UNIVERSITY OF CALGARY

Mechanisms of Atrophy and Myosin Heavy Chain Co-expression in Aging Muscle

by

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Abstract

Sarcopenia is the age related loss of skeletal muscle mass and skeletal muscle function. It causes impairment of mobility activities and can lead to reduced quality of life of elderly persons. Currently, the cause of sarcopenia is unknown and there are no effective methods for its treatment. Recent findings from our lab implicate denervation as the primary instigator of myofiber atrophy leading to the loss of skeletal muscle mass observed in sarcopenia. Using immunolabelling for MuRF1, MHCs and MHCf protein expression, we examined the involvement of the proteasomal protein degradation system in sarcopenia in the context of denervation, as well as the morphology of sarcopenic muscles of different myofiber type compositions. We found that MuRF1 expression was elevated in a denervation specific manner in sarcopenia, that sarcopenic muscle is characterised by marked MHC co-expression and muscle specific shifts in MHC expression, and that MHCs myofibers are not protected from atrophy.

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There are several people who have contributed greatly to the development and completion of this work who I wish to thank. Firstly, I wish to thank my supervisor, Dr. Russell Hepple, for his enthusiasm, encouragement and guidance throughout the entirety of this research experience. Secondly, I wish to thank Dr. David Severson and Rosalie Kolstad for their tireless support – they have served above and beyond their roles as liaisons of the Kinesiology Faculty. Thirdly, I wish to thank the members of the Hepple lab; Sharon Rowan for her assistance in learning and developing the immunolabelling techniques used herein, Dr. Melissa M. Thomas for her help with tissue collection and Rui Mi for sectioning the tissues. Finally, I wish to thank my supervisory committee for their dedication of time and energy.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
MHC	Myosin heavy chain
MHCs	Slow myosin heavy chain isoform
MHCf	Fast myosin heavy chain isoform
CSA	Cross sectional area
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
YA	Young adult
SEN	Senescent
F344BN	Fisher 344 X Brown Norway F1 rat
SOL	Soleus muscle
GASr	Red region of the gastrocnemius muscle
GASm	Mixed region of the gastrocnemius muscle

Chapter One: Introduction

1.1 Background

Sarcopenia is the age-related loss of skeletal muscle mass and skeletal muscle function. In humans, it is associated with impairment of mobility activities such as walking, climbing stairs, and getting out of bed, and can also compromise the ability to conduct activities of daily living such as eating, bathing, shopping, and household chores (Janssen *et al.* 2002). These impairments contribute to an increased risk of falls and bone fractures, decreased physical activity, and a loss of independence (Jones *et al.* 2009). Consequently, sarcopenia contributes to reduced quality of life of elderly persons (Spirduso & Cronin 2001) and is a significant burden on health care and social support systems (Motl & McAuley 2010).

A U.S. national health survey found that 69% of women over the age of 60 and 52% of men over the age of 60 exhibit a clinically significant degree of sarcopenia, defined here as a muscle mass lower than values for a healthy adult population of the same sex by at least one standard deviation (Janssen *et al.* 2002). Although these values are based on the U.S. population, similar population demographics in Canada imply similar prevalence in our population. With estimates that by the year 2026 those aged 65 years or older will comprise 20% of the total Canadian population (Health Canada, 2002), it follows that, by that time, over 10% of the population will suffer from a clinically significant degree of sarcopenia and its related risks. Clearly, research efforts to understand and treat this condition are both warranted and necessary.

There are two components of sarcopenic loss of muscle mass: loss of individual myofibers and atrophy of individual myofibers (Kirkendall & Garrett 1998). Using whole

muscle cross sections, Lushaj and colleagues found that in rat models of sarcopenia total muscle mass was reduced by as much as 50% by very old age (Lushaj *et al.* 2008). They also found that there was an increase in collagen levels within the muscle such that, by very old age, collagen accounted for greater than 20% of the muscle composition, indicating that the *functional* mass of the muscle was further reduced. Contributing to this loss of mass, they found that between 30% and 45% of myofibers were lost by very old age and the mean cross sectional area (CSA) of remaining myofibers was reduced by approximately 40% compared to young adult rats. At this point it is not known what events lead to the loss of individual myofibers in sarcopenia. However, we have recently shown that the accumulation of severly atrophied myofibers parallels the progression of sarcopenia, suggesting that individual myofibers undergo a period of atrophy prior to being lost from the population (Rowan *et al.* 2011). If this is the case, identifying the initial cause and mechanisms of myofiber atrophy in sarcopenia represents the first steps to combating its progression. Recent evidence from our lab implicates myofiber denervation as the primary instigator of individual myofiber atrophy in sarcopenia (Rowan *et al.* 2010). We have found that in senescence, denervated myofibers were atrophied by approximately 35% compared to innervated myofibers in aged muscle, but innervated myofibers were only 7% smaller than young adult myofibers where no denervation was observed. Further study is required to determine the cellular *mechanisms* by which denervation is causing muscle atrophy associated with age.

In surgical (Talmadge *et al.* 1999; Patterson *et al.* 2006) and disease (Borg *et al.* 1989) models of denervated skeletal muscle, the muscle morphology is characterized by severe atrophy of individual myofibers and pronounced co-expression of multiple myosin

heavy chain (MHC) isoforms within individual myofibers. Our lab has confirmed that this muscle morphology is also present in aging-associated denervation (data in review) and, furthermore, that accumulation of severely atrophied myofibers marks the accelerating phase of sarcopenia which precipitates the most serious complications arising from muscle atrophy (Rowan *et al.* 2011). However, there is limited documentation of the prevalence of MHC co-expression in aging muscle or its impact on the understanding of age-associated changes in myofiber size and type proportions. As such, further study is required to determine the extent to which the morphologies of denervated muscle and aging muscle are similar.

1.2 Objectives

Based on the aforementioned finding by our lab implicating myofiber denervation as the primary instigator of individual myofiber atrophy in sarcopenia (Rowan *et al.* 2010), the overriding hypothesis of this work is:

Denervation is the primary cause of myofiber atrophy in sarcopenic muscle and, therefore, dictates the activation of cellular atrophy mechanisms and changes in myofiber morphology observed with age.

Figure 1.1 presents a visual representation of this hypothesis.

Consequently, the objectives of this work are:

(1) To examine the *mechanisms* of age-associated myofiber atrophy following deneveration.

(2) To examine the *prevalence* of MHC co-expression associated with aging in muscles of different myofiber type compositions and to determine the *impact* of MHC co-expression on estimates of alterations in myofiber type proportions and estimates of type-specific alterations in myofiber size associated with age.

Combined, this research will provide novel insights into the mechanisms that cause muscle atrophy associated with aging and into how they impact different myofiber types. These insights will, in turn, contribute to a knowledge base from which treatments to counter or prevent sarcopenia can be developed.

1.3 Presentation

This thesis contains several chapters. Chapter One provides a brief introduction to the subject. Chapter Two is a literature review of current knowledge regarding denervation-associated and age-associated myofiber atrophy and muscle morphology. Chapter Three addresses objective (1) and Chapter Four addresses objective (2). Chapter Five provides an overall discussion of the findings, future directions and final conclusions of the research material presented herein.

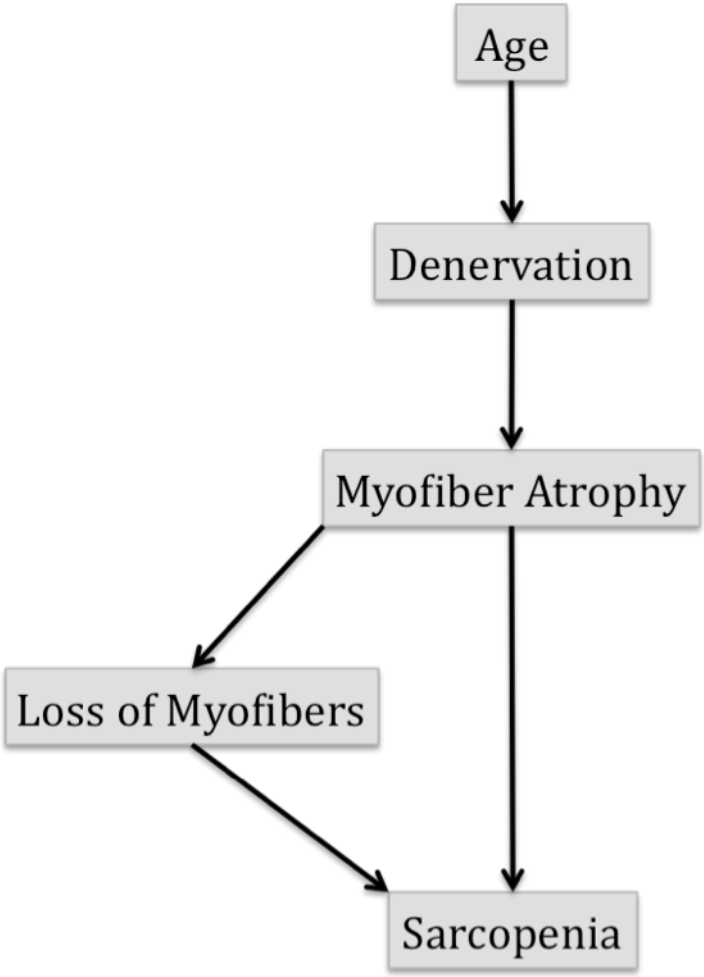

Figure 1.1 Schematic showing the overriding hypothesis of this thesis.

Chapter Two: Literature Review

2.1 Sarcopenia

Currently, sarcopenia is an unavoidable consequence of aging (Faulkner *et al.* 2007; Marzetti *et al.* 2009). In humans, its progression starts as early as the third decade of life (Lexell *et al.* 1988) and accelerates around the fifth decade such that, by the eighth decade of life, there is a reduction in muscle mass by approximately 50% (Lexell *et al.* 1988) and an even greater reduction in muscle strength (Goodpaster *et al.* 2006). This relative progression pattern is also observed in rat models of sarcopenia (Hepple *et al.* 2004; Lushaj *et al.* 2008). As outlined in Chapter 1, the reduction in muscle mass observed in sarcopenia is a result of both atrophy and loss of individual myofibers (Lexell *et al.* 1988; Lushaj *et al.* 2008). The reduction in muscle strength observed in sarcopenia is likely due to a combination of reduced functional muscle mass available to contribute to force production (Frontera *et al.* 2000b; Frontera *et al.* 2008) and reduced efficiency of force development as a result of neural impairment (Delbono 2003). There is also some evidence which suggests that the contractile ability of individual myofibers decreases with age and contributes to reduced specific force generation (Thompson 2009); however, in a longitudinal study of aged human muscle, Frontera and colleagues found no decrease in individual myofiber strength (2008). Although the morphological characteristics of aged muscle will be discussed in greater detail later in this review, briefly, sarcopenia is characterised by myofiber type grouping as a result of ongoing denervation/re-innervation (Lexell & Downham 1991), shifts in myosin heavy chain expression (Snow *et al.* 2005), an accumulation of severely atrophied myofibers (Rowan *et al.* 2011), marked myofiber size heterogeneity due to compensatory hypertrophy of

remaining "healthy" myofibers (Frontera *et al.* 2008) and an accumulation of connective tissue and collagen (Lushaj *et al.* 2008).

Many potential causes of sarcopenia have been posited in the literature. They range from broad, systemic theories of aging, to narrow, cellular causes of sarcopenia. Amongst the most often studied hypotheses is that sarcopenia is the result of mitochondrial dysfunction which eventually causes irreparable oxidative damage to the myofiber (Marzetti *et al.* 2009; Thompson 2009). Another is that sarcopenia is the result of an imbalance between protein synthesis and protein degradation which eventually compromises the myofiber's ability to survive (Clavel *et al.* 2006; Altun *et al.* 2010). While denervation has long been a proposed cause of the muscle atrophy observed in sarcopenia, in the past, there have not been studies which directly examine its contribution to sarcopenia (Faulkner *et al.* 2007) and it has not received much attention in the literature in recent years. To our knowledge, our finding that denervated myofibers in sarcopenic muscle are significantly atrophied, whereas innervated myofibers are only minimally atrophied (data in review), is the first to directly implicate denervation as a cause of the muscle atrophy seen in sarcopenia.

2.2 Denervation and its Involvement in Sarcopenia

Age-associated denervation is a well-documented phenomenon in both animal studies and human studies (Gutmann & Hanzlikova 1972/73; Luff 2006; McMullen & Andrade 2009). The general changes occurring in age-associated denervation of myofibers are as follows: 1) Alteration/remodeling of neuromuscular junctions; 2) Loss

of whole motor neurons to the muscle; 3) Reduced capacity of remaining motor neurons to compensate for those lost.

A simple schematic of a typical neuromuscular junction is shown in Figure 2.1. Alterations of individual neuromuscular junctions may be one of the precipitating events leading to age-associated denervation of myofibers. With age, neuromuscular junctions become increasingly complex. There is an increase in the number of axonal branches and an increase in their length and their complexity, alterations that are characteristic of ongoing denervation and re-innervation at the neuromuscular junction (Deschenes *et al.* 2010). As this process progresses, the interaction between the pre-synaptic endplate and post-synaptic folds is compromised, with increasing numbers of post-synaptic folds becoming exposed (Cardasis & LaFontaine 1987). Furthermore, the neuromuscular junction becomes increasingly fragmented (Cardasis & LaFontaine 1987; Prakash & Sieck 1998; Suzuki *et al.* 2009; Deschenes *et al.* 2010). These alterations may contribute first to "functional" denervation of the myofiber, then to physical denervation of the myofiber where contact between the neuron and the myofiber is completely lost (Suzuki *et al.* 2009). Recently, it has been shown that age-related alterations of the neuromuscular junction that are characteristic of denervation and re-innervation precede any identifiable changes in muscle morphology, perhaps indicating that the progression of sarcopenia starts at the neural level (Deschenes *et al.* 2010). For reasons that are unclear, the capacity for re-innervation of denervated myofibers becomes compromised with age, eventually resulting in permanently denervated myofibers (Pestronk *et al.* 1980). Overall, there is a reduction in the number of motor neurons to the muscle

(Carlson 2004), and remaining motor neurons are reduced in diameter (Edstrom *et al.* 2007).

Recently, novel evidence from our lab, in collaboration with the lab of Dr. Doug Turnbull at Newcastle University, directly links age-related denervation and myofiber atrophy. We have found that denervated myofibers are significantly atrophied in aged muscle, however, innervated aged myofibers are only minimally smaller than young adult myofibers and, furthermore, the amount of overall muscle atrophy corresponds to a loss of motor neurons in the spine (data in review). This implicates denervation as the primary instigator of age-associated muscle atrophy and suggests that individual myofibers are mere respondents. This novel finding is supported by the works of Carlson & Faulkner (1989) where they show that damaged, denervated aged muscle that is surgically transplanted into a young muscle host repairs itself in a manner indistinguishable from damaged, denervated young muscle, making an almost full recovery. Conversely, damaged, denervated young muscle that is surgically transplanted into an old muscle host has reduced capacity for repair (Carlson & Faulkner 1989). This suggests that the vitality of aged muscle is not limited by factors within individual myofibers, but rather, by other systemic factors which then have a downstream impact on individual myofibers. Given our recent evidence implicating denervation as that factor, our justification for objective (1) is based on a necessity to determine the mechanism by which denervation acts in the aged myofiber.

2.3 Mechanisms of Myofiber Atrophy Following Denervation

There are two systems that contribute to the atrophy of individual myofibers: the lysosomal autophagy system and the proteasomal protein degradation system. In denervated muscle, there is mounting evidence that the proteasomal system is a main contributor to myofiber atrophy. Pharmacological inhibition of the proteasomal system (Beehler *et al.* 2006), knock-out of proteasomal regulators (Mittal *et al.* 2010; Moresi *et al.* 2010) and knock-out of components of the proteasomal system (Bodine *et al.* 2001) all result in approximately 50% protection from myofiber atrophy in denervated muscles. Examining both the autophagy system and the proteasomal system exceeds the scope of this thesis, so, from here forward focus will be on the proteasomal system.

The proteasomal system is a method of intracellular protein degradation whereby damaged or unwanted proteins within the cell are selectively targeted and broken down into amino acids. This system is dictated by the complex interaction of four main components: ubiquitin-activating enzymes (E1's), ubiquitin-conjugating enzymes (E2's), ubiquitin ligases (E3's), and the 26S proteasome (Figure 2.2) (Glickman & Ciechanover 2002; Beehler *et al.* 2006; Eddins *et al.* 2011). Ubiquitin-activiating enzymes represent the first step in ubiquitination of a protein. In an ATP-dependent reaction they change ubiquitin from an inactive form to an active ubiquitin intermediate. Next, the active ubiquitin intermediate is transferred to an ubiquitin-conjugating enzyme which then transports it to an ubiquitin ligase-substrate protein complex. The active ubiquitin is then transferred to the substrate protein. This series of events occurs repeatedly until multiple ubiquitins are attached to the substrate protein at which point it is known as a "polyubiquitinated protein". The polyubiquitinated protein is recognised and absorbed by

the 26S proteasome where it is degraded into peptides which, along with the now inactivated ubiquitins, are released into the cell for reuse (Glickman & Ciechanover 2002). Within this process ubiquitin ligases play the important role of identifying target proteins for degradation and represent the main mechanism for specificity within the system. There are many ubiquitin ligases, each of which have an affinity for specific proteins (Glickman & Ciechanover 2002).

Two ubiquitin ligases have been identified for their role in muscle atrophy, MuRF1(Bodine *et al.* 2001) and MAFbx (also known as Atrogin-1) (Bodine *et al.* 2001; Gomes *et al.* 2001). Using rat models of denervation, immobilization and un-weighting, Bodine and colleagues (Bodine *et al.* 2001) showed that only these two genes were up-regulated in all three models of atrophy. Furthermore, they showed that knocking out MuRF1 and MAFbx protected skeletal muscle from atrophy following surgical denervation, and that over-expression of MAFbx induced atrophy. Since these initial findings, MuRF1 and MAFbx have been the focus of intense research and have been found to be involved in many different skeletal muscle atrophy scenarios, including fasting-induced atrophy, cancer cachexia, rheumatoid cachexia, diabetes, and uremia (Lecker *et al.* 2004; Foletta *et al.* 2011). The involvement of MuRF1 and MAFbx in such a diverse multitude of atrophy scenarios has led to them being considered broad markers of proteasomal involvement in muscle atrophy (Paul *et al.* 2010). Though the full extent of their cellular roles is unknown, it is now believed that MAFbx is involved in the regulation of myotube differentiation while MuRF1 is primarily involved in the degradation of structural and contractile proteins within the myofiber (Foletta *et al.* 2011). Due to their distinct contributions to muscle atrophy, MuRF1 and MAFbx may

each respond differently to different atrophy inducing scenarios. Recently, our lab examined the involvement of MAFbx in age-associated myofiber atrophy following denervation and found it to be elevated in some denervated myofibers in aging muscle, however, the involvement of MuRF1 has yet to be studied. As such, to address objective (1), we propose to examine the role of MuRF1 in age-associated myofiber atrophy in the context of denervation.

2.4 Characteristics of Denervated Muscle

Changes in the morphology and structure of skeletal muscles in response to different conditions provide important insight into the complex interactions and systems of organization that govern their function. In conditions of denervation, the most pronounced changes observed are alterations in the myosin heavy chain (MHC) composition of individual myofibers and progressive myofiber atrophy, followed by degeneration (Midrio 2006).

Myosin heavy chains are contractile proteins within myofibers. There are several different isoforms of myosin heavy chains, each with slightly different contractile capabilities. Through close coordination with cellular functional elements, myosin heavy chain expression serves as an indicator of the contractile and physiological capabilities of individual myofibers (Pette 2002). Loosely, adult myosin heavy chain isoforms can fall into one of two categories: fast myosin heavy chains (MHCf) and slow myosin heavy chains (MHCs). Myofibers that express predominantly MHCf, known as fast myofibers or type II myofibers, are characterised by high glycolytic capacity and are generally innervated by fast-twitch motor neurons (Malina *et al.* 2004). Myofibers that express

predominantly MHCs, known as slow myofibers or type I myofibers, are characterised by high oxidative capacity and are generally innervated by slow-twitch motor neurons (Malina *et al.* 2004). This scheme of fast and slow myofibers is often expanded to describe the contractile and physiological qualities of individual whole muscles (Patterson *et al.* 2006; Carter *et al.* 2010). Muscles that are innervated predominantly by fast-twitch motor neurons and are composed predominantly of fast myofibers are known as fast-twitch muscles. Muscle that are innervated predominantly by slow-twitch motor neurons and are composed predominantly of slow myofibers are known as slow-twitch muscles. Generally, fast-twitch muscles have high, explosive force generating capabilities but are easily fatigued relative to slow-twitch muscles. In contrast, slow-twitch muscles have lower force generating capabilities but are much more resistant to fatigue (Malina *et al.* 2004). Accordingly, fast-twitch muscles tend to be involved in voluntary movement where the performance demands are for high, intermittent force generation and slow-twitch muscles tend to be involved in maintaining posture where the performance demands are for low, constant force generation (Armstrong & Phelps 1984).

Following denervation, there are pronounced alterations in MHC expression that seem to occur in a muscle-specific manner (Pette & Staron 2000). In fast twitch muscles, such as the tibialis anterior, there is a tendency for MHC expression to shift towards a more slow profile (Huey & Bodine 1998; Raffaello *et al.* 2006). In contrast, in slow twitch muscles, such as the soleus, there is a tendency for MHC expression to shift towards a more fast profile (Huey & Bodine 1998; Talmadge *et al.* 1999). As a consequence of these shifts in MHC expression, there is a pronounced accumulation of MHC co-expressing myofibers. Patterson and colleagues (Patterson *et al.* 2006) found

that 50 days following denervation, MHC co-expressing myofibers accounted for > 75% of all myofibers in the fast-twitch extensor digitorum longus muscles and slow-twitch soleus muscles of rats. Furthermore, Talmadge and colleagues have found that these MHC co-expressing myofibers are still present 1 year following denervation, suggesting that MHC co-expressing myofibers are not indicative of a transitional state, but rather represent a "stable" sub-population of myofibers under conditions of denervation (Talmadge *et al.* 1999).

Marked myofiber atrophy as a result of denervation was documented as early as 1935 (Tower 1935) and since, has become a well established characteristic of denervated muscle (Pellegrino & Franzini 1963; Carlson 2004; Ashley *et al.* 2007). Myofiber atrophy progresses in a type-specific manner, with MHCf myofibers exhibiting steady atrophy immediately following denervation and MHCs myofibers atrophying only after prolonged denervation (several months) (Lu *et al.* 1997; Carlson 2004). These findings suggest that the mechanisms responsible for myofiber atrophy following denervation may *be* different, or *behave* differently, in different myofiber types.

2.5 Characteristics of Aged Muscle

Similar to the morphological and structural changes observed in muscles following denervation, the most prominent aging-associated changes are also alterations in myosin heavy chain composition of individual myofibers (Lexell 1995) and myofiber atrophy (Lexell & Taylor 1991). These similarities are not surprising given our recent finding implicating denervation as the primary cause of sarcopenic muscle atrophy. Our lab has recently shown that the accumulation of severely atrophied fibers marks the

accelerating phase of sarcopenia in both fast twitch muscles and slow twitch muscles (Rowan *et al.* 2011). In addition, we have also found that myofibers that express MHCf appear to be more susceptible to atrophy, whereas, pure MHCs myofibers display a degree of resistance to atrophy (data in review). These findings are also in accordance with the delayed atrophy of MHCs myofibers reported in studies of denervation (Carlson 2004).

In contrast to the characteristics reported in denervated muscle where there is a prominent emergence of MHC co-expressing myofibers and muscle-dependent shifts in MHCf and MHCs expression, the majority of sources indicate that there is a preferential shift from MHCf expressing myofibers to MHCs expressing myofibers in aging muscle (Kirkendall & Garrett 1998; Pette & Staron 2000; Brunner *et al.* 2007; Marzetti *et al.* 2009). Though this seems to suggest a discrepancy between denervation-related atrophy and sarcopenic atrophy, it may be primarily due to differences in methodology employed by the two research communities. In fact, very few studies of sarcopenia have allowed for the assessment of MHC co-expressing fibers in their methodological design and interpretation. Relatively recently, novel studies in senescent rats (Snow *et al.* 2005) and senescent humans (Andersen *et al.* 1999) have demonstrated that MHC co-expression is actually quite prolific in aged muscle, accounting for greater than 30% of all myofibers. Furthermore, previous data from our lab (Carter *et al.* 2010) and others (Monemi *et al.* 1999; Frontera *et al.* 2000a) suggest that age-associated shifts in MHC expression may be muscle-specific rather than follow a “fast to slow” rule. In light of our recent finding implicating denervation as the primary instigator of sarcopenia, these discrepancies

within the literature regarding age-associated changes in MHC expression serve as our justification for objective (2).

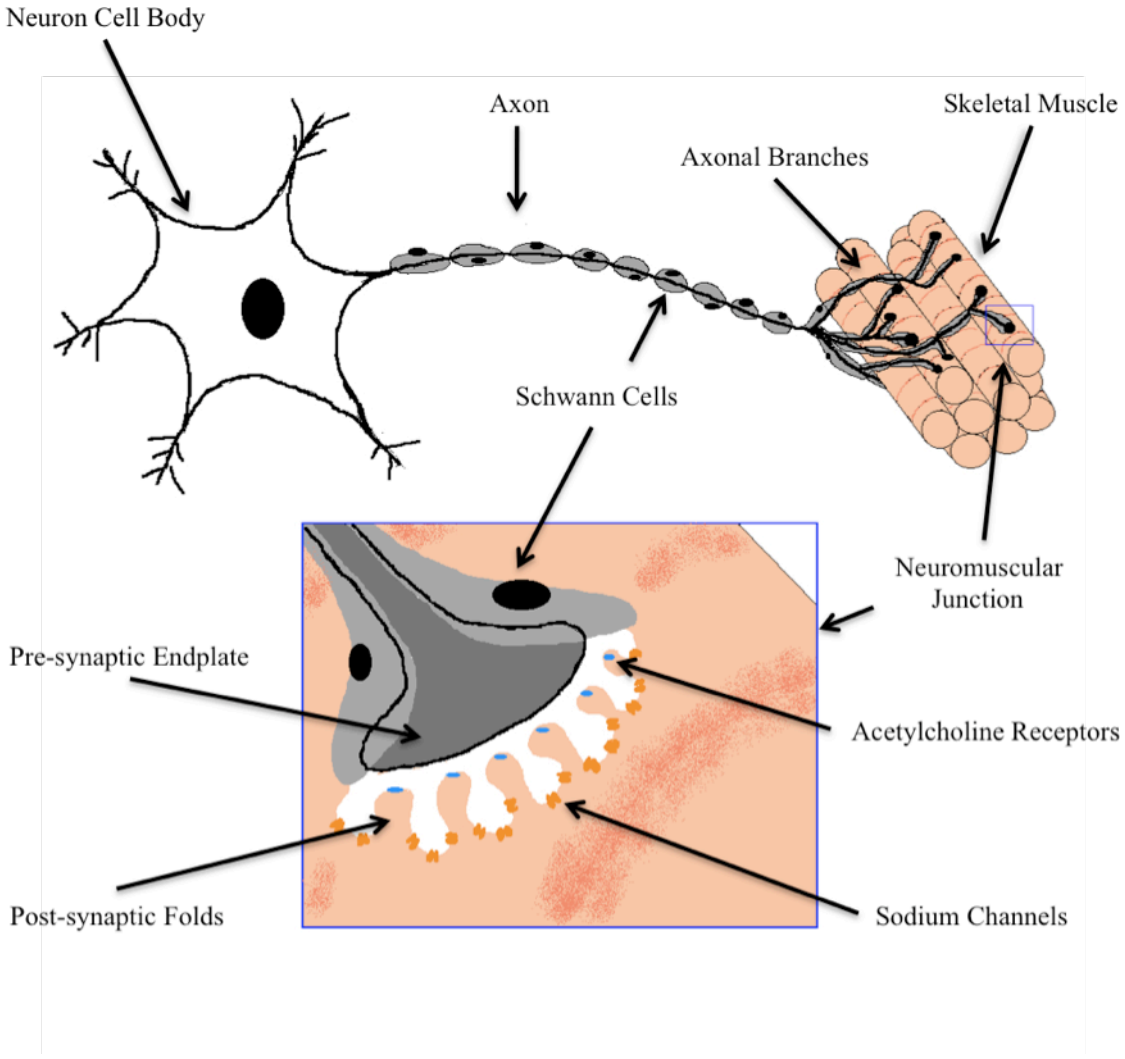

Figure 2.1 Schematic showing the basic structure of a neuromuscular junction.

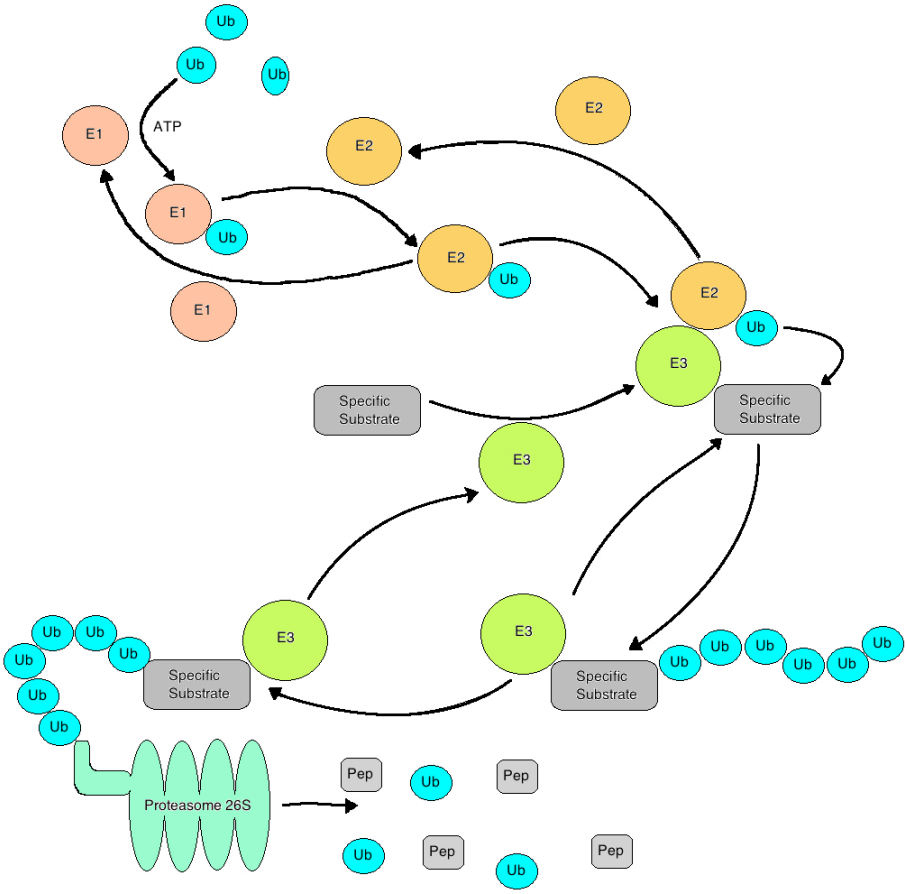

Figure 2.2 Schematic showing the process of substrate ubiquitination and breakdown via the proteasomal system. Step (1) ubiquitin is activated by ubiquitin-activating enzymes, E1's. Step (2) ubiquitin is transferred to ubiquitin-conjugating enzymes,E2's. Step (3) specific substrates (proteins) are targeted by specific ubiquitin ligases, E3's. Step (4) transfer of ubiquitin to the substrate is facilitated by an interaction between the E2's and E3's. Step (5) numerous ubiquitins are attached to the substrate so that it becomes "polyubiquitinated". Step (6) the polyubiquitinated protein is recognized by the 26S proteasome where it is absorbed and broken down into peptides. Step (7) the peptides and ubiquitins are released into the cell for recycling. This schematic is an adaptation from (Murton *et al.* 2008).

Chapter Three: MuRF1 Expression

These results are part of a manuscript that has been submitted for publication in a peer reviewed journal.

3.1 Introduction

Sarcopenia is the age-related loss of skeletal muscle mass and function which involves the atrophy and loss of individual myofibers (Lexell & Taylor 1991). Recent evidence from our lab implicates denervation as the primary instigator of individual myofiber atrophy in sarcopenia (Rowan *et al.* 2010). We have found that in senescent muscle, denervated myofibers are severely atrophied whereas innervated myofibers are similar in size to young adult myofibers. In light of this novel finding, we wished to examine the possible mechanisms of age-associated myofiber atrophy within denervated myofibers.

Despite consistent evidence for the involvement of MuRF1 and MAFbx in denervation-related muscle atrophy (outlined in Chapter Two), evidence for their involvement in sarcopenia is less conclusive. Some sources report an up-regulation of MuRF1 (Altun *et al.* 2010) and MAFbx in aged skeletal muscle (Clavel *et al.* 2006; Hepple *et al.* 2008), while others report a down-regulation (Edstrom *et al.* 2006). These conflicting results would seem to suggest that denervation-related atrophy and age-related atrophy occur via different mechanisms and, thus, are not one and the same. However, there are many factors which complicate the accuracy of this conclusion. Firstly, these prior results were based on whole muscle homogenates of different muscles. Secondly, some studies examined mRNA levels and others examined both mRNA and protein levels. Thirdly, the studies were conducted in rats of different ages and, therefore, at

different stages of sarcopenia. Fourthly, the studies were conducted without regard for the denervation status of individual myofibers. In considering denervation as the primary instigator of age-related atrophy, these factors, particularly the use of whole muscle homogenates, are of concern for the following reasons:

1) Different myofiber types may respond differently to denervation, therefore, homogenates may obscure true changes in MuRF1 and MAFbx expression in sarcopenia. Recent results from our lab indicate that pure MHCs myofibers that are denervated are the same size as pure MHCs myofibers from young adult that are not denervated, whereas MHCf expressing myofibers that are denervated exhibit marked atrophy (data in review), providing strong evidence that different myofiber type populations respond differently to denervation. This notion is further supported by the work of Moriscot and colleagues (Moriscot *et al.* 2010) where they show that MuRF1 is preferentially expressed in MHCf myofibers following denervation.

2) If concern 1 is the case, then differences in the myofiber type composition of different muscles may further obscure these changes. Significantly, Moriscot and colleagues also showed that when MuRF1 is knocked out, muscles composed predominantly of MHCf myofibers are most protected from atrophy following denervation (Moriscot *et al.* 2010).

3) The heterogeneous nature of age-associated denervation, in contrast to whole-muscle surgical or chemical denervation where all myofibers are impacted, likely results in a lower total level of MuRF1 and MAFbx expression within the muscle (compared to traditional denervation models of muscle atrophy). However, on a denervated myofiber to denervated myofiber level, their expression may be similar. In this scenario, the use of

homogenates may cause investigators to conclude that the proteasomal systems plays less of a role in sarcopenic atrophy than in traditional models of denervation related atrophy when that may not be the case.

Given these concerns, we feel that the previous literature examining the role of MuRF1 and MAFbx in age-related myofiber atrophy is insufficient to accurately elucidate their involvement. As such, our lab has examined the involvement of MAFbx in sarcopenic denervated and innervated myofibers (data in review) and found that it is elevated with age in MHCf expressing myofibers but does not change with denervation, however, in pure MHCs myofibers, MAFbx expression does increase with denervation (though remains at lower levels than in MHCf myofibers). The purpose of the current study was to examine the level of MuRF1 expression in sarcopenic denervated and innervated myofibers to gain insight into the significance of denervation in the elevation of MuRF1 with aging. Using antibodies for MuRF1 and dystrophin, we labelled sections of young adult and senescent red gastrocnemius muscle. Next, by comparing these sections to serial sections labelled for MHCs, MHCf and NAV1.5 (a neuromuscular junction-sodium channel expressed in adulthood only following denervation (Yang *et al.* 1991; Stocksley *et al.* 2005)), we determined the level of MuRF1 expression in single myofibers of young adult and senescent rats, and determined how these levels varied as a function of innervation status and MHC expression pattern. We hypothesized that MuRF1 would be up-regulated in denervated myofibers compared to innervated myofibers. We further hypothesised that MuRF1 would be preferentially up-regulated in denervated myofibers that express MHCf. Finally, we hypothesized that MuRF1 levels

would not be different between innervated myofibers in young adult muscle and innervated myofibers in senescent muscle.

3.2 Methods

3.2.1 Experimental Animals and Tissue Collection

Three young adult (YA) aged 8-10 months, and five senescent (SEN) aged 36 months, male Fisher 344 X Brown Norway F1 (F344BN) rats were obtained from the National Institute on Aging (NIA; Baltimore, MD). They were housed in the Biological Sciences vivarium at the University of Calgary in single cages (12/12 hour light/dark cycle, 21°C) and provided food and water *ad libitum*. On tissue harvest day, animals were anesthetised with sodium pentobarbital (55-65 mg x kg^{-1}) and the hind limb muscles were removed. These were dissected free of fat, weighed and mounted on cork in optimal cutting temperature compound. The muscle tissue was then frozen in liquid isopentane (cooled in liquid nitrogen) and stored at -80°C until sectioning. For the purposes of this study, 10-μm thick sections of gastrocnemius muscle were cut using a cryostat (-18°C) and mounted on lysine-coated slides (Superfrost). They were air dried for one hour and then stored at -80°C until use for immunolabelling. This is the standard procedure for muscle tissue collection used by our lab (Rowan *et al.* 2010; Thomas *et al.* 2010; Rowan *et al.* 2011).

3.2.2 Immunolabelling

The gastrocnemius muscle sections were labelled for MuRF1 expression, dystrophin, and myosin heavy chain slow isoform (MHCs) expression (to aid in

individual myofiber recognition) in keeping with the following immunolabelling procedure used previously by our lab (Rowan *et al.* 2010; Thomas *et al.* 2010; Rowan *et al.* 2011).

After reaching room temperature, stored tissue slides were fixed in paraformaldehyde solution (4% - 30 min), rehydrated in phosphate buffered saline (PBS; pH 7.4 - 5 min) and then incubated in permeabilization solution (0.1% Triton X-100 in PBS - 15 min). Next, slides were washed in PBS (3 x 5 min) and incubated in blocking solution (10% donkey serum, 1% bovine serum albumin in PBS – 30 min), and then in primary antibody solution (mouse anti-MHCs, Novocastra 1:10 dilution; mouse anti-dystrophin, Sigma 1:200 dilution; and goat anti-MuRF1, Genetex 1:100 dilution in blocking solution - overnight at 4°C). Slides were again washed in PBS (3 x 5 min) and incubated in blocking solution (30 min), then incubated in secondary antibody solution (donkey anti-goat AlexaFluor 546, Invitrogen 1:200 dilution and donkey anti-mouse AlexaFluor 488, Invitrogen 1:200 dilution in blocking solution – one hour at room temperature). Finally, slides were washed in PBS (3 x 5 min), rinsed in distilled water, mounted with Prolong Gold (Invitrogen) and stored at 4°C until imaging the following day. Negative control samples, where the primary antibody solution was omitted from the labelling procedure, are pictured in Figure 3.6.

3.2.3 Confocal Imaging and Image Analysis

Using an Olympus Fluoview confocal microscope, two images of MuRF1, dystrophin and MHCs or MHCf expression were obtained from the red region of the gastrocnemius muscle at 200x magnification, such that a minimum of 75 myofibers per

muscle was sampled. These images were serial images to ones previously obtained and analyzed by our lab (data in review) showing NAV1.5 expression, MHCs expression and MHCf expression. All images were then analysed offline in ImageJ software, using an internal reference frame within the image to prevent bias against large myofibers (Hepple & Mathieu-Costello 2001), to identify individual myofibers from the previously analyzed sections and to determine individual myofiber MuRF1 expression. MuRF1 expression was determined by quantifying the image intensity of MuRF1 labelling within the cytoplasmic area of each individual myofiber. In the previous analysis of MHCs expression and MHCf expression, myofibers that were negative for MHCf in the MHCf labelled sections were classified as pure MHCs myofibers, those negative for MHCs in MHCs labelled sections were classified as pure MHCf myofibers and all others were classified as MHC co-expressing myofibers. In the previous analysis of NAV1.5 expression, denervation status was identified as one of three possible conditions: 1) no NAV1.5 labelling indicating that the myofiber is negative for denervation (negative myofibers = innervated), 2) NAV1.5 labelling present along the circumference of the myofiber (ringed myofibers = denervated) and, 3) NAV1.5 labelling within the cytoplasm of the myofiber (cytoplasmic myofibers = denervated).

3.2.4 Statistics

Comparisons within young adult and senescent groups of MuRF1 expression between different myofiber types and between myofibers of different denervation status were made using two-way ANOVAs for myofiber type, denervation status and MuRF1 expression, followed by Holm-Sidak post-hoc tests. Comparisons of muscle mass and

MuRF1 expression between young adult and senescent groups were made using Student's T-tests or Mann-Whitney Rank Sum tests in conditions of unequal variance. For all tests, $\alpha = 0.05$. All values are expressed as means ± standard deviation (SD).

3.3 Results

3.3.1 Muscle Mass

In the gastrocnemius muscle, there was a 34.7% decrease in whole muscle mass between the YA and SEN groups (YA = 2004 ± 78 mg, SEN = 1309 ± 50 mg, $p < 0.05$), representing marked sarcopenia.

3.3.2 MuRF1 Expression and Denervation Status

Figure 3.1 shows examples of MuRF1 expression in YA and SEN muscles. Figure 3.2 is reproduced from the thesis of Sharon Rowan (Rowan 2011) and shows examples of the different NAV1.5 classifications. In YA muscle, all myofibers were innervated based on their NAV1.5 labelling characteristics. In SEN muscle, 154 myofibers analysed were distinctly NAV1.5 negative, 138 myofibers analysed displayed distinct NAV1.5 ringed labelling, and 87 myofibers analysed displayed distinct NAV1.5 cytoplasmic labelling. MuRF1 expression was significantly higher in NAV1.5 cytoplasmic myofibers compared to both NAV1.5 ringed ($p < 0.05$) and NAV1.5 negative myofibers ($p < 0.05$) in MHCf and MHC co-expressing myofibers, but only compared to NAV1.5 negative myofibers ($p < 0.05$) in MHCs myofibers. However, there was no significant difference in MuRF1 expression between NAV1.5 ringed and NAV1.5 negative myofibers (Figure 3.3).

3.3.3 MuRF1 Expression in Different Myofiber Types

In YA muscle, 195 myofibers analysed were pure MHCf, 110 myofibers analysed were pure MHCs, and 2 myofibers were MHC co-expressing. There was no significant difference in MuRF1 expression between pure MHCf and pure MHCs myofibers in YA muscle ($1-\beta = 0.549$). MuRF1 expression was significantly greater in MHC co-expressing myofibers compared to pure MHCf and pure MHCs myofibers ($p < 0.05$), however, the low sample number of co-expressing myofibers in YA muscle warrants caution in interpretation of this result (Figure 3.4).

In SEN muscle, 174 myofibers analysed were pure MHCf, 91 myofibers were pure MHCs, and 114 myofibers analysed were MHC co-expressing. After accounting for denervation status, there was no significant difference in MuRF1 expression between pure MHCf, pure MHCs and MHC co-expressing myofibers in SEN muscle (Figure 3.3).

3.3.4 MuRF1 Expressing and Age

To examine the effect of age on MuRF1 expression, we compared all myofibers negative for denervation in YA muscle to all myofibers negative for denervation in SEN muscle. MuRF1 expression was significantly higher in SEN myofibers compared to YA myofibers ($p < 0.05$) (Figure 3.5).

3.4 Discussion

The findings of this study are threefold: (1) MuRF1 expression is elevated in denervated myofibers of sarcopenic muscle; (2) MuRF1 is not preferentially expressed in pure MHCf myofibers; (3) MuRF1 expression is elevated with age.

3.4.1 MuRF1 Expression and Denervation in Sarcopenic Muscle

To our knowledge, this is the first time that MuRF1 expression has been examined in both denervated and innervated myofibers from within the same muscle. Consistent with our central hypothesis that denervation is the primary instigator of sarcopenic myofiber atrophy, we have found that MuRF1 protein immunoreactivity is elevated in denervated myofibers compared to innervated myofibers. This suggests that proteasomal protein degradation activity is elevated following denervation in sarcopenic muscle and contributes to the marked atrophy that we have observed. These results are not surprising given the extensive documentation in the literature that MuRF1 expression is elevated following denervation (Bodine *et al.* 2001; Sacheck *et al.* 2007; Moriscot *et al.* 2010). Interestingly, MuRF1 expression was only elevated in myofibers with cytoplasmic expression of NAV1.5 and not in myofibers with only ringed NAV1.5 expression. This suggests that the two categories of NAV1.5 expressing myofibers are, in some capacity, functioning differently. One explanation for this may be that myofibers with cytoplasmic expression of NAV1.5 have been denervated longer than those with only ringed NAV1.5 expression. In MuRF1 knockout mice, protection from atrophy was observed at 14 days following denervation (the last time point reported), but not at 7 days following denervation, suggesting that MuRF1 is most active at later stages of denervation (Bodine *et al.* 2001). Unfortunately, there have been no studies examining the protein expression of MuRF1 at different time points following denervation, so it is difficult to know if this is a reasonable explanation. In a study of MuRF1 and MAFbx/Atrogen1 mRNA expression at 1, 3, 14 and 28 days following denervation of mouse gastrocnemius muscle, it was observed that MuRF1 mRNA expression peaked at

3 days following denervation, then gradually declined to just above basal levels at 28 days following denervation (Sacheck *et al.* 2007). In contrast, while it was observed that MAFbx mRNA expression also peaked at 3 days following denervation, its degree of increase was twice that of MuRF1 and its expression had fallen to near basal levels by 14 days following denervation, when MuRF1 expression was still relatively high (Sacheck *et al.* 2007). They also found that atrophy occurred in two distinct phases; one phase of rapid atrophy immediately following denervation, and a second phase of slow, prolonged atrophy. As such, it may be that while MuRF1 is always contributing to atrophy following denervation, its relative contribution is not significant until later stages of denervation. Alternatively, there may be a discrepancy between mRNA levels of MuRF1 and protein levels of MuRF1 which contributes to a delayed impact of MuRF1 in atrophy following denervation. Further exploration is required to determine the functional differences of myofibers with ringed NAV1.5 expression and cytoplasmic NAV1.5 expression.

Another interesting finding of the current study is that the degree of MuRF1 immunoreactivity elevation is relatively small compared to the degree of atrophy observed. However, it is difficult to speculate on the significance of this finding. While it may indicate that MuRF1 is not contributing significantly to the atrophy observed in sarcopenia, it is also possible that only modest increases in MuRF1 protein expression are necessary to elicit a pronounced atrophy response. Furthermore, the progressive nature of sarcopenia may mean that at no time is MuRF1 expression uniformly elevated within a sub-population of myofibers. Decreases in mean myofiber cross sectional area begin at as early as 27 months of age in F344XBN rats (Lushaj *et al.* 2008) and by 36 months of age,

a significant accumulation of severely atrophied myofibers has already occurred (Rowan *et al.* 2011). If myofiber denervation is the cause of sarcopenic atrophy, it seems reasonable to suggest that it occurs relatively sporadically. By the time point examined in the present study, a significant amount, if not the majority, of severely atrophied myofibers, have already been denervated for a period in excess of the 3 to 28 day period following denervation when MuRF1 mRNA expression is most elevated (Sacheck *et al.* 2007) and protection from atrophy in MuRF1 knockout mice is observed (Bodine *et al.* 2001). The relatively small elevation in MuRF1 protein immunoreactivity observed in the sub-population of myofibers with cytoplasmic expression of NAV1.5 may simply be a reflection of this. Interestingly, while all denervation categories had similar SDs, approximately 5% of myofibers with cytoplasmic expression of NAV1.5 had MuRF1 immunoreactivity levels greater than 2 SD above the sub-population mean, while only 1% of those with ringed NAV1.5 expression and 0% of NAV1.5 negative myofibers had MuRF1 immunoreactivity levels greater than 2 SD above the sub-population mean. In contrast, no myofibers had MuRF1 immunoreactivity levels greater than 2 SD below their sub-population means. Combined, this demonstrates that the range of MuRF1 protein immunoreactivity in myofibers with cytoplasmic NAV1.5 expression is both greater and skewed towards higher levels of MuRF1 protein expression. This may be representative of myofibers within a more rapid phase of MuRF1 mediated atrophy.

3.4.2 MuRF1 Expression and Myofiber Type

Contrary to our hypothesis, we did not find that MuRF1 immunoreactivity was elevated in a myofiber type specific manner. This is in contrast to the findings of

Moriscot and colleagues (2010) that MuRF1 is preferentially expressed in MHCf myofibers compared to MHCs myofibers prior to and following denervation. The less than desired power value observed in our comparison of MHCs and MHCf myofibers warrants noting when interpreting the significance of this finding, however, we also observed that mean MuRF1 expression in MHCs myofibers was 2% lower than in MHCf myofibers, suggesting that the difference between our results and those of Moriscot and colleagues is real. There are two factors which may contribute to this discrepancy. Firstly, while Moriscot and colleagues (2010) have shown images of MuRF1 immunolabelling in serial sections which appear to demonstrate a myofiber type specific labelling pattern, the actual quantification of MuRF1 expression was done using muscle homogenates, forcing a certain degree of interpretation when concluding that there is a myofiber preference. In contrast to this approach, we have quantified MuRF1 immunoreactivity in individual myofibers, providing a much more sensitive measure of its expression in myofibers of different types. While it is unlikely that this accounts fully for the discrepancy, it is possible that Moriscot and colleagues have over-estimated the degree of myofiber type specificity which truly occurs. Secondly, the use of MuRF1 antibodies for immunolabelling in serial sections is not an established procedure and many relatively new and untested antibodies are available. It may be that differences in the MuRF1 antibodies used by Moriscot and colleagues and those used in the present study account for some of the discrepancies observed. In an attempt to validate our method, we have conducted experiments in serial sections of rat gastrocnemius muscle using a second MuRF1 antibody produced by a different manufacturer and in a different host animal (rabbit anti-MuRF1, Abcam). Notably, the staining patterns were similar using both

antibodies, demonstrating that they label consistently and providing us with confidence that they were appropriately labelling for MuRF1 (Figure 3.7).

3.4.3 MuRF1 Expression and Age

Again, contrary to our hypothesis, we found that MuRF1 immunoreactivity was elevated in SEN myofibers negative for NAV1.5 expression compared to YA myofibers negative for NAV1.5 expression. This indicates that, in addition to there being an effect of denervation on MuRF1 expression, there is also a more general effect of age. One possible explanation for this general effect is that, in addition to denervation-induced atrophy, there is also a certain degree of disuse atrophy occurring as a result of increasingly sedentary behaviour with age. In rats, it has been observed that MuRF1 mRNA expression is elevated following muscle disuse as a result of hind limb suspension (Bodine *et al.* 2001; Haddad *et al.* 2006) and hind limb casting (Krawiec *et al.* 2005). What is not clear, however, is whether the natural level of sedentary behaviour observed at advanced ages (Hagen *et al.* 2004) is sufficient to induce a muscle disuse program of atrophy.

Combined, our results show that overall MuRF1 protein immunoreactivity is elevated in sarcopenia. This is consistent with our previous finding in sarcopenic rat plantaris muscle (Hepple *et al.* 2008) and the findings of Altun and colleagues in aging rat gastrocnemius muscle (2010). Notably, there remains a significant amount of controversy regarding the mRNA response of MuRF1 in sarcopenia. Claval and colleagues report an increase in MuRF1 mRNA expression (2006), while recent works by Edstrom and colleagues (2006), Altun and colleagues (2010), and Gaugler and colleagues

(2011) report either a decrease or no change in MuRF1 mRNA expression in sarcopenia. The dual findings by Altun and colleagues that MuRF1 protein expression, but not mRNA expression, was elevated in sarcopenia provides evidence that a discrepancy between mRNA and protein expression can occur, and it highlights the importance of directly examining protein levels of cellular regulators before drawing any conclusions.

3.5 Conclusion

The purpose of this study was to examine the role of MuRF1 in sarcopenic myofiber atrophy within the context of denervation. This is a very complex issue to study which is compounded by the progressive nature of sarcopenia. Unlike models of total muscle denervation and disuse where the cause and effect is relatively clear, aging represents a situation where seemingly sporadic denervation is combined with progressive disuse, making it difficult to decipher what effect is attributable to what cause. Using rat gastrocnemius muscle, this study demonstrates that MuRF1 protein immunoreactivity is elevated in sarcopenic muscle and that this elevation corresponds, to a large degree, with the denervation status of myofibers. Though preliminary, this research supports the hypothesis that denervation acts as a main instigator of sarcopenic myofiber atrophy.

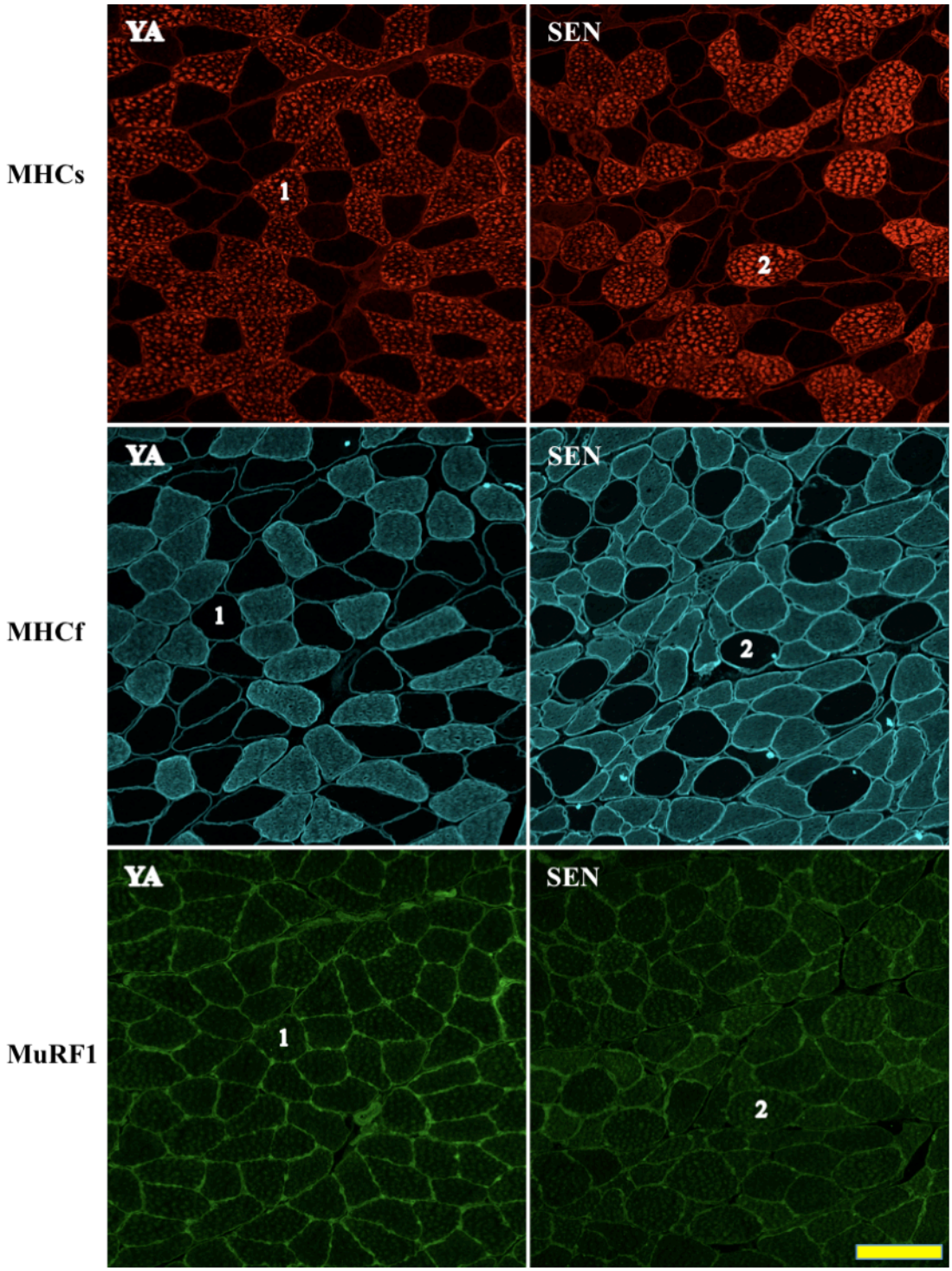

Figure 3.1 MHCs, MHCf and MuRF1 protein expression in the red region of the gastrocnemius muscle of YA and SEN rats. Numbers indicate the same myofiber in serial sections. Scale bar = 150 μm.

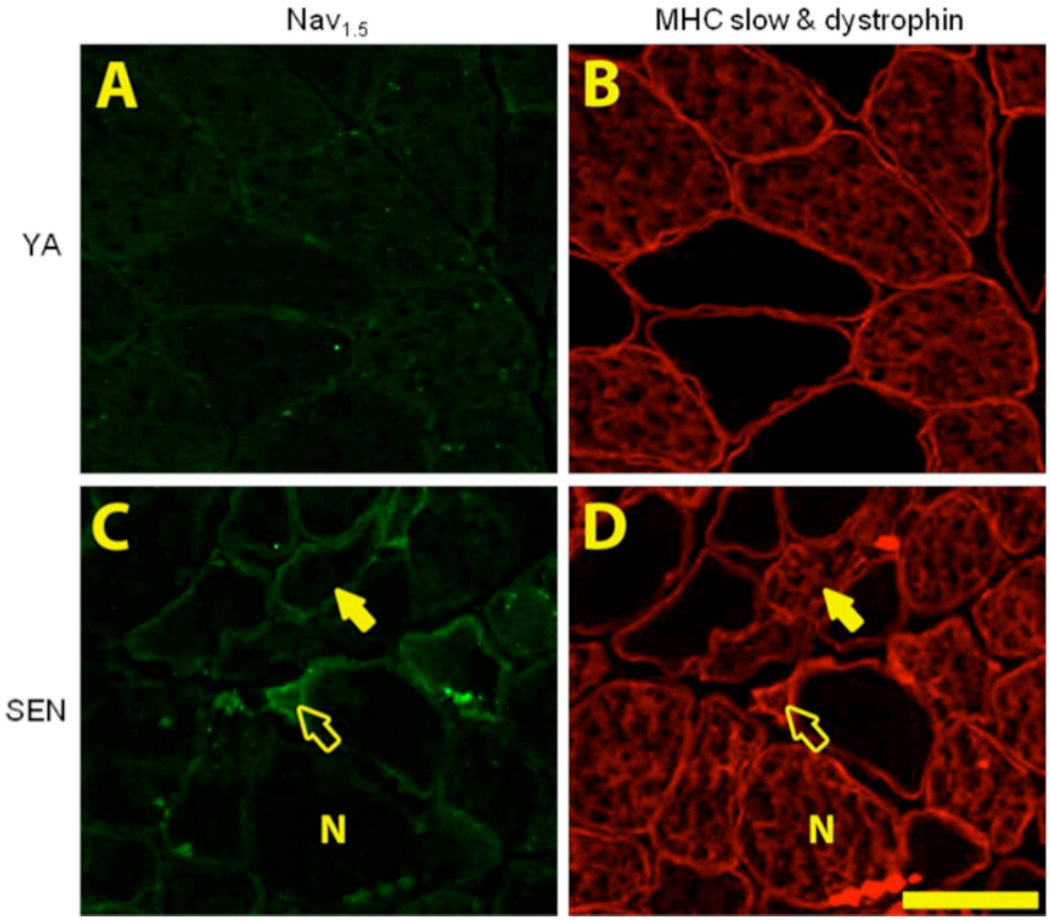

Figure 3.2 NAV1.5 and MHCs expression in the red region of the gastrocnemius muscle of YA and SEN rats. Figure reproduced from the thesis of Sharon Rowan (Rowan 2011). In the SEN sections, the yellow N indicates an example of a NAV1.5 negative myofiber, the solid arrow indicates a NAV1.5 ringed myofiber and the hollow arrow indicates a NAV1.5 cytoplasmic myofiber. Scale bar = 50 μm.

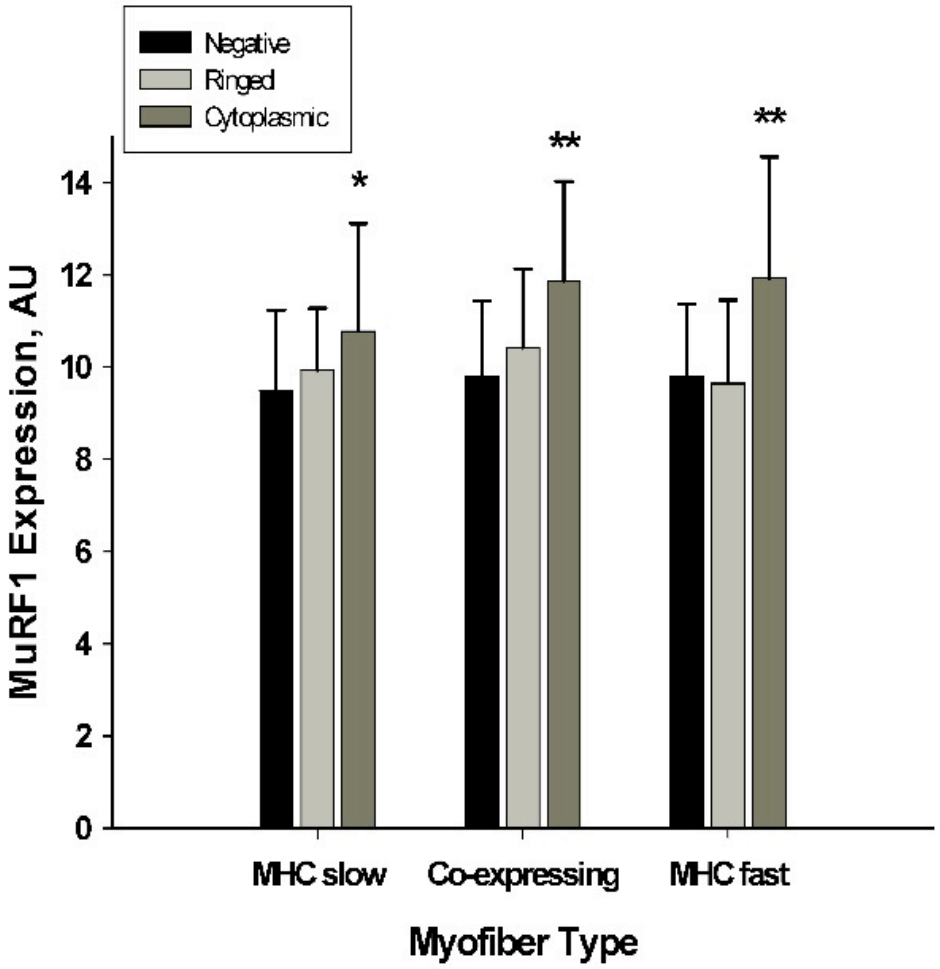

Figure 3.3 MuRF1 protein expression in myofibers of different denervation status and myofiber type in SEN red gastrocnemius muscle. * Indicates significant difference within myofiber type to NAV1.5 negative myofibers ($p < 0.05$). ** Indicates significant difference within myofiber type to both NAV1.5 negative and NAV1.5 ringed myofibers ($p < 0.05$). NAV1.5 negative n = 154, NAV1.5 ringed n = 138, NAV1.5 cytoplasmic n = 87, MHCs n = 91, MHCf n = 174, MHCco-expressing n = 114. Bars indicate standard deviation.

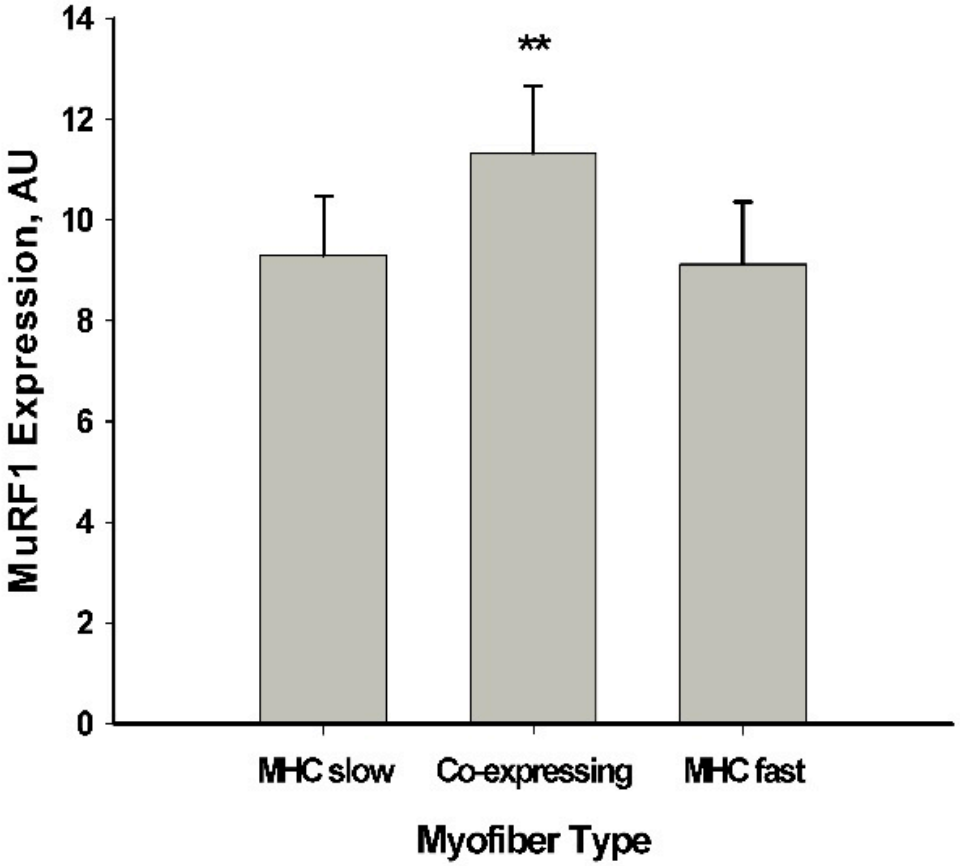

Figure 3.4 MuRF1 protein expression in myofibers of different myofiber type in YA red gastrocnemius muscle. ** Indicates significant difference compared to both MHCs and MHCf myofiber type ($p < 0.05$). MHCs n = 110, MHCf n = 195, MHCco-expressing n = 2, 1-β = 0.549. Bars indicate standard deviation.

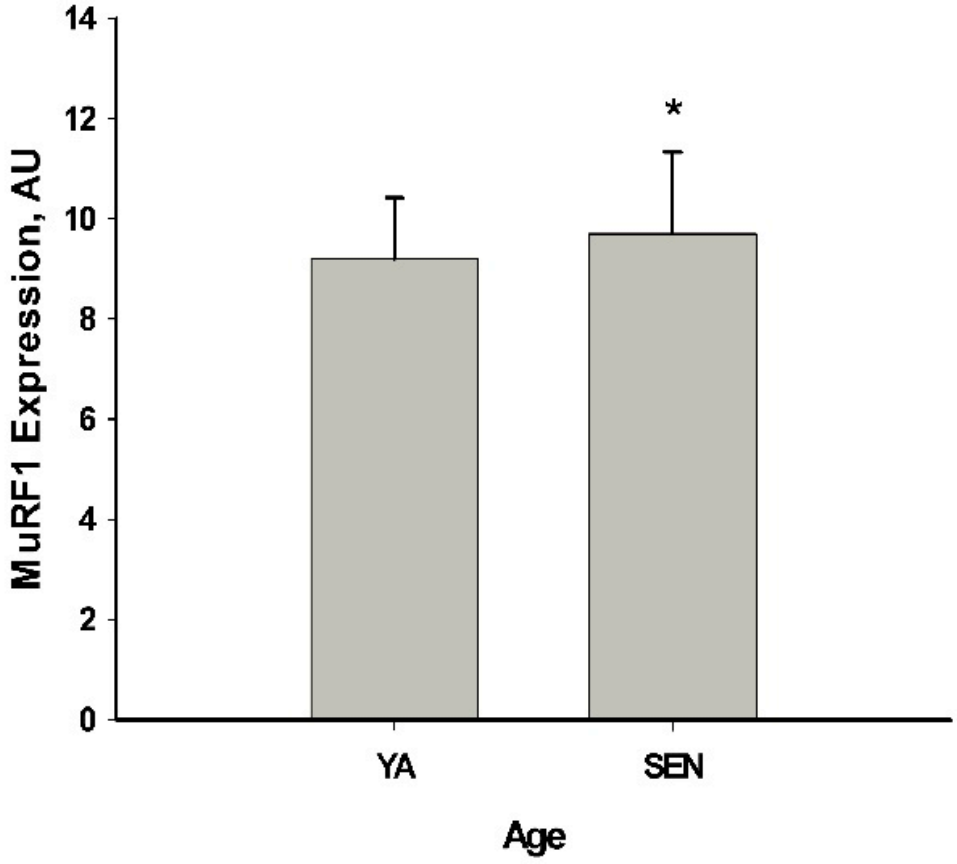

Figure 3.5 MuRF1 protein expression in NAV1.5 negative myofibers in YA and SEN red gastrocnemius muscle. * Indicates significant difference compared to YA myofibers ($p < 0.05$). YA n = 307, SEN n = 154. Bars indicate standard deviation.

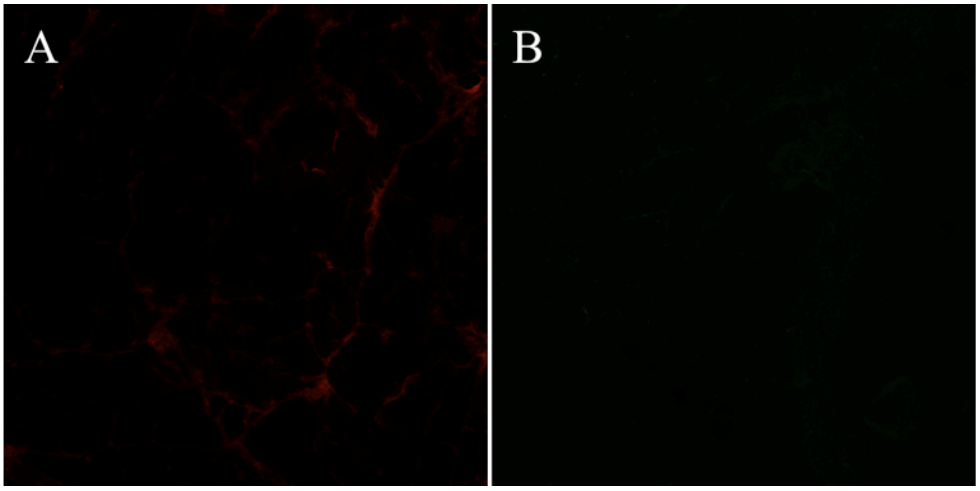

Figure 3.6 Negative control samples for MHCs and MuRF1 labelling. (A) is the negative control for MHCs and (B) is the negative control for MuRF1.

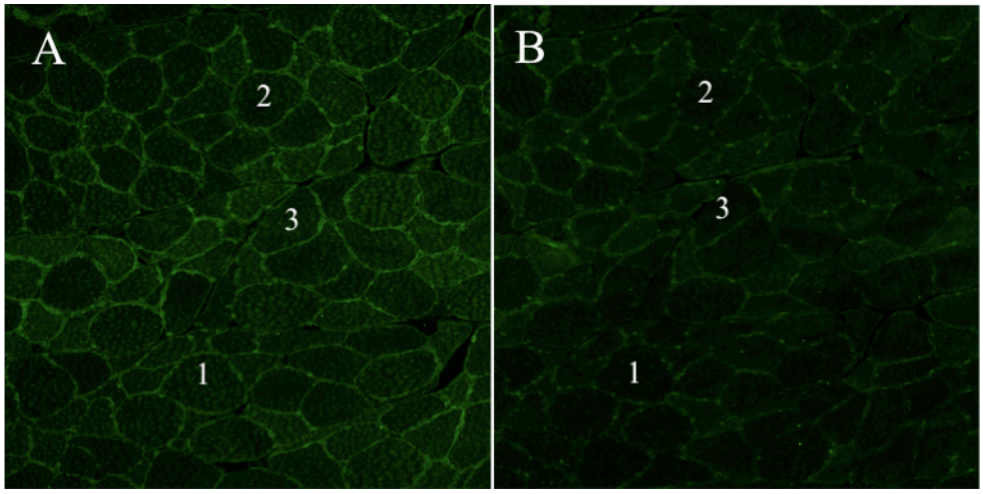

Figure 3.7 MuRF1 antibody comparison. (A) section labelled with goat anti-MuRF1. (B) section labelled with rabbit anti-MuRF1. Numbers indicate the same myofibers in different serial sections.

Chapter Four: Myosin Heavy Chain Co-Expression

A version of this manuscript has been submitted for publication in a peer reviewed journal.

4.1 Introduction

Sarcopenia, the age related loss of skeletal muscle mass and function (Janssen *et al.* 2002), is characterized by atrophy and loss of individual myofibers (Lexell & Taylor 1991) as well as changes in myosin heavy chain (MHC) expression (Lexell 1995). While the preponderance of sources indicate a relative shift in the proportion of fast MHC (MHCf) expressing (type II) myofibers towards an increase in slow MHC (MHCs) expressing (type I) myofibers with age and that MHCs myofibers are less susceptible to atrophy, the accuracy of these conjectures is complicated by the phenomenon of MHC co-expressing myofibers.

MHC co-expressing myofibers, also known as hybrid myofibers, are individual myofibers that simultaneously express two or more MHC isoforms (Pette & Staron 2000). Original estimates of MHC expression associated with age using myosin ATPase were reliant on the enzymatic behaviour of individual cells. This is a suspect indicator of true MHC identity (Andersen & Schiaffino 1997; Pette & Staron 2000), particularly given that the activity of many enzymes is altered in aging muscle (Thompson 2009). This makes the identification of MHC co-expressing myofibers difficult and confounds estimates of changes in myofiber type proportions and type-specific alterations in myofiber size associated with age. However, the emergence of MHC isoform-specific antibodies has allowed for the identification of co-expressing myofibers through the use of multiple MHC antibodies when categorizing myofibers. As such, while MHC co-

expressing myofibers are found in small numbers in young, healthy, skeletal muscle (Andersen 2003), they are found in abundance in states of denervation (Talmadge *et al.* 1999; Patterson *et al.* 2006), disease (Borg *et al.* 1989), and old age (Ansved & Larsson 1990; Andersen *et al.* 1999; Snow *et al.* 2005). Despite this evidence, studies within aging research often do not account for the occurrence of co-expressing myofibers in either methodological design or interpretation of results. This may have consequences on the accuracy of estimates regarding the myosin heavy chain characteristics of aged muscle.

The purpose of this study was to obtain an estimate of age related MHC co-expression in muscles of different myofiber type distributions. Additionally, we wished to determine the error in estimates of myofiber type proportions and type-specific alterations in myofiber size associated with age when not having accounted for MHC co-expressing myofibers. Specifically, we wished to determine the impact of this error on the understanding of MHCs myofiber and MHCf myofiber populations rather than in more distinct sub-populations of myofiber types (type I, type IIa, type IIx, and type IIb) because slow vs. fast myofiber proportions and characteristics are more reported in the literature and, therefore, have the most immediate relevance. Using antibodies for MHCs and MHCf, we labelled serial sections of young and old soleus (predominantly slow twitch in young adult), red gastrocnemius (mixed slow twitch and fast twitch in young adult), and mixed gastrocnemius (predominantly fast twitch in young adult) muscle (Armstrong & Phelps 1984). Next we determined the proportion and size of slow, fast and co-expressing myofibers in these three regions. We then determined the proportion and size of slow and fast myofibers in these regions when using only one antibody

(MHCs or MHCf). We hypothesized that MHC co-expression is extensive in very old age. We further hypothesized that the use of only one antibody for myofiber type identification would significantly impact estimates of both myofiber type proportions and myofiber type size in aged skeletal muscle.

4.2 Methods

4.2.1 Experimental Animals and Tissue Collection

Six young adult (YA) aged 8-10 months, and six senescent (SEN) aged 36 months, male Fisher 344 X Brown Norway F1 (F344BN) rats were obtained from the National Institute on Aging (NIA; Baltimore, MD). They were housed in the Biological Sciences vivarium at the University of Calgary in single cages (12/12 hour light/dark cycle, 21°C) and provided food and water *ad libitum*. On tissue harvest day, animals were anesthetised with sodium pentobarbital (55-65 mg x kg^{-1}) and the hind limb muscles were removed. These were dissected free of fat, weighed and mounted on cork in optimal cutting temperature compound. The muscle tissue was then frozen in liquid isopentane (cooled in liquid nitrogen) and stored at -80°C until sectioning. For the purposes of this study, 10-μm thick sections of soleus muscle and gastrocnemius muscle were cut using a crytostat (-18°C) and mounted on lysine-coated slides (Superfrost). They were air dried for one hour and then stored at -80°C until use for immunolabelling. This is the standard procedure for muscle tissue collection used by our lab (Rowan *et al.* 2010; Thomas *et al.* 2010; Rowan *et al.* 2011).

4.2.2 Immunolabelling

The soleus muscle and gastrocnemius muscle sections were labelled for myosin heavy chain slow isoform (MHCs) expression and myosin heavy chain fast isoform (MHCf) expression in keeping with the following procedure used previously by our group (Rowan *et al.* 2010; Thomas *et al.* 2010; Rowan *et al.* 2011).

After reaching room temperature, stored tissue slides were rehydrated in phosphate buffered saline (PBS; pH 7.4 - 5 min) and incubated in permeabilization solution (0.1% Triton X-100 in PBS - 15 min). Next, slides were washed in PBS (3 x 5 min) and incubated in blocking solution (10% goat serum, 1% bovine serum albumin in PBS – 30 min), and then in primary antibody solution (mouse anti-MHCs or mouse anti-MHCf, Novocastra 1:10 dilution; mouse anti-dystrophin, Sigma 1:200 dilution in blocking solution - overnight at 4°C). Slides were again washed in PBS (3 x 5 min) and incubated in blocking solution (30 min), then incubated in secondary antibody solution (goat anti-mouse AlexaFluor 633, Invitrogen 1:200 dilution in blocking solution – one hour at room temperature). Finally slides were washed in PBS (3 x 5 min), rinsed in distilled water, mounted with Prolong Gold (Invitrogen) and stored at 4°C until imaging the following day. Negative control samples, where the primary antibody solution was omitted from the labelling procedure, are pictured in Figure 4.9.

4.2.3 Confocal Imaging and Image Analysis

Using an Olympus Fluoview confocal microscope, two to four serial images of MHCs and MHCf expression were obtained from the soleus muscle (SOL), the red region of the gastrocnemius muscle (GASr) and the mixed region of the gastrocnemius muscle

(GASm) at each 100x and 200x magnification. In the SOL, images were taken through the entire area of the cross-section. In the GASr, one image was taken from the apex of the central area of the muscle, where the muscle has the highest concentration of MHCs myofibers, and one image was taken from either outer lobe with the highest concentration of MHCs myofibers. The GASm region was determined by working back from the apex of the central area of the muscle, used as a landmark, to the border of the white region of the muscle, where no MHCs myofibers are located. Two images were then taken along this border. Figure 4.1 gives a schematic of where these images were sampled from. All images were then analyzed offline in ImageJ software, using an internal reference frame within the image to prevent bias against large myofibers (Hepple & Mathieu-Costello 2001), to obtain individual myofiber cross sectional area (CSA) and to allow for identification of pure MHCs expressing myofibers, pure MHCf expressing myofibers and MHC co-expressing myofibers. Myofibers that were negative for MHCs in the MHCs labelled sections were classified as pure MHCf myofibers, those negative for MHCf in the MHCf labelled section were classified as pure MHCs myofibers and all others were classified as MHC co-expressing myofibers. In the case of using only MHCs antibody for myofiber type identification, myofibers positive for MHCs were classified as MHCs myofibers, and myofibers negative for MHCs were classified as MHCf myofibers. When using only MHCf antibody for myofiber type identification, myofibers positive for MHCf were classified as MHCf myofibers, and myofibers negative for MHCf were classified as MHCs myofibers.

4.2.4 Statistics

Comparisons between YA and SEN whole muscle mass and myofiber type proportions were made using student t-tests or Mann-Whitney rank sum tests when there were conditions of unequal variance. Comparisons within and between YA and SEN groups for myofiber type CSA were made using two-way ANOVA's for age, myofiber type and CSA followed by Holm-Sidak post-hoc tests. For all tests, $\alpha = 0.05$. All values are expressed as means ± standard deviation (SD).

4.3 Results

4.3.1 Abnormal Sample

Following imaging of immunolabelled sections, it was discovered that one YA soleus sample contained myofiber abnormalities know as "whorled fibers". These myofibers are characteristic of muscle regeneration and indicate that the muscle has experienced some sort of trauma (personal communication from Erin O-Farrell, clinical neurologist, McGill University). As such, this sample was removed from the data set. All YA soleus muscle results are based on 5 muscle samples. Due to the relatively rare occurrence of such "whorled" myofibers, the raw data and images of this abnormal sample are included in Appendix A for the sake of documentation.

4.3.2 Muscle Mass

In the soleus muscle, there was a 14.7% decrease in whole muscle mass between the YA and SEN groups (YA = 155 ± 6 mg, SEN = 132 ± 6 mg, $p < 0.05$). In the

gastrocnemius muscle, there was a 34.7% decrease in whole muscle mass between the YA and SEN groups (YA = 2004 ± 78mg, SEN = 1309 ± 50mg, $p < 0.05$).

4.3.3 Myosin Heavy Chain Expression in SOL, GASm and GASr Muscle

Figures 4.2, 4.4, and 4.6 show examples of MHCs and MHCf expression in young adult and senescent SOL, GASm, and GASr, respectively.

In the soleus muscle, a minimum of 450 myofibers per animal were sampled for myofiber type identification. When using both MHCs and MHCf antibodies in order to account for myosin heavy chain co-expression, there was no significant change in the proportion of pure MHCf myofibers between YA (1.2 ± 0.5%) and SEN (2.9 ± 3.2%) groups. There was a 47% decrease in pure MHCs myofibers with age (YA = 95.5 ± 1.5%, SEN = 50.3 ± 12.1%, $p < 0.05$) and the proportion of MHC co-expressing myofibers in SEN (46.8 ± 13.0%) was 14.2-fold that of YA (3.3 ± 1.9%; $p < 0.05$).

When using only MHCs antibody to identify myofiber type, while there was still no significant change in the proportion of MHCf myofibers between YA and SEN groups (YA = 1.2 ± 0.5%, SEN = 2.9 ± 3.2%), there was also no significant change in the proportion of MHCs myofibers between YA and SEN groups (YA = 98.8 ± 0.5%, SEN = 97.1 ± 3.2%). In contrast, when using only MHCf antibody to identify myofiber type, the proportion of MHCf myofibers in SEN (49.7 ± 12.1%) was 11-fold that of YA (4.5% ± 1.5%: $p < 0.05$), and there was a corresponding 47% decrease in MHCs myofibers (YA = 95.5 ± 1.5%, SEN = 50.3 ± 12.1%, $p < 0.05$). All results for the soleus muscle are represented in Figure 4.3.

In the red region of the gastrocnemius muscle, due to the limited area of the region, a minimum of 75 myofibers per animal were sampled for myofiber type identification. When using both MHCs and MHCf antibodies for myofiber type identification, there was a 26% decrease in the proportion of pure MHCf myofibers with age (YA = 64.3 ± 5.3%, SEN = 47.5 ± 6.4%, $p < 0.05$), a 37% decrease in the proportion of pure MHCs myofibers with age (YA = 35.5 ± 5.2%, SEN = 22.4 ± 7.7%, $p < 0.05$) and the proportion of MHC co-expressing myofibers in SEN (30.0 ± 7.7%) was 150-fold that of YA (0.2 ± 0.4%; $p < 0.05$).

When using only MHCs antibody to identify myofiber type, there was a 26% decrease in the proportion of MHCf myofibers with age (YA = 64.3 ± 5.3%, SEN = 47.5 ± 6.4%, $p < 0.05$) and the proportion of MHCs myofibers in SEN (52.5 ± 6.4%) was 1.5-fold that of YA (35.7 ± 5.3%; $p < 0.05$). In contrast, when using only MHCf antibody to identify myofiber type, the proportion of MHCf myofibers in SEN (77.6 ± 7.7%) was 1.2-fold that of YA (64.5 ± 5.2%; $p < 0.05$) and there was a 37% decrease in the proportion of MHCs myofibers (YA = 35.5 ± 5.2%, SEN = 22.4 ± 7.7%, $p < 0.05$). All results for the red region of the gastrocnemius muscle are represented in Figure 4.5.

In the mixed region of the gastrocnemius, a minimum of 450 myofibers per animal were sampled for myofiber type identification. When using both MHCs and MHCf antibodies for myofiber type identification, there was no significant difference in the proportion of pure MHCf myofibers between YA and SEN groups (YA = 80.5 ± 3.9%, SEN = 82.8 ± 9.3%). There was a 81% decrease in the proportion of pure MHCs myofibers with age (YA = 17.6 ± 5.3%, SEN = 3.4 ± 2.8%, $p < 0.05$) and the proportion

of MHC co-expressing myofibers in SEN (13.8 ± 8.2%) was 7.3-fold that of YA (1.9 ± 1.5%; $p < 0.05$).

When using only MHCs antibody for myofiber type identification there was no significant change in the proportion of MHCf myofibers (YA = 80.5 ± 3.9%, SEN = 82.8 ± 9.3%) or MHCs myofibers (YA = 19.5 ± 3.9%, SEN = 17.2 ± 9.3%) with age. In contrast, when using only MHCf antibody for myofiber type identification, the proportion of MHCf myofibers in SEN (96.6 ± 2.8%) was 1.2-fold that of YA (82.4 ± 5.3%; $p < 0.05$) and there was a 81% decrease in the proportion of MHCs myofibers with age (YA = 17.6 ± 5.3%, SEN = 3.4 ± 2.8%, $p < 0.05$). These results are represented in Figure 4.7.

4.3.4 Myofiber Size in SOL, GASr and GASm

In the soleus muscle, individual myofiber CSA was analyzed from a minimum of 100 myofibers per animal. When using both MHCs and MHCf antibodies for myofiber type identification, within the YA group both pure MHCf and co-expressing myofibers were significantly smaller than pure MHCs myofibers ($p < 0.05$ for both), however, there was no significant difference in mean CSA between pure MHCf and co-expressing myofibers (pure MHCf = 2186 ± 701μm^2, pure MHCs = 3881 ± 378μm^2, co-expressing = 2193 ± 749μm^2). Within the SEN group, there was a significant difference in mean CSA between all different myofiber types ($p < 0.05$ for all). Pure MHCf myofibers were the smallest, followed by co-expressing myofibers and then pure MHCs myofibers (pure MHCf = 881 ± 361μm^2, pure MHCs = 3146 ± 530μm^2, co-expressing = 1631 ± 217μm^2). Between the YA and SEN groups, there was a 60% decrease in the mean CSA of pure

MHCf myofibers with age ($p < 0.05$) and a 19% decrease in the mean CSA of pure MHCs myofibers ($p < 0.05$), but no significant difference among co-expressing myofibers.

When using only MHCs antibody for myofiber type identification, the mean CSA of MHCf myofibers was significantly smaller than that of MHCs myofibers within the YA (MHCf = 2186 ± 701μm^2, MHCs = 3828 ± 362μm^2, $p < 0.05$) and SEN (MHCf = 881 ± 361μm^2, MHCs = 2342 ± 392μm^2, $p < 0.05$) groups. Between the YA and SEN groups, there was 60% decrease in the mean CSA of MHCf myofibers with age ($p < 0.05$) and a 39% decrease in the mean CSA of MHCs myofibers with age ($p < 0.05$). When using only MHCf antibody for myofiber type identification, the mean CSA of MHCf myofibers was also significantly smaller than that of MHCs myofibers within the YA (MHCf = 2342 ± 612μm^2, MHCs = 3881 ± 378μm^2, $p < 0.05$) and SEN groups (MHCf = 1603 ± 200μm^2, MHCs = 3146 ± 530μm^2, $p < 0.05$). However, while there was still a significant decrease in the mean CSA of MHCf myofibers with age, it was only a 32% decrease ($p < 0.05$). Furthermore, while there was still a significant decrease in the mean CSA of MHCs myofibers with age, it was only a 19% decrease ($p < 0.05$). Results for myofiber type mean CSA within the soleus muscle are presented in Figure 4.3.

In the red region of the gastrocnemius muscle, individual myofiber CSA was measured from a minimum of 75 myofibers per animal. When using both MHCf and MHCs antibodies for myofiber type identification, within the YA group there was no significant difference in mean CSA between pure MHCf and pure MHCs myofibers, but both pure MHCf and pure MHCs myofibers were significantly larger than co-expressing myofibers ($p < 0.05$ for both comparisons) (pure MHCf = 2464 ± 382μm^2, pure MHCs =

2688 ± 320μm^2). Within the SEN group, while there was no significant difference in mean CSA between pure MHCf and co-expressing myofibers, pure MHCs myofibers were significantly larger than both pure MHCf and co-expressing myofibers ($p < 0.05$ for both) (pure MHCf = 1874 ± 371μm^2, pure MHCs = 2851 ± 691μm^2, co-expressing = 1538 ± 251μm^2). Between the YA and SEN groups, there was a 24.0% decrease in the mean CSA of pure MHCf myofibers ($p < 0.05$) with age, but no difference between the mean CSA of pure MHCs myofibers or co-expressing myofibers.

When using only MHCs antibody for myofiber type identification, there was no significant difference in mean CSA between MHCf and MHCs myofibers within the YA (MHCf = 2464 ± 382μm^2, MHCs = 2683 ± 324μm^2) or SEN (MHCf = 1874 ± 371μm^2, MHCs = 2104 ± 478μm^2) groups. Between YA and SEN groups, there was a 24.0% decrease in the mean CSA of MHCf myofibers ($p < 0.05$) and a 21.6% decrease in the mean CSA of MHCs myofibers ($p < 0.05$) with age. However, when using only MHCf antibody for myofiber type identification, while there was still no significant difference in mean CSA of MHCf and MHCs myofibers within the YA group (MHCf = 2461 ± 384μm^2, MHCs = 2688 ± 320μm^2), within the SEN group, MHCs myofibers were significantly larger than MHCf myofibers (MHCf = 1735 ± 300μm^2, MHCs = 2851 ± 691μm^2, $p < 0.05$). Between YA and SEN groups, there was no significant difference in the mean CSA of MHCs myofibers, but a 29.5% decrease in the mean CSA of MHCf myofibers ($p < 0.05$). These results are represented in Figure 4.5.

In the mixed region of the gastrocnemius muscle, individual myofiber CSA was measured from a minimum of 100 myofibers per animal. When using both MHCf and

MHCs antibodies for myofiber type identification, within the YA group, there was no significant difference in mean CSA between pure MHCs and pure MHCf or co-expressing myofibers; however, pure MHCf myofibers were significantly larger than co-expressing myofibers ($p < 0.05$) (MHCf = 2993 ± 499μm^2, MHCs = 2437 ± 314μm^2, co-expressing = 1975 ± 400μm^2). Within the SEN group, while there was a trend for co-expressing myofibers to be smaller, there was no significant difference in mean CSA among myofiber types (MHCf = 2055 ± 443μm^2, MHCs = 2025 ± 692μm^2, co-expressing = 1362 ± 413μm^2). Between the YA and SEN groups, there was a 31.3% decrease in the mean CSA of pure MHCf myofibers ($p < 0.05$) with age, but no significant difference in mean CSA of pure MHCs or co-expressing myofibers.

When using only MHCs antibody for myofiber type identification, MHCf myofibers were significantly larger than MHCs myofibers within both the YA (MHCf = 2993 ± 499μm^2, MHCs = 2416 ± 324μm^2, $p < 0.05$) and SEN (MHCf = 2055 ± 443μm^2, MHCs = 1515 ± 482μm^2, $p < 0.05$) groups. Between the YA and SEN groups, there was a 31.3% decrease in the mean CSA of MHCf myofibers ($p < 0.05$) and a 37.3% decrease in the mean CSA of MHCs myofibers ($p < 0.05$) with age. In contrast, when using only MHCf antibody for myofiber type identification, there was no significant difference in mean CSA between MHCf and MHCs myofibers within the YA (MHCf = 2979 ± 496μm^2, MHCs = 2437 ± 314μm^2) or SEN (MHCf = 1970 ± 405μm^2, MHCs = 2025 ± 692μm^2) groups. Between YA and SEN groups, there was a 33.9% decrease in the mean CSA of MHCf myofibers ($p < 0.05$), but no significant difference in the mean CSA of MHCs myofibers with age. These results are presented in Figure 4.7.

4.4 Discussion

The findings of this study are fourfold: (1) MHC co-expression increases with age in both the slow-twitch soleus muscle and the fast-twitch gastrocnemius muscle; (2) Pure MHCf myofibers are not preferentially lost with age; (3) MHCs myofibers are not protected from atrophy; (4) Not accounting for co-expressing myofibers significantly impacts estimates of myofiber type proportions and type-specific alterations in myofiber size associated with age.

4.4.1 Myosin Heavy Chain Expression Trends Associated with Age

In the soleus muscle, MHC co-expression in SEN was 14.2-fold that of YA. As a consequence, in old age, co-expressing myofibers accounted for 47% of all myofibers. This supports the findings of Snow and colleagues, (Snow *et al.* 2005) that, compared to 12-month old rat soleus, 36-month old rat soleus had approximately nine times as many myofibers co-expressing MHCs and MHCf, comprising over 50% of the total myofibers sampled. Also in line with their findings, our study found that increased MHC co-expression did not correspond to a decrease in pure MHCf myofibers, but rather a decrease in pure MHCs myofibers. This suggests that the majority MHCs/MHCf co-expressing myofibers in the old adult soleus muscle are derived from the population of pure MHCs myofibers in young adult soleus. In the red region of the gastrocnemius muscle, where the myofiber type distribution in the young adult rat is much more varied, co-expressing myofibers comprised 30% of all myofibers in old age and corresponded to similar decreases in pure MHCs and pure MHCf proportions. In the mixed region of the

gastrocnemius muscle, the proportion of myofibers co-expressing MHCs and MHCf in SEN was 7.3-fold that of YA and only corresponded to a decrease in the proportion of pure MHCs myofibers. Previous work from our lab with different cohorts of young adult and very old rats (Carter *et al.* 2010) found that, in the soleus muscle, there was an increase in overall MHCf expression associated with age, but in whole gastrocnemius muscle, there was an increase in overall MHCs expression. Similarly, in rat quadriceps muscle, Lushaj and colleagues found an increase in MHCs expression with age (Lushaj *et al.* 2008). Together, these results indicate that there is not a preferential fast-to-slow shift in myofiber type associated with age, but possibly a more muscle-specific shift. This is in contradiction to the position expressed in the literature that MHCf myofibers are preferentially lost with age (Kirkendall & Garrett 1998; Pette & Staron 2000; Andersen 2003; Brunner *et al.* 2007; Lushaj *et al.* 2008; Ryall *et al.* 2008; Marzetti *et al.* 2009). Evidence against the preferential loss of MHCf myofibers with age is not only present in the rat model of aging, but in human studies as well (Canepari *et al.* 2010). In the recently published results of a longitudinal study by Frontera and colleagues in elderly men and women (Frontera *et al.* 2008), it was found that there were no significant changes in myofiber type distribution in vastus lateralis muscle over an eight year follow up period (71 years to 80 years). In a previous report in men (65 years to 77 years) (Frontera *et al.* 2000a), a significant decrease in type I myofibers over a 12 year period was found. Monemi and colleagues also report this contradictory, seemingly muscle-specific aspect of myofiber type distribution associated with age, where in human masseter muscle there was an increase in MHCf expression, but in biceps brachii muscle, there was an increase in MHCs expression (Monemi *et al.* 1999). It is clear that a blanket

statement regarding the tendency of MHC expression to shift in one direction or another cannot be applied in the aging context and may be dependent on individual muscle composition or function.

4.4.2 Type-specific Alterations in Myofiber Size Associated with Age

In the soleus muscle and both regions of the gastrocnemius muscle, MHCf myofibers were significantly smaller with age. MHCf myofiber atrophy associated with age is well documented in studies of aging rats (Blough & Linderman 2000; Snow *et al.* 2005; Carter *et al.* 2010) and those of aging humans (Aniansson *et al.* 1986; Coggan *et al.* 1992; Andersen 2003). The impact of aging on MHCs myofiber size is less clear.

It has been reported that MHCs myofibers are more or less protected from atrophy until very old age (Porter *et al.* 1995). However, we found that pure MHCs myofibers were significantly smaller with age. Furthermore, since most of the 43% of myofibers that are co-expressing in the aged soleus muscle and most of the 43% of myofibers that are co-expressing in the red region of the gastrocnemius muscle must stem from a population of pure MHCs myofibers in the young adult, then it becomes clear that MHCs myofibers exhibit marked atrophy with aging. In the soleus muscle, the co-expressing myofibers were 58% smaller than young adult pure MHCs myofibers ($p < 0.05$). This indicates that a sub-population of formerly pure MHCs myofibers is atrophying substantially, albeit after co-expressing MHCf, which directly contradicts the position expressed in the literature (Kirkendall & Garrett 1998; Brunner *et al.* 2007; Marzetti *et al.* 2009) that MHCs myofibers are protected from atrophy associated with age. Strikingly, in the gastrocnemius, myofibers that maintained pure MHCs expression in old age were

very similar in size to young adult pure MHCs myofibers and in the soleus, atrophy was least pronounced in pure MHCs myofibers. This raises at least two possibilities worth further exploration: first, either MHCs myofibers that become co-expressing in old age (and have a greater capacity to atrophy) are fundamentally different from MHCs myofibers that do not become co-expressing; or second, something about the co-expression of MHCf allows for more pronounced atrophy. In both cases, the co-expression of MHC isoforms provides further insight into the complexity of muscle atrophy in sub-populations of muscle myofibers.

4.4.3 Co-Expression of Multiple Myosin Heavy Chain Fast Isoforms and Myonuclear Domain

The goal of this study was to determine the degree of MHCs and MHCf co-expression with age in muscles of different myofiber type distributions. However, it should be noted that the results reported here will underestimate the true occurrence of MHC co-expression with age for two reasons.

(1) It is thought that myofibers co-expressing MHC isoforms follow the "next neighbour rule" (Pette 2001) such that type I myofibers would co-express Type IIa MHC and Type IIa myofibers would co-express Type IIx MHC and vice versa, but type I and type IIx MHC would not co-exist within a myofiber, though rare exceptions to the rule have been observed (Andersen 2003). The use of the more general MHCs and MHCf antibodies for myofiber type identification eliminates the possibility of identifying myofibers that co-express multiple MHCf isoforms (Type IIa, Type IIx, Type IIb). This is of particular significance in muscle regions where there is a mixture of fast myofiber

types, such as the mixed and white regions of the gastrocnemius muscle (Armstrong & Phelps 1984). We believe that this explains the low observed occurrence of co-expressing myofibers in the mixed region of the gastrocnemius muscle where, increasingly, fast myofibers would be a mix of type IIa /IIx and type IIx/IIb. In future studies, individual MHC isoform antibodies will be used to shed light on this matter.

(2) In the present study we have used serial sections of whole muscle cross-sectional area for analysis, which provided opportunity to examine multiple characteristics of individual myofibers in the context of their physical position within the muscle. This limited our scope to one myonuclear domain per myofiber. This is not problematic if MHC expression is always uniform along the entire length of a myofiber, however, it has been suggested that under some conditions, including aging, a discoordination of MHC expression can occur among the many myonuclear domains within an individual myofiber (Andersen 2003). The extent to which this discoordination occurs in aging requires further documentation, but if there are multiple patterns of MHC expression within a single muscle myofiber in old age, then our results will underestimate MHC co-expression.

4.4.4 Impact of Two Antibody vs. One Antibody Method for Myofiber Type Identification

Given the occurrence of co-expressing myofibers with age, we thought it pertinent to examine the impact that failing to account for co-expression has on estimates of myofiber type proportions and size. In all muscle regions examined, the impact was dramatic, as shown in Figure 4.8.

When examining changes in myofiber type proportions in the cases of the soleus muscle and the mixed region of gastrocnemius muscle, use of only the MHCs antibody for myofiber type identification did not show any changes in myofiber type proportions associated with age, hiding the changes occurring in MHCs myofibers. Use of only the MHCf antibody reported a decrease in MHCs expressing myofibers. Neither of these scenarios is a true representation of the changes in MHC expression occurring in association with age, yet several previous studies have employed only one antibody in their myofiber type estimations in aging muscle (Wanagat *et al.* 2001; McKiernan *et al.* 2009; McKiernan *et al.* 2011). Similarly, in the red region of the gastrocnemius muscle, use of only the MHCs antibody showed a decrease in MHCf proportions and use of only the MHCf antibody showed a decrease in MHCs proportions. In actuality, MHCs and MHCf expression proportions remained stable.

When examining type-specific alterations in myofiber size, the impact appeared to be more muscle specific. In the soleus where the largest myofibers in young adult animals were MHCs myofibers, not accounting for co-expressing myofibers disguised the increased atrophy of formerly pure MHCs myofibers co-expressing MHCf and underestimated the degree of atrophy in pure MHCf myofibers when using only the MHCf antibody. In the gastrocnemius muscle where pure MHCs and pure MHCf myofibers had similar sizes in young adult, failure to account for co-expressing myofibers overestimated the degree of atrophy in MHCs myofibers when using only the MHCs antibody, and underestimated it when using only the MHCf antibody.

These findings illustrate that significant discrepancies exist between methods and warrant attention when interpreting previously published results and designing future

studies. That said, perhaps the most relevant impact of not accounting for co-expressing myofibers in methodological design and interpretation of results is that it masks an entire level of myofiber behaviour with age. The discovery of co-expressing myofibers in aged muscle leads to new questions to be explored. For example, why is it that only some myofibers co-express multiple MHC isoforms? Or, in the case of pure MHCs myofibers that go on to co-express MHCf, why is it that they then, apparently, have the capacity to atrophy?

4.5 Conclusion

The purpose of this study was to determine the degree of myofiber myosin heavy chain co-expression with age in examples of slow- and fast-twitch skeletal muscle and to elucidate the impact of these co-expressing myofibers on estimates of myofiber type proportions and type-specific alterations in myofiber size associated with age. Using rat soleus muscle and gastrocnemius muscles, this study demonstrates that co-expressing myofibers represent a significant proportion of myofibers in both slow-twitch and fast-twitch muscles in very old age. This study also demonstrates that these myofibers must stem from both populations of MHCf and populations of MHCs myofibers in the young adult rat, disproving the belief that fast myofibers are preferentially lost with age. Furthermore, this study shows that not accounting for co-expressing myofibers significantly impacts understanding of the dynamics of muscle atrophy with age as it pertains to myosin heavy chain expression and type-specific myofiber behaviour.

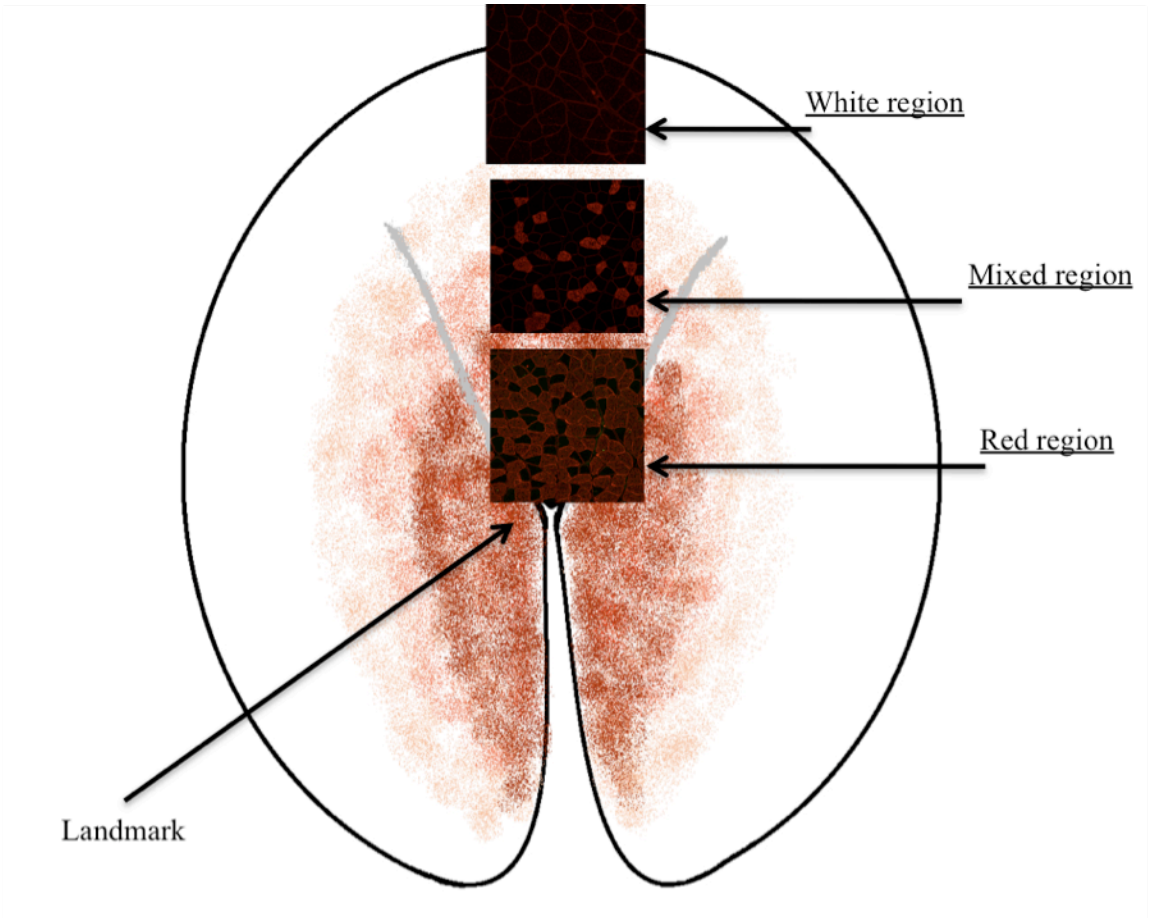

Figure 4.1 Schematic of the gastrocnemius muscle showing the different regions of the muscle. The fibers in the images are labelled for MHCs expression.

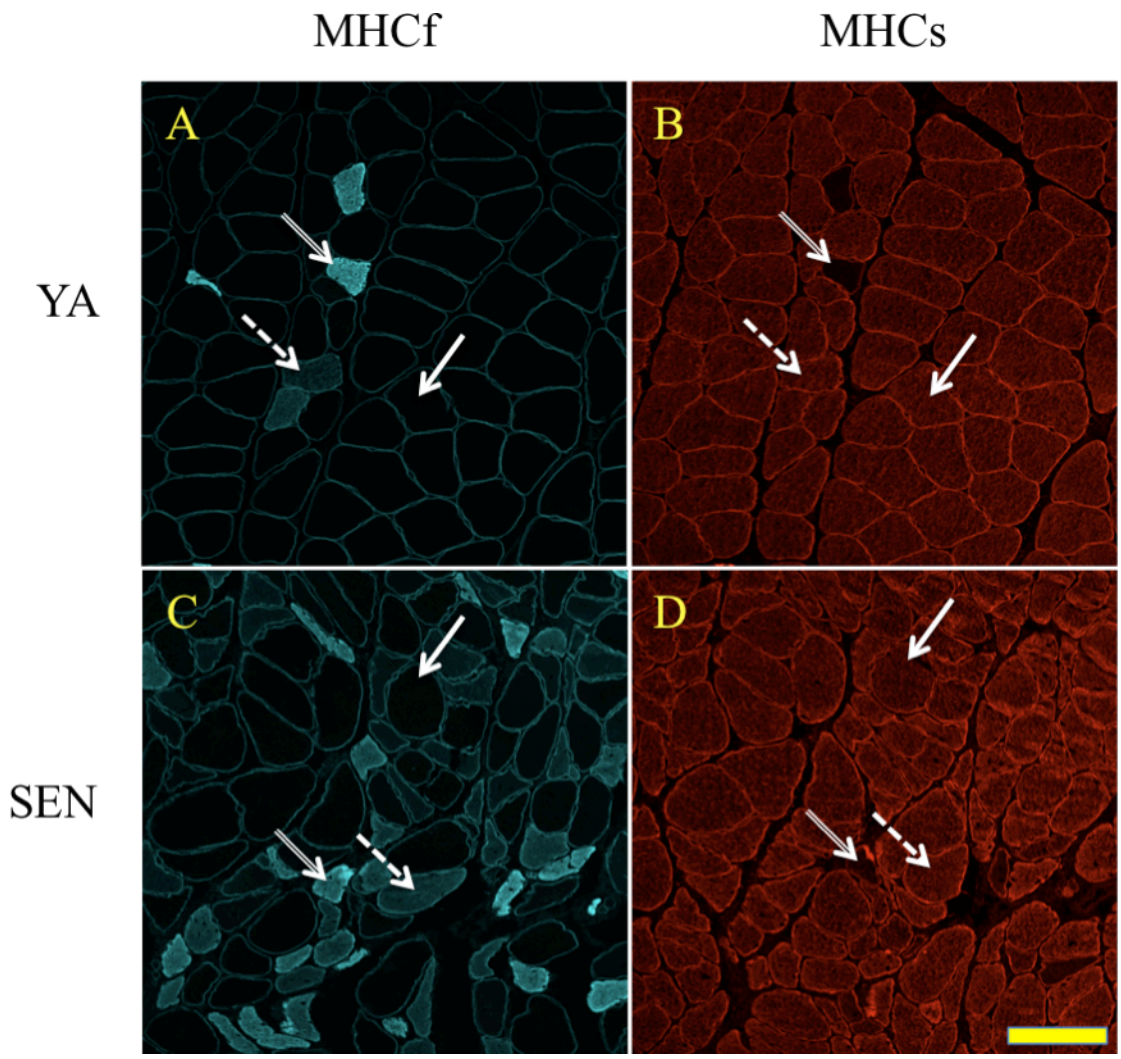

Figure 4.2 MHC expression between YA and SEN in SOL. Solid arrows indicate pure MHCs fibers, hollow arrows indicate pure MHCf fibers, dashed arrows indicate co-expressing fibers. Scale bar = 150 μm.

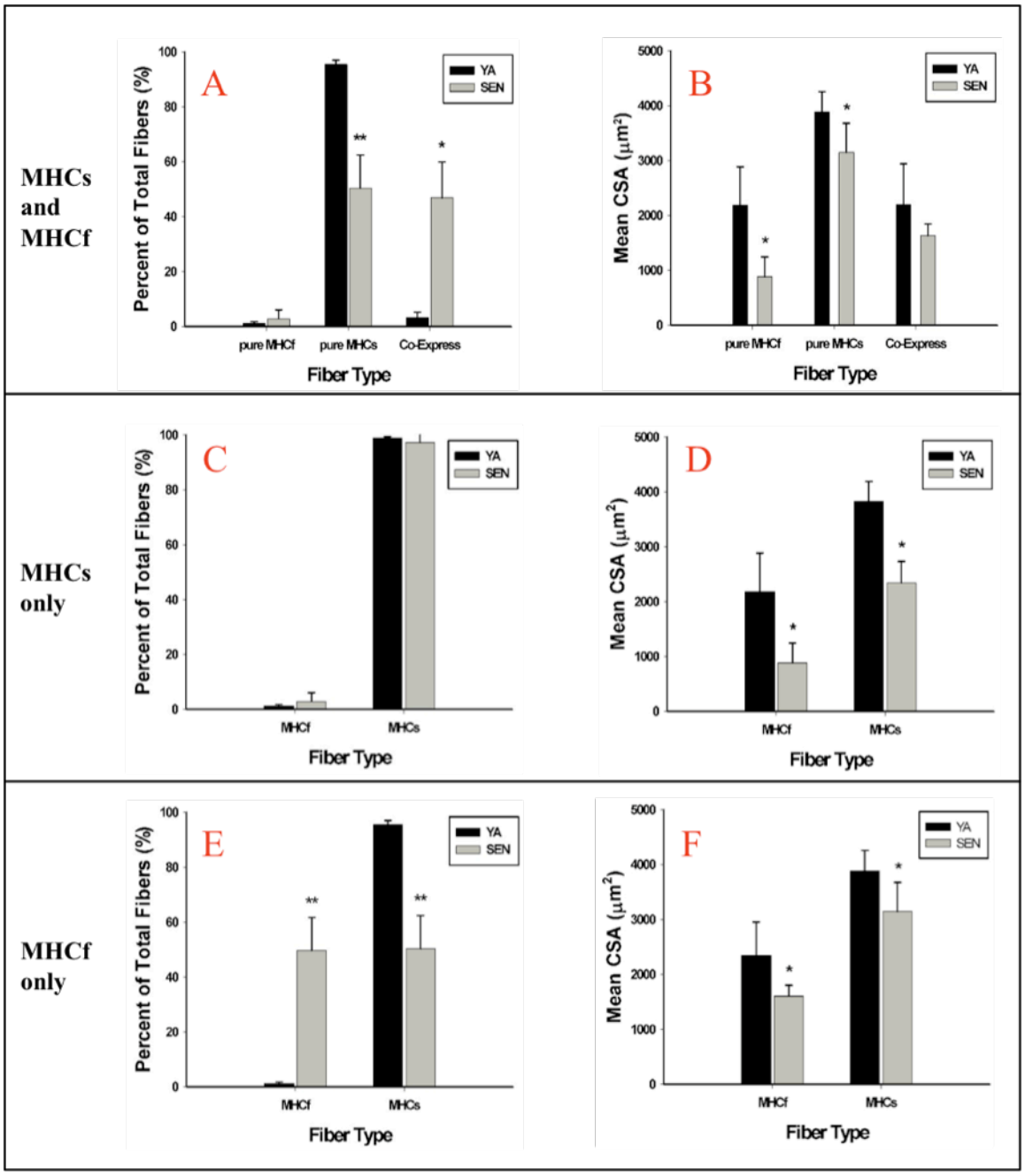

Figure 4.3 MHC expression and myofiber type CSA in SOL. A,B = results using both MHCf and MHCs antibodies. C,D = results using MHCs antibody only. E,F = results using MHCf antibody only. * Indicates significance between YA and SEN using a Student t-test. ** Indicates significance between YA and SEN using a Mann-Whitney rank sum test. Bars indicate standard deviation.

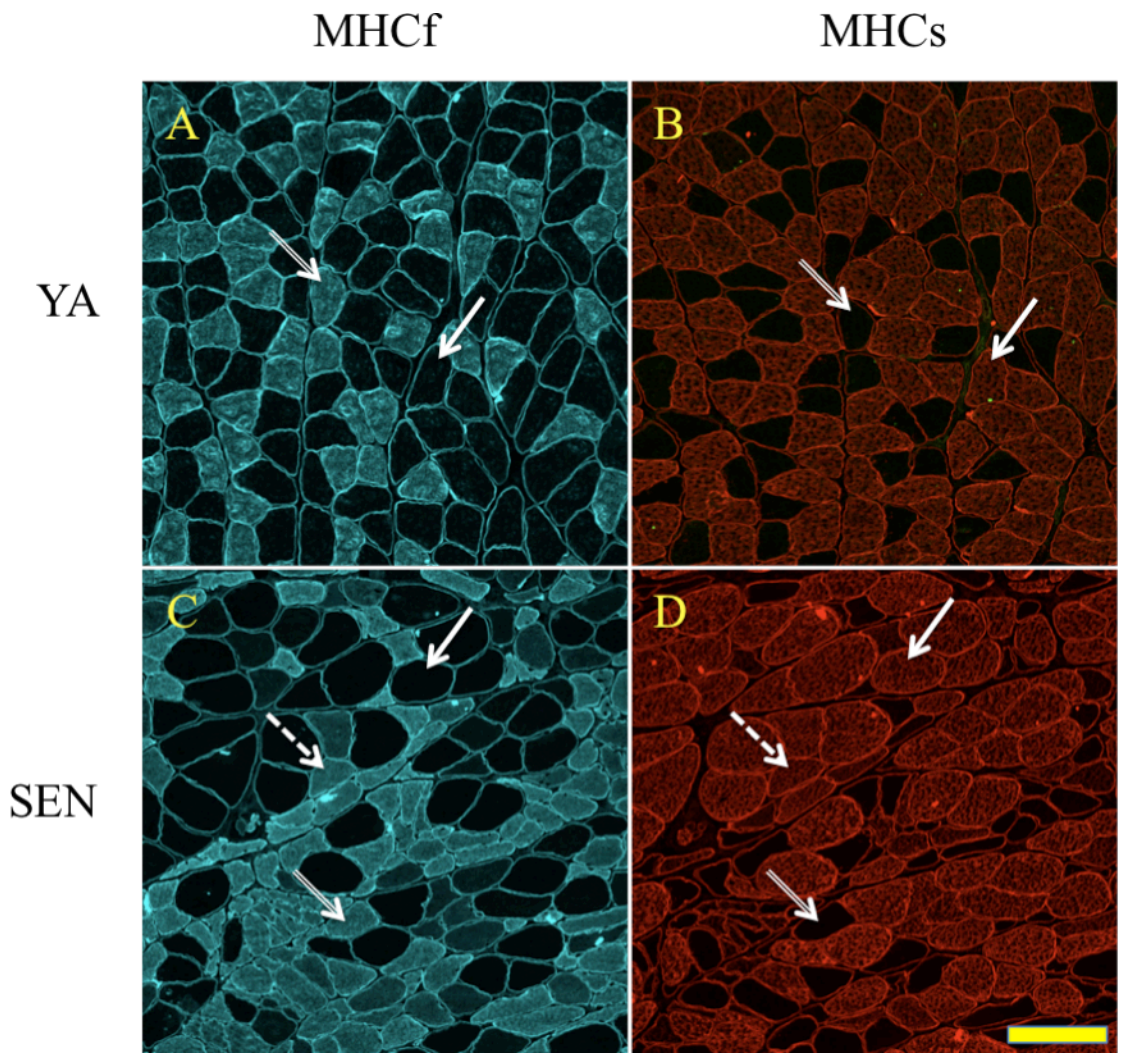

Figure 4.4 MHC expression between YA and SEN in GASr. Solid arrows indicate pure MHCs fibers, hollow arrows indicate pure MHCf fibers, dashed arrows indicate co-expressing fibers. Scale bar = 150 μm.

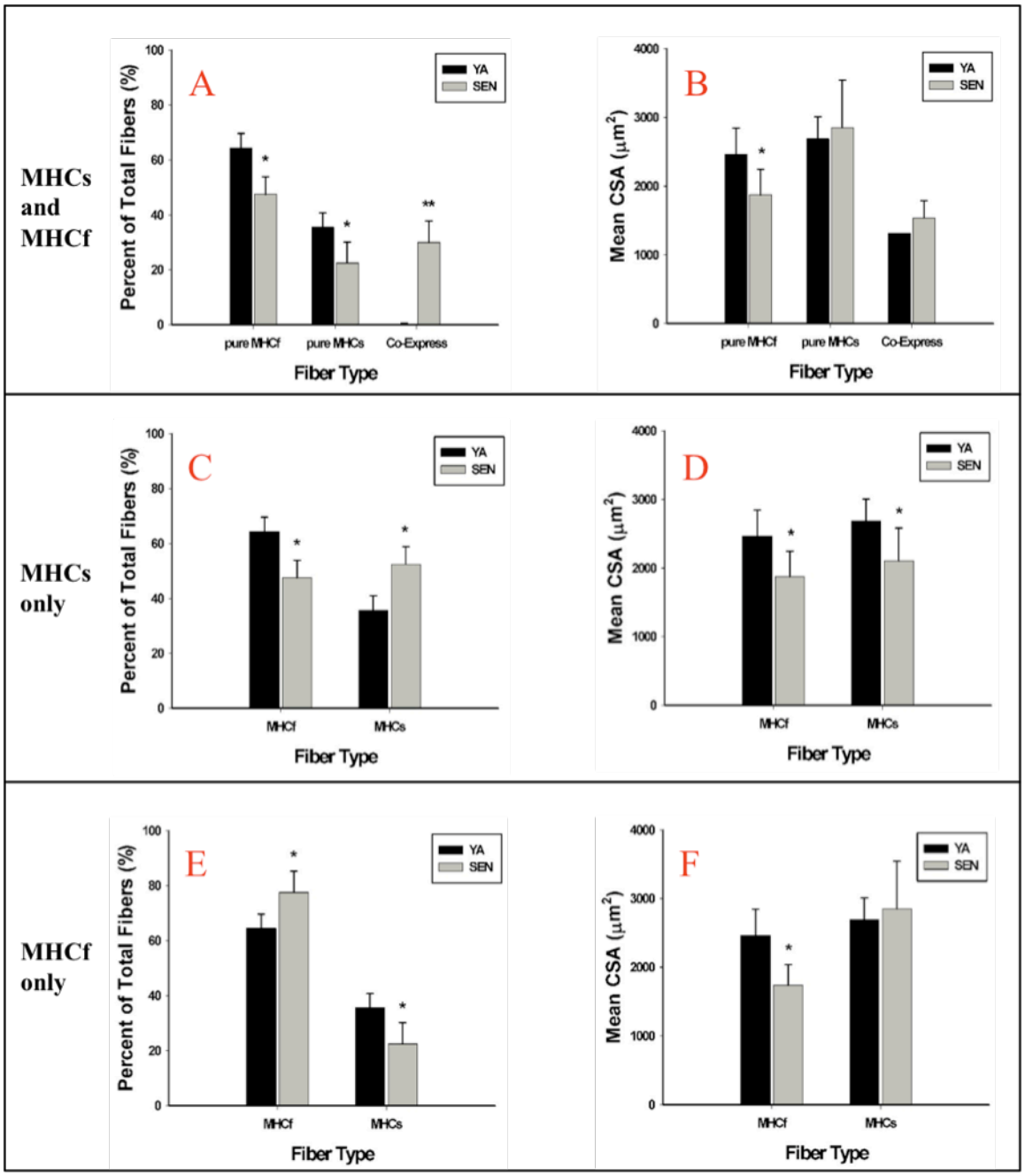

Figure 4.5 MHC expression and myofiber type CSA in GASr. A,B = results using both MHCf and MHCs antibodies. C,D = results using MHCs antibody only. E,F = results using MHCf antibody only. * Indicates significance between YA and SEN using a Student t-test. ** Indicates significance between YA and SEN using a Mann-Whitney rank sum test. Bars indicate standard deviation.

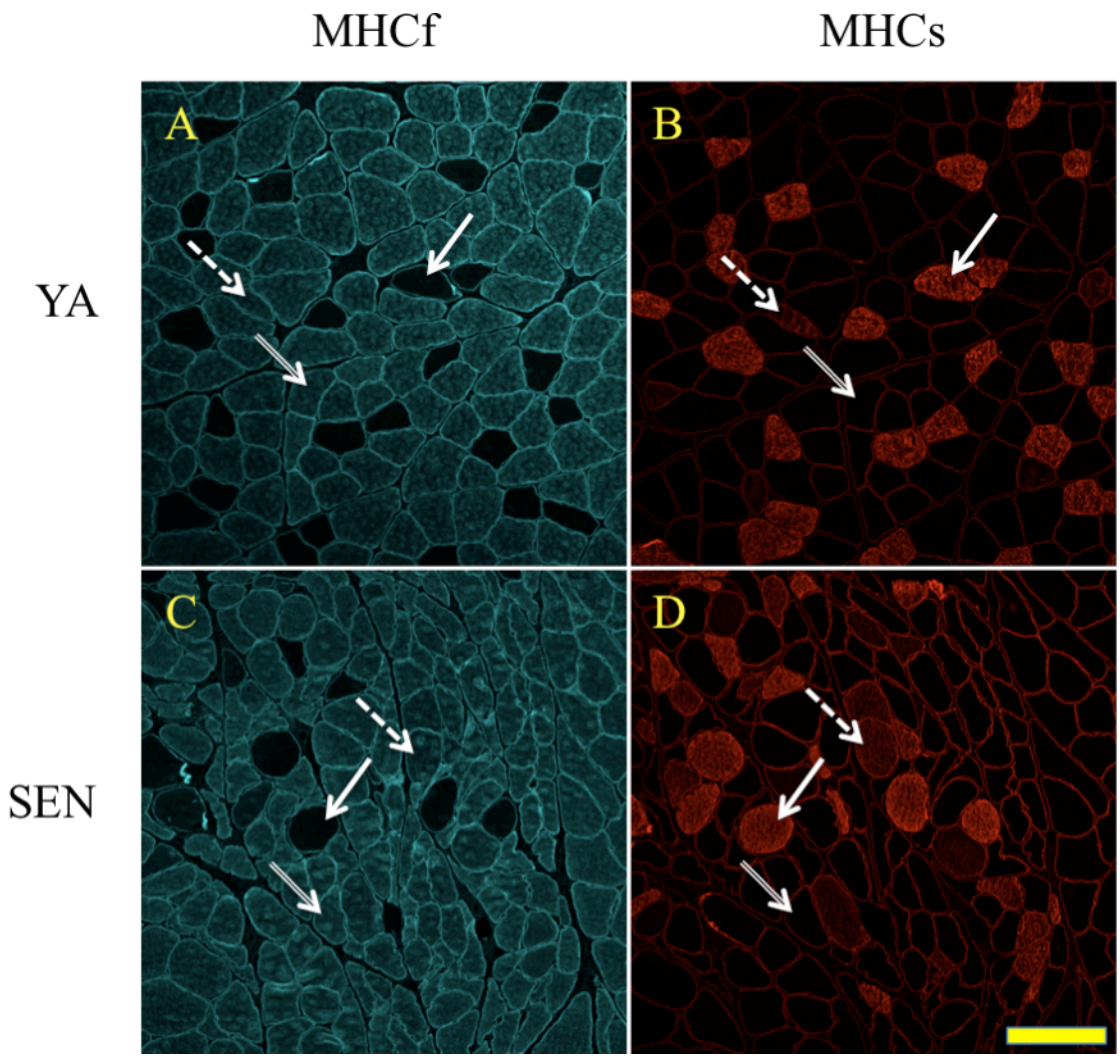

Figure 4.6 MHC expression between YA and SEN in GASm. Solid arrows indicate pure MHCs fibers, hollow arrows indicate pure MHCf fibers, dashed arrows indicate co-expressing fibers. Scale bar = 150 μm.

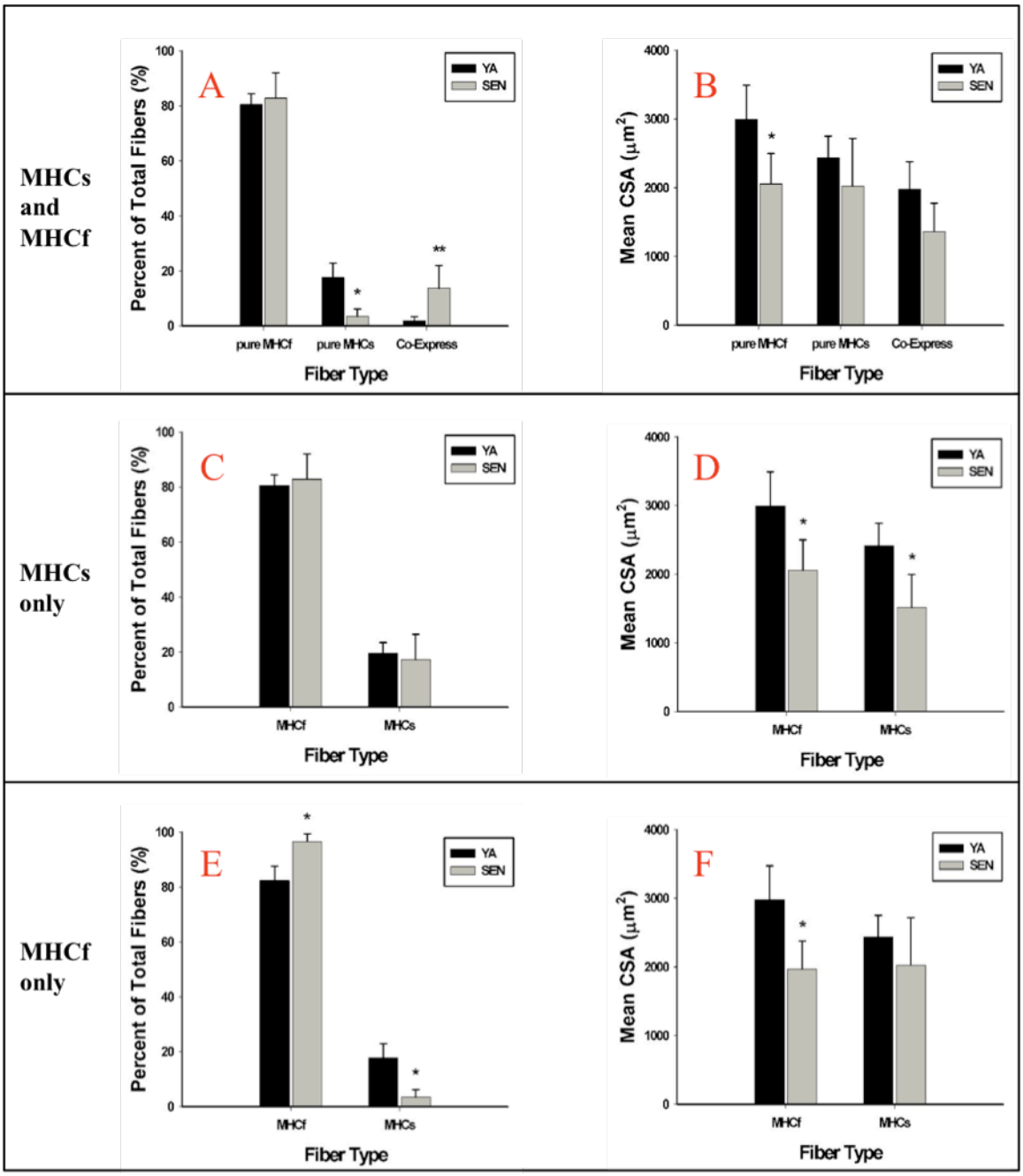

Figure 4.7 MHC expression and myofiber type CSA in GASm. A,B = results using both MHCf and MHCs antibodies. C,D = results using MHCs antibody only. E,F = results using MHCf antibody only. * Indicates significance between YA and SEN using a Student t-test. ** Indicates significance between YA and SEN using a Mann-Whitney rank sum test. Bars indicate standard deviation.

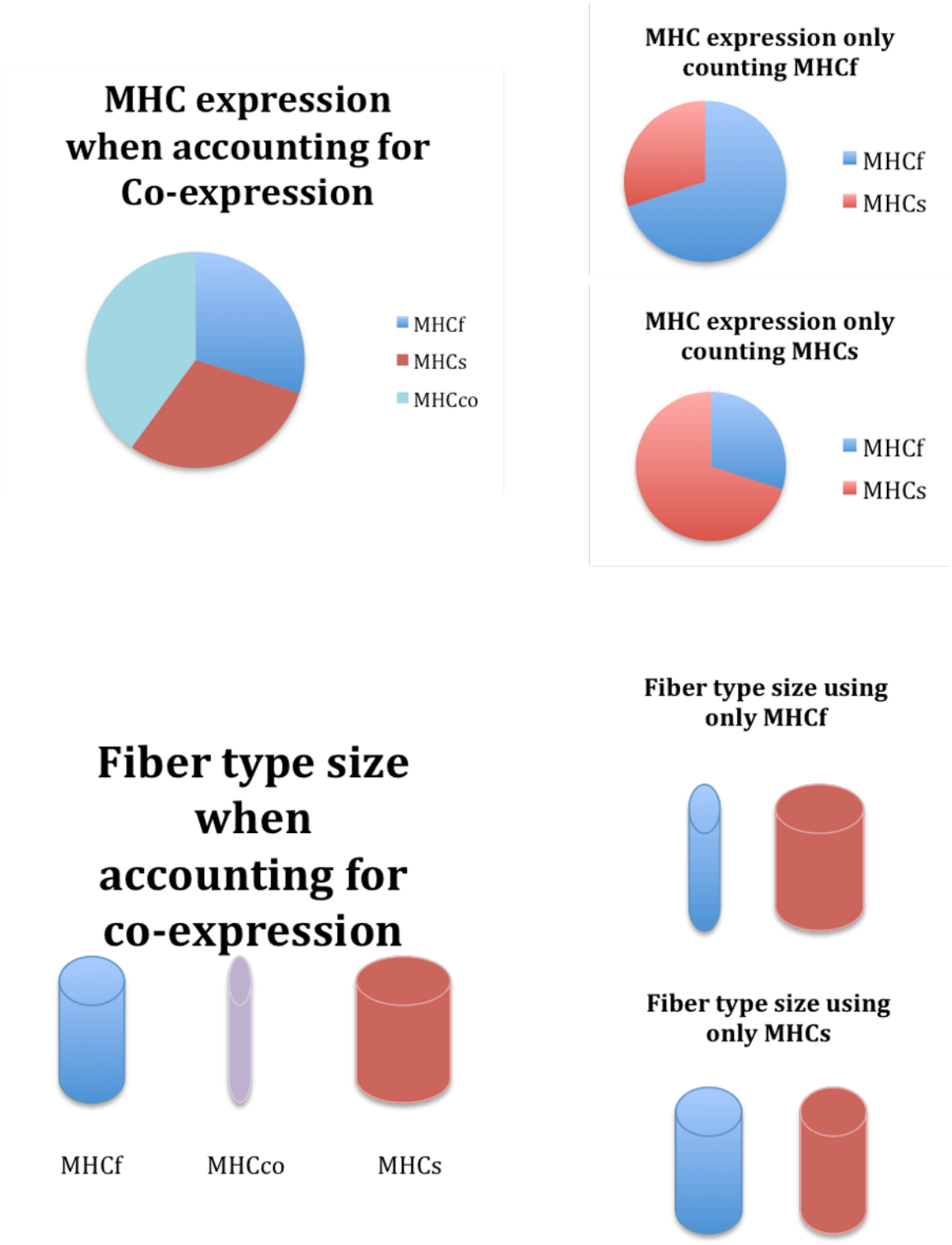

Figure 4.8 Impact of different methods for myofiber type identification on estimates of myofiber type and size.

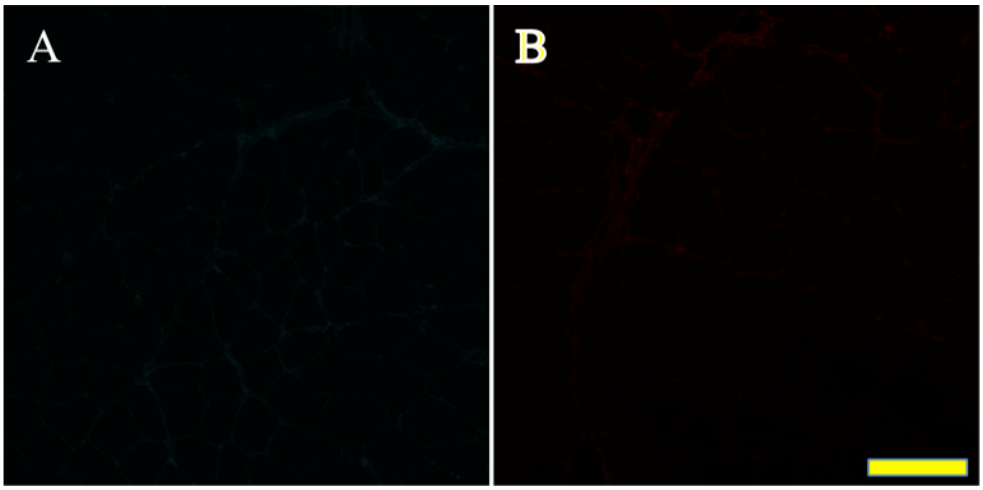

Figure 4.9 Negative control samples for MHC labelling. (A) is the negative control for MHCf antibody. (B) is the negative control for MHCs antibody. Scale bar = 150 μm.

Chapter Five: Discussion

The purpose of this work was to provide insight into the mechanisms and morphology of sarcopenia. We have found that MuRF1, a main contributor to muscle atrophy, is elevated in a denervation-specific manner in sarcopenic muscle. We have also found that the morphology of sarcopenic muscle is much more similar to that of denervated muscle than previously estimated in the literature. Both of these findings support the hypothesis that denervation plays a significant role in the progression of sarcopenia. These findings have implications for the future focus of sarcopenia research and for the development of effective treatments of sarcopenia.

5.1 Denervation as the Primary Instigator of Sarcopenia

There are several lines of evidence that implicate denervation as the primary cause of sarcopenic muscle atrophy. In fact, many of the morphological and functional characteristics of sarcopenia which make it appear as a unique form of muscle atrophy, are actually more indicative of denervation being its cause. These characteristics include myofiber type grouping, myofiber size heterogeneity, reduced specific force of muscles, muscle specific shifts in MHC expression, MHC co-expression and reduced atrophy of pure MHCs myofibers. These physiological changes have not been observed in atrophy scenarios where the nervous system is not compromised, such as cancer cachexia and rheumatoid arthritis cachexia.

One of the initial and unique morphological signs of sarcopenia is the appearance of myofiber type grouping as a result of ongoing denervation/reinnervation (Lexell & Downham 1991). Recently, it was demonstrated that remodelling of the neuromuscular

junction (Deschenes *et al.* 2010) occurs prior to any observable changes in muscle morphology and function. Combined, these findings establish that neural dysfunction is occurring early in the progression of sarcopenia and is possibly the initiating event in sarcopenia. Myofiber type grouping also demonstrates that degradation and loss of motor neurons does not occur in a uniform manner, but rather, in a relatively sporadic manner. This suggests that, while some myofibers will become denervated and respond accordingly (by atrophying), others will remain in a normal functioning state. Given the structure of a typical motor unit, denervation then also explains the myofiber size heterogeneity observed in sarcopenic muscle (Frontera *et al.* 2008) and the reduced specific force of muscles observed in sarcopenia (Frontera *et al.* 2008). Although we did not quantify these characteristics in the present work, myofiber type grouping and myofiber size heterogeneity associated with age can clearly be identified in Figures 4.2, 4.4, and 4.6.

As outlined in Chapter 4, age-associated shifts in MHC expression are a source of controversy and, previously, were not believed to align with the shifts observed following denervation. This work demonstrates that, like in models of denervation (Pette & Staron 2000), the shifts in MHC expression which occur in sarcopenia are muscle specific and towards greater expression of the non-dominant myosin type. Further, it demonstrates that MHC co-expressing myofibers, myofibers primarily associated with states of denervation or motor neuron trauma (Borg *et al.* 1989; Talmadge *et al.* 1999; Patterson *et al.* 2006), occur in abundance in sarcopenic muscle.

Finally, in sarcopenia, slow twitch muscles and pure MHCs myofibers exhibit reduced atrophy compared to their fast twitch/ MHCf expressing counterparts. Counter

intuitive to this finding, functionally, they exhibit the same degree, or greater, deterioration (Carter *et al.* 2010). However, these apparently contrasting findings are consistent with the involvement of denervation in sarcopenia. It has been reported that, with denervation, MHCs myofiber atrophy occurs at a much later time-point than MHCf myofiber atrophy (several months later in rat muscle) (Lu *et al.* 1997). Given the time course of sarcopenia and its acceleration during the late stages of life (Lexell *et al.* 1988), delayed atrophy of pure MHCs myofibers due to denervation explains both the apparent protection of slow twitch muscle and pure MHCs myofibers from atrophy and their reduced function.

Although there is limited data available on the topic, these distinct characteristics do not appear to be found in models of muscle atrophy where denervation is not involved. Cachexia is the loss of muscle mass, with or without the loss of fat mass, as a result of chronic disease such as cancer or rheumatoid arthritis (Diffee *et al.* 2002). In a study of the morphology of cachexia in mice afflicted with colon cancer, Aulino and colleagues (2010) found that atrophy occurred in a uniform manner across all muscles studied (soleus, extensor digitorum longus and tibialis anterior) and that myofiber size appeared to be homogeneous. This suggests that in cancer cachexia atrophy is similar in both pure MHCs and MHCf expressing myofibers and contrasts with the progression of denervation-associated atrophy and sarcopenia. They also found no decrease in the specific force of muscles compared to control animals, indicating that all myofibers contributing to muscle mass were also contributing to muscle function (Aulino *et al.* 2010). Similarly, Matschke and colleagues (2010) did not find any changes in the specific force of muscles in patients with rheumatoid cachexia compared to control subjects.

Neither study examined changes in myosin heavy chain expression so no comparisons to sarcopenia can be made.

Based on these lines of evidence and our findings regarding individual myofiber denervation status, atrophy, MAFbx protein expression and MuRF1 protein expression, our proposed progression of sarcopenia is represented in Figure 5.1. If denervation is the primary instigator of sarcopenia and myofiber atrophy is merely a response to denervation, then the current direction of sarcopenia research efforts will need to be re-evaluated.

5.2 Current Research and Treatments in Sarcopenia

At the present time, the majority of studies which research sarcopenia posit that loss of myofibers and myofiber atrophy are the result of dysfunction within the myofiber, such as mitochondrial dysfunction (Marzetti *et al.* 2009; Thompson 2009) or impaired protein synthesis/degradation balance (Clavel *et al.* 2006; Altun *et al.* 2010). Consequently, methods for treating sarcopenia that are currently available, or are being developed, aim to slow or halt myofiber atrophy directly, or even to cause myofiber hypertrophy (Glass & Roubenoff 2010). These treatment methods include, but are not limited to, exercise, the use of hormone therapy (such as testosterone or insulin-like-growth hormone) to promote protein synthesis, the use of proteasomal system inhibitors to slow protein degradation, and the use of antioxidants to counter oxidative damage which may be caused by dysfunctional mitochondria (Glass & Roubenoff 2010; Waters *et al.* 2010). Currently, there are no treatment methods which cure sarcopenia and those which have been developed appear to have limited efficacy in staying its progression. For

example, our lab recently examined the effectiveness of exercise training at preserving muscle function and mass with age (Thomas *et al.* 2010) and found that it had only minimal effect on individual muscle function and that it caused an increase in the number of atrophied myofibers (notably, exercise training has been found to improve whole limb and body function (Marzetti *et al.* 2008)). Similarly, while hormone therapy has been shown to improve muscle mass, these improvements do not translate into improved function (Marzetti *et al.* 2008; Waters *et al.* 2010). If denervation is the primary instigator of myofiber atrophy in sarcopenia, then the poor performance of these treatment methods is both expected and highlights two of the main problems associated with targeting muscle atrophy directly.

Firstly, methods which target muscle atrophy directly without regard for nerve health may inadvertently exacerbate the rate at which denervation occurs, contributing to the progression of sarcopenia. The observation that exercise training causes an increase in the number of atrophied myofibers associated with age (Thomas *et al.* 2010) suggests that either myofibers have a reduced capacity for training adaptation, or atrophied myofibers are not being engaged in exercise training. Since there is evidence that the regenerative capacity of muscle is not compromised with age (Edstrom & Ulfhake 2005), then the latter suggestion is the most likely. Keeping with our hypothesis that denervation is the primary cause of myofiber atrophy, it seems reasonable to suggest that increased stress due to exercise training causes premature failure of weaker motor neurons resulting in greater numbers of denervated myofibers and, thus, atrophied myofibers. Exercise may represent a unique treatment method where, in the interim, its whole body benefits to elderly persons far outweigh its consequences to sarcopenia (Spirduso & Cronin 2001;

Marzetti *et al.* 2008; Waters *et al.* 2010); however, future development of treatment methods for sarcopenia must focus on preserving motor neuron health with age if they are ultimately going to be successful.

Secondly, functionally, a large denervated myofiber is equally as ineffective as a small denervated myofiber. If denervation is the primary cause of myofiber atrophy, then any method with directly targets myofiber size in an attempt to treat sarcopenia, such as hormone therapy or inhibition of the proteasomal system, will fail to improve physical function or quality of life (the true measure of a successful sarcopenia treatment). In fact, treatments such as testosterone replacement are not recommended for treating sarcopenia because, despite their effectiveness in improving muscle mass, they have little effect on physical function (Waters *et al.* 2010). These disappointing results will continue until treatment methods are developed which improve the denervation status of individual myofibers, either by preventing myofiber denervation, or by facilitating myofiber re-innervation.

5.3 Future Directions of the Present Research

Based on the findings from this research, four main areas of interest are proposed for future work:

(1) Further research is needed to determine a comprehensive pathway from initial denervation of a myofiber to myofiber atrophy. In this work we have demonstrated that MuRF1 protein expression is elevated in a denervation specific manner in sarcopenic muscle. Our lab has also examined the involvement of MAFbx in age-associated myofiber atrophy (data in review). However, we believe that MuRF1 and MAFbx

represent only the last step in a complex pathway of cellular regulators and interactions from denervation to myofiber atrophy (figure 5.1). Furthermore, the proteasomal system may only be accountable for approximately 50% of the atrophy which occurs following denervation (Bodine *et al.* 2001). As such, significant research remains to be done before a clear understanding of how denervation causes myofiber atrophy can be reached. In one capacity, this research will include examining the role of lysosomal autophagy in sarcopenic muscle atrophy and probing for upstream regulators of both the proteasomal system and the autophagy system. In another capacity, it will include exploration of what the alterations in myosin heavy chain expression associated with age and denervation represent in terms of myofiber function and in terms of how myofibers respond to adverse conditions. The different atrophy characteristics of pure MHCs myofibers and of MHC co-expressing myofibers which were formerly pure MHCs myofibers indicate that myofiber atrophy is intimately tied to myosin heavy chain expression. More importantly, these characteristics indicate that the atrophy process is extremely dynamic. Exploring what processes allow for pure MHCs myofibers to become MHC co-expressing myofibers in sarcopenia will not only contribute to the research community's general knowledge of myosin heavy chain expression, but will likely provide complementary insight into the mechanisms which govern myofiber atrophy. If it is the case that denervation is the cause of sarcopenic muscle atrophy, then this research will provide significant insight not only into the mechanisms of sarcopenia, but into the mechanisms of all atrophy scenarios where denervation is implicated.

(2) Future work should examine the possible causes of denervation in sarcopenic muscle. As outlined in the previous section of this discussion, if denervation is the

primary instigator of myofiber atrophy in sarcopenic muscle, then preventing it from occurring or facilitating re-innervation represent the only methods of sarcopenia treatment which will be ultimately effective. Determining the cause, or perhaps causes, of age-associated denervation is the first step toward developing these treatments.

(3) Research should be undertaken to corroborate our findings, observed in an animal model of sarcopenia, with the progression of sarcopenia in humans. This study used the Fischer 344 X Brown Norway F1 rat model of sarcopenia. In addition to having a relatively short lifespan allowing for timely and less costly acquisition of results, the use of a rat model of sarcopenia allowed us to use more invasive techniques in order to gain a thorough understanding of the mechanisms occurring in sarcopenia. While the Fischer 344 X Brown Norway F1 rat model is considered the most synonymous rodent model of sarcopenia to that of human sarcopenia (Rice *et al.* 2005), it is possible that human sarcopenia does not occur via the same mechanisms as rodent sarcopenia (Foletta *et al.* 2011). Indirect evidence from the muscle morphology of sarcopenic human muscle (Scelsi *et al.* 1980; Andersen 2003) suggests that human sarcopenia is very similar to that of the rodent model we have used and is consistent with denervation being the primary cause of sarcopenic myofiber atrophy. Now that we have some preliminary data in the rat model, we can begin to test these findings in human muscle tissue samples.

(4) Future studies need to determine whether there is a sex effect in the role which denervation plays in sarcopenia. The prevalence and impact of sarcopenia is greater in females than males (Janssen *et al.* 2002). While this may be due to inherent differences between males and females in the mechanisms which cause sarcopenia, there are many confounding variables related to social differences between the sexes which could also

account for the higher prevalence and impact observed in females. Very little sarcopenia research has been preformed using females and what data is available is difficult to assign meaning to. Some studies have used both males and females, but made no distinction between the two groups (Scelsi *et al.* 1980), while others have used only females (Edstrom *et al.* 2006), making their findings difficult to interpret within the greater body of sarcopenia literature. However, one study which did compare the impact of sarcopenia on whole muscle strength and individual myofiber strength in males versus females found that, while there were inherent differences between males and females, these differences were not exacerbated by age, indicating that the mechanisms of sarcopenia are the same in males and females (Frontera *et al.* 2000b).

5.4 Conclusions

Combined, the findings of this thesis provide further evidence that the focus of sarcopenia research may require a fundamental shift from mechanisms which aim to treat muscle atrophy once it has occurred to mechanisms which aim to treat or prevent myofiber denervation. In reaching this conclusion, we have identified and addressed significant discrepancies in the methodologies used to determine MuRF1 expression in sarcopenic muscle and those used to examine myosin heavy chain expression characteristics in sarcopenic muscle. As a result, we have provided much needed clarity and novel insights into the mechanisms involved in sarcopenia. We have shown that MuRF1 protein expression is elevated with age in a denervation specific manner. We have also identified that formerly pure MHCs myofibers undergo significant atrophy following the expression of MHCf, suggesting that there is an inextricable relationship

between MHC expression and myofiber atrophy. Although our findings represent only the initial stages of a long research process, we hope that they will contribute to the future development of effective treatments for sarcopenia.

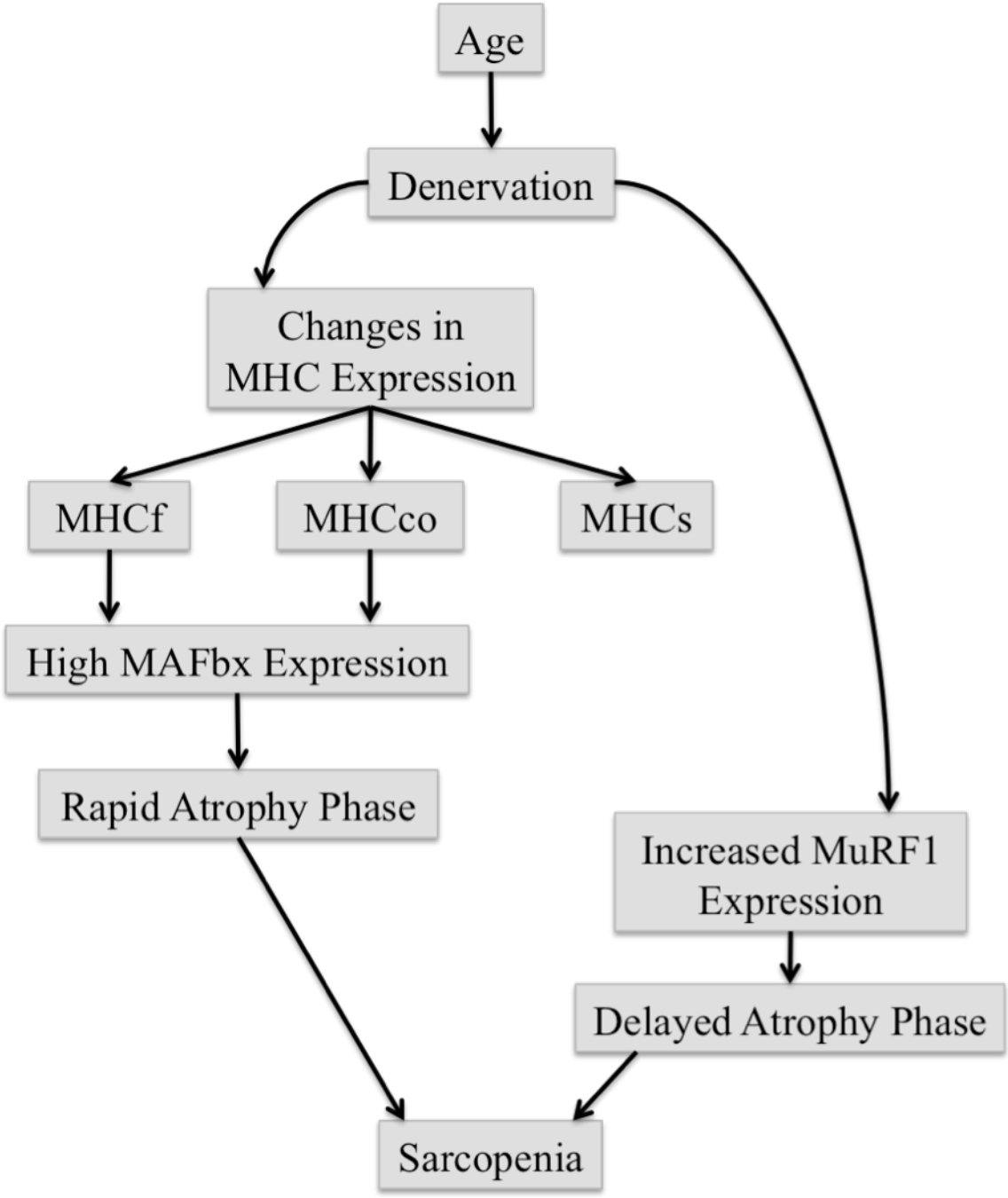

Figure 5.1 Schematic showing our proposed progression of sarcopenia.

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APPENDIX A: ABNORMAL SOL SAMPLE

Following imaging of immunolabelled sections for Chapter 4, it was discovered that one YA soleus sample contained myofiber abnormalities know as “whorled fibers”. These myofibers are characteristic of muscle regeneration and indicate that the muscle has experienced some sort of trauma (personal communication from Erin O-Farrell, clinical neurologist, McGill University). As such, this sample was removed from the data set. Due to the relatively rare occurrence of such “whorled” myofibers, the raw data for myofiber CSA of this abnormal sample is contained in Tables A.1, A.2 and A.3 and the images in Figures A.1, A.2, and A.3.

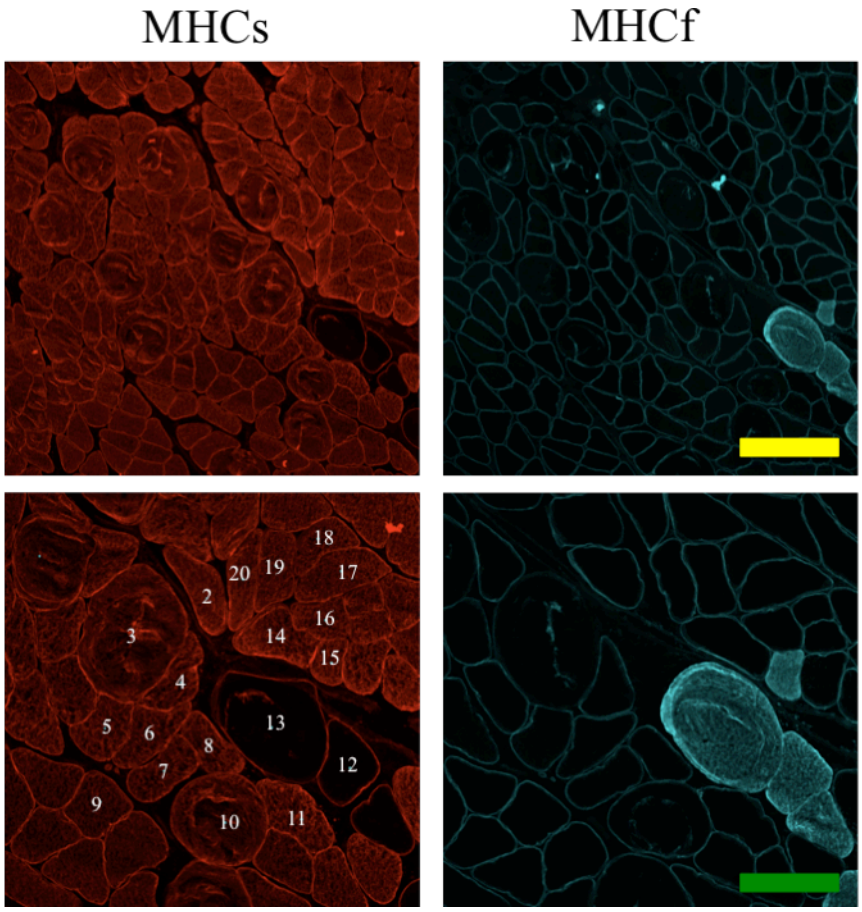

Figure A.1 SOL sample 1008 image 1. Images on the top are serial images taken at 100x magnification, the yellow scale bar = 300μm. Images on the bottom are serial images taken at 200x magnification, the green scale bar = 150μm. The numbers correspond to the myofiber numbers in table A.1. Myofibers 3, 10 and 13 are examples of "whorled fibers".

Myofiber Number	Myofiber CSA (μm^2)
1	N/A
2	6918.047
3	17213.817
4	5846.436
5	6113.017
6	6969.749
7	6513.37
8	4502.093
9	6684.62
10	14186.282
11	7098.811
12	7590.651
13	26318.043
14	7377.693
15	3332.94
16	4514.97
17	8033.095
18	4758.584
19	5695.27
20	5485.676

Table A.1 SOL sample1008 image 1 myofiber CSA. Myofiber number 1 was used as a reference point and was not included in the CSA analysis.

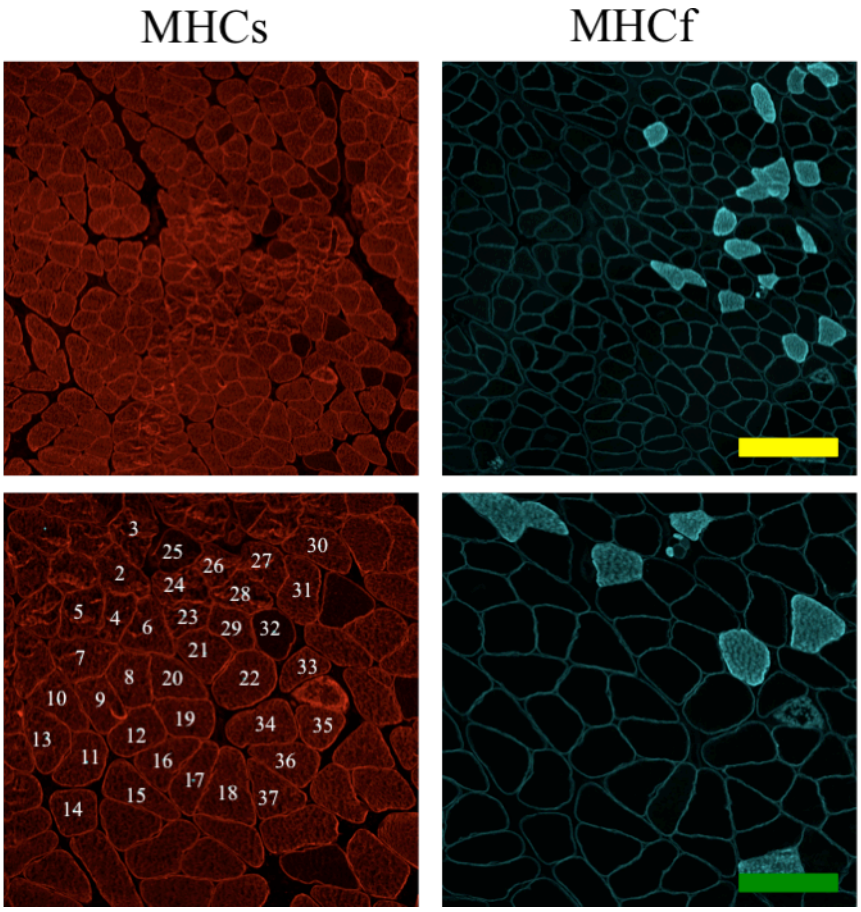

Figure A.2 SOL sample 1008 image 2. Images on the top are serial images taken at 100x magnification, the yellow scale bar = 300μm. Images on the bottom are serial images taken at 200x magnification, the green scale bar = 150μm. The numbers corresponds to the myofiber numbers in table A.2.

Myofiber Number	Myofiber CSA (μm^2)	Myofiber Number	Myofiber CSA (μm^2)
1	N/A	21	5396.592
2	5107.523	22	7326.376
3	4665.271	23	3738.194
4	4300.187	24	5145.002
5	5735.632	25	3834.678
6	6009.037	26	3878.019
7	6449.655	27	4456.349
8	5586.197	28	4890.241
9	5179.79	29	4132.012
10	5579.47	30	5353.539
11	5702.862	31	5325.574
12	5635.496	32	4639.131
13	6161.548	33	3312.951
14	4874.769	34	5814.627
15	5747.357	35	3467.672
16	5441.951	36	4464.422
17	4758.68	37	4106.545
18	6322.323		
19	5398.225		
20	5262.917		

Table A.2 SOL sample 1008 image 2 myofiber CSA. Myofiber number 1 was used as a reference point and was not included in the CSA analysis.

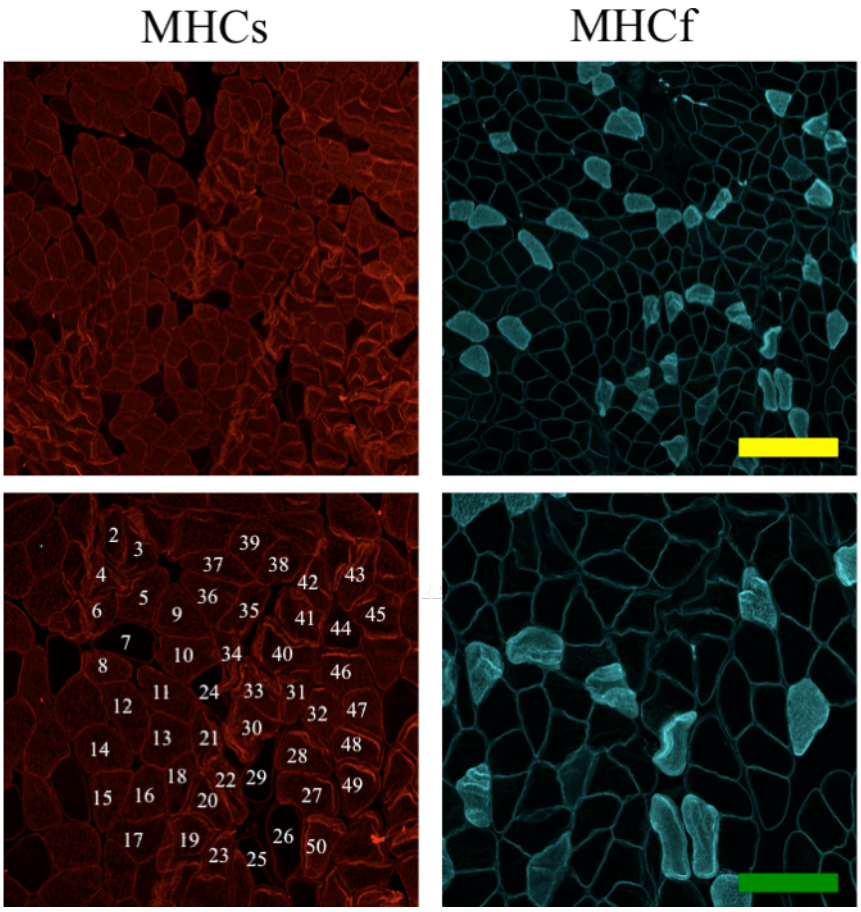

Figure A.3. SOL sample 1008 image 3. Images on the top are serial images taken at 100x magnification, the yellow scale bar = 300μm. Images on the bottom are serial images taken at 200x magnification, the green scale bar = 150μm. The numbers corresponds to the myofiber numbers in table A.3.

Myofiber Number	Myofiber CSA (μm^2)	Myofiber Number	Myofiber CSA (μm^2)
1	N/A	26	5277.139
2	3124.98	27	5103.391
3	4622.026	28	5338.259
4	2680.037	29	3829.777
5	4123.651	30	4062.243
6	3321.985	31	4149.502
7	3875.233	32	5139.524
8	2523.778	33	3928.952
9	4123.171	34	3777.499
10	3745.978	35	3769.619
11	3826.99	36	3511.494
12	3787.205	37	4034.47
13	4065.222	38	4405.224
14	4070.892	39	3493.908
15	3386.276	40	3622.297
16	4258.672	41	3391.081
17	5268.202	42	2634.966
18	2906.929	43	3989.88
19	4299.706	44	3464.117
20	3996.895	45	5220.825
21	4186.693	46	5553.907
22	3685.531	47	4101.644
23	3389.447	48	4499.979
24	3982.768	49	4435.592
25	5169.7	50	4841.326

Table A.3. SOL sample 1008 image 3 myofiber CSA. Myofiber number 1 was used as a reference point and was not included in the CSA analysis.