or activity are altered with age and if so, whether there is a significant potential for NO supplementation to activate satellite cells and stimulate muscle growth in older people.

Beyond 6 months-of-age, passive stretch of EDL muscles from normal mice did not elicit an increase in satellite cell activation. However, when LA was added to the whole-muscle culture medium of 8-month-old mice, satellite cell activation tended to rise, although not to significant levels, compared to the control group. Increasing sample size or testing the response to various doses of LA may have produced a statistically significant change. LA treatments have been used in single fiber cultures and in vivo experiments, including those on C57BL/6, mdx and $NOS^{-/-}$ mouse strains [27, 252, 295, 314]. Administration of LA resulted in both structural and functional improvements in studies that demonstrated that the NOS substrate increased satellite cell activation, increased utrophin and γ-sarcoglycan protein content, decreased eccentric contraction-induced damage, enhanced neuronal NOS activity, improved muscle regeneration

following crush injury, decreased exercise-induced muscle damage in pathological muscle tissue and increased distance traveled by mdx mice on a rodent wheel [27, 252, 295, 314]. However, Betters and colleagues showed that administration of LA to centrifuged single muscle fibers (isolated from the gastrocnemius muscle) from young, adult and old mice did not increase the number of $BrdU^+$ nuclei per fiber [29]. Our data confirm a deficient response in satellite cell activation to LA administration in muscle from both adult and old mice.

To our knowledge, this is the first time LA or ISDN have been combined with stretch to stimulate activation of satellite cells in cultured muscles from 8- and 18-month-old mice. Although LA did not increase mechanically-induced stretch activation in muscles from 8-month-old mice, administration of an exogenous source of NO (ISDN) did increase satellite cell activation significantly. These results suggest NOS-I expression, and possibly the DGC complex, may be altered in muscle as early as 8 months-of-age. ISDN does not have to be converted to NO by NOS-I activity, suggesting that satellite cell activation can be increased to greater levels with ISDN than LA treatment since the latter would require NOS activity to produce NO. In addition, when ISDN was combined with the NOS inhibitor LN, increased satellite cell activation still occurred. These data suggest, at 8 months-of-age, that satellite cells maintain the sensitivity to nitric oxide as an activating stimulus, since exogenous NO supplementation increased activation when combined with a mechanical stimulus.

Contrary to results from 8-month-old muscle, stretch and a variety of drug-treatment cocktails did not affect satellite cell activation in 18-month-old mice. It is possible that the DGC complex and NOS-I gene expression are more extensively altered

as age progresses which is an agreement with previous findings [39]. Disruption of the DGC complex, NOS-I expression and/or activity, as well as the activation of NOS-I by calcium calmodulin may require a greater dose of LA, compared to the dose used in the current study, in order to stimulate increased satellite cell activation. Also, with increasing age, the sensitivity of satellite cells to chemical stimuli may change, or the production of HGF by muscle fibers and sequestration of HGF in the extracellular matrix around fibers in aged muscle may be altered, and could also contribute, in part, to the absence of satellite cell activation observed after ISDN supplementation in cultures of muscle from older mice.

Regardless of the NO donor, endogenous and exogenous sources of nitric oxide have provided excellent structural and functional benefits to muscles studied in culture and in vivo. In muscle where NOS-I expression is perturbed (in *mdx* mouse muscular dystrophy), an exogenous source of nitric oxide would be the preferred treatment modality, since it does not require NOS-I activity to supply nitric oxide to the muscle. The challenge that lies ahead is to determine physiological levels of nitric oxide that would provide therapeutic benefits to muscle tissue without negatively affecting other tissues and organs. Since nitric oxide is involved in both the activation and quiescence of satellite cells, it has the potential to be effective in counteracting muscle wasting related to age, disease, or disuse.

4.5 Tables and Figures

Figure 4-1. Isolation of the extensor digitorum longus muscle
Precise dissection of the EDL muscle, here lifted gently away from the muscle bed in the hindlimb of a normal mouse. Careful removal of the EDL muscle is required to prevent stretch or damage of the muscle belly and the muscle fibers within it, which would result in activation of satellite cells [6].

Figure 4-2. Pinning of EDL muscle in FlexCell Plate™ in preparation for stretch experiments

Following isolation, EDL muscles were placed in PM and connective tissue was carefully removed under a dissecting microscope (50X magnification). Muscles were then placed in separate wells of a FlexCell™ plate and submerged in 2 ml of BGM. Under greater magnification (200X), tendons were carefully separated and then secured to the dish with insect pins. The proximal tendon of the EDL muscle was pinned to the rigid periphery of the well, while 3 insect pins were used to pin the distal tendons of the muscle toward the centre of the well. Muscles were pinned at resting length which was determined by observing length of muscle fibers at 200X magnification.

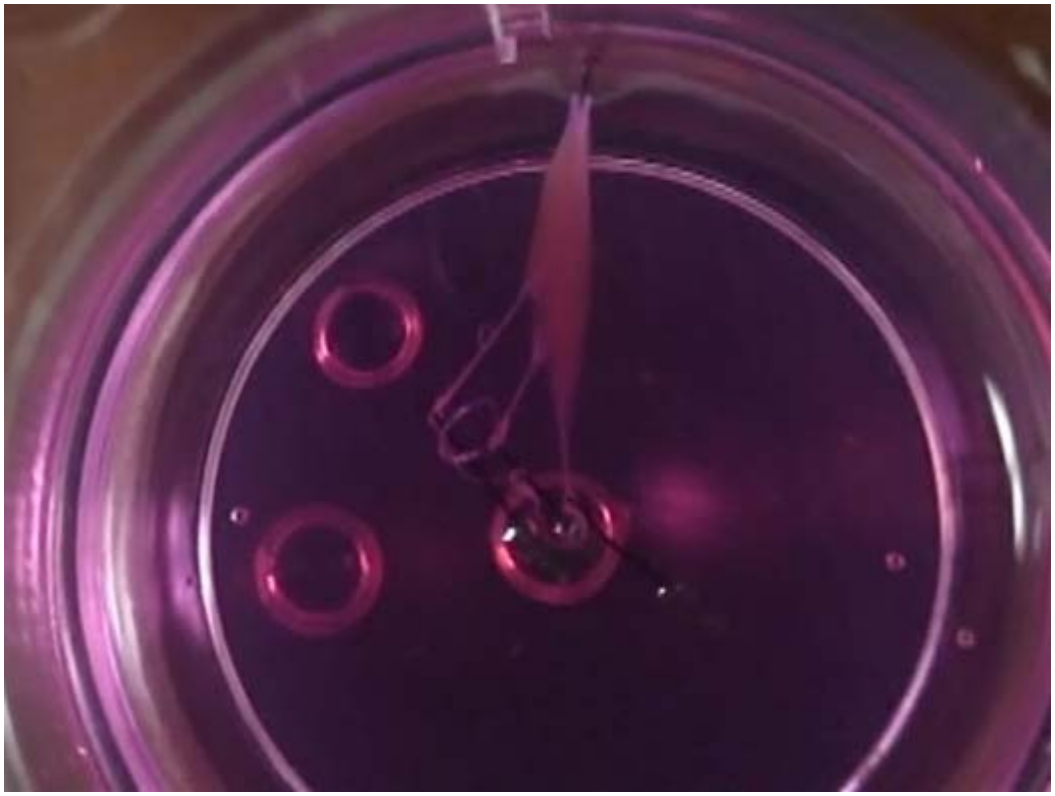


Figure 4-3. Effects of stretch magnitude on satellite cell activation in 6-month-old female mice

Percentage change in satellite cell activation of EDL muscles from normal, female 6-month-old mice with 10% and 20% stretch. Satellite cell activation was determined by scintillation counts standardized to μg of DNA. A 10% stretch, or lengthening of muscle, did not produce increased satellite cell activation whereas a 20% stretch increased satellite cell activation by 97% ($p<.05$). Sample size: n=7, except for n=6 in the 6-month-old US group. Values are expressed as means and SEM.

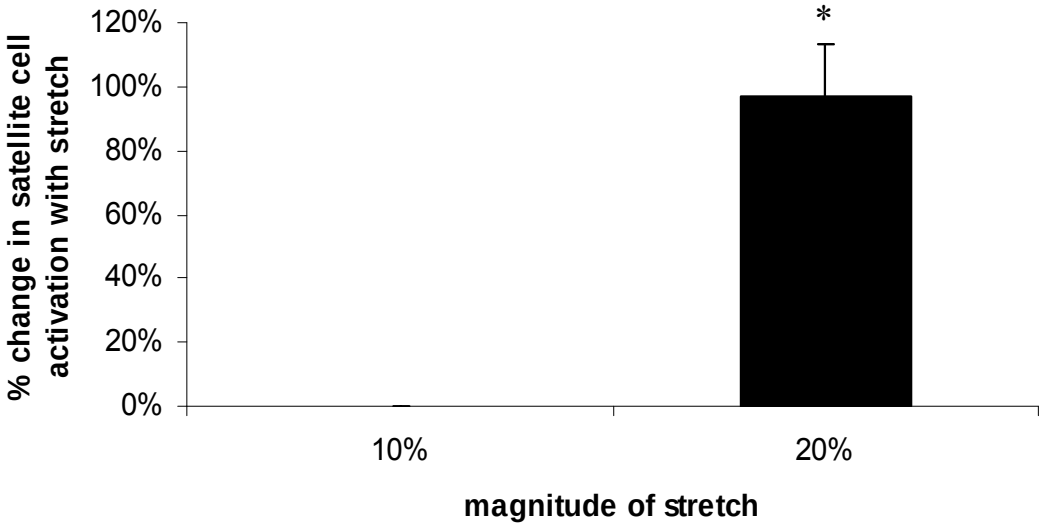


Figure 4-4. Effects of age on satellite cell activation in normal female mice
These data show the difference in satellite cell activation from the US EDL muscles of mice aged to 6 (n=13) weeks, 6- (n=6), 8- (n=11), 10- (n=7) and 18- (n=9) months-of-age. Muscles from 8-month-old-mice demonstrated greater satellite cell activation than all other age groups (*, $p<.01$). Activation of satellite cells was determined by measuring incorporation of tritiated thymidine into new DNA via scintillation counting. Satellite cell activation is expressed as dissociations per minute per microgram of DNA (DPM/μg DNA). Values are expressed as means and SEM.

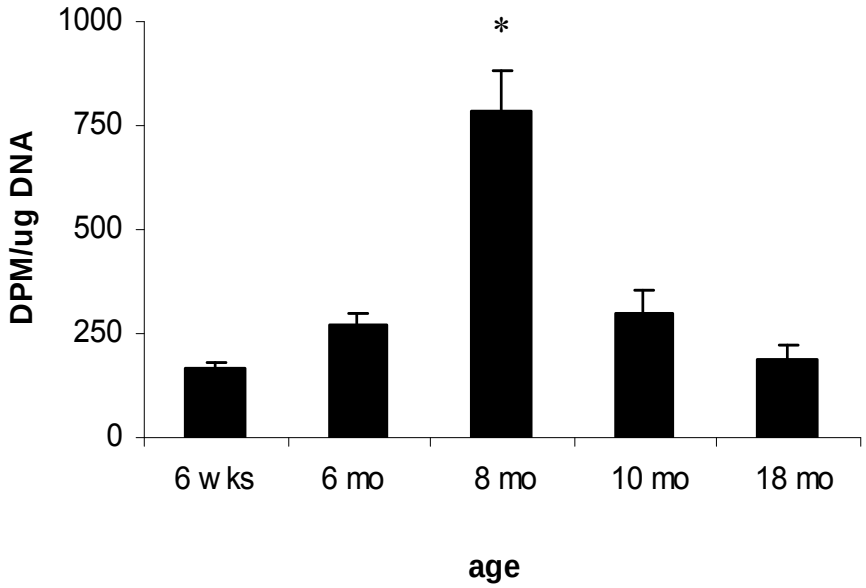


Figure 4-5. The effect of age on stretch-induced activation of satellite cells in cultured muscles

This graph shows the percent change in stretch-induced satellite cell activation in cultured EDL muscles isolated from normal female mice aged to 6 weeks (n= \14), or 6-(n=7), 8-(n=12), 10-(n=6) and 18-(n=11) months-of-age. EDL muscles from 6- week- and 6-month-old mice demonstrated a significant stretch-induced increase in satellite cell activation with a 10%- and 20%-length increase, respectively (*, $p<.01$). Muscles from mice aged to 8-, 10- and 18-months-of-age did not respond to the mechanical stretch stimulus. Values are expressed as means and SEM.

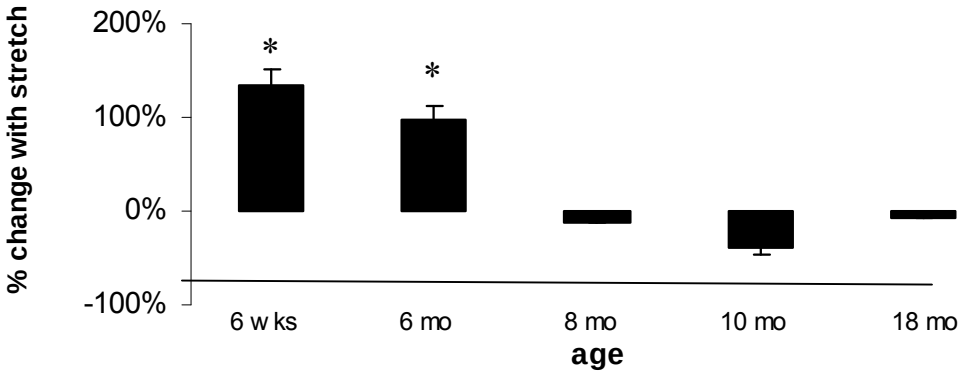


Figure 4-6. The effects of stretch and drug treatments on satellite cell activation in cultured EDL muscles prepared from 8-month-old mice

This graph demonstrates that stretch alone did not increase satellite cell activation. However, there was significantly greater satellite cell activation when stretch was combined with an exogenous source of NO (ISDN) or a combination of ISDN and the NO inhibitor (ISDNLN). A decrease in satellite cell activation was evident when S, LA and LN were combined (LALN). * indicates a significant difference from the unstretched (US) control group ($p<.01$). Values are expressed as means and SEM, sample size is indicated below treatment group on graph.

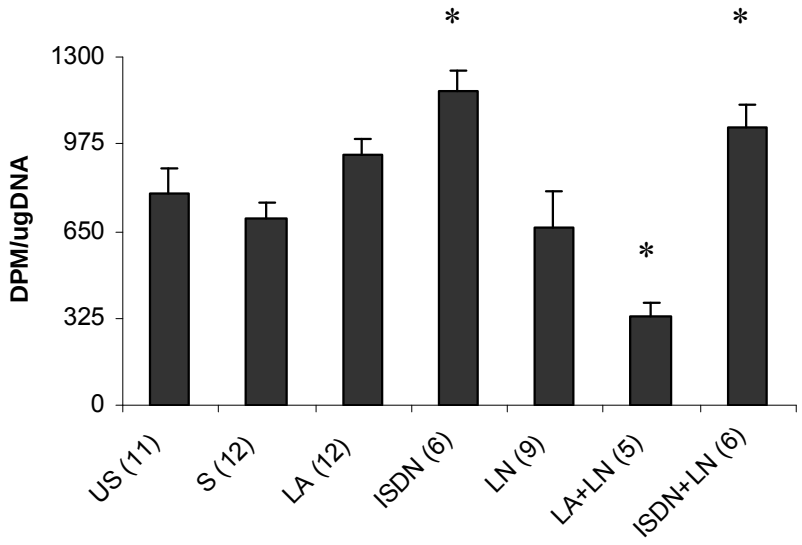


Table 4-1. The effects on satellite cell activation (DPM/ug DNA) of stretch and various drug treatments in cultured muscles from normal 8- and 18-month-old mice

(a) The effects of a mechanical stimulus and nitric oxide supplementation, inhibition, or combined treatments on satellite cell activation were investigated in whole-muscle cultures. Muscles treated with stretch and ISDN (ISDN), as well as, stretch, ISDN and LN (ISDNLN) had greater satellite cell activation than the unstretched control group. The combination of stretch, LA and LN (LALN) decreased satellite cell activation below the levels in muscles from the unstretched (US) control group (*, $p < .01$). Values are expressed as the mean and SEM of DPM/ug DNA. The number of muscles in each group is indicated by n.

(b) Muscle cultures from EDL muscles of 18-month-old mice subjected (or not) to stretch, or to stretch combined with nitric oxide supplementation, inhibition, or combined drug treatment. In contrast to results in muscles isolated from younger mice, satellite cells in muscle from 18-month-old mice did not demonstrate any change in satellite cell activation with stretch, or after stretch was combined with various drug treatments (same treatments as in table 4-1a). Values are expressed as the mean and SEM of DPM/ug DNA in each treatment group. Sample size of each group is indicated by n.

(a)

8-month-old mice							
	US	S	LA	ISDN	LN	LALN	ISDNLN
mean	786.86	697.86	931.6	1174.52*	666.00	330.83*	1039.70*
SEM	93.19	60.04	62.77	74.84	133.02	50.07	78.14
n	11	12	12	6	9	5	6

(b)

18-month-old mice							
	US	S	LA	ISDN	LN	LALN	ISDNLN
mean	187.48	173.25	127.90	105.51	129.93	181.23	189.76
SEM	34.3	13.32	14.24	10.31	11.49	50.14	78.47
n	9	11	6	5	5	5	5

CHAPTER 5. AGE-RELATED CHANGES IN STRUCTURE, FUNCTION, GENE EXPRESSION AND SATELLITE CELL ACTIVATION

5.1 Introduction

Sarcopenia is characterized by a loss of muscle CSA which is a secondary disease process to motor unit loss and 'wasting' of individual muscle fibers [375, 376]. Muscle mass is intimately associated with strength and since muscle strength and muscle mass decrease proportionately, the ability to function and perform activities of daily living decreases with age [248, 385]. Muscle satellite cells are the only cells capable of dividing and contributing to hypertrophy in postnatal muscle [217]. Adequate satellite cell pool size, normal sensitivity to activation stimuli and an unimpaired ability to add to the myonuclear number of a fiber is an essential cascade of events required to combat age-related muscle loss. Unfortunately, several studies report a decrease in satellite cell pool size with age in both rodents [22, 218, 277] and humans [443-445] although the results are not universal [22, 204, 207, 446, 447]. One possible reason for the lack of agreement between studies is the muscle being investigated. In 2006, Shefer and colleagues. reported a decline in the number of satellite cells associated with the EDL muscle of 12-month-old mice but decreased satellite cell pool size was not evident in the soleus muscle until 28-30 months-of-age [22]. A major difference between the two muscles is fiber type since the EDL muscle consists of mainly type II fibers (91 ± 13%) whereas the soleus muscle has a larger percentage of type I fibers (44 ± 4%) [65]. On average, type II muscle fibers have a larger CSA than type I fibers and consequently, are more affected by muscle atrophy [110].

In addition to a lack of agreement on changes in satellite cell pool size with age, muscle satellite cells from aged muscle demonstrate a long latent period between an

activating stimulus and activation, up to 24 hours [22-24]. This delayed activation of satellite cells from aged contractile tissue may account for the decreased capacity of satellite cells to repair or regenerate muscle to the same extent as in young animals [218, 278, 441]. We have also demonstrated that whole-muscle (EDL) cultures from mice aged to 6-months-of-age require twice the %length increase via passive stretch to increase satellite cell activation compared to 6-week-old mice (refer to section 4.2a). Taken together, the results of previous studies motivated us to investigate the structural and functional changes that occur in skeletal muscle with age to determine if these changes are related to protein content, and satellite cell activation and/or satellite cell pool size.

5.2 Methods

5.2a Animals

All study activities were approved by the Protocol Management and Review Committee at the University of Manitoba and were in accordance with the guidelines set forth by the Canadian Council on Animal Care. Four age groups of normal C57/BL6 female mice (3-, 8-, 12-, and 18-month-old) were used in the study and were bred and housed at the University of Manitoba's Central Animal Care Services with the exception of a population of mice imported from The Jackson Laboratory (Bar Harbor, Maine, USA) at 8 months-of-age. The Jackson Laboratory mice were not used in any study related activities for at least four months to ensure acclimatization to the new environment had occurred. Mice had access to water and food ad libitum and were exposed to an alternating 12-hour light/dark cycle.

5.2b Body Mass

Mice were weighed before the initiation of grip strength testing since values were used to normalize the quantity of muscle force production to body mass (g). Each group consisted of 6 animals and mice were weighed immediately before grip strength testing.

5.2c Grip Strength

The force produced by the forelimb muscles of mice aged to 3-, 8-, 12-, and 18-months-of-age was recorded with a calibrated [448] Chatillon strain gauge (Chatillon, DFM-2.0kg, Greensboro, North Carolina). Animals were firmly grasped by the tail, as close to the body as possible, removed from the cage and held in a vertical orientation over the trapeze bar of the strain gauge until the mouse proceeded to grasp the bar with both forelimbs. When a bilateral grip of the bar was obtained, the mouse was lowered to a horizontal orientation to ensure the forelimbs and the body of the mouse was parallel to the trapeze bar of the force measurement device (figure 5-1). Each mouse performed 3 sets of 5 repetitions within one minute, rested for 6 minutes and then executed 3 more repetitions until a total of 15 repetitions were completed. Between each set, mice were returned to the cage and allowed to drink and eat ad libitum. Total test time was approximately one hour. Absolute mean grip strength was obtained by taking the mean value of repetitions 1-3 from sets 1– \5. Absolute maximum grip strength was determined by extracting the highest force recorded in repetitions 1–3 from sets 1–5. Absolute mean and maximum grip strength values were adjusted for body mass to obtain relative mean- and relative maximum-grip strength.

5.2d Muscle Isolation

Mice were anaesthetized with PRAErrane Isoflourane USP (Baxter Corporation, Mississauga, ON) and then immediately sacrificed by cervical dislocation. The extensor digitorum longus (EDL) muscles were isolated with precise dissection and placed in proliferation medium (PM; 1X DMEM with 10% fetal bovine serum, 2% chick embryo extract, 1% antibiotic/antimytotic and 0.1% gentamycin). The connective tissue of EDL muscles was removed under a dissection microscope (5X magnification) and placed in individual wells of FlexCell^{TM} plates containing 2 ml basal growth medium (BGM; 1X DMEM with 2% controlled replacement serum-2 diluted in 18% 5X DMEM, 1% fetal bovine serum, 1% antibiotic/antimycotic and 0.1% gentamycin). Under greater magnification (20X), muscle tendons were secured with insect pins to ensure muscles were at resting length. Each well was treated with 4 µl of tritiated thymidine and placed in an incubator, set at 37° C, for 22- (3- and 8-month-old mice) or 44- (12-and 18-month-old mice) hours. Following 22 hours of incubation for 3- and 8-month-old mice and 44 hours for mice aged to 12- and 18-months-of-age, the tendons of EDL muscles were removed. Then, the muscle was washed in 1X cold PBS and weighed. EDLs were placed immediately on ice, minced with a razor blade and homogenized. Following DNA extraction (refer to Section 4.1e), 300 µl of the homogenate were placed in 5 ml of scintillation fluid for scintillation counting and 5 ul were added to 200 ul of Hoeschst dye for quantification of DNA (refer to Section 4.1e). Following these processes, dissociations per minute (DPM) were adjusted per ug of DNA. DPM/ug DNA was used as a measure of satellite cell activation.

5.2e Muscle Fiber CSA

Instantly proceeding EDL isolation, GAST muscles were removed from the hindlimb of each mouse. GAST muscles were sectioned transversely and placed in a cryomold (Tissue-Tek®, Sakura Finetek, Torrance, California) containing OCT compound (Shandon Cryomatrix^{TM}), submerged in isopentane (≤ -50° C), and placed in a -20° C freezer. Muscles were sectioned with a cryotome at a thickness of 8 um. Muscle sections were stained with Harris Hemotoxylin (Fisher, Hampton, New Hampshire) and Eosin. Slides were viewed with an Olympus BH2 fluorescence microscope (Olympus America Inc., Parkway, PA) and images were captured with a Sony 3 chip colour CCD camera (Sony, USA). Images were imported into NIH ImageJ software and the CSA of each muscle fiber was calculated. A minimum of 150 fibers from each animal and at least 1200 fibers from each age group were analyzed.

5.1f In Situ Hybridization

In situ hybridization for Pax7, myogenin and MyoD RNA was performed to identify all satellite cells/fiber since at least one of Pax7, myogenin and MyoD will be expressed during quiescence, activation and proliferation. Muscle sections of GAST muscle were cleaned in 1X PBS and incubated for 3 hours and then fixed for 15 minutes in 4% paraformeldahyde. GAST muscle sections were again cleaned in 1X PBS and incubated under cover slips with hybridization buffer for 1 hour at 42°C. Cover slips were removed and sections were rinsed in 1X PBS. The hybridization cocktail was prepared which included hybridization buffer (1:200 ng/ml), myogenin AS (Santa Cruz Biotechnology, Santa Cruz, CA; 300 ng/ml), MyoD AS (Santa Cruz Biotechnology, Santa Cruz, CA; 300ng/ml), and Pax7 AS (Santa Cruz Biotechnology, Santa Cruz, CA;

300 ng/ml). The hybridization cocktail was warmed for 15 minutes at 65°C; sections were covered uniformly with the cocktail; cover slips were carefully placed on each slide and sealed with rubber cement (Elmer's, Toronto, ON); and then slides were hybridized overnight in a humid chamber at 42° C. The following day, rubber cement was removed and slides were prepared for analysis.

The number of satellite cells expressing at least one of Pax7, myogenin or MyoD RNA was determined by viewing slides at 200X magnification under oil immersion. Only positive cells lying between the sarcolemma and basement membrane of the muscle fiber were included, interstitial satellite cells staining positive for Pax7, MyoD or myogenin were excluded. Values were expressed as the number of positive satellite cells per field and only fields filled entirely by muscle fibers were included.

5.2 g Westerns Blotting Experiments for NOS-I, myostatin, and Myf5

Protein was isolated from the section of GAST muscles that was not used for sectioning. Western blot analysis, as per standard immunodetection protocols, was performed to determine the expression of Myf5, NOS-I, and myostatin. Individual muscles were homogenized in lysis buffer, incubated on ice for 60 minutes and then centrifuged for 5 minutes at 10 000 rpm. To determine protein concentration, 1 µl of each sample was dyed with Bradford Reagent (Sigma Aldrich, St. Louis, MO) and analyzed with an Ultraspec 2100 pro UV/Visible Spectrophotometer (Biochrom Ltd, Cambridge, England). Following isolation of protein, samples (20 ug of protein) were loaded on a 12% acrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Osmonics, Minnetonka, Minnesota).

Membranes were stained with Ponceau (to ensure successful transfer of gel), blocked with buffer containing 5% skim milk powder (Nestle Carnation, USA) for 60 minutes, and incubated overnight with NOS-I antibody (Abcam, Cambridge, MA; 1:200) at 4°C. The following day, membranes were washed and then blocked in buffer with ECLTM Anti-rabbit Ig, Horseradish Peroxidase linked F primary antibody fragment from donkey (GE Healthcare, Little Chalfoot Buckinghampshire, UK; 1:2000) for one hour, washed again, and shaken in 2 ml of ECL Western Blotting Luminescence Reagent (Santa Cruz Biotechnolody, Santa Cruz, CA) for 1 min. PVDF membranes were exposed for various lengths of time depending on intensity of signal. Following NOS-I detection, membranes were stripped by being submerged in a stripping solution (70 °C) for 20 minutes. Membranes were washed in 1X PBS (3 X 10 min) and the same process as described above was executed with Myf5 antibody (1:200). Upon completion of Myf5 detection, membranes were stripped and prepared for detection of myostatin (1:200). The film was analyzed with a densitometer (accumulated densities), optical densities were calculated and background was subtracted. It should be noted that protein expression in GAST muscle from 18-month-old mice was scanned in a PC and then analyzed with NIH ImageJ software. The apparent changes in sensitivity of the anti-Myf5 antibody to detecting Myf5 (before and after lab relocation), explains the missing data point for Myf5 in the table for 18-month-old mice. Since protein expression in GAST muscle from 8-month-old mice was analyzed with both techniques, a calibration factor was used to standardize the protein expression from muscle of 18-month-old mice (6 samples for both NOS-I and myostatin). The standardization of data from one lab with data from another lab was done in as rigorous a manner as possible, considering that samples were aging

and that another set of 18-month-old animals could not be aged for that dataset. The original design of the experiment was to collect all the data on one set of animals, and that would not have been possible if there was a requirement for another set of animals. The calibration factor was calculated by using the mean optical density of protein expression of 8-month-old muscle (determined by densitometer) and dividing it by the optical density of protein expression from a different sample of muscle from 8-month-old mice (determined by NIH ImageJ software). Protein expression from the GAST muscle of 18-month-old mice (determined by NIH ImageJ software) was then multiplied by the calibration factor specific to each protein being analyzed. Considerations of the aging samples versus the need to age another whole set of animals interfered with completely consistent data collection.

5.2h Data Analysis

An analysis of variance (ANOVA) was incorporated to investigate the differences in body mass, muscle mass, muscle fiber CSA, expression of NOS-I, Myf5, and myostatin protein expression, absolute mean and maximum grip strength, relative mean and maximum grip strength, and the total number of satellite cells between age groups. In addition, a Chi squared analysis was performed to detect differences in the distribution of satellite cells per field between mice aged to 3-, 8-, 12- and 18-months-of-age. Fisher's least significant differences (LSD) post hoc tests were used to identify statistical difference between groups with a significance level of $p<.05$.

5.3 Results

5.3a Body Mass

The body mass of mice was greater at 8- and 12- months-of-age compared to animals 3 months-of-age (table 5-1). Mice aged to 18 months-of-age demonstrated similar values as 3- and 12-month-old mice but weighed less than 8-month-old mice ($p<.05$).

5.3b Muscle Mass

The mass of EDL muscles was greatest at in adulthood at 8- and 12-months-of-age such that the mass of muscles from 12 month-old-mice was greater than the muscle mass of animals aged to 3- and 18-months (table 5-1). EDL muscles from 18-month-old mice weighed significantly less than 8- and 12- month-old animals and atrophied below the mass of muscle from mice aged to 3 months-of-age (figure 5-2).

5.3c Grip Strength

Absolute mean grip strength was the average of 3 sets of 5 repetitions per mouse and 6 mice were in each age group. Therefore, absolute mean strength reflected the average of 90 repetitions for the 3-, 8-, 12-, and 18-month-old age groups. In general, absolute mean grip strength decreased with age. Three-month-old mice had significantly greater absolute mean grip strength than all other ages and 18-month-old mice had less than all other age groups, although there was no significant difference between 12- and 18-month-old mice (table 5-1). The absolute mean grip strength of mice aged to12 months-of-age was greater than 18-month-old mice but lower than 3- and 8-month-old mice.

The absolute maximum grip strength of 18-month-old mice was lower than 3-, 8- and 12-month-old mice. Three-month-old mice had greater absolute maximum grip strength than 12- and 18-month-old animals (table 5-1). Increasing age is associated with a decrease in absolute maximum strength and is most evident at 12- and 18-months-of-age (figure 5-3).

Relative mean grip strength was determined by standardizing mean absolute grip strength to body mass which is more related to function than absolute grip strength. The oldest mice had less relative grip strength than mice aged to 3- and 8-months-of-age but not statistically different from 12-month-old mice. Mice aged to 3 months-of-age had the greatest grip strength but there was no difference in relative mean grip strength between 8- and 12-month-old mice (table 5-1).

Relative maximum grip strength of 18-month-old mice was less than 3- but not 8-, and 12-month-old age groups. Relative maximum grip strength of 3-month-old mice was greater than all other age groups (table 5-1). Decreased body mass in 18-month-old mice may be an adaptation to maintain relative muscle strength since 18-month-old mice did not differ in relative maximum force production compared to mice aged to 8- and 12-months-of-age.

5.3d Muscle Fiber CSA

Eighteen-month-old mice demonstrated decreased muscle fiber CSA compared to mice aged to 3-, 8- and 12-months-of-age. Muscle fiber CSA peaked at 8 months-of-age and demonstrated a stepwise decrease in mice aged to 12- and 18-months-of-age (table 5-1, figure 5-4). Age-related atrophy of individual fibers is evident by one year and continues to decrease up to 18 months-of -age.

5.3e Number and distribution of Satellite Cells

Tissue sections from the GAST muscle of 18-month-old mice demonstrated a significantly fewer mean number of satellite cells per field than animals 3-, 8- and 12-months-of-age. Interestingly, the number of satellite cells per field was greatest at 12 months-of-age but was greatly reduced by 18 months-of-age. The number of satellite cells in the GAST muscle of 18-month-old mice was less than all other age groups.

A Chi squared analysis of satellite cell distribution demonstrated that 18-month-old mice (38.9 %) had significantly more ($p<.01$) fields without any cells positively stained for Pax7, MyoD and myogenin than 3- (17.8 %), 8- (9.9 %), and 12-month-old mice (4.2 %). This data suggests that muscle fibers from aged mice have fewer satellite cells per fiber compared to younger mice (figure 5-5). Fewer satellite cells per fiber indicate that satellite cell pool size does decrease with age in the GAST muscle.

5.3f Satellite Cell Activation

Satellite cell activation in the EDL muscles of 18-month-old mice was significantly less than 3-, 8- and 12-month-old mice. This is in agreement with whole muscle culture studies of EDL muscles (refer to Chapter 4) in which satellite cell activation in muscle from 18-month-old mice was significantly less than satellite cell activation in younger, 8-month-old mice but no difference between aged- and younger-mice. Mice aged to 8 months-of-age demonstrated greater satellite cell activation than all other age groups. However, at 12 months-of-age, satellite cell activation was less than 8-month-old mice. Satellite cell activation was decreased even further at 18 months-of-age; less than all other age groups (table 5-1, figure 5-6).

5.3g NOS-I, myostatin, and Myf5 Protein Content

There was no statistically significant difference in NOS-I expression between any of the age groups (figure 5-7). NOS-I protein content was greatest in muscle from 8- to 12-month-old-mice and lowest in mice aged to 3- and 18-months-of age (table 5-1).

There was an upregulation of myostatin in muscle from 18-month-old mice compared to protein levels of myostatin in muscle from mice aged to 8- and 12-months-of-age. There was no difference in the protein content of muscle in 3- and 18-month-old mice (figure 5-7, table 5-1).

Myf5 protein content in GAST muscle from 12-month-old mice was greater ($p<0.01$) than Myf5 levels in muscle from 8- but not 3-month-old mice (figure 5-7, table 5-1).

5.4 Discussion

Results demonstrated significant fiber atrophy and sarcopenia at 18-months-of age in normal female mice. These changes in muscle structure were accompanied by a significant reduction in satellite cell number and activation, as well as, reduced grip strength. Muscle from the GAST muscle of 18-month-old mice also showed an increase in myostatin, a gene that inhibits muscle growth. Taken together, the results of this set of experiments suggests that age-related changes in muscle structure and function are, to some extent, a result of a deficient contribution of muscle satellite cells to the processes of muscle maintenance and growth. In addition, basal levels of satellite cell activation may be inhibited by increased expression of myostatin with age.

In order to increase our understanding of the mechanisms responsible for sarcopenia, structural and functional measurements of skeletal muscle were combined

with investigations of gene expression (protein levels). NOS-I, Myf5 and myostatin were selected for analysis since the expression of all three genes is intimately linked with satellite cell activation and muscle growth. Increased NOS-I activity is associated with an increased production of NO which is a chemical that stimulates satellite cell activation. Myf5 is a myogenic regulatory factor with a key role in regulating the differentiation of satellite cells. Myostatin is a growth factor that inhibits the growth of muscle tissue. Changes in expression (protein levels) of these genes with age may be a limiting factor in muscle growth and contribute to the decline in muscle size and strength evident in older animals.

Results from the current study demonstrated that the EDL muscle mass of 18-month-old mice was less than all other age groups including mice aged to 12-months-of-age. Muscle mass peaked at 12 months-of-age but had decreased below all other age groups six months later. At 18 months-of-age, both the mass of EDL muscles and the CSA of GAST muscles were less than 3-, 8-, and 12-month-old age groups. The GAST and EDL muscles of wild type mice contain over 90% of type II fibers [65] and are affected by age to a greater extent than type I fibers [385]. In agreement with the results from Ludatschen and colleagues (1983), the GAST muscles of 18-month-old female C57BL/6 mice in this study demonstrated decreased muscle fiber CSA compared to younger animals [449]. Muscle fiber CSA was greatest at 8 months-of-age but was negatively correlated with age at 12- and 18-months-of-age. Ludatschen and colleagues (1983) reported that there was not much difference between the diameter of muscle fibers from 19- and 27-month-old mice, which combined with the results of this study, suggest age-related muscle atrophy begins as early as 1 year after birth [449]. It is also important

to note that body mass steadily decreased after 8 months-of-age and may be a compensatory aging mechanism to account for loss of force production in aging contractile tissue. All age groups were fed ad libitum and housed in equal sized cages, therefore, external influences did not cause the changes in body mass between age groups. The morphological changes in lower limb muscles were accompanied by decreased function in the forelimb muscles which suggests that age-related atrophy affects all weight bearing limbs. After 3 months-of-age, there is a negative association between absolute mean and maximum forelimb grip strength up to 18 months-of-age. By 18 months-of-age, maximum absolute grip strength decreased by over 30%. However, when maximum grip strength was adjusted for body mass (relative maximum grip strength) old mice did not show a difference in grip strength compared to younger, 8- and 12-month-old animals. Relative grip strength is more functionally relevant since force production, expressed in relation to body mass, indicates the ability of the animal to manipulate and lift body mass if necessary. These results suggest that C57/BL6 mice have undergone several age-related changes in muscle structure and function including decreased muscle mass, decreased fiber CSA and decreased absolute maximum grip strength. Although it has not been determined if structural changes are the cause or effect of functional deficits, the variables are closely linked and feedback on one another to play a major role in the progression of sarcopenia.

Although the mechanisms responsible for the progression of age related muscle wasting have yet to be realized, one fact is for certain, satellite cells from the host or donor must be activated by chemical or mechanical stimuli to initiate muscle growth and combat atrophy. The purpose of the current study was to not only investigate the

progression of structural and functional changes that occur with age but also to determine how satellite cell pool size and function (activation) are affected during the aging process. Triple in situ hybridization for Pax7, MyoD, and myogenin RNA indicated that the number of satellite cells in aged muscle declined by 18 month-of-age. In fact, almost 40% of the microscopic fields from 18-month-old mice did not contain any satellite cells associated with muscle fibers, in comparison to only 4.2% of the fields from 12-month-old mice. This does not mean there were no satellite cells on fibers in those fields, only that this sampling approach has demonstrated a decrease in the overall frequency of satellite cells in muscle from aged mice. Compounding the problem are the results that indicate satellite cell activation in 18-month-old mice is less than all the other age groups and that activation decreases significantly after 8 month-of-age. Therefore, aged muscle not only has fewer satellite cells, the satellite cells that are associated with muscle fibers demonstrate decreased activation patterns which may limit the ability of satellite cells to combat sarcopenia by becoming activated and contributing to muscle growth.

In addition to the changes in satellite cell number and activation with age, Myf5 and myostatin gene expression also differed between some of the age groups. Myostatin was upregulated in the GAST muscle from 18-month-old mice which suggests the inhibition of muscle growth is greater in older animals [13, 440-442]. This may account for decreased satellite cell activation and muscle fiber CSA in muscle from older mice compared to the contractile tissue from younger animals. Increased Myf5 protein content in muscle from 12-month-old animals suggests there are a greater number of activated satellite cells that have not entered S-phase compared to muscle from 8-month-old mice since Myf5 expression decreases during the S phase of the cell cycle [450]. This may

indicate that muscle from 12-month-old mice are in a 'compensatory state' in an attempt to combat age-related muscle atrophy by increasing the contribution of satellite cells to existing muscle fibers. Although this was not reflected in levels of satellite cell activation, satellite cell distribution profiles indicated that there was an increase in the percentage of microscopic fields that contained a higher number of satellite cells in muscle from 12-month-old mice compared to younger mice. This was further confirmation that satellite cells are being recruited in an attempt to prevent, or minimize, muscle atrophy.

Although there were no differences in NOS-I expression between any of the age groups, western analysis is not sensitive to enzyme activity. Therefore, the production of NO by NOS-I may differ between age groups and contribute to decreased levels of satellite cell activation in muscle from older mice. In addition, muscle atrophy in younger mice, via tail-suspension models of muscle disuse, is associated with a dislocation of NOS-I into the cytoplasm of muscle fibers [40]. This not only increases atrophic signaling in muscle fibers, but also may decrease the availability of NO to satellite cells which would decrease satellite cell activation. In this case, NOS-I protein content or activity may not change but the results of NO production may be contradictory to what takes place in muscle when NOS-I is not dislocated from the DGC. Future studies are required to determine if NOS-I activity changes with age or if NOS-I is in fact dislocated from the DGC into the cytoplasm of the muscle fiber.

Loss of muscle mass, fiber CSA and strength are changes in muscle that occur with age and have been well documented. However, the underlying mechanisms responsible for the progression of sarcopenia and the resultant wasting of muscle have yet

to be realized. A major reason as to why the mechanism responsible for age-related muscle atrophy has not been discovered is the interaction of the 12 other systems of the body with muscle tissue. Muscle is the most abundant insulin sensitive tissue in the body and accounts for up to 50% of body weight. At rest, it is responsible for 20-30% of oxygen consumption and 75–90% of glucose absorption [353]. Therefore, it is likely that muscle atrophy is a multifactoral process that requires further investigation in several disciplines to effectively and efficiently reverse the age-related negative changes in muscle.

5.5 Tables and Figures

Figure 5-1. Set-up for forelimb grip strength testing
Grip strength testing was performed with a Chatillon strain gauge (Chatillon, DFM-2.0kg, Greensboro, North Carolina). Mice were gently pulled by the tail when body and forelimbs were parallel to force transducer. Each mouse performed 5 sets of 3 repetitions for a total of 15 repetitions. Each set was performed within 1 minute with a minimum of 6 minutes between sets to eliminate fatigue as a variable.

Figure 5-2. The muscle mass of EDL muscle from mice aged to 3-, 8-, 12- and 18-months-of-age

All tendons were removed prior to weighing of the contractile tissue. Eighteen-month-old mice had significantly less muscle mass than 8- and 12-month-old mice and decreased to levels below mice aged to 3 months-of-age, although not significantly. Significantly different from mice aged to *3-, $^{+}$ 8-, $^{\#}$ 12- and $^{\sim}$ 18-months-of-age (p<. 01).

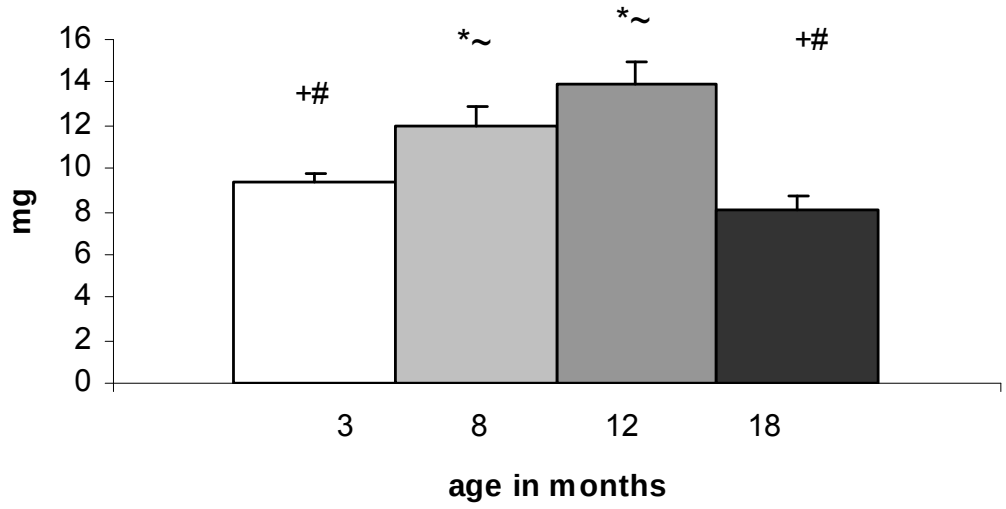


Figure 5-3. The effects of age on absolute maximum grip strength measurements

Absolute maximum grip strength of mice aged to 3-, 8-, 12- and 18-months-of-age was determined by extracting the highest recorded force value for repetitions 1-3 in sets 1-5. Eighteen-month-old mice recorded the lowest absolute maximum grip strength of all age groups. Significantly different from mice aged to *3-, $^{+}$8-, $^{\#}$12- and $^{\sim}$18-months-of-age ($p<.05$).

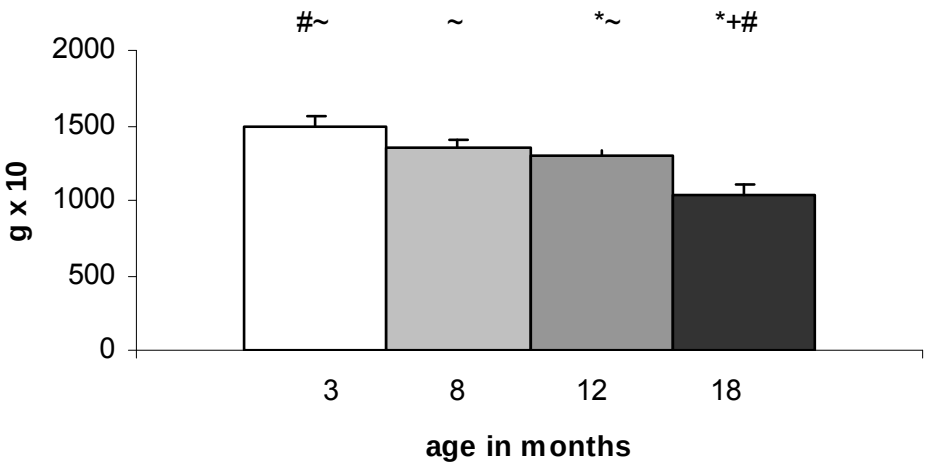


Figure 5-4. Muscle fiber CSA from the Gastrocnemius muscle of 3-, 8-, 12- and 18-month-old C57/BL6 mice

Muscle sections were cut with a cryotome at thickness of 8 um. The GAST muscles were isolated from 6 mice of each age group and a minimum of 1200 fibers (at least 150 from each mouse) were selected for analysis. Muscle CSA was determined with NIH ImageJ software and results indicate that muscle fibers from 18-month-old mice have significantly less CSA than mice from all other age groups. Muscle fiber CSA peaked at 8 months-of-age and then significantly declined in mice aged to 12- and 18-months-of-age. Significantly different from *3 months, $^{+}$ 8 months, $^{\#}$ 12 months and $^{\sim}$ 18 months (p<0.05).

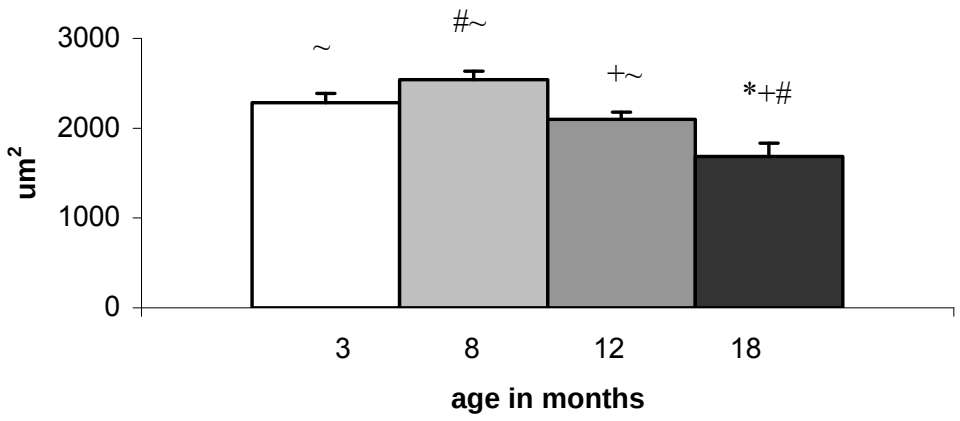


Figure 5-5. The effects of age on satellite distribution

Percentage of satellite cells per age group from the GAST muscles of 3-, 8-, 12- and 18-month-old mice. Positive cells stained for at least one of Pax7, MyoD, or myogenin RNA; only cells located in the satellite cell position were included, cells positioned in the interstitial space were excluded. A Chi squared analysis ($p<.01$) indicated that 18-month-old mice had the greatest number of fields without any associated satellite cells which suggests the satellite cell pool size of 18-month-old mice is less than all the other age groups.

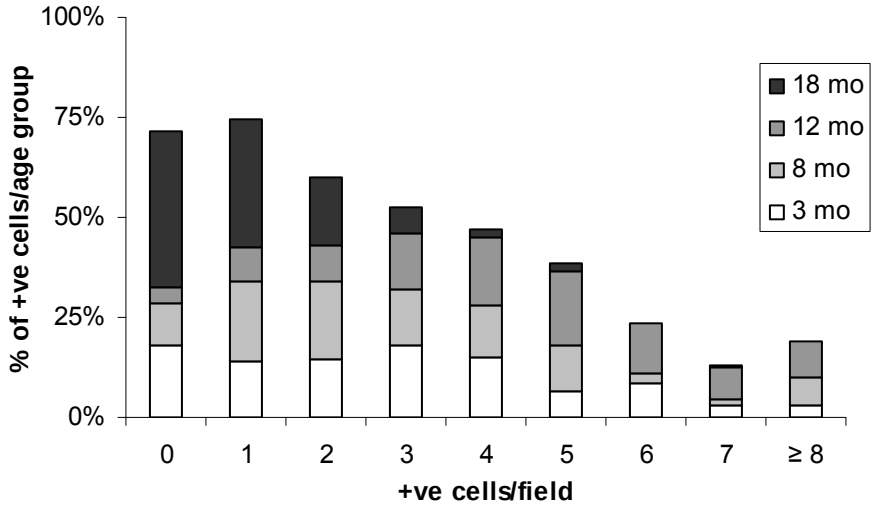


Figure 5-6. Age-related changes in satellite cell activation

Satellite cell activation was recorded in units of DPM/ug DNA and was determined by treating whole EDL muscles in culture with [^{3}]H-thymidine and harvesting muscles either 22- (3- and 8-month-old mice) or 44- (12- and 18-month-old) hours later. A DNA Hoechst assay was performed to determine ug of DNA in the sample used for scintillation counts. Similar to muscle mass and fiber CSA, 18-month-old mice demonstrated less SC activation than any other age group. Significantly different from mice aged to *3-, $^{+}$8-, $^{\#}$ 12- and $^{\sim}$18-months-of-age (p<0.05).

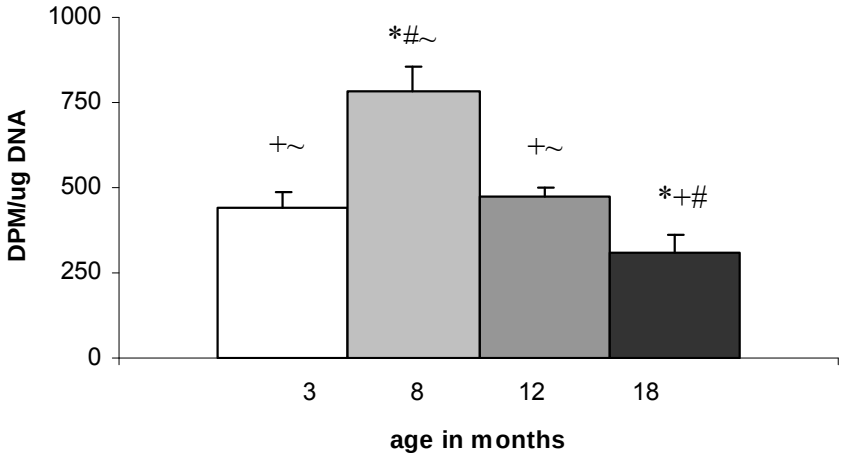


Figure 5-7. Myostatin, NOS-I and Myf5 protein content in GAST muscle from 3-, 8-, 12- and 18-month-old normal female mice

Twelve-month-old mice demonstrated increased Myf5 protein content in the GAST muscle compared to levels of Myf5 detected in muscle from 8- but not 3-month-old mice. Muscle from 18-month-old mice demonstrated increased myostatin protein content compared to muscle from 8- and 12-month-old mice. Significantly different from mice aged to *3-, $^{+}$ 8-, $^{\#}$ 12- and $^{\sim}$ 18-months-of-age (p<.05).
Note: Myf5 protein content could not be obtained from the muscle of 18-month-old mice due to technical difficulties.

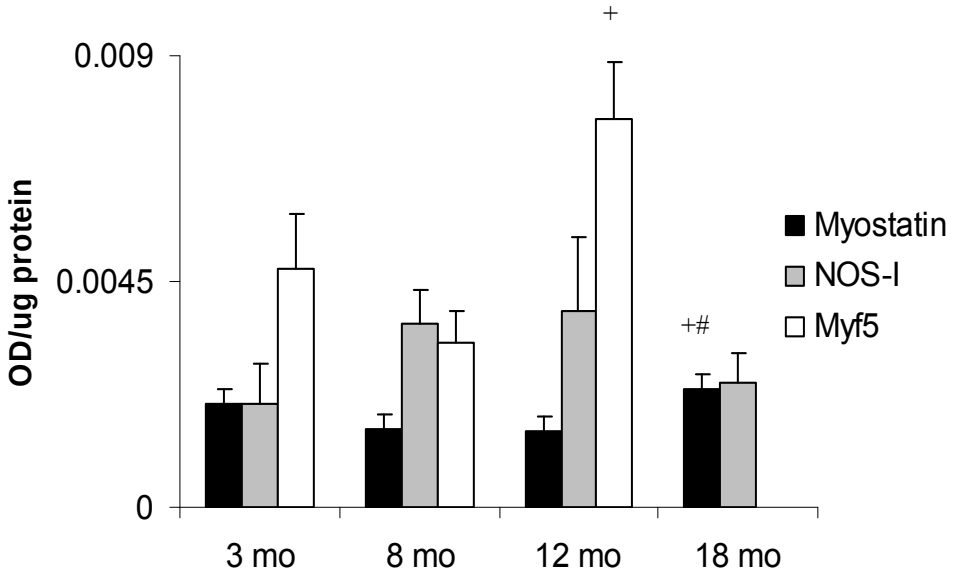


Table 5-1. Change in body mass, muscle mass, fiber CSA, grip strength, gene expression and satellite cell activation with age

Mean (SEM), and significance values for body mass, muscle mass, fiber CSA, absolute mean grip strength (Abs mean GS), absolute maximum grip strength (Abs max GS), relative mean grip strength (Rel mean GS), relative maximum grip strength (Rel max GS), NOS-I protein levels, Myf5 protein levels, myostatin protein levels, Pax7/myoD/myogenin positively stained cells, and satellite cell activation (SC activation) from 3-, 8-, 12-, and 18-month-old female C57/BL6 mice. Significantly different from mice aged to *3-, $^{+}$ 8-, $^{\#}$ 12- and $^{\sim}$ 18-months-of-age.
Note: Myf5 protein content could not be obtained from the muscle of 18-month-old mice due to technical difficulties.

	3 mo	8 mo	12 mo	18 mo	*p*
Body mass (g)	25.6 $^{+\#}$ (1.04)	34.57 $^{*\sim}$ (0.98)	32.22* (3.13)	28.47^{+} (1.05)	<.01
Muscle mass (mg) - EDL	9.43 $^{+\#\sim}$ (0.36)	11.93 $^{*\sim}$ (0.89)	13.93$^{*\sim}$ (1.03)	8.07$^{+\#}$ (0.70)	<.01
Abs mean GS (g x 10)	1123.33 $^{+\#\sim}$ (60.7)	1106.57 $^{*\sim}$ (25.6)	907.89* (39.36)	779.07 $^{*+}$ (17.17)	<.01
Abs max GS (g x 10)	1493.33 $^{\#\sim}$ (69.12)	1351.67 $^{\sim}$ (58.96)	1296.67 $^{\#\sim}$ (33.53)	1036.67 $^{*+\#}$ (59.93)	<.01
Rel mean GS (g x 10/BM)	43.83 $^{+\#\sim}$ (1.08)	32.07 $^{*\sim}$ (0.68)	29.15 * (2.28)	27.72 $^{*+}$ (0.71)	<.01
Rel max GS (g x 10/BM)	58.60 $^{+\#\sim}$ (2.95)	39.10 $^{\sim}$ (1.26)	42.36 * (4.57)	36.83 * (1.93)	<.01
Fiber CSA (um^2) - GAST	2284.64 $^{\sim}$ (101.18)	2541.72 $^{\#\sim}$ (91.79)	2098.26 $^{+\sim}$ (80.18)	1685.96 $^{*+}$# (144.31)	<.01
Pax7/Myf5/myogenin (+ cells/field) - GAST	2.87 $^{\#}$ (0.14)	3.18 (0.14)	4.37 $^{*\sim}$ (0.14)	1.11 $^{\#}$ (0.08)	<.01
SC activation (DPM/μg DNA) - EDL	443.50 $^{+\sim}$ (51.51)	848.61 $^{*\#\sim}$ (40.77)	471.52 $^{+\sim}$ (31.06)	310.67 $^{*+\#}$ (48.56)	<.01
NOS-I (OD/ug protein) - GAST	0.002 (0.0008)	0.0037 (0.0007)	0.0039 (0.00032)	0.0025 (0.0003)	
Myostatin (OD/ug protein) - GAST	0.0021 (0.0003)	0.0015 (0.0001)	0.0015 (0.0001)	0.0024 (0.003)	<.05
Myf5 (OD/ug protein) - GAST	0.005 (0.001)	0.0037 (0.0006)	.008^{+} (0.001)	unavailable	<.01

CHAPTER 6. MUSCLE-SPECIFIC CHANGES IN SATELLITE CELL ACTIVATION AND GENE EXPRESSION AFTER VOLUNTARY WHEEL RUNNING IN NORMAL ADULT MICE

6.1 Introduction

Although the age-related changes in muscle structure and function are well documented, the mechanisms responsible for these changes are not fully understood. Muscle satellite cells are the main contributor for muscle maintenance, growth and repair [5]. However, the ability of satellite cells from aged muscle to contribute to these processes is impaired [274, 441]. Satellite cells in muscle from young mice typically become activated within 10 minutes of a stimulus; however, aged muscle precursor cells demonstrate a 24 hour lag between a stimulus and activation [22, 23, 222]. The response of aged muscle to mechanical stimuli is also perturbed since older contractile tissue requires a greater magnitude of stretch and %length increase to activate satellite cells than muscle from younger mice (Leiter and Anderson, unpublished). Nitric oxide, produced mainly by NOS-I in muscle, is an important chemical messenger that activates satellite cells in response to stretch, exercise, denervation and trauma [4]. In aged muscle, NOS-I may be dislocated into the cytoplasm of the muscle fibers due to a disrupted dystrophin-glycoprotein complex (DGC) [39]. Thus, since impaired NO signaling and deficient satellite cell activation in aged muscle may play a role in age-associated muscle atrophy, it needs to be investigated further.

The DGC, which links the contractile component of the muscle fiber to the basement membrane, functions to sense mechanical forces impressed upon the sarcolemma [38, 39]. A disrupted DGC in aged muscle would not only contribute to the increased susceptibility of older muscle to contraction-induced injury but also help to

explain deficient response to the mechanical stresses placed upon it. Muscle atrophy following 14 days of tail suspension in young mice was associated with displacement of NOS-I into the cytoplasm of the muscle fiber. Since a bolus release of nitric oxide (NO) activates satellite cells [28, 31, 253, 266-269, 304], dislocation of NOS-I into the cytoplasm may not change the expression of NOS-I in muscle fibers but rather alter the concentration of NO available to satellite cells following diffusion from the sarcoplasm. Since pulsatile releases of NO maintain satellite cell quiescence, decreased NO availability to muscle precursor cells might tend to keep cells in a quiescent state rather than causing activation [20].

In addition to the inherent changes in skeletal muscle with age, the environment in which the satellite cells are housed also has a significant affect on muscle satellite cell function. Old muscle regenerates to nearly the same extent as young muscle following transplantation into a young host [277, 278]. The positive influence of a young environment on muscle repair, or conversely, the negative affect of an aged environment on young muscle has also been confirmed in heterochronic parabiosis experiments. These experiments confirmed that the regeneration capacity of aged muscle was increased when the blood supply of old mice was shared with the circulatory system of young animals and vice versa [278]. The vast range of systemic changes that occur in animals and humans with aging make it difficult to determine which environmental alterations within the body limit muscle maintenance, growth and repair. That being stated, the systemic benefits of exercise are also numerous; one purpose of this research was to determine if voluntary wheel running was capable of restoring a more youthful environment in muscle which would be conducive to successful muscle maintenance,

growth and repair [451, 452]. This study was undertaken to determine the effects of exercise on satellite cell activation, and NOS and MRF gene expression between muscle over time in young and older mice. We hypothesize that improved muscle satellite cell activation in aged muscle may be partially a result of the changes toward a more youthful environment in which muscle satellite cells habitate.

6.2 Materials

6.2a Animals and Exercise

All mice were housed at University of Manitoba Central Animal Care Services according to the guidelines set forth by the Canadian Council on animal care. Ethics approval was obtained prior to commencement of any study activities. The majority of mice involved in the study were bred and raised at the University of Manitoba but several of the aged mice were transported to Central Animal Care Services as retired breeders at 8 months-of-age from The Jackson Laboratory (Bar Harbor, Maine, USA). Animals from The Jackson Laboratory were housed at the University of Manitoba prior to any involvement in the study to allow for acclimatization to the new environment.

A total of 24 normal female C57/BL6 mice were involved in the study; 12 mice in each of the 8- and 18-month-old age groups. Within each age group, animals were randomly assigned to either the no exercise (NEX) or exercise (EX) group (n=6 in each group). Each mouse was housed individually in a see-through plastic rat cage. This isolation was physical although mice were in visual contact with others in the colony. Each cage contained a running wheel (locked or not), water bottle, shavings and normal diet of food so mice could eat and drink ad libitum.

6.2b Training Schedule

Animals randomized to the exercise group had access to a steel rodent wheel 24 hours/day for a total of three weeks. Body mass and grip strength measurements were taken prior to the exercise intervention (baseline) and at the end of each week (week 1, 2, and 3). Running wheel activity was recorded at baseline and throughout each week of the protocol. Data for the remainder of variables which included DNA synthesis, muscle fiber cross-sectional area, and protein expression were obtained at the completion of the three-week exercise protocol when mice were sacrificed.

6.2c Voluntary Exercise

Each mouse had access to a steel rodent wheel (12.7 cm in diameter) for the entire course of the study; however, the wheels in the cages of the NEX group were locked from rotating and could be used as a climbing structure only. The wheels were covered with a mesh between the horizontal bars to provide a continuous running surface for the rodent. Two small magnets (1 cm^2) were secured to horizontal bars located at opposite ends of an imaginary line bisecting the circular wheel. The magnets were used to trigger a counting device (Omron™ Type H7EC-BLM) via magnetic switches (Guard™) secured to the bars of the cage above the rodent wheel, approximately 2 cm above the path of the magnets. Each magnet triggered the counter once per revolution so that a total of 2 counts indicated one complete revolution of the wheel. Two magnets were used to capture as much data as possible over the recording period as "wheel activity" by a mouse does not necessarily result in one full revolution of the wheel. Data were recorded 5 times per week for 3 weeks. A total of 15 measurements per animal were taken during

the course of the study. The mean and maximum distance traveled over a 24-hour period was calculated for each week of exercise.

6.2d Grip Strength

Forelimb grip strength measurements were taken with a calibrated [448], strain gauge (Chatillon, DFM-2.0kg, Greensboro, North Carolina) by prompting the mouse to grip the trapeze bar with the forelimbs, and pulling the mouse by the tail (as proximal to the body as possible) parallel to the orientation of the strain gauge and trapeze bar. Grip strength measurements were obtained immediately prior to the initiation of the voluntary exercise program and at the end of each week thereafter for a total of 4 testing periods. Each mouse was required to pull on the bar 3 times over the course of a minute and then placed back into the cage to rest while the other mice in the group performed a set. A total of 3 sets of 5 repetitions were executed during each testing period with a minimum 6-minute rest period between each set. A total of 15 measurements were obtained for each testing period. Four variables were extracted from the measurements which included the following: (1) absolute mean grip strength was determined by taking the average measurement for all repetitions in sets 1-5, (2) relative mean grip strength was calculated by dividing absolute mean grip strength by the weight of the mouse (grams), (3) absolute maximum grip strength was the maximum peak tension recorded on the strain gauge over the 5 sets of 3 repetitions, and (4) relative maximum grip strength was calculated by dividing the absolute maximum grip strength by the mass of the mouse (grams).

6.2e Tissue Collection

Two hours prior to sacrifice, mice were weighed and then received an intraperitoneal injection of [^{3}H]-thymidine (2uCi/g body mass). Following completion of the training protocol mice were euthanized to obtain measures of muscle mass, muscle cross-sectional area, rates of DNA synthesis, and gene expression. Mice were removed from the cage, weighed and then placed into a container containing PRAErrane Isoflourane USP (Baxter Corporation, Mississauga, ON). Following administration of anaesthetic, a cervical dislocation was performed. The extensor digitorum longus- (EDL), gastrocnemius- (GAST), tibialis anterior- (TA), and quadriceps- (QUAD) muscles were isolated and carefully dissected. Each EDL muscle was sectioned transversely into two equal halves; one half was placed into a tube and stored at -20°C for analysis of DNA synthesis while the other half was placed into an eppendorf® tube (Eppendorf, Westbury, New York), snap frozen in liquid nitrogen and stored at -80°C for Western analysis. GAST muscles were grossly sectioned with a razor blade and oriented in a cryomold (Tissue-Tek®, Sakura Finetek, Torrance, California) filled with OCT compound (Shandon CryomatrixTM) submerged in isopentane (≤ -50° C), and placed in a -20° C freezer for later sectioning with a cryotome. TA- and QUAD-muscles were cut into halves and stored at -80° C to allow for assays of DNA synthesis and protein levels.

6.2f Muscle Cross Sectional Area

GAST muscles were sectioned in series at a thickness of 8 μm onto silinated slides. To determine the CSA of individual muscle fibers sections were stained with hematoxylin and eosin. Digital images were obtained at 200X magnification and the CSA of at least 50 fibers per section and 4 sections per muscle were captured with

ImageJ software. Individual fibers were numbered prior to the measurement of CSA which allowed the investigator to identify the CSA of each fiber retrospectively. Each of the four groups (2 age groups, 2 treatment groups) consisted of muscle samples from 6 mice; at least 1200 fibers per group were analyzed to determine the mean CSA.

6.2g Satellite Cell Activation

DNA synthesis, a quantification of [^{3}H]-thymidine incorporation into DNA, was used to determine satellite cell activation as reported previously [5, 252]. As described earlier, EDL, GAST, TA, and QUAD muscles were isolated from both hind limbs of each mouse immediately after sacrifice. A sample of each muscle was placed in a tube and stored at -20°C for analysis of DNA synthesis. Muscles were minced with a razor blade and homogenized in 1 ml of DNA extraction solution and warmed in a tissue bath for 10 minutes at 37.5°C. Samples were immediately placed on ice and a 600 µl aliquot of the sample was placed in a new tube containing 300 µl of acetic acid. Tubes were centrifuged at 2500 rpm for 30 min at a temperature of 4°C. The supernatant was removed and pippetted into a new tube. At this point, a 300 µl aliquot of each sample was added to 5 ml of Ready Safe™ scintillation fluid (Beckman, Fuller, CA), racked in a scintillation counter (LS-6500, Beckman-Coulter Inc.) and counted for 15 minutes. Results (DPM) were adjusted per ug DNA which was determined by adding 200 µl of Hoechst Assay Solution to 5 µl of each sample and analyzing the 96 well plate in a VICTOR2 (PerkinElmer, Waltham, Massachusetts) micro-plate reader [453]. DNA content of each well was calculated by determining the linear slope of 12 standards ranging in concentration from 5 ng/µl to 166.67 ng/µl.

6.2h Western Blot analysis for NOS-I, MyoD and myostatin

Protein was isolated from samples of the EDL-, GAST-, TA- and QUAD-muscles. Western blot analysis, as per standard immunodetection protocols, was performed with primary antibodies to determine the expression of MyoD (Santa Cruz Biotechnology, Santa Cruz, CA), NOS-I (Abcam, Cambridge, MA) and myostatin (Santa Cruz Biotechnology, Santa Cruz, CA). Individual muscles were homogenized in lysis buffer, incubated on ice for 60 minutes and then centrifuged for 5 minutes at 10,000 rpm. To determine protein concentration, 1 µl of each sample was dyed with Bradford Reagent (Sigma-Aldrich Co., Oakville, Ontario) and analyzed with a spectrophotometer against a standard dilution curve (R^2>0.99). Following isolation of protein, samples were loaded on a 12% reducing gel and transferred to a PVDF membrane. Membranes were stained with Ponceau (to ensure successful transfer of gel), blocked with buffer for 60 minutes, and incubated overnight with MyoD antibody (1:500) at 4°C. Expression of MyoD indicates commitment of satellite cell progeny to development or repair of myofibers [454]. The following day, membranes were washed and then blocked in buffer with Anti-rabbit Ig, Horseradish Peroxidase linked F primary antibody fragment from donkey (GE Healthcare, Little Chalfoot Buckinghampshire, UK; 1:2000), incubated for one hour, washed again, and agitated in a 50 ml Falcon™ conical tube (BD Biosciences, San Jose, CA) containing 2 ml of ECLTM Western Blotting Luminescence Reagent (Santa Cruz Biotechnolody, Santa Cruz, CA) for 1 min. PVDF membranes (Osmonics, Minnetonka, MN) were exposed for times depending on intensity of chemiluminescence signal. The optical density (OD) of each band was determined by scanning the developed photographic film into a PC and then analyzing areal density with NIH ImageJ software.

The optical density of each band was standardized with the same sized window (for each band on a gel) against the OD of GAPDH (determined by stripping and reprobing with anti-GAPDH) to account for loading errors.

6.2i Data Analysis

An analysis of variance (ANOVA) was incorporated to investigate the differences between age and treatment groups for muscle mass, rates of DNA synthesis, muscle CSA and gene expression. A repeated measures ANOVA was performed on the remaining variables because data was collected at multiple time-points for body mass, grip strength, and mean distance traveled on the wheel. Least Significant Difference (LSD) post-hoc tests were used and the significance level was $p<.05$.

6.3 Results

6.3a Body Mass

The body mass of 18-month-old mice was less than 8-month-old mice throughout the study regardless of the exercise intervention (table 6-1). One week of exercise in 8-month-old mice resulted in a decrease in body mass that continued throughout the exercise program. There was no change in body mass of the 8-month-old NEX group at any time-point. The 18 month-old exercise group demonstrated decreased body mass after two weeks of voluntary wheel running but this difference also appeared in the NEX group 1 week later. Weight loss in both the NEX and EX groups of 18-month-old mice suggested changes in body mass were due to aging and not to voluntary activity on the wheel.

6.3b Voluntary Exercise

Prior to analyzing the data it was noted that the 8-month-old group had two subgroups (3 mice each) that showed significantly different activity profiles. Based on this difference it was decided to break the 8-month-old group into low- and high-activity subgroups for data analysis. Therefore, the young age group had low- and high-activity level groups whereas the 18-month-old group did not. Baseline testing revealed that there was no difference in distance traveled between any of the age groups. During the second week of exercise, 8-month-old mice high activity group ran further than both the 8-month-old low activity group and the 18-month-old group. However, the activity level of the old EX mice continued to increase in a linear fashion ($r = 0.9988$) to the extent that there was no difference in distance traveled compared to the 8-month-old high activity at the end of the 3-week voluntary wheel running program. Both the 8-month-old high activity mice and the 18-month old mice ran further than the 8-month-old low activity group during week 3 of the exercise intervention (figure 6-1).

6.3c Grip Strength

There was an age-associated decrease in absolute maximum grip strength, the old mice exhibited less force production than younger mice throughout the study, regardless of exercise (table 6-2). Absolute maximum grip strength tended to decrease in 18-month-old mice throughout the study but the change only became significant at the end of week 3. There was no difference in forelimb grip strength between the NEX and EX groups of old mice.

Contrary to the older group, young mice demonstrated significant decreases in force production at weeks 1, 2 and 3 compared to baseline measurement at the start of the

experiment but again, no differences were evident between the NEX and EX groups (table 6-2).

When maximum absolute grip strength was adjusted to body mass, the age-related decreases in strength were eliminated (figure 6-2). Mice aged to 18 months-of-age had the same maximum relative grip strength per gram of body weight as the 8-month-old mice. Voluntary wheel running did not result in a change in relative maximum grip strength in either the 8- or 18 month-old mice.

6.3d Muscle Fiber Cross Sectional Area

A significant age-associated decrease in muscle fiber CSA was evident in the GAST muscles of 18-month-old mice (1503.88 ± 127.87 μm^2) compared to 8-month-old mice (2091.40 ± 159.87 μm^2, figure 6-3). Three weeks of voluntary wheel running did not affect muscle fiber CSA of the GAST muscle in either the 8- (2247.87 ± 295.78 μm^2) or 18-month-old (1406.83 ± 120.45) μm^2 age group.

6.3e Satellite Cell Activation

Satellite cell activation was measured in vivo in proximal- (QUAD), distal- (TA, EDL, and GAST), anterior- (QUAD, TA, EDL) and posterior- (GAST) muscles of the hindlimb. Old mice (18-month-old) had a lower level of satellite cell activation in the EDL-, TA- and QUAD-muscles compared to young mice, although there was no difference in activation in GAST muscle between the two age groups (table 6-3).

Interestingly, the QUAD muscle of 8-month-old mice was the only muscle that demonstrated a significant increase ($p<.01$) in satellite cell activation with exercise (1058.28 ± 53.33 DPM/μg DNA) compared to the non-exercise (625.65 ± 125.36

DPM/μg DNA) group (figure 6-4). Conversely, voluntary wheel running resulted in a strong trend toward decreased SC activation in the QUAD muscle of 18-month-old mice (449.73 ± 38.82 DPM/μg DNA) compared to the control group (669.33 ± 148.79 DPM/μg DNA).

6.3f NOS-I

NOS-I protein content was investigated in the EDL-, TA-, QUAD- and GAST- muscles from the NEX and EX groups of mice aged to 8- and 18 months-of-age. With exercise, NOS-I expression increased in the QUAD muscle of 8-month-old mice, consistent with the exercise-induced increase in satellite cell activation in the same muscle- and age-group (figure 6-5, table 6-4). Conversely, exercise did not change NOS-I protein content in the QUAD muscle of 18-month-old mice.

There was no difference in NOS-I expression between the NEX and EX groups, respectively, in the EDL-, TA- and GAST- muscles of 8-month-old mice (table 6-4). Similarly, exercise did not have an affect on NOS-I expression in the EDL-, TA- or GAST-muscles of 18-month-old mice.

6.3g MyoD

There was no difference in MyoD protein content between the NEX and EX groups, respectively in the QUAD and GAST- muscles of 8-month-old normal female mice (table 6-4). Similarly, there was no difference in MyoD protein content with exercise in the QUAD-, and GAST- muscles of 18-month-old mice. MyoD protein content for the TA muscle of 8-month-old mice and the EDL muscle from either age group could not be obtained due to technical difficulties.

6.3h Myostatin

Voluntary wheel running decreased myostatin expression in the QUAD muscle of 8-month-old mice compared to the NEX group (table 6-4). By contrast, the level of myostatin protein expression in the GAST muscles was increased in the same age group. Exercise did not change the level of myostatin protein expression in the TA muscles of younger mice or in the GAST-, TA- or QUAD-muscles of 18-month-old mice. Myostatin protein content from the EDL muscle of either age group could not be obtained due to technical difficulties during processing.

6.4 Discussion

This is the first study to investigate the effects of age and voluntary exercise on the structure, function, satellite cell activation, and gene expression of skeletal muscle from normal mice. It is also the first to study to investigate the effects of exercise on satellite cell activation in vivo in mice greater than 2-months-of-age. A subpurpose of the current investigation was to determine if the effects of age and exercise on satellite cell activation and gene expression differ between the proximal-, distal-, anterior- and posterior-skeletal muscles of the mouse hind limb. Voluntary exercise increased satellite cell activation and NOS-I expression, as well as decreased myostatin expression in the QUAD muscle of adult mice. Several experimental models have been used to stimulate myogenic stem cells from quiescence, including crush injury, denervation, stretch, hind limb suspension, neotoxin or cardiotoxin injection, and exercise [5, 7, 15, 27, 140, 244, 270], but few studies analyzed more than one- or two-muscles. Muscle stems cells are responsible for the maintenance, repair, and growth of skeletal muscle so the therapeutic benefits of maximizing activation, proliferation, differentiation and successful

regeneration are enormous given the severity of muscular disease (such as Duchenne Muscular Dystrophy) and the vast number of people disabled by muscle atrophy (from aging, disuse, spaceflight, limb immobilization and cachexia)[5]. The research presented here was therefore focused on age-related muscle atrophy from a therapeutic standpoint.

While maintaining satellite cell activation through exercise would be an optimal treatment intervention to muscle structure and function, mechanically-induced satellite cell activation is known to decrease with age [307]. Previous work in our lab demonstrated that an altered NO pathway, decreased satellite cell activation, and negative responses to NO supplementation and passive stretch (in terms of SC activation) are evident from experiments on whole-muscle cultures from aged mice (Leiter and Anderson, unpublished). However, satellite cells from aged muscle are known to activate in response to hepatocyte growth factor supplementation [307]. These results suggest that the NO pathway in aged muscle is disturbed since NO signaling is required for HGF release and may be a prime target to combat sarcopenia [16, 27, 28, 270]

There was a negative effect of age on body mass regardless of exercise. These findings are in agreement with a study be Betters and colleagues (2008) that demonstrated a significant reduction in body mass between adult and senescent C57/BL6 mice [307]. Although caloric intake was not recorded, each group of animals had full access to food and water ad libitum. Reduced body mass in a controlled environment may be an adaptive response by aged animals to compensate for decreased strength since there was no difference in relative grip strength between age groups when values were adjusted for body mass. Reductions in the muscle mass to body mass ratio confirm the adaptive decrease in body mass with age (Leiter and Anderson, unpublished, [307]).

Functionally, this appears to be an important adaptation as other findings from the same experiment suggest that older animals are able to perform strength-dependent activities to the same extent as younger animals.

The environment of satellite cells plays a significant role in their ability to be activated from the G0-phase of the cell cycle and contribute to muscle maintenance, growth and repair [277, 278]. With age, environmental influences negatively impact the ability of satellite cells to function. Fortunately, exercise results in several significant systemic changes that have been observed to restore some of the characteristics of a youthful environment [38, 452], although the impact of exercise or training on satellite cell activation in muscle from aged mice has not been investigated to date. The current study has demonstrated that 3 weeks of voluntary wheel running was sufficient to restore the exercise capacity of 18-month-old mice to levels evident in younger, 8-month-old-mice. However, despite significant improvements in running performance (i.e. distance) of aged mice, voluntary wheel running did not "rejuvenate" satellite cell activation in the EDL-, GAST-, TA- and QUAD-muscles of 18-month-old mice to levels evident in 8-month-old mice. These findings suggest that voluntary wheel running alone may not have a sufficiently positive impact on the systemic environment to stimulate satellite cell activation in muscle from aged animals. Alternatively, it is possible that the mechanical stimulus of voluntary wheel running was not sufficient to surpass a putative threshold for activation. This seems likely since our previous experiments on stretching whole-muscle cultures from old mice required double the length change to induce activation compared to muscles from younger mice (see section 4.1d). Similarly, a mechanically-induced increase in satellite cell activation was not demonstrated in single-fiber experiments from

22-month-old mice in which single fibers from the GAST muscle were centrifuged to induce activation [307]. Since voluntary wheel running may not contribute to muscle growth to the same extent as resistance training, it is possible that a greater mechanical stimulus, such as resistance training, or prolonged eccentric activity may be required to increase muscle satellite cell activation in older mice.

The only muscle to demonstrate increased satellite cell activation with exercise in vivo was the QUAD muscle from 8-month-old mice. The EDL-, TA- and GAST-muscles did not show increased satellite cell activation in response to voluntary wheel running in either age group. The results for satellite cell activation in the EDL muscle are in agreement with whole-muscle culture studies in which satellite cell activation in muscle from 18-month-old mice was significantly lower than satellite cell activation in muscle from younger, 8-month-old mice. In addition, the TA-muscles from the non-exercised group of 18-month-old mice had a significantly lower level of muscle precursor cell activation than 8-month-old mice. Therefore, it seems clear that satellite cell activation, with or without mechanical stimulation, changes with age and is muscle-specific. As a consequence, characteristics of one muscle group should not be applied to other muscles without caution. Structurally, the QUAD muscle was the largest of all the muscles that were investigated. For the most part, it acts eccentrically to control knee extension during running. Since large eccentric loads are imposed on the QUAD muscle during exercise, increased satellite cell activation in 8-month-old mice may be a result of a greater magnitude of mechanical stimulation and the nature of that stimulation. The lack of an increase in satellite cell activation in the QUAD muscle of 18-month-old mice suggests that older muscle lacks the ability to respond to a mechanical stimulus or

requires an even more intense stimulus to activate satellite cells. The unique architecture includes a significant sized sesamoid bone at the knee that also may mediate some of the muscle-specific responsiveness by the QUAD muscle to exercise. Another explanation may be the motor-planning strategy for voluntary exercise changes with age resulting in less mechanical strain in the QUAD muscle versus other muscles. Decreased satellite cell activation may be one reason why aged muscle does not hypertrophy to the same extent as young muscle when exposed to physical activity.

Age-related muscle loss in humans primarily affects the muscles surrounding weight-bearing limbs yet the extent to which satellite cell activation is implicated in this process is unknown. In addition, the question arises as to whether satellite cell activation is affected by muscle size, location, and primary action of the muscle and fiber type. The results of the current study indicate that satellite cell activation by mechanical activity is affected differently in various muscles, and that these changes are more evident as age increases. At 8 months-of-age, exercised animals demonstrated a significant increase (+40%) in satellite cell activation of the QUAD muscle with exercise but the opposite trend (- 33%) was evident in mice aged to 18 months-of-age. Older mice also displayed low SC activation in EDL- and TA-muscles after exercise (compared to non-exercised mice) but the GAST muscle did not show this effect. The deficient activation of satellite cells with exercise in vivo is consistent with results from single-fiber experiments in which centrifugation of fibers failed to mechanically stimulate muscle precursor cells [307]. However a thorough survey of muscle according to limb segment, proximally to distally, and by compartment (flexion vs. extension) and fiber type (superficial fast vs.

deep slow) is required to address these questions as findings would impact strategies to prevent or treat age-related atrophy.

Muscle fiber CSA from the GAST muscle of 18-month-old mice was less than 8-month-old mice; this is consistent with several studies investigating the age-related changes in muscle structure [295, 375-380]. Voluntary wheel running did not increase fiber CSA in either age group. By comparison an increase in fiber CSA in the rat plantaris muscle was demonstrated after 10-weeks of voluntary exercise [338]. This study only involved 3-weeks of voluntary wheel running and exercise was initiated at 8- or 18-months-of-age in our study compared to 5 weeks-of-age in the rat study. Thus, to increase muscle fiber CSA, a longer exercise intervention may be required and/or exercise should begin at a younger age. The longer exercise period would potentially demonstrate significant or further muscle-specific responses to exercise. However, changes in activation (producing more myonuclei) would likely be confounded even further with training responses and hypertrophy than in the 3-week study presented here. The extent of age-related muscle atrophy is largely dependent on activity levels over a lifetime such that chronic exercise decreases muscle wasting with age [455].

The dependence of satellite cell activation on nitric oxide and NOS-I expression was once again confirmed in this study. QUAD muscles from 8-month-old mice were the only muscle to demonstrate increased satellite cell activation with exercise and were also the only group to show an increase in NOS-I expression. There was no difference in satellite cell activation or NOS-I expression with exercise in the TA-, EDL- and GAST-muscles from either age group. Therefore, it seems increased satellite cell activation and increased NOS-I expression are tandem observations in mouse muscle following

exercise, consistent with Betters and colleagues. [307]. The lack of an increase in NOS-I with exercise may partly explain the deficient satellite cell activation in the aged muscles. In addition, the current study provides further evidence that satellite cell activation and NOS-I expression is muscle- and load-specific. For instance, only the QUAD muscle from 8-month-old mice was sensitive to the stimulus of exercise whereas the TA-, EDL-, and GAST-muscles were not which is in agreement with data from the plantaris muscle of aged rats [307].

In addition to increased satellite cell activation and NOS-I expression in the QUAD muscles of 8-month-old-mice, myostatin expression in the exercised group was significantly reduced compared to control mice. Myostatin expression is negatively correlated with muscle growth [13, 428, 456, 457]. Therefore, increased NOS-I expression, increased satellite cell activation and decreased myostatin expression indicate that the QUAD muscles of 8-month-old mice have responded to exercise by initiating growth. Conversely, exercise did not change myostatin expression in the TA-, EDL- or GAST-muscles of either age group. Resistance loading in humans has resulted in decreased myostatin expression in both young and old subjects with the exception of older females [13]. It is interesting to note that all mice in this study were female; therefore, muscle atrophy may be age-dependent, muscle-, and gender-specific.

Age-related changes in muscle structure and function have been relatively well addressed in the literature yet the mechanisms responsible for these changes have yet to be elucidated. Our research has demonstrated that a decrease in body mass with age may be an adaptive response that accommodates atrophic changes in muscle structure and function. This would have significant implications for the increase in obesity in our

population. Central to the processes of maintenance, growth and repair of voluntary contractile tissue is the muscle satellite cell. To contribute to these processes, precursor cells must be activated, proliferate and differentiate and fuse into mature muscle fibers. Our results suggest that satellite cell activation is age-dependent and muscle- specific. Deficits in satellite cell activation with age are more than likely a result of changes in the NO pathway including either a decrease in the availability of NO, a reduction in the expression of NOS-I, and/or a dislocation of NOS-I into the cytoplasm of muscle fibers. Future studies should investigate the effects of exercise and NO supplementation on satellite cell activation in vivo since increased levels of NO may overcome the threshold required to activate satellite cells in muscle of older mice. In addition, mechanical stresses placed on muscle in those experiments should incorporate eccentric exercise or resistance training since the lack of mechanically-induced satellite cell activation may be due to insufficient stimulation during voluntary wheel running. Stimulating satellite cells with exercise and NO supplementation, and finding the threshold for activation has the potential to provide information that would help maintain and/or restore age-related deficits in muscle structure and function.

6.5 Tables and Figures

Table 6-1. The effects of a 3-week voluntary wheel running program on the body mass of mice aged to 8- and 18-months

Body mass (g) was recorded at baseline, prior to the initiation of voluntary wheel running, and at the end of each week of exercise (weeks 1, 2, 3). Values are means and (SEM), n=6 mice per group at each time-point. A significant difference from baseline within the same age group is represented by *; a statistical difference between age groups is indicated by # ($p<.05$).

	baseline	Week 1	Week 2	Week 3
			8-month-old mice	
non-exercise	34.92	32.96	33.35	33.4
	(1.10)	(0.92)	(1.17)	(1.40)
exercise	34.57	31.40*	31.55*	31.61*
	(0.99)	(0.92)	(0.83)	(0.67)
			18-month-old mice	
non-exercise	27.83#	26.82#	27.38#	26.37*#
	(0.61)	(0.56)	(0.63)	(0.52)
exercise	28.47#	26.67#	26.05*#	26.33*#
	(1.04)	(0.98)	(0.59)	(0.66)

Table 6-2. Absolute maximum forelimb grip strength in the non-exercise- and exercise-groups of 8- and 18-month-old normal female mice

Eighteen-month-old mice demonstrated decreased grip strength compared to 8-month-old mice throughout the study when ages groups were not separate into NEX and EX groups. Compared to baseline, strength deficits in both the NEX- and EX-groups were evident in 8-month-old mice during weeks 1, 2 and 3. The three-week voluntary wheel running program did not have an affect on grip strength in either the 8- or 18-month-old age groups. Six mice were randomized to each age group and treatment. Values (g x 10) are expressed as mean and (SEM) (#, significant difference from 8-month-old mice, * significant difference from baseline within same age group; $p<.01$).

	Baseline	Week 1	Week 2	Week 3
		8-month-old mice		
non-exercise	1338.33	1190.00*	953.33*	1115.00*
	(71.06)	(43.44)	(92.10)	(71.50)
exercise	1351.67	1125.00*	1081.67*	1060.00*
	(58.96)	(55.48)	(42.14)	(41.31)
		18-month-old mice#		
non-exercise	1008.33	963.33	946.67	773.70
	(27.86)	(32.02)	(59.06)	(102.4)
exercise	1060.33	873.33	816.00	843.33
	(67.6)	(33.43)	(50.36)	(31.16)

Table 6-3. Effects of age on satellite cell activation in the EDL-, TA-, QUAD- and GAST-muscles

SC activation was determined by measuring DNA synthesis per ug of DNA (DPM/ug DNA). Aged animals (mean ± (SEM)) demonstrated decreased satellite cell activation compared to adult mice in EDL-, TA- and QUAD-muscles (*, $p<.05$). There was no difference in satellite cell activation in the GAST muscle between 8- and 18-month-old mice.

Muscle	8-month-old	18-month-old
EDL	39.80	24.74*
	(3.25)	(4.84)
TA	165.65	52.22*
	(21.98)	(13.12)
QUAD	841.96	559.93*
	(92.08)	(80.44)
GAST	317.13	275.64
	(26.78)	(25.89)

Table 6-4. NOS-I, MyoD and myostatin protein content in the TA, GAST, QUAD and EDL muscles of 8- and 18-month-old mice.

Western blot analysis was performed to determine the relative intensity (OD/GAPDH) of NOS-I (a), MyoD (b) and myostatin (c) protein content in the TA, GAST, QUAD and EDL muscles of 8- and 18-month-old mice subjected to 3 weeks of voluntary wheel running or not. *indicates significantly different from control value of the same muscle and same age group *($p<.05$)*.

(a)

	8-month-old		18-month-old	
Muscle	NEX	EX	NEX	EX
TA	1.19	1.20	2.31	2.29
	(0.26)	(0.26)	(0.27)	(0.17)
GAST	1.23	1.01	1.74	2.14
	(0.31)	(0.27)	(0.43)	(0.37)
QUAD	1.38	2.75*	1.52	1.30
	(0.25)	(0.78)	(0.59)	(0.37)
EDL	0.82	0.94	0.92	0.76
	(0.11)	(0.04)	(0.13)	(0.06)

(b)

	8-month-old		18-month-old	
Muscle	NEX	EX	NEX	EX
TA			1.27	2.13*
			0.15	0.40
GAST	0.63	0.52	0.97	0.73
	0.06	0.05	0.13	0.11
QUAD	0.73	0.81	0.92	0.72
	0.05	0.10	0.15	0.09

(c)

	8-month-old		18-month-old	
Muscle	NEX	EX	NEX	EX
TA	1.97	1.97	1.67	1.19
	0.48	0.56	0.62	0.27
GAST	0.74	1.18*	0.57	0.49
	0.06	0.11	0.08	0.07
QUAD	1.26	0.57*	1.70	1.70
	0.15	0.10	0.23	0.14

Figure 6-1. Difference in distance traveled between 18-month-old mice and the low and high activity subgroups in the 8-month-old age category

Distance traveled over the course of a 24-hour period (relative to body mass) was recorded in meters, 5 times per week (mean ± (SEM)). The 8-month-old high activity group ran further (total distance per 24 hours) than the 8-month-old low activity group and the 18-month-old group during week 2 of the exercise program (*, significantly different from the 8-month-old low activity group; #, significantly different from the 18-month-old age group; $p<.01$). However, the 18-month-old age group demonstrated a linear increase ($R^2 = 0.9977$) in distance run over the course of the study such that no difference was evident between the 18-month-old group and 8-month-old high activity group in week 3 of the exercise intervention. By contrast, both the 8-month-old high activity group and the 18-month-old mice ran further than the 8-month-old low activity group during week 3 of the voluntary wheel running protocol.

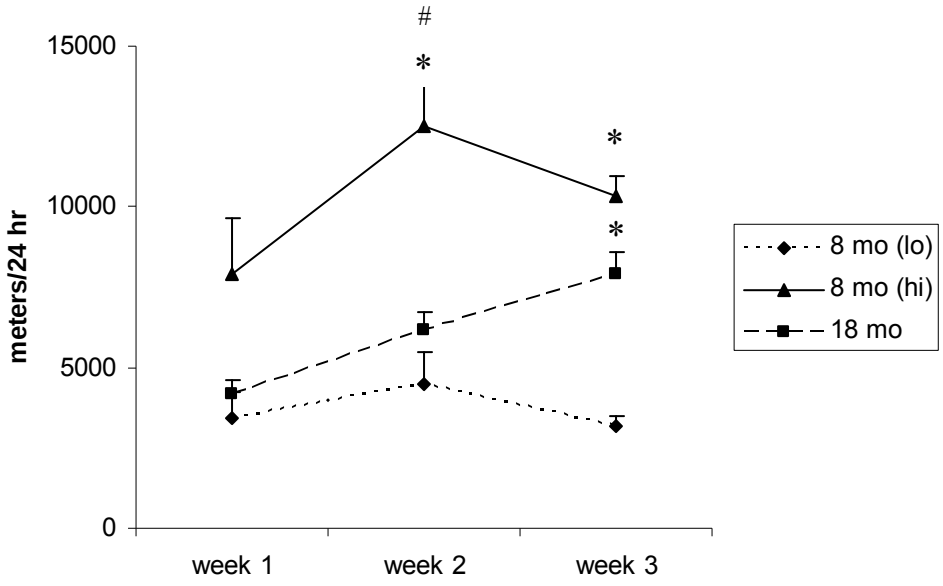


Figure 6-2. Absolute and relative forelimb grip strength values for all 18-month-old mice expressed as a percentage of values demonstrated in 8-month-old mice

Absolute maximum grip strength of 18-month-old mice was less than 8-month-old mice throughout the study. Conversely, when absolute grip strength was standardized to body mass (relative maximum grip strength), no differences were detected between the two age groups since maximum grip strength per gram of body mass for 18-month-old mice was just below, or slightly above 100% of the grip strength values achieved by 8-month-old mice. No differences were detected between the exercise- and non-exercise-groups in either of the age groups. (*, significantly different from 8-month-old mice; $p<.01$).

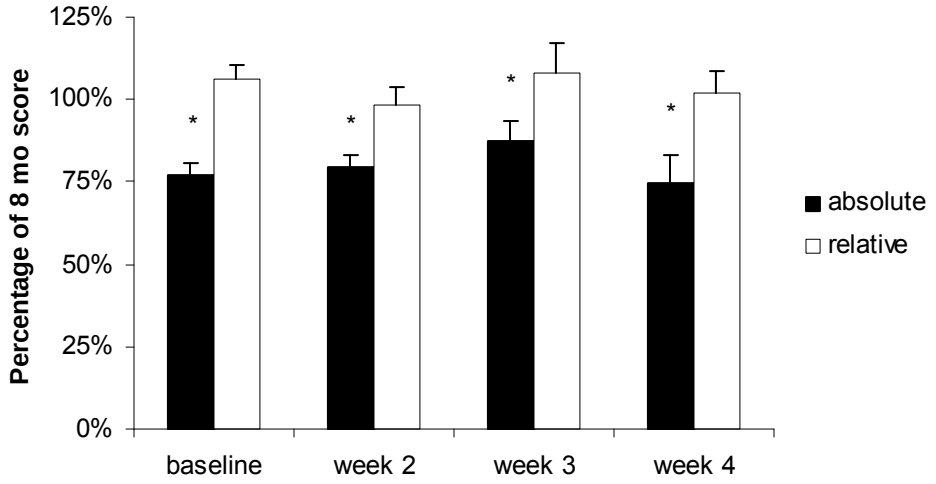
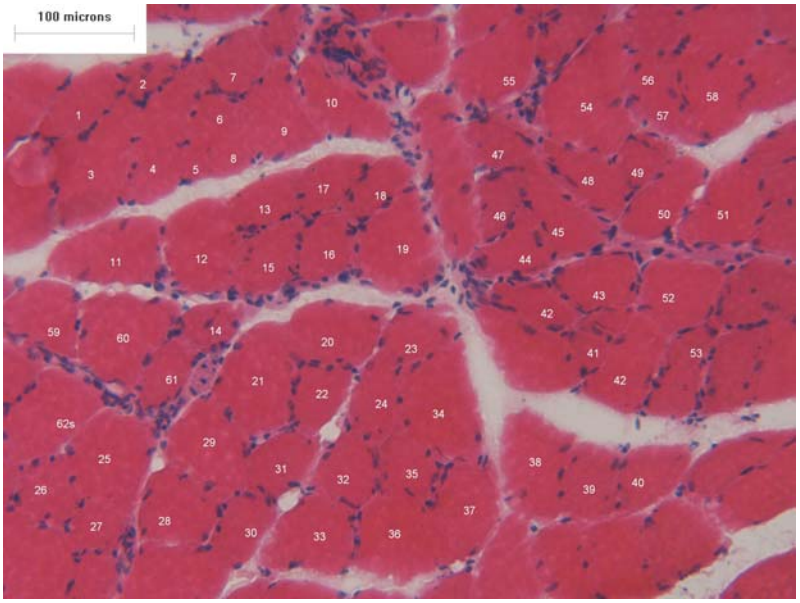


Figure 6-3. Hematoxylin and Eosin staining of GAST muscle isolated from (a) 8- and (b) 18-month-old mice

Fiber CSA analysis was performed using NIH ImageJ software. Muscle fibers from 18-month-old had less CSA than fibers from younger mice. Exercise did not have a significant impact on muscle fiber CSA in either 8- or 18-month-old mice.

(a)



(b)

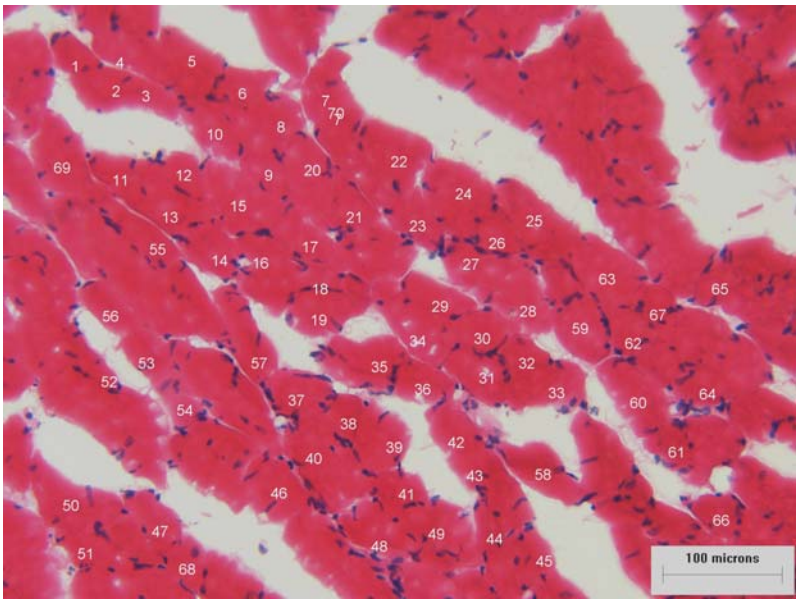


Figure 6-4. Changes in satellite cell activation with voluntary exercise in the EDL-, TA-, QUAD- and GAST-muscles of normal female 8-month-old mice

Voluntary wheel running increased satellite cell activation (mean ± (SEM)) in the QUAD muscle but did not have an affect on muscle precursor activation in the EDL-, TA- and GAST-muscles. Satellite cell activation was determined by scintillation counts standardized to μg of DNA and is expressed as DPM/ug of DNA. (*, $p<.05$).

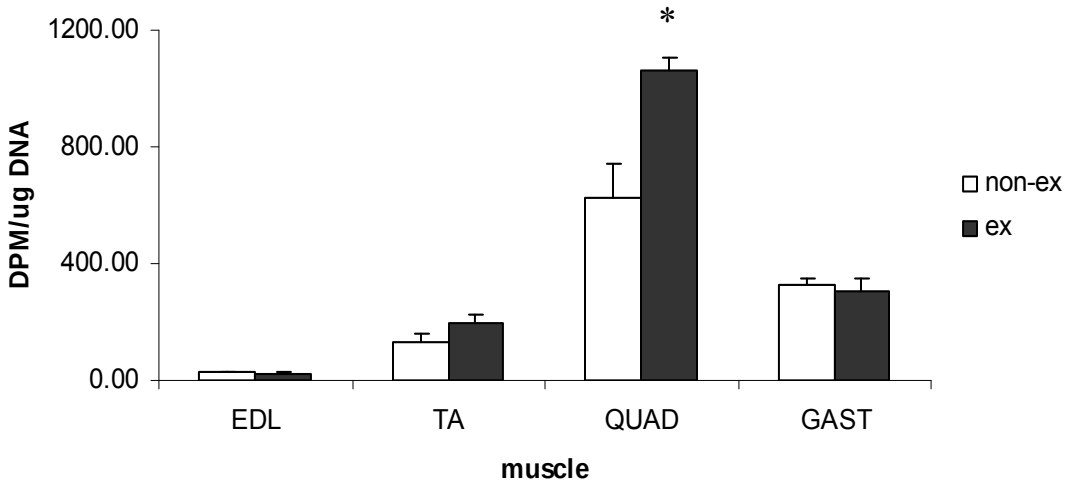
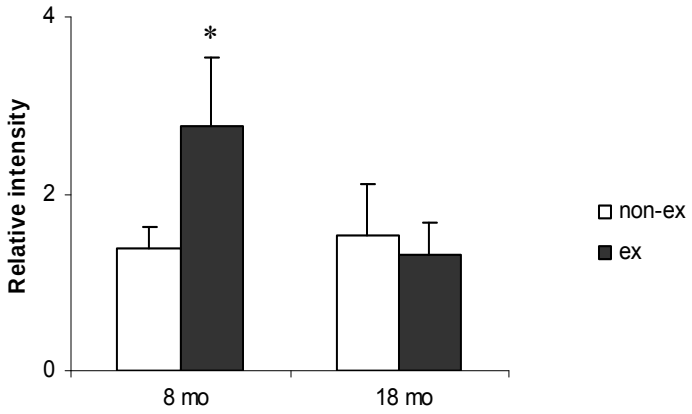


Figure 6-5. NOS-I protein content in the quadriceps muscle of the non-exercise and exercise groups in 8- and 18-month-old mice

NOS-I protein expression (mean ± (SEM)) increased with exercise, compared to the non-exercise group, in the quadriceps muscle of 8-month-old mice but had no affect in older, 18-month-old animals (*, $p<.05$).



CHAPTER 7. DISCUSSION

The purpose of this thesis was to increase our understanding of the mechanisms responsible for age-related muscle atrophy and although future studies are required that will ultimately lead to full determination, this is a contribution to solving the disabling condition of sarcopenia. The structural and functional changes that occur in muscle with aging were re-investigated, within the context of recent literature and our work on muscle cultures so pointed questions related to the regulation of satellite cell activation in aged muscle could be answered, to establish a timeline for the progression of atrophy and determine the age at which satellite cells are less responsive to chemical and mechanical stimulation.

This research was divided into three distinct sets of experiments and the results of each section will be discussed separately. After summarizing the three components of this work in isolation, the strengths and limitations of this research will be identified and discussed. Then, the results of each section will be integrated with one another and elaborated upon in reference and comparison to relevant literature. Finally, future directions for this research will be discussed.

7.1 Mechanical and chemical activation of satellite cells is perturbed in skeletal muscle from normal aged mice.

The experiments presented in Chapter 4 of this thesis were designed and executed to determine the effects of stretch, and stretch plus NO treatment on satellite cell activation and gene expression in whole muscle (EDL) cultures from young and old mice. The first major discovery from these experiments was that muscle from 6-month-old

mice required twice the mechanical stimulus (20%- vs. 10%-length increase) of muscle from 3-month-old mice to increase satellite cell activation compared to the unstretched group of the same age. Therefore, evidence suggests that mechanical activation of satellite cells (required for muscle maintenance, growth and repair) is perturbed as early as 6 months-of-age in normal mice which is an important time point to consider if age-related muscle loss is to be prevented. Further, by 8 months-of-age, this magnitude of stretch did not increase satellite cell activation even after applying a 20% length increase to EDL muscles in culture. Muscle from mice aged to 10- and 18-months-of-age did not respond to stretch by increasing satellite cell activation. Hence, in muscle isolated from mice 6 months-of-age or older mechanical stimulus alone, at least at the magnitude applied in these experiments, was not sufficient enough to surpass the threshold required to increase satellite cell activation. Mechanical stimulation produces a bolus release of the chemical messenger NO that subsequently activates satellite cells. It is possible that a greater stretch magnitude; applying electrical stimulation to the muscle to produce a lengthening contraction, which induces greater muscle damage; or administering NO would have increased satellite cell activation in muscle from older mice.

In addition to stretch, NO treatments were administered immediately prior to applying the mechanical stimulus to determine if chemical stimulation, combined with mechanical stimulation, was sufficient to increase satellite cell activation. Two sources of NO supplementation were utilized in an attempt to provoke muscle precursor cells to be activated and leave the quiescent state. An endogenous source of NO (LA), which requires the enzyme NOS-I to produce NO, and an exogenous source of NO (ISDN) were applied to muscles in culture from 8- and 18-month-old mice. Increased satellite cell

activation occurred with ISDN treatment but only in muscle from younger mice (8-month-old). NOS-I may be perturbed as early as 8 months-of-age since LA supplementation was unable to increase satellite cell activation with age. This was further confirmed by combining LA and ISDN treatments with the NOS-I inhibitor, LN. When LN was added in conjunction with ISDN, satellite cell activation still increased beyond control levels and verified that ISDN does not require NOS-I to produce NO. However, when LA was combined with LN, satellite cell activation decreased compared to basal levels of satellite cell activation which suggests that NOS-I not only converts LA to NO, but also plays an important role in maintaining baseline levels of satellite cell activation. Together, these experiments confirm that changes in NO and NOS-I activity contribute to the disruption of satellite cell activation in 8-month-old mice compared to that in younger mice.

Contrary to the effects of NO supplementation in younger mice, exogenous and endogenous sources of nitric oxide, combined with mechanical stretch, did not increase satellite cell activation in muscle from 18-month-old mice. These results suggest that NO production and NOS-I activity is also perturbed in older mice since LA treatment did not increase satellite cell activation. In addition, ISDN supplementation did not stimulate greater levels of satellite cell activation in 18-month-old mice. Therefore, satellite cells from older muscle may be less sensitive to NO with age and may require a greater concentration of NO to surpass the threshold for satellite cell activation.

This series of experiments demonstrated that prevention of muscle atrophy must be initiated in adult muscle (8-month-old), prior to the commencement of muscle loss, and that combined treatments of stretch and NO supplementation may be the most

beneficial. There was a negative effect of age on satellite cell activation but a positive association between increasing age and the magnitude of mechanical stimulation that needed to be applied to traverse the threshold for satellite cell activation.

7.2 Changes in the structure and function of normal skeletal muscle with age

The results of the experiments in Chapter 4 motivated us to re-visit the age-related changes in the structure and function of normal muscle to determine if these changes are synchronous with perturbed satellite cell activation via mechanical (stretch) and chemical (NO) stimulation. As discussed previously, mechanical stimulation of muscle satellite cells was reduced by 6 months-of-age and severely disrupted by 8-months-of-age such that increased satellite cell activation was not apparent at this age despite increased mechanical loads. Mechanical stretch and chemical stimulation with exogenous sources of NO (ISDN) were sufficient to increase satellite cell activation in younger muscle but failed to produce the same effects in older mice. Therefore, the purpose of this set of experiments was to determine the age at which the structure and function of normal muscle begins to decline.

Although this research investigated skeletal muscle, it is important to understand, or at the very least realize, that muscle is affected by the other systems that make up an organism as a whole. Body mass is not only a robust measure of overall health, but also an important variable in the assessment of muscle function, both morphologically and physiologically. In particular, muscle strength in isolation is of minimal importance if it is not related to function and the efficiency at which an organism can manipulate itself. Thus, body mass must be considered when determining the influence of muscle strength on performance of various activities. Therefore, for the purposes of this research, body

mass was assessed prior to the investigation of age-related changes in the structure and function of skeletal muscle.

In normal female C57/BL6 mice, body mass peaked at 8 months-of-age but was significantly decreased in 18-month-old mice. It is important to note that all age groups were given food and water ad libitum; nevertheless, older mice weighed less than mice 10 months younger. The significance of this finding is most relevant to the results of forelimb grip strength measurements. Although 18-month-old mice demonstrated decreased maximum grip strength compared to 8-month-old rodents, the difference was ameliorated when adjusted for body mass. In other words, because older mice weighed less, the animals were able to produce as much force per gram of body mass compared to younger, heavier mice. Older mice have the functional strength to manipulate their body weight to the same extent as adult mice; thus, compensatory weight loss counteracts decreased absolute grip strength in 18-month-old mice and maintains function.

Consistent with the results of body mass, the muscle mass of 18-month-old mice was less than younger mice. Reduced muscle mass and decreased fiber CSA in the GAST muscle of 18-month-old mice confirmed that reduced muscle mass is positively correlated with fiber CSA. The link between muscle structure and function was further established in aged muscle since decreased muscle mass and fiber CSA was associated with reduced absolute maximum grip strength (not adjusted for body mass). Our results confirmed that aging is related to decreased body mass, muscle mass and strength but to this point, the implications of altered satellite cell pool size or disrupted activation characteristics on age-related muscle degeneration have yet to be determined.

In addition to a decrease in body mass, muscle mass, muscle fiber CSA and muscle strength; the number of satellite cells was reduced in muscle from 18-month-old mice. Mice aged to 18 months-of-age had the greatest number of fibers without any associated satellite cells. In addition, aged muscle demonstrated the least mean number of satellite cells. The reduced number of satellite cells in EDL muscle from 18-month-old mice may have contributed to decreased satellite cell activation compared to muscle from 3-, 8-, and 18-month-old mice. Therefore, the results of this series of experiments demonstrate an association between a declining number of satellite cells, and decreased muscle mass, fiber CSA, and strength.

The intricate link between muscle structure and function, and the importance of muscle satellite cells in maintaining the youthful characteristics of contractile tissue has been well established here. These experiments have provided circumstantial evidence that reduced satellite cell pool size and decreased satellite cell activation are associated with decreased muscle mass and fiber CSA in aged muscle. Consequently, reduced muscle mass and decreased fiber CSA seem to play a role in decreased grip strength. Therefore, in order to combat age-related deficits in muscle structure and function, replenishment of the satellite cell pool size and rejuvenated satellite cell activation are essential.

7.3 Exercise-induced satellite cell activation, NOS-I expression, and myostatin regulation are muscle-dependent and age-specific

In vivo rodent experiments investigating the effects of exercise on satellite cell activation and gene expression usually analyze one or two muscles of the lower limb. Evidence suggests mechanically-induced satellite cell activation decreases with age but

caution is advised when extrapolating these results to all muscles of the body. Based on location, architecture, function, type of contraction and fiber type, the strain imposed on specific muscles during various activities may vary extensively. The purpose of this section of the thesis was not only to study the effects of voluntary wheel running and age on satellite cell activation and gene expression, but also to determine if various muscles of the lower limb were affected differently. This knowledge will be useful in considering how best to design exercise protocols to treat muscle atrophy. The type of activity and age of the animal or subject must also be considered for protocol development.

As mentioned in the previous section, body mass must be taken into account when assessing muscle function either by grip strength or in this case, voluntary wheel running. Eighteen-month-old mice weighed less than younger 8-month-old mice throughout the study protocol, regardless of exercise or not. Lighter, older mice would have required less muscle force to produce the actions of voluntary wheel running which may have contributed to an increase in the distance traveled over the course of the exercise intervention.

Similar to the results presented in Chapter 5, older mice demonstrated decreased absolute maximum grip strength compared to younger mice. Unexpectedly, the absolute grip strength of young mice decreased throughout the study but there was no difference between the non-exercise and exercise groups. When maximum grip strength was adjusted for body mass (relative maximum grip strength), the differences in force production between 8- and 18-month-old mice were ameliorated. Therefore, a compensatory decrease in body mass with age maintained maximum grip strength in older mice when compared to the younger age group. This again demonstrated that a

compensatory decrease in body mass assists animals to maintain function despite a decrease in muscle size and absolute muscle strength.

Prior to analyzing the voluntary wheel running data it was realized that the younger group could be split into two separate categories based on activity level. Conveniently enough, the 8-month-old group was split in half and 3 mice were placed in the high-activity group while the remaining 3 were allocated to the low-activity group. The older, 18-month-old group did not differ in activity level and all 6 mice were analyzed as one group. Although the young, high activity group ran further than the older age group during the second week of exercise, the older mice continued to increase the distance traveled over the course of the exercise intervention such that no difference was evident between two groups at the end of the exercise trial. These results demonstrate that older mice are capable of restoring exercise capacity to the level of younger mice after only 3-weeks of exercise. Again, decreased body mass may have contributed to the improvement of endurance capacity in the older mice over the course of the voluntary wheel running protocol.

Muscle fiber CSA from the GAST muscle of 18-month-old mice was decreased compared to values obtained from 8-month-old mice. Although exercise did not have a significant effect on fiber CSA, results demonstrated a trend toward increased and decreased CSA in young and old mice, respectively. These data suggest that younger muscle hypertrophies in response to exercise whereas older muscle tends to decrease in size. The trend towards increased fiber CSA in 8-month-old mice shows that younger muscle still has the ability, although reduced, to increase muscle size with exercise. Although the QUAD muscle was not selected for fiber CSA measurements, it is expected

that an exercise-induced increase in fiber CSA would be evident since satellite cell activation increased with voluntary wheel running. Similar to stretch experiments, mechanical stimulation of satellite cells was not sufficient enough to increase satellite cell activation. However, supplementing exercise with an NO donor may have increased muscle precursor cell activation to significant levels which would have been in agreement with the results from stretch and ISDN treatment in whole muscle cultures of 8-month-old mice. Voluntary wheel running showed a trend towards decreased fiber CSA in 18-month-old mice which may be a result of the diminished hypertrophic response of older muscle to mechanical activation. Contrary to younger mice, stretch and ISDN treatment decreased satellite cell activation in older mice which suggests that the NO pathway and sensitivity of satellite cells to NO may be impaired in older muscle. In order for exercise and NO treatment to be advantageous, the correct level of NO must be determined to increase satellite cell activation and thus, muscle growth, in old mice.

As mentioned previously, this is the first study to compare the effects of age and exercise on satellite cell activation in various muscles from the hind limb of normal mice. Results demonstrated very distinct differences among muscles and age groups with respect to satellite cell activation. Interestingly, the QUAD muscle of 8-month-old mice was the only muscle that showed increased satellite cell activation in response to voluntary wheel running. QUAD muscles from the older group had opposing results since satellite cell activation decreased with exercise. Previous research in our lab indicated that mechanical stimulation of muscle causes a bolus release of nitric oxide that in turn activates satellite cells via the release of HGF from the extracellular matrix. The liberated HGF then binds to its receptor c-met. An upregulation of NOS-I and associated

increase in satellite cell activation with exercise in the QUAD muscle from 8-month-old mice supported this finding. Results from the QUAD muscle of older mice suggest NO release, as a result of voluntary wheel running, had an opposite effect on satellite cells and, thus, decreased activation. Decreased muscle precursor cell activation in response to a bolus release of NO is similar to results demonstrated in *mdx* mice. Therefore, a disrupted DGC and/or displaced NOS-I may account for the difference in response of satellite cells to mechanical stimulation between young and old mice. If the DGC and NOS-I are disrupted, sufficient levels of NO may not be available to satellite cells or satellite cell response to NO in aged muscle may be perturbed. Regardless of the reason for this difference, it is evident that the correct volume and intensity of exercise, as well as the level of NO must be investigated further to determine which combination would have the greatest therapeutic benefit to treat age-related muscle atrophy.

It has been established that NO production is an intermediary step between mechanical stimulation and satellite cell activation in normal young muscle. Therefore, to activate satellite cells, NO availability must increase in response to exercise. NO is produced endogenously by the enzyme NOS-I since it converts LA to L-citrulline and NO. Deductively, in normal muscle, expression of NOS-I would increase to produce more NO in response to mechanical stimulation of muscle. If this occurs in normal muscle, satellite cells respond to the chemical messenger (NO) by becoming activated. Hence, increased NOS-I expression would transpire into increased satellite cell activation. This was supported by our study since increased NOS-I expression was evident in the QUAD muscles from the exercise group of 8-month-old mice; the same group that demonstrated increased satellite cell activation. Increased NOS-I expression

was not apparent in any of the other muscles, regardless of exercise, in either the 8- or 18-month-old age groups. Similar and complimentary to satellite cell activation, expression of NOS-I was age-dependent and muscle-specific. Exercise programs developed to prevent or combat sarcopenia must take into account the different responses of satellite cells and NOS-I expression in various muscles of the lower limb. In order to maximize the benefits of exercise training, mechanical loads that stimulate NOS-I expression and satellite cell activation must be determined for specific muscles and age groups.

Further confirming the role satellite cell activation and NO in the process of muscle growth was the complimentary downregulation of myostatin in the QUAD muscle from the 8-month-old exercise group. Myostatin is a member of the transforming growth factor-β superfamily and is negatively correlated with muscle growth. If myostatin increases, muscle growth is inhibited. Conversely, decreased expression of myostatin, as was evident in the QUAD muscle of the young exercise group, is associated with increased muscle growth. Therefore, the results from Chapter 6 demonstrated that an increase in NOS-I was associated with increased satellite cell activation and decreased levels of the muscle inhibitory factor, myostatin. Muscle was processed for Western blot analysis 24-or 48-hours after completion of the exercise protocol in 8- and 18-month-old mice, respectively. Muscle fiber CSA measurements were performed on the GAST muscle and did not demonstrate muscle growth. However, evidence suggests that if the QUAD muscle was selected for muscle fiber CSA measurements, muscle hypertrophy would have been apparent in the younger exercise group. Results from this section of the thesis confirm that NO treatment and exercise have the potential to stimulate satellite cell

activation and increase muscle growth. The challenge that lies ahead is to determine the mechanical load and NO concentration that would promote muscle growth in aged muscle.

7.4 Limitations and strengths

7.4a Limitations

One limitation of the studies presented in this thesis is the use of [^{3}]H-thymidine as a measure of satellite cell activation. Incorporation of 3H-thymidine into new DNA occurs as cells enter the G1 phase of the cell cycle from quiescence (GO). In normal young muscle, activation of satellite cells occurs within 10 minutes of an activating stimulus, therefore, when muscle is isolated 22 hrs (44 hours later in muscle aged to 12 months or beyond) later, assays for DNA synthesis may not accurately reflect what occurred 22 hours earlier since all activated satellite cells do not necessarily proliferate. In addition, this measure of satellite cell activation assumes all cells activate and proliferate at the same time because only incorporation of radioactivity into new cells at the time of muscle harvest is being captured. Satellite cells that proliferated earlier, or later, than the time of assay would have missed the "window of opportunity" to be included in DNA scintillation counts. Further, 3H-thymidine incorporation into new DNA is not specific to muscle satellite cells and would mark other proliferating cells, such as fibroblasts. However, this measurement of muscle precursor cell activation has been used extensively in our lab, allows for the processing of a large number of muscles, provides results quickly (within 24 hrs), and can produce consistent results if connective tissue and tendons are removed from muscle samples prior to homogenization (decreases the number of fibroblasts included in the muscle homogenate).

Another limitation to this research is the indirect measurement of NO production through western blot analysis for NOS-I. Increased NOS-I expression may be consistent with the increased endogenous production of NO but this may not necessarily be the case. NO production could increase or decrease without a change in NOS-I if the rate at which the enzyme produces NO was altered. In addition, the availability of LA would certainly affect the production rate of NO within a muscle. Since NO has an extremely short half-life (4-15 seconds), measuring quantities of NO within muscle is extremely challenging and time-consuming. Taken into account that Chapter 6 of this thesis investigated the effects of age and exercise on muscle in vivo, measuring NOS-I expression, rather than NO production, was the most useful data since it allowed for comparison with whole muscle culture experiments. Stretch experiments combined with NO treatments of whole muscle cultures also provided indirect information on the availability of NO and the function of NOS-I in 8- and 18-month old mice. Collectively, these experiments confirmed that NO is a key activator of satellite cells and that NO production in aged muscle is disturbed since satellite cell activation could not be increased with exercise, mechanical stretch or a combination of stretch and NO supplementation.

A longitudinal study design would have strengthened the results of this study as opposed to a cross-sectional design. If mice were followed from 6 weeks-of-age until 18-months-of-age, the progression of age-related muscle atrophy, and the mechanisms responsible for the changes in muscle structure and function could have been investigated with greater certainly since the variability between age groups would have been eliminated. However, longitudinal studies also have limitations and regardless the reproducibility of techniques, the variability of the results increases when techniques are

performed several months apart. In addition, several of the variables obtained within each of the studies could not have been obtained without sacrificing the mice to obtain muscle tissue. Although a longitudinal study design would have been beneficial for studying grip strength, endurance activity and body mass measurements, all other variables were obtained from muscle tissue post-mortem, thus a cross-sectional design was necessary and justified.

Due to a substantial increase in aging research over the last decade, acquiring aged animals for the purposes of research is extremely difficult and costly. Fortunately, for the purposes of our studies, we were able to obtain retired female C57/BL6 mice from Central Animal Care Services (University of Manitoba) and The Jackson Laboratory (Bar Harbor, Maine). All mice included in this thesis were female which makes it difficult, and unadvisable, to apply these results to male mice. However, the endocrinological period when estrus cycling ceases, taken as equivalent to menopause in humans, is associated with accelerated loss of muscle structure and function and occurs between the ages of 18- and 20-months in mice. This allowed us to assess age-related muscle atrophy at a younger age, when fewer co-morbidities are present, and at a time interval when muscle wasting has been shown to accelerate. Both of these factors increase the possibility of identifying mechanisms that are responsible for sarcopenia since changes in skeletal muscle are taking place rapidly and the influence of other systemic pathologies on contractile tissue are at a minimum.

7.4b Strengths

The major strength of this study was that the analysis of the effects of aging and mechanical stimulation on skeletal muscle was investigated in both whole muscle culture

and in vivo. Extending the research from a stretch model of isolated muscle to an in vivo model of voluntary wheel running significantly expanded the potential for application of this research from the bench to exercise training and rehabilitation programs. Contrary to previous studies, stretch and exercise are realistic, ethical, and safe models to not only investigate satellite cell activation, but also to disseminate knowledge form theses studies into real world settings of sarcopenia.

An additional strength of this study was the combination of variables, which included muscle structure, function, satellite cell activation and gene expression. To date, the argument still exists regarding muscle structure and function. Does muscle structure change in response to decreased magnitude and frequency of mechanical loads or do alterations in muscle structure precede force deficits? Recently, the effect of the environment on satellite cell activation in muscle has received a lot of attention yet the models of muscle transplantation and heterochronic parabiosis are not reasonable treatments for individual suffering from sarcopenia, although the results of these studies have provided significant insight environmental influences on satellite cell activation. Chapter 6 of this work incorporated exercise into the study protocol to determine if more realistic, lifestyle activities could influence the satellite cell environment in a positive manner and rescue the characteristics of young muscle that are impaired with age. The combination of muscle structure, function, satellite cell activation and gene expression provided insight into the mechanisms responsible for age-related atrophy and possible treatment options to prevent or reverse muscle wasting in older animals.

This was the first study to investigate the effects of age and exercise on several hindlimb muscles of 8- and 18-month-old rodents. The quantity and quality of data

collected from muscles with a different location, function, and fiber type expanded our knowledge in this area of research and revealed that the effects of exercise are age-dependent and muscle-specific. Results from previous studies have been generalized beyond the muscles being studied which introduced the risk of accepting hypothesis that were not universal and aborting treatments, such as exercise and NO supplementation, that were realistic, ethical and safe in animal models.

Together the strengths of this research vastly outweigh the weaknesses and have provided information that is interesting and applicable in whole muscle cultures, as well as, in vivo. The intent of this research was to make a connection between single fiber models, whole muscle cultures and in vivo experiments so that the gap between basic sciences research and clinical research could be narrowed. Our lab believes that the broad scope of these studies have assisted us in achieving the goal we set forth at the commencement of this project.

7.5 Conclusion

As age increases, muscle satellite cells require greater mechanical and chemical stimulation to become activated in order to contribute to muscle maintenance, growth and repair. Satellite cell activation, which is required to induce muscle hypertrophy, is both age-dependent and muscle-specific. Age-related deficits in mechanically-induced satellite cell activation lead to decrements in muscle structure and function that ultimately lead to sarcopenia. Voluntary exercise, stretch and NO treatments alone were not able to rescue muscle precursor cells from an age-induced quiescent state, but have the potential to prevent and treat muscle atrophy when used in combination.

Sarcopenia is an age-related loss of muscle mass and function characterized by a reduction in fiber CSA and loss of motor units [218, 375, 433]. With aging, muscle atrophy is accompanied by deficits in strength that lead to decreased function and increased disability [321, 435]. In the next 10 years over 50% of the workforce will retire and since up to 30% of muscle loss occurs after the fifth decade of life, sarcopenia will have an enormous impact on the health of Canadians and the economy of our country [436]. The goal of the research presented here was to identify the mechanisms responsible age-related muscle loss and propose therapies to prevent or treat this universally disabling condition.

With age muscles atrophy, but it has not been universally accepted that the number of satellite cells decrease with age. Previous studies indicate that the age-related change in the population of satellite cells is age-dependent and muscle-specific [22, 204, 207, 277]. In situ hybridization for Pax7, MyoD and myogenin indicated that the GAST muscle had a decreased satellite cell pool size compared to younger mice. Similar results were demonstrated previously in the EDL muscle of old animals [22, 204]. In humans, the results are also inconclusive as some studies demonstrate a decrease in satellite cell pool size [446, 458] whereas other investigators have shown an increase satellite cell number [443]. Therefore, in order to accurately assess the effects of age on satellite cell number, more muscles from the same animal need to be investigated. If the age-related change in satellite cell number is in fact muscle-specific, it would be important to determine which muscles are affected more intently. This would not only provide further information on the mechanisms of sarcopenia but also the most effective treatments for individual muscles or muscle groups.

In normal adult muscle, satellite cells are metabolically and mitotically quiescent, but upon activation, are the main contributor to muscle maintenance, growth and repair [5]. Satellite cells become activated in response to stretch, exercise, trauma and denervation but, as age increases, muscle precursor cell activation becomes delayed and/or perturbed [22, 23, 218, 222, 278, 441]. This was confirmed by our study in whole muscle cultures since satellite cell activation in the EDL muscle from normal female mice was greatest at 8 months-of-age but significantly decreased at, and beyond, 10 months-of-age. Although Betters and colleagues (2008) reported that satellite cell activation was not decreased in isolated single fibers from the GAST muscle of 10 month-old mice, a significant decrease was evident in single fibers from 22-month-old mice. This difference may be due to an inconsistency in the muscles that were investigated (EDL vs. GAST), as well as, the type of culture being studied (single-fiber vs. whole-muscle). In addition, in vivo experiments demonstrated perturbed satellite cell activation in the EDL-, QUAD- and TA-muscles from 18-month-old mice compared to 8-month-old animals. Although an aged systemic environment negatively affects satellite cell activation [278], consistency in results between whole muscle culture and in vivo experiments indicate that the inherent muscle environment, even in isolation, contributes to deficient satellite cell activation. However, despite decreased basal levels of satellite cell activation in several muscles, the effects of exercise and stretch on activation in aged animals has yet to be realized.

For the research presented here, stretch of whole-muscle in culture and exercise in vivo were selected as models of mechanical stimulation to activate satellite cells. Age-related deficits in satellite cell activation have been demonstrated using models of muscle

trauma [218, 261], however, the mechanisms of muscle repair may differ in response to severe trauma as apposed to stretch or exercise. In severe, non-physiological models of muscle damage (cardiotoxin injection) a side population of cells ($CD45^{+}$;$SCA1^{+}$) rather than satellite cells are recruited for repair [140]. However, the same group demonstrated that in young muscle, the primary responder to muscle damage caused by exercise is muscle satellite cells. The recruitment of different cell types may be a result of the obliteration of the satellite cell population with cardiotoxin injection or this may indicate that the severity of muscle damage may determine which cells are recruited for repair. Further, with mechanical stimulation, a threshold may have to be surpassed to recruit satellite cells. Our data support this theory since previous experiments in our lab demonstrated that muscle from 6-month-old mice required twice the length increase via stretch to activate satellite cells to the same extent as muscle from 3-month-old mice. Unfortunately, increasing the magnitude of stretch did not increase satellite cell activation in whole-muscle (EDL) culture from either 8- or 18-month-old mice. This data suggest that mechanical activation of satellite cells is perturbed as early as 8 months-of-age and that older muscle requires greater mechanical stimulation to induce activation. Betters and collegues (2008) demonstrated increased satellite cell activation in single fibers from 10-month-old mice but the stimulus used (centrifugation) to mechanically-activate satellite cells was more aggressive than a passive stretch. Alternatively, exercise-induced muscle damage compared to severe muscle damage has been shown to recruit a different type of cell (satellite cells vs. CD45+ Sca-1+) for regeneration in young mouse muscle [140]. Inflicting severe muscle damage to combat age-related muscle atrophy is neither realistic nor ethical; hence, effective therapies that activate satellite cells through stretch

and/or exercise must be developed. In addition to mechanical stimuli, chemically-induced satellite cell activation may be another potential therapy to treat sarcopenia.

To date, the only two chemical stimuli proven to activate satellite cells are nitric oxide and hepatocyte growth factor [5, 6, 23]. Our lab, and later corroborative studies by others, has demonstrated that a bolus release of nitric oxide, in response to the application of a mechanical stimulus, activates satellite cells in: myoblast cultures, single fiber preparations, and in vivo [28, 31]. The role of nitric oxide in satellite cell activation was confirmed in the current studies using stretch and NO treatments in whole-muscle cultures from 8- and 18-month-old mice. In 8-month-old mice, stretch alone did not increase satellite cell activation but, when stretch was combined with an exogenous source (ISDN) of nitric oxide, activation increased beyond control levels. When ISDN was combined with LN, a non-specific inhibitor of NOS, satellite cell activation still increased and confirmed the positive benefit of NO supplementation on muscle precursor activation. LA (an endogenous source of NO), on the other hand, did not increase satellite cell activation compared to unstretched muscles in the 8-month-old age group. These results are in agreement with a study by Betters et al. (2008) in which administration of DETA-NO increased $BrdU^{+}$ cells in 10-month-old mice but LA did not have an effect. The significance of these findings is two-fold. Firstly, the role of nitric oxide is confirmed with the administration of ISDN and the corresponding increase in satellite cell activation. When ISDN was combined with LN, activation still increased which corroborates that ISDN is independent of NOS activity. Secondly, and consistent with previous research[29], NOS activity is perturbed since LA, which requires NOS-I to

produce NO, did not result in an elevation of satellite cell activation compared to non-treated muscle.

Contrary to the effect of DETA-NO treatment in old mice [29], satellite cell activation was not increased or decreased with the addition of endogenous and exogenous sources of NO, or NOS inhibitors, in our 18-month-old mice. However, when ISDN was added to cultures and the muscle was stretched, a strong trend toward decreased satellite cell activation was evident. This suggested that the sensitivity to satellite cells in aged muscle differs from muscle in adult mice since ISDN treatment increased satellite cell activation occurred after ISDN supplementation in 8-month-old mice. We propose a hypothetical curve for satellite cell activation and NO concentration in aged mice (figure 7-1). The solid line represents the hypothetical curve for young muscle whereas the dashed line is an illustration of the hypothetical curve for aged muscle. As NO concentration increases in young muscle with stretch or NO supplementation, satellite cell activation increases. Conversely, when NO concentration decreases in young muscle, with LN treatment, satellite cell activation decreases. The hypothetical curve for aged muscle is shifted downward with less curvature. Satellite cell quiescence in muscle from older mice is maintained at a lower concentration of NO and as NO concentration increases, satellite cell activation actually decreases. On the contrary, when LN is added to muscle in culture, there is either no change or a slight increase in satellite cell activation since NO concentration is already decreased compared to normal young muscle.

This was the first study that demonstrated a muscle-specific, hypertrophic response (increased satellite cell activation) to mechanical stimulation via exercise in

normal adult mice. The QUAD muscle from 8-month-old mice was the only muscle that demonstrated an increase in satellite cell activation with 3-weeks of voluntary wheel running. There was no evidence of increased muscle precursor activation in the EDL-, TA-, and GAS- muscle of adult mice following exercise. Human studies also show muscle-specific responses in satellite cell activation to various levels or types of activity. Muscle biopsies taken from the trapezius-, biceps brachii-, masseter-, tibialis anterior- and vastus lateralis-muscle all demonstrated age-dependent and muscle-specific levels of satellite cell activation which can be attributed, in part, to various types and intensities of muscle activity [169, 439, 443, 444, 446]. Previous work in our lab did not demonstrate an exercise-induced, muscle-specific response in satellite cell activation between QUAD-, TA-, GAST- and diaphragm-muscles from the untreated group of 4-week-old *mdx* mice [252]. However, it is important to note that muscle from the *mdx* mouse model, a homologue to Duchenne Muscular Dystrophy in humans, is characterized by satellite cell hyperactivation, even without exercise. Therefore, direct comparison of results in young *mdx* mice to the effects of exercise on satellite cell activation in older muscle from normal mice is not feasible. Balon and Nadler (1997) reported an increase in NOS-I expression in the soleus muscle of rats after level treadmill running but the age of the rodents was not stated and only the soleus muscle was selected for analysis. Central nucleation, a morphological marker of muscle repair, has also demonstrated muscle-specificity. Muscle from normal mice (between 2-and 3-months-of-age), exposed to 4-weeks of uphill treadmill running, had a greater percentage of fibers with central nuclei compared to the control group. Increased central nucleation was evident in the plantaris-, tibialis anterior-, and extensor digitorum longus-muscle, but not the soleus muscle [253].

Although this study demonstrated a difference in central nucleation between muscles, the muscles were isolated from 2-3-month old mice compared to 8-month-old mice. We have demonstrated in our whole-muscle culture studies that mechanically-induced satellite cell activation via stretch was altered between 3- and 6-months-of-age since the latter age group required twice the mechanical stretch to increase satellite cell activation. Parise and colleagues (2008) subjected 8-10-week-old mice to 3 days of downhill treadmill running, which resulted in a peak of satellite cell activation in the TA muscle at 48-hours post-exercise. Satellite cell activation in other muscles was not investigated; therefore, the muscle specificity of satellite cell activation could not be determined. Together, these studies suggest several factors may contribute to the muscle-specific response in satellite cell activation to mechanical stimuli including: age of the animal, strain of the animal (C57/BL6 vs. *mdx*), type of exercise (treadmill-l versus voluntary wheel-running) and intensity of exercise (level- vs. uphill- vs. downhill-running). Similar statements were reported by Kadi and colleagues (2004), "the fact that satellite cells were investigated in different muscles is probably a major reason for the difference between studies" and "the four muscles are used differently, have different fiber type composition and are also affected differently by increasing age". Further studies are warranted to determine the extent to which these variables contribute to satellite cell activation in various muscles. It is important to consider the role of each muscle during specific activities based on the anatomical location, angle pennation, fiber-type, size, force requirements, and type of contraction of the muscle during various phases of the activity.

The effects of age and exercise on satellite cell activation in normal adult- and old-female mice have yet to be realized. As mentioned previously, exercise induced

satellite cell activation was not only muscle-specific but also age-dependent. Three-weeks of voluntary wheel running did not have an affect on satellite cell activation in the EDL-, QUAD-, TA- or GAST-muscle from 18-month-old mice. These results are in agreement with our whole-muscle culture studies in which passive stretch was not an adequate stimulus to activate satellite cells from the EDL muscle of 18-month-old-mice. Similar findings have been reported in single fiber experiments from old (22-month-old) muscle in which fibers were centrifuged to activate satellite cells [307]. Therefore, mechanical stimulation in single-fiber, whole-muscle culture, and in vivo-studies has been insufficient to activate satellite cells from old muscle (18-22 month-old). Mechanical stimuli have included passive stretch of whole muscle, voluntary wheel running and centrifugation of muscle fibers. The effects of mechanical stimulation on satellite cell activation decrease with age to the extent that various protocols of mechanical stress have been unable to activate muscle precursor cells beyond control levels. A more traumatic form of mechanical stimulation (contraction-induced injury) demonstrated an increase in satellite cell activation from old muscle, but this was also associated with an increase in pro-apoptotic markers. However, this study did not show a decrease in satellite cell activation with age, using only MyoD as a satellite cell marker, and muscle was not retrieved for analysis until 10 days post-injury [459]. It has been well documented, in young muscle, that mechanical stimuli such as shear forces, stretch, exercise, loading and injury cause a bolus release of nitric oxide [28, 31, 80, 246, 253, 266-269]. However, evidence suggests this mechanism of satellite cell activation is perturbed in aged muscle. Hence, it was important to determine if deficient levels of nitric oxide were responsible for the lack of response to hypertrophic stimuli.

Although stretch of whole muscle in culture and treatment with an exogenous NO donor (ISDN) increased satellite cell activation in 8-month-old mice, this cocktail of mechanical and chemical stimuli did not increase muscle precursor activation in 18-month-old mice. Results from the 8-month-old group are in agreement with a study by Betters and colleagues (2008) which demonstrated an increase in emanating satellite cells on single-fibers subjected to centrifugation and NO treatments (LA or DETA-NO). However, increased satellite activation ($BrdU^+$ cells) was demonstrated in all age groups (young, adult and old) with either treatment (LA or DETA-NO) which is inconsistent with the results from our lab. We did not demonstrate an elevation in satellite cell activation in response to stretch plus LA treatment in 8-month-old mice. In addition, stretch combined with LA or ISDN did not have an affect on satellite cell activation in old, 18-month-old muscle. A few variables may account for the discrepancy between studies. Firstly, we used tritiated thymidine incorporation (scintillation counts/ug DNA) into satellite cells as a marker for activation as opposed to $BrdU^+$ nuclei per fiber. Secondly, the concentrations of LA and the source of exogenous NO differed between studies. Thirdly, our studies used a whole-muscle culture model versus single fibers in culture and the muscle of study also differed, EDL versus GAST. Finally, the method of mechanical stimulation differed between studies. We used passive mechanical stretch (length increase of 20%) to activate satellite cells whereas Betters et al. (2008) centrifuged single fibers to induce muscle precursor cell activation. Although neither method of mechanical stimulation in isolation was able to induce satellite cell activation in aged muscle, it is quite possible that the amount of NO produced and released during

these activities differed. This could account for the difference in response to NO supplementation between the two studies.

As mentioned previously, mechanical stimulation of muscle causes a bolus release of nitric oxide, which, in normal young muscle, activates satellite cells. Therefore, we also investigated NOS-I expression in EDL, QUAD, TA and GAST muscle of adult and old mice subjected to 3-weeks of voluntary wheel running. Interestingly, the only group to demonstrate an increase NOS-I expression with exercise was the QUAD muscle from 8-month-old mice. This is the same muscle and age group that demonstrated an increase in satellite cell activation with exercise. The role of NO and satellite cell activation was further confirmed in whole muscle culture experiments in which exogenous sources of NO and stretch increased satellite cell activation, regardless of NOS inhibition, in muscle from adult mice. Therefore, we have extended the association between NO and satellite cell activation to whole muscle culture stretch experiments and in vivo exercise protocols that involved normal mice up to 8 months-of-age. Increased satellite cell activation in response to mechanical stimuli (centrifugation) and exogenous NO treatment has also recently been demonstrated in old (22 months-of-age) mice [307]. Taken together, these findings suggest that mechanical stimulation alone may not be enough to activate satellite cells beyond control levels in older mice, but could be very effective in stimulating muscle growth when combined with NO supplementation.

Further corroborating the role of NO and satellite cell activation with respect to the growth of muscle were the investigations of the effects of age and exercise on myostatin expression. Only the QUAD muscle from 8-month-old mice demonstrated a

downregulation of myostatin in response to exercise. This was the same group that had increased NOS-I expression and satellite cell activation after 3-weeks of voluntary wheel running. The use of a myostatin antagonist (Mstn-ant1) increased satellite cell activation and muscle regeneration following neotoxin injury to the TA muscle of aged animals [457]. Since myostatin acts to inhibit the progression of muscle precursor cells from the G0-to S-phase of the cell cycle, blocking the effects of myostatin should increase satellite cell activation [456]. Aged myostatin null mice have a greater number of satellite cells and an increased capacity for muscle regeneration compared to wild type mice [460]. Therefore, the downregulation of myostatin, upregulation of NOS-I and increased satellite cell activation in the 8-month-old exercise group indicate that voluntary wheel running surpassed the threshold for mechanically-induced satellite cell activation in the QUAD muscle of adult mice. Myostatin antagonists provide an additional potential therapeutic intervention since inhibiting myostatin in aged muscle has increased satellite cell activation and muscle regeneration. Consequently, because sarcopenia is a multifactoral process, potential therapies may combine various methods of activating satellite cells including mechanical stimuli (stretch and exercise), chemical stimuli (NO) and stimulation and/or inhibition of growth factors (HGF and myostatin, respectively).

Age-related changes in muscle structure, satellite cell pool size and satellite cell activation did not correspond to decreased muscle function. Forelimb grip strength indicated that the maximum grip strength of 18-month-old mice was less than 8-month-old animals; however, when absolute strength values were adjusted for body mass (relative grip strength) the difference in force production between the age groups was ameliorated. The body mass of old mice was less than adult mice and may have been an

adaptive response to aging to minimize the effects of muscle atrophy and associated strength deficits. Relative maximum grip strength is most relevant to function since this measure indicates the ability of an animal to manipulate body mass. For example, the ability to leg press 120 kilograms is insufficient if a person weighs 150 kilograms and wants to rise from a chair. Unfortunately, contrary to aging mice in this study, obesity rates in our population continue to rise which will ultimately confound the effects of strength loss due to aging.

At the initiation of the 3-week exercise protocol, old mice could not run as far as the high-activity adult mice, which was not unexpected. However, at the completion of the exercise protocol, there was no difference between the two groups and 18-month-old mice ran the same distance as the high-activity 8-month-old group. The older mice demonstrated a linear increase ($R^2 = 0.9977$) in exercise capacity over the course of the study, which was quite surprising considering the age of the animals and the fact that the high-activity 8-month-old group plateaued after week 2. These results suggest that senescent mice still have the ability to significantly improve exercise performance after only 1 month of a voluntary exercise intervention. Considering the vast array of health benefits associated with exercise, it is possible that long-term voluntary wheel running in aged mice could maintain or reverse muscle atrophy by positively altering the satellite cell environment.

Muscle atrophy is a significant pathology in our society whether it is a result of aging, injury, surgery, immobilization, cancer, disuse, space flight, or osteoarthritis, etc. This thesis has demonstrated that, in mice, the mechanisms that underlie muscle atrophy occur prior to changes in the structure and function of contractile tissue. Greater

magnitudes of mechanical stimulation and supplementation with chemical stimuli (NO) can rescue muscle satellite cells from quiescence in adult mice. However, stretch, exercise and NO supplementation used in this study were not able to rejuvenate satellite cell activation in muscle from old mice. However, the current research confirmed the role of NO and NOS-I activity in age-related muscle atrophy. Exercise, stretch and/or NO supplementation are a potential treatment for sarcopenia if therapeutic levels of each can be determined. Utilizing exercise and NO administration to treat muscle-wasting conditions would not only be realistic and inexpensive; it would also provide the individual with the many benefits of exercise to overall health.

7.6 Future Directions

The major finding of this thesis was that mechanically-induced satellite cell activation in normal female mice is age-dependent and muscle-specific. Older muscle requires a greater mechanical load than younger muscle to induce muscle precursor cell activation. We discovered that muscle from 6-month-old mice required twice the length increase as muscle from 3-month-old mice to increase satellite cell activation. However, this stimulus was not significant enough to increase satellite cell activation in muscle from older mice. Hence, future studies should investigate the threshold for mechanically-induced satellite cell activation in aged muscle. In addition to increasing the length change of a resting muscle in the whole muscle culture model (passive stretch), lengthening contractions (eccentric contractions) should also be performed to determine if greater contraction-induced injury stimulates satellite cell activation above control levels. A threshold should not only be established for each age group, but also each muscle being tested and the type of contraction or stretch being performed to induce muscle-hypertrophy. The oldest mice in our study were 18-months-of-age but future research should investigate mice 20- to 28-months-of-age.

We demonstrated that a combination of stretch and NO supplementation rescued satellite cells from a mechanically-resistant dormant state in muscle from 8-month-old mice. It is important to note that only NO supplementation with an exogenous source of NO (ISDN) had the capacity to increase satellite cell activation, which suggests NOS-I activity is impaired in old muscle. In spite of this, a cocktail of mechanical and chemical stimulation did not increase satellite cell activation from the EDL muscle of old mice. Whole-muscle culture experiments designed to determine a dose response for both

endogenous and exogenous sources of NO would not only establish a threshold for satellite cell activation but would also quantify the change in NOS-I activity with age. As mentioned previously, mice beyond 18 months-of-age should also be included in future studies to further assess the rate of progression of sarcopenia and establish age-specific NO supplementation protocols to combat muscle wasting.

Since NO supplementation with LA in whole muscle cultures of adult and old mice did not provide sufficient enough chemical stimulation to increase satellite cell activation, it is important to determine if dislocation of NOS-I from the DGC is responsible for this deficiency. If this is the case, and NOS-I has been relocated to the cytoplasm, atrophic signaling pathways may also be activated when a bolus release of NO occurs in response to mechanical stimulation [40]. Impending studies should determine if NOS-I is displaced from the DGC and dislocated into the cytoplasm of the muscle fiber, and more importantly, find out if NOS-I can be relocated to the DGC with mechanical and/or chemical stimulation. To remain consistent with the systematic approach to this thesis, establishing the age at which dislocation/relocation of NOS-I occur would provide insight into potential prevention and treatment options for muscle wasting diseases.

Voluntary wheel running in mice is similar to interval sprint training in humans yet this model of exercise only induced increased satellite cell activation in the QUAD muscle of adult mice. The EDL, TA and GAST muscles from adult mice did not respond to exercise in manner, nor did all four muscles from old mice. Additional data demonstrated that increased satellite cell activation in the QUAD muscle of adult mice was associated with increased NOS-I expression and downregulation of myostatin;

consistent with muscle hypertrophy. Subsequent research should be designed to determine why NOS-I expression was different between muscles of the same age group. In other words, was NOS-I relocated into the cytoplasm of muscle fibers that did not respond to exercise? It would also be important to establish if higher-intensity running programs (i.e. uphill and downhill treadmill running) increased hypertrophy-inducing changes in all muscles, not just the QUAD muscle.

In this thesis we combined passive stretch of muscle in culture with NO supplementation but did not combine in vivo exercise with NO treatments. Previous work in our lab has coalesced exercise and LA treatment in mdx mice and demonstrated improved functional performance with the treatment cocktail [252]. Future studies should investigate the effects of voluntary wheel running and NO supplementation (endogenous and exogenous) on inducing muscle hypertrophy in muscle from adult- and old-mice. Evidence from our whole-muscle culture studies suggest that exogenous NO supplementation and mechanical stimulation in vivo would increase satellite cell activation in muscle from adult mice. An in vivo model would provide further insight into the role of the aged satellite cell environment with respect to the mechanisms of age-related muscle atrophy. In addition, exercise and NO supplementation experiments in animals could be applied to humans since several products on the market today already contain LA, although side effects have not yet been established.

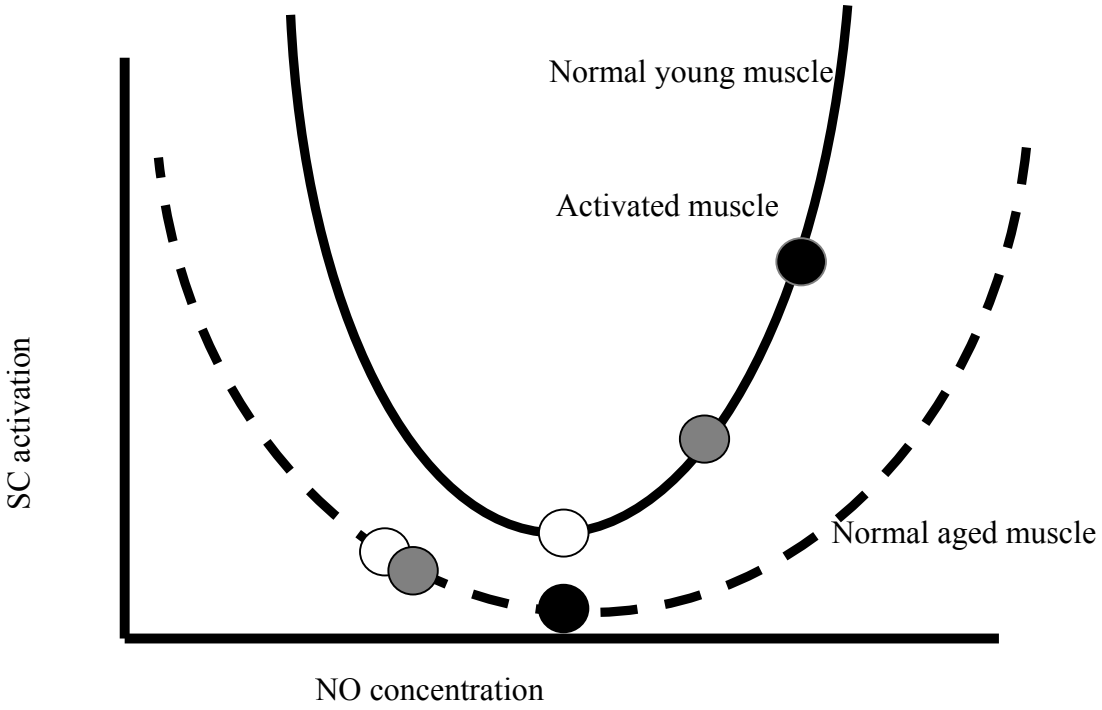
We have established that muscle response to mechanical stimuli in normal animals is muscle-dependent and age-specific. Similar results have been demonstrated in humans [169, 439], but further research is warranted because, to date, satellite cell activation agonist and antagonist muscle groups from the same individual have not been

selected for analysis. It is important to study the effects of in vivo exercise protocols on satellite cell activation and gene expression in agonist and antagonist musculature to determine if an inherent imbalance in these variables is correlated with differences in strength between opposing muscles of a joint (i.e. hamstring to quadriceps strength ratio). The mechanism of muscle atrophy in humans should be investigated to determine if the DGC and NOS-I are disrupted and dislocated, respectively, in aged muscle. This would provide insight into the role of NO and NOS-I activity in sarcopenia and increase the potential for therapeutic administration of NO and exercise to combat age-related muscle wasting. In addition, once baseline values are established for several joints and various age groups, the same protocol can be applied to numerous muscle and joint diseases that affect the structure and function of the human body (i.e. osteoarthritis, rotator cuff tears, etc.).

The future directions for this research are numerous since further insight into the mechanisms responsible for muscle atrophy is needed to develop effective prevention or treatment programs for sarcopenia. In addition, the application of such knowledge would extend well beyond sarcopenia and into other conditions and diseases that cause muscle loss including cancer, congestive heart failure, renal disease, osteoarthritis, AIDS, etc. We believe the findings of this thesis have moved aging research one step closer to finding treatment and/or cure for sarcopenia.

Figure 7-1. Satellite cell response to NO concentration in normal young- and old-muscle.

The solid line represents the hypothetical curve for young muscle whereas the dashed line is an illustration of the hypothetical curve for aged muscle. A darker circle indicates a greater concentration of NO. The gray circle represents NO concentration when satellite cells are in a quiescent state since a pulsatile release of NO maintains satellite cell quiescence in normal adult muscle. As NO concentration increases (black circle) in young muscle with stretch or NO supplementation, satellite cell activation increases. Conversely, when NO concentration decreases in young muscle (white circle) with LN treatment, satellite cell activation decreases. The hypothetical curve for aged muscle is shifted downward with less curvature. Satellite cell quiescence in muscle from older mice is maintained at a lower concentration of NO (gray circle) and as NO concentration increases, satellite cell activation actually decreases. On the contrary, when LN is added to muscle in culture, there is either no change or a slight increase in satellite cell activation since NO concentration is already decreased compared to normal young muscle.



CHAPTER 8. REFERENCES

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