Fibro-Adipogenic Progenitors and the Extracellular Matrix: Interactions in Skeletal Muscle Fibrosis

By

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DISSERTATION

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DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of California, Davis.

It has not been submitted for any degree or examination at any another University.

ABSTRACT

Fibro-adipogenic progenitors (FAPs) are the main source of extracellular matrix (ECM) deposition in skeletal muscle fibrosis. Fibrosis is the pathological accumulation of ECM, which impairs tissue function resulting in reduced muscle strength and increased passive stiffness. The factors that drive FAPs to develop a fibrotic phenotype are not well understood. The goal of this dissertation was to understand how the ECM and FAPs interact in fibrosis and contribute to fibrotic development. Particularly, we proposed there is a pro-fibrotic feedback loop between FAPs and the ECM leading to the progressive degeneration of muscle. We hypothesized that as fibrotic muscle becomes stiffer this leads to fibrotic myofibroblast activation in FAPs, and this myofibroblast activation in FAPs results in further fibrotic ECM deposition, impairing muscle regeneration and furthering fibrosis.

To investigate this loop, first we looked at the effect of the ECM on FAP activation into myofibroblasts. We demonstrated that increased stiffness, physiologically relevant to fibrotic skeletal muscle, leads to increased myofibroblast activation in FAPs. We found that this activation could be reduced with pharmacological intervention using a drug called verteporfin to block the yes-associated protein (YAP) transcription pathway involved in mechanosensing.

Next, we investigated the effect myofibroblast activation of FAPs has on ECM deposition and muscle satellite cell (MuSC) differentiation. We found that FAPs derived from a fibrotic environment or activated into myofibroblasts produce ECM that is more fibrotic, in terms of collagen deposition and collagen fiber size. Verteporfin significantly reduced collagen production in FAPs. Myogenesis was impaired by myofibroblast produced soluble factors and by FAP-produced ECM on fibrotic-like stiffnesses.

Lastly, we applied what we found to human studies to investigate the effect of fibrotic ECM stiffness on FAPs and MuSCs derived from children with cerebral palsy (CP). We found that CP MuSCs had impaired differentiation and increased myonuclear clustering with increasing

stiffness. The clustering could be slightly reduced with verteporfin treatment. CP FAPs were not mechanosensitive compared to their typically developing (TD) counterparts, suggesting a disruption in mechanosignaling pathways.

Overall, this dissertation demonstrates a profibrotic feedback loop wherein fibrotic ECM activates FAPs into myofibroblasts, which in turn leads to further fibrotic ECM deposition and impaired myogenesis. Fibrotic FAP phenotype can be partially reduced through targeting the YAP pathway, providing a therapeutic avenue to target fibrosis across diseases.

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CHAPTER 1 INTRODUCTION: FIBRO-ADIPOGENIC PROGENITORS IN SKELETAL MUSCLE FIBROSIS

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ABSTRACT

Fibro-adipogenic progenitors (FAPs) are key regulators of skeletal muscle regeneration and homeostasis. However, dysregulation of these cells leads to fibro-fatty infiltration across various muscle diseases. FAPs are the key source of extracellular matrix (ECM) deposition in muscle, and disruption to this process leads to a pathological accumulation of ECM, known as fibrosis. The replacement of contractile tissue with fibrotic ECM functionally impairs the muscle and increasing muscle stiffness. FAPs and fibrotic muscle form a progressively degenerative feedback loop where, as a muscle becomes fibrotic, it induces a fibrotic FAP phenotype leading to further development of fibrosis. In this review we summarize FAPs' role in fibrosis in terms of their activation, heterogeneity, contributions to fibrotic degeneration, and role across musculoskeletal diseases. We also discuss current research on potential therapeutic avenues to attenuate fibrosis by targeting FAPs.

NEW AND NOTEWORTHY

Fibro-adipogenic progenitors (FAPs) are key stem cells in supporting skeletal muscle health, but in pathologic conditions contribute to fibrosis. This review highlights the profibrotic positive feedback loop of activated FAPs producing more fibrotic components, those fibrotic components leading to tissue stiffness, and that stiffness activating even more FAPs to perpetuate muscle fibrosis.

INTRODUCTION

Fibrosis is the pathological accumulation of extracellular matrix (ECM) components, predominantly fibrillar collagens, which impair tissue function (1,2). Fibrosis is a consequence of a multitude of diseases across tissues where the regenerative processes are impaired (3–7). In skeletal muscle, the excessive accumulation of ECM in fibrosis takes the place of functional, contractile tissue, weakening the muscle and increasing muscle passive stiffness (8,9). The weakened muscle becomes more prone to injury leading to further degeneration and fibrosis as the muscle becomes chronically injured.

The main cell driver of fibrosis in skeletal muscle are fibro-adipogenic progenitors (FAPs). FAPs are muscle resident non-myogenic cells that reside in the interstitial space (10). FAPs play an essential role in supporting regeneration. In uninjured muscle, FAPs exist at relatively low levels but rapidly proliferate after injury (11,12). After rapid expansion in response to injury, FAPs deposit ECM components to replace the damaged matrix and release pro-myogenic factors to induce muscle differentiation (13,14). FAPs then rapidly undergo apoptosis, returning to pre-injury levels, which halts ECM deposition and allows for full regeneration of the muscle (12,15). FAPs become resistant to apoptosis in fibrotic conditions, remaining at chronically high levels and continuously depositing ECM resulting in fibrosis (11,12,16). The development of fibrosis triggers FAPs to develop a fibrotic phenotype leading to a progressively degenerative feedback loop. As FAPs become activated into a fibrotic phenotype, they contribute to fibrotic ECM deposition and aberrant cell signaling, leading to further fibrotic activation and degeneration. Research on FAPs has rapidly expanded in the past couple years, dramatically increasing our understanding. However, the mechanosensitivity of FAPs has only recently been defined and require further research to understand the interplay between FAPs and the fibrotic ECM. The goal of this review is to highlight the positive feedback loop of FAPs and fibrosis; what

contributes to this cyclic activation and methods to disrupt the feedback loop to stop the progression of fibrosis.

ACTIVATION AND PERSISTENCE OF FIBROTIC PHENOTYPE

FAPs were first defined by their ability to spontaneous differentiate into adipocytes and activate into myofibroblasts (10). Notably, there is considerable overlap between FAPs and what may also be referred to as a muscle resident fibroblast (17). Activation of myofibroblasts is often considered a marker of fibrosis with alpha smooth muscle action (αSMA), a myofibroblast marker, significantly increasing in fibrotic conditions and strongly correlating with increased ECM deposition (18–23). A series of soluble factors and mechanical signaling from the ECM trigger FAP activation into myofibroblasts. These activation signals create a progressively degenerative feedback loop; as FAPs become activated into myofibroblasts, ECM deposition and release of pro-fibrotic signals increase leading to additional induction of myofibroblast activation and fibrosis (Fig. 1).

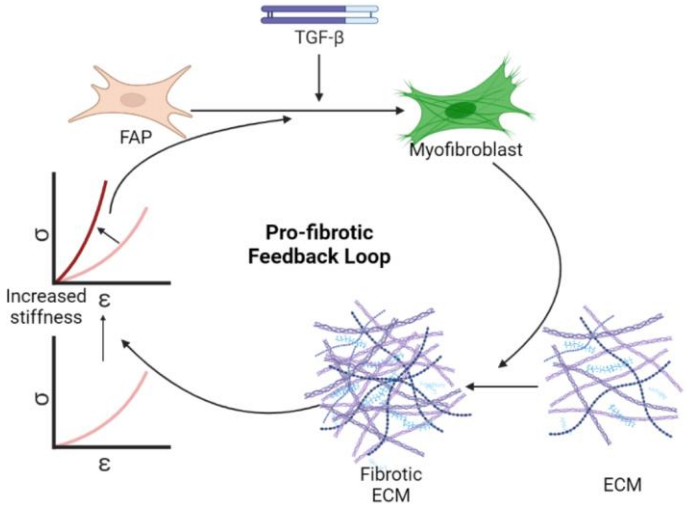

Figure 1.1: The profibrotic positive feedback loop between FAPs and fibrotic muscle. As FAPs are activated into myofibroblasts, they produce fibrotic levels of ECM leading to increased stiffness, which in turn activates FAPs further into myofibroblasts. TGF-β contributes to myofibroblast activation and ECM production, furthering the fibrotic loop. Graph axis are stress (σ) and strain (ε) indicating the stiffness of a material. Pink line indicates stiffness of healthy ECM and red line indicates stiffness of fibrotic ECM. Created with BioRender.com

SOLUBLE SIGNALING TO FAPS

Various soluble factors induce FAP activation into myofibroblasts. The most established one being transforming growth factor-beta (TGF-β). In skeletal muscle, TGF-β is released by both FAPs and macrophages, which play a key role in modulating FAP behavior through soluble factors (24–26). TGF-β is widely regarded as a strong pro-fibrotic signal, with its expression increased across fibrotic diseases (27). Exposure to TGF-β induces myofibroblast activation and is strongly correlated with fibrotic ECM deposition (12,16,27,28). Other soluble factors, downstream of TGF-β, increase myofibroblast activation, further implicating TGF-β has a strong pro-fibrotic factor. Connective tissue growth factor, also known as cellular communication network factor-2 (CTGF/CCN-2), downstream targets of TGF-β, increase αSMA when overexpressed in wildtype (*wt)* mice (29). In a rat model for an overuse fibrotic injury, TGF-β, αSMA, and CCN2 expression were all increased (30). Myostatin, which signals through the same SMAD pathway as TGF-β, increases FAP proliferation and αSMA expression (23). FAPs produce bone morphogenetic protein 1 (BMP1) and matrix metalloproteinase 14 (MMP14) which activate latent TGF-β into an active form (24). These leads to a feedforward loop as activated FAPs lead to the activation of TGF-β and therefore, more FAP activation.

TGF-β is involved in activating the Hedgehog (Hh) signaling pathway. This signaling pathway is known to block FAP differentiation into adipocytes after injury and enhance regeneration (31). However, ectopic activation of the Hh pathway leads to increase activation of FAPs into myofibroblasts, increased collagen deposition, and impaired regeneration (32). As with TGF-β, Hh signaling plays a key role in promoting regeneration in muscle, however, excessive and sustained expression leads to overactivation of FAPs and initiates fibrotic degeneration.

Platelet-derived growth factor receptor A (PDGFRα) is a tyrosine kinase receptor that is used as the canonical marker for FAP isolation (13,21,33). PDGFRα expression is strongly correlated with collagen expression in FAPs suggesting that the surface marker plays a fibrogenic role.

Exposure to PDGF-AA *in vitro* significantly inhibited FAP adipogenic potential while retaining fibrogenic potential and collagen expression (34,35). Constitutively active PDGFRα expression in an injured mouse model resulted in increased collagen deposition and fibrosis (35,36). Exposure to TGF-β decreases PDGFRα expression while increasing αSMA expression, suggesting FAPs lose their canonical marker as they commit to a fibrotic phenotype (19). PDGFRα modulates TGF-β induced myofibroblast activation, with blockage of PDGFRα decreasing myofibroblast activation and fibrosis, highlighting the cross-talk between these two play a key role in regulating FAPs' activation and fate (19,37). The persistence of FAPs in fibrosis leads to chronically high expression of PDGFRα which activates FAPs into a fibrotic phenotype, creating a positive feedback loop of fibrosis and fibrotic activation.

Excessive reactive oxygen species (ROS), particularly NADPH oxidase 4 (NOX4), have been implicated in myofibroblast activation in FAPs and other fibroblast cell types across tissues (38,39). TGF-β induces NOX4 expression across different cell types, indicating it as a downstream target of the profibrotic pathway in FAPs (38–40). In an *mdx* mouse, a model for skeletal muscle fibrosis, NOX4 expression is significantly higher than in *wt* mice. In a NOX4-KO *mdx* mouse model, the number of myofibroblast is decreased suggesting NOX4 plays a role in FAP activation (39). ROS levels are elevated in fibrotic skeletal muscle, therefore leading to cyclic activation of FAPs in an already fibrotic condition (41,42).

The persistence of a fibrotic phenotype is not only driven by FAP activation into myofibroblasts but also their resistance to apoptosis, resulting in the maintenance of chronically elevated levels of FAPs. Clearance of FAPs is an essential part of the regeneration and removes them from the profibrotic feedback loop. FAPs undergo apoptosis, mediated by tumor necrosis factor alpha (TNFα) after rapidly proliferating and depositing ECM post injury (12). This prevents excessive ECM deposition and keeps FAPs at relatively low levels in non-fibrotic and uninjured (11,12,43).

TGF-β blocks TNFα induced apoptosis, resulting in chronically high levels of FAPs and furthered fibrosis (12,16).

Mechanical Signaling to FAPs

The mechanical properties and organization of the ECM are altered in fibrosis. Excessive ECM deposition leads to increased stiffness, crosslinking, and altered alignment (44–46). Similar to mesenchymal stem cells (MSCs), FAPs are responsive to substrate stiffness (47,48). Increasing substrate stiffness dramatically increases αSMA expression in FAPs. Stiffness that are physiologically representative to fibrotic ECM have significantly more myofibroblast activation compared to stiffness representative of healthy ECM (48). In aged skeletal muscle, the increase in muscle stiffening leads to increased YAP/TAZ expression in muscle fibroblasts (49). YAP/TAZ are known to mediate TGF-β signaling in order to promote myofibroblast activation and collagen deposition (3,4). Therefore, the increase in ECM stiffness due to fibrosis induces FAP activation into myofibroblasts through the YAP/TAZ pathway. As a muscle becomes fibrotic it leads to further myofibroblast activation in FAPs, contributing to the progressively degenerative positive feedback loop.

When cultured in standard conditions, FAPs largely spontaneously activate into myofibroblasts as measured by αSMA stress fibers (10,13,19). Given the mechanosensitivity of FAPs, this could be due to the excessive high stiffness of tissue-cultured plastic. Therefore, culture conditions that are more physiologically representative of the ECM stiffness *in vivo* better represent FAP activation and behavior. A physiological substrate would thus include an elastic stiffness on the order of ~10 kPa of healthy muscle (50). However, mimicking the mechanical environment would also dictate transitioning from standard 2D culture systems to 3D culture which can influence the response to substrate stiffness (51). Further, MSCs respond not only to elastic stiffness, but also viscoelasticity (52). The specific impact of these more complex mechanical features on FAPs is still currently unknown.

Apoptosis provides a mechanism for FAPs to exit the profibrotic feedback loop and restore homeostatic conditions in skeletal muscle. However, profibrotic signaling such as TGF-β and increased stiffness, block this exit pathway leading to a cyclic process of profibrotic activation, further fibrotic signaling and activation, and fibrotic development. This profibrotic feedback loop leads to continuous fibrotic activation of FAPs and the progressively degenerative nature of fibrosis.

HETEROGENEITY WITHIN FAPS

FAPs overall are one of the main cell drivers of fibrosis. Recent studies have identified key subpopulations in FAPs that affect their fibrotic and regenerative capacities through single cell RNA sequencing (scRNAseq). Studies have divided these subpopulations by different markers, but overall, there is significant heterogeneity within FAPs that is altered in injury and disease.

Malecova et al. identified distinct subpopulations of FAPs that changed in injury and fibrosis. These subpopulations were defined by relative expression of two surface markers, vascular cell adhesion molecule 1 (Vcam1) and tyrosine kinase receptor (Tie2), that have unique transcriptional profiles. Vcam1 expression was significantly increased after acute injury and in *mdx* mice, but relatively low in *wt* mice. Tie2 expression levels were opposite, most FAPs expressed some level of Tie2 in healthy muscle and those levels significantly decreased after injury and in fibrosis (11). High expression of Vcam1+ FAPs was observed in other fibrotic conditions. In oculopharyngeal muscular dystrophy (OPMD), a genetic disorder affecting the muscles in the eyelids and pharynx, there was a higher abundance of Vcam1+ FAPs in the fibrotic muscle compared to nonfibrotic muscle (43). Human FAPs isolated from nonfibrotic muscles clustered differently from those isolated from fibrotic muscles, suggesting changes in gene expression in injury and disease (43). Vcam1 levels are upregulated in other tissue type diseases and associated with increased proliferation including in idiopathic pulmonary fibrosis and lung cancer (5,53). In lung fibroblasts, TGFβ significantly increased Vcam1 expression and

fibroblast proliferation (5). If TGFβ triggers Vcam1 expression in FAPs as well, it further implicates this subpopulation as the more fibrotic phenotype.

Type 2 diabetes (T2D) is a disease that is associated with fibro-fatty infiltration of skeletal muscle, with contractile tissue being replaced by both excessive ECM deposition and adipocytes (34). Farup et al. analyzed human FAPs isolated from patients with T2D clustered into four distinct subpopulations. A population high in αSMA expression, also differentially expressed collagens and other ECM proteins, indicating that the myofibroblast population of FAPs is primarily responsible for ECM deposition (6,34). FAPs from T2D patients clustered based on CD90+/- expression, with genes upregulated in CD90+ FAPs were also upregulated in T2D muscle. CD90+ FAPs had higher proliferative capacity and clonal formation, suggesting a progenitor phenotype while CD90- FAPs expressed a more adipogenic phenotype. CD90 positive and negative FAPs had distinct transcriptomes. CD90- FAPs were enriched for angiogenesis and regulation of endothelial proliferation while CD90+ subset was enriched for ECM proteins, collagen synthesis, and TGFβ response, suggesting this subset is a more fibrotic phenotype. Patients with T2D had significantly higher population of CD90+ FAPs, exclusively, implying this population is the primarily responsible for the fibrotic degeneration seen in T2D (34).

Davies et al. divided murine FAPs into subpopulations based on their expression of uncoupling protein 1 (UCP1), a precursor marker for beige adipose (54). UCP1+ FAPs released promyogenic exosomes while UCP1- FAPs were defined by profibrotic exosomes. FAPs isolated from humans with rotator cuff tears, an injury associated with fatty infiltration and fibrosis, clustered into six distinct subpopulations. These distinct subpopulations had differential expression of fibrogenic and adipogenic genes including COL1A1, PDGRFA, ADIPOQ, and UCP1 (54).

In cells isolated from patients with limb-girdle muscular dystrophy (LGMD), Depuydt et al. divided FAPS based on lumican (LUM+) and proteoglycan 4 (PRG4+) expression. LUM+ expression positively correlated with disease severity (55). In non-diseased human muscle, Rubenstein et al. also found FAPs to cluster based on LUM+ and PRG4+ markers. After using mouse samples to validate the human sequencing however, FAPs were divided into two subpopulations based on lumican (LUM+) and fibrillin 1 (FBN1+) expression, highlighting the heterogeneity between species. Both of these subpopulations had high expression of fibrillar collagens I and III, the predominant proteins in fibrosis, but LUM+ FAPs differentially expressed types IV and XV collagens while FBN1+ FAPs had heightened expressed of collage XIV.

Several studies have looked at FAP heterogeneity in the context of regeneration. While these do not give direct insights into FAP subpopulations in fibrosis, the subpopulation of FAPs responsible for ECM deposition and TGF-β signaling in regeneration likely share characteristics with FAPs that persist in fibrosis (56,57). Scott RW et al. classified FAPs into two subpopulations that differentially expressed genes for ECM components and cell signaling pathways (58). The temporal expression of these genes after injury with notexin (NTX) suggests a subset of the FAP population is primarily responsible for ECM deposition after injury. This same subpopulation, defined by ECM expression, may become persistent in chronic injury and disease leading to fibrosis. Oprescu et al. identified subpopulations of murine FAPs that are dynamically altered in homeostasis and cardiotoxin (CTX)- induced injury. After muscle damage, FAPs diverged into subpopulations, the markers of which are time dependent. At 21 DPI, FAPs could be divided into Osr1+ FAPs and fibroblasts enriched with collagen-I, which might represent the more persistent, fibrotic subset of FAPs (57). Osr1+ is associated with pro-regenerative signaling in FAPs and is upregulated in FAPs after acute injury (14,15). Leinroth et al. described six distinct subpopulations of FAPs with varying adipogenic potential and ability to respond to $BaCl_2$-induced injury. Osr1+ defined one subpopulation that expressed genes related

to stemness and development, indicating a precursor population (15,59). Two clusters defined by Adam12 and Gap43 had enhanced enrichment of fibrotic genes including TGFβ and Fibronectin 1 (Fn1) and downregulation of Ltbp4, a negative regulator of TGF-β (59).

FAPs are the primary contributors of fibrotic ECM deposition and play essential roles in regeneration and homeostasis. While ablating FAPs reduces excessive ECM deposition, it results in muscle atrophy and MuSC depletion, even under homeostatic conditions (60). Recent illumination of FAP subpopulations implies an opportunity for more targeted approaches to reduce fibrosis. Inhibiting a profibrotic subpopulation of FAPs while leaving a pro-regenerative subpopulation intact offers an avenue to maintain the supportive role of FAPs while attenuating fibrosis. However, there seems to be little consensus about how to define these subpopulations in both the context of regeneration and fibrosis. This is likely due to biological and technical variability that exist across samples, disease models, and analysis techniques. Despite different markers, these subpopulations share similarities across studies. For example, in healthy mice, Rubenstein et al. defined a subpopulation by FBN1+ expression, notably, the FBN1+ subset co-expressed TEK, the gene for Tie2, the defining marker for a subpopulation in the Malecova et al. study (11,61). While αSMA is the canonical marker of myofibroblasts it has not a distinguishing factor in profibrotic FAPs at the transcriptional level across studies in mouse and human (Table 1). Although the identifying markers can shift a consistent feature of profibrotic FAPs is increased expression of fibrillar collagens I and III as well as collagen crosslinking enzymes.

Table 1.1: Summary of FAP heterogeneity and potential markers for profibrotic populations, determined by high fibrillar collagen expression and relative expression in fibrotic conditions.

Profibrotic Subpopulation(s)	Other Subpopulation(s)	Sample	Condition	Ref
Vcam1+	Tie2+	mouse	fibrosis (*mdx*)	Malecova et al. (2018)
UCP1-	UCP1+	mouse	rotator cuff tear	Davies et al. (2022)
CD90+	CD90-	human	T2D	Farup et al. (2022)
Lumican+	Proteoglycan 4+	human	LGMD	Depuydt et al. (2022)
Lumican+ Fibrillin 1+		mouse, human	healthy, resting	Rubenstein et al. (2020)
Dlk1+ Fibroblasts	Dpp4+ Cxcl14+ Ors1+ Wisp1+	mouse	CTX-injury	Oprescu et al. (2020)
FAP1	FAP2	mouse	NTX-injury	Scott et al. (2019)
	Gli1 + Gli1-	mouse	NTX-injury	Yao et al. (2021)
Adam12+ Gap43+	Osr1+ Gli1+ Hsd11b1+ Clu+	mouse	$BaCl_2$ injury	Leinroth et al. (2022)
	Osr1+ Osr1-	mouse	Freeze injury, glycerol injury	Stumm et al. (2018)
MME+	MME-	human	HOA	Fitzgerald et al. (2023)

CONTRIBUTIONS TO FIBROSIS

In acute injury, FAPs play a key role in supporting healthy regeneration by both depositing ECM to replace the damaged matrix and signaling to MuSCs through the release of pro-myogenic signals. In a fibrotic state, these pathways get disrupted leading to excessive ECM deposition, fatty infiltration, and aberrant cell signaling. The fibrotic contributions of FAPs lead to further profibrotic activation, continually disrupting the proregenerative pathways and leading to progressively degenerative fibrosis.

ECM DEPOSITION

The defining characteristic of fibrosis is a pathological excess of ECM deposition, which stiffens the muscle and impairs contractile function (2,7,43–45,62). FAPs account for the majority of collagen expression in skeletal muscle both in regenerating injuries and fibrotic conditions

(10,12,13,19–21,31,63). The number of FAPs strongly correlate with muscle collagen levels (22,23). This is supported by the increase in expression of ECM proteins, particularly fibrillar collagens I and III, from FAPs derived from fibrotic conditions (58,64). Increased synthesis of various proteoglycans, including decorin and biglycan, found within the ECM is seen in both Duchenne muscular dystrophy (DMD) patients and *mdx* mice (64,65). Lumican and fibrillin, proteoglycans upregulated in FAPs are known to accumulate with fibrillar collagens, promote thicker collagen fibril formation, and induce myofibroblast activation through the TGF-β pathway in other fibrotic diseases (66–69). The increase in ECM deposition stiffens the muscle, leading to further myofibroblast activation in FAPs and fibrotic development (45,48).

The skeletal muscle ECM not only increases in content throughout fibrosis, but also the organization of the ECM and collagen fibrils is altered. Collagen crosslinking and alignment are increased in fibrotic muscle, indicating a change in how FAPs deposit and organize the ECM (44,70). FAPs express high levels of not only ECM genes, but also regulatory genes that control ECM degradation and organization. FAPs have increased expression of lysyl oxidases (LOXs), which promote collagen crosslinking (64,71,72). This makes the ECM stiffer and resistant to degradation, contributing to the continuous buildup of ECM and stiffening of the muscle (44,45,70). Fibrotic FAPs express CD147, an inducer of matrix metalloproteinases (MMPs), at higher levels than non-fibrotic FAPs (43,73,74). MMPs are a large class of proteinases, many of which degrade ECM and are considered antifibrotic. However, MMPs affect a large range of biological processes, and some have been identified as having pro-fibrotic properties. Particularly *mdx* FAPs had increased expression of MMP2 and MMP14, both of which are upregulated in the presence of TGF-β and have been implicated in the furthering of fibrosis (75–77). While it may be counterintuitive that MMPs that breakdown ECM are increased in fibrosis, the expression of tissue inhibitors of MMPs (TIMPs) are also overexpressed in fibrotic conditions that shift the balance toward ECM accumulation (78).

FAPs contribution to the fibrotic ECM perpetuates the positive feedback loop of FAPs and fibrosis. FAPs are sensitive to both the ECM stiffness and architecture, inducing myofibroblast activation (48). Fibrotic FAPs have altered expression of regulatory ECM genes affecting ECM organization and remodeling. As FAPs produce a more fibrotic ECM in terms of stiffness, content, and architecture, it induces further myofibroblast activation in the FAPs leading to more fibrotic ECM deposition. This cycle continues leading to continuing stiffer and more disorganized ECM as fibrosis progresses.

Fatty Infiltration

Fibrosis is, by definition, a pathological accumulation of ECM, which is a result of FAPs pro-fibrotic activation into myofibroblasts. However, the dysregulation of FAPs that occurs in fibrotic diseases, often results in fatty infiltration of the muscle too, as FAPs both differentiate into adipocytes and activate into myofibroblasts, highlighting the multipotency to these cells. The persistently high levels of FAPs due to their apoptotic resistance during fibrosis can lead to high levels of both fibroblastic and adipogenic activation.

Fibro-fatty infiltration is seen in various chronic injuries including rotator cuff tears and DMD. (79–81). MRI assessment of skeletal muscles reveals high fat fraction in DMD, predominantly in the hip and thigh muscles (82,83). The amount of fatty infiltration negatively correlates with muscle function and strength (84). Therefore, FAPs impairment of muscle function in fibrotic diseases is in part through the replacement of contractile tissue with not only ECM but also fatty connective tissue.

FAP activation into myofibroblasts and differentiation into adipocytes are often viewed as opposing sides of a coin with profibrotic factors, such as TGF-β, inhibiting adipogenesis while promoting fibrogenesis and pro-adipogenic factors having the opposite effect (85,86). However, the existence of fibro-fatty infiltration across various skeletal muscle diseases suggests a dual capacity of FAPs to activate in both directions under the same conditions. This is attributed in

part to the heterogeneity of FAPs, with certain subsets of FAPs accounting for almost all adipogenic differentiation in muscle (54,87). The dysregulation of signaling in fibrotic conditions may induce subsets of FAPs to undertake a fibrotic or adipogenic phenotype.

Aberrant Cell Signaling

The altered ECM provides a non-direct way in which FAPs signal to surrounding muscle cells. MuSCs are sensitive to their mechanical and architectural environment and therefore, affected by the FAP-produced ECM (88). The increased collagen cross-linking and stiffness, seen in fibrosis, impair MuSC differentiation (50,88,89). Since the fibrotic response and the regenerative response are opposing pathways to dealing with damage the inhibition of myogenesis propagates the progressive nature of fibrosis.

FAPs also signal to MuSCs through the release of soluble factors. In a regenerative state, FAPs release pro-myogenic exosomes that promote MuSC migration to injury site and differentiation into muscle (54,56). In fibrosis, these signaling pathways are disrupted, with FAPs impairing rather than promoting myogenesis (90,91). MuSCs cultured in conditioned media obtained from fibrotic myofibroblasts in skeletal muscle had declined proliferation and increased apoptosis, resulting in poor wound healing (37). Co-culturing of FAPs isolated from fibrotic conditions with MuSCs had a negative effect on fusion index compared to coculture of nonfibrotic FAPs, both in direct coculture and in indirect transwell experiments, indicating at least a portion of the negative effect comes from FAP soluble factor release rather than direct cell contact (14,43).

Fibrotic degeneration is a complex process involving various cell types, but FAPs play a main role in promoting deterioration through multiple pathways. The pro-regenerative nature of FAPs is switched to a degenerative phenotype of fibro-fatty infiltration and impaired myogenesis advancing fibrotic development across skeletal muscle diseases. FAPs also receive signaling from MuSCs, differentiated muscle fibers play a role in determining FAPs' adipogenic or fibrogenic fate (92,93). As FAPs' signaling to MuSCs becomes dysregulated in fibrosis so does

MuSCs signaling to FAPs as a result. Therefore, the cyclic nature of fibrosis continues leading to fibrotic activation of FAPs and aberrant cell signaling.

FAPS IN FIBROTIC CONDITIONS

Fibrosis is a consequence of nearly all skeletal muscle diseases and chronic injuries. While the causes of the disease may differ, a common feature is the persistent presence of FAPs and their contributions to fibrosis.

MUSCULAR DYSTROPHIES

One of the more commonly studied group of fibrotic diseases in skeletal muscles are muscular dystrophies, a group of genetic diseases that progressively weaken muscles. The most studied one being DMD. DMD is an x-linked recessive disorder resulting from a mutation in the dystrophin gene, a structural protein that provides mechanical stability and strength to the muscle. The muscles in DMD are more prone to injury without a fully functioning dystrophin protein and are significantly weaker leading to chronic damage and fibrosis. FAPs in DMD muscle are more highly activated into myofibroblasts and exist in larger numbers than in non-diseased muscle driving the progressively degenerative fibrosis (94). Profibrotic markers are enhanced in DMD FAPs including TGF-β, αSMA, and fibrillar collagens (11,34). Profibrotic miRNAs are elevated in FAPs isolated from DMD versus control muscle. These profibrotic miRNAs strongly correlate with TGF-β and ECM protein expression levels (94).

In limb-girdle muscular dystrophy (LGMD), is a disease caused by a mutation in the dysferlin gene, impairing muscle repair and leading to chronic injury. FAPs in LGMD, specifically the subpopulation expressing lumican (LUM), progressively increased with disease severity (55,95). LGMD muscle had upregulation of genes related to fibro-fatty infiltration while genes related to protein synthesis and regeneration were downregulated (55). Particularly FAPs contribute significantly to adipogenic replacement of contractile tissue in LGMD, impairing muscle function and furthering disease progression (95).FAPs isolated from patients with OPMD have significant

increased proliferative capacity and were more motile compared to control FAPs. OPMD FAPs have increased expression of ECM proteins and altered expression of genes related to cell signaling, which resulted in impaired fusion index of MuSCs (43).

Skeletal Muscle Diseases and Chronic Injuries

Skeletal muscle fibrosis is most commonly studied in the context of muscular dystrophies, however, almost any chronic skeletal muscle injury or disease results in some level of fibrosis. Across these diseases FAP levels are elevated and highly activated leading to increased ECM deposition and fibrosis. In cerebral palsy, a group of motor disorders, collagen expression is elevated in FAPs compared to typically developing controls (22,46,96). In a mouse model for amyotrophic lateral sclerosis (ALS), a motor neuron disease, αSMA expression is increased over wild type controls, indicating enhanced FAP activation into myofibroblasts (20). Chronic kidney disease (CKD) and kidney disfunction is strongly correlated with a progressively fibrotic muscle phenotype. This is purportedly due to decreased TNF-α expression leading to a persistently expanding pool of FAPs resulting in the number of FAPs nearly doubling in CKD muscle (8), or increased myostatin in CKD stimulating the differentiation of FAPs to myofibroblasts (23). In a mouse model for CKD, αSMA and ECM protein expressions are elevated (23). T2D is associated with fibro-fatty degeneration of skeletal muscle and increased PDGFRα expression. Genes related to ECM turnover and remodeling are differently expressed in FAPs, including increased collagen-I deposition (34).

FAPs are responsible for the high levels of fatty infiltration and fibrotic deposition observed in rotator cuff tears (81,97). FAP levels are elevated and apoptotic index is decreased in a mouse model for rotator cuff tears (80). TGF-β levels are increased after rotator cuff injury and increase FAP survival (80,97). Rotator cuff injuries develop fatty infiltration at a high rate and concentration than other muscles across diseases. This may in part be due to the heterogeneity of FAPs discussed above not only within a muscle but across anatomical regions. FAPs from

rotator cuff muscles reside at a higher concentration and demonstrate greater adipogenic potential than lower limb muscle FAPs (98).

Skeletal muscle diseases and chronic injuries vary in their cause, symptoms, and development. However, a common factor across diseases is the role FAPs play in the progressively degenerative nature of fibrosis. Therefore, targeting FAPs through various anti-fibrotic therapies offers an avenue to attenuate a broad range of fibrotic conditions (Fig. 2).

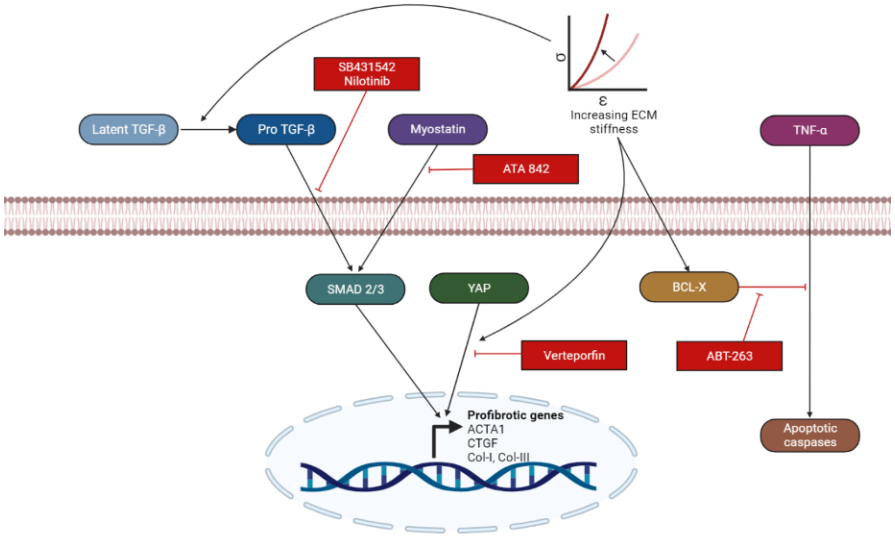

Figure 1.2: Signaling pathways involved in FAP fibrotic activation and potential therapeutic targets. Therapeutics to block fibrotic pathways are marked by the red boxes. Created with BioRender.com

ATTENUATION OF FIBROSIS THROUGH TARGETING FAPS

The positive feedback loop of FAPs and fibrosis causes muscles to progressively degenerate. To attenuate fibrosis and improve muscle function, this cycle needs to be disrupted otherwise FAPs will continually activate and contribute to fibrosis. Methods to break this cycle include blocking the soluble and mechanical signaling within the cycle as well as promoting apoptosis, to remove FAPs from the fibrotic feedback loop.

BLOCKAGE OF TGF-B SIGNALING

TGF-β plays a significant role in driving FAPs' fibrotic behavior by inducing proliferation, myofibroblast activation, and blocking apoptosis. Methods that inhibit TGF-β signaling to FAPs help promote apoptosis, restoring a key aspect of the regenerative cycle, and limit myofibroblast activation and ECM deposition. Small molecule inhibitors of TGF-β reduce FAP number and fibrotic signaling in mice (80,99). In a mouse model of a rotator cuff injury, SB431542, a small molecule TGF- β inhibitor reduced αSMA expression, collagen deposition, the number of FAP cells, and increased FAPs' apoptotic index (80). ITD-1, which targets TGF- β receptors, reduced TGF- β activity, FAP accumulation, and fibrotic area in *mdx* mice (99).Nilotinib, a tyrosine kinase inhibitor, blocks TGF-β signaling, restoring FAP apoptosis and as a result, reducing collagen expression (12,100).

The benefits of blocking TGF-β in order to attenuate fibrosis have been widely studied and proven successful in promoting FAP apoptosis and reduction of ECM deposition. However, these techniques need to be applied with caution as total inhibition can have adverse side effects. TGF-β plays a role in many different biological signaling pathways including myogenesis (101). Therefore, complete inhibition could impair regeneration, rendering the reduction in fibrosis functionally inconsequential. Targeting downstream targets of TGF-β specifically involved in myofibroblast activation provides a more specific method to attenuating fibrosis while leaving most broad signaling pathways intact. TGF-β acts on many different signaling pathways, with most fibrotic upregulation occurring through the SMAD2/3 pathway (4,38,43). Therefore, blocking downstream targets of TGF-β related to this pathway allows for inhibition of fibrotic signaling while leaving other important signaling pathways intact. Blocking CTGF/CCN-2, a downstream target of TGF-β/SMAD pathway, reduces αSMA expression and fibrosis in a rat overuse injury model (30). ATA 842, a drug that inhibits the myostatin/SMAD pathway, prevents

fibrosis progression, promotes apoptosis, and reduces myofibroblast activation in fibrotic FAPs (23,102).

Blocking Mechanosensing

The mechanical environment of the muscle is altered in fibrosis with stiffness increasing as fibrosis progresses. FAPs are sensitive to these increases in stiffness, inducing higher myofibroblast activation on increased stiffness (48). Blocking FAPs' ability to sense fibrotic stiffness provides an avenue to reduce myofibroblast activation in fibrotic muscle by stopping the profibrotic feedback loop between myofibroblast activation, ECM deposition, and stiffness. YAP/TAZ are proteins involved in mechanosensing, translocating to the nucleus on stiffer substrates, and are linked to myofibroblast activation in FAPs and other fibroblast-like cells (3,48,103). Inhibiting YAP/TAZ translocation to the nucleus prevents myofibroblast activation on stiffer substrates and therefore, could be applied to fibrotic conditions where there the muscles have elevated stiffness (4,48,104). Drugs that block YAP translocation to the nucleus shown preclinical efficacy as antifibrotic drugs including verteporfin (105) and a dopamine receptor agonist (106). However, to our knowledge YAP inhibitors have not been applied in the context of skeletal muscle tissue.

A stiff mechanical environment also inhibits apoptosis of pro-fibrotic myofibroblasts. Despite the presence of apoptotic signals being present stiffness induces the expression of anti-apoptotic protein BCL-X_L (107). To combat this pathway and reduce the number of myofibroblasts strategies to block BCL-X_L can be applied. The drug ABT-263 inhibits BCL-X_L and has demonstrated anti-fibrotic activity in the lung (108), liver (109), and heart (110), but similarly has not been applied to skeletal muscle.

Restoring Cell Signaling Pathways

In fibrosis, FAP signaling to MuSCs is disrupted with FAPs releasing inhibitory exosomes rather than pro-myogenic exosomes. Histone deacetylase inhibitors (HDACis) have emerged as a

therapeutic method to tune FAP signaling to a more pro-regenerative state. Extracellular vesicles (EVs) released from both human DMD FAPs and mouse *mdx* FAPs had altered expressions of miRNAs. HDACis increased expression of pro-regenerative miR-206 from FAPs which in turn increased differentiation in MuSCs and decrease the amount of collagen in the muscle (90,111). HDACis also inhibit FAPs adipogenic potential and increase FAP expression of follistatin, a glycoprotein known to inhibit myostatin, therefore blocking myofibroblast activation (112,113). Givinostat, an HDACi, has been used in clinical trials for DMD with successful reduction of fibrotic and fatty areas (114,115).

However, experiments in *mdx* mice showed HDACis were only effective in restoring FAPs' pro-regenerative signaling in young mice, whereas old *mdx* FAPs were resistant to HDACi treatment (112). Similarly, antibody treatments against myostatin increased muscle size and specific force, only when administered to young *mdx* mice, and had no effect on adult *mdx* mice (102). Fibrosis is largely considered irreversible, although there is some evidence to the contrary (106,116). Treatments the prevent the development of fibrosis rather than reversing already existing fibrosis tend to be more efficacious. Therefore, targeting FAPs to prevent pro-fibrotic activation before excessive fibrotic development provides the best method for improving muscle function.

PERSPECTIVES AND FUTURE STUDIES

Fibrosis is a progressively degenerative consequence of nearly all skeletal muscle diseases as a result of the profibrotic feedback loop that, not only activates FAPs into a profibrotic state, but keeps them there increasing fibrotic contributions (7,79,117). Soluble and mechanical signaling contribute to the activation of FAPs and block their exit from the profibrotic feedback loop (12,16,48,118). Investigations into methods to attenuate fibrosis must address ways to disrupt this loop otherwise the cyclic nature of FAPs and fibrosis will continue.

Two interesting avenues to break the profibrotic feedback loop are the relatively new discoveries of FAPs' mechanosensitivity and heterogeneity. FAPs' mechanosensitivity has only

recently been defined and adds insight into the cyclic nature of fibrotic tissue stiffening and the activation of myofibroblasts (48). However, the signaling pathways that induce myofibroblast activation of FAPs on stiff substrates are not fully defined and further investigation could provide more insight and therapeutic targets to disrupting FAP activation and fibrosis. Methods to block YAP/TAZ or other mechanosensing pathways *in vivo* have proved effective in other fibrotic tissue (104,119). Another prospective strategy to break the fibrotic feedback loop would be to target the ECM structure directly. Direct injection of collagenase to digest fibrotic collagen is performed in Dupuytren's contracture (120) and has been proposed as a method of reducing muscle stiffness in muscle contractures of children with cerebral palsy (121,122). Blocking collagen crosslinking is a potential target as well to increase the turnover by endogenous collagenase and decrease the stiffness of the collagen network (123). A range of collagen crosslinking inhibitors have been explored in other tissues (124) although a common collagen crosslinking inhibitor was found to be ineffective in blocking crosslinking in skeletal muscle. Methods to disrupt the profibrotic cycle through mechanosensing or altering the mechanics should be further developed in the skeletal muscle environment to determine their efficacy in treating skeletal muscle fibrosis.

The heterogeneity of FAPs in the context of regeneration and fibrosis offers various avenues for future studies to parse out the individual contributions of each subpopulation. Experimentation isolating out individual subpopulations and defining there matrisome and secretome would better define the profibrotic and proregenerative nature of the subpopulations. This could be explored through depletion experiments to ablate a specific subpopulation of FAPs and assess changes in skeletal muscle function and fibrosis *in vivo*.

CONCLUSIONS

FAPs play an essential role in regeneration and homeostasis of skeletal muscle through their contributions to the ECM and myogenic signaling. However, in disease, these pathways get

disrupted and FAPs quickly take on a pathogenic phenotype. The transient nature of FAPs after injury is essential for maintaining their pro-regenerative role. The persistence of FAPs and resistance to apoptosis leads to the chronic cycle of ECM deposition, fibrosis, and fibrotic activation of FAPs. Recent progress in RNAseq and transcriptomics has allowed insights into the vast heterogeneity of FAPs in healthy and diseased muscle. The importance of these subpopulations is not fully understood but may provide insights into the multidirectional nature of FAPs and more specific targets for anti-fibrotic therapies.

FAPs and fibrosis contribute to a progressively degenerative positive feedback loop. The development of fibrosis disrupts cell signaling leading to fibrogenic activation of FAPs, furthering fibrosis and, as a result, FAP activation and contributions to fibrosis. Disrupting this feedforward pathway is necessary to attenuate fibrosis and restore muscle function. Targeting the various signaling pathways that induce myofibroblast activation and inhibit apoptosis in FAPs will disrupt the fibrotic feedback loop.

CHAPTER 2: THE EFFECT OF THE ECM ON FAP ACTIVATION

This chapter was previously published as:

Loomis, Taryn, et al. "Matrix stiffness and architecture drive fibro-adipogenic progenitors' activation into myofibroblasts." **Scientific Reports** 12.1 (2022): 13582.

ABSTRACT

Fibro-adipogenic progenitors (FAPs) are essential in supporting regeneration in skeletal muscle, but in muscle pathologies FAPs are the are main source of excess extracellular matrix (ECM) resulting in fibrosis. Fibrotic ECM has altered mechanical and architectural properties, but the feedback onto FAPs of stiffness or ECM properties is largely unknown. In this study, FAPs' sensitivity to their ECM substrate was assessed using collagen coated polyacrylamide to control substrate stiffness and collagen hydrogels to engineer concentration, crosslinking, fibril size, and alignment. FAPs on substrates of fibrotic stiffnesses had increased myofibroblast activation, depicted by αSMA expression, compared to substrates mimicking healthy muscle, which correlated strongly YAP nuclear localization. Surprisingly, fibrosis associated collagen crosslinking and larger fibril size inhibited myofibroblast activation, which was independent of YAP localization. Additionally, collagen crosslinking and larger fibril diameters were associated with decreased remodeling of the collagenous substrate as measured by second harmonic generation imaging. Inhibition of YAP activity through verteporfin reduced myofibroblast activation on stiff substrates but not substrates with altered architecture. This study is the first to demonstrate that fibrotic muscle stiffness can elicit FAP activation to myofibroblasts through YAP signaling. However, fibrotic collagen architecture actually inhibits myofibroblast activation through a YAP independent mechanism. These data expand knowledge of FAPs sensitivity to ECM and illuminate targets to block FAP's from driving progression of muscle fibrosis.

BACKGROUND

Fibro-adipogenic progenitors (FAPs) are a mesenchymal-like cell population within skeletal muscle that have the capacity to differentiate down either an adipogenic or myofibroblast lineage. FAPs play a vital role in supporting homeostasis and proper regeneration in skeletal muscle. Under homeostatic conditions, depletion of FAPs results in decreases in the muscle stem cell (MuSC) pool, myofiber size, and functional capacity of muscle (60). FAPs support tissue repair through expression of key myogenic and immune signals, clearance of necrotic debris, and deposition of extracellular matrix (ECM) (13,125,126). Depletion of FAPs causes a regenerative deficiency and induces muscle atrophy, impairing proper muscle function (60,127). The role of FAPs in acute regeneration is transient with FAPs rapidly proliferating within the first few days post injury, activating into myofibroblasts, depositing ECM, and undergoing macrophage-mediated apoptosis once regeneration is complete (12). However, in chronic injury, FAPs become resistant to apoptosis and continuously produce ECM components resulting in a more fibrotic tissue (12,28). Myofibroblast progenitors in other tissues have demonstrated enhanced activation in more stiff fibrotic environments (128). Thus, FAPs' production of ECM can create a positive feedback loop for progressive fibrosis with increasingly fibrotic tissue leading to an increase in the number of fibrotic FAPs resulting in the deposition of more ECM.

Pathologically, fibrosis is the excess accumulation of ECM components, predominantly collagen. In skeletal muscle, the excess ECM takes the place of contractile material, weakening the muscle and increasing muscle stiffness (129). Fibrotic material impairs the functional capacity of the muscle, decreasing mobility and leading to contractures (130). Fibrosis is prevalent across many musculoskeletal diseases such as Duchenne muscular dystrophy, and in cases of severe injury that become chronic (7). A fibrotic ECM has altered mechanical and architectural properties compared to a healthy ECM, including increased stiffness and

crosslinking (44,70,131). MuSCs have been shown to be sensitive to their mechanical and architectural environment (50,132) , affecting their capacity for self-renewal and differentiation. Damaged myofibers increase in stiffness causing an increase in MuSC proliferation (89). Changes in collagen crosslinking and fibril size affect MuSCs' capacity to differentiate into myotubes (88). Mesenchymal stem cells (MSCs) are also known to be sensitive to their mechanical and architectural environment (47,133), although FAPs specifically have not been investigated. Therefore, investigation into FAPs' response to changes in mechanics and ECM architecture would provide novel insights into these cells' mechanosensitivity.

FAPs reside in the interstitial space and as a result are in direct contact with the ECM. Collagen, specifically type I collagen, makes up a large portion of the ECM environment, and is the primary load bearing molecule of the ECM (134). Type I collagen is used in cell culture to treat tissue culture plastic or construct hydrogels. Collagen hydrogels can be manipulated to mimic different aspects of the ECM environment including collagen concentration, crosslinking, fibril size, and fibril alignment (133,135,136). In this study, we fabricated collagen hydrogels to mimic architectural features of a fibrotic ECM to investigate FAPs' sensitivity to their architectural and mechanical environment. We hypothesized that there would be an increase in proliferation, myofibroblast activation, and yes-associated protein (YAP) nuclear localization, a measure of mechanosensitivity, on collagen gels that resemble a fibrotic ECM. The goal of this study was to determine if FAPs are sensitive to their mechanical environment and ECM architecture, and if FAPs' mechanosensitivity plays a role in the development of a regenerative versus fibrotic phenotype.

RESULTS

FAP Proliferation, Myofibroblast Activation, and Nuclear YAP Increase with Increasing Substrate Stiffness

FAPs can differentiate into adipocytes or activate into myofibroblasts in vivo. To confirm that these cells maintain their multilineage capacity after primary cell isolation, cells were grown in either adipogenic media or FAP media for 7 days on standard tissue culture plastic. Cells in adipogenic media increased perilipin expression, a marker of lipid droplets and adipogenic differentiation, while cells in the FAP media had increased alpha smooth muscle actin (αSMA) expression (Fig 1A). Fibrosis is associated with an increase in muscle stiffness. Healthy myofibers are typically 8kPa compared to fibrotic myofibers that are greater than 18 kPa (50,137). Therefore, to assess FAPs' response to changes in substrate stiffnesses that are physiologically relevant, a Matrigen Softwell plate with polyacrylamide hydrogels ranging from 0.2kPa to 50kPa was utilized. Polyacrylamide hydrogels were coated with 0.1% collagen to promote cell adhesion. YAP nuclear localization increased with increasing substrate stiffness after 3 days in culture (Fig 1B, C). Higher substrate stiffness and increased nuclear YAP correlated strongly with each other (Fig S2.1A). Therefore, we investigated if early YAP nuclear localization induced changes in FAP behavior, specifically proliferation at 3 days and myofibroblast activation at 7 days. Proliferation rate, measured by EdU incorporation correlated with substrate stiffness, but was not significantly different between any of the gels, although trended upward with increasing stiffness (Fig 1 D-E, Fig S1B). Myofibroblast activation was significantly higher on substrates with fibrotic stiffnesses compared to a physiologically, healthy modulus (Fig 1F, G). The increase in myofibroblast activation was strongly correlated with the increase in substrate moduli, indicating FAPs ability to detect their underlying substrate mechanics (Fig S2.1C). The increase in myofibroblast activation and YAP nuclear localization were highly correlated indicating nuclear YAP may be driving this change cell phenotype (Fig 1H). Recent studies have shown a subpopulation of FAPs marked by high Vcam1 expression is

significantly increased during injury and fibrosis in mice (11). To investigate if the increase in myofibroblast activation is driven by Vcam1 positive FAPs, we looked at Vcam1 and αSMA expression in FAPs cultured on substrates of increasing stiffness. There were similar levels of Vcam1 expression in αSMA+ and αSMA- FAPs (Fig S2.2A, B). As opposed to myofibroblast activation, the adipogenic differentiation of FAPs over 7 days was not sensitive to the range of substrate stiffnesses tested (Fig S2.2C). Overall, FAPs appear sensitive to changes in substrate stiffness through YAP localization and associated myofibroblast activation, although substrate stiffness did not impact Vcam1 expression nor adipogenesis.

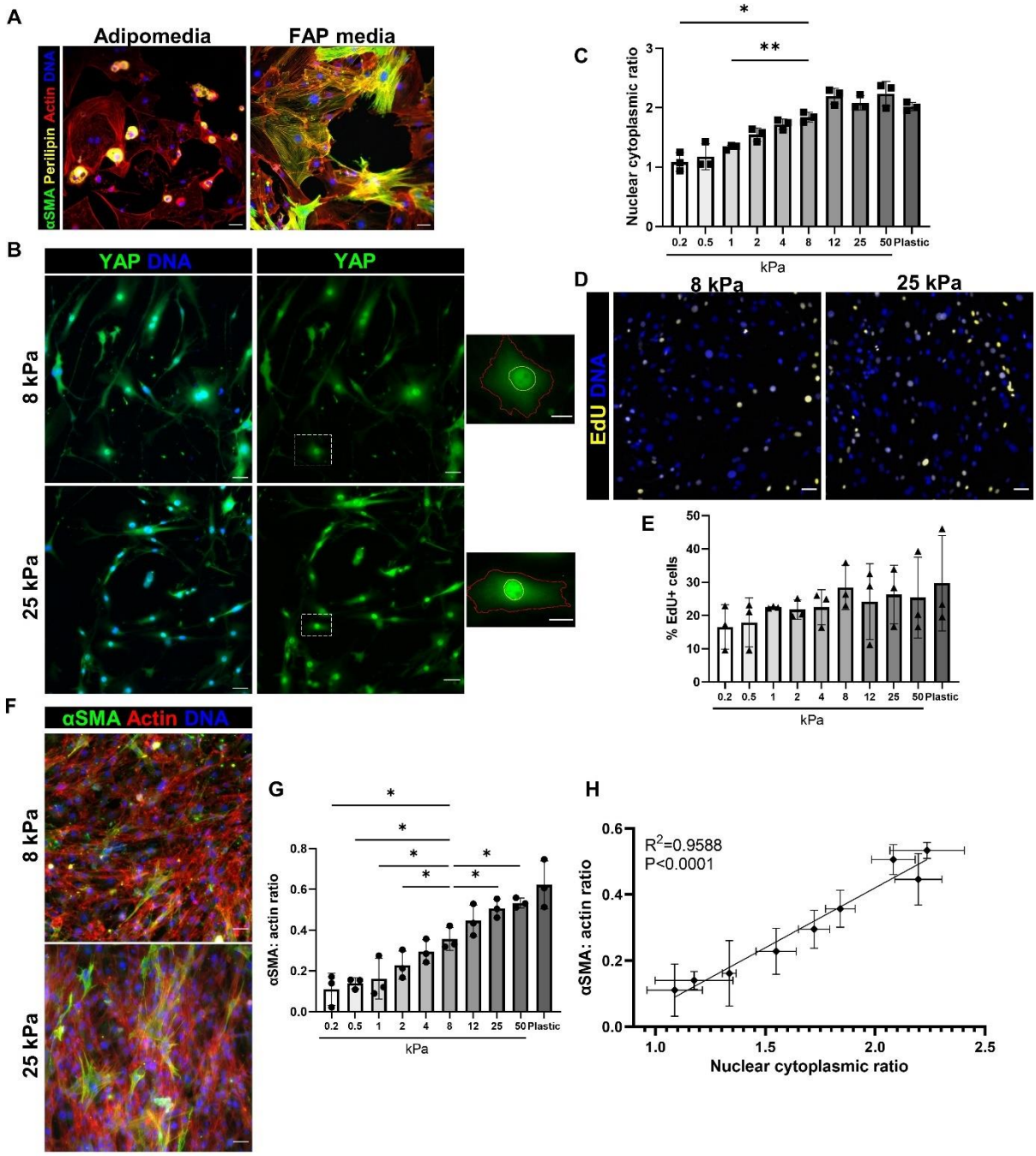

*Figure 2.1: FAPs' response to substrate stiffness. A) Expression of perilipin and αSMA in FAPs after 7 days in adipogenic or FAP media. B) YAP immunofluorescence of FAPs on polyacrylamide gels on 8kPa and 25kPa. Yellow outlines indicate nuclei on insets. C) Quantification of YAP signal intensity in nucleus and cytoplasm. D-E) EdU signal and quantification of percent proliferating cells after 24 hrs EdU treatment F) αSMA signaling as a measure of myofibroblast activation in FAPs. G) Quantification of αSMA and actin area ratio. H) Correlation of αSMA expression and YAP nuclear localization using linear regression. *P< 0.05, **P< 0.01, compared to 8kPa via one-way ANOVA using repeated measures with Dunnett correction. Scales bars set to 50μm. Scale bars for insets set to 20μm. N=3 mice and n=3 independent gels.*

YAP Localization Is Sensitive to Collagen Concentration

Fibrosis not only alters the mechanics of the ECM, but also the ECM content and architecture (7,44). Since fibrosis is associated with an increase in ECM components, specifically collagen, we investigated FAPs' response to telocollagen gels with increasing collagen concentrations of 1.5, 3.0, 4.5, and 6.0 mg/ml. FAPs were also grown on tissue cultured plastic, which represents a rigid substrate compared to the soft collagen gels. YAP nuclear localization was sensitive to changes in collagen concentration and stiffness (Fig 2A, B). Proliferation was not affected by collagen concentration but increased on plastic compared to all the collagen gels except the 6.0 mg/ml gels (Fig 2C, D). There was no difference in myofibroblast activation across the range of collagen gels, but activation did increase on plastic compared to all the gels (Fig 2E, F). Cellular and nuclear area were not significantly different across the substrate conditions, although cells on plastic tended to be more spread out and have higher cell density (Fig S2.3A-C). Overall, YAP nuclear localization was affected by collagen concentration, but not to an extent that altered FAP proliferation or activation into myofibroblasts.

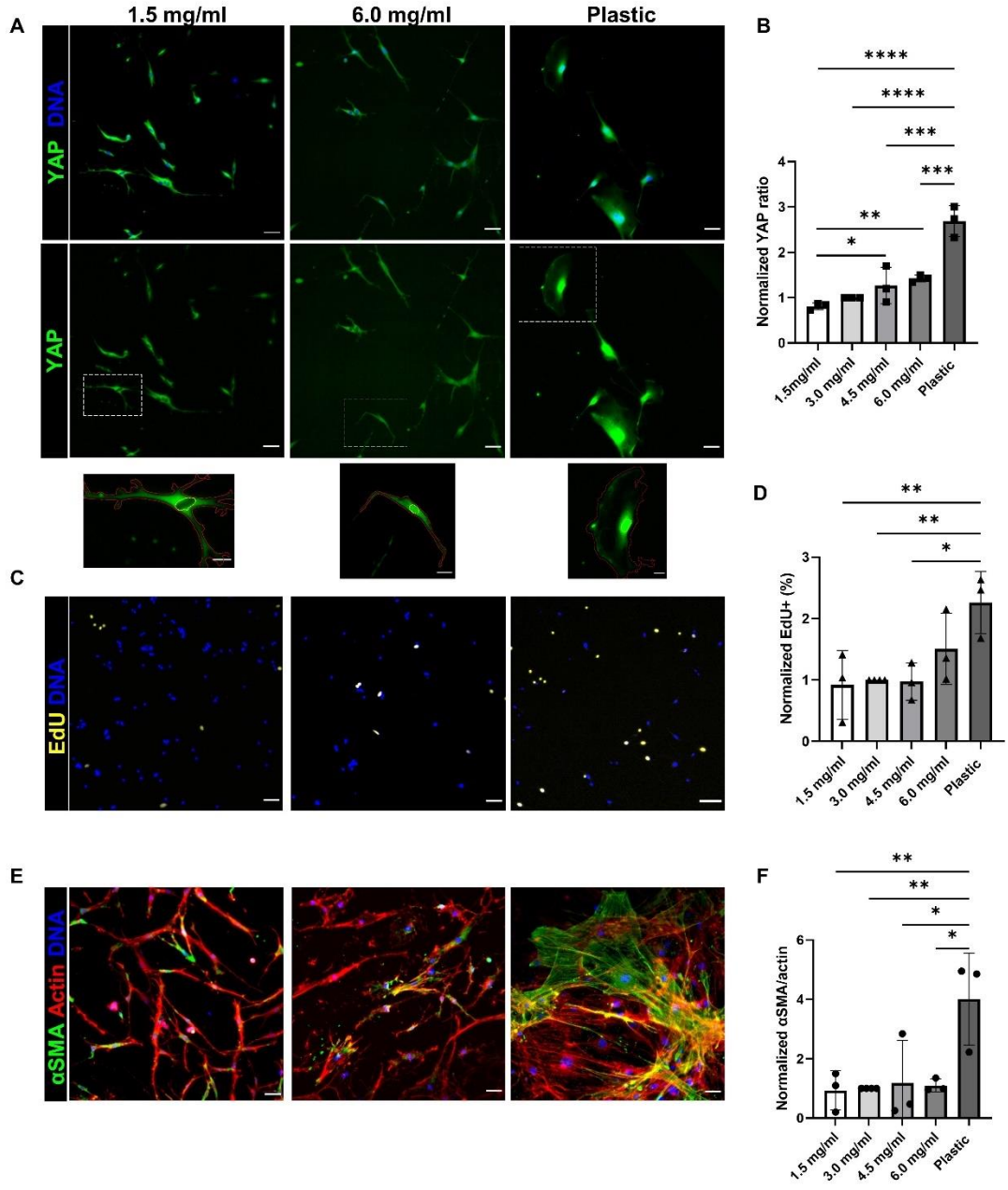

*Figure 2.2: FAP behavior in response to scaling collagen concentration. A) YAP immunofluorescence of FAPs on 1.5 and 6.0 mg/ml collagen gels and tissue cultured plastic. Yellow outlines indicate nuclei on insets. B) Quantification of YAP signal intensity as a nuclear to cytoplasmic ratio. C-D) EdU signal and quantification across collagen concentrations and plastic. E) αSMA signaling in FAPs after 7 days in FAP media. F) Quantification of αSMA and actin area ratio as a measure of myofibroblast activation. *p< 0.05, **p< 0.01, ***p=<0.001, ****p< 0.0001, One-way ANOVA with multiple comparisons with Dunnett correction. Data normalized to 3.0 mg/ml. Scales bars set to 50μm. Scale bars for insets set to 20μm. N=3 and n=3 for 1.5, 4.5, 6.0 mg/ml and plastic. N=4 and n=4 for 3.0 mg/ml. N=number of mice, n=number of independent gels.*

Collagen Crosslinking Inhibits FAP Activation into Myofibroblasts

Further investigation was conducted into several aspects of collagen architecture. Collagen crosslinking is known to increase in fibrotic conditions, including muscle fibrosis (70). Pyridinoline crosslinking of collagen fibrils occurs in the telopeptide region of the fibril (72,138). Atelocollagen is a collagen solution with the telopeptide region cleaved, preventing crosslinking. Atelocollagen was used alongside telocollagen to evaluate the effect of collagen crosslinking on FAP behavior. YAP localization was not affected by crosslinking (Fig 3A, B). Proliferation rate did not change between telocollagen and atelocollagen (Fig 3C, D). Myofibroblast activation significantly increased when crosslinking was prevented (Fig 3E, F). Surprisingly, this data demonstrates that collagen crosslinking blocks FAPs activation into myofibroblasts without a significant impact on proliferation or YAP localization.

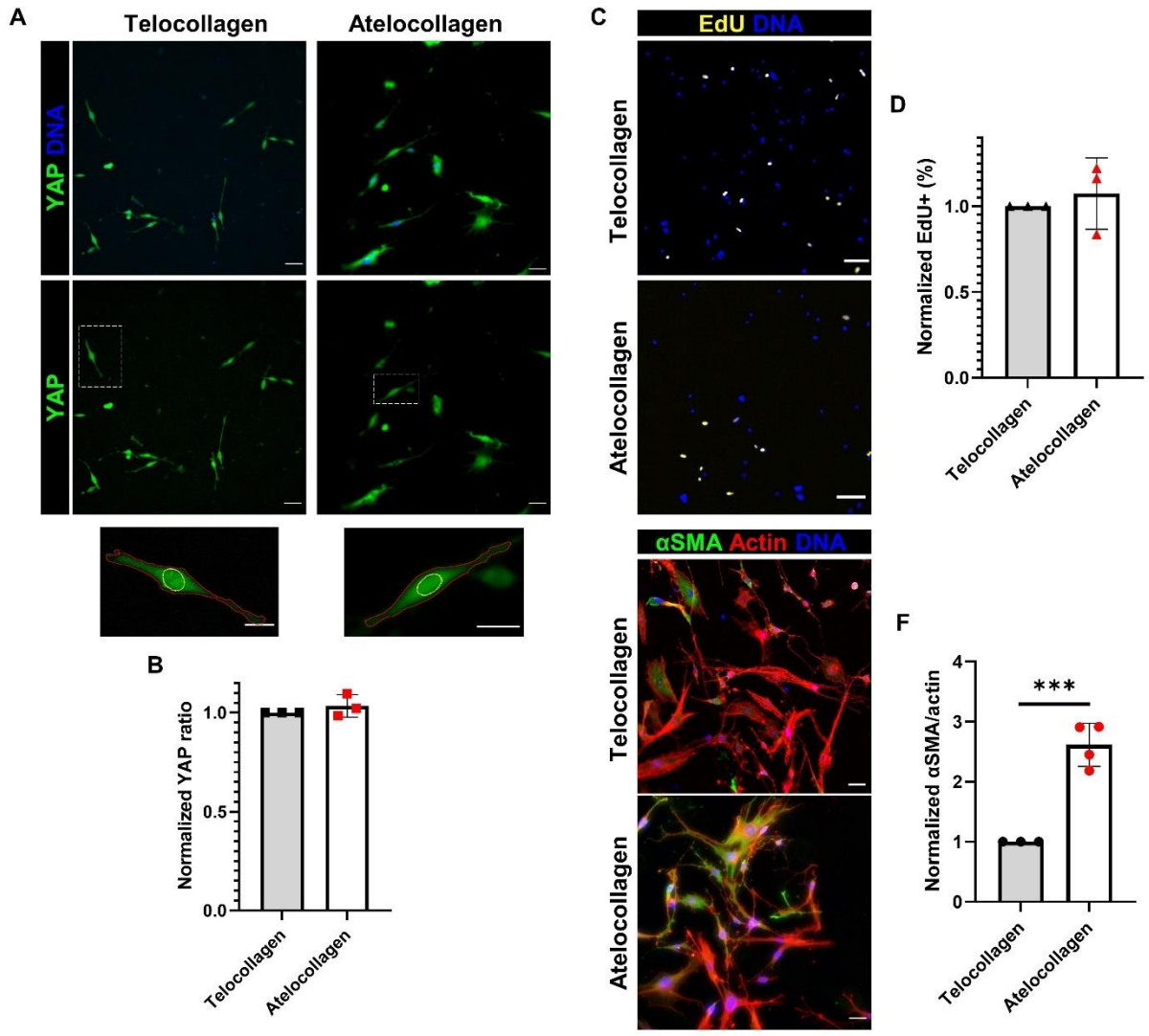

*Figure 2.3: FAPs' response to crosslinked and non-crosslinked collagen gels. A) YAP immunofluorescence of FAPs on telocollagen and atelocollagen gels. Yellow outlines indicate nuclei on insets. B) Quantification of YAP signal intensity to find nuclear to cytoplasmic ratio of YAP. C-D) EdU signal and quantification of percent proliferating cells after 24 hrs EdU treatment E) αSMA signaling as a measure of myofibroblast activation in FAPs. F) Quantification of αSMA and actin area ratio. ***P< 0.001. Data normalized to telecollagen gels. Scales bars set to 50μm. Scale bars for insets set to 20μm. N=3 mice and n=3 independent gels.*

FAPs Deform the Collagen Matrix

The decrease in myofibroblast activation with collagen crosslinking was unexpected due to the increase in crosslinking seen in fibrosis (44,70). We hypothesized that this disparity was due to

the increased ability of the FAPs to deform the atelocollagen, creating localized areas of higher stiffness and collagen content. To investigate this, we imaged telocollagen and atelocollagen gels using second harmonic generation (SHG) imaging after 2 and 7 days with FAPs to observe changes in collagen distribution across the gel. We hypothesized due to the contractility of myofibroblasts, these cells would be able to deform the matrix more than non-activated FAPs. Therefore, we looked at αSMA+ and αSMA- FAPs independently to observe differences in collagen distribution. There was an overall increase in collagen intensity under the cells, significantly on the day 2 atelocollagen and day 7 telocollagen gels. However, there was no difference in collagen intensity between at αSMA+ and αSMA- FAPs (Fig 4 A, C).

These cells showed consistency with previous myofibroblast activation results (Fig 4B). However, there was no significant difference in collagen intensity between the atelocollagen and telocollagen gels, suggesting the cells were not deforming the atelocollagen gels to a greater degree.

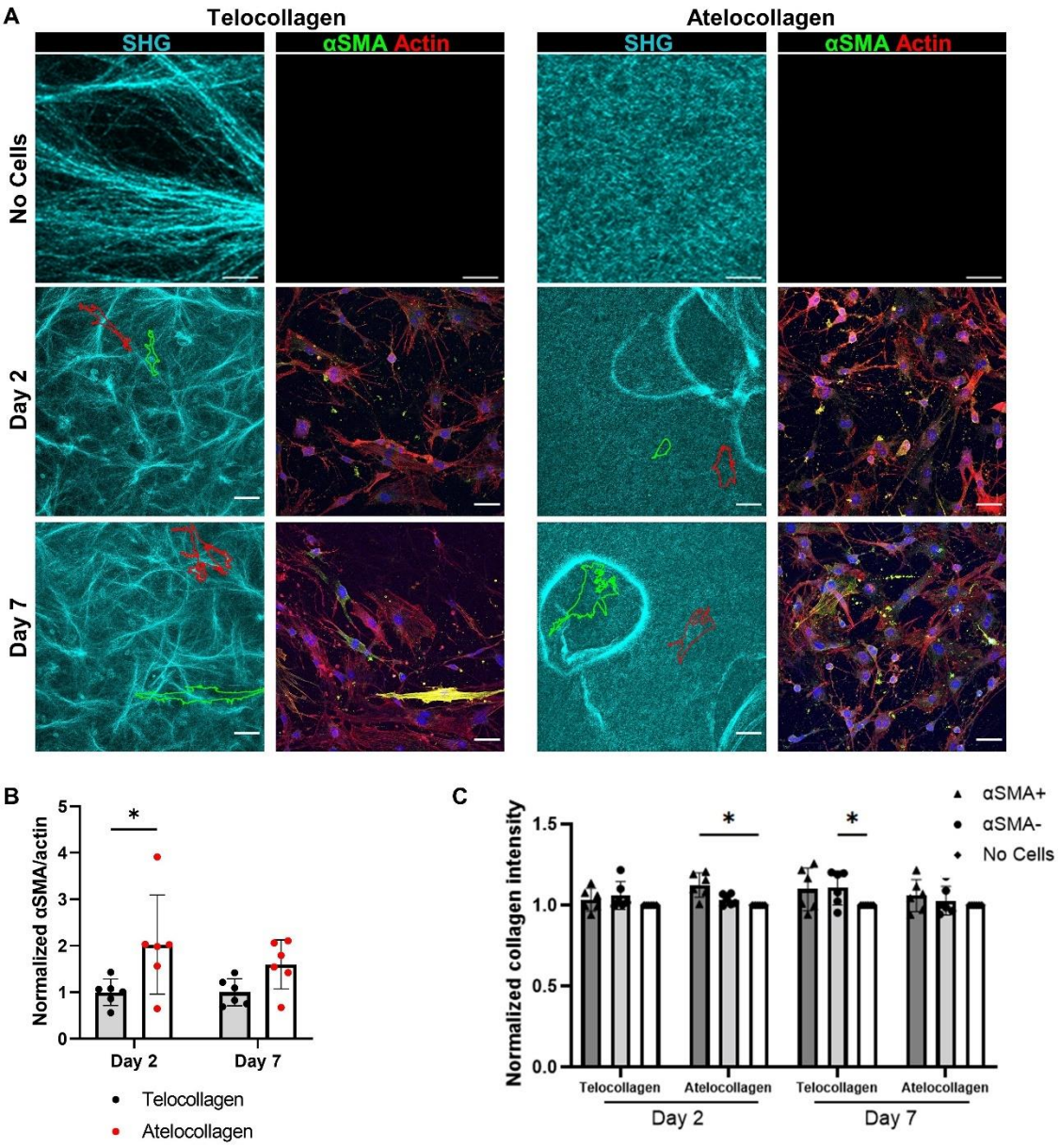

*Figure 2.4: SHG imaging of crosslinked and non-crosslinked collagen gels. A) SHG images of collagen and FAP cells. Red outlines indicate a αSMA- cells and green outlines indicate a αSMA+ cell. Scale bars on no cell images set to 10 μm. Scales bars on day 2 and day 7 images set to 50μm. B) αSMA and actin area ratio after 2 and 7 days on the collagen gels. C) Collagen intensity of gels under αSMA+ or αSMA- cells, or under no cells normalized to areas of collagen without any cells. *p< 0.05 using a two-way ANOVA with multiple comparisons using Tukey correction. N=2 independent gels and n=3 fields per gel.*

Larger Collagen Fibrils Inhibit FAP Activation into Myofibroblasts

Along with the abundance of collagen fibers in fibrosis is a shift to collagen fibers and fibrils of a larger diameter (139). Collagen fibril size in hydrogels is affected by polymerization temperature with a higher temperature resulting in more fibrils of smaller sizes than lower temperatures (140,141). Collagen gels were polymerized at 22°C along with 37°C to elucidate FAPs response to fibril size. Proliferation and YAP localization were not changed between the two conditions (Fig 5A-D). Smaller collagen fibrils resulted in significantly increased myofibroblast activation compared to the larger fibrils (Fig 5E, F). As with collagen crosslinking, surprisingly, increased collagen fibril size appeared to inhibit FAP activation into myofibroblast.

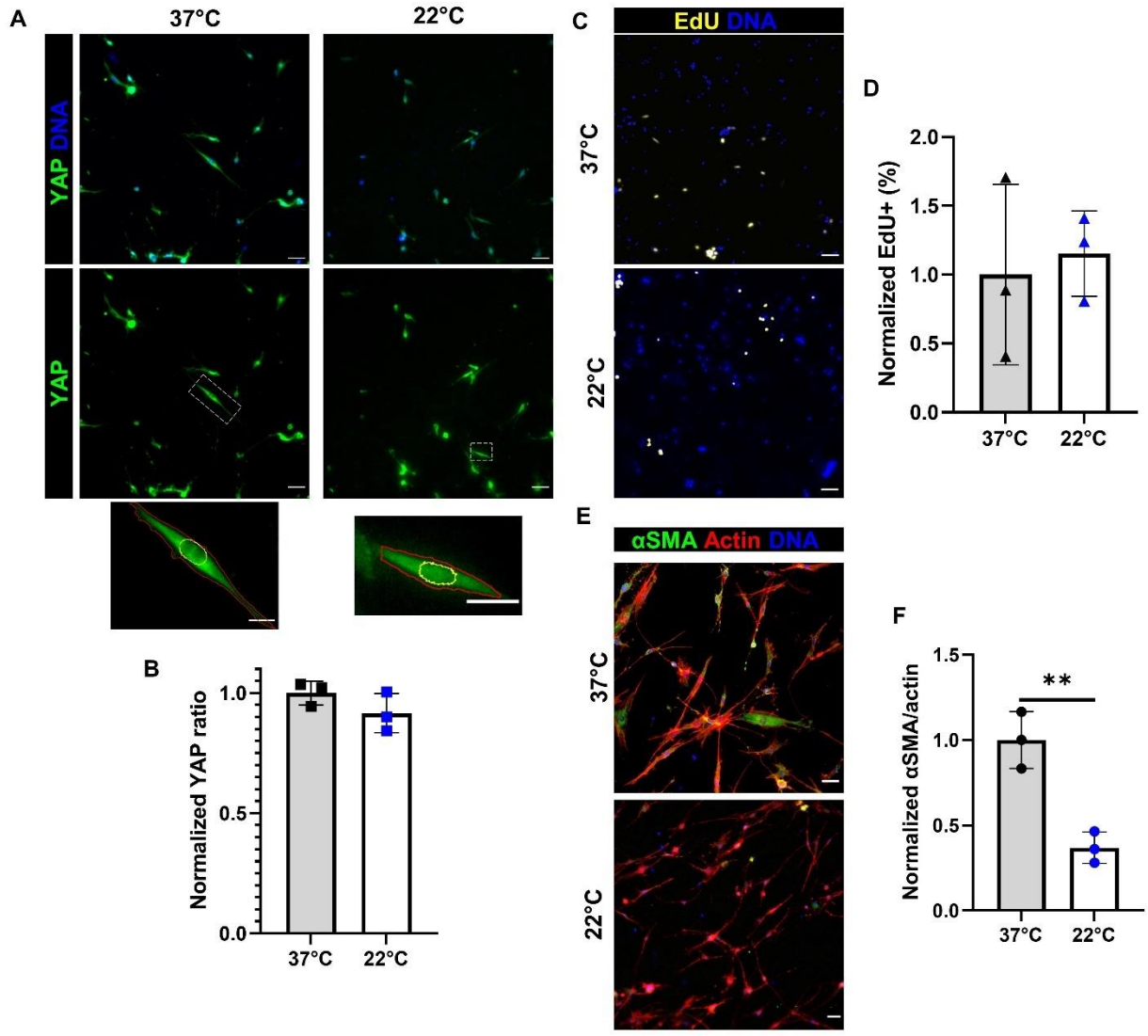

*Figure 2.5: Effect of fibril size on FAP behavior. A) YAP immunofluorescence of FAPs on telocollagen gels polymerized at 37 and 22°C. Yellow outlines indicate nuclei on insets. B) Quantification of YAP signal intensity to find nuclear to cytoplasmic ratio of YAP. C-D) EdU signal and quantification of percent proliferating cells after 24 hrs EdU treatment. E) αSMA signaling in FAPs after 7 days. Scales bars set to 50μm. F) Quantification of αSMA and actin area ratio. **p< 0.01. Data normalized to 37°C. Scales bars set to 50μm; insets set to 20μm. N=1 mouse and n=3 independent gels.*

FAPs Deform Smaller Collagen Fibrils

Smaller collagen fibrils are more deformable and malleable to cell deformation (133). We investigated if the FAPs were deforming the collagen gels polymerized at 37°C more than those

polymerized at 22°C, which may have contributed to the differences in myofibroblast activation. SHG imaging of the collagen gels revealed an increase in collagen intensity under cells on the 37°C gels compared to areas without cells. This suggests that the cells were able to pull the matrix and localize the collagen. There was no significant difference in collagen distribution across the 22°C gels (Fig 6A, C). The decrease in myofibroblast activation on the 22°C gels was consistent with previous results (Fig 6B). It appeared that the FAPs, regardless of whether activated into myofibroblasts or not, were able to deform the 37°C collagen gels but not the 22°C gels. Together, with the data on myofibroblast activation on non-crosslinked gels, this implicates substrate malleability as a potential factor in myofibroblast activation.

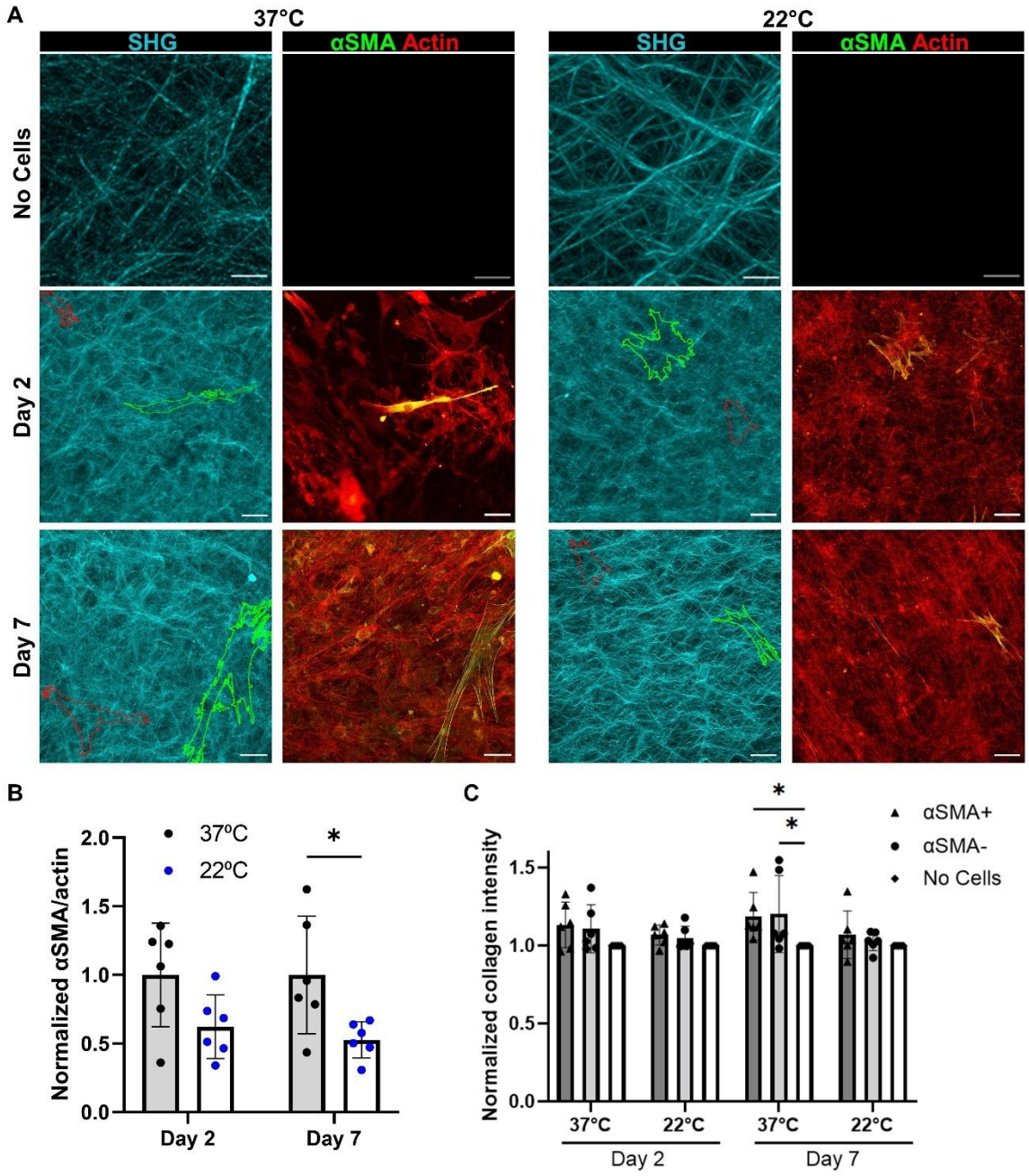

*Figure 2.6: SHG imaging of collagen gels polymerized at different temperatures. A) SHG images of collagen gels polymerized at 37°C and 22°C at day 2 and day 7 after cell seeding. Scale bars on no cell images set to 10 μm. Scales bars on day 2 and day 7 images set to 50μm. Red outlines indicate a αSMA- cell and green outlines indicate a αSMA+ cell. B) Myofibroblast activation at day 2 and day 7 across the gels. C) Collagen intensity under no cells, αSMA+, or αSMA- cells. Intensity normalized to areas of gels without cells. *p< 0.05 using a two-way ANOVA with multiple comparisons using Tukey correction. N=2 independent gels and n=3 fields per gel.*

Collagen Alignment Does Not Impact FAP Proliferation, Differentiation, or YAP Localization

Collagen gels were aligned using magnetic beads during initial stages of polymerization (135). FAPs were plated on aligned or unaligned telocollagen gels to determine the effect of alignment on FAP behavior. FAPs were able to sense their underlying substrate and elongate their nuclei along the axis of collagen alignment (Fig. S2.4A, B). Proliferation and YAP nuclear localization was consistent across the two conditions after 2 days (Fig S2.4C-F). Myofibroblast activation did not change with alignment over the course of a week (Fig S2.4G, H). Skeletal muscle is typically highly anisotropic (44,142), and FAPs showed preference to align their nuclei with the direction of the fibrils, but that alignment did not significantly impact the FAP response in the other measures investigated.

YAP Inhibition Reduces Myofibroblast Activation on Stiff Substrates

Given the strong correlation between YAP nuclear localization and myofibroblast activation on stiff substrates, we investigated if blocking YAP activity would reduce αSMA expression in FAPs. FAPs were cultured on collagen-coated polyacrylamide gels at 0.2, 8, 25 kPa, and plastic as well as on collagen gels polymerized at 37°C and 22°C. There was a significant increase in αSMA expression with increasing substrate stiffness. With the addition of verteporfin, an inhibitor of YAP activity, YAP localization to the nucleus decreased (Fig 7A). After 7 days, verteporfin led to the elimination of mechanosensitivity as αSMA expression was significantly reduced in FAPs on the 25kPa gel and on plastic (Fig 7B, C). However, blocking YAP did not impact FAP sensitivity to collagen fibril diameter as verteporfin did not appear to have an effect on FAPs cultured on collagen gels polymerized at different temperatures (Fig 7B, D).

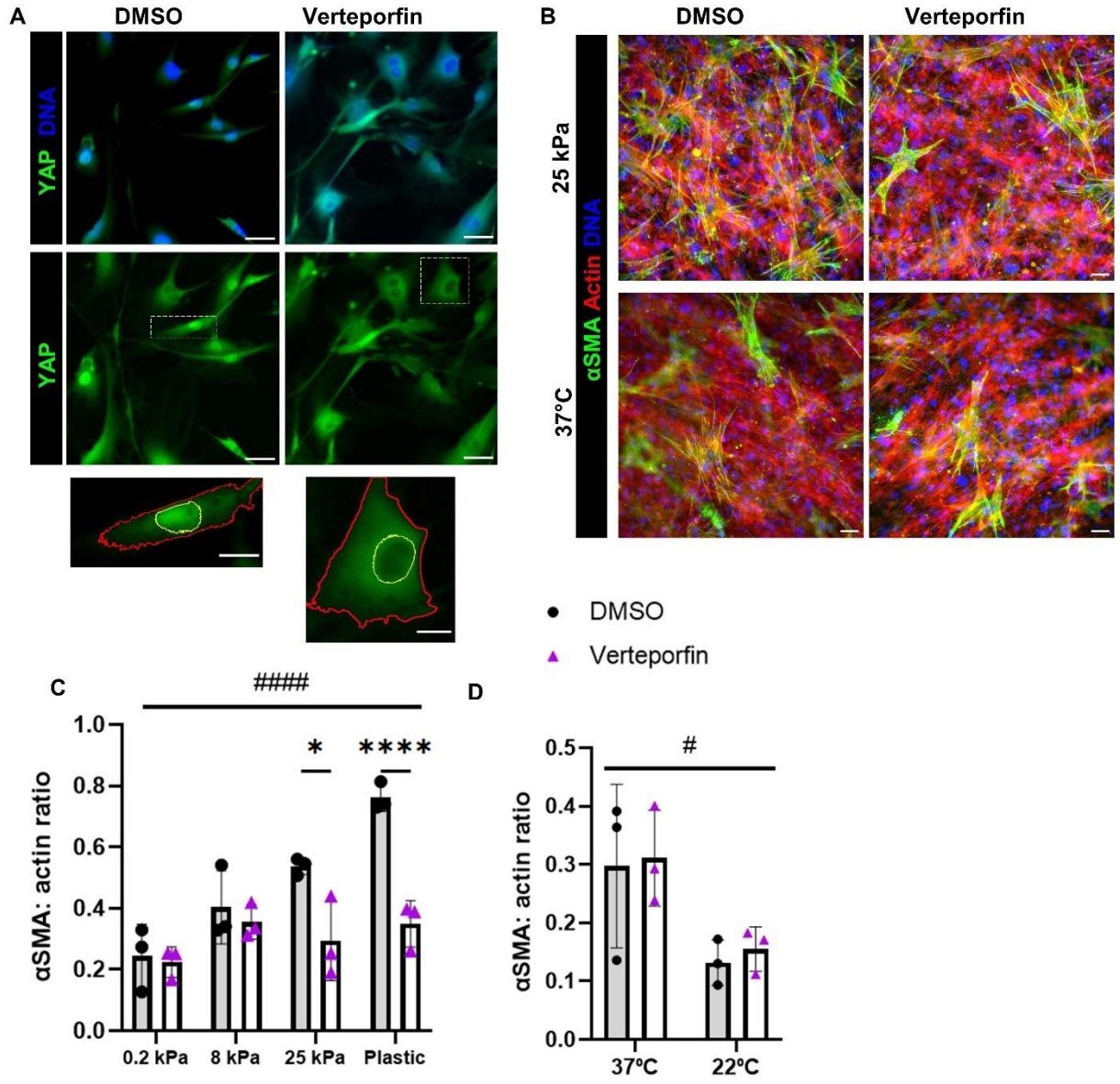

*Figure 2.7: YAP inhibition reduces myofibroblast activation. A) YAP localization in FAPs cultured on plastic in DMSO or 0.5μM verteporfin for 2 days. Scale bars are 50μm, insets are 20μm. Yellow outlines indicate nuclei and red outlines indicate cytoplasm on insets. B) Myofibroblast activation in FAPs cultured for 7 days with 0.5μM verteporfin or 0.1 % DMSO on collagen-coated polyacrylamide gels at 25kPa and 3.0 mg/ml telocollagen gels polymerized at 37°C. Scale bars set to 50μm. C) Quantification of myofibroblast activation of FAPs in DMSO or 0.5μM verteporfin on collagen-coated polyacrylamide gels. D) Quantification of myofibroblast activation of FAPs in DMSO or 0.5μM verteporfin on collagen gels polymerized at different temperatures. *p<0.05, ****p<0.0001 between DMSO and verteporfin treatment. # p<0.05, #### p<0.0001 between substrates.*

DISCUSSION

The goal of this study was to investigate FAPs' sensitivity to their underlying substrate mechanics and architecture. The mechanosensitivity of FAPs has not, to our knowledge, been investigated. Sensitivity to their mechanical and architectural environment may play a key role in FAP's contribution to fibrosis, with fibrotic muscle inducing a more fibrotic FAP phenotype. We focused on proliferation and myofibroblast activation as measures of a fibrotic phenotype and YAP localization as a potential mechanosensing pathway. Polyacrylamide hydrogels with collagen coating were used to investigate a range of physiologically relevant stiffnesses. Collagen gels were engineered to manipulate collagen concentration, crosslinking, fibril size, and alignment. YAP nuclear localization increased on substrates with increasing stiffness. Proliferation was increased on plastic compared to collagen gels but was not affected by architectural changes in substrate. Myofibroblast activation was inhibited on gels with crosslinking, larger fibrils, or lower stiffness. Alignment affected the orientation of nuclei but had no significant effect on proliferation or myofibroblast activation. Increased YAP nuclear localization correlated with higher myofibroblast activation levels when associated with altered mechanics. However, YAP localization was not correlated with changes induced via architecture. Using verteporfin to block YAP activity, resulted in a reduction of myofibroblasts on stiff substrates but not on substrates with altered architecture. Overall, this study demonstrates that FAPs are sensitive to aspects of both their mechanical and architectural environment with implications for their role in propagating fibrosis.

Fibrosis is associated with an increase in tissue stiffness, with skeletal muscle bundles increasing from around 8kPa in healthy muscle to > 20kPa in fibrosis (7,50). To investigate if this change in stiffness altered FAP behavior, collagen-coated polyacrylamide gels ranging from 0.2 to 50 kPa were utilized. YAP nuclear localization and myofibroblast activation scaled with increasing stiffness. There were significant differences in myofibroblast activation between 8kPa

and 25kPa. The increase in YAP nuclear localization was highly correlated with the increase in myofibroblast activation indicating that YAP may be driving this change. Previous studies have shown that YAP nuclear localization is tied to fibroblast activation and proliferation (4,143,144). Blockage of YAP activity with verteporfin reduced myofibroblast activation on 25kPa gels and on plastic. This supports previous studies that have shown a reduction in YAP activity, αSMA expression , and fibrosis with the addition of verteporfin (4,104,119). The increase in myofibroblast activation supports the concept of a feedforward pathway of fibrosis. A fibrotic environment induces FAP activation into myofibroblasts, which in turn increases ECM deposition making the tissue increasingly fibrotic (125,143). The expression of Vcam1 is significantly upregulated in FAPs during injury and fibrosis, suggesting it may play a role in fibrotic development (11). However, Vcam1 levels were similar across FAPs cultured on different stiffness and between αSMA+ and αSMA- cells, suggesting Vcam1 is not driving the changes induced by stiffness. Due to the limited availability of primary FAPs, cells were cultured on tissue-cultured plastic for 1-2 weeks before plating on substrates of altered stiffness. It is possible that this time on rigid plastic induced activation of Vcam1 across all cells that was maintained even on soft substrates. In addition to driving production of fibrotic material as myofibroblasts, FAPs in diseased conditions also undergo adipogenesis leading to fatty infiltration of muscle. While we hypothesized this would be supported on softer substrates in adipogenic media there was no difference in the FAP produced adipocytes with stiffness. However, mechanosensitivity of adipogenesis is largely regulated by cyclic strain which was not incorporated into our system (145). This is the first study to specifically demonstrate FAP's substrate mechanosensitivity, with stiff environments reinforcing YAP associated pathways toward myofibroblasts.

The ECM architecture, as well as mechanics, are altered in a fibrotic muscle. Fibrosis is largely tied to an increase in ECM content, specifically collagen. Therefore, we manipulated collagen

hydrogels to mimic different aspects of the ECM architecture altered in fibrosis that FAPs residing in the interstitial matrix would experience. The range of collagen concentrations investigated in this study are representative of in vivo concentrations, although the in vivo organization of collagen fibrils into cables is not reconstituted in vitro (138,146,147). While the modulus of the collagen gels scaled with concentration, overall, the stiffnesses of the gels (0.1-0.4 kPa) were well below the physiological range (7,88). YAP nuclear localization scaled with increasing collagen concentration and therefore, stiffness. However, this change in YAP did not correlate well with changes in proliferation or myofibroblast activation. As YAP translocates to the nucleus in response to increased substrate stiffness, there may be a concentration threshold needed to observe phenotypic changes. This threshold is reached on plastic, where the significant increase in nuclear YAP compared to collagen gels correlated with an increase in proliferation and myofibroblast activation.

Collagen crosslinking increases in skeletal muscle fibrosis (44,70). Surprisingly, myofibroblast activation increased with the removal of collagen crosslinking. Collagen stiffness and crosslinking are often associated with each other, but studies have suggested they do not strongly correlate in skeletal muscle (148). The stiffness of the collagen gels used here has been shown to remain relatively similar with or without crosslinking, which is supported by similar YAP nuclear to cytoplasmic ratios across the gels (88,148). This suggests that the increase in myofibroblast activation may be driven by a signaling pathway independent of YAP. Crosslinked collagen gels have been shown to have increased resistance to deformation when compared to gels without crosslinking due to the contraction of the crosslinked collagen fibrils (149). Despite increased resistance to deformation, there was no significant difference in matrix remodeling between the atelocollagen and telocollagen gels. FAPs did appear to pull the matrix around them regardless of cell fate. MSCs have improved differentiation on reduced crosslinked collagen substrates due to an enhanced ability of cells to contract (150). FAPs may activate

into myofibroblasts on the atelocollagen gels to compensate for the lack of crosslinks by producing a more crosslinked matrix. TGF-β is a predominant driver of fibrosis in vivo, inducing the activation of FAPs into myofibroblasts and causing the cells to be resistant to apoptosis. Soluble factors, such as TGF-β signaling, may dominate over the architectural cues from crosslinking in fibrotic tissue (12). Contrary to our initial hypothesis, this result implicates a YAP and stiffness independent mechanism of myofibroblast activation that is diminished in response to collagen crosslinking.

To investigate the effect of fibril size, collagen gels were polymerized at 22ºC, to produce larger fibrils, compared to the standard gel formed at 37ºC (140). MSCs are known to have increased proliferation and increased adipogenic differentiation on collagen gels polymerized at 37ºC compared to 22ºC (133). Similarly, our study showed that gels with higher polymerization temperatures resulted in more myofibroblast activation but did not change their proliferation rate. This increase in myofibroblast activation was not correlated with changes in YAP localization, and the inhibition of YAP activity did not appear to have any effect on αSMA expression. This indicates that the increase in myofibroblast activation is driven by a YAP-independent mechanism. The change in myofibroblast activation may be due to an increase in intrafibrillar crosslinking at 22ºC due to the larger fibril diameter. Higher polymerization temperatures result in smaller, longer fibrils allowing for increased focal adhesions and cell spreading (140,141,151). Despite no changes in overall bulk stiffness, fibrils polymerized at higher temperatures are more flexible and malleable to cell deformation (18,20). MSCs that can remodel their substrate have increased cell spreading, which is linked to the expression of myofibroblast markers including αSMA (133,152,153). In this study, we found that FAPs may be able to deform the collagen matrices with smaller fibrils, which correlated to higher αSMA expression. Alternatively, the increase in collagen intensity may also be caused by an increase in collagen secretion from FAPs on the gels polymerized at higher temperatures. FAPs'

activation into myofibroblasts may be driven not only by mechanical and architectural sensing, but also the cells' ability to remodel the surrounding matrix.

Another key aspect of collagen architecture is alignment of fibrils. Collagen fibrils are highly aligned in skeletal muscle and altered in fibrosis (44). Alignment of collagen fibrils does not affect overall stiffness of the gels, resulting in similar YAP localization (88,135). ECM alignment is important in skeletal muscle for force transmission and proper muscle fiber regeneration. Despite the highly aligned nature of skeletal muscle, there was no change in FAP behavior with alignment except for the cells orienting their nuclei along the axis of alignment. MSCs grown on collagen gels showed no difference in osteogenic or adipogenic differentiation with or without collagen alignment despite cells orienting with the direction of the fibrils (154). While alignment of the ECM is important in regeneration to promote properly aligned myofibers, it does not appear to affect FAPs' cell fate.

Collagen hydrogels provide an opportunity to investigate in vivo changes in architecture, yet also pose several limitations. FAPs reside in the interstitial space in vivo and are completely surrounded by ECM (155). This study investigated FAPs' response to collagen architecture in a 2D environment rather than a 3D environment, which may have influenced cell-matrix interactions (156–158). This in vitro study allows us to isolate the influence individual aspects of the ECM architecture play on FAPs but does not reconstitute the complex in vivo environment. While we have demonstrated stiffness, collagen crosslinking, and fibril size are capable of dictating FAPs' response, how these signals are interpreted among the complexity of the environment in vivo requires further study. Additionally, due to limited number of FAPs isolated from a single dissection, cells were grown up on tissue cultured plastic before being passaged to the collagen gels for the myofibroblast activation assay. Previous reports have shown different cell lines, including adipose stem cells and MSCs, retain mechanical memory of their initial substrate stiffness (159,160). MSCs, which share many similarities to FAPs, have been

shown to maintain mechanosensitivity despite mechanical memory (160). Additionally, the differences observed in myofibroblast activation across the collagen gels, despite being cultured on plastic first, indicate that FAPs do retain mechanosensitivity regardless of potential mechanical memory. The results of this study have implications in musculoskeletal diseases such as muscular dystrophies where fibrosis is prevalent. FAPs play a central role in the development and progression of fibrosis. The development of fibrotic ECM provides mechanical and architectural cues to FAPs to further fibrosis through activation into myofibroblasts. YAP appears to dictate FAPs' response to changes in mechanics, but not the response to architecture suggesting another signaling pathway drives architectural sensing. While more research is needed to uncover this alternative pathway, it could represent a target for novel anti-fibrotic therapy. The ECM is an important target in treating fibrosis to make the environment more conducive to both a regenerative FAP phenotype and MuSC regeneration.

CONCLUSIONS

Overall, this study provides novel insights into the mechanosensitivity of FAPs, which has not to our knowledge been investigated. This study shows that FAPs' sensitivity to their substrate expands beyond mechanics to architecture as well. YAP has been a well-known target for mechanosensitivity investigations. Yet, in this study, we show that YAP does not correlate with changes induced by substrate architecture. This study provides novel therapeutic ECM targets to modulate FAP behavior in fibrosis.

METHODS

Animal Handling

Mice were obtained from Jackson Laboratory and bred at a UC Davis animal facility. The animals were given food and water ad libitum and kept on a 12-h light/dark cycle. Wild-type male and female mice aged 6-12 weeks were used for primary cell collection, animals were selected randomly from the pool of cages. CO¬2 followed by cervical dislocation was used to

euthanize the animals prior to muscle collection. All animal experiments were in compliance with ARRIVE guidelines, approved by the University of California Davis Institutional Animal Care and Use Committee, and performed in accordance with the relevant guidelines and regulations.

FAP Isolation and Culture

To isolate FAPs, the lower limb skeletal muscles of wild type mice were dissected. Muscle tissue pieces were digested in an enzyme mix from Skeletal Muscle Dissociation Kit (Miltenyi Biotec) in two 30 min intervals at 37^{o}C under continuous rotation. Mechanical disruption via a tissue homogenizer was used between intervals. The digested muscle solution was filtered using a 70 µm filter, and red blood cell lysis solution was added to remove erythrocytes. CD140a (PDGFRα) magnetic beads (Miltenyi Biotec 130-101-502) were added to filtered cells to label FAPs for positive selection through a MACs Column and Separator (Miltenyi Biotec). The isolated FAPs were added to either tissue culture treated plastic plates or directly onto collagen gels in FAP media (DMEM, 20% FBS (Biowest S1620), 10% HS (Fisher SH3007403), 1% Penicillin-Streptomycin (Fisher 15-140-122), 50ng/ml bFGF (Fisher PHG6015)). For adipogenic differentiation, cells were grown in DMEM containing 10% FBS, 1% Penicillin-Streptomycin, 0.25 µM dexamethasone (Fisher 1126100), 0.5 mM IBMX (VWR 102516-252), 5 µM troglitazone (Fisher 501150786), and 1 µg/mL insulin (Fisher MP219390025) for 7 days (2). Cells were fed with fresh media every 2-3 days.

Collagen Gel Fabrication

Glass bottom petri dishes were treated before the addition of collagen gel solution to ensure collagen adherence (161). Dishes were treated by adding 2% aminopropyltriethoxysilane (Sigma A36648) for 15 min, washing with deionized water for 5 min, air-drying for 15 min, 0.1% glutaraldehyde (Sigma G5882) for 15 min, and rinsing with deionized water three times. Collagen gel solutions were prepped over ice to prevent polymerization before addition to treated dishes. Solutions were made with either telocollagen (Telecol 10, Advanced Biomatrix

5226) or atelocollagen (Fibricol, Advanced Biomatrix 5133) and diluted to desired concentration 10% 10X PBS diluted with deionized water to 1X PBS and 1N NaOH to a pH of 7.0. All collagen gels were made at a concentration of 3.0 mg/ml except in the case of altered collagen concentrations. Aligned collagen gels were prepared with the addition of 1% magnetic Polystyrene magnetic particles (Spherotech PM-20-10) (22). Collagen solutions were pipetted onto treated glass dishes and topped with a glass coverslip to ensure a flat surface for cell adhesion and imaging. Collagen gels were left to polymerize at either 37°C for 90 min or 22°C for 4 hours. Aligned collagen gels were placed against a strong magnet for to pull beads in one direction, which took less than 1 minute, and then transferred to 37°C to finish polymerization. After polymerization, coverslips were removed, and gels were either used immediately or stored in 1X PBS at 4°C for up to 3 weeks. Unaligned, 3mg/ml telocollagen gels polymerized at 37°C were considered the control across each experimental condition. A Softwell 96-well plate with polyacrylamide hydrogels ranging in stiffnesses from 0.2kPa to 50 kPa was utilized (Matrigen). Hydrogels were coated in collagen at a concentration of 0.1% telocollagen, left to polymerized at 37°C, and then excess solution was removed before use.

EdU Assay

FAPs were seeded on collagen gels in a 6mm cloning cylinder in FAP media at a concentration of 35,000 cells/mm2immediately following cell isolation. Cells were given 48 hrs to adhere, and then the media was replaced with fresh FAP media plus 0.1% EdU (Invitrogen). Cells were incubated with EdU for 24 hrs, and then fixed with 4% paraformaldehyde (Fisher AA433689M). Cells were labeled for EdU incorporation with a solution of 100mM Tris (Fisher BP152 500), 1mM CuSO4 (Fisher 60-045-185), 10 μM Alexa647-Azide (Fisher A10277), and 100 mM ascorbic acid (Fisher AC401475000) for 30 minutes.

Myofibroblast Activation Assay

FAPs were cultured on tissue-cultured plastic for 7 days before assay to ensure high enough cell count. FAPs were then seeded on collagen gels in a 6mm cloning cylinder in FAP media at a concentration of 175,000 cells/mm2. Cells were fed fresh FAP media every 2-3 days and then fixed with 4% paraformaldehyde after 7 days.

YAP Inhibition Assay

FAPs were cultured in FAP media with 0.5μM Verteporfin (Sigma SML0534) solubilized in DMSO or in 0.1% DMSO alone. FAPs were cultured on collagen-coated polyacrylamide gels and collagen gels at a concentration of 175,000 cells/mm2. Cells were fed fresh FAP media every 2-3 days supplemented with Verteporfin or DMSO and fixed with 4% paraformaldehyde after 7 days.

Immunostaining

Cells were fixed with 4% paraformaldehyde for 15 min, washed in 0.1% bovine serum albumin (BSA) (Fisher BP1600 100) in PBS, and blocked with 5% BSA for 30 min. Samples were immunostained with YAP1 in association with the EdU assay, αSMA in association with the myofibroblast activation assay, or αSMA and Vcam1 on substrates with altered stiffnesses. Samples were stained with a primary antibody overnight in 5% BSA followed by a secondary fluorophore and Acti-stain 555 Phalloidin for 90 minutes, and Hoechst 33342 in 0.1% BSA for 15 min. Table 2.1 indicates the primary antibodies used for immunofluorescence.

Table 2.1: Antibodies used for immunostaining.

Antigen	Vendor	Cat #	Dilution	Host Species
αSMA	Fisher	MS113P0	1:800	Mouse
Perilipin	Cell Signaling	9349S	1:200	Rabbit
YAP1	Santa Cruz Biotechnology	sc-101199	1:1000	Mouse
Vcam1	abcam	Ab134047	1:500	Rabbit
EdU	Fisher	A10044	1:1000	
Acti-stain	Fisher	50646254	1:250	
Hoechst	Fisher	H3570	1:2000	

Imaging and Analysis

Samples of immunostained cells were imaged using an inverted Leica DMi8. Images were captured with a dry 20X/0.40 objective using a Leica DFC9000 GTC camera and LAS X software. At least 200 nuclei were imaged per sample. Each set of samples were captured using the same light intensity and exposure time to ensure consistency during image processing and analysis. Images were analyzed using custom macros in FIJI: ImageJ 2.1.051. To determine proliferation rate, the fraction of EdU+ nuclei was determined. The number of individual nuclei was identified, and an intensity threshold was set for the EdU signal to determine the number of EdU+ nuclei. To perform the myofibroblast activation analysis, the area ratio of αSMA to Acti-stain/F-actin was determined as the signals colocalize on myofibroblasts. To determine YAP localization, individual nuclei were identified, and the YAP intensity of each nucleus was measured as the mean gray value. Each nuclear YAP intensity was divided by the average cytoplasmic YAP intensity to give a nuclear to cytoplasmic ratio. Cytoplasmic area was defined as actin positive and DNA negative area.

Collagen gels were visualized using SHG imaging on a Leica TCS SP8 with a multiphoton laser tuned to 866nm, using a 25X water immersion objective. Three independent image stacks per

gel were taken with a depth of 100μm and a slice thickness of 1 μm. SHG images were analyzed in FIJI: ImageJ to get the average collagen intensity under αSMA+ or αSMA- cells.

STATISTICAL ANALYSIS

All statistical analysis was done using GraphPad Prism 9.3.1. One-way ANOVA with multiple comparisons or unpaired t-tests were used where appropriate to analyze differences between collagen gels. Data was normalized to the control, 3.0mg/ml unaligned telocollagen gel polymerized at 37°C. For the polyacrylamide gels, one-way ANOVA with repeated measures using Dunnett's correction and multiple comparisons were used. Correlation graphs were fitted using a nonlinear regression least squares fit, and correlation was done using Pearson Correlation. For SHG image analysis, a two-way ANOVA with multiple comparisons using a Tukey correction was utilized. Significance was set at $p<0.05$. Data was presented as individual values with mean and standard deviation. Outliers were determined using the Grubb's test, no data points were excluded.

SUPPLEMENTAL INFORMATION

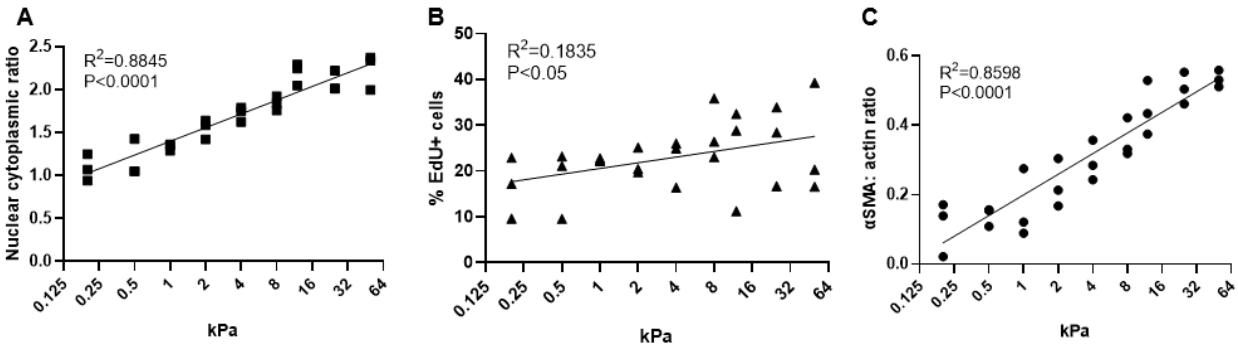

Figure S2.1 Pearson correlation of substrate stiffness with FAP behavior. A-C) Correlation of substrate stiffness with YAP nuclear localization, proliferation, and αSMA expression, respectively. Lines fitted with semi log line (x is log2, y is linear) using least squares fit. N=3 mice and n=3 independent gels.

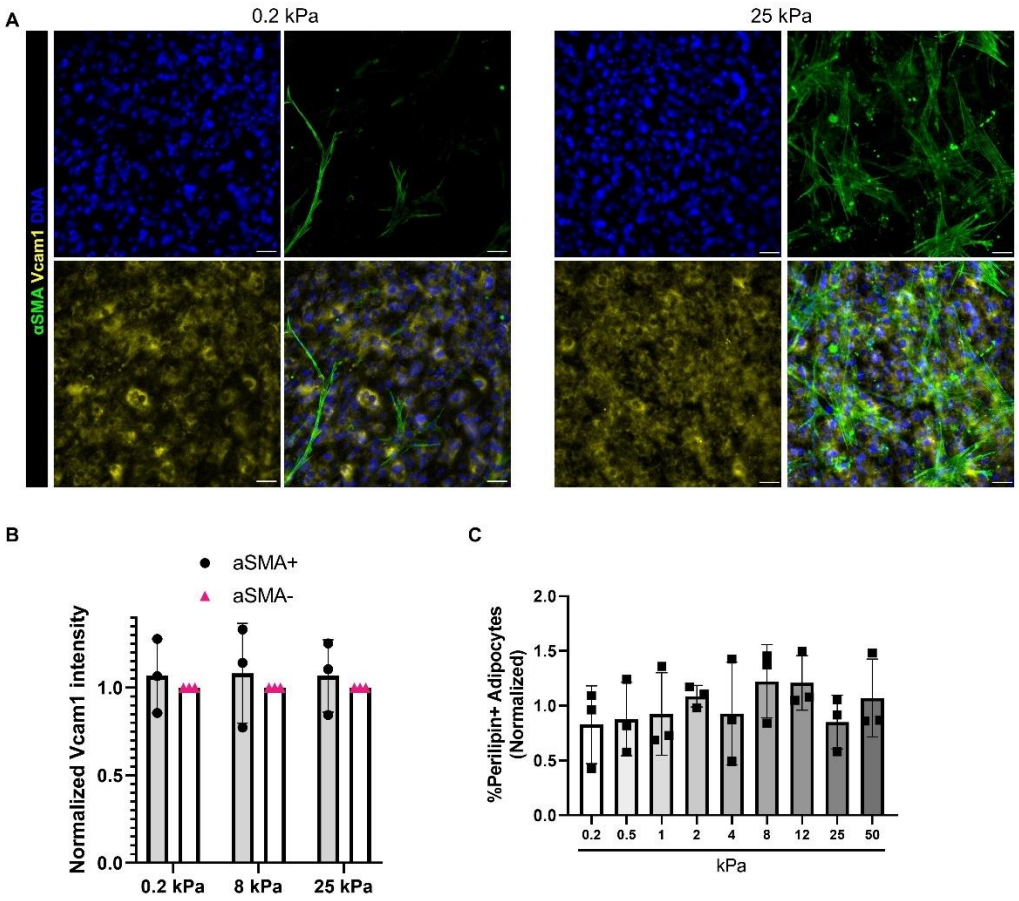

Figure S2.2 Vcam1 Expression in FAPs. A) αSMA and Vcam1 expression in FAPs cultured on 0.2 and 25 kPa collagen-coated polyacrylamide gels. B) Vcam1 intensity in αSMA+ and αSMA- FAPs, normalized to αSMA- FAP intensity. Scales bars set to 50μm. N=1 mouse and n=3 independent gels. C) Number of adipocytes measured by perilipin staining across range of stiffnesses. Data normalized to mean.

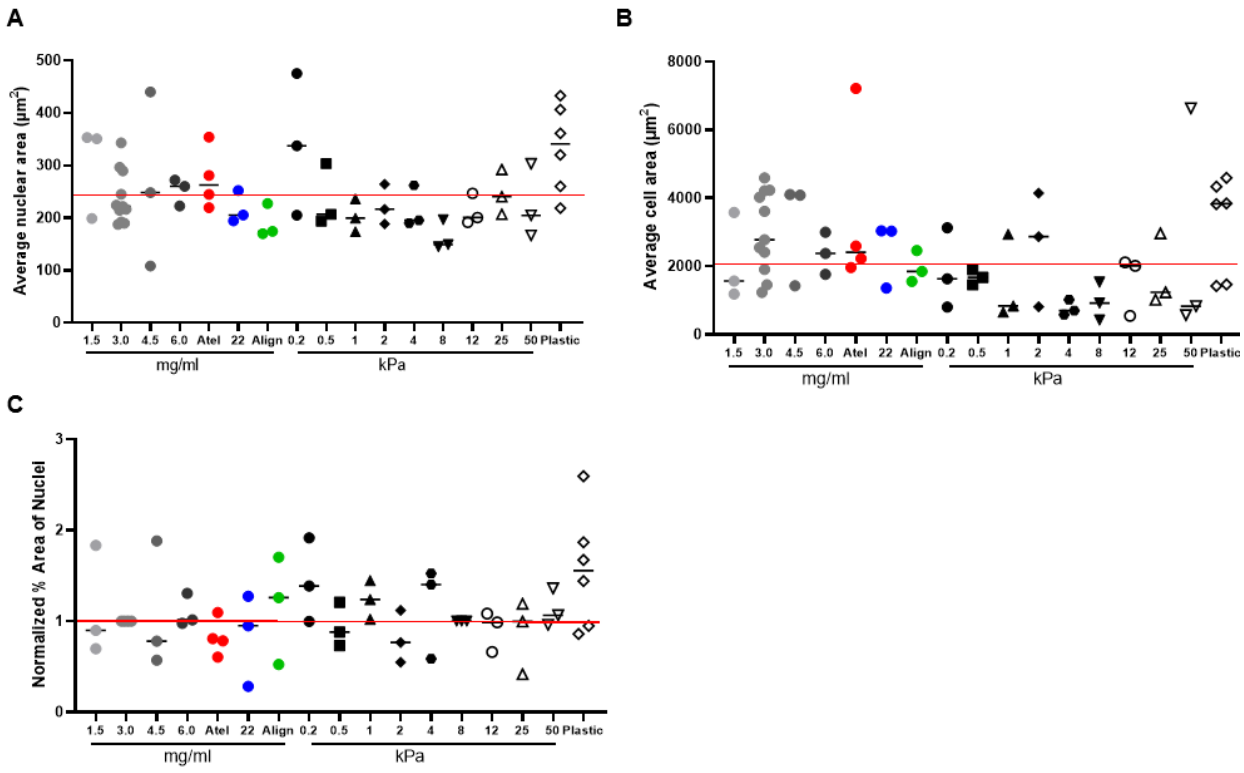

Figure S2.3 Nuclear and Cellular Area. A) Average nuclear area across substrate conditions. B) Average cellular area across substrates. C) Percent nuclear area. Data normalized to experimental control (3mg/ml telocollagen unaligned polymerized at 37°C for collagen gels, 8kPa for polyacrylamide gels). Red line indicates average on graphs. Each data point represents average nuclear area per individual gel. 0.2-50kPa=collagen coated polyacrylamide gels, all other substrates are 3mg/ml telocollagen gel, unless otherwise indicated. Atel=atelocollagen, 22=polymerized at 22°C, Align=aligned with magnetic beads. No significant difference ($p<0.05$) was determined between any of the conditions using a one-way ANOVA with Dunnett's correction.

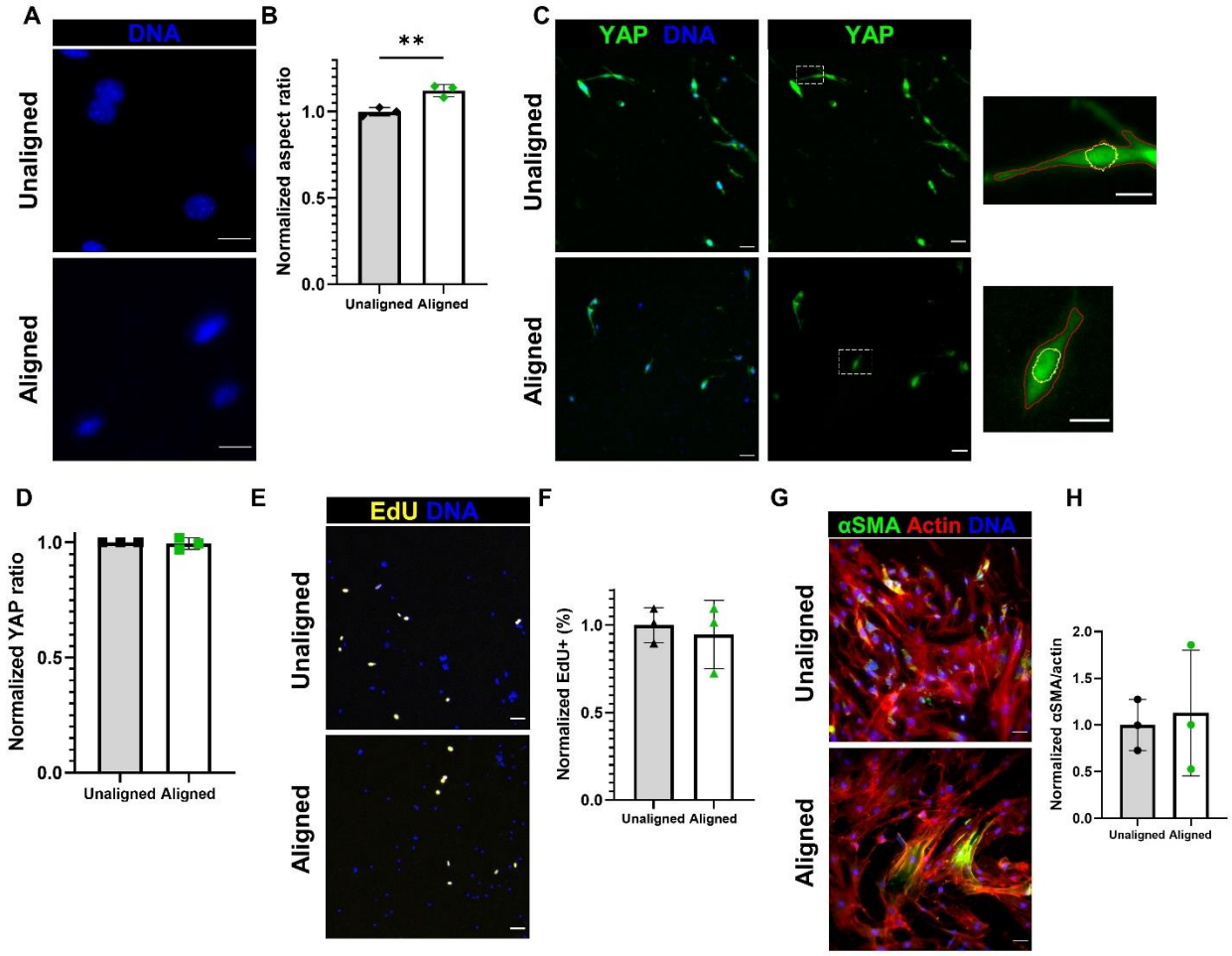

*Figure S2.4 FAPs' response to changes in collagen alignment. A) Alignment of nuclei with 3mg/ml collagen gels polymerized with or without magnetic bead alignment. N=2 mice and n=3 independent gels. B) Quantification of nuclear aspect ratio. C) YAP immunofluorescence of FAPs. Yellow outlines indicate nuclei and red outlines indicate cytoplasm on insets. D) Quantification of YAP signal intensity inside nucleus and cytoplasm. N=3 mice and n=3 independent gels. E-F) EdU signal and quantification of percent proliferating cells after 24 hrs EdU treatment. N=1 mouse and n=3 independent gels. G) αSMA signaling in FAPs. H) Quantification of αSMA and actin area ratio. N=1 mouse and n=3 independent gels. **P< 0.01. Data normalized to unaligned gels. Scales bars set to 50μm, inset scale bars set to 20μm.*

CHAPTER 3: THE EFFECT OF FAP ACTIVATION ON THE ECM AND MYOGENESIS

This chapter is being prepared for submission as:

Loomis, Taryn et al. “Fibrotic Fibro-Adipogenic Progenitors Produce Altered Extracellular Matrix and Impair Myogenesis.”

ABSTRACT

The source of fibrosis in skeletal muscle is fibro-adipogenic progenitors (FAPs) which deposit the majority of the extracellular matrix (ECM). FAPs play a key role in supporting regeneration and myogenesis in healthy muscle through myogenic signaling and ECM deposition, but this becomes disrupted in fibrosis. The goal of this study was to determine the direct effect of fibrotic FAPs on ECM deposition and myogenesis. To do so we utilized cell-derived matrices (CDMs) to synthesize FAP-derived ECM and transwell co-culture systems to interrogate the effect of FAP ECM and soluble factors on myogenesis. We found that fibrotic FAPs, either derived from *mdx* mice, or induced using TGF-β, produced ECM with altered organization than that of healthy FAPs. Verteporfin, a drug that blocks myofibroblast activation, significantly impaired FAPs ability to produce collagen fibers. FAPs on fibrotic-like stiffnesses produced ECM that impaired myogenesis compared to healthy stiffnesses. Additionally, the activation into myofibroblasts, modulated by TGF-β, significantly impaired myogenesis in a transwell co-culture experiment. Taken together, fibrotic FAPs contribute altered ECM and impair regeneration in muscle and fibrotic ECM deposition is attenuated with verteporfin treatment.

INTRODUCTION

Fibrosis is the pathological and excess accumulation of extracellular matrix (ECM) components in a tissue. In skeletal muscle, fibrosis is a consequence of many diseases including Duchenne muscular dystrophy (DMD), which affects 1 in every 5,000 live male births (28,114,162,163). Fibrosis impairs muscle function by limiting contractility, mobility, and disrupting key cell signaling pathways (125,164,165).

The source of the excess accumulation of ECM in skeletal muscle is a non-myogenic stem cell population called fibro-adipogenic progenitors (FAPs). (10,22). FAPs play a key role in promoting regeneration in acute injury by depositing ECM to replace the damaged matrix and releasing of pro-myogenic factors to promote MuSC differentiation (15,125). In fibrosis, these pathways become disrupted. FAPs activate more readily into myofibroblasts, becoming resistant to apoptosis, leading to chronically elevated cell numbers, consistent and excessive ECM deposition, and disrupted signaling to MuSCs. (12,16,22,48,93,166).

MuSCs are sensitive to changes in ECM stiffness and architecture that occur in fibrosis. (50,88,89). Fibrotic-like stiffnesses and fibrotic features of the ECM such as increased collagen cross-linking and fiber size impair myogenesis in MuSCs (88,89). The direct effect of FAP-derived ECM on MuSC differentiation is unknown. FAPs are known to signal to MuSCs through soluble factors, but their influence on myogenesis through ECM deposits has not been investigated.

In this study, we investigated how FAP-based ECM deposition may be influencing myogenesis. We utilized FAP cell-derived matrices (CDMs) which allow for the generation of a three-dimensional ECM mimetic to interrogate protein deposition and cellular response to the ECM. FAPs were isolated from *wt* and *mdx* mice, a model for DMD, to interrogate healthy and fibrotic FAP ECM deposition. FAP activation into myofibroblasts was modulated using verteporfin and TGF- β as well as varying substrate stiffnesses. Verteporfin is a small molecule drug that we have previously shown blocks FAP activation into myofibroblasts (48). TGF-β expression is elevated in DMD and other fibrotic diseases and promotes FAP activation into myofibroblasts (19,80). Therefore, we investigated whether FAP ECM deposition and its effect on MuSCs was altered by the state of FAP activation and if fibrotic ECM deposition could be rescued with a small molecule treatment.

METHODS

Animal Handling

Mice were obtained from Jackson laboratory and housed and bred at a UC Davis animal facility. Animals were given food and water ad libitum with a 12-hour light/dark cycle. DBA/2J (*wt*) and D2.B10-Dmdmdx/J (*mdx*) mice were used for primary culture at ages 8-16 weeks. Euthanasia was performed via CO2 followed by cervical dislocation. All animal experiments were approved by the University of California, Davis Institutional Animal Care and Use Committee, and performed in accordance with relevant guidelines and regulations.

Primary Cell Isolation and Culture

To isolate primary muscle stem cells, the lower limb skeletal muscles of wt and mdx mice were dissected and digested in an enzyme mix (Miltenyi Biotec 130-098-305) in two 30-minute intervals of continuous rotation followed by mechanical digestion via a tissue homogenizer (Miltenyi Biotec 130-093-235). Erythrocytes were removed via a red blood cell lysis buffer (Miltenyi Biotec 130-094-183). Cells were isolated by magnetic activated cell sorting. FAPs were isolated using positive selection with CD140a (PDGFRα) magnetic beads (Miltenyi Biotec 130-101-502). FAPs were plated in FAP growth media (DMEM (Fisher 11-965-092), 20% FBS (Biowest S1620), 10% HS (Fisher SH3007403), 75 ng/ml hFGF (Fisher PHG6015), and 1% Penicillin-Streptomycin (Fisher 15-140-122)). MuSCs were isolated using negative selection with a satellite cell isolation kit (Miltenyi Biotec 130-104-268). MuSCs were plated in myoblast growth media (DMEM, F-10 (Fisher 11550043), 20% FBS, 50 ng/ml hFGF, 1% Penicillin-Streptomycin, 0.5% amphotericin-B (Fisher 15290018), and 0.5% gentamicin (Millipore Sigma G1272-10ML). FAPs and MuSCs were fed every 2-3 days with fresh media and passaged once 80% confluent for 2-3 passages until ready for use.

CDM SYNTHESIS AND SOLUBILIZATION

Plates were treated with 0.2% gelatin solution in Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS+; Fisher 14040133) for 1 hour at 37°C, 1% glutaraldehyde (Sigma G5882-10X10ML) solution in DPBS+ (Fisher 14040133) for 30 minutes at room temperature, and 1 M ethanolamine (Fisher AC149582500) in Di-H2O for 30 minutes at room temperature with three 5-minute washes with DPBS+ between each step.

FAPs were plated on pre-treated plates at a concentration of 2.5e5 cells/mL in CDM media (DMEM, 20% FBS, and 1% Penicillin-Streptomycin). 50µ/mL ascorbic acid (Fisher AC401475000) in DPBS+ was added to the media fresh. The media was replaced fresh every 24 hours for 14 days to allow cells to deposit a matrix.

FAPs were removed from the matrix after 14 days using an extraction buffer of phosphate-buffered saline (PBS-; Fisher 20 012 043), 0.5% (v/v) TritonX-100 (Fisher BP151 500), and 20 mM NH4OH (Fisher AC255210010) for 5 minutes at 37°C. Once cells were lysed, extraction buffer was diluted in two to three times the volume of PBS- and left overnight at 4°C. The next day, the liquid was removed, and the matrix was treated with 10U/mL of DNase I (Fisher FEREN0521) in DPBS+ for 30 minutes at 37°C. CDMs were then washed twice with DPBS+ with 1% Penicillin-Streptomycin, wrapped in parafilm and left at 4°C until ready to use.

For solubilization, after decellularization, CDMs were treated with a solubilization reagent of Di-H_2O, 5 M guanidine-hydrochloride (Sigma SIAL-G4505-100G), and 10mM dithiothreitol (Fisher BP172 5) on ice for 5 minutes. CDMs were manually removed from the dish using a cell scraper. The matrix solubilization mixture was added to a microcentrifuge tube and rotated for 1 hour at 4°C. The solution was then centrifuged for 15 minutes at 12000 g at 4°C. The supernatant was transferred to a 10K dialysis cassette (Fisher 66382) to remove solubilization reagent while maintaining ECM proteins. Dialysis cassette was left in PBS- (Gibco 20-012-043) overnight. Solubilized matrix was plated for 1 hour at 37°C prior cell plating.

MuSC Differentiation Assays

MuSCs were plated on decellularized CDMs, solubilized CDMs, and in the bottom of 0.4μm transwells at a concentration of 5e5 cells/mL. FAPs were plated on the top of the transwell at a concentration of 5e5 cells/mL. For ECM select assay, MuSCs were plated on an ECM Select Array Kit Ultra-36 (Advanced Biomatrix 5170) at a concentration of 2.5e5 cells/mL.

All cells were plated in myoblast growth media for one day and then switched to differentiation media (DMEM, 5% HS, and 1 % Penicillin-Streptomycin) for 5 days. Cells were then fixed with 4% paraformaldehyde (Fisher AA433689M).

TGF-B and Verteporfin Assays

FAPs plated for CDMs were treated with either 5 ng/mL TGF-β (Sigma T7039-2UG) or 0.5μM Verteporfin (Sigma SML0534) every other day during the two-week CDM synthesis. Negative controls were treated with the solubilization agent for the treatments, dimethyl sulfoxide (DMSO; Fisher BP231100). FAPs pretreated with TGF-β or Verteporfin for two weeks in FAP growth prior to plating on transwells. Once in the transwells, FAPs were plated in differentiation media without TGF-β or Verteporfin to exclude any influence of the treatments themselves on the MuSCs.

Immuno- and Picrosirius Red Staining

Fixed cells were permeabilized with 0.1% TritonX-100 for 10 minutes and blocked with 5% bovine serum albumin (BSA; Fisher BP1600 100) for 30 minutes at room temperature. MuSCs were treated with primary antibody myosin heavy chain (MyHC:MF20) (DHSB MF20-c; 1:500) and FAPs were treated with αSMA (Fisher MS113P0; 1:800) in 5% BSA overnight at 4°C. Secondary fluorophore anti-mouse 488 (Fisher A32766; 1:500) and Acti-stain Phalloidin (Fisher 50646254; 1:250) in 0.1% BSA were added for 90 minutes at room temperature followed by Hoechst 33342 (Fisher H3570; 1:2000) for 15 minutes.

For picrosirius red staining, fixed cells were rinsed with distilled water for 5 minutes three times, air dried for 15 minutes, and stained with picrosirius red solution for 1 hour. Samples were then washed with acidified water for 1 minute two times, followed by 100% ethanol washes for 1 minute three times, and then cleared in Citrisolv (Fisher 22 143 975) for 3 minutes.

Imaging and Analysis

Second harmonic generation (SHG) images of the CDMs were taken using a Leica TCS SP8 with a multiphoton laser tuned to 848 nm and a 25X water immersion objective. Fluorescent and picrosirius red images taken using an inverted Leica DMi8, Leica DFC9000 GTC camera, and LAS X software at 20X.

Orientation index was determined using the OrientationJ in FIJI: ImageJ with minimum coherency and minimum energy set to 1% using a 5-pixel window and cubic spine gradient. Collagen fiber size was determined using a custom MATLAB script.

MuSC differentiation and FAP activation were determined by custom FIJI macros. MuSC differentiation was measured as the fraction of nuclei positive for MyHC. FAP activation was measured as an area ratio of αSMA to actin area.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 10.1.0. An unpaired t-test was used to measure differences in CDM architecture. One-way ANOVA with post-hoc analysis of multiple comparisons with Tukey correction was used to analyze differences in MuSC differentiation or CDM architecture. A simple linear regression was performed to investigate correlations between FAP activation and MuSC differentiation. Significance was set at $p<0.05$.

RESULTS

MDX AND WT FAPS PRODUCE ECM WITH DIFFERENT COLLAGEN ARCHITECTURE.

SHG imaging allows for visualization of collagen fibers without labeling. Here, we used SHG to reveal differences in the collagen architecture produced by wt and mdx FAPs. Collagen fibers were slightly less aligned in the CDMs produced by mdx FAPs as compared to the wt CDMs (Fig. 1A, B). Collagen fibers in mdx CDMs were significantly thicker than that of wt CDMs (Fig. 1A, C). Overall, the collagen architecture produced by mdx FAPs was altered than that in wt FAPs.

MDX AND WT FAPS DID NOT AFFECT MUSC DIFFERENTIATION.

Based on the differences in the architecture of the CDMs from wt and mdx FAPs, we investigated if MuSCs responded differently to the CDMs in terms of their differentiation potential. MuSCs may respond to either the CDM architecture or proteins in the CDMs, therefore we examined MuSC differentiation on FAP-derived CDMs and solubilized CDMs, respectively. CDMs were decellularized prior to MuSC culture to remove any cell-cell interactions (Fig. S1A). However, despite differences in CDM architecture there was not a difference in MuSC differentiation between mdx and wt FAPs in any of the conditions (Fig. 1D).

FAP ACTIVATION INTO MYOFIBROBLASTS IMPAIRS MUSC DIFFERENTIATION.

Despite little difference between mdx and wt FAPs on MuSC differentiation, we noticed variability in the activation of FAPs into myofibroblasts between experiments. Therefore, we looked at the correlation of FAP activation and MuSC differentiation. FAPs that were highly activated into myofibroblasts produced CDMs that were less conducive to MuSC differentiation than FAPs that were less activated (Fig. 1E). The level of myofibroblast activation, in terms of αSMA expression, was negatively correlated with MuSC differentiation across conditions (Fig. 1F). This suggests that myofibroblast activation in FAPs impairs myogenesis.

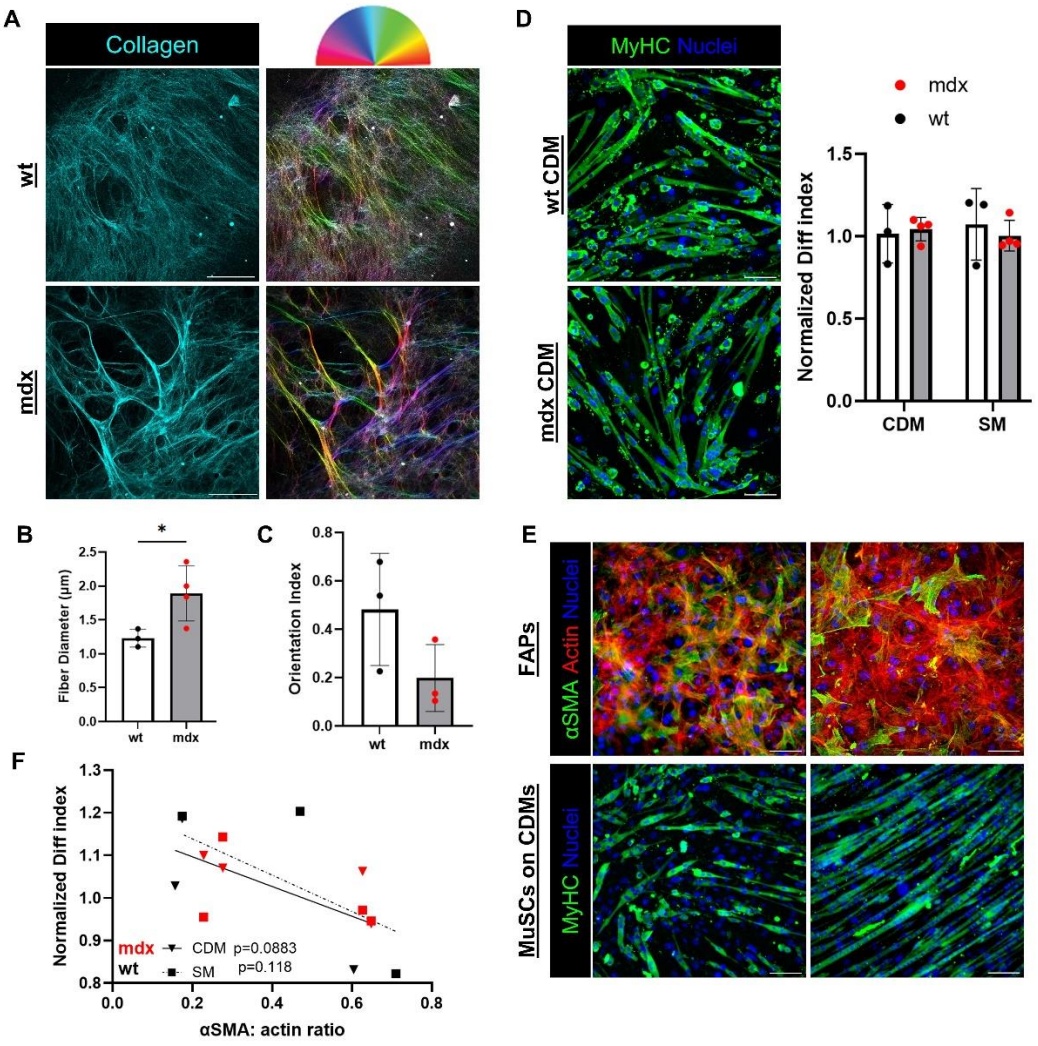

*Figure 3.1: mdx and wt FAPs produce different collagen matrices. A) SHG imaging of decellularized CDMs synthesized from wt and mdx FAPs (top) and orientation analysis using OrientationJ in FIJI (bottom). B) Average collagen fiber diameter based on SHG images. C) Orientation index of wt and mdx CDMs using OrientationJ. D) Representative images of MuSC differentiation, as measured by MyHC expression on either wt or mdx CDMs and quantification of MuSC differentiation on CDMs and soluble matrices (SM) normalized to tissue-cultured treated plastic. E) High (left) and low (right) myofibroblast activation in FAPs and MuSC differentiation on the corresponding CDMs synthesized from these FAPs. F) Correlation of FAP activation and corresponding MuSC differentiation.*p<0.05. Scale bars are 100µm.*

Myofibroblast activation in FAPs alters ECM deposition.

To further interrogate the role of FAP activation into myofibroblasts on ECM deposition, CDMs were synthesized with TGF-β or verteporfin, to induce or block myofibroblast activation,

respectively. Verteporfin works to block myofibroblast activation by inhibiting YAP translocation to the nucleus, which was confirmed by immunofluorescence (Fig. 2A). Verteporfin slightly decreased myofibroblast activation while TGF-β significantly increased the amount of myofibroblast activation in FAPs, compared to DMSO control- treated FAPs (Fig. 2B, C). With treatment of TGF-β, CDMs had altered collagen deposition based on SHG imaging (Fig. 2B, D). Verteporfin, on the other hand, appeared to prevent the formation of collagen fibers with minimal collagen visible with SHG imaging (Fig. 2B, D). Despite changes in overall collagen deposition, there were not changes in collagen organization between conditions (Fig. 2E, F).

MuSC differentiation on CDMs and solubilized matrices was investigated in the context of FAPs treated with TGF-β or verteporfin. CDMs and solubilized matrices from either TGF-β or Verteporfin-treated FAPs did not have a significant impact on MuSC differentiation when normalized to a tissue culture treated plastic control (Fig. 2G). There was a negative correlation between myofibroblast activation and MuSC differentiation (Fig. 2H).

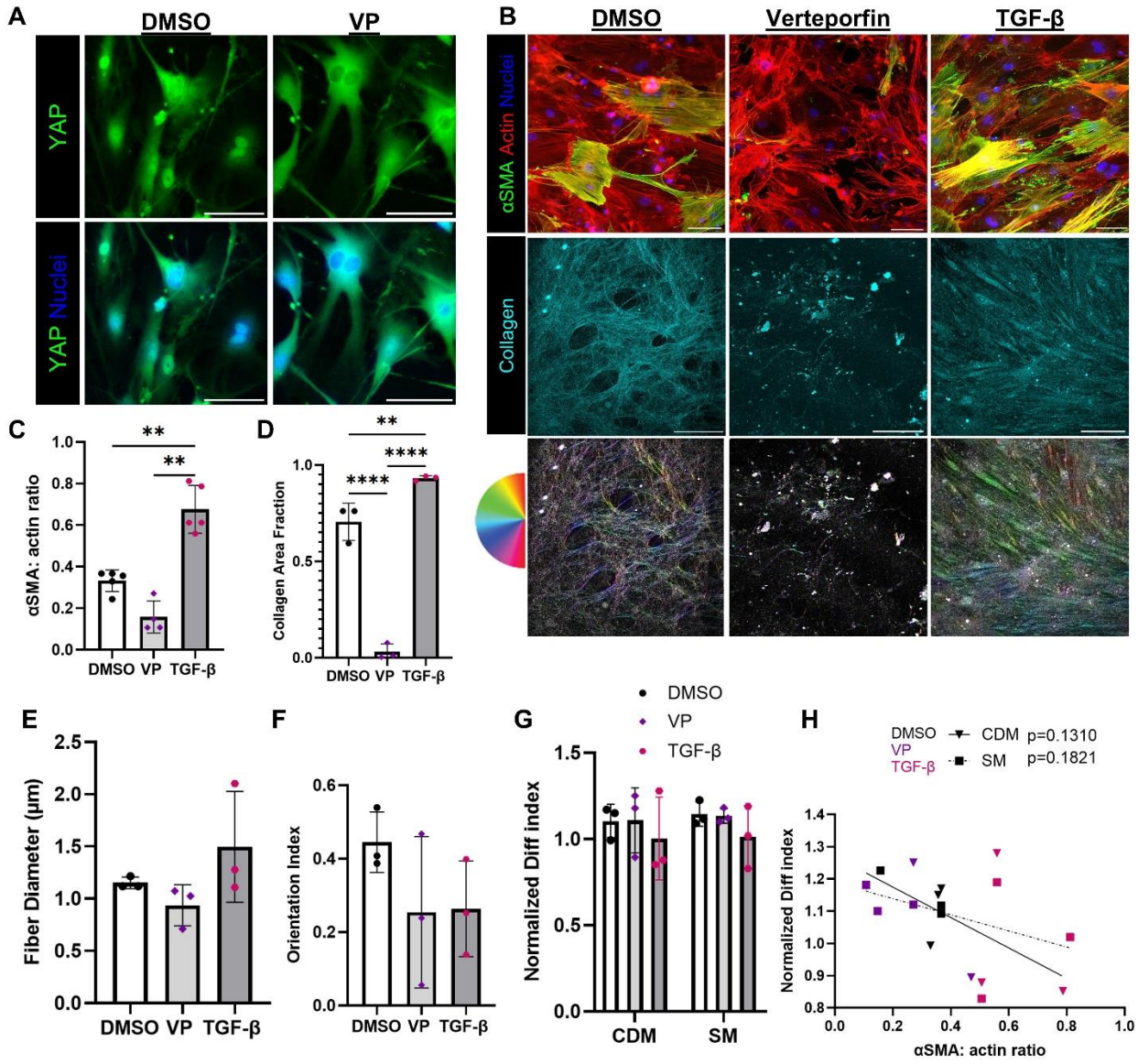

*Figure 3.2 TGF-β FAPs are more highly activated and produce altered collagen. A) YAP nuclear localization of verteporfin treated FAPs. B) Myofibroblast activation in FAPs (top row) and the corresponding CDM imaged using SHG (middle row) along with orientation analysis using OrientiationJ in FIJI (bottom row). C) Quantification of myofibroblast activation in FAPs treated with either 5ng/ml TGF-β or 0.5μM Verteporfin (VP). D) Quantification of total collagen area per image. E) Orientation index quantified using OrientationJ in FIJI. F) Average collagen fiber diameter of SHG CDMs. G) Quantification of MuSC differentiation on CDMs and solubilized matrices (SM) derived from FAPs treated with DMSO, Verteporfin, or TGF-β. H) Correlation of myofibroblast activation and MuSC differentiation. **p<0.01, ***p<0.001, ****p<0.0001. Scale bars are 100μm.*

FAP ECM DEPOSITION IMPAIRS MYOGENESIS ON FIBROTIC-LIKE STIFFNESSES.

Previous research in our lab has indicated that myofibroblast activation in FAPs is strongly correlated with stiffness (11). Given the negative effect myofibroblast activation on ECM deposition, we investigated if MuSC differentiation and CDM deposition would be different in CDMs synthesized across a range of stiffnesses. Collagen-coated polyacrylamide gels at stiffness of 0.2, 8, and 25 kPa were used to represent a soft, healthy, and fibrotic stiffness, as well as a collagen-coated plastic control.

MuSCs on these stiffnesses alone did not have significantly altered differentiation (Fig. 3A,B). However, on CDMs synthesized on these stiffnesses, MuSCs had significantly impaired differentiation on 25 kPa compared to on 25kPa without a CDM or to other stiffnesses (Fig. 3A, B). Interestingly, the impairment of differentiation at 25 kPa did not hold for the significantly stiffer plastic (Fig. 3A, B).

To determine the effect of CDMs derived on 25 kPa on MuSC differentiation, we investigated differences in ECM deposition that may be affecting MuSC differentiation. We conducted Sirius red staining, due to inability to perform SHG imaging on the softwells, and fluorescent fibronectin stain to interrogate CDM architecture. We found no distinct differences in CDM architecture that would explain the differences in differentiation (Fig. 3C).

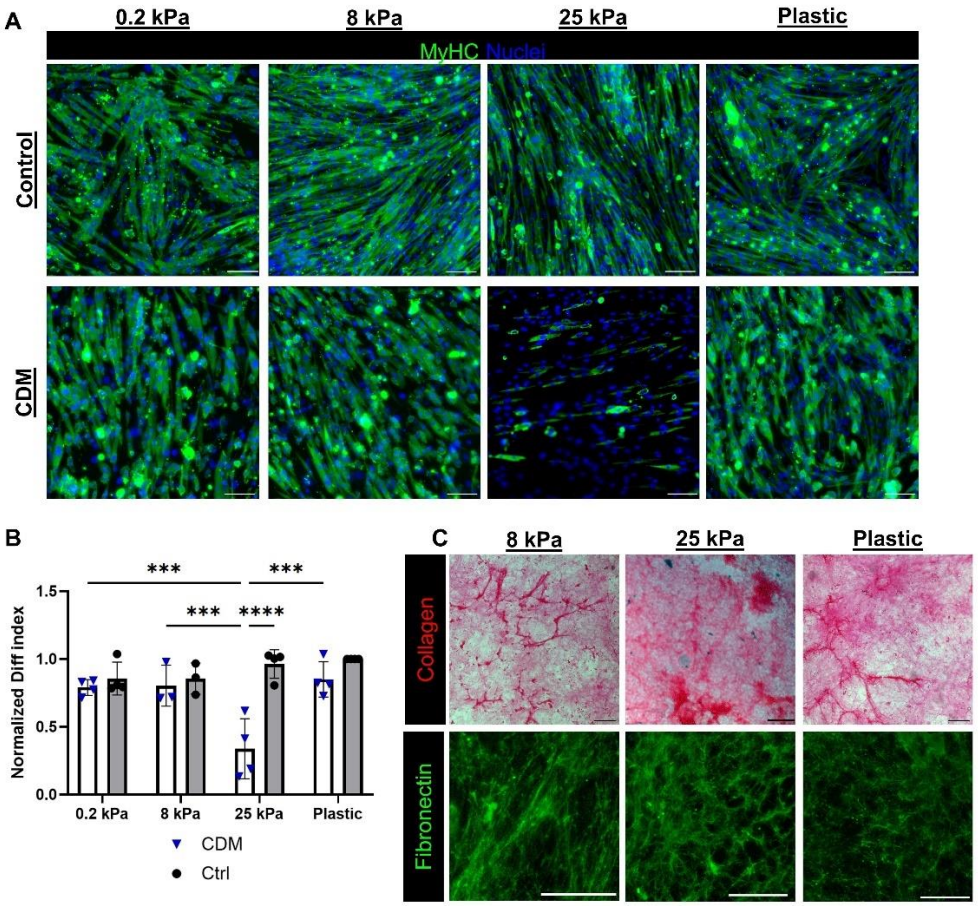

*Figure 3.3 CDMs are altered on different stiffnesses. A) MuSC differentiation on CDMs derived on different stiffness of collagen-coated polyacrylamide gels and on control collagen-coated polyacrylamide gels without CDMs. B) Quantification of MuSC differentiation. C) Sirius Red and IF Fibronectin images of CDMs on 8 kPa, 25 kPa, and plastic. ***p<0.001, ****p<0.0001. Scale bars are 100μm.*

ECM PROTEINS ALTER MUSC ADHESION AND DIFFERENTIATION.

We conducted an ECM screening assay to look at cell attachment and differentiation of MuSCs across a range of various proteins found in the ECM to elucidate potential ECM proteins that were impairing myogenesis. We looked at coatings that included collagen I, as all the softwells were coating with collagen I as a control. Several coatings that included collagen I plus another protein, including collagen V, VI, laminin, and tropoelastin significantly reduced the number of MuSCs that were able to adhere (Fig. 4A, B).

Differentiation index was assessed only on coatings with sufficient number of cells to provide an accurate analysis, therefore, low adhesion coatings were excluded. Collagen IV when paired with collagen I increased differentiation compared to on collagen I alone (Fig. 4A, C). This suggests that these proteins may be lacking on the 25 kPa CDM or are produced in more abundance on the other stiffness to results in a disparity in differentiation. Overall, changes in stiffness appeared to affect FAP ECM deposition which in turn altered myogenesis.

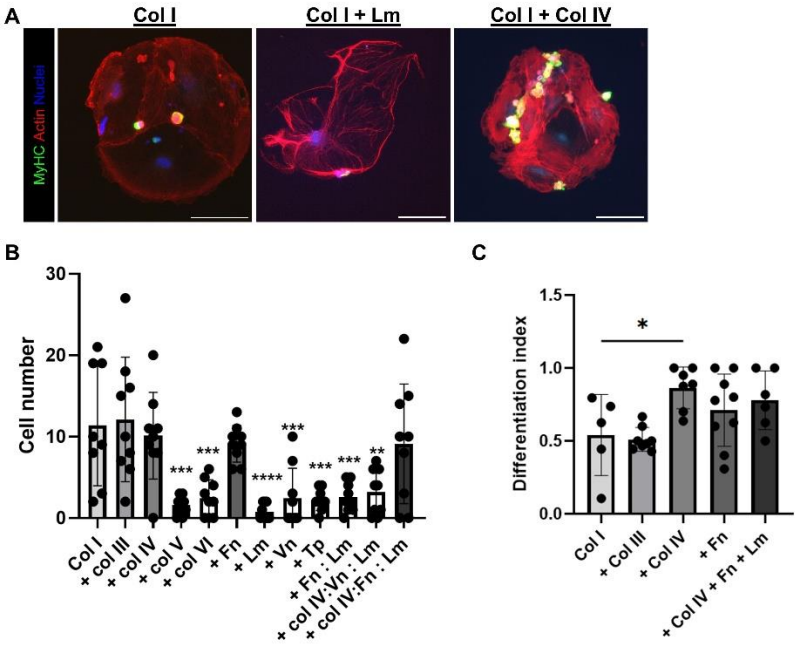

*Figure 3.4 MuSC adhesion and differentiation is affected by ECM proteins. A) Example of MuSC differentiation on ECM select array on three different coatings with collagen I. B) Quantification of MuSC adhesion on coatings with collagen I plus additional coatings. Each point represents number of cells on an individual coating dot. Asterisk represent significantly different from collagen I only coating. C) Quantification of MuSC differentiation on coatings that had at least total 50 nuclei. All coatings include collagen I. **p<0.01, ***p<0.001, ****p<0.0001. Scale bars are 100μm.*

TGF-B ACTIVATED FAPS IMPAIR MYOGENESIS THROUGH SOLUBLE FACTORS.

Given the mixed results of FAP-derived ECM on myogenesis, we investigated other ways FAPs may be signaling to MuSCs. We set up a transwell co-culture assay to determine the effect of soluble signaling on myogenesis. Using either wt or mdx FAPs did not appear to affect

myogenesis (Fig. 5A, B), although there was a slight negative correlation of myofibroblast activation and MuSC differentiation (Fig. 5C). Given the potential effect of myofibroblast activation, we pretreated FAPs with verteporfin or TGF-β prior to transwell co-culture. MuSCs in the transwell with TGF-β- treated FAPs had significantly impaired myogenesis compared to the DMSO control or verteporfin treated FAPs (Fig. 5D, E). There was a significant negative correlation of myofibroblast activation and myogenesis (Fig. 5F). This indicates that myofibroblast activation in FAPs impairs myogenesis primary through soluble factor signaling.

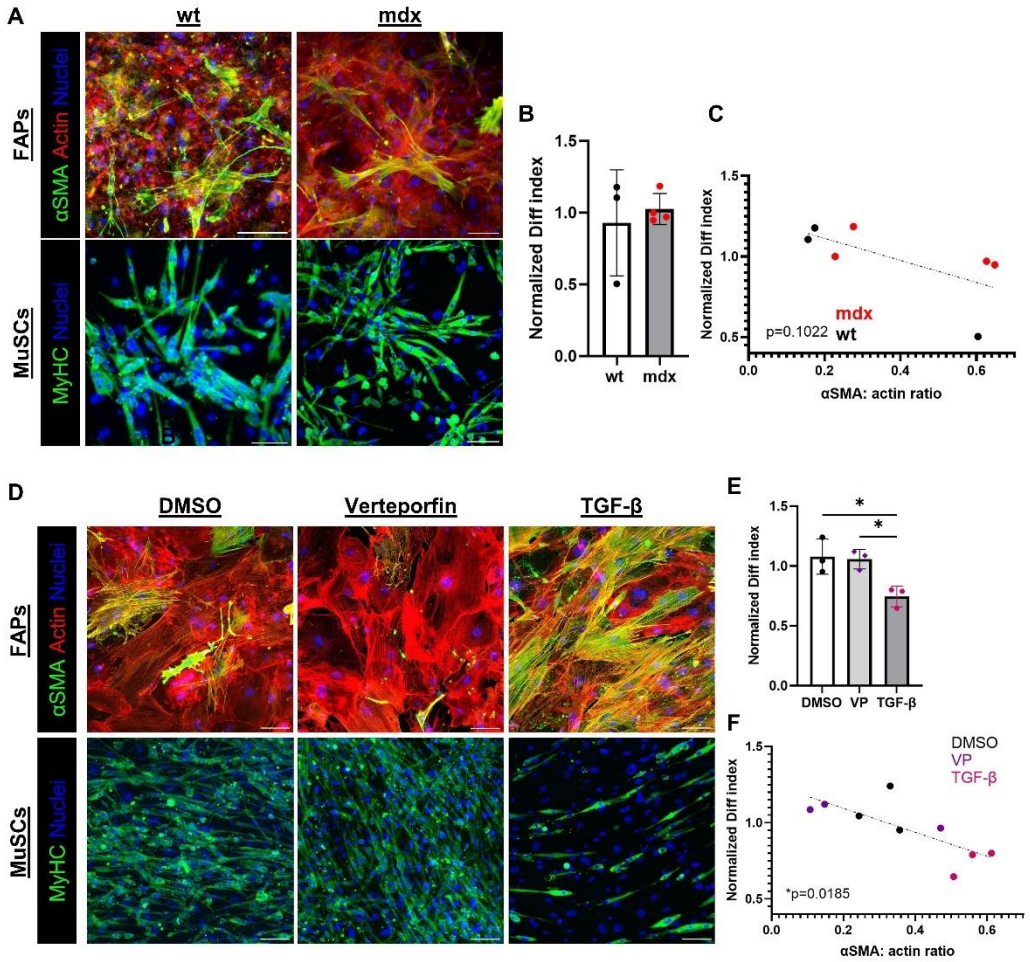

*Figure 3.5 Myofibroblasts produce soluble factors that impair myogenesis. A) FAP activation (top) and corresponding MuSC differentiation (bottom) in transwells from wt or mdx FAPs. B). Quantification of differentiation index in transwells. C) Correlation of myofibroblast activation and MuSC differentiation from wt or mdx FAPs. D) Transwell co-culture of FAPs (top) and MuSCs (bottom) with FAPs pretreated with either DMSO, Verteporfin, or TGF-β. E) Quantification of differentiation index in transwells. F) Correlation of myofibroblast activation and MuSC differentiation from FAPs pretreated with either DMSO, Verteporfin, or TGF-β. *p<0.05. Scale bar 100um. Data normalized to a tissue-cultured treated plastic control.*

DISCUSSION

The goal of this study was to determine the effect of fibrotic FAPs on ECM deposition and MuSC differentiation. MuSCs are mechanosensitive, and therefore, may be influenced by the ECM that FAPs produce in fibrotic and healthy conditions (50,88). To investigate the role of the FAP produced ECM on MuSCs, we synthesized CDMs. CDMs allow for *in vitro* modeling of the 3D microenvironment of the ECM and were used to interrogate ECM architecture and effect of the ECM on MuSCs. To our knowledge, the direct effect of FAP-produced ECM on myogenesis has not been investigated. We found that *mdx* FAPs produced ECM with altered architectural features compared to *wt* FAPs, but these CDMs did not significantly affect MuSC differentiation. FAPs on fibrotic-like stiffnesses produced ECM that impaired myogenesis but was not directly related to substrate stiffness. FAPs release pro-myogenic soluble factors in acute injury, but this gets disrupted in fibrosis (125). Myofibroblast activation of the FAPs appeared to have the most significant effect on MuSC differentiation, with differentiation significantly decreasing in transwells with myofibroblasts. Overall, our data indicate that fibrotic, activated FAPs produce altered ECM and soluble factors that impair muscle cell differentiation.

In fibrosis, the ECM architecture, along with the content, is altered. Collagen fiber diameter may increase, and alignment of fibers are altered (44,139). Here we found this to be the case with *mdx* FAPs, which are isolated from a fibrotic *in vivo* environment. These FAPs produce CDMs with thicker collagen fibers and slightly less aligned fibers, indicating a more fibrotic ECM. However, these CDMs did not have a differential impact on MuSC differentiation, despite previous work in the lab that found MuSC differentiation is impaired on larger collagen fibers (88). This disparity may be attributed to a couple different factors. One, the diameter of fibers in collagen gels is much smaller than those in the CDMs. All CDMs had collagen fibers that were on the upper end of the size seen in collagen gels. Larger fibers are more difficult for cells to remodel, which may affect their regenerative capacity (133). It is possible the fibers in both

CDMs are too big for the MuSCs to easily remodel. Two, collagen gels are not representative of the complex mix of proteins that are in the ECM, while CDMs reconstitute the full protein makeup of the ECM. Other proteins in the CDMs, such as fibronectin, may compensating for the larger collagen fiber size in terms of differentiation response.

Due to the lack of response of the MuSCs to either *wt* or *mdx* FAPs, we investigated further into the fibrotic phenotype of FAPs, myofibroblasts. We found with *wt* and *mdx* FAPs a slight negative correlation between myofibroblast activation and MuSC differentiation, which was not dependent on phenotype. To validate this, we treated FAPs with TGF-β, which is known to induce myofibroblast activation (19), or verteporfin, a small molecule drug that inhibits myofibroblast activation by inhibiting mechanosensing signaling on stiff substrates (48,167). We found that verteporfin removed FAPs' ability to produce collagen fibers, even when having only a moderate effect on myofibroblast activation. Verteporfin, therefore, may act as an anti-fibrotic by blocking collagen fiber synthesis in FAPs. FAPs treated with TGF-β produced clustered collagen fibers that were slightly thicker and appeared more clustered around the cells, indicating myofibroblast activation affects FAP collagen synthesis and organization. This in turn could contribute to fibrotic development through not only increased ECM deposition but contributing to the disorganized architecture observed in fibrosis (44,48).

Previous work in our lab has shown that FAPs are mechanosensitive, with myofibroblast activation increasing with stiffness (48). Therefore, we determined whether increasing stiffness affects CDM deposition and MuSC differentiation. We found a decrease in differentiation on CDMs synthesized on 25 kPa polyacrylamide gels compared to other stiffnesses and to 25 kPa gels without CDMs. Surprisingly, this deficit in differentiation was rescued on plastic, suggesting this disparity is not directly related to stiffness or myofibroblast activation (48). We found that MuSC adherence, confluency, and differentiation are influenced by various ECM proteins suggesting the protein makeup of CDMs derived from FAPs on 25 kPa stiffness is different than

on other stiffnesses. 25 kPa is equivalent to fibrotic skeletal muscle stiffnesses *in vivo,* indicating in fibrotic muscle the increase in stiffness may drive altered ECM expression in the FAPs (7,50,132). The basal lamina, where MuSCs reside, is rich in collagen IV and laminin proteins (168,169). However, laminin significantly reduced MuSC cell number in this study indicating an inability to differentiate. Previous work has indicated that laminin scarring in fibrosis significantly impairs adhesion, remodeling, and regeneration in MuSCs (170). Therefore, it is possible that laminin deposition is dysregulated in FAPs on fibrotic-like stiffnesses producing a disorganized ECM that MuSCs are unable to remodel to differentiate. On the other hand, collagen IV significantly increased MuSC differentiation compared to collagen I alone, indicating a promotion of myogenesis and therefore, may be lacking on the 25 kPa CDMs. This motivates further studies to determine the composition of FAP-derived ECM and the influence of myofibroblast activation and stiffness on ECM deposition.

MuSCs are mechanosensitive and therefore, responsive to mechanical cues as FAPs deposit ECM. However, FAPs and MuSCs do not reside in direct contact in muscle, with FAPs residing the interstitial space and MuSCs residing in the basal lamina (10,171). Therefore, these cells could also be signaling to each other through soluble signaling and myofibroblast activation in FAPs affects this signaling. We found that FAPs pre-treated with TGF-β significantly impaired myogenesis in a transwell co-culture experiment meaning that FAPs activated into myofibroblasts *via* the TGF-β pathway release soluble factors that significantly impair myogenesis. TGF-β expression and myofibroblast activation are increased in skeletal muscle fibrosis indicating FAP activation affects ECM accumulation and impaired regeneration seen in fibrosis (18,80,125,172).

CDMs offer a way to investigate the 3D architecture and cellular response to ECM deposition *in vitro* (173). However, there are limitations including the thickness of the CDMs may interfere with mechanosensing of the cells. The CDMs are only ~15μM thick so the MuSCs seeded on

top may be able to sense the underlying plastic stiffness interfering with the direct response to the CDMs. This is one possible explanation as to why we do not see an effect of the FAP-derived CDMs on MuSC differentiation when synthesized on plastic but see a difference in softwells where MuSCs are differentiated across a range of stiffnesses. While more research is required to fully elucidate the composition and mechanics of FAP-derived CDMs, this study elucidates the role of FAP activation on ECM deposition and myogenesis, which can be used to identify therapeutic targets to treat fibrosis and improve muscle regeneration.

CONCLUSION

Overall, this work indicates that fibrotic FAPs produce altered extracellular matrix compared to non-activated FAPs in terms of collagen fiber synthesis and collagen fiber diameter. The activation of FAPs into myofibroblasts significantly impairs myogenesis, primarily through soluble factor release. Therefore, targeting myofibroblast activation is a potential method to reduce fibrosis and improve regeneration in skeletal muscle.

SUPPLEMENTAL INFORMATION

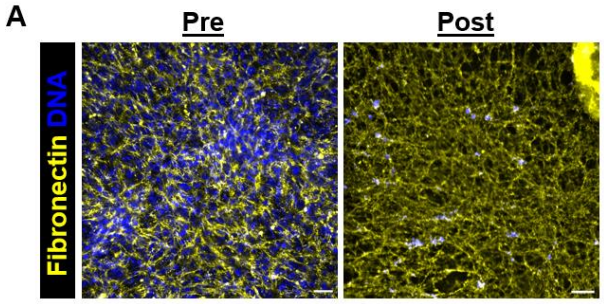

Figure S3.1 Verification of decellularization protocol. A) Immunofluorescence of CDMs pre and post decellularization. Scale bars are 100μm.

CHAPTER 4: HUMAN STEM CELL MECHANOSENSING IN CEREBRAL PALSY

This chapter was provisionally accepted for publication as:

Loomis, Taryn, et al. "Muscle Satellite Cells and Fibro-Adipogenic Progenitors from Muscle Contractures of Non-Ambulatory Children with Spastic Cerebral Palsy Have Impaired Regenerative Capacity." **Developmental Medicine and Child Neurology**. (2024)

ABSTRACT

Aim: To evaluate the mechanosensitivity of muscle satellite cells (MuSCs) and fibro-adipogenic progenitors (FAPs) in cerebral palsy (CP) and the efficacy of the drug verteporfin in restoring cells' regenerative capacity.

Method: Muscle biopsies were collected from six children with CP and six typically developing (TD) children. MuSCs and FAPs were isolated and plated on collagen-coated polyacrylamide gels at 0.2, 8, and 25 kPa stiffnesses. Cells were treated with verteporfin to block mechanosensing or with dimethyl sulfoxide (DMSO) as a negative control. MuSC differentiation and FAP activation into myofibroblasts were measured via immunofluorescent staining.

Results: Surprisingly, MuSC differentiation was not affected by stiffness, but stiff substrates resulted in large myonuclear clustering. Across all stiffness, CP MuSCs had less differentiation than their TD counterparts. FAP activation into myofibroblasts was significantly higher in CP than TD but was not affected by stiffness. Verteporfin did not affect differentiation or activation in either cell population, but slightly decreased myonuclear clustering on stiff substrates.

Interpretation: CP cells appeared less regenerative and more fibrotic compared to their TD counterparts, with MuSCs having a sensitivity to increases in stiffness. Mechanosensitivity of MuSCs and FAPs, therefore, may represent a novel target to improve differentiation and activation in CP muscle.

INTRODUCTION

Cerebral Palsy (CP) describes a group of permanent disorders of the development of movement and posture that are attributed to non-progressive disturbances that occurred in the fetal or infant brain (1). Disturbances to the motor neuron impairs signaling to skeletal muscle, resulting in pathological development (2). Muscles in CP are often spastic and form contractures, limiting range of motion and mobility (2). A defining characteristic of contractures is an excessively high stiffness (2,3). While the influence of substrate stiffness on stem cell migration, proliferation, and differentiation has been reported previously (4,5), the impact of high stiffness on the stem cells in the context of contractures is unknown. Stem cells are responsible for creating muscle tissue structures during development. Understanding the effect of stiffness on their development provides insights into the development of contractures.

Muscle satellite cells (MuSCs) and fibro-adipogenic progenitors (FAPs) are two key cell populations that work together to form muscle during development and regeneration and maintain homeostatic muscle mass (6,7). MuSCs are the cells that differentiate into muscle fibers. They activate after injury and during muscle development to form muscle fibers, which combine together to form whole muscles (7,8). FAPs are cells that reside in the muscle, but do not directly form muscle. Rather, they deposit extracellular matrix (ECM) to provide structure and support to the muscle. FAPs also signal to the MuSCs to promote muscle formation (6,9,10). In diseased conditions, such as CP, the signaling to these cells is disrupted leading to pathological development. The stem cell pool is depleted, and cells have lower myogenic capacity in contractured CP muscle compared TD MuSC population (11–13). MuSCs from early development of contractures in CP show enhanced fusion index, indicating an overall alteration of MuSC phenotype in CP (14). In diseased conditions, FAPs become chronically activated into myofibroblasts, leading to excessive ECM deposition known as fibrosis (9,15,16). FAP

population levels have been shown to be similar in CP and TD, however, little is known regarding the activation of FAPs in contractures of children with CP (17).

Previous reports established that MuSCs and FAPs isolated from mice are mechanosensitive (15,18–20). MuSCs differentiate optimally on stiffness mimicking healthy muscle and FAPs activate into myofibroblast more frequently on stiff substrates (15,20). Given that excessive stiffness is a defining feature of fixed muscle contractures, the mechanosensitivity of stem cells in the stiff environment have profound implications for muscle development. The increase in stiffness seen in fibrotic conditions impairs murine MuSC differentiation and increases FAP activation into myofibroblasts (15,18). Yet, the mechanosensitivity of these cells in the context of muscle contractures in humans is not known.

If mechanosensitivity plays a key role in impairing muscle development in contracture, then blocking mechanosensing could be a viable therapeutic target. Verteporfin is an FDA-approved drug used in photodynamic therapy that effectively blocks cellular mechanosensing. Verteporfin works by preventing Yes-associated protein 1 (YAP1) from translocating to the nucleus. When a cell senses a stiff substrate, YAP translocates to the nucleus to induce transcriptional changes including increasing myofibroblast activation in fibroblast cells like FAPs and activating MuSCs (21–23). Verteporfin reduces myofibroblast activation in murine FAPs and other fibroblast-like cells on fibrotic-like stiffnesses, although its use in CP has yet to be investigated (15,21). To address this knowledge gap, we assessed the mechanosensitivity of CP and TD stem cells and the ability of verteporfin to rescue CP cells.

METHODS

MUSCLE COLLECTION

Ethical approval for this study was obtained through Shriners Children's and the WCG IRB (www.wcgirb.com). Samples were coded before receipt and given an identifier to protect patient identities.

Consent was obtained from parents and age-appropriate assent was obtained from the participants. Participants were children with CP undergoing hamstring or hip adductor longus lengthening surgeries (Table 4.1). Typically developing (TD) children, children with no known neurological disorders, undergoing elective Anterior Cruciate Ligament (ACL) reconstruction with hamstring tendon autograft were recruited as controls, providing access to distal gracilis muscle tissue that was routinely discarded during the surgery. Biopsies (200-400 mg) were collected from the surgical sites and stored in phosphate buffered saline (PBS) on ice until processing.

*Table 4.1: Patient Demographics including muscle biopsy was collected from, age, race/ethnicity, and sex of patient, and Gross Motor Function Classification System (GMFCS) for patients with CP. *p<0.05 for average age.*

Group	Muscle Collected	Age	Race/Ethnicity	Sex	GMFCS	Type	Average Age +/- SD*
TD	Gracilis	16	White	M	-	N/A	14 years and 6 months +/- 1 year and 3 months
TD	Gracilis	15	Other/Hispanic	F	-		
TD	Gracilis	12	White	F	-		
TD	Gracilis	15	White	F	-		
TD	Gracilis	14	White/Hispanic	M	-		
TD	Gracilis	15	White/Hispanic	F	-		
CP	Adductor Longus	4	White/Hispanic	M	V	Spastic quadriplegic	8 years and 6 months +/- 4 years and 2 months
CP	Adductor Longus	4	White/Hispanic	M	V		
CP	Gracilis	8	Asian	M	V		
CP	Gracilis	8	White	F	IV		
CP	Gracilis	11	White	F	IV		
CP	Gracilis	16	White	M	IV		

TISSUE DIGESTION

Biopsy tissue was digested using an enzyme mix (Miltenyi Biotec) at 37 °C for 1 hour under continuous rotation. Samples were mechanically digested using a tissue homogenizer for 30 seconds every 30 minutes. The digested solution was processed through a 70 µm filter, and hemolysis was induced using a red blood cell lysis solution (Miltenyi Biotec). Cell debris was removed using a Debris Removal Solution (Miltenyi Biotec). Cells were frozen in 900 µL of fetal bovine serum (FBS) with 100 µL of dimethyl sulfoxide (DMSO) in liquid nitrogen until time for cell isolation.

Cell Isolation and Culture

Individual cell types were isolated using magnetic-activated cell sorting (MACS). Cell solutions was incubated with a blocking reagent and then cells were labeled with CD140a (PDGFRα) magnetic beads (Miltenyi Biotec) to label the FAPs. The labeled cell solution was added to a MACS Column and Separator (Miltenyi Biotec), and FAPs were isolated via positive selection. The remaining cells were labeled with a Satellite Cell Isolation Kit (Miltenyi Biotec) and added to the MACS Column and Separator. MuSCs were collected via negative selection through the column. FAPs and MuSCs were then plated on tissue culture plastic in FAP media (F-10 (Fisher 11550043), 20% FBS (Biowest S1620), 1% Penicillin/Streptomycin (Fisher 15-140-122), 10 ng/mL fibroblast growth factor 2 (FGF2) (Fisher PHG6015), and 0.2 µg/mL dexamethasone (Fisher 1126100)) or Myoblast Growth media (DMEM (Fisher 11-965-092), 15% Medium 199 (Fisher 11150067), 20% FBS, 1% Penicillin/Streptomycin, 25 µg/mL fetuin (Fisher 3415061GM), 5 ng/mL human epidermal growth factor (Fisher PHG0311), 0.5 ng/mL FGF2, 5 µg/mL insulin (Fisher MP219390025), and 0.2 µg/mL dexamethasone), respectively. FAPs were cultured in either Adipogenic (DMEM, 10% FBS, 1% Penicillin/Streptomycin, 0.25 µM dexamethasone, 0.5 mM IBMX (VWR 102516-252), 5 µM troglitazone (Fisher 501150786), and 1 µg/mL insulin) or Fibrogenic Media (FAP media, 5 ng/mL TGF-β (Sigma T7039-2UG) to validate FAPs' multipotency. Cells were fed fresh media every 2-3 days while undergoing proliferation and passaged when 60-80% confluent for 2-3 passages before experiments.

Mechanosensitivity and Verteporfin Assay

Polyacrylamide gels were obtained from Matrigen as stiffnesses of 0.2, 8, and 25 kPa, to represent physiologically relevant stiffnesses. Gels and tissue cultured plastic (~10,000 kPa) were coated with a 0.1% collagen-I (Advanced Biomatrix 5226) solution for 1 hour at 37 °C prior to seeding. Cells were plated at a concentration of 1.5e5 cells/cm2. MuSCs were switched to

serum-free Differentiation media (DMEM, 50 µg/mL gentamicin (Fisher 15710064), and 10 µg/mL insulin). All cells were fixed on day 5 with 4% paraformaldehyde (Fisher AA433689M).

For the verteporfin assay, cells were treated with verteporfin (Sigma SML0634) at a concentration of 0.5 µM solubilized in DMSO or DMSO alone as a control. Media with verteporfin or DMSO was replaced every other day and cells were fixed in 4% paraformaldehyde on day 5.

Immunostaining

After fixation, cells were permeabilized with Triton-X (Fisher BP151 500) for 10 minutes, washed with 0.1% bovine serum albumin (BSA) for 5 minutes, and blocked with 5% BSA for 30 minutes. Primary antibodies were added to the cells in 5% BSA and left overnight. FAPs were stained with alpha smooth muscle actin (αSMA) primary antibody (Fisher MS113P1; 1:800 dilution) and perilipin (Cell Signaling 9349S; 1:200 dilution), and MuSCs were stained with myosin heavy chain (MyHC: Fisher 14-6503-82; 1:500 dilution) primary antibody. Cells treated with verteporfin were stained with a YAP1 primary antibody (Cell Signaling 14074; 1:1000 dilution). After two washes with 0.1% BSA and a secondary fluorophore and Acti-stain 555 Phalloidin (Fisher 50646254; 1:250 dilution) were added in 0.1% BSA for 90 minutes. Hoechst 33342 (Fisher H3570; 1:2000 dilution) in 0.1% BSA was added for 15 minutes, and cells were washed in 0.1% BSA twice and kept in PBS at 4°C.

Imaging and Image Analysis

Cells were imaged using an inverted Leica DMi8 microscope at 20X dry objective. Images were captured using a Leica DFC9000 GTC camera and LAS X software. Images were analyzed using custom macros in FIJI: ImageJ to determine FAP activation and MuSC differentiation. FAP activation into myofibroblasts was determined by the area of αSMA expression. Differentiation index was determined as the fraction of nuclei within a MyHC+ myotube.

Myotube Analyzer in MATLAB was used to assess myonuclei clustering. A nuclear cluster was defined as four or more myonuclei that were all within 4 μm of each other (24).

Statistical Analysis

Statistical analysis was completed using GraphPad Prism 10.1.2 for Windows, GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com. A two-way ANOVA with followed by a Tukey method for post-hoc pairwise comparisons was used to analyze the differences between stiffness and condition (CP vs. TD). A three-way ANOVA followed by a Tukey method for post-how pairwise comparisons was used to analyze differences between stiffness, condition, and treatment (VP vs. DMSO). Normality was tested using a Shapiro-wilk test for all ANOVAs and was confirmed to be normal. Homoscedasticity was tested using linear regression and visual analysis of plots. MuSC differentiation and myonuclear clustering data were homoscedastic. FAP differentiation data required a Box-Cox transformation to obtain homoscedasticity. A p value of <0.05 was set as significant.

RESULTS

MuSCs from CP Have Impaired Differentiation.

MuSCs differentiate best on stiffnesses that are physiologically relevant to healthy skeletal muscle (20). Therefore, we assessed if MuSCs differentiate better on substrates possessing a physiologically relevant stiffness of 8 kPa and whether MuSCs cultured on substrates with a fibrotic stiffness of 25 kPa exhibit impaired differentiation. We did not detect any significant effect of stiffness on differentiation index (Fig. 1A, B). Across stiffnesses, CP MuSCs had significantly lower differentiation than TD (Fig. 1B), in agreement with prior work. Overall, MuSCs from CP had impaired differentiation and less readily formed myotubes.

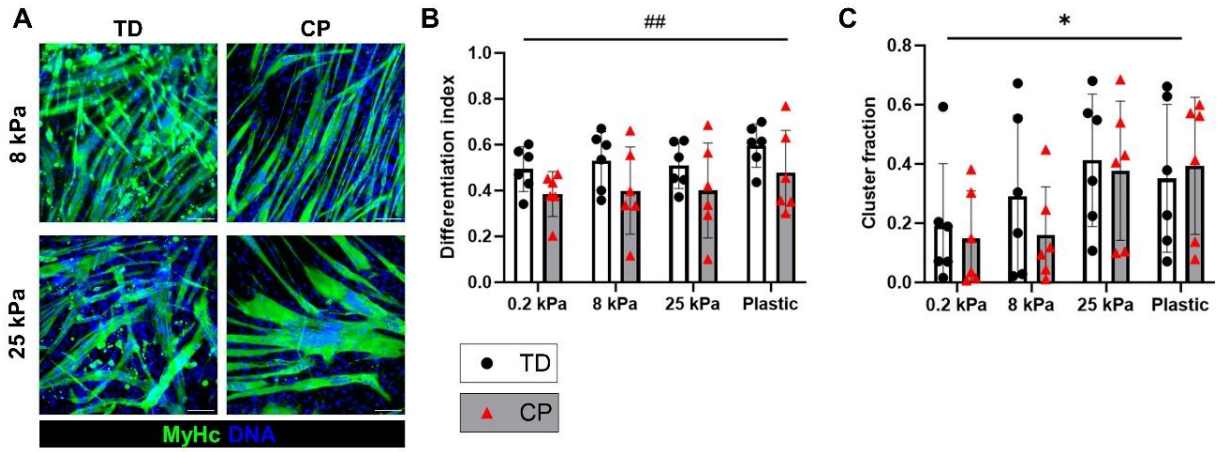

*Figure 4.1: MuSC differentiation is lower in CP and myonuclear clustering increases on stiffer substrates. (A) MuSC differentiation on 8 and 25 kPa collagen-coated polyacrylamide gels demonstrated by myosin heavy chain (MyHC) staining (MyHC in green, nuclei in blue). (B) Quantification of percent of nuclei within myotubes. (C) Quantification of number of myonuclei with clusters. *p<0.05 main effect of stiffness in a two-way ANOVA, ## p<0.01 main effect between CP and TD cells on substrates of different stiffnesses. Scale bars are 100 μm. Data are mean ± SD (n=6).*

Increased Stiffness Caused Myonuclei Clustering.

While the differentiation index of MuSCs was not sensitive to changes in stiffness, we observed a noticeable change in the morphology of the myotubes as stiffness increased, most notably in the CP cells. CP MuSCs formed thicker myotubes with more branching and high nuclear clustering on stiff substrates of 25 kPa and tissue culture plastic (Fig. 1A, C). TD MuSCs also exhibited increased nuclear clustering on stiffer substrates but less degree of branching and thickening of the myotubes compared to CP MuSCs. The cluster fraction increased with increasing stiffness (Fig. 1C) for both cell populations. MuSCs appeared sensitive to fibrotic stiffnesses, which altered their differentiation into myotubes.

FAP Activation into Myofibroblasts Was Higher in CP Cells.

Myofibroblast activation increases on stiffer substrates. Therefore, we assessed if FAPs more readily activate into myofibroblasts on fibrotic-like stiffnesses and whether myofibroblast activation is higher in CP. The multipotency of FAPs was confirmed by culturing FAPs in both

adipogenic and fibrogenic media to assess adipogenic and myofibroblast activation (Fig. 2A). TD FAPs exhibited relatively low αSMA expression across stiffnesses, with an increase in expression on stiffer substrates (Figs. 2B-C; S3B). CP FAPs more readily activated into myofibroblasts, with αSMA expression being five to ten times higher than that of TD FAPs (Fig. 2C). Myofibroblasts were more spread than non-activated FAPs, although cell area was not affected by stiffness (Fig. S4.1A-C). CP FAPs appeared less sensitive to changes in stiffness but were more primed overall to activate into a fibrotic state than TD FAPs.

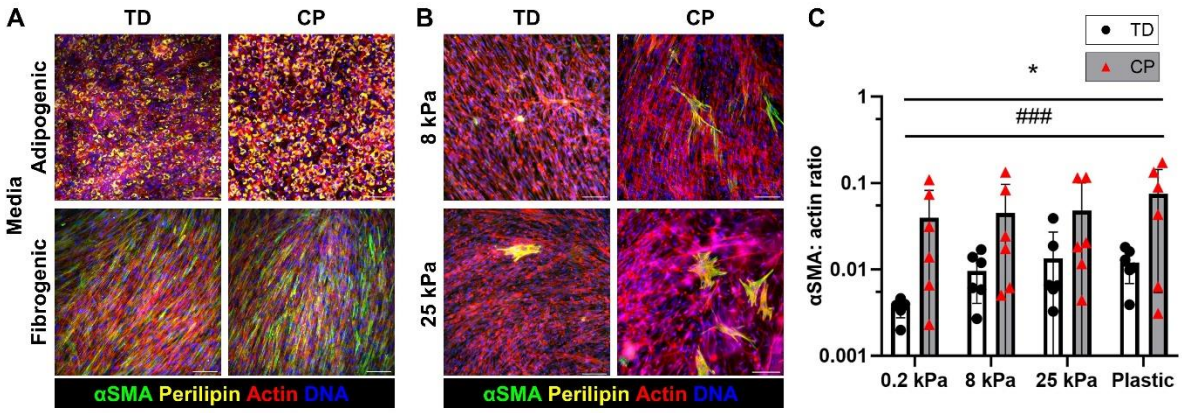

Figure 4.2: FAP activation into myofibroblasts is higher in CP cells. (A) Expression of perilipin and αSMA in FAPs after 5 days in adipogenic or fibrogenic media on tissue-cultured plastic. (B) Myofibroblast activation in FAPs on 8 and 25 kPa collagen-coated polyacrylamide gels. (C) Quantification of myofibroblast activation. Data are mean ± SD (n=6). ### p<0.001 main effect between CP and TD in a two-way ANOVA. Scale bars are 100 μm.

YAP Nuclear Localization Is Lower in CP Cells and May Affect Myonuclear Clustering.

Due to the increased clustering seen in myotubes on stiffer substrates, we interrogated the effect of blocking cellular mechanosensing as a potential method to restore the regenerative capacity of CP cells. FAPs and MuSCs were treated with 0.5 μM verteporfin to reduce myofibroblast activation and nuclear clustering. Both MuSCs and FAPs had overall lower YAP nuclear localization in CP compared to TD (Figs. 3A, 4A). On stiff substrates, verteporfin did not

affect MuSC differentiation but did reduce YAP nuclear localization and slightly reduced nuclear clustering, although not significantly (Fig.3A-D, S2B).

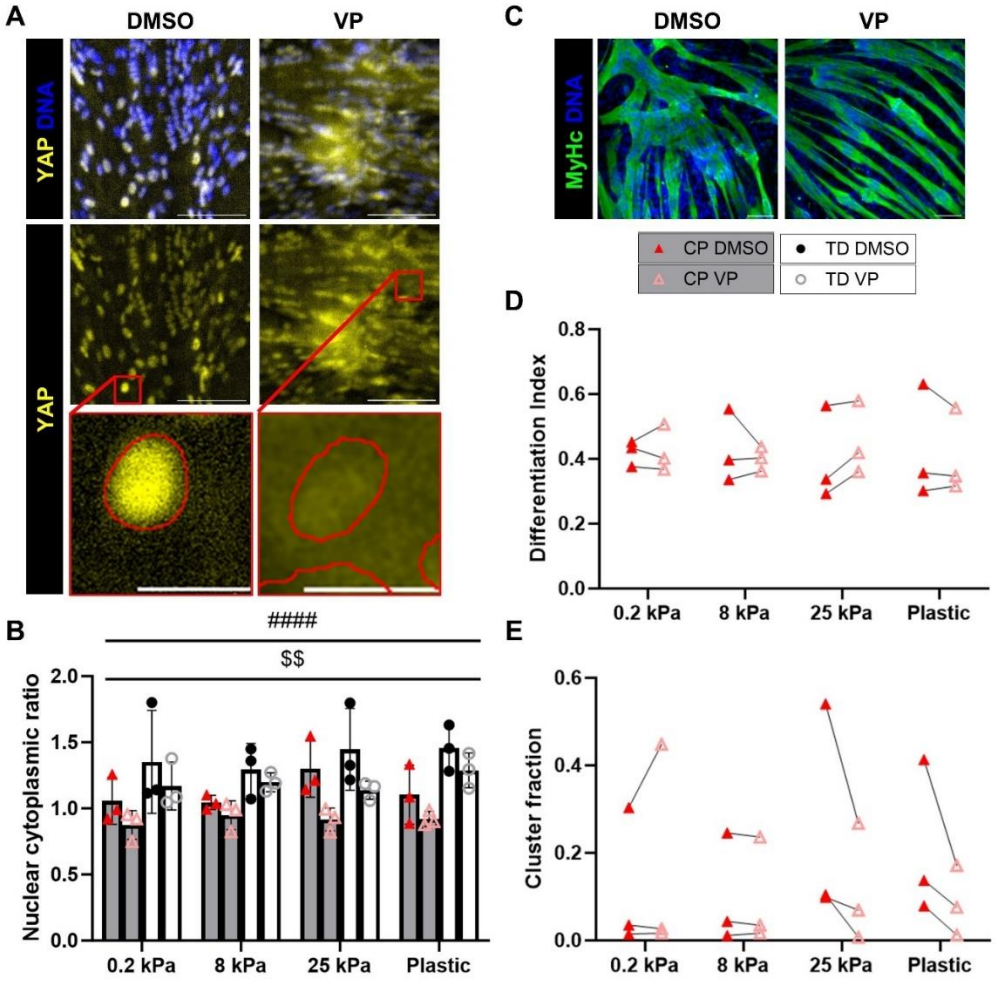

Figure 4.3: Verteporfin treatment in MuSCs. (A) YAP nuclear expression in DMSO control or verteporfin treatment on 25 kPa stiffness in CP MuSCs. Insets show individual nuclei expression, red outlines highlight nuclei. (B) Quantification of YAP nuclear cytoplasmic ratio. (C) CP MuSC myotubes at 25 kPa. (D) CP MuSC differentiation index with verteporfin treatment. (E) CP MuSC myonuclear clustering after verteporfin treatment, p=0.112 for treatment. Data are mean ± SD (n=3). #### p<0.0001 main effect between CP and TD. $$p<0.01 main effect between DMSO and VP treatment in a 3-way ANOVA. Scale bars are 100 μm; scale bars for inset images are 20 μm.

YAP nuclear localization in FAPs was sensitive to changes in stiffness, with higher nuclear localization on stiffer substrates. TD FAPs were more mechanosensitive than CP FAPs, with a

more significant increase in YAP nuclear localization with increasing stiffness (Fig. 4A, B). CP FAPs appeared resistant to verteporfin with little change in YAP nuclear localization or myofibroblast activation (Fig. 4A, C).

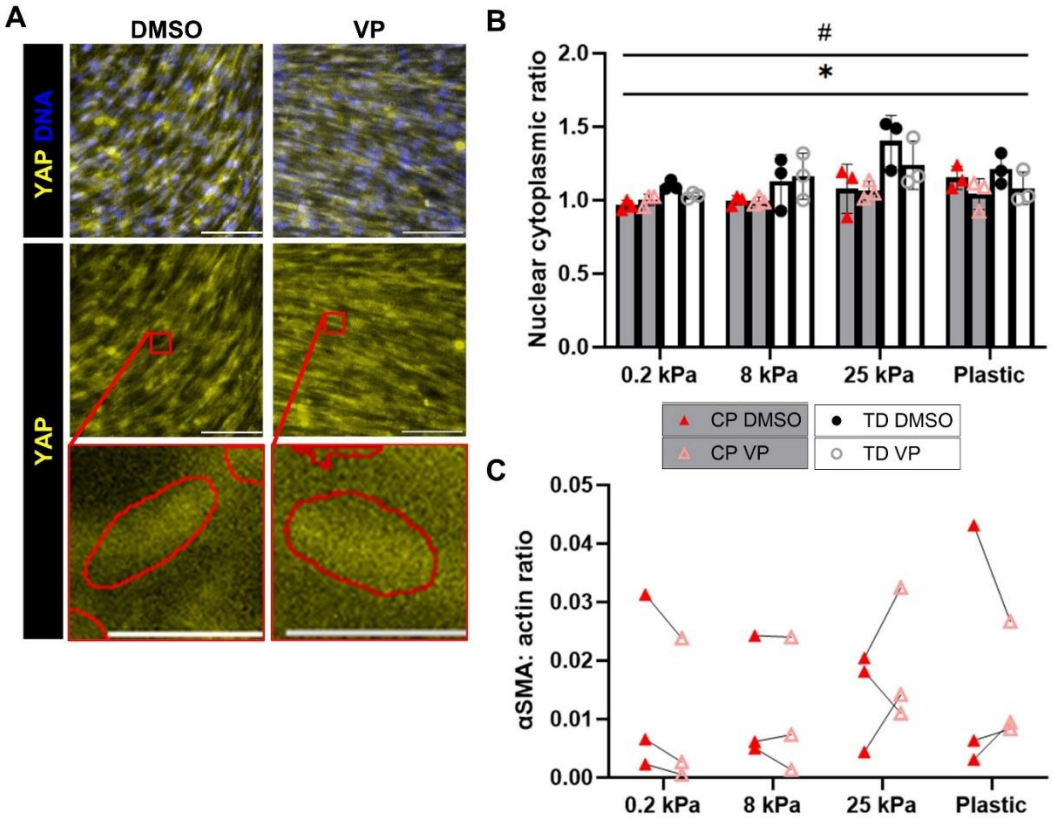

*Figure 4.4: Verteporfin treatment in FAPs. (A) YAP nuclear expression at 25 kPa in CP FAPs and quantification of nuclear cytoplasmic ratio. Red boxes indicate insets, red outline indicates nuclei within inset. (B) Quantification of YAP nuclear cytoplasmic ratio. (C) Myofibroblast activation in CP FAPs after verteporfin treatment. Data are mean ± SD (n=3). # p<0.05 main effect between CP and TD cells, *p<0.05 main effect of stiffness in a 3-way ANOVA. Scale bars are 100 μm; scale bars for inset images are 20 μm.*

DISCUSSION

The aim of this study was to determine the mechanosensitivity of human muscle stem cells in the context of fibrosis and CP. The focus was on MuSC differentiation into muscle, a necessary part of regeneration, and FAP activation into myofibroblasts, an instigator of fibrosis. We used

an established culture substrate, collagen-coated polyacrylamide gels, across a range of stiffnesses that were physiologically relevant to healthy and fibrotic muscle (18,20). Surprisingly, neither MuSC differentiation nor FAP-myofibroblast activation appeared particularly sensitive to the changes in stiffness. The lack of mechanosensitivity in the CP cells is attributed to the overall lower YAP expression compared to TD cells, indicating the signaling pathways have been disrupted or cells maintain a level of mechanical memory based on their in vivo environments (25). Overall, CP MuSCs possessed lower differentiation capacity compared to TD controls, and MuSCs in both conditions on fibrotic-like stiffnesses had altered phenotypes with high myonuclear clustering. CP FAPs more readily activated into myofibroblasts compared to TD FAPs. Verteporfin was explored to restore the regenerative capacity of CP cells but had negligible effect on MuSC differentiation or FAP myofibroblast activation, although slightly decreased myonuclear clustering. Overall, stem cells from CP muscle appeared to adapt a more fibrotic and less regenerative phenotype compared to TD controls that were less dependent on YAP signaling.

While cerebral palsy is a nonprogressive brain disturbance, the skeletal muscles demonstrate progressive contracture. The proper functioning of MuSCs is necessary to maintain muscle mass and recover from injury. The impaired differentiation index in CP MuSCs compared to their TD counterparts, as revealed herein, suggests these cells do not regenerate as well, leading to loss in muscle function. Previous studies have shown similar loss in differentiation potential and linked it to changes in DNA methylation (26,27). Changes in methylation patterns have been associated with changes in matrix stiffness (28). CP MuSCs may have altered methylation patterns due to the increase of stiffness in contracture and maintain a level of mechanical memory, impairing differentiation in vitro (25). Further studies could elucidate the mechanisms and potential therapeutic targets to induce regeneration. CP muscle pathology is largely heterogeneous. In this study, the CP gracilis and adductor longus muscle biopsies were

obtained from non-ambulatory children classified as Gross Motor Function Classification System (GMFCS) levels 4 and 5. A previous study in ambulatory patients with CP, GMFCS levels 1-3, found increased fusion index in CP MuSCs from the medial gastrocnemius, highlighting the vast heterogeneity in CP (14). Disparities in findings can be attributed to ambulatory status, age, and heterogeneity within individual muscles (12).

While the differentiation capacity of CP MuSCs has been previously studied, the phenomenon of nuclear clustering in response to stiffness has not, to our knowledge, been reported. Previous report of nuclear clustering did not find differences in expression of genes involved in myoblast fusion, although other genes may be involved in myonuclei clustering (17). Myofibers are composed of nuclei that should be evenly distributed, their positioning driven by microtubules (29,30). However, on stiff substrates, we observed that myonuclei cluster in large groups, leaving large sections of the myofiber without a nucleus close by. This has implications in muscle function. The observation of nuclear clusters indicates the myonuclear domain is disrupted. The even distribution of nuclei along a myofiber allows each nucleus to oversee maintenance of a certain section of the myofiber. Altering the positioning of myonuclei results in reduced contractility of the myofibers (29,31). The assembly of sarcomeres is dependent on close proximity to nuclei (32). This suggests the large areas of the myofibers, apart from the nuclear clusters, are without properly formed sarcomeres, resulting in reduced contractility and strength. Coupled with impaired differentiation, MuSCs in patients with CP appear to be forming muscle at a slower rate than their TD counterparts (26), and the muscle that is formed is weaker and less contractile due to nuclear clustering. This motivates further research into nuclear clustering in CP, potential mechanisms, and methods to reduce clustering.

Both MuSCs from patients with CP and TD counterparts appeared to form nuclear clusters on stiff, contracture-mimetic substrates, suggesting mechanosensing pathways are involved. Therefore, the cells were treated with verteporfin, a drug known to block cellular

mechanosensing capabilities through the degradation of YAP (21). As expected, verteporfin did not influence MuSC differentiation due to the lack of change in differentiation across the different stiffnesses. We observed a slight decrease in nuclear clustering in CP cells on stiff substrates, suggesting some nuclear clustering is rescued through mechanosensing targets. However, the limited sample size due to patient availability and heterogeneity between patients and muscle types makes it difficult to make definitive conclusions on the effectiveness of this method in restoring MuSCs' regenerative capacity. Higher concentrations of verteporfin or pretreatment before differentiation may yield different results. Other mechanosensing activators, such as TAZ, may play a more significant role in MuSC differentiation. Future studies can elucidate further the signaling pathway that drives the clustering.

MuSC differentiation is dependent on a variety of myogenic signaling pathways, some of which is derived from FAPs. FAPs and MuSCs work together in healthy muscle to restore the ECM and muscle, respectively, after injury. FAPs release myogenic signals to MuSCs post-injury to induce their migration to the injury site and differentiation into muscle (10,33). However, in disease conditions, FAPs activate into myofibroblasts and can release soluble factors that impair rather than promote myogenesis, as well as deposit fibrotic ECM leading to increased stiffness (34). The increased activation into myofibroblasts seen in CP FAPs compared to TD FAPs suggests these cells take on a more pathologic rather than regenerative role in CP muscle and impair MuSC differentiation. Myofibroblast activation was high in CP FAPs regardless of stiffness, suggesting activation is not mechanosensitive. However, TD FAPs increased markers of myofibroblast activation as substrate stiffness increased, although such indicators remained relatively low compared to CP FAPs. Overall, myofibroblast activation was low in both CP and TD FAPs compared to studies in murine models, yet their activation into both myofibroblasts and adipocytes suggests the cells are indeed a relatively pure population of FAPs (15,35). Myofibroblast activation is sensitive to increases in stiffness (13). CP FAPs face

other dysregulation in vivo that disrupts their mechanosensing pathways, making them less sensitive to changes in stiffness, explaining why verteporfin did not appear to influence the FAPs. Further research is needed to determine if FAPs are profibrotic in the contracture due to the low activation observed in this study. Identification of YAP-independent pathways for myofibroblast activation would better identify targets to reduce activation of FAPs in CP.

The use of human cells in in vitro experiments offers an opportunity for more translational research compared to immortalized or other animal cell lines but comes with its limitations. The limited number of biopsies and cells available limits the potential to have large sample groups to account for heterogeneity across muscle types, as both adductors and gracilis muscle were used, and variability in ages. The effectiveness of verteporfin as a treatment to restore the regenerative capacity of MuSCs is difficult to determine with the small sample size presented in this study, especially due to the inherent variability between patients. The role of FAP activation into myofibroblasts is limited by the sample size due to the low activation rates and large variability between patients.

This study shows the dysregulation of muscle stem cells in CP. MuSCs and FAPs from children with CP appear to take on a less regenerative and more fibrotic-like phenotype than their TD counterparts. The identification of myonuclear clustering on stiff substrates provides insights into the loss of contractility seen in CP. Verteporfin offers a potential avenue to reduce this clustering, yet further research is needed to validate its usage in the context of CP. The apparent lack of mechanosensitivity from CP MuSCs and FAPs in the context of differentiation and activation suggests other therapeutic targets may yield improved results.

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SUPPLEMENTAL INFORMATION

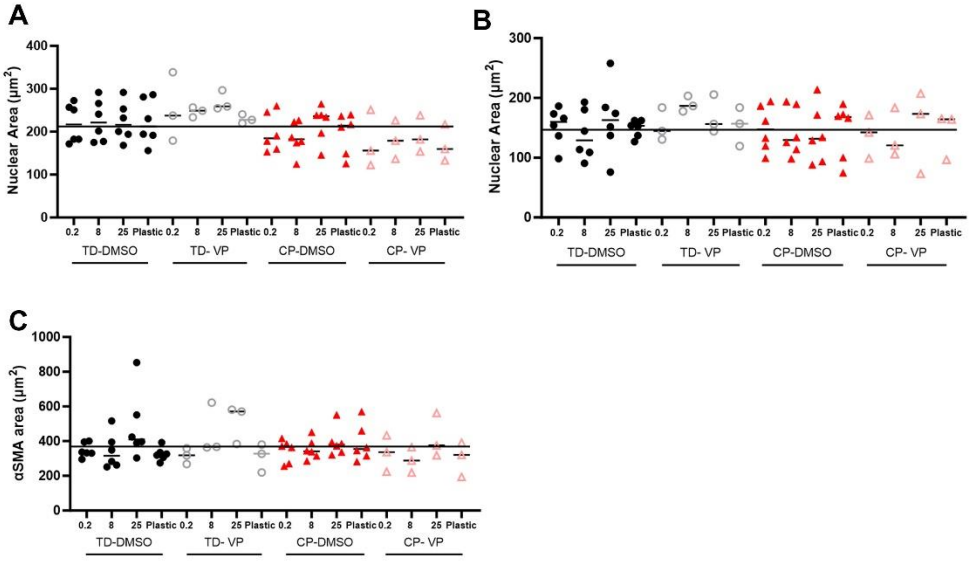

Figure S4.1 Average nuclear and cellular area did not change across conditions. (A) Average nuclear area of MuSCs. (B) Average nuclear area of FAPs. (C) Average myofibroblast area.

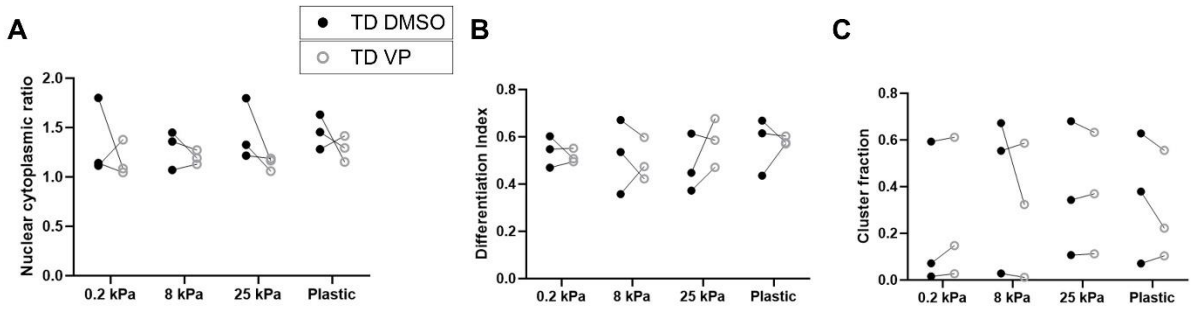

Figure S4.2 Verteporfin treatment to TD MuSCs. (A) YAP nuclear localization with verteporfin treatment or DMSO. (B) MuSC differentiation after treatment with verteporfin or DMSO. (C) Myonuclear clustering with verteporfin treatment.

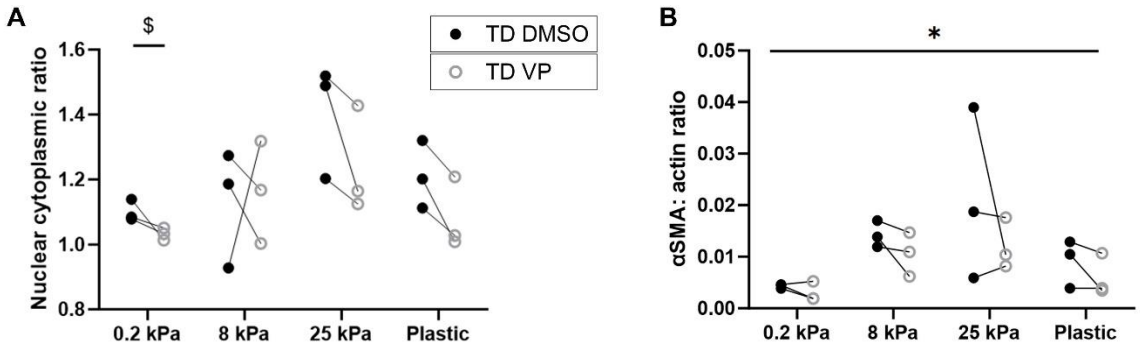

*Figure S4.3 Verteporfin treatment to TD FAPs. (A) YAP nuclear localization with verteporfin treatment or DMSO. (B) Myofibroblast activation after treatment with verteporfin or DMSO. $ p<0.05 between verteporfin and DMSO treatment. *p<0.05 across stiffness.*

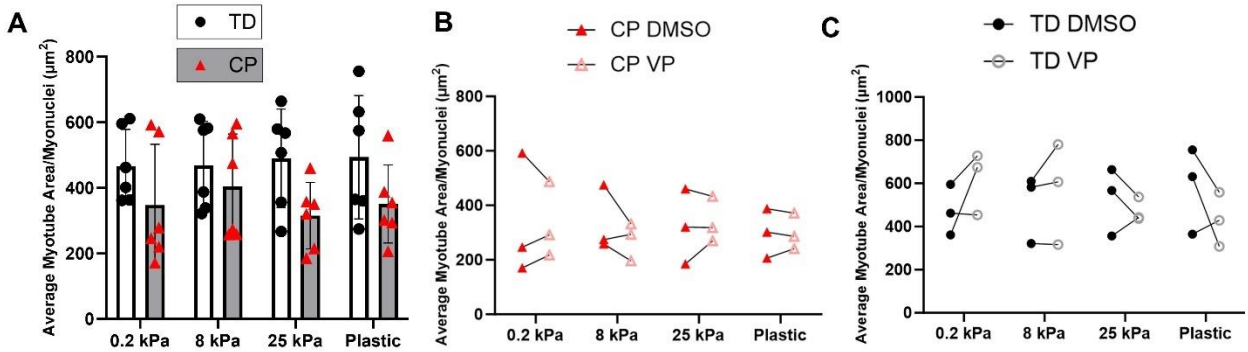

Figure S4.4 Myotube Area per myonuclei. (A) Average myotube area per myonuclei in microns between CP and TD MuSCs. (B) Effect of verteporfin on myotube area in CP. (C) Effect of verteporfin on myotube area in TD MuSCs.

CHAPTER 5: DISCUSSION

BACKGROUND AND MOTIVATION OF DISSERTATION RESEARCH

Fibrosis is the excessive and pathological accumulation of ECM components and is a consequence of a myriad of diseases across tissues, impairing tissue function and leading to increased morbidity. In skeletal muscle, fibrosis is a consequence of diseases such as Duchenne muscular dystrophy and cerebral palsy, resulting in contractures, impaired mobility, and decreased strength (9,164,165). Despite the prevalence of skeletal muscle fibrosis, there are limited therapies to attenuate or reverse fibrosis resulting in an unmet need to identify mechanisms and therapeutic targets for skeletal muscle fibrosis.

The fibrotic ECM has altered mechanical and architectural properties, including increased stiffness, altered collagen alignment, and increased collagen fiber size (44,70,139). The fibrotic ECM impairs MuSCs' ability to differentiate, limiting the muscle's ability to regenerate after chronic injury (90,93,174). Damaged muscle fibers are replaced by noncontractile fibrotic tissue leading to progressive weakness, chronic damage, and more fibrotic development. The main source of the pathological accumulation of ECM in skeletal muscle is FAPs (22,127). In acute injury and homeostasis, FAPs play vital roles in maintaining muscle fibers, promoting myogenesis after injury, and replacing damaged ECM (12,166). However, in fibrosis, cell signaling and commitment gets disrupted and FAPs become chronically activated into myofibroblasts, leading to excess ECM deposition (16,37,166). Understanding FAPs' activation into myofibroblasts has primarily focused on soluble factor signaling (19). However, the role of mechanosensing on FAP activation is unknown and may influence fibrotic activation in FAPs. Additionally, FAPs play a positive role on myogenesis in acute injury, but how this role is disrupted in fibrosis, and the role of FAP-derived ECM on myogenesis, is not fully understood.

The motivation for this dissertation was to determine how the ECM and FAPs interact in fibrosis, particularly how ECM drives myofibroblast activation and how myofibroblast activation affects

ECM deposition and myogenesis. This provides key insights into therapeutic targets for fibrosis, which could be used to improve muscle function and as a combinatorial treatment to address causes of fibrotic diseases.

DISCUSSION OF RESULTS

Fibrosis is pathologically degenerative, with the development of fibrosis leading to further fibrotic tissue development and progressive loss of tissue function (28,99,175). This is in part due to dysregulation of FAPs, resulting in the development of a fibrotic rather than regenerative phenotype (11,12). In **chapter 1**, we proposed a pro-fibrotic feedback loop wherein fibrotic ECM induces myofibroblast activation in FAPs, which in turn leads to further fibrotic ECM deposition resulting in additional myofibroblast activation in FAPs. We investigated this loop in **chapter 2** and **3** of this dissertation, alongside ways to disrupt this loop, with a translational application to patients in **chapter 4.**

The passive stiffness of muscle can increase dramatically in fibrotic diseases such as DMD or CP (9,44–46). In **chapter 2** of this dissertation, we investigated how this increase in stiffness and other alterations in ECM architecture affect FAP activation. We found that fibrotic-like stiffness induces myofibroblast activation to a significant higher degree when compared to healthy-like stiffnesses. We found that this increase in myofibroblast activation was strongly correlated with YAP nuclear localization, suggesting a mechanistic cause of the activation. To test this, we treated FAPs with verteporfin, a drug known to inhibit YAP translocation to the nucleus. We found that verteporfin significantly reduced myofibroblast activation on stiff substrates, providing a way to mitigate fibrotic development. Additionally, we found that FAPs have increased activation on collagen gels with smaller fibers or reduced cross-linking along with increased matrix deformation indicating a reciprocal relationship between FAPs and the ECM. This activation was not linked to YAP expression as the collagen gels all maintained the same bulk stiffness indicating myofibroblast activation is induced by multiple pathways.

Altogether, the data from **chapter 2** indicate that fibrotic ECM development affects FAP activation into myofibroblasts partially through the YAP signaling pathway, suggesting YAP inhibition as a potential therapeutic target to reduce fibrotic activation.

The increase in myofibroblast activation in FAPs likely affects FAPs' two key functions, ECM production and myogenic signaling. In **chapter 3** of this dissertation, we examined the effect of myofibroblast activation on FAP ECM production through CDM synthesis and the consequential effects on myogenesis. We found that *mdx* FAPs or FAPs treated with TGF-β had altered collagen organization compared to control *wt* FAPs, indicating fibrotic FAPs not only produce more ECM but contribute to the disorganized nature of fibrotic ECM (44,139,141). When treated with verteporfin, FAPs lost the ability to form robust collagen fibers, therefore, is a therapeutic method to halt the development of fibrosis.

We suspected the changes in ECM organization would impair myogenesis based on MuSCs' sensitivity to changes in ECM stiffness and architecture (50,88). FAP-produced CDMs impaired myogenesis when synthesized at 25 kPa, a physiologically relevant fibrotic stiffness. This trend did not hold true on plastic, suggesting myofibroblast activation alone, which increases with stiffness, does not solely control ECM deposition. Analysis of MuSC differentiation on different ECM coatings revealed preferential differentiation on certain ECM proteins, such as collagen IV, while other proteins, such as laminin, significantly impaired MuSC adhesion and regeneration. Laminin scarring has been shown to impair regeneration in MuSCs suggesting a possible change in FAP-derived ECM composition on fibrotic stiffnesses (170).

FAPs and MuSCs are known to communicate through soluble factors (93,112). TGF-β induced myofibroblast activation in FAPs resulted in significantly impaired myogenesis in the MuSCs in a transwell co-culture system, suggesting myogenic signaling between FAPs and MuSC is driven by soluble factors. Myofibroblast activation in FAPs had a particularly strong negative correlation with myogenesis indicating an impairment of differentiation in fibrosis.

Taken together, the data from **chapter 3** indicate that myofibroblast activation in FAPs and FAPs on physiologically relevant fibrotic stiffness result in fibrotic ECM deposition. Myogenesis is impaired by the ECM of FAPs on fibrotic-like stiffness and by soluble factors when FAPs are activated into myofibroblasts. Verteporfin is a way to block fibrotic development by limiting collagen fiber production.

Chapters 2 and 3 of this dissertation were completed using primary cells isolated from *wt* and *mdx* mice, a model for DMD. While these can provide valuable insights into the mechanisms and behaviors of FAPs and MuSCs in fibrosis, there is no good animal model to replicate the contractures and spasticity seen in CP muscle (176,177). Therefore, in **chapter 4** of this dissertation, we investigated the mechanosensitivity of muscle resident stem cells derived from muscle biopsies from children with CP. We found that CP FAPs activated much more readily into myofibroblasts compared to their TD counterparts but showed less mechanosensitivity. CP MuSCs showed less myogenic activity than TD MuSCs, with differentiation index not sensitive to stiffness. However, on fibrotic-like stiffnesses, the myonuclei in CP MuSCs formed large nuclear clusters indicating a level of mechanosensitivity that disrupts proper myotube formation. The consequences of the observed myonuclear clustering are not understood but has implications in maintenance of myofibers and myonuclear domain (178–180). The myonuclear clustering is potentially involved in the limited sarcomerogenesis observed in CP that drives the development of contractures (9,176). While CP FAPs showed little mechanosensitivity, CP MuSCs were slightly responsive to verteporfin with myonuclear clustering slightly decreasing. Therefore, targeting the myonuclear clustering through YAP or other mechanosensing pathway is a potential therapeutic pathway for improving proper myofiber function in CP. Overall, **chapter 4** shows that in a fibrotic environment FAPs and MuSCs have impaired regenerative capacity that is sustained *in vitro* through higher activation of FAPs and impaired myogenesis.

LIMITATIONS AND FUTURE AREAS OF RESEARCH

This dissertation provides exciting insights into FAPs and the ECM interactions in fibrosis. There are a few areas of research that are interesting for future research to expand on what was presented here.

First, the mechanosensing assays conducted here were all two-dimensional (2D). FAPs *in vivo* reside in the interstitial space and therefore, are completely surrounded by ECM in a three-dimensional (3D) environment (10). Mechanosensing pathways are altered between 2D and 3D environments (157). The work in **chapter 2** lays the foundation for FAP mechanosensitivity on hydrogels. It would be interesting to build on this work by imbedding FAPs in hydrogels and characterize their response and activation in 3D to further model the *in vivo* fibrotic environment.

This work focused on the cell-matrix interactions that occur in fibrosis, focusing on mechanosensing pathways and direct interactions. While cell-matrix interactions are likely to be a driving force for fibrotic development, the *in vivo* environment is much more complex that what is modeled here. **Chapter 2** proved that blocking mechanosensing pathways reduces myofibroblast activation *in vitro*. Further work is needed to determine whether this is feasible *in vivo* or if other fibrotic signaling such as TGF-β would override any therapeutic effects of treatments such as verteporfin (93).

The CDMs described in **chapter 3** are a novel way to investigate ECM deposition and response *in vitro*. This dissertation investigated CDM architecture using SHG and fluorescence imaging to characterize collagen and fibronectin organization. However, the complete make-up of the CDMs is unknown. Mass spectrometry would allow for full characterization of FAPs' ECM deposits and broaden our understanding of how ECM composition is altered in fibrosis and with myofibroblast activation. The mechanics of the CDMs is another unknown to characterized, which could be characterized through atomic force microscopy (AFM). AFM would allow for a better understanding of how well CDMs recapitulate the mechanics of the muscle ECM and how

these mechanics change in fibrosis and with drug treatments. Along with SHG imaging, mass spectrometry and AFM would allow for a fuller picture of FAPs' ECM deposition under healthy or fibrotic conditions and inform their influence on myogenesis.

In **chapter 3**, we observed impaired myogenesis on CDMs synthesized at 25kPa but not on plastic indicating stiffnesses is not the only driving factor of this change. Full analysis of the composition and mechanics of these CDMs will further elucidate the underlying cause. It is possible that plastic is too far outside the physiologically realm of stiffnesses that it disrupts FAPs and MuSCs in a way that is not physiologically relevant. The lack of effect on myogenesis of CDMs derived on plastic compared to CDMs derived on 25kPa may explain why we did not see any effect on CDMs derived from either *mdx* FAPs or FAPs treated with TGF-β or verteporfin. All these CDMs were synthesized on tissue-cultured plastic. Therefore, further experiments synthesizing CDMs on physiologically relevant stiffnesses to healthy and fibrotic muscle may further elucidate if the activation state of FAPs impairs myogenesis through ECM deposition.

Chapter 4 of this dissertation focused on the application of FAPs and mechanosensing in the context of human disease, specifically CP. Whether the cell behavior observed here holds true across fibrotic skeletal muscle diseases including DMD is unknown. CP is much more prevalent than DMD, 1 in every 350 births compared to 1 in every 5000 male births, making CP biopsies more readily available and thus were used in this work (181,182). However, using cells isolated from DMD patients would provide a much more comparable model to what is observed in the *mdx* mice and would allow for more direct translation to the development of DMD treatments.

Single-cell RNAseq has opened a whole area of research to dive into the diversity within cell populations. There has been some research that suggests subpopulations of FAPs hold differing pro-regenerative or pro-fibrotic capabilities (11,34,87). FAPs are necessary for homeostatic regulation of skeletal muscle and regeneration after acute injury (12,33,183).

Therefore, complete ablation of the cell type to prevent fibrosis is not feasible (33). Identification of the function and transiency of these subpopulations, particularly those more prone to myofibroblast activation, could provide direct therapeutic targets to reduce solely the pro-fibrotic phenotype in FAPs while maintaining FAPs' pro-regenerative features.

CONCLUSION

This dissertation demonstrates that FAPs and the ECM have significant interactions in the context of skeletal muscle fibrosis. Fibrotic ECM, in terms of stiffness, activates FAPs into myofibroblasts. In turn, myofibroblasts produce a more fibrotic ECM and inhibit myogenesis. Disrupting this loop can reduced fibrosis in terms of myofibroblast activation and ECM deposition.

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