INVESTIGATING FUSION-INDEPENDENT ROLES OF MUSCLE PROGENITOR CELLS IN RESPONSE TO EPS-INDUCED MYOTUBE DAMAGE

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TITLE:	Investigating fusion-independent roles of muscle progenitor cells in response to EPS-induced myotube damage
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LAY ABSTRACT

When muscle damage occurs, whether through rigorous exercise or physical trauma, the muscle relies on a specific group of stem cells to help repair itself. These stem cells, termed satellite cells, can migrate to specific sites of muscle damage, differentiate into myoblasts, and donate nuclei and genetic material to the injured muscle. This increase in nuclear content helps the muscle synthesize more protein to rebuild and regenerate and promotes muscle growth. However, when the satellite cell becomes dysfunctional, as seen in aging muscle and certain genetic conditions, the muscle struggles to repair itself in response to damage and cannot grow in response to exercise. Satellite cell biology has clearly defined the role of nuclear donation in muscle function, however very little is known about how this stem cell 'talks' to the muscle through signaling molecules. As such, this thesis elucidates the effect of myoblast signaling on electrically stimulated damaged immature muscle fibers, otherwise known as myotubes, by preventing myoblast-myotube physical interactions in cell culture experimentation. Interestingly, the data presented here demonstrate that myoblast exposure to damaged myotubes may increase muscle protein breakdown as myotube diameters are reduced in size acutely post-damage, likely resulting from the increase in protease and autophagy protein expression markers. Additionally, myoblast exposure to damaged myotubes may increase mitochondrial fatty acid oxidation to generate energy, which is the fuel of choice during muscle regeneration.

ABSTRACT

INTRODUCTION: Following damaging stimuli, skeletal muscle exhibits coordinated interplay between intra- and extra-cellular processes resulting in satellite cell (SC) recruitment. SCs are known to play a central role in muscle plasticity post-injury by differentiating into myoblasts (MBL) and fusing with damaged tissue to donate myonuclei. Yet, their role within skeletal muscle remodeling through paracrine signaling remains to be fully elucidated. Thus, the purpose of this project was two-fold: 1) develop an in vitro model of MBL intercellular communication following myotube damage and 2) to determine if MBL proximity alone is adequate for improving tissue repair and reducing cellular stress during recovery. **METHODS:** C2C12 myotubes were exposed to 1 hour of electrical pulse stimulation (EPS) with 15Hz pulse for 5s and 5Hz pulse for 5s, separated by a 5s break. Myotubes were then introduced to non-electrically stimulated (NS) MBL adhered to a porous cell insert to allow paracrine signaling and samples were collected at varying timepoints post-EPS. **RESULTS:** EPS induced Z line sarcomeric disorganization and creatine kinase release into the cell culture media, which was mitigated in MBL+ groups ($p<0.05$). A significant main effect of MBL exposure was observed in EPS myotubes where MBL+ myotubes had greater Hsp70 gene expression, calpain 3 protein and gene expression, and t-ACC, p-ACC^{Ser79}, t-ULK, p-ULK^{Ser555} protein expression than MBL- myotubes when recovering from EPS ($p<0.05$). A main effect of time was observed where β-dystroglycan and p-mTOR^{Ser2448} protein expression decreased in the EPS myotubes, and myotube diameter only decreased in the MBL+ condition ($p<0.05$). **CONCLUSION:** MBL signaling to damaged myotubes is evident and may increase catabolic processes through upregulating contraction-mediated protease activity and autophagy, as well as increase ATP generation through oxidative phosphorylation during regeneration.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

MPS	Muscle protein synthesis	mTORC1	mTOR complex 1
MPB	Muscle protein breakdown	mTORC2	mTOR complex 2
SC	Satellite cell	S6K1	S6 kinase 1
ECC	Excitation contraction coupling	EIF3	Eukaryotic initiation factor 3
Ca2+	Calcium ion	4E-BP1	4E-binding protein 1
EIMD	Exercise induced muscle damage	CD8	Cluster of differentiation 8
DAPC	Dystrophin associated protein complex	MRF	Myogenic regulatory factors
DAG1	Dystroglycan 1	Pax7	Paired box transcription factor 7
CK	Creatine kinase	Myf5	Myogenic factor 5
PCr	Phospho-creatine	MyoD	Myoblast determination protein 1
DOMS	Delayed onset muscle soreness	*Mymk*	Myomaker
DHPR	Dihydhropyridine receptor	CCR2	CC chemokine receptor 2
RyR1	Ryanodine receptor	IL-6	Interleukin-6
SERCA	Sarco-endoplasmic reticulum calcium ATPase	TNF-α	Tumour necrosis factor α
CAPN3	Calpain 3	IGF-1	Insulin growth factor 1
UPS	Ubiquitin proteasome system	EV	Extracellular vesicles
HSPs	Heat shock proteins	*Mmp9*	Matrix metalloprotein 9
HSP70	Heat shock protein 70	ECM	Extracellular matrix
HSPA1A	Heat shock protein 70-1	MPC	Muscle progenitor cells
HSPA1B	Heat shock protein 70-2	Rbp1	Retinol binding protein 1
ROS	Reactive oxygen species	Tcf4	Transcription factor 4
ALS	Autophagy-lysosome system	eMyHC	Embryonic myosin heavy chain
ULK1	Unc-51-like kinase 1	PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1- α
mTOR	Mammalian target of rapamycin	GLUT4	Glucose transporter type 4
PI3K	Phosphoinositide 3-kinase	EPS	Electrical pulse stimulation
Ser	Serine	NS	Unstimulated myotubes
AMP	Adenosine monophosphate	MBL+	Myoblast exposure
ATP	Adenosine triphosphate	MBL-	No myoblast exposure
AMPK	AMP-activated protein kinase	MBL	Myoblast
ACC	Acetyl-coA carboxylase	Thoc5	THO complex subunit 5
CPT1	Carnitine-palmitoyl transferase 1	CPT1b	Carnitine-palmitoyl transferase 1b
$^{2}H_2O$	Heavy water – deuterium dioxide	*Mdx*	Mouse model of Duchenne muscular dystrophy

DECLARATION OF ACADEMIC ACHIEVEMENT

The contents of this thesis have been prepared in accordance with the guidelines provided by the School of Graduate Studies at McMaster University which includes an introduction, detailed methods, results and discussion section. Magda Alexandra Lesinski was the principal contributor to completing experiments, data collection, data analysis and interpretation throughout the duration of this project. Dr. Gianni Parise formed the vision of this project and assisted with interpreting results and planning next steps.

LITERATURE REVIEW

Introduction

Skeletal muscle comprises nearly 40% of total body mass and is highly plastic [1]. The size of skeletal muscle depends on the net balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). Multiple lifestyle factors including physical activity, nutrition, injury, and disease highly influence these processes. Skeletal muscle is directly responsible for voluntary movement by converting chemical energy into mechanical energy. Furthermore, skeletal muscle serves as the body's largest reservoir of amino acids and glycogen. As such, muscle health directly impacts quality of life, where severe decreases in skeletal muscle size and strength often impact daily physical movements and increases the risk of frailty in advanced age [2]. Lifelong exercise training is strongly associated with reduced aging-related muscle weakness, improved balance and mobility, and a greater quality of life [2,3].

Exercise has long been identified as a key therapeutic for many states of muscular impairment, such as diabetes, muscular dystrophies, and progressive aging [4–6]. However, the molecular mechanisms underlying the beneficial impact of exercise in these unique conditions remain to be fully elucidated. Skeletal muscle stem cells, termed satellite cells (SCs), were first discovered by Alexander Mauro in 1961 and since then, their role in muscle regeneration and myofiber development has been thoroughly examined [7]. SCs have been found to contribute to resistance exercise-mediated muscle hypertrophy, although the extent of their contribution has been the subject of intense debate for several years [8,9]. Dysfunction of the SC population is seen as a contributing factor in age-related sarcopenia where SCs seem to be incarcerated by increased laminin deposition, which may affect their ability to migrate and promote muscle growth [10]. As such, insights into the fusion-independent effects of SCs through intercellular communication may

lead to alternative strategies to promote muscle repair under various conditions. Beyond the satellite cell, characterizing paracrine signaling within the skeletal muscle niche is paramount to understanding the complex network of systems contributing to healthy muscle preservation.

Skeletal muscle contraction

Skeletal muscle comprises multiple bundles of muscle fibres known as fasciculi [1]. Each fibre contains many myofibrils segmented into sarcomeres – the contractile units of skeletal muscle. Each sarcomere is separated into distinct regions bordered by a dark Z line, from which thin actin filaments originate and project inwards towards a centrally located M line [1]. The M line contains the thick myofilament myosin that projects outwards towards the Z line and interacts with the actin filament when stimulated (Fig. 1) [1]. Between the Z and M line extends the large titin protein which acts as a molecular spring responsible for passive elasticity [11]. The I band is the region between the M line and Z line where myosin is not present. At rest, actin filaments are tightly associated with tropomyosin, an accessory protein that blocks the actin binding sites and prevents the myosin heads from binding to actin. Tropomyosin binds directly to the troponin complex consisting of 3 subunits (Fig. 1) [12]. Together, these proteins work to regulate muscle contraction.

The excitation-contraction coupling mechanism (ECC) is a vital process for skeletal muscle contraction. Calcium ion (Ca^{2+}) release from the sarcoplasmic reticulum is initially triggered by the propagation of an action potential from a motor neuron. This action potential results in acetylcholine neurotransmitter release from the neuromuscular junction to acetylcholine receptors on the muscle post-synaptic membrane [13]. When cytosolic Ca^{2+} concentrations are low, the tropomyosin/troponin complex prevents actin-myosin binding, known as cross-bridging. However, when calcium levels increase during muscle contraction, Ca^{2+} binds to troponin subunit C, which rotates tropomyosin away from the myosin-binding sites on actin, allowing for actin-myosin

interactions [12]. Myosin heads can then act as motors to bind and release actin filaments through ATP hydrolysis, ultimately increasing muscle tension.

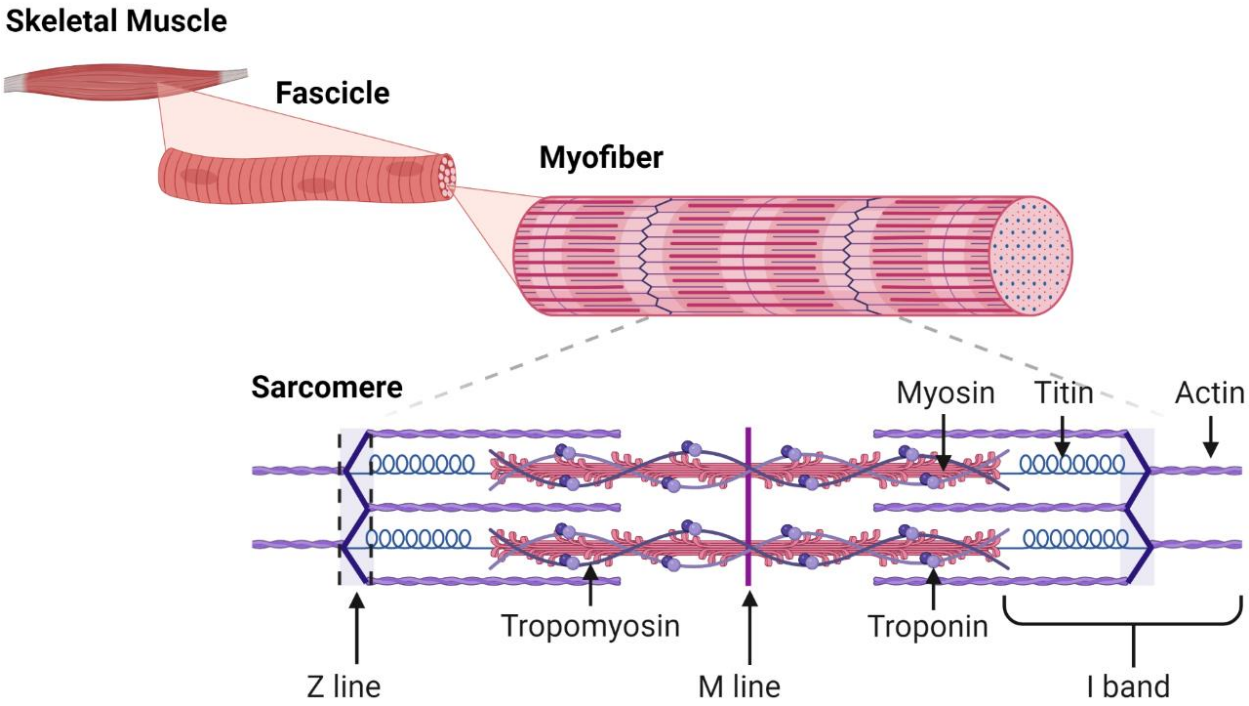

Figure 1: Skeletal muscle composition. Skeletal muscle is composed of multiple bundles of fibers known as fasciculi which are further composed of many myofibrils. Each myofiber is segmented into sarcomeres, which are the contractile units of skeletal muscle. A sarcomere is bordered by the Z line which anchors titin and the thin myofilament actin, which projects inward towards the M line. The M line contains the thick myofilament myosin which is tightly associated with the tropomyosin and troponin complexes. The region between the Z and M lines that does not contain myosin is the I band. Muscle contraction is regulated by the overlapping regions of actin and myosin where actin-myosin cross-bridging inwards towards the M line increases muscle tension.

Exercise induced skeletal muscle damage

Exercise-induced muscle damage (EIMD) often occurs with the overstretching of sarcomeres during eccentric contractions, when the skeletal muscle contracts during elongation, resulting in actin and myosin filaments extending beyond where they would normally overlap [14]. This causes not only structural damage to the M line and adjacent regions but also to the titin filament, which extends between the Z and M line. These ultrastructural changes have been observed *in vivo* where sarcomeres become disorganized, and Z line streaming is evident [15]. Damage to the sarcomere and the sarcolemma allows for a series of signaling cascades that together clearly characterize EIMD.

a. Membrane and sarcomeric stability

Membrane stability during muscle contraction is important in preventing contraction-induced muscle injury. There are many factors that contribute to sarcolemmal stability, but perhaps none of which are more important than the dystrophin-associated protein complex (DAPC). This heteromeric complex contains transmembrane and membrane-associated proteins that help anchor the extracellular matrix with the intracellular cytoskeleton. Significant dysregulation of any accompanying proteins results in various muscular dystrophies, including Duchenne muscular dystrophy (DMD) and some types of limb-girdle muscular dystrophy [16,17]. A core component of the DAPC is dystroglycan, a protein encoded by the *dystroglycan 1 (DAG1)* gene which is post-translationally cleaved to produce two subunits, α- and β-dystroglycan [17]. β-dystroglycan is important as it directly links extracellular α-dystroglycan to intracellular dystrophin [17]. Mutations in the *DAG1* locus, particularly within the α-subunit encoding region, result in a group of disorders known as dystroglycanopathies [18].

Additionally, conditional deletion of *DAG1* via tamoxifen-induced knock-out resulted in increased contraction-induced force deficits in mice, demonstrating the importance of the dystroglycan subunits on sarcomeric stability [16]. Previous inquiries on *DAG1* transcription in DMD have implicated the dystroglycans in preventing myofiber necrosis through their linkage of the extracellular matrix to the sarcolemma, without which the disease severity would increase [16,17]. However, membrane permeability and desmin content were unaffected in the inducible-*DAG1* mouse model following lengthening contractions while titin abundance decreased, suggesting the dystroglycans have a greater role in maintaining the sarcomere than the sarcolemma [17,19]. Further, selective reduction of the α-dystroglycan subunit did not display any changes from wild-type animals, demonstrating the importance of β-dystroglycan in sarcomeric stability and force production [16,17]

Sarcomeric instability is a hallmark sign of EIMD. The Z line is considered one of the more complex macromolecular structures in biology, composed of hundreds of proteins [20]. The electron-dense structure of the Z line allows for clear visualization with transmission electron microscopy, appearing as dark rectangular plates interspersed between long sections of striated myofilaments [21]. The Z line can range from roughly 40nm in fast twitch muscle and 120nm in slow twitch muscle and has long been regarded as exclusively involved in the mechanical stability of the sarcomere [22]. After a damaging bout of eccentric contractions, the Z line configuration is altered, resulting in a zigzag appearance, otherwise known as Z line streaming. As such, exercise-induced muscle damage contributes to sarcomeric adaptations, contraction deficits, and membrane instability.

b. Creatine kinase

The increase in myofibrillar instability after exercise results in elevated muscle protein breakdown and membrane permeability due to stretch-mediated processes, remodeling, and

immune infiltration [23]. As such, protein and enzyme secretion into the blood serum are common and can be used as indirect measures of skeletal muscle damage. Creatine kinase (CK) is an essential catalyst within the phosphocreatine (PCr) system and is highly abundant in the muscle cytosol, composing nearly 20% of soluble sarcoplasmic protein content in some muscles [24]. Cytosolic CK is responsible for ATP generation and is closely linked to glycolysis. Within the mitochondria, ATP generated from oxidative phosphorylation allows for donating an inorganic phosphate to creatine to generate PCr. This PCr is then supplied to the cytosol, where CK dephosphorylates creatine to supply ATP for muscle activity. As CK is often localized to the I band of the sarcomere and the M line of the sarcoplasmic reticulum, it's use as a marker of muscle damage, as opposed to disruption of other muscle processes or myofibrillar remodeling, can be questioned [24,25]. Nevertheless, CK is highly elevated in response to eccentric exercise-induced microtears in myofibrillar structures when delayed onset muscle soreness (DOMS) is most evident, making it a consistent marker of such damage.

c. Calcium signaling

The t-tubular system is composed of invaginations of the sarcolemma that propagate the muscle action potential and are rich in receptors required for signal transduction, such as dihydhropyridine receptor (DHPR) and ryanodine receptor (RyR1) [26]. The ECC required for muscle contraction is regulated through calcium release from the tightly associated terminal cisternae of the sarcoplasmic reticulum. The t-tubule membrane contains the DHPR voltage-gated calcium channels, and the adjacent sarcoplasmic reticulum has the RyR1 calcium release channel [27,28]. DHPR is activated by the action potential to initiate muscle contraction and activates RyR1 in two ways: 1 - DHPR can release minimal Ca^{2+} ions to activate RyR1, or more canonically 2 - by physically interacting and changing the conformational state of RyR1 to release more calcium

[29,30]. During muscle relaxation, the sarco-endoplasmic reticulum calcium ATPase pumps (SERCA), also located on the sarcoplasmic reticulum, allow for the reuptake of Ca^{2+} when sarcomeres shorten to reduce intracellular content. Muscle contraction is also modulated by the influx of Ca^{2+} ions into the cytosol by activating stretch-gated ion channels. These channels are sensitive to mechanical strain and are thus implicated in EIMD [14]. Fundamentally, skeletal muscle damage can be tightly associated with the dysregulation of Ca^{2+} signaling.

Excessive Ca^{2+} concentrations in the cytosol of skeletal muscle can lead to muscle damage by activating proteases [31,32]. One notable protease involved in EIMD and sarcolemmal remodeling is calpain 3. The muscle-specific isoform of the Ca^{2+}-dependent calpain cysteine protease family, calpain 3, has been implicated in muscle maintenance and function as mutations throughout the associated *CAPN3* gene result in limb-girdle muscular dystrophy type 2A [33]. The proteolytic function of calpain 3 is inactive under normal conditions but is enhanced in response to elevated levels of intracellular calcium, which occurs after repeated eccentric contractions and muscle damage [34]. While it remains unclear exactly how the proteolytic activity of calpain 3 is regulated, the 94 kDa calpain isoform localizes on the titin M and Z line regions along sarcomeres when inactive and may require dissociation for its IS1 domain to be autolyzed [35]. This allows further intramolecular proteolysis of additional sites to activate calpain 3's proteolytic activity [36].

When minor damage caused by regular wear and tear of daily physical activity occurs, the recruitment of intracellular vesicles to patch damaged membranes is required. The dysferlin membrane repair complex tethers lysosomes to the cellular membrane, allowing for their exocytosis while triggering the endocytosis of pores at the site of damage [37]. Desmoyokin, a large protein associated with the dysferlin complex, requires cleavage by calpain 3 for membrane patch repair to proceed [38]. Further, calpain 3 has been implicated in sarcomere maintenance given that

calpain 3 deficient primary myotubes lack well-defined sarcomeric structure and immature Z line formation [39]. These findings demonstrate the importance of calpain 3 both in the proper functioning of skeletal muscle and its recovery following stress.

Calpain 3 activity is upregulated and salient within fully differentiated myotubes and myofibres. Unlike ubiquitous calpains, this isoform does not participate in myoblast proliferation and the lack of calpain 3 proteolytic activity in developed myofibres leads to muscle degeneration. These findings suggest that calpain 3's involvement in skeletal muscle adaptation is more crucial than development [36,40]. Additionally, calpain 3 is not implicated in the initial disassembly of myofibrils, usually coordinated by ubiquitous calpains and the ubiquitin proteasome system (UPS) but is upregulated following this degradation to promote sarcomere remodeling [41–43]. It has been shown that calpain 3 deficient muscle fails to regain muscle mass after primary injury, possibly due to the decrease in ubiquitination of proteins in said muscle, suggesting a role for calpain 3 upstream of the UPS [43]. Together, the data proposes the role of calpain 3 in skeletal muscle adaptation after injury through the potential recovery of muscle force, strength and size.

d. Heat shock protein response

The resulting sarcomeric disruption from EIMD reduces muscle force production and increases intracellular stress, notably in heat shock proteins (HSPs) [44]. HSPs are a group of chaperone proteins that regulate appropriate protein folding and unfolding in response to heat and oxidative stress. Heat shock protein 70 (HSP70) is the predominant responder to such stressors in skeletal muscle. There are 13 different genes that encode for distinct HSP70 proteins, all sharing a common domain [45]. The most studied gene products originate from the *HSPA1A* and *HSPA1B* loci encoding proteins HSP70-1, HSP70-2 respectively [45]. HSP70-1 and HSP70-2 have been identified as interchangeable stress-inducible HSP70s and are sometimes referred to collectively

as HSP70, Hsp72 or HSP70i [46]. HSP70 protein expression has been shown to increase following physical (i.e., exercise), chemical (i.e., changes in pH), and physiological (i.e., oxidative stress) stimuli [47]. More specifically, HSP70 proteins are particularly sensitive to skeletal muscle contraction and are highly responsive to the accumulation of reactive oxygen species (ROS).

Nevertheless, HSP70 does not seem to increase in skeletal muscle following low frequency, low intensity stimulation, which may suggest an intensity-dependent relationship [48,49]. The activity of these chaperones can maintain cellular homeostasis by preventing premature apoptosis, participating in energy metabolism by increasing anaerobic glycolysis, and promoting muscular adaptations in response to stress [50]. It is of the utmost importance that HSP70 increases in response to a stressful stimulus and appropriately decreases when the stress is resolved to prevent excessive protein misfolding. In adult male mice, it has been shown that HSP70 protein expression increases with treadmill speed, suggesting that HSP70 responds to exercise intensity [51]. In addition to regulating appropriate protein folding, Hsp70 has been shown to participate in signaling proteins for degradation [50]. As such, HSP70 has been identified as a potential marker for exercise-induced stress, alongside creatine kinase through its involvement in proper protein coordination.

e. Exercise-induced catabolism

Effective skeletal muscle adaptations to exercise also require adequate protein breakdown. A careful balance between protein synthesis and protein breakdown is required to ensure dysfunctional components are cleared and sufficiently replaced, resulting in appropriate remodeling. While the ubiquitin-proteasome system (UPS) degrades short-lived soluble proteins, the autophagy-lysosome system (ALS) is required to break down long-lived, insoluble proteins and even organelles [52]. Autophagy is regulated by the unc-51-like kinase 1 (ULK1) complex which initiates the formation of an immature autophagosome, otherwise known as a phagophore [53–55].

This is necessary to clear dysfunctional organelles, such as mitochondria, as well as misfolded or aggregated proteins. Under normal conditions, the mammalian target of rapamycin (mTOR), a serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family, inhibits autophagy by phosphorylating ULK1 on serine (Ser)-757 and thereby reducing its activity. However, after an acute bout of exercise, the changes in molecular messengers associated with muscle contraction, such as calcium, adenosine monophosphate/triphosphate (AMP/ATP) ratios, nicotinamide adenine dinucleotide (NAD+), and ROS, lead to the activation of AMP-activated protein kinase (AMPK). AMPK activation inhibits mTOR, preventing mTOR-mediated ULK1 inhibition and increasing autophagy [56]. AMPK is also responsible for directly activating ULK1 through phosphorylation on Ser-555 [57]. ULK1 activation ultimately leads to the fusion of cargo-carrying autophagosomes to lysosomes, specialized organelles that hydrolyze proteins. The resulting increase in the amino acid pool potentiates an autophagic negative feedback loop whereby the activation of mTOR complex 1 inhibits ULK1 [57]. Crosstalk between the ALS and UPS has been established, where the downregulation of one degradative system leads to the compensatory increase in the other [58]. However, evidence suggests a more salient autophagic response to damage incurred from strenuous exercise, and this response is seen in both endurance and resistance training models [59–61].

Catabolism is further promoted during muscle contraction by AMPK inhibition of acetyl-CoA carboxylase (ACC) [62]. ACC is involved in energy metabolism by regulating beta oxidation. Under homeostatic conditions, ACC upregulates malonyl-CoA production required for fatty acid biosynthesis and inhibition of carnitine-palmitoyl transferase 1 (CPT1), located on the outer membrane of the mitochondria, which is required for fatty acid uptake [63]. When AMPK is activated in response to muscle contraction, it inhibits ACC inhibiting fatty acid biosynthesis and activating

CPT1, resulting in fatty acid transfer into the mitochondria. Increased fatty acid availability leads to increased beta oxidation, where fatty acids are broken down to generate acetyl-CoA, further used in the tricarboxylic acid cycle to generate ATP [64]. However, additional pathways independent of ACC may be involved in regulating fatty acid oxidation as the lack of AMPK phosphorylation sites on ACC in exercised mice still displayed comparable oxidation levels and exercise capacity as wild-type mice [64]. Together, catabolism is a crucial component in mediating exercise-induced adaptations helping to meet energetic demand and by promoting appropriate protein turnover for remodeling.

f. Muscle protein synthesis

As mentioned previously, skeletal muscle mass and hypertrophy in response to resistance exercise depend on the balance between MPS and MPB. Muscle recovery post-exercise requires myofibrillar remodeling involving protein breakdown, which then allows for the replacement of essential proteins with MPS [43,65,66]. It is well understood that the rates of MPS during training are correlated with the degree of hypertrophy observed following the completion of a strength training regime. The increased ratio of MPS/MPB during an exercise program results in greater muscle growth [66]. Surprisingly the initial rate of MPS seen after the first bout of resistance exercise is not correlated to muscle hypertrophy seen after a training program is completed [66]. Further, the damage to skeletal muscle and the associated DOMS after a single bout of unaccustomed resistance exercise is inconsistent during a prolonged resistance exercise training program [67]. Moreover, myofibrillar protein synthesis, as measured with labelled heavy water (^2H_2O), increased in response to damaging eccentric contractions [68]. This suggests that acute damage from resistance exercise may affect muscle protein balance in a manner that does not result in hypertrophy, but to support the tissue repair needed in exercise naïve muscle [69].

The MPS response seen during EIMD is regulated by mTOR, often referred to as the master regulator of protein synthesis. Functionally and mechanistically distinct complexes, mTORC1 and mTORC2, share the same mTOR protein but contain different components [56]. mTORC1 signaling ultimately results in the regulation of protein synthesis, while mTORC2 signaling affects cell survival and metabolism [70]. As the catalytic subunit for both processes, the activation of mTOR has significant effects on skeletal muscle development, growth, and homeostasis under a variety of conditions. mTORC1 activity is sensitive to changes in nutrient and oxygen availability, energy levels, and growth factors, but it can be inhibited by extensive stress [70]. mTORC1 regulates MPS by activating mTOR-specific downstream targets such as S6 Kinase 1 (S6K1), which is involved in activating the eukaryotic initiation factor 3 (EIF3) translation initiation complex and inhibiting 4E-binding protein 1 (4E-BP1), which is a translation initiation inhibitor [56].

After acute resistance and aerobic exercise in untrained men, mTOR phosphorylation and activation is seen as soon as 1- and 4-hours post exercise, respectively with downstream S6K1 activation and 4E-BP1 inhibition demonstrating immediate responses in MPS for repair [65]. Additionally, mTOR substrate phosphorylation is highest in response to acute resistance exercise, where subsequent training sessions do not seem to activate the pathway to the same degree unless a sedentary period is introduced [71]. Altogether, mTOR activation may play a role in both the immediate need for myofibrillar protein remodeling in response to acute damage to skeletal muscle and promoting hypertrophy long term.

g. Inflammation and skeletal muscle damage

Though not a potent contributor to *in vitro* models of muscle damage, inflammation is acutely required for appropriate skeletal muscle adaptation after injury *in vivo*. Unaccustomed exercise consisting of eccentric contractions often results in muscle damage as previously

mentioned; however, the adaptations that occur thereafter are not solely contained within the myofiber. Broadly, muscle cytokines, termed myokines, are released from cells within the skeletal muscle niche to signal for the infiltration of various immune cells [72]. The acute response to muscle damage involves the appearance of neutrophils, which are responsible for inducing necrosis and phagocytosis to clear damaged tissue [73]. Pro-inflammatory macrophages are the next to enter the skeletal muscle niche, which further promotes phagocytosis and proliferation of satellite cells. The switch to anti-inflammatory macrophages signals for myogenin expression in satellite cells leading to myotube formation [74]. Between 4-24 hours after damage, CD8 and T regulatory lymphocytes infiltrate the niche and increase SC expansion and fibre maturation [72]. All these immune cells secrete important factors that regulate skeletal muscle repair and adaptation post-injury and are thus potent contributors to the characterization of EIMD.

The roles of satellite cells

Satellite cells (SC) are a lineage of multipotent muscle stem cells that reside between the basal lamina and sarcolemma of muscle fibres. They remain in a quiescent state until an injury occurs, whereafter they activate and proliferate at damage sites in response to cytokine release from immune cells and the damaged muscle [7]. It is well established that SC play a crucial role in muscle hypertrophy, regeneration, and repair post-injury. Extreme injuries caused by trauma or extensive physical activity induce the activation and differentiation of SC into myoblasts, which fuse to existing muscle fibres or form new ones [75]. Alternatively, SC may undergo proliferation and return to quiescence to expand the SC pool [76].

The proper functioning of SCs is regulated by a group of transcription factors collectively known as myogenic regulatory factors (MRFs) (Fig. 2). Quiescent SCs are identified by their expression of paired box transcription factor 7 (Pax7); however, 90% of quiescent SC express

myogenic factor 5 (Myf5), which indicates a commitment to the myogenic lineage [77]. In response to injury or exercise, the activation of SCs requires the positive expression of myoblast determination protein 1 (MyoD) and Pax7 and Myf5. SCs are then able to proliferate and can either return to quiescence to replenish the SC pool or begin expressing myogenin which inhibits Pax7 and promotes the terminal differentiation of myoblasts into myocytes, characterized as MyoD+/Myogenin+/Mrf4+ [75]. A fully differentiated myocyte can fuse to damaged muscle fibres to donate myonuclei, or myocytes can fuse to create *de novo* myotubes and, subsequently, myofibres. Myocyte fusion capacity is regulated by the consistent expression of myogenin, MyoD, and Myf5, where inhibition arrests fusion, likely by preventing the transcription of fusion-essential genes [78]. Further, other myogenic stem cells, such as mesenchymal stem cells, have also been identified as having undergone myogenic differentiation to provide similar aids to regeneration, though this is less common [79,80].

Muscle degeneration post-injury is important for SC recruitment. Damaged muscle fibres become necrotic, allowing for increased myofibre permeability that is evident through increases in creatine kinase levels in plasma [81]. This necrosis further triggers calcium release from the sarcoplasmic reticulum and is associated with calcium-dependent proteolysis involving the calcium-activated protease, calpain 3 [82]. Muscle degradation then activates an inflammatory response allowing macrophage infiltration further activating SC proliferation and differentiation.

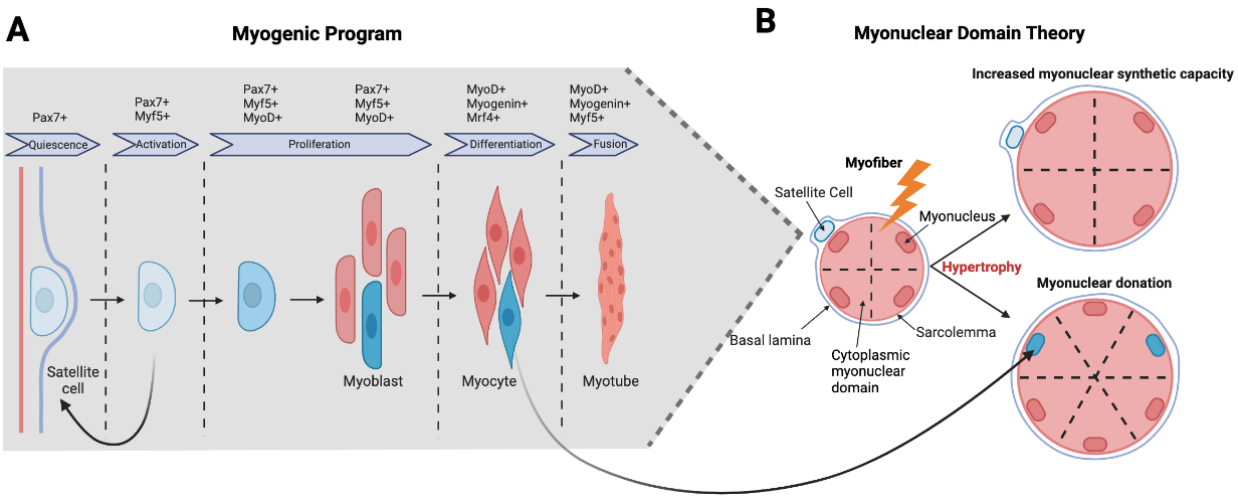

Figure 2. The role of the myogenic program in muscle hypertrophy. A) Satellite cells (SC), also known as muscle stem cells, remain in quiescence until an activating stimulus, such as skeletal muscle damage, is presented. Once activated, SCs progress through the myogenic program to proliferate and terminally differentiate into myocytes, at which point they either donate their nuclei to existing myofibers or fuse to form *de novo* myotubes. Alternatively, undifferentiated SCs generated through mitosis can return to replenish the SC pool. B) SCs are at the core of the Myonuclear Domain theory, which suggests that in response to a hypertrophic stimulus, a myofiber can grow in size by first expanding the transcriptional capacity of existing myonuclei, resulting in an increased cytosolic volume per nucleus. However, as nuclei can only support a certain volume of the cytosol, once this threshold is met, an increase in nuclear content is required for maximal hypertrophy, which differentiated SC provide.

a. Fusion-dependent roles of satellite cells

The increased demand for MPS after exercise is not only required for repair but for muscle growth. The myonuclear domain theory suggests that individual myonuclei have a threshold for synthetic capacity and are transcriptionally responsible for specific cytoplasmic volumes [83]. This theory postulates that hypertrophy can initially be addressed with an increase in nuclear synthetic capacity to increase cytoplasmic volume; however, further growth must be compensated for by increasing myonuclear content as supplied through SC nuclear donation (Fig. 2) [84]. It is speculated that the increase in nuclear content is required to meet the transcriptional demand for MPS to maximize hypertrophy [85]. With resistance exercise training, there is an increase in myonuclear content and myonuclear domain [86].

The SC's remarkable capacity for skeletal muscle regeneration through fusion events has been repeatedly demonstrated. SC fusion to generate new myofibers has been notably demonstrated through experimentation on irradiated immunodeficient dystrophic mice. The transplantation of a single healthy SC to these SC deficient mice can induce *de novo* myofibre development through SC proliferation and differentiation, consequently increasing the SC pool as well [87]. SC fusion is heavily influenced by the increase in circulating factors such as interleukin-6, follistatin, angiopoietin, testosterone, and growth factors during and after exercise [88–91]. While initial studies on muscle development and fusion identified the formation of a fusion pore through cytoskeletal remodeling in *Drosophila,* the mechanism of myoblast fusion in human skeletal muscle remains unclear [92]. Although many membrane-associated factors such as actin regulators, cadherins, integrins, and caveolins may be important in muscle development, myomaker has been identified as a crucial transmembrane protein involved in embryonic skeletal muscle formation and adult fusion-mediated hypertrophy [93–98]. Myomaker is predominantly expressed on the cell membrane but also localizes to intracellular vesicles, the endoplasmic reticulum, and the Golgi apparatus [99]. Regulation of myomaker is still largely unknown, however, chromatin immunoprecipitation sequencing has identified high binding affinity of MyoD and myogenin to the promoter region of the myomaker encoding gene, *Mymk,* during differentiation [100]. *In vitro* culture of myoblasts isolated from myomaker null embryos display a reduced fusion index compared to wild type myoblasts [93].

Additionally, cardiotoxin-induced skeletal muscle injury in adult mice augments myomaker expression during regeneration and is absent after myofibre formation and repair [100]. The emerging data suggests that myomaker is crucial for forming skeletal muscle during embryonic development, for fusion-mediated repair, and regeneration in adult skeletal muscle.

However, it does not appear to regulate myoblast differentiation or the progression of the myogenic program [92].

Myomixer is another recently discovered micropeptide that has also been implicated in the skeletal muscle fusion processes [101]. Its expression patterns and transcriptional regulation seem to mirror that of myomaker. Genetically modified mice lacking Myomixer expression display a similar muscle phenotype and lack of multinucleated myofibre development as Myomaker deficient mice [101]. Additionally, conditional deletion of Myomixer in SCs results in a lack of regenerative fibres identified through centralized nuclei, lower muscle mass, and a loss of muscle cells after cardiotoxin injury [102] . Co-immunoprecipitation has elucidated Myomaker binding to Myomixer; however, their interaction is not required for myoblast fusion, and both proteins function independently during this process [103]. Experimentation with a lipid-labeled population of C2C12s exposed to an unlabeled population has identified Myomaker as crucial for hemifusion (mixing membrane lipids of fusing cells). Myomixer regulates membrane fusion pore expansion by generating mechanical stress [103]. Myomixer-deficient myoblasts undergo lipid mixing but cannot complete myoblast fusion, implicating its downstream function in fusion progression [103]. While it is clear that fusion regulatory factors such as Myomixer and Myomaker are crucial for skeletal muscle development and repair, it is still worth exploring alternative pathways involved in fusion and fusion-independent processes to assess muscle regeneration as a whole.

b. Satellite cell participation in intercellular communication

A vast array of literature characterizes the various cell types that contribute to skeletal muscle regeneration through intercellular signaling. Evidence has demonstrated that muscle releases chemokines that recruit inflammatory cell types into the muscle microenvironment. CC chemokine receptor 2 (CCR2) has been identified as a crucial signaling molecule required to

promote monocyte infiltration, which later differentiate into pro-inflammatory macrophages [104]. CCR2 deficiency in mice has demonstrated a profound impact on muscle regeneration, decreased myofiber size, and reduced macrophage presence [104]. Moreover, these same pro-inflammatory macrophages secrete interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α), which have been shown to promote SC proliferation during regeneration [105,106]. Non-immune cells such as fibro-adipogenic progenitor cells also increase in response to muscle damage and have been implicated as regulators of SC differentiation through IL-6 and insulin growth factor 1 (IGF-1) [107]. There is abundant evidence demonstrating that intercellular signaling occurs to recruit non-muscle cells to sites of muscle damage and that these cells further signal toward SCs to promote regeneration. However, research into SC paracrine signaling towards other cell populations is limited and thus requires further investigation.

While the primary role of SCs is to facilitate myofiber regeneration through fusion events and myonuclear donation, some evidence suggests SCs also contribute to paracrine signaling. Extracellular vesicles (EVs) have been of recent interest in characterizing paracrine signaling. EVs have been widely accepted as lipid membrane-enclosed cargo-carrying units for nucleic acids, proteins, and metabolites, and can be broadly classified by their size and formation process. As SCs are heavily involved in skeletal muscle adaptation, it is important to understand their paracrine contributions to the skeletal muscle microenvironment [108].

SCs have been shown to release EVs taken up by mouse myofibres during load-induced hypertrophy [109]. Lineage tracing utilized in fusion incompetent SCs co-cultured with myotubes demonstrated the increase of SC-derived markers within myotubes during load-induced hypertrophy, indicating the uptake of SC-released EVs [109]. Further profiling of mouse muscle tissue discovered that these extracellular vesicles might be involved in transporting miRNAs that

regulate matrix metalloprotein 9 (*Mmp9)* expression, which is important for ECM remodeling during anabolic stimuli [109]. This data broadly identifies a role for SC paracrine signaling in regulating the skeletal muscle contributions to the ECM.

Of late, research into exosome secretions, the smallest sub-population of EVs, from muscle progenitor cells (MPCs) has identified a role for these cells in extracellular signaling to adjacent, namely fibrogenic, cells. Evidence has shown an increase in muscle extracellular matrix (ECM) deposition after mechanical overload, during repair, and aging when SC are depleted from skeletal muscle [110–112]. The depletion of SC in mice undergoing hypertrophic mechanical overload demonstrated an increase in fibrotic tissue and a blunted hypertrophic response due to a lack of exosomal miR-206 release [111]. miR-206 has been implicated in MPC-to-fibrogenic cell communication where it inhibits retinol-binding protein 1 (Rbp1), a master regulator of collagen biosynthesis, the loss of which results in excessive collagen deposition in the skeletal muscle microenvironment [111]. SC depletion has also been shown to reduce the expansion of transcription factor 4 (Tcf4) positive fibroblasts, and the reciprocal depletion of these fibroblasts resulted in reduced SC differentiation and skeletal muscle embryonic myosin heavy chain (eMyHC) expression in regenerating muscle [112]. Together, the data suggests that MPCs can directly impact fibrogenic cell function and expansion by releasing secretory factors, and other cell populations can influence SC function. It is evident that SC are extremely important for muscle hypertrophy and recovery through fusion-mediated events and that their paracrine signaling influences skeletal muscle fibrosis and ECM coordination; however, their fusion-independent role in myofiber communication in response to a damaging stimulus remains to be elucidated.

There are still several gaps in the literature as to the role of paracrine signaling by muscle progenitor cells during damage to muscle tissue. Identifying feasible and efficient models to

demonstrate the effects of this paracrine signaling is required to provide insight into the role of muscle progenitor cells in muscle regeneration. Further, examining the impact of muscle progenitor cell-mediated paracrine signaling on specific proteins involved in muscle hypertrophy, cytoskeletal remodeling, and necrosis is important to characterize the entire muscle regeneration process and highlight the specific pathways affected.

Electrical pulse stimulation as an *in vitro* model of exercise

It is evident that physical activity is a potent intervention against several states of muscular impairment, however, the exact mechanisms involved under different conditions are difficult to ascertain. *In vitro* experimentation allows for directly examining relationships of distinct criteria without the possibility of interference by other biological systems and tissues. It provides complete control when identifying important relationships prior to committing to more extensive *in vivo* experimentation.

While all the mechanisms implicated in exercise-induced adaptations cannot be addressed by one particular *in vitro* model, these experiments aimed to develop a cell culture model resembling aspects of the *in vivo* muscle microenvironment after exercise-induced muscle damage. Part of this process includes accounting for important signaling factors widely accepted as "exercise-related". For example, skeletal muscle contraction is regulated by intracellular calcium ion concentrations, changes in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and glucose transporter type 4 (GLUT4) transcription and translation, increased AMPK signaling, mTOR signaling, and morphological changes such as myofiber hypertrophy [113]. Electrical pulse stimulation (EPS) is one of the few commonly used *in vitro* models of exercise that induce skeletal muscle cell contraction.

EPS has been utilized as an *in vitro* model of muscle contraction by replacing neuronal activation to stimulate myotube contraction, however, the accuracy in imitating specific exercise modalities has yet to be verified [114]. Despite this, there is some agreement that short-term, high-frequency stimulation may mimic an acute high-intensity bout of resistance exercise resulting in greater glucose uptake, lactate production and decreased cellular ATP and phosphocreatine in cultured myotubes to levels comparable to *in vivo* exercise [115]. Experimentation with chronic low-frequency stimulation demonstrates the reorganization of the cytoskeleton and new sarcomeric structures in myotubes [115,116]. Further protocols have successfully inflicted visible, physical damage to myotubes in culture through the development of EPS-induced lesions, as identified through positive staining of xin – a sarcomeric damage marker [117]. The administration of EPS following a protocol delivering a 5s tetanic hold during continuous pulses at 15 Hz, 5 seconds delay, 5 seconds of pulses at 5 Hz followed by another 5 seconds delay caused focal lesion formation almost immediately after a stimulus is delivered, which continue to grow in size over time [117]. Altogether, these findings suggest that EPS may accurately recapitulate aspects of exercise through an electrically stimulated contraction.

Study objectives and hypotheses

A plethora of evidence supports the fusion-dependent role of SC in skeletal muscle size and repair. However, the role of these myogenic cells through paracrine signaling remains to be fully elucidated. Therefore, the objective of this study was to investigate the potential role of secretory factors from skeletal muscle myoblasts to determine if fusion-independent mechanisms broadly participate in damage recovery or in the reduction of cellular stress *in vitro.* We sought to characterize the morphological and physiological adaptations associated with EPS-induced

myotube contraction and damage and the concomitant effects of myoblast paracrine signaling thereafter.

It was hypothesized that after 1 hour of electrical pulse stimulation, myotubes in proximity to muscle progenitor cells would:

1) Increase the expression of stress-response and repair-associated factors between stimulation groups.

2) Increase stress-response and repair-associated genes and proteins after the stimulus was administered and as myotubes continued to incubate over time, both within and between stimulation groups, compared to groups not near muscle progenitor cells.

3) Protect the structural morphology compared to groups not near muscle progenitor cells.

METHODS

General cell culture

C2C12 myoblasts were cultured in growth medium (GM) containing Dulbecco's Modified Eagle Medium (DMEM) High Glucose 1x (Thermo Fisher Scientific; 11965092) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen; 12483020), and 1% penicillin-streptomycin (Invitrogen; 15140122). Cells were allowed to adhere and grow to 60% confluency within a 37˚C incubator at 5% CO_2 before passaging. Following a 1x PBS wash, 1x trypsin (Invitrogen; 15090046) was added for 3 min at 37˚C. One volume of GM was added to neutralize the trypsin before cells were collected and centrifuged at 200g for 5 min at 20˚C. The supernatant was then replaced with fresh GM, and cells were plated in 6-well plates. For myoblast differentiation, cultures were plated at 200,000 cells/well and allowed to grow to 100% confluency in GM within 2 days, after which the media was changed to differentiation medium (DM) composed of DMEM supplemented with 2% Horse Serum (Invitrogen; 16050122) and 1% penicillin/streptomycin. DM was changed every 48 hours for 6 days prior to experimentation.

Transwell experiments

Cells were plated and differentiated as described above. Myoblasts were plated at 20,000 cells per transwell insert sized to fit 6-well plates (Thermo Fisher Scientific, 140660) and incubated with GM for twelve hours prior to differentiation day 6. On the day of experimentation, electrical pulse stimulation (EPS) was administered to the differentiated myotubes with the C-Pace EM (IonOptix) at 23V with 15Hz pulse for 5s and 5Hz pulse for 5s, separated by a 5s break. After 1 hour of EPS, electrodes were removed, and the transwell inserts were immediately inserted for 1, 3, 8, 12, or 24 hours to allow for intercellular signaling without fusion. The 0-hr time point was collected immediately following the EPS protocol. Control groups underwent the same

intervention either without stimulation and/or using transwell inserts only containing GM without cells.

Immunocytochemistry

Experiments were conducted as previously stated, however, 6-well plates contained coverslips prior to myoblast plating. Myotubes were washed 3 times with 1x PBS prior to a 10-minute fixation period with 4% paraformaldehyde (PFA, Sigma-Aldrich; P6148-500G). Myotubes were again washed with 1x PBS and permeabilized for 15 minutes with 1% BSA (BioShop Canada; ALB007.500), 0.1% Triton X-100 (Sigma-Aldrich; X100-1L) in 1x PBS. The permeabilization solution was removed, and coverslips were washed for 5 minutes, 3 times with 1x PBS prior to a 1-hour block in 5% goat serum (Sigma-Aldrich; G9023-10ML), 0.1% Tween-20 (Thermo Fisher Scientific; BP337500) in 1x PBS (1x PBST). Myotubes were incubated overnight at 4˚C in calpain 3 primary antibody (Table 1), diluted to 1:50 in a 1% BSA in 1x PBST solution. The next day, myotubes were washed in 1X PBS and incubated for 2 hours at room temperature with Alexa-594 goat anti-mouse secondary antibody (1:500, Thermo Fisher Scientific; A11005). For myosin heavy chain I (MHCI) staining, myotubes were washed in 1X PBS, re-fixed in 4% PFA for 10 minutes and incubated overnight in MHCI (neat; DSHB; clone 5.8, mouse) at 4°C. Myotubes were washed in 1X PBST, and incubated in the appropriate secondary antibody. 4′,6-diamidino-2-phenylindole (DAPI, 1:50,000) was applied prior to mounting slides using fluorescent mounting medium (Dako; S3023) on microscope slides (Corning; 294875X25). Images were captured using the Nikon Eclipse Ti2 at 10x magnification. NIS thresholding analysis was used to determine myotube and nuclear-specific calpain 3 expression. For each sample, the diameter of approximately 300 myotubes was evaluated by

averaging the length of 5 lines drawn across the width of each myotube. Bifurcations were considered new myotubes.

Protein isolation and media collection

Myotubes were washed with ice-cold 1x PBS. RIPA buffer (Sigma-Aldrich; R0278-500ML) containing phosphatase (Sigma-Aldrich; P5726-1ML) and protease inhibitors (Thermo Fisher Scientific; A32965) was added to myotubes and flash-frozen in a shallow liquid nitrogen bath prior to storage at -80°C. All time points within the same experiment were then processed together. After thawing on ice, samples were sonicated for 5 seconds with the Branson SFX150 Ultrasonic Processor (Cole-Parmer) 3 times, followed by further agitation with FastPrep-24 (MP Biomedicals). Samples were centrifuged at 14,000 x g for 15 minutes, and the supernatant was transferred to new 1.5ml microcentrifuge tubes for protein quantification and western blot sample preparation.

Protein quantification and western blotting

The Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific cat: 23225) was used to measure protein content per the manufacturer's instructions. All protein lysates were then diluted to 1μg/μl, and 10μg of each sample was loaded into individual lanes of a 4-15% Criterion TGX precast protein gel (Bio-Rad Laboratories; 5678085). Following protein separation via electrophoresis, gels were activated using the ChemiDoc MP Imaging System (Bio-Rad Laboratories), followed by protein transfer to a nitrocellulose membrane (Bio-Rad Laboratories; 1620115). Stain-free imaging of nitrocellulose membranes was performed to visualize loading normalization, and Ponceau S solution (Sigma-Aldrich; P7170) was performed for band visualization. Blots were then washed to remove ponceau and placed in blocking solution (5%

BSA in 1x TBST) for 1 hour prior to overnight incubation at 4°C with the relevant primary antibody (Table 1). Blots were washed in 1x TBST prior to a 2-hour incubation at room temperature in the appropriate diluted secondary antibody. Luminol-based ECL reagent (Bio-Rad Laboratories; 1705062) was used for band visualization with the ChemiDoc MP Imaging System. All images were quantified using Image Lab software, version 6.1.0 (Bio-Rad Laboratories). Bands were normalized to total protein obtained from stain-free images or ponceau.

Table 1: Primary antibodies used for western blotting

Primary Antibody	Source	Catalog Number	Secondary Antibody
Calpain 3	Santa Cruz Biotechnology	sc-365277	Mouse
Hsp70	Santa Cruz Biotechnology	sc-24	Mouse
β-dystroglycan	Developmental Studies Hybridoma Bank	MANDAG2(7D11)	Mouse
p-ULK^{Ser555}	Cell Signaling Technology	5869S	Rabbit
t-ULK	Cell Signaling Technology	8054S	Rabbit
p-mTOR^{Ser2448}	Cell Signaling Technology	2971S	Rabbit
t-mTOR	Cell Signaling Technology	2972S	Rabbit
p-ACC^{Ser79}	Cell Signaling Technology	3661S	Rabbit
t-ACC	Cell Signaling Technology	3676S	Rabbit
Desmin	Abcam	ab32362	Rabbit
p-4e-bp1$^{Thr37/46}$	Cell Signaling Technology	2855S	Rabbit
t-4e-bp1	Cell Signaling Technology	9452S	Rabbit

RNA isolation, cDNA synthesis, and qRT-PCR

After the EPS and unstimulated protocols were completed, myotubes were washed with ice-cold 1x PBS 3 x for 5 minutes each. Samples were collected and processed as suggested by the E.Z.N.A Total RNA Kit I (VWR; R6834-02). Isolated RNA was stored at -80°C until further processing. RNA content was quantified using the Nanodrop Spectrophotometer. For cDNA synthesis, High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific; 4368814)

was used according to the manufacturer's instructions. PCR reactions using Taqman assay probes were performed with Taqman Master Mix (Fisher Scientific; 44-445-57) and the relevant FAM assay mix. PCR reactions using primers were performed with GoTaq Master Mix (Manufacturer) and 5μM of forward and reverse primers. All assays and primers used are listed in Table 2.

Table 2: Primers and assays used for qPCR

Gene Primer	**Source**	**Forward 5'-3'**	**Reverse 5'-3'**
18S	Sigma	CGCCGCTAGAGG-TGAAATC	CCAGTCGGCAT-CGTTTATGG
β-Actin	Sigma-Genosys	GACAGGATGCAG-AAGGAGATTACT	TGATCCACATC-TGCTGGAAGGT
Taqman Assay	**Source**	**Lot**	**Assay ID**
Hspa1a	Applied Biosystems	2042101	Mm01159846_s1
Clpn3	Applied Biosystems	1925544	Mm00482985_m1

Creatine kinase assay

Conditioned media collected from the transwell experiments were centrifuged at 200g for 5 min to pellet cellular debris, after which the supernatant was transferred to new microcentrifuge tubes and stored at -80°C. Creatine kinase activity in the cell culture media was assessed using the colorimetric Creatine Kinase Activity Assay Kit (Abcam; ab155091) per manufacturer instructions.

Transmission electron microscopy

Myoblasts were plated and differentiated as previously described under 'Transwell Experiments.' After the EPS and unstimulated protocols were completed, myotubes were washed with 1x PBS 3 x for 5 minutes each. The monolayers were then fixed with 2% glutaraldehyde and

kept at 4°C for further processing at the Canadian Centre for Electron Microscopy (CCEM) at McMaster University.

Statistics

Data were analyzed using GraphPad Prism 8.0.2. A two-way ANOVA was used for all outcomes when comparing MBL+ and MBL- cells within conditions (NS or EPS). A three-way ANOVA was used for calpain 3 fluorescent intensity and myotube diameter, followed by a two-way ANOVA for each condition (NS or EPS). Analyses utilized the Tukey post hoc, and $p < 0.05$ was deemed statistically significant.

RESULTS

Myotube recovery after electrical pulse stimulation (EPS)–induced damage was improved with myoblast exposure

The aim of this project was to develop an *in vitro* model of muscle progenitor cell paracrine signaling and investigate its effects on damaged muscle tissues. To inflict damage, fully differentiated C2C12 myotubes were subjected to electrical pulse stimulation (EPS) for 1 hour. Unstimulated control samples (NS) underwent 1 hour of incubation with disconnected EPS electrodes. Immediately after stimulation, transwell inserts that contained adhered myoblasts (MBL+) or no cells (MBL-) were introduced to the myotubes (Fig. 3A). Myotube damage was identified by the presence of Z-band streaming, as visualized through transmission electron microscopy (Fig. 3B). Immediately after EPS, the sarcomeres within the EPS group displayed obvious signs of contraction-mediated stress as Z-band streaming was evident. At the same time, the NS condition did not show the same degree of sarcomeric disorganization.

The presence of creatine kinase (CK) in the media was used as an indirect marker of myotube damage, as the cytosolic protein is highly elevated in blood serum after extraneous exercise due to increased muscle membrane permeability. Following the same protocol, media was collected at the 0- 1-, 8-, and 12-hour time points post-EPS to measure CK activity. Significant differences over time in both the MBL+ and MBL- groups were observed where the 0-, 1-, and 8 hour time points post-EPS had lower CK activity in comparison to the 12-hour time point ($p<0.05$, Fig. 3C). Additionally, there were significant differences between MBL+ and MBL- groups at the 8- and 12-hours post-EPS ($p<0.05$, Fig.3C).

As exercise and other stimuli that result in skeletal muscle damage often increase intracellular stress and thus the expression of chaperone proteins to assist with protein folding, Hsp70 protein content and gene expression were assessed. Expression of the Hsp70 encoding gene *Hspa1a* showed a main effect of time ($p<0.05$, Fig. 3G), indicating that EPS appeared to increase *Hspa1a* expression over time in both MBL groups. Additionally, there was a significant main effect of MBL presence ($p < 0.05$) such that regardless of time point, MBL+ conditions had greater *Hspa1a* expression. Hsp70 protein content, as determined through western blotting, did not change significantly between MBL+ and MBL- groups under the NS condition (Fig. 3D,F); however, under the EPS condition did result in a main effect of time where protein expression seemed to decrease ($p<0.05$, Fig. 3G). These results show that MBL exposure may increase protein chaperone expression in response to damaging stimuli.

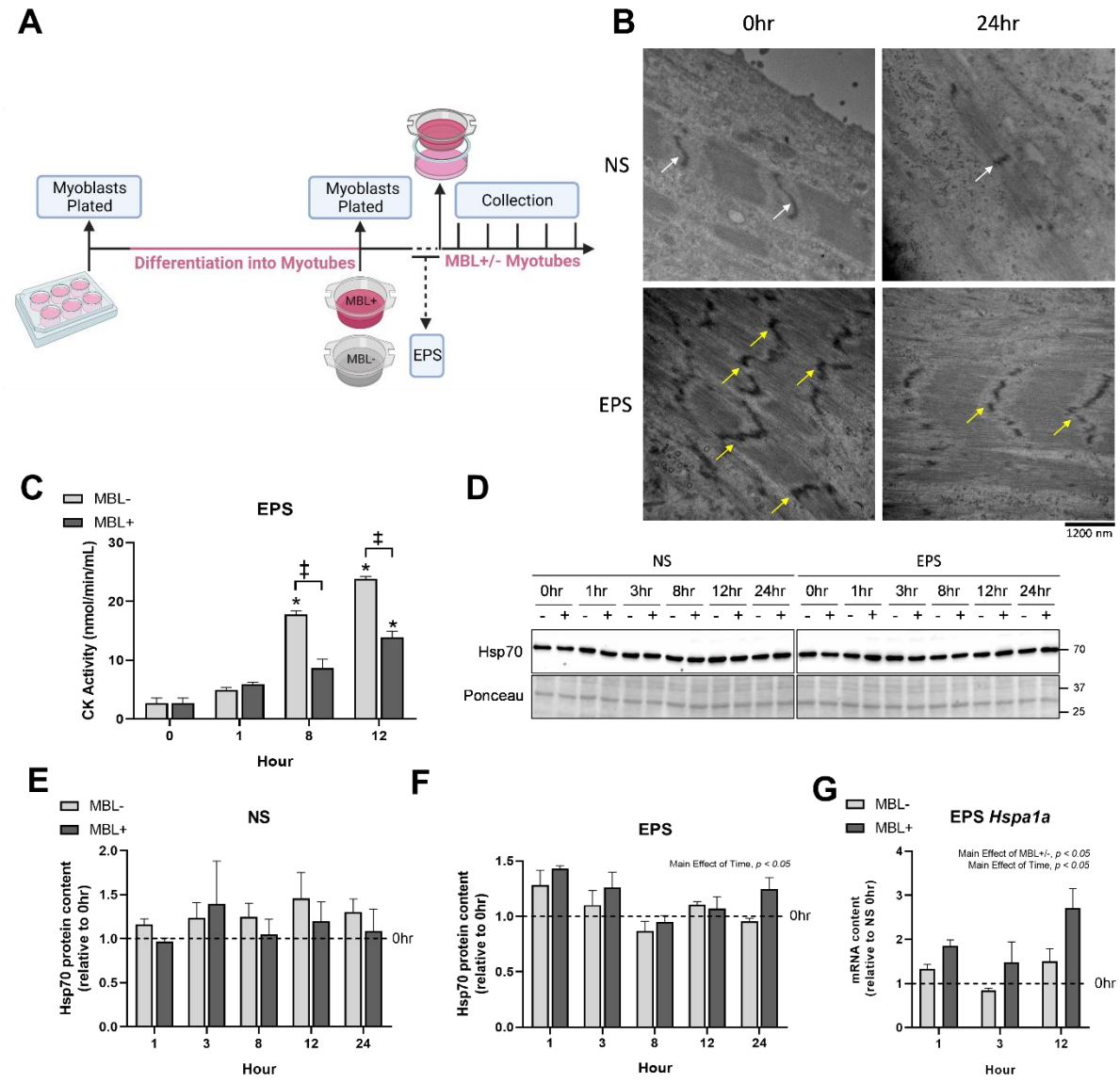

Figure 3. Myotube recovery after electrical pulse stimulation (EPS)–induced damage is improved with myoblast exposure. A) A visual schematic of the study design. Differentiated myotubes and media were collected following 1 hour of EPS or unstimulated incubation (NS) in fresh growth media. Transwells with (MBL+) or without (MBL-) myoblasts were inserted into the culture at 0-hours post-EPS, and cell lysate was collected at various time points thereafter. **B)** Transmission electron microscopy images of unstimulated C2C12 myotubes and immediately after and 24hr post-EPS. White arrows identify an intact Z line, and yellow arrows denote Z line streaming. Scale bar = 1200µm. **(C)** Graphical summaries of creatine kinase (CK) activity in MBL+ and MBL- myotubes following EPS **D)** Representative western blot of heat shock protein 70 (Hsp70) expression. A Ponceau stain is displayed below to indicate equal loading. **(E & F)** Summary of Hsp70 protein content in MBL+ and MBL-, NS (**E)** and EPS **(F)** myotubes expressed relative to their 0hr timepoint. **G)** *Heat shock protein family A member 1A* (*Hspa1a*) mRNA content in MBL+ and MBL- EPS myotubes, normalized to NS 0hr. n = 3. Two-way ANOVA. * vs 0hr within group $p<0.05$, ‡ between group difference $p<0.05$.

Myoblast exposure to myotubes may not affect sarcolemmal integrity post-EPS

It was next prudent to assess whether other membrane-associated proteins were affected by EPS and by MBL exposure, as their increased presence is often associated with greater membrane stability. Western blotting identified no significant differences between MBL+ and MBL- in β-dystroglycan protein content under the NS condition (Fig. 4B). A main effect of time was observed where desmin protein expression tended to decrease ($p<0.05$, Fig. 4D). When EPS was administered to myotubes, β-dystroglycan decreased significantly over time in the MBL- group where 24 hr differed from 1 hour ($p<0.05$, Fig. 4C) however desmin protein expression was not different between MBL+ and MBL- groups (Fig. 4E). The data demonstrates no effect of MBL exposure on select membrane stability-associated markers.

Calpain 3, transcriptional and translational response may differ with the presence of myoblasts

The evidence of sarcomeric damage after EPS indicated a potential for increased myotube membrane permeability. As such, calpain-3 was assessed to determine sarcolemmal integrity since its proteolytic activity is associated with membrane patch repair. The expression of the calpain 3 encoding gene *Clpn3* showed a main effect of time where EPS appeared to decrease *Clnp3* expression ($p<0.05$, Fig. 5D). Further, calpain 3 protein content, as identified through western blotting (Fig. 5A), demonstrated no significant differences between MBL+ and MBL- groups under the NS condition (Fig. 5B) however under the EPS condition did result in a main effect of MBL exposure where calpain 3 protein expression increased ($p<0.05$, Fig. 5C). Complementary calpain 3 immunofluorescent staining was done (Fig. 5E), however, both the total fluorescent intensity for calpain 3 in myotubes (Fig. 5F) and the myonuclear associated calpain 3 fluorescence

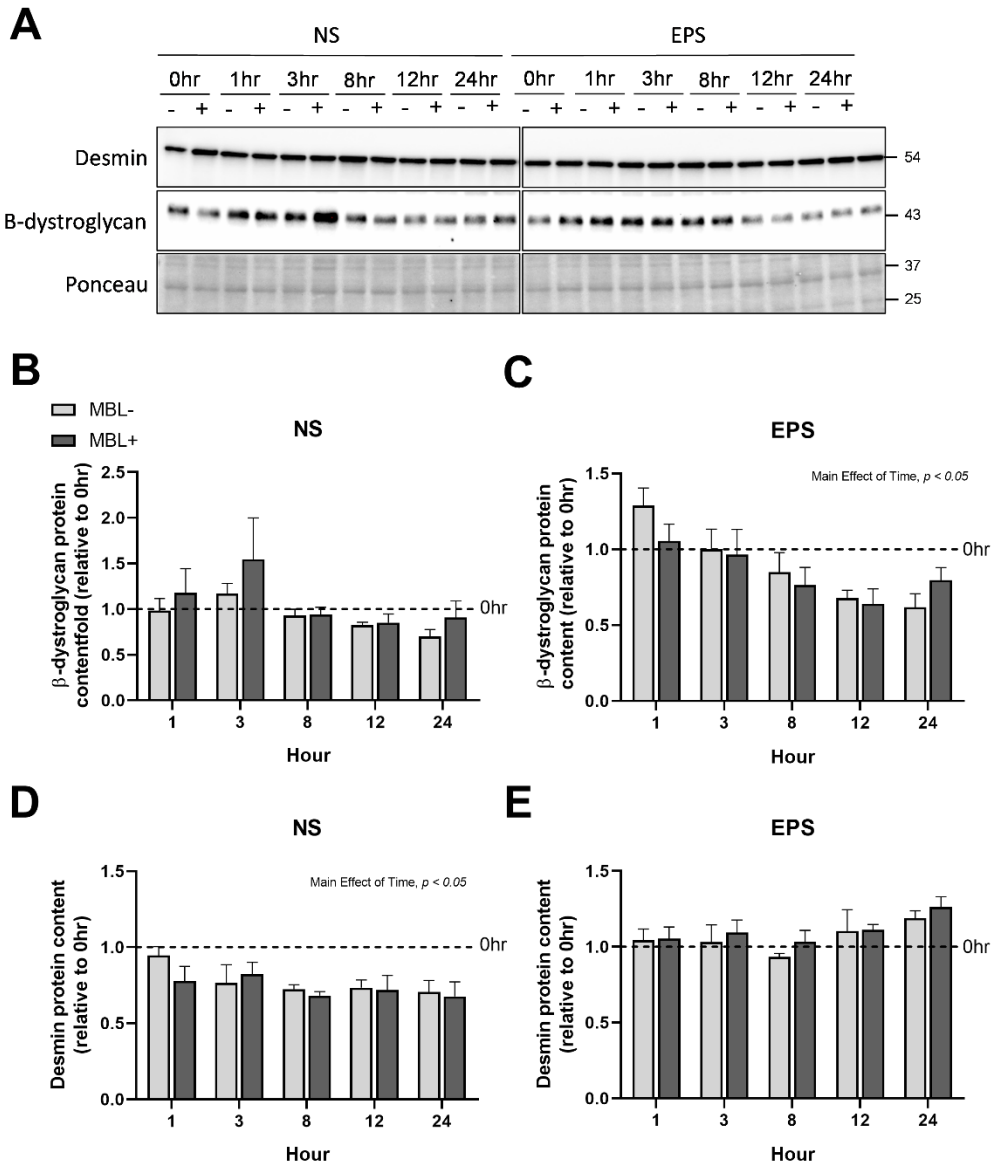

Figure 4: Myoblast exposure to myotubes does not affect sarcolemma-associated proteins after EPS. (A) Representative western blot of desmin and β-dystroglycan protein content. A Ponceau stain is displayed below to indicate equal loading. **(B & C)** β-dystroglycan protein content in MBL+ and MBL- NS **(B)** and EPS **(C)** myotubes. **(D & E)** Desmin protein content in MBL+ and MBL- NS **(D)** and EPS myotubes. All bar graphs are expressed relative to the appropriate 0hr timepoint. n = 3. Two-way ANOVA.

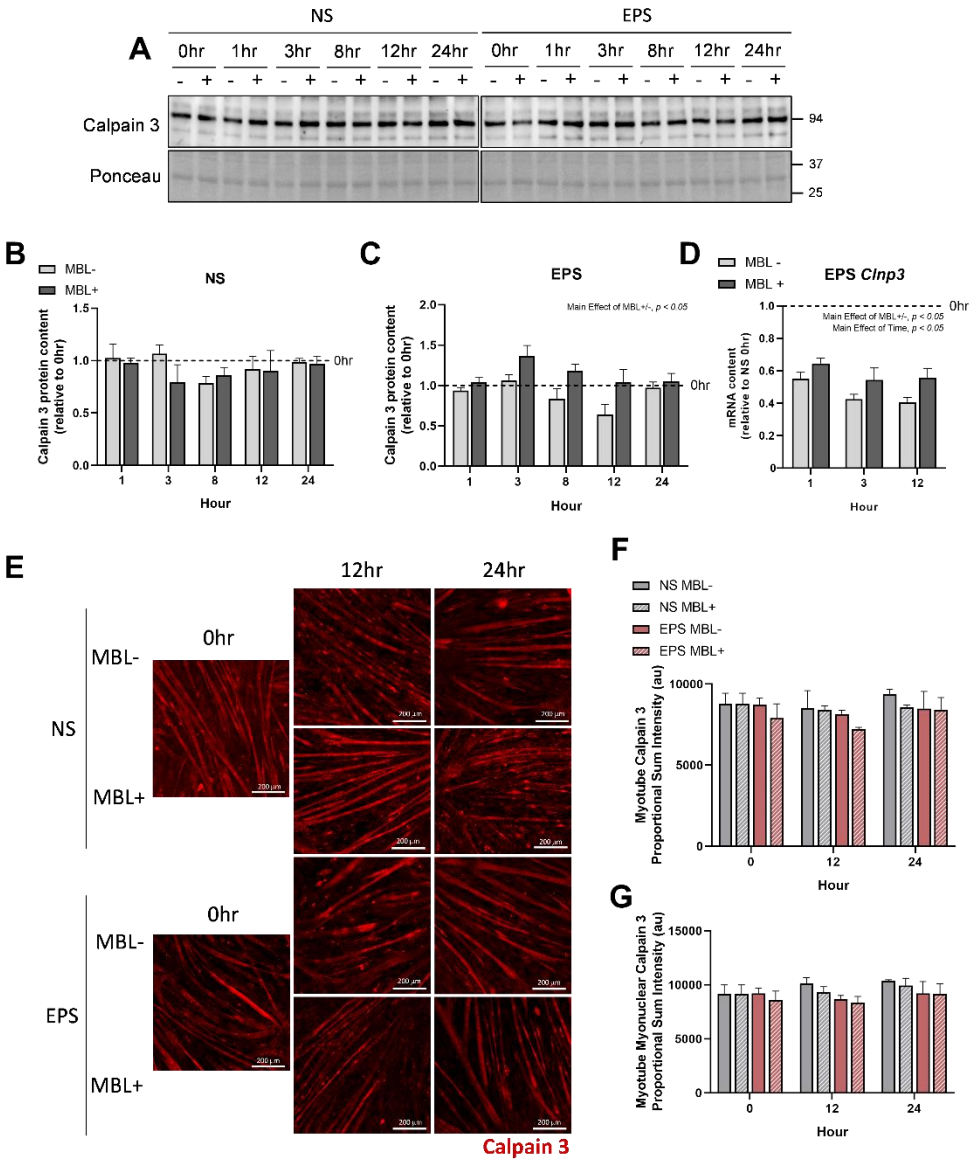

Figure 5: Calpain-3 transcriptional and translational response to EPS may differ with the presence of myoblasts. **A)** Representative western blot of Calpain-3 protein content. A Ponceau stain is displayed below to indicate equal loading. **(B & C)** Summary of Hsp70 protein content in MBL+ and MBL- NS **(B)** and EPS **(C)** myotubes expressed relative to the appropriate 0hr timepoint. **(D)** *Calpain 3 (Clnp3)* mRNA content in MBL+ and MBL- EPS myotubes normalized to NS 0hr. **(E)** Representative immunofluorescence images of calpain 3 (red) in MBL+ and MBL-, NS and EPS myotubes. Scale bar = 200µm. **(F)** Myotube-associated calpain 3 immunofluorescent intensity proportional to myotube surface area. **(G)** Myonuclear calpain 3 immunofluorescent intensity proportional to DAPI surface area. n = 3. Two-way ANOVA, Three-way ANOVA.

(Fig. 5G) were not significantly different between the NS and EPS conditions, nor the associated MBL+ or MBL- groups. Together, MBL exposure may subtly increase calcium-dependent proteolysis for remodeling in response to EPS.

mTOR signaling and myotube diameter are affected by EPS

Previous work with different EPS protocols has demonstrated hypertrophic effects correlated with increased mammalian target of rapamycin (mTOR) protein content and activity. To determine if the current EPS protocol resulted in increased muscle protein synthesis, and if MBL exposure could further mediate this process, the expression of muscle protein synthesis markers, mTOR and its downstream target 4e-binding protein 1 (4e-bp1), were compared through western blotting (Fig.6A). Under the NS conditions, there was no effect of time or MBL exposure in protein content of phosphorylated-mTOR (p-mTOR^{Ser2448}), total-mTOR (t-mTOR), phosphorylated-4e-bp1$^{Thr37/46}$ (p-4e-bp1$^{Thr37/46}$), or total-4e-bp1 (t-4e-bp1; Fig. 6B,D,F,H). When EPS was administered, only p-mTOR^{Ser2448} tended to decrease over time regardless of MBL presence ($p < 0.05$; Fig. 6C), while t-mTOR, p-4e-bp1$^{Thr37/46}$, and t-4e-bp1 remained unchanged (Fig. 6E, G, I). Myotube diameter was assessed as a measure of hypertrophy (Fig. 6J) and MBL+ EPS myotubes showed a significant decrease in average myotube diameter 12 hours after EPS compared to immediately post-EPS, followed by a recovery in size 24 hours later (Fig. 6K). Together, mTOR-mediated processes are negatively regulated by EPS; however, they may not be further influenced by MBL.

Myoblast exposure may increase autophagy and β-oxidation in electrically stimulated myotubes

As MBL exposure did not seem to influence anabolic processes through mTOR signaling, but as catabolic calpain 3 protease activity seemed to be affected and myotube diameter decreased in the EPS MBL+ group, further insight into catabolism was prudent. Catabolism plays a crucial role in skeletal muscle remodeling *in* vivo, as it allows for the clearance of dysfunctional proteins and organelles through autophagic processes. As such, western blotting of Unc-51, like autophagy-

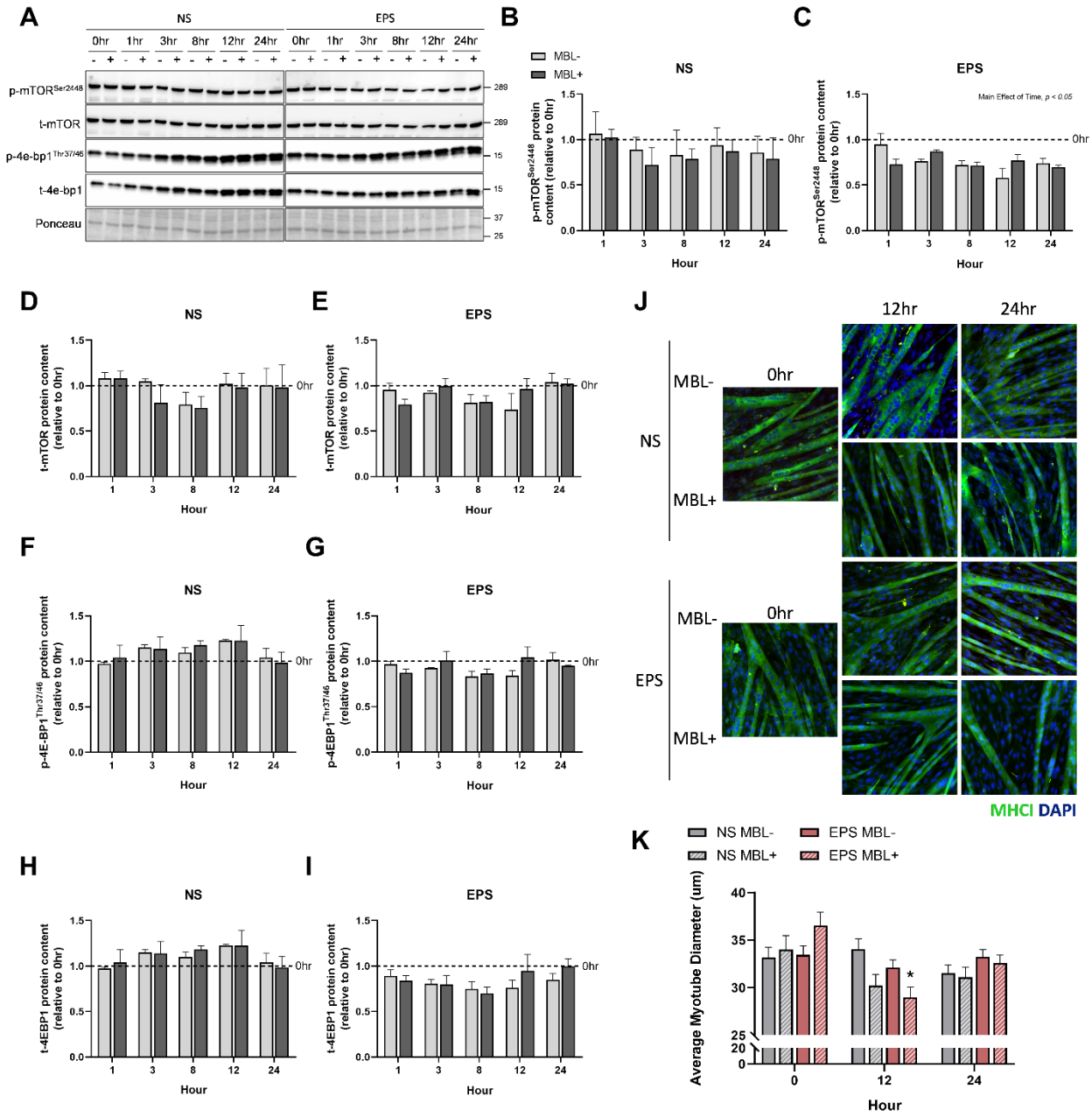

Figure 6: mTOR signaling and myotube diameter are affected by EPS. (A) Representative western blot of phosphorylated mammalian target of rapamycin (p-mTOR^{Ser2448}), total (t)-mTOR), phosphorylated 4e-binding protein 1 (p-4e-bp1$^{Thr37/46}$), and total (t) - 4e-bp1. **(B & C)** p-mTOR^{Ser2448} protein content in MBL+ and MBL-, NS **(B)** and EPS **(C)** myotubes. **(D & E)** t-mTOR protein content in MBL+ and MBL-, NS **(D)** and EPS **(E)** myotubes. **(F & G)** p-4e-bp1$^{Thr37/46}$ protein content in MBL+ and MBL- NS **(F)** and EPS **(G)** myotubes. **(H & I)** t-4e-bp1 protein content in MBL+ and MBL- NS **(H)** and EPS **(I)** myotubes. All bar graphs derived from western blotting are expressed relative to the appropriate 0hr timepoint **(J)** Representative images of myosin heavy chain I (MHCI; green) to outline myotubes and DAPI (blue) to denote myonuclei in MBL+ and MBL-, NS and EPS myotubes. Scale bar = 100 μm. **(K)** Average diameter of the top 25 largest myotubes per sample. n = 3. Two-way ANOVA, Three-way ANOVA.

activating kinase (ULK) was assessed as a catabolic marker. A significant main effect of time was observed in both incubation conditions where activated p-ULK^{Ser555} decreased 3-hours post-EPS ($p<0.05$, Fig 7B-C). A significant main effect of MBL exposure in the EPS myotubes was also evident, where the MBL+ condition increases p-ULK^{Ser555} protein content ($p<0.05$, Fig. 7C). Significant changes in time were observed in total ULK (t-ULK) protein content in both the NS and EPS myotubes where the highest expression was seen 24-hours post-incubation ($p<0.05$, Fig. 7D-E). A significant main effect of MBL exposure in EPS myotubes was observed where t-ULK increased when MBL were present ($p<0.05$, Fig 7E). The data suggest that MBL exposure may increase autophagic processes in response to EPS-induced damage.

Finally, as myotubes predominantly rely on oxidative phosphorylation for energy, it was of interest to investigate acetyl coA carboxylase (ACC) protein content (Fig. 7A), as it is involved in regulating fatty acid oxidation. A main effect of MBL exposure on activated p-ACC^{Ser79} protein levels was observed in both conditions, where MBL exposure decreased protein levels in the NS myotubes (Fig. 7F), but increased p-ACC^{Ser79} in the EPS myotubes (Fig. 7G). There were significant differences over time where the EPS myotubes expressed higher levels of p-ACC^{Ser79} 8- and 24-hours post-EPS in comparison to 0-hours ($p<0.05$, Fig. 7G). Total-ACC (t-ACC) protein levels shared similar trends to p-ACC^{Ser79} (Fig.7H-I). Our data demonstrate the potential for increases in fatty acid oxidation in response to EPS damage when MBL are present.

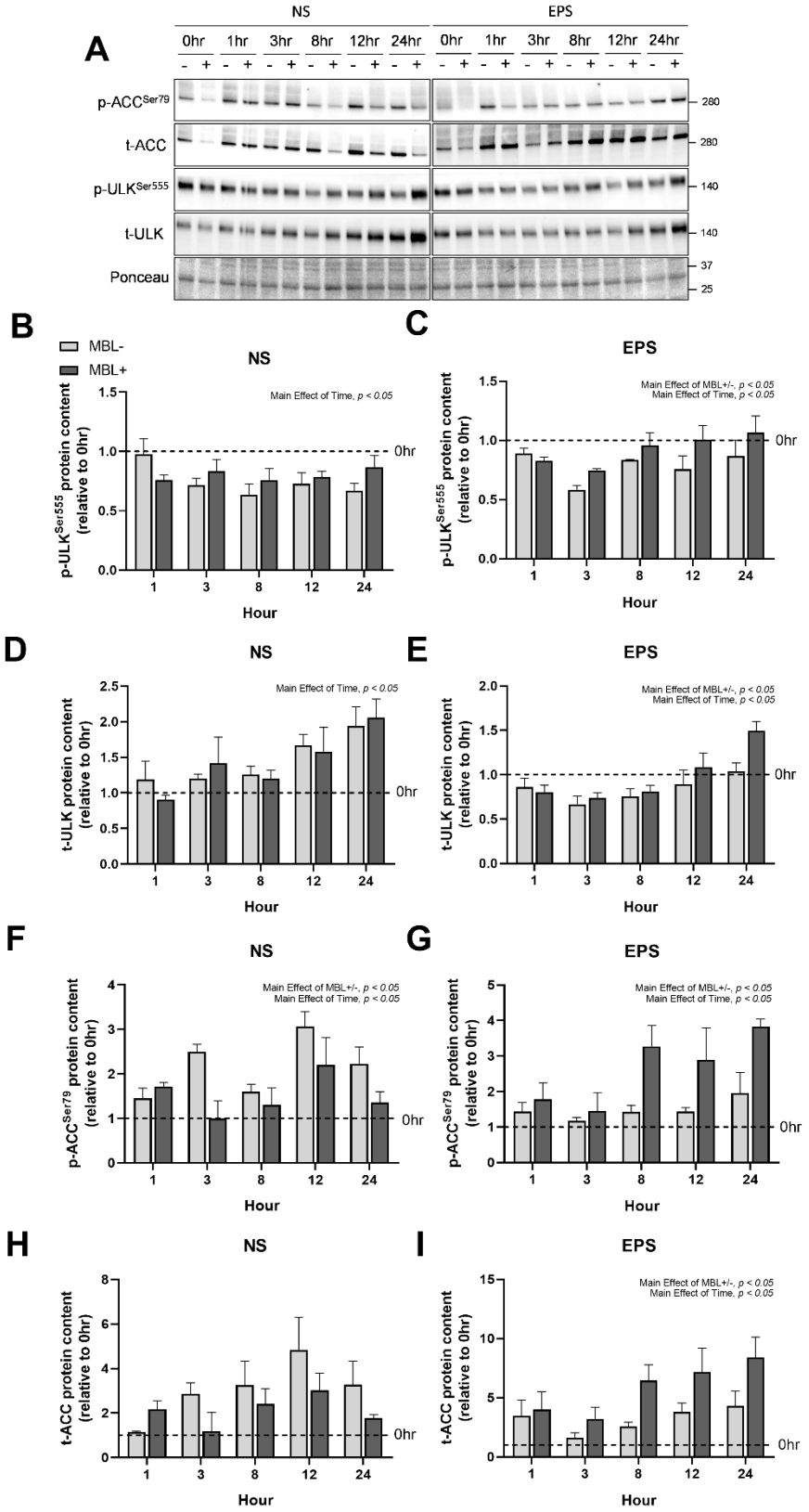
A
NS
EPS
0hr 1hr 3hr 8hr 12hr 24hr
p-ACC^Ser79
t-ACC
p-ULK^Ser555
t-ULK
Ponceau
280
280
140
140
37
25
B
C
MBL-
MBL+
NS
EPS
Main Effect of Time, p < 0.05
Main Effect of MBL+/-, p < 0.05
p-ULK^Ser555 protein content (relative to 0hr)
Hour
D
E
t-ULK protein content (relative to 0hr)
F
G
p-ACC^Ser79 protein content (relative to 0hr)
H
I
t-ACC protein content (relative to 0hr)
0hr
1
3
8
12
24

Figure 7: Myoblast exposure may increase autophagy and β-oxidation in electrically stimulated myotubes. (A) Representative western blot of phosphorylated acetyl coA carboxylase (p-ACC^{Ser79}), total (t)-ACC, phosphorylated Unc-51 like autophagy activating kinase (p-ULK^{Ser555}), and total (t)-ULK. **(B & C)** p-ULK^{Ser555} protein content in MBL+ and MBL-, NS **(B)** and EPS **(C)** myotubes. **(D & E)** t-ULK protein content in MBL+ and MBL-, NS **(D)** and EPS **(E)** myotubes. **(F & G**) p-ACC^{Ser79} protein content in MBL+ and MBL-, NS **(F)** and EPS **(G)** myotubes. **(H & I)** t-ACC protein content in MBL+ and MBL-, NS **(H)** and EPS **(I)** myotubes. All bar graphs are expressed relative to the appropriate 0hr timepoint n = 3. Two-way ANOVA.

DISCUSSION

The aim of the current investigation was to broadly identify the role of satellite cell (SC) signaling in damaged muscle cells independent of SC fusion-dependent processes. An *in vitro* model of intercellular communication utilized two populations of cells; differentiated C2C12 myotubes that underwent contraction-mediated damage through electrical pulse stimulation (EPS) and proliferating C2C12 myoblasts. Data from this study suggests that EPS was able to simulate contraction-mediated muscle damage as identified through sarcomeric disorganization, creatine kinase increases in conditioned media, and increases in autophagy-associated protein expression under all conditions. The presence of MBL regulated these processes by reducing CK levels and increasing protease, autophagic, and beta oxidative activity in the EPS myotubes. Together, this demonstrates the potential for intercellular signaling between myoblasts and myotubes to promote tissue remodeling in response to damage.

The EPS protocol used in this study was adapted from Orfanos and colleagues (2016) where they identified the formation of sarcomeric lesions on myotubes, *in vitro,* that began to form as quickly as 1-hour with continuous EPS and continued to grow thereafter [117] . Further, the newly formed lesions within their investigations displayed non-contractile properties while surrounding areas could contract, providing strong evidence that sarcomeric inactivity is a reason for delayed onset muscle soreness (DOMS) [117].

As this EPS protocol resulted in sarcomeric instability and lesion formation in previous studies, Z line streaming was identified with transmission electron microscopy in the present study aligning with previously reported findings. Z line streaming was qualitatively more prominent in the EPS group (EPS) compared to the unstimulated control group (NS). As seen *in vivo*, sarcomeric disorganization was most obvious after eccentric contractions, even at low intensities and was evident as early as 1-day post-exercise [118]. This disruption was due to the overstretching of myosin-actin binding regions within the M line, which can overload the sarcolemma, activating stretch-activated channels, membrane disruption, and excessive calcium influx [67]. As sarcomeric damage was observed, it was hypothesized that damage associated with creatine kinase (CK) release into the cell culture media would be elevated post-EPS. It is well established that, *in vivo*, conditions of muscle damage releases cytosolic CK into the blood serum [119] . While it is often suggested that membrane permeability is the reason behind CK release, emerging evidence suggests that CK may have a more prominent role in sarcomeric and myofibrillar remodeling in response to damage, as it is often localized to the sarcomere as well as the sarcolemma [119]. However, in this investigation, CK did accumulate in the media of EPS myotubes over time, and there was a significant decrease in this accumulation in the MBL+ group. *In vivo*, CK in blood plasma increases up to 3 days after a bout of damaging exercise, declining thereafter. We expected a similar profile in the present study, and the observed decrease in CK with MBL exposure suggests MBL are capable of releasing factors that may acutely improve myofibrillar integrity in response to EPS.

The observed sarcomeric damage and creatine kinase release into media may suggest a role for calcium-dependent protease activity, as calcium flux is heavily involved in contraction-mediated damage. As muscle contraction relies on calcium influx into the cytosol, damaging eccentric contractions often results in excess cytosolic calcium concentrations, which persists due

to the activation of stretch-gated ion channels. These channels are sensitive to mechanical strain and can dysregulate ion flux. The increase in cytosolic calcium concentrations activates calpain 3, a calcium-dependent protease. Calpain 3 has been implicated in several repair-associated processes, such as the dysferlin membrane repair complex, where it is responsible for degrading the cytoskeleton at sites of damage to allow the fusion of intracellular vesicles to patch the area, as well as sarcomeric remodeling. Calpain 3 expression did show a main effect of MBL exposure, where after EPS was administered, the MBL+ group had elevated calpain 3 protein expression and *Clnp3* gene expression. This provides some evidence that MBL may promote myotube protease activity through paracrine signaling mechanisms in response to damage associated with excess Ca^{2+} release. As such, it was expected that calpain 3 expression would increase in response to increased damage. However, no direct interactions were observed, and calpain 3 immunofluorescence also showed no differences between groups. It remains a possibility that the EPS protocol induced sarcomeric damage resulting in slightly increased calpain 3 protein and gene expression for sarcomeric remodeling. However, the EPS protocol could have inflicted mild membranous damage that induced the response of ubiquitous calpains instead. Alternatively, calpain 3 activation is complex; therefore, the transcriptional and translational regulation of the gene and protein may not indicate function. The protein contains two insertion sequences that regulate its function and activity: IS1 and IS2 [35,36]. Calpain 3 is normally localized to the Z-line regions of the sarcomere, and it is theorized that dissociation from this site is required to expose the IS1 domain of calpain 3. This site is where autolysis occurs, segmenting calpain 3 into two distinct subunits, which are re-associated through noncovalent bonds [35,36]. While calpain 3 is consistently activated in non-muscle tissue, it is thought that the protease binds selectively to titin in skeletal muscle, which prevents IS1 autolysis, making titin a regulator of calpain 3 activity [35] .

Together, calpain 3 activity may be regulated by MBL exposure in response to EPS damage to promote cytoskeletal remodeling, however, an investigation into its activity through alternative means and perhaps through proxy measures such a titin levels, would provide more concise insight [35].

Contraction-induced muscle damage, and other stressors on skeletal muscle, can also be monitored through the quantification of heat shock proteins. Hsp70 is a prominent heat shock chaperone protein expressed in skeletal muscle responsible for preventing protein aggregate formation, promoting appropriate protein folding, and/or signaling for protein breakdown. It is elevated in response to aerobic and resistance exercise, where changes in pH, reactive oxygen species generation, mechanical stretching, and Ca2+ influx contribute to intracellular stress resulting in protein dysregulation [120]. We hypothesized that Hsp70 protein and gene expression would increase in response to EPS, and that exposure to MBL would augment this expression pattern to increase proper protein turnover. Our results demonstrate a main effect of MBL exposure in *Hspa1a* gene expression where MBL+ myotubes expressed higher transcript levels than MBL- myotubes. Moreover, there was a significant main effect of time in both Hsp70 protein and *Hspa1a* gene expression, however, the patterns seem to oppose one another, where protein expression decreases while gene expression increases over time post-EPS. It has been shown that HSP70 mRNA translation in HeLa cells is unaffected by heat shock responses, however, these stimuli are required to prolong the half-life of the chaperone protein, which may explain the mild increases in expression seen at hours 1 and 3 post-EPS (Fig. 3H) [121]. While *Hspa1a* is intron-less, resulting in the absence of post-transcriptional splicing, its export from the nucleus is mediated by THO complex subunit 5 (Thoc5) mRNA transport protein which, therefore, may be a limiting factor for Hsp70 protein expression in the EPS condition, resulting in the observed opposing expression

patterns [121]. Despite these differences, Hsp70 protein expression seems to be upregulated in myotubes when MBL are present, suggesting a role of intercellular signaling resulting in augmented stress response.

Other membrane stability measures were assessed by quantifying β-dystroglycan and desmin protein expression as these proteins are implicated in tethering the cell membrane to the extracellular matrix (ECM) and the sarcomere, respectively. Neither protein changed expression levels in MBL+ myotubes under NS or EPS conditions. However, β-dystroglycan protein content decreased in the EPS conditions, while a significant main effect of time was observed in the unstimulated NS condition, where desmin content seemed to decrease over time. Previous work in dystroglycan-deficient mice demonstrated increased contraction-induced injury, however, this was not accompanied by increased necrosis, excitation-contraction uncoupling, or sarcolemmal instability. Instead, there was excessive dysfunction in the sarcomere, where decreased titin content was evident [19]. Interestingly, the increase in Z line streaming seen in EPS myotubes was hypothesized to increase β-dystroglycan protein expression in a compensatory fashion. However, β-dystroglycan translation may increase in response to EPS but decrease due to the induced damage and perhaps increased muscle protein breakdown.

Protein synthesis was important to consider in response to myotube damage as the increase in synthesis is required for remodeling to replace degraded components. Indicators of protein synthesis remained relatively unchanged in the myotubes under all conditions. It was expected that after EPS, the master regulator of protein synthesis, mTOR, would increase, as seen after resistance exercise training *in vivo*, along with its activating phosphorylation at serine 2448 [122]. mTOR complex 1 (mTORC1), relieves translation inhibition by 4e-bp1. As such, 4e-bp1 total protein content was expected decrease after EPS and activating phosphorylation at threonine 37 and 46

would increase to allow translation to proceed. While total mTOR protein content did not change, the activated phosphorylated state of mTOR decreased over time in the EPS condition. Further, myotube diameter seemed to decrease in the EPS MBL+ condition at 12-hours post-EPS compared to immediately after the stimulus. This is suggestive of reduced muscle protein synthesis to muscle protein breakdown ratios immediately post-EPS, resulting in decreased myotube diameter. Previous work identifying hypertrophy-inducing EPS protocols on primary myotubes exhibited increases in p-mTOR^{Ser2448} protein expression [123]. Their work observed no changes in AMP-activated protein kinase (AMPK) activation, a potent regulator of mTORC1 activity and an energy-sensing biochemical. As such, our specific damaging EPS protocol, which has been shown to cause sarcomeric lesion formation, may not activate biological processes resulting in muscle hypertrophy but may initiate catabolic processes seen during the early stages of regeneration [117].

In the present study, the observed changes in AMPK substrate activity, combined with reduced mTOR activation and a decrease in myotube diameter, together implies that the EPS protocol may be more catabolic in nature. AMPK is a direct regulator of autophagic protein activity as it directly phosphorylates and activates ULK1 and relieves ULK's inhibition by mTOR. As mentioned previously with calpain 3 protease activity, appropriate protein breakdown and organelle degradation, is important for appropriate cell remodeling as it allows for the clearance of dysfunctional components. While total ULK1 protein content did seem to increase over time even in the NS control myotubes, and rates of ULK1 phosphorylation showed a significant main effect of time, this may be indicative of the adaptive response to gradual serum depletion and the autophagy of the remaining myoblasts in culture. Of particular interest are the adaptive responses in the EPS condition. Total ULK content increased 24 hours after EPS was concluded in MBL+ myotubes, and there was a main effect demonstrating that total ULK content was higher in MBL+

than MBL- myotubes. The same trend was shown with ULK1 phosphorylation, though absolute levels did not increase as obviously from 0 hrs. This suggests that MBL may be secreting factors that preferentially upregulate autophagy-associated processes, perhaps by mediating AMPK activity. However, these conclusions are limited to ULK activity, and a clear detailing of autophagy requires looking into additional markers.

Metabolic processes may also be under direct regulation through MBL-secreted factors. Our results indicate that NS myotubes in control conditions may preferentially generate ATP through beta oxidation, regulated through ACC expression and phosphorylation. This aligns with the literature that has repeatedly demonstrated that the SC predominantly uses mitochondrial oxidative phosphorylation when in quiescence, where it then switches to glycolysis when proliferating, then returning to oxidative phosphorylation when differentiating and in terminal differentiation. To reiterate, myotubes generate the majority of energy through oxidative phosphorylation, while in myoblasts, that accounts for only 30% of energy production. [124] Low-intensity EPS has been shown to increase both glycogen oxidation and complete fatty acid oxidation along with carnitine palmitoyl transferase b (CPT1b) protein expression, responsible for fatty acid transport into the mitochondria, which is regulated by ACC phosphorylation [125]. This increase in beta-oxidation is seen, *in vivo,* where endurance-trained athletes tend to have increased lipid turnover and fatty acid oxidation than their sedentary counterparts [126]. Myotubes cultured from satellite cells derived from endurance trained athletes and sedentary individuals do demonstrate the same increase in beta oxidation [127].

In alignment with the previous studies, when myotubes experienced EPS in the present study, there was a significant increase in ACC protein content and activation exclusively in the MBL+ group that increased post-EPS, while the MBL- group remained relatively unchanged over

time. As with the changes in autophagic ULK1 and MPS-regulating mTOR, there seems to be a greater influence of AMPK-mediated processes in response to EPS and with MBL exposure.

Skeletal muscle damage has previously been shown to initially reduce citrate synthase activity, which is often used as a marker for mitochondrial content, and this reduction corresponds with the proliferation of resident myoblasts. Citrate synthase activity then seems to drastically increase several days thereafter when myoblasts terminally differentiate [128–130]. As such, our observed increases in ACC protein expression may also indicate an increase in mitochondrial content correlated with the later stages of the myogenic program and regeneration.

Muscle regeneration has been reported to be impaired when mitochondrial protein synthesis is inhibited, further suggesting its reliance on oxidative phosphorylation to meet energy demands [129]. Previous work on C2C12 myoblasts treated with chloramphenicol – an antibiotic that selectively prevents mitochondrial protein synthesis – demonstrated impaired differentiation independent of ATP synthesis as glycolysis could compensate [131]. As such, the fuel usage of skeletal muscle in response to muscle damage may closely mimic that of the responding myoblasts and, therefore may be involved in regulating the myogenic program and nuclear donation from terminally differentiated myoblasts.

While the literature does not present much information regarding the relative basal levels of glycogen and fatty acid oxidation, it maintains that the mitochondria play a more important role in differentiation and skeletal muscle regeneration than glycolysis. Though direct comparisons to glucose metabolism are lacking, our results demonstrate the importance of fatty acid oxidation in EPS myotubes, which is further upregulated in response to MBL intercellular signaling.

Our primary aim in the current investigation was to determine the fusion-independent role of muscle progenitor cells during skeletal muscle repair following a damaging stimulus. There is

a plethora of evidence *in vitro* and *in vivo* to suggest that SCs and other skeletal muscle precursors are involved in paracrine communication through the release of signaling molecules, including micro-RNAs and proteins, many of which may be secreted via extracellular vesicles such as *Mmp9* within exosomes [132]. For example, the basal secretome of C2C12 myoblasts and myotubes share components classified under biological processes such as vesicle-mediated transport, endocytosis, cellular membrane organization, and small GTPase-mediated signal transduction. Common molecular functions include cytoskeletal protein binding, unfolded protein binding, and calcium ion binding [133]. Furthermore, differentiated skeletal muscle myotubes *in vitro* stimulated or treated with an exercise mimetic release hundreds of proteins, including the proteasome subunit β type 6, superoxide dismutase, fatty acid binding proteins, heat shock protein 70, tropomyosin, desmin, and several protease inhibitors [134]. While these progenitor cells directly experience an exercise intervention rather than respond to another population, it is plausible that skeletal muscle fibres may also secrete molecules to SCs to initiate repair mechanisms. Given these findings, the presence of myoblasts in indirect co-culture with EPS myotubes may release proteins involved in proteasome formation to promote protein breakdown, reducing reactive oxygen species stressors by providing antioxidants and heat shock proteins, promoting sarcomeric stabilization, and regulating fatty acid metabolism. Collectively, our data further confirms and expands on the findings that muscle progenitor cells are involved in paracrine communication.

Limitations and Future Directions

In the present study, it was observed that EPS was able to simulate some features of contraction-mediated exercise-induced muscle damage as identified through sarcomeric disorganization, creatine kinase increases in conditioned media, and increases in autophagy-

associated protein expression. In response to MBL exposure, CK levels after EPS decreased while protease, autophagic, and beta oxidative activity, as identified by select markers, increased. The data presents evidence of MBL intercellular signaling to muscle cells *in vitro.*

EPS has been utilized as an *in vitro* exercise model by replacing neuronal activation, however, the accuracy in mimicking specific exercise modalities has yet to be verified [125]. There is some agreement that short-term, high-frequency stimulation may mimic an acute high-intensity bout of resistance exercise while chronic low-frequency stimulation may represent endurance-like exercise [125]. However, despite the inconsistencies in these claims, the resulting reorganization of the cytoskeleton and new sarcomeric structures in myotubes, along with other metabolic changes seen in response to EPS suggests a useful model in characterizing contraction-mediated exercise benefits in humans [125,135]. While the present EPS protocol was adapted from Orfanos et al. (2016) where myotubular sarcomeric lesions were prominent and contractile properties altered in response to the stimulus, it was unclear whether other markers of EIMD would be altered. Our protocol was acute and used high-frequency stimulation that induced both tetanic holds and pulses as done previously, and it was expected this would result in a "resistance exercise"- like phenotype [117]. However, it is difficult to ascertain the specific type of contraction-mediated damage that occurred, as eccentric contractions are not possible in basic *in vitro* models as myotubes cannot stretch appropriately on the culture plates, and concentric contractions are also not fully possible as the myotubes are adhered to the dish and do not shorten along the whole length of the cell [117]. As such, future work would benefit from plating myotubes on flexible substrates allowing for stretch during contractions.

Some antibiotics have been shown to have effects on myotube differentiation and function. Streptomycin, an antibiotic used in the growth media, has previously been identified as a stretch-

activated channel inhibitor that can prevent membrane depolarization after damage and therefore could affect calcium efflux [32]. However, while ion flux may have been disrupted in the TA of rats, it was concluded that streptomycin did not induce changes in force or contraction-mediated injury. However, treatment with streptomycin in *mdx* mice after eccentric contractions mitigated force decreases and membrane permeability compared to wild type counterparts [136]. As such, perhaps changes in calpain 3 protease activity were blunted due to streptomycin present in the cell culture media. Future work would benefit from measuring ion flux in response to myotube contraction or careful selection of antibiotics.

Lastly, in the present study, myoblasts were plated at ~20,000 cells per transwell insert. A confluent 6-well dish can contain ~250, 000 cells prior to differentiation and fusion into myotubes, which represents a rough 12.5% proportionality. While there is variation between muscle groups, *in vivo* satellite cells make up 2-7% of myonuclei within a muscle [137]. While our proportions may present as physiological, perhaps the concentration, *in vitro,* needs to be increased to account for the lack of migratory capacity, increased physical distance from the myoblasts in the transwell insert to the myotubes below, and the lack of an ECM that may help propagate signals. Future work would benefit from utilizing 3D models of skeletal muscle *in vitro*.

Conclusion

In summary, the primary objective of this study was to investigate the role of secretory factors from skeletal muscle myoblasts to determine if fusion-independent mechanisms participate in damage recovery or the reduction of cellular stress. Based on our results, it is evident that myoblast signaling towards myotubes is present and may be responsible for reducing membrane permeability after EPS damage, increasing calcium-dependent protease activity to promote

remodeling and increasing autophagy and beta-oxidation protein expression, potentially through regulating AMPK activity.

Alongside further characterizing paracrine signaling in the skeletal muscle environment, this work provides insight into the biological processes within the skeletal muscle influenced by myogenic progenitor cell signaling. Previous work has identified a significant impact of MPC signaling on fibrogenic cell function, and other studies that have looked at skeletal muscle communication have only identified ECM-associated protein regulation. In this study, we identified four pathways influenced by MBL signaling that may affect the myogenic program, bioenergetics, and regeneration.

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