UNIVERSITY OF CALIFORNIA, SAN DIEGO

Skeletal Muscle Adaptation in a Model of Massive Rotator Cuff Tear

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Bioengineering

by

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The Dissertation of Eugene Julius Sato is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To Mom and Dad,

Thank you for all the love and support you have given me throughout my life's pursuits.

To my grandparents,

For being my biggest cheerleaders from half a world away.

To Narangika,

For being my best friend,

For your unconditional love and support,

I love you!

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LIST OF ABBREVIATIONS

BTX	Botulinum toxin A
ECM	Extracellular matrix
IS	Infraspinatus
MHC	Myosin heavy chain
MW	Molecular weight
PCSA	Physiological cross-sectional area
SS	Supraspinatus
TT	Tenotomy

LIST OF TABLES AND FIGURES

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Sato EJ, Killian ML, Choi AJ, Lin E, Choo AD, Rodriguez-Soto AE, Lim CT, Thomopoulos S, Galatz LM, Ward SR. Architectural and Biochemical Adaptations in Skeletal Muscle and Bone Following Rotator Cuff Injury in a Rat Model. J Bone Joint Surg. 2015 Apr: 1;97(7):565-73. doi: 10.2106

Meyer GA, Farris AL, **Sato E**, Lane JG, Ward SR, Enger AJ. Muscle Progenitor Cell Regenerative Capacity in the Torn Rotator Cuff. J Orthop Res. 2014 Nov 20. doi: 10.1002

Choo A, McCarthy M, Pichika R, **Sato E**, Lieber R, Schenk S, Lane J, Ward S. Muscle Gene Expression Patterns in Human Rotator Cuff Pathology. J Bone Joint Surg Am. 2014 Sep 17;96(18):1558-65. doi: 10.2106

Sato EJ, Killian ML, Choi AJ, Lin E, Esparza MC, Galatz LM, Thomopoulos S, Ward SR. Skeletal Muscle Fibrosis and Stiffness Increase after Rotator Cuff Tendon Injury and

Neuromuscular Compromise in a Rat Model. J Orthop Res. 2014 Sep;32(9):1111-6. doi: 10.1002

Swan MA, **Sato E**, Galatz L, Thomopoulos S, Ward SR. **The Effect of Age on Rat Rotator Cuff Muscle Architecture**. J Shoulder Elbow Surg. 2014 Dec;23(12):1786-91. doi: 10.1016.

Li Z, Haynes R, **Sato E**, Shields MS, Fujita Y, Sato C. **Microbial community analysis of a single chamber microbial fuel cell using potato wastewater.** Water Environ Res. 2014 Apr;86(4):324-30 PMID:24851328

Killian ML, Cavinatto L, Shah SA, **Sato E**, Ward SR, Havlioglu N, Galatz LM, Thomopoulos S. **The Effects of Repair-Site Gapping and Chronic Unloading on Tendon-to-Bone Healing in a Rat Model of Massive Rotator Cuff Tears.** J Orthop Res. 2014 Mar;32(3):439-47. doi: 10.1002.

ABSTRACT OF THE DISSERTATION

Skeletal Muscle Adaptation in a Model of Massive Rotator Cuff Tear

by

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Doctor of Philosophy in Bioengineering

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Professor Samuel Ward, Chair

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Rotator cuff (RC) tears are a degenerative condition that affects ~30% of people over the age of 60, resulting in pain and loss of function in the shoulder. Chronically torn muscles are prone to atrophy, fatty infiltration, and an increase in tissue stiffness. These changes are insensitive to rehabilitation and are associated with poor patient and surgical outcomes.

Although human clinical studies of RC tears are common, they are limited by a lack of experimental control and logistical difficulties. Thus, animal models have been utilized to study this injury. However, data describing the muscle architecture and mechanics in these models are limited. Therefore, in chapter 2, we

characterized muscle architectural adaptations in a rat model of RC injury. We discovered that tenotomy alone leads to mild changes in muscle architecture, while adding a secondary chemical nerve injury causes extensive muscle changes.

Various studies have demonstrated increased passive stiffness of the whole muscle after RC tear, but the mechanisms causing this phenomenon were unknown. Therefore, in Chapter 3, we measured stiffness at the individual fiber and fiber bundle levels. Fiber bundles stiffness increased after a combined tendon and nerve injury, but was unchanged after tenotomy alone. These results suggest that stiffness changes are due to adaptations in the extracellular matrix.

Recent studies have reported functional deficits in tenotomized RC muscles that are not predicted by architectural adaptations. A potential explanation for this could be that muscle strain to sarcomere strain transmission is altered after injury. In chapter 4, we investigated this discrepancy and found that strain transmission was not altered due to tenotomy at low strains. However, sarcomere lengths were significantly shorter in tenotomized muscles at high strains. These results fail to predict the presence of functional deficits in tenomized muscles and do not reconcile the reported deficits.

Overall, these data suggest that this model of RC injury exhibits mild changes that are not representative of the human injury. Future research may need to pursue alternative models (aged animals, alternative species, etc.) of RC tear that result in muscle adaptations that are comparable to what is observed in human patients.

CHAPTER 1 : INTRODUCTION

The human shoulder is the most mobile joint in the human body[1] allowing for a large range of motion, while still providing the necessary strength for performing tasks of daily living[2, 3]. In simplistic terms, the glenohumeral joint is classified as a ball-and-socket joint, however the amount of surface area contact is minimal and the joint is more analogous to a golf ball resting on a shallow golf tee[4]. The minimal bony surface area in contact at the joint allows the shoulder to have a large range of motion, but also makes the joint anatomically unstable[5]. In order to stabilize the joint, the shoulder is supported by soft tissue structures, namely the joint capsule and the muscles of the rotator cuff, which are susceptible to soft tissue injury.

1.1 Rotator Cuff Tears

The rotator cuff is a set of four muscles, the supraspinatus, infraspinatus, teres minor, and subscapularis, which all originate on the scapula and whose distal tendons encapsulate the humeral head (Figure 1). Together, the rotator cuff muscles function to provide dynamic stability to the shoulder by actively compressing the head of the humerus into the glenoid of the scapula[6]. as well as assisting in shoulder motion[1].

Rotator cuff tears are injuries to one or more of the tendons of the rotator cuff, and can be a result of acute trauma or develop over time. Traumatic insults to the shoulder, such as a shoulder dislocation[7], have been linked to tearing of the rotator cuff. However, more commonly impingement and abrasion of the rotator

cuff tendons against bony elements in the shoulder[8] lead to a torn cuff over time. In these cases, it is hypothesized that the supraspinatus tendon, which lies inferior to the acromion and superior to the glenoid, comes into physical contact with the acromion[9], or glenoid[10], during shoulder motion. The repeated mechanical abrasion of the tendon is thought to lead to the tendon damage and eventual tearing in both young overhead athletes[11-13], or in the aging general population[14, 15].

Clinically, RC tears are the most common shoulder injury and are particularly common within the elderly population—over 30% of people over 60 years of age reportedly have a torn RC in at least one shoulder[14, 15]. RC tears are highly correlated with age and tend to have an insidious onset, as many patients have asymptomatic tears that progress to showing symptoms later in the disease process[14-18]. Patient symptoms primarily include pain, and loss of strength and range of motion in the affected shoulder.

Conservative treatment, including use of anti-inflammatories, rest, and physical therapy, can be used to initially treat rotator cuff injuries[19, 20], but the success rates have been highly variable, ranging between 31-80%[21-23]. Several factors have been suggested as predictors of treatment failure, including reduced starting shoulder strength, range of motion, and muscle atrophy[24, 25]. However, continued investigation is required to better predict which patients will respond to conservative treatment. If conservative treatment fails to treat a patient's symptoms, they may elect for surgical treatment.

Every year, 250,000 rotator cuff repair surgeries are performed in the United States[26], with the number of procedures performed increasing in recent years[27]. Rotator cuff repair surgeries involve pulling the retracted muscle-tendon unit back onto the greater tuberosity of the humerus, and suturing the tendon back onto the bone. This procedure attempts to restore the original muscle length and mechanical load to the muscle thus restoring function; but follow-up in patients studies have reported high repair failure rates (20%-90% depending tear severity) and poor clinical outcomes[28-30].

1.2 Muscle Architecture

Skeletal muscle architecture, or the study of the arrangement of muscle fibers in relation to the force-generating axis of the muscle[31, 32], can be used to predict muscle function[32-34]. For example, the architectural parameter physiological cross sectional area (PCSA) is a calculated estimate of whole muscle fiber area arranged in parallel. PCSA has been previously shown to be the best predictor of muscle force producing capacity[34]. Similarly, normalized fiber length, analogous to the number of sarcomeres arranged in series in a muscle, provides the best estimate of muscle excursion and velocity[33, 35]. Finally, the sarcomere lengths within the muscles are important in understanding muscle force production, as muscles are length sensitive[36]. Therefore, sarcomere length can be used to predict the amount of isometric force that can be produced by the muscle throughout its range of lengths (Figure 2)[36].

Architectural evaluations of the rotator cuff muscles have shown that the muscles tend to have short fibers and large physiological cross-sectional areas, which support the idea that these muscles functional as stabilizing muscles[37]. In addition, it was found that the supraspinatus and infraspinatus muscles had relatively long sarcomere lengths (3.2 μm vs. 2.7 μm optimal length in humans) in the anatomically neutral shoulder position, which suggest that these muscles are under relatively high passive tension in this position compared to other muscles and may be particularly sensitive to stretch[37].

1.3 Pathological Changes in the Torn Rotator Cuff

Non-invasive imaging techniques (mainly, magnetic resonance imaging and computer tomography) have been used to study the health of the rotator cuff muscles in patients. These studies have noted decreased muscle cross sectional area[38], a sign of muscle atrophy, and the presence of "fatty degeneration"[39] in muscles after a rotator cuff tear. While many studies assessing muscle atrophy and "fatty degeneration" utilize qualitative scoring schemes, one study compared the cross sectional area of muscles from torn shoulders with the healthy contralateral shoulder in patients and showed a nearly 40% deficit in muscle cross sectional area.[40] Similarly, severe cases of fatty degeneration may result in 50% or more of the muscle's cross sectional area replaced by fatty tissue[39]. More recent studies have highlighted increasing age[41, 42] and tear severity[42-44] as predictive factors leading to muscle atrophy and "fatty degeneration". Furthermore, pre-

operative muscle atrophy and “fatty degeneration” have been correlated with increased re-tear rates[45, 46] and poor functional outcomes.[38, 47]

Studies in human cadavers suggest high tensions at the repair site lead to tendon re-tears,[48, 49] with failures tending to occur at both the suture-tendon interface and in the suture failure itself.[50, 51] Architectural studies in human cadavers have measured shortened muscle fibers in torn muscles[52, 53] which could potentially lead to high tensions when the muscle is strained during repair. This hypothesis was further supported by a seminal study by Hersche and Gerber[54], which directly measured muscle stiffness in patients undergoing rotator cuff repair, and showed that patients with torn rotator cuffs had stiffer muscles than untorn cuffs.

However, repair integrity alone does not necessarily correlate with positive patient outcomes.[29, 55] Longitudinal studies of patients who have undergone rotator cuff repairs have shown that many of the chronic changes measured preoperatively, such as muscle atrophy[40] and “fatty degeneration” do not improve after repair[46, 56] In addition, poorly understood aspects of rotator cuff injuries, such as the possible involvement of a suprascapular nerve injury in a subset of patients,[57-59] may contribute to poor outcomes. However, the degree to which this injury is present in patients and how it influences muscle health is still controversial.[57, 60-62] Together, these studies highlight the need to better understand the biological mechanisms leading to irreversible muscle changes such

that novel therapies can be proposed to prevent or reverse these muscle adaptations and improve patient outcomes.

1.4 Animal Models of Rotator Cuff Tear

Numerous clinical studies have attempted to investigate RC injuries in terms of patient outcomes. However, several limitations exist when investigating biological or physiological changes to the muscle in human patients due to limited experimental controls over the injury period and the logistical difficulties related to conducting human studies. In addition, patient studies have many variables that are difficult to control, such as the magnitude or chronicity of the injury. In order to address these limitations animal models have been utilized to study rotator cuff injuries due to their convenience and the experimental control allowed by these models. A large animal sheep model has been used to recapitulate many aspects of the human injury, including decreased function, muscle atrophy, increase in connective tissue and fatty infiltration that does not resolve after RC repair[63-65]. Unfortunately, these models are expensive and are not easily accessible to many researchers.

In order to overcome these limitations, small animals models using rats were introduced by Soswlowsky, et al after demonstrating the anatomical similarity of the rat rotator cuff to the human rotator cuff. The authors compared 34 different metrics relating to musculature, bony anatomy, articulations, and motions. Importantly, particular attention was paid to the location of the supraspinatus tendon with respect to the acromion[66]. A later study comparing the muscle

architecture of the rotator cuff of 10 commonly studied species found that after primates, rats were most similar to humans based on a comparison of fiber length-to-muscle length ratio, fiber length-to-moment arm ratio, and the fractional distribution of physiological cross-sectional area of the rotator cuff muscles[67].

Rotator cuff tear studies in rat models emulate tendon injury by surgical transection of the supraspinatus, and infraspinatus tendons. Initial studies using this model highlighted altered tendon mechanical properties, increased collagen disorganization, and increased cellularity after tenotomy[68-70]. Similar studies investigating tendon repair-site properties have shown poor tissue integrity, including disorganized histology and impaired mechanical properties compared to the uninjured tendon-bone insertion site[71]. Additionally, mechanical studies of the muscle-tendon unit simulating rotator cuff repairs measured increased repair tensions after chronic tenotomy that inhibited tendon healing[72, 73]. Furthermore, these impairments are exacerbated when the repair is delayed[74].

In contrast, fewer studies have focused on the changes in the rotator cuff muscles. Early studies measured muscle atrophy (~15% decrease in muscle mass, 10% decrease in PCSA, 66% decrease in muscle fiber area) in the first few weeks after injury, but these studies also reported signs of recovery by 8 weeks and almost complete recovery to normal values at 16 weeks[75, 76]. Although these studies suggest muscle changes occur soon after injury, rotator cuff tears in humans tend to be chronic (on the order of years) and thus highlight the need to study this model at long time-points (months) relative to the animal's lifespan. Additionally, further

study is still required to fully characterize the chronic effects of tenotomy in this animal model.

More recent studies have incorporated variations of the RC injury models using tenotomy, suprascapular nerve injury (physical or chemical denervation), or a combination of the two to induce a more severe rotator cuff muscle injury. In general, these studies have shown much greater atrophy as a result of nerve injury. Studies using denervation injuries typically show greater than 50% reduction in muscle mass, compared to roughly 20% or smaller changes in mass due to tendon injury alone[77-79]. Histological evaluations of the combined tenotomy and denervation models has shown qualitative increases in muscle fibrosis[80], and mild fatty infiltration[81, 82]. However, these studies have suffered from poor or unknown stereological methods, calling into question the validity of extrapolating these findings to the entire muscle.

Multiple efforts to study the biological mechanisms underlying muscle atrophy, fibrosis, and fatty infiltrations have examined gene and protein expression of several major cellular pathways associated with these processes. Liu et al[77] measured reduced activity in a key pathway for muscle hypertrophy after tenotomy. Interestingly, they also measured increased activity in the same hypertrophy pathway but also increased expression of in the muscle atrophy pathway after denervation alone[77]. A separate study using a combined tenotomy and denervation model measured increased hypertrophy signaling in these muscles[83]. These studies suggested that the muscle response to injury was highly dependent on

the mechanism of injury. In a different study, Killian et al also measured an injury-dependent response to rotator cuff injury, as upregulation of myogenic and adipogenic pathway genes were observed after chemically denervation of RC muscles, but not after tendon injury alone[79]. Therefore, the specific protein degradation/atrophy mechanisms that are active in these muscles also appear to be injury-dependent as well. Increased activity in the autophagy pathway has been measured in tenotomized muscles, while increased activity in the ubiquitin-proteasome pathway was observed after denervation[78]. These studies suggest that the different injury mechanisms lead to slightly different tissue-level responses, but through distinct and separate biological responses in the muscle.

In addition to muscle-specific pathways, upregulation of genes related to tissue fibrosis have been measured after tenotomy[79], while similar increases in protein expression have been measured in a study of the combined injury (tenotomy and denervation) models[80]. However, these studies were limited, as they did not concurrently measure tissue-level fibrosis, such as increases in collagen content or extracellular matrix, within the muscles.

Although these studies have begun to investigate the gene and protein expression changes within the muscle, there is still limited knowledge on how the measured changes manifest as structural or functional adaptations at the whole muscle level. Understanding the muscle-wide structural adaptions would help contextualize the gene and protein expression patterns from these studies. Data

presented in Chapter 2 of this dissertation will characterize the whole muscle architectural changes in the rat model of rotator cuff injury.

As previously mentioned, evidence suggests that increased passive stiffness of the whole muscle following tenotomy injury contributes to repair failure. Recently, a study analyzing human biopsies concluded that increased passive tension is due to changes in the connective tissue, or extracellular matrix, that binds muscle bundles together, and not due to changes in muscle fiber stiffness[84]. While small animals studies have also shown increased whole muscle stiffness[72, 85] akin to human studies, no well controlled studies have been conducted to determine the source of passive stiffness in these muscles. An increased understanding of which tissue components change mechanical properties after injury could allow for targeted therapies to reverse muscle stiffness changes and improve repair outcomes. Chapter 3 of this dissertation will investigate the passive mechanical properties of muscle fibers and muscle fiber bundles in the chronic tenotomy model.

A few recent studies have evaluated muscle function after rotator cuff tears. These studies measured decreased isometric force[86] and a narrowing of the whole muscle length tension curve (decreased excursion)[85], which suggest impaired muscle function. However, the mild architectural changes measured in Chapter 2 of this dissertation are not predictive of these functional changes. Due to the relationship between sarcomere length and force production described previously[36], the deficit in muscle force production in these experiments could be explained by differences in sarcomere lengths between normal and tenotomized

muscles during functional testing. A fundamental problem in these studies is that sarcomere length is not measured directly, but predicted based on muscle length. However, the relationship between muscle strain and sarcomere length in these muscles (healthy and tenotomized) is unknown. Therefore, we hypothesized that one possible explanation for this discrepancy could be due to differences in sarcomere lengths as the muscle is stretched during these experiments. Chapter 4 of this dissertation will investigate the hierarchical strain distribution from the muscle to the sarcomere in the rotator cuff tenotomy model.

In summary, the rat model has become an increasingly popular model to study rotator cuff injury. Recent studies have begun to assess muscle mechanics and to characterize the biological mechanisms of atrophy, fibrosis, and fatty infiltration after injury. However, the effect of tendon injury on rat muscle architecture and passive stiffness and how these changes compare to the human rotator cuff injury process, remains unknown. For example, increased whole muscle stiffness has been found in both rat model and humans after injury. However, the presence of these changes at the muscle bundle level and the tissue adaptations (fibrosis) that lead to increased stiffness are unknown. Additionally, in several previous studies the suprascapular nerve has been injured as means to provoke muscle changes/damage. Furthermore, the magnitude of this injury relative to tenotomy alone in the rat model has not been directly compared. Finally, the relationship between muscle strain and sarcomere length in these muscles, which is important for interpreting muscle function, has yet to be defined. Further research into each of these areas will

improve our understanding of the injury model, and may suggest novel strategies for reversing muscle pathology in the rotator cuff.

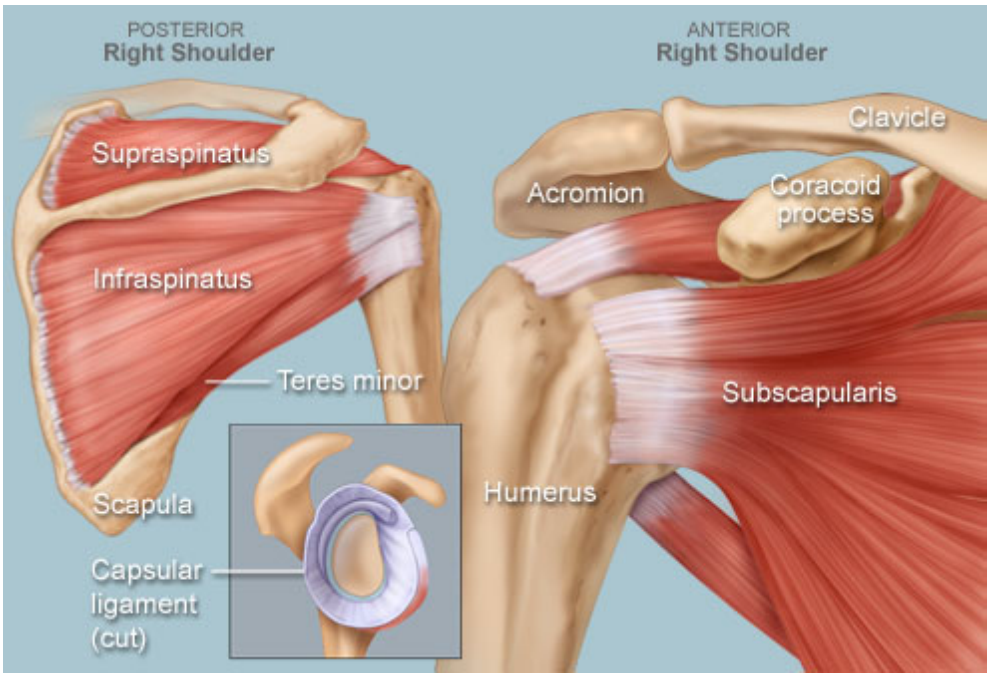

Figure 1.1 [87] The Rotator Cuff

Anterior and posterior views of the scapula and the rotator cuff muscles and tendons. The rotator cuff is made up of four muscles: supraspinatus, infraspinatus, subscapularis, and teres minor. Together these muscles provide dynamic stability to the glenohumeral joint.

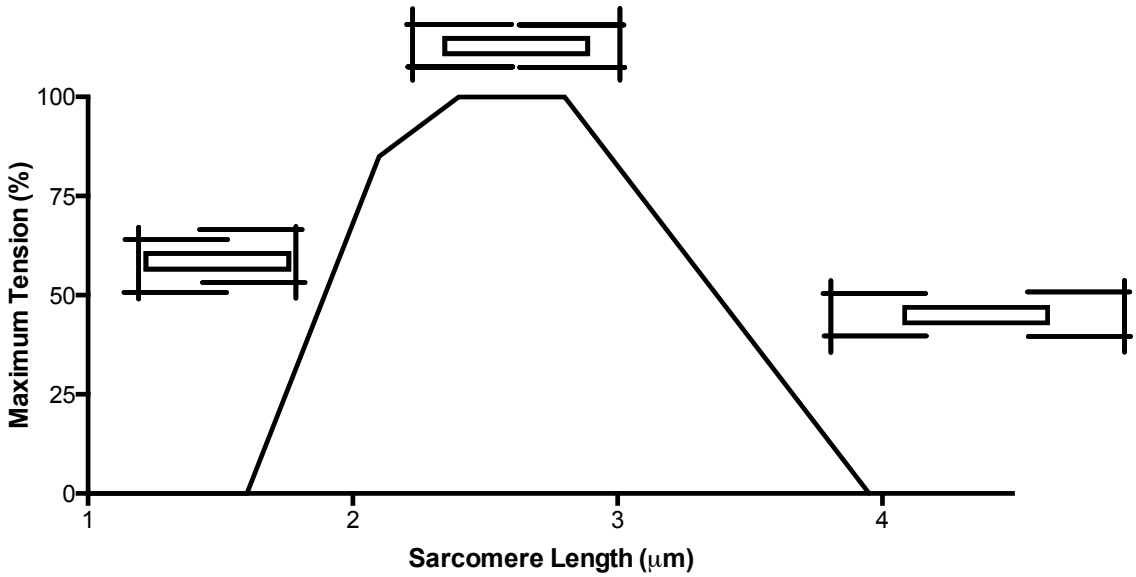

Figure 1.2 Example sarcomere length-tension curve (Rat).
Sarcomeres are the smallest functional unit of muscle. Force is generated by the interaction of the thick (rectangle) and thin (horizontal lines) protein filaments, which make up the sarcomere. The length of the sarcomere corresponds to the amount of overlap of the filaments and thus the amount of force generated by the sarcomere.[86] In the schematic above, maximum overlap of the filaments, and thus maximum tension, occurs at the plateau between 2.40 and 2.80 μm. As the sarcomere is stretched, filament overlap decreases and as a result, the tension decreases. Similarly, as sarcomeres shorten, force is reduced due to physical interactions of the filaments resisting shortening. Note that the resting sarcomere length of the supraspinatus muscle in humans (3.2μm) lies on the descending limb of the curve.

1.5 References

1. Hoppenfeld S, deBoer, P., Buckley, R.: **The Shoulder**. In: *Surgical Exposures in Orthopaedics: The Anatomic Approach.* 4 edn: Lippincott Williams & Wilkins; 2009.

2. Magermans DJ, Chadwick EKJ, Veeger HEJ, van der Helm FCT: **Requirements for upper extremity motions during activities of daily living**. *Clinical Biomechanics* 2005, **20**(6):591-599.

3. Veeger HEJ, van der Helm FCT: **Shoulder function: The perfect compromise between mobility and stability**. *Journal of Biomechanics* 2007, **40**(10):2119-2129.

4. Williams GR, Iannotti, J.P.: **Biomechanics of the Glenohumeral Joint: Influence on Shoulder Arthroplasty**. In: *Disorders of the Shoulder: Diagnosis & Management, Volume 1.* Edited by Iannotti JP, Williams, G. R.: Lippincott Williams & Wilkins; 2007.

5. Soslowsky LJ, Flatow EL, Bigliani LU, Mow VC: **Articular Geometry of the Glenohumeral Joint**. *Clinical Orthopaedics and Related Research* 1992, **285**:181-190.

6. Lippitt SB, Vanderhooft JE, Harris SL, Sidles JA, Harryman Ii DT, Matsen Iii FA: **Glenohumeral stability from concavity-compression: A quantitative analysis**. *Journal of Shoulder and Elbow Surgery* 1993, **2**(1):27-35.

7. Neviaser RJ, Neviaser TJ, Neviaser JS: **Concurrent rupture of the rotator cuff and anterior dislocation of the shoulder in the older patient**. *J Bone Joint Surg Am* 1988, **70**(9):1308-1311.

8. Neer CS: **Anterior Acromioplasty for the Chronic Impingement Syndrome in the Shoulder**. *A PRELIMINARY REPORT* 1972, **54**(1):41-50.

9. Burns WC, 2nd, Whipple TL: **Anatomic relationships in the shoulder impingement syndrome**. *Clin Orthop Relat Res* 1993(294):96-102.

10. Davidson PA, Elattrache NS, Jobe CM, Jobe FW: **Rotator cuff and posterior-superior glenoid labrum injury associated with increased glenohumeral motion: a new site of impingement**. *J Shoulder Elbow Surg* 1995, **4**(5):384-390.

11. Glaser DL, Sher, J. S., Ricchetti, E. T., Williams, G. R., Soslowsky, L. J.: **Anatomy, Biomechanics, and Pathophysiology of Rotator Cuff Disease**. In: *Disorders of the Shoulder: Diagnosis & Management, Volume 1.* Edited by Iannotti JP, Williams, G. R., vol. 1: Lippincott Williams & Wilkins; 2007.

12. Jobe FW, Pink M: **Classification and treatment of shoulder dysfunction in the overhead athlete**. *J Orthop Sports Phys Ther* 1993, **18**(2):427-432.

13. Jobe FW, Kvitne RS, Giangarra CE: **Shoulder pain in the overhand or throwing athlete. The relationship of anterior instability and rotator cuff impingement**. *Orthopaedic review* 1989, **18**(9):963-975.

14. Tempelhof S, Rupp S, Seil R: **Age-related prevalence of rotator cuff tears in asymptomatic shoulders**. *J Shoulder Elbow Surg* 1999, **8**(4):296-299.

15. Yamaguchi K, Ditsios K, Middleton WD, Hildebolt CF, Galatz LM, Teefey SA: **The demographic and morphological features of rotator cuff disease. A comparison of asymptomatic and symptomatic shoulders**. *J Bone Joint Surg Am* 2006, **88**(8):1699-1704.

16. Yamamoto A, Takagishi K, Osawa T, Yanagawa T, Nakajima D, Shitara H, Kobayashi T: **Prevalence and risk factors of a rotator cuff tear in the general population**. *J Shoulder Elbow Surg* 2010, **19**(1):116-120.

17. Mall NA, Kim HM, Keener JD, Steger-May K, Teefey SA, Middleton WD, Stobbs G, Yamaguchi K: **Symptomatic Progression of Asymptomatic Rotator Cuff Tears**, vol. 92; 2010.

18. Moosmayer S, Tariq R, Stiris M, Smith H-J: **The Natural History of Asymptomatic Rotator Cuff Tears**, vol. 95; 2013.

19. Itoi E: **Rotator cuff tear: physical examination and conservative treatment**. *Journal of Orthopaedic Science* 2013, **18**(2):197-204.

20. Kuhn JE, Dunn WR, Sanders R, An Q, Baumgarten KM, Bishop JY, Brophy RH, Carey JL, Holloway BG, Jones GL *et al*: **Effectiveness of physical therapy in treating atraumatic full-thickness rotator cuff tears: a multicenter prospective cohort study**. *Journal of Shoulder and Elbow Surgery* 2013, **22**(10):1371-1379.

21. Merolla G, Paladini P, Saporito M, Porcellini G: **Conservative management of rotator cuff tears: literature review and proposal for a prognostic. Prediction Score**. *Muscles, Ligaments and Tendons Journal* 2011, **1**(1):12-19.

22. Itoi E, Tabata S: **Conservative treatment of rotator cuff tears**. *Clin Orthop Relat Res* 1992(275):165-173.

23. Wolfgang GL: **Surgical Repair of Tears of the Rotator Cuff of the Shoulder**. *FACTORS INFLUENCING THE RESULT* 1974, **56**(1):14-26.

24. Harris JD, Pedroza A, Jones GL, The MSG: **Predictors of Pain and Function in Patients With Symptomatic, Atraumatic Full-Thickness Rotator Cuff Tears: A Time-Zero Analysis of a Prospective Patient Cohort Enrolled in a Structured Physical Therapy Program**. *Am J Sports Med* 2012, **40**(2):359-366.

25. Tanaka M, Itoi E, Sato K, Hamada J, Hitachi S, Tojo Y, Honda M, Tabata S: **Factors related to successful outcome of conservative treatment for rotator cuff tears**. *Upsala Journal of Medical Sciences* 2010, **115**(3):193-200.

26. Mather RC, Koenig L, Acevedo D, Dall TM, Gallo P, Romeo A, Tongue J, Williams G: **The Societal and Economic Value of Rotator Cuff Repair**. *The Journal of Bone & Joint Surgery* 2013, **95**(22):1993-2000.

27. Colvin AC, Egorova N, Harrison AK, Moskowitz A, Flatow EL: **National Trends in Rotator Cuff Repair**. *J Bone Joint Surg Am* 2012, **94**(3):227-233.

28. Harryman DT, 2nd, Mack LA, Wang KY, Jackins SE, Richardson ML, Matsen FA, 3rd: **Repairs of the rotator cuff. Correlation of functional results with integrity of the cuff**. *J Bone Joint Surg Am* 1991, **73**(7):982-989.

29. Galatz LM, Ball CM, Teefey SA, Middleton WD, Yamaguchi K: **The Outcome and Repair Integrity of Completely Arthroscopically Repaired Large and Massive Rotator Cuff Tears**. *The Journal of Bone & Joint Surgery* 2004, **86**(2):219-224.

30. Le BTN, Wu XL, Lam PH, Murrell GAC: **Factors Predicting Rotator Cuff Retears: An Analysis of 1000 Consecutive Rotator Cuff Repairs**. *Am J Sports Med* 2014, **42**(5):1134-1142.

31. Gans C: **Fiber architecture and muscle function**. *Exercise and Sport Science Reviews* 1982, **10**:160-207.

32. Lieber RL, Friden J: **Functional and clinical significance of skeletal muscle architecture**. *Muscle Nerve* 2000, **23**(11):1647-1666.

33. Bodine SC, Roy RR, Meadows DA, Zernicke RF, Sacks RD, Fournier M, Edgerton VR: **Architectural, histochemical, and contractile characteristics of a unique biarticular muscle: the cat semitendinosus**. *J Neurophysiol* 1982, **48**:192-201.

34. Powell PL, Roy RR, Kanim P, Bello M, Edgerton VR: **Predictability of skeletal muscle tension from architectural determinations in guinea pig hindlimbs**. *Journal of Applied Physiology* 1984, **57**:1715-1721.

35. Winters TM, Takahashi M, Lieber RL, Ward SR: **Whole muscle length-tension relationships are accurately modeled as scaled sarcomeres in rabbit hindlimb muscles**. *J Biomech* 2011, **44**(1):109-115.

36. Gordon AM, Huxley AF, Julian FJ: **The variation in isometric tension with sarcomere length in vertebrate muscle fibres**. *Journal of Physiology (London)* 1966, **184**:170.

37. Ward SR, Hentzen ER, Smallwood LH, Eastlack RK, Burns KA, Fithian DC, Friden J, Lieber RL: **Rotator cuff muscle architecture: implications for glenohumeral stability**. *Clin Orthop Relat Res* 2006, **448**(Jul):157-163.

38. Shen PH, Lien SB, Shen HC, Lee CH, Wu SS, Lin LC: **Long-term functional outcomes after repair of rotator cuff tears correlated with atrophy of the supraspinatus muscles on magnetic resonance images**. *J Shoulder Elbow Surg* 2008, **17**(1 Suppl):1S-7S.

39. Goutallier D, Postel JM, Bernageau J, Lavau L, Voisin MC: **Fatty muscle degeneration in cuff ruptures. Pre- and postoperative evaluation by CT scan**. *Clin Orthop* 1994, **304**(304):78-83.

40. Shimizu T, Itoi E, Minagawa H, Pradhan RL, Wakabayashi I, Sato K: **Atrophy of the rotator cuff muscles and site of cuff tears**. *Acta Orthopaedica Scandinavica* 2002, **73**(1):40-43.

41. Melis B, DeFranco MJ, Chuinard C, Walch G: **Natural History of Fatty Infiltration and Atrophy of the Supraspinatus Muscle in Rotator Cuff Tears**. *Clinical Orthopaedics and Related Research* 2010, **468**(6):1498-1505.

42. Barry JJ, Lansdown DA, Cheung S, Feeley BT, Ma CB: **The relationship between tear severity, fatty infiltration, and muscle atrophy in the supraspinatus**. *J Shoulder Elbow Surg* 2013, **22**(1):18-25.

43. Kim HM, Dahiya N, Teefey SA, Keener JD, Galatz LM, Yamaguchi K: **Relationship of Tear Size and Location to Fatty Degeneration of the Rotator Cuff**. *The Journal of Bone & Joint Surgery* 2010, **92**(4):829-839.

44. Rulewicz GJ, Beaty S, Hawkins RJ, Kissenberth MJ: **Supraspinatus atrophy as a predictor of rotator cuff tear size: an MRI study utilizing the tangent sign**. *Journal of Shoulder and Elbow Surgery* 2013, **22**(6):e6-e10.

45. Goutallier D, Postel J-M, Gleyze P, Leguilloux P, Van Driessche S: **Influence of cuff muscle fatty degeneration on anatomic and functional outcomes after simple suture of full-thickness tears**. *Journal of Shoulder and Elbow Surgery* 2003, **12**(6):550-554.

46. Deniz G, Kose O, Tugay A, Guler F, Turan A: **Fatty degeneration and atrophy of the rotator cuff muscles after arthroscopic repair: does it improve, halt or deteriorate?** *Arch Orthop Trauma Surg* 2014, **134**(7):985-990.

47. Gladstone JN, Bishop JY, Lo IKY, Flatow EL: **Fatty Infiltration and Atrophy of the Rotator Cuff Do Not Improve After Rotator Cuff Repair and Correlate With Poor Functional Outcome**. *Am J Sports Med* 2007, **35**(5):719-728.

48. Haering D, Blache Y, Raison M, Begon M: **Mechanical risk of rotator cuff repair failure during passive movements: A simulation-based study**. *Clinical biomechanics (Bristol, Avon)* 2015.

49. Burkhart SS, Johnson TC, Wirth MA, Athanasiou KA: **Cyclic loading of transosseous rotator cuff repairs: tension overload as a possible cause of failure**. *Arthroscopy* 1997, **13**(2):172-176.

50. Cummins CA, Murrell GA: **Mode of failure for rotator cuff repair with suture anchors identified at revision surgery**. *J Shoulder Elbow Surg* 2003, **12**(2):128-133.

51. Baums MH, Buchhorn GH, Gilbert F, Spahn G, Schultz W, Klinger HM: **Initial load-to-failure and failure analysis in single- and double-row repair techniques for rotator cuff repair**. *Arch Orthop Trauma Surg* 2010, **130**(9):1193-1199.

52. Itoi E, Hsu HC, Carmichael SW, Morrey BF, An KN: **Morphology of the torn rotator cuff**. *J Anat* 1995, **186 (Pt 2)**:429-434.

53. Tomioka T, Minagawa H, Kijima H, Yamamoto N, Abe H, Maesani M, Kikuchi K, Shimada Y, Itoi E: **Sarcomere length of torn rotator cuff muscle**. *J Shoulder Elbow Surg* 2009, **18**(6):955-959.

54. Hersche O, Gerber C: **Passive tension in the supraspinatus musculotendinous unit after long-standing rupture of its tendon: a preliminary report**. *J Shoulder Elbow Surg* 1998, **7**(4):393-396.

55. Russell RD, Knight JR, Mulligan E, Khazzam MS: **Structural Integrity After Rotator Cuff Repair Does Not Correlate with Patient Function and Pain**. *A Meta-Analysis* 2014, **96**(4):265-271.

56. Goutallier D, Postel JM, Bernageau J, Lavau L, Voisin MC: **Fatty infiltration of disrupted rotator cuff muscles**. *Revue du rhumatisme (English ed)* 1995, **62**(6):415-422.

57. Bachasson D, Singh A, Shah SB, Lane JG, Ward SR: **The role of the peripheral and central nervous systems in rotator cuff disease**. *Journal of Shoulder and Elbow Surgery* 2015, **24**(8):1322-1335.

58. Asami A, Sonohata M, Morisawa K: **Bilateral suprascapular nerve entrapment syndrome associated with rotator cuff tear**. *Journal of Shoulder and Elbow Surgery* 2000, **9**(1):70-72.

59. Costouros JG, Porramatikul M, Lie DT, Warner JJ: **Reversal of suprascapular neuropathy following arthroscopic repair of massive supraspinatus and infraspinatus rotator cuff tears**. *Arthroscopy* 2007, **23**(11):1152-1161.

60. Shi LL, Boykin RE, Lin A, Warner JJ: **Association of suprascapular neuropathy with rotator cuff tendon tears and fatty degeneration**. *J Shoulder Elbow Surg* 2014, **23**(3):339-346.

61. Berhouet J, Collin P, Benkalfate T, Le Du C, Duparc F, Courage O, Favard L: **Massive rotator cuff tears in patients younger than 65 years. Epidemiology and characteristics**. *Orthopaedics & traumatology, surgery & research : OTSR* 2009, **95**(4 Suppl 1):S13-18.

62. Collin P, Treseder T, Lädermann A, Benkalfate T, Mourtada R, Courage O, Favard L: **Neuropathy of the suprascapular nerve and massive rotator cuff tears: a prospective electromyographic study**. *Journal of Shoulder and Elbow Surgery* 2014, **23**(1):28-34.

63. Gerber C, Meyer DC, Schneeberger AG, Hoppeler H, von Rechenberg B: **Effect of Tendon Release and Delayed Repair on the Structure of the Muscles of the Rotator Cuff: An Experimental Study in Sheep**. *The Journal of Bone & Joint Surgery* 2004, **86**(9):1973-1982.

64. Meyer DC, Gerber C, Von Rechenberg B, Wirth SH, Farshad M: **Amplitude and strength of muscle contraction are reduced in experimental tears of the rotator cuff**. *Am J Sports Med* 2011, **39**(7):1456-1461.

65. Gerber C, Meyer DC, Frey E, von Rechenberg B, Hoppeler H, Frigg R, Jost B, Zumstein MA: **Neer Award 2007: Reversion of structural muscle changes caused by chronic rotator cuff tears using continuous musculotendinous traction. An experimental study in sheep**. *Journal of Shoulder and Elbow Surgery* 2009, **18**(2):163-171.

66. Soslowsky LJ, Carpenter JE, DeBano CM, Banerji I, Moalli MR: **Development and use of an animal model for investigations on rotator cuff disease**. *J Shoulder Elbow Surg* 1996, **5**(5):383-392.

67. Mathewson MA, Kwan A, Eng CM, Lieber RL, Ward SR: **Comparison of rotator cuff muscle architecture between humans and other selected vertebrate species**. *The Journal of Experimental Biology* 2014, **217**(2):261-273.

68. Carpenter JE, Thomopoulos S, Flanagan CL, DeBano CM, Soslowsky LJ: **Rotator cuff defect healing: A biomechanical and histologic analysis in an animal model**. *Journal of Shoulder and Elbow Surgery* 1998, **7**(6):599-605.

69. Gimbel JA, Van Kleunen JP, Mehta S, Perry SM, Williams GR, Soslowsky LJ: **Supraspinatus tendon organizational and mechanical properties in a chronic rotator cuff tear animal model**. *Journal of Biomechanics* 2004, **37**(5):739-749.

70. Dourte LM, Perry SM, Getz CL, Soslowsky LJ: **Tendon Properties Remain Altered in a Chronic Rat Rotator Cuff Model**. *Clinical Orthopaedics and Related Research* 2010, **468**(6):1485-1492.

71. Galatz LM, Sandell LJ, Rothermich SY, Das R, Mastny A, Havlioglu N, Silva MJ, Thomopoulos S: **Characteristics of the rat supraspinatus tendon during tendon-to-bone healing after acute injury**. *Journal of Orthopaedic Research* 2006, **24**(3):541-550.

72. Gimbel JA, Mehta S, Van Kleunen JP, Williams GR, Soslowsky LJ: **The tension required at repair to reappose the supraspinatus tendon to bone rapidly increases after injury**. *Clin Orthop Relat Res* 2004(426):258-265.

73. Gimbel JA, Van Kleunen JP, Lake SP, Williams GR, Soslowsky LJ: **The role of repair tension on tendon to bone healing in an animal model of chronic rotator cuff tears**. *J Biomech* 2007, **40**(3):561-568.

74. Galatz LM, Rothermich SY, Zaegel M, Silva MJ, Havlioglu N, Thomopoulos S: **Delayed repair of tendon to bone injuries leads to decreased biomechanical properties and bone loss**. *Journal of Orthopaedic Research* 2005, **23**(6):1441-1447.

75. Ward SR, Sarver JJ, Eng CM, Kwan A, Wurgler-Hauri CC, Perry SM, Williams GR, Soslowsky LJ, Lieber RL: **Plasticity of muscle architecture after supraspinatus tears**. *J Orthop Sports Phys Ther* 2010, **40**(11):729-735.

76. Barton ER, Gimbel JA, Williams GR, Soslowsky LJ: **Rat supraspinatus muscle atrophy after tendon detachment**. *J Orthop Res* 2005, **23**(2):259-265.

77. Liu X, Joshi SK, Samagh SP, Dang YX, Laron D, Lovett DH, Bodine SC, Kim HT, Feeley BT: **Evaluation of Akt/mTOR activity in muscle atrophy after rotator cuff tears in a rat model**. *J Orthop Res* 2012, **30**(9):1440-1446.

78. Joshi SK, Kim HT, Feeley BT, Liu X: **Differential ubiquitin-proteasome and autophagy signaling following rotator cuff tears and suprascapular nerve injury**. *J Orthop Res* 2014, **32**(1):138-144.

79. Killian ML, Lim CT, Thomopoulos S, Charlton N, Kim HM, Galatz LM: **The effect of unloading on gene expression of healthy and injured rotator cuffs**. *Journal of Orthopaedic Research* 2013, **31**(8):1240-1248.

80. Liu X, Joshi SK, Ravishankar B, Laron D, Kim HT, Feeley BT: **Upregulation of transforming growth factor-beta signaling in a rat model of rotator cuff tears**. *J Shoulder Elbow Surg* 2014, **23**(11):1709-1716.

81. Liu X, Manzano G, Kim HT, Feeley BT: **A rat model of massive rotator cuff tears**. *J Orthop Res* 2011, **29**(4):588-595.

82. Kim HM, Galatz LM, Lim C, Havlioglu N, Thomopoulos S: **The effect of tear size and nerve injury on rotator cuff muscle fatty degeneration in a rodent animal model**. *Journal of Shoulder and Elbow Surgery* 2012, **21**(7):847-858.

83. Joshi SK, Liu X, Samagh SP, Lovett DH, Bodine SC, Kim HT, Feeley BT: **mTOR regulates fatty infiltration through SREBP-1 and PPARgamma after a combined massive rotator cuff tear and suprascapular nerve injury in rats**. *J Orthop Res* 2013, **31**(5):724-730.

84. Silldorff MD, Choo AD, Choi AJ, Lin E, Carr JA, Lieber RL, Lane JG, Ward SR: **Effect of supraspinatus tendon injury on supraspinatus and infraspinatus muscle passive tension and associated biochemistry**. *J Bone Joint Surg Am* 2014, **96**(20):e175.

85. Mannava S, Plate JF, Whitlock PW, Callahan MF, Seyler TM, Koman LA, Smith TL, Tuohy CJ: **Evaluation of in vivo rotator cuff muscle function after acute and chronic detachment of the supraspinatus tendon: an experimental study in an animal model**. *J Bone Joint Surg Am* 2011, **93**(18):1702-1711.

86. Ditsios K, Boutsiadis A, Kapoukranidou D, Chatzisotiriou A, Kalpidis I, Albani M, Christodoulou A: **Chronic massive rotator cuff tear in rats: in vivo evaluation of muscle force and three-dimensional histologic analysis**. *J Shoulder Elbow Surg* 2014, **23**(12):1822-1830.

87. WebMD: **Picture of the Shoulder**. In. http://www.webmd.com/pain-management/picture-of-the-shoulder: WebMD, LLC; 2014.

CHAPTER 2 : ARCHITECTURAL AND BIOCHEMICAL ADAPTATIONS IN SKELETAL MUSCLE AND BONE FOLLOWING ROTATOR CUFF INJURY IN A RAT MODEL

2.1 Abstract

Background: Injury to the rotator cuff can cause irreversible changes to the structure and function of the associated muscles and bones. The temporal progression and pathomechanisms associated with these adaptations are unclear. The purpose of this study was to investigate the time course of structural muscle and osseous changes in a rat model of a massive rotator cuff tear.

Methods: Supraspinatus and infraspinatus muscle architecture and biochemistry and humeral and scapular morphological parameters were measured three days, eight weeks, and sixteen weeks after dual tenotomy with and without chemical paralysis via botulinum toxin A (BTX).

Results: Muscle mass and physiological cross-sectional area increased over time in the age-matched control animals, decreased over time in the tenotomy+BTX group, and remained nearly the same in the tenotomy-alone group. Tenotomy+BTX led to increased extracellular collagen in the muscle. Changes in scapular bone morphology were observed in both experimental groups, consistent with reductions in load transmission across the joint.

Conclusions: These data suggest that tenotomy alone interferes with normal age-related muscle growth. The addition of chemical paralysis yielded profound

structural changes to the muscle and bone, potentially leading to impaired muscle function, increased muscle stiffness, and decreased bone strength.

Clinical Relevance: Structural musculoskeletal changes occur after tendon injury, and these changes are severely exacerbated with the addition of neuromuscular compromise.

2.2 Introduction

Rotator cuff tears are a common degenerative condition found in approximately 30% of individuals over sixty years of age [1] and resulting in pain and loss of functional range of motion in the shoulder [2]. While surgical treatment and repair of the tendon are possible, failure rates have been reported to be high as 20% to 94%, with an increasing prevalence of failure associated with increases in the size of the tear and the age of the patient [3,4].

Muscle atrophy is associated with chronic, massive rotator cuff tears and has been documented with magnetic resonance imaging and computed tomography (CT) in humans and animal models [5-7]. Likewise, decreased muscle weight, volume, and/or fiber length have been observed in both animal models and human cadavers with rotator cuff injuries [8, 9]. Previous work with a sheep model demonstrated a correlation between active force production and muscle atrophy following rotator cuff injury [7]. However, muscle mass, volume, and fiber length are overall poor indicators of muscle function. In contrast, architectural parameters such as physiological cross-sectional area have been previously shown to be good predictors of muscle force production [10]. Similarly, normalized fiber length (i.e.,

the number of sarcomeres in series) provides the best estimate of muscle excursion and velocity [11, 12]. Shorter fibers, which become highly strained and result in larger forces at the repair site, have been implicated as one of the obstacles to the repair of massive rotator cuff tears [13, 14] and to the achievement of good tendon-to-bone healing [15]. Protein level adaptations, such as increased collagen content5 and adaptations in the intramyocellular protein titin [16], may also influence muscle stiffness at the time of repair. Previous work with a rat model of a single rotator cuff tendon injury demonstrated transient changes in supraspinatus physiological cross-sectional area and sarcomere number [17]. This finding supports the concept that shorter fibers may lead to a stiffer muscle because higher sarcomere strains are needed to achieve anatomical repair. However, to our knowledge, there have been no quantitative measurements of collagen and titin in these muscles to support the idea of material property changes, which would further increase muscle stiffness after injury.

The contribution of suprascapular neuropathy or neurapraxia to muscle trophic changes associated with massive, retracted tears has not been clearly established. Alterations in nerve function may influence the clinical deterioration of cuff muscles and have been associated with massive rotator cuff tears [18,19]. Similarly, rotator cuff arthropathy in the setting of chronic, massive rotator cuff tears can lead to alterations in the osseous architecture of the shoulder (e.g., osteopenia, cartilage loss, proximal migration of the humeral head, abnormal bone wear, and osteophyte formation) [20]. These changes theoretically may be due to

mechanical unloading and/or changes in trophic factor interactions among bone, muscle, and tendon. We are not aware of any animal studies assessing osseous architecture changes in the shoulder following a rotator cuff injury. Although some animal model data suggest that a combined tendon and nerve injury leads to more muscle changes than does a tendon injury alone [21-24], to our knowledge no study has correlated changes in bone and muscle architecture to the severity of tendon and muscle injury.

In the current study, botulinum toxin A (BTX) was used in conjunction with tendon injury to mimic a chronic, massive rotator cuff injury leading to severe muscle atrophy. The objective of the study was to investigate the short-term (three-day) and long-term (eight and sixteen-week) muscle and bone adaptations that occur in a rodent model of a massive rotator cuff tear. Specifically, we focused on muscle structural parameters that are believed to influence active (contractile) and passive force generation. We hypothesized that injury to the rotator cuff would result in radial and longitudinal muscle atrophy, increased collagen content, and decreased scapular fossa depth and trabecular number and thickness. We also hypothesized that these changes would be exacerbated by additional BTX injury and chronicity. This information is clinically useful because a better understanding of the mechanisms by which rotator cuff muscles and the surrounding bone degenerate following injury may lead to therapeutic interventions that can one day improve clinical results following repair.

2.3 Materials and Methods

Animal Model and Surgical Methods

Fifty-five male Sprague-Dawley rats were used for this study. The animals were divided into three groups: bilateral dual tenotomy of the supraspinatus and infraspinatus tendons only (tenotomy-alone group), bilateral dual tenotomy of the supraspinatus and infraspinatus tendons with concomitant chemical denervation of the muscles induced with BTX (tenotomy+BTX group), and age-matched uninjured controls (control group). Following approval from the university's Animal Studies Committee, the surgical procedures were performed after induction of anesthesia with isoflurane and a 1% oxygen carrier. Under sterile conditions, a 2-cm vertical incision was made over the scapulohumeral joint and the deltoid was detached from the cranial and lateral aspects of the acromion with use of electrocautery. The acromion was elevated with use of a 3-0 Vicryl (polyglactin) suture passed through the acromial notch to expose the underlying rotator cuff tendons. The supraspinatus tendon was exposed by supination of the forearm, and a number-11 blade was used to transect the supraspinatus tendon at its insertion on the humeral head. The forearm was then internally rotated 45° to expose the infraspinatus tendon, which was transected from the humeral head with use of a number-11 blade. Retraction of the tendons was confirmed visually by the surgeon (C.T.L.). In the tenotomy+BTX group, BTX diluted in sterile saline solution (~9 U/kg) was injected into the supraspinatus muscle belly at the time of surgery. In the sixteen-week tenotomy+BTX group, a second injection of BTX was administered into the

supraspinatus muscle belly at eight weeks postsurgery. No injections were performed in the tenotomy-alone group. The deltoid and trapezius muscles were then reattached with use of 3-0 Vicryl suture, and the skin was closed with staples. Postoperative animal care was administered by an animal care technician.

Animals were killed at three days, eight weeks, or sixteen weeks after injury (Table I). At the time of sacrifice, the supraspinatus and infraspinatus muscles from one shoulder were individually dissected, snap-frozen in liquid nitrogen, and stored at −80°C for biochemical analysis. In a subset of these animals, the contralateral shoulder was dissected en bloc and pinned in its anatomical orientations for evaluation of muscle architecture and bone morphology. All musculature except the supraspinatus and infraspinatus was then removed, and the shoulders were fixed in 4% paraformaldehyde overnight. Samples were then stored in 70% ethanol for further analysis of muscle architecture and bone morphology measurements. Because the osseous anatomy was disrupted on dissection (e.g., the acromion or scapular spine broke), eight shoulders were excluded from bone morphometric analyses (Table I).

Muscle Architecture

Specimens were sharply dissected from the scapulae to isolate the supraspinatus and infraspinatus muscles and were stored in phosphate-buffered saline solution. Muscle specimens were removed from the saline solution, gently blotted dry, and weighed. Muscle fiber sarcomere length, normalized muscle fiber length, and physiological cross-sectional area were measured as previously

described for rat rotator cuff muscles [**17**]. Fiber length was normalized to a sarcomere length of 2.4 μm, which represents the optimum sarcomere length for rat muscle based on actin and myosin filament lengths [**25**].

Bone Morphology

Following fixation, micro-CT (SkyScan 1076; SkyScan, Aartselaar, Belgium) was performed with a cone beam, 36-μm voxel resolution, 45-kV (177-μA) energy, standard resolution, and 300-msec integration time. Bone morphometric parameters, including total volume, bone volume fraction (bone volume divided by total volume), trabecular thickness, trabecular number, and trabecular spacing, were measured in the humeral head with use of Scanco Medical software (Brüttisellen, Switzerland). Bone architecture (scapular fossa depth) was measured with use of OsiriX 32-bit imaging software (open source version 5.5). Following imaging, specimens were stored in 70% ethanol at 4°C for further analysis.

Titin Molecular Weight Determination

Titin molecular weight was quantified with use of a previously developed method utilizing sodium dodecyl sulfate-vertical agarose gel electrophoresis (SDS-VAGE) [26]. Details of the method are available in the Appendix.

Collagen Content

The hydroxyproline content was measured with a modification of a previously published protocol to determine the collagen content (μg collagen/mg wet weight tissue) of the supraspinatus and infraspinatus muscles [27]. The measured hydroxyproline content was used to calculate the collagen amount by

using the constant 7.46, which corresponds to the average number of hydroxyproline residues in a collagen molecule [28]. Details of the methods are available in the Appendix.

Statistical Analysis

After the data were screened for normality and homogeneity of variances, two-way analysis of variance was used to compare groups for each dependent measure. Post-hoc Sidak tests were performed to identify specific group differences. Statistical analyses were performed with use of SPSS software 20.0 (IBM, Armonk, New York) and Prism 6.0b (GraphPad, La Jolla, California). Significance was set at $p < 0.05$, and all data are presented as the mean and standard deviation.

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2.4 Results

Muscle Architecture

As expected, the control animals had significantly larger supraspinatus ($p = 0.001$) and infraspinatus ($p < 0.001$) muscle mass at eight weeks compared with the muscle mass at three days. The mass of both muscles was also increased at sixteen weeks, compared with the three-day value, in the control group ($p < 0.001$) (Figs. 1-A and 1-B). Tenotomy alone did not yield a significant reduction in supraspinatus or infraspinatus muscle mass over time, but these muscles were significantly smaller

than the control muscles at sixteen weeks ($p < 0.001$). In contrast, the addition of BTX produced significant reductions in supraspinatus ($p = 0.013$) and infraspinatus ($p = 0.034$) muscle mass at eight weeks, as well as at sixteen weeks ($p < 0.001$), compared with the muscle mass at three days. Because of this active atrophy, the muscles in the tenotomy+BTX group had a significantly reduced mass compared with those in the controls ($p < 0.001$ for all comparisons) and compared with those in the tenotomy-alone group at eight weeks ($p < 0.001$ for the supraspinatus and $p = 0.002$ for the infraspinatus) and at sixteen weeks ($p < 0.001$).

At three days, sarcomere length was significantly reduced, compared with the length in the control group, in the supraspinatus in both tenotomy groups (with and without BTX) ($p < 0.01$) and in the infraspinatus in the tenotomy+BTX group ($p = 0.003$) (Figs. 1-C and 1-D). These findings were consistent with muscle retraction. However, in both injury groups the sarcomere length recovered by eight weeks, and in the tenotomy+BTX group it slightly exceeded the control value at sixteen weeks. These data confirm the initial tenotomy-induced retraction of the muscles and suggest adaptation of longitudinal sarcomere lengths over time.

The normalized fiber length in both muscles remained constant over time in the control group. In the tenotomy+BTX group, the normalized fiber length was reduced in both the supraspinatus ($p = 0.044$) and the infraspinatus ($p = 0.006$), compared with the values in the control group, at sixteen weeks and in the infraspinatus at eight weeks ($p = 0.025$). The normalized muscle fiber length in the tenotomy-alone group became, on average, smaller than that in the control group

over time, but the difference reached significance ($p = 0.020$) only in the infraspinatus muscle at eight weeks (Figs. 1-E and 1-F).

The physiological cross-sectional area followed a pattern similar to that of the muscle mass. In the control group, the physiological cross-sectional area was significantly increased at eight weeks in both the supraspinatus ($p = 0.005$) and the infraspinatus ($p = 0.001$) and at sixteen weeks in both muscles ($p < 0.001$) compared with the values at three days (Figs. 1-G and 1-H). Tenotomy alone did not yield significant reductions in the physiological cross-sectional area over time, but these values remained constant so the values for both the supraspinatus ($p = 0.001$) and the infraspinatus ($p = 0.015$) were significantly lower than the control values by sixteen weeks. However, the physiological cross-sectional areas of the supraspinatus and infraspinatus in the tenotomy+BTX group were significantly lower than the control or tenotomy-alone values at eight ($p < 0.001$) and sixteen ($p < 0.001$) weeks.

Bone Morphology

Bone architectural and morphometric parameters were evaluated with use of axial, sagittal, and coronal views of the scapula and humeral head (Figs. 2-A, 2-B, and 2-C). In the control animals, the supraspinatus and infraspinatus fossa depths were increased at eight weeks ($p < 0.001$) and sixteen weeks ($p < 0.001$) compared with the depths at three days (Figs. 2-D and 2-E). Tenotomy alone did not significantly decrease fossa depth over time; however, there were significant decreases, compared with the control values, in the fossa depths at eight weeks ($p =$

0.013 for the supraspinatus fossa and $p = 0.005$ for the infraspinatus fossa) and at sixteen weeks ($p < 0.001$ and $p = 0.004$, respectively) in the tenotomy-alone group. Scapulae from the tenotomy+BTX group demonstrated significant reductions in fossa depth at eight weeks and sixteen weeks compared with those in the control and tenotomy-alone groups ($p < 0.001$ for all comparisons). These data closely matched the changes in muscle mass.

The humeral head bone volume fraction (bone volume divided by total volume) was significantly reduced, compared with the control value, in the tenotomy-alone group ($p = 0.002$) and tenotomy+BTX group ($p < 0.001$) at eight weeks and in both groups ($p < 0.001$) at sixteen weeks (Fig. 3-A). These changes tracked the changes in muscle mass, increasing over time in the control animals, remaining nearly constant over time in the tenotomy-alone group, and decreasing over time in the tenotomy+BTX group. Trabecular spacing was increased at eight weeks ($p = 0.033$) and sixteen weeks ($p = 0.005$), compared with the value at three days, only in the tenotomy+BTX group (Fig. 3-B). Tenotomy+BTX also led to significantly greater trabecular spacing compared with that in the control group at eight weeks ($p = 0.001$) and sixteen weeks ($p = 0.006$).

In the control group, trabecular thickness increased over time, with a higher value at eight weeks ($p = 0.001$) and sixteen weeks ($p < 0.001$) than at three days, whereas trabecular thickness remained nearly constant over time in both injury groups (Fig. 3-C). There was a significant reduction in trabecular thickness, compared with the control value, at eight weeks in the tenotomy-alone group ($p =$

0.016) and the tenotomy+BTX group ($p < 0.001$) and at sixteen weeks in the tenotomy-alone group ($p = 0.001$) and the tenotomy+BTX group ($p < 0.001$), but the injury groups did not differ significantly from each other. The trabecular number was also significantly reduced, compared with the control value, at eight weeks and sixteen weeks in both the tenotomy-alone ($p < 0.05$) and the tenotomy+BTX ($p < 0.001$) group (Fig. 3-D).

Titin Molecular Weight Determination

Titin molecular weight was significantly reduced in both the supraspinatus ($p = 0.017$) and the infraspinatus ($p = 0.011$) muscles at eight weeks in the tenotomy-alone group compared with the values in the control group (Figs. 4-A and 4-B). These reductions were also observed in the supraspinatus muscle at three days ($p = 0.044$). The addition of BTX had no further effect on titin molecular weight.

Collagen Content

The most profound increases in muscle collagen content relative to the controls were observed in the tenotomy+BTX group at eight weeks ($p = 0.005$ for the supraspinatus and $p < 0.001$ for the infraspinatus) and sixteen weeks ($p < 0.001$ for both muscles) (Figs. 4-C and 4-D). Although there was some increase in the infraspinatus collagen content in the tenotomy-alone group compared with the control value at eight weeks ($p = 0.034$), this increase did not remain significant at the sixteen-week time point.

2.5 Discussion

The purpose of this study was to characterize adaptations in muscle and bone architecture as well as muscle biochemistry in response to a massive rotator cuff tear with and without muscle paralysis. Tenotomy with muscle paralysis in our rat model resulted in significant and progressive radial muscle atrophy (a decrease in physiological cross-sectional area) over sixteen weeks. Tenotomy with muscle paralysis induced substantially more severe muscle changes than tenotomy alone, a finding that is consistent with previous rodent studies [21]. In fact, tenotomy alone did not yield significant decreases in muscle dimensions over time. However, when compared with age-matched controls, the tenotomized muscles were significantly smaller, indicating that they had failed to grow normally over time. Tenotomy with muscle paralysis resulted in only mild longitudinal muscle atrophy (reductions in muscle fiber length), at the later time points, compared with the control values. This finding may be related to the fact that sarcomere lengths were only slightly reduced three days after the tenotomy, indicating that muscle retraction, although present, was mild. These findings agree with previous observations of only small decreases in muscle dimensions following single-muscle (supraspinatus) tenotomies in the rats [17] and with observations in human cadaver shoulders with rotator cuff tears [9]. Taken together, these changes suggest severe impairment of muscle force production and more minor impairment of muscle excursion and velocity. These data do not support the idea that the high passive tensions observed during anatomical surgical reconstruction result from higher strains in muscles with

shorter fibers. Future studies should be undertaken to investigate whether these muscle adaptations are reversible following tendon repair and/or rehabilitation.

Interestingly, changes in humeral head trabecular architecture were consistent with muscle unloading in both injury groups, while scapular fossa depth closely followed muscle architectural changes, which differed between the two injury groups. For example, decreased trabecular thickness and trabecular number were observed in both injury groups, suggesting that humeral head bone morphology is less robust when the supraspinatus and infraspinatus muscles are no longer connected to the humerus (and therefore no longer transmitting force to the humerus). In contrast, scapular fossa depths were more severely reduced in the tenotomy+BTX group compared with the tenotomy-alone group, and these differences tracked the changes measured in muscle mass and volume. A possible explanation for this differential osseous effect could be cross-talk between muscle and bone via paracrine factors leading to osseous adaptations that depend on the severity of the muscle degeneration [29]. This paracrine-mediated hypothesis warrants further experiments.

Also interestingly, small but distinct biochemical changes were observed in both injury groups and both muscles. Titin is a large intracellular structural protein in the sarcomere that has been implicated in determining the stiffness of single muscle fibers [16]. Decreases in molecular weight would be expected to increase stiffness at the single-fiber (cell) level [16]. These changes in titin molecular weight suggest that titin molecular weight may be regulated, at least in part, by the absence

of mechanical loading or by changes in muscle fiber length, as would result from detachment of tendon from bone. Although it is appealing to speculate that muscle retraction would lead to shorter sarcomeres and therefore reduced titin length (molecular weight), our sarcomere data do not support this idea.

In contrast to tenotomy alone, the addition of muscle paralysis led to progressive increases in muscle collagen content, which suggests that proper innervation has a unique role in the prevention of rotator cuff muscle fibrosis. Increases in collagen content may also increase muscle stiffness [5, 8], but this simple correlation should be interpreted with caution, as the relationship between muscle collagen content and stiffness is weak [30]. Nevertheless, the sources of increased muscle stiffness after rotator cuff injury are a major focus of current work in this area, as increased muscle stiffness has been implicated in rotator cuff repairs that are difficult to perform in human patients. Rodent model systems may allow further exploration of the molecular mechanisms and sources of fibrosis, despite the fact that they do not exactly recapitulate the human condition.

The current study has several limitations. First, tenotomy in the animal model does not mimic the magnitude of muscle retraction observed in complete human rotator cuff tears, potentially mitigating longitudinal atrophy of the muscles. Second, the biochemical changes observed in the muscles imply passive stiffness changes at multiple size scales (titin indicates increased stiffness at the single-cell size scale and increased collagen content indicates increased stiffness at the muscle-fiber-bundle and whole-muscle size scales). However, we did not directly measure

muscle passive mechanical changes implied by the biochemical findings. Third, the use of BTX in this model to exacerbate muscle atrophy may not directly recapitulate the unloading conditions seen in human patients. In contrast to neurotomy, which completely disrupts nerve structure and function and is used in some rotator cuff injury models [21, 31, 32], injection of BTX temporarily disrupts cholinergic communication between the nerve and muscle (chemical denervation) while preserving noncholinergic communication. For this reason, it could be argued that neurotomy does not recapitulate the human condition as well as BTX, although neither injury is a compression neuropathy model. However, both models yield severe atrophy similar to what is observed in humans [23, 31]. Finally, active mechanics were not tested in this study; thus, we are unable to quantify the physiological force-producing capacity of the muscle implied by the decreased physiological cross-sectional area.

In conclusion, these data suggest that the addition of muscle paralysis to massive tendon tears yields profound structural changes to the muscle and bone. These changes would presumably impair muscle active force-generating capacity, muscle stiffness, and bone strength. Further study is required to assess the mechanisms associated with the changes and to reconcile these findings with those observed in patients. The profound effects of advanced atrophy in this study highlight the importance of diagnosing and treating rotator cuff tears at risk for developing chronic degenerative changes before advanced changes occur. Further research is needed to understand the mechanisms of (1) aggressive muscle atrophy

and (2) muscle-bone interactions under conditions of combined tendon and nerve injury.

2.6 Appendix

Titin Molecular Weight Determination

Titin molecular weight was quantified with a previously developed method utilizing SDS-VAGE [26]. Protein homogenates were prepared from frozen samples and loaded into 1% agarose gels. A small 12.8% acrylamide plug was placed at the bottom of the gel apparatus to hold the agarose gel in place. Human soleus and rat cardiac muscles were used as titin standards. Gels were run two at a time at 25 mA for 4.5 hours at 4 ^{o}C. Gels were stained according to the Bio-Rad Silver Stain Plus kit protocol, and bands were identified and quantified with densitometry (Quantity One; Bio-Rad). Collagen Content Hydroxyproline content was used to determine the collagen content (mg collagen/mg wet weight tissue) of the supraspinatus and infraspinatus muscles with use of a modification of a previously published protocol [27]. Tissue samples were hydrolyzed in 6-mol/L HCl at 110 ^{o}C overnight and neutralized with NaOH to pH 6.98 to 7.04. Samples were then incubated with a chloramine-T solution for twenty minutes at room temperature, followed by addition of a p-dimethylaminobenzaldehyde solution and incubation at 60 ^{o}C for thirty minutes. The hydroxyproline concentration was determined with spectrophotometry at 550 nm and was normalized to the wet mass of the original tissue sample. Standard solutions provided a calibration curve for spectrophotometry. The measured hydroxyproline content was used to calculate the

collagen amount by using the constant 7.46, corresponding to the average number of hydroxyproline residues in a collagen molecule [28].

2.7 Acknowledgements

Chapter 2 is nearly identical to a peer-reviewed article entitled "Architectural and biochemical adaptations in skeletal muscle and bone following rotator cuff injury in a rat model" by Sato EJ, Killian ML, Choi AJ, Lin E, Choo AD, Rodriguez-Soto AE, Lim CT, Thomopoulos S, Galatz LM, Ward SR. The article was published in the *Journal of Bone and Joint Surgery* 2015, **97**(7):565-573. The authors acknowledge the technical support of Ki-Seok Lee, MD. The sources of funding for this study were: NIH R01 AR057836, NIH R24 HD050837, NIH P30 AR057235, NIH T32 AR060712.

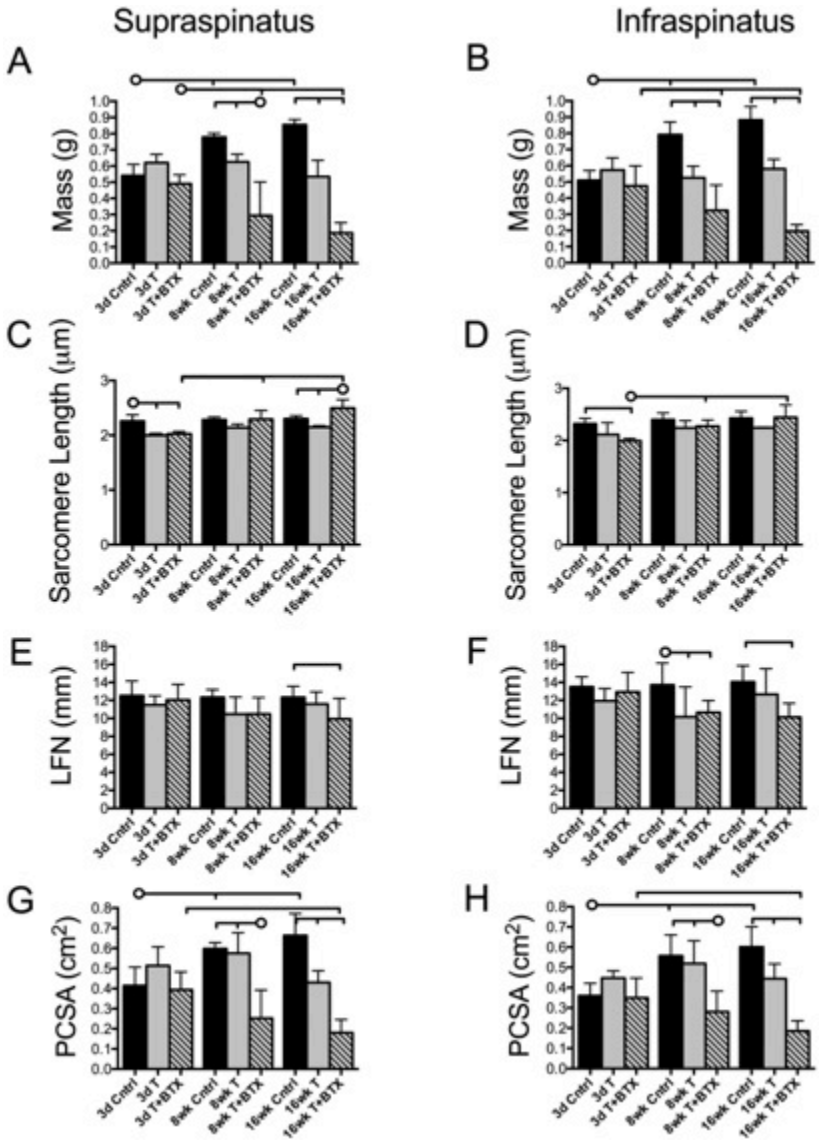

Figure 2.1 Architectural measurements of the supraspinatus and infraspinatus muscles.

Architectural measurements of the supraspinatus and infraspinatus muscles indicate that the mass and physiological cross-sectional area were progressively reduced in the tenotomy+BTX group. Muscle mass (Figs. 1-A and 1-B), sarcomere length (Figs. 1-C and 1-D), normalized fiber length (LFN) (Figs. 1-E and 1-F), and physiological cross-sectional area (PCSA) (Figs. 1-G and 1-H) are shown for each group at each time point. The horizontal lines without circles at the tops of the panels indicate significant differences ($p < 0.05$) between all groups with a vertical tick mark. The horizontal lines with circles indicate significant differences ($p < 0.05$) between the group identified with the circle and the groups identified with a vertical tick mark but no significant difference between the groups identified with the tick mark. Cntrl = control, T = tenotomy, BTX = botulinum toxin A, 3d = three-day, 8wk = eight-week, and 16wk = sixteen-week.

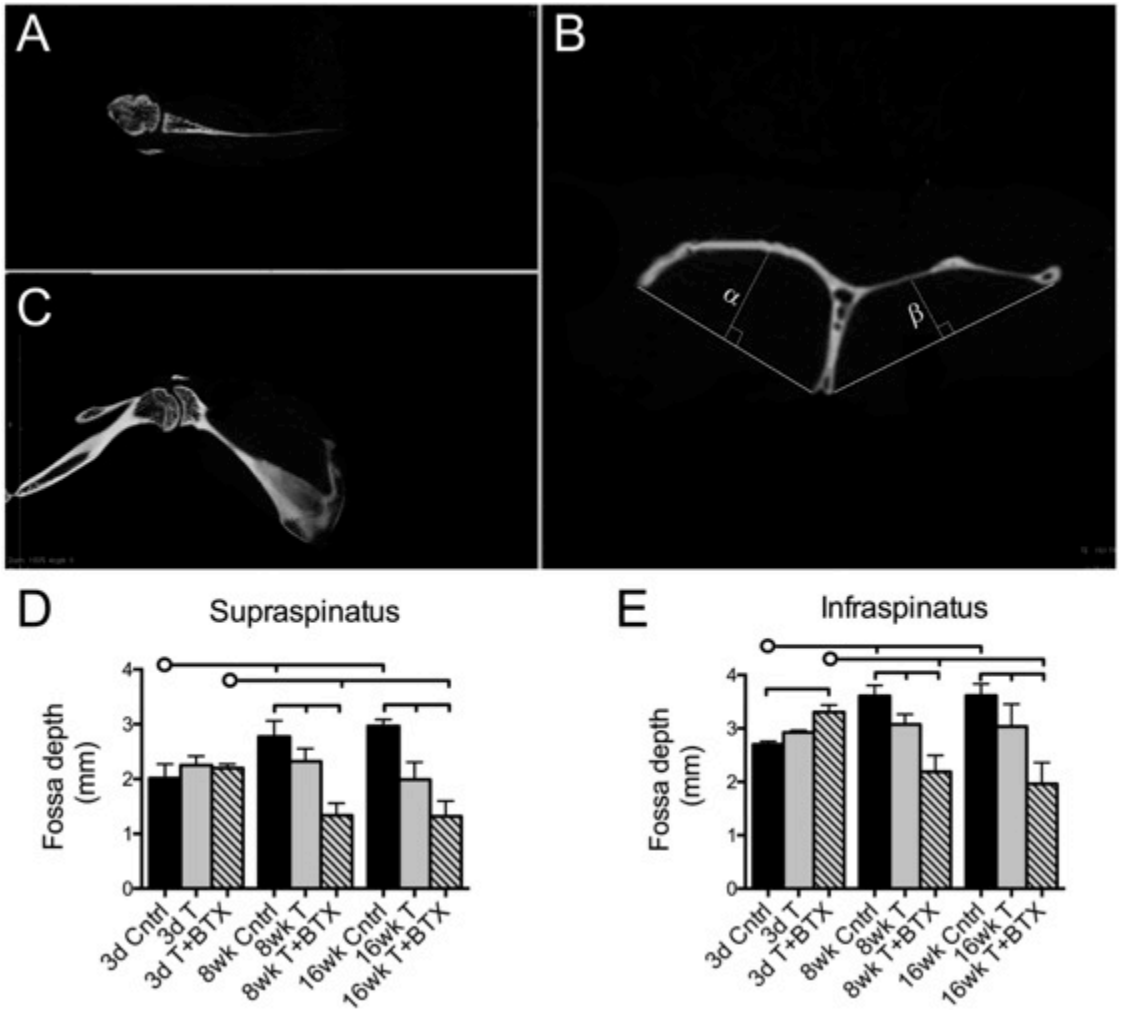

Figure 2.2 Representative micro-CT images and fossa depth measurements
Representative micro-CT images depicting axial (Fig. 2-A), sagittal (Fig. 2-B), and coronal (Fig. 2-C) views of the scapula. The sagittal oblique view (Fig. 2-B) was used to measure the infraspinatus fossa depth (α) and supraspinatus fossa depth (β). At eight and sixteen weeks, the supraspinatus and infraspinatus fossa depths were reduced, compared with the control values, in the tenotomy-alone and tenotomy+BTX groups and the fossa depths in the tenotomy+BTX group were significantly reduced compared with those in the tenotomy-alone group (Figs. 2-D and 2-E). The horizontal lines without circles at the tops of the panels indicate significant differences ($p < 0.05$) between all groups with a vertical tick mark. The horizontal lines with circles indicate significant differences ($p < 0.05$) between the group identified with the circle and the groups identified with a vertical tick mark but no significant difference between the groups identified with the tick mark. Cntrl = control, T = tenotomy, BTX = botulinum toxin A, 3d = three-day, 8wk = eight-week, and 16wk = sixteen-week.

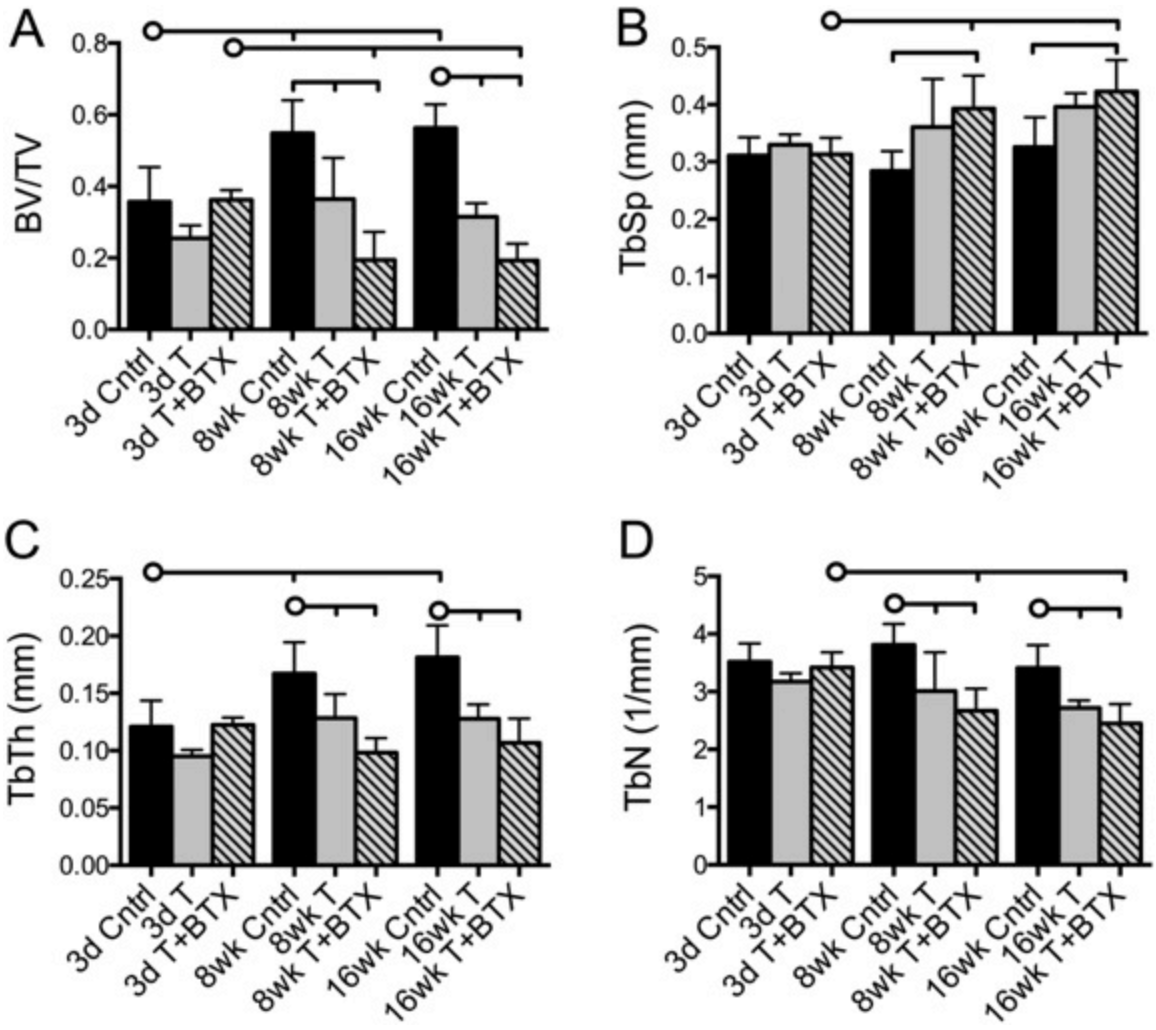

Figure 2.3 Humeral head bony measurements

Humeral head measurements for bone volume fraction (bone volume [BV]/total volume [TV]) (Fig. 3-A), trabecular spacing (TbSp) (Fig. 3-B), trabecular thickness (TbTh) (Fig. 3-C), and trabecular number (TbN) (Fig. 3-D). The bone volume fraction was significantly reduced in the tenotomy+BTX group compared with the tenotomy-alone group at eight weeks, while trabecular spacing, trabecular thickness, and trabecular number were uniformly changed in both injury groups. The horizontal lines without circles at the tops of the panels indicate significant differences ($p < 0.05$) between all groups with a vertical tick mark. The horizontal lines with circles indicate significant differences ($p < 0.05$) between the group identified with the circle and the groups identified with a vertical tick mark but no significant difference between the groups identified with the tick mark. Cntrl = control, T = tenotomy, BTX = botulinum toxin A, 3d = three-day, 8wk = eight-week, and 16wk = sixteen-week.

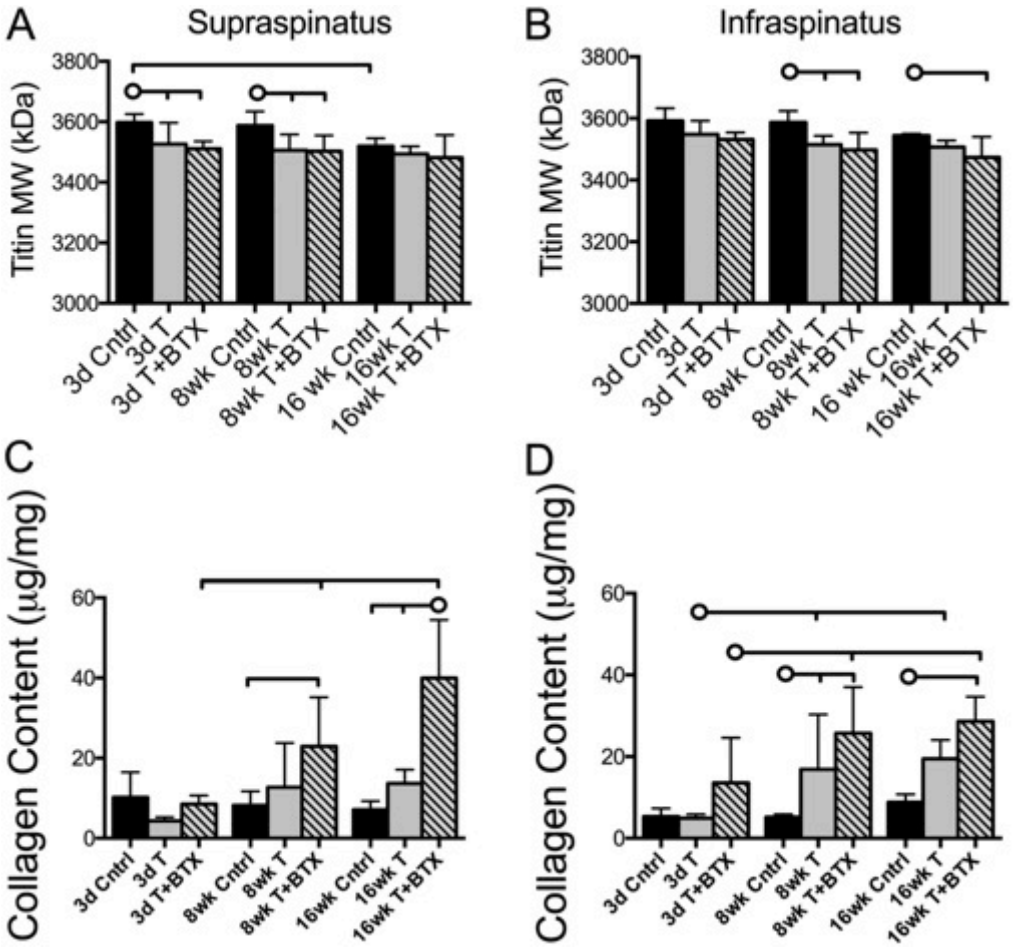

Figure 2.4 Titin molecular weight and collagen content

Titin molecular weight (MW) was decreased in the supraspinatus (Fig. 4-A) and infraspinatus (Fig. 4-B) muscles following tenotomy, compared with the control values, but with different time courses. The addition of nerve injury (tenotomy+BTX) did not yield any further change in titin. Collagen content was increased in the supraspinatus (Fig. 4-C) at eight weeks and sixteen weeks in the tenotomy+BTX group, compared with the control values, and similar changes were observed in the infraspinatus (Fig. 4-D). Tenotomy alone increased collagen content in the infraspinatus muscle at eight weeks, but this did not remain significant at the sixteen-week time point. The horizontal lines without circles at the tops of the panels indicate significant differences ($p < 0.05$) between all groups with a vertical tick mark. The horizontal lines with circles indicate significant differences ($p < 0.05$) between the group identified with the circle and the groups identified with a vertical tick mark but no significant difference between the groups identified with the tick mark. Cntrl = control, T = tenotomy, BTX = botulinum toxin A, 3d = three-day, 8wk = eight-week, and 16wk = sixteen-week.

Table 2.1 Number of Animals and Number of Shoulder Specimens Used in Each Analysis

TABLE I Number of Animals and Number of Shoulder Specimens Used in Each Analysis

	Total No. of Animals	No. of Shoulder Specimens Used in Analysis		
		Biochemistry	Architecture	Bone Morphology
3 days				
Control	6	6	6	6
Tenotomy	6	6	4	3
Tenotomy+BTX	5	5	4	4
8 weeks				
Control	6	6	6	6
Tenotomy	6	6	5	5
Tenotomy+BTX	8	8	8	5
16 weeks				
Control	6	6	6	4
Tenotomy	6	6	5	6
Tenotomy+BTX	6	6	6	4

2.8 References

1. Lehman C, Cuomo F, Kummer FJ, Zuckerman JD: **The incidence of full thickness rotator cuff tears in a large cadaveric population**. *Bull Hosp Jt Dis* 1995, **54**(1):30-31.

2. Fuchs S, Chylarecki C, Langenbrinck A: **Incidence and symptoms of clinically manifest rotator cuff lesions**. *Int J Sports Med* 1999, **20**(3):201-205.

3. Galatz LM, Ball CM, Teefey SA, Middleton WD, Yamaguchi K: **The Outcome and Repair Integrity of Completely Arthroscopically Repaired Large and Massive Rotator Cuff Tears**. *The Journal of Bone & Joint Surgery* 2004, **86**(2):219-224.

4. Harryman DT, 2nd, Mack LA, Wang KY, Jackins SE, Richardson ML, Matsen FA, 3rd: **Repairs of the rotator cuff. Correlation of functional results with integrity of the cuff**. *J Bone Joint Surg Am* 1991, **73**(7):982-989.

5. Gerber C, Meyer DC, Schneeberger AG, Hoppeler H, von Rechenberg B: **Effect of tendon release and delayed repair on the structure of the muscles of the rotator cuff: an experimental study in sheep**. *J Bone Joint Surg Am* 2004, **86-A**(9):1973-1982.

6. Goutallier D, Postel JM, Bernageau J, Lavau L, Voisin MC: **Fatty muscle degeneration in cuff ruptures. Pre- and postoperative evaluation by CT scan**. *Clin Orthop* 1994, **304**(304):78-83.

7. Meyer DC, Gerber C, Von Rechenberg B, Wirth SH, Farshad M: **Amplitude and strength of muscle contraction are reduced in experimental tears of the rotator cuff**. *Am J Sports Med* 2011, **39**(7):1456-1461.

8. Safran O, Derwin KA, Powell K, Iannotti JP: **Changes in rotator cuff muscle volume, fat content, and passive mechanics after chronic detachment in a canine model**. *J Bone Joint Surg Am* 2005, **87**(12):2662-2670.

9. Tomioka T, Minagawa H, Kijima H, Yamamoto N, Abe H, Maesani M, Kikuchi K, Shimada Y, Itoi E: **Sarcomere length of torn rotator cuff muscle**. *J Shoulder Elbow Surg* 2009, **18**(6):955-959.

10. Powell PL, Roy RR, Kanim P, Bello M, Edgerton VR: **Predictability of skeletal muscle tension from architectural determinations in guinea pig hindlimbs**. *Journal of Applied Physiology* 1984, **57**:1715-1721.

11. Bodine SC, Roy RR, Meadows DA, Zernicke RF, Sacks RD, Fournier M, Edgerton VR: **Architectural, histochemical, and contractile characteristics of a unique biarticular muscle: the cat semitendinosus**. *J Neurophysiol* 1982, **48**:192-201.

12. Winters TM, Takahashi M, Lieber RL, Ward SR: **Whole muscle length-tension relationships are accurately modeled as scaled sarcomeres in rabbit hindlimb muscles**. *J Biomech* 2011, **44**(1):109-115.

13. Hersche O, Gerber C: **Passive tension in the supraspinatus musculotendinous unit after long-standing rupture of its tendon: a preliminary report**. *J Shoulder Elbow Surg* 1998, **7**(4):393-396.

14. Gimbel JA, Mehta S, Van Kleunen JP, Williams GR, Soslowsky LJ: **The tension required at repair to reappose the supraspinatus tendon to bone rapidly increases after injury**. *Clin Orthop Relat Res* 2004(426):258-265.

15. Gimbel JA, Van Kleunen JP, Lake SP, Williams GR, Soslowsky LJ: **The role of repair tension on tendon to bone healing in an animal model of chronic rotator cuff tears**. *J Biomech* 2007, **40**(3):561-568.

16. Prado LG, Makarenko I, Andresen C, Kruger M, Opitz CA, Linke WA: **Isoform diversity of giant proteins in relation to passive and active contractile properties of rabbit skeletal muscles**. *The Journal of general physiology* 2005, **126**(5):461-480.

17. Ward SR, Sarver JJ, Eng CM, Kwan A, Wurgler-Hauri CC, Perry SM, Williams GR, Soslowsky LJ, Lieber RL: **Plasticity of muscle architecture after supraspinatus tears**. *J Orthop Sports Phys Ther* 2010, **40**(11):729-735.

18. Mallon WJ, Wilson RJ, Basamania CJ: **The association of suprascapular neuropathy with massive rotator cuff tears: a preliminary report**. *J Shoulder Elbow Surg* 2006, **15**(4):395-398.

19. Costouros JG, Porramatikul M, Lie DT, Warner JJ: **Reversal of suprascapular neuropathy following arthroscopic repair of massive supraspinatus and infraspinatus rotator cuff tears**. *Arthroscopy* 2007, **23**(11):1152-1161.

20. Kannus P, Leppala J, Lehto M, Sievanen H, Heinonen A, Jarvinen M: **A rotator cuff rupture produces permanent osteoporosis in the affected extremity, but not in those with whom shoulder function has returned to normal**. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 1995, **10**(8):1263-1271.

21. Kim HM, Galatz LM, Lim C, Havlioglu N, Thomopoulos S: **The effect of tear size and nerve injury on rotator cuff muscle fatty degeneration in a rodent animal model**. *Journal of Shoulder and Elbow Surgery* 2012, **21**(7):847-858.

22. Liu X, Laron D, Natsuhara K, Manzano G, Kim HT, Feeley BT: **A mouse model of massive rotator cuff tears**. *J Bone Joint Surg Am* 2012, **94**(7):e41.

23. Killian ML, Lim CT, Thomopoulos S, Charlton N, Kim HM, Galatz LM: **The effect of unloading on gene expression of healthy and injured rotator cuffs**. *Journal of Orthopaedic Research* 2013, **31**(8):1240-1248.

24. Samagh SP, Kramer EJ, Melkus G, Laron D, Bodendorfer BM, Natsuhara K, Kim HT, Liu X, Feeley BT: **MRI quantification of fatty infiltration and muscle atrophy in a mouse model of rotator cuff tears**. *J Orthop Res* 2013, **31**(3):421-426.

25. Schmutz S, Fuchs T, Regenfelder F, Steinmann P, Zumstein M, Fuchs B: **Expression of Atrophy mRNA Relates to Tendon Tear Size in Supraspinatus Muscle**. *Clinical Orthopaedics and Related Research* 2009, **467**(2):457-464.

26. Warren CM, Krzesinski PR, Greaser ML: **Vertical agarose gel electrophoresis and electroblotting of high-molecular-weight proteins**. *Electrophoresis* 2003, **24**:1695-1702.

27. Edwards CA, O'Brien Jr WD: **Modified assay for determination of hydroxyproline in a tissue hydrolyzate**. *Clinica Chimica Acta* 1980, **104**(2):161-167.

28. Neuman RE, Logan MA: **THE DETERMINATION OF COLLAGEN AND ELASTIN IN TISSUES**. *Journal of Biological Chemistry* 1950, **186**(2):549-556.

29. Karsenty G, Ferron M: **The contribution of bone to whole-organism physiology**. *Nature* 2012, **481**(7381):314-320.

30. Mannava S, Plate JF, Whitlock PW, Callahan MF, Seyler TM, Koman LA, Smith TL, Tuohy CJ: **Evaluation of in vivo rotator cuff muscle function after acute and chronic detachment of the supraspinatus tendon: an experimental study in an animal model**. *J Bone Joint Surg Am* 2011, **93**(18):1702-1711.

31. Liu X, Joshi SK, Samagh SP, Dang YX, Laron D, Lovett DH, Bodine SC, Kim HT, Feeley BT: **Evaluation of Akt/mTOR activity in muscle atrophy after rotator cuff tears in a rat model**. *J Orthop Res* 2012, **30**(9):1440-1446.

32. Joshi SK, Kim HT, Feeley BT, Liu X: **Differential ubiquitin-proteasome and autophagy signaling following rotator cuff tears and suprascapular nerve injury**. *J Orthop Res* 2014, **32**(1):138-144.

CHAPTER 3 : SKELETAL MUSCLE FIBROSIS AND STIFFNESS INCREASE AFTER ROTATOR CUFF TENDON INJURY AND NEUROMUSCULAR COMPROMISE IN A RAT MODEL

3.1 Abstract

Rotator cuff tears can cause irreversible changes (e.g., fibrosis) to the structure and function of the injured muscle(s). Fibrosis leads to increased muscle stiffness resulting in increased tension at the rotator cuff repair site. This tension influences repairability and healing potential in the clinical setting. However, the micro- and meso-scale structural and molecular sources of these whole-muscle mechanical changes are poorly understood. Here, single muscle fiber and fiber bundle passive mechanical testing was performed on rat supraspinatus and infraspinatus muscles with experimentally induced massive rotator cuff tears (Tenotomy) as well as massive tears with chemical denervation (Tenotomy+BTX) at 8 and 16 weeks post-injury. Titin molecular weight, collagen content, and myosin heavy chain profiles were measured and correlated with mechanical variables. Single fiber stiffness was not different between controls and experimental groups. However, fiber bundle stiffness was significantly increased at 8 weeks in the Tenotomy+BTX group compared to Tenotomy or control groups. Many of the changes were resolved by 16 weeks. Only fiber bundle passive mechanics was weakly correlated with collagen content. These data suggest that tendon injury with concomitant neuromuscular compromise results in extracellular matrix production

and increases in stiffness of the muscle, potentially complicating subsequent attempts for surgical repair.

3.2 Introduction

Rotator cuff tears are a common degenerative condition found in approximately 30% of patients over 60 years of age [1]. Symptoms have been reported in approximately 7% of all elderly patients, resulting in pain and loss of functional range of motion in the shoulder [2]. While surgical treatment and repair of the tendon is possible, structural failure rates have been reported as high as 20–94%, with increasing rates of failure coinciding with the size of the tear and the age of the patient [3, 4].

Increased passive stiffness of the rotator cuff muscle-tendon unit has been measured in both human and animals models and has been implicated as one reason for the difficultly in repairing massive rotator cuff tears [5, 6] and for poor tendon-to-bone healing [7]. Previous work in sheep and canine models has suggested an association between increased passive stiffness and the proliferation of collagen in muscle following chronic rotator cuff tears [8, 9]. However, it is unclear if the observed increases in whole muscle stiffness were due to changes at the single fiber and/or the fiber-bundle hierarchical levels of the muscles. Fiber- and fiber bundle-level changes in passive stiffness likely contribute to degenerative changes in rotator cuff muscle and are driven by accumulation and/or altered structure of a number of proteins such as titin [10] and collagen [11]. Likewise,

traction to the suprascapular nerve and subsequent denervation of the rotator cuff muscles has been associated with massive rotator cuff tears, and may accelerate degenerative changes in the muscle and contribute to poor clinical outcomes [12, 13]. Determining the scale and protein-level sources of passive tension following rotator cuff injury may yield insight into therapeutic targets for mitigating tear-related fibrosis in the rotator cuff and improve healing potential after surgical repair.

To address the aforementioned questions, a controlled investigation using a rodent model of massive rotator cuff injury was performed [14]. The objectives of our study were to identify muscle passive mechanical outcomes and protein-level adaptations at the single fiber and fiber bundle scales in a rodent model of massive rotator cuff tear with or without nerve injury. We hypothesized that fiber and fiber bundle passive stiffness would both increase with time and injury severity, and that these mechanical changes would be associated with smaller titin isoforms at the fiber level and increased collagen content at the bundle level.

3.3 Materials and Methods

Animal Model and Surgical Methods

All animal procedures were approved by the institutional Animal Studies Committee. Twenty-four male Sprague-Dawley rats (506.6 ± 69.1g at the time of sacrifice) were used in this study. In order to investigate the effect of a large rotator cuff tendon injury with and without denervation, animals were subjected to bilateral dual tenotomy of the supraspinatus (SS) and infraspinatus (IS), with chemical

denervation via botulinum toxin A (BTX) injection on the left shoulder and without BTX on the right shoulder. All surgeries were performed under sterile conditions. Briefly, animals were anesthetized using isofluorane carried by 1% oxygen. The injury site was sterilized with iodine preparation. Dual-tendon injury for both Tenotomy and Tenotomy+BTX shoulders was performed as follows: a 2-cm vertical incision was made over the scapulohumeral joint, and the deltoid was detached from the cranial and lateral aspects of the acromion using electrocautery. The acromion was elevated using a 3-0 Vicril suture passed around the spine of the scapula. The SS and IS tendons were exposed by supination of the forearm and transected via No.11 blade at the insertion on the humeral head. For the left shoulders (Tenotomy+BTX group), the above tendon transections were performed following BTX injections, whereby BTX was diluted in sterile saline and injected into both the SS and IS muscle bellies (2.5U/125μl per muscle). The right SS and IS muscles of each animal were injected with a volume-matched injection of saline (Tenotomy group). Following tenotomy and injections, the deltoid and trapezius muscles were then reattached using 3-0 Vicryl suture and the skin was closed using staples. Postoperative animal care was administered by an animal care technician, staples were removed within 10 days post-injury, and animals were allowed free cage activity until sacrifice time points of either 8 wk (animal age ~4 months) or 16 wk (animal age ~ 6 months) post-surgery. Normal, uninjured animals were time-zero age-matched to serve as comparative controls (N=6). Control animals did not undergo sham surgery.

At the time of sacrifice, SS and IS muscles were individually dissected and divided in half along their intramuscular tendons. The superior half of each muscle was placed in storage solution for passive mechanical testing and the inferior half was snap frozen in liquid nitrogen-chilled isopentane and stored at −80°C for subsequent biochemical analysis.

Passive Mechanics of Single Fibers and Fiber Bundles

Single fiber and fiber bundle testing was carried out as previously described [15]. Detailed methods and representative stress-strain curves (Fig. S1) are available in the Supplemental Materials.

Tangent modulus was defined as the tangent to the quadratic fit of the stress-strain curves between 2.0 and 4.0 μm. Tangent modulus was taken at the strain corresponding to a sarcomere length of 3.2 μm, as this is sarcomere length of human SS and IS muscles in the anatomical position [16]. This is within the physiological range of sarcomere lengths for rat skeletal muscle [17]. Three fibers and three fiber bundles were tested from each muscle sample and moduli were averaged. Samples were discarded if they did not produce a clear diffraction pattern, if any irregularities appeared along their length, or if they were severed or slipped at suture attachment points during testing.

Titin Molecular Weight Determination

Titin molecular weight was quantified using a previously developed method utilizing SDS-VAGE18. Detailed methods and a representative gel image (Fig. S2) are available in the Supplemental Materials.

Collagen Content

The hydroxyproline content of the muscles was used to determine collagen content (μg collagen/mg wet weight tissue) using a modification of a previously published protocol [19]. Detailed methods are available in the Supplemental Materials.

Myosin Heavy Chain

Muscle fiber type was estimated using myosin heavy chain (MHC) composition as previously described [20]. Detailed methods and a representative gel image (Fig. S3) are available in the Supplemental Materials.

Statistical Analysis

After screening the data for normality and homogeneity of variances, two-way ANOVA with post-hoc Sidak tests were used to determine significant differences between treatments, time-points, and between individual groups within each time-point. Linear regression was used to determine correlations between mechanical and protein-level variables. Statistical analyses were performed using SPSS 20.0 (IBM, Armonk, NY) and Prism 6.0b (GraphPad Inc, La Jolla, CA). Significance was set at $p < 0.05$ and all data are presented as Mean ± SD.

3.4 Results

Single Fiber Passive Mechanics

At the single fiber level, the SS and IS muscles became less stiff between 8 and 16 weeks ($p = 0.018$ and $p = 0.001$, respectively), regardless of injury (Fig. 1A–B). In the SS muscle, there was also a significant interaction between time and injury

(p=0.001); muscle fibers from the Tenotomy+BTX injured muscles tended to be more stiff at 8 weeks and less stiff at 16 weeks. Although there were no significant differences between injury types at 8 weeks, by 16 weeks the Tenotomy (12.68±3.86 kPa/μm) and Tenotomy+BTX (6.59±2.35 kPa/μm) groups were significantly less stiff ($p<0.05$) than controls (22.48±10.87 kPa/μm; Fig. 1A). When comparing individual groups across time, the Tenotomy+BTX group was significantly less stiff ($p<0.001$) at 16 weeks compared to 8 weeks (23.26±6.77 kPa/μm). In the IS muscle, there were no injury type main effects or time main effects, but fiber stiffness significantly decreased from 8 weeks to 16 weeks in both Tenotomy (p=0.041) and Tenotomy+BTX groups (p=0.022), Fig. 1B).

Titin Isoform

In the SS muscle, at 16 weeks, Tenotomy (3521±35 kDa) and Tenotomy+BTX (3540±36 kDa) resulted in significantly smaller titin isoforms compared to controls (3633±110 kDa) (Fig. 1C). There was an observed time effect in the IS muscle, as titin molecular weight significantly increased from 8 to 16 weeks (p=0.002; Fig. 1D). Specifically, controls increased in titin molecular weight from 8 to 16 weeks (p=0.002), with a similar trend observed in Tenotomy+BTX groups (p=0.055). At 16 weeks, Tenotomy alone (3591±67 kDa) showed a trend (p=0.070) toward decreased titin molecular weight, while Tenotomy+BTX (3586±38 kDa) resulted in significantly decreased titin molecular weight compared to Control (3668±83 kDa; p=0.049).

Muscle Fiber Bundle Passive Mechanics

In SS and IS muscles at both 8 and 16 weeks, Tenotomy+BTX fiber bundles tended to have increased stiffness compared to controls or Tenotomy alone (Fig. 2A–B). At 8 weeks, Tenotomy+BTX (SS=186.60±44.54 kPa/μm; IS=122.2±37.64 kPa/μm) had significantly stiffer fiber bundles than controls (SS=60.96±16.57 kPa/μm, $p<0.001$; IS=58.67±8.58 kPa/μm, $p=0.002$) or Tenotomy alone (SS=64.90±19.01 kPa/μm, $p<0.001$; IS=54.61±21.71 kPa/μm, $p=0.001$). In the SS muscle at 16 weeks, Tenotomy+BTX fiber bundles (90.18±56.72 kPa/μm) were significantly stiffer than controls (33.89±7.73 kPa/μm) but not different than Tenotomy only. The SS muscles of all three groups were less stiff at 16 weeks than at 8 weeks ($p< 0.001$). In the IS muscle at 16 weeks, there was a trend for Tenotomy+BTX (64.00±50.39 kPa/μm) fiber bundles to be stiffer than Tenotomy alone (22.90±11.18 kPa/μm, $p=0.066$), but were similar to control fiber bundles. Fiber bundle passive mechanics of the IS muscle in all three groups were less stiff at 16 weeks than at 8 weeks ($p<0.001$).

Collagen Content

In SS and IS muscles, control (SS $p=0.003$; IS $p=0.008$) and Tenotomy (SS $p=0.002$; IS $p=0.001$) muscle collagen content significantly increased from 8 to 16 weeks, respectively (Fig. 2C–D). In SS muscle at 8 weeks, the Tenotomy+BTX (22.80±7.93 μg/mg) fiber bundles had significantly increased collagen content compared to control (12.10±4.08 μg/mg) and Tenotomy alone (11.79±4.52 μg/mg) (Fig. 2C). In the IS muscle, there were no significant differences in collagen content between groups at 8 or 16 weeks (Fig. 2D).

Myosin Heavy Chain Composition

Results, figure (Fig. S4) and discussion related to myosin heavy chain composition are summarized in the Supplementary Materials.

Passive Mechanics and Biochemical Correlations

There was no observed correlation between single fiber titin molecular weight and single fiber tangent modulus across both muscles and time points (Fig. 3A). Similarly, there was no correlation between fiber bundle tangent modulus and collagen content across all muscles and time points. However, there was a significant, but weak, correlation between fiber bundle tangent modulus and collagen content within the 8-week samples (R^2=0.20, p=0.0046; Fig. 3B).

3.5 Discussion

The purpose of this study was to characterize the adaptations in rotator cuff muscle passive mechanics after injury at the micro- and mesoscale and to correlate these changes with the underlying protein-level adaptations in the muscles. Contrary to our hypothesis, results suggest that increases in muscle stiffness after injury do not originate at the single fiber level and are not supported by changes in titin molecular weight. We did observe a decrease in titin molecular weights at 16 weeks in both Tenotomy and Tenotomy+BTX groups compared to controls. We expected this to be associated with stiffer muscle fibers [10] compared to controls, but our micromechanical data at the fiber level did not support this relationship. However, results demonstrated that muscle fiber bundles had increased stiffness after injury when (BTX) was used in conjunction with tenotomy. Consistent with

this change, collagen content was elevated in this group, and positively correlated with fiber bundle stiffness at 8 weeks. At 16 weeks, collagen content was elevated in all groups, including controls, but the magnitude of the increased bundle stiffness was diminished. The observation that collagen content was elevated following massive tendon injury with BTX, indicating muscle fibrosis, supports previous whole-muscle experiments in rotator cuff muscles [8, 9]. This is consistent with our recent work showing that collagen content is weakly correlated to fiber bundle stiffness [21, 22]. However, the weak correlations suggest that the structural adaptations which lead to increased stiffness in rotator cuff tears may not simply be due to an increased collagen fraction in the muscle. Alternative sources and mechanisms of stiffness should therefore be explored. Sources of increased muscle stiffness may include changes to collagen cross-linking, increased collagen fibril diameter, or alternations in collagen isoform expression and are appropriate future experimental directions. Furthermore, the cellular sources and targets for suppression of fibrosis remain unknown.

The passive tangent stiffness data in this experiment were analyzed at a sarcomere length of 3.2 μm because this represents the length of the SS and IS muscles measured in humans in the anatomical position [16], and is a meaningful length in terms of passive tension generation in muscle. Recent data from our laboratory have measured the sarcomere lengths of rat SS and IS muscles in the anatomical position to be closer to 2.3–2.4 μm [23]. However, when we analyzed the muscle tangent stiffness at 2.4 and 2.8 μm, we found no change in the pattern of

differences between groups. Therefore, we believe that our conclusions, based on our measurements at 3.2 μm, are independent of our analytical methods.

Contrary to the original hypothesis, the passive stiffness of Tenotomy+BTX muscles decreased from 8 to 16 week. This surprising outcome may be due to a combination of factors, including aging and recovery of the muscle from a singular BTX injury over time. Recent data from our lab show that muscle architecture features of uninjured rotator cuff muscles in rats change with age [23], so it is possible that biochemistry and passive mechanical properties also change as a result of aging. Prior animals studies using BTX injections to paralyze skeletal muscle have shown that the effects of BTX last beyond 8 weeks [24-26], with some functional recovery observed after 100 days post-injection [27]. Further experiments are necessary to uncouple the effects of aging and recovery from BTX from changes in passive mechanical properties.

Clinically, repair of chronic, large rotator cuff tears becomes increasingly difficult over time. Chronic changes in the muscles include atrophy, fat accumulation, and general loss of plasticity. Beyond a certain level of retraction, the cuff becomes very difficult to mobilize and restore normal length. As a result, tears potentially become irreparable or repairs are under such high tension that mechanical failure of repairs results. These changes likely have implications to muscle function as well, making it difficult to restore strength even after successful repair. This study demonstrates increased stiffness at the bundle level associated with a massive tear, especially when accelerated by further chemical impairment of

neuromuscular function. While the exact mechanism remains unknown, we have established a model that can be used for further study. Furthermore, we also found a decrease in fiber stiffness with injury, both with and without BTX. This may reflect pathologic changes secondary to mechanical unloading and/or neuromuscular disruption that may have implications with regards to muscle function and deterioration with time.

The current study has several limitations. First, our study did not test whole muscle-tendon unit passive mechanics, which would ultimately determine the amount of passive stress in the muscle during repair. Whole muscle passive mechanical measurements in addition to fiber and bundle mechanics may be useful in corroborating the clinical manifestations of these observed changes. However, previous reports have already described increased tension in rotator cuff tendon-muscle units after tenotomy, including in the rat [5, 6]. Second, our assay for fibrosis, the hydroxyproline assay, only measures the changes in fractional amounts of collagen within the tissue. Future studies will be required to parse out the potential contributing factors leading to increased passive stiffness, including collagen type, collagen organization, and other non-collagenous components which compose the muscle extracellular matrix. Third, although BTX is frequently used to produce chronic and severe rotator cuff degeneration in rat models, the causes of degeneration in patients with rotator cuff disease are not fully understood. This is an experimental issue that should be explored prior to searching for a source of increased passive mechanical stiffness in BTX-induced denervation models. Last, we

studied a relatively short time periods. Chronic muscle changes take years to develop in humans, and we studies short-term responses. A longer time frame may have more effectively replicated the clinical scenario, but this is a limitation of animal modeling.

Conclusions

In summary, massive rotator cuff tears, combined with chemical denervation, result in increased passive stiffness at the muscle fiber bundle level in SS and IS muscles. Our findings suggest that adaptations leading to increased stiffness of the rotator cuff muscles occur not within the muscle fibers themselves, but at the fiber bundle level via contributions from newly deposited extracellular matrix. However, the molecular and protein-level mechanisms that lead to this increase in stiffness were only partially elucidated by the current study. Further study of these mechanisms of fibrosis will be required to determine therapeutics targets to mitigate muscle fibrosis and ultimately improve the success rate of rotator cuff repairs.

3.6 Supplemental Materials

Detailed Methods for Passive Mechanics of Single Fibers and Fiber Bundles

Single fiber and fiber bundle testing was carried out as previously described [15]. Briefly, the dissected fiber or fiber bundle segment was secured on both sides to 125 μm titanium wires using 10-0 silk sutures. One wire was secured to an ultrasensitive force transducer (Model 405A, sensitivity 10 V/g, Aurora Scientific, Ontario, Canada) and the other was secured to a micromanipulator (Model-405A,

Aurora Scientific, Aurora, Ontario, Canada). Sarcomere length measurements were measured in real-time by laser diffraction using a 7mW diode laser (Coherent Laser, Auburn, CA), as previously described [23]. Samples were lengthened until force registered on the force transducer, which was defined as the baseline load, and slack sarcomere length (L_0) was then measured. Initial sample diameters were optically measured with a cross-hair reticule mounted on a dissecting microscope and micromanipulators on an x–y mobile stage. Force-displacement data were generated for each mounted sample in 250 μm increments after which stress-relaxation was permitted for 3 min and both sarcomere length and tension were again recorded. Segments were elongated until samples demonstrated a measured sarcomere length greater than 4.0 μm. Force data were converted to stress by dividing force by the baseline cross-sectional area and displacement was converted to strain by subtracting sarcomere length (L) from the baseline slack sarcomere length and then dividing by the baseline slack sarcomere length value ($\Delta L/ L_0$).

Tangent modulus was defined as the tangent to the quadratic fit of the stress-strain curves (Fig. S1) between 2.0 and 4.0 μm. Tangent modulus was taken at the strain corresponding to a sarcomere length of 3.2 μm, as this is sarcomere length of human SS and IS muscles in the anatomical position [16]. This is within the physiological range of sarcomere lengths for rat skeletal muscle [17]. Three fibers and three fiber bundles were tested from each muscle sample and moduli were averaged. Samples were discarded if they did not produce a clear diffraction pattern,

if any irregularities appeared along their length, or if they were severed or slipped at suture attachment points during testing.

Detailed Methods for Titin Molecular Weight Determination

Titin molecular weight was quantified using a previously developed method utilizing SDS-VAGE [18]. Protein homogenates were prepared from frozen samples and loaded into 1% agarose gels. A small 12.8% acrylamide plug was placed at the bottom of the gel apparatus to hold the agarose gel in place. Human soleus and rat cardiac muscles were used as titin standards. Gels were run two at a time at 25 mA for 4.5 h at 4 °C. Gels were stained according to the BioRad Silver Stain Plus kit protocol, and bands were identified and quantified using densitometry (Quantity One; Bio-Rad). Representative gel lanes are presented in Fig. S2.

Detailed Methods for Collagen Content

The hydroxyproline content of the muscles was used to determine collagen content (μg collagen/mg wet weight tissue) using a modification of a previously published protocol [19]. Frozen samples were hydrolyzed in 6 mol l–1 HCl at 110°C overnight and neutralized with NaOH to pH 6.98–7.04. Samples were then incubated with a chloramine T solution for 20 min at room temperature, followed by addition of a *p*-dimethylaminobenzaldehyde solution and incubation at 60 °C for 30 min. Hydroxyproline concentration was determined by spectrophotometry at 550 nm and was normalized to the wet mass of the original tissue sample. Standard solutions provided a calibration curve for spectrophotometry. Measured hydroxyproline content was used to calculate collagen amount using the constant

7.46, corresponding to the average number of hydroxyproline residues in a collagen molecule [24].

Detailed Methods for Myosin Heavy Chain Composition

MHC composition was quantified using a previously developed method [25]. Homogenized protein solution was resuspended to 0.125 μg μl−1 protein (BCA protein assay, Pierce, Rockford, IL, USA) in a sample buffer consisting of 100 mmol l−1 DTT, 2% SDS, 80 mmol l−1 Tris-base pH 6.8, 10% glycerol and 0.01% w/v Bromophenol Blue. Samples were boiled (2 min) and stored at 80°C. Prior to being loaded on the gel, the protein solution was further diluted 1:20 (0.006 μg μl−1) in the sample buffer and10 μl of each sample were loaded in each lane. Acrylamide concentrations in the gels were 4% and 8% in the stacking and resolving gels, respectively (bisacrylamide, 1:50). Gels (16×22 cm, 0.75 mm thick) were run at a constant current of 10 mA for 1 h, and then at a constant voltage of 275 V for 20 h at 4–6°C. Gels were stained according to the BioRad Silver Stain Plus kit protocol, and bands were identified and quantified using densitometry (Quantity One; Bio-Rad), as previously described [20]. Mouse soleus was prepared (as described above) and utilized as a standard lane on all gels showing all four MHC bands (IIa, IIx,IIb, and I). Representative gel lanes are presented in Fig. S3.

Results of Myosin Heavy Chain Composition

There was a significant decrease in type I MHC isoform contribution in the SS from 8 to 16 weeks in Tenotomy (8wk = 10.1±2.1%; 16wk = 2.9±3.5%) and Tenotomy+BTX (8wk = 9.4±2.0%; 16wk = 4.5±4.7%; Fig S4A). Corresponding

differences between time points in both injury groups were observed in the IS muscle for type I MHC composition (8wk Tenotomy = 10.1±2.7% vs. 16wk Tenotomy = 3.9±3.5%; 8wk Tenotomy+BTX = 9.9±2.6% vs. 16wk Tenotomy+BTX = 3.2±2.4%; Fig S4B). There were no differences in type IIa MHC composition in either muscle. In the SS muscle, the Tenotomy+BTX SS muscles were composed of a higher proportion of type IIx MHC isoform compared to control ($p=0.048$) and Tenotomy only ($p=0.005$) groups at 8 weeks and compared to Tenotomy alone group at 16 weeks. Increased type IIx MHC isoform composition corresponded with decreased type IIb MHC isoform composition following Tenotomy+BTX injury compared to controls at 8 weeks and compared to Tenotomy alone at 16 weeks, indicating that the muscle was "slower" after nerve injury (BTX). There were no observed effects in myosin type IIb MHC isoform composition in the IS muscle. In the SS and IS muscles, for all groups including control, the relative fraction of type I MHC isoform decreased from 8 to 16 weeks ($p<0.001$; Fig S4A–B).

Discussion of Myosin Heavy Chain Composition

There were few changes observed in MHC isoform composition. We observed a time-dependent decrease in type I MHC isoform composition in the injury groups. In addition, there was an observed a shift of type IIb isoform to type IIx isoform in the nerve injured (BTX) muscles. It is possible that the observed shift in MHC composition to slower isoforms may be specific to the use of BTX as our method of neuromuscular impairment, as this shift has been observed previously in other muscles following injury induced via BTX [26, 27], but not following neurotomy [28].

3.7 Acknowledgements

Chapter 3 is nearly identical to a peer-reviewed article entitled “Skeletal muscle fibrosis and stiffness increase after rotator cuff tendon injury and neuromuscular compromise in a rat model” by Sato EJ, Killian ML, Choi AJ, Lin E, Esparza MC, Galatz LM; Thomopoulos S, and Ward SR. The article was published in the *Journal of Orthopaedic Research* 2014, **32**(9):1111-1116. The authors also wish to acknowledge the technical support of Drs. Chanteak Lim, Leonardo Cavinatto, and Ki-Seok Lee. The sources of funding for this study were; NIH R01 AR057836, NIH R24 HD050837, NIH P30 AR057235, NIH T32 AR060712.

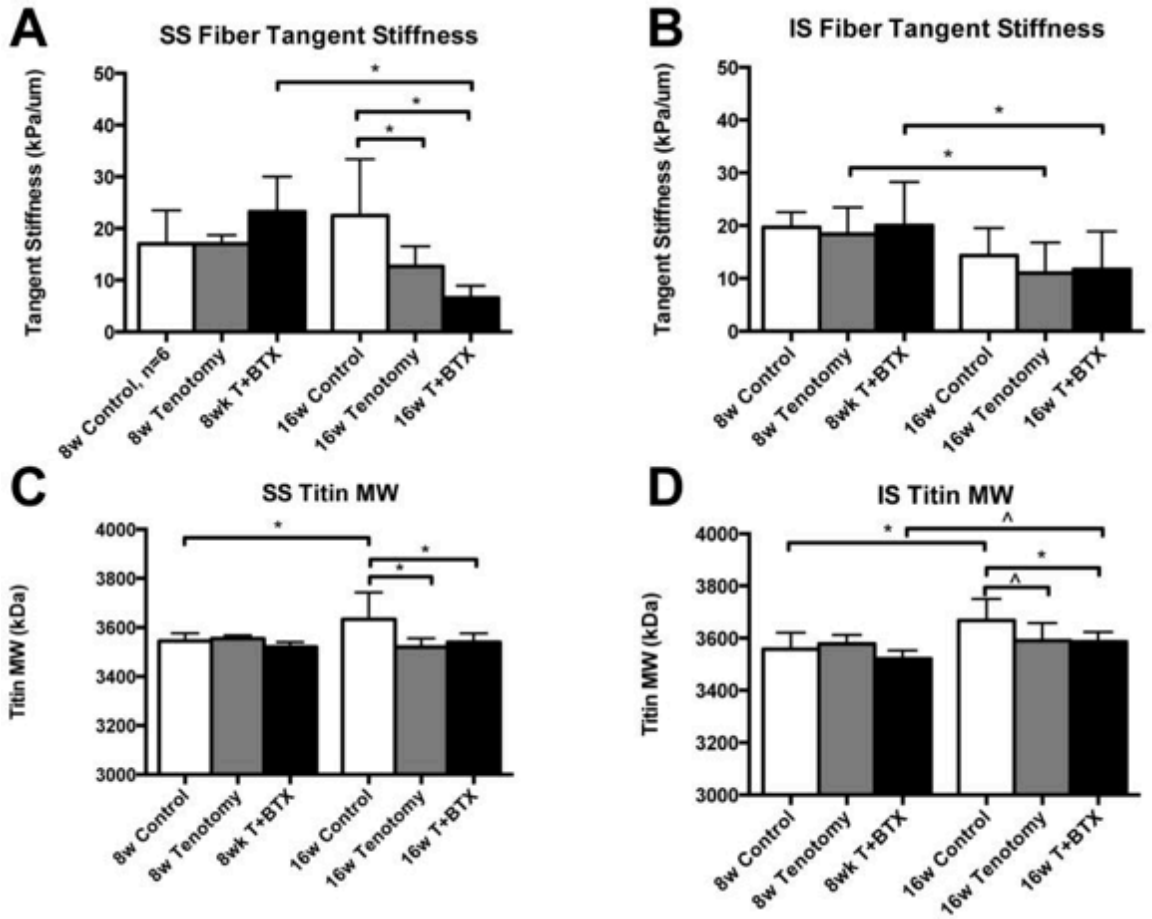

Figure 3.1 Single fiber tangent stiffness and titin molecular weight
The SS (A) and IS (B) single muscle fibers became less stiff between 8 and 16 weeks, regardless of injury. Whole muscle titin molecular weight was lower in the injured SS (C) and IS (D) muscles compared to controls at 16 weeks. In both muscles, this effect appears to be related to larger titin isoforms in the control group at 16 weeks compared to 8 weeks. $N = 6$ for each group. * Indicates $p < 0.05$ for individual comparisons, and ^ indicates $p < 0.1$.

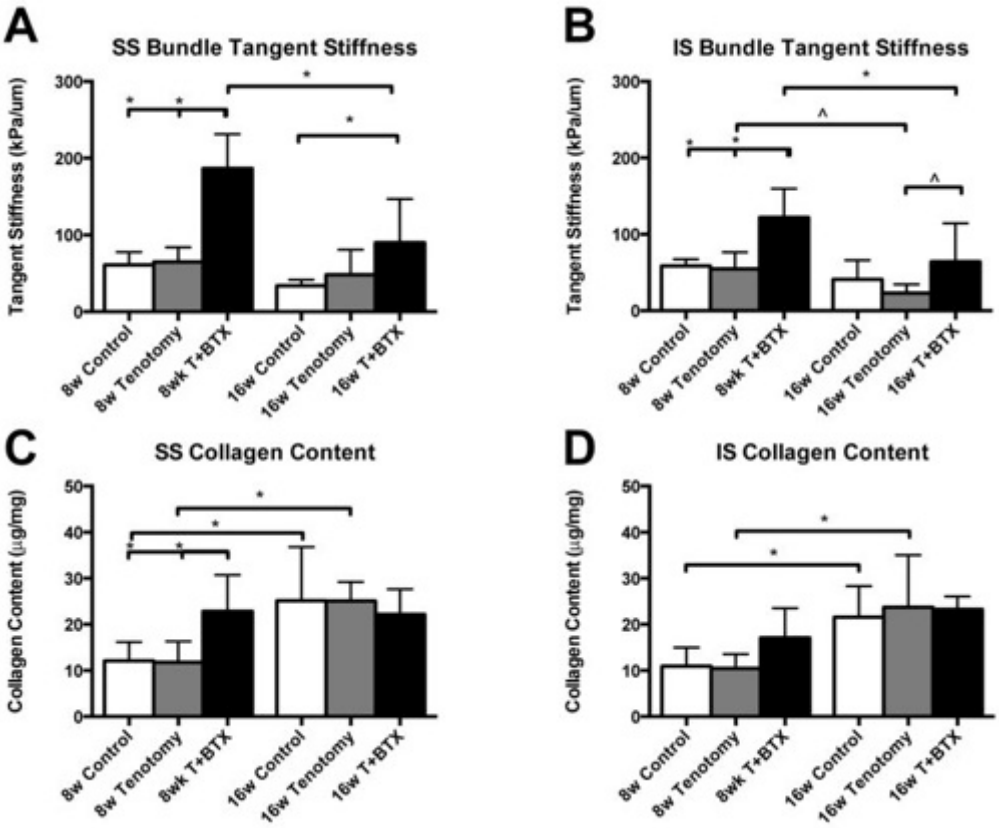

Figure 3.2 Bundle tangent stiffness and collagen content
The SS (A) and IS (B) muscle fiber bundles were more stiff in the Tenotomy + BTX group at both 8 and 16 weeks, although this effect was attenuated by 16 weeks. At 8 weeks, there was also an increase in collagen content in the SS muscle (C). This trend was observed in the IS muscle (D), but it does not achieve significance. By 16 weeks, there were no differences between groups as control and Tenotomy groups had great collagen content at this later time point. $N = 6$ for each group. *$p < 0.05$ for individual comparisons, and ^ $p < 0.1$.

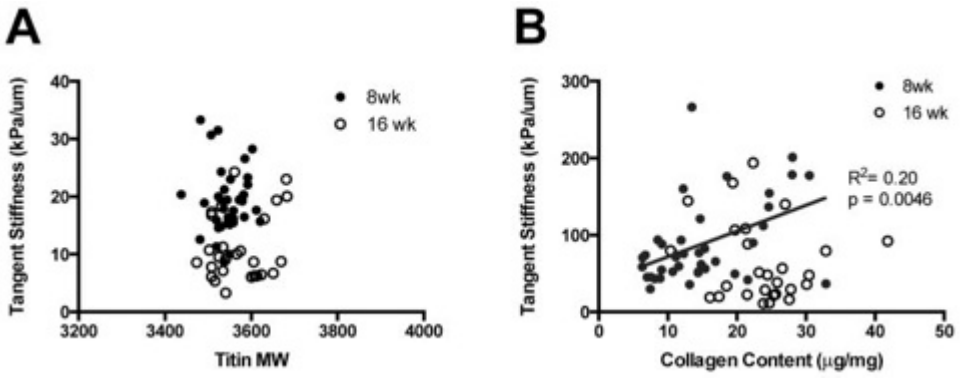

Figure 3.3 Correlations between biochemical measures and passive stiffness
Single fiber stiffness was not related to titin molecular weight at 8 or 16 weeks (A). However, was a positive linear relationship (R^2 = 0.20, p = 0.0046) between collagen content and muscle fiber bundle tangent modulus at 8 weeks, but not at 16 weeks (B).

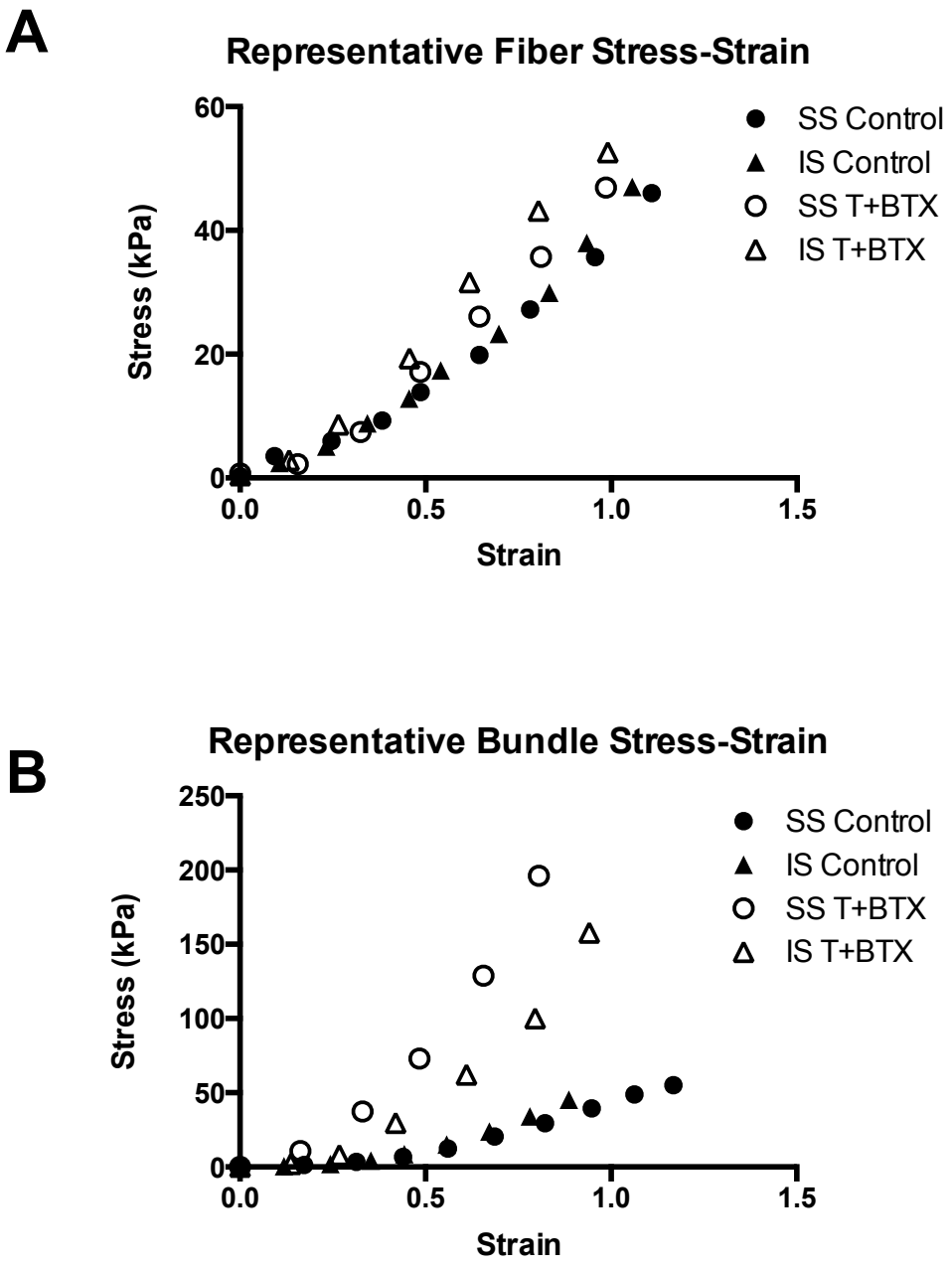

Figure 3.S1 Representative stress-strain data for control and T+BTX groups. (A) Muscle fiber data for SS and IS muscles are animal matched. (B) Muscle fiber bundle data for SS and IS muscles are animal matched. (● = Control SS; ○=T+BTX SS; ▲=Control IS; ‣=T+BTX IS)

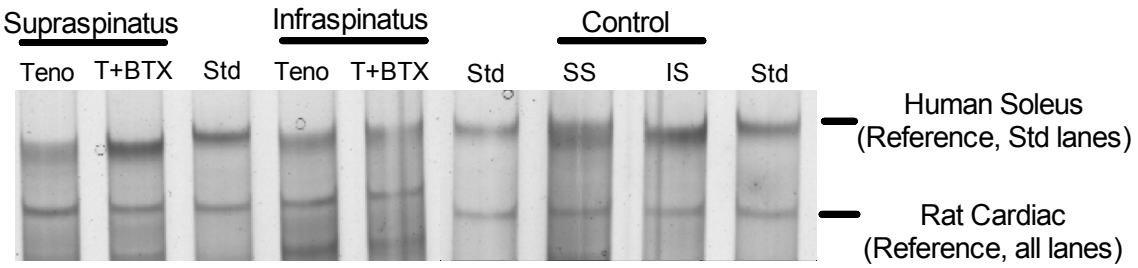

Figure 3.S2 Representative images of titin gels used for titin molecular weight determination.
Representative images of titin gels used for titin molecular weight determination. Injured data are animal matched and represent data from the 16 wk time point.

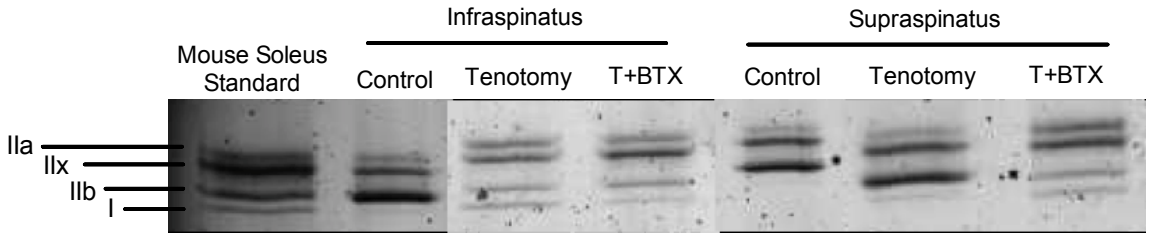

Figure 3.S3 Representative image of MHC gels used to determine relative MHC composition.

Representative image of MHC gels used to determine relative MHC composition. Injured data are animal matched and represent data from the 8 wk time point.

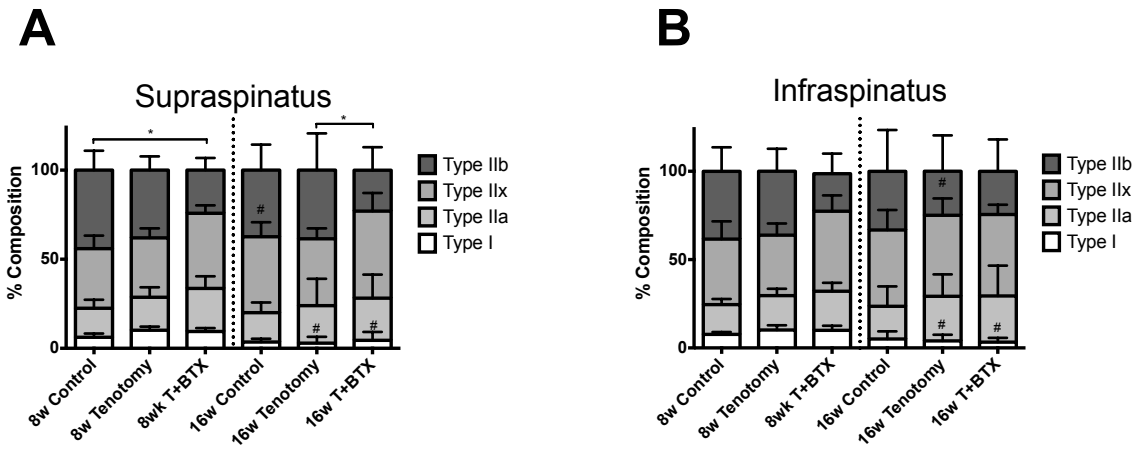

Figure 3.S4 Myosin heavy-chain composition
In the SS muscle (A), there was an increase in type IIx MHC in the Tenotomy+BTX group compared to Control or Tenotomy at 8 weeks and compared to Tenotomy at 16 weeks. There was also significant decrease in type I MHC in the SS (A) and IS (B) muscles in both injury groups between 8 and 16 weeks. * $p<0.05$ for individual comparisons, and # $p<0.05$ versus

3.8 References

1. Lehman C, Cuomo F, Kummer FJ, Zuckerman JD: **The incidence of full thickness rotator cuff tears in a large cadaveric population**. *Bull Hosp Jt Dis* 1995, **54**(1):30-31.

2. Fuchs S, Chylarecki C, Langenbrinck A: **Incidence and symptoms of clinically manifest rotator cuff lesions**. *Int J Sports Med* 1999, **20**(3):201-205.

3. Galatz LM, Ball CM, Teefey SA, Middleton WD, Yamaguchi K: **The Outcome and Repair Integrity of Completely Arthroscopically Repaired Large and Massive Rotator Cuff Tears**. *The Journal of Bone & Joint Surgery* 2004, **86**(2):219-224.

4. Harryman DT, 2nd, Mack LA, Wang KY, Jackins SE, Richardson ML, Matsen FA, 3rd: **Repairs of the rotator cuff. Correlation of functional results with integrity of the cuff**. *J Bone Joint Surg Am* 1991, **73**(7):982-989.

5. Hersche O, Gerber C: **Passive tension in the supraspinatus musculotendinous unit after long-standing rupture of its tendon: a preliminary report**. *J Shoulder Elbow Surg* 1998, **7**(4):393-396.

6. Gimbel JA, Mehta S, Van Kleunen JP, Williams GR, Soslowsky LJ: **The tension required at repair to reappose the supraspinatus tendon to bone rapidly increases after injury**. *Clin Orthop Relat Res* 2004(426):258-265.

7. Gimbel JA, Van Kleunen JP, Lake SP, Williams GR, Soslowsky LJ: **The role of repair tension on tendon to bone healing in an animal model of chronic rotator cuff tears**. *J Biomech* 2007, **40**(3):561-568.

8. Safran O, Derwin KA, Powell K, Iannotti JP: **Changes in rotator cuff muscle volume, fat content, and passive mechanics after chronic detachment in a canine model**. *J Bone Joint Surg Am* 2005, **87**(12):2662-2670.

9. Gerber C, Meyer DC, Schneeberger AG, Hoppeler H, von Rechenberg B: **Effect of tendon release and delayed repair on the structure of the muscles of the rotator cuff: an experimental study in sheep**. *J Bone Joint Surg Am* 2004, **86-A**(9):1973-1982.

10. Prado LG, Makarenko I, Andresen C, Kruger M, Opitz CA, Linke WA: **Isoform diversity of giant proteins in relation to passive and active contractile properties of rabbit skeletal muscles**. *The Journal of general physiology* 2005, **126**(5):461-480.

11. Williams PE, Goldspink G: **Connective tissue changes in immobilised muscle**. *J Anat* 1984, **138**:343.

12. Mallon WJ, Wilson RJ, Basamania CJ: **The association of suprascapular neuropathy with massive rotator cuff tears: a preliminary report**. *J Shoulder Elbow Surg* 2006, **15**(4):395-398.

13. Costouros JG, Porramatikul M, Lie DT, Warner JJ: **Reversal of suprascapular neuropathy following arthroscopic repair of massive supraspinatus and infraspinatus rotator cuff tears**. *Arthroscopy* 2007, **23**(11):1152-1161.

14. Kim HM, Galatz LM, Lim C, Havlioglu N, Thomopoulos S: **The effect of tear size and nerve injury on rotator cuff muscle fatty degeneration in a rodent animal model**. *Journal of Shoulder and Elbow Surgery* 2012, **21**(7):847-858.

15. Fridén J, Lieber RL: **Spastic muscle cells are shorter and stiffer than normal cells**. *Muscle Nerve* 2003, **27**:157-164.

16. Ward SR, Hentzen ER, Smallwood LH, Eastlack RK, Burns KA, Fithian DC, Friden J, Lieber RL: **Rotator cuff muscle architecture: implications for glenohumeral stability**. *Clin Orthop Relat Res* 2006, **448**(Jul):157-163.

17. ter Keurs HE, Luff AR, Luff SE: **Force--sarcomere-length relation and filament length in rat extensor digitorum muscle**. *Adv Exp Med Biol* 1984, **170**:511-525.

18. Warren CM, Krzesinski PR, Greaser ML: **Vertical agarose gel electrophoresis and electroblotting of high-molecular-weight proteins**. *Electrophoresis* 2003, **24**:1695-1702.

19. Edwards CA, O'Brien Jr WD: **Modified assay for determination of hydroxyproline in a tissue hydrolyzate**. *Clinica Chimica Acta* 1980, **104**(2):161-167.

20. Talmadge RJ, Roy RR: **Elecrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms**. *Journal of Applied Physiology* 1993, **75**:2337.

21. Ward SR, Tomiya A, Regev GJ, Thacker BE, Benzl RC, Kim CW, Lieber RL: **Passive mechanical properties of the lumbar multifidus muscle support its role as a stabilizer**. *J Biomech* 2009, **42**(10):1384-1389.

22. Smith LR, Lee KS, Ward SR, Chambers HG, Lieber RL: **Hamstring contractures in children with spastic cerebral palsy result from a stiffer extracellular matrix and increased in vivo sarcomere length**. *J Physiol* 2011, **589**(Pt 10):2625-2639.

23. Swan MA, Sato E, Galatz LM, Thomopoulos S, Ward SR: **The Effect of Age on Rat Rotator Cuff Muscle Architecture**. *Journal of Shoulder and Elbow Surgery* in press.

24. Han N, Kim HD, Eom MJ, You JM, Han J, Kim HK, Kang MS: **Proteomic changes in rat gastrocnemius muscle after botulinum toxin a injection**. *Annals of rehabilitation medicine* 2013, **37**(2):157-166.

25. Thacker BE, Tomiya A, Hulst JB, Suzuki KP, Bremner SN, Gastwirt RF, Greaser ML, Lieber RL, Ward SR: **Passive mechanical properties and related proteins change with botulinum neurotoxin A injection of normal skeletal muscle**. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2011.

26. Killian ML, Lim CT, Thomopoulos S, Charlton N, Kim HM, Galatz LM: **The effect of unloading on gene expression of healthy and injured rotator cuffs**. *Journal of Orthopaedic Research* 2013, **31**(8):1240-1248.

27. Billante CR, Zealear DL, Billante M, Reyes JH, Sant'Anna G, Rodriguez R, Stone RE: **Comparison of neuromuscular blockade and recovery with botulinum toxins A and F**. *Muscle & Nerve* 2002, **26**(3):395-403.

28. Lieber RL, Yeh Y, Baskin RJ: **Sarcomere length determination using laser diffraction. Effect of beam and fiber diameter**. *Biophysical journal* 1984, **45**(5):1007-1016.

29. Neuman RE, Logan MA: **THE DETERMINATION OF COLLAGEN AND ELASTIN IN TISSUES**. *Journal of Biological Chemistry* 1950, **186**(2):549-556.

30. Tirrell TF, Cook MS, Carr JA, Lin E, Ward SR, Lieber RL: **Human skeletal muscle biochemical diversity**. *The Journal of Experimental Biology* 2012, **215**(15):2551-2559.

31. Legerlotz K, Matthews KG, McMahon CD, Smith HK: **Botulinum toxin-induced paralysis leads to slower myosin heavy chain isoform composition and reduced titin content in juvenile rat gastrocnemius muscle**. *Muscle Nerve* 2009, **39**(4):472-479.

32. Rowshan K, Hadley S, Pham K, Caiozzo V, Lee TQ, Gupta R: **Development of fatty atrophy after neurologic and rotator cuff injuries in an animal model of rotator cuff pathology**. *J Bone Joint Surg Am* 2010, **92**(13):2270-2278.

CHAPTER 4 : HIERARCHICAL STRAIN DISTRIBUTIONS IN A RAT MODEL OF ROTATOR CUFF TEAR

4.1 Abstract

Background: Rotator cuff tears have been shown to impair muscle function in small animal models. However, the mild muscle structure changes associated with these injuries do not support the observed functional changes. One possible reason for the departure between muscle structure and function in this model is that the underlying length-tension behavior of the muscle after a tendon tear is unknown. Therefore, the purpose of this study was to understand the strain relationships between whole muscle, fibers, and sarcomeres in a rat model of massive rotator cuff tear.

Methods: Supraspinatus (SS) muscles (N=60) eight weeks after dual tenotomy injury or sham surgery were stretched and formalin fixed at 0%, 5%, 10%, and 20% of whole muscle strain. Fixed SS muscles were then dissected, allowing muscle mass and length, and muscle fiber and sarcomere lengths to be measured from seven predefined regions. A separate set of muscles were set aside for histological examination of extracellular matrix area fraction, muscle fiber area, and centralized nuclei. Data were analyzed using t-tests to compare whole muscle mass, muscle length, baseline sarcomere length and serial sarcomere number. Two-way ANOVAs were used to compare groups for fiber length and sarcomere length, and two-way repeated measures ANOVA (region x injury group) were used to compare muscle fiber area, centralized nuclei, and ECM area fraction in histology.

Results: Overall, muscle mass and length decreased after tenotomy. Tenotomy did not result in shorter muscle fibers or sarcomeres at baseline. Fiber length and sarcomere length were not different between groups at 0%, 5%, and 10% whole muscle strains. However, at 20% strain tenotomized muscles had significantly shorter muscle fibers (14.21±0.40 mm vs 15.95±0.41 mm, p=0.002) and sarcomere lengths (3.16±0.07 μm vs 3.49±0.07 μm, p=0.004). Quantification of histology showed no differences in extracellular matrix area fraction, muscle fiber area, or number of centralized nuclei.

Conclusion: These data suggest that chronically torn muscles exhibit normal sarcomere strain behavior at low muscle strains, but fail to lengthen at high strains. This is likely due to impaired strain transmission from the whole muscle to the fibers. This may have clinical implications in situations when muscles may be subjected to high strains, such as during a rotator cuff repair.

4.2 Introduction

Rotator cuff tears are a common orthopaedic condition that affects 30% of patients over 60 years of age[1], resulting in pain and functional deficits in the shoulder[2]. While surgical repair of the tendon is often possible, 20-94% of these repairs fail, with increasing incidence of failure associated with increasing patient age and the size of the initial tear[3, 4].

While descriptive and clinical studies are prevalent in the literature, scientific studies in human patients are difficult to control due to the asymptomatic onset of many rotator cuff tears[5, 6]. To improve experimental control, the rat model of

rotator cuff tear has been used extensively due to its anatomical similarity to humans[7]. While human and large animal studies have shown both decreased muscle cross sectional area[8-11] (a predictor of force producing capacity of the muscle) and fiber length[12] (a predictor of muscle excursion and velocity[13, 14]) in torn rotator cuff muscles, analogous studies in the rat model have shown only mild architectural changes[15, 16].

Studies investigating supraspinatus (SS) function in animals have shown reduced isometric force production[17] and possible narrowing of the length tension curve[18]. These functional deficits are counter to what would be predicted by the mild changes in muscle architecture. One possible explanation for this discrepancy could be due to differences in sarcomere lengths as the muscle is stretched during these experiments. The magnitude of isometric force produced by a muscle is determined by the sarcomere length,[19] thus the deficit in muscle force production in these experiments could be explained by differences in sarcomere lengths between normal and injured muscles when the muscle is stretched during functional testing. However, the relationship between muscle strain and sarcomere length in these muscles is unknown. Thus, the objective of this study was to investigate the transmission of strain from the whole muscle level to muscle fibers, and to the sarcomeres following a chronic tenotomy injury.

4.3 Materials and Methods

Animal Model and Surgical Methods

Three month old male Sprague-Dawley rats (N=60) were used for this study. Animals were separated into two experimental groups: dual-tenotomy to the supraspinatus (SS) and infraspinatus (IS) tendons (TT starting weight = 402.6 ± 3.951g) and a control Sham surgery group (starting weight = 401.7 ± 8.407g). Following approval from the University's Animal Studies Committee, surgeries were performed under isofluorane-induced anesthesia using 1% oxygen carrier. Under sterile conditions, a 2-cm vertical incision was made over the scapulohumeral joint and the deltoid was cut transversely to access the rotator cuff. The SS tendon was exposed by supination of the forearm and a No.11 blade was used to transect the SS tendon at its insertion on the humeral head. The forearm was then internally rotated 45 degrees to expose the IS tendon, which was then transected from the humeral head using a No.11 blade. The surgeon visually confirmed retraction of the tendons. In the Sham animals, the tendons were identified but were not transected. The deltoid and trapezius muscles were then reattached using 4-0 PGA suture and the skin was closed subcutaneously using 4-0 PGA suture. Post-operative animal care was administered by an animal care technician.

Supraspinatus Strain

Animals were sacrificed 8 weeks after injury (Sham animal mass= 612.7 ± 10.86g; TT animal mass = 593.4 ± 13.26g). At the time of sacrifice, the entire left limb was transected *en bloc*, all superficial musculature was removed to expose the supraspinatus muscle. Subsequently, the acromion and clavicle were removed to visualize the distal pole of the SS muscle. Scar tissue was observed filling the lesion

between the humeral head and the SS/IS muscles in the tenotomized muscle. Muscle length was measured as the distance between the most proximal muscle fibers to the most distal muscle fibers, with the shoulder in neutral position. The tendons/scar tissue attaching the scapula to the humeral head were transected to isolate the scapula and its musculature. Then, the infraspinatus and subscapularis muscles were dissected, leaving only the supraspinatus muscle intact on the scapula.

Using a custom-designed device, the scapula was stabilized by clamping the infraspinatus fossa while a second clamp was used to connect the supraspinatus muscle-tendon junction (Figure 1). The muscle was stretched and held at lengths corresponding to 0% (n=5 per group), 5% (n=2 per group), 10% (n=8 per group) or 20% (n=8 per group) of its initial, predissection muscle length. The stretched samples were then fixed in 4% paraformaldehyde for 36 hours.

In a subset of animals (n=3 per group), the entire limb was fixed *en bloc* in 4% paraformaldehyde for measurements of baseline muscle properties and as a negative control for our device fixed muscles. In a separate subset of these animals (n=4 per group) muscles from the affected shoulder were instead individually dissected, snap frozen in liquid nitrogen, and stored at -80°C for biochemical and histological analysis.

Muscle Architecture

To confirm muscle strain was maintained throughout fixation, final muscle length was measured post-fixation. Specimens were sharply dissected from scapulae to isolate the supraspinatus muscle. The muscle was gently blotted dry,

and weighed. Muscle fiber, sarcomere length and sarcomere number were measured or computed from seven different regions of the muscle (4 anterior, 3 posterior) using methods previously described (Figure 2).[15] Because we did not measure resting sarcomere length differences between regions, they were averaged to obtain whole muscle values for each parameter.

In addition to plotting group data, linear regression of individual data points were plotted to create scatterplots of muscle strain-fiber length and fiber length-sarcomere length. Linear regression was used to measure the slope and intercept of the data points and ANCOVA analysis was used to compare slope and intercept between Sham and Tenotomy data.

Histology

Whole muscle cross sections were taken from the distal third, midbelly, and proximal third of the muscle, embedded in OCT, and sectioned for histological analysis. Hematoxylin and eosin (H&E) and Gomori Trichrome stains were used to qualitatively evaluate overall tissue composition and structure[20]. Trichrome stained slides were used for calculating the relative fraction of connective tissue in muscle cross-sections. A custom-written macro on ImageJ (NIH, Bethesda, MD) was employed to automatically quantify the relative fraction muscle fiber tissue and connective tissue based on staining color and intensity for the full cross-section of each sample[21]. Tendon area was subtracted from the connective tissue fraction to provide the fraction of the cross-section that was extracellular matrix (ECM area fraction).

Laminin and DAPI staining was carried out to quantify muscle fiber area and centralized nuclei as previously described[22]. Fiber cross-sectional areas and number of fibers containing centralized nuclei were counted automatically using a custom-written macro in ImageJ (NIH, Bethesda, MD) from 6 randomly selected fields per section.[23] Filtering criteria were applied to ensure measurement of actual muscle fibers and to exclude neurovascular structures and 'optically fused' fibers. Fiber cross-sectional area of the tenotomized muscles was corrected for differences in baseline sarcomere length under the assumption that total volume is conserved, such that $CS_1 \times Ls_1 = CS_2 \times Ls_2$.

Statistical Analysis

After screening the data for normality and homogeneity of variances, t-tests were used to compare whole muscle mass, muscle length, baseline sarcomere length and serial sarcomere number. Two-way ANOVAs (strain x injury group) were used to compare groups for fiber length and sarcomere length. Linear regression analysis was used to investigate correlations between muscle strain, fiber length, and sarcomere length. ANCOVA was used to compare the slopes of linear fits to the data. Two-way repeated measures ANOVA (region x injury group) were used to compare muscle fiber area, centralized nuclei, and ECM area fraction in histology. Post-hoc Sidak tests were performed to identify specific group differences. Statistical analyses were performed using SPSS 20.0 (IBM, Armonk, NY) and Prism 6.0b (GraphPad Inc, La Jolla, CA). Significance was set at $p < 0.05$ and all data are presented as Mean ± SEM.

4.4 Results

Baseline Whole Muscle Properties

In the tenotomized shoulders, scar tissue formation filling in the gap between humeral head and distal tendon stump was observed at time of harvest (Figure 3). Eight weeks of tenotomy resulted in a small but significant decrease in whole muscle mass (Sham=0.701±0.012 g, TT=0.607±0.015 g, $p<0.0001$; Figure 1A) as well as muscle length (Sham = 30.15 ± 0.2343 mm, TT = 28.01 ± 0.2392 mm, $p<0.0001$; Figure 4B). Measurements of fiber length and sarcomere length from shoulders fixed in neutral shoulder position showed no differences in sarcomere length between injury groups (Figure 4C-D).

Muscle Fiber and Sarcomere Length During Strain

Muscle fiber length was compared between injury groups at the four different strains. Muscle fiber lengths after tenotomy were not different from each other at 0%, 5% and 10% whole muscle strain (Figure 5A). However, at 20% of whole muscle strain, muscle fibers were significantly shorter in the tenotomy group (14.21±0.40 mm) than in the sham group (15.95±0.41 mm, $p=0.002$).

Similar to muscle fiber length measurements, sarcomere lengths between injury groups were not different from each other at 0%, 5%, and 10% strain (Figure 5B). However, Tenotomy muscles had significantly shorter sarcomere lengths (3.16±0.07 μm) at 20% muscle strain as compared to Sham (3.49±0.07 μm, $p=0.004$).

Theoretically, transmission of whole muscle strain to sarcomere strain requires effective strain transmission from the whole muscle to the muscle fibers, and subsequently from the muscle fibers to the sarcomeres. To investigate whether differences between injury groups at 20% strain were due to differences in the relationship between whole muscle strain-fiber length or muscle fiber length-sarcomere length, individual data points were plotted on graphs of muscle strain against fiber length and fiber length against sarcomere length. As expected, significant linear relationships were found between muscle strain and fiber length in Sham animals ($p < 0.0001$, $R^2=0.79$; Figure 6A). In Tenotomy, the linear relationship between muscle strain and fiber length showed greater variability in the data ($p=0.0002$, $R^2=0.49$), significant differences in y-intercept coefficient (Sham=2.41 ± 0.06 μm, TT = 2.35 ± 0.09μm, $p=0.0088$), and a trend toward a decreased slope compared to Sham (Sham=0.059 ± 0.005, TT=0.047 ± 0.007, $p=0.06$). Significant linear relationships between fiber length and sarcomere length were observed in both Sham and Tenotomy ($p<0.0001$; Figure 6B) muscles, with no differences between regression lines ($p=0.71$).

Histology

H&E staining showed no gross structural differences between groups. Analysis of trichrome stained sections showed no differences in ECM area fraction between Sham and Tenotomy groups (Figure 7A) or between regions (distal, middle, or proximal) of the muscle. Muscle sections stained for laminin and DAPI were used for the measurement of muscle fiber area and centralized nuclei. Fiber cross

sectional area of the tenotomy muscles was corrected for differences in baseline sarcomere length, resulting in a 4.5% reduction from the measured cross-sectional area. There were no differences between regions for either muscle fiber area or centralized nuclei. Muscle fiber areas were not different between injuries (Figure 7B). The number of centralized nuclei in the cross sections also remained unchanged following tenotomy (Figure 7C).

4.5 Discussion

The purpose of this study was to characterize the relationship between supraspinatus whole muscle strain, muscle fiber length and sarcomere length in a model of massive rotator cuff tear. Baseline muscle architecture data showed generally mild adaptations after tenotomy, which are consistent with previous studies measuring muscle dimensions at chronic time points[15, 24, 25]. While Tenotomy and Sham fiber length and sarcomere length were not different at baseline, muscle length was observed to be shorter in tenotomized muscles. Thus, we normalized the length change of each muscle as strain when analyzing the stretch data.

Functional data from Mannava et al. suggested that the active length-tension curves of the supraspinatus narrows following tenotomy[18], resulting in a smaller operating range for the muscle. However, when muscles were strained in this study, chronically tenotomized muscles behaved similarly to sham controls at low strains. However, at 20% of muscle strain, muscle fiber and sarcomere lengths were shorter in the Tenotomy muscles. Contrary to previous literature, these data would suggest

a widening of the length-tension curves, as sarcomere lengths were comparatively short at high muscle strains (Figure 8). Additional studies that carefully match sarcomere lengths during length-tension experiments are required to reconcile these results.

In this study, we compared how strain was transmitted from the whole muscle to the fibers and then the subsequently to the sarcomeres between groups using linear regression. There were no differences between injury groups when comparing strain transmission between fibers and sarcomeres. However, we observed significant differences in y-intercept and a trend for different slopes in muscle to fiber strain transmission between Sham and Tenotomy. These data suggest that abnormal transmission of muscle strain to the muscle fiber may be the cause of shorter observed sarcomeres at 20% strain in the tenotomized muscles.

Altered transmission of muscle strain to fibers suggests changes in extracellular components of the muscle (i.e. extracellular matrix, internal tendon), which typically transmit load to the muscle fibers. Our current study and prior studies have shown no differences in the properties of the extracellular matrix between muscle fibers, both in terms of the amount of collagen in the muscle[16, 25] or the mechanical stiffness in muscle bundles[26]. An alternative mechanism that would lead to impaired strain transmission is altered mechanical properties of the serial elastic components of the muscle. These would include the components that transmit load and strain between the muscle fiber and the distal tendon, including the internal tendon or aponeurosis and the myotendinous junctions. Strain-to-

failure experiments in whole muscles have shown that structural failure occurs at the muscle-tendon junction,.[27, 28] suggesting the vulnerability of these regions to injury during high muscle strains. Additional experiments are required to investigate whether structural failure may have occurred at these locations.

Quantitative evaluation of muscle histology showed no differences between groups or muscle regions within groups. Quantitative analysis of trichrome stained sections found no differences in ECM area fraction as a result of tenotomy. This agrees with previous findings showing no differences in collagen content from muscle homogenates after chronic tenotomy[16, 26]. Somewhat surprisingly, muscle fiber area was unchanged eight weeks post-tenotomy. These results are in contrast to decreased fiber area measured in human rotator cuff biopsies[29]. A previous study by Barton et al. in the rat model has shown that fiber area decreases initially before gradually recovering to normal values by 16 weeks after injury[25]. While this study measured reduced fiber area eight weeks after injury, it is important to note that the fiber areas were recovering towards normal values by this time point[25]. In addition, Barton et al. calculated fiber areas indirectly, which may account for the discrepancy with our results. Overall, these results suggest that the tenotomized muscle appears to be relatively healthy and would not suggest any functional deficits. This continues to reinforce the pattern of mild biological effects seen in this model as compared to the human injury.

These results may have clinical implications as muscle-tendon units are stretched during rotator cuff repairs. The fibers of the supraspinatus muscle are

relatively short compared to the whole muscle, and thus the fibers and sarcomeres undergo large strains relative to the whole muscle during strain. These high strains have previously been implicated in causing high passive tensions during rotator cuff repair[30, 31], but high strains may also suggest poor functional outcomes due to altered strain and potentially force transmission throughout the muscle. In addition, a study by Davis et al. showed strain-induced muscle fiber damage during rotator cuff repair in an animal model[32]. Taken together, these data imply that muscle strain needs to be carefully considered during rotator cuff repair, as large strains can result in structural and functional impairment.

The current study has several limitations. First, tenotomy in the animal model does not mimic the magnitude of muscle architectural or structural changes observed in human rotator cuff tears. The mild adaptations following tenotomy injury in this animal model is perhaps due to the tendency for the distal tendon stump to form scar tissue to fill the lesion, permitting partial load onto the muscle. These effects are likely compounded by continued growth of these animals during this time frame.[33] Second, we only looked at a limited number of discrete muscle strains in our experiment. Anatomical and technological limitations preclude us from measuring sarcomere length during muscle stretch without dissection, thus preventing us from obtaining multiple length measurements per muscle. The upper limit of twenty percent strain was chosen as this closely approximates the gap distance required to perform a rotator cuff repair in the Tenotomy animals. Third, our study did not measure the passive forces imparted on the muscle during strain.

Monitoring passive forces during stretching would have allowed us to parse out whether the shorter sarcomere lengths at large strains in the tenotomized muscles were due to increased compliance of the muscle at high strains or tissue failure.

In conclusion, these data suggest that tenotomized muscles exhibit abnormal strain transmission to the sarcomere at only high strains. This may have clinical implications in situations when muscles may be subjected to high strains, such as during a rotator cuff repair. Further research is needed to understand the mechanism driving these changes at large strains and how this may impact muscle function.

4.6 Supplemental Materials

The transmission of whole muscle strain down to the sarcomere can be separated into two distinct steps. First, muscle strain must be transmitted through the internal tendon to the muscle fibers. Second, muscle fiber strain must be transmitted intracellularly to the sarcomeres themselves. The results in Aim 3 suggest that transmission of strain from the muscle fiber to the sarcomere is conserved in both sham and tenotomized muscles, and that there is abnormal transmission of muscle strain to the muscle fibers at 20% strain.

One possible mechanism that could decouple muscle-fiber strain transmission could be structural damage/failure at several key structures and interfaces within the muscle. When the supraspinatus muscle is strained the structural integrity of the internal tendon, muscle-tendon junction, the muscle fiber, and the muscle fiber-bony at the surface of the scapula must be maintained to

ensure proper strain transmission. Using the remaining tissue from Aim 3, we looked for evidence of macroscopic structural damage of the muscle fibers, the muscle-tendon junction, and the internal tendon. The interface of the muscle and scapula was not investigated as the tissue had already been dissected off of the scapula for previous analysis.

Methods

Previously dissected muscle fiber bundles (11 bundles from 3 different muscles per group) were visualized optically under light microscopy at 40x magnification. The distance spanning 10 consecutive sarcomeres were measured and divided by the number of sarcomeres to obtain average sarcomere length. Three muscle fibers were investigated per bundle and averaged after the coefficient of variation between fibers was determined to be less five percent. Sarcomere lengths obtained optically were compared with the sarcomere lengths obtained using the laser diffraction method on the same sample.

Remaining fixed tissue from 20% strain groups (n=4/group) were sectioned (10μm thickness) longitudinally and stained with H&E in order to visualize the internal tendon and muscle-tendon junction. Two samples from each group were damaged during preparation and were excluded from the analysis. Samples from the mid-portion of the muscle were visually inspected along the internal tendon. The number of discontinuities between the muscle fiber and internal tendon at the muscle-tendon junction, and the frequency of discontinuities within the internal tendon were then quantified. Polarized-light microscopy was used to visualize the

presence of crimp within the tendon. Longitudinal sections from unstrained muscles were used as a negative control to confirm the presence of crimp structure when the muscle is unstrained.

Results

Muscle samples inspected under light microscopy did not show signs of damage along the length of individual muscle fibers (Figure S1). Sarcomere lengths measured optically agreed with lengths measured by laser diffraction (ICC=0.958).

There were no differences between groups in the fraction of discontinuous fibers at the muscle tendon junction (Sham = 0.32 ± 0.02,Tentomy = 0.14 ± 0.06; Figure S2) or the frequency of internal tendon discontinuities (Sham = 0.57 ± 0.39 mm^{-1}, Tentomy = 0.33 ± 0.15 mm^{-1}; Figure S3). Tendon crimp was observed in the unstrained muscles in both groups, and there were no differences in crimp frequency between groups (0.021±0.008 μm^{-1}; 0.030±0.006 μm^{-1}; Figure S4). In the 20% strained samples, crimping was not observed in either group.

Discussion

Sarcomere lengths measured using laser diffraction are an average of sarcomere lengths from many muscle fibers. Thus a muscle fiber bundle composed of fibers with long sarcomere lengths (strained) and short sarcomere lengths (damaged and shortened) may result in a short sarcomere length measurement using laser diffraction. However, there was no evidence of fiber damage, and sarcomere length variation was less than five percent between muscle fibers within a fiber bundle. Additionally, the sarcomere length values measured optically were

in good agreement with sarcomere lengths obtained via the laser diffraction method. These data confirm the values obtained via laser diffraction, and suggest that muscle fibers are not damaged at 20% muscle strain.

Previous experiments have shown that whole muscles fail at the myotendinous junction when under high strains.[27, 28] Thus, we hypothesized that strain transmission to muscle fibers may be disrupted at the muscle-tendon junction or within the internal tendon. High rates of structural damage or discontinuities within these structures would impair the transmission of strain throughout the muscle. Interestingly, we observed, on average, higher frequency of damage at the muscle tendon junction and the internal tendon in Sham-treated muscles. This effect was opposite of what was expected, and suggests that observed structural damage may be an artifact of tissue processing. Alternatively, differences in the material properties of these structures may contribute to the differences in damage frequency observed between groups. Thus future studies may focus on measuring the changes in the material properties of the internal tendon or muscle-tendon junction.

Unstrained and under-tensioned tendons exhibit a crimping structure, which is visible under polarized light, and this crimping structure disappears or decreases in frequency when the tendon is placed under strain.[34, 35] Thus, the presence of crimping structure in the internal tendons of strained muscles may suggest a loss of tension within the structure, leading to impaired strain transmission within the muscle. Internal tendons from unstretched muscles from both groups had

observable crimping structure. On average, tendons in the tenotomy group had a higher crimp frequency than Sham controls, which may suggest that the tendons in these muscles have altered mechanical properties at baseline and may warrant future study. When muscles were stretched to 20% strain, crimping was not observed in tendons from either group. This suggests that the tendons in both groups were still transmitting tension and strain within the muscle during the experiment.

The preceding analysis focused primarily on macroscopic defects within the serially compliant components within the muscle. Future studies should aim to measure the material properties of these serial components by recording the whole muscle passive tension. Loss of tension due to tissue failure and subsequent study of the failed tissue may elucidate the mechanism leading to altered strain transmission within tenotomized muscles.

4.7 Acknowledgements

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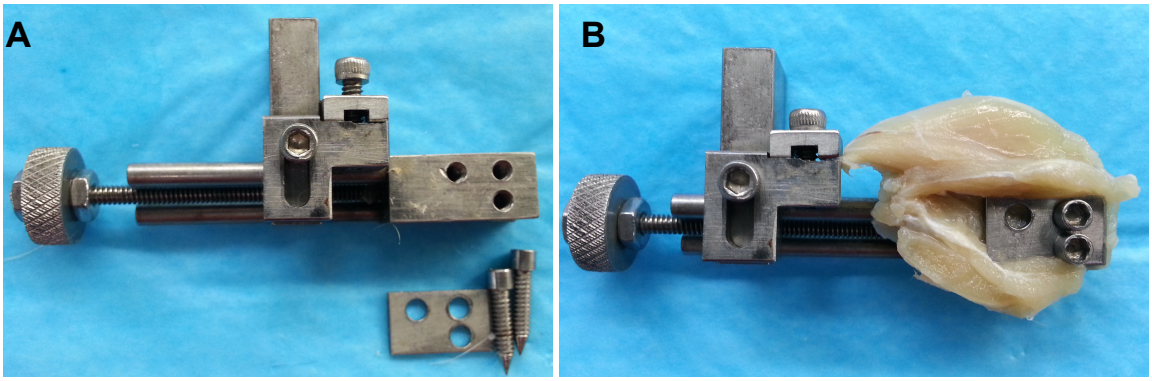

Figure 4.1 Image of supraspinatus muscle strain
Device designed for stabilizing the scapula and straining the supraspinatus muscle during fixation (A). Example image of a muscle after formalin fixation on the device (B).

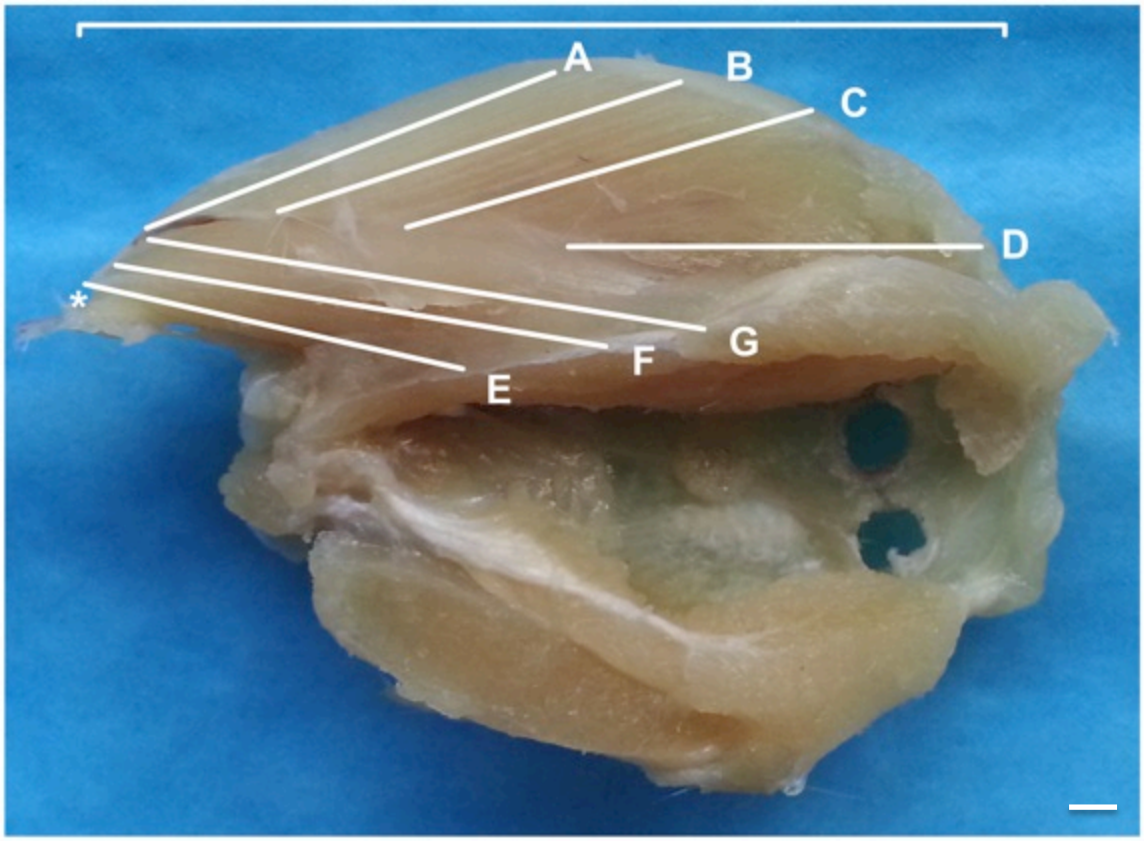

Figure 4.2 Representative posterior image of the left scapula after formalin fixation in the muscle strain device.
Final supraspinatus muscle length was measured after fixation (bracket). Muscle fiber length and sarcomere length were measured from 7 regions (solid lines, A-G) for analysis. * denotes the muscle-tendon junction.

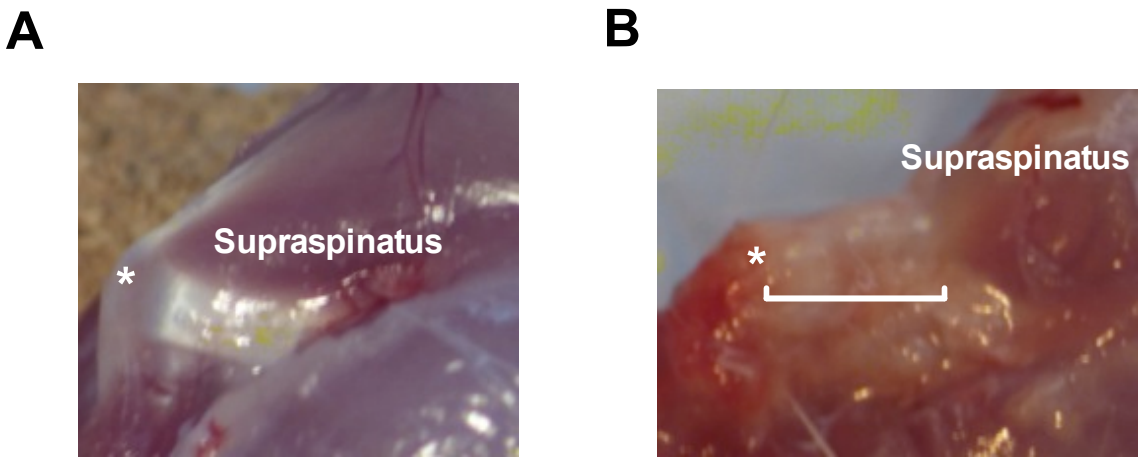

Figure 4.3 Representative images of the distal pole of the supraspinatus muscle at sacrifice.
Sham-operated shoulders (A) exhibited intact tendon insertion onto the humeral head. Scar formation was observed to fill in the gap (brackets) created by the retracted muscle in the tenotomized shoulders (B). * denotes the location of the humeral head.

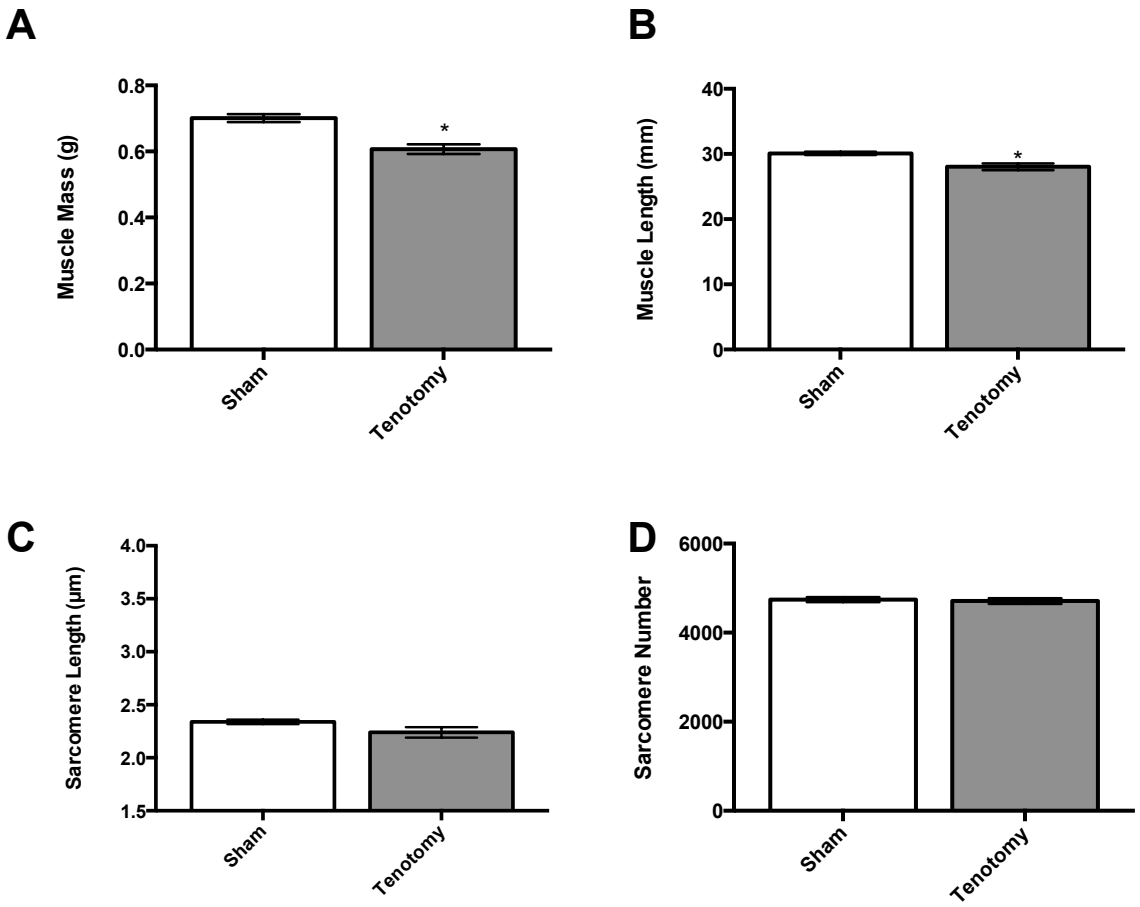

Figure 4.4 Architectural measurements in the supraspinatus muscle indicate mild changes in whole muscle properties.
Architectural measurements in the supraspinatus muscle indicate mild changes in whole muscle properties. Tenotomized muscles had significantly reduced muscle mass (A) and muscle length (B). Sarcomere length (C) and serial sarcomere number (D) were unchanged due to tenotomy. * $p<0.05$

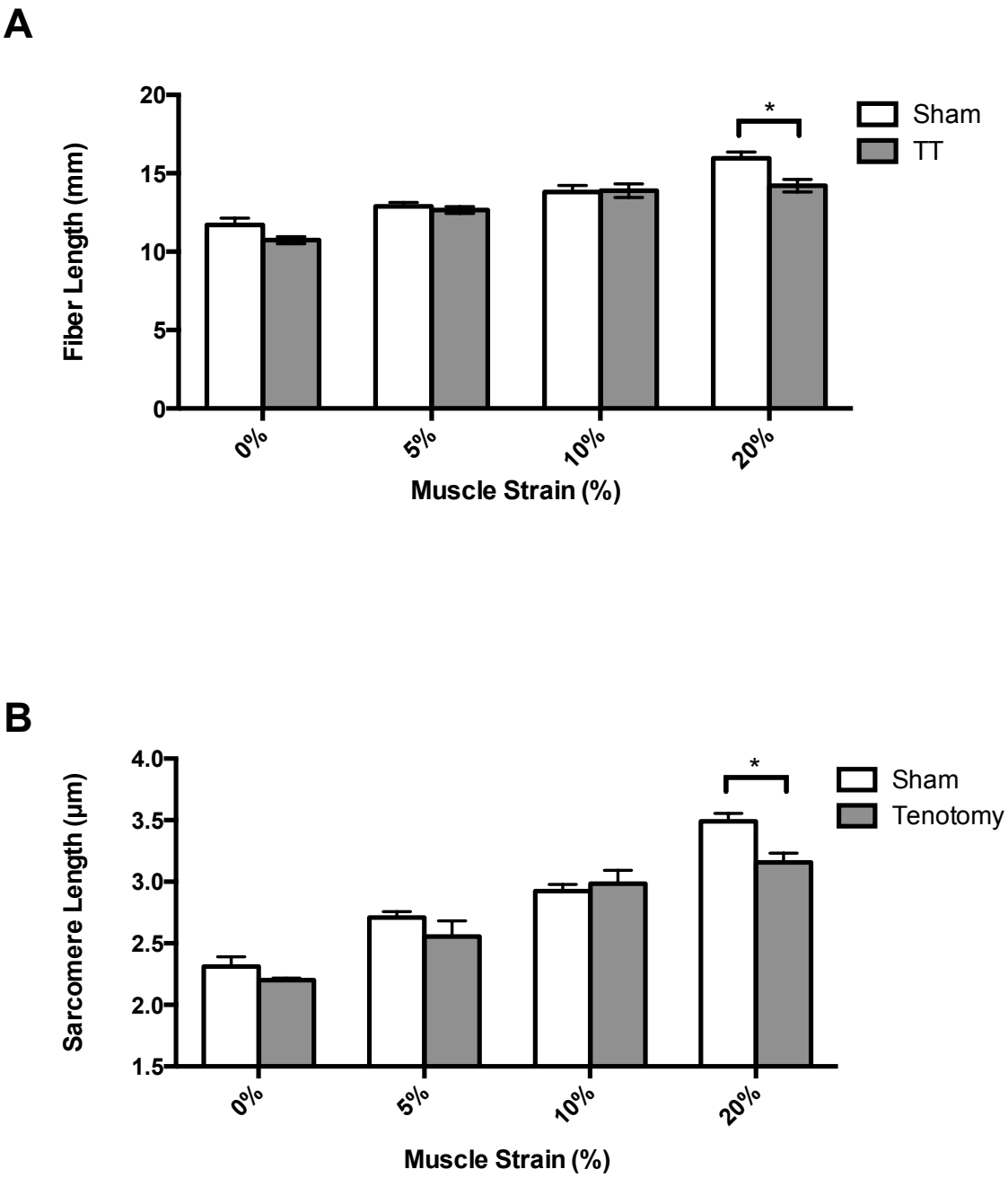

Figure 4.5 Average supraspinatus muscle fiber length and sarcomere length after 0%, 5%, 10%, and 20% strain

Average supraspinatus muscle fiber length (A) and sarcomere length (B) after 0%, 5%, 10%, and 20% strain. Sham (open bars) and Tenotomy (black bars) fiber and sarcomere lengths were not different from each other at low strains. Tenotomy muscles had significantly shorter muscle fiber and sarcomere lengths at 20% strain. * $p<0.05$

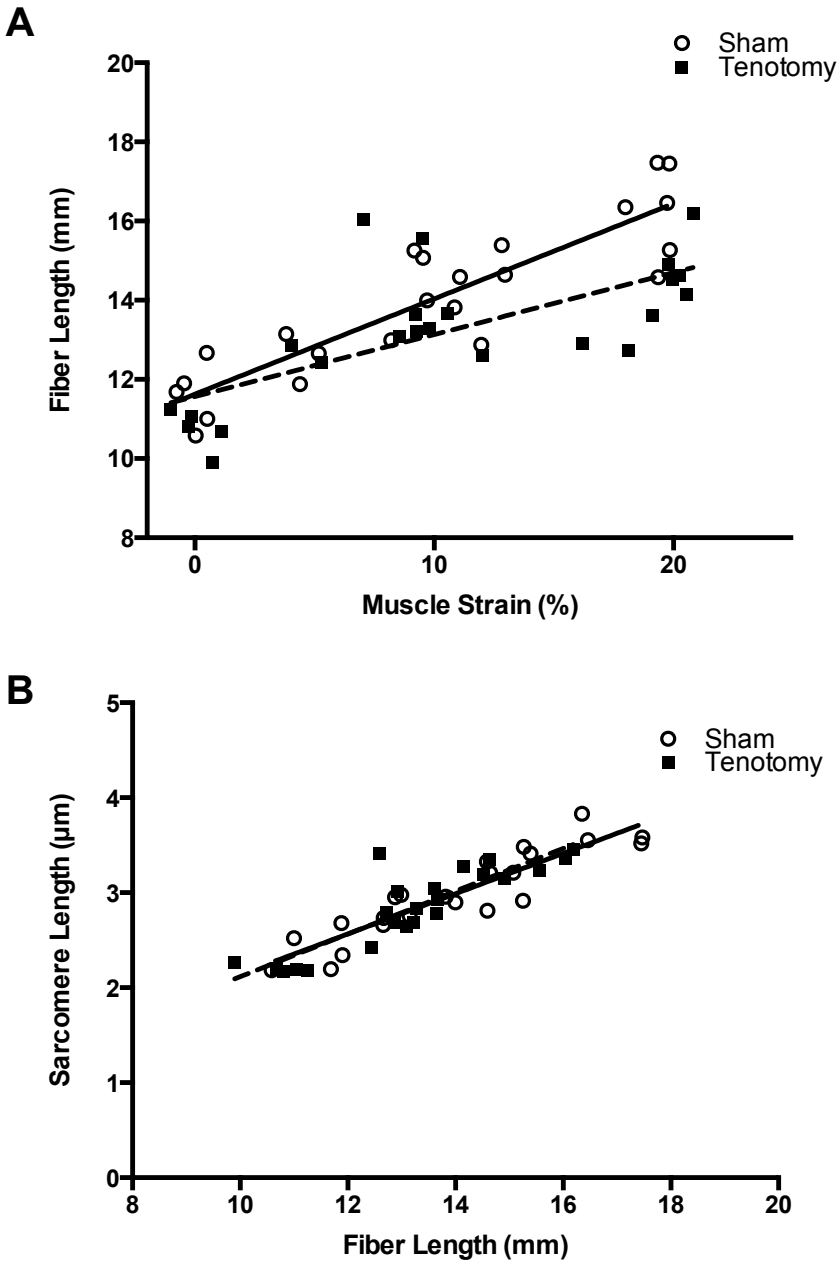

Figure 4.6 Scatter plots of muscle strain v. fiber length and fiber length v. sarcomere length

Scatter plot of muscle strain versus fiber length (A) and fiber length versus sarcomere length (B). Significant linear relationships are denoted by solid lines for sham, and dashed lines for tenotomy. (A) Comparison of regression lines show significantly different y-intercepts and a trend for different slopes. (B) Regression lines were not different from each other.

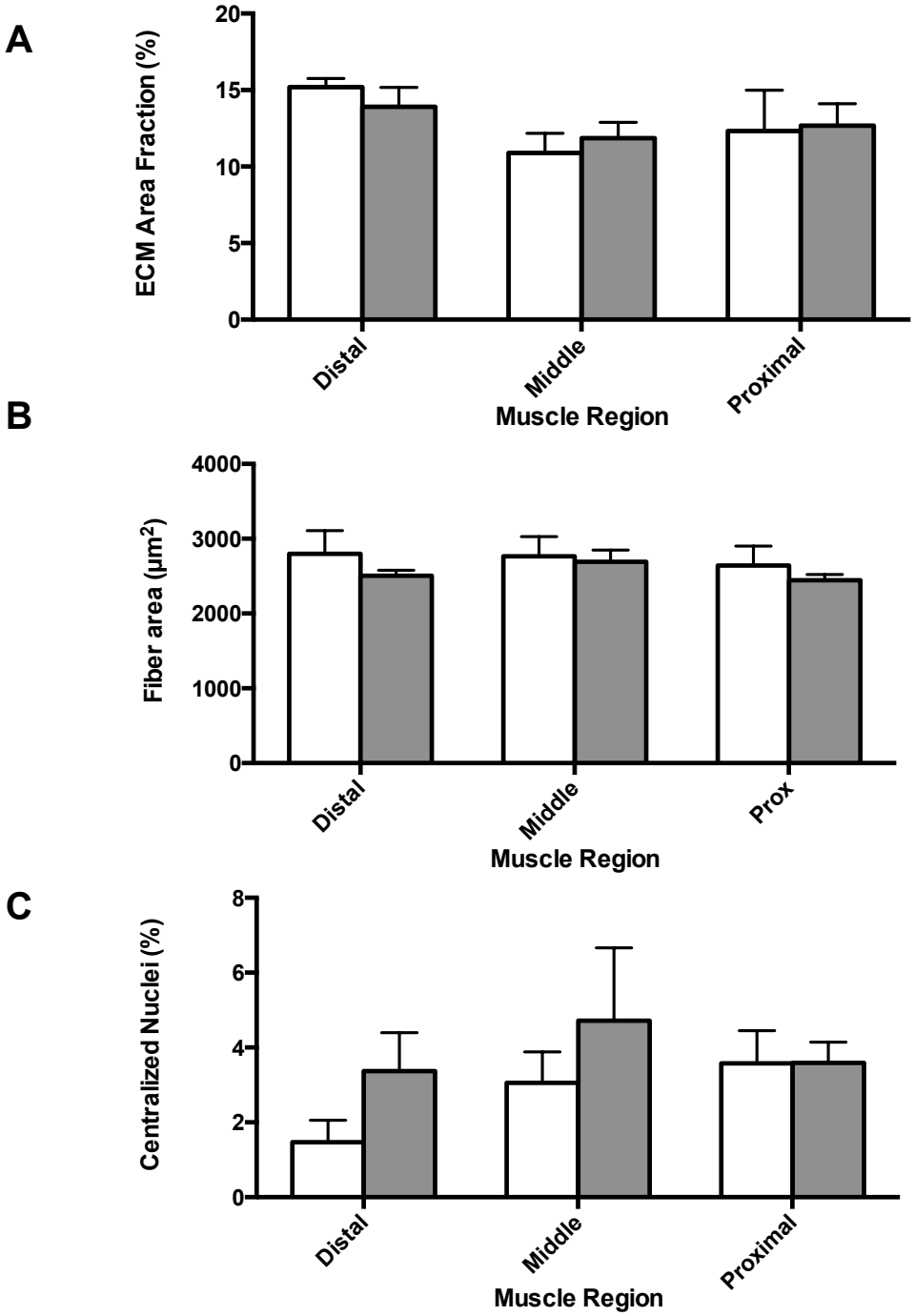

Figure 4.7 Quantitative analysis of histology
Quantitative analysis of histology showed no differences in ECM area fraction (A), muscle fiber area (B), or number of centralized nuclei (C) between injury groups or between distal, middle, or proximal muscle regions.

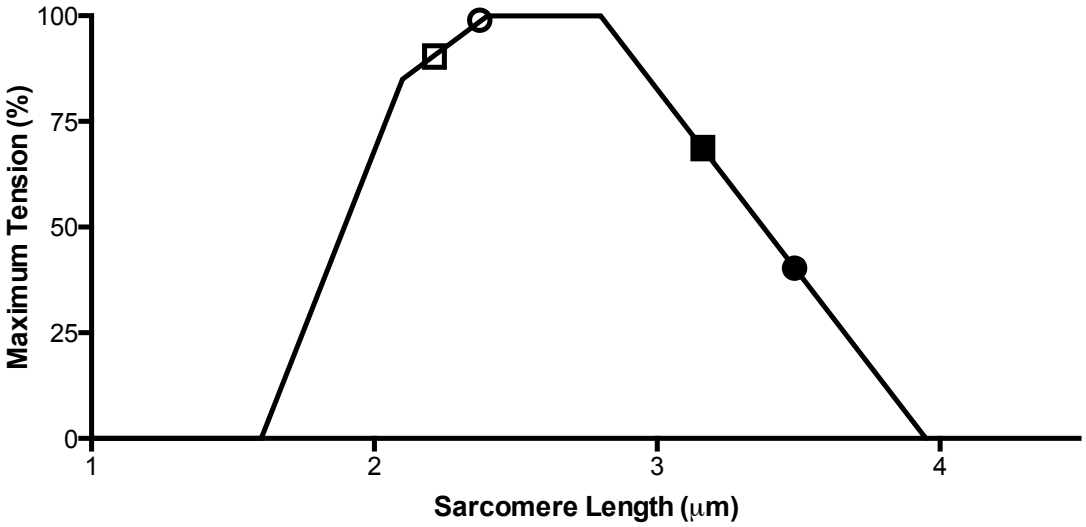

Figure 4.8 Theoretical sarcomere length-tension curve for rat muscle.
Sham (open circle) and tenotomy (open square) muscles at resting muscle length sit near the plateau of the length tension curve. However, when strained to 20%, Sham (filled circle) muscles have significantly longer sarcomere lengths compared to tenotomy (filled square) muscles and lie further down the descending limb of the length-tension curve. This would suggest that tenotomy muscles would be able to generate more force than sham muscles at large strains.

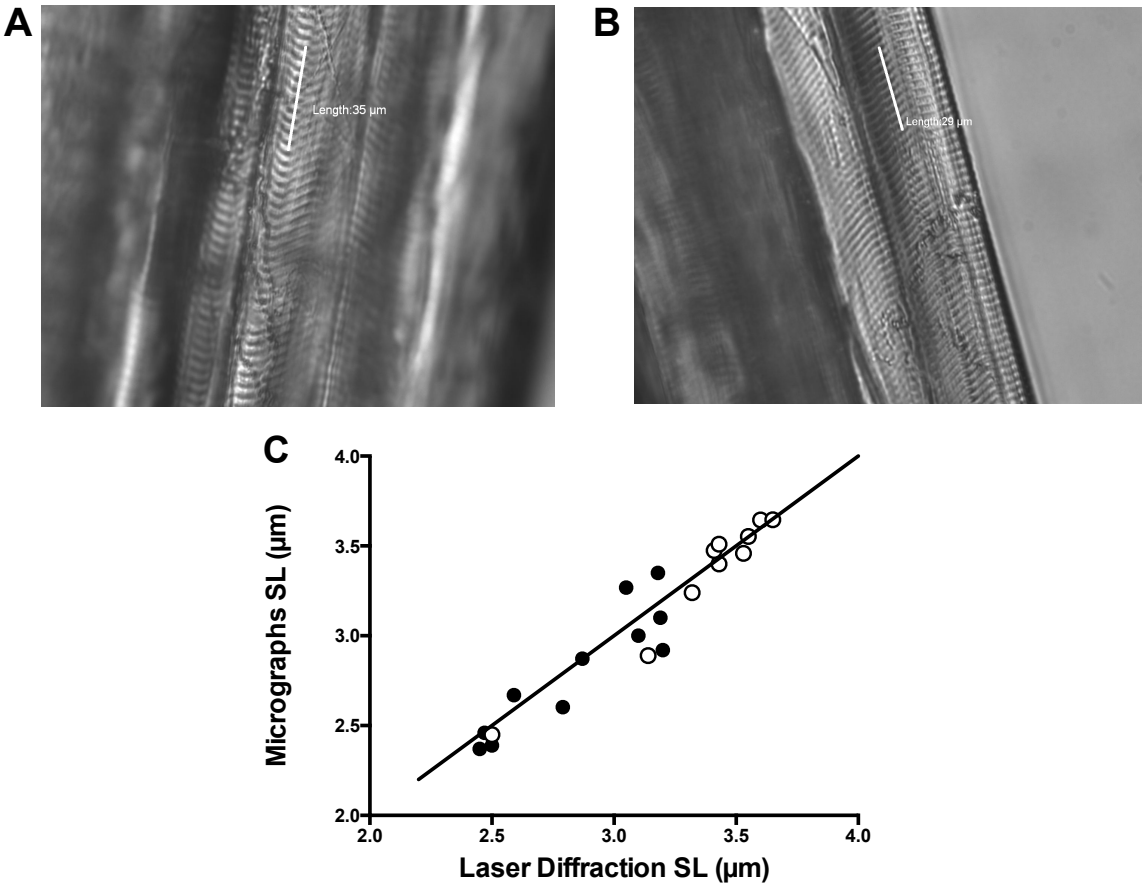

Figure 4.S1 Muscle fiber structural integrity
Representative images of muscle fiber bundles dissected from (A) Sham and (B) Tenotomized muscles. Damage to the mid-substance of the muscle fibers were not observed. (C) Sarcomere lengths measured optically were in good agreement with sarcomere lengths measured via laser diffraction method (ICC: 0.958).

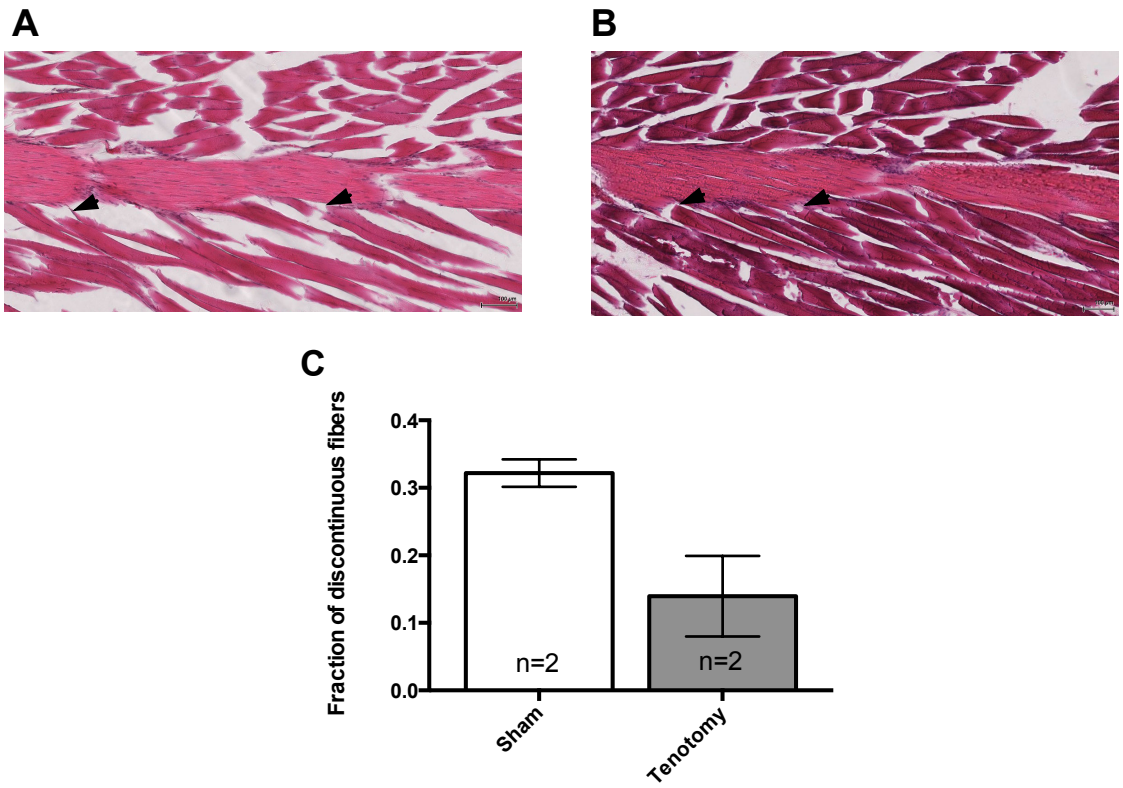

Figure 4.S2 Muscle-tendon junction structural integrity
Representative images of supraspinatus longitudinal sections near the muscle-tendon junction in the (A) Sham and (B) Tenotomized muscles. Black arrow-heads denote discontinuities at the muscle-tendon junction. (C) On average, the fraction of discontinuous fibers at the muscle-tendon junction was larger in Sham treated muscles than Tenotomy.

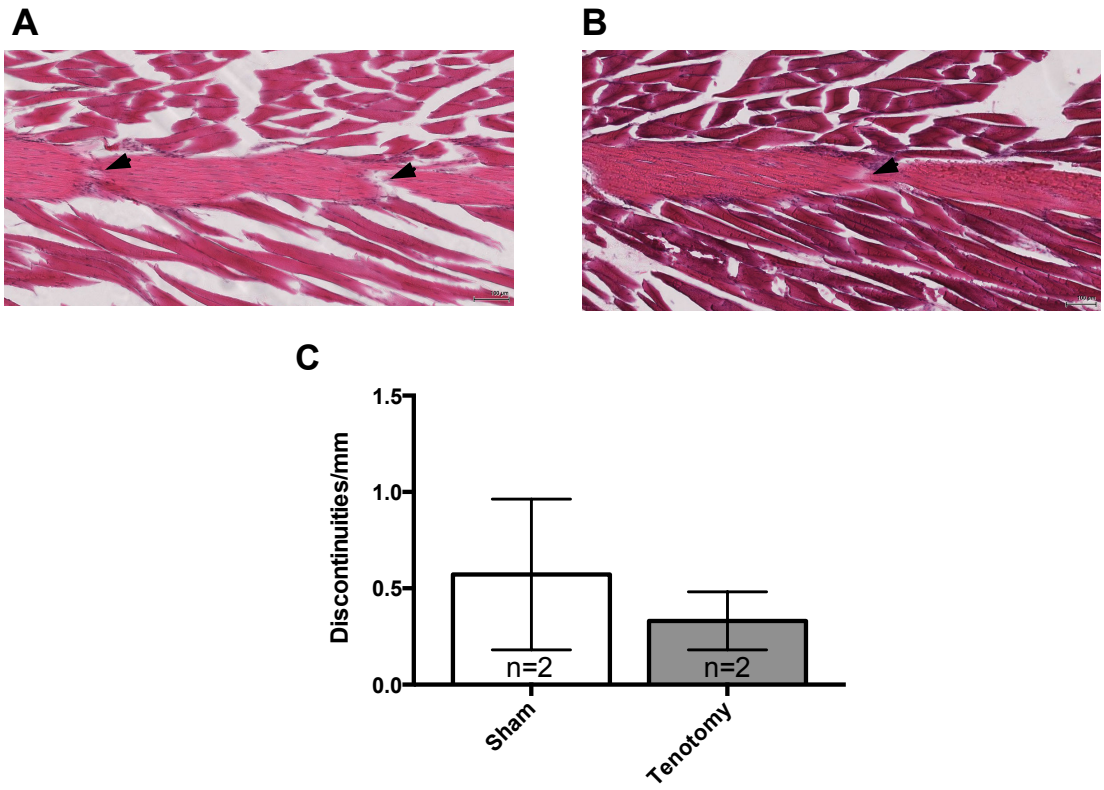

Figure 4.S3 Internal tendon structural integrity

Representative images of supraspinatus longitudinal sections showing the internal tendon in (A) Sham and (B) Tenotomized muscles. Black arrow-heads denote discontinuities within the tendon. (C) On average, the frequency of tendon discontinuities was greater in Sham treated muscles than Tenotomy.

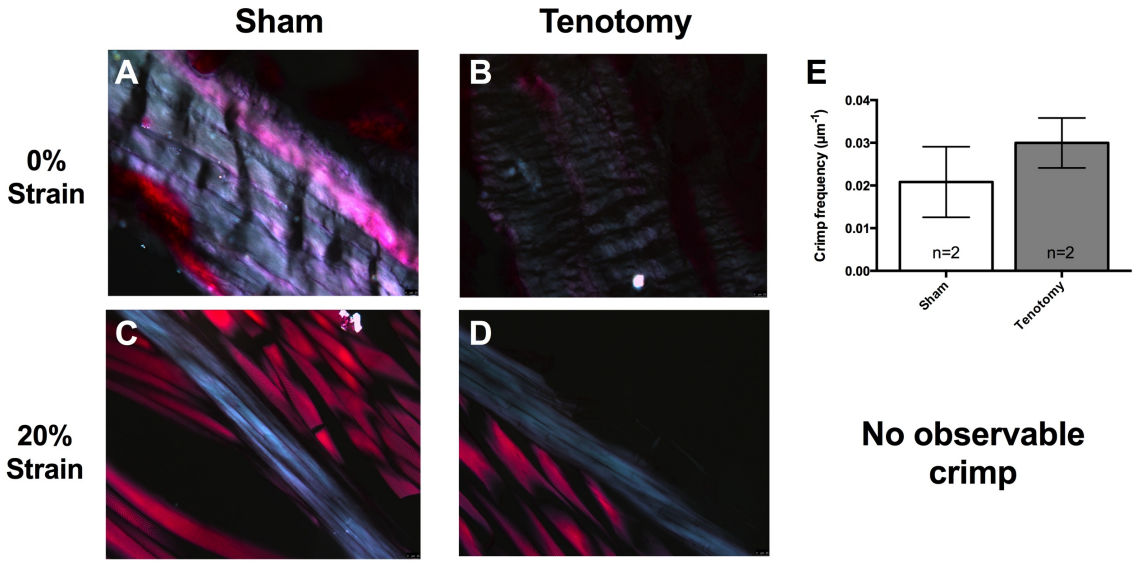

Figure 4.S4. Internal tendon crimp
Representative images of supraspinatus longitudinal sections showing the internal tendon under polarized light in (A, C) Sham and (B, D) Tenotomized muscles at 0% and 20% strain. (E) On average, the frequency of tendon crimp was greater in Tenotomy muscles. No tendon crimp was observed when the muscle was stretched to 20% strain.

4.8 References

1. Lehman C, Cuomo F, Kummer FJ, Zuckerman JD: **The incidence of full thickness rotator cuff tears in a large cadaveric population**. *Bull Hosp Jt Dis* 1995, **54**(1):30-31.

2. Fuchs S, Chylarecki C, Langenbrinck A: **Incidence and symptoms of clinically manifest rotator cuff lesions**. *Int J Sports Med* 1999, **20**(3):201-205.

3. Galatz LM, Ball CM, Teefey SA, Middleton WD, Yamaguchi K: **The Outcome and Repair Integrity of Completely Arthroscopically Repaired Large and Massive Rotator Cuff Tears**. *The Journal of Bone & Joint Surgery* 2004, **86**(2):219-224.

4. Harryman DT, 2nd, Mack LA, Wang KY, Jackins SE, Richardson ML, Matsen FA, 3rd: **Repairs of the rotator cuff. Correlation of functional results with integrity of the cuff**. *J Bone Joint Surg Am* 1991, **73**(7):982-989.

5. Mall NA, Kim HM, Keener JD, Steger-May K, Teefey SA, Middleton WD, Stobbs G, Yamaguchi K: **Symptomatic Progression of Asymptomatic Rotator Cuff Tears**. *The Journal of Bone & Joint Surgery* 2010, **92**(16):2623-2633.

6. Tempelhof S, Rupp S, Seil R: **Age-related prevalence of rotator cuff tears in asymptomatic shoulders**. *J Shoulder Elbow Surg* 1999, **8**(4):296-299.

7. Soslowsky LJ, Carpenter JE, DeBano CM, Banerji I, Moalli MR: **Development and use of an animal model for investigations on rotator cuff disease**. *J Shoulder Elbow Surg* 1996, **5**(5):383-392.

8. Barry JJ, Lansdown DA, Cheung S, Feeley BT, Ma CB: **The relationship between tear severity, fatty infiltration, and muscle atrophy in the supraspinatus**. *J Shoulder Elbow Surg* 2013, **22**(1):18-25.

9. Gerber C, Meyer DC, Schneeberger AG, Hoppeler H, von Rechenberg B: **Effect of Tendon Release and Delayed Repair on the Structure of the Muscles of the Rotator Cuff: An Experimental Study in Sheep**. *The Journal of Bone & Joint Surgery* 2004, **86**(9):1973-1982.

10. Meyer DC, Gerber C, Von Rechenberg B, Wirth SH, Farshad M: **Amplitude and strength of muscle contraction are reduced in experimental tears of the rotator cuff**. *Am J Sports Med* 2011, **39**(7):1456-1461.

11. Goutallier D, Postel JM, Bernageau J, Lavau L, Voisin MC: **Fatty muscle degeneration in cuff ruptures. Pre- and postoperative evaluation by CT scan**. *Clin Orthop* 1994, **304**(304):78-83.

12. Itoi E, Hsu HC, Carmichael SW, Morrey BF, An KN: **Morphology of the torn rotator cuff**. *J Anat* 1995, **186 (Pt 2)**:429-434.

13. Bodine SC, Roy RR, Meadows DA, Zernicke RF, Sacks RD, Fournier M, Edgerton VR: **Architectural, histochemical, and contractile characteristics of a unique biarticular muscle: the cat semitendinosus**. *J Neurophysiol* 1982, **48**:192-201.

14. Winters TM, Takahashi M, Lieber RL, Ward SR: **Whole muscle length-tension relationships are accurately modeled as scaled sarcomeres in rabbit hindlimb muscles**. *J Biomech* 2011, **44**(1):109-115.

15. Ward SR, Sarver JJ, Eng CM, Kwan A, Wurgler-Hauri CC, Perry SM, Williams GR, Soslowsky LJ, Lieber RL: **Plasticity of muscle architecture after supraspinatus tears**. *J Orthop Sports Phys Ther* 2010, **40**(11):729-735.

16. Sato EJ, Killian ML, Choi AJ, Lin E, Choo AD, Rodriguez-Soto AE, Lim CT, Thomopoulos S, Galatz LM, Ward SR: **Architectural and biochemical adaptations in skeletal muscle and bone following rotator cuff injury in a rat model**. *J Bone Joint Surg Am* 2015, **97**(7):565-573.

17. Ditsios K, Boutsiadis A, Kapoukranidou D, Chatzisotiriou A, Kalpidis I, Albani M, Christodoulou A: **Chronic massive rotator cuff tear in rats: in vivo evaluation of muscle force and three-dimensional histologic analysis**. *J Shoulder Elbow Surg* 2014, **23**(12):1822-1830.

18. Mannava S, Plate JF, Whitlock PW, Callahan MF, Seyler TM, Koman LA, Smith TL, Tuohy CJ: **Evaluation of in vivo rotator cuff muscle function after acute and chronic detachment of the supraspinatus tendon: an experimental study in an animal model**. *J Bone Joint Surg Am* 2011, **93**(18):1702-1711.

19. Gordon AM, Huxley AF, Julian FJ: **The variation in isometric tension with sarcomere length in vertebrate muscle fibres**. *Journal of Physiology (London)* 1966, **184**:170.

20. Miller JL, Watkin KL, Chen MF: **Muscle, adipose, and connective tissue variations in intrinsic musculature of the adult human tongue**. *Journal of speech, language, and hearing research : JSLHR* 2002, **45**(1):51-65.

21. Abràmoff MD, Magalhães, P. J. & Ram, S. J.: **Image processing with ImageJ**. *Biophotonics International* 2004, **11**(7):36-42.

22. Minamoto VB, Suzuki KP, Bremner SN, Lieber RL, Ward SR: **Dramatic changes in muscle contractile and structural properties after 2 botulinum toxin injections**. *Muscle & Nerve* 2015:n/a-n/a.

23. Minamoto VB, Hulst JB, Lim M, Peace WJ, Bremner SN, Ward SR, Lieber RL: **Increased efficacy and decreased systemic-effects of botulinum toxin A injection after active or passive muscle manipulation**. *Developmental Medicine & Child Neurology* 2007, **49**(12):907-914.

24. Kim HM, Galatz LM, Lim C, Havlioglu N, Thomopoulos S: **The effect of tear size and nerve injury on rotator cuff muscle fatty degeneration in a rodent animal model**. *Journal of Shoulder and Elbow Surgery* 2012, **21**(7):847-858.

25. Barton ER, Gimbel JA, Williams GR, Soslowsky LJ: **Rat supraspinatus muscle atrophy after tendon detachment**. *J Orthop Res* 2005, **23**(2):259-265.

26. Sato EJ, Killian ML, Choi AJ, Lin E, Esparza MC, Galatz LM, Thomopoulos S, Ward SR: **Skeletal muscle fibrosis and stiffness increase after rotator cuff tendon injury and neuromuscular compromise in a rat model**. *J Orthop Res* 2014, **32**(9):1111-1116.

27. Garrett WE, Safran MR, Seaber AV, Glisson RR, Ribbeck BM: **Biomechanical comparison of stimulated and nonstimulated skeletal muscle pulled to failure**. *Am J Sports Med* 1987, **15**(5):448-454.

28. Garrett WE, Nikolaou PK, Ribbeck BM, Glisson RR, Seaber AV: **The effect of muscle architecture on the biomechanical failure properties of skeletal muscle under passive extension**. *Am J Sports Med* 1988, **16**(1):7-12.

29. Lundgreen K, Lian Ø B, Engebretsen L, Scott A: **Lower muscle regenerative potential in full-thickness supraspinatus tears compared to partial-thickness tears**. *Acta Orthopaedica* 2013, **84**(6):565-570.

30. Hersche O, Gerber C: **Passive tension in the supraspinatus musculotendinous unit after long-standing rupture of its tendon: a preliminary report**. *J Shoulder Elbow Surg* 1998, **7**(4):393-396.

31. Gimbel JA, Mehta S, Van Kleunen JP, Williams GR, Soslowsky LJ: **The tension required at repair to reappose the supraspinatus tendon to bone rapidly increases after injury**. *Clin Orthop Relat Res* 2004(426):258-265.

32. Davis ME, Stafford PL, Jergenson MJ, Bedi A, Mendias CL: **Muscle fibers are injured at the time of acute and chronic rotator cuff repair**. *Clin Orthop Relat Res* 2015, **473**(1):226-232.

33. Swan MA, Sato E, Galatz LM, Thomopoulos S, Ward SR: **The effect of age on rat rotator cuff muscle architecture**. *J Shoulder Elbow Surg* 2014.

34. Miller KS, Connizzo BK, Feeney E, Tucker JJ, Soslowsky LJ: **Examining differences in local collagen fiber crimp frequency throughout mechanical**

testing in a developmental mouse supraspinatus tendon model. *J Biomech Eng* 2012, **134**(4):041004.

35. Franchi M, Fini M, Quaranta M, De Pasquale V, Raspanti M, Giavaresi G, Ottani V, Ruggeri A: **Crimp morphology in relaxed and stretched rat Achilles tendon**. *Journal of Anatomy* 2007, **210**(1):1-7.

CHAPTER 5: SUMMARY AND SIGNIFICANCE

5.1 Summary of Findings

Muscle architecture

Changes to whole muscle structure and architecture can be used as a measure of muscle health and to infer muscle function. Chapter 2 of this dissertation characterized the adaptations in muscle architecture following tenotomy with or without nerve compromise (via BTX). Muscle architectural changes in the animal model were overall mild after tenotomy. For example, atrophy, as measured by muscle mass, was mildly reduced after tenotomy in all studies. Interestingly, mass was reduced by 20% after 8 weeks and 40% after 16 weeks of tenotomy, when compared to age-matched control muscles. However, mass did not decrease as a function of time in the tenotomized muscles, but it increased in the uninjured shoulders. Although this would appear to be an important reduction in mass, implying atrophy, our ability to carefully capture aged-matched controls leads to a finding related more to growth arrest that to atrophy. In contrast, large changes in muscle mass were observed after the addition of BTX injury as muscle mass continually decreased over time, and was ~60% smaller after 8 weeks and ~80% smaller after 16 weeks when compared to uninjured shoulders. Physiological cross sectional area mirrored the patterns observed in muscle mass. Raw fiber length and serial sarcomere numbers changes after tendon injuries were also small throughout each study. Significant loss of serial sarcomere number was observed at 16 weeks of

tenotomy and BTX injury. These data are in sharp contrast to the adaptations observed in human patients (see below).

Patient imaging and human cadaver studies have measured decreased muscle anatomical cross-sectional area[1, 2] and shortened muscle fiber in muscles with tendon tears[3, 4]. While most studies do not directly quantify muscle atrophy (clinical scoring systems are commonly used), a 40% decrease in muscle cross sectional area has been measured in torn rotator cuff muscles when compared to an uninjured contralateral shoulder. Direct comparison of muscle atrophy between human and animal studies suggest that tendon injury alone does not result in the same degree of muscle injury observed in human patients, and that additional nerve injury is required to attain the severe muscle atrophy observed in human patients. This may be due, at least in part, to the tendency for small animals to fill the lost tendon space with scar tissue, which has been previous observed[5], and the propensity for these animals to grow throughout their lifetime[6]. As mentioned above, muscle mass deficits measured between control and tenotomized muscles were due to increasing muscle mass in the age-matched control over time. In contrast to adult humans who have reached skeletal maturity, the adult rat and consequently the rotator cuff muscles continue to grow over the duration of the study period[6]. The continued increase in mass of the control muscle paired with the absence of muscle mass increases in tenotomized muscles results in the observed muscle atrophy in these studies. Meanwhile, the addition of the BTX injury results in muscle mass loss over the duration of the study. These data suggest that

tenotomy does not result in a decrease in muscle mass but rather limits the typical muscular growth in these animals. This will likely be a confounding factor when comparing the model with the human condition, and will require additional study. Overall, these data suggest tenotomy in this animal model would lead to minimal physiological deficits, and that a nerve injury may be required to achieve the structural adaptations that occur in human pathology.

Muscle Passive Mechanics

Increased stiffness of the muscle-tendon unit in rotator cuff tears has been measured in both human[7, 8] and animal studies, and has been implicated leading to poor tendon repair integrity[9, 10]. Chapter 3 of this dissertation studied the microscale passive mechanics in rotator cuff muscles after tendon injury. This insult to the rotator cuff muscles did not result in an increase in fiber or bundle stiffness relative to control muscles. However, the passive stiffness of fiber bundles increased over two-fold after combined tenotomy and BTX injury.

Studies in human cadavers have suggested that high mechanical loading at the repair site can lead to repair failure and retearing of the tendon[11, 12]. Meanwhile, increased passive tension has been measured during rotator cuff repairs in patients[7]. Similar studies of the rat supraspinatus have reported increased stiffness at the whole muscle level after rotator cuff tenotomy[9, 10, 13]. A recent study examining muscle biopsies from patients measured increased stiffness in fiber bundles, and that this correlated with the amount of collagen in the sample[8].

These data suggest that stiffness increases are due to extracellular matrix components of the muscle.

Although the addition of BTX increased bundle stiffness as well as collagen content, increased bundle stiffness was not measured after tenotomy alone. These results are in contrast with the aforementioned study in patients, which showed bundle level stiffness changes after tendon injury. This suggests that tissue stiffness changes in rat supraspinatus muscle occurs at larger structural levels, such as the fascicle or the whole muscle levels.

Muscle fibrosis and fatty infiltration

Overall, there was limited evidence of tissue fibrosis or fatty infiltration within the muscle in the rat model throughout this dissertation. Tissue fibrosis, measured by collagen content from tissue homogenates and extracellular matrix area fraction via histology, was not observed after tenotomy across all studies. Increased collagen content was measured only after the addition of BTX injury. Similarly, histological evaluation of the tissue in Chapter 4 did not reveal accumulation of adipocytes within the muscle. These data agree with past studies in this model, that have previously demonstrated increased connective tissue and fatty infiltration occur only after a nerve injury[14-16].

Increased tissue stiffness, which correlates with tissue fibrosis[8], has been implicated in leading to failed repairs[7, 9-12, 17]. However, these studies were unable to produce evidence for fibrosis or fatty infiltration after tendon injury. Similarly, fatty infiltration in patients is used as a muscle health marker, with the

most severe cases scoring 50% or greater replacement of muscle with fat.[2, 18] Both muscle atrophy and fatty infiltration have been shown to correlate with poor outcomes[1, 19-21], and thus it is important that model systems reflect the severe muscle pathologies. However, these studies show that tenotomy alone does not lead to comparable muscle injury in the animal model, and that alternative injury mechanisms may be required to recapitulate the degree of injury in humans. Unfortunately, this poses additional challenges as these alternative injury mechanisms (i.e. nerve injury) may lead to unique biological responses that may be difficult to reconcile when comparing animals models to the rotator cuff injury in humans.

Hierarchical strain transmission

The length-dependent nature of muscle function requires the reliable transmission of force and length (strain) between the sarcomeres, muscle fibers, and the whole muscle itself. Thus changes in how strain is transmitted between these hierarchical levels may result in altered force production and excursion capacity of the muscle, and may explain functional impairments observed after rotator cuff tears. In order to investigate the hierarchical strain transmission within the muscle in chapter 4, we designed a device that would allow us to stretch and chemically fix the supraspinatus at varying strains and measure the corresponding length changes in the muscle fibers and sarcomeres.

Sarcomere lengths measured at low, discrete muscle strains showed no differences between tenotomized and control muscles. Relatively, short muscle

fiber and sarcomere lengths were measured at strains approaching 20%, but the precise mechanism for this change is unknown. Measurement of other architectural parameters also showed small differences between injury groups. Despite whole muscles being shorter and smaller in mass, muscle fiber length and physiological cross-sectional area were the same as controls.

Recent studies by other groups suggest that whole muscle functional is impaired in the form of decreased force production and excursion of the muscle after tenotomy[13, 22]. However, these studies lacked additional data to support potential mechanisms for these functional changes. Furthermore, data from Chapters 2 and 3 of this dissertation suggest that neither radial nor longitudinal atrophy of the muscle contribute to these observed changes. Thus an alternative mechanism that could lead to abnormal muscle function could involve changes in the relationship between muscle strain and sarcomere length. However, the results showed small differences between tenotomized and control muscles. In fact, the data may suggest that tenotomized muscles may have increased excursion, as sarcomere lengths were shorter at high strains in the tenotomized muscles.

A potential mechanism for impaired muscle strain transmission to the sarcomere could involve decoupling of muscle fiber length strains with sarcomere length strains. However, this appears to be unlikely as there was a strong correlation between fiber length and sarcomere length. An alternative mechanism could be due to changes in the serial elastic components of the muscle that transmit force and length change from whole muscle to individual fibers, including changes

to the internal tendon, aponeurosis, or muscle-tendon junction. However, no gross structural differences were found in the internal tendon of torn muscles compared to controls at high strains (data not shown). Unfortunately, there are currently no other studies that have investigated these tissue structures in a tenotomy injury model, and thus may merit further study.

In addition, no differences in fiber cross-sectional area were observed. Although we did not measure force in these studies, this result would suggest that a decrease in the number of parallel sarcomeres is unlikely to be the cause for decreased force production. Decreased muscle fiber cross sectional area has been measured in human supraspinatus muscle biopsies[23], but decreases in fiber area in the rat model have only been observed within the first few weeks after injury due to the gradual recovery of fiber area over time[24]. Interestingly, studies of skinned human/rat muscles fibers from torn rotator cuff muscles measured decreased specific force production, but further study is required to understand the underlying mechanism causing this difference[25, 26]. In summary, these findings would predict minimal functional changes at small muscle strains, and may suggest increased excursion for muscles after tenotomy.

5.2 Significance of Findings

The work presented in this dissertation demonstrates that skeletal muscle adaptations in this injury model (tenotomy) are mild. Significant changes comparable to what is observed in human pathology were only found after the addition of a secondary nerve compromise. Other studies investigating BTX only

injury to muscle have shown profound changes, such as increase in collagen content, due to BTX alone[27-29]. These studies suggest that nerve injury independently accounts for most of the pathology observed in the combined injury models.

Also, both the mechanism and magnitude of the nerve injury models are unlike what is thought to occur in clinically. Suprascapular neuropathy is thought to be a mechanical compression on the nerve, but experimental nerve injuries either completely severe the nerve (neurotomy) or chemically block the communication between the muscle and nerve—leading to complete or near complete denervation of the muscle and resulting in a much more severe injury. After considering the controversial nature of the prevalence of this injury[30], the usefulness of the nerve injury model to study rotator cuff tear pathology is questionable.

Chapter 4 of this dissertation highlighted an important aspect of muscle architecture relevant to rotator cuff surgical treatment. Rotator cuff repairs require stretching the muscle and tendon back to its original position. The data in this experiment highlighted the presence of relatively short muscle fibers compared to the whole muscle, and thus the relatively large strains that muscle fibers and sarcomere must undergo during lengthening procedures like a rotator cuff repair. This concept is particularly interesting when considering the resting sarcomere length of the human supraspinatus has been measured to be longer (3.2 μm) than optimal sarcomere length (2.7 μm), which places the muscle on the descending limb of the muscle length-tension curve. It is conceivable that stretching a retracted

muscle in this case could result in unnaturally long sarcomere lengths, which could be damaging to the repaired muscle and impair force production.

In summary, there was no evidence throughout this dissertation (using a rat model) to suggest increased fibrotic or fatty tissue accumulation within the muscle after tenotomy alone, nor has there been substantial architectural or passive mechanical defects that would suggest pathology akin to human injuries or even large animal models. Taking into consideration the mild effects of tenotomy as well as the large size scale differences between rodents and humans, coupled with the propensity for healing and growth in these animals, these studies highlight the overall limitations of using this model to study human rotator cuff muscle injury.

5.3 Future Directions

The work presented in this dissertation highlight the limitations of using this model for future work studying rotator cuff tears. However, there are several avenues of additional research that are promising. The results from Chapter 2 suggest that continual growth of these animals is an important consideration when studying this model. The data also suggest that while active atrophy was not apparent after tenotomy injury, there was a cessation of normal muscle growth. Studies investigating the mechanisms that oppose the normal growth signal in these muscles may provide insight into how muscle mass is regulated in general. Alternatively, few studies in the literature have investigated the effects of injury in elderly rats. Although these animals are difficult to obtain and less convenient to

use, continued study of these animals may be more relevant than the commonly used adult aged rat.

Also in Chapter 2, we observed changes to bony parameters in the scapula and humeral head that closely mirrored the changes observed in the muscle. These effects appear to be driven by factors in addition to unloading of the bone—the addition of BTX lead to larger decreases in bony parameters, despite being mechanically unloaded. This highlights a potential interaction between muscle and bone health mediated by paracrine factors. Future research into this hypothetical mechanism may highlight new mechanisms for regulating musculoskeletal health.

There are still several unexplained phenomena in the literature related to this model. The whole muscle active and passive mechanical deficits (decreased isometric force, narrowing of the length-tension curve) observed in other studies are left unexplained by data presented in this dissertation (mild radial and longitudinal atrophy. A comprehensive study investigating many of the variables presented in this dissertation concurrently with functional experiments may be required to resolve these conflicting results. Finally, the mechanism behind the abnormal sarcomere length behavior at large muscle strains presented in Chapter 4 remains unresolved. While the data suggest mechanical decoupling between the whole muscle and muscle fibers, further research into muscle-tendon junction properties is likely required to understand this phenomenon.

5.4 References

1. Shen PH, Lien SB, Shen HC, Lee CH, Wu SS, Lin LC: **Long-term functional outcomes after repair of rotator cuff tears correlated with atrophy of the supraspinatus muscles on magnetic resonance images**. *J Shoulder Elbow Surg* 2008, **17**(1 Suppl):1S-7S.

2. Barry JJ, Lansdown DA, Cheung S, Feeley BT, Ma CB: **The relationship between tear severity, fatty infiltration, and muscle atrophy in the supraspinatus**. *J Shoulder Elbow Surg* 2013, **22**(1):18-25.

3. Itoi E, Hsu HC, Carmichael SW, Morrey BF, An KN: **Morphology of the torn rotator cuff**. *J Anat* 1995, **186 (Pt 2)**:429-434.

4. Tomioka T, Minagawa H, Kijima H, Yamamoto N, Abe H, Maesani M, Kikuchi K, Shimada Y, Itoi E: **Sarcomere length of torn rotator cuff muscle**. *J Shoulder Elbow Surg* 2009, **18**(6):955-959.

5. Ward SR, Sarver JJ, Eng CM, Kwan A, Wurgler-Hauri CC, Perry SM, Williams GR, Soslowsky LJ, Lieber RL: **Plasticity of muscle architecture after supraspinatus tears**. *J Orthop Sports Phys Ther* 2010, **40**(11):729-735.

6. Swan MA, Sato E, Galatz LM, Thomopoulos S, Ward SR: **The effect of age on rat rotator cuff muscle architecture**. *J Shoulder Elbow Surg* 2014.

7. Hersche O, Gerber C: **Passive tension in the supraspinatus musculotendinous unit after long-standing rupture of its tendon: a preliminary report**. *J Shoulder Elbow Surg* 1998, **7**(4):393-396.

8. Silldorff MD, Choo AD, Choi AJ, Lin E, Carr JA, Lieber RL, Lane JG, Ward SR: **Effect of supraspinatus tendon injury on supraspinatus and infraspinatus muscle passive tension and associated biochemistry**. *J Bone Joint Surg Am* 2014, **96**(20):e175.

9. Gimbel JA, Van Kleunen JP, Lake SP, Williams GR, Soslowsky LJ: **The role of repair tension on tendon to bone healing in an animal model of chronic rotator cuff tears**. *J Biomech* 2007, **40**(3):561-568.

10. Gimbel JA, Mehta S, Van Kleunen JP, Williams GR, Soslowsky LJ: **The tension required at repair to reappose the supraspinatus tendon to bone rapidly increases after injury**. *Clin Orthop Relat Res* 2004(426):258-265.

11. Haering D, Blache Y, Raison M, Begon M: **Mechanical risk of rotator cuff repair failure during passive movements: A simulation-based study**. *Clinical biomechanics (Bristol, Avon)* 2015.

12. Burkhart SS, Johnson TC, Wirth MA, Athanasiou KA: **Cyclic loading of transosseous rotator cuff repairs: tension overload as a possible cause of failure**. *Arthroscopy* 1997, **13**(2):172-176.

13. Mannava S, Plate JF, Whitlock PW, Callahan MF, Seyler TM, Koman LA, Smith TL, Tuohy CJ: **Evaluation of in vivo rotator cuff muscle function after acute and chronic detachment of the supraspinatus tendon: an experimental study in an animal model**. *J Bone Joint Surg Am* 2011, **93**(18):1702-1711.

14. Liu X, Manzano G, Kim HT, Feeley BT: **A rat model of massive rotator cuff tears**. *J Orthop Res* 2011, **29**(4):588-595.

15. Liu X, Joshi SK, Ravishankar B, Laron D, Kim HT, Feeley BT: **Upregulation of transforming growth factor-beta signaling in a rat model of rotator cuff tears**. *J Shoulder Elbow Surg* 2014, **23**(11):1709-1716.

16. Kim HM, Galatz LM, Lim C, Havlioglu N, Thomopoulos S: **The effect of tear size and nerve injury on rotator cuff muscle fatty degeneration in a rodent animal model**. *Journal of Shoulder and Elbow Surgery* 2012, **21**(7):847-858.

17. Gimbel JA, Van Kleunen JP, Mehta S, Perry SM, Williams GR, Soslowsky LJ: **Supraspinatus tendon organizational and mechanical properties in a chronic rotator cuff tear animal model**. *Journal of Biomechanics* 2004, **37**(5):739-749.

18. Goutallier D, Postel JM, Bernageau J, Lavau L, Voisin MC: **Fatty muscle degeneration in cuff ruptures. Pre- and postoperative evaluation by CT scan**. *Clin Orthop* 1994, **304**(304):78-83.

19. Gladstone JN, Bishop JY, Lo IKY, Flatow EL: **Fatty Infiltration and Atrophy of the Rotator Cuff Do Not Improve After Rotator Cuff Repair and Correlate With Poor Functional Outcome**. *Am J Sports Med* 2007, **35**(5):719-728.

20. Goutallier D, Postel J-M, Gleyze P, Leguilloux P, Van Driessche S: **Influence of cuff muscle fatty degeneration on anatomic and functional outcomes after simple suture of full-thickness tears**. *Journal of Shoulder and Elbow Surgery* 2003, **12**(6):550-554.

21. Deniz G, Kose O, Tugay A, Guler F, Turan A: **Fatty degeneration and atrophy of the rotator cuff muscles after arthroscopic repair: does it improve, halt or deteriorate?** *Arch Orthop Trauma Surg* 2014, **134**(7):985-990.

22. Ditsios K, Boutsiadis A, Kapoukranidou D, Chatzisotiriou A, Kalpidis I, Albani M, Christodoulou A: **Chronic massive rotator cuff tear in rats: in vivo evaluation of muscle force and three-dimensional histologic analysis**. *J Shoulder Elbow Surg* 2014, **23**(12):1822-1830.

23. Lundgreen K, Lian Ø B, Engebretsen L, Scott A: **Lower muscle regenerative potential in full-thickness supraspinatus tears compared to partial-thickness tears**. *Acta Orthopaedica* 2013, **84**(6):565-570.

24. Barton ER, Gimbel JA, Williams GR, Soslowsky LJ: **Rat supraspinatus muscle atrophy after tendon detachment**. *J Orthop Res* 2005, **23**(2):259-265.

25. Gumucio JP, Davis ME, Bradley JR, Stafford PL, Schiffman CJ, Lynch EB, Claflin DR, Bedi A, Mendias CL: **Rotator cuff tear reduces muscle fiber specific force production and induces macrophage accumulation and autophagy**. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2012, **30**(12):1963-1970.

26. Mendias CL, Roche SM, Harning JA, Davis ME, Lynch EB, Sibilsky Enselman ER, Jacobson JA, Claflin DR, Calve S, Bedi A: **Reduced muscle fiber force production and disrupted myofibril architecture in patients with chronic rotator cuff tears**. *J Shoulder Elbow Surg* 2015, **24**(1):111-119.

27. Billante CR, Zealear DL, Billante M, Reyes JH, Sant'Anna G, Rodriguez R, Stone RE: **Comparison of neuromuscular blockade and recovery with botulinum toxins A and F**. *Muscle & Nerve* 2002, **26**(3):395-403.

28. Thacker BE, Tomiya A, Hulst JB, Suzuki KP, Bremner SN, Gastwirt RF, Greaser ML, Lieber RL, Ward SR: **Passive mechanical properties and related proteins change with botulinum neurotoxin A injection of normal skeletal muscle**. *J Orthop Res* 2012, **30**(3):497-502.

29. Minamoto VB, Suzuki KP, Bremner SN, Lieber RL, Ward SR: **Dramatic changes in muscle contractile and structural properties after 2 botulinum toxin injections**. *Muscle & Nerve* 2015:n/a-n/a.

30. Bachasson D, Singh A, Shah SB, Lane JG, Ward SR: **The role of the peripheral and central nervous systems in rotator cuff disease**. *Journal of Shoulder and Elbow Surgery* 2015, **24**(8):1322-1335.