8-2019

Investigation of the Effect of Age on Regenerative Outcomes Following Treatment of Volumetric Muscle Loss Injuries

John Taehwan Kim
University of Arkansas, Fayetteville

Investigation of the Effect of Age on Regenerative Outcomes Following Treatment of Volumetric Muscle Loss Injuries

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Biomedical Engineering

by

John Taehwan Kim
University of Arkansas
Bachelor of Science in Chemical Engineering, 2009

August 2019
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

Jeffrey C. Wolchok, Ph.D.
Dissertation Director

Kartik Balachandran, Ph.D.
Committee Member

Kyle P. Quinn, Ph.D.
Committee Member

Tyrone Washington, Ph.D.
Committee Member

Abstract

Volumetric muscle loss (VML) is a traumatic injury in skeletal muscle resulting in the bulk loss of more than 20% of the muscle's volume. Included in the bulk loss of muscle is the skeletal muscle niche comprised of nerve bundles, vasculature, local progenitor cells, basal lamina, and muscle fibers, overwhelming innate repair mechanisms. The hallmark of VML injury is the excessive accumulation of non-contractile, fibrotic tissue and permanent functional deficits. Though predominant in the younger demographic, the elderly population is also captured within VML injuries. There are many factors that change with aging in skeletal muscle that may further hinder recovery and regeneration following VML. In an attempt to further our understanding on how age affects VML treatments, comparisons between young and aged animals following VML injury and repair were made. Presented in this dissertation is a summary of the current state of the tissue engineering field in skeletal muscle and explores strategies for repairing not only VML but also understanding what age-associated changes in skeletal muscle preclude effective tissue repair. The future directions and potential approaches to further the field's understanding of VML repair in the aging microenvironment along with the remaining challenges in skeletal muscle tissue engineering are presented within.

Acknowledgements

I would like to, first and foremost, thank my peers in the Regenerative Biomaterials Laboratory at the University of Arkansas. Benjamin Kasukonis, Kevin Roberts, and Grady Dunlap, your collaboration and assistance in these studies made this research possible. A special thanks is due to the members of my dissertation committee: Dr. Balachandran, Dr. Quinn, and Dr. Washington for their constructive feedback and time.

To my advisor, my deepest gratitude and thanks to Dr. Jeffrey Wolchok, for providing me the opportunity to conduct research and gain valuable skills to take with me as I start my journey as a scientist. Last, but not least, I would like to thank our funding sources (NIH Award# R15AR065581 and the Arkansas Biosciences Institute) that made this work possible.

Dedication

I would like to dedicate this work to my mother and father, for their unconditional love and support and the countless sacrifices that were made to make this journey possible. I love you both deeply and hope to continue my journey to become a person of whom you can continue to be proud.

Table of Contents

List of Published Papers

Chapter 2:

Kim, J.T., Kasukonis, B.M., Brown, L.A., Washington, T.A., Wolchok, J.C. (2016). Recovery from volumetric muscle loss injury: A comparison between young and aged rats. *Experimental Gerontology,* 83:37-46, doi: 10.1016/j.exger.2016.07.008

Chapter 3:

Kim, J.T., Kasukonis, B.M., Dunlap, G., Perry, R., Washington, T.A., Wolchok, J.C. (2019). Regnerative Repair of Volumetric Muscle Loss Injuries is Sensitive to Age. *Tissue Engineering: Part A*, doi: 10.1089/ten.TEA.2019.0034

Chapter 1
Introduction

A. Skeletal Muscle Review

Comprising approximately 40% of total body weight, skeletal muscle is one of the most plastic and dynamic tissues in the human body. Additionally, it is seen as one of the biggest reservoirs for amino acids, accounting for roughly 50-75% of all body proteins (Frontera & Ochala, 2015). Mechanically, skeletal muscle has the main function of providing locomotor movement as well as providing skeletal stability, preventing damage and misalignment at the joints. Structurally, skeletal muscle is a highly organized, hierarchical tissue composed of tightly apposed bundles of multi-nucleated fibers. Generally, skeletal muscle is comprised of bundles of muscle fibers, resident muscle cells, nerve fibers, blood vessels, non-contractile tissue such as fat, and the extracellular matrix (ECM).

Individual muscles are surrounded by a sheath of connective tissue called the epimysium. Within those muscles are fibers that are arranged into bundles that are surround by their own layer of connective tissue called the perimysium. Further down are single muscle fibers that are surrounded by a cell membrane, or the sarcolemma. The bundle containing these connective tissue sheaths and membranes are typically referred to as a fascicle and originate at proximally located tendon plates and terminate at distal insertion points (Lieber & Friden, 2000). For the purposes of this paper, the aforementioned connective tissues will be referred to as the ECM unless noted otherwise.

Connecting the sarcolemma to the myofibrils is a complex of myofilament proteins, referred to as a sarcomere, that is necessary for normal muscle function (Frontera & Ochala, 2015). The sarcomere is considered to be the basic contractile unit of skeletal muscle. The main

components of the sarcomere include the thin filament protein actin, thick filament protein myosin, intermediate linking proteins providing mechanical stability such as dystrophin, titin, and nebulin (Frontera & Ochala, 2015; Monroy et al., 2012; Ottenheijm & Granzier, 2010), and the Z disk which functions as an anchor point for myofilaments within the sarcomere (Frontera & Ochala, 2015). Any absence or dysfunction of one of these proteins are associated with instability in muscle structure leading to sarcolemmal damage or muscle weakness. A common example of this is the absence of dystrophin found in neuromuscular disorders such as Becker and Duchenne muscular dystrophies (G. D. Thomas, 2013).

The basic mechanism of muscle contraction is known as the sliding filament theory involving the transmission of forces generated by the actin-myosin cross-bridges within the muscle fiber (A. F. Huxley & Niedergerke, 1954; H. Huxley & Hanson, 1954). Adenosine triphosphate (ATP) is the main source of energy used in muscle action, which is produced by muscle cells via the metabolism of carbohydrates and fats (Romijn et al., 1993). To describe briefly, in the resting state myosin is tightly bound to actin at a 45º angle with respect to each other. As ATP binds to myosin, it is dissociated from actin and proceeds to hydrolyze ATP into ADP and a phosphate group. As a result, the head of the myosin molecule swings and weakly binds to actin on a different binding site. Release of the phosphate group bound to the myosin molecule initiates the power stroke of myosin, causing it to rotate on its hinge and pushing the actin filament past it. At the end of the stroke, ADP is released from myosin causing it to resume its previous tightly bound state on actin. The generated force is transmitted all the way down to the z disk and movement is finally produced when the force reaches the myotendinous junction, tendons, and finally the joints (Frontera & Ochala, 2015; A. F. Huxley & Niedergerke, 1954; H. Huxley & Hanson, 1954).

B. Muscle Injury and Repair

Following damage such as tears, contusions, and lacerations (Garrett, 1996; M. J. Jarvinen & Lehto, 1993), skeletal muscle has the capacity to repair itself through a series of critical events in the repair process. These events can broadly be categorized into the following phases: degradation, inflammation, repair, remodeling, and maturation (Charge & Rudnicki, 2004; T. A. Jarvinen, Jarvinen, Kaariainen, Kalimo, & Jarvinen, 2005). Degradation occurs immediately following injury as the sarcolemma (muscle cell plasma membrane) is ruptured causing an influx of cytosolic calcium ions which activate calcium-dependent proteases, further degrading the membrane (Fredsted, Gissel, Madsen, & Clausen, 2007; Tidball, 2011). Additionally, as necrosis of damaged muscle fibers begin, a structure comprised of cytoskeletal material referred to as the contraction band acts to seal the area off to localize the necrosis as well as forming a protective barrier to allow for the repair of the damaged sarcolemma (T. A. Jarvinen et al., 2005).

The inflammatory phase is initiated as activated satellite cells, fibroblasts, and the damaged and necrotized ends of myofibers release host biomolecules that act as chemotractants for immune cell extravasation via damaged blood vessels at the site of injury (Baoge et al., 2012; T. A. Jarvinen et al., 2005; Tidball, 1995, 2011; Toumi & Best, 2003). During the acute phase of inflammation, the first and most abundant cell population consist of polymorphonuclear leukocytes such as neutrophils that act to debride the damaged site (Brickson, Hollander, Corr, Ji, & Best, 2001; Brickson et al., 2003; Hurme, Kalimo, Lehto, & Jarvinen, 1991). Within 24 hours, the concentration of polymorphonuclear leukocytes rapidly decline and are replaced with invading monocytes that ultimately transform into phagocytic macrophages, commonly referred to as M1 macrophages, reaching peak concentrations at 48 hours post-injury (Ochoa et al., 2007;

St Pierre & Tidball, 1994; Tidball, 2011). M1 macrophages are considered to be pro-inflammatory, releasing cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) among others in order to further promote immune cell migration, activation of inflammatory signaling via NF-κβ, and furthering muscle damage via increased nitric oxide production (Nguyen & Tidball, 2003; Villalta, Nguyen, Deng, Gotoh, & Tidball, 2009).

Following M1 macrophages are their non-phagocytic, anti-inflammatory counterparts, the M2 macrophages, which reach peak concentrations approximately 4 days post-injury (Krippendorf & Riley, 1993; St Pierre & Tidball, 1994). M2 macrophages have been known however to linger up to 2 weeks at the injury site, which also coincides with the terminal differentiation of muscle satellite cells, suggesting a role of M2 macrophages in regulating muscle repair and regeneration (St Pierre & Tidball, 1994). This is consistent with what was reported in a macrophage depletion study using the loading/re-loading model in mice. The study demonstrated that the depletion of macrophages during re-loading of muscle resulted in a significant reduction in growth and differentiation as well as prevention of the repair of damaged muscle cell membranes (Tidball & Wehling-Henricks, 2007).

Coinciding with shifts in the inflammatory response, the repair phase of regeneration occurs, primarily involving the migration, proliferation, and differentiation myogenic progenitor cells including satellite cells at sites of muscle injury. Satellite cells are widely accepted as the main contributor to skeletal muscle regeneration (Zammit et al., 2002). Satellite cells normally reside between the plasma and basement membrane of myofibers in a quiescent state, until they are activated by muscle injury (Cossu & Biressi, 2005). A hallmark of satellite cells is their self-renewal ability, which replenishes the remaining satellite cell population either by proliferation or withdrawal from the cell cycle and returning to a quiescent state. Although satellite cells

account for only 1-5% of total myonuclei (Alameddine, Dehaupas, & Fardeau, 1989), they have been demonstrated to be extremely competent in generating new myofibers following muscle injury (Collins et al., 2005). Following injury, a mixture of wound healing cytokines released by immune cells as well as mitogens from damaged muscle activate and induce the proliferation of satellite cells as well as migration towards the injury site (Allen & Boxhorn, 1989; Bischoff, 1986). In addition, a family of muscle-specific transcription factors referred to as myogenic regulatory factors (MRFs) regulate the proliferation and differentiation of satellite cells (Pavlath, Dominov, Kegley, & Miller, 2003; Suelves, Lluis, Ruiz, Nebreda, & Munoz-Canoves, 2004). Following injury, activated satellite cells are characterized by the high expression of MyoD and Myf5, committing them to differentiation (Cooper et al., 1999; Pavlath et al., 2003; Smith, Janney, & Allen, 1994; Yablonka-Reuveni & Rivera, 1994). Transient down-regulation of MyoD and Myf5 give rise to increasing expression of myogenin (MyoG) and MRF4, characterizing the terminal differentiation of myoblasts(Karalaki, Fili, Philippou, & Koutsilieris, 2009). Myogenin has been associated with terminal differentiation and the fusion of myogenic precursor cells to new or existing myofibers while MRF4 promotes early differentiation and myotube formation (Launay et al., 2001).

Concurrent with the repair phase, the extracellular matrix at the site of injury is remodeled and turned-over through the actions of metalloproteinases (MMPs) and their antagonists, tissue inhibitors of metalloproteinases (TIMPs). The regulation of MMPs and TIMPs are highly regulated throughout the muscle repair process as the balance of ECM degradation and synthesis is vital to normal muscle regeneration (Casar et al., 2004; Velleman, 1999). The degradation of ECM into fragments and the growth factors that are released during this process play an important role in the recruitment of a variety of cell types to the site of injury

(K. Thomas, Engler, & Meyer, 2015). Among these cells, the resident myofibroblasts are the main contributors to skeletal muscle ECM. They are responsible for the deposition of the two predominant fibrous collagen types in interstitial connective tissue (type I and III) but have also been shown to secrete collagens found in the basal lamina (type IV and VI) (Kuhl et al., 1984; Zou, Zhang, Sabatelli, Chu, & Bonnemann, 2008).

In skeletal muscle, the two main MMPs during remodeling have been identified as MMP-2 and MMP-9 and it has been proposed that these MMPs are differentially expressed transiently throughout the regenerative process (Kherif et al., 1999). Specifically, MMP-2 is concomitantly activated with *de novo* myofiber formation and MMP-9 is expressed in the early stages of regeneration involving inflammation and satellite cell activation (Kherif et al., 1999). Interestingly, activated satellite cells are capable of synthesizing and secreting MMP-2 and 9, suggesting that satellite cells are involved in the degradation of their own niche, allowing them to migrate to the site of injury (Kuhl et al., 1984; Zou et al., 2008).

C. Volumetric Muscle Loss

When extreme trauma occurs resulting in the bulk loss of tissue (<20% of muscle's mass), the regenerative capacity of muscle is overwhelmed leading to excessive scar tissue deposition and permanent functional deficits (Garg, Ward, et al., 2015; Mase et al., 2010; Terada, Takayama, Yamada, & Seki, 2001; Turner & Badylak, 2012). These injuries, referred to as volumetric muscle loss (VML), can result from both military (Corona, Rivera, Owens, Wenke, & Rathbone, 2015; B. D. Owens et al., 2008) and civilian trauma (Grogan, Hsu, & Skeletal Trauma Research, 2011; MacKenzie et al., 2000). Though VML primarily occurs through initial

trauma to the soft tissues, the condition can be further exacerbated as a consequence of excision and debridement of necrotic muscle tissue in cases involving extremity compartment syndrome from crush injuries. The absence of the contractile properties of the remaining muscle was reviewed by Turner and Badylak in 2012 where they reported that due to a dense cap of scar tissue forming between the damaged myofibers, denervation of the distal tissue occurs due to the absence of neuromuscular junctions (Turner & Badylak, 2012). Aside from the permanent loss of contractile tissue, the most challenging clinical problem is the multi-factorial nature of VML resulting in the concomitant loss of the myogenic cell population as well as changes to the muscle's architecture and composition (Corona et al., 2018; Corona, Garg, et al., 2013; Corona, Wu, et al., 2013).

With VML injuries, treating the injury itself may not be sufficient as there may be injury-associated changes to the remaining musculature that may preclude effective repair. A study highlighting the complexity of VML injuries was performed by Garg et al. found that the magnitude of functional deficits from VML were dependent on joint angle and muscle length (Garg, Ward, et al., 2015). Specifically, they reported that strength deficits were significantly worse at joint angles where the muscle was in a shortened position (i.e. dorsiflexion of the TA muscle). In addition, reductions in myofiber length, increased intramuscular collagen and scarring, and reduction in range-of-motion were observed. "This study highlights the complexity of VML injury: Not only the gross loss of muscle tissue but also architectural and histologic adaptations of the remaining tissue underlie the persistent functional deficits" (Garg, Ward, et al., 2015). Regeneration of de novo muscle fibers would be insufficient in restoring functional capacity to muscle as demonstrated by this study and would "require therapies that maximize the functional capacity of the remaining muscle tissue, promote de novo regeneration of muscle

tissue, and integrate these tissues to re-establish the intrinsic properties of the original muscle" (Garg, Ward, et al., 2015; Mase et al., 2010; J. G. Owens et al., 2011).

The current surgical standard of care for VML is the use of autologous muscle flap transfers in addition to rehabilitative regiments (Greene & Beatty, 1988; Mase et al., 2010; J. G. Owens et al., 2011). Flap transfer procedures have been used in various limbs including the forearm (Fan et al., 2008), elbow (Vekris et al., 2008), and lower extremities (C. H. Lin, Lin, Yeh, & Chen, 2007) following traumatic injuries requiring limb salvage. However, due to the size of the tissue required, these complex procedures are often associated with significant donor site morbidity. Moreover, the majority of flap transfers are non-functional and are mainly for cosmetic purposes though functional free muscle transplantations have been explored with limited success. Flap and free autografts are distinguished by flap autografts remaining attached to the muscle origin to preserve systemic blood supply (Klebuc & Menn, 2013).

Muscle Flap Transfers

First performed experimentally in 1970 by Tamai et al. and clinically by Harii et al. in 1976, free muscle transplantation with microsurgical techniques became widely used for the reconstruction of soft-tissue defects and functional replacement of damaged muscles (Harii, Ohmori, & Torii, 1976; Tamai, Komatsu, Sakamoto, Sano, & Sasauchi, 1970). Muscle flap transfer procedures have been explored for their advantages over local skin and fasciocutaneous flaps for the treatment of complex soft tissue traumas. Muscle flaps demonstrate the ability to fill in the dead space remaining in wound sites while the dense capillary network facilitates reperfusion into the surrounding tissue via anastomosis (Klebuc & Menn, 2013). Additionally,

muscle flaps have demonstrated rapid collagen deposition and allows for greater tissue ingrowth when compared to skin or fasciocutaneous flaps (Calderon, Chang, & Mathes, 1986; Chang & Mathes, 1982; Gosain, Chang, Mathes, Hunt, & Vasconez, 1990).

There have been many conflicting reports on the reinnervation of transplanted muscles and their effects on functional recovery and muscle maintenance (Brunelli, Monini, & Brunelli, 1990; Chiu, Chen, Spielholtz, & Beasley, 1991; McNamara, Garrett, Seaber, & Goldner, 1987). It was initially believed that the motor nerve was solely responsible for contractility and muscle maintenance (TOWER, 1931, 1939). However, it was also suggested that the trophic influence was a function of the sensory nerve fibers (FORBES & COBB, 1926). In 1945, Gutmann experimentally showed that motor nerve repair promptly after denervation mitigated loss to contractility and trophic maintenance of the affected muscle (Gutmann, 1945). Gutmann further demonstrated that sensory muscle reinnervation by neurorrhaphy of a sensory nerve to a motor nerve was insufficient to maintain muscle mass or contractility, supporting the claim that the motor nerve is responsible for muscle maintenance and contractility (Gutmann, 1945). Later in 1970, Zalewski's findings were in agreement with Gutmann when experiments showed that reinnervation by sensory or symphathetic neurons could not preserve mass or contractility (Zalewski, 1970). These studies were performed in an ideal setting with unaltered anatomic position of the muscle as well as with an undisturbed vascular pedicle. The findings from Gutmann and Zalewski prompted Thompson in 1971(Thompson, 1971a, 1971b) to explore the effects of innervation of transplanted muscle which prompted other researchers in the area to follow suit.

Interestingly, the previous findings supporting the motor nerve as the sole contributor to muscle contractility and maintenance did not translate into muscle transplantation procedures as

was demonstrated by Harii et al. (Harii et al., 1976). Harii et al. used autologous gracilis muscle transplantation to treat a patient with facial palsy and reported that despite prompt motor reinnervation of the transplanted muscle, the muscle atrophied by approximately 50% following 8 months of recovery. Similarly, in a rat model of muscle transplantation used by Zhang et al., gracilis muscle flap transplantation was used to study the effects of reinnervation of transplanted muscle using various microsurgical techniques including anastomosis and neurorrhaphy (Zhang et al., 1997). They found that denervated gracilis muscle *in situ* and transplanted decreased to 28.4% and 31.6% of their initial masses, respectively. Interestingly, motor neurotization of muscle *in situ* yielded bulk preservation of 83.2%, however, motor and sensory neurotization of transplanted muscle resulted in only 38.5% and 38.4% preservation, respectively and not significantly better than the denervated control flaps (Zhang et al., 1997). Though Zhang's findings demonstrated that motor nerves had better bulk preservation and trophic influence on muscle, it was also the first study to experimentally show that sensory neurons could still contribute to muscle maintenance, albeit to a lesser degree. These findings motivated further studies into sensory neurotization as a potential therapeutic treatment for the bulk preservation of denervated muscle.

Though sensory innervation was previously shown to be not as effective in muscle preservation, it was not until the past decade where a clinically relevant procedure using sensory nerves was developed. Termed 'sensory protection', the Bain group in 2001 first demonstrated that sensory neurotization of denervated gastrocnemius and soleus muscles in rats resulted in higher preservation of mass and superior histological appearance compared to non-protected muscles (Bain, Veltri, Chamberlain, & Fahnestock, 2001). However, isometric force did not improve but stayed constant, affirming previous observations by Gutmann suggesting that

sensory neurons are incapable of forming functional neuromuscular junctions (Gutmann, 1945). Despite this, Bain et al. reported that temporary sensory protection followed by motor neurotization can significantly improve force production in muscle and the first clinical application of this technique was reported in 2008 by the same group (Bain, Hason, Veltri, Fahnestock, & Quartly, 2008). This technique is not without drawbacks as there are still concerns regarding changes in the nerve microenvironment with the engraftment of sensory neurons that may create a sub-optimal environment for axonogenesis and myelination (Sulaiman et al., 2002). Furthermore, the bulk of the evidence for the preservation ability of sensory neurons in muscle is provided by very few sources. Though a significant amount of progress has been made in the field, there still remains a need for more rigorous clinical studies examining the effects of sensory neurotization and its effect on facilitating reinnervation and mass preserveration. As such, the use of muscle flap transfers for treating severe soft tissue traumas such as VML will remain ineffective in restoring functional capacity.

Rehabilitation Therapy

In the military medical system, the main form of therapeutic strategy for VML management is physical rehabilitation as most tissue engineering strategies being researched have yet to be realized in the clinic (Mase et al., 2010; J. G. Owens et al., 2011). By using aggressive rehabilitation programs as reported by Owens et al., all of the patients in the study were able to return to weight lifting while 80% of the cohort were able to return to running activities (J. G. Owens et al., 2011). Immediately after discharge from the hospital, patients underwent strength training to develop the force-generating capacity that is required for sports activities and then transitioned into plyometrics, agility, and run training as long as there was no

abnormal increase in pain with regimen progression (J. G. Owens et al., 2011). Even with the wide variety of injuries from explosive devices, high-velocity gunshot wounds, and motor vehicle collisions, the fact that the majority of the patients were able to return to sports activities and re-deploy in a few of the cases lends clinical value to rehabilitation programs, especially if they were to be used in conjunction with a tissue-engineered repair strategy.

There is no guarantee that general rehabilitation programs will impart beneficial effects in terms of muscle recovery following VML and VML-like injuries. Though physical rehabilitation has been investigated for the recovery of skeletal muscle damage due to conditions such as age(Betik, Baker, Krause, McConkey, & Hepple, 2008; Seene & Kaasik, 2012) and metabolic conditions like diabetes(Armstrong & Ianuzzo, 1977; Suga et al., 2014), there still remains a need for such investigations specifically for VML. In a study performed by Aurora et al. in 2014, the response of VML injured muscle to physical activity was investigated in order to aid in developing an appropriate rehabilitation regimen for VML injuries (Aurora, Garg, Corona, & Walters, 2014). The primary findings from the study reported that voluntary wheel running male adult Lewis rats following VML promoted approximately a 17% increase in maximal isometric torque as well as a 13% increase in weight of the injured muscle compared to sedentary controls, reflecting a 31% recovery of the functional deficit which is similar to force recovery following decellularized ECM transplantation following VML (Aurora et al., 2014; Corona, Wu, et al., 2013). However, no morphological changes were observed with regards to hypertrophy and hyperplasia, which may have indicated that the observed functional improvements were due to improved force transmission rather than force generation (Aurora et al., 2014). As demonstrated by the group before in an earlier study (Corona, Wu, et al., 2013), ECM remodeling and deposition at the defect site may be an adaptation for improved force transmission. The findings

from the study provided valuable insight into the changes in VML injured muscle following physical rehabilitation and brought attention to other important parameters to investigate such as magnitude of physical activity in the form of resistance exercise and timing of the implementation of the rehabilitation program following injury.

D. Tissue Engineered Strategies

Great strides have been made in the advancement of tissue engineering as a field in the past couple of decades, but despite the extensive effort of researchers there are still many obstacles in translating exploratory strategies to the clinic. Cell and scaffold-based strategies have been extensively employed in the repair and regeneration of a variety of tissues including cartilage, skin, cardiac, and bone in the clinic (Chambers, Rimington, Rajani, Hodson, & Shabbo, 2007; Falanga & Sabolinski, 1999; Harding, Sumner, & Cardinal, 2013; Oryan, Alidadi, Moshiri, & Maffulli, 2014; Song & Ott, 2011). However, there is a lack of therapies available for the repair and regeneration of muscle following extensive musculoskeletal trauma, though there are a number of research groups developing cell and scaffold-based technologies to address this issue (Carosio et al., 2013; Mertens, Sugg, Lee, & Larkin, 2014; Tedesco & Cossu, 2012). Similar to therapies in other tissues, skeletal muscle constructs must be biocompatible with the host environment, promote cell adhesion, proliferation, and differentiation, and also be able to deliver additional biomolecules such as growth factors. The challenge, however, comes when examining the components of skeletal muscle that need to be recapitulated through therapy.

Repair constructs must be able to mimic the highly aligned architecture of native skeletal muscle and be able to form functional tissue post-implantation (Turner & Badylak, 2012;

Valentin, Turner, Gilbert, & Badylak, 2010). In pre-clinical animal models where engineered skeletal muscle constructs were used, contractile function restoration was demonstrated via remodeling of the construct and formation of functional fibrotic structures that retained continuity in muscle architecture allowing for effective force transmission through the muscle (Aarimaa et al., 2004; Corona, Wu, et al., 2013). Construct design must also accommodate sufficient diffusion of oxygen and nutrients to the muscle if they are to provide a sustainable environment for seeded cell viability (Cittadella Vigodarzere & Mantero, 2014). Lastly, due to the permanent denervation of the distal fibers following VML and VML-like injuries, it is essential for repair strategies to be able to regenerate not only *de novo* muscle tissue but new motor endplates as well to restore functional capability in order to avoid atrophy (T. A. Jarvinen et al., 2007; T. A. Jarvinen et al., 2005). The importance of neurogenesis during muscle regeneration was investigated in the 1970s and 1980s, first by Benoit and Belt then by Foster and Carlson in studies using bupivacaine to induce muscle necrosis while preserving satellite cells, nerves, and vasculature (Benoit & Belt, 1970; Foster & Carlson, 1980). In the model, new myofiber formation was observed 3 days after bupivacaine injection with presence of newly forming motor endplates resulting in restoration of muscle function (Jirmanova & Thesleff, 1972). However, in a separate study where bupivacaine was injected in addition to denervation, the regenerating muscle was devoid of any contractile function (B. M. Carlson & Faulkner, 1996, 1998). In addition to conferring control to new muscle, motor endplates have also been shown to have chemotropic and stimulating effects influencing muscle fiber type, alignment, and size (Donghui et al., 2010; Grubic, Komel, Walker, & Miranda, 1995; Lefeuvre, Crossin, Fontaine-Perus, Bandman, & Gardahaut, 1996).

Despite these challenges, the biological and synthetic scaffolds being used in pre-clinical animal models have been demonstrated to be reliable platforms upon which regenerative therapies can be developed. These strategies aim to improve muscle regeneration by not only providing the mechanical/architectural cues absent following injuries, but they also act as a substrate for host and transplanted cells to carry out their regenerative processes. Biological scaffolds such as decellularized ECM scaffolds have been shown to promote muscle regeneration and functional improvement in patients suffering from traumatic injuries (Porzionato et al., 2015; Sicari et al., 2014). The combination of cells such as myogenic precursors, mesenchymal stem cells, and muscle satellite cells with scaffolds have also shown great promise as they similarly contribute to myogenesis, modulate the inflammatory response, and help maintain an environment conducive for regeneration (Borselli, Cezar, Shvartsman, Vandenburgh, & Mooney, 2011; B. N. Brown, Valentin, Stewart-Akers, McCabe, & Badylak, 2009; Garg, Ward, Rathbone, & Corona, 2014; Machingal et al., 2011). Synthetic scaffolds such as PGA (polyglycolic acid), PLA (polylactic acid), and PLGA (poly(lactic-co-glycolic acid)) (Cronin et al., 2004; Hoque et al., 2009; Lesman et al., 2011) are popular as they can be formed into constructs with specific patterning or alignment in order to manipulate cell behavior (Guex, Birrer, Fortunato, Tevaearai, & Giraud, 2013). Another benefit of synthetic constructs is that they can be easily engineered to facilitate the controlled release of growth factors and drugs to aid in muscle regeneration (Grasman, Do, Page, & Pins, 2015). However, the main disadvantage of synthetic scaffolds compared to decellularized xenogeneic and autologous tissue scaffolds is that they have poorer affinity for cellular binding, often requiring protein coatings to promote adhesion, and have higher risk of a foreign body response due to degradation products or the polymer itself (Yang, Jao, McNally, & Anderson, 2014).

Decellularized ECM Scaffold Strategies

Decellularized tissue scaffolds for tissue repair in recent years has been of particular interest due to the scaffolds sharing similar properties of the native tissue as well as being able to facilitate remodeling of *de novo* tissue and host cell infiltration (Merritt et al., 2010; Turner et al., 2010). Cellular antigens are recognized by the host system as foreign bodies and therefore induce an immune response. However, components of the ECM are remarkably conserved across species post-decellularization, which have made decellularized ECM scaffolds an attractive option in the area of regenerative medicine. The challenge and goal of decellularization protocols is to be able to retain the composition, bioactivity, and mechanical integrity of the remaining ECM during the decellularization process (Gilbert, Sellaro, & Badylak, 2006). Other factors to consider carefully are the host of origin of the tissue from which the ECM will be isolated and the method of decellularization as these factors can affect composition and structure of the ECM leading to changes in host response to the implanted scaffolds (Gilbert et al., 2006). The most effective protocols incorporate a combination of physical, chemical, and enzymatic treatments in order to lyse the cell membranes, separate the ECM from the cellular components, and solubilize the components for removal with subsequent rinse and sterilization steps (Gilbert et al., 2006).

These biologic scaffolds have seen success in both pre-clinical animal studies and in human clinical trial applications (S. F. Badylak, 2004; F. Chen, Yoo, & Atala, 1999; Dellgren, Eriksson, Brodin, & Radegran, 1999; Kolker, Brown, Redstone, Scarpinato, & Wallack, 2005; Wainwright, 1995). Based out of the University of Pittsburgh, Dr. Stephen Badylak and his group were among the first researchers exploring the value of tissue-based scaffolds and grafts as regenerative biomaterials in small and medium pre-clinical animal models. The focus of his work has been on the use of decellularized porcine small intestinal submucosa (SIS) as a biomaterial

(Stephen F. Badylak, 1993). Prior to his investigations into orthopedic soft tissue applications, his investigations included the use of SIS in the repair of small and large diameter arteries (S. F. Badylak, Lantz, Coffey, & Geddes, 1989; Lantz, Badylak, Coffey, Geddes, & Blevins, 1990), veins (Sandusky, Badylak, Morff, Johnson, & Lantz, 1992), and skin (Stephen F. Badylak, 1993).

In one of the first studies exploring the feasibility of using SIS as a biomaterial to repair localized orthopedic injuries, Badylak and colleagues evaluated the tissue reponse to SIS when it was used to repair an Achilles tendon defect in a canine model (S. F. Badylak et al., 1995). In the study, a 1.5cm long segment of Achilles tendon was excised from 20 dogs and the resulting defect was filled with a cylindrical piece of SIS that was sutured into place. Animals were sacrificed at 1, 2, 4, 8, 16, 24, and 48 weeks post-repair and neotendons were collected to monitor mechanical, biochemical, and morphological parameters (S. F. Badylak et al., 1995). Mechanically, the repaired tendons were observed to be stronger than both the musculotendinous origin and boney insertion, bearing loads of at least 1000N before rupturing when compared to the non-repaired group (S. F. Badylak et al., 1995). At one and two weeks post-repair, excised tendon samples showed dense infiltration of immune cells as well as a “richly vascular connective tissue matrix material” and was only observed within the anastomosis site and the area immediately adjacent (S. F. Badylak et al., 1995). At the later timepoints, the connective tissue appeared to have been remodeled into organized sheets that were aligned to the weight-bearing load. Additionally, the absence of significant populations of lymphoid and plasma cells was inconsistent with immune rejection of the SIS material, lending great value and potential of the material to be investigated in other repair applications (S. F. Badylak et al., 1995). Most importantly, the study found that the SIS material was degraded over time and allowed for host

tissue integration making it an attractive option for development as it was not associated with any of the complications normally associated with synthetic and other biomaterials.

Subsequent studies involving the use of the porcine SIS scaffold led to investigations in the repair of urinary tract replacement procedures (Shalhav et al., 1999), meniscal regeneration (Cook, Tomlinson, Kreeger, & Cook, 1999), and bone regeneration (Suckow, Voytik-Harbin, Terril, & Badylak, 1999; Winkler, Swaim, Sartin, Henderson, & Welch, 2002). However, the first use of porcine SIS in muscle repair was performed in 1995 by Prevel et al. in a rodent model of abdominal wall defects (Prevel et al., 1995). A 2x2-cm full-thickness defect was excised from the rodent abdominal wall. A patch of porcine SIS graft material was then sutured into the defect and the animals were left to recover for 1 week, 2 weeks, 4 weeks, 2 months, and 3 months prior to euthanization. To assess treatment efficacy, histological analysis was performed and it was revealed that the SIS graft had integrated into the host tissue with minimal inflammation by 2 months. Similar to studies using SIS biomaterials, there was no evidence indicating a host-rejection response to the material (Prevel et al., 1995).

In the realm of VML repair, in 2012 the Badylak group developed a murine model of VML in the quadriceps muscle and demonstrated the response to repair using a bioscaffold-based approach (Sicari et al., 2012). In the study, female C57/BL/6 mice aged 6-8 weeks had a 4x3mm full thickness segment of their tensor fasciae latae muscle along with a portion of the underlying rectus femoris muscle resected and then the defect was filled with a 4x4x3mm porcine SIS-ECM sheet implant. Histological analysis at the repair site at 7 days post-surgery showed dense populations of neutrophils and mononuclear cells around the defect site and the outer edges of the scaffold. In addition, evidence of angiogenesis and neurogenesis was found in the remodeling scaffold at day 7 and remained until the 56 day endpoint. After 28 days, host-

derived ECM was found to be integrating with the remodeled ECM implant and appeared to be cellularized. At 56 days, the ECM was completely populated with cells including desmin-positive skeletal muscle cells. On the contrary, the unrepaired group in the study exhibited deposits of dense connective tissue consistent with scar tissue formation with a distinct layer of host-derived neo-matrix at the margins of the defect site (Sicari et al., 2012). This study clearly demonstrated the potential of decellularized matrix scaffolds as therapies for VML injuries as the implanted scaffolds were able to integrate with the surrounding host tissue, promoted constructive remodeling of the defect site, and allowed for the recruitment of immune and progenitor cells to the site without illiciting an overt foreign body response.

Extending their previous research into VML repair in both mice and humans (Sicari et al., 2014), the Badylak group conducted a clinical study using several different tissue-derived ECM scaffolds of porcine origin to repair VML injuries in a 13-paitent cohort (Dziki et al., 2016). The injured muscle compartment of each patient was identified wherein scar tissue debridement and selective tenolysis was performed prior to the implantation of one of three ECM bioscaffolds: porcine urinary bladder matrix (UBM, MatriStem, ACell, Columbia, MD, USA), porcine SIS (BioDesign, Cook Medical, Bloomington, IN, USA) or porcine dermis (XenMatrix, C.R. Bard, Warwick, RI, USA) (Dziki et al., 2016). All bioscaffolds were suture with contact to adjacent native healthy tissue and secured under tension with suture. Tissue biopsies taken at 6-8 weeks, 10-12 weeks, and 24-28 weeks post-surgery showed progressive evidence of *de novo* muscle formation and neovascularization verified by the presence of desmin+ cells with centrally located nuclei and CD146+NG2+ perivascular stem cells. The presence of β-III tubulin+ nerve bundles by 6 months after surgery within the repair site was suggestive of innervation and neurogenic cell activity. Post-operative improvements in force production was measured via dynamometer

ranging from 20-140% at 6 months post-surgery which was attributed to ECM implantation. However, all patients underwent aggressive physical therapy programs pre-operatively and during recovery from VML repair which may have had a positive impact on bioscaffold remodeling leading to increased vascularization and innervation. These findings are supported by previous studies investigating the effects of mechanical loading following VML repair with ECM scaffolds (Ballotta, Driessen-Mol, Bouten, & Baaijens, 2014; Gentile et al., 2014; Gilbert et al., 2007; Turner, Badylak, Weber, & Badylak, 2012). However, the improved functional outcomes are likely attributed to improved force transduction via scar tissue remodeling as documented by Aurora and Corona (Aurora, Roe, Corona, & Walters, 2015; Corona, Wu, et al., 2013). Further, the use of acellular bioscaffolds such as the urinary bladder matrix used in this study alone were demonstrated to be insufficient in generating an appreciable amount of *de novo* skeletal muscle and was suggested that for significant muscle regeneration to occur, repair strategies needed to incorporate progenitor cell populations and develop more effective myoconductive scaffolds (Aurora et al., 2015).

Decellularized Muscle-Derived ECM Scaffold Strategies

Though decellularization of tissues has been shown to be non-immunogenic and acellular, there may be benefits to using a scaffold derived from the target tissue of interest. Specifically for muscle, the remnant structure following decellularization may facilitate the integration of peripheral nerves due to the already existing neural pathways in the scaffold (Borschel, Dennis, & Kuzon, 2004). Further, the remaining vascular system in acellular skeletal muscle scaffolds could be used to perfuse the constructs allowing for improved growth and force production as well as recellularizing the vascular bed (Borschel et al., 2004). Current trends in

skeletal muscle tissue engineering have shown a favorable response to the use of skeletal muscle-derived ECM strategies for muscle regeneration with extensive work being performed assessing their host bioactivity and histocompatibility (Borschel et al., 2004; Corona, Wu, et al., 2013; DeQuach et al., 2012; Perniconi et al., 2011).

In a study by Borschel et al., the myogenic potential and force production capability of acellular muscle scaffolds seeded with and without C2C12 myoblasts was explored (Borschel et al., 2004). Acellular scaffolds were created from extensor digitorum longus muscles excised from adult C57-BL6 mice. Repopulated and acellular scaffolds were cultured for a period of 3 days in growth medium and then for 3 weeks in differentiation medium. Force production in the myoblast populated muscle scaffolds was substantially lower than normal EDL muscle output but was enough to demonstrate that the seeded cells were able to proliferate and differentiate into fused myofibers within the acellular muscle scaffolds. These findings were further substantiated with histological analysis using toluidine blue staining to confirm cell proliferation and electron micrograph imaging to examine microscale structures within the populated and non-populated acellular scaffolds. The non-populated scaffolds were devoid of the key muscle cytoskeletal elements actin and myosin as well as having a loosely organized matrix. However, with the populated scaffolds, researchers found the existence of the filamentous proteins actin and myosin and further observed that they had assembled into a sarcomeric pattern (Borschel et al., 2004). Though tested *in vitro*, these findings were significant in that they addressed some of the major hurdles facing the development of constructs that are to be clinically relevant.

While it was demonstrated previously to be feasible to produce highly complex organs such as the rodent heart *in vitro* (Ott et al., 2008), an *in vivo* skeletal muscle transplantable had yet to be developed. To that end, Perniconi et al. produced acellular scaffolds from tibialis

anterior (TA) muscles of adult sex-matched BALB/C mice and transplanted them into syngeneic hosts to be assessed for histocompatibility, bioactivity, and host integration in a murine model (Perniconi et al., 2011). An incision was made in the hindlimb to expose the TA epimysium to allow for the excision of the TA. The distal tendon and a fragment of the proximally inserted muscle on the TA were left intact to anchor the scaffold. Mice were given 2 and 4 weeks to recover after which the grafted materials were harvested for histological analysis. Immunostaining revealed that at 2 weeks post-implantation there were signs overt inflammation within and around the scaffold, which was further specified to be infiltration of CD45+ cells comprised mostly of macrophages via flow cytometry, indicating that immune cell infiltration was restricted to the implanted material. In 4 week grafted material, the degree of inflammation appeared to be decreased to control levels. The presence of muscle interstitial stem cells was also confirmed within grafted materials. Scattered nascent myofibers exhibiting centrally located nuceli preferentially located in the mid-belly and distal regions of the scaffold demonstrated its myogenic capability. These findings were further validated with protein expression of myosin heavy chain and sarcoglycan and mRNA expression of muscle-specific late differentiation markers (Ott et al., 2008). Taken with other studies that use ECM from other tissues to regenerate muscle that morphologically and functionally mimic native tissue (Turner et al., 2010; Valentin et al., 2010; Vindigni et al., 2004), ECM has consistently shown the ability to form *de novo* tissue, in part due to contributions of the migration of tissue-resident progenitor cells to the implant site. The added value of this study is that it demonstrates that muscle-derived ECM itself has pro-myogenic qualities supporting an environment favorable for myofiber formation. Findings from other researchers highlighting the impact of niche regulation on tissue-resident stem cell function suggest that muscle-derived ECM may possess to some degree muscle niche

properties that aid in its myogenic activity (Ferraro, Celso, & Scadden, 2010; Pelosi et al., 2007; Vindigni et al., 2004).

The use of muscle-derived ECM scaffolds in VML repair has been of great interest to the military due to the high incidence of extremity trauma in veterans returning from active war zones and the lack of an effective treatment to restore functional tissue following reconstructive surgery (Andersen et al., 2014; Mase et al., 2010; B. D. Owens et al., 2008; J. G. Owens et al., 2011; Ramasamy, Harrisson, Clasper, & Stewart, 2008). However, trauma research at the U.S. Army Institute of Surgical Research (USAISR) led by the Walters and Corona groups have made great advancements in the development of VML repair strategies using muscle-derived ECM scaffolds. The Walters group's initial work in VML yielded a standardized rat model of VML (Wu, Corona, Chen, & Walters, 2012) upon which various treatment strategies have been tested (Aurora, Corona, & Walters, 2016; Corona, Garg, et al., 2013; Corona, Ward, Baker, Walters, & Christ, 2014). Specifically, the model involves the creation of a VML defect (~20% of muscle's mass) in the tibialis anterior muscle of a rat. More importantly, there has been thorough documentation of the response to VML injury itself and to the implantation of muscle ECM scaffolds during VML repair (Aurora et al., 2015; Corona et al., 2014; Corona, Wu, et al., 2013; Garg, Corona, & Walters, 2015). Wu et al. demonstrated in the standardized rat VML model that following VML injury at 2 and 4 months, significant atrophy to the unrepaired muscles occurred (22.7% and 19.5% reduction in mass compared to uninjured controls at 2 and 4 months) while also being accompanied by a reduction in peak isometric torque of 28.4% and 32.5% at 2 and 4 months respectively (Wu et al., 2012). In addition, they observed the characteristic deposition of scar tissue at the VML injury site as well as ongoing remodeling and repair as indicated by the presence of centrally nucleated muscle fibers at the injury site (Wu et al., 2012). These results

have been observed to be consistent across similar VML studies (Corona, Wu, et al., 2013; Kasukonis, Kim, Washington, & Wolchok, 2016).

In terms of functional recovery following repair, scaffold-only repair has been incapable of promoting an appreciable amount of muscle regeneration resulting in significant increases to contractile function (Aurora et al., 2015; Corona et al., 2014). Despite reports of improved functional outcomes following VML repair with muscle-derived ECM and other ECM scaffolds, the force improvements are most likely attributed to a "bridging" of the defect site by scar tissue deposition as demonstrated by Corona et al. (Corona, Wu, et al., 2013). In that study performed by Corona et al., a VML defected created in the tibialis anterior muscle of a rat was left untreated, repaired with muscle ECM, or repaired with muscle ECM with a delayed injection of bone marrow stem cells (Corona, Wu, et al., 2013). Interestingly, it was found that untreated VML injury resulted in extensive fiber damage and remodeling that visibly extended from the defect site to the underlying musculature. However, repair with the muscle ECM (with and without BMSC delivery) appeared to attenuate the damage to the remaining musculature which was associated with the observed functional improvements at 2 and 4 months post-treatment. No significant *de novo* myofiber formation was observed in this study, though there have been varying reports on the degree of myofiber generation following scaffold-mediated repair ranging from abundant and modest levels (Corona et al., 2012; Machingal et al., 2011; Merritt et al., 2010) to low or non-existent levels (Conconi et al., 2005; Turner et al., 2010). However, the characteristic deposition of mostly collagen I-enriched scar tissue at the VML repair site was observed; findings that are consistent with the use of ECM scaffolds to repair similar complex injuries (Gamba et al., 2002; Kim, Kasukonis, Brown, Washington, & Wolchok, 2016; Turner et al., 2012).

Cell-based Strategies

The use of cells in tissue-engineered therapies can have tremendous value compared to acellular scaffolds when considering rejuvenating the native cell population in cases such as VML injuries. Due to their multipotency, mesenchymal stem cells have the ability to aid in the repopulation and subsequent proliferation and differentiation into target tissue cells once implanted. In various studies, the delivery of quiescent muscle satellite cells have shown to promote greater regeneration compared to culture-derived myoblasts (DiMario & Stockdale, 1995; Rossi et al., 2011). These studies, however, focused on the therapeutic effects that the degree of maturation of immature cells (quiescent vs. activated cells) may have whereas the therapeutic potential in delivering a construct in combination with active precursor cells has been explored to a lesser extent. The greatest advantage of using a tissue-engineered scaffold in conjunction with myogenic precursor cells is that such a strategy aids in recapitulating the cellular and mechanical environment following VML injury. For instance, a study led by Corona from the Christ research group from Wake Forest University is particularly interesting as it combines both acellular scaffold and *in vitro* cultured myofbers and cells (Corona et al., 2012). The treatment strategy included the use of a porcine urinary bladder matrix, which was seeded with a mix of primary cells isolated from tibialis anterior and soleus muscle. The seeded constructs were then pre-conditioned in a stretch bioreactor in order to proliferate and differentiate the cells (Corona et al., 2012). The three separate constructs tested in the study consisted of a single seeding of cells that were proliferated during pre-conditioning (TEMR-1SP), a seeding of cells that were differentiated under pre-conditioning (TEMR-1SPD), and 2 seedings of cells that were proliferated and differentiated (TEMR-2SPD). Significant functional recovery following 1 and 2 months post-implantation was detected for all groups compared to

non-repaired controls with the TEMR-2SPD group exhibiting the highest force recovery. It was also observed that both magnitude and time course of recovery was dependent on the TEMR construct used with the TEMR-1SPD and TEMR-2SPD constructs providing a 62% and 110% increase in peak force values when compared to non-repaired controls at 2 months post-injury. Additionally, TEMR-1SPD and TEMR-2SPD groups exhibited a 39% and 28% improvement in peak force between 1 and 2 months post-injury. Morphologically, TEMR-1SPD and TEMR-2SPD repaired muscle samples appeared to have an increased cellular presence within the scaffold area, an increase in muscle tissue formation at both the repair site and within the scaffold, and a presence of vascular and neural structures at the tissue-construct interface (Corona et al., 2012).

These findings are particularly interesting as they illustrate the unique benefits of a hybrid construct containing both pre-conditioned proliferating and differentiating cell populations. Specifically, Corona et al. found that actively proliferating cells contributed to accelerated functional recovery while the presence of differentiated myotubes is critical in maintaining continuous functional recovery (Corona et al., 2012). Compared to strategies using acellular scaffolds to form *de novo* muscle tissue, it is plausible that hybrid constructs such as the TEMR would require a shorter time to promote new tissue growth contributing to functional recovery ((Corona et al., 2012)). An alternative to the delivery of stem or progenitor cells is the use of autologous minced muscle grafts as a means of introducing not only muscle progenitor cells but other muscle-native cells and native ECM to the VML site (Corona, Garg, et al., 2013; Goldman & Corona, 2017; Goldman, Henderson, & Corona, 2017; Hurtgen et al., 2017; B. Kasukonis et al., 2016).

The first experiments detailing the regenerative capacity of minced muscle fragments were performed by Studitsky (Studitsky, 1964) and Carlson (B. M. Carlson, 1968) in their respective seminal publications. Studitsky's most significant finding was that minced muscle tissue was observed to enter a state of plasticity and thereby increased the survivability and development of transplanted minced tissue in injury sites (Studitsky, 1964). Carlson's study in 1968 confirmed Studitsky's previous findings by implanting minced muscle to regenerate rat and frog muscles following complete removal of the gastrocnemius muscle (B. M. Carlson, 1968). Carlson confirmed the regeneration of muscle and restoration of anatomical organization in both frog and rat specimens, though it was noted that regeneration appeared to occur at approximately twice the rate in the rats compared to the frogs (B. M. Carlson, 1968). An important finding from the study, however, was the observation of normal regeneration in the acute phases of healing but then cycles of degeneration of newly formed myofibers within the tissue regenerates. At the end of the study, it was reported that most of the muscle regenerates were reduced to "a tendinous structure with only a few scattered muscle fibers…" (B. M. Carlson, 1968). This was attributed in part to the lack of innervation in the late phases of regeneration as previous reports have demonstrated in detail that early muscle differentiation can occur independent of innervation but is necessary for the latter stages of differentiation (Striganova, 1958; Zhenevskaya, 1962).

In recent reports, the delivery of satellite cells via transplantation of single muscle fibers has been demonstrated to be a potent technique to not only produce myogenically competent progeny but to increase satellite cell engraftment and survivability as well (Collins et al., 2005; Hall, Banks, Chamberlain, & Olwin, 2010; Tateyama, Fujihara, Misu, & Itoyama, 2009). However, the delivery of progenitor cells via minced muscle grafts may be more advantageous

due to the simplicity of the procedure compared to single myofiber isolation and lends itself to facile integration with treatment strategies for use in the clinic. Corona et al. in 2013 first explored the use of autologous minced muscle grafts for VML repair (Corona, Garg, et al., 2013). Following VML defect creation in the TA muscle of Lewis rats, it was observed that orthotopic transplantation of minced muscle autografts into the wound bed promoted myofiber regeneration by 8 and 16 weeks post-repair. Interestingly, by 8 weeks the regenerated fibers exhibited signs of innervation, which was further validated via a glycogen depletion study. Fibers were also reported to have similar cross-sectional areas as uninjured muscle fibers when compared to literature (Corona, Garg, et al., 2013). Functional capacity was restored to approximately 55% of the functional deficit present in unrepaired TA muscles (Corona, Garg, et al., 2013). In an earlier study that transplanted decellularized muscle-derived ECM to repair a VML defect (Corona, Wu, et al., 2013), functional recovery at 16 weeks post-repair was reported to be approximately 20%. It is clear that minced muscle autografts contribute to an increase in functional tissue as evidenced by the two-fold increase in functional recovery over scaffold-alone repair.

Building off the minced muscle graft approach for VML repair, Kasukonis et al. demonstrated that the combinatorial therapy of minced muscle grafts and decellularized ECM scaffolds was able to significantly improve functional outcomes as well as exert transcriptional regulation over myogenesis following VML repair (B. Kasukonis et al., 2016). The use of a 25% minced muscle graft in this study was motivated by previous work from Corona et al. exploring minced muscle graft volume expansion approaches for the treatment of VML (Ward, Ji, & Corona, 2015). In that study, Corona et al. observed that using approximately 50% of the VML defect tissue as minced muscle in a hydrogel carrier was comparable in promoting functional

recovery to that of a 100% minced muscle graft with hydrogel carrier (Ward et al., 2015). By utilizing a <50% minced muscle graft, Kasukonis et al. hoped to evaluate whether a decellularized skeletal muscle scaffold (DSM) could improve the regenerative abilities of the sub-optimal amount of minced muscle graft used in the study. Not only did DSM and 25% minced muscle repair improve functional outcomes that were comparable to what was observed with the use of 50% minced muscle by Corona et al. (~50% force recovery), significant improvements in the restoration of muscle mass and upregulation of myogenic gene expression were also observed (B. Kasukonis et al., 2016). These findings warrant further investigation into the development of DSM scaffolds as carriers for minced muscle grafts. Specifically, it is of great interest whether these scaffolds act merely as vehicles for the minced muscle or if there are any significant matrix-minced muscle interactions that modulate satellite cell activity for the benefit of myogenesis and VML repair.

Synthetic and Natural Polymer Strategies

The benefit in using natural and synthetic polymers in the development of engineered skeletal muscle constructs is the ability to control the mechanical and spatial properties of the material during fabrication. Another advantages of synthetic materials is the ability to incorporate growth factors into the bulk structure of these polymers allowing for controlled release of these factors into the site of injury (Niu, Feng, Wang, Guo, & Zheng, 2009; Prabaharan & Jayakumar, 2009). Biomaterial development for skeletal muscle tissue engineering has explored a large variety of materials ranging from synthetic polymers such as poly(glycolic acid) (PGA) (Saxena, Marler, Benvenuto, Willital, & Vacanti, 1999; Saxena, Willital, & Vacanti, 2001), poly(lactic acid) (PLA) (Cronin et al., 2004), and poly-ε-caprolactone (PCL)(Choi, Lee,

Christ, Atala, & Yoo, 2008); to natural polymers such as alginate (Borselli et al., 2010), collagen (Hurd, Bhatti, Walker, Kasukonis, & Wolchok, 2015; Kroehne et al., 2008), and fibrin (Marcinczyk et al., 2018; Page et al., 2011).

The spatial patterning of constructs, especially highly aligned patterns of electrospun nanofibers mimicking the native architecture of muscle, provides control over cellular behavior, which can be of great benefit in terms of being able to enhance certain phases of myogenesis. *In vitro* studies exploring spatially patterned constructs for musculoskeletal engineering applications have demonstrated that highly aligned cultures of myoblasts seeded onto aligned nanofiber scaffolds have increased proliferation and more readily fuse into multi-nucleated myofibers (Choi et al., 2008; Guex et al., 2013; Zatti et al., 2012) than cultures using randomly-oriented fibers. These effects are augmented when additional scaffold properties are incorporated, as was the case with Chen et al. who fabricated electrically conductive PCL/polyaniline (PCL/PANi) nanofiber scaffolds to evaluate their potential use in engineering regenerative strategies for skeletal muscle (M. C. Chen, Sun, & Chen, 2013). Chen et al. observed synergistic effects of topographical and electrical cues on myotube formation with significant increases in myotube formation and maturation (15% and 18% increases, respectively) when compared with non-conductive or randomly oriented conductive scaffolds. Similar electrically conductive scaffolds have been demonstrated to increase differentiation and myogenic gene expression in myoblasts (Jun, Jeong, & Shin, 2009), increase alignment and proliferation in osteoblasts (Shao et al., 2011), and increase adhesion and proliferation of cardiac myoblasts (Bidez et al., 2006). Synthetic constructs that provide multiple environmental cues will be indispensable in the further development of tissue engineering strategies due to the immensely complex nature of the *in vivo* environment.

Despite imparting beneficial effects on functional outcomes, synthetic biomaterials are limited due to their poor bioactivity, often requiring the addition of coatings to promote cell adhesion or incorporation of natural polymers to attenuate the host immune response (Choi et al., 2008; Wolf et al., 2014). An interesting approach exploring the development of a more bioactive scaffold leveraged the porous architecture of polyurethane (PU) foams to act as a sacrificial template for the deposition of ECM from rat myoblasts (Hurd et al., 2015). Myoblasts were cultured on fibronectin coated PU foams for 4 weeks after which the foams were dissolved in solvent to isolate the resulting ECM material. Collagen I, collagen IV, and laminin were all detected to be present in the resulting material, all of which are present in the basement membrane of skeletal muscle (Gillies & Lieber, 2011). Materials such as these are uniquely suited to be used in the repair of VML and VML-like injuries as they can provide the bioactive signaling cues that are missing due to the disruption to the basement membrane following VML injury. However, Hurd et al. reported the mechanical strength of the foam template ECM to be approximately one-third of decellularized muscle ECM, which may lack the structural support to withstand mechanical loading during regeneration (Hurd et al., 2015).

Unlike their synthetic counterparts, natural polymers intrinsically possess bioactivity as well as the ability to form molecules such as proteoglycans which enhance cell migration, proliferation, and differentiation via binding of growth factors (Bidarra, Barrias, & Granja, 2014; A. C. Brown & Barker, 2014; Walters & Stegemann, 2014). Among these biopolymers, alginate (Hill, Boontheekul, & Mooney, 2006b; Liu et al., 2013), collagen (Chevallay & Herbage, 2000; Rhim et al., 2007), and fibrin (Heher et al., 2015; Marcinczyk et al., 2018; Matsumoto et al., 2007) have been predominantly explored in muscle regenerative applications.

Initial exploration into the applications of alginate-based biomaterials were to utilize them as encapsulating agents for the delivery of proteins in order to mimic paracrine signaling between cells (Edelman, Mathiowitz, Langer, & Klagsbrun, 1991; Sugamori & Sefton, 1989). Due to its ability to maintain steady release profiles of desired proteins and its tunable mechanical properties (Boontheekul, Kong, & Mooney, 2005; Drury, Boontheekul, & Mooney, 2005), it remains as an ideal candidate for controlled delivery systems for various tissue engineering applications such as the delivery of pancreatic islet cells (Opara, Mirmalek-Sani, Khanna, Moya, & Brey, 2010), to promote osteogenesis via mesenchymal stem cell delivery (Zhou & Xu, 2011), and revascularization of various tissues (Drewa, Adamowicz, & Sharma, 2012; Iwasa, Engebretsen, Shima, & Ochi, 2009; Macchiarini et al., 2008). Within skeletal muscle regeneration, *in vitro* investigations found that the stiffness of the gels (~45 kPa) significantly increased myoblast proliferation and differentiation (Boontheekul, Hill, Kong, & Mooney, 2007). As a delivery vehicle, alginate has been demonstrated to be able to transport and release various growth factors crucial to muscle regeneration including VEGF (Shvartsman et al., 2014; Wang et al., 2014), IGF-1 (Borselli et al., 2010), and HGF and FGF-2 (Hill, Boontheekul, & Mooney, 2006a; Hill et al., 2006b). *In vivo*, the combined delivery of VEGF and IGF-1 in an alginate gel carrier was observed to improve host progenitor cell survival and vascularization in the acute phases of regeneration (Borselli et al., 2010; Shvartsman et al., 2014). Further, delivery of both VEGF and IGF-1 increased force production approximately 4-5 fold compared to blank control gels (Borselli et al., 2010). However, this study used a less severe injury model than a VML with ischemia induced muscle injury. Additionally, alginate gels have only, thus far, been implanted on the surface of injury sites (Borselli et al., 2011) and even though they have been proven effective in improving vascularization and myoblast proliferation and differentiation, no

conclusions can be drawn on their efficacy when presented with a more complex *in vivo* environment as in VML cases.

In skeletal muscle, collagen I gels have been used as cell delivery vehicles for myoblasts which have been shown to promote cell migration out of the porous gels to the ends of existing myofibers (Carnio et al., 2011). Without modifications to collagen assembly within gels, the result of implantation following injury is a suboptimal healing response characterized by increased immune cell infiltration into the site and an absence of myogenesis (van Wachem, Brouwer, & van Luyn, 1999). However, collagen constructs with tunable internal structures such as the parallel pore collagen sponge developed by Korehne et al. can promote aligned myotube growth and development *in vitro*, which can later be transplanted into an *in vivo* injury site (Kroehne et al., 2008). In the study, 6mm x 3mm x 2.5mm collagen sponges were seeded with C2C12 myoblasts which were allowed to proliferate for 1 day before switching to differentiation medium for an additional 5-6 days, after which the cultured regenerates were sutured into the muscle bed where the tibialis anterior muscle was located prior to excision. It was observed that the implanted regenerates were able to integrate into the surrounding host tissue as well as having the ability to contract under stimulation (Kroehne et al., 2008). These results suggest that rather than the material itself, the alignment of the constructs used to repair large tissue defects appear to impart greater effects to improve myogenesis compared to similar materials with no alignment. In addition, with the absence of any significant regenerative outcomes resulting from the implantation of collagen materials/scaffolds, their uses are limited to *in vitro* development of partial tissue regenerates and acting as vehicles for the delivery of growth factors such as VEGF (Frey, Jansen, Raschke, Meffert, & Ochman, 2012) or HGF, IGF-1, and FGF2, which have been shown to promote satellite cell recruitment (Ju, Atala, Yoo, & Lee, 2014).

Fibrin, a branched fibrillary polymer, is another attractive biopolymer material upon which muscle regenerative strategies can be developed due to its role *in vivo* as the provisional matrix that is deposited during wound healing as well as its inherent bioactivity (A. C. Brown & Barker, 2014; Chiron et al., 2012; Clark, 2001). Though its biodegradation has been observed to be approximately 3 weeks *in vivo*, its structural integrity can be augmented via incorporation of polymers such as PLLA/PLGA or polyethylene glycol (PEG) to slow the degradation rate (Lesman et al., 2011). Additionally, an alternative to hybridizing fibrin with synthetic polymers is manipulating the degree of cross-linking (ultraviolet or chemically-induced) (Grasman, Pumphrey, Dunphy, Perez-Rogers, & Pins, 2014). Several studies have demonstrated the ability of myoblast seeded fibrin gels to improve myoblast survival post-transplantation and promote differentiation into myofibers that are able to integrate into the uninjured host tissue (Beier, Kneser, Stern-Strater, Stark, & Bach, 2004; Beier et al., 2006; Gerard, Forest, Beauregard, Skuk, & Tremblay, 2012). Further, it was found that implanting a myoblast-loaded fibrin gel sheaths in the proximity of native blood vessels increased vascular perfusion into the constructs (Borschel, Dow, Dennis, & Brown, 2006). In addition, the resulting tissue explants isolated from the gel sheaths were found to resemble functional muscle tissue capable of contraction while maintaining their mechanical integrity through testing (Borschel et al., 2006). Despite these findings, with the lack of *in vivo* functional measurements it is difficult to say whether the amount of regeneration and angiogenesis resulting from treatment would be effective in treating larger volumetric defects in muscle.

In a VML injury setting, fibrin has been utilized in many forms including microthreads (Grasman, Page, & Pins, 2017), hydrogels (Gilbert-Honick, Iyer, et al., 2018), and microbeads (Lalegul-Ulker, Seker, Elcin, & Elcin, 2019). The benefit of these materials is that they are able

to deliver growth factors (Cornwell & Pins, 2010; Grasman et al., 2015) and cells (Gilbert-Honick, Ginn, et al., 2018; Page et al., 2011) to the site of injury to augment the regenerative process. In a study that leveraged both the delivery capability and tunable structure of fibrin, researchers were able to seed adult adipose stem cells (ASCs) onto fibrin microfiber bundles prior to implantation into a VML defect in a murine model (Gilbert-Honick, Ginn, et al., 2018). Interestingly, *in vitro* experiments assessing the pro-myogenic potential of the ASC seeded fibrin microfiber bundles revealed that the seeded constructs were able to produce aligned and elongated ASCs that expressed markers for skeletal muscle but failed to mimic native muscle characteristics. When the bundles were implanted in place of both TA and EDL muscles *in vivo*, a significant reduction in fibrosis at the implant site was observed (Gilbert-Honick, Ginn, et al., 2018). Further, compared to acellular fiber bundles, seeded bundles exhibited increased markers for embryonic myosin as well as the mature muscle marker myosin heavy chain. However, due to the lack of functional muscle measurements as well as histological analysis of the regenerating construct/tissue explants, no meaningful conclusions can be drawn from a clinically relevant standpoint. As the majority of non-modified fibrin materials degrade *in vivo* within 3 weeks upon implantation, the resulting *de novo* myofibers are often randomly oriented and disorganized unlike the highly aligned surrounding host tissue, giving rise to poor functional outcomes and regeneration. Despite this, the development of biopolymer scaffolds such as fibrin and those mentioned previously still hold great value when thinking towards combinatorial tissue engineering approaches to repair large tissue defects like VML.

E. Aging Skeletal Muscle

Closely associated with aging is the gradual decline of cellular activity ultimately leading to physiological changes that contribute to the impairment of normal regenerative processes. This has been documented in various tissues such as skin during wound healing (D. R. Thomas, 2001), angiogenesis in various tissues and organs (Edelberg & Reed, 2003; Lahteenvuo & Rosenzweig, 2012), and remyelination of axons in the central nervous system (Franklin, Zhao, & Sim, 2002; Sim, Zhao, Penderis, & Franklin, 2002). In skeletal muscle, the predominant change with aging is the overall loss of muscle strength, muscle mass, and decline in regenerative potential. Underlying the loss in strength is a reduction in the number of functional motor units, particularly fast-fatigueable motor units (Kung et al., 2014; Larsson, 1995). The progressive loss in muscle mass, often referred to as sarcopenia, and decline in regenerative capacity is largely due in part to the reduced activity and numbers of satellite cells associated with aging (Brack & Rando, 2007; Conboy & Rando, 2005; Snow, 1977). Specifically, a decrease in sensitivity to signals initiating the activation, proliferation, and renewal capability of satellite cells has been documented with aging (Brack & Rando, 2007; Gopinath & Rando, 2008; Kuang, Gillespie, & Rudnicki, 2008). Also, associated with sarcopenia is the marked decrease in capillary density and circulating levels of angiogenic factors that may have negative implications regarding metabolism, endocrine function, and muscle contractility (Olfert, Baum, Hellsten, & Egginton, 2016). Though these major changes in aging skeletal muscle have implications regarding the regenerative capacity of muscle, it is equally as important to understand the more subtle changes in the host environment and satellite cell niche that also contribute to the reduction in regenerative ability.

Extracellular Matrix

One of the major changes in the extracellular matrix with aging is the significant increase in interstitial connective tissue (Marshall, Williams, & Goldspink, 1989). According to Marshall et al., there is approximately a two-fold increase in endomysial collagen when comparing mice aged 3 weeks and 26 weeks (Marshall et al., 1989). Further, there is also a marked increase in the fibrotic response during muscle regeneration (B. M. Carlson & Faulkner, 1989; Ullman, Ullman, Sommerland, Skottner, & Oldfors, 1990). Concomitant with increased fibrosis is the thickening of the external lamina surrounding satellite cells resulting in reduced satellite cell numbers (Snow, 1977). This increased fibrogenesis in aging muscle has been attributed to increased Wnt signaling and possibly to Wnt-like proteins present in the serum (Brack et al., 2007). Specifically, Brack et al. exposed young and aged satellite cells to serum in a heterochronic pairing. When aged cells were exposed to serum from young animals, there was a significant reduction in the myogenic-to-fibrogenic conversion of the aged satellite cells. Assessing the effects of Wnt signaling on myogenic cell fate, Wnt3A protein was added to young serum which resulted in an increase in the fibrogenic conversion of young satellite cells while administration of a Wnt inhibitor into aged serum decreased the fibrogenic conversion of aged progenitors *in vitro* (Brack et al., 2007). Though it was discovered that age-related changes in the muscle microenvironment were responsible for increased fibrosis and altered cell fate in aging muscle progenitors, this study identified a potential therapeutic target to improve the regenerative response while reducing fibrosis.

Aside from fibrosis, ECM and ECM-associated molecules also play a direct role in communicating with the cells that interact with it in order to regulate the migration, proliferation, and differentiation of myoblasts (Grounds, 1991; Grounds & Yablonka-Reuveni, 1993). These

include laminins that are associated with the external lamina (B. M. Carlson, 1995; Maley, Davies, & Grounds, 1995), proteoglycans that are necessary for the binding of specific growth factors to their receptors (Muramatsu, Muramatsu, & Kojima, 2006; Schultz & Wysocki, 2009), and proteolytic fragments such as collagen I and IV, fibronectin, and elastin which induce chemotaxis of inflammatory cells (Adair-Kirk & Senior, 2008). Changes in ECM composition and remodeling activity also are affected with aging. It was found that due to the increased levels of matrix metalloproteinases (MMP) and reduced basement membrane protein synthesis, there is a thinning of the basement membrane (Callaghan & Wilhelm, 2008; Frantz, Stewart, & Weaver, 2010). Moreover, as a result of cellular senescence, fibroblasts in aged tissue are resistant to apoptotic cues and typically express increased levels of ECM-associated proteins such as fibronectin, MMPs, growth factors, and cytokines in addition to elevated levels of plasminogen activator inhibitor (PAI)(Coppe, Desprez, Krtolica, & Campisi, 2010) and reactive oxygen species (ROS)(Untergasser, Madersbacher, & Berger, 2005). Together, these factors result in a chronic inflammatory environment in aging tissue which can further lead to elastin degradation and a modified collagen network in the ECM resulting in aberrant cellular function (Callaghan & Wilhelm, 2008; Freund, Orjalo, Desprez, & Campisi, 2010; Nomura, 2006; Sprenger, Plymate, & Reed, 2008).

Innervation

Accompanying sarcopenia, myofiber atrophy, and reduction in muscle strength and quality is the age-related deterioration of the neuromusculuar junction, decline in motor unit numbers, and neuromuscular junction instability (Hepple & Rice, 2016). Gutmann and Hanzlikova first demonstrated that there is indeed a morphological deterioration of

neuromuscular junctions as was observed in aged rodents (Gutmann & Hanzlikova, 1966). Their findings were later confirmed as the same changes were observed in eldery humans in a study conducted by the Oda group (Oda, 1984). Specifically, in older subjects increased fragmentation of end-plates and increased pre-terminal axon branching at the end-plates were observed, which may explain the increased fatigability associated with aging skeletal muscle.

Closely associated with the progressive loss in muscle strength with aging is the decline in the number of functional motor units. Previously, electrophysiological techniques were used to estimate numbers of motor units in the tibaialis anterior muscle of three age cohorts: young adults (~25 years) older adults (~65 years), and very old adults (~80 years) (McNeil, Doherty, Stashuk, & Rice, 2005). When comparing motor unit estimates between young and older adults which were separated by ~40 years, there was approximately a 40% decline in motor unit numbers in the older adults. In the very old group, which represented a 15-year difference compared to the older adult group, there were 33% fewer motor units. These results support the notion that a rapid decline in muscle function and muscle mass follows the loss of motor units. Interestingly, studies have found that a decline in muscle strength with aging occurs prior to significant losses in muscle mass (Goodpaster et al., 2006). Goodpaster et al. observed that even after significant muscle loss had occurred, the reduction in strength was far greater than what could be accounted for solely from muscle atrophy (Goodpaster et al., 2006). Overall, these observations implicate motor unit loss as the contributor to loss of muscle strength preceding the loss of muscle mass.

Through much of the life span of an adult, muscle has been shown to undergo repeated cycles of denervation and reinnervation leading to alterations in the neuromuscular junction. This process involves a temporary decoupling of an individual myofiber from its motor neuron

preceded by reinnervation by either the original motor neuron associated with the myofiber or through axonal branching of an adjacent motor neuron (Hepple & Rice, 2016). At the cellular level, this cycle of denervation-reinnervation result in significant changes in the components of the neuromuscular junction, namely in the narrowing of terminal axons (Jang & Van Remmen, 2011), altered distribution of post-synaptic membrane proteins (Samuel, Valdez, Tapia, Lichtman, & Sanes, 2012), and excessive sprouting of terminal axons (Balice-Gordon, 1997). An interesting study of note, Deschenes et al. set out to determine whether myofiber atrophy was secondary to age-related myofiber denervation and whether the activity level in muscle affected its sensitivity to age-related denervation (Deschenes, Roby, Eason, & Harris, 2010). Muscles from aged rats that were lightly recruited exhibited signs of denervation with no change in myofiber size and composition while muscles that were heavily recruited showed no signs of denervation nor changes in myofiber profile. The researchers were able to confirm that changes in neuromuscular junction did indeed occur prior to myofiber atrophy and that increased activity could possibly delay age-related denervation and atrophy (Deschenes et al., 2010). However, as previous studies have demonstrated that the early phases of regeneration can progress independent of innervation until the late differentiation and fusion phases, the effects of poor innervation ostensibly should have little impact on the regenerative process but will pose a challenge later in overall muscle maintenance and myofiber survival (B. M. Carlson, 1995; McGeachie & Grounds, 1989).

Vasculature

Resulting from regions of impaired or altered hemodynamics as is observed in aging skeletal muscle (Socha & Segal, 2018), atherosclerosis creates a pro-inflammatory environment

and severely compromises endothelial cell (EC) and smooth muscle cell function (SMC) (Dai et al., 2004). ECs in flow regions promoting atherosclerotic plaque development have been shown to be more prone to apoptosis as well as upregulating the activation of inflammatory NF-κβ signaling and preventing the expression of transcription factors (KLF4) acting in an atheroprotective manner (Dai et al., 2004; Y. Z. Jiang et al., 2014). In advanced atherosclerosis, SMCs exhibited low proliferation rates and increased senescence attributed to growth factor insensitivity and increased expression of cell cycle inhibitors (Patel et al., 2001). Moreover, senescent SMCs promote a pro-inflammatory environment via release of pro-inflammatory cytokines and MMPs while depositing reduced amounts of ECM into the vessel walls (Gardner, Humphry, Bennett, & Clarke, 2015). This inflammatory environment is especially detrimental to regenerative signaling pathways in aging skeletal muscle. Specifically, high levels of TGF-ß and its effector pSmad3 found in aging muscle was observed to increase the expression of several cyclin dependent kinase inhibitors that lead to the reduced activation of aged satellite cells (M. E. Carlson, Hsu, & Conboy, 2008). Additionally, endogenous TGF-ß/pSmad3 was found to have an antagonistic relationship with Notch, a protein involved in molecular signaling regulating satellite cell activity. The Notch signaling pathway and its role in regulating satellite cell activity as well as changes in Notch signaling with advanced aging is discussed in further detail in the following section.

Satellite Cells and Regeneration

The gradual decline in regenerative capacity with age in skeletal muscle can be attributed to the decline in satellite cell activity as they are largely responsible for the regenerative potential of skeletal muscle (Zammit et al., 2002). The view that satellite cell numbers are altered with age

has produced many confounding reports ranging from a reduction in numbers (Bockhold, Rosenblatt, & Partridge, 1998), to no change (Conboy, Conboy, Smythe, & Rando, 2003), and to an increase in numbers (Gibson & Schultz, 1983). Regardless, there is a consensus that more important than satellite cell numbers is the ability of satellite cells to produce fusion-competent progeny that can contribute to muscle regeneration, which carries greater value than any observed perturbations in the number of muscle satellite cells. The age-associated decline in satellite cell function is thought to be caused by either intrinsic changes such as cellular senescence leading to insensitivity to environmental signals or changes in the environmental cues directing satellite cells down the myogenic program. One of the more well-documented mechanisms by which satellite cell activity is regulated involves the Notch signaling pathway, which has been shown to exert regulatory control over the cell cycle of many cells including satellite cells (Charge & Rudnicki, 2004; Conboy et al., 2003; C. Jiang et al., 2014).

There are marked changes associated with aging in the Notch signaling pathway that may account for the reduction in satellite cell activity in skeletal muscle. Under normal conditions such as in younger muscle, there is an upregulation of both Notch and its ligand, Delta, following muscle injury promoting satellite cell proliferation and inhibiting differentiation (Conboy & Rando, 2002). Once a pool of proliferating satellite cells have formed, the Notch antagonist, Numb, is upregulated while Notch is downregulated, causing satellite cells to exit the cell cycle and commit to differentiation (Conboy & Rando, 2002). However, in response to injury in aged skeletal muscle, it is reported that there is a failure to upregulate Delta leading to diminished myogenic potential (Conboy et al., 2003). Additionally, there is a distinct difference in the levels of Notch and Numb between young (high levels) and aged (low levels) muscles that highlight the importance of Notch signaling in regulating satellite cell activity (Carey, Farnfield, Tarquinio, &

Cameron-Smith, 2007; Conboy et al., 2003). *In vitro* forced activation of Notch was able to restore the proliferative abilities of aged satellite cell leading to efficient tissue repair similar to what was observed in young satellite cells. Similar results were observed when Notch was activated *ex vivo* regardless of Delta ligand upregulation (Conboy et al., 2003). These findings are significant as Conboy et al. demonstrate that the diminished regenerative potential in aging skeletal muscle is not an irreversible process and they identify a potential target for therapeutic strategies restoring regenerative potential in aging muscle.

Moreover, the previously reported results suggest that a decline in environmental cues and not dysfunction intrinsic to the cell is responsible for the impaired regenerative potential in aging muscle. These observations are in agreement with previous reports proposing the age-associated decline in environmental cues as the cause of the decline in regenerative capacity in old skeletal muscle (B. M. Carlson & Faulkner, 1989; Zacks & Sheff, 1982). In these studies, muscles were transplanted heterochronically (old to young, young to old) and isochronically (old to old, young to young) in order to identify whether intrinsic or extrinsic changes associated with aging were the cause of declining regeneration in aging muscle. It was found that old muscles grafted into young hosts recovered just as well as young muscles grafted into young hosts. Further, skeletal muscle mass and strength were well preserved in aged muscles grafted into young hosts similar to observations from young muscles grafted into young hosts (B. M. Carlson & Faulkner, 1989). Conboy et al. utilized a similar heterochronic transplantation model in mice to determine whether the young environment contains systemic factors that contribute to myogenesis and if the aged-associated decline in satellite cell activity can be rescued by exposure to factors found in the young environment (Conboy et al., 2005). Young mice (2-3 months) were transgenic for green fluorescent protein (GFP) in order to differentiate which

host's cells contributed to regeneration. The young host heterochronic and isochronic pairings exhibited robust regeneration as demonstrated by the presence of centrally located nuclei and embryonic myosin heavy chain (eMHC) expressing myofibers. The aged mice (19-26 months) in isochronic pairings failed to form myotubes and contained large regions of fibrotic repair tissue. However, in the aged heterochronic parabionts, regeneration was comparable to that of both young heterochronic and isochronic pairings (Conboy et al., 2005). Notably, the improved regeneration in the aged muscle was exclusively due to the contribution of the aged satellite cells as only 0.1% of regenerated myotubes were GFP+. Further, an upregulation of Delta in aged satellite cells from aged heterochronic parabionts was observed and were similar to Delta levels in both young pairings, though a slight inhibition of Delta expression was observed in young heterochronic parabionts. Together, the data suggests that there are factors in the young environment that are able to restore molecular signaling pathways critical to muscle regeneration in aging muscle and that the majority of the intrinsic regenerative capabilities of aged progenitors are conserved but the systemic environment in which they reside prohibit efficient tissue repair (Conboy et al., 2005).

Hormonal Changes

With increasing age, there is a decrease in the levels of circulating hormones and anabolic stimuli such as testosterone (Harman et al., 2001), growth hormone, and IGF-1 (Abbasi, Drinka, Mattson, & Rudman, 1993) that contribute to the loss of muscle mass and strength possibly as a result of decreased satellite cell activity. Due to this, androgenic interventions have been found to be attractive options to mitigate age-related decline in skeletal muscle. In a study comparing the regenerative response to testosterone supplementation between 2-month and 24-

month old mice, various improvements in aging muscle regeneration was reported (Serra et al., 2013). Researchers observed an increase in satellite cell activation and in the number of proliferating satellite cells in the injured tibialis anterior muscles of castrated 2-month and 24-month old mice as well as increased cross-sectional area in regenerating fibers (Serra et al., 2013). The sham counterparts on the other hand exhibited significantly lower levels of satellite cell activation and proliferation, which reduced/delayed the rate of regeneration in aged muscles. Despite the fact that this investigation was limited to only the early stages of myogenesis, it demonstrates the potential application of this type of treatment in augmenting the declining regenerative response observed in aging skeletal muscle.

Testosterone has been shown to stimulate various molecular signaling pathways to improve satellite cell activity and muscle mass maintenance. A report by Kovacheva et al. demonstrated testosterone's ability to stimulate Notch signaling while preventing apoptotic signaling in muscle (Kovacheva, Hikim, Shen, Sinha, & Sinha-Hikim, 2010). One of the more salient findings from this study was that testosterone administration in aging animals clearly inhibited the activation of JNK signaling, which is responsible for inducing cell apoptosis following injury or in aging (Braga et al., 2008; Sinha-Hikim, Braga, Shen, & Sinha Hikim, 2007). Additionally, there was an observed decrease in myostatin expression which antagonizes Akt signaling-mediated skeletal muscle hypertrophy (Amirouche et al., 2009; Morissette et al., 2006) and decreased 4-HNE expression which is a marker of oxidative stress that decreases satellite cell life-span and proliferative capacity (Renault, Thornell, Butler-Browne, & Mouly, 2002). However, no effect on p38 MAPK signaling was found with testosterone in aging animals, which is consistent with previous studies indicating that the regulation of cellular homeostasis through p38 MAPK signaling is more complex and varies widely with age and

target tissue (Hsieh, Rosenblatt, & Papaconstantinou, 2003; A. Lin & Dibling, 2002). Finally, an increase in Notch 1 expression in aged muscle was observed with testosterone administration with concomitant increases in proliferating cell nuclear antigen (PCNA) and myogenin expression, indicating increased satellite cell proliferation and differentiation (Kovacheva et al., 2010). Taken together, these results suggest that testosterone supplementation in aging muscle could rescue the regenerative potential of satellite cells by stimulating molecular signaling pathways that are akin to what would be found in a younger skeletal muscle microenvironment.

Testosterone derivatives such as nandrolone decanoate have also been shown to improve muscle regeneration in low testosterone conditions. Following contusion injuries, nandrolone decanoate was found to increase muscle twitch and tetanic force 2 weeks post-injury and improved the growth of regenerating myofibers 25 days post-injury (Ferry et al., 1999). In another study, nandrolone injection in castrated mice exhibited increased expression of IGF-1, MyoD, and cyclin D1 following bupivacaine-induced muscle injury (White, Baltgalvis, Sato, Wilson, & Carson, 2009). Based on these results, researchers suggested that nandrolone improved muscle regeneration through an IGF-1-mediated mechanism. IGF-1 is known to promote myoblast proliferation and differentiation (Coolican, Samuel, Ewton, McWade, & Florini, 1997; Jacquemin, Butler-Browne, Furling, & Mouly, 2007) and its receptor in muscle (IGF-1R) appears to be essential in mediating the effects of testosterone on progenitor cell proliferation and differentiation (White et al., 2009). Notwithstanding the beneficial effects to skeletal muscle regeneration, the main drawback of nandrolone is that it has potent anabolic effects while its androgenic effects are weak and its use is associated with various side effects including reducing native testosterone biosynthesis (Barone et al., 2017; Kicman, 2008; Nagata et al., 1999; Pomara et al., 2016; Takahashi, Tatsugi, & Kohno, 2004).

F. Objective

The goal of this research is to provide evidence to the following fundamental premise: age-associated changes in skeletal muscle will negatively affect muscle recovery following VML injury and the response to repair with tissue engineered strategies. The following investigations were performed to explore our fundamental premise:

1) Examine the effect of age on the response to recovery following volumetric muscle loss.

Hypothesis: Age will increase the force loss following VML injury.

2) Examine how age affects the response to repair of VML injury using minced muscle autografts co-delivered with DSM (DSM+MM).

Hypothesis: Age will decrease the amount of force recovered following VML repair.

3) Investigate the regenerative outcomes following VML repair with co-delivery of the anabolic steroid nandrolone decanoate in aged animals.

Hypothesis: Nandrolone decanoate administration will reverse the decrements to force recovery following VML repair in aged animals.

VML investigations thus far have utilized various young animal models in developing tissue repair strategies. With what is known about the declining regenerative potential and various deleterious changes in skeletal muscle associated with aging, it is a possibility that treatment strategies that have proven successful in younger animal models will not be effective in older animal models. As per this gap in knowledge, we saw an opportunity to address the potential differences in recovery after VML injury and VML repair in the aging animal population when compared to the young population.

The use of the DSM+MM repair strategy has been demonstrated previously to increase myogenic gene expression, reduce fibrosis during regeneration, and significantly improve functional outcomes in a young rat model of VML. We hope to study the changes in the microenvironment of aging animals and how they affect the response to VML injury and repair in order to better inform future research in developing not only tissue engineering strategies for VML repair but also the effects of aging on muscle regeneration in VML. Moreover, many researchers have employed the use of testosterone and other hormone replacement therapies to combat age-associated muscle wasting and functional decline. Testosterone has also been shown to improve regenerative outcomes in aging animals, restoring the response to injury to levels seen in younger animals. We will use this as an adjunct to our current DSM+MM therapy in order to further improve regeneration in aging animals.

A brief outline of our approach is as follows:

- Develop an aged model of VML injury and investigate differences in VML recovery between young and aged animals.
- Investigate differences in the regenerative and functional outcomes following VML repair using DSM co-delivered with MM autografts between young and aged animals.
- The final study will use the anabolic steroid nandrolone decanoate as an adjunctive treatment to DSM+MM repair and explore the effects on regenerative and functional outcomes between young and aged animals.

References

Aarimaa, V., Kaariainen, M., Vaittinen, S., Tanner, J., Jarvinen, T., Best, T., & Kalimo, H. (2004). Restoration of myofiber continuity after transection injury in the rat soleus. *Neuromuscul Disord, 14*(7), 421-428. doi: 10.1016/j.nmd.2004.03.009

Abbasi, A. A., Drinka, P. J., Mattson, D. E., & Rudman, D. (1993). Low circulating levels of insulin-like growth factors and testosterone in chronically institutionalized elderly men. *J Am Geriatr Soc, 41*(9), 975-982.

Adair-Kirk, T. L., & Senior, R. M. (2008). Fragments of extracellular matrix as mediators of inflammation. *Int J Biochem Cell Biol, 40*(6-7), 1101-1110. doi: 10.1016/j.biocel.2007.12.005

Alameddine, H. S., Dehaupas, M., & Fardeau, M. (1989). Regeneration of skeletal muscle fibers from autologous satellite cells multiplied in vitro. An experimental model for testing cultured cell myogenicity. *Muscle Nerve, 12*(7), 544-555. doi: 10.1002/mus.880120705

Allen, R. E., & Boxhorn, L. K. (1989). Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. *J Cell Physiol, 138*(2), 311-315. doi: 10.1002/jcp.1041380213

Amirouche, A., Durieux, A. C., Banzet, S., Koulmann, N., Bonnefoy, R., Mouret, C., . . . Freyssenet, D. (2009). Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. *Endocrinology, 150*(1), 286-294. doi: 10.1210/en.2008-0959

Andersen, R. C., Wilson, K. W., Bojescul, J. A., Mickel, T. J., Gordon, W. T., & Potter, B. K. (2014). Open, combat-related loss, or disruption of the knee extensor mechanism: treatment strategies, classification, and outcomes. *J Orthop Trauma, 28*(11), e250-257. doi: 10.1097/BOT.0000000000000121

Armstrong, R. B., & Ianuzzo, C. D. (1977). Exercise-induced muscle glycogen depletion and repletion in diabetic rats. *Life Sci, 20*(2), 301-308.

Aurora, A., Corona, B. T., & Walters, T. J. (2016). A Porcine Urinary Bladder Matrix Does Not Recapitulate the Spatiotemporal Macrophage Response of Muscle Regeneration after Volumetric Muscle Loss Injury. *Cells Tissues Organs, 202*(3-4), 189-201. doi: 10.1159/000447582

Aurora, A., Garg, K., Corona, B. T., & Walters, T. J. (2014). Physical rehabilitation improves muscle function following volumetric muscle loss injury. *BMC Sports Sci Med Rehabil, 6*(1), 41. doi: 10.1186/2052-1847-6-41

Aurora, A., Roe, J. L., Corona, B. T., & Walters, T. J. (2015). An acellular biologic scaffold does not regenerate appreciable de novo muscle tissue in rat models of volumetric muscle loss injury. *Biomaterials, 67*, 393-407. doi: 10.1016/j.biomaterials.2015.07.040

Badylak, S. F. (1993). Small Intestinal Submucosa (SIS): A Biomaterial Conducive to Smart Tissue Remodeling. In E. Bell (Ed.), *Tissue Engineering: Current Perspectives* (pp. 179-189). Boston, MA: Birkhäuser Boston.

Badylak, S. F. (2004). Xenogeneic extracellular matrix as a scaffold for tissue reconstruction. *Transpl Immunol, 12*(3-4), 367-377. doi: 10.1016/j.trim.2003.12.016

Badylak, S. F., Lantz, G. C., Coffey, A., & Geddes, L. A. (1989). Small intestinal submucosa as a large diameter vascular graft in the dog. *J Surg Res, 47*(1), 74-80.

Badylak, S. F., Tullius, R., Kokini, K., Shelbourne, K. D., Klootwyk, T., Voytik, S. L., . . . Simmons, C. (1995). The use of xenogeneic small intestinal submucosa as a biomaterial for Achilles tendon repair in a dog model. *J Biomed Mater Res, 29*(8), 977-985. doi: 10.1002/jbm.820290809

Bain, J. R., Hason, Y., Veltri, K., Fahnestock, M., & Quartly, C. (2008). Clinical application of sensory protection of denervated muscle. *J Neurosurg, 109*(5), 955-961. doi: 10.3171/JNS/2008/109/11/0955

Bain, J. R., Veltri, K. L., Chamberlain, D., & Fahnestock, M. (2001). Improved functional recovery of denervated skeletal muscle after temporary sensory nerve innervation. *Neuroscience, 103*(2), 503-510.

Balice-Gordon, R. J. (1997). Age-related changes in neuromuscular innervation. *Muscle Nerve Suppl, 5*, S83-87.

Ballotta, V., Driessen-Mol, A., Bouten, C. V., & Baaijens, F. P. (2014). Strain-dependent modulation of macrophage polarization within scaffolds. *Biomaterials, 35*(18), 4919-4928. doi: 10.1016/j.biomaterials.2014.03.002

Baoge, L., Van Den Steen, E., Rimbaut, S., Philips, N., Witvrouw, E., Almqvist, K. F., . . . Vanden Bossche, L. C. (2012). Treatment of skeletal muscle injury: a review. *ISRN Orthop, 2012*, 689012. doi: 10.5402/2012/689012

Barone, R., Pitruzzella, A., Marino Gammazza, A., Rappa, F., Salerno, M., Barone, F., . . . Pomara, C. (2017). Nandrolone decanoate interferes with testosterone biosynthesis altering blood-testis barrier components. *J Cell Mol Med, 21*(8), 1636-1647. doi: 10.1111/jcmm.13092

Beier, J. P., Kneser, U., Stern-Strater, J., Stark, G. B., & Bach, A. D. (2004). Y chromosome detection of three-dimensional tissue-engineered skeletal muscle constructs in a syngeneic rat animal model. *Cell Transplant, 13*(1), 45-53.

Beier, J. P., Stern-Straeter, J., Foerster, V. T., Kneser, U., Stark, G. B., & Bach, A. D. (2006). Tissue engineering of injectable muscle: three-dimensional myoblast-fibrin injection in the syngeneic rat animal model. *Plast Reconstr Surg, 118*(5), 1113-1121; discussion 1122-1114. doi: 10.1097/01.prs.0000221007.97115.1d

Benoit, P. W., & Belt, W. D. (1970). Destruction and regeneration of skeletal muscle after treatment with a local anaesthetic, bupivacaine (Marcaine). *J Anat, 107*(Pt 3), 547-556.

Betik, A. C., Baker, D. J., Krause, D. J., McConkey, M. J., & Hepple, R. T. (2008). Exercise training in late middle-aged male Fischer 344 x Brown Norway F1-hybrid rats improves skeletal muscle aerobic function. *Exp Physiol, 93*(7), 863-871. doi: 10.1113/expphysiol.2008.042069

Bidarra, S. J., Barrias, C. C., & Granja, P. L. (2014). Injectable alginate hydrogels for cell delivery in tissue engineering. *Acta Biomater, 10*(4), 1646-1662. doi: 10.1016/j.actbio.2013.12.006

Bidez, P. R., 3rd, Li, S., Macdiarmid, A. G., Venancio, E. C., Wei, Y., & Lelkes, P. I. (2006). Polyaniline, an electroactive polymer, supports adhesion and proliferation of cardiac myoblasts. *J Biomater Sci Polym Ed, 17*(1-2), 199-212.

Bischoff, R. (1986). A satellite cell mitogen from crushed adult muscle. *Dev Biol, 115*(1), 140-147.

Bockhold, K. J., Rosenblatt, J. D., & Partridge, T. A. (1998). Aging normal and dystrophic mouse muscle: analysis of myogenicity in cultures of living single fibers. *Muscle Nerve, 21*(2), 173-183.

Boontheekul, T., Hill, E. E., Kong, H. J., & Mooney, D. J. (2007). Regulating myoblast phenotype through controlled gel stiffness and degradation. *Tissue Eng, 13*(7), 1431-1442. doi: 10.1089/ten.2006.0356

Boontheekul, T., Kong, H. J., & Mooney, D. J. (2005). Controlling alginate gel degradation utilizing partial oxidation and bimodal molecular weight distribution. *Biomaterials, 26*(15), 2455-2465. doi: 10.1016/j.biomaterials.2004.06.044

Borschel, G. H., Dennis, R. G., & Kuzon, W. M., Jr. (2004). Contractile skeletal muscle tissue-engineered on an acellular scaffold. *Plast Reconstr Surg, 113*(2), 595-602; discussion 603-594. doi: 10.1097/01.PRS.0000101064.62289.2F

Borschel, G. H., Dow, D. E., Dennis, R. G., & Brown, D. L. (2006). Tissue-engineered axially vascularized contractile skeletal muscle. *Plast Reconstr Surg, 117*(7), 2235-2242. doi: 10.1097/01.prs.0000224295.54073.49

Borselli, C., Cezar, C. A., Shvartsman, D., Vandenburgh, H. H., & Mooney, D. J. (2011). The role of multifunctional delivery scaffold in the ability of cultured myoblasts to promote muscle regeneration. *Biomaterials, 32*(34), 8905-8914. doi: 10.1016/j.biomaterials.2011.08.019

Borselli, C., Storrie, H., Benesch-Lee, F., Shvartsman, D., Cezar, C., Lichtman, J. W., . . . Mooney, D. J. (2010). Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. *Proc Natl Acad Sci U S A, 107*(8), 3287-3292. doi: 10.1073/pnas.0903875106

Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., & Rando, T. A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science, 317*(5839), 807-810. doi: 10.1126/science.1144090

Brack, A. S., & Rando, T. A. (2007). Intrinsic changes and extrinsic influences of myogenic stem cell function during aging. *Stem Cell Rev, 3*(3), 226-237.

Braga, M., Sinha Hikim, A. P., Datta, S., Ferrini, M. G., Brown, D., Kovacheva, E. L., . . . Sinha-Hikim, I. (2008). Involvement of oxidative stress and caspase 2-mediated intrinsic pathway signaling in age-related increase in muscle cell apoptosis in mice. *Apoptosis, 13*(6), 822-832. doi: 10.1007/s10495-008-0216-7

Brickson, S., Hollander, J., Corr, D. T., Ji, L. L., & Best, T. M. (2001). Oxidant production and immune response after stretch injury in skeletal muscle. *Med Sci Sports Exerc, 33*(12), 2010-2015.

Brickson, S., Ji, L. L., Schell, K., Olabisi, R., St Pierre Schneider, B., & Best, T. M. (2003). M1/70 attenuates blood-borne neutrophil oxidants, activation, and myofiber damage following stretch injury. *J Appl Physiol (1985), 95*(3), 969-976. doi: 10.1152/japplphysiol.00005.2003

Brown, A. C., & Barker, T. H. (2014). Fibrin-based biomaterials: modulation of macroscopic properties through rational design at the molecular level. *Acta Biomater, 10*(4), 1502-1514. doi: 10.1016/j.actbio.2013.09.008

Brown, B. N., Valentin, J. E., Stewart-Akers, A. M., McCabe, G. P., & Badylak, S. F. (2009). Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. *Biomaterials, 30*(8), 1482-1491. doi: 10.1016/j.biomaterials.2008.11.040

Brunelli, G., Monini, L., & Brunelli, F. (1990). [Direct muscular neurotization]. *Ann Chir Main Memb Super, 9*(4), 290-295.

Calderon, W., Chang, N., & Mathes, S. J. (1986). Comparison of the effect of bacterial inoculation in musculocutaneous and fasciocutaneous flaps. *Plast Reconstr Surg, 77*(5), 785-794.

Callaghan, T. M., & Wilhelm, K. P. (2008). A review of ageing and an examination of clinical methods in the assessment of ageing skin. Part 2: Clinical perspectives and clinical methods in the evaluation of ageing skin. *Int J Cosmet Sci, 30*(5), 323-332. doi: 10.1111/j.1468-2494.2008.00455.x

Carey, K. A., Farnfield, M. M., Tarquinio, S. D., & Cameron-Smith, D. (2007). Impaired expression of Notch signaling genes in aged human skeletal muscle. *J Gerontol A Biol Sci Med Sci, 62*(1), 9-17.

Carlson, B. M. (1968). Regeneration fo the completely excised gastrocnemius muscle in the frog and rat from minced muscle fragments. *J Morphol, 125*(4), 447-472. doi: 10.1002/jmor.1051250405

Carlson, B. M. (1995). Factors influencing the repair and adaptation of muscles in aged individuals: satellite cells and innervation. *J Gerontol A Biol Sci Med Sci, 50 Spec No*, 96-100.

Carlson, B. M., & Faulkner, J. A. (1989). Muscle transplantation between young and old rats: age of host determines recovery. *Am J Physiol, 256*(6 Pt 1), C1262-1266. doi: 10.1152/ajpcell.1989.256.6.C1262

Carlson, B. M., & Faulkner, J. A. (1996). The regeneration of noninnervated muscle grafts and marcaine-treated muscles in young and old rats. *J Gerontol A Biol Sci Med Sci, 51*(1), B43-49.

Carlson, B. M., & Faulkner, J. A. (1998). Muscle regeneration in young and old rats: effects of motor nerve transection with and without marcaine treatment. *J Gerontol A Biol Sci Med Sci, 53*(1), B52-57.

Carlson, M. E., Hsu, M., & Conboy, I. M. (2008). Imbalance between pSmad3 and Notch induces CDK inhibitors in old muscle stem cells. *Nature, 454*(7203), 528-532. doi: 10.1038/nature07034

Carnio, S., Serena, E., Rossi, C. A., De Coppi, P., Elvassore, N., & Vitiello, L. (2011). Three-dimensional porous scaffold allows long-term wild-type cell delivery in dystrophic muscle. *J Tissue Eng Regen Med, 5*(1), 1-10. doi: 10.1002/term.282

Carosio, S., Barberi, L., Rizzuto, E., Nicoletti, C., Del Prete, Z., & Musaro, A. (2013). Generation of eX vivo-vascularized Muscle Engineered Tissue (X-MET). *Sci Rep, 3*, 1420. doi: 10.1038/srep01420

Casar, J. C., Cabello-Verrugio, C., Olguin, H., Aldunate, R., Inestrosa, N. C., & Brandan, E. (2004). Heparan sulfate proteoglycans are increased during skeletal muscle regeneration: requirement of syndecan-3 for successful fiber formation. *J Cell Sci, 117*(Pt 1), 73-84. doi: 10.1242/jcs.00828

Chambers, J. B., Rimington, H. M., Rajani, R., Hodson, F., & Shabbo, F. (2007). A randomized comparison of the Cryolife O'Brien and Toronto stentless replacement aortic valves. *J Thorac Cardiovasc Surg, 133*(4), 1045-1050. doi: 10.1016/j.jtcvs.2006.10.071

Chang, N., & Mathes, S. J. (1982). Comparison of the effect of bacterial inoculation in musculocutaneous and random-pattern flaps. *Plast Reconstr Surg, 70*(1), 1-10.

Charge, S. B., & Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiol Rev, 84*(1), 209-238. doi: 10.1152/physrev.00019.2003

Chen, F., Yoo, J. J., & Atala, A. (1999). Acellular collagen matrix as a possible "off the shelf" biomaterial for urethral repair. *Urology, 54*(3), 407-410.

Chen, M. C., Sun, Y. C., & Chen, Y. H. (2013). Electrically conductive nanofibers with highly oriented structures and their potential application in skeletal muscle tissue engineering. *Acta Biomater, 9*(3), 5562-5572. doi: 10.1016/j.actbio.2012.10.024

Chevallay, B., & Herbage, D. (2000). Collagen-based biomaterials as 3D scaffold for cell cultures: applications for tissue engineering and gene therapy. *Med Biol Eng Comput, 38*(2), 211-218.

Chiron, S., Tomczak, C., Duperray, A., Laine, J., Bonne, G., Eder, A., . . . Coirault, C. (2012). Complex interactions between human myoblasts and the surrounding 3D fibrin-based matrix. *PLoS One, 7*(4), e36173. doi: 10.1371/journal.pone.0036173

Chiu, D. T., Chen, L., Spielholtz, N., & Beasley, R. W. (1991). A comparative electrophysiological study on neurotisation in rats. *J Hand Surg Br, 16*(5), 505-510.

Choi, J. S., Lee, S. J., Christ, G. J., Atala, A., & Yoo, J. J. (2008). The influence of electrospun aligned poly(epsilon-caprolactone)/collagen nanofiber meshes on the formation of self-aligned skeletal muscle myotubes. *Biomaterials, 29*(19), 2899-2906. doi: 10.1016/j.biomaterials.2008.03.031

Cittadella Vigodarzere, G., & Mantero, S. (2014). Skeletal muscle tissue engineering: strategies for volumetric constructs. *Front Physiol, 5*, 362. doi: 10.3389/fphys.2014.00362

Clark, R. A. (2001). Fibrin and wound healing. *Ann N Y Acad Sci, 936*, 355-367.

Collins, C. A., Olsen, I., Zammit, P. S., Heslop, L., Petrie, A., Partridge, T. A., & Morgan, J. E. (2005). Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell, 122*(2), 289-301. doi: 10.1016/j.cell.2005.05.010

Conboy, I. M., Conboy, M. J., Smythe, G. M., & Rando, T. A. (2003). Notch-mediated restoration of regenerative potential to aged muscle. *Science, 302*(5650), 1575-1577. doi: 10.1126/science.1087573

Conboy, I. M., Conboy, M. J., Wagers, A. J., Girma, E. R., Weissman, I. L., & Rando, T. A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature, 433*(7027), 760-764. doi: 10.1038/nature03260

Conboy, I. M., & Rando, T. A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell, 3*(3), 397-409.

Conboy, I. M., & Rando, T. A. (2005). Aging, stem cells and tissue regeneration: lessons from muscle. *Cell Cycle, 4*(3), 407-410. doi: 10.4161/cc.4.3.1518

Conconi, M. T., De Coppi, P., Bellini, S., Zara, G., Sabatti, M., Marzaro, M., . . . Nussdorfer, G. G. (2005). Homologous muscle acellular matrix seeded with autologous myoblasts as a tissue-engineering approach to abdominal wall-defect repair. *Biomaterials, 26*(15), 2567-2574. doi: 10.1016/j.biomaterials.2004.07.035

Cook, J. L., Tomlinson, J. L., Kreeger, J. M., & Cook, C. R. (1999). Induction of meniscal regeneration in dogs using a novel biomaterial. *Am J Sports Med, 27*(5), 658-665. doi: 10.1177/03635465990270051901

Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J., & Florini, J. R. (1997). The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J Biol Chem, 272*(10), 6653-6662.

Cooper, R. N., Tajbakhsh, S., Mouly, V., Cossu, G., Buckingham, M., & Butler-Browne, G. S. (1999). In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. *J Cell Sci, 112 (Pt 17)*, 2895-2901.

Coppe, J. P., Desprez, P. Y., Krtolica, A., & Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol, 5*, 99-118. doi: 10.1146/annurev-pathol-121808-102144

Cornwell, K. G., & Pins, G. D. (2010). Enhanced proliferation and migration of fibroblasts on the surface of fibroblast growth factor-2-loaded fibrin microthreads. *Tissue Eng Part A, 16*(12), 3669-3677. doi: 10.1089/ten.TEA.2009.0600

Corona, B. T., Flanagan, K. E., Brininger, C. M., Goldman, S. M., Call, J. A., & Greising, S. M. (2018). Impact of volumetric muscle loss injury on persistent motoneuron axotomy. *Muscle Nerve, 57*(5), 799-807. doi: 10.1002/mus.26016

Corona, B. T., Garg, K., Ward, C. L., McDaniel, J. S., Walters, T. J., & Rathbone, C. R. (2013). Autologous minced muscle grafts: a tissue engineering therapy for the volumetric loss of skeletal muscle. *Am J Physiol Cell Physiol, 305*(7), C761-775. doi: 10.1152/ajpcell.00189.2013

Corona, B. T., Machingal, M. A., Criswell, T., Vadhavkar, M., Dannahower, A. C., Bergman, C., . . . Christ, G. J. (2012). Further development of a tissue engineered muscle repair construct in vitro for enhanced functional recovery following implantation in vivo in a murine model of volumetric muscle loss injury. *Tissue Eng Part A, 18*(11-12), 1213-1228. doi: 10.1089/ten.TEA.2011.0614

Corona, B. T., Rivera, J. C., Owens, J. G., Wenke, J. C., & Rathbone, C. R. (2015). Volumetric muscle loss leads to permanent disability following extremity trauma. *J Rehabil Res Dev, 52*(7), 785-792. doi: 10.1682/JRRD.2014.07.0165

Corona, B. T., Ward, C. L., Baker, H. B., Walters, T. J., & Christ, G. J. (2014). Implantation of in vitro tissue engineered muscle repair constructs and bladder acellular matrices partially restore in vivo skeletal muscle function in a rat model of volumetric muscle loss injury. *Tissue Eng Part A, 20*(3-4), 705-715. doi: 10.1089/ten.TEA.2012.0761

Corona, B. T., Wu, X., Ward, C. L., McDaniel, J. S., Rathbone, C. R., & Walters, T. J. (2013). The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. *Biomaterials, 34*(13), 3324-3335. doi: 10.1016/j.biomaterials.2013.01.061

Cossu, G., & Biressi, S. (2005). Satellite cells, myoblasts and other occasional myogenic progenitors: possible origin, phenotypic features and role in muscle regeneration. *Semin Cell Dev Biol, 16*(4-5), 623-631. doi: 10.1016/j.semcdb.2005.07.003

Cronin, E. M., Thurmond, F. A., Bassel-Duby, R., Williams, R. S., Wright, W. E., Nelson, K. D., & Garner, H. R. (2004). Protein-coated poly(L-lactic acid) fibers provide a substrate for differentiation of human skeletal muscle cells. *J Biomed Mater Res A, 69*(3), 373-381. doi: 10.1002/jbm.a.30009

Dai, G., Kaazempur-Mofrad, M. R., Natarajan, S., Zhang, Y., Vaughn, S., Blackman, B. R., . . . Gimbrone, M. A., Jr. (2004). Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. *Proc Natl Acad Sci U S A, 101*(41), 14871-14876. doi: 10.1073/pnas.0406073101

Dellgren, G., Eriksson, M., Brodin, L. A., & Radegran, K. (1999). The extended Biocor stentless aortic bioprosthesis. Early clinical experience. *Scand Cardiovasc J, 33*(5), 259-264.

DeQuach, J. A., Lin, J. E., Cam, C., Hu, D., Salvatore, M. A., Sheikh, F., & Christman, K. L. (2012). Injectable skeletal muscle matrix hydrogel promotes neovascularization and muscle cell infiltration in a hindlimb ischemia model. *Eur Cell Mater, 23*, 400-412; discussion 412.

Deschenes, M. R., Roby, M. A., Eason, M. K., & Harris, M. B. (2010). Remodeling of the neuromuscular junction precedes sarcopenia related alterations in myofibers. *Exp Gerontol, 45*(5), 389-393. doi: 10.1016/j.exger.2010.03.007

DiMario, J. X., & Stockdale, F. E. (1995). Differences in the developmental fate of cultured and noncultured myoblasts when transplanted into embryonic limbs. *Exp Cell Res, 216*(2), 431-442. doi: 10.1006/excr.1995.1054

Donghui, C., Shicai, C., Wei, W., Fei, L., Jianjun, J., Gang, C., & Hongliang, Z. (2010). Functional modulation of satellite cells in long-term denervated human laryngeal muscle. *Laryngoscope, 120*(2), 353-358. doi: 10.1002/lary.20796

Drewa, T., Adamowicz, J., & Sharma, A. (2012). Tissue engineering for the oncologic urinary bladder. *Nat Rev Urol, 9*(10), 561-572. doi: 10.1038/nrurol.2012.158

Drury, J. L., Boontheekul, T., & Mooney, D. J. (2005). Cellular cross-linking of peptide modified hydrogels. *J Biomech Eng, 127*(2), 220-228.

Dziki, J., Badylak, S., Yabroudi, M., Sicari, B., Ambrosio, F., Stearns, K., . . . Rubin, J. P. (2016). An acellular biologic scaffold treatment for volumetric muscle loss: results of a 13-patient cohort study. *NPJ Regen Med, 1*, 16008. doi: 10.1038/npjregenmed.2016.8

Edelberg, J. M., & Reed, M. J. (2003). Aging and angiogenesis. *Front Biosci, 8*, s1199-1209.

Edelman, E. R., Mathiowitz, E., Langer, R., & Klagsbrun, M. (1991). Controlled and modulated release of basic fibroblast growth factor. *Biomaterials, 12*(7), 619-626.

Falanga, V., & Sabolinski, M. (1999). A bilayered living skin construct (APLIGRAF) accelerates complete closure of hard-to-heal venous ulcers. *Wound Repair Regen, 7*(4), 201-207.

Fan, C., Jiang, P., Fu, L., Cai, P., Sun, L., & Zeng, B. (2008). Functional reconstruction of traumatic loss of flexors in forearm with gastrocnemius myocutaneous flap transfer. *Microsurgery, 28*(1), 71-75. doi: 10.1002/micr.20449

Ferraro, F., Celso, C. L., & Scadden, D. (2010). Adult stem cels and their niches. *Adv Exp Med Biol, 695*, 155-168. doi: 10.1007/978-1-4419-7037-4_11

Ferry, A., Noirez, P., Page, C. L., Salah, I. B., Daegelen, D., & Rieu, M. (1999). Effects of anabolic/androgenic steroids on regenerating skeletal muscles in the rat. *Acta Physiol Scand, 166*(2), 105-110. doi: 10.1046/j.1365-201x.1999.00549.x

FORBES, A., & COBB, S. (1926). PHYSIOLOGY OF SYMPATHETIC NERVOUS SYSTEM IN RELATION TO CERTAIN SURGICAL PROBLEMS. *Journal of the American Medical Association, 86*(25), 1884-1886. doi: 10.1001/jama.1926.02670510006002

Foster, A. H., & Carlson, B. M. (1980). Myotoxicity of local anesthetics and regeneration of the damaged muscle fibers. *Anesth Analg, 59*(10), 727-736.

Franklin, R. J., Zhao, C., & Sim, F. J. (2002). Ageing and CNS remyelination. *Neuroreport, 13*(7), 923-928.

Frantz, C., Stewart, K. M., & Weaver, V. M. (2010). The extracellular matrix at a glance. *J Cell Sci, 123*(Pt 24), 4195-4200. doi: 10.1242/jcs.023820

Fredsted, A., Gissel, H., Madsen, K., & Clausen, T. (2007). Causes of excitation-induced muscle cell damage in isometric contractions: mechanical stress or calcium overload? *Am J Physiol Regul Integr Comp Physiol, 292*(6), R2249-2258. doi: 10.1152/ajpregu.00415.2006

Freund, A., Orjalo, A. V., Desprez, P. Y., & Campisi, J. (2010). Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol Med, 16*(5), 238-246. doi: 10.1016/j.molmed.2010.03.003

Frey, S. P., Jansen, H., Raschke, M. J., Meffert, R. H., & Ochman, S. (2012). VEGF improves skeletal muscle regeneration after acute trauma and reconstruction of the limb in a rabbit model. *Clin Orthop Relat Res, 470*(12), 3607-3614. doi: 10.1007/s11999-012-2456-7

Frontera, W. R., & Ochala, J. (2015). Skeletal muscle: a brief review of structure and function. *Calcif Tissue Int, 96*(3), 183-195. doi: 10.1007/s00223-014-9915-y

Gamba, P. G., Conconi, M. T., Lo Piccolo, R., Zara, G., Spinazzi, R., & Parnigotto, P. P. (2002). Experimental abdominal wall defect repaired with acellular matrix. *Pediatr Surg Int, 18*(5-6), 327-331. doi: 10.1007/s00383-002-0849-5

Gardner, S. E., Humphry, M., Bennett, M. R., & Clarke, M. C. (2015). Senescent Vascular Smooth Muscle Cells Drive Inflammation Through an Interleukin-1alpha-Dependent Senescence-Associated Secretory Phenotype. *Arterioscler Thromb Vasc Biol, 35*(9), 1963-1974. doi: 10.1161/ATVBAHA.115.305896

Garg, K., Corona, B. T., & Walters, T. J. (2015). Therapeutic strategies for preventing skeletal muscle fibrosis after injury. *Front Pharmacol, 6*, 87. doi: 10.3389/fphar.2015.00087

Garg, K., Ward, C. L., Hurtgen, B. J., Wilken, J. M., Stinner, D. J., Wenke, J. C., . . . Corona, B. T. (2015). Volumetric muscle loss: persistent functional deficits beyond frank loss of tissue. *J Orthop Res, 33*(1), 40-46. doi: 10.1002/jor.22730

Garg, K., Ward, C. L., Rathbone, C. R., & Corona, B. T. (2014). Transplantation of devitalized muscle scaffolds is insufficient for appreciable de novo muscle fiber regeneration after volumetric muscle loss injury. *Cell Tissue Res, 358*(3), 857-873. doi: 10.1007/s00441-014-2006-6

Garrett, W. E., Jr. (1996). Muscle strain injuries. *Am J Sports Med, 24*(6 Suppl), S2-8.

Gentile, N. E., Stearns, K. M., Brown, E. H., Rubin, J. P., Boninger, M. L., Dearth, C. L., . . . Badylak, S. F. (2014). Targeted rehabilitation after extracellular matrix scaffold transplantation for the treatment of volumetric muscle loss. *Am J Phys Med Rehabil, 93*(11 Suppl 3), S79-87. doi: 10.1097/PHM.0000000000000145

Gerard, C., Forest, M. A., Beauregard, G., Skuk, D., & Tremblay, J. P. (2012). Fibrin gel improves the survival of transplanted myoblasts. *Cell Transplant, 21*(1), 127-137. doi: 10.3727/096368911X576018

Gibson, M. C., & Schultz, E. (1983). Age-related differences in absolute numbers of skeletal muscle satellite cells. *Muscle Nerve, 6*(8), 574-580. doi: 10.1002/mus.880060807

Gilbert-Honick, J., Ginn, B., Zhang, Y., Salehi, S., Wagner, K. R., Mao, H. Q., & Grayson, W. L. (2018). Adipose-derived Stem/Stromal Cells on Electrospun Fibrin Microfiber Bundles Enable Moderate Muscle Reconstruction in a Volumetric Muscle Loss Model. *Cell Transplant*, 963689718805370. doi: 10.1177/0963689718805370

Gilbert-Honick, J., Iyer, S. R., Somers, S. M., Lovering, R. M., Wagner, K., Mao, H. Q., & Grayson, W. L. (2018). Engineering functional and histological regeneration of vascularized skeletal muscle. *Biomaterials, 164*, 70-79. doi: 10.1016/j.biomaterials.2018.02.006

Gilbert, T. W., Sellaro, T. L., & Badylak, S. F. (2006). Decellularization of tissues and organs. *Biomaterials, 27*(19), 3675-3683. doi: 10.1016/j.biomaterials.2006.02.014

Gilbert, T. W., Stewart-Akers, A. M., Sydeski, J., Nguyen, T. D., Badylak, S. F., & Woo, S. L. (2007). Gene expression by fibroblasts seeded on small intestinal submucosa and subjected to cyclic stretching. *Tissue Eng, 13*(6), 1313-1323. doi: 10.1089/ten.2006.0318

Gillies, A. R., & Lieber, R. L. (2011). Structure and function of the skeletal muscle extracellular matrix. *Muscle Nerve, 44*(3), 318-331. doi: 10.1002/mus.22094

Goldman, S. M., & Corona, B. T. (2017). Co-delivery of micronized urinary bladder matrix damps regenerative capacity of minced muscle grafts in the treatment of volumetric muscle loss injuries. *PLoS One, 12*(10), e0186593. doi: 10.1371/journal.pone.0186593

Goldman, S. M., Henderson, B. E. P., & Corona, B. T. (2017). Evaluation of bone marrow mononuclear cells as an adjunct therapy to minced muscle graft for the treatment of volumetric muscle loss injuries. *Stem Cell Res Ther, 8*(1), 142. doi: 10.1186/s13287-017-0589-z

Goodpaster, B. H., Park, S. W., Harris, T. B., Kritchevsky, S. B., Nevitt, M., Schwartz, A. V., . . . Newman, A. B. (2006). The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study. *J Gerontol A Biol Sci Med Sci, 61*(10), 1059-1064.

Gopinath, S. D., & Rando, T. A. (2008). Stem cell review series: aging of the skeletal muscle stem cell niche. *Aging Cell, 7*(4), 590-598. doi: 10.1111/j.1474-9726.2008.00399.x

Gosain, A., Chang, N., Mathes, S., Hunt, T. K., & Vasconez, L. (1990). A study of the relationship between blood flow and bacterial inoculation in musculocutaneous and fasciocutaneous flaps. *Plast Reconstr Surg, 86*(6), 1152-1162; discussion 1163.

Grasman, J. M., Do, D. M., Page, R. L., & Pins, G. D. (2015). Rapid release of growth factors regenerates force output in volumetric muscle loss injuries. *Biomaterials, 72*, 49-60. doi: 10.1016/j.biomaterials.2015.08.047

Grasman, J. M., Page, R. L., & Pins, G. D. (2017). (*) Design of an In Vitro Model of Cell Recruitment for Skeletal Muscle Regeneration Using Hepatocyte Growth Factor-Loaded Fibrin Microthreads. *Tissue Eng Part A, 23*(15-16), 773-783. doi: 10.1089/ten.TEA.2016.0440

Grasman, J. M., Pumphrey, L. M., Dunphy, M., Perez-Rogers, J., & Pins, G. D. (2014). Static axial stretching enhances the mechanical properties and cellular responses of fibrin microthreads. *Acta Biomater, 10*(10), 4367-4376. doi: 10.1016/j.actbio.2014.06.021

Greene, T. L., & Beatty, M. E. (1988). Soft tissue coverage for lower-extremity trauma: current practice and techniques. A review. *J Orthop Trauma, 2*(2), 158-173.

Grogan, B. F., Hsu, J. R., & Skeletal Trauma Research, C. (2011). Volumetric muscle loss. *J Am Acad Orthop Surg, 19 Suppl 1*, S35-37.

Grounds, M. D. (1991). Towards understanding skeletal muscle regeneration. *Pathol Res Pract, 187*(1), 1-22. doi: 10.1016/S0344-0338(11)81039-3

Grounds, M. D., & Yablonka-Reuveni, Z. (1993). Molecular and cell biology of skeletal muscle regeneration. *Mol Cell Biol Hum Dis Ser, 3*, 210-256.

Grubic, Z., Komel, R., Walker, W. F., & Miranda, A. F. (1995). Myoblast fusion and innervation with rat motor nerve alter distribution of acetylcholinesterase and its mRNA in cultures of human muscle. *Neuron, 14*(2), 317-327.

Guex, A. G., Birrer, D. L., Fortunato, G., Tevaearai, H. T., & Giraud, M. N. (2013). Anisotropically oriented electrospun matrices with an imprinted periodic micropattern: a new scaffold for engineered muscle constructs. *Biomed Mater, 8*(2), 021001. doi: 10.1088/1748-6041/8/2/021001

Gutmann, E. (1945). The reinnervation of muscle by sensory nerve fibres. *J Anat, 79*(Pt 1), 1-8 4.

Gutmann, E., & Hanzlikova, V. (1966). Motor unit in old age. *Nature, 209*(5026), 921-922.

Hall, J. K., Banks, G. B., Chamberlain, J. S., & Olwin, B. B. (2010). Prevention of muscle aging by myofiber-associated satellite cell transplantation. *Sci Transl Med, 2*(57), 57ra83. doi: 10.1126/scitranslmed.3001081

Harding, K., Sumner, M., & Cardinal, M. (2013). A prospective, multicentre, randomised controlled study of human fibroblast-derived dermal substitute (Dermagraft) in patients with venous leg ulcers. *Int Wound J, 10*(2), 132-137. doi: 10.1111/iwj.12053

Harii, K., Ohmori, K., & Torii, S. (1976). Free gracilis muscle transplantation, with microneurovascular anastomoses for the treatment of facial paralysis. A preliminary report. *Plast Reconstr Surg, 57*(2), 133-143.

Harman, S. M., Metter, E. J., Tobin, J. D., Pearson, J., Blackman, M. R., & Baltimore Longitudinal Study of, A. (2001). Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. *J Clin Endocrinol Metab, 86*(2), 724-731. doi: 10.1210/jcem.86.2.7219

Heher, P., Maleiner, B., Pruller, J., Teuschl, A. H., Kollmitzer, J., Monforte, X., . . . Fuchs, C. (2015). A novel bioreactor for the generation of highly aligned 3D skeletal muscle-like constructs through orientation of fibrin via application of static strain. *Acta Biomater, 24*, 251-265. doi: 10.1016/j.actbio.2015.06.033

Hepple, R. T., & Rice, C. L. (2016). Innervation and neuromuscular control in ageing skeletal muscle. *J Physiol, 594*(8), 1965-1978. doi: 10.1113/JP270561

Hill, E., Boontheekul, T., & Mooney, D. J. (2006a). Designing scaffolds to enhance transplanted myoblast survival and migration. *Tissue Eng, 12*(5), 1295-1304. doi: 10.1089/ten.2006.12.1295

Hill, E., Boontheekul, T., & Mooney, D. J. (2006b). Regulating activation of transplanted cells controls tissue regeneration. *Proc Natl Acad Sci U S A, 103*(8), 2494-2499. doi: 10.1073/pnas.0506004103

Hoque, M. E., San, W. Y., Wei, F., Li, S., Huang, M. H., Vert, M., & Hutmacher, D. W. (2009). Processing of polycaprolactone and polycaprolactone-based copolymers into 3D scaffolds, and their cellular responses. *Tissue Eng Part A, 15*(10), 3013-3024. doi: 10.1089/ten.TEA.2008.0355

Hsieh, C. C., Rosenblatt, J. I., & Papaconstantinou, J. (2003). Age-associated changes in SAPK/JNK and p38 MAPK signaling in response to the generation of ROS by 3-nitropropionic acid. *Mech Ageing Dev, 124*(6), 733-746.

Hurd, S. A., Bhatti, N. M., Walker, A. M., Kasukonis, B. M., & Wolchok, J. C. (2015). Development of a biological scaffold engineered using the extracellular matrix secreted by skeletal muscle cells. *Biomaterials, 49*, 9-17. doi: 10.1016/j.biomaterials.2015.01.027

Hurme, T., Kalimo, H., Lehto, M., & Jarvinen, M. (1991). Healing of skeletal muscle injury: an ultrastructural and immunohistochemical study. *Med Sci Sports Exerc, 23*(7), 801-810.

Hurtgen, B. J., Ward, C. L., Leopold Wager, C. M., Garg, K., Goldman, S. M., Henderson, B. E. P., . . . Corona, B. T. (2017). Autologous minced muscle grafts improve endogenous fracture healing and muscle strength after musculoskeletal trauma. *Physiol Rep, 5*(14). doi: 10.14814/phy2.13362

Huxley, A. F., & Niedergerke, R. (1954). Structural changes in muscle during contraction; interference microscopy of living muscle fibres. *Nature, 173*(4412), 971-973.

Huxley, H., & Hanson, J. (1954). Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature, 173*(4412), 973-976.

Iwasa, J., Engebretsen, L., Shima, Y., & Ochi, M. (2009). Clinical application of scaffolds for cartilage tissue engineering. *Knee Surg Sports Traumatol Arthrosc, 17*(6), 561-577. doi: 10.1007/s00167-008-0663-2

Jacquemin, V., Butler-Browne, G. S., Furling, D., & Mouly, V. (2007). IL-13 mediates the recruitment of reserve cells for fusion during IGF-1-induced hypertrophy of human myotubes. *J Cell Sci, 120*(Pt 4), 670-681. doi: 10.1242/jcs.03371

Jang, Y. C., & Van Remmen, H. (2011). Age-associated alterations of the neuromuscular junction. *Exp Gerontol, 46*(2-3), 193-198. doi: 10.1016/j.exger.2010.08.029

Jarvinen, M. J., & Lehto, M. U. (1993). The effects of early mobilisation and immobilisation on the healing process following muscle injuries. *Sports Med, 15*(2), 78-89.

Jarvinen, T. A., Jarvinen, T. L., Kaariainen, M., Aarimaa, V., Vaittinen, S., Kalimo, H., & Jarvinen, M. (2007). Muscle injuries: optimising recovery. *Best Pract Res Clin Rheumatol, 21*(2), 317-331. doi: 10.1016/j.berh.2006.12.004

Jarvinen, T. A., Jarvinen, T. L., Kaariainen, M., Kalimo, H., & Jarvinen, M. (2005). Muscle injuries: biology and treatment. *Am J Sports Med, 33*(5), 745-764. doi: 10.1177/0363546505274714

Jiang, C., Wen, Y., Kuroda, K., Hannon, K., Rudnicki, M. A., & Kuang, S. (2014). Notch signaling deficiency underlies age-dependent depletion of satellite cells in muscular dystrophy. *Dis Model Mech, 7*(8), 997-1004. doi: 10.1242/dmm.015917

Jiang, Y. Z., Jimenez, J. M., Ou, K., McCormick, M. E., Zhang, L. D., & Davies, P. F. (2014). Hemodynamic disturbed flow induces differential DNA methylation of endothelial Kruppel-Like Factor 4 promoter in vitro and in vivo. *Circ Res, 115*(1), 32-43. doi: 10.1161/CIRCRESAHA.115.303883

Jirmanova, I., & Thesleff, S. (1972). Ultrastructural study of experimental muscle degeneration and regeneration in the adult rat. *Z Zellforsch Mikrosk Anat, 131*(1), 77-97.

Ju, Y. M., Atala, A., Yoo, J. J., & Lee, S. J. (2014). In situ regeneration of skeletal muscle tissue through host cell recruitment. *Acta Biomater, 10*(10), 4332-4339. doi: 10.1016/j.actbio.2014.06.022

Jun, I., Jeong, S., & Shin, H. (2009). The stimulation of myoblast differentiation by electrically conductive sub-micron fibers. *Biomaterials, 30*(11), 2038-2047. doi: 10.1016/j.biomaterials.2008.12.063

Karalaki, M., Fili, S., Philippou, A., & Koutsilieris, M. (2009). Muscle regeneration: cellular and molecular events. *In Vivo, 23*(5), 779-796.

Kasukonis, B., Kim, J., Brown, L., Jones, J., Ahmadi, S., Washington, T., & Wolchok, J. (2016). Codelivery of Infusion Decellularized Skeletal Muscle with Minced Muscle Autografts Improved Recovery from Volumetric Muscle Loss Injury in a Rat Model. *Tissue Eng Part A, 22*(19-20), 1151-1163. doi: 10.1089/ten.TEA.2016.0134

Kasukonis, B. M., Kim, J. T., Washington, T. A., & Wolchok, J. C. (2016). Development of an infusion bioreactor for the accelerated preparation of decellularized skeletal muscle scaffolds. *Biotechnol Prog, 32*(3), 745-755. doi: 10.1002/btpr.2257

Kherif, S., Lafuma, C., Dehaupas, M., Lachkar, S., Fournier, J. G., Verdiere-Sahuque, M., . . . Alameddine, H. S. (1999). Expression of matrix metalloproteinases 2 and 9 in regenerating skeletal muscle: a study in experimentally injured and mdx muscles. *Dev Biol, 205*(1), 158-170. doi: 10.1006/dbio.1998.9107

Kicman, A. T. (2008). Pharmacology of anabolic steroids. *Br J Pharmacol, 154*(3), 502-521. doi: 10.1038/bjp.2008.165

Kim, J. T., Kasukonis, B. M., Brown, L. A., Washington, T. A., & Wolchok, J. C. (2016). Recovery from volumetric muscle loss injury: A comparison between young and aged rats. *Exp Gerontol, 83*, 37-46. doi: 10.1016/j.exger.2016.07.008

Klebuc, M., & Menn, Z. (2013). Muscle flaps and their role in limb salvage. *Methodist Debakey Cardiovasc J, 9*(2), 95-99.

Kolker, A. R., Brown, D. J., Redstone, J. S., Scarpinato, V. M., & Wallack, M. K. (2005). Multilayer reconstruction of abdominal wall defects with acellular dermal allograft (AlloDerm) and component separation. *Ann Plast Surg, 55*(1), 36-41; discussion 41-32.

Kovacheva, E. L., Hikim, A. P., Shen, R., Sinha, I., & Sinha-Hikim, I. (2010). Testosterone supplementation reverses sarcopenia in aging through regulation of myostatin, c-Jun NH2-terminal kinase, Notch, and Akt signaling pathways. *Endocrinology, 151*(2), 628-638. doi: 10.1210/en.2009-1177

Krippendorf, B. B., & Riley, D. A. (1993). Distinguishing unloading- versus reloading-induced changes in rat soleus muscle. *Muscle Nerve, 16*(1), 99-108. doi: 10.1002/mus.880160116

Kroehne, V., Heschel, I., Schugner, F., Lasrich, D., Bartsch, J. W., & Jockusch, H. (2008). Use of a novel collagen matrix with oriented pore structure for muscle cell differentiation in cell culture and in grafts. *J Cell Mol Med, 12*(5A), 1640-1648. doi: 10.1111/j.1582-4934.2008.00238.x

Kuang, S., Gillespie, M. A., & Rudnicki, M. A. (2008). Niche regulation of muscle satellite cell self-renewal and differentiation. *Cell Stem Cell, 2*(1), 22-31. doi: 10.1016/j.stem.2007.12.012

Kuhl, U., Ocalan, M., Timpl, R., Mayne, R., Hay, E., & von der Mark, K. (1984). Role of muscle fibroblasts in the deposition of type-IV collagen in the basal lamina of myotubes. *Differentiation, 28*(2), 164-172.

Kung, T. A., Cederna, P. S., van der Meulen, J. H., Urbanchek, M. G., Kuzon, W. M., Jr., & Faulkner, J. A. (2014). Motor unit changes seen with skeletal muscle sarcopenia in oldest old rats. *J Gerontol A Biol Sci Med Sci, 69*(6), 657-665. doi: 10.1093/gerona/glt135

Lahteenvuo, J., & Rosenzweig, A. (2012). Effects of aging on angiogenesis. *Circ Res, 110*(9), 1252-1264. doi: 10.1161/CIRCRESAHA.111.246116

Lalegul-Ulker, O., Seker, S., Elcin, A. E., & Elcin, Y. M. (2019). Encapsulation of bone marrow-MSCs in PRP-derived fibrin microbeads and preliminary evaluation in a volumetric muscle loss injury rat model: modular muscle tissue engineering. *Artif Cells Nanomed Biotechnol, 47*(1), 10-21. doi: 10.1080/21691401.2018.1540426

Lantz, G. C., Badylak, S. F., Coffey, A. C., Geddes, L. A., & Blevins, W. E. (1990). Small intestinal submucosa as a small-diameter arterial graft in the dog. *J Invest Surg, 3*(3), 217-227.

Larsson, L. (1995). Motor units: remodeling in aged animals. *J Gerontol A Biol Sci Med Sci, 50 Spec No*, 91-95.

Launay, T., Armand, A. S., Charbonnier, F., Mira, J. C., Donsez, E., Gallien, C. L., & Chanoine, C. (2001). Expression and neural control of myogenic regulatory factor genes during regeneration of mouse soleus. *J Histochem Cytochem, 49*(7), 887-899. doi: 10.1177/002215540104900709

Lefeuvre, B., Crossin, F., Fontaine-Perus, J., Bandman, E., & Gardahaut, M. F. (1996). Innervation regulates myosin heavy chain isoform expression in developing skeletal muscle fibers. *Mech Dev, 58*(1-2), 115-127.

Lesman, A., Koffler, J., Atlas, R., Blinder, Y. J., Kam, Z., & Levenberg, S. (2011). Engineering vessel-like networks within multicellular fibrin-based constructs. *Biomaterials, 32*(31), 7856-7869. doi: 10.1016/j.biomaterials.2011.07.003

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

Lin, A., & Dibling, B. (2002). The true face of JNK activation in apoptosis. *Aging Cell, 1*(2), 112-116.

Lin, C. H., Lin, Y. T., Yeh, J. T., & Chen, C. T. (2007). Free functioning muscle transfer for lower extremity posttraumatic composite structure and functional defect. *Plast Reconstr Surg, 119*(7), 2118-2126. doi: 10.1097/01.prs.0000260595.85557.41

Liu, G., Pareta, R. A., Wu, R., Shi, Y., Zhou, X., Liu, H., . . . Zhang, Y. (2013). Skeletal myogenic differentiation of urine-derived stem cells and angiogenesis using microbeads loaded with growth factors. *Biomaterials, 34*(4), 1311-1326. doi: 10.1016/j.biomaterials.2012.10.038

Macchiarini, P., Jungebluth, P., Go, T., Asnaghi, M. A., Rees, L. E., Cogan, T. A., . . . Birchall, M. A. (2008). Clinical transplantation of a tissue-engineered airway. *Lancet, 372*(9655), 2023-2030. doi: 10.1016/S0140-6736(08)61598-6

Machingal, M. A., Corona, B. T., Walters, T. J., Kesireddy, V., Koval, C. N., Dannahower, A., . . . Christ, G. J. (2011). A tissue-engineered muscle repair construct for functional restoration of an irrecoverable muscle injury in a murine model. *Tissue Eng Part A, 17*(17-18), 2291-2303. doi: 10.1089/ten.TEA.2010.0682

MacKenzie, E. J., Bosse, M. J., Kellam, J. F., Burgess, A. R., Webb, L. X., Swiontkowski, M. F., . . . McCarthy, M. L. (2000). Characterization of patients with high-energy lower extremity trauma. *J Orthop Trauma, 14*(7), 455-466.

Maley, M. A., Davies, M. J., & Grounds, M. D. (1995). Extracellular matrix, growth factors, genetics: their influence on cell proliferation and myotube formation in primary cultures of adult mouse skeletal muscle. *Exp Cell Res, 219*(1), 169-179. doi: 10.1006/excr.1995.1217

Marcinczyk, M., Dunn, A., Haas, G., Madsen, J., Scheidt, R., Patel, K., . . . Garg, K. (2018). The Effect of Laminin-111 Hydrogels on Muscle Regeneration in a Murine Model of Injury. *Tissue Eng Part A*. doi: 10.1089/ten.TEA.2018.0200

Marshall, P. A., Williams, P. E., & Goldspink, G. (1989). Accumulation of collagen and altered fiber-type ratios as indicators of abnormal muscle gene expression in the mdx dystrophic mouse. *Muscle Nerve, 12*(7), 528-537. doi: 10.1002/mus.880120703

Mase, V. J., Jr., Hsu, J. R., Wolf, S. E., Wenke, J. C., Baer, D. G., Owens, J., . . . Walters, T. J. (2010). Clinical application of an acellular biologic scaffold for surgical repair of a large, traumatic quadriceps femoris muscle defect. *Orthopedics, 33*(7), 511. doi: 10.3928/01477447-20100526-24

Matsumoto, T., Sasaki, J., Alsberg, E., Egusa, H., Yatani, H., & Sohmura, T. (2007). Three-dimensional cell and tissue patterning in a strained fibrin gel system. *PLoS One, 2*(11), e1211. doi: 10.1371/journal.pone.0001211

McGeachie, J. K., & Grounds, M. D. (1989). The onset of myogenesis in denervated mouse skeletal muscle regenerating after injury. *Neuroscience, 28*(2), 509-514.

McNamara, M. J., Garrett, W. E., Jr., Seaber, A. V., & Goldner, J. L. (1987). Neurorrhaphy, nerve grafting, and neurotization: a functional comparison of nerve reconstruction techniques. *J Hand Surg Am, 12*(3), 354-360.

McNeil, C. J., Doherty, T. J., Stashuk, D. W., & Rice, C. L. (2005). Motor unit number estimates in the tibialis anterior muscle of young, old, and very old men. *Muscle Nerve, 31*(4), 461-467. doi: 10.1002/mus.20276

Merritt, E. K., Cannon, M. V., Hammers, D. W., Le, L. N., Gokhale, R., Sarathy, A., . . . Farrar, R. P. (2010). Repair of traumatic skeletal muscle injury with bone-marrow-derived mesenchymal stem cells seeded on extracellular matrix. *Tissue Eng Part A, 16*(9), 2871-2881. doi: 10.1089/ten.TEA.2009.0826

Mertens, J. P., Sugg, K. B., Lee, J. D., & Larkin, L. M. (2014). Engineering muscle constructs for the creation of functional engineered musculoskeletal tissue. *Regen Med, 9*(1), 89-100. doi: 10.2217/rme.13.81

Monroy, J. A., Powers, K. L., Gilmore, L. A., Uyeno, T. A., Lindstedt, S. L., & Nishikawa, K. C. (2012). What is the role of titin in active muscle? *Exerc Sport Sci Rev, 40*(2), 73-78. doi: 10.1097/JES.0b013e31824580c6

Morissette, M. R., Cook, S. A., Foo, S., McKoy, G., Ashida, N., Novikov, M., . . . Rosenzweig, A. (2006). Myostatin regulates cardiomyocyte growth through modulation of Akt signaling. *Circ Res, 99*(1), 15-24. doi: 10.1161/01.RES.0000231290.45676.d4

Muramatsu, T., Muramatsu, H., & Kojima, T. (2006). Identification of proteoglycan-binding proteins. *Methods Enzymol, 416*, 263-278. doi: 10.1016/S0076-6879(06)16017-6

Nagata, S., Kurosawa, M., Mima, K., Nambo, Y., Fujii, Y., Watanabe, G., & Taya, K. (1999). Effects of anabolic steroid (19-nortestosterone) on the secretion of testicular hormones in the stallion. *J Reprod Fertil, 115*(2), 373-379.

Nguyen, H. X., & Tidball, J. G. (2003). Interactions between neutrophils and macrophages promote macrophage killing of rat muscle cells in vitro. *J Physiol, 547*(Pt 1), 125-132. doi: 10.1113/jphysiol.2002.031450

Niu, X., Feng, Q., Wang, M., Guo, X., & Zheng, Q. (2009). Porous nano-HA/collagen/PLLA scaffold containing chitosan microspheres for controlled delivery of synthetic peptide derived from BMP-2. *J Control Release, 134*(2), 111-117. doi: 10.1016/j.jconrel.2008.11.020

Nomura, Y. (2006). Structural change in decorin with skin aging. *Connect Tissue Res, 47*(5), 249-255. doi: 10.1080/03008200600846606

Ochoa, O., Sun, D., Reyes-Reyna, S. M., Waite, L. L., Michalek, J. E., McManus, L. M., & Shireman, P. K. (2007). Delayed angiogenesis and VEGF production in CCR2-/- mice during impaired skeletal muscle regeneration. *Am J Physiol Regul Integr Comp Physiol, 293*(2), R651-661. doi: 10.1152/ajpregu.00069.2007

Oda, K. (1984). Age changes of motor innervation and acetylcholine receptor distribution on human skeletal muscle fibres. *J Neurol Sci, 66*(2-3), 327-338.

Olfert, I. M., Baum, O., Hellsten, Y., & Egginton, S. (2016). Advances and challenges in skeletal muscle angiogenesis. *Am J Physiol Heart Circ Physiol, 310*(3), H326-336. doi: 10.1152/ajpheart.00635.2015

Opara, E. C., Mirmalek-Sani, S. H., Khanna, O., Moya, M. L., & Brey, E. M. (2010). Design of a bioartificial pancreas(+). *J Investig Med, 58*(7), 831-837. doi: 10.231/JIM.0b013e3181ed3807

Oryan, A., Alidadi, S., Moshiri, A., & Maffulli, N. (2014). Bone regenerative medicine: classic options, novel strategies, and future directions. *J Orthop Surg Res, 9*(1), 18. doi: 10.1186/1749-799X-9-18

Ott, H. C., Matthiesen, T. S., Goh, S. K., Black, L. D., Kren, S. M., Netoff, T. I., & Taylor, D. A. (2008). Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med, 14*(2), 213-221. doi: 10.1038/nm1684

Ottenheijm, C. A., & Granzier, H. (2010). Lifting the nebula: novel insights into skeletal muscle contractility. *Physiology (Bethesda), 25*(5), 304-310. doi: 10.1152/physiol.00016.2010

Owens, B. D., Kragh, J. F., Jr., Wenke, J. C., Macaitis, J., Wade, C. E., & Holcomb, J. B. (2008). Combat wounds in operation Iraqi Freedom and operation Enduring Freedom. *J Trauma, 64*(2), 295-299. doi: 10.1097/TA.0b013e318163b875

Owens, J. G., Blair, J. A., Patzkowski, J. C., Blanck, R. V., Hsu, J. R., & Skeletal Trauma Research, C. (2011). Return to running and sports participation after limb salvage. *J Trauma, 71*(1 Suppl), S120-124. doi: 10.1097/TA.0b013e3182219225

Page, R. L., Malcuit, C., Vilner, L., Vojtic, I., Shaw, S., Hedblom, E., . . . Dominko, T. (2011). Restoration of skeletal muscle defects with adult human cells delivered on fibrin microthreads. *Tissue Eng Part A, 17*(21-22), 2629-2640. doi: 10.1089/ten.TEA.2011.0024

Patel, V. A., Zhang, Q. J., Siddle, K., Soos, M. A., Goddard, M., Weissberg, P. L., & Bennett, M. R. (2001). Defect in insulin-like growth factor-1 survival mechanism in atherosclerotic plaque-derived vascular smooth muscle cells is mediated by reduced surface binding and signaling. *Circ Res, 88*(9), 895-902.

Pavlath, G. K., Dominov, J. A., Kegley, K. M., & Miller, J. B. (2003). Regeneration of transgenic skeletal muscles with altered timing of expression of the basic helix-loop-helix muscle regulatory factor MRF4. *Am J Pathol, 162*(5), 1685-1691. doi: 10.1016/S0002-9440(10)64303-9

Pelosi, L., Giacinti, C., Nardis, C., Borsellino, G., Rizzuto, E., Nicoletti, C., . . . Musaro, A. (2007). Local expression of IGF-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines. *FASEB J, 21*(7), 1393-1402. doi: 10.1096/fj.06-7690com

Perniconi, B., Costa, A., Aulino, P., Teodori, L., Adamo, S., & Coletti, D. (2011). The pro-myogenic environment provided by whole organ scale acellular scaffolds from skeletal muscle. *Biomaterials, 32*(31), 7870-7882. doi: 10.1016/j.biomaterials.2011.07.016

Pomara, C., Barone, R., Marino Gammazza, A., Sangiorgi, C., Barone, F., Pitruzzella, A., . . . Turillazzi, E. (2016). Effects of Nandrolone Stimulation on Testosterone Biosynthesis in Leydig Cells. *J Cell Physiol, 231*(6), 1385-1391. doi: 10.1002/jcp.25272

Porzionato, A., Sfriso, M. M., Pontini, A., Macchi, V., Petrelli, L., Pavan, P. G., . . . De Caro, R. (2015). Decellularized Human Skeletal Muscle as Biologic Scaffold for Reconstructive Surgery. *Int J Mol Sci, 16*(7), 14808-14831. doi: 10.3390/ijms160714808

Prabaharan, M., & Jayakumar, R. (2009). Chitosan-graft-beta-cyclodextrin scaffolds with controlled drug release capability for tissue engineering applications. *Int J Biol Macromol, 44*(4), 320-325. doi: 10.1016/j.ijbiomac.2009.01.005

Prevel, C. D., Eppley, B. L., Summerlin, D. J., Jackson, J. R., McCarty, M., & Badylak, S. F. (1995). Small intestinal submucosa: utilization for repair of rodent abdominal wall defects. *Ann Plast Surg, 35*(4), 374-380.

Ramasamy, A., Harrisson, S. E., Clasper, J. C., & Stewart, M. P. (2008). Injuries from roadside improvised explosive devices. *J Trauma, 65*(4), 910-914. doi: 10.1097/TA.0b013e3181848cf6

Renault, V., Thornell, L. E., Butler-Browne, G., & Mouly, V. (2002). Human skeletal muscle satellite cells: aging, oxidative stress and the mitotic clock. *Exp Gerontol, 37*(10-11), 1229-1236.

Rhim, C., Lowell, D. A., Reedy, M. C., Slentz, D. H., Zhang, S. J., Kraus, W. E., & Truskey, G. A. (2007). Morphology and ultrastructure of differentiating three-dimensional mammalian skeletal muscle in a collagen gel. *Muscle Nerve, 36*(1), 71-80. doi: 10.1002/mus.20788

Romijn, J. A., Coyle, E. F., Sidossis, L. S., Gastaldelli, A., Horowitz, J. F., Endert, E., & Wolfe, R. R. (1993). Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol, 265*(3 Pt 1), E380-391. doi: 10.1152/ajpendo.1993.265.3.E380

Rossi, C. A., Flaibani, M., Blaauw, B., Pozzobon, M., Figallo, E., Reggiani, C., . . . De Coppi, P. (2011). In vivo tissue engineering of functional skeletal muscle by freshly isolated satellite cells embedded in a photopolymerizable hydrogel. *FASEB J, 25*(7), 2296-2304. doi: 10.1096/fj.10-174755

Samuel, M. A., Valdez, G., Tapia, J. C., Lichtman, J. W., & Sanes, J. R. (2012). Agrin and synaptic laminin are required to maintain adult neuromuscular junctions. *PLoS One, 7*(10), e46663. doi: 10.1371/journal.pone.0046663

Sandusky, G. E., Jr., Badylak, S. F., Morff, R. J., Johnson, W. D., & Lantz, G. (1992). Histologic findings after in vivo placement of small intestine submucosal vascular grafts and saphenous vein grafts in the carotid artery in dogs. *Am J Pathol, 140*(2), 317-324.

Saxena, A. K., Marler, J., Benvenuto, M., Willital, G. H., & Vacanti, J. P. (1999). Skeletal muscle tissue engineering using isolated myoblasts on synthetic biodegradable polymers: preliminary studies. *Tissue Eng, 5*(6), 525-532. doi: 10.1089/ten.1999.5.525

Saxena, A. K., Willital, G. H., & Vacanti, J. P. (2001). Vascularized three-dimensional skeletal muscle tissue-engineering. *Biomed Mater Eng, 11*(4), 275-281.

Schultz, G. S., & Wysocki, A. (2009). Interactions between extracellular matrix and growth factors in wound healing. *Wound Repair Regen, 17*(2), 153-162. doi: 10.1111/j.1524-475X.2009.00466.x

Seene, T., & Kaasik, P. (2012). Role of exercise therapy in prevention of decline in aging muscle function: glucocorticoid myopathy and unloading. *J Aging Res, 2012*, 172492. doi: 10.1155/2012/172492

Serra, C., Tangherlini, F., Rudy, S., Lee, D., Toraldo, G., Sandor, N. L., . . . Bhasin, S. (2013). Testosterone improves the regeneration of old and young mouse skeletal muscle. *J Gerontol A Biol Sci Med Sci, 68*(1), 17-26. doi: 10.1093/gerona/gls083

Shalhav, A. L., Elbahnasy, A. M., Bercowsky, E., Kovacs, G., Brewer, A., Maxwell, K. L., . . . Clayman, R. V. (1999). Laparoscopic replacement of urinary tract segments using biodegradable materials in a large-animal model. *J Endourol, 13*(4), 241-244. doi: 10.1089/end.1999.13.241

Shao, S., Zhou, S., Li, L., Li, J., Luo, C., Wang, J., . . . Weng, J. (2011). Osteoblast function on electrically conductive electrospun PLA/MWCNTs nanofibers. *Biomaterials, 32*(11), 2821-2833. doi: 10.1016/j.biomaterials.2011.01.051

Shvartsman, D., Storrie-White, H., Lee, K., Kearney, C., Brudno, Y., Ho, N., . . . Mooney, D. J. (2014). Sustained delivery of VEGF maintains innervation and promotes reperfusion in ischemic skeletal muscles via NGF/GDNF signaling. *Mol Ther, 22*(7), 1243-1253. doi: 10.1038/mt.2014.76

Sicari, B. M., Agrawal, V., Siu, B. F., Medberry, C. J., Dearth, C. L., Turner, N. J., & Badylak, S. F. (2012). A murine model of volumetric muscle loss and a regenerative medicine approach for tissue replacement. *Tissue Eng Part A, 18*(19-20), 1941-1948. doi: 10.1089/ten.TEA.2012.0475

Sicari, B. M., Rubin, J. P., Dearth, C. L., Wolf, M. T., Ambrosio, F., Boninger, M., . . . Badylak, S. F. (2014). An acellular biologic scaffold promotes skeletal muscle formation in mice and humans with volumetric muscle loss. *Sci Transl Med, 6*(234), 234ra258. doi: 10.1126/scitranslmed.3008085

Sim, F. J., Zhao, C., Penderis, J., & Franklin, R. J. (2002). The age-related decrease in CNS remyelination efficiency is attributable to an impairment of both oligodendrocyte progenitor recruitment and differentiation. *J Neurosci, 22*(7), 2451-2459. doi: 20026217

Sinha-Hikim, I., Braga, M., Shen, R., & Sinha Hikim, A. P. (2007). Involvement of c-Jun NH2-terminal kinase and nitric oxide-mediated mitochondria-dependent intrinsic pathway signaling in cardiotoxin-induced muscle cell death: role of testosterone. *Apoptosis, 12*(11), 1965-1978. doi: 10.1007/s10495-007-0120-6

Smith, C. K., 2nd, Janney, M. J., & Allen, R. E. (1994). Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. *J Cell Physiol, 159*(2), 379-385. doi: 10.1002/jcp.1041590222

Snow, M. H. (1977). The effects of aging on satellite cells in skeletal muscles of mice and rats. *Cell Tissue Res, 185*(3), 399-408.

Socha, M. J., & Segal, S. S. (2018). Microvascular mechanisms limiting skeletal muscle blood flow with advancing age. *J Appl Physiol (1985), 125*(6), 1851-1859. doi: 10.1152/japplphysiol.00113.2018

Song, J. J., & Ott, H. C. (2011). Organ engineering based on decellularized matrix scaffolds. *Trends Mol Med, 17*(8), 424-432. doi: 10.1016/j.molmed.2011.03.005

Sprenger, C. C., Plymate, S. R., & Reed, M. J. (2008). Extracellular influences on tumour angiogenesis in the aged host. *Br J Cancer, 98*(2), 250-255. doi: 10.1038/sj.bjc.6604144

St Pierre, B. A., & Tidball, J. G. (1994). Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension. *J Appl Physiol (1985), 77*(1), 290-297. doi: 10.1152/jappl.1994.77.1.290

Striganova, A. R. (1958). [Reactive changes of the intact muscle in atrophy and restoration of opposite denervated muscle in rats]. *Arkh Patol, 20*(9), 11-17.

Studitsky, A. N. (1964). Free Auto- and Homografts of Muscle Tissue in Experiments on Animals. *Ann N Y Acad Sci, 120*, 789-801.

Suckow, M. A., Voytik-Harbin, S. L., Terril, L. A., & Badylak, S. F. (1999). Enhanced bone regeneration using porcine small intestinal submucosa. *J Invest Surg, 12*(5), 277-287.

Suelves, M., Lluis, F., Ruiz, V., Nebreda, A. R., & Munoz-Canoves, P. (2004). Phosphorylation of MRF4 transactivation domain by p38 mediates repression of specific myogenic genes. *EMBO J, 23*(2), 365-375. doi: 10.1038/sj.emboj.7600056

Suga, T., Kinugawa, S., Takada, S., Kadoguchi, T., Fukushima, A., Homma, T., . . . Tsutsui, H. (2014). Combination of exercise training and diet restriction normalizes limited exercise capacity and impaired skeletal muscle function in diet-induced diabetic mice. *Endocrinology, 155*(1), 68-80. doi: 10.1210/en.2013-1382

Sugamori, M. E., & Sefton, M. V. (1989). Microencapsulation of pancreatic islets in a water insoluble polyacrylate. *ASAIO Trans, 35*(4), 791-799.

Sulaiman, O. A., Midha, R., Munro, C. A., Matsuyama, T., Al-Majed, A., & Gordon, T. (2002). Chronic Schwann cell denervation and the presence of a sensory nerve reduce motor axonal regeneration. *Exp Neurol, 176*(2), 342-354.

Takahashi, M., Tatsugi, Y., & Kohno, T. (2004). Endocrinological and pathological effects of anabolic-androgenic steroid in male rats. *Endocr J, 51*(4), 425-434.

Tamai, S., Komatsu, S., Sakamoto, H., Sano, S., & Sasauchi, N. (1970). Free muscle transplants in dogs, with microsurgical neurovascular anastomoses. *Plast Reconstr Surg, 46*(3), 219-225.

Tateyama, M., Fujihara, K., Misu, T., & Itoyama, Y. (2009). CCR7+ myeloid dendritic cells together with CCR7+ T cells and CCR7+ macrophages invade CCL19+ nonnecrotic muscle fibers in inclusion body myositis. *J Neurol Sci, 279*(1-2), 47-52. doi: 10.1016/j.jns.2008.12.024

Tedesco, F. S., & Cossu, G. (2012). Stem cell therapies for muscle disorders. *Curr Opin Neurol, 25*(5), 597-603. doi: 10.1097/WCO.0b013e328357f288

Terada, N., Takayama, S., Yamada, H., & Seki, T. (2001). Muscle repair after a transsection injury with development of a gap: an experimental study in rats. *Scand J Plast Reconstr Surg Hand Surg, 35*(3), 233-238.

Thomas, D. R. (2001). Age-related changes in wound healing. *Drugs Aging, 18*(8), 607-620. doi: 10.2165/00002512-200118080-00005

Thomas, G. D. (2013). Functional muscle ischemia in Duchenne and Becker muscular dystrophy. *Front Physiol, 4*, 381. doi: 10.3389/fphys.2013.00381

Thomas, K., Engler, A. J., & Meyer, G. A. (2015). Extracellular matrix regulation in the muscle satellite cell niche. *Connect Tissue Res, 56*(1), 1-8. doi: 10.3109/03008207.2014.947369

Thompson, N. (1971a). Autogenous free grafts of skeletal muscle. A preliminary experimental and clinical study. *Plast Reconstr Surg, 48*(1), 11-27.

Thompson, N. (1971b). Investigation of autogenous skeletal muscle free grafts in the dog with a report on a successful free graft of skeletal muscle in man. *Transplantation, 12*(5), 353-363.

Tidball, J. G. (1995). Inflammatory cell response to acute muscle injury. *Med Sci Sports Exerc, 27*(7), 1022-1032.

Tidball, J. G. (2011). Mechanisms of muscle injury, repair, and regeneration. *Compr Physiol, 1*(4), 2029-2062. doi: 10.1002/cphy.c100092

Tidball, J. G., & Wehling-Henricks, M. (2007). Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. *J Physiol, 578*(Pt 1), 327-336. doi: 10.1113/jphysiol.2006.118265

Toumi, H., & Best, T. M. (2003). The inflammatory response: friend or enemy for muscle injury? *Br J Sports Med, 37*(4), 284-286.

TOWER, S. S. (1931). A SEARCH FOR TROPHIC INFLUENCE OF POSTERIOR SPINAL ROOTS ON SKELETAL MUSCLE, WITH A NOTE ON THE NERVE FIBRES FOUND IN THE PROXIMAL STUMPS OF THE ROOTS AFTER EXCISION OF THE ROOT GANGLIA. *Brain, 54*(1), 99-110. doi: 10.1093/brain/54.1.99

TOWER, S. S. (1939). PERSISTENCE OF FIBRILLATION IN DENERVATED SKELETAL MUSCLE AND ITS NONOCCURRENCE IN MUSCLE AFTER TENOTOMY. *Archives of Neurology & Psychiatry, 42*(2), 219-223. doi: 10.1001/archneurpsyc.1939.02270200039003

Turner, N. J., Badylak, J. S., Weber, D. J., & Badylak, S. F. (2012). Biologic scaffold remodeling in a dog model of complex musculoskeletal injury. *J Surg Res, 176*(2), 490-502. doi: 10.1016/j.jss.2011.11.1029

Turner, N. J., & Badylak, S. F. (2012). Regeneration of skeletal muscle. *Cell Tissue Res, 347*(3), 759-774. doi: 10.1007/s00441-011-1185-7

Turner, N. J., Yates, A. J., Jr., Weber, D. J., Qureshi, I. R., Stolz, D. B., Gilbert, T. W., & Badylak, S. F. (2010). Xenogeneic extracellular matrix as an inductive scaffold for

regeneration of a functioning musculotendinous junction. *Tissue Eng Part A, 16*(11), 3309-3317. doi: 10.1089/ten.TEA.2010.0169

Ullman, M., Ullman, A., Sommerland, H., Skottner, A., & Oldfors, A. (1990). Effects of growth hormone on muscle regeneration and IGF-I concentration in old rats. *Acta Physiol Scand, 140*(4), 521-525. doi: 10.1111/j.1748-1716.1990.tb09029.x

Untergasser, G., Madersbacher, S., & Berger, P. (2005). Benign prostatic hyperplasia: age-related tissue-remodeling. *Exp Gerontol, 40*(3), 121-128. doi: 10.1016/j.exger.2004.12.008

Valentin, J. E., Turner, N. J., Gilbert, T. W., & Badylak, S. F. (2010). Functional skeletal muscle formation with a biologic scaffold. *Biomaterials, 31*(29), 7475-7484. doi: 10.1016/j.biomaterials.2010.06.039

van Wachem, P. B., Brouwer, L. A., & van Luyn, M. J. (1999). Absence of muscle regeneration after implantation of a collagen matrix seeded with myoblasts. *Biomaterials, 20*(5), 419-426.

Vekris, M. D., Beris, A. E., Lykissas, M. G., Korompilias, A. V., Vekris, A. D., & Soucacos, P. N. (2008). Restoration of elbow function in severe brachial plexus paralysis via muscle transfers. *Injury, 39 Suppl 3*, S15-22. doi: 10.1016/j.injury.2008.06.008

Velleman, S. G. (1999). The role of the extracellular matrix in skeletal muscle development. *Poult Sci, 78*(5), 778-784. doi: 10.1093/ps/78.5.778

Villalta, S. A., Nguyen, H. X., Deng, B., Gotoh, T., & Tidball, J. G. (2009). Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. *Hum Mol Genet, 18*(3), 482-496. doi: 10.1093/hmg/ddn376

Vindigni, V., Mazzoleni, F., Rossini, K., Fabbian, M., Zanin, M. E., Bassetto, F., & Carraro, U. (2004). Reconstruction of ablated rat rectus abdominis by muscle regeneration. *Plast Reconstr Surg, 114*(6), 1509-1515; discussion 1516-1508.

Wainwright, D. J. (1995). Use of an acellular allograft dermal matrix (AlloDerm) in the management of full-thickness burns. *Burns, 21*(4), 243-248.

Walters, B. D., & Stegemann, J. P. (2014). Strategies for directing the structure and function of three-dimensional collagen biomaterials across length scales. *Acta Biomater, 10*(4), 1488-1501. doi: 10.1016/j.actbio.2013.08.038

Wang, L., Cao, L., Shansky, J., Wang, Z., Mooney, D., & Vandenburgh, H. (2014). Minimally invasive approach to the repair of injured skeletal muscle with a shape-memory scaffold. *Mol Ther, 22*(8), 1441-1449. doi: 10.1038/mt.2014.78

Ward, C. L., Ji, L., & Corona, B. T. (2015). An Autologous Muscle Tissue Expansion Approach for the Treatment of Volumetric Muscle Loss. *Biores Open Access, 4*(1), 198-208. doi: 10.1089/biores.2015.0009

White, J. P., Baltgalvis, K. A., Sato, S., Wilson, L. B., & Carson, J. A. (2009). Effect of nandrolone decanoate administration on recovery from bupivacaine-induced muscle injury. *J Appl Physiol (1985), 107*(5), 1420-1430. doi: 10.1152/japplphysiol.00668.2009

Winkler, J. T., Swaim, S. F., Sartin, E. A., Henderson, R. A., & Welch, J. A. (2002). The effect of a porcine-derived small intestinal submucosa product on wounds with exposed bone in dogs. *Vet Surg, 31*(6), 541-551.

Wolf, M. T., Carruthers, C. A., Dearth, C. L., Crapo, P. M., Huber, A., Burnsed, O. A., . . . Badylak, S. F. (2014). Polypropylene surgical mesh coated with extracellular matrix mitigates the host foreign body response. *J Biomed Mater Res A, 102*(1), 234-246. doi: 10.1002/jbm.a.34671

Wu, X., Corona, B. T., Chen, X., & Walters, T. J. (2012). A standardized rat model of volumetric muscle loss injury for the development of tissue engineering therapies. *Biores Open Access, 1*(6), 280-290. doi: 10.1089/biores.2012.0271

Yablonka-Reuveni, Z., & Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev Biol, 164*(2), 588-603. doi: 10.1006/dbio.1994.1226

Yang, J., Jao, B., McNally, A. K., & Anderson, J. M. (2014). In vivo quantitative and qualitative assessment of foreign body giant cell formation on biomaterials in mice deficient in natural killer lymphocyte subsets, mast cells, or the interleukin-4 receptoralpha and in severe combined immunodeficient mice. *J Biomed Mater Res A, 102*(6), 2017-2023. doi: 10.1002/jbm.a.35152

Zacks, S. I., & Sheff, M. F. (1982). Age-related impeded regeneration of mouse minced anterior tibial muscle. *Muscle Nerve, 5*(2), 152-161. doi: 10.1002/mus.880050213

Zalewski, A. A. (1970). Effects of reinnervation on denervated skeletal muscle by axons of motor, sensory, and sympathetic neurons. *Am J Physiol, 219*(6), 1675-1679. doi: 10.1152/ajplegacy.1970.219.6.1675

Zammit, P. S., Heslop, L., Hudon, V., Rosenblatt, J. D., Tajbakhsh, S., Buckingham, M. E., . . . Partridge, T. A. (2002). Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. *Exp Cell Res, 281*(1), 39-49.

Zatti, S., Zoso, A., Serena, E., Luni, C., Cimetta, E., & Elvassore, N. (2012). Micropatterning topology on soft substrates affects myoblast proliferation and differentiation. *Langmuir, 28*(5), 2718-2726. doi: 10.1021/la204776e

Zhang, F., Lineaweaver, W. C., Ustuner, T., Kao, S. D., Tonken, H. P., Campagna-Pinto, D., & Buncke, H. J. (1997). Comparison of muscle mass preservation in denervated muscle and transplanted muscle flaps after motor and sensory reinnervation and neurotization. *Plast Reconstr Surg, 99*(3), 803-814.

Zhenevskaya, R. P. (1962). Experimental histologic investigation of striated muscle tissue. *Rev Can Biol, 21*, 457-470.

Zhou, H., & Xu, H. H. (2011). The fast release of stem cells from alginate-fibrin microbeads in injectable scaffolds for bone tissue engineering. *Biomaterials, 32*(30), 7503-7513. doi: 10.1016/j.biomaterials.2011.06.045

Zou, Y., Zhang, R. Z., Sabatelli, P., Chu, M. L., & Bonnemann, C. G. (2008). Muscle interstitial fibroblasts are the main source of collagen VI synthesis in skeletal muscle: implications for congenital muscular dystrophy types Ullrich and Bethlem. *J Neuropathol Exp Neurol, 67*(2), 144-154. doi: 10.1097/nen.0b013e3181634ef7

Chapter 2

Recovery from Volumetric Muscle Loss Injury: A Comparison between Young and Aged Rats

Submitted as an original article by:

John T. Kim[1], Benjamin Kasukonis[1], Lemuel Brown[2], Tyrone Washington[2], and Jeffrey C. Wolchok PhD[1]

[1] Department of Biomedical Engineering, University of Arkansas, Fayetteville, Arkansas, 72701

[2] Department of Health, Human Performance, and Recreation, College of Education and Health Professions, University of Arkansas, Fayetteville, AR, Fayetteville, Arkansas 72701

Corresponding Author:

Jeffrey C. Wolchok, PhD

Department of Biomedical Engineering

University of Arkansas

John A. White, Jr. Engineering Hall, Suite 120, Fayetteville, Arkansas

Phone: 479-575-4667

Email: jwolchok@uark.edu

Highlights

- Comparison between young and aged rats to volumetric muscle loss injury (VML).
- Age does not exacerbate the post-VML functional response.
- Age affects ECM composition and results in a more fibrotic post-VML environment.

Abstract

Termed volumetric muscle loss (VML), the bulk loss of skeletal muscle tissue either through trauma or surgery overwhelms the capacity for repair, leading to the formation of non-contractile scar tissue. The myogenic potential, along with other factors that influence wound repair are known to decline with age. In order to develop effective treatment strategies for VML injuries that are effective across a broad range of patient populations, it is necessary to understand how the response to VML injury is affected by aging. Towards this end, this study was conducted to compare the response of young and aged animal groups to a lower extremity VML injury. Young (3 months, n=12) and aged (18 months, n=8) male Fischer 344 rats underwent surgical VML injury of the tibialis anterior muscle. Three months after VML injury it was found that young TA muscle was on average 16% heavier than aged muscle when no VML injury was performed and 25% heavier when comparing VML treated young and aged animals ($p<0.0001$, $p<0.0001$). Peak contractile force for both the young and aged groups was found to decrease significantly following VML injury, producing 65% and 59% of the contralateral limbs' peak force, respectively ($p<0.0001$). However, there were no differences found for peak contractile force based on age, suggesting that VML affects muscle's ability to repair, regardless of age. In this study, we used the ratio of collagen I to MyoD expression as a metric for fibrosis vs. myogenesis. Decreasing fiber cross-sectional area with advancing age ($p<0.005$) coupled with the ratio of collagen I to MyoD expression, which increased with age, supports the thought that regeneration is impaired in the aged population in favor of fibrosis ($p=0.0241$). This impairment

is also exacerbated by the contribution of VML injury, where a 77-fold increase in the ratio of collagen I to MyoD was observed in the aged group ($p<0.0002$). The aged animal model described in this study provides a tool for investigators exploring not only the development of VML injury strategies but also the effect of aging on muscle regeneration.

Keywords: orthopedics, musculoskeletal, animal model, tibialis anterior, aging, VML

A. Introduction

Our multidisciplinary team is exploring the development of implantable biomaterials targeting the repair of damaged skeletal muscle (Hurd et al., 2015; B. Kasukonis, J. Kim, T. Washington, & J. Wolchok, 2016). Towards that end we are particularly interested in understanding the response of muscle to volumetric muscle loss injury (Corona et al., 2015; Grogan, Hsu, & Skeletal Trauma Research, 2011b). In response to mild injuries like strains and contusions, in which the myocytes are damaged but the underlying muscle structure and satellite cell population is preserved, muscle has a strong capacity for regeneration (M. Hill et al., 2003; Mauro, 1961). Muscle regeneration is characterized by progressive and coordinated inflammation, repair, and remodeling phases (Charge & Rudnicki, 2004b; Mann et al., 2011b; Turner & Badylak, 2012b). During the first hours and days after injury, phagocytic neutrophils and macrophages are recruited to the wound site to clear the injury of dead cells and necrotic tissue during what is termed the inflammatory phase. Transition from the inflammatory phase to the repair phase, is characterized by a shift in the phenotype of the macrophages from the inflammatory M1 phenotype, responsible for the digestion of necrotic tissues and promotion of satellite cell proliferation, to tissue remodeling M2 macrophages responsible for myoblast proliferation, growth and differentiation (Tidball, 2005a; Tidball & Villalta, 2010). During the repair phase, satellite cells proliferate and differentiate into myoblasts that fuse with other myoblasts or with existing fibers to form new skeletal muscle (Turner & Badylak, 2012b). The remodeling phase is a continuation of the repair phase where myofibers mature and start to adhere to both native and newly remodeled extracellular matrix (ECM). In muscle, the regenerative process peaks anywhere from 7 to 30+ days depending on the type and severity of

the injury (Benoit & Belt, 1970; Nikolaou, Macdonald, Glisson, Seaber, & Garrett, 1987; Warren et al., 2007).

Alternatively, when significant skeletal muscle volume is lost (trauma or surgical resection) the regenerative cues provided by the underlying ECM and satellite cell pool is missing, and regeneration is poor. Termed volumetric muscle loss (VML), the bulk loss of muscle tissue (>20% by mass) overwhelms the capacity for regeneration, leading to the formation of non-contractile scar tissue at the defect site and functional impairment for the patient (Grogan et al., 2011b; Terada et al., 2001). The poor clinical outcome following VML injury combined with a lack of effective treatment options motivates our exploration of VML regeneration. To better understand the sequence of events that follow VML injury and aid in the development of effective treatment strategies, various pre-clinical animal models have been developed (X. K. Chen & Walters, 2013; Hou, Gong, & Zhu, 2016; Turner et al., 2012; Wu, Corona, Chen, & Walters, 2012b; J. Zhang et al., 2016). Among these, the hind limb muscles of the rat are a reliable pre-clinical model that recapitulates healing with non-contractile scar tissue observed in humans following VML injury (Wu et al., 2012b). In particular, the rat hind limb has become a standard model for military research (Aurora et al., 2014; Aurora et al., 2015; Corona, Garg, et al., 2013; Corona, Ward, Baker, Walters, & Christ, 2014a). This group of investigators is seeking treatments for VML injuries caused by devastating battlefield trauma. With the active military population in mind, the response to VML injury has typically been explored in young (<50% of the median lifespan = 24 months) rat models. However, in addition to traumatic injuries, the surgical removal of muscle as a treatment for soft tissue sarcoma is another anticipated target for VML repair strategies (Fischer et al., 2015). This group of patients is

typically older, and therefore an improved understanding of the response to VML injury in aged muscle may provide unique insights that could guide future treatment strategies.

With increasing age, both animals and humans develop pathological changes to muscle tissue which includes decreased muscle volume and increased adipose infiltration which reduces muscle strength and can increase the risk of injury (Freemont & Hoyland, 2007). These aged-associated changes in muscle physiology can act as obstacles to muscle regeneration (Grounds, 1998a). We suspect that age related changes in muscle physiology might significantly influence the response to VML injury. It is well established that muscle resident satellite cells account for the majority of skeletal muscle's regenerative capacity following injury (Conboy & Rando, 2005). However, aging leads to an overall decrease in myogenic capacity mostly in part due to reduced satellite cell activity (Brack & Rando, 2007b; Conboy & Rando, 2005; Snow, 1977). Specifically, it appears there is an age-related loss in satellite cell functionality, possibly indicating that the "aging" of the satellite cells renders them less responsive to regenerative cues (Brack & Rando, 2007a; Charge & Rudnicki, 2004b; Gopinath & Rando, 2008b). In addition, a decreased responsiveness to the stimuli needed to induce activation and proliferation of satellite cells has been observed with aging (Brack et al., 2007a; Gopinath & Rando, 2008b; Kuang et al., 2008; Vinciguerra, Musaro, & and Rosenthal, 2010). When taken as a whole, the literature supports the conclusion that aging diminishes the regenerative capacity of muscle.

While aging associated changes in muscle regeneration are known to exist, the effects of aging on VML recovery has yet to be explored to our knowledge. With that gap in mind, an aged animal model of VML injury was developed to investigate and compare the regenerative response following VML injury of the hind limb tibialis anterior (TA) muscle. Two populations of Fisher 344 rats aged 3 months and 18 months were used in this study. These ages are

representative of adolescent (25% of mean lifespan) and early elderly (75% of mean lifespan) rat lifespans. In order to assess the response to injury; muscle strength, mass, histology, and gene expression profiles were measured for both aged and young animals three-months post VML injury, a commonly explored VML recovery time point (Aurora et al., 2015; Corona, Garg, et al., 2013; Corona et al., 2014b; Merritt, Cannon, et al., 2010b; Wu et al., 2012b). Overall, the study was designed to compare the response between the two age groups and test the hypothesis that aged animals recover differently from VML injury when compared to young animals as might be evidenced by differences in myogenesis, contractile force, muscle atrophy, and tissue fibrosis. Somewhat similar to VML in terms of functional outcomes, crush injuries result in similar functional deficits in the muscle or in severe cases, complete amputation of the limb due to inflammation, tissue swelling and necrosis, a condition known as acute compartment syndrome (ACS) (Via, Oliva, Spoliti, & Maffulli, 2015). Although this may seem a worthwhile issue to address, the effects of surgical VML don't lead to other myopathies and conditions as ACS does. Additionally, due to high mortality rates associated with ACS (Schwartz et al., 1989), it would not be guaranteed that all animals used in the study would survive to the end of the study.

The results generated by this study not only provide some of the first insights into recovery from VML injury in aged muscle, but also provide baseline recovery measures from which to compare future VML injury strategies targeting aged patients.

B. Methods

Volumetric Muscle Loss Injury

In vivo VML injury studies were performed with young (3 month) and aged (18 months) male Fisher 3 44 rats. All contralateral limbs to the VML injured limb served as internal controls for comparison and are denoted as the normal groups in the study. The young rats (n=12) were purchased commercially from Harlan Laboratories (Indianapolis, IN) and the aged rats (n=8) were obtained through the National Institute on Aging (Bethesda, MD). Surgical procedures and implant preparation methods were performed in accordance with protocols approved by the University of Arkansas IACUC (Protocol #14044) and guided by published methods (Wu et al., 2012b). Anesthesia was induced using isoflurane (1-3%) in oxygen. The implant site was surgically exposed through a 1-2 cm incision running parallel to the tibia. The TA was identified and a partial thickness VML defect (8mm diameter x 3mm deep) was created using a sterile biopsy punch **(Figure 1)**. Data that was previously collected correlated animal body weight to TA mass for each animal group (**Figure 2**). From this relationship, the percentage of total TA mass excised during surgery was calculated for each animal, to help insure consistent defect creation across all animals. The average size of the VML defect was 18.7±0.6% and 19.5±0.6% for the young and aged animal groups respectively (Corona et al., 2014a). The difference in defect size between groups was not statistically significant (p=0.36). The contralateral limb was left untreated to serve as an internal comparative control. The deeper periosteum and surface skin layers were separately closed using an interrupted stich with a 5-0 absorbable suture (Vicryl, Ethicon, Summerville, MA). Postoperative analgesia consisted of 0.1mg/kg buprenorphine administered subcutaneously via injection (neck scruff) twice daily for two days. Animals also received anti-inflammatory medication (Carprophan) via a dietary gel cup (Medigel CPF,

ClearH$_2$O, Westbrook, ME) added as needed to each cage out to 7 days. Following surgery, animals were housed in standard-sized rat cages with unrestricted movement. The animals were allowed to bear weight on the operative extremity as tolerated. All animals were housed for a 12-week recovery period.

In-situ Contractile Force Measurement

Peak tetanic contraction produced by the TA muscle of both young (n=12) and aged (n=8) rats were measured isometrically *in vivo* using similar procedures previously described[18]. After rats were anesthetized (2-2.5% isoflurane), the left hindlimb was prepared for measurement. To eliminate force contributions from the synergist muscle, distal tenotomies were performed on the extensor digitorum longus (EDL) and extensor halluces longus (EHL). The knee was stabilized in place using a custom mounting system and the knee and ankle were positioned at a right angle. The foot was attached to a foot-plate with surgical tape, which was attached to the shaft of a servomotor (Aurora Scientific, Inc., Mod. 305b) and was in turn controlled by a computer. Percutaneous needle electrodes were inserted into the anterior compartment of the TA to stimulate the peroneal nerve. Optimal voltage (2 – 5 V) was determined using a series of tetanic contractions (150Hz, 0.1 ms pulse width, 400 ms train). Average peak tetanic force for each animal was calculated from the average of 5 contractions. To minimize muscle fatigue, 1-minute rest periods were taken between contraction cycles. Commercial muscle physiology software (Aurora Scientific, Inc., Ontario, Canada) was used to collect contractile force versus time data. At the conclusion of electrophysiological testing, all animals were euthanized through carbon dioxide inhalation in accordance with guidelines provided by the 2013 AVMA Panel on Euthanasia of Animals.

Tissue Histology and Image Analysis

VML treated TA muscles along with contralateral untreated TA muscles were harvested from young (n=12) and aged (n=8) and trimmed to remove fascia and tendon, then weighed. EDL muscles were also collected and weighed for comparison between groups. Muscles were rinsed in sterile PBS, dabbed dry, and then weighed. Harvested muscles were flash frozen in liquid nitrogen, and stored at -80°C for histological analysis. Tissue cross-sections were obtained with the aid of a cryostat (Leica BioSystems) and maintained at a temperature between -25 to -20°C and thickness of 7 µm. Cross-sectional slices were taken from the top third of the TA muscle, the region where the surgical VML injury was created. Prior to immunostaining, slides were permeabilized in 0.1% 10X Triton then rinsed in phosphate-buffered solution (PBS, pH 7.4). Slides were then blocked in PBS containing 4% goat serum and 0.05% sodium azide for 1h at room temperature prior to incubation in primary antibodies including mouse-anti-collagen I IgG (1:500, Sigma Aldrich), rabbit polyclonal anti-collagen III (1:1000, Abcam), and mouse-anti-myosin IgG_{2B} (MF-20, 1:10, Developmental Studies Hybridoma Bank, Iowa City, IA) for 4h at 4°C. Following PBS washes, slides were incubated in the appropriate corresponding Alexa Fluor 488 and 596 (1:500, Life Technologies) labeled secondary antibodies for 30 minutes at room temperature. Additional tissue sections were stained using commercially available Hematoxylin and Eosin (H&E) and Masson's Trichrome staining kits following manufacturers' guidelines (Sigma). All sections were mounted onto microscope slides and digitally imaged. Treated sections were evaluated for myofiber average cross-sectional area, formation, and organization as well as the formation and extent of VML site repair tissue for comparison to normal contralateral muscle tissue sections.

Collagen type I and III accumulation, a potential indicator of diffuse muscle fibrosis, in tissue regions away from the VML defect area was estimated using measures of collagen I (Sigma Aldrich, St. Louis, MO) and collagen III (Abcam, Cambridge, MA) immunoreactivity and guided by published methods (Bedair et al., 2007). Tissue immunoreactivity to collagen I was calculated from fluorescent microscopic images (200X) using image analysis software (Image J, NIH). All images were converted to 8-bit, gray scale, and uniformly thresholded across all samples to isolate collagen type I and III positive tissue regions. From these images the percent collagen type I and III positive tissue area was calculated for all tissue sections. Similar image analysis methods were used to compute muscle fiber cross-sectional area from magnified (200X) images. Images were converted to gray scale and thresholded to isolate individual muscle fibers. Fiber cross sectional area (um^2) for all fibers within an individual image (typically 50+ fibers) were calculated. Representative tissue sections collected from four animals per group were used for all calculations. Within each section, three separate regions were imaged and analyzed to calculate the average collagen type I and III percent area as well as average fiber cross-sectional area for each sample. A total of twelve images were analyzed for each treatment group. All image analysis was performed using a protocol with set parameters to process each image under the same conditions as well as eliminating any user bias.

Gene Expression

Real-time PCR was performed using the protocol reported by Washington et al (Washington et al., 2013). In preparation for RT-PCR, samples of muscle from the defect site of the right and left TA (n=4 animals/experimental group) were homogenized with Trizol (Ambion, Carlsbad, CA)/choloroform (Sigma Aldrich, St. Louis, MO). Samples were treated with DNase (Invitrogen, Carlsbad, CA) and then RNA was extracted using an RNAeasy kit (Invitrogen, Carlsbad, CA). After quantification of RNA with a plate reader (BioTek, Winooski, VT), RNA was converted to cDNA with a kit (Invitrogen, Carlsbad, CA). TAQMAN primers (Invitrogen, Carlsbad, CA) for MyoD, Collagen I, Collagen III, ratios of Collagen I to MyoD and Collaen I to Collagen III, and 18S rRNA housekeeping were used to quantify the expression of desired genes. Experimental sample group expressions were normalized to 18S rRNA and then referenced to the contralateral normal limb. Gene expression levels are reported as fold change using the $2^{-(\Delta\Delta Ct)}$ method.

Data Analysis

All data is presented as the mean ± standard error and was tested for normality using the Shapiro-Wilks Test. In addition, the data is also presented as mean ± standard deviation for reference in Table 1. The key outcome measures, muscle mass, peak tetanic force, percent collagen I / III areas, fiber cross-sectional area, and gene expression fold changes were analyzed with two-way ANOVA. *Post hoc* comparison testing was performed using Tukey's HSD test. All statistical analyses were performed using commercial statistical analysis software (JMP 11 Pro). Animal growth and defect size comparisons were made using a two-sided Students t-test. A standard $p<0.05$ level of significance was used for all statistical tests.

C. Results

Animal Body Weight

Both aged and young animals tolerated the surgery well and there were no signs of post-surgical infection. At one week post-implantation, all animals were fully ambulatory with no noticeable gait differences between groups. At the end of the study period, the young animals increased in mass by an average of 36% (312±3 vs. 424±7 g, $p<0.0001$) whereas the aged animals showed no significant change in body weight (**Figure 3)**. Also, by the end of the 12-week study period the difference in average mass between the aged and young groups had narrowed to 3%. The difference in mass between young and aged animals at the end of the study period was not statistically significant ($p=0.38$). Over the study period, the young animals put on mass at a growth rate of 9.3±0.4 g/week while the aged animals lost mass at a negative growth rate of 2.3±0.9 g/week. Age (young vs. old) was shown to have a statistically significant effect on animal growth rate ($p<0.05$).

TA Peak Tetanic Contractile Force

Tetanic contraction plots for all muscles were characterized by a sharp rise in force, followed by a plateau at the peak force, and then a return to a no force resting state (**Figure 4**). There was a main effect of treatment with VML injury to decrease peak contractile force, regardless of age ($p<0.0001$). Based on age, peak contractile force showed a decreasing trend moving from the young to the aged group ($p=0.08$). The young and aged VML groups produced 65% (2.16±0.07 N/kg vs. 1.39±0.11 N/kg, $p<0.0001$) and 59% (2.02±0.05 N/kg vs 1.19±0.12 N/kg, $p<0.0001$) of their respective contralateral normal muscles in response to VML injury (Figure 4A).

TA and EDL Muscle Mass

Upon gross examination the defect site was visible on TA muscle samples collected from both young and aged VML treated animals. Morphologically, there is a drastic difference in overall muscle appearance and size following VML injury (**Figure 5**). EDL muscles were not surgically disrupted during the TA VML defect treatment. Superficially, the EDL muscles associated with both the normal and VML treated TA muscles appeared normal, with the VML side EDL appearing slightly larger in size than the normal EDL. However, treated muscles were noticeably larger in size. There was a main effect of age and treatment to decrease TA muscle mass normalized to body weight, but no interaction effect was observed ($p<0.0001$, $p<0.0001$, **Figure 5**). Based on age, the young normal group compared to the aged normal group exhibited a 16% increase in normalized TA weight (1.61±0.04 mg/g vs. 1.37±0.05 mg/g, $p<0.0001$). A similar response to age was exhibited by the young VML group compared to the aged VML group with a 25% increase in normalized TA weight (1.27±0.02 mg/g vs. 0.99±0.02 mg/g, $p<0.0001$). In response to treatment within the young group, young normal TA mass was on average 24% heavier than young VML TA muscle (1.61±0.04 mg/g vs. 1.27±0.04 mg/g, $p<0.0001$, Figure 5). Similarly, within the aged group, aged normal TA mass on average was 32% heavier than aged VML TA muscle (1.37±0.05 mg/g vs 0.99±0.02 mg/g, $p<0.0001$, **Figure 5**).

Interestingly for the normalized EDL weights, the main effect of age decreased EDL mass whereas treatment was found to have an effect to increase EDL mass ($p<0.0001$ and $p<0.008$, respectively). However, an interaction effect was not observed. Based on age, the aged normal group compared to the young normal group exhibited a 25% decrease in normalized EDL weight (0.4±0.01 mg/g vs. 0.31±0.02 mg/g, $p<0.0001$, Figure 5). Similarly, the aged VML group

compared to the young VML group exhibited a 20% decrease in normalized EDL weight (0.43±0.01 mg/g vs. 0.35±0.02 mg/g, $p<0.0001$). Within the young group, there was an increase of 7% in EDL mass in response to VML injury (0.4±0.01 mg/g vs. 0.43±0.01 mg/g, $p<0.008$). Similarly, within the aged group in response to VML injury, there was an increase of 12% in EDL weight (0.31±0.02 mg/g vs. 0.35±0.02 mg/g, $p<0.008$).

VML Site Histology

Normal TA muscle histology for both young and aged groups (**Figure 6A/B, E/F**) was characterized by ordered circular bundles of MHC positive myofibers surrounded by collagen positive perimysium extending completely out to the anterior surface of the muscle. Twelve-weeks following VML injury, tissue sections prepared from young animals were characterized by collagen-dense repair layer located at the muscle surface. For the young VML muscles, MHC positive muscle fibers were observed up to the border of the repair layer, but did not extend into the repair tissue itself (**Figure 6C,D**). A similar distinct layer of collagen enriched repair tissue was not typically observed within the aged animal groups. Aged animal sections prepared from VML site tissue samples were frequently characterized by a region of diffuse collagen I deposition, as well as an overall appearance indicating increased MHC positive myofibers disorganization in the repair region (**Figure 6G,H**). While MHC positive muscle fibers were present they were generally less closely packed than in normal TA muscle sections.

Collagen Percent Area and Fiber Cross-sectional Area

Percent area positively stained for collagen I and III provided a quantitative measure of collagen content in the TA muscle regions outside the defect area (**Figure 7A,B**). There were no

changes in percent area stained for collagen I between young normal and VML muscles (7.6±1.6% vs. 8.3±1.0%, **Figure 7C**). Similarly, aged normal and VML treated muscles showed no changes in percent collagen I area (6.0±1.3% vs. 8.3±0.9%).

There was a main effect of VML injury to decrease collagen III percent area in the young group and increase in the aged group (p=0.011, **Figure 7D**). Collagen III area decreased by 13% (19.2%±0.2% and 16.8%±1.6%) for the young normal group compared to the young VML group. Collagen III percent area increased by 10% (20.8%±1.6% and 23%±3.0%) in the aged VML group compared to the aged normal group.

Similarly, age had a significant effect to decrease average TA fiber cross-sectional area (p=0.0365, **Figure 8C**). Average cross-sectional area decreased by 17% (2299±154 μm^2 and 1926±122 μm^2) for the aged normal group compared to the young normal group. For the aged VML group compared to the young VML group, fiber cross-sectional area decreased by 28% (2123±100 μm^2 and 1585±318 μm^2).

Gene Expression

There was a main effect of VML injury to decrease MyoD gene expression regardless of age in rat TA muscle (p=0.0231, **Figure 9A**). There was approximately a 13-fold decrease (1±0.58 and 0.08±0.04) in MyoD expression for the young VML group compared to the young normal group (**Figure 9A**). Comparing the aged VML group to the aged normal group, there was a 10-fold decrease in MyoD expression (1±0.21 vs. 0.1±0.03). For collagen I an interaction effect of age and VML injury was found to increase collagen I expression (p<0.003, **Figure 9B**). There were no differences found in collagen I expression within the young group, however, between the aged normal (1±0.33) and aged VML group (14±4), a 14-fold increase in collagen I

expression was found ($p<0.0009$) exhibiting a treatment effect. Between the young VML (1.55±0.14) and aged VML (14±4) groups, a 9-fold increase in collagen I expression in the aged VML group compared to the young VML group was observed ($p<0.0014$, **Figure 9B**). Regarding collagen III gene expression, there was an interaction effect of age and VML injury on expression levels ($p=0.0164$, **Figure 9C**). Post-hoc comparison revealed that the young VML group responded with a 2-fold increase in collagen III expression over the young normal group to VML injury (1±0.14 vs. 2.14±0.25, $p=0.034$). While not exhibiting significance for VML injury within the aged group, the aged VML group exhibited a significantly lower response compared to the young VML group with a 3.5-fold decrease in collagen III expression (2.14±0.25 vs. 0.61±0. 26, $p=0.013$, **Figure 9C**).

There was an interaction effect of age and treatment on the ratio of collagen I to MyoD ($p<0.011$). Post-hoc comparison revealed that the aged VML group responded with a 77-fold increase in the collagen I to MyoD ratio over the aged normal group to VML injury (0.95±0.25 vs 74.5±18.8, $p<0.0002$, **Figure 9D**). While not exhibiting significant differences for VML injury, the young group did exhibit significantly lower ratios of collagen I to MyoD compared to the aged VML group. Compared to the young VML group, the aged VML group exhibited a 2-fold increase in collagen I to MyoD ratio over the young VML group exhibiting an age effect (32.9±10.5 vs. 74.5±18.8, $p=0.0241$, **Figure 9D**).

Similarly, there was an interaction effect of age and VML injury on the ratio of collagen I to collagen III gene expression. Post-hoc comparison revealed that the aged VML group responded with an 8-fold increase in the collagen I to collagen III ratio over the aged normal group to VML injury (3.33±2.18 vs 27.8±5.21, $p<0.0001$, **Figure 9E**). While not exhibiting significant differences for VML injury, the young group did exhibit significantly lower ratios of

collagen I to collagen III compared to the aged VML group. Compared to the young VML group, the aged VML group exhibited a 38-fold increase in collagen I to collagen III ratio over the young VML group exhibiting an age effect (0.736±0.044 vs. 27.8±5.2, $p<0.0001$, **Figure 9D**).

D. Discussion

Extracellular matrix (ECM) remodeling is a critical aspect of successful resolution of the regenerative response in skeletal muscle. Skeletal muscle has a robust capacity to recover from injury in which there is not a significant loss of muscle mass. However, when there is traumatic muscle injury and more than 20% of the muscle is lost (VML), the regenerative process will not resolve and there will be a functional deficit (Corona et al., 2015; Garg, Corona, & Walters, 2014; Grasman, Zayas, Page, & Pins, 2015; Grogan et al., 2011b). Indicative of such injuries, it is expected for there to be nerve damage or even complete denervation of the muscle. Interestingly, even with temporary denervation of the muscle, it has been reported that muscle virtually recovers completely given that reinnervation is allowed to proceed unhindered (Lowrie, Krishnan, & Vrbova, 1982). However, even if innervation is preserved, unless there is transmission from the nerve to the muscle, degenerative changes have been observed in the muscle (Drachman, 1974; Gordon, Perry, Tuffery, & Vrbova, 1974). Though VML itself has been investigated, the response to VML in the aged population has yet to be explored. To our knowledge this is the first study to investigate how aging modulates the response to VML. The current study reports that aging muscle modifies the ECM response post-VML. The current study reports that the ECM response post-VML is modified in muscle from aged rats. It is of vital importance to understand how aged muscle response to VML so effective tissue engineering treatment strategies can be developed for this population to aid in skeletal muscle regeneration.

Sarcopenia is associated with decreased muscle mass and force deficits (Blough & Linderman, 2000; Carson, Lee, McClung, & Hand, 2002; Washington, Healey, Thompson, Lowe, & Carson, 2014). Our data is consistent with the literature in that we demonstrate an aged-dependent decrease in muscle mass in both the TA and the EDL. Additionally we demonstrate a trend for an age-dependent decrease in peak tetanic force. VML is associated with incomplete skeletal muscle regeneration. We are the first to demonstrate how aging affects skeletal muscle post-VML. 3 months post-VML skeletal muscle mass had only returned to 75% of the contralateral control limb regardless of age. Additionally, muscle force only returned to approximately 60% of the contralateral control limb 3 months post-VML in young and aged rats. Taken together, the similar muscle mass loss and force deficit between young and aged rats, suggests age does not exacerbate the post-VML functional response.

Skeletal muscle regeneration is a complex process that is characterized by inflammation, ECM remodeling, and myofiber growth (L. A. Brown et al., 2015; Washington et al., 2011). Impairments to any one of these processes can lead to incomplete skeletal muscle regeneration (Washington et al., 2011). The ECM plays an important role during skeletal muscle regeneration and acts as a functional link between skeletal muscle and bone. In skeletal muscle, the ECM is composed primarily of collagen I and collagen III (Kovanen, 2002). Previous research has demonstrated that 28 days post-VML there is a larger induction of collagen III compared to collagen I gene expression in young rats (Garg, Corona, et al., 2014). Our data is in agreement with this observation. We demonstrate an increase in collagen III gene expression with no change in collagen I gene expression 3 months post-VML. However, muscle from aged rats had a different response to VML. In muscle from aged rats, there was an increase in collagen I gene expression with no change in collagen III gene expression 3 months post-VML. The increased

expression of collagen I gene expression may contribute to the impaired regenerative response associated with aged muscle. The mdx mouse is a model for Duchenne muscular dystrophy (DMD). DMD is a degenerative muscular disease associated with accumulation of ECM material (Fadic, 2005). It has been demonstrated that collagen I is elevated during the lifespan in this animal (McDonald, Hebert, Kunz, Ralles, & McLoon, 2015). Collagen I inhibits muscular regeneration and supports the production of more collagen (Alexakis, Partridge, & Bou-Gharios, 2007). It has been demonstrated that type I collagen inhibits differentiation of C2C12 myoblasts (Alexakis et al., 2007). Differentiation of C2C12 myoblasts and myotubes is associated with a reduction in transcription of collagen I mRNA (Alexakis et al., 2007). The increased collagen I gene expression in aged muscle post-VML could negatively affect the ability of myoblasts to proliferate and differentiate and contribute to regeneration of aged skeletal muscle.

There was a difference in gene expression and protein expression, as determined by immunofluorescence, of collagen I and III post-VML. The collagen constitutive rate of synthesis is very low due to the long half-life (45-70 days) (Rucklidge, Milne, McGaw, Milne, & Robins, 1992). Therefore, changes in gene expression are a more accurate representation of the acute effects of VML. Collagen I and collagen III are produced from 1-2 different genes and coalesce into a triple-helix pro-collagen molecule. Further processing is done leading to the mature collagen molecule. Collagen is degraded by a family of enzymes referred to as matrix metalloproteinases (MMPs). There is evidence that muscle MMP expression differs as a function of age (Dennis et al., 2008; Robert et al., 1997; Sharples et al., 2012). Therefore, several factors can regulate collagen expression post-transcriptionally and explain the discrepancy between gene expression and protein expression.

In order to employ quantitative measures to assess myogenic capability and fibrosis, we calculated two ratios: the ratios of collagen I to MyoD and collagen I to collagen III expression. Due to MyoD's role in regulating muscle differentiation and myogenesis, exploring the proportion of a pro-regenerative marker to a fibrotic marker such as collagen I can assist in assessing the degree of regeneration. Satellite cells/myoblasts are critical to optimal skeletal muscle regeneration. Upon activation satellite cells proliferate and differentiate into myoblasts and begin expressing myoD (Hawke & Garry, 2001). In aged animals, satellite cell number as well as activation and proliferation are depressed (Barani, Durieux, Sabido, & Freyssenet, 2003; Kadi, Charifi, Denis, & Lexell, 2004). This would reduce the inherent regenerative capacity of aged skeletal muscle. VML was associated with an increase in the Collagen I to MyoD ratio regardless of age. However, there was a much larger increase in this ratio in the aged VML group. The increase is suggestive of a switch from myogenesis to fibrosis. There are numerous studies that demonstrate increased collagen deposition and fibrosis following VML with limited skeletal muscle regeneration (Corona et al., 2014a; Corona, Wu, et al., 2013; VanDusen, Syverud, Williams, Lee, & Larkin, 2014). In addition, increased intermuscular adipose tissue (IMAT) has been found to be related to decreased whole body glucose metabolism, particularly in individuals who have experienced an injury resulting in loss of muscle function (Addison, Marcus, Lastayo, & Ryan, 2014). However, the larger induction in the aged group suggests a more of an accelerated and more robust fibrotic response.

The ratio of collagen I to collagen III gene expression can provide insight in post-VML ECM composition changes. As skeletal muscle regeneration involves the migration of satellite cells and inflammatory cells through the ECM to the site of injury, the composition of the ECM is critical for an efficient regenerative response (Keely & Nain, 2015). Garg et. al. demonstrated

an impaired ability of satellite cells to migrate into the VML site (Aurora et al., 2014). Collagen I is a triple helix that polymerizes into fibrils that are stabilized by covalent cross-links contributing to its high tensile strength. Collagen III forms a looser network and is ideal for maintaining structural integrity and distensibility of the collagen network. Higher ratios of collagen I to collagen III is indicative of a stiffer scar region due to the mechanical properties of collagen I whereas a lower ratio could mean greater flexibility and a greater tensility during recovery leading to a more well-defined tissue reformation. 3 months post-VML, muscle from aged rats had an increased collagen I to collagen III ratio whereas in young rats there was no difference. This increased ratio has been demonstrated in skin from aged individuals (Goldspink, Fernandes, Williams, & Wells, 1994). Due to the long half-life of collagen molecules (45-70 days) this sustained response 3 months post-VML could lead to an altered collagen I and collagen III protein expression at a later time point. This would suggest an altered ECM composition that could ultimately affect the regenerative capacity of skeletal muscle. Aged tissue is associated with accumulation of advanced glycation end products (AGEs) and their receptors (RAGEs) (Odetti et al., 1998). AGEs have been demonstrated to induce expression of collagen I and inhibit collagen III expression (Tang et al., 2008). AGEs and RAGE signaling have been implicated in chronic inflammation which is a characteristic associated with aging (Chuah, Basir, Talib, Tie, & Nordin, 2013; Goto et al., 2004; Kislinger et al., 2001). Taken together, AGEs and chronic inflammation could alter the muscle microenvironment and contribute to the altered collagen III to collagen I ratio observed post-VML in aging skeletal muscle. As the functional outcome was similar between young and aged rats, the altered ECM response has implications for treatment strategies. This data implies treatment strategies in a young animal may not be effective in an older animal possibly due to the altered ECM composition post-VML.

E. Conclusion

In summary, this study is the first to describe the response to VML in muscle from aged rats. By characterizing and understanding the responses to VML by both young and aged muscle, various VML injury strategies can target areas that have a direct impact on myogenesis. With the age range of the young and aged rats being equivalent to the human age range of 9-18 years old and 54-63 years old, our aged model is effective in discriminating young vs aged responses to VML (Andreollo, Santos, Araujo, & Lopes, 2012). We clearly demonstrate an altered response to VML between young and aged rats. The aged rats had a much different ECM response as compared to the young rats. This would suggest that tissue engineering strategies aimed at treating VML should account for the age of the host.

References

Addison, O., Marcus, R. L., Lastayo, P. C., & Ryan, A. S. (2014). Intermuscular fat: a review of the consequences and causes. *Int J Endocrinol, 2014*, 309570. doi: 10.1155/2014/309570

Alexakis, C., Partridge, T., & Bou-Gharios, G. (2007). Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen overproduction. *Am J Physiol Cell Physiol, 293*(2), C661-669. doi: 10.1152/ajpcell.00061.2007

Andreollo, N. A., Santos, E. F., Araujo, M. R., & Lopes, L. R. (2012). Rat's age versus human's age: what is the relationship? *Arq Bras Cir Dig, 25*(1), 49-51.

Aurora, A., Garg, K., Corona, B. T., & Walters, T. J. (2014). Physical rehabilitation improves muscle function following volumetric muscle loss injury. *BMC Sports Sci Med Rehabil, 6*(1), 41. doi: 10.1186/2052-1847-6-41

Aurora, A., Roe, J. L., Corona, B. T., & Walters, T. J. (2015). An acellular biologic scaffold does not regenerate appreciable de novo muscle tissue in rat models of volumetric muscle loss injury. *Biomaterials, 67*, 393-407. doi: 10.1016/j.biomaterials.2015.07.040

Barani, A. E., Durieux, A. C., Sabido, O., & Freyssenet, D. (2003). Age-related changes in the mitotic and metabolic characteristics of muscle-derived cells. *J Appl Physiol (1985), 95*(5), 2089-2098. doi: 10.1152/japplphysiol.00437.2003

Bedair, H., Liu, T. T., Kaar, J. L., Badlani, S., Russell, A. J., Li, Y., & Huard, J. (2007). Matrix metalloproteinase-1 therapy improves muscle healing. *J Appl Physiol (1985), 102*(6), 2338-2345. doi: 10.1152/japplphysiol.00670.2006

Benoit, P. W., & Belt, W. D. (1970). Destruction and regeneration of skeletal muscle after treatment with a local anaesthetic, bupivacaine (Marcaine). *J Anat, 107*(Pt 3), 547-556.

Blough, E. R., & Linderman, J. K. (2000). Lack of skeletal muscle hypertrophy in very aged male Fischer 344 x Brown Norway rats. *J Appl Physiol (1985), 88*(4), 1265-1270.

Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., & Rando, T. A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science (New York, N.Y.), 317*(5839), 807-810. doi: 317/5839/807 [pii]

Brack, A. S., & Rando, T. A. (2007a). Intrinsic changes and extrinsic influences of myogenic stem cell function during aging. *Stem Cell Rev, 3*(3), 226-237.

Brack, A. S., & Rando, T. A. (2007b). Intrinsic changes and extrinsic influences of myogenic stem cell function during aging. *Stem cell reviews, 3*(3), 226-237. doi: SCR:3:3:226 [pii]

Brown, L. A., Lee, D. E., Patton, J. F., Perry, R. A., Jr., Brown, J. L., Baum, J. I., . . . Washington, T. A. (2015). Diet-induced obesity alters anabolic signalling in mice at the onset of skeletal muscle regeneration. *Acta Physiol (Oxf), 215*(1), 46-57. doi: 10.1111/apha.12537

Carson, J. A., Lee, W. J., McClung, J., & Hand, G. A. (2002). Steroid receptor concentration in aged rat hindlimb muscle: effect of anabolic steroid administration. *J Appl Physiol (1985), 93*(1), 242-250. doi: 10.1152/japplphysiol.01212.2001

Charge, S. B., & Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiological Reviews, 84*(1), 209-238. doi: 10.1152/physrev.00019.2003 [doi]

Chen, X. K., & Walters, T. J. (2013). Muscle-derived decellularised extracellular matrix improves functional recovery in a rat latissimus dorsi muscle defect model. *J Plast Reconstr Aesthet Surg, 66*(12), 1750-1758. doi: 10.1016/j.bjps.2013.07.037

Chuah, Y. K., Basir, R., Talib, H., Tie, T. H., & Nordin, N. (2013). Receptor for advanced glycation end products and its involvement in inflammatory diseases. *Int J Inflam, 2013*, 403460. doi: 10.1155/2013/403460

Conboy, I. M., & Rando, T. A. (2005). Aging, stem cells and tissue regeneration: lessons from muscle. *Cell Cycle, 4*(3), 407-410. doi: 10.4161/cc.4.3.1518

Corona, B. T., Garg, K., Ward, C. L., McDaniel, J. S., Walters, T. J., & Rathbone, C. R. (2013). Autologous minced muscle grafts: a tissue engineering therapy for the volumetric loss of skeletal muscle. *Am J Physiol Cell Physiol, 305*(7), C761-775. doi: 10.1152/ajpcell.00189.2013

Corona, B. T., Rivera, J. C., Owens, J. G., Wenke, J. C., & Rathbone, C. R. (2015). Volumetric muscle loss leads to permanent disability following extremity trauma. *J Rehabil Res Dev, 52*(7), 785-792. doi: 10.1682/JRRD.2014.07.0165

Corona, B. T., Ward, C. L., Baker, H. B., Walters, T. J., & Christ, G. J. (2014a). Implantation of in vitro tissue engineered muscle repair constructs and bladder acellular matrices partially restore in vivo skeletal muscle function in a rat model of volumetric muscle loss injury. *Tissue Eng Part A, 20*(3-4), 705-715. doi: 10.1089/ten.TEA.2012.0761

Corona, B. T., Ward, C. L., Baker, H. B., Walters, T. J., & Christ, G. J. (2014b). Implantation of in vitro tissue engineered muscle repair constructs and bladder acellular matrices partially restore in vivo skeletal muscle function in a rat model of volumetric muscle loss injury. *Tissue engineering.Part A, 20*(3-4), 705-715. doi: 10.1089/ten.TEA.2012.0761 [doi]

Corona, B. T., Wu, X., Ward, C. L., McDaniel, J. S., Rathbone, C. R., & Walters, T. J. (2013). The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. *Biomaterials, 34*(13), 3324-3335. doi: 10.1016/j.biomaterials.2013.01.061

Dennis, R. A., Przybyla, B., Gurley, C., Kortebein, P. M., Simpson, P., Sullivan, D. H., & Peterson, C. A. (2008). Aging alters gene expression of growth and remodeling factors in human skeletal muscle both at rest and in response to acute resistance exercise. *Physiol Genomics, 32*(3), 393-400. doi: 10.1152/physiolgenomics.00191.2007

Drachman, D. B. (1974). Trophic functions of the neuron. 3. Mechanisms of neurotrophic interactions. The role of acetylcholine as a neurotropic transmitter. *Ann N Y Acad Sci, 228*(0), 160-176.

Fadic, R. (2005). Cell surface and gene expression regulation molecules in dystrophinopathy: mdx vs. Duchenne. *Biol Res, 38*(4), 375-380.

Fischer, S., Soimaru, S., Hirsch, T., Kueckelhaus, M., Seitz, C., Lehnhardt, M., . . . Daigeler, A. (2015). Local tendon transfer for knee extensor mechanism reconstruction after soft tissue sarcoma resection. *J Plast Reconstr Aesthet Surg, 68*(5), 729-735. doi: 10.1016/j.bjps.2015.01.002

Freemont, A. J., & Hoyland, J. A. (2007). Morphology, mechanisms and pathology of musculoskeletal ageing. *The Journal of pathology, 211*(2), 252-259. doi: 10.1002/path.2097 [doi]

Garg, K., Corona, B. T., & Walters, T. J. (2014). Losartan administration reduces fibrosis but hinders functional recovery after volumetric muscle loss injury. *J Appl Physiol (1985), 117*(10), 1120-1131. doi: 10.1152/japplphysiol.00689.2014

Goldspink, G., Fernandes, K., Williams, P. E., & Wells, D. J. (1994). Age-related changes in collagen gene expression in the muscles of mdx dystrophic and normal mice. *Neuromuscul Disord, 4*(3), 183-191.

Gopinath, S. D., & Rando, T. A. (2008). Stem cell review series: aging of the skeletal muscle stem cell niche. *Aging Cell, 7*, 590-598.

Gordon, T., Perry, R., Tuffery, A. R., & Vrbova, G. G. G. (1974). Possible mechanisms determining synapse formation in developing skeletal muscles of the chick. *Cell Tissue Res, 155*(1), 13-25.

Goto, K., Mizutani, A., Shingu, C., Hasegawa, A., Hidaka, S., Ito, K., . . . Noguchi, T. (2004). [Colforsin daropate does not affect the cerebral blood-flow in cardiac surgery patients under cardiopulmonary bypass]. *Masui, 53*(4), 385-390.

Grasman, J. M., Zayas, M. J., Page, R. L., & Pins, G. D. (2015). Biomimetic scaffolds for regeneration of volumetric muscle loss in skeletal muscle injuries. *Acta Biomater, 25*, 2-15. doi: 10.1016/j.actbio.2015.07.038

Grogan, B. F., Hsu, J. R., & Skeletal Trauma Research, C. (2011). Volumetric muscle loss. *The Journal of the American Academy of Orthopaedic Surgeons, 19 Suppl 1*, S35-37. doi: 19/suppl_1/S35 [pii]

Grounds, M. D. (1998). Age-associated changes in the response of skeletal muscle cells to exercise and regeneration. *Ann N Y Acad Sci, 854*, 78-91.

Hawke, T. J., & Garry, D. J. (2001). Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol (1985), 91*(2), 534-551.

Hill, M., Wernig, A., & Goldspink, G. (2003). Muscle satellite (stem) cell activation during local tissue injury and repair. *J Anat, 203*(1), 89-99.

Hou, L., Gong, C., & Zhu, Y. (2016). In vitro construction and in vivo regeneration of esophageal bilamellar muscle tissue. *J Biomater Appl, 30*(9), 1373-1384. doi: 10.1177/0885328215627585

Hurd, S. A., Bhatti, N. M., Walker, A. M., Kasukonis, B. M., & Wolchok, J. C. (2015). Development of a biological scaffold engineered using the extracellular matrix secreted by skeletal muscle cells. *Biomaterials, 49*, 9-17.

Kadi, F., Charifi, N., Denis, C., & Lexell, J. (2004). Satellite cells and myonuclei in young and elderly women and men. *Muscle Nerve, 29*(1), 120-127. doi: 10.1002/mus.10510

Kasukonis, B., Kim, J., Washington, T., & Wolchok, J. (2016). Development of an infusion bioreactor for the accelerated preparation of decellularized skeletal muscle scaffolds. *Biotechnol Prog*. doi: 10.1002/btpr.2257

Keely, P., & Nain, A. (2015). Capturing relevant extracellular matrices for investigating cell migration. *F1000Res, 4*. doi: 10.12688/f1000research.6623.1

Kislinger, T., Tanji, N., Wendt, T., Qu, W., Lu, Y., Ferran, L. J., Jr., . . . Schmidt, A. M. (2001). Receptor for advanced glycation end products mediates inflammation and enhanced expression of tissue factor in vasculature of diabetic apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol, 21*(6), 905-910.

Kovanen, V. (2002). Intramuscular extracellular matrix: complex environment of muscle cells. *Exerc Sport Sci Rev, 30*(1), 20-25.

Kuang, S., Gillespie, M. A., & Rudnicki, M. A. (2008). Niche regulation of muscle satellite cell self-renewal and differentiation. *Cell stem cell, 2*(1), 22-31. doi: 10.1016/j.stem.2007.12.012 [doi]

Lowrie, M. B., Krishnan, S., & Vrbova, G. (1982). Recovery of slow and fast muscles following nerve injury during early post-natal development in the rat. *J Physiol, 331*, 51-66.

Mann, C. J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A. L., & Munoz-Canoves, P. (2011). Aberrant repair and fibrosis development in skeletal muscle. *Skeletal muscle, 1*(1), 21-5040-5041-5021. doi: 10.1186/2044-5040-1-21 [doi]

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol, 9*, 493-495.

McDonald, A. A., Hebert, S. L., Kunz, M. D., Ralles, S. J., & McLoon, L. K. (2015). Disease course in mdx:utrophin+/- mice: comparison of three mouse models of Duchenne muscular dystrophy. *Physiol Rep, 3*(4). doi: 10.14814/phy2.12391

Merritt, E. K., Cannon, M. V., Hammers, D. W., Le, L. N., Gokhale, R., Sarathy, A., . . . Farrar, R. P. (2010). Repair of traumatic skeletal muscle injury with bone-marrow-derived mesenchymal stem cells seeded on extracellular matrix. *Tissue engineering.Part A, 16*(9), 2871-2881. doi: 10.1089/ten.TEA.2009.0826 [doi]

Nikolaou, P. K., Macdonald, B. L., Glisson, R. R., Seaber, A. V., & Garrett, W. E., Jr. (1987). Biomechanical and histological evaluation of muscle after controlled strain injury. *Am J Sports Med, 15*(1), 9-14.

Odetti, P., Aragno, I., Garibaldi, S., Valentini, S., Pronzato, M. A., & Rolandi, R. (1998). Role of advanced glycation end products in aging collagen. A scanning force microscope study. *Gerontology, 44*(4), 187-191.

Robert, V., Besse, S., Sabri, A., Silvestre, J. S., Assayag, P., Nguyen, V. T., . . . Delcayre, C. (1997). Differential regulation of matrix metalloproteinases associated with aging and hypertension in the rat heart. *Lab Invest, 76*(5), 729-738.

Rucklidge, G. J., Milne, G., McGaw, B. A., Milne, E., & Robins, S. P. (1992). Turnover rates of different collagen types measured by isotope ratio mass spectrometry. *Biochim Biophys Acta, 1156*(1), 57-61.

Schwartz, J. T., Jr., Brumback, R. J., Lakatos, R., Poka, A., Bathon, G. H., & Burgess, A. R. (1989). Acute compartment syndrome of the thigh. A spectrum of injury. *J Bone Joint Surg Am, 71*(3), 392-400.

Sharples, A. P., Player, D. J., Martin, N. R., Mudera, V., Stewart, C. E., & Lewis, M. P. (2012). Modelling in vivo skeletal muscle ageing in vitro using three-dimensional bioengineered constructs. *Aging Cell, 11*(6), 986-995. doi: 10.1111/j.1474-9726.2012.00869.x

Snow, M. H. (1977). The effects of aging on satellite cells in skeletal muscles of mice and rats. *Cell Tissue Res, 185*(3), 399-408.

Tang, M., Zhong, M., Shang, Y., Lin, H., Deng, J., Jiang, H., . . . Zhang, W. (2008). Differential regulation of collagen types I and III expression in cardiac fibroblasts by AGEs through TRB3/MAPK signaling pathway. *Cell Mol Life Sci, 65*(18), 2924-2932. doi: 10.1007/s00018-008-8255-3

Terada, N., Takayama, S., Yamada, H., & Seki, T. (2001). Muscle repair after a transsection injury with development of a gap: an experimental study in rats. *Scand J Plast Reconstr Surg Hand Surg, 35*(3), 233-238.

Tidball, J. G. (2005). Inflammatory processes in muscle injury and repair. *American journal of physiology.Regulatory, integrative and comparative physiology, 288*(2), R345-353. doi: 288/2/R345 [pii]

Tidball, J. G., & Villalta, S. A. (2010). Regulatory interactions between muscle and the immune system during muscle regeneration. *American journal of physiology.Regulatory, integrative and comparative physiology, 298*(5), R1173-1187. doi: 10.1152/ajpregu.00735.2009 [doi]

Turner, N. J., Badylak, J. S., Weber, D. J., & Badylak, S. F. (2012). Biologic scaffold remodeling in a dog model of complex musculoskeletal injury. *J Surg Res, 176*(2), 490-502. doi: 10.1016/j.jss.2011.11.1029

Turner, N. J., & Badylak, S. F. (2012). Regeneration of skeletal muscle. *Cell and tissue research, 347*(3), 759-774. doi: 10.1007/s00441-011-1185-7 [doi]

VanDusen, K. W., Syverud, B. C., Williams, M. L., Lee, J. D., & Larkin, L. M. (2014). Engineered skeletal muscle units for repair of volumetric muscle loss in the tibialis anterior muscle of a rat. *Tissue Eng Part A, 20*(21-22), 2920-2930. doi: 10.1089/ten.TEA.2014.0060

Via, A. G., Oliva, F., Spoliti, M., & Maffulli, N. (2015). Acute compartment syndrome. *Muscles Ligaments Tendons J, 5*(1), 18-22.

Vinciguerra, M., Musaro, A., & and Rosenthal, N. (2010). Regulation of muscle atrophy in aging and disease. *Adv Exp Med Biol*(694), 211-233.

Warren, G. L., Summan, M., Gao, X., Chapman, R., Hulderman, T., & Simeonova, P. P. (2007). Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models. *J Physiol, 582*(Pt 2), 825-841. doi: jphysiol.2007.132373 [pii]

Washington, T. A., Brown, L., Smith, D. A., Davis, G., Baum, J., & Bottje, W. (2013). Monocarboxylate transporter expression at the onset of skeletal muscle regeneration. *Physiol Rep, 1*(4), e00075. doi: 10.1002/phy2.75 [doi]

Washington, T. A., Healey, J. M., Thompson, R. W., Lowe, L. L., & Carson, J. A. (2014). Lactate dehydrogenase regulation in aged skeletal muscle: Regulation by anabolic steroids and functional overload. *Exp Gerontol, 57*, 66-74. doi: 10.1016/j.exger.2014.05.003

Washington, T. A., White, J. P., Davis, J. M., Wilson, L. B., Lowe, L. L., Sato, S., & Carson, J. A. (2011). Skeletal muscle mass recovery from atrophy in IL-6 knockout mice. *Acta Physiol (Oxf), 202*(4), 657-669. doi: 10.1111/j.1748-1716.2011.02281.x

Wu, X., Corona, B. T., Chen, X., & Walters, T. J. (2012). A Standardized Rat Model of Volumetric Muscle Loss Injury for the Development of Tissue Engineering Therapies *BioResearch Open Access, 1*(6), 280-290.

Zhang, J., Hu, Z. Q., Turner, N. J., Teng, S. F., Cheng, W. Y., Zhou, H. Y., . . . Badylak, S. F. (2016). Perfusion-decellularized skeletal muscle as a three-dimensional scaffold with a vascular network template. *Biomaterials, 89*, 114-126. doi: 10.1016/j.biomaterials.2016.02.040

Figure Legends

Figure 1. Surgical VMLs are gross defect creations in the left TA muscle of the rat. Surgical site was cleaned and disinfected prior to a vertical incision that was made to expose underlying muscle (A). An 8mm biopsy punch was used to create a defect to a depth of 2mm and the amount of muscle removed is approximately equivalent to 20% of the TA weight (B). Surgical defects were created in the rat left hindlimb and the contralateral limbs were untouched, serving as internal controls. The surgical site was then sutured with 5-0 absorbable sutures (C).

Figure 2. Correlation of animal weight and TA weight. (A) Aged animal (n=8) terminal weight vs TA weight. (B) Young animal (n=12) terminal weight vs TA weight. Linear regression analysis performed for all cases provided information to approximate TA weight given animal weight. From the TA weight, the size of the defect (20% of TA weight) can be determined.

Figure 3. Young and aged Fisher 344 rat weight (g) prior to surgery and at the end of the study period. The younger animals experienced significant weight gain post-surgery, whereas the aged animals lost weight. Values are mean+SEM. * represents $p<0.05$ Student's t-test for the young group.

Figure 4. Peak tetanic force measurements of Young and Aged rats via electrophysiology. (A) Percutaneous needle electrodes were placed in the anterior compartment of the TA to stimulate the peroneal nerve. The knee was stabilized and the foot was attached to a muscle lever system for isometric measurements. (B) In order to account for normal growth over the 3-month period post-surgery, contractile force measurements were normalized to body weight (N/kg). The contralateral normal limbs for both age groups yielded similar values as well as showing a similar decrease in force production after VML injury. Values are mean+SEM. * represents $p<0.05$ two-way ANOVA for normal vs VML injury.

Figure 5. Comparison of TA and EDL muscle weight between normal and VML injury groups between Young (n=12) and Aged (n=8) rats. Gross morphological images of young normal (A), young VML (B), aged normal (C), and aged VML (D) TA muscles. At the end of the 3 month study period, terminal weights for TA (E) and EDL (F) of both young and aged normal and VML groups were measured. Values are mean+SEM. # represents $p<0.05$ two-way ANOVA for young vs aged. * represents $p<0.05$ two-way ANOVA for normal vs VML injury.

Figure 6. Comparison of histological cross-sections of young normal (A, B) and VML (C, D), aged normal (E, F) and VML (G, H) TA muscles. The inset depicting the TA shows from where the histological cross-sections were taken. Masson's trichrome stained sections imaged at 100X magnification (A, C, E, G) depict collagen in blue/purple colored regions whereas muscle is shown in red/pink. Sections stained for collagen I (green) and myosin heavy chain (MHC, red)

at 100X magnification (B, D, F, H). * indicates the collagen I rich scar region in the young muscles treated with VML (D). ** indicates the VML region for the aged group which is characterized by diffuse collagen I deposition and disorganized myofiber bundles compared to the young VML group (H). Masson's trichrome was stained using a commercially available kit. Primary antibodies used were anti-collagen I mouse IgG (1:500) and anti-myosin mouse IgG_{2B} (1:10). Secondary antibodies used were goat anti-mouse IgG (H+L) Alexa Fluor 488 and goat anti-mouse IgG_{2B} Alexa Fluor 594.

Figure 7. Representative TA cross-sections immunostained for Collagen I (A, green) and Collagen III (B, red). Stained images were then analyzed in ImageJ software to calculate % area of the stains as a function of cross-sectional area. No difference was found for collagen I area (C) but a decrease in collagen III area was found for the young group after VML injury (D). On the contrary, the aged group had an increase in collagen III following VML. Primary antibody stains used were anti-collagen I mouse IgG (1:500) and anti-collagen III rabbit polyclonal (1:500). Secondary antibody stains used were goat anti-mouse IgG (H+L) Alexa Fluor 488 and goat anti-rabbit IgG (H+L) Alexa Fluor 594. * represents $p<0.05$ two-way ANOVA for normal vs VML injury.

Figure 8. Rat TA muscle cross-sections of young (A) and aged (B) rats stained with H&E and imaged at 200X magnification. Average fiber cross-sectional areas were calculated using ImageJ software for young normal, young VML, aged normal, and aged VML groups (n=3, C). It was observed that fiber cross-sectional area decreases significantly with age, regardless of VML injury. # represents $p<0.05$ two-way ANOVA for young vs aged.

Figure 9. Normal gene expression compared to expression in response to VML. Expression of MyoD (A), Collagen I (B), Collagen III (C), ratio of Collagen I to MyoD (D), and ratio of Collagen I to Collagen III (E) are presented as fold-changes obtained using RT-PCR. Except for MyoD, which experienced only a treatment effect, interaction effects of age and treatment were found for all other genes/ratios tested. Values are mean+SEM. * represents $p<0.05$ two-way ANOVA for normal vs VML injury and # represents $p<0.05$ two-way ANOVA for young vs aged. *Post-hoc* analysis was performed upon finding significant interaction effects.

Figures

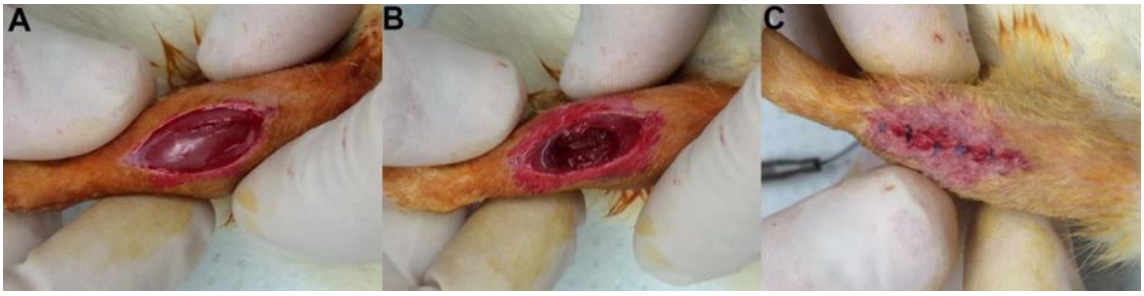

Figure 1

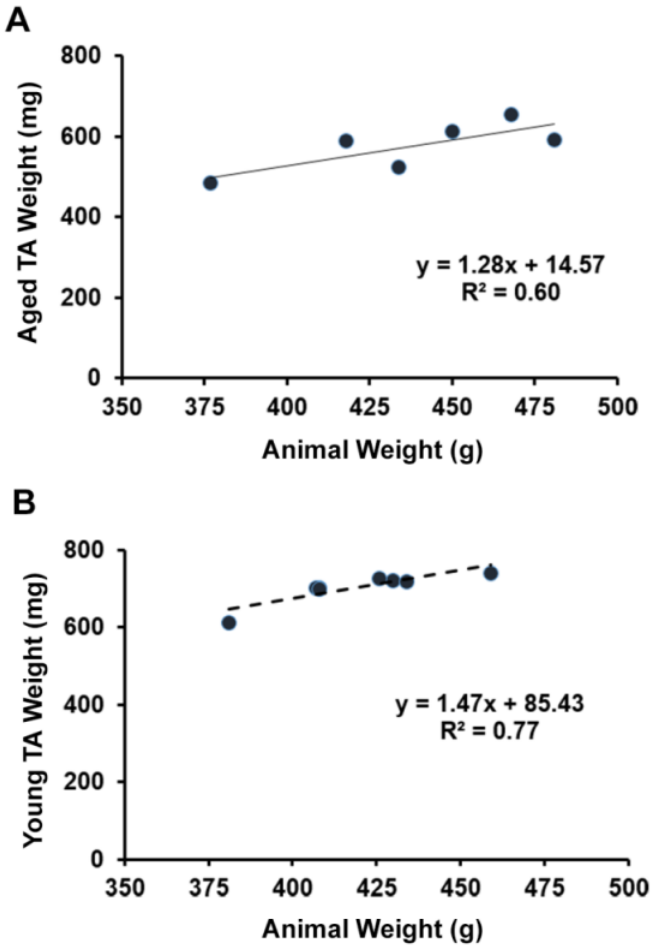

Figure 2

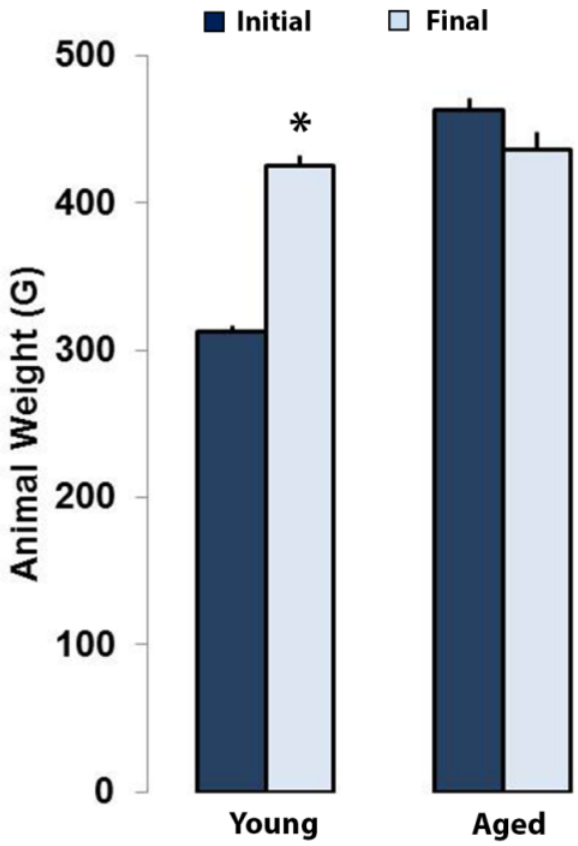

Figure 3

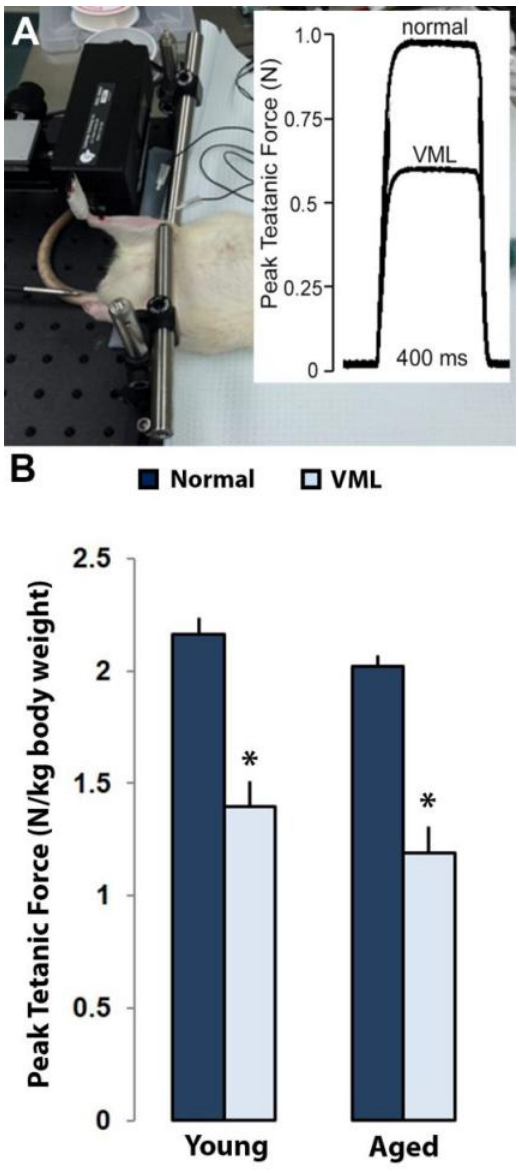

Figure 4

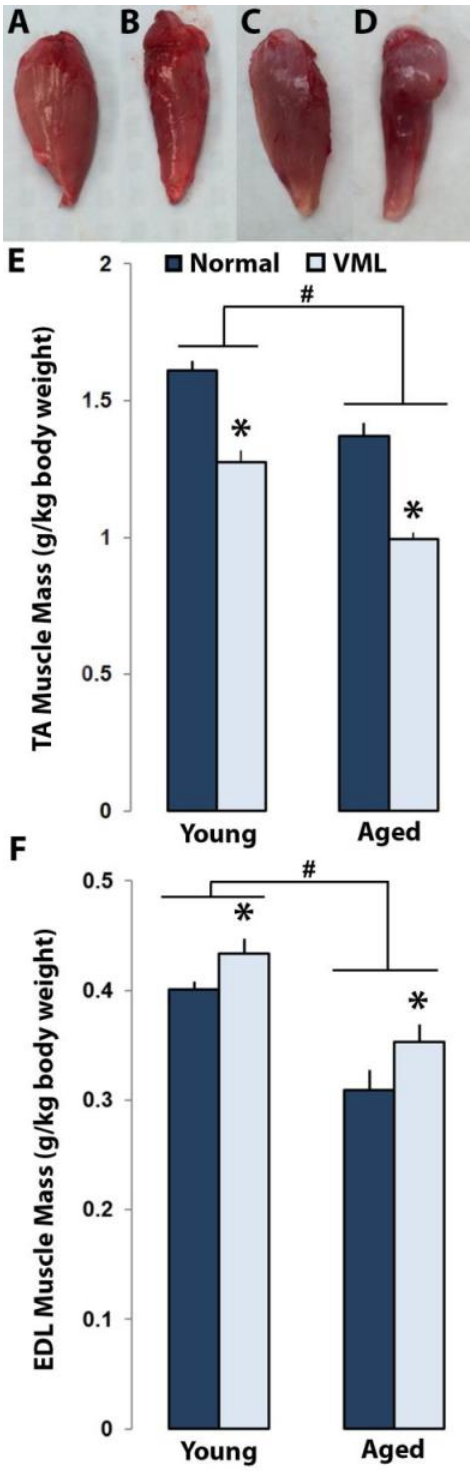

Figure 5

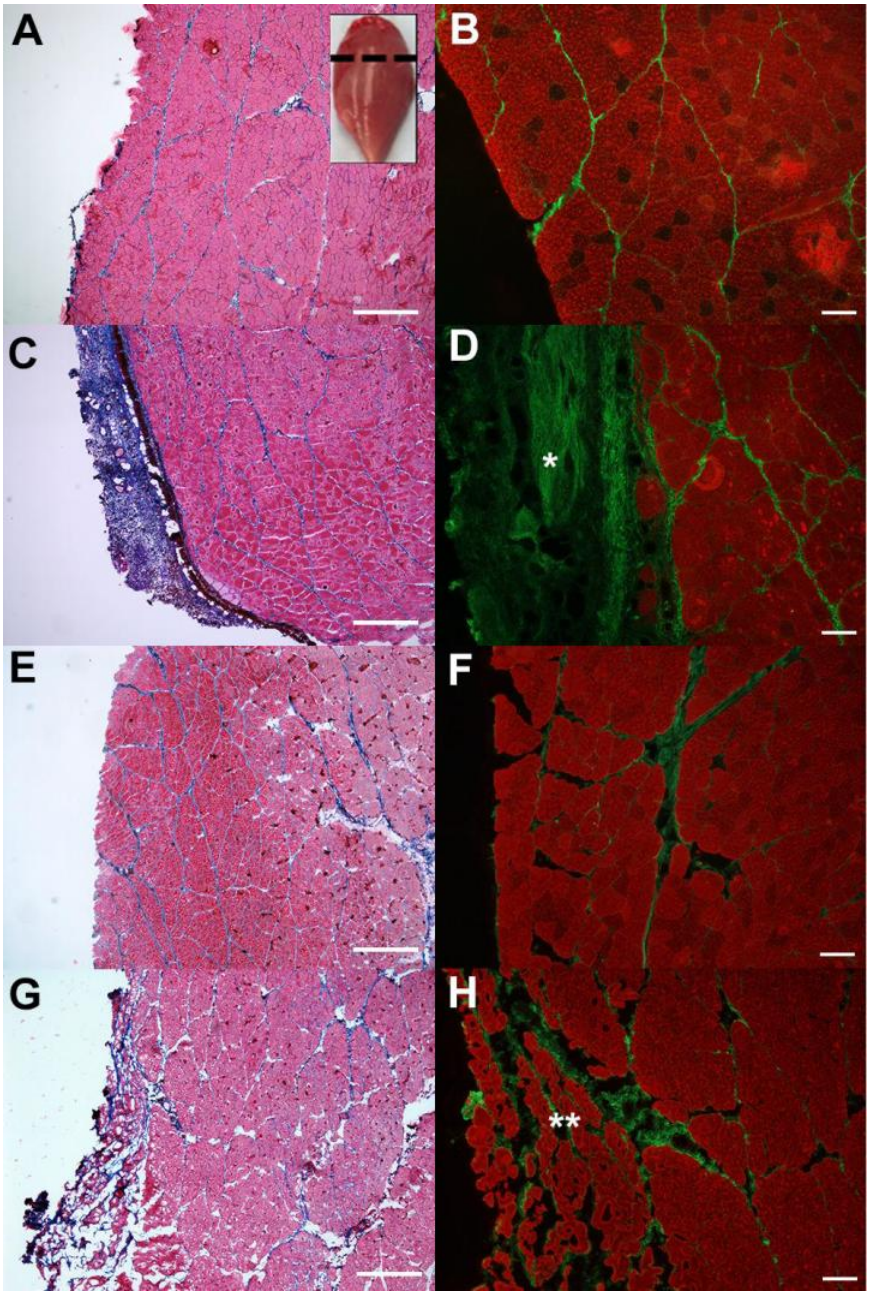

Figure 6

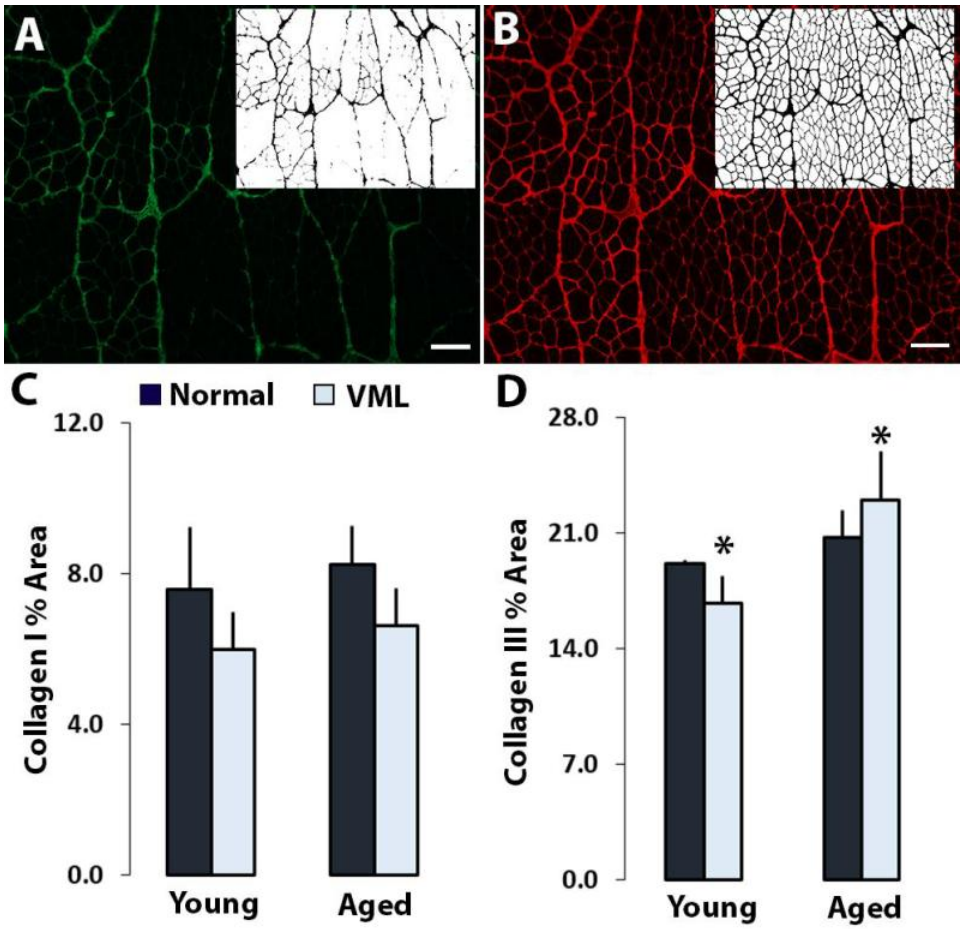

Figure 7

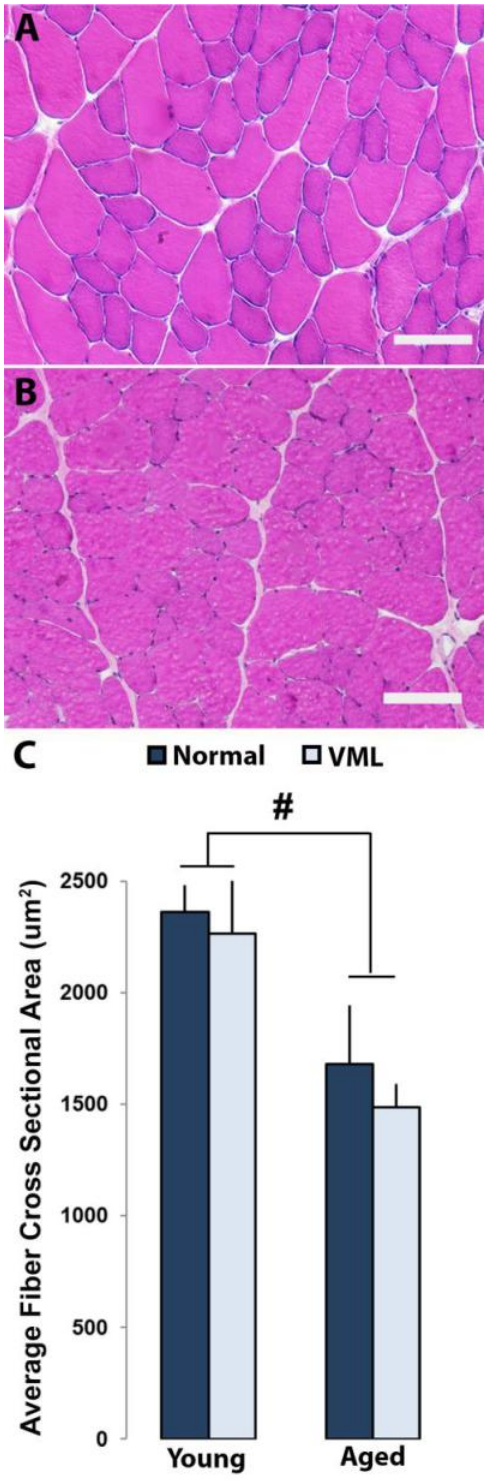

Figure 8

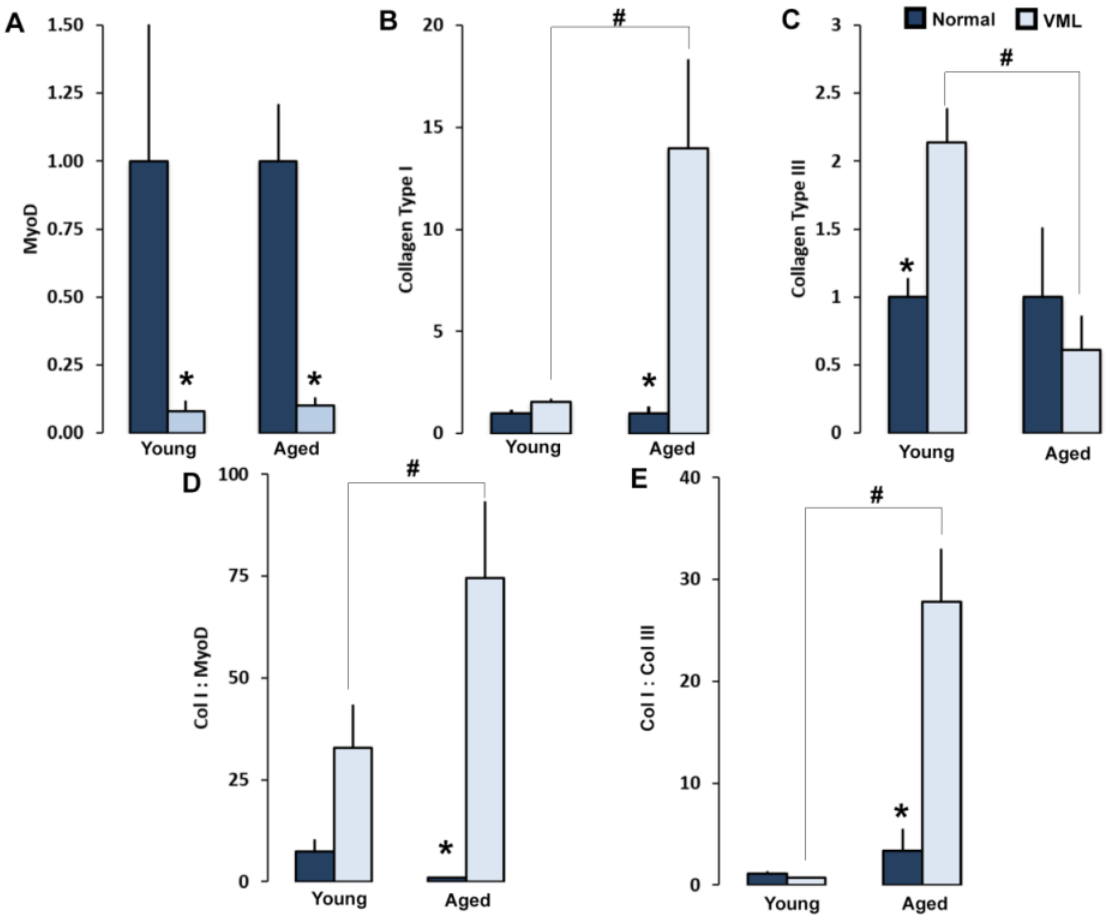

Figure 9

Chapter 3

Regenerative Repair of Volumetric Muscle Loss Injury is Sensitive to Age

Submitted as an original article by:

John T. Kim[1], Benjamin Kasukonis[1], Grady Dunlap[1], Richard Perry[2], Tyrone Washington[2], and Jeffrey C. Wolchok PhD[1]

[1] Department of Biomedical Engineering, University of Arkansas, Fayetteville, Arkansas, 72701

[2] Department of Health, Human Performance, and Recreation, College of Education and Health Professions, University of Arkansas, Fayetteville, AR, Fayetteville, Arkansas 72701

Corresponding Author:

Jeffrey C. Wolchok, PhD

Department of Biomedical Engineering

University of Arkansas

John A. White, Jr. Engineering Hall, Suite 120, Fayetteville, Arkansas

Phone: 479-575-4667

Email: jwolchok@uark.edu

Abstract

In this study, the influence of age on effectiveness of regenerative repair for the treatment of volumetric muscle loss (VML) injury was explored. Tibialis anterior (TA) VML injuries were repaired in both 3-month and 18-month aged animal models (Fischer 344 rat) using allogeneic decellularized skeletal muscle (DSM) scaffolds supplemented with autologous minced muscle paste (MM). Within the 3-month animal group, TA peak contractile force was significantly improved (79% of normal) in response to DSM+MM repair. However, within the 18-month animal group, muscle force following repair (57% of normal) was not significantly different from unrepaired VML controls (59% of normal). Within the 3-month animal group, repair with DSM+MM generally reduced scarring at the site of VML repair, whereas scarring and a loss of contractile tissue was notable at the site of repair within the 18-month group. Within 3-month animals, expression of myogenic genes (MyoD, MyoG), ECM genes (Col I, Col III, TGF-ß), and key wound healing genes (TNF-α and IL-1ß) were increased. Alternatively, expression was unchanged across all genes examined within the 18-month animal group. The findings suggest that a decline in regenerative capacity and increased fibrosis with age may present an obstacle to regenerative medicine strategies targeting VML injury.

Keywords: orthopedics, musculoskeletal, animal model, tibialis anterior, VML

Impact Statement

This study compared the recovery following VML injury repair using a combination of minced muscle paste and decellularized muscle ECM carrier in both a younger (3 months) and older (18 months) rat population. Currently, VML repair research is being conducted with the young patient population in mind but our group is the first to look at the effects of age on the efficacy of VML repair. Our findings highlight the importance of considering age-related changes in response to VML when developing repair strategies targeting an elderly patient population.

A. Introduction

Volumetric muscle loss (VML) injury is commonly characterized as the bulk loss (>20%) of muscle tissue resulting in incomplete or aberrant muscle regeneration. Current soft tissue repair techniques and traditional rehabilitation have not been able to reverse the pathological changes that occur following VML injury. To reverse the pathobiology of VML injury, the use of regenerative medicine strategies to recapitulate the cellular and structural cues during regeneration have shown efficacy in animal models (Corona, Henderson, Ward, & Greising, 2017; Corona, Wu, et al., 2013; J. Dziki et al., 2016; Garg, Ward, et al., 2014; Goldman & Corona, 2017; Goldman, Henderson, Walters, & Corona, 2018; Li, Willett, Uhrig, Guldberg, & Warren, 2014; Nakayama et al., 2018). Among these strategies, our group has demonstrated that the implantation of a decellularized skeletal muscle scaffold (DSM) co-delivered with a minced muscle (MM) autograft paste is capable of recovering half of the contractile force lost to VML injury (B. Kasukonis, J. Kim, L. Brown, et al., 2016). Other groups have shown similar improvements in muscle recovery using various combinations of scaffolds with and without myogenic cell sources (Corona et al., 2012; Corona et al., 2014b; Dziki, Sicari, Wolf, Cramer, & Badylak, 2016; Merritt, Cannon, et al., 2010a; Valentin et al., 2010). When taken as a whole, our findings and others suggest that the use of regenerative medicine may have therapeutic potential as a treatment for VML injury.

The examination of regenerative medicine strategies for VML repair has been limited thus far to young animal models (Gilbert-Honick, Ginn, et al., 2018; Goldman et al., 2018; Nakayama et al., 2018; Quarta et al., 2017). It remains unclear whether the VML findings observed in these animal models will be predictive of clinical performance in the elderly. It is reasonable to speculate that the age of the VML injured patient could influence the effectiveness

of any VML treatment. Specifically, the decrease in myogenic capacity that occurs with age could present a significant hurdle to muscle regeneration in an older patient population (Conboy & Rando, 2005; Domingues-Faria, Vasson, Goncalves-Mendes, Boirie, & Walrand, 2016). Traditionally, battlefield injuries to the extremities have been the primary clinical target motivating the development of VML repair strategies (Mase et al., 2010; B. D. Owens et al., 2008; J. G. Owens et al., 2011). Regarding civilian trauma, VML injuries are not as well-documented or tracked due to the lack of a standard therapy (Corona, Wenke, & Ward, 2016). Despite this fact, there are approximately 150,000 documented cases of open fractures (type III open fracture) each year in the United States, with the majority of cases associated with the concomitant loss of the surrounding soft tissues (Court-Brown & McBirnie, 1995). Moreover, there is likely a high proportion of the VML-afflicted patient population (both civilian and military) suffering from chronic pain and disability due to a lack of a standard of care for VML. We suggest that an improved understanding of the relationship between age and VML treatment performance would provide valuable insights that could help elucidate the appropriateness of current VML repair strategies for a broader patient population.

As a first step towards reaching this level of understanding, our group has developed an aged rat VML model and demonstrated that age impacts muscle healing following VML injury (Kim et al., 2016). Specifically, our findings suggested that extracellular matrix (ECM) gene expression and protein deposition is significantly altered following VML injury in an aged model (18 months), resulting in a more fibrotic environment when compared to non-aged controls (3 months). While changes in muscle wound healing with age were identified and appeared consistent with findings in humans (Parker et al., 2017), the study did not examine whether these age-related differences in VML healing would in turn influence the response to regenerative

repair. The purpose of this study was to examine whether the improved functional outcomes that have been observed following VML repair in non-aged models are sensitive to aging. Specifically, this study was designed to test the hypothesis that age will dampen the beneficial effects of regenerative treatment. This study presents those performance results along with comparisons to non-aged animal model findings.

B. Methods

DSM Scaffold Preparation

To prepare DSM samples for implantation, whole tibialis anterior (TA) muscle was decellularized using a previously reported (B. M. Kasukonis et al., 2016) infusion system (**Figure 1**). TA muscles were harvested from rats (Sprague Dawley) that had been euthanized at the completion of an unrelated study. The TA samples were infused overnight (approximately 12 hours) with 1% SDS at a flow rate of 5mL/hr. Following infusion, samples were incubated overnight in a DNAse solution (1kU/ml DNAse in 10mM Tris-HCL buffer; 2.5mM $MgCL_2$ + 0.5mM $CaCl_2$) and then incubated (8 hours) in a 1X penicillin/streptomycin solution to reduce the risk of infection. Samples were rinsed thoroughly (a total of six 24-hour wash steps) in PBS between each preparation step. Following completion of decellularization, the DNA concentration of representative DSM implants (n=4) was measured with the aid of a commercial quantification kit (Qubit, Fisher Scientific). DSM implants were lyophilized and stored at -20ºC until utilized for implantation.

Volumetric Muscle Loss Injury and Minced Muscle

In vivo VML studies were performed using male Fischer 344 rats with ages of either 3 months (20% of the median lifespan) or 18 months (75% of their median lifespan). The 3-month rats were purchased commercially from Envigo (Indianapolis, IN) and the 18-month rats were obtained through the National Institute on Aging (Bethesda, MD). Surgical procedures and implant preparation methods were performed in accordance with protocols approved by the University of Arkansas IACUC (protocol #14044) and as described previously (B. Kasukonis, J. Kim, L. Brown, et al., 2016). Briefly, VML defects (8mmx3mm) were created in the tibialis anterior using a biopsy punch (**Figure 2A,B**). 25% of the removed defect plugs were retained for use as MM autografts following hand mincing of the removed muscle (**Figure 2C**). DSM scaffolds were fully coated in the minced muscle paste and then implanted into the VML defect site (**Figure 2D-F)**. Animals were randomly assigned to one of two treatment groups (n=8/treatment group): unrepaired VML defect (VML), or VML defect repaired with DSM scaffold supplemented with MM (DSM+MM). All contralateral limbs were left untreated to serve as comparative controls. All animals were housed for a 12-week recovery period.

In vivo Contractile Torque Measurement

Peak tetanic contractions produced by the TA muscle were measured isometrically ***in vivo*** using procedures as previously described by Corona and familiar to our group (Corona, Garg, et al., 2013; B. Kasukonis, J. Kim, L. Brown, et al., 2016; Kim et al., 2016). Briefly, the lower limb of the animals were stabilized at 90º of knee flexion via a custom alignment jig. The foot was secured to the lever arm of a dual-mode lever system (Aurora Scientific). Prior to nerve stimulation, distal tenotomies of the extensor digitorum longus (EDL) and extensor hallucis

longus (EHL) was performed to isolate the force contribution of the TA muscle. Percutaneous needle electrodes were inserted into the anterior compartment of the tibialis anterior muscle and the peroneal nerve was stimulated via physiological stimulator (Grass; S88). Optimal voltage (2-5 V) was determined using a series of tetanic contractions (150Hz, 0.1 ms pulse width, 400ms train (3-month animals) and 200ms train (18-month animals)). The mean isometric torque values (N cm) were calculated from 5 consecutive contractions with 1 minute rest periods in between each contraction. The weight normalized (N cm/kg) and contralateral untreated limb normalized (% normal) force data is reported in the results section.

Tissue Histology

VML and DSM+MM treated TA muscles along with contralateral untreated TA muscles were harvested and trimmed to remove fascia, rinsed in sterile PBS, dabbed dry, and then weighed. Harvested muscles were flash frozen in isopentane (2-methylbutane) chilled in liquid nitrogen, and stored at -80°C. Tissue cross-sections (8µm) of the repair site were obtained via cryostat (Leica BioSystems) maintained at a temperature between -25 and -20°C. Prior to immunostaining, slides were permeabilized in 0.1% 100X triton and then rinsed in phosphate-buffered solution (PBS, pH 7.4). Sections were blocked in PBS containing 4% goat serum and 0.05% sodium azide for 1h at room temperature prior to incubation in primary antibodies including mouse-anti-collagen I IgG (1:500, Sigma-Aldrich), rabbit polyclonal anti-collagen III (1:1000, Abcam), and mouse-anti-myosin IgG_{2B} (MF-20, 1:10, Developmental Studies Hybridoma Bank) for 2h at room temperature. Following PBS washes, slides were incubated in the appropriate fluorescently labeled secondary antibodies (AlexaFluor, 1:500, Life Technologies) for 30 minutes at room temperature. Additional tissue sections were stained using a Masson's Trichrome kit following the manufacturer's guidelines (Sigma-Aldrich).

Image Analysis

Collagen type I accumulation within VML injured and DSM+MM repaired muscle tissue was quantified via immunostaining for collagen I (Sigma-Aldrich) with myosin heavy chain (Developmental Studies Hybridoma Bank) as a counter stain. Representative tissue sections collected from four animals per group were used for all calculations. A total of twelve sections (4 animals x 3 sections/animal, each section separated by approximately 500-700μm) were imaged (100X) at the site of injury or repair. All images were converted to 8-bit greyscale and a uniform threshold was applied to isolate collagen type I positive tissue regions within each section. Tissue immunoreactivity to collagen I as a percentage of total tissue area (% Col I) was calculated using image analysis software (ImageJ, NIH) using established techniques familiar to our group (Kim et al., 2016). Similar image analysis methods were used to compute collagen III percent area (% Col III) and fiber cross-sectional area (μm^2) using magnified (100X) collagen III images. Collagen III immunostained images were converted to greyscale and thresholded to isolate the borders of individual muscle fibers (typically 300+ fibers / field of view).

Gene Expression

RT-PCR was performed using the protocol reported by Washington et al (Washington et al., 2013). In prep for RT-PCR, TA muscle tissue (~30mg) was collected from the site of injury/treatment (n=4 animals/group) and homogenized with Trizol (Ambion)/choloroform (Sigma Aldrich). Homogenized samples were treated with DNase (Invitrogen) and then RNA was extracted using an RNeasy kit (Invitrogen). After quantification of RNA with UV spectrophotometry (BioTek), cDNA was reverse transcribed from 1μg of total RNA (Invitrogen). TAQMAN primers (Invitrogen) for Collagen I, Collagen III, TGF-β1, IGF-1, Pax7, MyoD,

MyoG, IL-1β, TNF-α, and 18S rRNA housekeeping were used to quantify the expression of desired genes. 18s rRNA expression was consistent between all groups. Experimental sample group expressions were normalized to 18S rRNA and then referenced to the contralateral normal limb. Gene expression levels are reported as fold change using the $2^{-(\Delta\Delta Ct)}$ method.

Statistical Analysis

Data is presented as the mean ± standard deviation and was tested for normality using the Shapiro-Wilks Test. All dependent variables (animal growth; TA and EDL muscle mass; peak tetanic force, %Col I and %Col III, fiber cross-sectional area) were analyzed using a two-way (age and treatment) ANOVA. Post-hoc comparisons were performed using Tukey's test and if present, interactions effects were reported. Pre-planned comparison between 3-month and 18-month repair groups were conducted by Student's t-test. Chi-square analysis was performed to compare fiber area distributions. All analyses were performed using commercial statistical analysis software (JMP 13). A standard $p<0.05$ level of significance was used for all statistical tests.

C. Results

Animal Growth

All animals tolerated the surgery well and post-surgical complications requiring euthanasia were absent. After one week post-implantation, all animals were ambulatory with no discernable gait differences between treatment groups. There was a significant effect with age to decrease animal growth ($p<0.0001$). On average, the 3-month animals gained 101±17g by the completion of the 12-week study period. Alternatively, the 18-month animals lost an average of

28±29g by the end of the study period. Although age significantly affected animal growth, we did not detect a significant effect on growth with treatment. Both the 3-month VML (8.8±1.7g/week) and DSM+MM (8.1±1.2g/week) groups gained weight at a similar rate. Similarly the 18-month VML and DSM+MM groups lost weight at comparable rates of -2.6±2.9 g/week and -1.7±0.80 g/week respectively.

Tibialis Anterior Appearance and Mass

Compared to normal TA muscle morphology, untreated VML and DSM+MM repaired muscles collected from both 3-month and 18-month aged groups appeared atrophied when compared to contralateral normals and were characterized by a distinct defect visible at the site of VML injury or repair (**Figure 3A and B**). An interaction effect of age and treatment (p=0.03) was detected for TA mass. 18-month TA samples across both VML and DSM+MM groups were on average 23% and 34% smaller than their 3-month counterparts, respectively. The 3-month VML and DSM+MM muscle masses were 81.7±6.3% and 87.2±8.8% of their normal contralateral TA mass respectively **(Figure 3C**) while their 18-month counterparts were 75.1±7.8% and 72.1±5.4% of the normal contralateral mass, respectively (**Figure 3C**). Within the 3-month age group, DSM+MM repair lead to a 7% increase in muscle mass while repair in the 18-month group resulted in a 5% decrease in muscle mass.

Peak Contractile Torque

In-situ muscle force recordings were characterized by a rapid rise in torque, followed by a stable tetanic peak torque plateau, and a similarly sharp descent to a resting state. Similar to the outcomes observed for muscle mass, a significant interaction between age and treatment was

detected within the torque data (**Figure 3D**, *p*=0.02). Mean VML and DSM+MM peak torque values (percent contralateral normal) for the 3-month age group were on average 20% (5.6±1.2 Ncm/kg vs. 4.6±1.1 Ncm/kg) and 47% greater (6.8±1.0 Ncm/kg vs. 4.2±0.6 Ncm/kg) respectively when compared to their 18-month counterpart torque values. In addition, the peak torque in response to DSM+MM repair within the 3-month age group was significantly increased when compared to the 3-month VML group (6.8±1.0 Ncm/kg vs. 5.6±1.2 Ncm/kg). Specifically, 3-month mean peak torque values reached 79.9±11.2% of normal in response to DSM+MM repair (6.8±1.0 Ncm/kg vs. 9.0±1.3 Ncm/kg); a 45% recovery in peak torque when compared to 3-month VML values (5.6±1.2 Ncm/kg vs. 9.0±1.3 Ncm/kg). Alternatively, within the 18-month age group, DSM+MM repair had a negligible effect on peak torque (57±8.7% of normal, 4.2±0.6 Ncm/kg) when compared to VML values (58.6±14% of normal,4.6±1.1 Ncm/kg). However, a significant decrease in peak torque was detected in both 18-month VML and repair groups when compared to the 3-month DSM+MM repair group.

Weight Change and Peak Torque Correlation

When the relationship between peak TA torque and weight change (% of initial weight) was examined within the 3-month age group, we did not detect a correlation (**Figure 4B**, r^2=-0.07). However, within the 18-month animal group there was a modest positive correlation (**Figure 4C**, r^2=0.49) between peak torque and body weight change. The findings suggest that increased weight loss during the 12-week recovery period was associated with decreased peak torque outcomes for the 18-month age group (**Figure 4)**. The absence of a correlation between weight change and peak torque in the 3-month age group possibly suggests that peak torque becomes more sensitive to changes in body weight with age progression.

Gene Expression

Among the transcription factors related to myogenesis (**Figure 5)**, MyoD (p=0.006) and MyoG (p=0.03) were expressed significantly higher in the 3-month repair group compared to the 18-month repair group by approximately 3-fold (4.52±1.43 fold change vs. 1.48±0.29 fold change) and 8-fold (16.3±9.8 fold change vs. 2.0±1.6 fold change), respectively. No difference was detected in Pax7 expression as both 3-month and 18-month groups exhibited muted expression (1.9±1.1 fold change vs. 1.7±0.3 fold change).

Significantly higher levels of expression of collagen I (p=0.044) and collagen III (p=0.047) were found in the 3-month repair group compared to the 18-month group. Collagen I was expressed approximately 35-fold higher in the 3-month repair group compared to the 18-month repair group (88.6±51.2 fold change vs. 2.5±1.9 fold change). Collagen III was expressed 600-fold higher in the 3-month repair group compared to 18-month repair (86.0±39.19 fold change vs. 0.1±0.0). Notably when examining the ratio of collagen I to collagen III gene expression, the 3-month repair group exhibited a significantly lower collagen I to collagen III ratio (p=0.009) of 1.1±0.2 whereas the 18-month repair group responded with a ratio of 17.5±8.6. TGF-ß expression was also significantly higher in the 3-month group compared to the 18-month repair group (10.5±4.9 fold change vs. 1.4±0.4 fold change, p=0.01).

Significant differences were detected in the expression of IL-1ß and TNF-α between the 3-month and 18-month repair groups. The 3-month repair group was found to express IL-1ß at levels 8.5-fold higher than its 18-month counterpart (9.7±5.9 fold change vs. 1.1±0.4 fold change, p=0.02). TNF-α was expressed approximately 13-fold higher in the 3-month repair group compared to the 18-month repair group (13.7±10.8 fold change vs. 1.2±1.2 fold change, p=0.04). Expression of the growth factor IGF-1, though not significantly different between the

two age groups (p=0.08), was expressed at elevated levels in the 3-month repair group compared to the 18-month repair group (5.3±3.9 fold change vs. 1.3±0.5 fold change).

VML Site Histology

Both 3-month and 18-month normal TA histology was characterized by tightly apposed bundles of MHC positive myofibers extending to the anterior surface of the muscle (**Figure 6A,B "Normal"**). When compared to normal muscle, both 3-month and 18-month VML wound site tissue was characterized by a fibrotic surface region and elevated collagen staining extending into the muscle body (**Figure 6A,B "VML"**). The repair site region was notably shallower than the initial VML defect for both 3-month and 18-month groups, typically extending a few hundred microns into the muscle, suggesting atrophy of the muscle at the injury site. Following DSM+MM repair, 18-month animal muscle was similarly characterized by a collagen enriched repair region, with the highest density near the anterior surface of the repair site (**Figure 6B "Repair").** Within the collagen dense region, there was diffuse and disorganized MHC positive tissue with increasing fiber disorganization towards the surface of the muscle. 3-month DSM+MM repair site tissue was notably devoid of collagen repair tissue and while the muscle as a whole was atrophied, the collagen staining at the fiber and bundle level appeared qualitatively similar to controls (**Figure 6A "Repair")**. There was no evidence of immature fibers with centrally located nuclei for both 3-month and 18-month groups, suggesting either negligible new fiber formation, or very likely, resolution and maturation of any new fiber by the 12-week time point.

Collagen I and III Area

A main effect of age (p=0.0009) was detected for collagen I % area. Within the 3-month age group, collagen I % area measurements were similar across all treatment groups. Specifically, 3-month normal, VML, and DSM+MM repair tissue contained collagen I % areas of 6.2±2.0%, 6.5±1.6%, and 5.4±1.2%, respectively (Figure 7A-C,G). Alternatively, exhibiting collagen I % area measurements of approximately 2-3 folds higher than their 3-month counterparts, 18-month normal, VML, and DSM+MM repair tissues comprised on average 10.4±4.5%, 17.9±4.5%, and 17.6+9.3% of the total tissue area, respectively (Figure 7D-F,G).

In contrast to collagen I % area, a main effect of treatment was detected for collagen III % area (p=0.0454). 3-month normal, VML, and DSM+MM repair groups were comprised of collagen III % areas of 19.8±3.5%, 21.4±4.4%, and 16.9±1.2%, respectively (Figure 8C). 18-month normal, VML, and DSM+MM groups were comprised of collagen III % areas of 17.7±1.9%, 22.2±1.5%, and 18.4±0.9%, respectively (Figure 8C). Within both 3-month and 18-month age groups, a significant decrease in collagen III % area was detected between VML and DSM+MM repair groups.

Fiber Cross-Sectional Area

The mean muscle fiber area at the site of injury (VML) or repair (DSM+MM) was not significantly different for either the 3-month or 18-month age groups (**Figure 9**). No effect of treatment was detected nor were fiber sizes significantly different between the age groups. Mean fiber diameter for both 3-month (2085±245μm^2) and 18-month (1993±400μm^2) DSM+MM repair tissue was within 10% of VML injured values for both 3-month (2123±200μm^2) and 18-month (2146±89μm^2) groups. No differences were detected between the distribution of fiber

sizes for 3-month and 18-month DSM+MM repair groups. A summary of the key outcome measures (mean ± standard deviation) is provided in **Table 1**.

D. Discussion

In this *in vivo* study, we observed significant effects with age on the response to regenerative repair of VML injury as well as significant interactions between age and repair. Specifically, the repair of VML defects using a combination of decellularized skeletal muscle scaffolds and minced muscle paste recovered approximately half (45%) of the force lost to VML injury when examined in the 3-month old rat model (3 months = 12.5% of median lifespan), but was ineffective (recovery=-1%) when tested in the 18 month old model (18 months = 75% of median lifespan). The age-related decrease in regenerative therapy effectiveness is meaningful when one considers the broad age range of patients that could benefit from VML treatment strategies. Overall, the results of this study suggest that the successful translation of VML injury treatment strategies to the clinic may be compromised by patient age.

Generally, a sustained inflammatory response has been associated with chronic inflammatory myopathies and disorders such as muscular dystrophy and myositis, which have been shown to lead to excessive fibrosis and stagnation of the normal regenerative program (Loell & Lundberg, 2011; Porter et al., 2002). However, recent studies have also demonstrated important roles for inflammatory cytokines such as TNF-α and IL-1ß in the regulation of the regenerative process. TNF-α attracts satellite cells to the injury site as well as promotes satellite cell proliferation and myoblast differentiation via activation of NF-κß and p38 MAPK signaling, respectively (S. E. Chen, Jin, & Li, 2007; Peterson, Bakkar, & Guttridge, 2011). These findings are consistent with our observations in functional recovery and gene expression data where there

was significant upregulation of TNF-α and MyoD in the 3-month repair group as well as a significant increase in peak torque values compared to the older 18-month repair group. The low expression of TNF-α in the 18-month repair group could be suggestive of low- or in-activation of p38 MAPK activity which has been demonstrated to downregulate the expression of muscle differentiation markers such as MyoD and MyoG (S. E. Chen et al., 2007; Zhan, Jin, Chen, Reecy, & Li, 2007). Similar to TNF-α, IL-1ß is able to stimulate migration, proliferation, and differentiation of mybolasts, albeit indirectly, by stimulating the secretion of IL-6 from skeletal muscle cells (Luo, Hershko, Robb, Wray, & Hasselgren, 2003; Munoz-Canoves, Scheele, Pedersen, & Serrano, 2013; Porter et al., 2002). Gene expression in the 3-month repair group compared to the 18-month group mirrors the observations of TNF-α expression and may speak to the aberrant inflammatory response and muscle homeostatic maintenance of the older 18-month rat group (Joe et al., 2010; Kami & Senba, 1998; C. Zhang et al., 2013).

In addition to an overall reduction in the pool of available satellite cells within aged muscle there is also a phenotypic shift of satellite cells from a myogenic to fibrogenic lineage (B. M. Carlson, 1995; Grounds, 1998b). This shift in lineage is attributed to dysfunction in satellite cell proliferative capacity (Conboy et al., 2005) and divergence towards fibrogenicity (Brack et al., 2007b), which is largely influenced by soluble factors in the aging tissue microenvironment. The age-related shift in satellite cell behavior towards a pro-fibrotic phenotype is consistent with our observed results. Histological and quantitative %NCT measurements revealed regions of increased collagen deposition within and around the area of injury and repair when compared to the non-aged model repair site. Additionally, the heightened pro-fibrotic response is capable of further impairing muscle fiber regeneration and force recovery via deregulation of satellite cells driven by interactions with fibrotic ECM (Alnaqeeb, Al Zaid, & Goldspink, 1984; Mann et al.,

2011a; Serrano et al., 2011). Furthermore, it has been demonstrated that increased collagen deposition can decrease growth factor diffusion within the repair site and hinder satellite cell migration, leading to incomplete regeneration at the site of injury (Hindi, Shin, Ogura, Li, & Kumar, 2013). The composition of the ECM at the site of repair can also have a negative effect on muscle regeneration (Lehto, Sims, & Bailey, 1985). Within tissue collected at the site of repair from the 18-month rats, the ratio of collagen I to collagen III gene expression was markedly elevated with approximately a 17:1 ratio while tissue from the 3-month rats exhibited a ratio closer to 1:1. Unusually high ratios of collagen I to collagen III have been associated with hypertrophic scar formation in wound healing as well as decreased sensitivity to growth factors (Garner, Karmiol, Rodriguez, Smith, & Phan, 1993).

The regenerative medicine strategy that was used as the test bed for the examination of age sensitivity to VML repair was similar in concept to strategies under examination by other groups. Specifically, we utilized a combination of allogeneic decellularized skeletal muscle scaffolds and autologous minced muscle paste to restore the architectural and cellular cues lost to VML injury. The regenerative capacity of minced muscle fragments has been demonstrated previously by Studitsky and Carlson in their respective seminal reports (B. M. Carlson, 1968; Studitsky, 1964) but not until recently has minced muscle been incorporated into tissue engineering strategies aimed at VML repair (Corona, Garg, et al., 2013; Goldman et al., 2018; B. Kasukonis, J. Kim, L. Brown, et al., 2016; Ward et al., 2015). Additionally, the effects of minced muscle transplantation on regeneration are comparable in its overall goal compared to the delivery of pure populations of progenitor cells explored by others (Merritt, Cannon, et al., 2010a; Quarta et al., 2017). Similarly, the ECM scaffolds used in this study are comparable to those which have been examined extensively by other groups (Corona et al., 2014b; Gentile et

al., 2014; Merritt, Cannon, et al., 2010a; Merritt, Hammers, et al., 2010; Sicari et al., 2012; Turner et al., 2012; Urciuolo et al., 2018). This is likely due to the consistency of the overwhelming inflammatory and fibrotic response to VML injury leading to functional deficits, regardless of scaffold composition or host species of origin. The broad similarities between our test bed and others, provides confidence that the age-related differences we observed in this study are relevant to more recent cell- and tissue-based regenerative medicine strategies under investigation by research teams (Gilbert-Honick, Iyer, et al., 2018; Goldman et al., 2018; Qiu et al., 2018; Quarta et al., 2017) and would suggest that similar age-related deficiencies would exist for these strategies.

Lastly, a limitation to this study that deserves discussion is the lack of an early-post implantation time point that would provide the data needed to identify specific wound healing events responsible for the age-related differences in functional outcome. The 12-week endpoint examined in this study, while appropriate for the examination of functional recovery, misses the key pro-myogenic milestones that occur during the first few weeks of muscle healing (Tidball, 2005b, 2017). In particular, the examination of acute time points may reveal key age-sensitive differences in cellular and soluble factor (cytokines and growth factors) responses that occur following regenerative VML repair (Tidball, 1995, 2011). The gene expression results measured in this study demonstrate that pro-myogenic transcription pathways and cytokines are elevated at 12 weeks in the non-aged animal model suggesting ongoing regenerative processes, but it remains unclear whether the muted myogenic response observed at 12 weeks in the aged animal was similarly muted during early healing. Early time points could reveal whether all or only specific key myogenic milestones are impaired within the VML repair site of aging muscle, and could provide valuable insights that would inform age-targeted therapeutic strategies.

E. Conclusion

In summary, this study is the first to demonstrate an age-dependent response to repair of a VML injury utilizing a tissue-engineered therapy. Notably, the repair of VML defects using a combination of tissue scaffolds and minced muscle paste recovered approximately half of the force lost to VML injury when examined in a 3-month rat model, but was ineffective when tested in the 18-month model. The 18-month animal model was characterized by an increase in scarring at the site of repair and a reduction in the expression of key myogenic genes. When taken together the findings suggest that a decline in regenerative capacity with age may present an obstacle to regenerative medicine strategies targeting VML injury.

References

Dziki, J., Badylak, S., Yabroudi, M., Sicari, B., Ambrosio, F., Stearns, K., Turner, N., Wyse, A., Boninger, M.L., Brown, E.H.P., andRubin, J.P. An acellular biologic scaffold treatment for volumetric muscle loss: results of a 13-patient cohort study. NPJ Regenerative medicine 1, 16008, 2016.

Corona, B.T., Wu, X., Ward, C.L., McDaniel, J.S., Rathbone, C.R., andWalters, T.J. The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. Biomaterials 34, 3324, 2013.

Goldman, S.M., Henderson, B.E.P., Walters, T.J., andCorona, B.T. Co-delivery of a laminin-111 supplemented hyaluronic acid based hydrogel with minced muscle graft in the treatment of volumetric muscle loss injury. PloS one 13, e0191245, 2018.

Goldman, S.M., andCorona, B.T. Co-delivery of micronized urinary bladder matrix damps regenerative capacity of minced muscle grafts in the treatment of volumetric muscle loss injuries. PloS one 12, e0186593, 2017.

Corona, B.T., Henderson, B.E., Ward, C.L., andGreising, S.M. Contribution of minced muscle graft progenitor cells to muscle fiber formation after volumetric muscle loss injury in wild-type and immune deficient mice. Physiol Rep 52017.

Garg, K., Ward, C.L., Rathbone, C.R., andCorona, B.T. Transplantation of devitalized muscle scaffolds is insufficient for appreciable de novo muscle fiber regeneration after volumetric muscle loss injury. Cell and tissue research 358, 857, 2014.

Li, M.T., Willett, N.J., Uhrig, B.A., Guldberg, R.E., andWarren, G.L. Functional analysis of limb recovery following autograft treatment of volumetric muscle loss in the quadriceps femoris. Journal of biomechanics 47, 2013, 2014.

Nakayama, K.H., Alcazar, C., Yang, G., Quarta, M., Paine, P., Doan, L., Davies, A., Rando, T.A., andHuang, N.F. Rehabilitative exercise and spatially patterned nanofibrillar scaffolds enhance vascularization and innervation following volumetric muscle loss. NPJ Regenerative medicine 3, 16, 2018.

Kasukonis, B., Kim, J., Brown, L., Jones, J., Ahmadi, S., Washington, T., andWolchok, J. Codelivery of Infusion Decellularized Skeletal Muscle with Minced Muscle Autografts Improved Recovery from Volumetric Muscle Loss Injury in a Rat Model. Tissue engineering Part A 22, 1151, 2016.

Dziki, J.L., Sicari, B.M., Wolf, M.T., Cramer, M.C., andBadylak, S.F. Immunomodulation and Mobilization of Progenitor Cells by Extracellular Matrix Bioscaffolds for Volumetric Muscle Loss Treatment. Tissue engineering Part A 22, 1129, 2016.

Valentin, J.E., Turner, N.J., Gilbert, T.W., andBadylak, S.F. Functional skeletal muscle formation with a biologic scaffold. Biomaterials 31, 7475, 2010.

Corona, B.T., Machingal, M.A., Criswell, T., Vadhavkar, M., Dannahower, A.C., Bergman, C., Zhao, W., andChrist, G.J. Further development of a tissue engineered muscle repair construct in vitro for enhanced functional recovery following implantation in vivo in a murine model of volumetric muscle loss injury. Tissue engineering Part A 18, 1213, 2012.

Corona, B.T., Ward, C.L., Baker, H.B., Walters, T.J., andChrist, G.J. Implantation of in vitro tissue engineered muscle repair constructs and bladder acellular matrices partially restore in vivo skeletal muscle function in a rat model of volumetric muscle loss injury. Tissue engineering Part A 20, 705, 2014.

Merritt, E.K., Cannon, M.V., Hammers, D.W., Le, L.N., Gokhale, R., Sarathy, A., Song, T.J., Tierney, M.T., Suggs, L.J., Walters, T.J., andFarrar, R.P. Repair of traumatic skeletal muscle injury with bone-marrow-derived mesenchymal stem cells seeded on extracellular matrix. Tissue engineering Part A 16, 2871, 2010.

Quarta, M., Cromie, M., Chacon, R., Blonigan, J., Garcia, V., Akimenko, I., Hamer, M., Paine, P., Stok, M., Shrager, J.B., andRando, T.A. Bioengineered constructs combined with exercise enhance stem cell-mediated treatment of volumetric muscle loss. Nature communications 8, 15613, 2017.

Gilbert-Honick, J., Ginn, B., Zhang, Y., Salehi, S., Wagner, K.R., Mao, H.Q., andGrayson, W.L. Adipose-derived Stem/Stromal Cells on Electrospun Fibrin Microfiber Bundles Enable Moderate Muscle Reconstruction in a Volumetric Muscle Loss Model. Cell transplantation, 963689718805370, 2018.

Domingues-Faria, C., Vasson, M.P., Goncalves-Mendes, N., Boirie, Y., andWalrand, S. Skeletal muscle regeneration and impact of aging and nutrition. Ageing research reviews 26, 22, 2016.

Conboy, I.M., andRando, T.A. Aging, stem cells and tissue regeneration: lessons from muscle. Cell cycle 4, 407, 2005.

Owens, J.G., Blair, J.A., Patzkowski, J.C., Blanck, R.V., Hsu, J.R., andSkeletal Trauma Research, C. Return to running and sports participation after limb salvage. The Journal of trauma 71, S120, 2011.

Mase, V.J., Jr., Hsu, J.R., Wolf, S.E., Wenke, J.C., Baer, D.G., Owens, J., Badylak, S.F., andWalters, T.J. Clinical application of an acellular biologic scaffold for surgical repair of a large, traumatic quadriceps femoris muscle defect. Orthopedics 33, 511, 2010.

Owens, B.D., Kragh, J.F., Jr., Wenke, J.C., Macaitis, J., Wade, C.E., andHolcomb, J.B. Combat wounds in operation Iraqi Freedom and operation Enduring Freedom. The Journal of trauma 64, 295, 2008.

Corona, B.T., Wenke, J.C., andWard, C.L. Pathophysiology of Volumetric Muscle Loss Injury. Cells, tissues, organs 202, 180, 2016.

Court-Brown, C.M., andMcBirnie, J. The epidemiology of tibial fractures. The Journal of bone and joint surgery British volume 77, 417, 1995.

Kim, J.T., Kasukonis, B.M., Brown, L.A., Washington, T.A., andWolchok, J.C. Recovery from volumetric muscle loss injury: A comparison between young and aged rats. Experimental gerontology 83, 37, 2016.

Parker, L., Caldow, M.K., Watts, R., Levinger, P., Cameron-Smith, D., andLevinger, I. Age and sex differences in human skeletal muscle fibrosis markers and transforming growth factor-beta signaling. European journal of applied physiology 117, 1463, 2017.

Kasukonis, B.M., Kim, J.T., Washington, T.A., andWolchok, J.C. Development of an infusion bioreactor for the accelerated preparation of decellularized skeletal muscle scaffolds. Biotechnology progress 32, 745, 2016.

Corona, B.T., Garg, K., Ward, C.L., McDaniel, J.S., Walters, T.J., andRathbone, C.R. Autologous minced muscle grafts: a tissue engineering therapy for the volumetric loss of skeletal muscle. American journal of physiology Cell physiology 305, C761, 2013.

Washington, T.A., Brown, L., Smith, D.A., Davis, G., Baum, J., andBottje, W. Monocarboxylate transporter expression at the onset of skeletal muscle regeneration. Physiological reports 1, e00075, 2013.

Loell, I., andLundberg, I.E. Can muscle regeneration fail in chronic inflammation: a weakness in inflammatory myopathies? Journal of internal medicine 269, 243, 2011.

Porter, J.D., Khanna, S., Kaminski, H.J., Rao, J.S., Merriam, A.P., Richmonds, C.R., Leahy, P., Li, J., Guo, W., andAndrade, F.H. A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. Human molecular genetics 11, 263, 2002.

Peterson, J.M., Bakkar, N., andGuttridge, D.C. NF-kappaB signaling in skeletal muscle health and disease. Current topics in developmental biology 96, 85, 2011.

Chen, S.E., Jin, B., andLi, Y.P. TNF-alpha regulates myogenesis and muscle regeneration by activating p38 MAPK. American journal of physiology Cell physiology 292, C1660, 2007.

Zhan, M., Jin, B., Chen, S.E., Reecy, J.M., andLi, Y.P. TACE release of TNF-alpha mediates mechanotransduction-induced activation of p38 MAPK and myogenesis. Journal of cell science 120, 692, 2007.

Luo, G., Hershko, D.D., Robb, B.W., Wray, C.J., andHasselgren, P.O. IL-1beta stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-kappa B. American journal of physiology Regulatory, integrative and comparative physiology 284, R1249, 2003.

Munoz-Canoves, P., Scheele, C., Pedersen, B.K., andSerrano, A.L. Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? The FEBS journal 280, 4131, 2013.

Zhang, C., Li, Y., Wu, Y., Wang, L., Wang, X., andDu, J. Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration. The Journal of biological chemistry 288, 1489, 2013.

Joe, A.W., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., Rudnicki, M.A., andRossi, F.M. Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. Nature cell biology 12, 153, 2010.

Kami, K., andSenba, E. Localization of leukemia inhibitory factor and interleukin-6 messenger ribonucleic acids in regenerating rat skeletal muscle. Muscle & nerve 21, 819, 1998.

Carlson, B.M. Factors influencing the repair and adaptation of muscles in aged individuals: satellite cells and innervation. The journals of gerontology Series A, Biological sciences and medical sciences 50 Spec No, 96, 1995.

Grounds, M.D. Age-associated changes in the response of skeletal muscle cells to exercise and regeneration. Annals of the New York Academy of Sciences 854, 78, 1998.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L., andRando, T.A. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature 433, 760, 2005.

Brack, A.S., Conboy, M.J., Roy, S., Lee, M., Kuo, C.J., Keller, C., andRando, T.A. Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. Science 317, 807, 2007.

Alnaqeeb, M.A., Al Zaid, N.S., andGoldspink, G. Connective tissue changes and physical properties of developing and ageing skeletal muscle. Journal of anatomy 139 (Pt 4), 677, 1984.

Serrano, A.L., Mann, C.J., Vidal, B., Ardite, E., Perdiguero, E., andMunoz-Canoves, P. Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease. Current topics in developmental biology 96, 167, 2011.

Mann, C.J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A.L., andMunoz-Canoves, P. Aberrant repair and fibrosis development in skeletal muscle. Skeletal muscle 1, 21, 2011.

Hindi, S.M., Shin, J., Ogura, Y., Li, H., andKumar, A. Matrix metalloproteinase-9 inhibition improves proliferation and engraftment of myogenic cells in dystrophic muscle of mdx mice. PloS one 8, e72121, 2013.

Lehto, M., Sims, T.J., andBailey, A.J. Skeletal muscle injury--molecular changes in the collagen during healing. Research in experimental medicine Zeitschrift fur die gesamte experimentelle Medizin einschliesslich experimenteller Chirurgie 185, 95, 1985.

Garner, W.L., Karmiol, S., Rodriguez, J.L., Smith, D.J., Jr., andPhan, S.H. Phenotypic differences in cytokine responsiveness of hypertrophic scar versus normal dermal fibroblasts. The Journal of investigative dermatology 101, 875, 1993.

Carlson, B.M. Regeneration fo the completely excised gastrocnemius muscle in the frog and rat from minced muscle fragments. Journal of morphology 125, 447, 1968.

Studitsky, A.N. Free Auto- and Homografts of Muscle Tissue in Experiments on Animals. Annals of the New York Academy of Sciences 120, 789, 1964.

Ward, C.L., Ji, L., andCorona, B.T. An Autologous Muscle Tissue Expansion Approach for the Treatment of Volumetric Muscle Loss. BioResearch open access 4, 198, 2015.

Turner, N.J., Badylak, J.S., Weber, D.J., andBadylak, S.F. Biologic scaffold remodeling in a dog model of complex musculoskeletal injury. The Journal of surgical research 176, 490, 2012.

Sicari, B.M., Agrawal, V., Siu, B.F., Medberry, C.J., Dearth, C.L., Turner, N.J., andBadylak, S.F. A murine model of volumetric muscle loss and a regenerative medicine approach for tissue replacement. Tissue engineering Part A 18, 1941, 2012.

Gentile, N.E., Stearns, K.M., Brown, E.H., Rubin, J.P., Boninger, M.L., Dearth, C.L., Ambrosio, F., andBadylak, S.F. Targeted rehabilitation after extracellular matrix scaffold transplantation for the treatment of volumetric muscle loss. American journal of physical medicine & rehabilitation 93, S79, 2014.

Merritt, E.K., Hammers, D.W., Tierney, M., Suggs, L.J., Walters, T.J., andFarrar, R.P. Functional assessment of skeletal muscle regeneration utilizing homologous extracellular matrix as scaffolding. Tissue engineering Part A 16, 1395, 2010.

Urciuolo, A., Urbani, L., Perin, S., Maghsoudlou, P., Scottoni, F., Gjinovci, A., Collins-Hooper, H., Loukogeorgakis, S., Tyraskis, A., Torelli, S., Germinario, E., Fallas, M.E.A., Julia-Vilella, C., Eaton, S., Blaauw, B., Patel, K., andDe Coppi, P. Decellularised skeletal muscles allow functional muscle regeneration by promoting host cell migration. Scientific reports 8, 8398, 2018.

Gilbert-Honick, J., Iyer, S.R., Somers, S.M., Lovering, R.M., Wagner, K., Mao, H.Q., andGrayson, W.L. Engineering functional and histological regeneration of vascularized skeletal muscle. Biomaterials 164, 70, 2018.

Qiu, X., Liu, S., Zhang, H., Zhu, B., Su, Y., Zheng, C., Tian, R., Wang, M., Kuang, H., Zhao, X., andJin, Y. Mesenchymal stem cells and extracellular matrix scaffold promote muscle regeneration by synergistically regulating macrophage polarization toward the M2 phenotype. Stem cell research & therapy 9, 88, 2018.

Tidball, J.G. Inflammatory processes in muscle injury and repair. American journal of physiology Regulatory, integrative and comparative physiology 288, R345, 2005.

Tidball, J.G. Regulation of muscle growth and regeneration by the immune system. Nature reviews Immunology 17, 165, 2017.

Tidball, J.G. Mechanisms of muscle injury, repair, and regeneration. Comprehensive Physiology 1, 2029, 2011.

Tidball, J.G. Inflammatory cell response to acute muscle injury. Medicine and science in sports and exercise 27, 1022, 1995.

Acknowledgements

This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number R15AR064481 as well as the Arkansas Biosciences Institute.

Disclosure Statement

No competing financial interests exist.

Figure Legends

Figure 1: Whole tibialis anterior (TA) muscles were decellularized using an infusion bioreactor. During decellularization, SDS solution was delivered to the mid-belly region of the TA muscle via a hypodermic needle and syringe pump (A). The bioreactor was designed to accommodate four side-by-side decellularization units each capable of accommodating a single muscle tissue sample (B). Representative whole TA muscle appearance prior to and following infusion decellularization treatment (C left and right), illustrates the appearance of the DSM scaffolds following removal of intracellular myoglobin. Infusion prepared DSM scaffolds, when viewed in thin section (scale bar = 100um) with H&E staining, retained the aligned network structure of native muscle ECM (D). (arrow = approximate direction of muscle contraction)

Figure 2: Surgical VML and repair procedure. The surgical site was cleaned and disinfected and a 1-2 cm incision was made to expose the underlying TA muscle (A). A 8mm biopsy punch was used to create a VML injury to a depth of 3mm. Approximately 20% of the TA muscle was excised (B). MM autografts were prepared using 25% (by mass) of the removed muscle plug. The muscle tissue was minced using a scalpel and scissors (C). The resulting MM paste was used to coat a DSM scaffold prior to implantation into the defect site (D). The MM coated DSM scaffold was packed into the defect with scaffold alignment oriented to direction of muscle contraction (E). The surgical site was closed using both deep and surface sutures (F). The uninjured contralateral limb served as a comparative control.

Figure 3: Gross morphology of 3-month (A) and 18-month (B) normal, VML, and DSM+MM repaired TA muscles. Dashed circle indicates the approximate location and boundary of the defect site. TA muscle wet weight (C) and peak torque (D) was normalized to animal weight (Nmm/kg) and calculated relative to normal contralateral TA muscle values (%normal) for each animal tested. Representative *in-vivo* TA torque recording (D inset). Relative muscle wet weight and peak torque data are presented. Data is presented as group means + SD; n=7-8/group; * denotes statistically significant ($p<0.05$) differences between groups.

Figure 4: Animal weight was recorded at the time of surgery (initial) and sacrifice (final) for each age and treatment group. Animal weight change (% initial) was quantified using initial and final animal weight values (A). Data is presented as group means + SD; n=7-8/group; * denotes statistically significant ($p<0.05$) differences between groups. Scatter plots, least square method linear correlation fits, and sample coefficients of determination (r^2) for animal weight change versus peak torque data are shown for both the 3-month (B) and 18-month DSM+MM repair groups (C).

Figure 5: Comparison of relative gene expression for 3-month and 18-month DSM+MM repair groups using RT-PCR. The expression of myogenic (Pax7, MyoD, MyoG), ECM (COL I and COL III), ECM regulatory (TGFβ1), and inflammation genes (IGF1, IL-1β and TNFα) were

measured using muscle tissue harvested from the DSM+MM repair site. Expression is presented as fold change normalized to contralateral normal muscle expression. Group means + SD are presented, n=4/group. * denotes statistically significant (p<0.05) differences between groups.

Figure 6: TA muscle cross-sections were stained with Masson's Trichrome. Representative 3-month (A) and 18-month (B) normal, VML, and DSM+MM repair groups are presented. Representative whole TA cross-sections and magnified (100X) images are shown. Arrows highlight regions and bands of fibrotic collagen accumulation. Inset indicates approximate location of magnified image within the TA cross-section. Arrow indicates anterior direction. Scale bar = 100μm unless noted.

Figure 7: TA tissue cross-sections were immunostained for collagen I (green) and counterstained against MHC (red). Representative 3-month (A) and 18-month (B) normal, VML, and DSM+MM repaired tissue images are shown. Inset indicates approximate location of magnified (100X) image within TA tissue section. Arrow indicates anterior direction. Open arrows highlight regions and bands of fibrotic collagen I accumulation. Scale bar = 100μm. Cross-sections were quantified for area fraction of collagen type I (C). Group means + SD are presented; n=4/group. * denotes statistically significant (p<0.05) differences between groups.

Figure 8: TA tissue cross-sections were immunostained for collagen III (red). Representative 3-month (A) and 18-month (B) DSM+MM repair site images are shown. Inset indicates approximate location of magnified (100X) image within TA tissue section. Arrow indicates anterior direction. Scale bar = 100μm. Cross-sections were quantified for area fraction (%) collagen type III (C). Group means + SD are presented; n=4/group. * denotes statistically significant (p<0.05) differences between groups.

Figure 9: Individual myofiber areas were measured from Collagen III stained tissue cross-sections. Group data for myofiber cross-sectional area (A) and area frequency distribution are shown (B). Group means + SD are presented; n=4/group; ns = no significance (p>0.05).

Figures

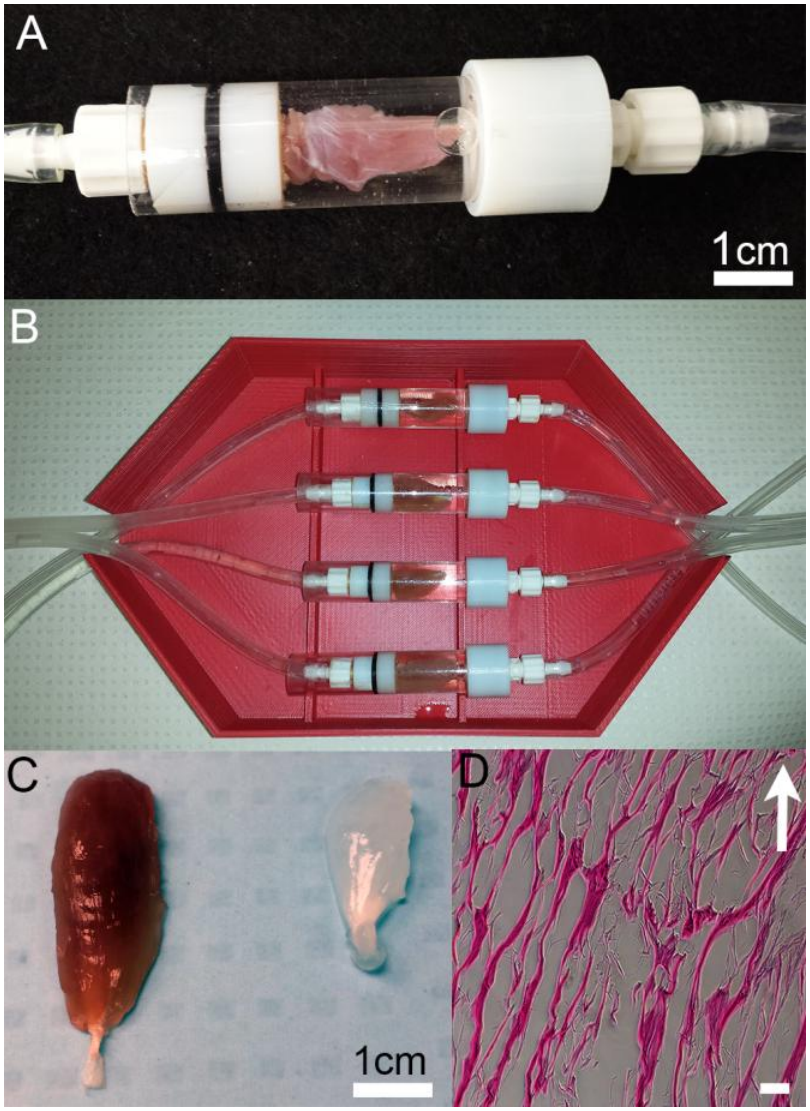

Figure 1

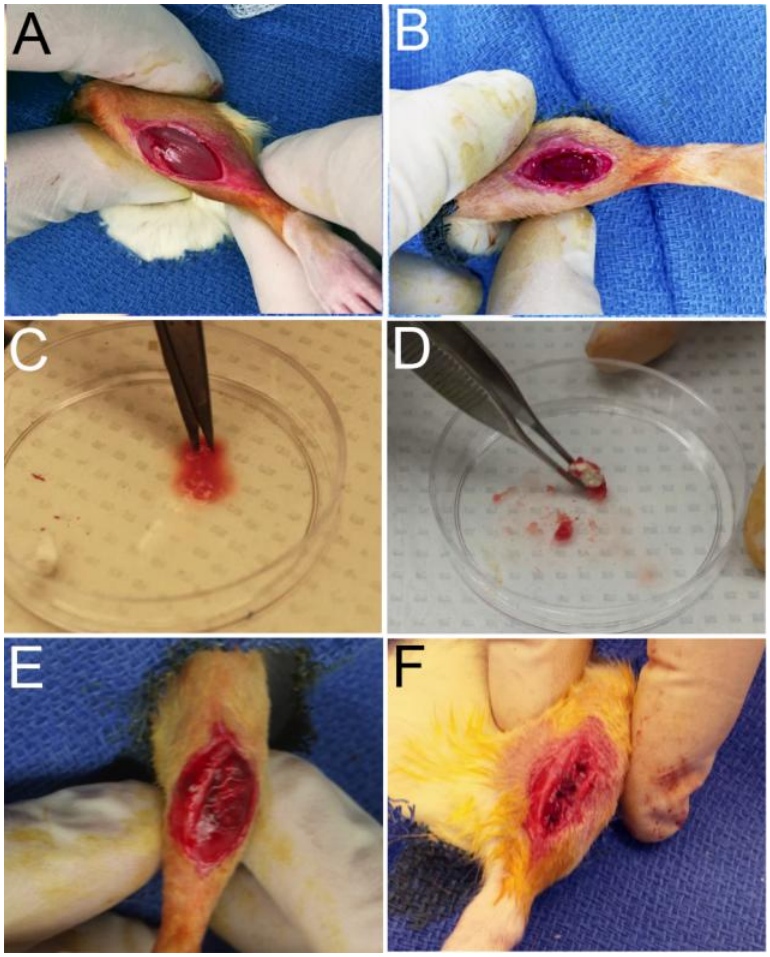

Figure 2

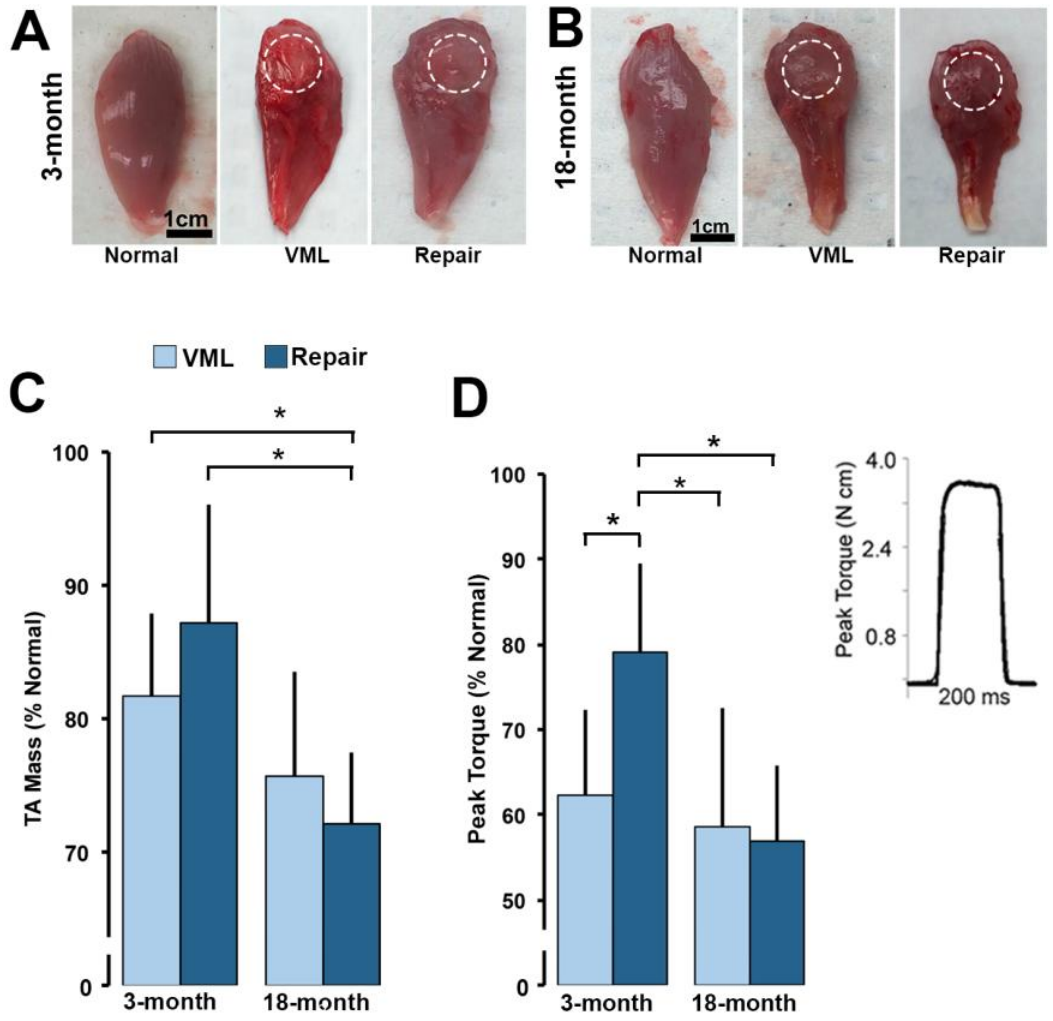

Figure 3

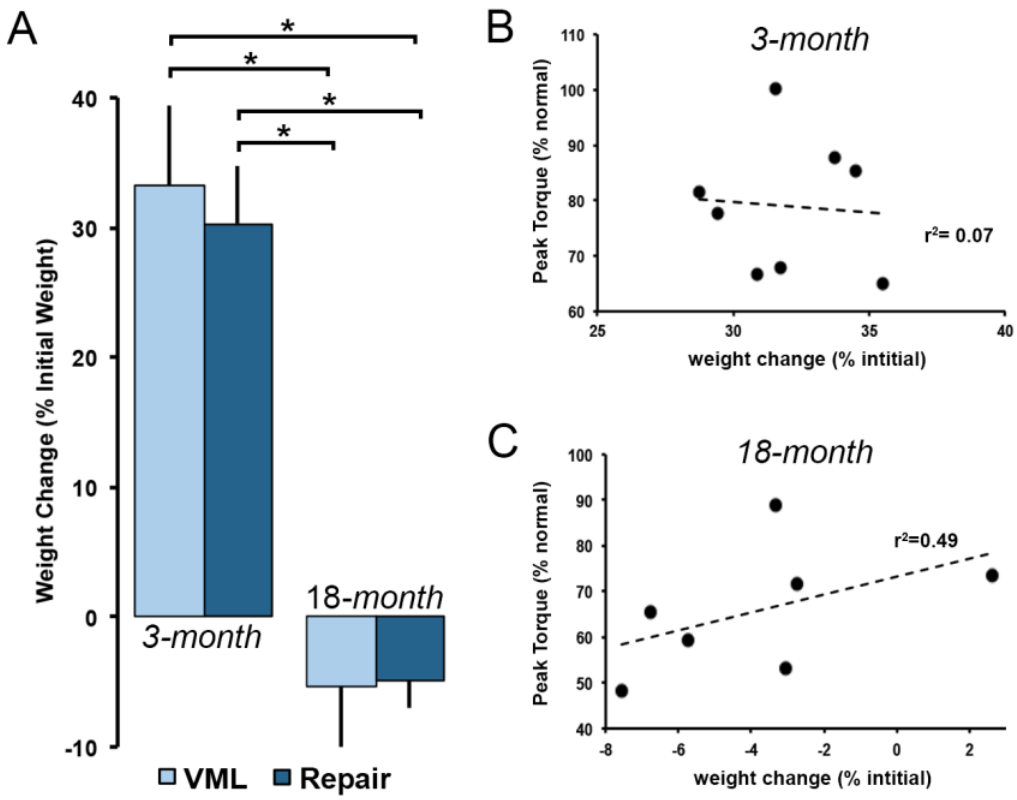

Figure 4

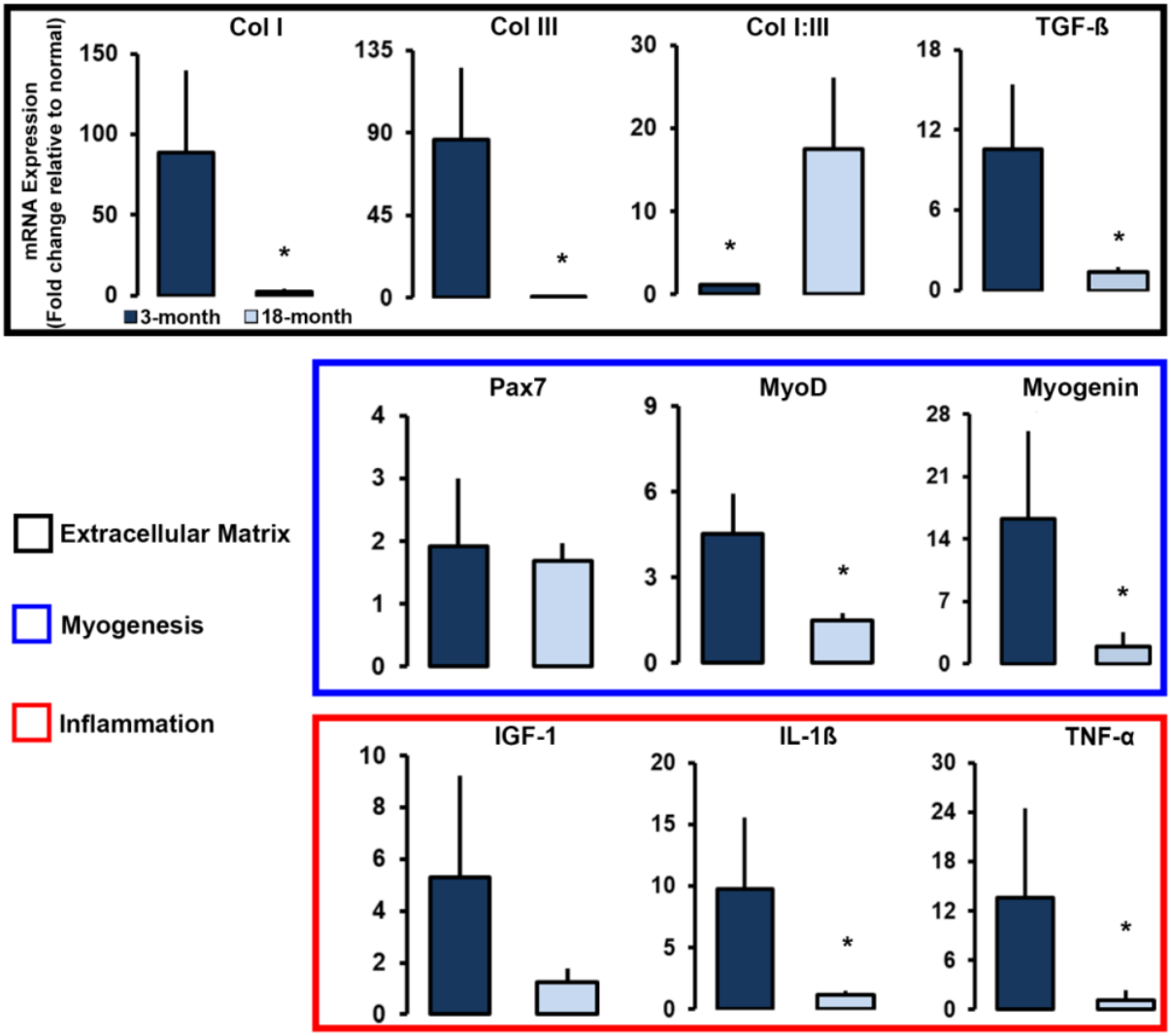

Figure 5

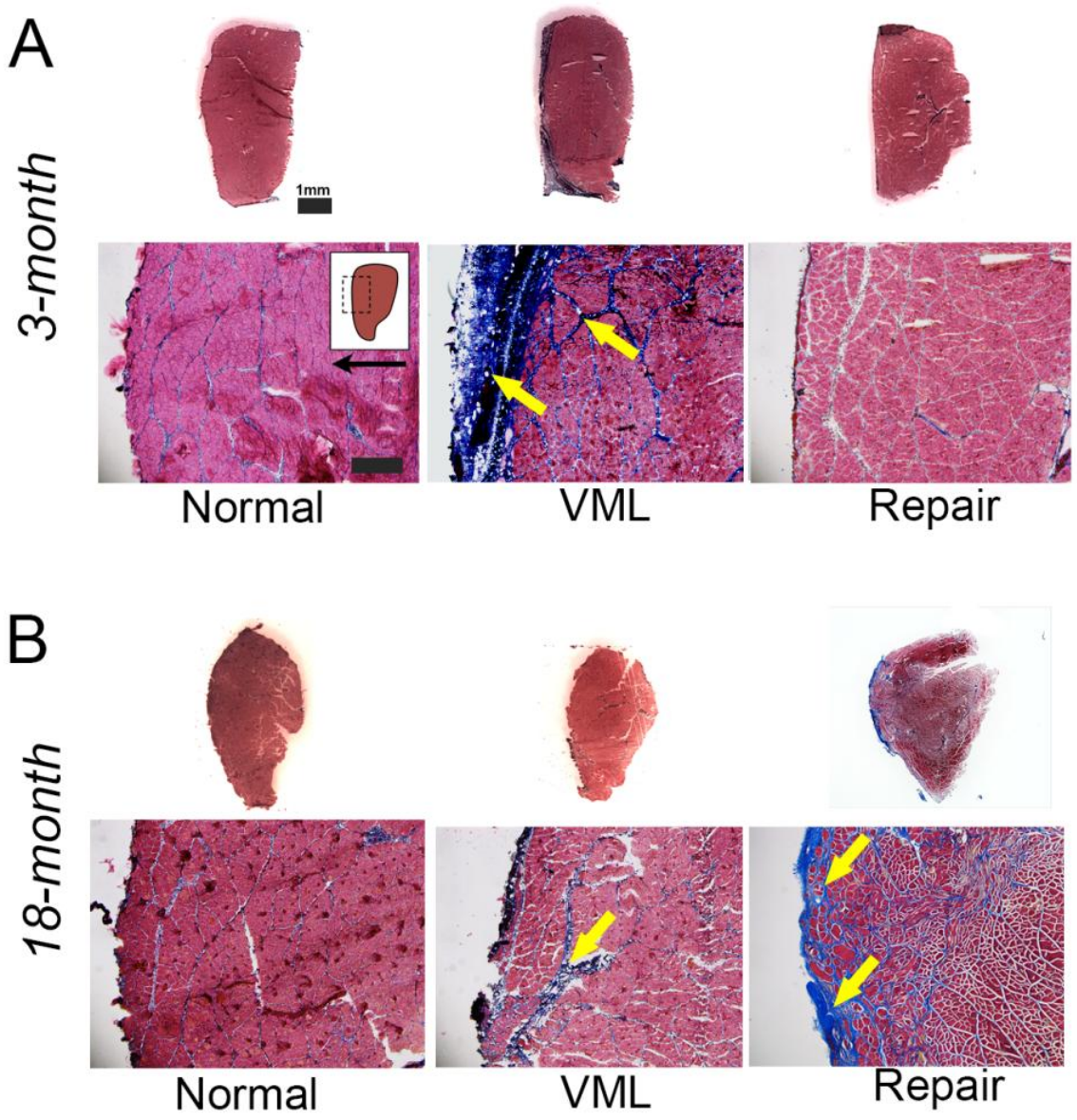

Figure 6

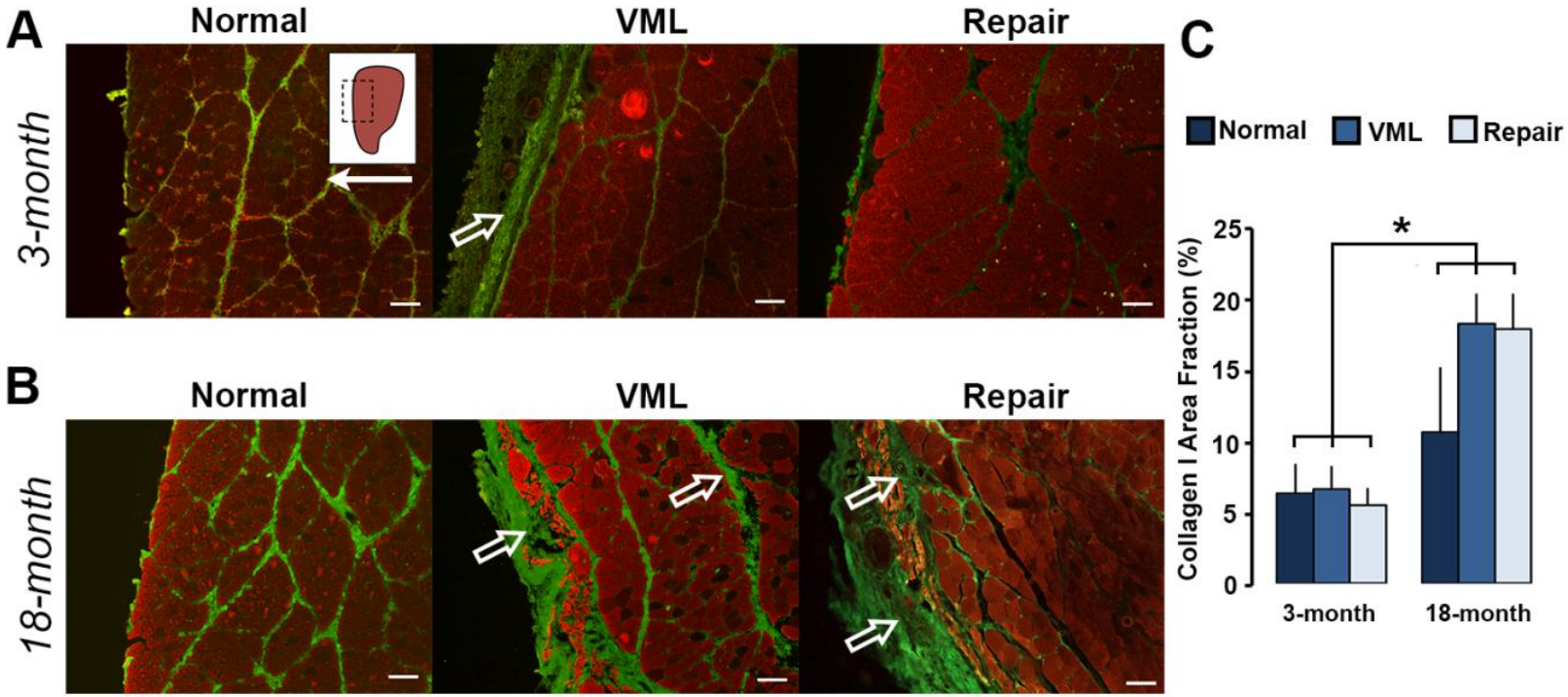

Figure 7

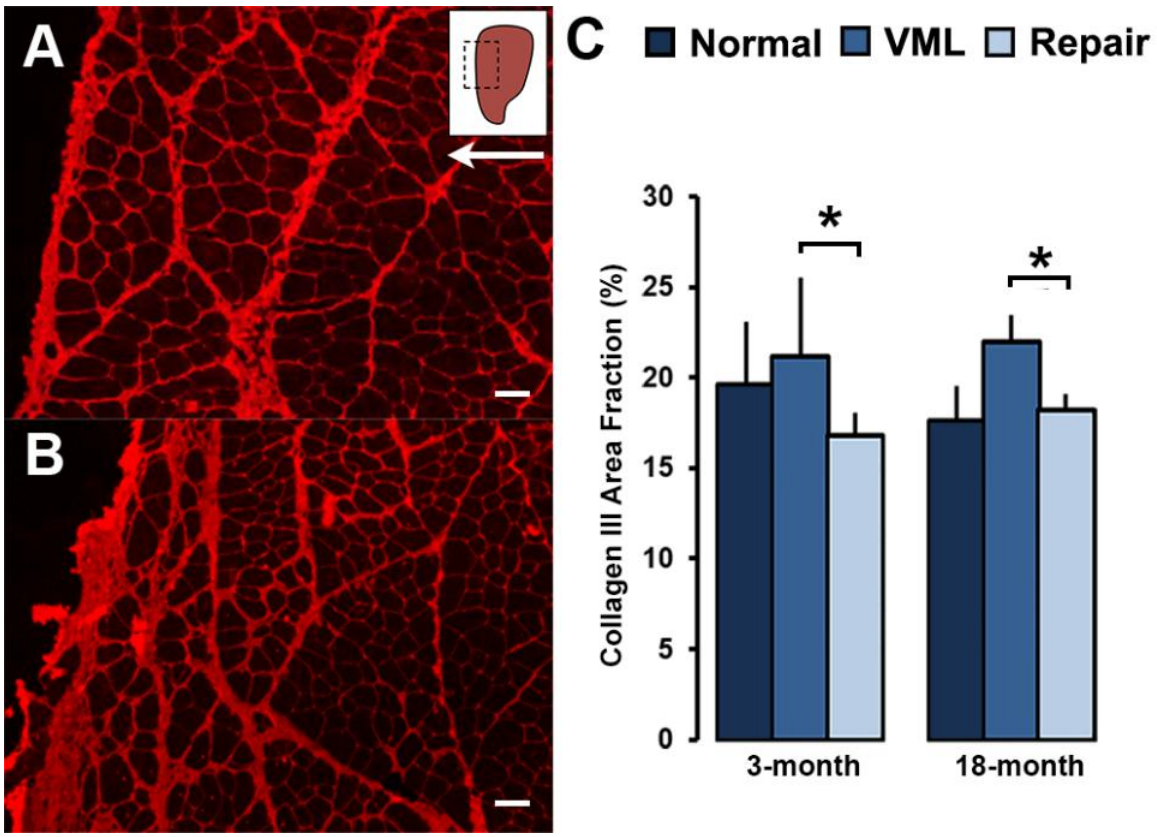

Figure 8

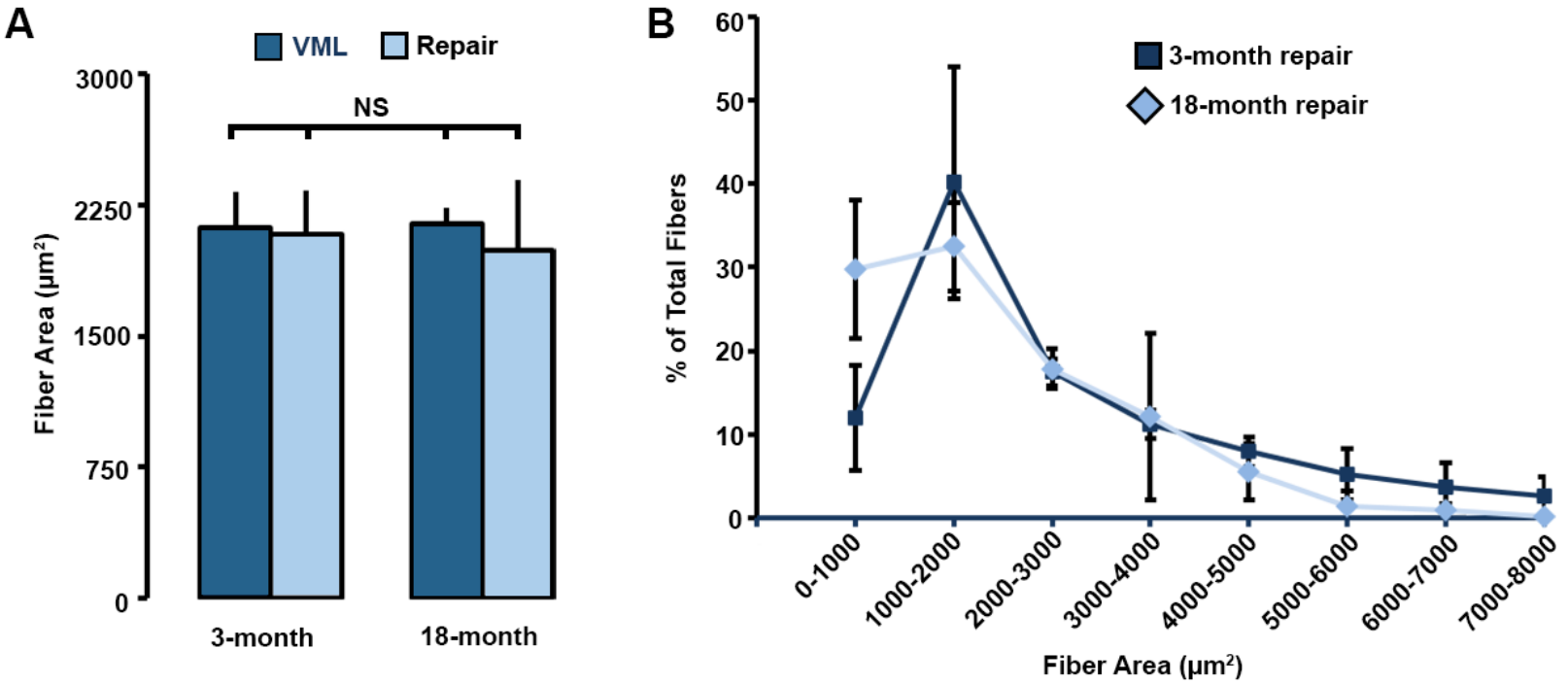

Figure 9

Chapter 4

Nandrolone Decanoate Does Not Improve Regenerative Repair of Volumetric Muscle Loss Injuries in Aging Rats

Submitted as an original article by:

John T. Kim[1], Kevin Roberts[1], Richard Perry[2], Tyrone Washington[2], and Jeffrey C. Wolchok PhD[1]

[1] Department of Biomedical Engineering, University of Arkansas, Fayetteville, Arkansas, 72701

[2] Department of Health, Human Performance, and Recreation, College of Education and Health Professions, University of Arkansas, Fayetteville, AR, Fayetteville, Arkansas 72701

Corresponding Author:

Jeffrey C. Wolchok, PhD

Department of Biomedical Engineering

University of Arkansas

John A. White, Jr. Engineering Hall, Suite 120, Fayetteville, Arkansas

Phone: 479-575-4667

Email: jwolchok@uark.edu

Abstract

It has been previously demonstrated that a combinatorial repair strategy for volumetric muscle loss (VML) consisting of autologous minced muscle (MM) grafts co-delivered with a decellularized muscle scaffold (DSM) was not able to promote a pro-myogenic environment leading to effective tissue repair nor restore muscle contractile function in an 18-month aged animal model (Fischer 344). In this study, the use of the anabolic steroid nandrolone decanoate (ND) was explored as an adjunct to DSM+MM repair in an aged model of VML injury (n=15/treatment group). We observed no significant improvements in peak isometric torque between ND(+) (64.5±18.3% of uninjured contralateral muscle) and ND(-) (80.9±37.9%) repair groups. Histological findings revealed a significant reduction in collagen dense repair tissue at the site of injury. These findings were also reflected in collagen I area fraction (%) measurements with both ND(+) uninjured (6.1+1.1%) and ND(+) repair (9.3+3.2%) groups having significantly lower collagen I area fraction compared to the ND(-) repair (15.6+5.5%) group. Gene expression data revealed no significant changes in myogenic gene expression with ND injection but did upregulate collagen III expression. The expression of transcription factors related to Notch (Notch1, Jag1) and insulin (TRIM72, FOXO1, IRS-1) signaling were found to be downregulated by age, independent of ND treatment. These findings suggest that ND treatment may not be an appropriate addition to existing VML repair strategies to enhance age-associated decline in regenerative potential.

A. Introduction

Volumetric muscle loss (VML) is a traumatic injury that overwhelms the regenerative ability of skeletal muscle leading to permanent functional loss and excessive scarring. Currently, surgical interventions for VML are limited to autologous muscle flap transfers which have been known to cause donor site morbidity, further exacerbating the patient (Fan et al., 2008; Lin, Lin, Yeh, & Chen, 2007; Vekris et al., 2008). However, the use of implantable biological scaffolds as a regenerative medicine strategy have shown encouraging results in small animal models (Corona et al., 2013; Corona, Ward, Baker, Walters, & Christ, 2014; Gentile et al., 2014; Kasukonis, Kim, Washington, & Wolchok, 2016). VML injuries can result from both battlefield (Corona, Rivera, Owens, Wenke, & Rathbone, 2015; Owens et al., 2008) and civilian trauma (Grogan, Hsu, & Skeletal Trauma Research, 2011; MacKenzie et al., 2000), with the latter encompassing a wide range of age groups. With current investigations into regenerative medicine strategies for the repair of VML being conducted primarily in young animal models, there still exists a need for investigations into how age affects regenerative outcomes following VML treatment.

There are well-documented aging-related changes in muscle function and quality including reduction in muscle mass, reduced metabolism, and increased fatty tissue infiltration into the muscle (Blaauw, Schiaffino, & Reggiani, 2013; Collino et al., 2013; Fielding et al., 2011; Gheller, Riddle, Lem, & Thalacker-Mercer, 2016). Factors contributing to these changes have been shown to be a combination of intrinsic changes in muscle such as the deterioration of skeletal muscle quality (fiber composition, muscle capillarization, mitochondrial dysfunction) (Coggan et al., 1992; Conley, Jubrias, & Esselman, 2000; Croley et al., 2005; Miller, Robinson, Bruss, Hellerstein, & Hamilton, 2012; Suetta et al., 2007) and extrinsic factors such as dietary

intake and physical activity (Donini, Savina, & Cannella, 2003; Straight, Brady, & Evans, 2015; Thalacker-Mercer, Fleet, Craig, & Campbell, 2010). Coupled with the multi-factorial nature of VML injuries, regenerative medicine strategies have proven unsuccessful in eliciting a significant regenerative response in aging skeletal muscle as previously demonstrated by our group (J. Kim et al., 2019). A potential solution to combat the age-related changes in aging skeletal muscle is the use of exogenous testosterone.

The use of testosterone and other anabolic stimuli in aging animal models has been documented to improve muscle regeneration. Specifically, testosterone has been demonstrated in several studies to induce muscle hypertrophy via increased protein accumulation and myonuclear accretion in addition to rescuing aging muscle from wasting (Axell et al., 2006; Kadi, Bonnerud, Eriksson, & Thornell, 2000; Kovacheva, Hikim, Shen, Sinha, & Sinha-Hikim, 2010; Sinha-Hikim et al., 2002; Sinha-Hikim, Cornford, Gaytan, Lee, & Bhasin, 2006). Results suggest that androgens can stimulate the proliferation of satellite cells and ultimately differentiation and fusion into myofibers. This is further supported by studies showing increased satellite cell numbers following treatment in numerous species including humans (Sinha-Hikim et al., 2006; Sinha-Hikim, Roth, Lee, & Bhasin, 2003), rodents(Joubert & Tobin, 1995), and chickens (Allouh, Jarrar, Asfour, Said, & Shaqoura, 2017). Testosterone has also demonstrated the ability to inhibit Notch signaling through its inhibitor, Numb, which has been shown to promote satellite cell differentiation during myogenic development and repair (Conboy & Rando, 2002; Jory et al., 2009; Zilian et al., 2001). These findings are likely attributed to the role of the androgen receptor on muscle cell proliferation and differentiation in both *in vitro* and *in vivo* environments (Dalbo et al., 2017; Diel, Baadners, Schlupmann, Velders, & Schwarz, 2008; Hughes et al., 2016; D. K. Lee, 2002; Sinha-Hikim, Taylor, Gonzalez-Cadavid, Zheng, & Bhasin, 2004).

There still remains the question of whether a reversal of aging-related pathologies via testosterone treatment used in conjunction with ECM-based therapies to repair VML injuries will create an environment that is conducive to muscle regeneration. To that end, an aged animal model of VML developed by our group (Kim, Kasukonis, Brown, Washington, & Wolchok, 2016) was used to investigate the response to VML repair with and without anabolic steroid supplementation. Fischer 344 rats aged 18 months were used in this study, which represent the mature to early elderly rat lifespan (ref). The purpose of this study was to test the hypothesis that testosterone supplementation in conjunction with our minced muscle and ECM-based VML repair therapy will counteract age-related muscle wasting in addition to stimulating myogenic activity, thus creating an environment more receptive to regenerative treatment. This study presents the performance of our previously reported regenerative strategy (J. Kim et al., 2019), with and without testosterone supplementation in an aged animal model.

B. Methods

Anabolic Steroid Administration

The anabolic steroid nandrolone decanoate (ND) (Steraloids, Inc, Newport, RI) was used in this study due to its long half-life (6-12 days) and has been demonstrated to have potent myotrophic effects in skeletal muscle (Carson, Lee, McClung, & Hand, 2002; Tsika, Herrick, & Baldwin, 1987; Washington, Healey, Thompson, Lowe, & Carson, 2014). Sesame oil was used as a carrier for ND and was administered intramuscularly into the vastus lateralis muscle at a concentration of 9 mg/kg body weight once weekly. Animal weights were recorded prior to injections to adjust ND dosages to be consistent with the 9 mg/kg body weight dose. ND(-)

animal groups were administered depot injections of sesame oil intramuscularly. Both ND(+) and ND(-) groups were administered either ND or depot injections at the time of surgical treatment and once every 7 days following the date of injection.

hDSM Preparation

To prepare human DSM samples (hDSM) for implantation, whole human tibialis anterior (TA) muscle was portioned into smaller fragments for decellularization. TA muscles were donated from Science Care, Inc (Phoenix, AZ). The TA samples were incubated in 1% SDS for approximately 2 weeks with a fresh exchange of SDS every 24 hrs. Following infusion treatment, samples were incubated overnight in a DNAse solution (1kU/ml DNAse in a 10mM Tris-HCL buffer; 2.5mM $MgCL_2$ + 0.5mM $CaCl_2$) and then incubated (8 hours) in a 1X penicillin/streptomycin solution to reduce the risk of infection. Samples were rinsed thoroughly (a total of six 24-hour wash steps) in PBS between each preparation step. Following completion of the entire decellularization protocol, the hDSM implants were lyophilized and stored at -20ºC.

Volumetric Muscle Loss Injury and Minced Muscle

In vivo VML studies were performed with aged (18 months) male Fisher 344 rats. The aged rats were obtained through the National Institute on Aging (Bethesda, MD). Surgical procedures and implant preparation methods were performed in accordance with protocols approved by the University of Arkansas IACUC (protocol #18052) and guided by published methods. Anesthesia was administered using isoflurane (1-3%) in oxygen. The implant site was exposed via a parallel incision (1-2cm) along the tibia (**Figure 1)**. The TA was then identified

and a partial thickness VML defect (8mm diameter x 3mm deep) was created using a sterile biopsy punch. Defect site tissue was then removed and weighed to ensure consistent defect mass values (average defect weight = 93.4mg). Defect mass values (20% of TA mass) were based on pilot study data measuring TA muscle mass (average TA mass = 470±17mg). The removed defect plugs were retained for use as minced muscle (MM) autografts (25% of VML defect mass). Animals were randomly assigned to one of two treatment groups (n=15/treatment group): VML defect repaired with hDSM scaffold supplemented with MM autografts followed by testosterone injection (ND(+)) or repair with no injection (ND(-)). All contralateral limbs to the ND(+) and ND(-) only limbs served as internal controls for comparison and are denoted as the normal group in the study.

A single infusion-prepared DSM scaffold was cut to size (average DSM mass = 4.9±0.7mg) using surgical scissors. Separately, 25% of the defect muscle plug was hand-minced into a loose paste using a scalpel and scissors until MM fragments larger than ~1mm^3 could not be visually detected. The DSM scaffolds were then manually rolled in the MM autograft paste until all surfaces were visibly coated. DSM+MM constructs were implanted into the VML defect. Care was taken to ensure DSM implant network alignment matched that of the surrounding muscle. Unrepaired VML defects served as negative controls. To remain consistent with previous MM repair techniques, the DSM scaffolds were sized to fill, but were not sutured to the surrounding tissue. The deep fascia and skin layers were separately closed using interrupted stitches with a 5-0 adsorbable suture (Vicryl, Ethicon). Post-operative analgesia consisted of 0.1mg/kg buprenorphine administered subcutaneously through injection twice daily for 2 days. The anti-inflammatory medication Carprofen was readily available to the animals via a dietary gel cup (MediGel CPF, ClearH_20, Westbrook, ME), which was added to each animal's

cage after surgery. Animal consumption of the gel was voluntary and any uneaten gel was removed from the cage at 1-week post-surgery. Following surgery, animals were house in standard-sized rat cages with unrestricted movement and were allowed to bear weight on the operated extremity as tolerated. All animals were housed for a 12-week recovery period.

In-vivo Contractile Force Measurement

Peak tetanic contractions produced by the TA muscle were measured isometrically *in vivo* using procedures described by Corona and familiar to our group (Corona et al., 2013; B. Kasukonis et al., 2016). Animals were anesthetized (isoflurane) and the lower limb was stabilized at 90° of knee flexion (tibia parallel to the benchtop) using a custom made alignment jig. The ankle was flexed to 90° and the foot was secured (surgical tape) to the lever arm of a dual-mode muscle lever systems (Aurora Scientific, Ontario, Canada,). To isolate the contribution of the TA during force measurement, distal tenotomies were performed on the extensor digitorum longus (EDL) and extensor halluces longus (EHL). TA peak isometric tetanic force was measured by stimulating the peroneal nerve with the aid of a physiological stimulator (Grass; S88). Optimal voltage (2 – 5 V) was determined using a series of tetanic contractions (150Hz, 0.1 ms pulse width, 200 ms train). Average peak tetanic force for each animal was calculated from an average of 5 contractions. All contractions were separated by one minute of rest. Raw peak tetanic contractile force (N) was recorded from both the treated and contralateral control limb of each animal and normalized to animal weight (N/kg). The weight normalized (N/kg) and contralateral limb normalized (% normal) force data is reported in the results section. At the conclusion of electrophysiological testing, all animals were euthanized through carbon

dioxide inhalation in accordance with guidelines provided by the 2013 AVMA Panel on Euthanasia of Animals.

Gene Expression

RT-PCR was performed using the protocol reported by Washington et al (Washington et al., 2013). In preparation for RT-PCR, samples of muscle from the defect site of the right and left TA ($n \leq 5$ animals/group) were homogenized with Trizol (Ambion, Carlsbad, CA)/choloroform (Sigma Aldrich, St. Louis, MO). Samples were treated with DNase (Invitrogen, Carlsbad, CA) and then RNA was extracted using an RNAeasy kit (Invitrogen, Carlsbad, CA). After quantification of RNA with a plate reader (BioTek, Winooski, VT), RNA was converted to cDNA with a kit (Invitrogen, Carlsbad, CA). TAQMAN primers (Invitrogen, Carlsbad, CA) for Collagen I, Collagen III, TGF-β, IGF-1, Pax7, MyoD, MyoG, IL-1β, TNF-α, IFN-γ, TRIM72, IRS-1, FOXO1, Notch1, Jag1, and 18S rRNA housekeeping were used to quantify the expression of desired genes. Experimental sample group expressions were normalized to 18S rRNA and then referenced to the contralateral normal limb. Gene expression levels are reported as fold change using the $2^{-(\Delta\Delta Ct)}$ method.

Non-contractile Tissue and Fiber Cross-Sectional Area Measurements

Collagen type I accumulation was quantified within normal and treated muscle tissue sections using measures of Collagen I (Sigma Aldrich, St. Louis, MO) immunoreactivity with myosin heavy chain (Developmental Studies Hybridoma Bank, Iowa City, IA) as a counter stain. Representative tissue sections were imaged (200X), converted to 8-bit greyscale, and a uniform threshold was applied across all samples to isolate collagen type I positive tissue regions within

each section. From each image, tissue immunoreactivity to collagen I as a percentage of total tissue area was calculated with the aid of image analysis software (ImageJ, NIH) and is reported in the results as percent non-contractile tissue (% NCT). Similar image analysis methods were used to compute mean muscle fiber cross-sectional area (μm^2) using magnified (200X) collagen III immunoreactivity images. Collagen III immunostained images were similarly converted to greyscale and thresholded to isolate the borders of individual muscle fibers (typically 50+ fibers per image). Representative tissue sections collected from four animals per group were used for all calculations. Three sections were imaged and analyzed to calculate the mean %NCT and fiber cross-sectional area for each animal. A total of twelve images (4 animals X 3 sections/animal) were analyzed for each treatment group. All image analysis was performed following a protocol established by our group (Kim et al., 2016) with set parameters to minimize any user bias.

Serum Testosterone Analysis

The Ultra-Sensitive Mouse & Rat LH ELISA is an in-house method based on a manuscript published by Steyn et al (Steyn et al., 2013). This assay allows for a LH measurement in 2.5 – 5ul of serum, plasma or whole blood. The capture monoclonal antibody (anti-bovine LH beta subunit, 518B7) is provided by Janet Roser, University of California. The detection polyclonal antibody (rabbit LH antiserum, AFP240580Rb) is provided by the National Hormone and Peptide Program (NHPP). HRP-conjugated polyclonal antibody (goat anti-rabbit) is purchased from DakoCytomation (Glostrup, Denmark; D048701-2). Mouse LH reference prep (AFP5306A; NHPP) is used as the assay standard. The Limit of Quantitation (Functional Sensitivity) is defined as the lowest concentration that demonstrates accuracy within 20% of expected values and intra-assay coefficient of variation (%CV) <20%, and was determined by

serial dilutions of a defined sample pool. Intra-assay %CV is 2.2%. Inter-assay %CVs are 7.3% (Low QC, 0.13 ng/ml), 5.0% (Medium QC, 0.8 ng/ml) and 6.5% (High QC, 2.3 ng/ml). Functional sensitivity is 0.016 ng/ml.

Statistical Analysis

Data is presented as the mean ± standard deviation and was tested for normality using the Shapiro-Wilks test. The following dependent variables were analyzed using a two-way ANOVA: body weight; TA mass; gastrocnemius mass; EDL mass; peak isometric torque; non-contractile tissue percentage, fiber cross-sectional area; myogenic, ECM, and inflammatory gene expression. Post-hoc comparisons were performed using Tukey's HSD test and if detected, interaction effects were reported. Comparisons between 3-month ND(-) repair, 18-month ND(-) repair, and 18-month ND(+) repair groups were conducted via one-way ANOVA with a Tukey's HSD post test. Comparison between ND(-) and ND(+) group means were conducted by Student's t-test. Chi-square analysis was performed to compare body weight curves, survival curves, and fiber area frequency distributions. All analyses were performed using commercial statistical analysis software (JMP 13). A standard $p<0.05$ level of significance was used for all statistical tests.

C. Results

Animal Growth

Following VML injury repair, all animals appeared to tolerate surgery well and were fully ambulatory within 1 week of the procedure. No surgical complications that required euthanasia were observed. Over the 8-week study period, ND(-) repair animals on average lost a

total of 18.1±25.2g while the ND(+) repair animals lost a total of 26.1±8.4g. ND(-) and ND(+) repair groups lost weight at similar rates of -2.3±3.2g/wk and -3.3±1.2g/wk, respectively (**Figure 2D)**. No statistical differences were found for both total weight loss and growth rate between the two groups.

TA, EDL, and Gastrocnemius Muscle Mass

Compared to normal TA morphology, the repaired muscles for both the ND(-) and ND(+) groups appeared significantly atrophied with a visible defect at the site of VML repair (**Figure 3A**). A main effect of treatment (p<0.0001) to reduce TA mass was detected. Post hoc analysis revealed that within their respective treatment groups, normal TA mass for the ND(+) (1.34±0.10 g/kg, p<0.0001) and ND(-) (1.32±0.07 g/kg, p=0.0005) groups were significantly higher than their ND(-) counterparts (1.07±0.08 g/kg and 1.12±0.09 g/kg)(**Figure 3B**). However, no significant differences in TA mass were detected between the ND(+) and ND(-) groups.

In the gastrocnemius muscles, a main effect of treatment (p=0.0002) was detected to increased gastrocnemius mass (**Figure 3C**). Specifically, ND(+) uninjured gastroc muscles weighed significantly less compared to ND(+) repair muscles (4.14±0.08 vs. 4.52±0.08, p=0.0074) while no significant differences were detected within the ND(-) group. For EDL mass, an interaction effect between treatment and testosterone (p=0.0358) was detected to decrease EDL mass (**Figure 3D**). Post hoc analysis revealed that EDL mass from the ND(-) repair group was significantly higher than the ND(+) repair group (0.41±0.03 g/kg vs. 0.37±0.03 g/kg, p=0.0219).

In vivo Peak Isometric Torque Measurements

In vivo torque readings were characterized by a rapid rise torque followed by a stable plateau at the peak value and then concluding with a sharp descent to a normal resting state (**Figure 4A**). Similar to our previous aging studies, there was a main effect of treatment to decrease peak isometric torque ($p<0.0001$)(**Figure 4B**). Post hoc analysis indicated that the ND(+) repair group TA produced significantly less force than its control counterpart (1.17±0.29 N/kg vs 1.87±0.38 N/kg, $p<0.0006$). Despite the absence of a significant difference in peak torque between the ND(-) control and repair groups, a decreasing trend in force production was observed with VML repair. These findings are consistent with previous findings utilizing this aged animal model. Additionally, no difference was detected between % normal torque between the ND(+) (64.5±18.3%) and ND(-) groups (80.9±37.9%)(**Figure 4C**).

Heart and Testes Mass

Heart mass measurements revealed that there was a significant increase in mass in the ND(+) group compared to the ND(-) group (2.77±0.18 g/kg vs. 2.59±0.17 g/kg, $p=0.0068$)(**Figure 5A**). Additionally, it was revealed that ND injections significantly decreased testicular mass in the animals when compared to the ND(-) animal group (5.84±0.84g/kg BW vs. 9.34±3.53 g/kg BW, $p=0.0018$)(**Figure 5B**).

Serum Testosterone

Interestingly, serum testosterone concentration was detected to be significantly higher in the ND(-) group compared to the ND(+) repair group (p=0.0063)(**Figure 5C**). Specifically, the ND(-) repair group's serum testosterone concentration (93.1±30.7 ng/dL) was approximately 38% higher than the ND(+) repair group (63.5±11.4 ng/dL).

Histology

Masson's trichrome staining revealed tightly apposed muscle fibers throughout both ND(-) and ND(+) uninjured groups, with no sign of disorganized or abnormal tissue (**Figure 6**). However, the respective repair groups were characterized with areas of dense collagen-rich scar regions as well as diffuse non-contractile tissue infiltration extending medially from the anterior surface of the muscle. By comparison, however, the ND(-) repair group had significantly more scarring at the anterior surface than its ND(+) counterpart. Additionally, while the ND(+) repair group maintained myofiber presence within and surrounding the repair site, the ND(-) repair tissue was largely devoid of myofibers, suggesting a greater degree of atrophy compared to its ND(+) counterpart.

Fiber Cross-sectional Area and Non-contractile Tissue Measurements

Regions immune-reactive to collagen I provided a semi-quantitative measure of scarring or percentage non-contractile tissue (**Figure 7A**). Myofiber cross-sectional area distribution histograms (**Figure 7B**) for all groups were characterized by a frequency peak at myofiber areas

in the range of 1000-2000μm^2. Though no significant differences were detected between fiber area distributions for all groups, the greatest difference in fiber area frequency (~7%) was observed in the 1000-2000μm^2 range between the ND(+) repair (35.9%) and ND(+) uninjured (28%) groups while ND(-) uninjured and repair groups exhibited similar fiber area frequencies (32% vs. 32.4%). Following statistical analysis, an interaction effect of treatment and drug was detected. Within the ND(-) group, a significant increase in percent non-contractile tissue was measured in the repair TA compared to the uninjured TA (15.6±5.5% vs. 6.4±0.8%, p<0.0001)(**Figure 7C**). Within the ND(+) however, no differences were detected between the uninjured and repair groups. *Post hoc* analysis revealed that both the ND(+) uninjured and ND(+) repair groups contained significantly less percentages of non-contractile tissue than the ND(-) repair group (6.1±1.1% and 9.3±3.2% vs. 15.6±5.5%, p<0.0001 and p=0.0046 respectively). Interestingly, though no significant differences were detected in No significant differences in myofiber cross-sectional area was detected with all groups containing similar mean cross-sectional areas ranging from 2100 μm^2 to 2200 μm^2 (**Figure 7D**).

Gene Expression

Myogenic gene expression (Pax7, MyoD, MyoG, IGF-1) within the ND(-) and ND(+) was not found to be significantly different (**Figure 8**). However, though not significantly different, MyoD expression had an elevated trend in the ND(+) control group compared to the ND(+) repair group as well as their ND(-) counterparts. Overall, expression for myogenic genes was similarly muted across all groups. Interestingly, among the inflammatory markers TNF-α, IFN-γ, and IL-1ß, IL-1ß was the only gene to be expressed at detectable levels. An effect of treatment was found to increase IL-1ß expression following repair in both non-ND (1.0±0.6 fold

change vs. 4.2±3.6 fold change) and ND (0.6±0.5 fold change vs. 2.8±1.1 fold change) groups (p=0.0198).

Among ECM-related genes (Col I, Col III, TGF-ß), Col III was the only target to be differentially expressed, exhibiting an effect of treatment to increase its expression (**Figure 8**). Specifically, the ND(+) repair group (16.4±18.1 fold change) expressed Col III approximately 47-fold higher than the ND control group (0.4±0.01 fold change). TGF-ß expression was not found to be significantly different across all groups, though there was a trend of increasing expression following repair for both non-ND and ND groups. An interesting trend in expression was found for Col I, though no significant differences were found. While treatment appeared to result in lower expression in the non-ND repair group compared to the non-ND control group, the opposite response was observed for the ND repair group compared to the ND control group with the ND repair group trending towards higher Col I expression by approximately 4-fold.

While examining the expression of genes related to IGF-1 signaling and atrophy (TRIM72, IRS-1, FOXO1), a main effect of age was detected to decrease the expression of IRS-1 and FOXO1 (p<0.0001 and p<0.0001, respectively) in the 18-month ND(-) and ND(+) groups compared to the 3-month ND(-) counterpart (**Figure 9**). Specifically, FOXO1 was expressed approximately 33-fold and 50-fold lower in the 18-month non-ND (0.03±0.01 fold change, p<0.0001) and ND (0.02±0.01 fold change, p<0.0001) groups, respectively than the 3-month non-ND group (1.0±0.2 fold change). IRS-1 was expressed 100-fold lower in both the 18-month non-ND (0.01±0.01 fold change, p<0.0001) and ND (0.01±0.004 fold change, p<0.0001) groups compared to the 18-month non-ND group (1.0±0.1 fold change). No differences were found in TRIM72 expression across the three groups, but there was a trend of higher expression in the 18-

month non-ND and ND groups (1.6±0.9 fold change and 1.1±0.3 fold change, respectively) compared to the 3-month non-ND counterpart (1.0±0.5 fold change).

Associated with satellite cell activity and myogenic regulation, the expression of the Notch signaling receptor Notch1 and its ligand, Jag1, were measured between 3-month –ND, 18-month –ND, and 18-month ND treatment groups (**Figure 9**). No significant differences were detected between groups for both Notch1 and Jag1. However, an increasing trend of Notch1 expression was observed in the 18-month ND group (1.38±1.30 fold change) compared to both 18-month –ND (1.17±1.13 fold change) and 3-month –ND groups (1.00±0.56 fold change). Interestingly, Jag1 expression in both 3-month -ND and 18-month ND groups were similarly expressed at levels approximately 2.5 folds more than (1.00±0.27 vs. 0.94±0.92 fold change, respectively) the 18-month –ND group (0.39±0.25 fold change).

D. Discussion

In this *in vivo* study we observed modest improvements in recovery following VML repair with testosterone administration in 18-month rats. Specifically, there appeared to be reduced scarring following VML repair in the ND treated animals compared to their -ND counterparts. However, no significant changes were found in force production and force recovery following VML repair as a result of ND administration. In addition, there were no observations to indicate muscle hypertrophy as a result of ND administration. These findings are meaningful when considering anabolic steroid supplementation to VML repair as they alone may not be able to elicit a strong regenerative response and overcome the dysfunctional environment formed by age-related changes in muscle as well as VML injury. The use of testosterone in less traumatic

injury models in both young and aged animals have resulted in beneficial effects to myogenesis but the results have varied from model to model (Beiner, Jokl, Cholewicki, & Panjabi, 1999; Ferry et al., 1999; White, Baltgalvis, Sato, Wilson, & Carson, 2009). Overall, the results of this study suggest that the treatment strategy used would prove more effective if used in conjunction with rehabilitative strategies in order to improve muscle quality and create an environment that is more receptive to treatment and recovery.

The use of ND has been explored extensively as a means to combat muscle wasting as a result of various conditions including disuse atrophy (Camerino et al., 2015; Chen, Lin, Chen, & Hsu, 2013), sarcopenia (Serra et al., 2013), and cachexia (Gold et al., 2006; Mulligan et al., 2005). These studies demonstrated improvements to body weight and lean body mass with the added benefit of improving physical activity in test subjects. However, we observed no such improvements to body weight maintenance within this study nor any improvements to peak tetanic contractions in the repaired TA muscles. Conflicting results across studies using ND as a therapeutic to combat wasting can be attributed to differences in species used in the studies, frequency of ND administration, and dosage, among other variables. Interestingly, it has also been reported that chronic administration of ND had no effects on body weight and in some cases decreased body weight (Oda & El-Ashmawy, 2012; Shahraki, Mirshekari, & Shahraki, 2015), which is in agreeance with our observations. Additionally, consistent with the use of anabolic steroids, chronic ND use has also been reported to cause decreased bioavailable testosterone (Nagata et al., 1999; Takahashi, Tatsugi, & Kohno, 2004), impaired spermatogenesis, depletion of Leydig cells, and reduction in fertility (Svechnikov, Izzo, Landreh, Weisser, & Soder, 2010).

In both recreational and clinical use, testosterone has a well-documented effect of not only inducing skeletal muscle hypertrophy but also increasing the number of satellite cells and myonuclei in young and older subjects in a dose-dependent manner(Bouhlel, Joumaa, & Leoty, 2003; Kadi et al., 2000; Sinha-Hikim et al., 2002; Sinha-Hikim et al., 2006; Sinha-Hikim et al., 2003). Bhasin et al (Serra et al., 2013) also reported increased fiber cross-sectional area after treatment with testosterone, though fiber hypertrophy was limited to the early stages of myofiber regeneration following cardiotoxin injury. The lack of peak tetanic contraction improvement observed in this study may be attributable to a lack of myofiber hypertrophy as evidenced by an absence of change in fiber cross-sectional area between treatment groups in the current study. It is likely that any regenerating fibers fully developed and matured by the end of the 8-week study endpoint. Non-contractile tissue percentages were observed to decrease following ND administration, which is in agreement with previous studies investigating the use of anabolic steroids in the clinic to aid in recovery following rotator cuff repair in sheep and rabbits (Gerber et al., 2015; Gerber, Meyer, Nuss, & Farshad, 2011). Gerber et al. found that administration of nandrolone decanoate immediately following rotator cuff tendon repair resulted in a significant reduction in intramuscular fat accumulation during recovery as well as attenuation of muscle atrophy. Taken together, these findings are confounding, given the ability of anabolic steroids to increase muscle protein synthesis and hypertrophy primarily through the activation of the mammalian target of rapamycin (mTOR) pathway (Basualto-Alarcon, Jorquera, Altamirano, Jaimovich, & Estrada, 2013; Favier, Benoit, & Freyssenet, 2008).

Androgens and anabolic steroids have been shown to regulate Wnt and Notch signaling pathways in skeletal muscle, which are crucial in determining myogenic stem cell fate and regulating muscle satellite cell activation and proliferation (Brack, Conboy, Conboy, Shen, &

Rando, 2008; Brack et al., 2007; Conboy & Rando, 2002; Cossu & Borello, 1999). Further, Notch signaling is essential for the maintenance of satellite cell quiescence and renewal during postnatal myogenesis (Bjornson et al., 2012; Fukada et al., 2011; Mourikis et al., 2012). Our observations of Notch1 gene expression indicated no differences between the 3-month –ND, 18-month –ND, and 18-month ND groups though there was an increasing trend of Notch1 expression following ND administration. In addition, Jag1 expression was similar between the 3-month –ND and 18-month ND groups, with both groups following a decreasing trend in expression. These results are in agreement with a study conducted by Carey et al. in which reduced expression of Notch signaling genes was only observed in human skeletal muscle from older individuals (60-75 years old) compared to muscle from younger men (18-25 years old) (Carey, Farnfield, Tarquinio, & Cameron-Smith, 2007). This suggests that ND administration in aging skeletal muscle following VML repair is able to stimulate Notch signaling. However, the near basal levels of Pax7 expression in the 18-month ND group belie the observed Notch1 expression in the same group. Moreover, the absence of myofiber hypertrophy and *de novo* fiber formation in both ND(-) and ND(+) treated groups suggests poor anabolic sensitivity in aging muscle.

A previous investigation into elderly human subjects' response to anabolic stimuli revealed a reduced response of muscle protein synthesis to anabolic factors in addition to dysfunction in the activation of translation factors contributing to protein metabolism (Guillet et al., 2004). A similar resistant response to anabolic stimuli has been demonstrated in adult and old rats (Baillie & Garlick, 1992; Dardevet, Sornet, Attaix, Baracos, & Grizard, 1994). Based on the regulation of protein synthesis by insulin and amino acids (Shah, Anthony, Kimball, & Jefferson, 2000) and the trophic action of testosterone on IGF-1 (Ashton, Degnan, Daniel, & Francis,

1995), it is possible that the absence of muscle protein accretion observed in this study is due to a defect in the insulin signal transduction pathway. Dysfunction in insulin/IGF-1 signaling occurs via degradation of the insulin receptor substrate-1 (IRS-1) due to its role as a critical node through which subsequent activation of various signaling pathways occur. Aside from insulin/IGF-1 signaling, IRS-1 is also responsible for activation of the PI3k/AKT/mTOR pathway (Bodine et al., 2001; Bohni et al., 1999; Rommel et al., 2001). The mechanism of IRS-1 degradation is still widely debated though a common observation by researchers is that it occurs through a negative feedback loop within the PI3K/AKT/mTOR signaling axis (Haruta et al., 2000; A. V. Lee, Gooch, Oesterreich, Guler, & Yee, 2000; Tzatsos & Kandror, 2006; Zhande, Mitchell, Wu, & Sun, 2002). An alternative mechanism of IRS-1 degradation involves the muscle-specific mitsugumin 53 (MG53; also called TRIM72) marking IRS-1 for ubiquitination (C. S. Lee et al., 2010; Song et al., 2013). The low expression of IRS-1 and increasing trend in TRIM72 expression in the 18-month animals compared to their 3-month counterparts seems to suggest that ubiquitin-mediated degradation of IRS-1 may be occurring and supports the lack of animal growth and functional improvements observed in the study. These findings, however, present a new set of challenges in developing VML repair techniques in the elderly population as insulin/IGF-1 pathway dysfunction is closely associated with insulin resistance in metabolic syndrome and type 2 diabetes (Eckel, Grundy, & Zimmet, 2005).

The results of this study would have benefitted from the addition of earlier time points in order to investigate the mechanistic changes as a result of the VML repair treatment utilized. It has been well-established that muscle satellite cell contribution during regeneration accounts for the majority of the regenerative capacity of skeletal muscle (Conboy & Rando, 2005). During muscle regeneration, the majority of the events take place within the first 7 days (Tidball, 2011)

and as such, the effects of nandrolone on satellite cell activity as well as on myogenic cell cycle regulatory factors such as MRFs (Lu, Jenster, & Epner, 2000) were not captured within this study. An important factor of aging skeletal muscle that was not considered in this study is the role of metabolism and how perturbations to aging skeletal muscle metabolism modulate muscle stem cell function and homeostasis. Of particular interest is nutrition with a focus on calorie restriction as it has been well-documented to be an effective strategy in increasing satellite cell abundance in aging skeletal muscle as well as increasing mitochondrial abundance and inducing conservation of metabolic and longevity regulators (Barger, Walford, & Weindruch, 2003; Cerletti, Jang, Finley, Haigis, & Wagers, 2012; Shyh-Chang, Daley, & Cantley, 2013).

E. Conclusion

In summary, the present study is the first to assess the treatment outcomes from administering nandrolone decanoate in conjunction with a minced muscle loaded decellularized muscle ECM scaffold to repair VML in aging rats. VML repair followed by nandrolone injection reduced collagen I accumulation at the defect site, which was reinforced with the downregulation of the transcription factor for collagen I and upregulation of collagen III. Further, downregulation of transcription factors related to Notch and insulin signaling independent of nandrolone injection but sensitive to age suggested dysfunction within these pathways, which may have caused abnormal cellular events resulting in sub-optimal regenerative conditions.

References

Allouh, M. Z., Jarrar, A. A., Asfour, H. A., Said, R. S., & Shaqoura, E. I. (2017). Sustanon induces dose-independent hypertrophy and satellite cell proliferation in slow oxidative fibers of avian skeletal muscle. *Histol Histopathol, 32*(11), 1151-1159. doi: 10.14670/HH-11-871

Andreollo, N. A., Santos, E. F., Araujo, M. R., & Lopes, L. R. (2012). Rat's age versus human's age: what is the relationship? *Arq Bras Cir Dig, 25*(1), 49-51.

Ashton, W. S., Degnan, B. M., Daniel, A., & Francis, G. L. (1995). Testosterone increases insulin-like growth factor-1 and insulin-like growth factor-binding protein. *Ann Clin Lab Sci, 25*(5), 381-388.

Axell, A. M., MacLean, H. E., Plant, D. R., Harcourt, L. J., Davis, J. A., Jimenez, M., . . . Zajac, J. D. (2006). Continuous testosterone administration prevents skeletal muscle atrophy and enhances resistance to fatigue in orchidectomized male mice. *Am J Physiol Endocrinol Metab, 291*(3), E506-516. doi: 10.1152/ajpendo.00058.2006

Baillie, A. G., & Garlick, P. J. (1992). Attenuated responses of muscle protein synthesis to fasting and insulin in adult female rats. *Am J Physiol, 262*(1 Pt 1), E1-5. doi: 10.1152/ajpendo.1992.262.1.E1

Barger, J. L., Walford, R. L., & Weindruch, R. (2003). The retardation of aging by caloric restriction: its significance in the transgenic era. *Exp Gerontol, 38*(11-12), 1343-1351.

Basualto-Alarcon, C., Jorquera, G., Altamirano, F., Jaimovich, E., & Estrada, M. (2013). Testosterone signals through mTOR and androgen receptor to induce muscle hypertrophy. *Med Sci Sports Exerc, 45*(9), 1712-1720. doi: 10.1249/MSS.0b013e31828cf5f3

Beiner, J. M., Jokl, P., Cholewicki, J., & Panjabi, M. M. (1999). The effect of anabolic steroids and corticosteroids on healing of muscle contusion injury. *Am J Sports Med, 27*(1), 2-9. doi: 10.1177/03635465990270011101

Bjornson, C. R., Cheung, T. H., Liu, L., Tripathi, P. V., Steeper, K. M., & Rando, T. A. (2012). Notch signaling is necessary to maintain quiescence in adult muscle stem cells. *Stem Cells, 30*(2), 232-242. doi: 10.1002/stem.773

Blaauw, B., Schiaffino, S., & Reggiani, C. (2013). Mechanisms modulating skeletal muscle phenotype. *Compr Physiol, 3*(4), 1645-1687. doi: 10.1002/cphy.c130009

Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., . . . Yancopoulos, G. D. (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol, 3*(11), 1014-1019. doi: 10.1038/ncb1101-1014

Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B. F., . . . Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. *Cell, 97*(7), 865-875.

Bouhlel, A., Joumaa, W. H., & Leoty, C. (2003). Nandrolone decanoate reduces changes induced by hindlimb suspension in voltage-dependent tension of rat soleus muscle. *Jpn J Physiol, 53*(2), 77-87.

Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J., & Rando, T. A. (2008). A temporal switch from notch to Wnt signaling in muscle stem cells is necessary for normal adult myogenesis. *Cell Stem Cell, 2*(1), 50-59. doi: 10.1016/j.stem.2007.10.006

Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., & Rando, T. A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science, 317*(5839), 807-810. doi: 10.1126/science.1144090

Camerino, G. M., Desaphy, J. F., De Bellis, M., Capogrosso, R. F., Cozzoli, A., Dinardo, M. M., . . . Pierno, S. (2015). Effects of Nandrolone in the Counteraction of Skeletal Muscle Atrophy in a Mouse Model of Muscle Disuse: Molecular Biology and Functional Evaluation. *PLoS One, 10*(6), e0129686. doi: 10.1371/journal.pone.0129686

Carey, K. A., Farnfield, M. M., Tarquinio, S. D., & Cameron-Smith, D. (2007). Impaired expression of Notch signaling genes in aged human skeletal muscle. *J Gerontol A Biol Sci Med Sci, 62*(1), 9-17.

Carson, J. A., Lee, W. J., McClung, J., & Hand, G. A. (2002). Steroid receptor concentration in aged rat hindlimb muscle: effect of anabolic steroid administration. *J Appl Physiol (1985), 93*(1), 242-250. doi: 10.1152/japplphysiol.01212.2001

Cerletti, M., Jang, Y. C., Finley, L. W., Haigis, M. C., & Wagers, A. J. (2012). Short-term calorie restriction enhances skeletal muscle stem cell function. *Cell Stem Cell, 10*(5), 515-519. doi: 10.1016/j.stem.2012.04.002

Chen, C. T., Lin, S. H., Chen, J. S., & Hsu, Y. J. (2013). Muscle wasting in hemodialysis patients: new therapeutic strategies for resolving an old problem. *ScientificWorldJournal, 2013*, 643954. doi: 10.1155/2013/643954

Coggan, A. R., Spina, R. J., King, D. S., Rogers, M. A., Brown, M., Nemeth, P. M., & Holloszy, J. O. (1992). Histochemical and enzymatic comparison of the gastrocnemius muscle of young and elderly men and women. *J Gerontol, 47*(3), B71-76.

Collino, S., Martin, F. P., Karagounis, L. G., Horcajada, M. N., Moco, S., Franceschi, C., . . . Offord, E. (2013). Musculoskeletal system in the old age and the demand for healthy ageing biomarkers. *Mech Ageing Dev, 134*(11-12), 541-547. doi: 10.1016/j.mad.2013.11.003

Conboy, I. M., & Rando, T. A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell, 3*(3), 397-409.

Conboy, I. M., & Rando, T. A. (2005). Aging, stem cells and tissue regeneration: lessons from muscle. *Cell Cycle, 4*(3), 407-410. doi: 10.4161/cc.4.3.1518

Conley, K. E., Jubrias, S. A., & Esselman, P. C. (2000). Oxidative capacity and ageing in human muscle. *J Physiol, 526 Pt 1*, 203-210.

Corona, B. T., Garg, K., Ward, C. L., McDaniel, J. S., Walters, T. J., & Rathbone, C. R. (2013). Autologous minced muscle grafts: a tissue engineering therapy for the volumetric loss of skeletal muscle. *Am J Physiol Cell Physiol, 305*(7), C761-775. doi: 10.1152/ajpcell.00189.2013

Corona, B. T., Rivera, J. C., Owens, J. G., Wenke, J. C., & Rathbone, C. R. (2015). Volumetric muscle loss leads to permanent disability following extremity trauma. *J Rehabil Res Dev, 52*(7), 785-792. doi: 10.1682/JRRD.2014.07.0165

Corona, B. T., Ward, C. L., Baker, H. B., Walters, T. J., & Christ, G. J. (2014). Implantation of in vitro tissue engineered muscle repair constructs and bladder acellular matrices partially restore in vivo skeletal muscle function in a rat model of volumetric muscle loss injury. *Tissue Eng Part A, 20*(3-4), 705-715. doi: 10.1089/ten.TEA.2012.0761

Cossu, G., & Borello, U. (1999). Wnt signaling and the activation of myogenesis in mammals. *EMBO J, 18*(24), 6867-6872. doi: 10.1093/emboj/18.24.6867

Croley, A. N., Zwetsloot, K. A., Westerkamp, L. M., Ryan, N. A., Pendergast, A. M., Hickner, R. C., . . . Gavin, T. P. (2005). Lower capillarization, VEGF protein, and VEGF mRNA response to acute exercise in the vastus lateralis muscle of aged vs. young women. *J Appl Physiol (1985), 99*(5), 1872-1879. doi: 10.1152/japplphysiol.00498.2005

Dalbo, V. J., Roberts, M. D., Mobley, C. B., Ballmann, C., Kephart, W. C., Fox, C. D., . . . Beck, D. T. (2017). Testosterone and trenbolone enanthate increase mature myostatin protein expression despite increasing skeletal muscle hypertrophy and satellite cell number in rodent muscle. *Andrologia, 49*(3). doi: 10.1111/and.12622

Dardevet, D., Sornet, C., Attaix, D., Baracos, V. E., & Grizard, J. (1994). Insulin-like growth factor-1 and insulin resistance in skeletal muscles of adult and old rats. *Endocrinology, 134*(3), 1475-1484. doi: 10.1210/endo.134.3.8119189

Diel, P., Baadners, D., Schlupmann, K., Velders, M., & Schwarz, J. P. (2008). C2C12 myoblastoma cell differentiation and proliferation is stimulated by androgens and associated with a modulation of myostatin and Pax7 expression. *J Mol Endocrinol, 40*(5), 231-241. doi: 10.1677/JME-07-0175

Donini, L. M., Savina, C., & Cannella, C. (2003). Eating habits and appetite control in the elderly: the anorexia of aging. *Int Psychogeriatr, 15*(1), 73-87.

Eckel, R. H., Grundy, S. M., & Zimmet, P. Z. (2005). The metabolic syndrome. *Lancet, 365*(9468), 1415-1428. doi: 10.1016/S0140-6736(05)66378-7

Fan, C., Jiang, P., Fu, L., Cai, P., Sun, L., & Zeng, B. (2008). Functional reconstruction of traumatic loss of flexors in forearm with gastrocnemius myocutaneous flap transfer. *Microsurgery, 28*(1), 71-75. doi: 10.1002/micr.20449

Favier, F. B., Benoit, H., & Freyssenet, D. (2008). Cellular and molecular events controlling skeletal muscle mass in response to altered use. *Pflugers Arch, 456*(3), 587-600. doi: 10.1007/s00424-007-0423-z

Ferry, A., Noirez, P., Page, C. L., Salah, I. B., Daegelen, D., & Rieu, M. (1999). Effects of anabolic/androgenic steroids on regenerating skeletal muscles in the rat. *Acta Physiol Scand, 166*(2), 105-110. doi: 10.1046/j.1365-201x.1999.00549.x

Fielding, R. A., Vellas, B., Evans, W. J., Bhasin, S., Morley, J. E., Newman, A. B., . . . Zamboni, M. (2011). Sarcopenia: an undiagnosed condition in older adults. Current consensus

definition: prevalence, etiology, and consequences. International working group on sarcopenia. *J Am Med Dir Assoc, 12*(4), 249-256. doi: 10.1016/j.jamda.2011.01.003

Fukada, S., Yamaguchi, M., Kokubo, H., Ogawa, R., Uezumi, A., Yoneda, T., . . . Yamamoto, H. (2011). Hesr1 and Hesr3 are essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers. *Development, 138*(21), 4609-4619. doi: 10.1242/dev.067165

Gentile, N. E., Stearns, K. M., Brown, E. H., Rubin, J. P., Boninger, M. L., Dearth, C. L., . . . Badylak, S. F. (2014). Targeted rehabilitation after extracellular matrix scaffold transplantation for the treatment of volumetric muscle loss. *Am J Phys Med Rehabil, 93*(11 Suppl 3), S79-87. doi: 10.1097/PHM.0000000000000145

Gerber, C., Meyer, D. C., Fluck, M., Benn, M. C., von Rechenberg, B., & Wieser, K. (2015). Anabolic Steroids Reduce Muscle Degeneration Associated With Rotator Cuff Tendon Release in Sheep. *Am J Sports Med, 43*(10), 2393-2400. doi: 10.1177/0363546515596411

Gerber, C., Meyer, D. C., Nuss, K. M., & Farshad, M. (2011). Anabolic steroids reduce muscle damage caused by rotator cuff tendon release in an experimental study in rabbits. *J Bone Joint Surg Am, 93*(23), 2189-2195. doi: 10.2106/JBJS.J.01589

Gheller, B. J., Riddle, E. S., Lem, M. R., & Thalacker-Mercer, A. E. (2016). Understanding Age-Related Changes in Skeletal Muscle Metabolism: Differences Between Females and Males. *Annu Rev Nutr, 36*, 129-156. doi: 10.1146/annurev-nutr-071715-050901

Gold, J., Batterham, M. J., Rekers, H., Harms, M. K., Geurts, T. B., Helmyr, P. M., . . . Investigators, E. S. (2006). Effects of nandrolone decanoate compared with placebo or testosterone on HIV-associated wasting. *HIV Med, 7*(3), 146-155. doi: 10.1111/j.1468-1293.2006.00358.x

Grogan, B. F., Hsu, J. R., & Skeletal Trauma Research, C. (2011). Volumetric muscle loss. *J Am Acad Orthop Surg, 19 Suppl 1*, S35-37.

Guillet, C., Prod'homme, M., Balage, M., Gachon, P., Giraudet, C., Morin, L., . . . Boirie, Y. (2004). Impaired anabolic response of muscle protein synthesis is associated with S6K1 dysregulation in elderly humans. *FASEB J, 18*(13), 1586-1587. doi: 10.1096/fj.03-1341fje

Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P. M., . . . Kobayashi, M. (2000). A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. *Mol Endocrinol, 14*(6), 783-794. doi: 10.1210/mend.14.6.0446

Hughes, D. C., Stewart, C. E., Sculthorpe, N., Dugdale, H. F., Yousefian, F., Lewis, M. P., & Sharples, A. P. (2016). Testosterone enables growth and hypertrophy in fusion impaired myoblasts that display myotube atrophy: deciphering the role of androgen and IGF-I receptors. *Biogerontology, 17*(3), 619-639. doi: 10.1007/s10522-015-9621-9

Jory, A., Le Roux, I., Gayraud-Morel, B., Rocheteau, P., Cohen-Tannoudji, M., Cumano, A., & Tajbakhsh, S. (2009). Numb promotes an increase in skeletal muscle progenitor cells in the embryonic somite. *Stem Cells, 27*(11), 2769-2780. doi: 10.1002/stem.220

Joubert, Y., & Tobin, C. (1995). Testosterone treatment results in quiescent satellite cells being activated and recruited into cell cycle in rat levator ani muscle. *Dev Biol, 169*(1), 286-294. doi: 10.1006/dbio.1995.1144

Kadi, F., Bonnerud, P., Eriksson, A., & Thornell, L. E. (2000). The expression of androgen receptors in human neck and limb muscles: effects of training and self-administration of androgenic-anabolic steroids. *Histochem Cell Biol, 113*(1), 25-29.

Kasukonis, B., Kim, J., Brown, L., Jones, J., Ahmadi, S., Washington, T., & Wolchok, J. (2016). Codelivery of Infusion Decellularized Skeletal Muscle with Minced Muscle Autografts Improved Recovery from Volumetric Muscle Loss Injury in a Rat Model. *Tissue Eng Part A, 22*(19-20), 1151-1163. doi: 10.1089/ten.TEA.2016.0134

Kasukonis, B. M., Kim, J. T., Washington, T. A., & Wolchok, J. C. (2016). Development of an infusion bioreactor for the accelerated preparation of decellularized skeletal muscle scaffolds. *Biotechnol Prog, 32*(3), 745-755. doi: 10.1002/btpr.2257

Kim, J., Kasukonis, B., Dunlap, G., Perry, R., Washington, T., & Wolchok, J. (2019). Regenerative Repair of Volumetric Muscle Loss Injury is Sensitive to Age. *Tissue Eng Part A*. doi: 10.1089/ten.TEA.2019.0034

Kim, J. T., Kasukonis, B. M., Brown, L. A., Washington, T. A., & Wolchok, J. C. (2016). Recovery from volumetric muscle loss injury: A comparison between young and aged rats. *Exp Gerontol, 83*, 37-46. doi: 10.1016/j.exger.2016.07.008

Kovacheva, E. L., Hikim, A. P., Shen, R., Sinha, I., & Sinha-Hikim, I. (2010). Testosterone supplementation reverses sarcopenia in aging through regulation of myostatin, c-Jun NH2-terminal kinase, Notch, and Akt signaling pathways. *Endocrinology, 151*(2), 628-638. doi: 10.1210/en.2009-1177

Lee, A. V., Gooch, J. L., Oesterreich, S., Guler, R. L., & Yee, D. (2000). Insulin-like growth factor I-induced degradation of insulin receptor substrate 1 is mediated by the 26S proteasome and blocked by phosphatidylinositol 3'-kinase inhibition. *Mol Cell Biol, 20*(5), 1489-1496.

Lee, C. S., Yi, J. S., Jung, S. Y., Kim, B. W., Lee, N. R., Choo, H. J., . . . Ko, Y. G. (2010). TRIM72 negatively regulates myogenesis via targeting insulin receptor substrate-1. *Cell Death Differ, 17*(8), 1254-1265. doi: 10.1038/cdd.2010.1

Lee, D. K. (2002). Androgen receptor enhances myogenin expression and accelerates differentiation. *Biochem Biophys Res Commun, 294*(2), 408-413. doi: 10.1016/S0006-291X(02)00504-1

Lin, C. H., Lin, Y. T., Yeh, J. T., & Chen, C. T. (2007). Free functioning muscle transfer for lower extremity posttraumatic composite structure and functional defect. *Plast Reconstr Surg, 119*(7), 2118-2126. doi: 10.1097/01.prs.0000260595.85557.41

Lu, S., Jenster, G., & Epner, D. E. (2000). Androgen induction of cyclin-dependent kinase inhibitor p21 gene: role of androgen receptor and transcription factor Sp1 complex. *Mol Endocrinol, 14*(5), 753-760. doi: 10.1210/mend.14.5.0461

MacKenzie, E. J., Bosse, M. J., Kellam, J. F., Burgess, A. R., Webb, L. X., Swiontkowski, M. F., . . . McCarthy, M. L. (2000). Characterization of patients with high-energy lower extremity trauma. *J Orthop Trauma, 14*(7), 455-466.

Miller, B. F., Robinson, M. M., Bruss, M. D., Hellerstein, M., & Hamilton, K. L. (2012). A comprehensive assessment of mitochondrial protein synthesis and cellular proliferation with age and caloric restriction. *Aging Cell, 11*(1), 150-161. doi: 10.1111/j.1474-9726.2011.00769.x

Mourikis, P., Sambasivan, R., Castel, D., Rocheteau, P., Bizzarro, V., & Tajbakhsh, S. (2012). A critical requirement for notch signaling in maintenance of the quiescent skeletal muscle stem cell state. *Stem Cells, 30*(2), 243-252. doi: 10.1002/stem.775

Mulligan, K., Zackin, R., Clark, R. A., Alston-Smith, B., Liu, T., Sattler, F. R., . . . Infectious Diseases Adult, A. C. T. G. (2005). Effect of nandrolone decanoate therapy on weight and lean body mass in HIV-infected women with weight loss: a randomized, double-blind, placebo-controlled, multicenter trial. *Arch Intern Med, 165*(5), 578-585. doi: 10.1001/archinte.165.5.578

Nagata, S., Kurosawa, M., Mima, K., Nambo, Y., Fujii, Y., Watanabe, G., & Taya, K. (1999). Effects of anabolic steroid (19-nortestosterone) on the secretion of testicular hormones in the stallion. *J Reprod Fertil, 115*(2), 373-379.

Oda, S. S., & El-Ashmawy, I. M. (2012). Adverse effects of the anabolic steroid, boldenone undecylenate, on reproductive functions of male rabbits. *Int J Exp Pathol, 93*(3), 172-178. doi: 10.1111/j.1365-2613.2012.00814.x

Owens, B. D., Kragh, J. F., Jr., Wenke, J. C., Macaitis, J., Wade, C. E., & Holcomb, J. B. (2008). Combat wounds in operation Iraqi Freedom and operation Enduring Freedom. *J Trauma, 64*(2), 295-299. doi: 10.1097/TA.0b013e318163b875

Rommel, C., Bodine, S. C., Clarke, B. A., Rossman, R., Nunez, L., Stitt, T. N., . . . Glass, D. J. (2001). Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat Cell Biol, 3*(11), 1009-1013. doi: 10.1038/ncb1101-1009

Sengupta, P. (2013). The Laboratory Rat: Relating Its Age With Human's. *Int J Prev Med, 4*(6), 624-630.

Serra, C., Tangherlini, F., Rudy, S., Lee, D., Toraldo, G., Sandor, N. L., . . . Bhasin, S. (2013). Testosterone improves the regeneration of old and young mouse skeletal muscle. *J Gerontol A Biol Sci Med Sci, 68*(1), 17-26. doi: 10.1093/gerona/gls083

Shah, O. J., Anthony, J. C., Kimball, S. R., & Jefferson, L. S. (2000). 4E-BP1 and S6K1: translational integration sites for nutritional and hormonal information in muscle. *Am J Physiol Endocrinol Metab, 279*(4), E715-729. doi: 10.1152/ajpendo.2000.279.4.E715

Shahraki, M. R., Mirshekari, H., & Shahraki, A. R. (2015). Chronic Administration of High Doses of Nandrolone Decanoate on the Pituitary-Gonadal Axis in Male Rats. *Int J High Risk Behav Addict, 4*(3), e24419. doi: 10.5812/ijhrba.24419

Shyh-Chang, N., Daley, G. Q., & Cantley, L. C. (2013). Stem cell metabolism in tissue development and aging. *Development, 140*(12), 2535-2547. doi: 10.1242/dev.091777

Sinha-Hikim, I., Artaza, J., Woodhouse, L., Gonzalez-Cadavid, N., Singh, A. B., Lee, M. I., . . . Bhasin, S. (2002). Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. *Am J Physiol Endocrinol Metab, 283*(1), E154-164. doi: 10.1152/ajpendo.00502.2001

Sinha-Hikim, I., Cornford, M., Gaytan, H., Lee, M. L., & Bhasin, S. (2006). Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men. *J Clin Endocrinol Metab, 91*(8), 3024-3033. doi: 10.1210/jc.2006-0357

Sinha-Hikim, I., Roth, S. M., Lee, M. I., & Bhasin, S. (2003). Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. *Am J Physiol Endocrinol Metab, 285*(1), E197-205. doi: 10.1152/ajpendo.00370.2002

Sinha-Hikim, I., Taylor, W. E., Gonzalez-Cadavid, N. F., Zheng, W., & Bhasin, S. (2004). Androgen receptor in human skeletal muscle and cultured muscle satellite cells: up-regulation by androgen treatment. *J Clin Endocrinol Metab, 89*(10), 5245-5255. doi: 10.1210/jc.2004-0084

Song, R., Peng, W., Zhang, Y., Lv, F., Wu, H. K., Guo, J., . . . Xiao, R. P. (2013). Central role of E3 ubiquitin ligase MG53 in insulin resistance and metabolic disorders. *Nature, 494*(7437), 375-379. doi: 10.1038/nature11834

Steyn, F. J., Wan, Y., Clarkson, J., Veldhuis, J. D., Herbison, A. E., & Chen, C. (2013). Development of a methodology for and assessment of pulsatile luteinizing hormone secretion in juvenile and adult male mice. *Endocrinology, 154*(12), 4939-4945. doi: 10.1210/en.2013-1502

Straight, C. R., Brady, A. O., & Evans, E. (2015). Sex-specific relationships of physical activity, body composition, and muscle quality with lower-extremity physical function in older men and women. *Menopause, 22*(3), 297-303. doi: 10.1097/GME.0000000000000313

Suetta, C., Aagaard, P., Magnusson, S. P., Andersen, L. L., Sipila, S., Rosted, A., . . . Kjaer, M. (2007). Muscle size, neuromuscular activation, and rapid force characteristics in elderly men and women: effects of unilateral long-term disuse due to hip-osteoarthritis. *J Appl Physiol (1985), 102*(3), 942-948. doi: 10.1152/japplphysiol.00067.2006

Svechnikov, K., Izzo, G., Landreh, L., Weisser, J., & Soder, O. (2010). Endocrine disruptors and Leydig cell function. *J Biomed Biotechnol, 2010*. doi: 10.1155/2010/684504

Takahashi, M., Tatsugi, Y., & Kohno, T. (2004). Endocrinological and pathological effects of anabolic-androgenic steroid in male rats. *Endocr J, 51*(4), 425-434.

Thalacker-Mercer, A. E., Fleet, J. C., Craig, B. A., & Campbell, W. W. (2010). The skeletal muscle transcript profile reflects accommodative responses to inadequate protein intake in younger and older males. *J Nutr Biochem, 21*(11), 1076-1082. doi: 10.1016/j.jnutbio.2009.09.004

Tidball, J. G. (2011). Mechanisms of muscle injury, repair, and regeneration. *Compr Physiol, 1*(4), 2029-2062. doi: 10.1002/cphy.c100092

Tsika, R. W., Herrick, R. E., & Baldwin, K. M. (1987). Effect of anabolic steroids on skeletal muscle mass during hindlimb suspension. *J Appl Physiol (1985), 63*(5), 2122-2127. doi: 10.1152/jappl.1987.63.5.2122

Tzatsos, A., & Kandror, K. V. (2006). Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. *Mol Cell Biol, 26*(1), 63-76. doi: 10.1128/MCB.26.1.63-76.2006

Vekris, M. D., Beris, A. E., Lykissas, M. G., Korompilias, A. V., Vekris, A. D., & Soucacos, P. N. (2008). Restoration of elbow function in severe brachial plexus paralysis via muscle transfers. *Injury, 39 Suppl 3*, S15-22. doi: 10.1016/j.injury.2008.06.008

Washington, T. A., Brown, L., Smith, D. A., Davis, G., Baum, J., & Bottje, W. (2013). Monocarboxylate transporter expression at the onset of skeletal muscle regeneration. *Physiol Rep, 1*(4), e00075. doi: 10.1002/phy2.75 [doi]

Washington, T. A., Healey, J. M., Thompson, R. W., Lowe, L. L., & Carson, J. A. (2014). Lactate dehydrogenase regulation in aged skeletal muscle: Regulation by anabolic steroids and functional overload. *Exp Gerontol, 57*, 66-74. doi: 10.1016/j.exger.2014.05.003

White, J. P., Baltgalvis, K. A., Sato, S., Wilson, L. B., & Carson, J. A. (2009). Effect of nandrolone decanoate administration on recovery from bupivacaine-induced muscle injury. *J Appl Physiol (1985), 107*(5), 1420-1430. doi: 10.1152/japplphysiol.00668.2009

Zhande, R., Mitchell, J. J., Wu, J., & Sun, X. J. (2002). Molecular mechanism of insulin-induced degradation of insulin receptor substrate 1. *Mol Cell Biol, 22*(4), 1016-1026.

Zilian, O., Saner, C., Hagedorn, L., Lee, H. Y., Sauberli, E., Suter, U., . . . Aguet, M. (2001). Multiple roles of mouse Numb in tuning developmental cell fates. *Curr Biol, 11*(7), 494-501.

Figure Legends

Figure 1: Surgical VML and repair procedure. The surgical site was cleaned and disinfected and a 1-2 cm incision was made to expose the underlying TA muscle (A,B). An 8mm biopsy punch was used to create a VML injury to a depth of 3mm (C). Approximately 20% of the TA muscle was excised (D). MM autografts were prepared using 50% (by mass) of the removed muscle plug and hand mincing with scissors and scalpel (E). The resulting MM paste was used to coat a DSM scaffold prior to implantation into the defect site. The MM coated DSM scaffold was packed into the defect with the scaffold oriented to the direction of muscle alignment (F). The surgical site was closed using both deep and surface sutures (G). Depending on treatment group, either ND or sesame oil depot injections were administered in the vastus lateralis muscle (H) followed by subcutaneous injection of buprenorphine. The uninjured contralateral limb served as a comparative control.

Figure 2: Animal body mass and survival analysis. Animal weights as a percentage of initial weight was tracked throughout the study period (A). Survival curve for animals (n=15/group) over the 8-week study period is presented (B). Growth rate (C) was calculated on a weekly basis and final body weight at the end of 8 weeks was compared between all groups (D).

Figure 3: Gross morphology of ND(-) uninjured, ND(-) repair, ND(+) uninjured, and ND(+) repair TA muscles. Dashed circle approximates the location of the defect site. TA (B), gastrocnemius (C), and EDL (D) wet weights were normalized to animal body weight (g/kg) for each group and presented as means + SD; n=7-13/group; * denotes statistically significant differences between groups ($p<0.05$).

Figure 4: Representative *in vivo* peak force readings for each treatment group (A). Peak isometric torque was normalized to animal body weight (Nmm/kg) for each group (B) and was also calculated relative to uninjured contralateral TA muscle values (% normal) for each animal tested (C). Data is presented as means + SD; n=7-13/group; * denotes statistically significant differences between groups ($p<0.05$).

Figure 5: Animal heart (A) and testes (B) wet weights were normalized to body weight (g/kg). Additionally, serum isolated from whole blood was analyzed for serum testosterone concentrations for all animals tested (C). Data is presented as means + SD; n=7-13/group; * denotes statistically significant differences ($p<0.05$).

Figure 6: TA muscle cross-sections stained with Masson's Trichrome. Representative whole TA sections and magnified (100X). TA cross-sections were also immunostained with myosin heavy chain (red) and counterstained with collagen I (green). Open arrows highlight regions and bands of fibrotic collagen accumulation. Arrow indicates anterior direction. Scale bar = 100μm.

Figure 7: Myofiber cross-sectional area and non-contractile tissue (%) was measured from TA muscle cross-sections immunostained for collagen I and collagen III (A). Images were acquired at a magnification of 100X. Fiber area frequency distributions (B) and group data for non-contractile tissue (%)(C) and fiber cross-sectional area (D) are shown. Scale bar = 100μm. Data is presented as group means + SD; n=4/group; * indicates significant differences based on treatment and # indicates significant differences based on drug (ND) ($p<0.05$).

Figure 8: Comparison of relative gene expression for ND(-) uninjured, ND(-) repair, ND(+) uninjured, and ND(+) repair groups using RT-PCR. The expression of myogenic (Pax7, MyoD, MyoG), ECM and ECM regulatory (collagen I, collagen III, TGF-ß), and inflammatory genes (IL-1ß, TNF-α, IGF-1) were measured using tissue harvested from the VML repair site. Expression is presented as $\log_2$ fold change normalized to ND(-) uninjured muscle expression. Data is presented as group means + SD; n=4/group; * indicates statistically significant differences based on treatment (uninjured vs. repair) and # indicates significant differences based on drug (ND(-) vs. ND(+)) ($p<0.05$).

Figure 9: Comparison of relative gene expression for 3-month ND(-) repair, 18-month ND(-) repair, and 18-month ND(+) repair groups using RT-PCR. The expression of genes related to satellite cell activity (Notch-1, Jag-1) and insulin signaling (TRIM72, FOXO1, IRS-1) were measured using tissue harvested from the site of VML repair. Expressionis presented as $\log_2$ fold change normalized to 3-month ND(-) repair muscle expression. Data is presented as groups means + SD; n=4/group; * indicates statistically significant differences between groups ($p<0.05$).

Figures

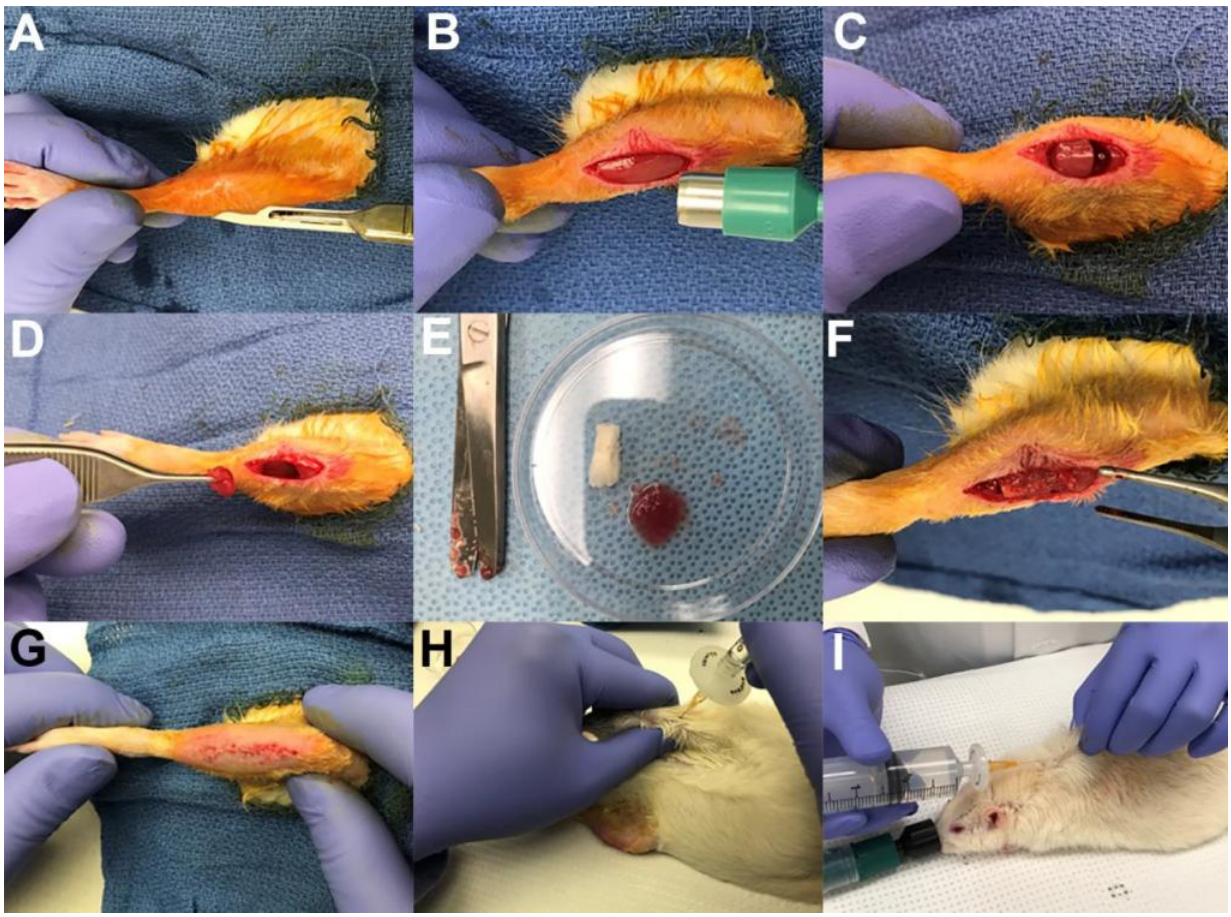

Figure 1

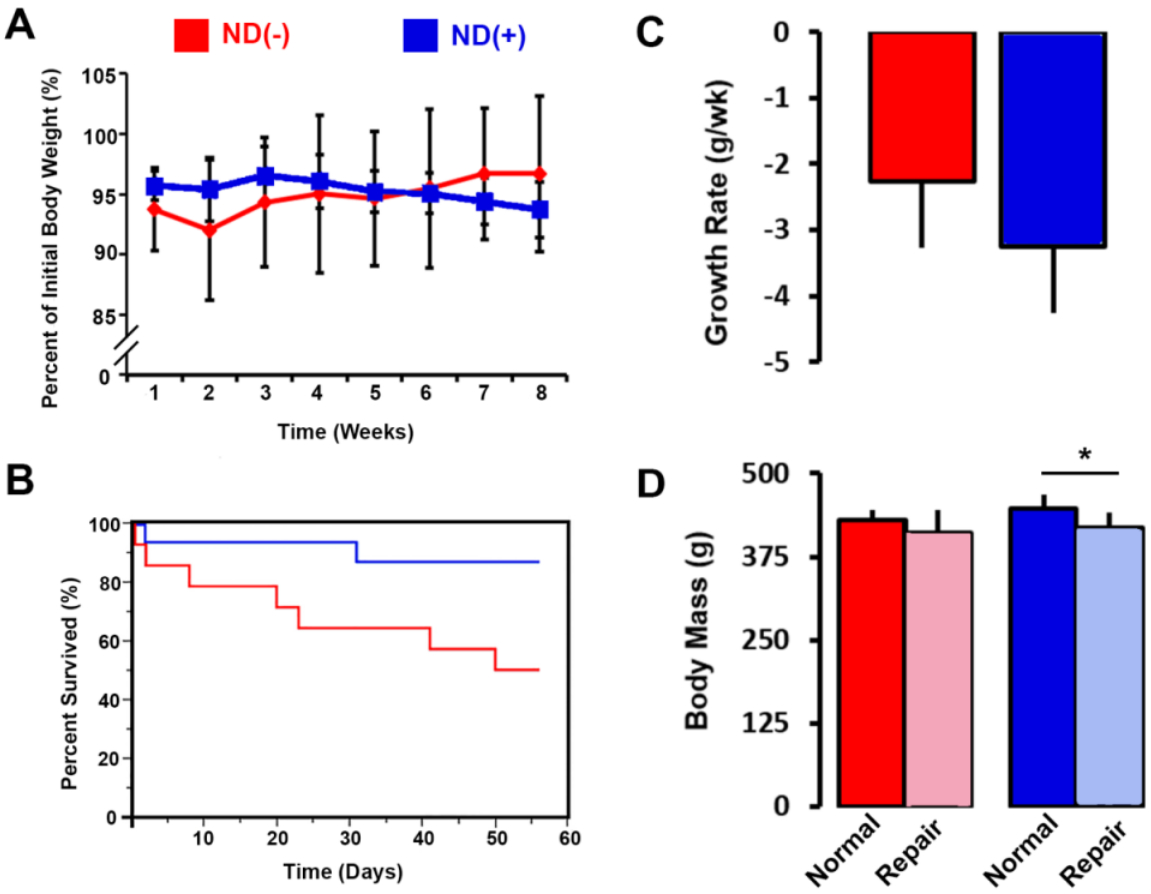

Figure 2

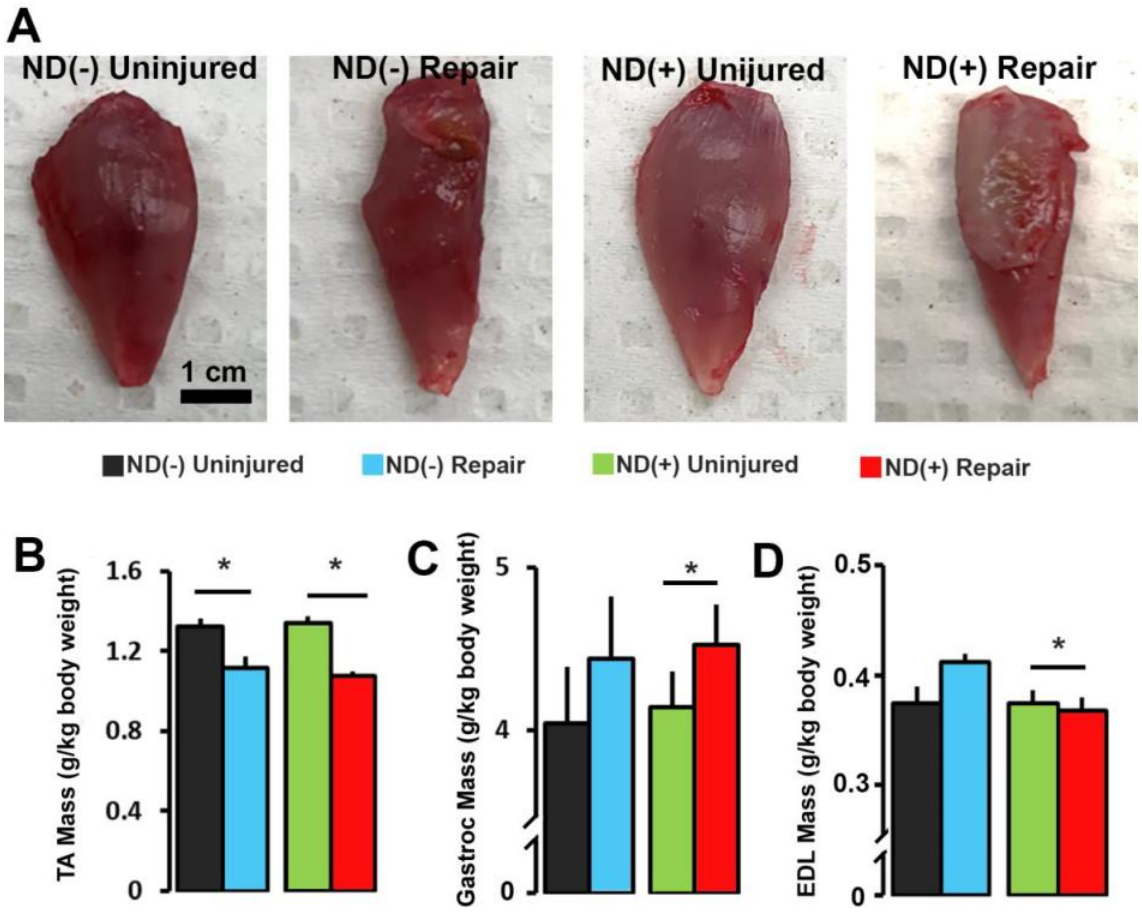

Figure 3

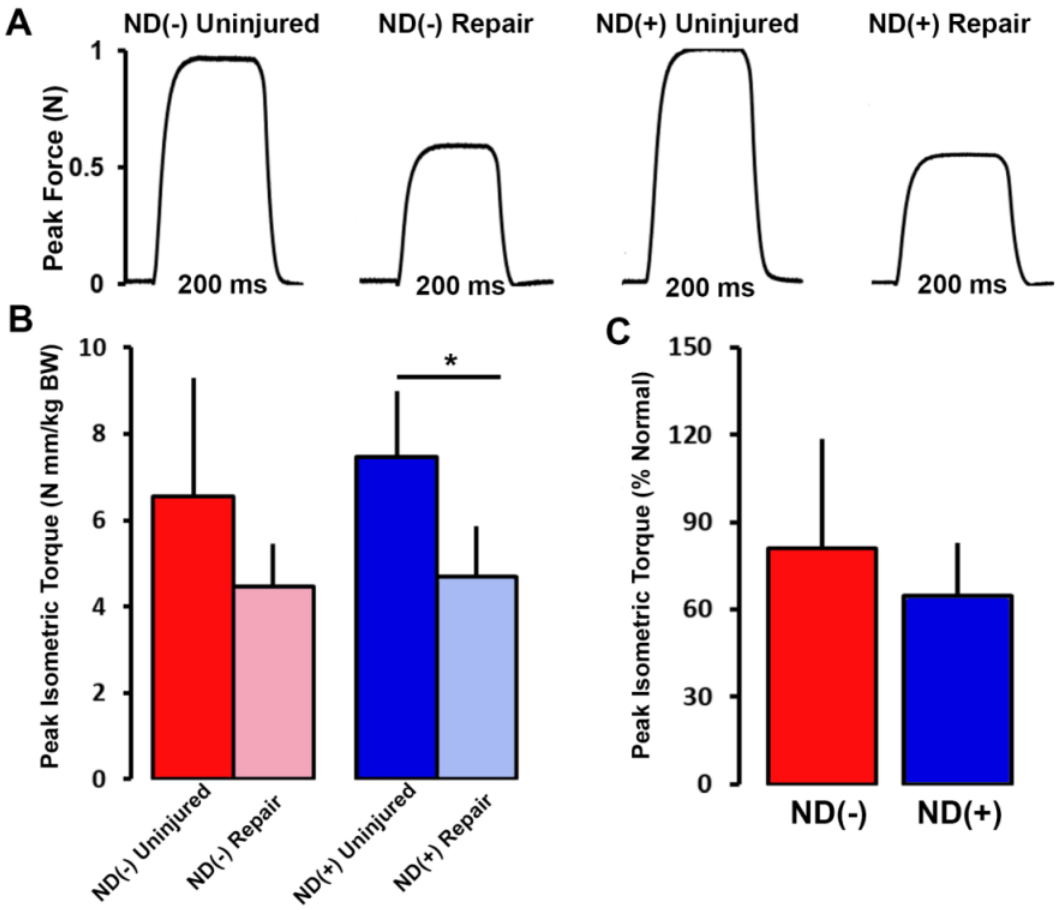

Figure 4

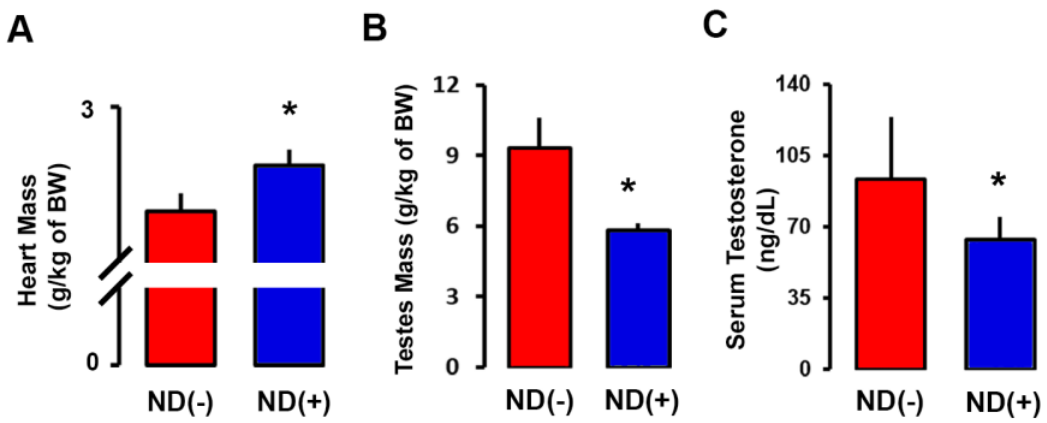

Figure 5

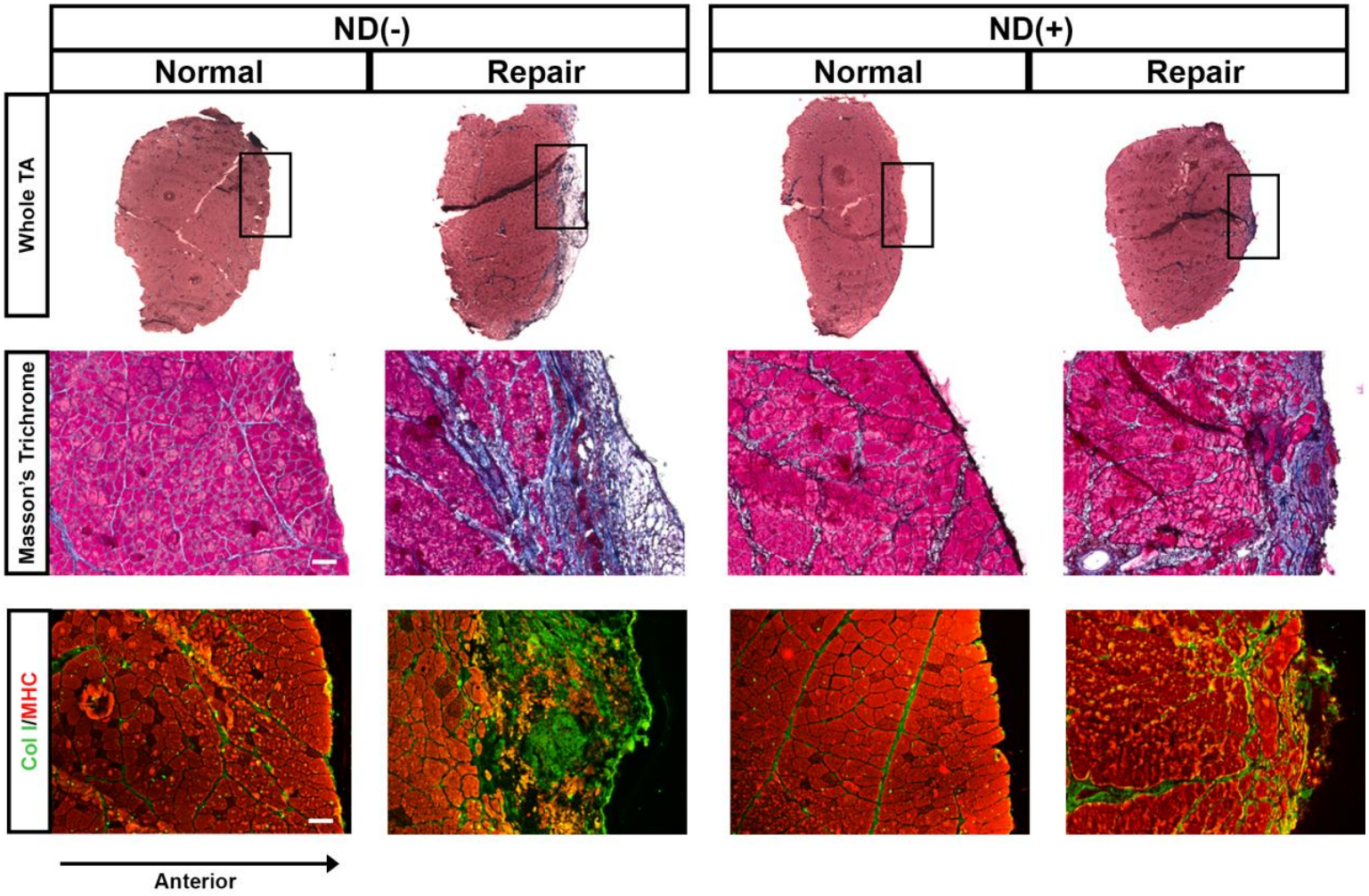

Figure 6

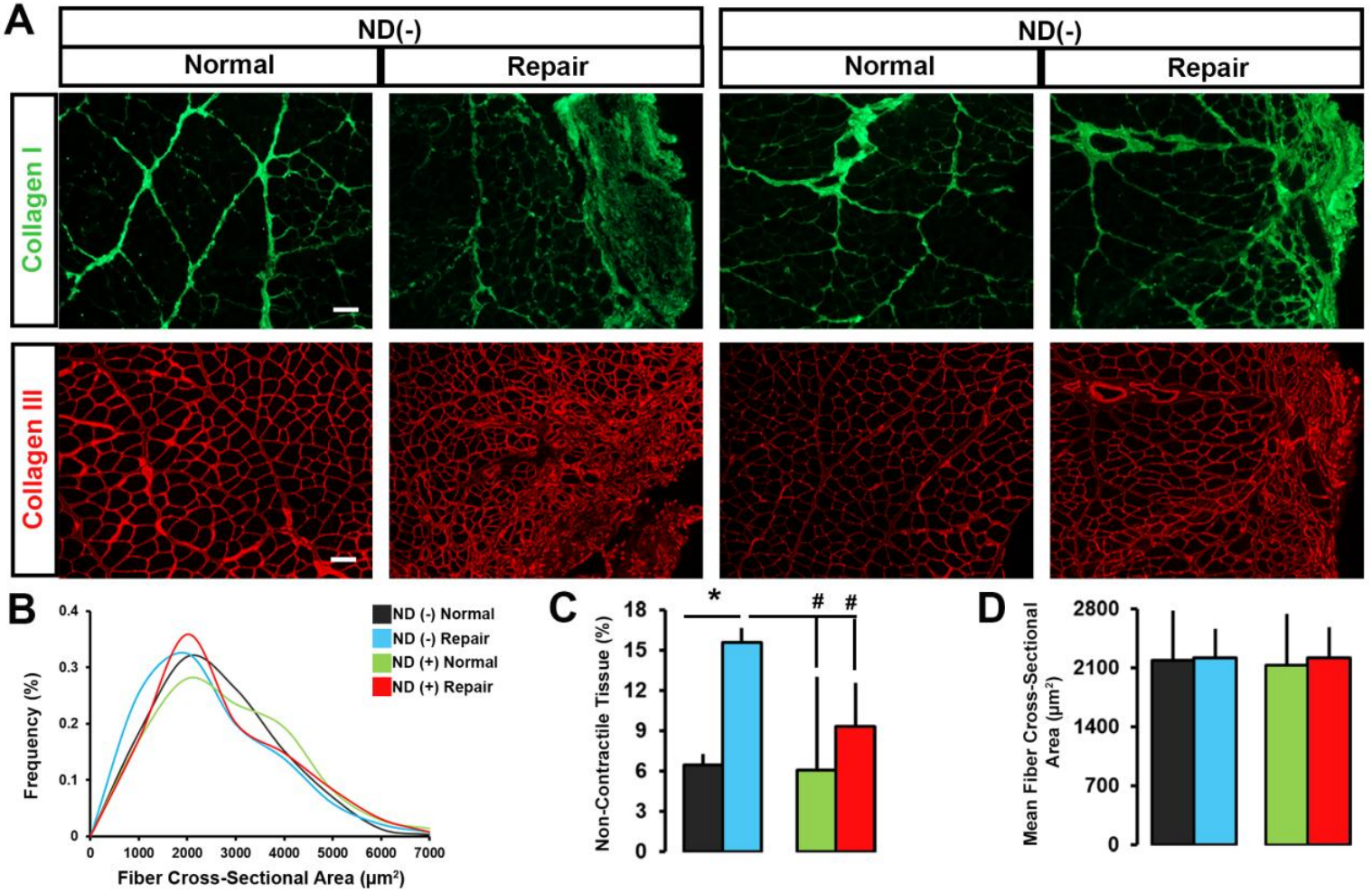

Figure 7

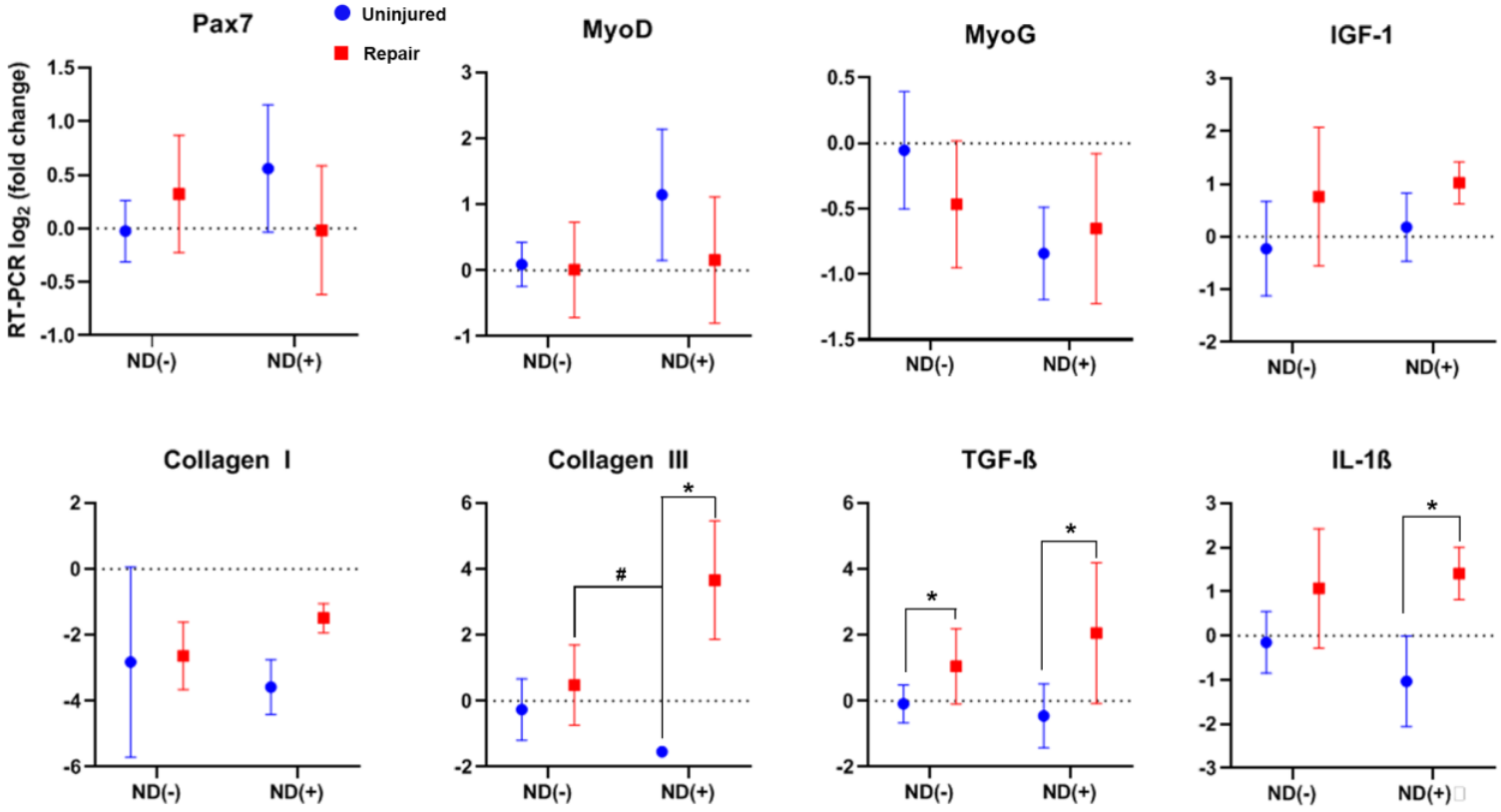

Figure 8

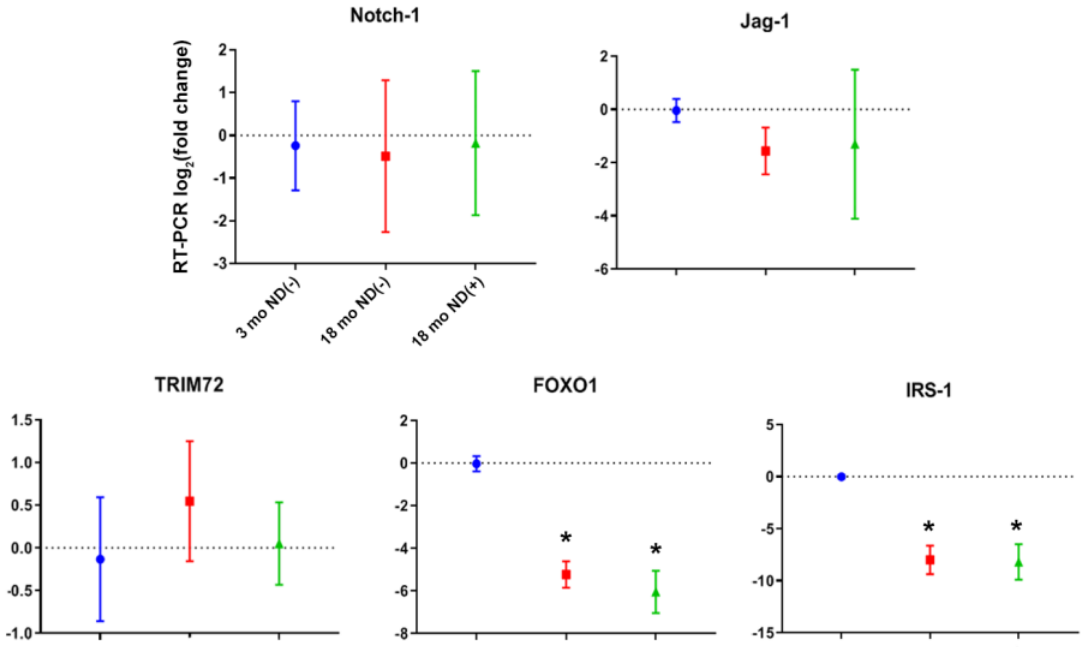

Figure 9

Chapter 5

Conclusion/Perspectives

A. Future Directions

Age-associated changes in skeletal muscle have been well-documented for many decades, with the first studies focusing on morphological changes with aging (Fudel'-Osipova, 1963; Ingelmark & Gustafsson, 1957; Lohmann, Wielepp, & Nocker, 1957) and eventually moving towards understanding the mechanisms of dysfunction in aging muscle that preclude effective regeneration and function (Buliakova, 1987; Herrmann, 1987; Lewis & Schmalbruch, 1995; Sadeh, 1988; Snow, 1977; White & Esser, 1989). With a wealth of foundational research available, it would be of great benefit to incorporate such mechanistic methodologies into aging VML repair investigations. It would not be unreasonable to assume that that the aging VML microenvironment would appear even more dysfunctional than in lesser injury models as VML has been demonstrated to result in an overwhelmingly inflammatory and fibrotic response resembling various pathological conditions such as muscle dystrophy (Greising et al., 2017; Larouche, Greising, Corona, & Aguilar, 2018; Mann et al., 2011). As there is no current research being pursued in age-associated changes in regeneration following VML injury and repair, understanding the broad changes in aging skeletal muscle and how they affect the regenerative process in a VML environment would be indispensable in the pursuit of developing effective repair strategies for VML injuries in aging muscle.

Cell-ECM Interaction

ECM, specifically skeletal muscle derived acellular scaffolds, has been previously demonstrated to provide a pro-myogenic environment, exerting control over cell migration, proliferation, and differentiation leading to constructive tissue remodeling and repair (Faulk, Johnson, Zhang, & Badylak, 2014; Perniconi et al., 2011). The mechanical and compositional characteristics of the ECM directly affect satellite cell activity as well as their self-renewal and proliferative capacity (Gilbert et al., 2010; Montarras et al., 2005; Urciuolo et al., 2013). Myogenic progenitors can also exert control over the local microenvironment (El Fahime, Torrente, Caron, Bresolin, & Tremblay, 2000; Guerin & Holland, 1995) as well as their own cell fate in a self-regulatory manner (Kuang, Gillespie, & Rudnicki, 2008). However, in an altered microenvironment as is observed with aging, there are alterations in ECM composition that may negatively affect cell behavior and function. ECM derived from senescent mesenchymal stem cells (MSCs) was found to have an altered composition of proteins implicated in cell-ECM interactions including laminin, collagens, TGF-ß, and LOXL-1 (Pohlers et al., 2009; Rodriguez, Rodriguez-Sinovas, & Martinez-Gonzalez, 2008). As a result, the altered ECM has been demonstrated to "re-program" gene expression patterns in cells such as fibroblasts and MSCs (H. R. Choi et al., 2011; Choi, Kurtz, & Stamm, 2011). However, the exact mechanism of these interactions are still unknown and warrant further investigation.

Hormonal and Secretory Alterations

There are distinct changes in the levels of circulating hormones associated with the decline in regenerative potential with aging muscle (Thorley et al., 2015). This is due in part to

alterations affecting several hormonal networks involved in inflammation, muscle regeneration, and prtotein synthesis (Vitale, Cesari, & Mari, 2016). Significant reductions in the levels of circulating factors such as testosterone, IL-6, growth hormone (GH), and IGF-1 have been detected in serum samples from aged subjects (Gordon et al., 2014; Kojo et al., 2014; Sattler, 2013). Epidemiological studies have shown that decreased levels of GH secretion are correlated with sarcopenia as well as other hallmarks of aging including increased adipose tissue accumulation, osteopenia, etc. (Thorley et al., 2015; Veldhuis, 2008). GH has also been shown to stimulate IGF-1, which can modulate myoblast proliferation (Brisson & Barton, 2012) and differentiation (Matheny & Nindl, 2011) through the signaling of the MAPK and ERK1/2 pathways (Brisson & Barton, 2012; Troy et al., 2012). Also associated with IGF-1 is the androgen, testosterone, which promotes anabolism and IGF-1 secretion in muscle while reducing adipose-tissue formation (M. A. Gentile et al., 2010). *In vitro* treatment of C2C12 myoblasts with androgen-induced secreted factors (IGF-1Ea and mechano growth factor (MGF)) led to nuclear accumulation of ß-catenin, a pro-myogenic, anti-adipogenic stem cell regulatory factor (M. A. Gentile et al., 2010). In addition to changes in levels of circulating hormones with aging, the local muscle secretome is also affected, which may act on neighboring host cells in an autocrine/paracrine manner (Thorley et al., 2015).

Stress Pathways

As satellite cells age they become more susceptible to apoptosis and senescence, rendering them less resistant to cellular stresses that limit their ability to function properly (Chakkalakal, Jones, Basson, & Brack, 2012; Sousa-Victor et al., 2014). Recently, multiple

groups have identified multiple molecular pathways associated with cellular stress including p38 MAPK (Kodama et al., 2013; Mandal, Blanpain, & Rossi, 2011), JAK-STAT3 (Park, Dahmer, & Quasney, 2012), and $p16^{INK4A}$ (Ramsey & Sharpless, 2006). Increased signaling in these stress pathways not only induce mitochondrial dysfunction and increase the production of reactive oxygen species leading to DNA damage, they have been demonstrated to limit the proliferative capacity of aged satellite cells as well as impairment of cell engraftment and myotube formation (Cerletti, Jang, Finley, Haigis, & Wagers, 2012). Some of these deleterious effects can be reversed, however, as was demonstrated in a study where introducing a p38 MAPK inhibitor to satellite cell cultures *in vitro* restored their proliferative ability while improving satellite cell engraftment and myotube formation when cells treated with the inhibitor were transplanted into immunodeficient mice compared to control groups (Charville et al., 2015). These observations provide a possible cause as to why DSM+MM treatment in a previous study presented in this dissertation was not able to elicit a significant myogenic response as was detected by RT-PCR and improve muscle force production. Nonetheless, pathways such as these that inhibit satellite cell functional output provide future therapeutic targets that may help mitigate cellular stress and rejuvenate their functional capabilities.

B. Evaluation of Myogenesis in the Aging Niche

The current criteria for assessing the success of VML treatment strategies currently rely largely on functional measurements, histology, and the biomolecular profile of the treated muscle. However, for evaluating success in an aging model, the previously mentioned factors and mechanistic methodologies would prove useful in better understanding the response to VML

treatment in aging animals. As demonstrated by the significant differences in treatment outcomes presented in the studies in this dissertation, there is a need for investigating the source of dysfunction in the aging animals that prevent successful VML repair. By incorporating some of the techniques and molecular targets found in traditional gerontological and current VML research into future aged VML studies, a more comprehensive understanding of age-associated impedance to effective tissue repair can be obtained so that more effective treatment strategies addressing the multi-factorial nature of not only VML but age-associated decline in regenerative potential can be resolved.

Histology

Current histological protocols allow for the evaluation of myogenesis, fibrosis and inflammation, angiogenesis, and innervation of skeletal muscle tissue. Immunofluorescence (IF) staining of muscle-specific motor proteins such as sarcomeric myosin heavy chain (MHC), embryonic myosin heavy chain (eMHC), and satellite cell related transcription factors such as Pax7 and Ki67 provide researchers with the ability to assess the degree of myogenesis and myofiber development and maturation while also providing information regarding satellite cell activation and proliferation. Angiogenesis can also be assessed via IF staining of angiogenic proteins such as vascular endothelial growth factor (VEGF), CD31, and anti-hypoxia-induced factor-1α (HIF-1α). A quantitative measure of innervation can be obtained by the use of α-bungarotoxin, a competitive antagonist to the acetylcholine receptors located at neuromuscular junctions. Fibrosis can be assessed qualitatively via IF staining for collagen I or using dye based staining protocols such as Masson's Trichrome or hematoxylin and eosin staining. Semi-

quantitative measures of fibrosis can be obtained using image analysis techniques to calculate the percentage of the muscle area occupied by immunopositive regions of collagen I.

An interesting addition to current histological protocols would be the use of muscle fiber typing as there have been documented changes to fiber type composition in response to external stimuli (Miljkovic, Lim, Miljkovic, & Frontera, 2015). For instance, type I fibers are susceptible to atrophy induced by disuse or denervation whereas type II fibers are affected by pathological conditions such as cancer, diabetes, heart failure, and aging (Wang & Pessin, 2013). Based on information from fiber typing, potential targets within the microenvironment and signaling pathways involved in potentiating the compositional changes in aging muscle fibers can help guide the development of more effective treatment strategies.

Biomolecular Analysis

There are a wide variety of quantitative methods available in the field of tissue engineering and biology that provide information on the biomolecular response in tissues to treatment strategies. In VML, one of the more commonly used analytical methods is RT-PCR for examining gene expression relating to myogenesis, inflammation, angiogenesis, and innervation. One disadvantage of PCR techniques, however, is that it only provides a single temporal snapshot of the biomolecular changes initiated in response to treatment. Moreover, relative gene expression at a certain time point may not account for potential post-transcriptional or post-translational modifications following translocation of the factor to the nucleus. This raises the issue of genotype vs. phenotype and the difficulty in discerning whether the phenotypical changes observed in the tissue are a direct result of the measured transcriptional activity. This

issue however can be resolved with the incorporation of multiple time points, especially short time points, for an improved temporal understanding of the transcriptional response to treatment. As stated previously, the terminal differentiation of myoblasts and myofiber formation culminate at approximately 14 days post. Short time points would provide valuable information regarding satellite cell-mediated regeneration and could provide indications of dysfunction within the myogenic program.

Western blotting is another biomolecular analytical tool providing information on protein expression captured at a single time point. With the ability to detect single proteins of interest, the technique provides researchers with the ability to investigate cell signaling pathways, whose activation or inhibition is paramount for maintaining tissue homeostasis and regulating cellular function. The presence or absence of specific proteins involved signaling pathways will point to dysfunction or inhibition of the pathway, often leading to aberrant changes that result in limited cell functional output (e.g. reduced satellite cell activation, increased apoptosis, cellular senescence) that translate to poor treatment outcomes at the tissue level.

To date, very few groups in the VML field have leveraged the power of whole transcriptome sequencing techniques such as RNASeq (Aguilar et al., 2018). Though the technique has been around for approximately a decade, it was not until recently that it was applied in the field of skeletal muscle tissue engineering. The study performed by Aguilar et al. sequenced minced muscle graft repair tissue and control tissue from 3, 7, 14, 28, 56 days post-injury, providing their group with a detailed and quantitative time course of tissue-level changes in response to regenerative therapy (Aguilar et al., 2018). The one unique aspect of RNASeq that will make it a ubiquitous technique used in research moving forward is the vast amount of data acquired detailing the expression of every single transcript within samples, post-transcriptional

modifications, gene fusion, mutations, temporal changes in gene expression, and differences in expression between treatment groups (Maher et al., 2009). Such a powerful tool will be indispensable in furthering our understanding of major changes occurring within the tissue following VML repair and providing interesting targets for future therapies that may not be captured with traditional biomolecular techniques.

Satellite Cell Engraftment

A question that remains with the aging VML repair studies presented in this dissertation is whether the transplanted cells in the form of the minced muscle graft that is co-delivered with DSM scaffolds actually contribute significantly to tissue repair. Studies have shown that the maintenance of the satellite cell niche improves the survival and engraftment capabilities of transplanted cells into a site of injury. Lesser injury models such as cryoinjury (Grounds & Yablonka-Reuveni, 1993) or myotoxin-induced injury models (Harris, 2003) result in local cell and myofiber death while preserving the satellite cell niche (basal lamina, nerves, vessels, etc.) which in principle should preserve engraftment, survival, and proliferation of transplanted satellite cells. A study performed by Boldrin et al. used genetically modified mice overexpressing ß-galactosidase (ß-gal) in myonuclei and satellite cells in order to identify donor cells within graft muscles (Boldrin, Neal, Zammit, Muntoni, & Morgan, 2012). The group injured muscle through various methods including myotoxin-induced injury and irradiation and found several interesting findings. If the grafted muscle's host satellite cells survived the injury, they were able to more effectively contribute to tissue regeneration compared to the transplanted donor cells. Conversely, if the host cells were destroyed following injury, the transplanted cells

were able to take over tissue repair and contributed to the regeneration of *de novo* muscle tissue. They did observe, however, that if the engraftment of the transplanted cells were not prompt, engraftment was observed to be extremely poor implying that the satellite cell niche comprised of existing myofibers, ECM, and satellite cells are required to allow for donor cell engraftment and subsequent regenerative events to occur (Boldrin et al., 2012). These results suggest that transplanted cells in not only VML but the aging satellite cell niche would not be effective in contributing to myogenesis due to the VML-associated bulk loss of tissue (muscle tissue, nerves, vasculature, basal lamina, satellite cells) and age-associated perturbations to the niche as mentioned previously. It would be interesting to definitively demonstrate the engraftment capabilities of minced muscle grafts in aging VML models because it would provide specific targets within the niche that could be modulated or enhanced in order to augment tissue repair.

Rehabilitation/Exercise

Exercise has been demonstrated in both rodents (Song, Kwak, & Lawler, 2006) and humans (Melov, Tarnopolsky, Beckman, Felkey, & Hubbard, 2007) to reverse or mitigate several age-associated changes in skeletal muscle. In both young(Carroll et al., 2015) and aged (Gosselin, Adams, Cotter, McCormick, & Thomas, 1998) rats, exercise has been demonstrated to reduce the amount of collagen cross-linking allowing for improved mechanical properties and mechanotransduction to resident satellite cells (Garg & Boppart, 2016), improved metabolism (Schultz et al., 2013), and increased satellite cell proliferation and differentiation (Smith & Merry, 2012). Until now, there have been only three published clinical reports in which decellularized ECM scaffolds were used to treat VML injuries combined with physical therapy

pre- and post-repair (N. E. Gentile et al., 2014; Mase et al., 2010; Sicari et al., 2014). However, these studies reported mixed results in improvements in contractile output in the affected muscles. Interestingly, Corona et al. reported a significant increase (~100%) in the recovery of TA functional output following minced muscle graft repair combined with running wheel exercise when compared to minced graft repair only animals (Corona et al., 2013). However, Aurora et al. reported contrasting results when using an acellular porcine urinary bladder matrix in conjunction with rehabilitation therapy (Aurora, Roe, Corona, & Walters, 2015). Not only was the scaffold found to be unsuitable for regenerative purposes, physical rehabilitation did not improve functional response nor tissue remodeling (Aurora et al., 2015). Results from both studies suggest that in order for physical activity/rehabilitation to improve muscle function, the regenerative therapy used needs to be able to elicit a regenerative response resulting in the formation of *de novo* myofibers so that they may contribute to overall force production in the muscle.

C. Remaining Challenges in Aging Myogenesis

Androgen Insensitivity

Despite the fact that as a cohort aging men have lower levels of serum testosterone, many older men have normal to low-normal levels of testosterone, suggesting they are insensitive to the anabolic effects of testosterone (Kuhne, Mikulas, Paul, & Weidinger, 1989). This may be due in part to the observed reduction in androgen receptor numbers and weak affinity for the receptor associated with aging rats (Bhasin & Bremner, 1997; Haji, Kato, Nawata, & Ibayashi, 1981). In stark contrast in aging human males, there is an increased sensitivity to androgen (Greenstein,

1979). In the last of the VML studies presented in this dissertation, the potent anabolic steroid nandrolone decanoate (ND) was administered in combination with DSM+MM for VML repair. The results from this study are consistent with what has been seen in aging rats and their insensitivity to androgens. There was no improvement in muscle functional output nor maintenance of body mass compared to control groups not receiving ND. However, there were improvements in animal survival rates and reduced fibrosis at the site of VML injury associated with the ND receiving repair groups. This could suggest that an aged rat model of VML may not be the appropriate model for exploring the regenerative effects of androgen/anabolic steroid therapy as there is an inherent bias towards insensitivity. Another possibility is that the anabolic steroid that was used in the study was not appropriate as chronic ND abused has been shown to reduce bioavailable testosterone *in vivo* (Barone et al., 2017). Further investigations exploring the intrinsic and extrinsic (environmental) changes in response to androgen/anabolic supplementation needs to be conducted to determine whether this form of therapy can be meaningful in long-term VML recovery and enhance regeneration in aging skeletal muscle.

Innervation

One of the biggest challenges in skeletal muscle tissue engineering, especially in VML and aging VML research, is the permanent denervation of distal myofibers due to the bulk loss of the local muscle niche as well as excessive fibrotic deposition in the injury site. Though decellularlized scaffolds have the porous structure needed to accommodate proper cell migration and vascularization to occur, re-innervation can be a challenge if the distance between the ends of the transected nerves is too large or if there is lack of available neighboring nerve bundles to

attach to (Kuiken, Barlow, Hargrove, & Dumanian, 2017). Complicating neurogenesis with age are a confluence of changes that lead to poor innervation. These include decrease nerve conduction velocity, sensory discrimination, and decline in the amount of trophic and tropic factors secreted by Schwann cells (Verdu, Ceballos, Vilches, & Navarro, 2000). Perhaps one of the major changes affecting nerve regeneration in aging muscle is metabolism. The progressive reduction in neural blood supply (Kihara, Nickander, & Low, 1991) leads to low availability of nutrients supporting mitochondrial ATP production which is needed for axonal transport and polymerization of microtubules and neurofilaments (Brunetti, Miscena, Salviati, & Gaiti, 1987; Frolkis, Tanin, Marcinko, Kulchitsky, & Yasechko, 1985; McQuarrie, Brady, & Lasek, 1989). Secondary effects are decreased membrane excitability and action potential propagation, which may explain decrease in muscle output associated with aging (Adinolfi, Yamuy, Morales, & Chase, 1991).

Fortunately, a number of these adverse changes in the aging environment can be attenuated through the use of physical activity as previously mentioned. However, the use of rehabilitation/physical therapy regimens have yet to be explored in aging VML studies but these treatments have been demonstrated to be effective in young VML models (Quarta et al., 2018; Quarta et al., 2017). By pursuing an aggressive exercise regimen along with their repair strategy, Quarta et al. observed enhanced maturation of innervation and force production at 30 days following VML treatment. Further, the group also observed increased vascularization and reduced fibrosis in *de novo* tissue generated from repair (Quarta et al., 2017). The discrepancy between Quarta et al. and other groups that may have not observed such improvements may lie in the treatment methodologies used. Quarta et al. used multi-cellular (freshly isolated satellite cells and other muscle resident cells) tubular ECM-based hydrogels whereas the previously

mentioned studies either used pure cell-based (minced muscle graft) or scaffold based (decellularized ECM) for treatment. Regardless, Quarta et al.'s results demonstrate the synergistic effects of physical exercise and regenerative therapy in VML repair and presents a very promising therapeutic strategy that has the possibility to be translated into the clinic fairly easily. Moreover, these results provide an additional treatment methodology that can be easily incorporated into future aging VML repair research that has the capability of addressing many of the factors, not just innervation, in the aging microenvironment that preclude effective regeneration.

Clinical Designation of Volumetric Muscle Loss

Due to the lack of a clinical standard of care for volumetric muscle loss injuries, the majority of extremity traumas involving massive soft tissue loss go undocumented as VML injuries and are categorized rather as varying degrees of open fractures (Lin, Lin, Yeh, & Chen, 2007), compartment syndrome (Badge & Hemmady, 2011), or general limb injury (Dziki et al., 2016). Current emphasis in the clinic for severe extremity trauma is addressing bone healing, despite the fact that upon successful limb salvage following bone repair there exists soft-tissue associated functional deficits leading to chronic disability. This lack of VML documentation and limited number of relevant clinical case studies act as hindrances in the development of clinically relevant treatments for the future. Due to the disconnect between clinicians and academic researchers, a shared database where clinical case studies can be shared between the two groups may prove invaluable in identifying real-world VML trauma and provide researchers with new insights that may impact future development of therapies and treatments targeting such traumas.

References

Adinolfi, A. M., Yamuy, J., Morales, F. R., & Chase, M. H. (1991). Segmental demyelination in peripheral nerves of old cats. *Neurobiol Aging, 12*(2), 175-179.

Aguilar, C. A., Greising, S. M., Watts, A., Goldman, S. M., Peragallo, C., Zook, C., . . . Corona, B. T. (2018). Multiscale analysis of a regenerative therapy for treatment of volumetric muscle loss injury. *Cell Death Discov, 4*, 33. doi: 10.1038/s41420-018-0027-8

Aurora, A., Roe, J. L., Corona, B. T., & Walters, T. J. (2015). An acellular biologic scaffold does not regenerate appreciable de novo muscle tissue in rat models of volumetric muscle loss injury. *Biomaterials, 67*, 393-407. doi: 10.1016/j.biomaterials.2015.07.040

Badge, R., & Hemmady, M. (2011). Forearm Compartment Syndrome following Thrombolytic Therapy for Massive Pulmonary Embolism: A Case Report and Review of Literature. *Case Rep Orthop, 2011*, 678525. doi: 10.1155/2011/678525

Barone, R., Pitruzzella, A., Marino Gammazza, A., Rappa, F., Salerno, M., Barone, F., . . . Pomara, C. (2017). Nandrolone decanoate interferes with testosterone biosynthesis altering blood-testis barrier components. *J Cell Mol Med, 21*(8), 1636-1647. doi: 10.1111/jcmm.13092

Bhasin, S., & Bremner, W. J. (1997). Clinical review 85: Emerging issues in androgen replacement therapy. *J Clin Endocrinol Metab, 82*(1), 3-8. doi: 10.1210/jcem.82.1.3640

Boldrin, L., Neal, A., Zammit, P. S., Muntoni, F., & Morgan, J. E. (2012). Donor satellite cell engraftment is significantly augmented when the host niche is preserved and endogenous satellite cells are incapacitated. *Stem Cells, 30*(9), 1971-1984. doi: 10.1002/stem.1158

Brisson, B. K., & Barton, E. R. (2012). Insulin-like growth factor-I E-peptide activity is dependent on the IGF-I receptor. *PLoS One, 7*(9), e45588. doi: 10.1371/journal.pone.0045588

Brunetti, M., Miscena, A., Salviati, A., & Gaiti, A. (1987). Effect of aging on the rate of axonal transport of choline-phosphoglycerides. *Neurochem Res, 12*(1), 61-65.

Buliakova, N. V. (1987). [The role of tissue regulators in skeletal muscle regeneration in animals of various ages]. *Dokl Akad Nauk SSSR, 296*(3), 763-765.

Carroll, C. C., Martineau, K., Arthur, K. A., Huynh, R. T., Volper, B. D., & Broderick, T. L. (2015). The effect of chronic treadmill exercise and acetaminophen on collagen and cross-linking in rat skeletal muscle and heart. *Am J Physiol Regul Integr Comp Physiol, 308*(4), R294-299. doi: 10.1152/ajpregu.00374.2014

Cerletti, M., Jang, Y. C., Finley, L. W., Haigis, M. C., & Wagers, A. J. (2012). Short-term calorie restriction enhances skeletal muscle stem cell function. *Cell Stem Cell, 10*(5), 515-519. doi: 10.1016/j.stem.2012.04.002

Chakkalakal, J. V., Jones, K. M., Basson, M. A., & Brack, A. S. (2012). The aged niche disrupts muscle stem cell quiescence. *Nature, 490*(7420), 355-360. doi: 10.1038/nature11438

Charville, G. W., Cheung, T. H., Yoo, B., Santos, P. J., Lee, G. K., Shrager, J. B., & Rando, T. A. (2015). Ex Vivo Expansion and In Vivo Self-Renewal of Human Muscle Stem Cells. *Stem Cell Reports, 5*(4), 621-632. doi: 10.1016/j.stemcr.2015.08.004

Choi, H. R., Cho, K. A., Kang, H. T., Lee, J. B., Kaeberlein, M., Suh, Y., . . . Park, S. C. (2011). Restoration of senescent human diploid fibroblasts by modulation of the extracellular matrix. *Aging Cell, 10*(1), 148-157. doi: 10.1111/j.1474-9726.2010.00654.x

Choi, Y. H., Kurtz, A., & Stamm, C. (2011). Mesenchymal stem cells for cardiac cell therapy. *Hum Gene Ther, 22*(1), 3-17. doi: 10.1089/hum.2010.211

Corona, B. T., Garg, K., Ward, C. L., McDaniel, J. S., Walters, T. J., & Rathbone, C. R. (2013). Autologous minced muscle grafts: a tissue engineering therapy for the volumetric loss of skeletal muscle. *Am J Physiol Cell Physiol, 305*(7), C761-775. doi: 10.1152/ajpcell.00189.2013

Dziki, J., Badylak, S., Yabroudi, M., Sicari, B., Ambrosio, F., Stearns, K., . . . Rubin, J. P. (2016). An acellular biologic scaffold treatment for volumetric muscle loss: results of a 13-patient cohort study. *NPJ Regen Med, 1*, 16008. doi: 10.1038/npjregenmed.2016.8

El Fahime, E., Torrente, Y., Caron, N. J., Bresolin, M. D., & Tremblay, J. P. (2000). In vivo migration of transplanted myoblasts requires matrix metalloproteinase activity. *Exp Cell Res, 258*(2), 279-287. doi: 10.1006/excr.2000.4962

Faulk, D. M., Johnson, S. A., Zhang, L., & Badylak, S. F. (2014). Role of the extracellular matrix in whole organ engineering. *J Cell Physiol, 229*(8), 984-989. doi: 10.1002/jcp.24532

Frolkis, V. V., Tanin, S. A., Marcinko, V. I., Kulchitsky, O. K., & Yasechko, A. V. (1985). Axoplasmic transport of substances in motoneuronal axons of the spinal cord in old age. *Mech Ageing Dev, 29*(1), 19-28.

Fudel'-Osipova, S. I. (1963). [Some biomorphological regularities in aging of skeletal muscles]. *Vestn Akad Med Nauk SSSR, 18*, 60-70.

Garg, K., & Boppart, M. D. (2016). Influence of exercise and aging on extracellular matrix composition in the skeletal muscle stem cell niche. *J Appl Physiol (1985), 121*(5), 1053-1058. doi: 10.1152/japplphysiol.00594.2016

Gentile, M. A., Nantermet, P. V., Vogel, R. L., Phillips, R., Holder, D., Hodor, P., . . . Ray, W. J. (2010). Androgen-mediated improvement of body composition and muscle function involves a novel early transcriptional program including IGF1, mechano growth factor, and induction of {beta}-catenin. *J Mol Endocrinol, 44*(1), 55-73. doi: 10.1677/JME-09-0048

Gentile, N. E., Stearns, K. M., Brown, E. H., Rubin, J. P., Boninger, M. L., Dearth, C. L., . . . Badylak, S. F. (2014). Targeted rehabilitation after extracellular matrix scaffold transplantation for the treatment of volumetric muscle loss. *Am J Phys Med Rehabil, 93*(11 Suppl 3), S79-87. doi: 10.1097/PHM.0000000000000145

Gilbert, P. M., Havenstrite, K. L., Magnusson, K. E., Sacco, A., Leonardi, N. A., Kraft, P., . . . Blau, H. M. (2010). Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. *Science, 329*(5995), 1078-1081. doi: 10.1126/science.1191035

Gordon, S. E., Kraemer, W. J., Looney, D. P., Flanagan, S. D., Comstock, B. A., & Hymer, W. C. (2014). The influence of age and exercise modality on growth hormone bioactivity in women. *Growth Horm IGF Res, 24*(2-3), 95-103. doi: 10.1016/j.ghir.2014.03.005

Gosselin, L. E., Adams, C., Cotter, T. A., McCormick, R. J., & Thomas, D. P. (1998). Effect of exercise training on passive stiffness in locomotor skeletal muscle: role of extracellular matrix. *J Appl Physiol (1985), 85*(3), 1011-1016. doi: 10.1152/jappl.1998.85.3.1011

Greenstein, B. D. (1979). Androgen receptors in the rat brain, anterior pituitary gland and ventral prostate gland: effects of orchidectomy and ageing. *J Endocrinol, 81*(1), 75-81.

Greising, S. M., Rivera, J. C., Goldman, S. M., Watts, A., Aguilar, C. A., & Corona, B. T. (2017). Unwavering Pathobiology of Volumetric Muscle Loss Injury. *Sci Rep, 7*(1), 13179. doi: 10.1038/s41598-017-13306-2

Grounds, M. D., & Yablonka-Reuveni, Z. (1993). Molecular and cell biology of skeletal muscle regeneration. *Mol Cell Biol Hum Dis Ser, 3*, 210-256.

Guerin, C. W., & Holland, P. C. (1995). Synthesis and secretion of matrix-degrading metalloproteases by human skeletal muscle satellite cells. *Dev Dyn, 202*(1), 91-99. doi: 10.1002/aja.1002020109

Haji, M., Kato, K. I., Nawata, H., & Ibayashi, H. (1981). Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat. *Brain Res, 204*(2), 373-386.

Harris, J. B. (2003). Myotoxic phospholipases A2 and the regeneration of skeletal muscles. *Toxicon, 42*(8), 933-945. doi: 10.1016/j.toxicon.2003.11.011

Herrmann, V. (1987). [Histogenesis, degeneration, regeneration and adaptation, aging and compensation in the skeletal muscles]. *Zentralbl Allg Pathol, 133*(5), 391-411.

Ingelmark, B. E., & Gustafsson, L. (1957). The aging of calf muscles in women; a morphological and roentgenological study. *Acta Morphol Neerl Scand, 1*(2), 173-187.

Kihara, M., Nickander, K. K., & Low, P. A. (1991). The effect of aging on endoneurial blood flow, hyperemic response and oxygen-free radicals in rat sciatic nerve. *Brain Res, 562*(1), 1-5.

Kodama, R., Kato, M., Furuta, S., Ueno, S., Zhang, Y., Matsuno, K., . . . Kamata, T. (2013). ROS-generating oxidases Nox1 and Nox4 contribute to oncogenic Ras-induced premature senescence. *Genes Cells, 18*(1), 32-41. doi: 10.1111/gtc.12015

Kojo, G., Yoshida, T., Ohkawa, S., Odamaki, M., Kato, A., Takita, T., . . . Kumagai, H. (2014). Association of serum total testosterone concentration with skeletal muscle mass in men under hemodialysis. *Int Urol Nephrol, 46*(5), 985-991. doi: 10.1007/s11255-013-0543-7

Kuang, S., Gillespie, M. A., & Rudnicki, M. A. (2008). Niche regulation of muscle satellite cell self-renewal and differentiation. *Cell Stem Cell, 2*(1), 22-31. doi: 10.1016/j.stem.2007.12.012

Kuhne, K. D., Mikulas, J., Paul, W., & Weidinger, V. (1989). [Measuring the age dependent psychophysical functional status of elderly humans]. *Z Arztl Fortbild (Jena), 83*(8), 419-422.

Kuiken, T. A., Barlow, A. K., Hargrove, L., & Dumanian, G. A. (2017). Targeted Muscle Reinnervation for the Upper and Lower Extremity. *Tech Orthop, 32*(2), 109-116. doi: 10.1097/BTO.0000000000000194

Larouche, J., Greising, S. M., Corona, B. T., & Aguilar, C. A. (2018). Robust inflammatory and fibrotic signaling following volumetric muscle loss: a barrier to muscle regeneration. *Cell Death Dis, 9*(3), 409. doi: 10.1038/s41419-018-0455-7

Lewis, D. M., & Schmalbruch, H. (1995). Effects of age on aneural regeneration of soleus muscle in rat. *J Physiol, 488 (Pt 2)*, 483-492.

Lin, C. H., Lin, Y. T., Yeh, J. T., & Chen, C. T. (2007). Free functioning muscle transfer for lower extremity posttraumatic composite structure and functional defect. *Plast Reconstr Surg, 119*(7), 2118-2126. doi: 10.1097/01.prs.0000260595.85557.41

Lohmann, D., Wielepp, G., & Nocker, J. (1957). [Changes of the skeletal muscles with age in man]. *Z Alternsforsch, 10*(3), 233-238.

Maher, C. A., Kumar-Sinha, C., Cao, X., Kalyana-Sundaram, S., Han, B., Jing, X., . . . Chinnaiyan, A. M. (2009). Transcriptome sequencing to detect gene fusions in cancer. *Nature, 458*(7234), 97-101. doi: 10.1038/nature07638

Mandal, P. K., Blanpain, C., & Rossi, D. J. (2011). DNA damage response in adult stem cells: pathways and consequences. *Nat Rev Mol Cell Biol, 12*(3), 198-202. doi: 10.1038/nrm3060

Mann, C. J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A. L., & Munoz-Canoves, P. (2011). Aberrant repair and fibrosis development in skeletal muscle. *Skelet Muscle, 1*(1), 21. doi: 10.1186/2044-5040-1-21

Mase, V. J., Jr., Hsu, J. R., Wolf, S. E., Wenke, J. C., Baer, D. G., Owens, J., . . . Walters, T. J. (2010). Clinical application of an acellular biologic scaffold for surgical repair of a large, traumatic quadriceps femoris muscle defect. *Orthopedics, 33*(7), 511. doi: 10.3928/01477447-20100526-24

Matheny, R. W., Jr., & Nindl, B. C. (2011). Loss of IGF-IEa or IGF-IEb impairs myogenic differentiation. *Endocrinology, 152*(5), 1923-1934. doi: 10.1210/en.2010-1279

McQuarrie, I. G., Brady, S. T., & Lasek, R. J. (1989). Retardation in the slow axonal transport of cytoskeletal elements during maturation and aging. *Neurobiol Aging, 10*(4), 359-365.

Melov, S., Tarnopolsky, M. A., Beckman, K., Felkey, K., & Hubbard, A. (2007). Resistance exercise reverses aging in human skeletal muscle. *PLoS One, 2*(5), e465. doi: 10.1371/journal.pone.0000465

Miljkovic, N., Lim, J. Y., Miljkovic, I., & Frontera, W. R. (2015). Aging of skeletal muscle fibers. *Ann Rehabil Med, 39*(2), 155-162. doi: 10.5535/arm.2015.39.2.155

Montarras, D., Morgan, J., Collins, C., Relaix, F., Zaffran, S., Cumano, A., . . . Buckingham, M. (2005). Direct isolation of satellite cells for skeletal muscle regeneration. *Science, 309*(5743), 2064-2067. doi: 10.1126/science.1114758

Park, S. K., Dahmer, M. K., & Quasney, M. W. (2012). MAPK and JAK-STAT signaling pathways are involved in the oxidative stress-induced decrease in expression of surfactant protein genes. *Cell Physiol Biochem, 30*(2), 334-346. doi: 10.1159/000339068

Perniconi, B., Costa, A., Aulino, P., Teodori, L., Adamo, S., & Coletti, D. (2011). The pro-myogenic environment provided by whole organ scale acellular scaffolds from skeletal muscle. *Biomaterials, 32*(31), 7870-7882. doi: 10.1016/j.biomaterials.2011.07.016

Pohlers, D., Brenmoehl, J., Loffler, I., Muller, C. K., Leipner, C., Schultze-Mosgau, S., . . . Wolf, G. (2009). TGF-beta and fibrosis in different organs - molecular pathway imprints. *Biochim Biophys Acta, 1792*(8), 746-756. doi: 10.1016/j.bbadis.2009.06.004

Quarta, M., Cromie Lear, M. J., Blonigan, J., Paine, P., Chacon, R., & Rando, T. A. (2018). Biomechanics show stem cell necessity for effective treatment of volumetric muscle loss using bioengineered constructs. *NPJ Regen Med, 3*, 18. doi: 10.1038/s41536-018-0057-0

Quarta, M., Cromie, M., Chacon, R., Blonigan, J., Garcia, V., Akimenko, I., . . . Rando, T. A. (2017). Bioengineered constructs combined with exercise enhance stem cell-mediated treatment of volumetric muscle loss. *Nat Commun, 8*, 15613. doi: 10.1038/ncomms15613

Ramsey, M. R., & Sharpless, N. E. (2006). ROS as a tumour suppressor? *Nat Cell Biol, 8*(11), 1213-1215. doi: 10.1038/ncb1106-1213

Rodriguez, C., Rodriguez-Sinovas, A., & Martinez-Gonzalez, J. (2008). Lysyl oxidase as a potential therapeutic target. *Drug News Perspect, 21*(4), 218-224. doi: 10.1358/dnp.2008.21.4.1213351

Sadeh, M. (1988). Effects of aging on skeletal muscle regeneration. *J Neurol Sci, 87*(1), 67-74.

Sattler, F. R. (2013). Growth hormone in the aging male. *Best Pract Res Clin Endocrinol Metab, 27*(4), 541-555. doi: 10.1016/j.beem.2013.05.003

Schultz, R. L., Kullman, E. L., Waters, R. P., Huang, H., Kirwan, J. P., Gerdes, A. M., & Swallow, J. G. (2013). Metabolic adaptations of skeletal muscle to voluntary wheel running exercise in hypertensive heart failure rats. *Physiol Res, 62*(4), 361-369.

Sicari, B. M., Rubin, J. P., Dearth, C. L., Wolf, M. T., Ambrosio, F., Boninger, M., . . . Badylak, S. F. (2014). An acellular biologic scaffold promotes skeletal muscle formation in mice and humans with volumetric muscle loss. *Sci Transl Med, 6*(234), 234ra258. doi: 10.1126/scitranslmed.3008085

Smith, H. K., & Merry, T. L. (2012). Voluntary resistance wheel exercise during post-natal growth in rats enhances skeletal muscle satellite cell and myonuclear content at adulthood. *Acta Physiol (Oxf), 204*(3), 393-402. doi: 10.1111/j.1748-1716.2011.02350.x

Snow, M. H. (1977). The effects of aging on satellite cells in skeletal muscles of mice and rats. *Cell Tissue Res, 185*(3), 399-408.

Song, W., Kwak, H. B., & Lawler, J. M. (2006). Exercise training attenuates age-induced changes in apoptotic signaling in rat skeletal muscle. *Antioxid Redox Signal, 8*(3-4), 517-528. doi: 10.1089/ars.2006.8.517

Sousa-Victor, P., Gutarra, S., Garcia-Prat, L., Rodriguez-Ubreva, J., Ortet, L., Ruiz-Bonilla, V., . . . Munoz-Canoves, P. (2014). Geriatric muscle stem cells switch reversible quiescence into senescence. *Nature, 506*(7488), 316-321. doi: 10.1038/nature13013

Thorley, M., Malatras, A., Duddy, W., Le Gall, L., Mouly, V., Butler Browne, G., & Duguez, S. (2015). Changes in Communication between Muscle Stem Cells and their Environment with Aging. *J Neuromuscul Dis, 2*(3), 205-217. doi: 10.3233/JND-150097

Troy, A., Cadwallader, A. B., Fedorov, Y., Tyner, K., Tanaka, K. K., & Olwin, B. B. (2012). Coordination of satellite cell activation and self-renewal by Par-complex-dependent

asymmetric activation of p38alpha/beta MAPK. *Cell Stem Cell, 11*(4), 541-553. doi: 10.1016/j.stem.2012.05.025

Urciuolo, A., Quarta, M., Morbidoni, V., Gattazzo, F., Molon, S., Grumati, P., . . . Bonaldo, P. (2013). Collagen VI regulates satellite cell self-renewal and muscle regeneration. *Nat Commun, 4*, 1964. doi: 10.1038/ncomms2964

Veldhuis, J. D. (2008). Aging and hormones of the hypothalamo-pituitary axis: gonadotropic axis in men and somatotropic axes in men and women. *Ageing Res Rev, 7*(3), 189-208. doi: 10.1016/j.arr.2007.12.005

Verdu, E., Ceballos, D., Vilches, J. J., & Navarro, X. (2000). Influence of aging on peripheral nerve function and regeneration. *J Peripher Nerv Syst, 5*(4), 191-208.

Vitale, G., Cesari, M., & Mari, D. (2016). Aging of the endocrine system and its potential impact on sarcopenia. *Eur J Intern Med, 35*, 10-15. doi: 10.1016/j.ejim.2016.07.017

Wang, Y., & Pessin, J. E. (2013). Mechanisms for fiber-type specificity of skeletal muscle atrophy. *Curr Opin Clin Nutr Metab Care, 16*(3), 243-250. doi: 10.1097/MCO.0b013e328360272d

White, T. P., & Esser, K. A. (1989). Satellite cell and growth factor involvement in skeletal muscle growth. *Med Sci Sports Exerc, 21*(5 Suppl), S158-163.

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Jeffrey Wolchok

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee

DATE: May 14, 2014

SUBJECT: IACUC APPROVAL
Expiration date: May 14, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED protocol 14044: ": Engineering a Muscle Mimetic Biomaterial" . We have listed the start date as May 15, 2014.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 14, 2017 you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

Request for Modification of an Approved Animal Use Protocol (MR)

IACUC use only:			
Protocol number:	_____	Category(s) of animal use:	
Date Received:	_____	☐ Agricultural	
Approval Date:	_____	☒ Biomedical	
Start Date:	_____	☐ Field	
End Date:	_____	LATA Training Verified	☐ Yes ☐ No

Instructions:

- *This form is required for minor modifications of an Animal Use Protocol (AUP) which is currently approved by the IACUC. There are the following exceptions:*
  - *These require only a memo (or email) to the Chairman of the IACUC. Requests for:*
    - *an extension of the study period (not to exceed 3 years from original approval date).*
    - *changes of personnel (be sure all new personnel have complete the required LATA modules), or*
    - *an increase of animal numbers not to exceed 10% of originally approved numbers*
  - *Major modifications will require a new AUP.*
- *In completing this MR, briefly state the Objective(s) of the original approved AUP and how the proposed modification(s) would serve to further satisfy the Objective(s).*
- *Explain the modification(s) so it can be clearly understood how it (or they) fit in the Experimental Design as described in the Approved AUP*
- *It is preferred that this document explain the proposed procedures adequately so that the reviewers do not need a copy of the Approved AUP. However, if necessary refer to the Approved AUP as needed so the reviewers can clearly understand the proposed modification(s).*
- *This is a MicroSoft Word (MSWord) "form". Use MSWord to fill in the information asked for in the blanks ("_____") provided. You can put as much information in the blanks as you need to.*

Approved Animal Use Protocol # 14044

Title:

Project Title of Original Protocol: Engineering a Muscle Mimetic Biomaterial

Start and End Dates of Approved Protocol:

Start Date 5/15/2014 End Date 5/14/2017

Principal Investigator

Name: Jeff Wolchok
Department/Division: Biomedical Engr.
Campus Mail Address: 125 Engineering Hall
Telephone: 479-575-2850
Fax: 479-575-4346
E-mail: jwolchok@uark.edu

Objective:

- Objective of the Original Protocol: Examination of tissue derived and cell derived biomaterials for the repair of damaged skeletal muscle
- Objective of the Modification: No change in protocol objective.
-

REQUESTED CHANGES

- **Animals:**
  If applicable:
  - *List any changes in species to be used* - None
  - *List numbers of animals required by this modification* - None
- **Methods:**
  If applicable:
  - *List any changes in Housing* - None
  - *List any changes in Experimental Design (such as treatment agents, procedures, etc.)* - None

- *List any changes in Non-Surgical Procedures* - None
- *List any changes in Surgical Procedures* - None
- *List any changes in Euthanasia* - None

- **Personnel:**

  If applicable:
  - *List any additional personnel that will be involved that are not listed on the Approved AUP, fill in the table below.*
  - *Include any additional qualifications here →* _____

LATA Training Documentation

Person:	*List Names →*	John Kim	____	____
Responsibility – PI, Tech, Student, or Other		☐ ☐ ☒ ☐ PI T S O	☐ ☐ ☒ ☐ PI T S O	☐ ☐ ☐ ☐ PI T S O
Required Modules:				
Base Modules				
The Humane Care and Use of Laboratory Animals	*Date Completed →*	8/28/14	____	____
Policy and Procedures	*Date Completed →*	8/28/14	____	____

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Jeffrey Wolchok
Fr:	Craig Coon
Date:	February 14th, 2018
Subject:	IACUC Approval
Expiration Date:	February 13th, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18052:** *Tesosterone Supplementation of Regenerative VML Repair Strategies*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond February 13th, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Jeffrey Wolchok, Tyrone Washington, John Kim, Kevin Roberts, and Tai Hyunh. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp