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Chronic muscle injury and corresponding myogenic niche changes affect skeletal muscle progenitor cell phenotype maintenance ex vivo

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The dissertation of Kelsey Alison Thomas is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE DISSERTATION

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Skeletal muscle progenitor cells (SMPs) are critical for muscle repair in response to injury. In vivo, SMPs reside in a niche under the basement membrane in muscle fibers and will activate and proliferate or differentiate in response to soluble factors released during injury. Here we sought to use two human muscle disorders, chronic rotator cuff tendon (RCT) tears and Duchenne muscular dystrophy (DMD), to characterize changes to the SMP niche in vivo and corresponding changes in SMP phenotype maintenance ex vivo. Tears in RCTs have known long-term effects on RC muscle atrophy, fibrosis, and fatty infiltration, with lasting damage even after surgical tendon repair. The inability of the RC muscles to recover from chronic RC tear indicates possible defects in muscle repair mechanisms.

We investigated if muscle injury state was a crucial factor during human SMP expansion and differentiation ex vivo. SMPs were isolated from muscles in patients with no, partial-thickness (PT), or full-thickness (FT) RCT tears. Despite using growth factors, physiological niche stiffness, and muscle-mimetic extracellular matrix (ECM) proteins, we found that SMPs isolated from human RC muscle with RCT tears proliferated slower but fused into myosin heavy chain (MHC)-positive myotubes at higher rates than SMPs from untorn RCTs. Proteomic analysis of RC muscle tissue revealed shifts in muscle composition with pathology, as muscle from massive RCTs had increased ECM deposition compared with no tear RC muscle. Similarly, a proteomic analysis of muscle tissue taken from DMD patients revealed corresponding shifts in ECM and cytoskeletal protein expression with disease. Both analyses illustrate potential changes in the SMP niche, with increased fibrosis and decreases in basement membrane proteins. Together these data underscore the importance of how the niche, both in and ex vivo, prime SMPs for expansion, self-renewal and differentiation.

Chapter 1

Extracellular matrix regulation in the muscle satellite cell niche

Stem cells live in a specialized microenvironment in tissues, commonly referred to as the niche. However, the niche is much more than an anatomical location; it is a dynamic circuit board transmitting mechanical and chemical signals that continuously relay the status and requirements of the tissue to its regenerative cell source. It protects the inactive, quiescent stem cell population from depletion and conveys signals for activation, proliferation and differentiation in response to tissue damage. In fact, the interplay between the stem cell and its niche is so important that alterations to components of the niche have been shown to result in defective regeneration in nearly every stem cell compartment in the body (for review, see [61]).

A growing body of evidence supports extracellular matrix (ECM) components as essential signal mediators in the niche, both for maintaining stem cell identity and regulating activation. One of the major roles of this ECM is to provide structural integrity to the niche, physically separating the stem cell pool from other tissue resident cells and interstitial matrix [52]. However, it also plays a role in localizing molecules such as growth factors and glycoproteins that regulate the balance between activation and quiescence. Stem cells are also able to sense and respond to the composition, porosity and stiffness of the ECM in their niche as they directly interact with it through focal adhesions [63, 37, 32]. Though the

multifaceted three-dimensional nature of the in vivo niche makes it difficult to isolate individual regulators, experiments using engineered niches have demonstrated that variations in the ECM, in absence of compounding factors, are capable of influencing the proliferation [42], migration [87, 114] and differentiation of stem cells [63, 93]. More recently, matrix cues have been examined in combination, in 3D and with growth factors, all of which complicate the niche and do not necessarily have additive effects [37, 92].

Understanding how environmental factors modulate tissue regeneration is critical for successful strategies in regenerative medicine, and this is particularly evident in skeletal muscle in conditions that cause high tissue turnover and poor regeneration, e.g. muscular dystrophy. Healthy skeletal muscle is one of the most adaptive and regenerative tissues in the adult body. Its regeneration capacity is so robust that, following widespread destruction of its myofibers by experimental myotoxin injection [113], mechanical crush [36], prolonged freeze injury [115] or ex-vivo mincing and replacement [12], a muscle is able to regain near normal morphology and force production in a matter of weeks. The primary source for regeneration in skeletal muscle is the satellite cell. These cells reside in a distinct niche and express a unique panel of surface markers and transcription factors, making them relatively easy to identify, target and isolate. This fact, combined with the spectrum of muscle-specific transgenic animals, the variety of muscle injury models and access to patient biopsies make the satellite cell niche a unique model system to study ECM regulation and cell-matrix interactions. A better understanding of the role of the ECM in regulating satellite cell function will provide new directions and targets for therapies aimed at improving muscle regeneration and tissue regeneration as a whole; this forms the focus of our review below, specifically introducing the satellite cell, its niche, how it is activated and how it responds in disease models.

1.1 The satellite cell niche

The satellite cell (SC) is an undifferentiated, unipotent muscle progenitor that resides within the basal lamina adjacent to the plasma membrane of a muscle fiber (Figure 1.1).

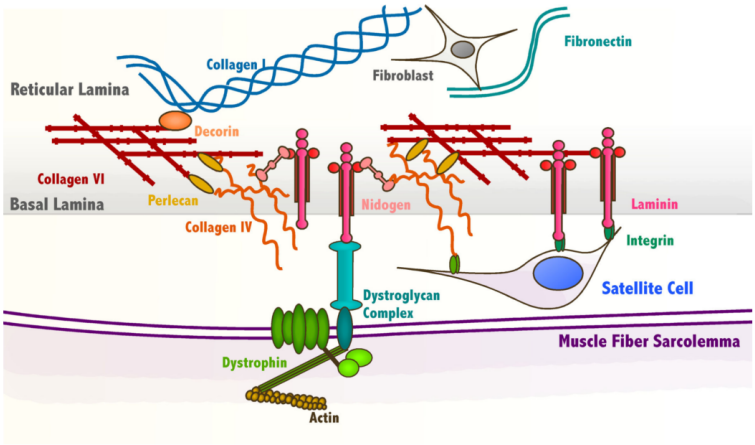

Figure 1.1: Schematic diagram of the satellite cell (SC) niche. SCs reside between the basal lamina (BL) and the muscle fiber sarcolemma where they interact with matrix components of the niche. Through integrins, SCs bind to collagen type IV and laminin. The ECM protein nidogen helps cross-link these two components into a matrix. They in turn bind to collagen type VI and several proteoglycans including perlecan and decorin. Collagen type VI integrates the BL with the reticular lamina composed primarily of collagen types I and III and fibronectin. On the other side of the SC niche, the muscle fiber sarcolemma links to the BL through the dystroglycan complex, which binds to the actin cytoskeleton thorough dystrophin and to laminin in the BL.

The majority of SCs are quiescent, but in response to increases in loading or tissue damage, SCs become activated and begin to divide. A fraction of these activated SCs will continue to proliferate and migrate as myoblasts before terminally differentiating and fusing into muscle fibers [99]. Though they share many behavioral characteristics, quiescent SCs, activated SCs and myoblasts are transcriptionally and functionally distinct. Much of our understanding of SC behavior has come about from animal models, but considerable inter-species variability in tissue composition and cell behavior may lead to species-to-species differences. Therefore, care will be taken in this discussion to differentiate in vitro from in vivo

studies and those in animal models from those in humans.

The ECM surrounding muscle fibers is composed of collagens, laminins, fibronectin and glycosaminoglycans (GAGs), short polysaccharide chains which bind to a protein core to form proteoglycans (for review, see [95]). This matrix is known as the basement membrane (BM), and has two layers, the basal lamina (BL) and the reticular lamina. SCs reside in the BL, whose two primary constituents, collagen type IV and laminin-2 (α2, β1, and γ1 chains), assemble into two cross-linked networks, which are then linked by the glycoprotein nidogen. The concentration of these two components of the BL varies as a function of muscle fiber type. The predominately slow Soleus muscle of adult rats has twice the concentration of collagen IV and less than half the concentration of laminin-2 in the BL compared with the predominately fast rectus femoris [65]. Interestingly, the concentration of SCs in slow rat muscle fibers is also double what is measured in fast muscle fibers [97]. Whether or how this is related to the differences in the ECM of the niche is unknown.

In addition to collagen IV and laminin-2, there are several other critical components of BM worth noting. Perlecan, a heparin sulfate proteoglycan, and decorin, an SLRP proteoglycan, are distributed throughout the BM [109]. These negatively charged proteoglycans bind and sequester a variety of growth factors, giving them both structural and signaling roles in the BM. Perlecan binds to collagen type IV and laminin-2 in the BL while decorin binds to collagen type I in the reticular lamina. In addition to collagen type IV, the BL also contains collagen type VI, which connects the BL to the reticular lamina. Fibronectin is primarily localized to the reticular lamina, with which SCs are typically not in contact, but may be transiently expressed and localized to the BL during regeneration [94]. The BL is also linked to the cytoskeleton of the muscle fiber at repeating assemblies of proteins called dystroglycan complexes (DGC). Within the muscle fiber, dystrophin links actin to the DGC, and in the basal lamina β-dystroglycan then binds to laminin. This integration further stabilizes the structure of the SC niche.

Cells adhere to the ECM not only for structural stability but also for sig-

naling, beginning with integrins, a family of cell surface receptors which bind to ECM proteins and from which focal adhesions form. Assembled within these structures are proteins that both serve structural and force-sensitive signaling roles, e.g. vinculin (for review, see [58]); these proteins also allow for so called outside-in signaling to relay outside conditions to inside the cell [50]. Integrins $\alpha 7$ and $\beta 1$ are the major isoforms expressed by SCs and together form a receptor complex that binds to laminin-2 in the BL [4]. However, integrin expression varies as a function of the activation state of SCs. Activated, but not quiescent, mouse satellite cells express integrin 3 which likely complexes with integrin v to form a receptor for proteins bearing an exposed Arg-Gly-Asp (RGD) tripeptide including fibronectin, osteopontin and some degraded laminins and collagens [73]. Activated human myoblasts also express integrin $\alpha 5$ in vitro which, in combination with $\beta 1$, is a receptor for fibronectin [5]. The temporal variation of integrin expression in SCs suggests that they may have unique regulatory roles in muscle, some promoting the initiation of myogenesis and some maintaining homeostasis.

1.2 Remodeling of the niche

Muscle BM substantially remodels during regeneration post injury (transition from left to center panels in Fig 1.2). Experimentally controlled ECM remodeling has demonstrated a critical role for modifications in restorating strength and morphology following injury (for review, see [101]). Focal muscle damage frequently involves an initial insult to the BM followed by further degradation by proteases. ECM fragments and growth factors that are released during the process of matrix degradation play a critical role in the migration and homing of inflammatory, endothelial and myogenic cell types. Furthermore, the reconstruction of the damaged matrix can scavenge these same factors, signaling the end of cellular infiltration and differentiation. Transcriptional profiles conducted using a cardiotoxin injury rodent model show a consistent temporal pattern of gene expression marked by a peak in expression of matrix degrading enzymes followed by upregulation of numerous ECM components of the BL [46, 62]. This is a dynamic process involving

the interplay between a variety of cell types in and around the SC niche.

The major enzymes responsible for the physiological breakdown of ECM are matrix metalloproteinases (MMPs), which work in tandem with the urokinase plasminogen activator. Two isoforms, MMP2 and MMP9, target collagen type IV and laminin in the BL and are significantly upregulated during muscle regeneration [64, 72]. Cultured human myoblasts constitutively synthesize and secrete MMP2 and the urokinase plasminogen activator and can be induced to secrete MMP9 [48, 35]. This is consistent with in vivo data from a regenerating mouse model, localizing expression of MMP2 and MMP9 to activated SCs [68, 128]. This data suggests that SCs are involved in the breakdown of their own niche, allowing them to leave the niche and migrate to the site of injury.

Resident muscle fibroblasts are considered to be the main contributor to the ECM of skeletal muscle. In addition to secreting the primary fibrous collagens found in the interstitial connective tissue (types I and III), fibroblasts have also been shown to secrete the major basal lamina collagens (types IV and VI) [68, 128]. Zou and co-workers took advantage of a mutation to the collagen type VI gene which caused the protein to be synthesized, but not secreted by cells [128]. They observed large significant collagen VI staining in interstitial fibroblasts and an absence of positive staining in myogenic cells suggesting that fibroblasts are the major, if not the only, contributor of collagen VI in human skeletal muscle.

However, fibroblasts are not the only contributor to the SC niche ECM. In addition to participating in the degradation of their surrounding ECM, SCs also secrete a variety of BL components in addition to MMPs. Cross-species co-cultures of mouse-derived fibroblasts with quail-derived myoblasts show collagen IV incorporation into the BL of myotubes of both mouse and quail origin [68], suggesting that both myogenic cells and fibroblasts contribute collagen IV to the SC niche. Studies in vitro and in vivo have also demonstrated expression and secretion of laminin and fibronectin by mouse myoblasts [96, 3]. In fact, Bentzinger and co-workers demonstrated that not only do proliferating SCs express fibronectin, but expression by SCs is important for efficient regeneration [3]. In this study, fibronectin knockdown in SCs decreased engraftment efficiency upon injection into

recipient mice, demonstrating that SC-derived fibronectin specifically is important for SC engraftment and function. ECM proteins collagen IV, decorin, perlecan, laminin chains α2 and β1, and nidogen are more highly expressed in quiescent mouse SCs compared with activated SCs [3, 40]. These data provide evidence that quiescent SCs may reside in a different niche than either activated or proliferating SCs and that they may require a complex combination of ECM proteins for optimal function and survival. It should also be noted that the time scale for ECM assembly is greater than 24-48 hours [100] and thus most of the in vitro data examining adhesion may not observe the same differential effects as longer-term or in vivo assays. Regardless of amount, assembly state and composition, the field is emerging around the concept that matrix does indeed establish and control a niche and the stem cells within it.

1.3 ECM regulation of SC quiescence and activation

One of the most important functions of the progenitor/stem cell niche is maintaining the balance between quiescence and activation. When proliferation is inhibited, tissue regeneration is severely blunted. On the other hand, overexuberant proliferation of SCs would lead to overpopulation of the niche and potential tumorigenesis. Interactions with the ECM have been suggested to regulate the switch between symmetric and asymmetric division in a variety of stem cell niches (for review, see [120]). However, BL ECM is an intricate composite with a variety of matrix components and complex geometry. Determining the component or combination of components that control cellular behavior is difficult.

In order to study cell-matrix interactions in a controlled environment, many researchers have turned to in vitro cell culture models where specific components or attributes of the ECM can be easily manipulated. Unfortunately, one of the strongest pieces of evidence for the SC niche being involved in the maintenance of quiescence is that when SCs are removed from their niche, they quickly withdraw from quiescence, enter the cell cycle and lose their myogenic properties [42]. How-

ever, several studies have identified matrix cues that promote the maintenance of quiescence in vitro. When cultured on various ECM coatings, a larger fraction of freshly isolated murine and porcine SCs were shown to express two transcriptional markers of quiescence, Pax7 and Pax3, in response to matrigel (a purified BM secreted by murine Engelbreth-Holm-Swarm tumor cells) or laminin compared with collagen I, gelatin or fibronectin [47, 116]. Also, when grown on matrigel with the addition of native collagen VI, a greater percentage of murine SCs expressed Pax7 than in cultures without collagen VI [111]. Furthermore, when collagen VI knockout fibers were cultured on collagen VI plus matrigel, SCs were better able to maintain Pax7 expression compared with matrigel alone. Although Matrigel is a heterogeneous mixture of ECM proteins and growth factors, several of its major constituents, including laminin, are primary components of the skeletal muscle BL. Taken together, these data suggest that the ECM composition of the BL may play a role in maintaining SC quiescence in vivo.

In addition to ligand cues, the stiffness of the culture substrate influences SC quiescence. Gilbert et al. cultured freshly isolated mouse SCs on tunable polyethylene glycol (PEG) hydrogels of different elastic moduli, and found greater SC survival and expression of Pax7 on hydrogels that approximate the physiological stiffness of muscle [42]. Furthermore, when these cells were injected back into regenerating mouse muscle, the SCs grown on the hydrogels of muscle-like stiffness were able to repopulate the native niche at rates similar to freshly isolated SCs. This ability is rapidly lost in SCs cultured on stiff tissue culture plastic. Furthermore, culturing aged mouse SCs on soft hydrogel substrates was shown to improve their functional capacity when transplanted into recipient muscle [23]. These data underscore the importance of understanding and mimicking the niche in moving forward with tissue engineered and cell-based therapies that rely on extensive culture periods.

Symmetric division, in which a SC divides into two identical daughter cells, and asymmetric division, in which one of the progeny remains a SC and the other daughter cell differentiates, are critical for balancing maintenance of the SC pool with the need for muscle repair (for review, see [66]). Emerging evidence has

pointed to the importance of the SC niche as a regulator of symmetric and asymmetric division. Polarity achieved through exposure to the basal lamina vs. the apical side of the SC niche has been established as a driver of asymmetric division [39]. The apical side of the SC expresses m-cadherin receptors, which allow the cell to interact with the muscle fiber, whereas the basal side expresses the laminin receptor integrin $\alpha 7\beta 1$. Studies in mice have demonstrated apical-basal oriented SC divisions where the daughter cell that remains in contact with the BL remains to repopulate the niche while the daughter cell closest to the fiber differentiates [67].

Fibronectin as an individual ECM protein component of the SC niche is critical for maintenance of the SC pool. Knockdown of fibronectin in mouse SCs leads to a drop in symmetric cell division in SCs, reducing the fraction of Pax 7 positive cells in vitro [3]. The regulation of symmetric cell division by fibronectin is achieved through the interaction of the fibronectin receptor syndecan-4 and the Wnt7a receptor frizzled-7. Collagen VI is another ECM component shown to be important in maintenance of the SC pool. In mouse muscle lacking collagen VI the SC pool is depleted, as SCs fail to sufficiently self-renew [111]. Furthermore, this defect was able to be rescued by injecting collagen VI expressing fibroblasts into the affected muscle indicating that the absence of this ECM protein, as opposed to a SC intrinsic defect, was driving the aberrant SC behavior. In addition to the fibrous matrix components, proteoglycans in the muscle BL play a role in regulating SC behavior. Heparan sulfate proteoglycans syndecan-3 and syndecan-4 have been shown to regulate SC activation and proliferation as SC self-renewal is impaired in mouse knockout muscle [22]. Furthermore, matrix resident proteoglycans such as perlecan and decorin can bind signaling molecules such as Notch and Wnts, known to influence SC asymmetric/symmetric division [8]. Thus, it is evident that the interactions between ECM proteins and cell surface receptors are necessary for striking the balance between differentiation and self-renewal, and that further research is needed to examine the synergistic effects of multiple ECM proteins present in the SC niche.

1.4 ECM regulation of differentiation

The ability of SCs to repair damaged muscle hinges on their capacity to differentiate and fuse into myofibers. Thus, there is great interest in determining the factors that promote SC differentiation both in vitro and in vivo. ECM factors regulating myogenic differentiation include specific ECM ligands, soluable growth factors sequestered within the matrix and the physical properties of the matrix itself.

Differentiation of SCs involves a temporal sequence of transcription factor expression, notably myf-5, myoD, desmin and myogenin, followed by fusion with neighboring myoblasts or with existing myotubes. Each of these steps is affected by the ECM in some fashion. Primary mouse myoblasts cultured on substrates of gelatin, Matrigel, laminin, fibronectin, collagen I or collagen IV fused most robustly on Matrigel [47, 74]. Similarly, porcine myoblasts cultured on substrates of collagen type I, gelatin, fibronectin, Matrigel, and laminin [116] expressed the late myogenic marker, myogenin, at lowest levels on collagen type I and at the highest level on Matrigel. Thus, it is evident that some constituents of Matrigel possess myogenic properties, and that single substrates are insufficient to promote the levels of myogenic differentiation seen with Matrigel; however, as the exact composition of Matrigel is undefined, the factors involved in SC proliferation and differentiation remain unclear.

To further elucidate the in-vivo ECM components ideal for SC culture in-vitro, murine SCs were cultured on an enactin-laminin-collagen (ECL) substrate, collagen IV, poly-D-lysine, and laminin [98, 7]. Higher myotube fusion rates were observed on poly-D-lysine or laminin than on collagen IV or ECL. However, ECL substrates in combination with glycosaminoglycans (GAGs) promoted myotube fusion better than ECL, GAGs, collagen type I, or laminin single substrates [68, 90]. Thus, GAGs, already known to be important components of the ECM, are also an important component of the SC niche. However, as ECL substrate and GAGs are both heterogeneous mixtures of numerous proteins, they are still undefined substrates in these culture systems, making it difficult to distinguish the contributions of individual proteins to the SC niche. Further research is needed to disentangle

the combinatorial effects of multiple ECM proteins on SC culture and to determine which components of Matrigel and ECL are instrumental in SC differentiation.

In addition to playing a role in regulating SC proliferation, proteoglycans participate in SC differentiation. Heparan sulfate proteoglycans (HSPGs) interact with a large number of growth factors in the muscle BL including insulin-like growth factor (IGF), fibrobast growth factor (FGF), hepatocyte growth factor (HGF) and transforming growth factor beta (TGF-β), all known to influence SC proliferation and differentiation [1, 49]. HSPGs can increase the local concentration of growth factors, or sequester them away from cells and even participate in their function by complexing with them, dramatically affecting the local environment and driving cell behavior.

The mechanical properties of the in vitro culture environment have also been shown to affect myoblast differentiation and fusion. Engler et al. induced fusion in immortalized mouse myoblasts on polyacrylamide gels of varying stiffness and found maximal myosin heavy chain striation, a marker of myotube maturity, on gels of muscle-like stiffness (11kPa) [31]. Similarly, primary mouse SCs cultured on tunable PEG hydrogels of muscle-like stiffness (12kPa) exhibited significantly greater engraftment efficiency upon injection into recipient mice than SCs cultured on tissue culture plastic [42]. These data not only underscore the need to mimic the mechanics of the in vivo environment in culture models, but also suggest that the progressive stiffening of the ECM that is a feature of many muscle diseases may have a significant negative impact on the SC population.

1.5 Pathological alterations to the SC niche

Under ideal conditions, damaged muscle would be completely replaced with fresh, healthy contractile fibers, resulting in a full recovery of force production and function. This is usually the case. Bouts of high intensity or unaccustomed exercise cause microtears, or disruptions to the sarcomeric structure, followed by an inflammatory response and ultimate reconstruction of the damaged area, frequently with gains in muscle mass and strength. However, the ability of the muscle to respond

to such cues for regeneration and growth can be dramatically affected by aging and disease. Both of these processes are typically characterized by pathological changes to muscle ECM, including increased deposition, density and stiffness (for review, see [101, 43]). Though alterations to the BL have received less focus than those to the interstitial matrix, significant changes to BL components have also been noted as a function of age and disease (Figure 1.2, far right).

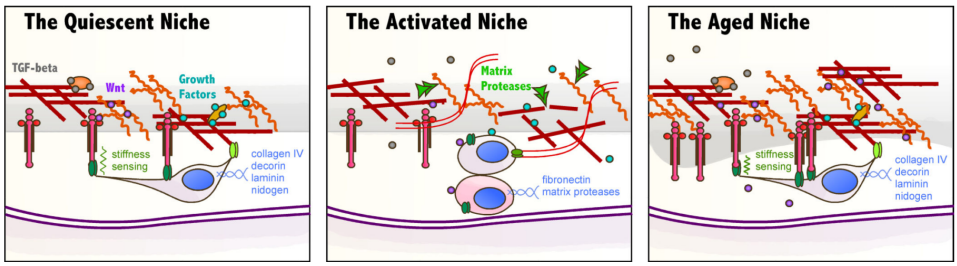

Figure 1.2: Schematic diagrams illustrating some differences between the quiescent, activated and aged satellite cell (SC) niche. (Far left) The quiescent SC senses the stiffness of its niche through integrins and expresses various matrix proteins to maintain its extracellular matrix (ECM). Within this matrix, growth factors and signaling molecules such as Wnts and TGF- are sequestered, maintaining the quiet state. (Center) In response to injury, components of the basal lamina are degraded by matrix proteases which results in the release of signaling molecules that play a role in activation and proliferation of the SC. The activated SC divides and some daughter cells begin to differentiate. (Far right) In the aged niche, matrix components accumulate to form a denser and thicker basal lamina. The stiffness sensing and sequestration of signaling molecules may be affected by this change.

1.6 Aging

The regenerative potential of skeletal muscle declines significantly with age. Studies in human muscle have come to conflicting conclusions about whether this decline is associated with intrinsic changes to the SC pool such as a reduction in SC numbers or proliferative capacity [91, 89]. However, studies that expose aged SCs to a young environment, either in vitro or by heterochronic parabiosis in vivo suggest that at least some of the deficit is environmental [21, 18]. Studies examining the basal lamina of aged muscle and other tissues have demonstrated

a pronounced thickening with a loss of laminated structure, becoming irregular and amorphous [9, 105]. Collagen IV concentration increases preferentially in slow muscles with age, while laminin increases preferentially in fast muscles [65]. This could affect the ability of the BL to store and release growth factors and other signaling molecules involved in maintaining the niche. Increased concentration of the glycoprotein osteopontin has been documented in the BL of mouse muscle with age [80]. This cytokine negatively regulates myoblast differentiation in vitro and muscle regeneration in vivo. Increased levels of other matrix associated negative regulators of myogenesis such as TGF- and Wnt have also been documented in the aged SC niche [8, 11].

1.7 Disease

A striking number of primary myopathies originate in mutations to components or to proteins that bind to components of the BL (for review, see [13]). The most common and well studied of these are the muscular dystrophies, a heterogeneous group of inherited progressive disorders characterized by pronounced muscle weakness, fibrosis and fatty infiltration. The most prevalent dystrophy is Duchenne Muscular Dystrophy (DMD), arising from a mutation in the gene encoding dystrophin, which participates in the linkage between the muscle fiber cytoskeleton and the BL. Loss of this integration is thought to cause destabilization of the fiber membrane resulting in repeated cycles of degeneration and regeneration, which eventually exhaust the regenerative potential of the SC pool.

Pathological alterations are seen in the BL of muscle from DMD patients including decreased accumulation of laminin 2 and 1 and increased accumulation of collagen IV [53]. This is consistent with gene expression studies showing lower expression of laminin 2 [106], higher expression of collagen IV [124] and higher expression of integrin 7 [56] in primary DMD myoblast cultures. In addition to changes in laminin and collagen IV, gene expression studies also show increased expression of TGF- and osteopontin, pro-fibrotic cytokines that inhibit myogenesis [51]. Interestingly, osteopontin was recently identified as determining factor of

disease severity in DMD patients, with lower levels of osteopontin correlated with greater weakness and earlier loss of ambulation [82].

In addition to DMD, there are a variety of less well-known dystrophies resulting from mutations to laminin (congenital muscular dystrophy type 1A), collagen IV (Walker-Warburg syndrome), collagen VI (Ulrich congenital muscular dystrophy and Bethlem myopathy) and components of the dystroglycan complex (dystroglycanopathies). These disorders are all characterized by progressive muscle weakness, from mild to debilitating depending on severity, and frequently exhibit disruptions to the BM. Studies in mice lacking collagen VI, the mouse model for Bethlem myopathy, demonstrate a significant reduction in muscle stiffness, impaired muscle regeneration and a progressive depletion of the SC pool [111]. Interestingly, this effect could be rescued by the transplantation of collagen VI expressing wildtype fibroblasts, which, within 12 days resulted in significant increases in collagen VI deposition, muscle stiffness and SC concentration. This suggests that modulation of the SC niche could have a positive effect on SCs even after long-term exposure to a diseased environment. Understanding the remodeling that occurs in the SC niche with the progression of muscle disease is critically important for therapies targeting the SC niche both those that seek to repopulate it with exogenous cells and those that seek to modify the behavior of the resident SCs.

1.8 Therapeutic implications

A large quantity of data has now been collected pointing to the critical role the BM plays in the maintenance of muscle integrity. In all of the dystrophies described above, muscle develops normally, but then progressively degenerates as a result of the loss of some reticular lamina BL cytoskeleton linkage. This makes these disorders excellent candidates for interventional regenerative therapies. A variety of therapeutic approaches are currently under investigation to either target or supplement the SC population, and some of the most promising involve the creation of tissue engineered niches mimicking those in healthy muscles.

As discussed above, the ECM composition and stiffness of the substrate on which SCs are grown in culture can have a dramatic effect on their ability to fuse into myotubes in vitro as outlined below. A natural next step for this finding is to use these model culture systems to condition cells for engraftment prior to injection into muscle, a strategy that has already shown promise in mice [42]. Taking this one step further, the engineered niche environment could be injected or implanted with the SCs to further promote survival and engraftment.

ECM scaffolds have shown considerable promise in the repair or replacement of a variety of diseased tissues, including muscle. When cross-sections of the rat abdominal wall were reconstructed with porcine-derived ECM constructs, force production and fatigue resistance was returned to native tissue levels, compared with polypropylene mesh reconstruction which was unable to improve muscle function [112]. Even though these constructs were acellular at the time of implantation, they promoted cellular infiltration of the injured area, resulting in the formation of new muscle fibers within the scaffold. Differences in regenerative efficiency have been noted for scaffolds of different materials highlighting the need for a thorough understanding of the signaling effects of different ECM proteins on SC activation, migration and differentiation [55, 26].

Decellularized matrix may also be milled and lyophilized into a powder, which can then be reconstituted and injected for minimally invasive applications. One such material is matrigel, which will form a solid gel in response to physiological temperatures. As discussed above, matrigel promotes maintenance of the SC pool and myoblast fusion in vitro making it an excellent candidate for this type of therapy. Furthermore, injectable hydrogels derived from skeletal muscle matrices have been shown to promote infiltration of muscle progenitors and tissue repair in a rat hindlimb ischemia model over those composed only of collagen, highlighting the importance of recapitulating the native ECM properties in artificial constructs [26].

ECM-mimicking hydrogels have the advantage that their shape and material properties can be precisely controlled. As discussed above, in addition to responding to ligand cues, SCs are sensitive to the stiffness of their environment. Hy-

drogels have been developed with precisely tunable mechanical properties [110, 114] even some with stiffness that changes temporally to mimic the changes seen in developing or regenerating tissue [122]. Furthermore, hydrogels can be designed to mimic complex 3-dimensional environments using 3-D printing, enabling precise arrangement of cells and growth factors (for review, see [34]).

Chapter 1 was a review published in Connective Tissue Research titled "Extracellular matrix regulation in the muscle satellite cell niche". I acknowledge Gretchen Meyer and Adam Engler as co-authors in the work.

Chapter 2

Rotator cuff tear state modulates self-renewal and differentiation capacity of human skeletal muscle progenitor cells

2.1 Introduction

Approximately 30% of the population 60+ years of age has a tear of at least one rotator cuff tendon (RCT) [108], typically either the supraspinatus tendon or both supraspinatus and infraspinatus tendons. Such injuries led to nearly 300,000 surgical interventions in the US in 2006 [20]. Given the often chronic presentation of RCT injuries, supraspinatus and infraspinatus muscles can degenerate, leading to fibrosis, fatty infiltration, and muscle loss [38, 41]. Fatty infiltration frequently occurs in the infraspinatus muscle even when only the supraspinatus tendon is torn as a result of altered muscle loading [17]. Furthermore, muscle damage, which occurs during chronic RCT injuries. does not often improve following tendon repair, and repair failure is correlated with continued progression of muscle atrophy and fatty infiltration [44]. Thus, chronic RCT injury can result in permanently altered muscle, indicating possible deficits in intrinsic muscle repair mechanisms.

Skeletal muscle progenitor cells (SMPs) are responsible for muscle growth and repair in response to injury [103]. While SMPs transition from quiescent to active in response to soluble factors released by injured muscle in vivo [121], their activation can also be modulated by insoluble factors within the niche itself [70, 111], due to their location under the basement membrane surrounding muscle fibers [76]. Niche characteristics typically include substrate stiffness [7], which for healthy muscle can range from 10-20 kiloPascals (kPa, a unit of stiffness) [19, 31], extracellular matrix (ECM) protein composition, including basement membrane collagens and laminins [78, 79], and soluble growth and signals factors [123, 60, 119, 29], including Notch regulation [121], HFG [119], IGF-1 [60], oxytocin [29], and p38 MAP kinase (MAPK) pathway activation [23]. Since SMPs are sensitive to these environmental cues, it is likely that tendon tear activates SMPs in RC muscles, as shown by an increase in the SMP population in muscle from partial RCT tears [77]. Whereas most murine or human studies focus on substantially younger populations than ours [108], the chronicity, tear severity, and advanced age of our patient population have previously been associated with less SMP activation resulting in lower regenerative capacity [77]. However, other muscle groups appear to maintain their regenerative capacity to some degree; indeed, aged murine intact myofibers contain fewer SMPs but tend to be more proliferative [69]. Replating SMPs in niche with aged characteristics can even reprogram young cells to resemble aged SMP characteristics [69]. Thus poor RCT surgical outcomes could be due to unique deficits in RC muscles created by ECM and growth factor composition of RC muscles.

To determine if the lower regenerative capacity of SMPs in muscle from torn RCTs can be rejuvenated by the restoration of normal niche characteristics, we examined whether or not SMPs isolated from supraspinatus, infraspinatus, and deltoid muscles from varying RCT tear states could be culture-expanded in muscle-mimetic niches. Using substrate stiffness [7, 19, 31, 42], ECM protein composition [78, 79], and soluble signals and growth factors [123, 119, 60, 29], we quantified to what extent disease state influenced expansion and subsequent differentiation, finding that tear state alone had a substantial and long-lasting effect on SMP phe-

notype; tear-derived SMPs fused into multinucleated myotubes at greater rates but were less proliferative than controls despite normal niche conditions. These data correlated with ECM compositional differences between tear states, suggesting that intrinsic niche differences may have permanently reprogrammed SMPs, thus impairing repair post-reloading of muscle.

2.2 Materials and Methods

2.2.1 Tissue Biopsies

Muscle biopsies were obtained from the distal third of the supraspinatus (SS), infraspinatus (IS), and/or deltoid (D) muscles from 15 patients of mixed gender undergoing arthroscopic or open shoulder surgery. RCTs were classified as having no tear (NT) with patients typically presenting with bursitis or instability, or tears of varying severity classified intraoperatively by the surgeon as a partial thickness tear (PT), full thickness tear (FT), or massive tear (MT). Patients classified as PT had torn one or more tendons partially but not completely through the sagittal plane of the tendon. Conversely, patients classified as FT had completely torn through the sagittal plane of the tendon. MT was categorized by FT of more than two tendons with medial retraction. Patients were classified into these groups by the operating surgeon and biopsies of approximately 10 mg of tissue were obtained using an arthroscopic rongeur. All biometric data and case notes are provided in Table 2.1 with average age of 54.2 $\pm$15.3 years and body mass index of 26.8 $\pm$4.1 kg/m^2. There was no significant difference in body mass index between torn and intact patients ($p = 0.16$), but age between torn and intact patients was significantly different ($p = 0.001$). This difference is consistent with the reported increasing incidence of rotator cuff tears with age [102]. The institutional review board of the University of California, San Diego Human Research Protection Program approved this study (approval 090829); all participants gave written informed consent to participate.

2.2.2 Skeletal Muscle Progenitor (SMP) isolation

Muscle samples were digested using 0.25% collagenase (Worthington Biochemical) and dispase (Stem Cell Technologies) for 30 minutes at 37°C, before being minced and digested for a subsequent 10 minutes. Cells were passed through a 70 μm filter (BD) and centrifuged at 2000 RPM for 10 minutes at 4°C. Cells were then resuspended in FACS buffer (2.5% normal goat serum and 1mM EDTA in PBS) and stained using PE mouse anti human NCAM (BD 561903), eFluor450 mouse anti human CD31 (eBioscience 48-0319-42), and FITC mouse anti-human CD45 (BioLegend 304017) for 20 minutes on ice. Cells were centrifuged at 2000 rpm for 4 minutes, resuspended in FACS buffer, and sorted using a FACSAria 2 cell sorter (BD). Following sorting, SMPs were kept in 20% FBS in one well of a 24-well plate and passaged when confluent. Medium was changed every other day.

2.2.3 Polyacrylamide gels

Polyacrylamide (PA) gels were used in all proliferation studies. PA gels were fabricated as described, with concentrations used to create a hydrogel with stiffness of 11 kiloPascal (kPa) [110]. Briefly, coverslips were functionalized using methacrylate (Sigma-Aldrich). A polyacrylamide solution of 10% acrylamide and 0.1% bis-acrylamide in PBS was polymerized using ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). Slides were treated with dichlorodimethylsilane (DCDMS) to spread the PA solution in a uniform layer on the coverslip and protect it from air during the polymerization process. Gels were detached from the DCDMS slides, rinsed twice with sterile PBS, and treated with UV light and sulfo-SANPAH (ThermoFisher scientific) at 0.2 μg in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer for 10 minutes. Gels were then washed twice with HEPES buffer and subsequently incubated with ECM proteins of choice at a 10 g/ml total protein concentration in water with 1% antibiotic/antimicotic (invitrogen) overnight at 37°C. Gels were UV sterilized for 30 minutes and washed twice with sterile PBS prior to use in cell culture.

2.2.4 Proliferation Assays

SMPs were maintained in 20% FBS on tissue culture plastic prior to being used for proliferation studies at passage 3 or 4, when sufficient cell number had been reached. For substrate studies, SMPs and C2C12s were plated onto 11 kPa PA gels with either collagen type IV (COL IV), laminin-111 (LM-111), and collagen type I (COL I) or collagen type IV and laminin-111 at a density of 10 cells/mm^2. Cells were passaged every 3-4 days, and proliferation was assessed using a hemocytometer.

For growth factor studies, SMPs were seeded on 11 kPa gels with COL IV and LM-111 only and passaged 5 times in the experiment. 20% FBS in DMEM (Invitrogen 11885) was supplemented with growth factors in the following concentrations: 30 ng/mL FGF2 [123] (Prospec), 30 ng/mL FGF6 [123] (PeproTech), 30 ng/mL FGF19 [123] (PeproTech), 2.5 ng/mL HGF [119] (Prospec), 50 ng/mL IGF-1 [60] (Peptide Sciences), and 30 nM oxytocin [29] (BACHEM). For the condition with all growth factors, individual growth factor concentrations were same as above. SMPs were grown on glass with 20% FBS with no added growth factors as a control. Medium was changed daily.

2.2.5 Differentiation Assay

Differentation assays were conducted at the last passage of each experiment. SMPs were seeded at 220 cells/mm^2 on LM-111 and COL IV on glass and cultured in myogenic differentiation medium (5% horse serum and 10 μg/ml insulin) for five days. Coverslips were fixed in ice-cold absolute methanol for 10 minutes at room temperature and rinsed three times with 1 mM $MgCl_2$MgCl 2 in PBS. Mouse anti-MHC primary antibody (Developmental Studies Hybridoma Bank MF20) was diluted 1:30 in 2% bovine serum albumin (BSA) in 1 mM $MgCl_2$MgCl 2 in PBS, and the coverslips were incubated for 1 hour at 37°C. Coverslips were washed with 1 mM $MgCl_2$MgCl 2 in PBS and then incubated with secondary antibody goat anti mouse Alexa Fluor 647 (Life Technologies A21235) 1:250 for 30 minutes 37°C. Hoescht (Life Technologies) was used at 1:1000 in di water for 2 minutes at room temperature to visualize nuclei. 70 images were taken per coverslip using ScanSlide

in Metamorph software. Using CellProfiler, differentiation was quantified as a percentage of nuclei in MHC-positive myotubes. CellProfiler output was analyzed using Matlab (Mathworks) and R software.

2.2.6 Proteomic analysis of human muscle tissues

Proteomic analysis of muscle tissues was conducted using supraspinatus muscle samples from patients with either no tear (NT) (n=4) or massive tear (MT) (n=3). All biopsies were flash frozen with liquid nitrogen shortly after time of biopsy. Tissue was prepared for mass spectroscopy analysis using an ECM enrichment strategy from Hill and coworkers [54]. Briefly, 10-50 mg of tissue was cut from each sample and homogenized using a Tissue Tearer (Biospec Products) in a high salt buffer (50 mM Tris-HCl, 0.25% CHAPS, 25 nM EDTA, 3 M NaCl, 10 μL/mL protease inhibitor). The tissue was spun at 14,000 rpm at 4°C and the supernatant was removed. Next, the pellet was resuspended in urea extraction buffer (8 M Urea, 100 mM ammonium bicarbonate, 25 nM tris(2-carboxyethyl) phosphine, passed over Amberlite IRN 150 mixed ion exchange resin) and vortexed for 30 minutes. The soluble fraction was collected and further processed using the filter-aided sample preparation [117] kit (Expedeon). Samples were denatured using heat and dithiothreitol before being carboxyamidomethylated with iodoacetamide. Samples were then washed in a spin filter using urea and ammonium bicarbonate. Next, samples were digested with trypsin protease overnight at 37°C and then washed with ammonium bicarbonate. Prior to high-pressure liquid chromatography (HLPC), samples were pipetted through ZipTips with C18 resin (Millipore) to desalt the solution and to limit the amount of peptides loaded. ZipTip-processed samples were dried in a SpeedVac and resuspended in 5% acetonitrile and 2% formic acid for analysis.

Nanospray ionization experiments were performed using a TripleTof 5600 hybrid mass spectrometer (ABSCIEX) interfaced with nano-scale reversed-phase HPLC (Tempo) using a 10 cm-100 micron ID glass capillary packed with 5 μm C18 Zorbax beads (Agilent Technologies, Santa Clara, CA). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (560%)

of acetonitrile using Buffer A (98% H2O, 2% ACN, 0.2% formic acid, and 0.005% TFA) and Buffer B (100% ACN, 0.2% formic acid, and 0.005% TFA). A column flow rate of 250 l/min was used for 1 hour to collect peptides. MS/MS data were acquired in a data-dependent manner in which the MS1 data was acquired for 250 ms at m/z from 400 to 1250 Da and the MS/MS data was acquired from m/z from 50 to 2,000 Da. For independent data acquisition, MS1-TOF of 250 ms was followed by 50 MS2 events of 25 ms each. The independent data acquisition criteria include being over 200 counts threshold and a charge state +2-4 with 4 seconds exclusion.

The collected data were analyzed using MASCOT (Matrix Sciences) and Protein Pilot 4.0 (ABSCIEX) for peptide identifications. Normalized spectral abundance factors (NSAFs) were calculated to correct spectral counts for proteins length and for the total peptide content of each run[81].

2.2.7 Histological Analysis

Muscle tissue from donors described above was blocked in OCT compound (Sakura) and sectioned on a cryostat in 10 m-thick sections. Sections were stained with picrosirus red to identify collagen content. Sections were fixed in ice cold acetone for 10 minutes and rehydrated in 100% - 95% - 70% ethanol solutions before being washed with distilled water and stained with 0.1% picrosirius red in piric acid (Electron Microscopy Sciences) for 1 hour. Slides were washed with two changes of 0.5% glacial acetic acid and three changes of 100% ethanol before being mounted in Cytoseal 60 (Thermo Scientific).

2.2.8 Statistical analysis

SMP proliferation was analyzed using unsupervised hierarchical clustering in R [86], with distance metric of correlation and complete linkage calculated. Heat maps were generated using the gplots package in R. Approximately unbiased (AU) p values for hierarchical clustering were calculated using the pvclust R package [107]. For substrate studies with C2C12s, a one way repeated measures analysis of

variance (ANOVA) was used. SMP substrate studies were analyzed using a two-way repeated measures ANOVA. Proliferation data was analyzed using a two-way repeated measures ANOVA, with factors medium and tear state. For differentiation studies, a two way ANOVA with factors medium and tear state was used. HPLC-MS/MS data was analyzed using a custom Matlab script. A mixed effects model for predicting NSAFs with fixed effects tear state and GO term and random effect patient revealed significant tear state * GO term interaction ($p<0.0001$), indicating that the abundance of proteins with ECM or cytoskeletal GO terms varies with tear state. Data were split according to GO term association (ECM, cytoskeletal, or other), and submodels with fixed effect tear and random effect patient were calculated. Tukeys honest significant difference post hoc testing was used to determine differences between factor levels for all ANOVAs. Statistical significance was set to $p < 0.05$.

2.3 Results

2.3.1 Ex vivo human SMP expansion is affected by rotator cuff tear state

Murine SMPs have successfully been expanded on polyacrylamide (PA) hydrogels with a stiffness of 11 kPa [19, 31, 42], so for human SMP expansion, we coated 11 kPa hydrogels with laminin-111 and type IV collagen [78, 79] and selectively with type I collagen to mirror previous descriptions of the in vivo mouse niche [28]. While C2C12 mouse myoblast expansion readily occurs in both of these conditions, C2C12s are insensitive to these niche variations (Figure 2.2A). Conversely, NCAM positive human SMPs (Figure 2.1) with the same niche combinations failed to proliferate over several passages (Figure 2.2B); ECM protein composition of the culture substrate again made no significant difference in cell proliferation rates.

Since ECM stiffness and composition were not sufficient to induce proliferation, we next decided to also culture SMPs in the presence of growth factors to

Table 2.1: Patient Demographics. (SS = supraspinatus, IS = infraspinatus, D = deltiod, NT = no tear, PT = partial tear, FT = full tear, MT = massive tear, SMP = skeletal muscle progenitor cell experiments, HPLC MS/MS = mass spectroscopy experiments, - = missing data)

ID	**Tear state**	**Sex**	**BMI**	**Age**	**Assay Usage**
62SS	NT	M	28.48	56	SMP
33IS	FT	F	34.70	62	SMP
82SS	PT	M	31.59	60	SMP
68D	PT	M	37.97	53	SMP
80IS	FT	M	22.81	62	SMP
65IS	NT	F	20.67	49	SMP
63D	NT	F	23.91	53	SMP
1D	NT	-	25.30	52	SMP
6SS	FT	F	24.24	55	SMP
57D	NT	M	27.37	57	SMP
47SS	FT	F	24.56	57	SMP
34SS	NT	M	28.89	38	SMP
5D	NT	-	25.57	56	SMP
MM49D	NT	F	24.53	50	SMP
MM49IS	NT	F	24.53	50	SMP
MM49SS	NT	F	24.53	50	SMP
MM50SS	NT	F	27.95	35	SMP
K23SS	NT	F	26.33	57	HPLC MS/MS
K25SS	NT	F	28.71	51	HPLC MS/MS
K26SS	NT	M	26.10	27	HPLC MS/MS
K30SS	NT	F	20.19	20	HPLC MS/MS
K8SS	MT	M	28.20	81	HPLC MS/MS
K12SS	MT	F	25.63	81	HPLC MS/MS
K14SS	MT	M	26.97	80	HPLC MS/MS

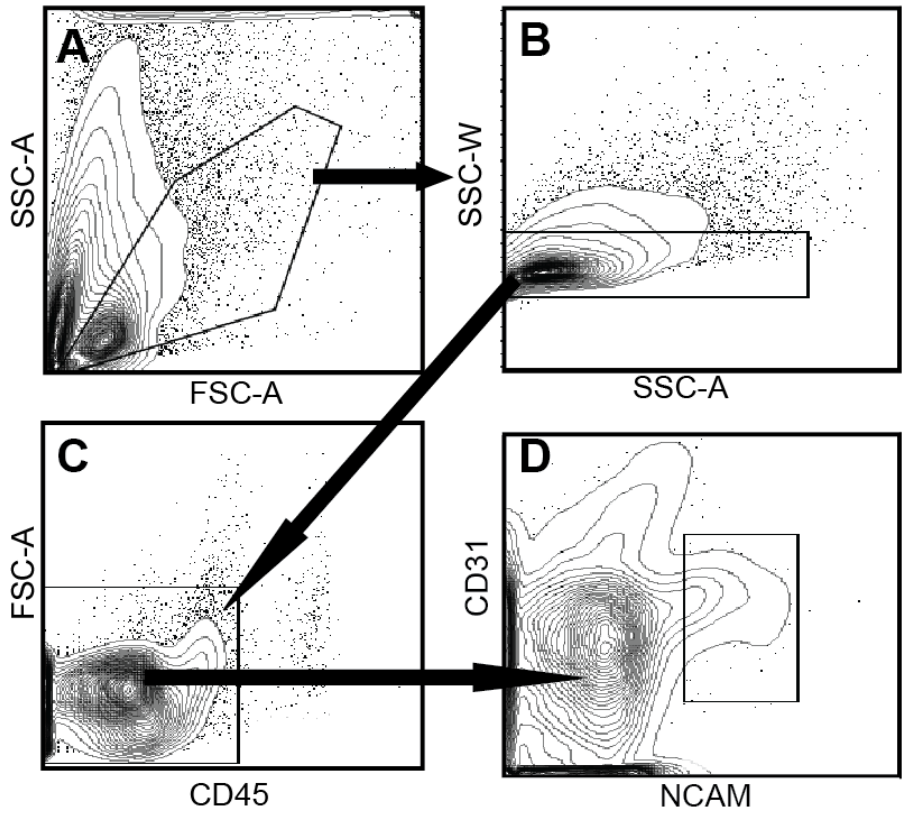

Figure 2.1: FACS isolation of human SMPs. Flow cytometry process used to isolate SMPs starts with separation by (A) size via gating of forward and side scatter amplitude as well as (B) side scatter width. (C) CD45 negative cells are then gated for (D) NCAM positive CD31 negative expression for SMPs.

test whether a more complete niche could maintain the SMP phenotype. Based on previous literature, FGF2 [123], FGF6 [123], FGF19 [123], HGF [119], IGF-1 [60], and oxytocin [29] were added individually, in combination, or not at all to growth medium. Again SMPs were cultured on 11 kPa PA hydrogels with collagen IV and laminin-111 as well as on glass. SMPs were quantified at each of 5 passages via hemocytometer. The growth factor effect was significant (Figure 2.3A; $p < 10\text{-}4$), with SMPs grown in FGF2 having the greatest average proliferation rate compared to SMPs grown without exogenously added growth factors (Figure 2.3B; $p < 0.05$, two way repeated measures ANOVA with Tukey post hoc testing); effects with other growth factors were not significantly better than proliferation rates without added growth factors, despite contrary observations with

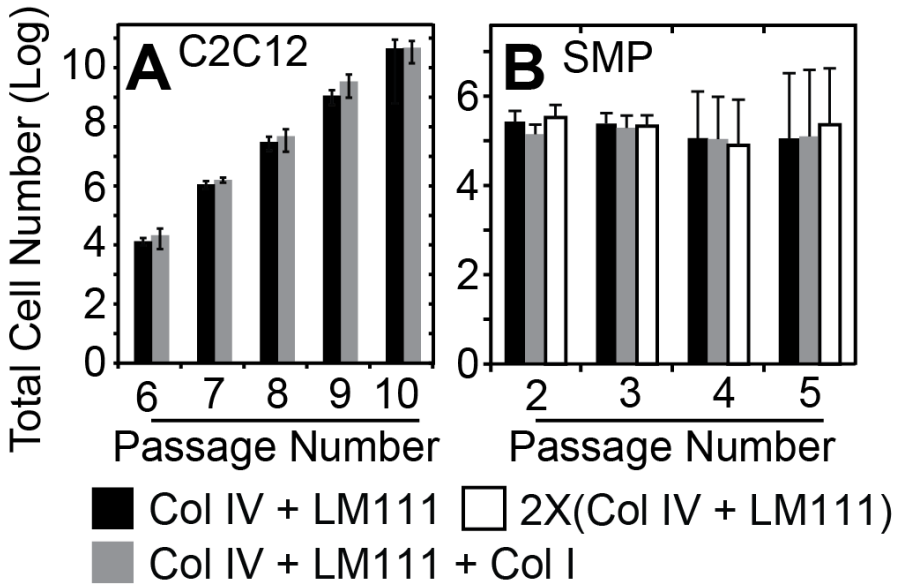

Figure 2.2: ECM proteins do not significantly affect proliferation rate. (A). C2C12 mouse myoblasts were cultured on condition 1: 11 kPa polyacrylamide gels, laminin-111, collagen type IV and condition 2: 11 kPa polyacrylamide gels, laminin-111, collagens type I and IV. n = 3 technical replicates with p-value = 0.32. (B). SMPs were cultured on conditions as described above. n = 3 biological replicates with p-value = 0.33. Data were analyzed using one way (A) or two way (B) repeated measures ANOVA with Tukey post hoc. Error bars are standard error of the mean (SEM).

mouse SMPs [123, 119, 60, 29] (Figure 2.3). However, tear state also had a significant effect on proliferation rates across growth factor conditions, with partial and full tear samples proliferating more slowly than no tear samples ($p < 10\text{-}4$). Unsupervised hierarchical clustering also revealed that SMPs from muscles with torn RCTs (either partial or full) were generally were less proliferative than SMPs from cases lacking a tear (Figure 2.3A). Approximately unbiased (AU) p-values calculated by multiscale nonparametric bootstrapping using the pvclust R package [107] indicated high confidence for the clustering observed (Figure 2.4). In the specific case of FGF2 where the highest average expansion occurred, it is worth noting that SMPs from muscles with torn RCTs were 100-fold less proliferative than the untorn counterparts (Figure 2.3B), indicating how dominant RCT tear state is as a predictor of SMP proliferation.

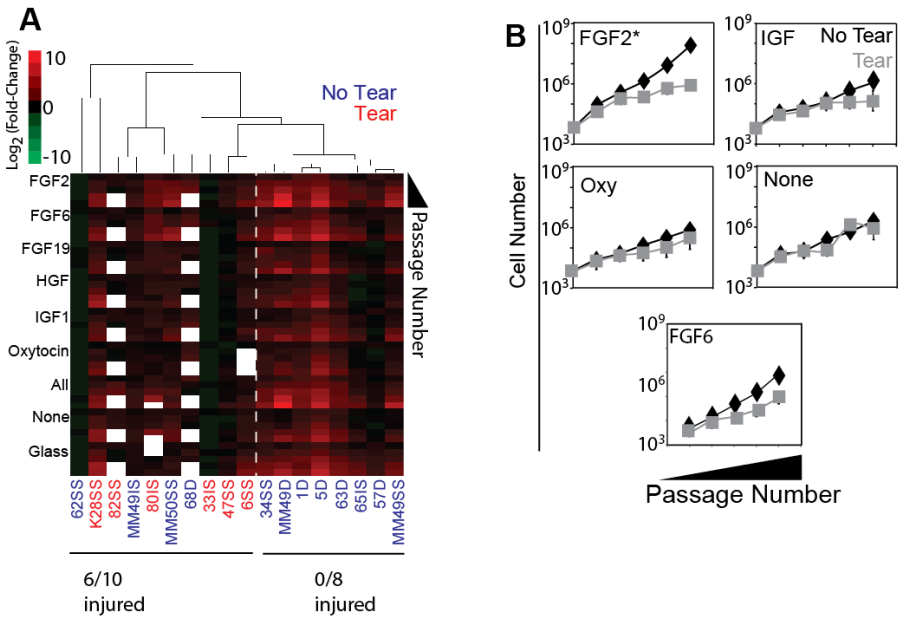

Figure 2.3: Growth factor and tear state affect long term SMP proliferation rates. (A). SMPs were grown on matrices of collagen IV and laminin-111 with the addition of growth factors. Unsupervised hierarchical clustering was used to order patient data at the third passage. Growth factor effect $p < 0.001$, tear effect $p < 0.001$. (B). FGF2 significantly enhanced SMP proliferation over adding no growth factors ($p < 0.01$). Data were analyzed using a two way repeated measures ANOVA with Tukey post hoc. Error bars are standard error of the mean (SEM).

2.3.2 Myotube fusion rate trends with rotator cuff tear state and growth medium

While the RCT injury niche may reprogram SMPs to limit their expansion, poor clinical outcomes 5 might further suggest limits on the ability of existing SMPs to fuse into and repair mature muscle. Thus, after expansion in a muscle mimetic niche, SMP differentiation potential was assessed for each growth factor. We found that differentiation rates, calculated as the percentage of nuclei within myosin heavy chain (MHC) positive myotubes (Figure 2.5A) after 5 days in differentiation media, were significantly affected by tear state of the RCT and specific growth factors used during cell expansion. When unsupervised hierarchical clustering was performed and cluster confidence was evaluated using the Pvclust R package [107], AU p-values from multiscale nonparametric bootstrapping indicated high

confidence (>95%) for each cluster generated (Figure 2.6). Differentiation rates were significantly greater for SMPs from torn cuffs than for SMPs from intact cuffs (Figure 2.5B,C; $p < 10^{-4}$, two way ANOVA). Growth factor effects were also significant ($p < 0.01$), as was the medium and tear state interaction ($p = 0.005$). Thus, the effects of expansion in IGF1, which produced the highest average rates of differentiation, were not uniform across tear state. The dependence of SMP expansion and differentiation on tear state and growth factors, as well as prior observations that injury can affect SMP expansion in vivo [77], suggests that expansion may differently affect SMPs underlying self-renewal status, i.e. the difference between proliferating and differentiating SMPs [121, 6]. These data suggest that specific niche conditions could prime cells for differentiation after expansion, which would impact the ability of differentiated progeny of SMPs in RC muscles to repair post injury.

Since FGF2 and IGF1 produced the most robust expansion and MHC positive fibers, respectively, we next determined the concentrations that optimized SMP proliferation and subsequent differentiation to ascertain if specific niche conditions could improve both. Using FGF2 and IGF1 concentrations above and below those reported to affect murine SMPs [123, 119], we found a significant growth factor effect in the no tear sample ($p < 10\text{-}4$, one way ANOVA) and the highest average SMP proliferation for FGF2, consistent with Figure 2.3. While there were few significant effects within FGF2 or IGF1 concentrations, maximal expansion for SMPs from muscle from torn RCTs occurred at 30 ng/mL IGF1 ($p = 0.037$, Tukey post hoc following one way ANOVA, Figure 2.7A). To determine if there was a concentration dependence on subsequent differentiation, we assessed myotube fusion based on the number of nuclei per MHC positive cell. SMPs from intact RCT muscles were not impacted by growth factor condition versus media without exogenous growth factors, consistent with Figure 2.3. However for SMPs from torn RC muscles, we again found that IGF1 produced a more robust response than FGF2, but specifically with an optimum at 50 ng/mL (Figure 2.7B,C). Thus, 50 ng/mL IGF1 appears to prime SMPs from torn RC muscles, while not affecting for SMPs from untorn RCT muscles. Given prior observations that injury can af-

fect SMP expansion in vivo [77] and our current observation that niche conditions affect expansion, these data implicate specific growth factor dosing in combination could better prime or encourage post-injury SMP expansion and differentiation.

2.4 Analysis of protein composition changes in muscle with rotator cuff tear state

Given that the niche significantly affected SMP behavior, we next characterized differences in the niche in vivo to correlate it with matrix and growth factor combinations used in vitro. We used high-pressure liquid chromatography coupled with tandem mass spectroscopy (HPLC-MS/MS) and an ECM enrichment strategy for sample preparation [54] to evaluate bulk protein changes in supraspinatus muscle from massive tear (n = 3) and untorn (n = 4) cases. 10,252 unique tryptic peptides were detected (Supplemental Table 2.1) accounting for a total of 447 (massive tear) and 337 (no tear) non-redundant proteins (Supplemental Table 2.2); of these proteins, 277 were common between tear states. ECM and cytoskeletal proteins were the most abundant gene ontology (GO) terms based on BioMart annotations [104] and in accordance with the ECM enrichment strategy [54]. Comparisons of normalized spectral abundance factors (NSAFs) [81] for each protein common between tear states showed ECM protein enrichment in biopsies of massive tears, i.e. a shift away from equal expression between cuff states (Figure 2.8A, red; upward and leftward shift).

A mixed effects model for predicting NSAFs with fixed effects tear state and GO term and random effect patient revealed significant tear state * GO term interaction ($p < 0.0001$), indicating that the abundance of proteins with ECM or cytoskeletal GO terms varies with tear state. In a subsequent model with the cytoskeletal fraction of the data with fixed effect tear and random effect patient, cytoskeletal proteins were significantly increased in no tear muscle ($p = 0.046$). To quantitatively illustrate this, differential expression was computed as distance of each proteins NSAF from the y=x line, plotted in the order of decreasing distance. For proteins with greater expression in massive tear samples (Figure 2.8B; Table

2.2), ECM GO terms comprised 48% of the 25 most differentially expressed proteins. The most abundant matrix proteins are also listed, with fibrillar collagens highlighted in bold. Given their relatively high abundance, the presence of several fibrillar collagens indicates possible niche remodeling. Conversely for no tear samples (Figure 2.8C; Table 2.3), cytoskeletal GO terms comprised 44% of the 25 most differentially expressed proteins whereas only 4 of the top 100 terms were ECM. Furthermore, the few ECM proteins enriched in no tear RC muscle were laminins, fibronectin, and nidogen, all of which are associated with the SMP niche. These data indicate that fibrosis may substantially change the composition of the SMP niche in vivo in muscle with RCT tears, and to confirm this, picrosirius red staining of the same samples was performed. Staining indicated that while muscle from untorn RCTs had some degree of fibrosis possibly associated with bursitis, there was substantial collagen deposition within muscle from torn RCTs (Figure 2.8D), consistent again with an altered SMP niche in vivo. These data further suggest that changes within the niche during the chronic phase of remodeling could negatively impact SMP repair ability post-surgery.

2.5 Discussion

While most murine and human studies with SMPs report age effects to varying degrees [14, 21, 23], our results highlight the importance of disease state and cell culture conditions in the ability of the SMP to proliferate and differentiate. While the former was assessed in vitro, it is possible that the significant differences we observed within the niche itself could have longer-term implications for repairing chronically torn RCTs.

2.5.1 Human versus murine differences in SMP behavior

Previous studies have implicated numerous growth factors, including FGF2 [123], FGF19 [123], FGF6 [123], HGF [119], IGF1 [60], and oxytocin [29], in maintaining the proliferative state of murine SMPs in vitro. Using human SMPs, however, we found only FGF2 to significantly improve cell expansion. Additionally, the

Table 2.2: Top 25 enriched proteins in muscle from massive tear RCs.

GI ID	**Protein name**	**Distance from y=x**
153946395	Tenascin C precursor	4.94
40217843	Cartilage oligomeric matrix protein precursor	4.20
61743954	AHNAK nucleoprotein isoform 1	3.13
55743098	Alpha 3 type VI collagen isoform 1 precursor	3.00
4507467	Transforming growth factor, beta-induced, 68kDa	2.78
93141047	Collagen, type XII, alpha 1 long isoform precursor	2.48
4502067	Alpha-1-microglobulin/bikunin precursor	2.36
51173715	Actin-binding LIM protein 1 isoform c	2.25
4557321	Apolipoprotein A-I preproprotein	2.22
4505763	Phosphoglycerate kinase 1	2.17
70906435	Fibrinogen, beta chain preproprotein	2.16
157419126	Laminin, alpha 4 isoform 2 precursor	2.14
27436946	lamin A/C isoform 1 precursor	2.10
32307172	Dermatopontin precursor	2.10
48762934	Alpha 2 type I collagen	1.98
4503689	Fibrinogen, alpha polypeptide isoform alpha-E preproprotein	1.91
110349772	Alpha 1 type I collagen preproprotein	1.91
5901944	Elastin microfibril interfacer 1	1.79
88853069	Vitronectin precursor	1.75
67782336	Tenascin XB isoform 1	1.70
4506041	Proline arginine-rich end leucine-rich repeat protein precursor	1.70
169218200	PREDICTED: hypothetical protein	1.68
70906439	Fibrinogen, gamma chain isoform gamma-B precursor	1.67
4758040	Cytochrome c oxidase subunit VIc proprotein	1.64

Table 2.3: Top 25 enriched proteins in muscle from untorn RCs.

GI ID	**Protein name**	**Distance from y=x**
4501891	Actinin, alpha 1	1.71
12025678	Actinin, alpha 4	1.64
4501885	Beta actin	1.55
21536274	Calsequestrin 1	1.53
28559088	Laminin alpha 2 subunit isoform a precursor	1.35
14589866	Aspartate beta-hydroxylase isoform a	1.27
40807491	Acyl-CoA synthetase long-chain family member 1	1.24
5454152	Ubiquinol-cytochrome c reductase binding protein	1.10
4505357	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4, 9kDa	1.08
156151369	Cell death-regulatory protein GRIM19	1.06
4507615	Troponin C, slow	1.03
4506911	Sarcoglycan, alpha (50kDa dystrophin-associated glycoprotein)	1.03
119703755	Laminin, beta 2 precursor	0.99
31542301	Sorting and assembly machinery component 50 homolog	0.92
114155140	Tropomyosin 3 isoform 1	0.91
15451856	Caveolin 1	0.91
156104903	Myomesin 2	0.90
66472922	Hypothetical protein LOC347273	0.90
4501893	Actinin, alpha 2	0.89
4507435	Telethonin	0.88
21359867	Cytochrome c-1	0.88
4758790	NADH dehydrogenase (ubiquinone) Fe-S protein 5, 15kDa (NADH-coenzyme Q reductase)	0.87
169217576	PREDICTED: similar to nebulin	0.87
39930527	Troponin T1, skeletal, slow	0.86
110349719	Titin isoform N2-A	0.86

effects of muscle-mimetic substrate stiffness and ECM protein composition were not sufficient to prolong human SMP proliferation, in contrast to murine models [42]. However, such comparisons can be problematic due to population differences resulting from SMP isolation [6, 25, 24, 126].

Previous studies have implicated numerous growth factors, including FGF2 [123], FGF19 [123], FGF6 [123], HGF [119], IGF1 [60], and oxytocin [29], in maintaining the proliferative state of murine SMPs in vitro. Using human SMPs, however, we found only FGF2 significantly improved cell expansion and IGF1 significantly improved cell fusion. Additionally, the effects of muscle-mimetic substrate stiffness and ECM protein composition were not sufficient to prolong human SMP proliferation, in contrast to murine models [42]. However, such comparisons may be problematic due to population differences resulting from SMP isolation [6, 25, 24, 126]. While populations can be evaluated for Pax7 expression, isolation differences could result different in Pax7+ subsets and thus different outcomes as we observed, e.g. growth factor concentrations reported in mouse literature resulted in different outcomes here; thus, additional combinations of niche conditions could further improve human SMPs expansion and their regenerative capacity [121, 6].

2.5.2 RC tear state affects SMP phenotype maintenance ex vivo

Here we showed that SMPs from muscle with torn RCTs proliferated at slower rates over several passages in culture but differentiated significantly better than SMPs from untorn RCTs. Of note is the difficultly of decoupling tear effects from any possible muscle group effects. As a RCT tear generally involves the supraspinatus or both of the supraspinatus and infraspinatus tendons, these muscles will be directly unloaded following injury, while the deltoid remains mechanically unaffected. The data presented here include some deltoid samples as intact control muscle, so it is possible that some of the differential clustering observed is due to differences between muscle groups. However, previous studies have investigated human SMPs from different muscle groups and pooled results

with success [16], indicating that SMPs from diverse muscle groups may have similar properties. However as that study had a patient cohort with substantially different demographics from this RCT repair cohort, exact comparisons to their expansion and engraftment may be difficult. Furthermore while the mean age of the tear group was significantly greater than that of the no tear group ($p = 0.001$, 65 and 45 years, respectively), it is not known to what degree such an age difference affects SMP qualities when both ages are neither juvenile nor geriatric. Regardless, our finding that tear state influenced the growth factor response, specifically with FGF2 and IGF1, indicates that subsequent analysis requires more careful tissue analysis and consideration of injury status. Moreover it suggests that possible clinical intervention with these specific factors in acute tears could be beneficial by maximizing expansion and repair, though more direct in vivo evidence is required.

2.5.3 RCT tear state affects SMP niche components

As the SMP niche has a demonstrated importance in maintaining the SMP phenotype, it is likely that the altered niche SMPs encounter in an injury or pathological state affects SMP quality. Indeed, mouse models bear this out; knock out of collagen VI impaired regeneration and reduced SMP self-renewal after injury [111]. Conversely, excessive fibrosis also limits SMP renewal through chronic inflammatory responses that block entry into muscle fibers [75]. Our examination of proteomic changes in muscles from torn versus untorn RCTs illustrates the increased ECM deposition and loss of cytoskeletal proteins seen in RCT tear muscles, which suggests that fibrotic responses can alter the SMP niche. Severe, chronic RCT injuries could then impair SMP self-renewal within the niche [77] as well as in culture, as we observed. Despite all of these significant remodeling events, especially those associated with the basement membrane that surrounds muscle fibers in vivo, it is important to note that HPLC-MS/MS evaluates bulk level protein expression in the sample. Thus the changes we observed were likely due to global differences in the connective tissue of the muscle belly rather than specific differences within the SMP niche. Myofibroblast-associated matrix could make SMP niche changes in response to injury difficult to detect. Although histological

analyses are an alternative to HPLC-MS/MS, the changes we observed in vivo and their ability to be modulated in vitro with growth factors that inhibit fibrosis lead us to conclude that HPLC-MS/MS provided a reasonable snapshot of the RC muscles.

2.6 Conclusion

This study demonstrates the importance of the SMP niche in maintaining proliferation and differentiation capacity in vitro. We show the difficulties of translating findings in soluble factors for murine SMPs to human SMPs, as only FGF2 substantially improves long-term expansion ex vivo. Furthermore, we establish a relationship between the injury state of the muscle used for SMP isolation and SMP phenotype maintenance ex vivo. Our data indicate that SMPs from muscles with a RCT tear proliferate more slowly but differentiate at greater rates after several passages than SMPs from muscles without RCTs. HPLC-MS/MS analysis of proteomic changes in response to RCT tear shows an accumulation of ECM proteins and a decrease in cytoskeletal proteins in massive RCT tear muscle. These shifts in protein expression could alter the in vivo niche for SMPs that affect their ability to expand in vitro, irrespective of culture conditions. These data suggest the importance of context and injury-specific considerations in treating RCTs with exogenous factors to expand and prime SMPs in vivo.

Chapter 2 was a paper submitted to Journal of Orthorpaedic Research titled "Rotator cuff tear state modulates self-renewal and differentiation capacity of human skeletal muscle progenitor cells". I acknowledge Michael Gibbons, John Lane, Anshuman Singh, Samuel Ward, and Adam Engler as co-authors in this work.

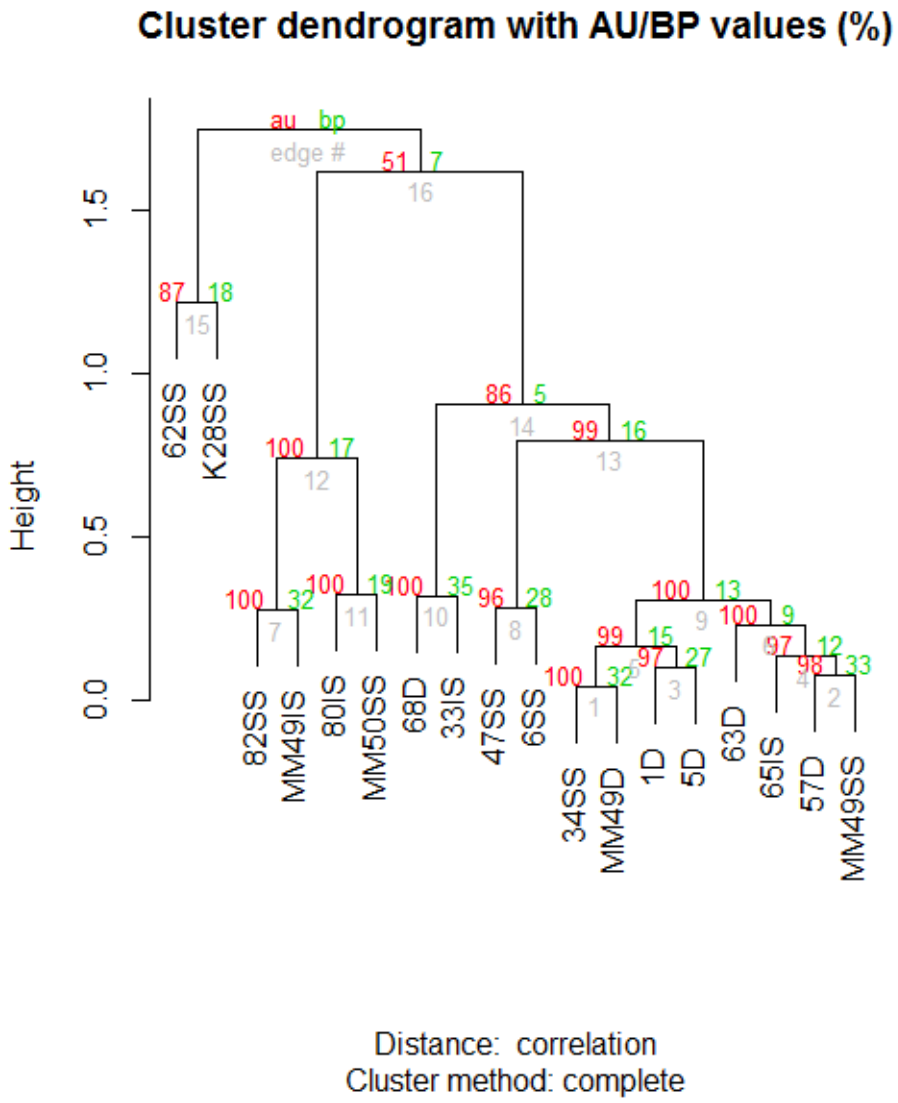

Figure 2.4: Cluster dendrogram for SMP proliferation. Pvclust R package and unsupervised hierarchical clustering was used to cluster SMP proliferation data. Approximately unbiased (AU) p-values calculated by multiscale nonparametric bootstrapping are shown in red and bootstrap probability (BP) values are shown in green.

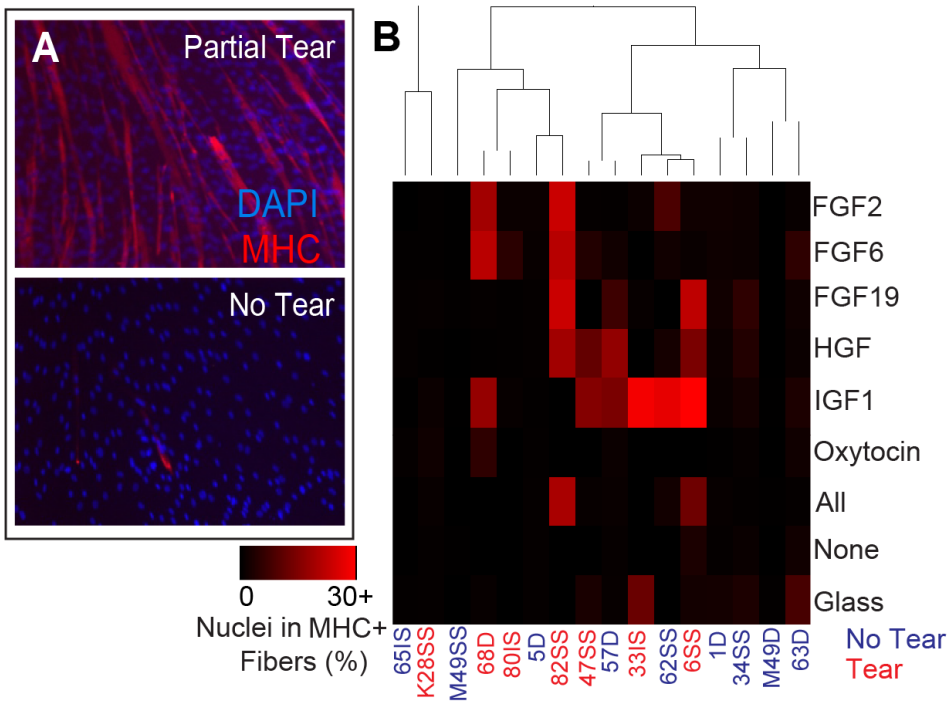

Figure 2.5: SMP differentiation capacity varies by tear state and proliferation medium. (A) Cells were seeded at high confluency at the end of the proliferation experiment (P7-8) and allowed to differentiate for 5 days in differentiation medium (5% horse serum and 10 µg/ml insulin). Representative images of cells are shown from partial tear and no tear. (B) Differentiation was quantified as the number of nuclei that were in myosin heavy chain (MHC)-positive myotubes. Growth factor effect p = 0.00698, tear effect p = 2.62E-5, growth factor*tear interaction p = 0.00547. Data were analyzed using a two way ANOVA.

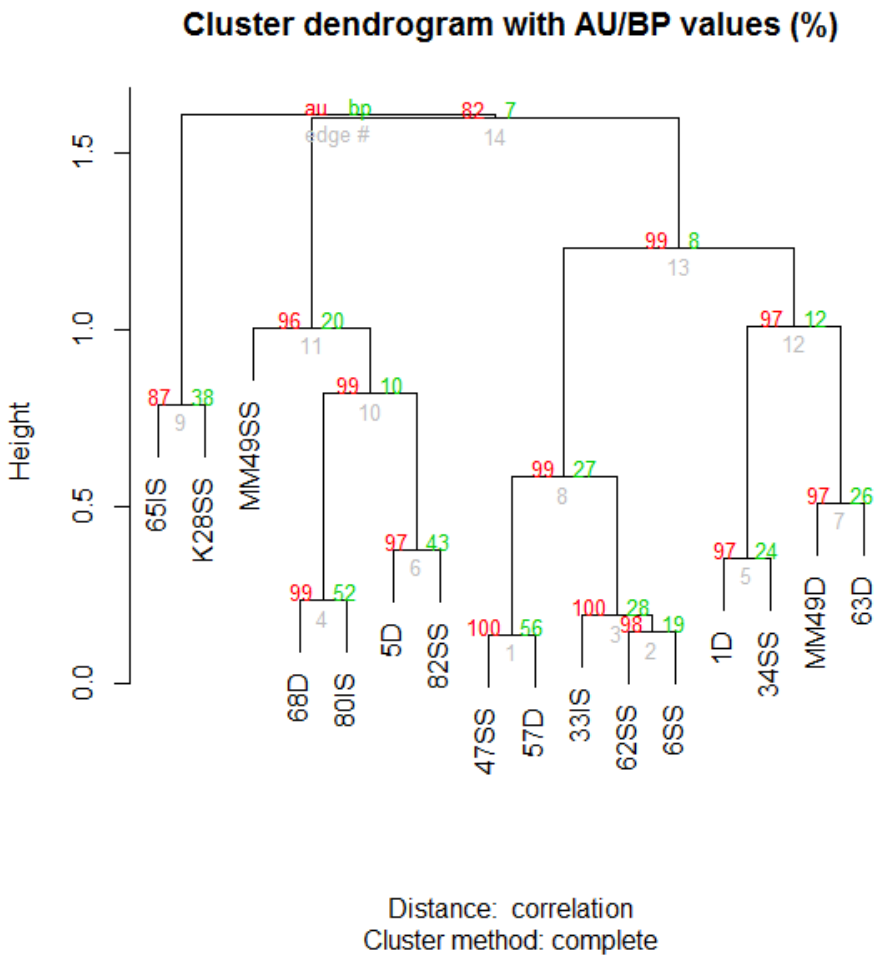

Figure 2.6: Cluster dendrogram for SMP differentiation. Pvclust R package and unsupervised hierarchical clustering was used to cluster SMP differentiation data. Approximately unbiased (AU) p-values calculated by multiscale nonparametric bootstrapping are shown in red and bootstrap probability (BP) values are shown in green.

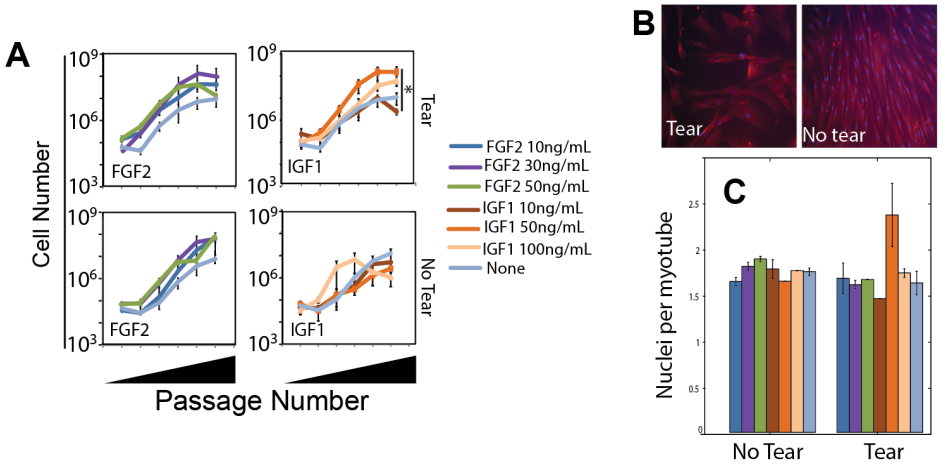

Figure 2.7: Growth factor dose effects. (A) SMPs were expanded on 11 kPa polyacrylamide gels, laminin-111, collagens type I and IV in the presence of the indicated growth factors and their concentrations. Data is plotted as total cell number versus passage number. n =3 technical replicates with one biological replicate per tear state. (B) Immunofluorescent staining for MHC from SMPs with the indicated tear state. (C) Number of nuclei per MHC positive cell plotted for the growth factors and concentrations indicated in panel A. *p <0.05 for comparisons to all other conditions using a post hoc Tukey test. Error bars are SEM.

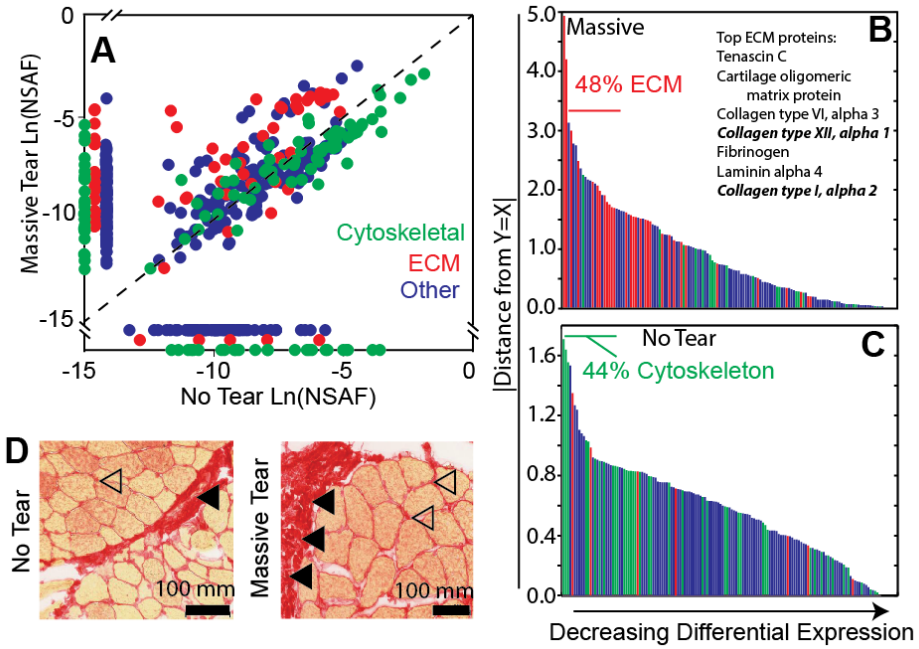

Figure 2.8: Mass spectroscopy reveals that muscle composition varies with disease state. (A) Natural log of mean normalized spectral abundance factors (NSAFs) plotted for massive tear (n = 3) vs. no tear (n = 4). Dashed line is y=x reference line. Proteins are colored by gene ontology (GO) terms for cytoskeletal (green), ECM (red), and other (blue). Proteins expressed in either massive tear or no tear samples but not both are located along each respective axis. (B, C) The absolute value of the distance from each point to the y=x reference line was calculated to indicate differential protein expression. Panel B indicates those proteins expressed at higher levels in massive tear samples (i.e. to the left of the y=x line), while panel C indicates those expressed at higher levels in no tear samples. (D) Representative images of picrosirius red staining of no tear and massive tear samples show increased ECM content and collagen deposition in massive tear samples, with some collagen deposition in no tears likely due to bursitis.

Chapter 3

Altered ECM and cytoskeletal content in dystrophic muscle: A proteomics study

3.1 Introduction

Duchenne Muscular Dystrophy (DMD) is a muscle disease affecting 1 in 3500 live male births [30]. It is caused by mutations in dystrophin, a protein linking the actin cytoskeleton and extracellular matrix (ECM) [83]. Loss of functional dystrophin results in instability of muscles contractile apparatus and skeletal muscle deterioration. A hallmark of disease progression in DMD is fatty infiltration and fibrosis in skeletal muscle [118, 127].

Work exploring altered ECM composition in human DMD muscle due to fibrosis is limited, conflicting, and is primarily focused on RNA expression [124, 84, 51], making it hard to discern how protein levels correspond. Human gene expression studies have demonstrated an upregulation of ECM components, including fibrillar collagens, basement membrane proteins, and crosslinking ECM proteins, in mRNA from DMD patient samples [124, 84]. However, conflicting results in gene and protein expression analyses indicate that glycoproteins decorin and biglycan may be upgegulated [33], downregulated [125], or relatively unchanged

and variable from sample to sample [125]. Mouse models, notably the mdx mouse, are excellent tissue sources to examine ECM protein expression in a dystrophic state, but as with most animal models of disease, the severity of human DMD is not fully recapitulated [27]. Thus, there remains a need to evaluate ECM composition at the protein level in human muscle samples.

To investigate the aberrant ECM protein expression in humna dystrophic muscle, we utilized high pressure liquid chromatography coupled with tandem mass spectroscopy (HPLC-MS/MS) on muscle biopsies obtained from DMD patient autopsies and from healthy donors undergoing reconstructive knee surgery. Relative protein content was quantified using the normalized spectral abundance factor (NSAF) [81]. Results indicate upregulation of fibrillar collagens (types I and III) and cross linking proteins in the ECM (for example, decorin), with basement membrane proteins, including laminins and collagen type IV, downregulated or unchanged in DMD muscle.

3.2 Materials and Methods

3.2.1 Proteomic analysis of human muscle tissues

Mass spectroscopy studies were conducted using 6 total samples from 3 DMD patients post-mortem, ages 12, 17, and 24 years, and 5 total samples from 4 healthy patients undergoing reconstructive knee surgery, ages 14-16 years. DMD biopsies were taken from the tibialis anterior, deltoid, biceps brachii, or quadriceps muscles, while biopsies from healthy donors were taken from the semitendinosus or gracilis muscles (Table 3.1). All biopsies were flash frozen with liquid nitrogen prior to being shipped to our lab. The tissues were prepared for mass spectroscopy analysis using the filter-aided sample preparation (FASP) [117] kit (Expedeon). Briefly, 10-50 mg of tissue was cut from each sample and placed in a 1% sodium dodecyl sulfate (SDS) solution overnight on an orbital shaker to enrich for ECM proteins before being homogenized in 1% SDS in PBS. The soluble fraction was collected and further processed using FASP. Samples were denatured using heat and dithiothreitol before being carboxyamidomethylated with iodoacetamide. Samples were

Table 3.1: Patient Information

Patient	**Disease state**	**Age (years)**	**Muscle Group**
1	DMD	24	Deltoid
1	DMD	24	Tibialis anterior
1	DMD	24	Biceps brachii
2	DMD	17	Deltoid
2	DMD	17	Quadracep
3	DMD	12	Quadracep
4	healthy	14	Semitendinosus
4	healthy	14	Gracilis
5	healthy	16	Semitendinosus
6	healthy	15	Semitendinosus
7	healthy	15	Gracilis

then washed in a spin filter using urea and ammonium bicarbonate. Next, samples were digested with trypsin protease overnight at 37°C and then washed with ammonium bicarbonate. Prior to high-pressure liquid chromatography (HLPC), samples were pipetted through ZipTips with C18 resin (Millipore) to desalt the solution and to limit the amount of peptides loaded. ZipTip-processed samples were dried in a SpeedVac and resuspended in 5% acetonitrile and 2% formic acid for analysis.

Nanospray ionization experiments were performed using a TripleTof 5600 hybrid mass spectrometer (ABSCIEX) interfaced with nano-scale reversed-phase HPLC (Tempo) using a 10 cm-100 micron ID glass capillary packed with 5 μm C18 Zorbax beads (Agilent Technologies). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5% 60%) of acetonitrile using Buffer A (98% water, 2% ACN, 0.2% formic acid, and 0.005% TFA) and Buffer B (100% ACN, 0.2% formic acid, and 0.005% TFA). A column flow rate of 250 l/min was used for 1 hour to collect peptides. MS/MS data were acquired in a data-dependent manner in which the MS1 data was acquired for 250 ms at m/z from 400 to 1250 Da and the MS/MS data was acquired from m/z from 50 to 2,000 Da. For independent data acquisition, MS1-TOF of 250 ms was followed by 50 MS2 events of 25 ms each. The independent data acquisition criteria include being over 200 counts threshold and a charge state +2-4 with 4 seconds exclusion.

The collected data were analyzed using MASCOT (Matrix Sciences) and

Table 3.2: Antibody information

Antigen	**Product number**	**Concentration**
Collagen I	Abcam ab34710	1:500
Collagen II	Thermo scientific 5B2.5	1:500
Collagen III	Abcam ab7778	1:500
Decorin	Abcam ab54728	1:500
Laminin	Abcam ab11575	1:500

Protein Pilot 4.0 (ABSCIEX) for peptide identifications. Normalized spectral abundance factors (NSAFs) were calculated to correct spectral counts for proteins length and for the total peptide content of each run [81]. Samples were run in duplicate from independent sample preparations to evaluate reproducibility.

3.2.2 Histological analysis

Muscle tissue from donors described above was blocked in OCT compound (Sakura) and sectioned on a cryostat in 10 μm-thick sections. Sections were stained with hematoxylin and eosin (H&E) or fixed in 3.7% formaldehyde in PBS for 15 minutes at room temperature and then washed with PBS for 15 minutes. Sections were blocked in 2% bovine serum albumin (BSA) in 1 mM $MgCl_2$MgCl 2 in PBS for 1 hour. Primary antibodies (Table 3.2) were diluted in 2% bovine serum albumin (BSA) in 1 mM $MgCl_2$MgCl 2 in PBS, and sections were incubated at 4°C overnight. Sections were stained with donkey anti rabbit Alexa Fluor 488 (Invitrogen A-11008) or donkey anti mouse Alexa Fluor 488 (Invitrogen A-21202) secondary antibody at a 1:200 diultion in 2% bovine serum albumin (BSA) in 1 mM $MgCl_2$MgCl 2 in PBS for 30 minutes at room temperature. Hoescht (1:1000 dilution in water) was used to stain for nuclei. Slides were mounted in Fluoromount (Southern Biotech) and allowed to dry overnight before imaging.

3.2.3 Statistical analysis

HPLC-MS/MS data was graphed using a custom Matlab script. A mixed effects model for predicting NSAFs with fixed effects disease state and GO term and random effect sample ID revealed a significant tear state * GO term interaction

($p < 0.0001$), indicating that the abundance of proteins with ECM or cytoskeletal GO terms varies with tear state. Data were split according to GO term association (ECM, cytoskeletal, or other), and submodels with fixed effect disease state and random effect sample ID were calculated. Models were fit using the nlme package [85] in R [86]. Statistical significance was set to $p < 0.05$.

3.3 Results

We used high-pressure liquid chromatography coupled with tandem mass spectroscopy (HPLC-MS/MS) on muscle samples from DMD (n = 6 muscles) or healthy (n = 5 muscles) to evaluate changes in ECM and cytoskeleton composition. 7,806 unique tryptic peptides were detected (Supplemental Table 3.1), mapping to 772 proteins (Supplemental Table 3.2); of these proteins, 171 were common between DMD and healthy samples, while DMD had 325 unique proteins and healthy had 276. ECM proteins were the most abundant gene ontology (GO) terms for DMD samples, and cytoskeletal proteins were most abundant for healthy samples (Figure 3.1A). Comparisons of normalized spectral abundance factors (NSAFs) [81] for each protein common between disease and healthy showed ECM protein enrichment in DMD muscle (Figure 3.1B, red; upward and leftward shift).

We fit a mixed effects model for predicting NSAFs with fixed effects disease state and protein GO term and random effect sample ID. A significant disease state * GO term interaction ($p < 0.0001$) indicated that the abundance of proteins with ECM or cytoskeletal GO terms varies with the presence of DMD. Fitting submodels with fixed effect disease state with ECM, cytoskeletal, and other fractions of the data showed significant disease effects for ECM proteins ($p = 0.0178$), cytoskeletal proteins ($p = 7E\text{-}4$), and other proteins ($p = 0.0024$). Thus, there were significantly more ECM and other proteins in DMD muscle, while healthy muscle had significantly more cytoskeletal proteins. As loss of dystrophin leads to sarcolemmal instability and cystoskeletal degradation, this data corresponds with established DMD muscle characteristics.

To graphically illustrate this, differential expression was computed as dis-

tance of each proteins mean NSAF from the y=x line and plotted in the order of decreasing distance (Figure 3.1C, D). The top 10 differentially expressed proteins higher in healthy muscle were largely cytoskeletal (titin, troponin, myotilin), while laminin α2 chain, which forms muscle laminin isoform 211 in the basement membrane [59], was the only ECM protein in the top 10 (Table 3.3). Furthermore, the few additional ECM proteins enriched in no tear RC muscle were other laminin chains (β2 and γ1), collagen type IV α2 chain, perlecan, and nidogen, all of which are associated with the basement membrane. In DMD muscle, the 10 top enriched proteins included a collagen type VI isoform and prolargin, which binds to collagens type I and II and the basement membrane heparan sulfate proteoglycan perlecan [2] (Table 3.4). Other ECM proteins enriched in dystrophic muscle include collagens type I and III, decorin, and tenascin c.

The reproducibility of results was evaluated by independently preparing 10/11 samples a second time and completing a second run. Plotting run 1 versus run 2 illustrates that, while proteins of high abundance tended to be identified in both runs, a substantial portion of proteins identified in one run were not present in the other run of the same sample (Figure 3.3). Furthermore, overall correlation values of 0.56 for healthy and 0.66 for DMD indicate that protein composition may vary even within a single muscle sample and could argue for increased sample sizes in proteomic studies of muscle.

Staining of DMD and healthy muscle sections confirmed our findings (Figure 3.2). Hematoxylin and eosin (H&E) staining showed the extent of structural alterations in DMD muscle compared with healthy. In dystrophic muscle, collagens type I, II, and III, decorin, and laminin were diffuse throughout the muscle, with less organization as seen in healthy muscle.

3.4 Discussion

Fibrosis and altered ECM content of dystrophic muscle have long been established in the DMD literature, with numerous studies quantifying proteomic changes seen in mdx or other mouse models of DMD [88, 71, 57, 10]; here, we

Table 3.3: Top 10 enriched proteins in healthy muscle.

GI ID	**Protein name**	**Distance from y=x**
388998877	Titin isoform IC	4.2339
28559088	Laminin subunit alpha-2 isoform a precursor	3.8197
4507621	Troponin I, fast skeletal muscle isoform 1	3.3231
94981553	Slow cardiac myosin regulatory light chain 2	3.2355
4557305	Fructose-bisphosphate aldolase A isoform 1	2.9936
4507879	Voltage-dependent anion-selective channel protein 1	2.9106
5032009	Glycogen phosphorylase, muscle form isoform 1	2.8245
5803106	Myotilin isoform a	2.7548
188595687	Filamin-C isoform b	2.6497
33286422	Pyruvate kinase, muscle isoform 2	2.6455

Table 3.4: Top 10 enriched proteins in DMD muscle.

GI ID	**Protein name**	**Distance from y=x**
4506041	Proline arginine-rich end leucine-rich repeat protein precursor (Prolargin)	4.9659
4557871	Transferrin	4.2266
55743106	Alpha 3 type VI collagen isoform 5 precursor	3.7548
29788785	Tubulin beta chain	3.3158
530398069	Apolipoprotein A-I isoform X2	3.1872
61743954	Neuroblast differentiation-associated protein AHNAK isoform 1	3.1350
7661704	Osteoglycin preproprotein	2.9098
15451856	Caveolin-1 isoform alpha	2.8298
23111005	Microfibril-associated glycoprotein 4 isoform 2 precursor	2.7038
4504165	Gelsolin isoform a precursor	2.6209

performed mass spectroscopy on human muscle samples which largely corresponded with the mouse model data. We found that human DMD skeletal muscle tissue has increased ECM deposition relative to healthy tissue, with fibrillar collagens I and III enriched, as in mdx studies [10]. Mirroring results in the mdx mouse [57], we found an increase in several collagen type VI chains in DMD muscle. Furthermore, loss of dystrophin leads to instability in the cytoskeleton and the significant loss of critical muscle cytoskeletal proteins, including titin, actin, and myosin. Although the mdx mouse fails to fully recapitulate the severity of DMD, our data validates the use of the mdx mouse in proteomic studies.

Reproducibility of results between independently prepared samples taken from the same larger muscle samples was lower than ideal, with a correlation coefficient of 0.3 for comparing NSAFs from the first run with the NSAFs from the second run for all samples. This indicates several possible scenarios. First, it is possible (even likely) that the proteomic composition of muscle is variable, and, particularly for highly fatty and fibrotic dystrophic muscle, protein content may vary across a small muscle sample. This could argue for an average of multiple samples taken per muscle for future studies. Secondly, given the high number of low abundance proteins identified in one run but not the other, we are limited by the detection threshold of the mass spectrometer, and further characterization could be aided by using an instrument with higher sensitivity.

3.5 Conclusion

Here we performed HPLC-MS/MS on human skeletal muscle samples and evaluated proteomic changes seen in DMD. We found a significant enrichment of ECM proteins and decrease of cytoskeletal proteins in DMD muscle compared with healthy muscle. Critical components of the muscle cytoskeleton were decreased in dystrophic muscle, while fibrillar collagens and other components of fibrosis were upregulated. Healthy muscle had higher levels of laminins, including chains of muscle laminin isoform 211, and other basement membrane proteins. Our data corresponds with previous findings in mouse models of muscular dystrophy.

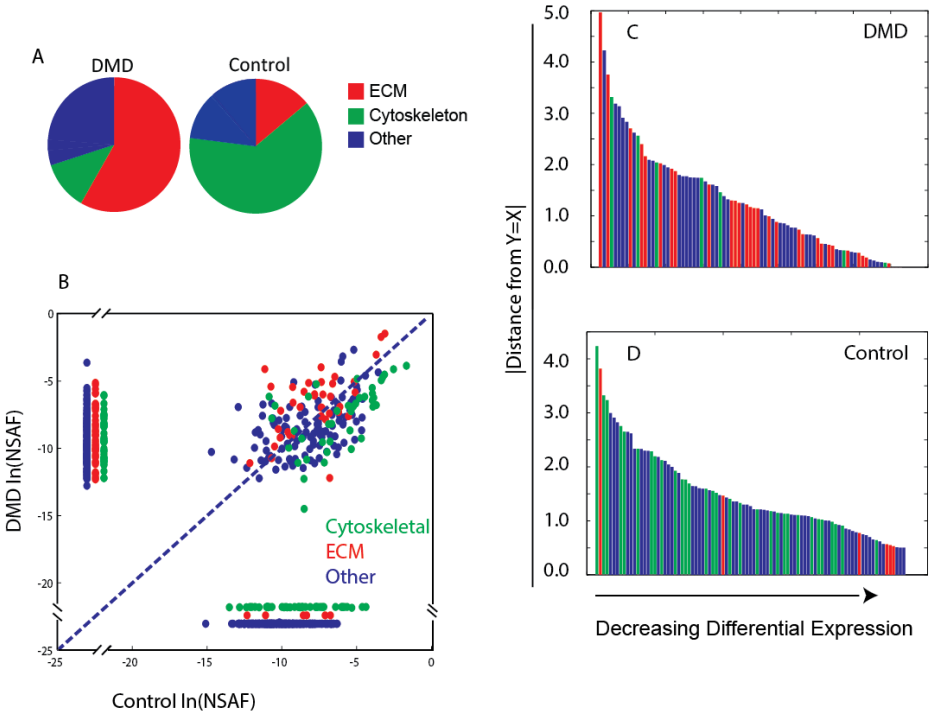

Figure 3.1: Mass spectroscopy reveals that muscle ECM and cytoskeletal content is affected by DMD. (A) ECM comprises over 50% of peptide hits in DMD muscle samples (3-fold more than WT). (B) Natural log of mean normalized spectral abundance factors (NSAFs) plotted for DMD (n = 6 muscles) vs. healthy (n = 5 muscles). Dashed line is y=x reference line. Proteins are colored by gene ontology (GO) terms for cytoskeletal (green), ECM (red), and other (blue). Proteins expressed in either DMD or healthy samples but not both are located along each respective axis. (C,D) The absolute value of the distance from each point to the y=x reference line was calculated to indicate differential protein expression. Panel C indicates those proteins expressed at higher levels in DMD samples (i.e. to the left of the y=x line), while panel D indicates those expressed at higher levels in healthy samples.

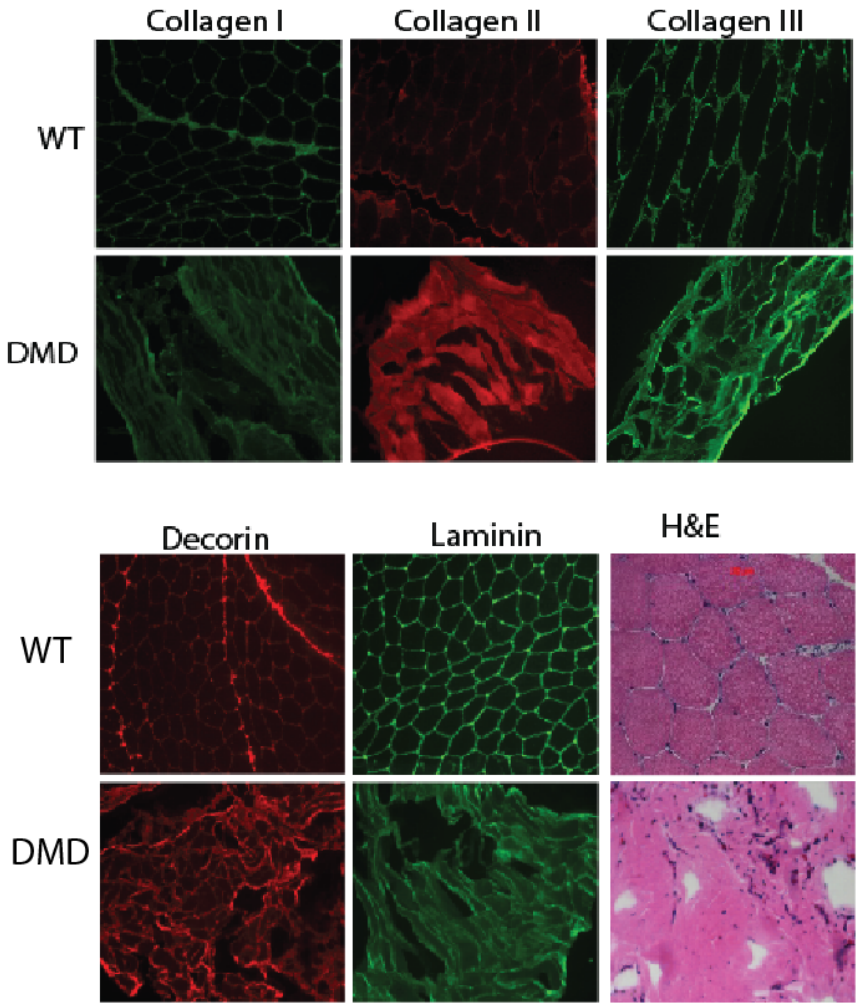

Figure 3.2: Immunoflurescent staining validates mass spectroscopy data. DMD and healthy muscle sections were stained with collagens type I, II, and III, decorin, laminin, and HE. The altered structure of the DMD muscle is evident across stains, with increased deposition of ECM proteins.

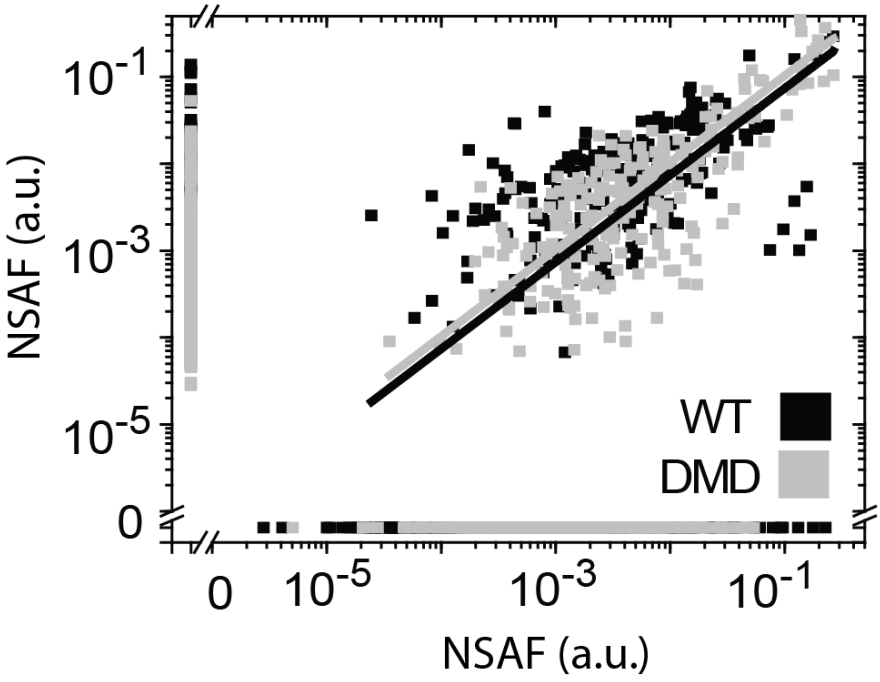

Figure 3.3: Reproducibility of mass spectroscopy data. Proteins identified in run 1 (x axis) and run 2 (y axis) have a correlation of 0.56 for healthy and 0.66 for DMD.

Chapter 4

Conclusions and Future Directions

Proteomic analyses of muscle changes in response to disease illustrate the changes in ECM and cytoskeleton composition during chronic injury. Although due to different disease mechanisms, skeletal muscle tissue from RCT tears and from DMD exhibited similar deposition of fibrillar collagens and decreases in basement membrane proteins, including laminins and collagen type IV. Additionally, cytoskeletal proteins, including actin and titin, were enriched in the control for each group (healthy and no tear for DMD and RC tears, respectively). Interestingly, the DMD samples appeared to show a more severe phenotype relative to RCT tear samples, with distances from the y=x line starting at 1.6 for proteins enriched in no tear and 4.5 for proteins enriched in healthy (both largely cytoskeletal). These data show that chronic muscle injuries lead to similar muscle phenotypes, even if the initial mechanism of disease differs.

SMPs, then, will likely find an altered cell niche in both the RC tear scenario and in the DMD scenario, as healthy muscle tissue is crowded out with fibrotic and fatty tissue. In DMD, severe muscle wasting occurs once the resident SMP population has been depleted due to constant muscle repair [83]. Here we showed that SMPs from RC muscles with a torn tendon have a greater tendency to differentiate when expanded ex vivo. Furthermore, the sensitivity of SMPs to soluble elements in the extracellular environment, such as growth factors, also varied depending on

the tear state of the tendon; SMPs from no tear patients proliferated at greater rates in response to treatment with FGF2, while SMPs from tear patients did not respond to FGF2 in the same magnitude. This data could shed light on why, even after tendon repair, patients with RCT tears often have continued muscle fibrosis and fatty infiltration in the RC muscle [45].

A major limitation of the SMP study lies in the size of the muscle specimens collected. As the specimens were approximately 15 - 25 mg and the SMP population was typically less than 1% of cells, the NCAM positive fraction cells collected after isolation typically numbered 300 - 1200. The SMPs remained cultured on plastic for 5-6 weeks, or until a large enough cell number had been reached to seed cells in all of the growth factor conditions. Thus, the SMPs were not exposed to the niche conditions of 11kPa hydrogel with collagen IV and laminin-111 and growth factors until roughly 6 weeks after isolation. Most other studies on murine and human SMPs measure proliferation and differentiation within the first two weeks of isolation, as phenotype loss occurs rapidly [42, 60, 23, 29]. Accordingly, we potentially lost substantial information in our study by waiting to measure any SMP characteristic until several weeks ex vivo. A critical next step would be to obtain larger human samples to isolate SMPs in greater numbers and then collect proliferation and differentiation data earlier in the culturing period. Furthermore, roughly half of samples obtained failed to generated SMP populations that survived in culture. Investigating deeper into the characteristics of the muscle samples that fail to yield viable SMP populations, and correlating those characteristics with patient outcome, may be of interest.

There existed a significant difference between the ages of patients with tears and without tears (65 years and 45 years, $p = 0.001$). Most (murine) studies of SMPs use younger sources for SMPs, as SMPs from geriatric mice are known to be both fewer in number and less responsive to injury cues in vivo [15, 23, 21, 69]. However, it is not known whether a mean age difference of 65 vs. 45 years would be biologically meaningful. That is, is the age difference between our groups a large factor in explaining the SMP phenotype differences we observed? A further study would benefit from expanding the patient population we currently have and

specifically including younger patients as they are available.

Additionally, the SMP study is limited by the inclusion of the deltiod muscle as a source of SMPs. In a RCT tear, the deltoid remains largely unaffected, with fibrosis and fatty infiltration in the supraspinatus and often the infraspinatus muscles [17]. Accordingly, all of our tear samples were suspraspinatus or infraspinatus, but 5 out of 11 no tear samples were from the deltiod. Human SMPs from multiple muscle groups have previously been grouped together while studying SMP characteristics [16], but specific analyses of muscle group differences has not been conducted. Future experiments would benefit from comparing across a single muscle group, as was done with the RCT tear mass spectroscopy experiment, comparing supraspinatus vs. supraspinatus.

Heterogeneity of the protein composition within the muscle may also be a subject for future proteomics investigation. Due to the low correlation coefficient between NSAFs of independent sample preparations of muscle in the DMD study ($r = 0.56$ for healthy and $r = 0.66$ for DMD), it is evident that either our assay had a high signal-to-noise ratio or there exists substantial protein variability within the muscle (or, likely, a little of both). Signal-to-noise ratio has long been an issue in mass spectroscopy, with newer instruments allowing for greater sensitivity to low abundance proteins. As many peptide hits in a sample may be for a single protein (for instance, collagen type I peptides comprised roughly one third of total peptide hits in the DMD muscle samples), lower abundance proteins can remain undetected. This issue could account for some of the low correlation, with many low abundance proteins present in one sample preparation and not the other. Instrument sensitivity issues aside, tissue heterogeneity in fibrotic muscle tissue is evident on H&E staining of tissue sections. A reasonable approach for further investigation could be to prepare several samples of the same muscle to characterize variability within the muscle, particularly for fibrotic or fatty samples.

Furthermore, while the RC tear study examined only the supraspinatus muscle for both massive tear and no tear groups, the DMD study used any DMD muscle tissues available from the National Disease Research Interchange, which included the deltiod, tibialis anterior, biceps brachii, and quadraceps. In an effort

to age-match the controls to the DMD cohort, we used gracilis and semitendinosus muscles obtained during reconstructive knee surgeries. Thus, the RC tear study gives us a snapshot of muscle changes seen in a single muscle group, while the DMD study may be applicable across a broad range of muscle groups but introduces another potential source of variability. To eliminate or evaluate muscle group variability, further investigations would benefit from comparing between a single muscle group for DMD and healthy samples or from comparing between different muscle groups within a disease state.

Here we presented evidence of SMP niche changes in muscle that is chronically injured or deloaded, and evaluated the effects this may have on SMP phenotype maintenance ex vivo. We found that SMPs from no tear RC muscles proliferate more rapidly and respond to growth factor FGF2 significantly more than SMPs from tear RC muscles. On the other hand, SMPs from tear RC muscles differentiated at significantly greater rates than SMPs from no tear RC muscles. Again the growth factor response depended on tear state, as IGF1 primed tear SMPs but not no tear SMPs for differentiation. SMPs may be affected by changes to their niche in vivo, as the RC muscle from torn RCTs contained fewer basement membrane proteins, particularly muscle laminin-211, and more fibrillar collagens. HPLC-MS/MS data on muscle from DMD patients corresponded with these findings, as the fibrotic DMD muscle was also enriched for fibrillar collagens and collagen cross-linking proteins and had a decrease in basement membrane proteins. Taken together, these data indicate that, irrespective of the mechanism of injury, chronic muscle injury results in an altered SMP niche, and that, in the case of the RC, the SMPs themselves are altered in vitro. This may in part explain why RC patients often have continued muscle degeneration and fatty infiltration following tendon repair. These studies indicate that lasting muscle damage is a complex relationship between pathological changes in the muscle and the resident muscle stem cells needed to repair the damage.

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