Examining the Role of L-type Amino Acid Transporter 1 (SLC7A5) in Myoblasts

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Abstract

Skeletal muscles represent the largest tissue mass within the body and are primarily involved in the generation of force for voluntary movement. Skeletal muscles have a remarkable capacity to repair, due primarily to the actions of muscle stem cells (MuSCs). MuSCs are normally quiescent in adult skeletal muscle; however, in response to myotrauma (trauma to muscle tissue) from muscle injury or exercise, MuSCs become activated, either undergo self-renewal to replenish the quiescent population or commit to the myogenic lineage as myoblasts, proliferate, and differentiate into myotubes *in vitro* or fuse to existing myofibers *in vivo*. This process of generating new myofibers from quiescent MuSCs is termed myogenesis and a full understanding of how myogenesis is regulated remains to be understood. Mounting evidence suggests that amino acids, particularly the essential amino acid leucine, play a role in MuSC regulation. Leucine is specifically translocated and sensed by the L-type amino acid transporter 1 (LAT1); which facilitates leucine uptake in mature myofibers. Inside the cell, leucine activates mammalian or mechanistic target of rapamycin complex 1 (mTORC1) to stimulate cell growth, proliferation, and protein synthesis. Whether leucine has direct effects on myoblast function via LAT1 is unknown. Thus, our overall objective was to begin to characterize the role of LAT1 in myogenesis. Our results indicate that myoblasts differentially expressed LAT1 throughout myogenesis with peak protein content occurring during differentiation ($p<0.05$ vs. early proliferation). Further, our results indicate thatpharmacological LAT1 inhibition reduced myoblast expansion and differentiation *in vitro* (both $p<0.05$ vs. control). Interestingly, myoblast LAT1 protein content did not change in response to leucine supplementation *in vitro*; however, was lower under *in vitro* atrophic conditions ($p<0.05$ vs. control). Based on these findings, we conclude that LAT1 plays an important role in regulating myogenesis. As such, we uncover a novel role for LAT1 in regulating muscle mass via contributing to the control of MuSC function.

CHAPTER 1

Introduction

Amino acid transport plays a vital function in cell survival (46) by delivering key substrates necessary for cell division and protein synthesis (32). Within skeletal muscle, amino acid transport contributes to maintaining intramyocellular amino acid pools (32), which compose roughly 40% of body weight and encompass 50–75% of all proteins in the human body (20). The L-Type amino acid transporter 1 (LAT1), also known as the solute carrier family 7 member 5 (SLC7A5), is a key amino acid transporter in skeletal muscle (32) due to its specific ability to transport branched-chain amino acids (BCAAS), particularly leucine (32). For proper function, LAT1 must form a heterodimer with CD98 that concurrently exports glutamine (32). Leucine influx plays a vital role in stimulating protein synthesis (11, 20, 60, 71), by activating mechanistic target of rapamycin complex 1 (mTORC1) (32). Thus, interventions using amino acid supplementation, particularly those enriched with leucine, have been investigated as a means of preventing muscle atrophy and promoting muscle hypertrophy (55).

Satellite cells or muscle stem cells (MuSCs) are undifferentiated myogenic stem cells that are able to form mature myocytes and regulate skeletal muscle development, maintenance, and regeneration (35, 63). MuSC decline or dysfunction leads to reduced muscle regeneration and atrophy (30). Skeletal muscle atrophy contributes to weakness of respiratory and peripheral muscles, leading to increased fatigue, compromised respiratory function, decreased quality of life, and poor immune and metabolic health (61). It is known that mTORC1 signaling regulates MuSC function (58); however, results from human studies examining the role of altering dietary amino acid content in regulating MuSCs are equivocal (23, 24, 66). Short term low protein consumption with inactivity has no effects on satellite cell quantity (66), whereas protein consumption combined with resistance training leads to an increase in satellite cell quantity (24).

An important gap in the literature that could help to clarify these discrepant findings is whether myoblasts express LAT1 and can thus respond directly to amino acid supplementation.

Therefore, our overall aim is to uncover the responsibility for LAT1 in maintaining muscle mass by investigating the role and regulation of LAT1 in MuSCs during myogenesis. A greater understanding would provide targets for novel therapeutic interventions for enhancing myogenesis in muscle-related morbidities.

Satellite Cells: Precursors to Skeletal Muscle

Skeletal muscle is the largest tissue by mass within the body, and plays a pivotal role in metabolism, postural support, and locomotion. Unlike smooth and cardiac muscles, skeletal muscles can be voluntarily controlled, and are composed of many striated myofiber bundles (9). Skeletal muscle is highly adaptable to disease, exercise, and inactivity due, in part, to the actions of skeletal muscle stem cells (MuSCs). MuSCs are primarily quiescent in adult skeletal muscle, and are necessary for muscle regeneration, remodeling, and repair. MuSCs are undifferentiated myogenic stem cells (26), which can also be termed 'satellite cells' due to their residence in indentations between the basal lamina on the periphery of muscle fibers and the sarcolemma (44, 48). MuSCs have the ability to enter the cell cycle to self-renew asymmetrically (5), or donate new myonuclei and form new myofibers through the process of myogenesis (6, 30, 56). Myogenesis consists of four distinct phases: 1. Quiescence, 2. Activation, 3. Proliferation, and 4. Differentiation/fusion (30). Intrinsic regulators aid in maintaining MuSCs in a quiescent state (22), which is composed of two functional phases; G_0 state and G_{alert} (58). G_0 state is seen as the resting phase, whereas G_{alert} is a pre-activation state for satellite cells primed to enter proliferation, which has been characterized by activated mammalian target rapamycin complex 1 (mTORC1) (58).

In response to myotrauma or growth stimuli, MuSCs become activated, proliferate then commit to the myogenic lineage to become myoblasts (62). There are many regulatory factors implicated in the differentiation pathway including pair-box transcription factor 7 (Pax7) which is exclusively expressed by MuSCs from quiescence through to terminal differentiation at which point Pax7 is downregulated (63). When activated and transitioning to proliferating myoblasts,

Pax7 stimulates the expression of the myogenic regulatory factors, myogenic factor 5 (Myf5) and myogenic determining factor (MyoD) (36). Myf5 is the earliest of the myogenic regulatory factors to be expressed and is a regulator of muscle development (40), while MyoD represses self-renewal promoting terminal differentiation (45), ultimately marking myoblast commitment (73). When myogenic differentiation occurs, transcription factors such as MRF4 and myogenin (MyoG) are up-regulated, with Pax7 downregulated (30, 67). In response to muscle damage, myoblasts repair myofibers by integrating their nuclei within damaged myofibers in a process termed fusion (56). Chemokines released from damaged myofibers signal myoblasts to migrate towards regions of damage via a chemical gradient (30). Once myoblasts reach the damaged region, they fuse to incorporate their nuclei with damaged muscle fibres (62). The complete set of factors regulating MuSC-mediated myofibre repair and plasticity remains unknown (6, 30, 56) (Figure 1).

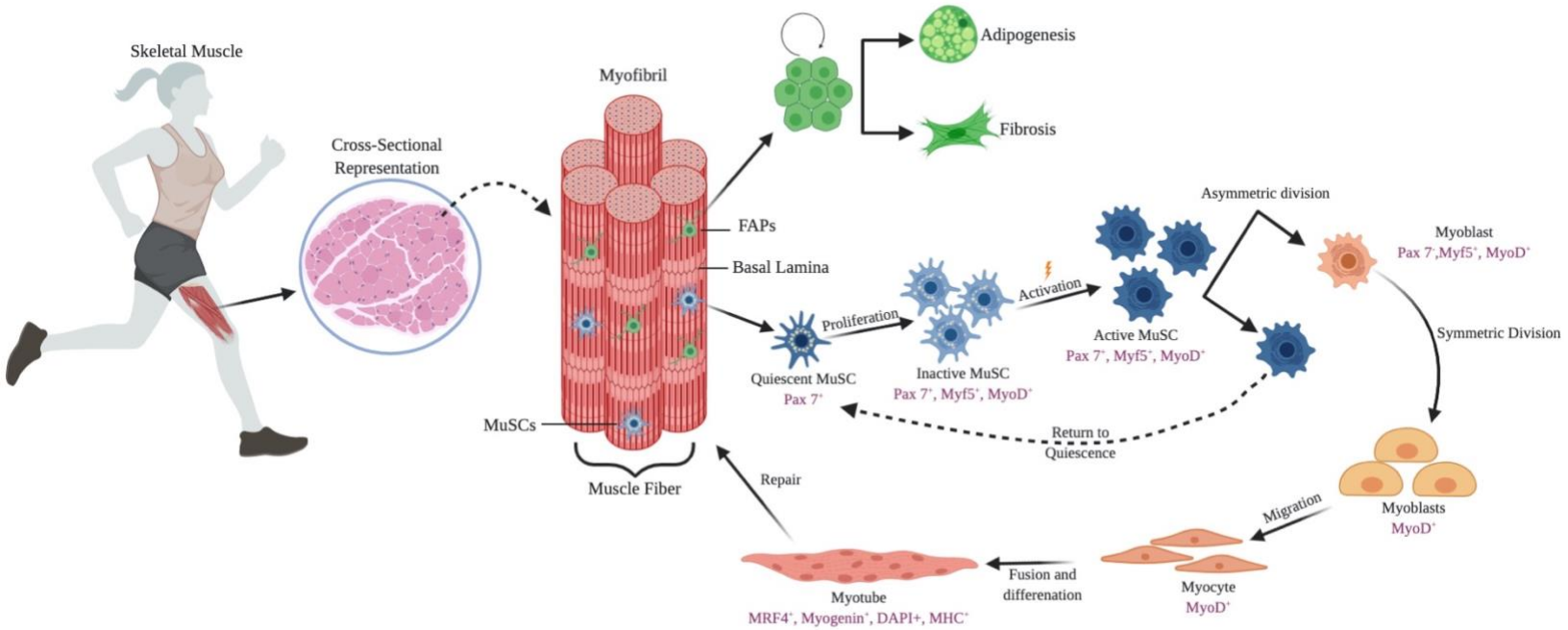

Figure 1. Schematic of myogenesis. MuSCs (Pax7+) reside in between the sarcolemma and the basal lamina, and are required for repair, regeneration, and hypertrophy of myofiber following skeletal muscle trauma or injury (e.g., resistance training). MuSCs become activated from their quiescent state, proliferate, undergo upregulation of the myogenic regulatory factors Myf5 and MyoD and asymmetric cell division. MuSCs will then either self-renew the quiescent population or go through symmetric division. Newly formed myoblasts or myocytes will migrate to the

damaged region and fuse with damaged fibers or produce new myofibers. Non-satellite stem cells in muscle, such as FAPs, secrete factors that aid in MuSCs activation.

Leucine: Important Substrate and Stimulator of Muscle Protein Synthesis

Amino acids are organic compounds composed of amine and carboxyl functional groups (70). As an important macronutrient, amino acids are essential building blocks for proteins. Broadly, amino acids can be classified into 2 groups; non-essential amino acids (NEAA) which develop in *de novo* synthesis and the essential amino acids (EAA) which cannot be synthesized within the body of mammals (46), and as such are derived from dietary protein (2). Evidence indicates a positive correlation between an increase in amino acid availability, specifically EAAs, and greater activation of anabolic pathways responsible for muscle protein synthesis (52). Protein supplementation with low EAA versus high EAA content has shown that higher content promotes muscle protein synthesis in the elderly (51) which translates into improved physical performance (33). Mounting evidence suggests that higher consumption of dietary protein amplifies resistance exercise-induced increases in strength and muscle size (13, 47, 68). The amino acid leucine is pivotal, often considered the most important EAA (61) for stimulating muscle growth because it serves as both a substrate for new protein production and a signal to activate muscle protein synthesis (2, 8, 60, 71). Leucine is the only EAA that stimulates phosphorylation of mTORC1 and its downstream targets: ribosomal S6 kinase 1 (S6K1), and 4E binding protein 1 (4E-BP1), leading to a stimulation of protein synthesis (2). Phosphorylation of mTORC1, a serine/threonine protein kinase, is important for regulation of *de novo* anabolic metabolism (lipids, nucleotides, proteins), needed for cell homeostasis, proliferation, and growth (2, 28). Thus, through regulating protein metabolism, leucine promotes cell growth, survival, and proliferation (72). Interestingly, mTORC1 signaling is involved in the early transition of quiescent MuSCs to activation (59). As such, leucine might control myoblast activity through activation of mTORC1 signaling.

Amino Acid Transporters: Gates for Amino Acid Transport

Amino acids, including leucine, are sensed and transported into various cell types including myofibers by transmembrane amino acid transporters. Although there are several amino acid transporters that are specific for groups of amino acids, the focus of this literature review will be on LAT1 as it is the only transporter responsible for sensing and transporting leucine into skeletal muscle (17). It is important to note; however, that LAT1 is a dedicated transporter for other essential amino acids as well, such as valine, isoleucine, and tyrosine (65). LAT1 requires its co-transporter CD98 for proper function. CD98 a 68 kDa type II glycoprotein and LAT1 a 55 kDa integral membrane protein (10) form a heterodimeric protein complex. This heterodimeric protein work in tandem to form an amino acid permease (50); a channel that allows for transport of specific molecules in or out of the cell (69). LAT1 acts as a hetero-exchanger with a 1:1 ratio, facilitating intracellular uptake of leucine in exchange for other amino acids, most often glutamine, in order to maintain an equilibrium of the amino acid pool (34). CD98 transports glutamine out of the cells (34) and stabilizes LAT1 (50). The LAT1/CD98 heterodimer is exclusively responsible for leucine transport into skeletal muscle (17, 34).

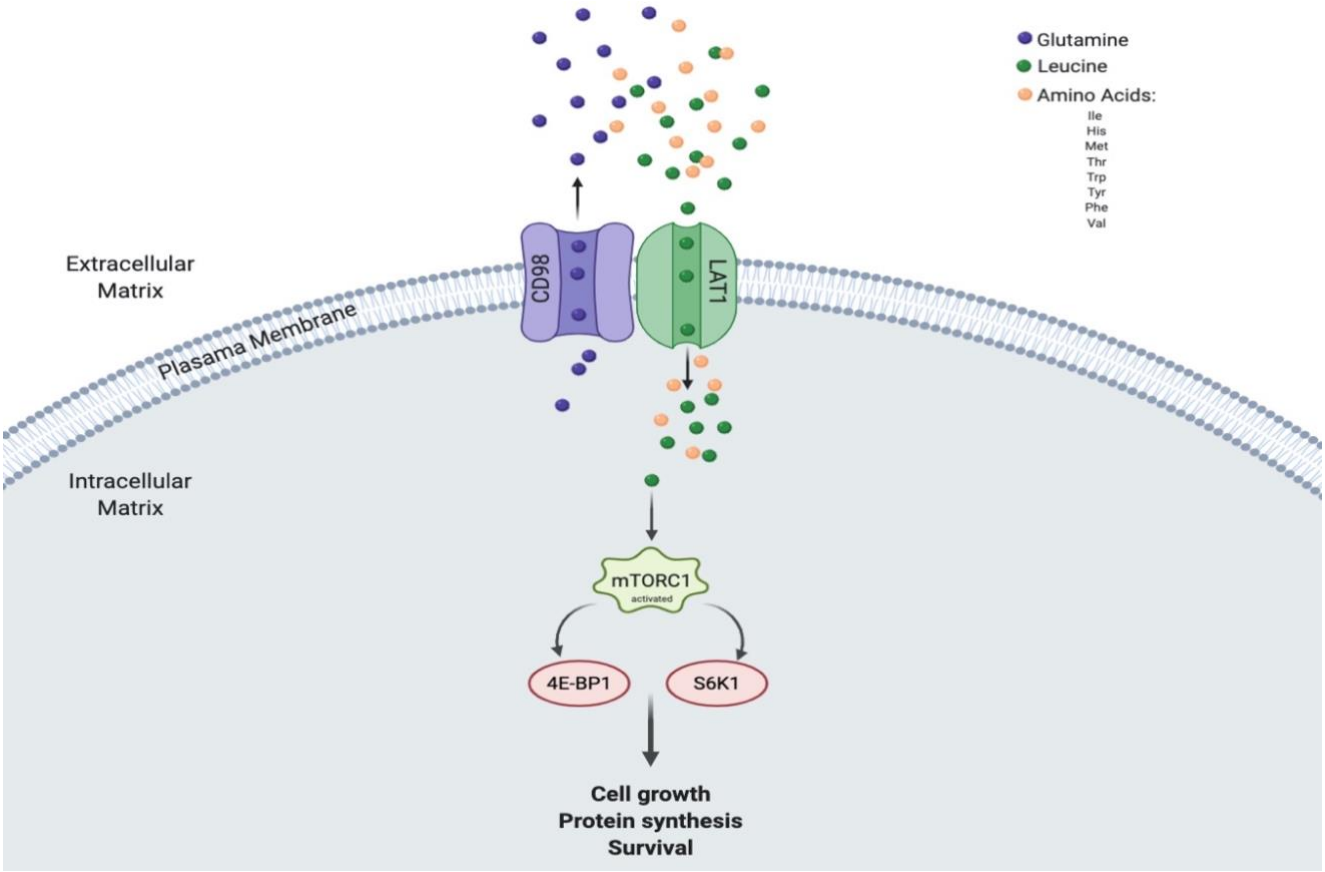

Figure 2. Schematic of LAT1 and co-transporter CD98. At a 1:1 ratio, CD98 exports glutamine, and LAT1 imports leucine and other EAAs (i.e Val, Met, Phe). Leucine activates mTORC1, which then stimulates protein synthesis.

LAT1 expression in skeletal muscle muscle is responsive to various anabolic and catabolic stimuli. Amino acid supplementation in conjunction with exercise, particularly resistance exercise, are two primary anabolic stimuli for skeletal muscle (1, 16). In response to an acute bout of resistance exercise coupled with EAA ingestion, LAT1 gene expression was significantly increased at one hour into recovery (15). Similarly, LAT1 protein content and gene expression were upregulated with or without resistance training, 3- and 6-hours following protein ingestion (4, 15, 19). As for catabolic stimuli, several inducers of muscle atrophy have been described, including age, inactivity, and disease. One pharmacological means of inducing muscle atrophy is via high dose, or prolonged glucocorticoid (GC) treatment. GCs are commonly used as a therapeutic agent to combat diseases often associated with inflammation (e.g. sepsis, cancer,

cachexia, starvation, and severe insulinopenia) (7, 27). Although acute GC treatment is beneficial in these conditions, high dose or prolonged GC therapy paradoxically results in skeletal muscle atrophy (27), particularly of fast-twitch or type II muscle fibers(14, 25, 61). GCs induce fibre atrophy by creating an imbalance between protein synthesis and protein breakdown that favors breakdown (43, 61) and by decreasing MuSC proliferation and differentiation *in vitro* and *in vivo* (18). GCs reduce protein synthesis by inhibiting amino acid transport, particularly leucine, into the muscle (39, 42, 64), via downregulation of LAT1, and inhibit mTORC1 (31). Furthermore, GCs inhibit the stimulatory action of insulin, IGF-I, and amino acids, specifically leucine, by abolishing the amino acid-induced phosphorylation of 4E binding protein 1 (4E-BP1) (42, 64). Thus, these actions indicate that both anabolic and catabolic processes in skeletal muscle modulate LAT1 content with increases in LAT1 seen during hypertrophy, and reductions in LAT1 observed during atrophy. In all of the above studies, LAT1 has been reported in whole muscle homogenate or on muscle fibres; however, whether LAT1 is expressed by MuSCs remains unknown.

While no previous studies have directly examined the role of LAT1 in MuSCs/myoblasts, several previous studies have investigated the role of LAT1 in other highly proliferative cell types. Leucine transport through LAT1 is essential for cell proliferation and survival *in vitro*, as specific inhibition of LAT1 by 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) inhibited proliferation and induced apoptosis of human oral epidermoid carcinoma cells (KB cells), Saos2 human osteogenic sarcoma cells and C6 rat glioma cells (37, 38). Thus, functional LAT1 seems to be essential for cell proliferation and survival. Whether leucine transport by LAT1 performs the same necessary functions in adult myoblasts which have similar properties to cancer cells remains unknown.

The Role of Leucine in Myogenesis

Myogenesis, as previously stated, is a process resulting in the formation of new myofibers or repair of damaged myofibers (6, 30, 56). Pax7+ MuSCs are activated and give rise to myoblasts, which express specific MRFs, then differentiate into myocytes and fuse (62). mTORC1 functions

as a regulator of cell growth by responding to changes in cellular energy status (29), growth factor signaling, and nutrient availability (57). Amino acids are required for mTORC1 activation, making these nutrients the dominant input for cell growth (29). Leucine, increases activation of mTORC1 (53) and its downstream targets, S6K1 and 4E-BP1 (41). Previous literature has investigated the effects of leucine supplementation on myoblast activity during myogenesis. C2C12 myoblasts cultured in leucine-depleted media demonstrate impaired differentiation via the upregulation of Myf5 gene expression and reduction of MyoD protein (3). Further, protein synthesis during myogenic differentiation in response to leucine deprivation was decreased resulting in impaired myogenesis (67). Using Sprague-Dawley rat MuSC isolates, Dai and colleagues (12), reported increased MyoD and MoyG expression with leucine treatment, and dose-dependent response to leucine, possibly affecting proliferation and differentiation. Interestingly, a pre-activation state for satellite cells primed to enter proliferation, termed G_{alert}, was recently identified, and was characterized by activated mTORC1 (58). These findings further support a role for leucine in regulating satellite cell fate (58). Results from animal studies of myogenesis and *in vitro* studies seem to support the idea that leucine supplementation amplifies myogenesis, enhancing myoblast differentiation and proliferation (12, 21). Pereira and colleagues (54), gave two-month and 20-24 month old male rats oral gavage of leucine supplementation, and subjected them to cryolesion of the soleus muscle. They determined that leucine improved regeneration of skeletal muscles, increasing proliferating satellite cells and size of regenerating myofibers (54). Perry and colleagues (55), found that mTORC1 content was higher with or without injury, and increased 4E-BP1 content post-injury in aged mice after leucine supplementation. These data indicate that leucine supplementation in aged, regenerating muscle enhances regeneration via increasing in anabolic signaling (55).

Although the animal and *in vitro* literature consistently demonstrate beneficial effects of leucine supplementation on myoblast function, human investigations into the role of protein supplementation on MuSC content and function remains ambiguous. Snijders and colleagues

demonstrated that short term consumption of a low protein diet had no effects on MuSC quantity in response to a period of inactivity (66). Conversely, Farup and colleagues observed an enhanced satellite cell response to an acute bout of damaging exercise with protein supplementation (23), and that protein supplementation combined with resistance training lead to an increase in satellite cell quantity (24). Muyskens and colleagues quantified MuSCs in patients undergoing total knee arthroplasty (TKA) during surgery and at several time points in recovery following ingesting of 20 g of EAA twice daily for 7 days prior to surgery and 6 weeks post-surgery (49). The authours did not detect any significant difference in MuSC content during recovery, and that EAA supplementation post-TKA did not mitigate muscle atrophy (49). Although clinical data are inconsistent, *in vitro* and *in vivo* findings using models that modulate leucine content (i.e. via supplementation or depletion) suggest that leucine may enhance *in vitro* myogenesis and *in vivo* muscle regeneration indicating a potential direct effect of leucine on MuSCs. However, uncertainty regarding the effects of leucine on myogenesis remains, in part, because no previous studies have examined the expression and role of LAT1 in MuSCs. Addressing this key research gap will shed light on the equivocal findings from human studies regarding the effects of leucine on MuSC content and function, and provide a novel role of LAT1 in muscle plasticity.

Research Aim

The purpose of this study is to begin to characterize the role of LAT1 in myogenesis. Our specific aims are to determine the extent to which:

1. LAT1 is expressed on myoblasts and if expression changes throughout different stages of myogenesis.
2. LAT1 regulates myoblast proliferation and differentiation, presuming existence on myoblasts.
3. LAT1 expression on myoblasts is altered in response to anabolic and catabolic stimuli.

Hypotheses

Our specific hypotheses are:

1. We hypothesize that LAT1 will be expressed on myoblasts during all stages of myogenesis with peak expression occurring during differentiation.
2. We hypothesize that LAT1 inhibition will impair myoblast proliferation and differentiation.
3. We hypothesize that exposure to higher concentrations of leucine will increase LAT1 content on myoblasts and exposure to atrophic conditions will reduce LAT1 expression.

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CHAPTER 2

LAT1 (SLC7A5) Transporter Regulates in vitro Myogenesis

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Abstract

Muscle stem cells (MuSCs) support muscle regeneration, remodeling, and repair through their activation, proliferation, and differentiation; however, how dietary factors regulate this process remains unknown. The L-Type amino acid transporter 1 (LAT1) transports amino acids, particularly leucine, into mature myofibers, which then stimulates protein synthesis. However, whether LAT1 is expressed on myoblasts and is involved in regulating myoblast function is unknown. Thus, presuming its existence on myoblasts, our purpose was to characterize the role of LAT1 in myoblast fate. We aimed to determine if LAT1 is expressed on myoblasts, if LAT1 expression changed throughout myogenesis *in vitro*, and if LAT1regulates myoblast proliferation and differentiation *in vitro*. Using C2C12 and human primary myoblasts, we determined that LAT1 protein content was differentially expressed throughout myogenesis, peaking during differentiation ($p<0.05$ vs. early proliferation). LAT1 inhibition impaired myoblast expansion and differentiation *in vitro* (both $p<0.05$ vs. control). LAT1 protein content did not change *in vitro* with exposure to leucine supplementation; however, LAT1 expression was lower under atrophic conditions *in vitro* ($p<0.05$ vs. control). Together, these findings identify a novel role for LAT1 as one key amino acid sensor on myoblasts that plays an important role in regulating myogenesis.

Introduction

Muscle stem cells (MuSCs) are undifferentiated myogenic stem cells with the capacity to differentiate into mature myocytes and regulate skeletal muscle maintenance, development, and regeneration (27, 44). MuSC decline or dysfunction leads to reduced muscle regeneration and skeletal muscle loss (23). MuSCs can enter the cell cycle to self-renew asymmetrically (6), or donate new myonuclei and form new myofibers through the process of myogenesis (7, 23, 42). The formation of new myofibers through myogenesis, occurs as MuSCs progress from quiescence, through activation and proliferation, to terminal differentiation and cell fusion (54). A group of muscle-specific transcription factors, collectively referred to as the myogenic regulatory factors (MRFs), regulate sequential progression through the various stages of myogenesis (54). Among the MRFs, Myf5 and MyoD are involved in muscle specification, whereas myogenin (MyoG) and MRF4 appear later in myogenesis aiding in myotube formation and maturation (54). In addition to these intrinsic factors, several cell non-autonomous factors have been implicated in regulating myogenesis. For example, insulin-like growth factors (IGFs) and myostatin (MSTN) are two key growth factors that regulate MuSC function (54). A complete understanding of the extrinsic factors, particularly dietary-derived factors, that regulate MuSCs remains to be described.

Amino acids, particularly the essential amino acid leucine, are necessary for skeletal muscle maintenance and hypertrophy. Leucine is unique amongst amino acids in that it serves as both a substrate for muscle protein synthesis (1, 8, 43, 53), and as a signaling molecule activating anabolic signaling through direct regulation of mammalian target rapamycin complex 1 (mTORC1) (35). While an extensive body of literature has described the anabolic effects of leucine on skeletal muscle fibres (21), relatively little information is available on the role of leucine and other amino acids on MuSCs (45). Previous literature has investigated the effects of leucine supplementation on animal and human myoblast activity during myogenesis. Animal studies of myogenesis tend to support the concept that leucine supplementation amplifies myogenesis,

increasing myoblast proliferation, differentiation (10, 14), and increasing myofiber size (39). Results from human studies inspecting the role of leucine supplementation in regulating MuSCs, however, have been equivocal (16, 17, 46). Protein consumption in combination with resistance training increases MuSC quantity (17), but acute low protein intake coupled with inactivity had no effects on MuSC quantity (46). The equivocal findings could be explained by developing a better understanding of whether MuSCs can directly sense and respond to leucine.

Leucine is sensed and transported into cells specifically by LAT1 along with its co-transporter CD98 (35). LAT1 is expressed on various tissues in the body, including mature myofibers, and transports other amino acids like tyrosine, isoleucine, valine and phenylalanine (12); however, LAT1 is the only transporter of leucine into cells (24). What remains unknown, is whether MuSCs express LAT1 and if manipulating leucine transport through LAT1 influences MuSC function. Thus, the purpose of this study was to examine the role of LAT1 transporter in myoblast fate. Specifically, we wanted to determine if MuSCs express LAT1, and if expression changes throughout different stages of myogenesis. Further, we sought to determine if manipulating leucine transport through LAT1 influenced myoblast proliferation and differentiation and whether LAT1 expression on myoblasts was altered in response to anabolic and catabolic stimuli. We hypothesized that LAT1 would be expressed on myoblasts during all stages of myogenesis with peak expression occurring during differentiation, and that LAT1 inhibition will impair myoblast proliferation and differentiation. Finally, we hypothesized that exposure to higher concentrations of leucine would increase LAT1 content on myoblasts, while exposure to atrophic conditions will reduce LAT1 expression.

Materials and Methods

Cell Culture

C2C12 mouse muscle-derived myoblasts (ATCC; Manassas, VA) were cultured in growth media (GM; 10% fetal bovine serum/1% penicillin/streptomycin/Dulbecco's modified Eagle's medium (DMEM)) at 37℃ in 5% CO_2. For quiescence induction, myoblasts were expanded to

~75-80% confluence then media was changed to serum free conditions (SFM; 99% DMEM, 1% penicillin/streptomycin). Quiescence was confirmed by a significant increase in the proportion of myoblasts in G0/G1 phase of the cell cycle by flow cytometry (Supplemental Figure 1). After 24 hours in SFM, a subset of cells for SFM day 0 (D0) were harvested for total protein and the remaining cells were treated with GM. At 24 (GM D1) and 72 (GM D3) hours post-treatment with GM, cells were harvested for total protein isolation. In order to induce differentiation, myoblasts were expanded to ~ 95% confluence in GM. Media was then changed to differentiation media (DM; 2% Horse Serum/ 1% penicillin/streptomycin/DMEM) (19). Cells were harvested for total protein after 1 (DM1), 4 (DM4), and 7 days (DM7) in DM. To examine the effects of LAT1 inhibition on myoblast differentiation, C2C12 myoblasts at ~95-100% confluence were plated with or without 25 mM BCH in DM. Total protein was harvested and quantified by Bradford assay.

To determine LAT1 expression in response to leucine supplementation *in vitro*, proliferating myoblasts were expanded to roughly 70-90% confluency in growth media (GM). Cells were repeatedly washed with PBS, then incubated with fresh GM containing 5 mM of leucine (Sigma, Aldrich, MO, USA). To determine the use of 5mM dose of leucine, a leucine dose-response was conducted on C2C12 which yielded no significant difference observed from doses 0 mM to 5 mM (Supplemental Figure 3) At 0, 0.5, 1, and 2 hours of leucine supplementation at 5 mM, cells were collected, and immunoblotting procedure was performed to evaluate LAT1 content. For atrophy induction, following 5 days of differentiation, C2C12 cells treated with dexamethasone (DEX, Sigma-Aldrich, MO, USA) at 10^{-6} M in DM for 24 hours and compared to control cells in DEX-free DM (20).

Immunoblotting

C2C12 cells from each time point: SFM D0, GM D1, GM D3, DM D1, DM D4, and DM D7 were homogenized in ice-cold RIPA buffer (50mM Tris-HCL pH 7.4, 150mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), with protease inhibitor tablet

(Roche Complete Mini with EDTA) and a phosphatase inhibitor tablet (Roche PhosSTOP). Homogenates were centrifuged at 13,000 rpm for 5 min at 4˚C, and the supernatant containing the total protein fraction was collected. Protein concentration was determined by Bradford assay. Samples were prepped in 4x Lammeli buffer (1M Tris-HCl pH 6.8, SDS, 100% glycerol, 14.7M β-mercaptoethanol, and bromophenol blue) and were heated at 95˚C for 5 min for denaturation. Equal amounts of protein (45 µg) from each sample were separated on SDS-PAGE gels then transfer onto polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 5% skim milk then incubated in primary antibody, anti-SLC7A5 (LAT1; Bioss Inc., Woburn, MA) at 1:1000 in 5% milk in a mixture of tris-buffered saline and Polysorbate 20 (TBST) overnight at 4˚C. After multiple washes, membranes were incubated at room temperature in anti-rabbit HRP-conjugated secondary antibody for 1 hour, then in Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL). Blots were imaged using a ChemiDoc Imaging System (Bio-Rad, California, United States). Blots were then stripped of anti-SLC7A5 by incubation in stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL) for 20 min at room temperature. Membranes were washed, blocked in 5% milk in TBST for 1 hour, and then re-probed for anti- α-tubulin (abcam, Cambridge, MA) at 1:5000 in 5% milk in TBST overnight at 4˚C. Images were analyzed using ImageJ software (NIH). After background correction, LAT1 expression was normalized to α-tubulin (abcam, Cambridge, MA).

Gene Expression analysis

RNA was extracted from C2C12 cells at each time point: SFM D1, GM D1, GM D3, DM D1, DM D4, and DM D7 as previously described (26). Briefly, total RNA was extracted in 1 mL Trizol followed by 0.2 mL of chloroform. Cells were then centrifuged at 12,000 x *g* at 4°C for 15 min for collection of the upper aqueous phase. The extracted RNA was washed with 70% ethanol, then purified using the Qiagen Mini Kit protocol (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA was reverse transcribed to cDNA using the High Capacity cDNA Kit (Applied Biosystems) according to manufacturer's instructions. Briefly, a master mix containing

10x RT buffer, 25x dNTP, 10x Random Primers, Mutliscribe RT, ddH20 and the extracted RNA sample were placed into a thermocycler for the following settings: 25˚C for 10min, 37˚C for 120min, 85˚C for 5min, and cooled at 4˚C then stored at -20₀C until analysis. Spectrophotometric quantification of isolated RNA was conducted using the NanoDrop (Thermo Scientific, Wilmington DE). Quantitative real-time PCR (qPCR) was conducted using Taqman chemistry (Applied Biosystems, USA). The following genes were analyzed across the time course from SFM through to DM7: LAT1 (Assay ID: Mm00448764_m1), CD98 (Assay ID: Mm00441516_m1) and rps11 (Assay ID: Mm02601829_g1). qPCR analyses were conducted using the ABI 7900HT Fast Real Time PCR System (Applied Biosystems, USA) and analyzed using SDS 2.4 Software (Applied Biosystems, USA). Changes in gene expression of the genes of interest were determined using the $2^{-\Delta\Delta CT}$ method (32) relative to the housekeeping gene rps11.

MTT Analysis

Cell quantity was assessed via MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay as previously described (11). MTT based Cell Growth Determination Kit (Sigma-Aldrich) solution was added in accordance with the instructions of the manufacturer at 10% of culture volume and left to incubate at 37°C in 5% CO_2 for 3hrs. the supernatant was removed and then 100µL of MTT solvent was added to each well. The plate was read on PolarStar Omega (BMG Labtech, Guelph, Canada) at 570nm and 690nm to aaccount for background absorbance. Obtained values were expressed as a fold change relative to myoblasts treated with SFM for the same amount of time.

Immunofluorescence and Myogenic analysis

Control, BCH, and DEX treated cells were subjected to immunofluorescence staining with MHC antibody (MF20 – red color indicative of myotubes) and visualized with anti-mouse IgG (H+L) Alexaflour 594 goat. The nuclei were counterstained with DAPI (blue). C2C12 cells were washed twice with PBS, fixed with 4% PFA in PBS for 10 min. Cells were rewashed with PBS then permeabilized with 0.5% Triton-X-100 in PBS for 10 min. After two washes with PBS the cells

were blocked in 10% goat serum in PBS-T (blocking buffer) for 30 minutes and incubated with primary antibody against myosin heavy chain (MyHC) MF20 at 2.5 µg/ml in blocking buffer for 1 h. After two washes with PBS the samples were incubated with secondary antibodies containing Alexa flour 594 goat anti-mouse immunoglobulin G (IgG) (1:300) in blocking buffer for 1 h. After additional washes, cells were counterstained with DAPI for 5 minutes and washed again. The cells were fixed with mounting media and imaged using the Celldiscover7 (Zeiss, Germany) with ZEN 2.3 software (Zeiss, Germany) with subsequent analyses conducted with ImageJ (NIH, USA). Well edges and regions which did not show cell adhesion were excluded from analysis (4). Myogenic differentiation of immunofluorescence stained myotubes was determined by quantifying the differentiation index, fusion index, maturation index, nuclei per myotube, myotube per area, and myotube density. The differentiation index was defined as the ratio of nuclei number counted within myotubes expressing myosin heavy chain by the total nuclei counted in each field. The fusion index represents the ratio of the nuclei number in myotubes expressing myosin heavy chain with two or greater nuclei divided by the total number of nuclei (33), and the maturation index is the proportion of myotubes having five or more nuclei in a single myotube (28). The nuclei per myotube is the total counted nuclei within myotubes divided by the total number of myotubes within a specific region of interest (ROI). Four identical ROI per image were analyzed, counting all myotubes within the determined area (m_2). The myotube per area was determined by the number of myotubes containing 2 or more myonuclei, divided by the area measured. Finally, myotube density was defined as the number of nuclei counted inside myotubes with 2 or more nuclei, within the unit area measured (mm_2) (31). To determine myotube diameter, cultures were photographed under phase contrast at 10 or 20x magnification using an EVOS fluorescent microscope (Westburg, Leusden, The Netherlands). Myotube diameter was measured on 40-60 myotubes, per condition from four independent samples using ImageJ (National Institutes of Health, Frederick, MD, USA) (38). For each myotube that was analyzed (avg. 15), three random measurements were performed along the length of the myotube (n = 3 measurements/myotube).

The average of these three measurements was taken to represent one single value (3). All analyses were conducted by an investigator blinded to the experimental condition.

Human Sample Acquisition

Human skeletal muscle samples were collected as part of a previously published study for human MuSC and fibro/adipogenic progenitor (FAP) isolation (50). Participant characteristics were presented in our previous publication (50). Briefly, participants were ten healthy young men (mean ± SEM age: 21 ± 1 year) who regularly perform resistance exercise training (mean ± SEM training years: 5 ± 1 year) who volunteered for the study. The study was approved by the Institutional Review Board at the University of Illinois at Urbana-Champaign and conformed to standards for the use of human participants in research as outlined in the Declaration of Helsinki.

Muscle biopsies were collected from the central region of the vastus lateralis with a Bergstrom needle modified for suction under local anesthesia. Muscle samples (~50 mg) were separated from visible fat mechanically minced with scissors, then enzymatically digested with 0.2% type 2 collagenase for 45 minutes at 37_{o}C, with trituration at 30 minutes. Minced tissue was filtered through 70 and 50 µm filters. Filtered samples were spun at 500 x *g* for 10 minutes. Red blood cells were lysed in NH_4Cl buffer for 10 minutes at 4_{o}C. Samples were then centrifuged at 500 x *g* for 10 minutes. Finally, samples were plated into a 24 well dish for primary cell expansion. Culture expanded human muscle explants were processed as previously described (49). Once human cells reached 70-80% confluence in growth medium they were aspirated and washed with PBS. Adherent cells were detached with 0.05% trypsin-EDTA then centrifuged for 5 minutes at 430 x *g*. Cell concentration was adjusted to 5 $x10_6$ cells/mL, then incubated with PE-conjugated mouse anti-human CD56 (1:20) (Miltenyi Biotec, clone: AF12-7H3), biotinylated goat polyclonal anti-human PDGFRα (final concentration of 2.5 µg/ml) (R&D, cat#: BAF322) for 30 min at 4°C in the dark. Samples were washed, then incubated with streptavidin-PE/Cy5 (1:200) in the dark for 30 minutes. Samples were washed and strained through a 40 µm cell strainer immediately prior

to sorting using an iCyt Reflection flow sorter (iCyt; Champaign, IL). Sorting gates were established based on isotype and secondary only single-stained controls. Sorted cells were preserved in freeze media (50 mL – 20% FBS, 10% DMSO in DMEM) until future analysis. Sorted cell purity was confirmed via gene expression analysis for the myoblast markers: Pax3, Pax7, and MyoD, and the FAP marker: PDGFRα.

Statistical Analyses

Results are reported as the mean ± standard error of the mean (SEM) with all statistical analyses conducted in GraphPad Prism 8.1.0 (San Diego, CA, USA). For comparisons between two groups, paired or unpaired t-tests were used to determine significant differences between two groups, while one-way ANOVA (time) was used to determine changes across time points. In all cases, a $p<0.05$ was used considered statistically significant.

Results

LAT1 expression changes during key transition stages of myogenesis

Representative images of LAT1 and α-tubulin western blot are presented in Figure 1A. No significant differences in LAT1 protein content were detected throughout the different stages of myogenesis (Fig. 1B). However, when isolating key transition stages of myogenesis; i.e. quiescence (SFM) to proliferation (GM1), and proliferation to differentiation (DM1), LAT1 content was significantly increased at early differentiation (DM1) compared to early proliferation (GM1) ($p=0.00587$; Fig. 1C). CD98 western blot with α-tubulin loading control also extrapolated a few findings. CD98 protein content was not significantly different across all stages of myogenesis (Fig. 1D). Comparison of CD98 content at the time when LAT1 was at its highest (DM1) to control (SFM), revealed an increase ($p=0.00402$; Fig. 1E).

LAT1 gene expression was not significantly different between GM1 and SFM ($p=0.1355$; Fig. 1F). LAT1 gene expression was decreased in early (DM1; $p=0.00199$; Fig. 1G) and late differentiation (DM4; $p=0.00222$; Fig. 1G) both compared to late proliferation. CD98 gene expression was increased in early proliferation (GM1) compared to SFM ($p=0.00668$; Fig. 1H).

CD98 gene expression significantly increased in early differentiation (DM1; p=0.000659; Fig. 1I) and was not statistically significant at late differentiation (DM4; Fig. 1I) compared to late proliferation.

Representative images of LAT1 on human CD56+ myoblasts cells and fibroblasts western blot are presented in Figure 1J, confirming that LAT1 protein is expressed in human myoblasts. Protein content was lower in human MuSCs compared to human fibroblasts. (p=0.02; Fig. 1K).

LAT1 regulates myoblast proliferation and differentiation.

C2C12 myoblasts cultured with BCH, a LAT1-specific inhibitor, had significantly reduced proliferation at 24, 48, and 72 hours compared to CON (p=0.00035, p=0.00111, p=0.013746 respectively; Fig. 2A). C2C12 myoblasts cultured in DM with BCH had reduced total protein content, a marker of differentiation, which demonstrated a downward trend at day 3 (p=0.07; Fig. 2B) but was reduced at day 5 (p=0.000183; Fig. 2B). BCH-treated myoblasts had reduced diameter based on visual inspection (Fig. 2C). Significant reductions in differentiation index (p=0.0147, Fig.2E), fusion index (p=0.0005, Fig. 2F), maturation index (p=0.0001, Fig. 2G), nuclei per myotube (p=0.0001, Fig. 2H), myonuclei density (p=0.0001, Fig. 2I), and myotube per area (p=0.0001 Fig. 2J) were detected in BCH-treated differentiating myoblasts compared to control.

LAT1 content in myoblasts changes during catabolic but not anabolic conditions

LAT1 protein was quantified in C2C12 myoblasts exposed to 5mM of leucine in GM for 0, 0.5, 1, and 2 hours (Fig. 3A), to see if the high end of the dose response would cause an effect. No significant difference was observed at any time point on the time course (Fig. 3B).

Catabolic conditions were created by using DEX-supplemented differentiation media followed by immunofluorescence staining with MHC antibody for markers of differentiation (Fig. 4A). Differentiation index and fusion index were not significantly affected by DEX treatment (Fig. 4B-C), as we expected they would be. However, maturation index (p=0.0003, Fig. 4D), nuclei per myotube (p=0.0002, Fig. 4E, myonuclei density (p=0.001, Fig. 4F), and myotube per area (p=0.0086, Fig. 4G) were all lower in DEX treated myoblasts compared to CON. Myotube

diameters measured from the phase-contrast representative images (Fig. 4H) demonstrated a decrease in DEX treated myoblasts compared to CON (p=0.0002, Fig. 4I). A strong trend for reduced LAT1 protein (Fig. 4J) was observed in DEX compared to CON (p=0.0563, Fig. 4K).

Discussion

Our overall objective was to determine the role of LAT1 in myoblast function. Specifically, we aimed to determine if LAT1 was expressed on myoblasts and if this expression changed throughout the different phases of myogenesis *in vitro*, if LAT1 played a role in regulating myoblast differentiation and proliferation, and if LAT1 content was altered under anabolic and catabolic conditions *in vitro.* We met our objectives and uncovered that LAT1 protein is expressed on myoblasts, LAT1 protein content was differentially expressed throughout myogenesis in C2C12 myoblasts, with the peak occurring during differentiation. Further, pharmacological LAT1 inhibition reduces myoblast expansion and differentiation. Lastly, LAT1 protein content was not significantly modified *in vitro* with exposure to leucine supplementation; however, LAT1 content demonstrated a strong trend to be reduced under atrophic conditions *in vitro*, which was accompanied by extensive, negative morphological changes. LAT1 was ultimately demonstrated to be a key amino acid sensor on myoblasts, playing an important role in regulating myogenesis.

This study is the first characterizing the expression of LAT1 on myoblasts throughout all stages of myogenesis. Our findings agree with our overall hypothesis, that LAT1 would be expressed throughout every stage of myogenesis and play an important role in myoblast function. LAT1 protein was expressed throughout myogenesis with the level of expression peaking at DM1. These findings are consistent with changes in LAT1 gene expression which were highest at late proliferation (GM3) and early differentiation (DM1). Up-regulation of LAT1 during differentiation corresponds to a stage of myogenesis in which a requirement for an influx of amino acids, including leucine, are necessary to sustain increasing levels of protein synthesis to form myotubes (34). Protein content and gene expression of CD98, the second protein in the heterodimer stabilizing LAT1 (36), did not mirror the results for LAT1. This lack of uniformity may be due to

higher basal levels of LAT1 within myoblasts, or that CD98 has other functions besides being a co-transporter for LAT1 (15, 18, 48). For example, within mammalian embryonic stem cells, CD98 has been observed to regulate integrin-mediated cell spreading, adhesion, and cell migration (15, 18, 48).

We confirmed our results in C2C12 myoblasts by showing that LAT1 was also expressed in human primary myoblasts. Interestingly, myoblasts express significantly lower levels of LAT1 than the fibroblasts population. The reasons for differential LAT1 expression between myoblasts and fibroblasts are not completely clear. Fibroblasts are the main connective tissue cells in the body and help form the structural framework of tissues, as well as playing a significant role in tissue repair (51). Tyrosine, an AA, is the precursor of dopamine biosynthesis, plays a role in fibroblast transport velocity, and LAT1 is pivotal in transport for tyrosine within fibroblasts (25, 37, 52). As such, perhaps proliferating fibroblasts upregulate LAT1 to increase fibrogenesis; however, this speculation requires further investigation.

Having demonstrated that LAT1 is differentially expressed by myoblast throughout myogenesis, we next used a pharmacological approach to investigate the role of inhibiting LAT1 function on myoblast proliferation and differentiation. BCH, a LAT1 specific inhibitor, blocks amino acid transport through LAT1, and has previously been shown to inhibit LAT1 in various cancer cell lines (30). A 25 mM dose of BCH was used to inhibit LAT1, as it has been shown previously to block 50% of leucine uptake (29). BCH-mediated inhibition of LAT1 significantly reduced myoblast proliferation and differentiation. These findings are similar to previous research demonstrating that myoblast differentiation was inhibited with the removal of Leucine from media (2). Thus, functional LAT1 is necessary for normal myoblast function.

Having established an important role of LAT1 in regulating myoblast function, we next investigated the role of anabolic and catabolic conditions in regulating LAT1. Previous work has shown that increasing extracellular leucine concentration from 0 mM (CON) to 1 mM stimulated protein synthesis, promoting proliferation in C2C12 myoblasts (9). Further, supplementation with

proteins high in leucine increase LAT1 content in human skeletal muscle (5, 13). LAT1 content in our study; however, did not significantly increase following treatment with growth media supplemented with leucine which was contrary to our initial hypothesis. These data align with past *in vitro* studies demonstrating no change in LAT1 protein in proliferating myoblasts treated with leucine (14). Growth media contains leucine (~0.8 mM), thus it is possible that this amount of leucine in growth media could be sufficient enough to maximally stimulate LAT1 expression in myoblasts. Indeed, in the basal state, plasma leucine concentration is ~0.1 mM in humans and can increase to ~0.35 mM postprandially (41). Thus, the supraphysiological leucine dose used in the present study (5 mM) should have been more than sufficient to induce a response. These findings, combined with our finding that LAT1 content peaks during early differentiation suggests that LAT1 content is regulated by amino acid need, rather than availability in growth conditions.

Conversely, under catabolic conditions, our results indicate that LAT1 content is reduced in myotubes. Myotube catabolism was induced using DEX, a synthetic glucocorticoid that is commonly used to induce myotube atrophy *in vitro* (20), and causes muscle atrophy *in vivo* with high doses or prolonged use (40). DEX induces myotube atrophy by inhibiting protein synthesis and increasing protein catabolism (22). Similar to previous literature, we observed a reduction in myotube diameter and differentiation (20). Myotube atrophy and impaired differentiation in this model was accompanied by a reduction in LAT1 protein content. These findings may aid to explain the reduced ability of skeletal muscles to increase protein synthesis (anabolic resistance) in response to amino acids in most catabolic conditions (47). Combined with our leucine supplementation experiments, these findings also suggest that LAT1 content may be more strongly regulated by myotube diameter than by amino acid availability. This speculation requires further investigation.

To summarize, our results show that myoblasts directly sense and respond to amino acids in their environment. Amino acid transport through LAT1 plays a pivotal role for normal myogenesis, and LAT1 content is more strongly regulated by catabolic as opposed to anabolic

stimuli. As such, our results demonstrate a novel role of LAT1 in myoblast function *in vitro*, and provide support for the further exploration of the role of LAT1 in regulating MuSC function *in vivo*.

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Figures Legends

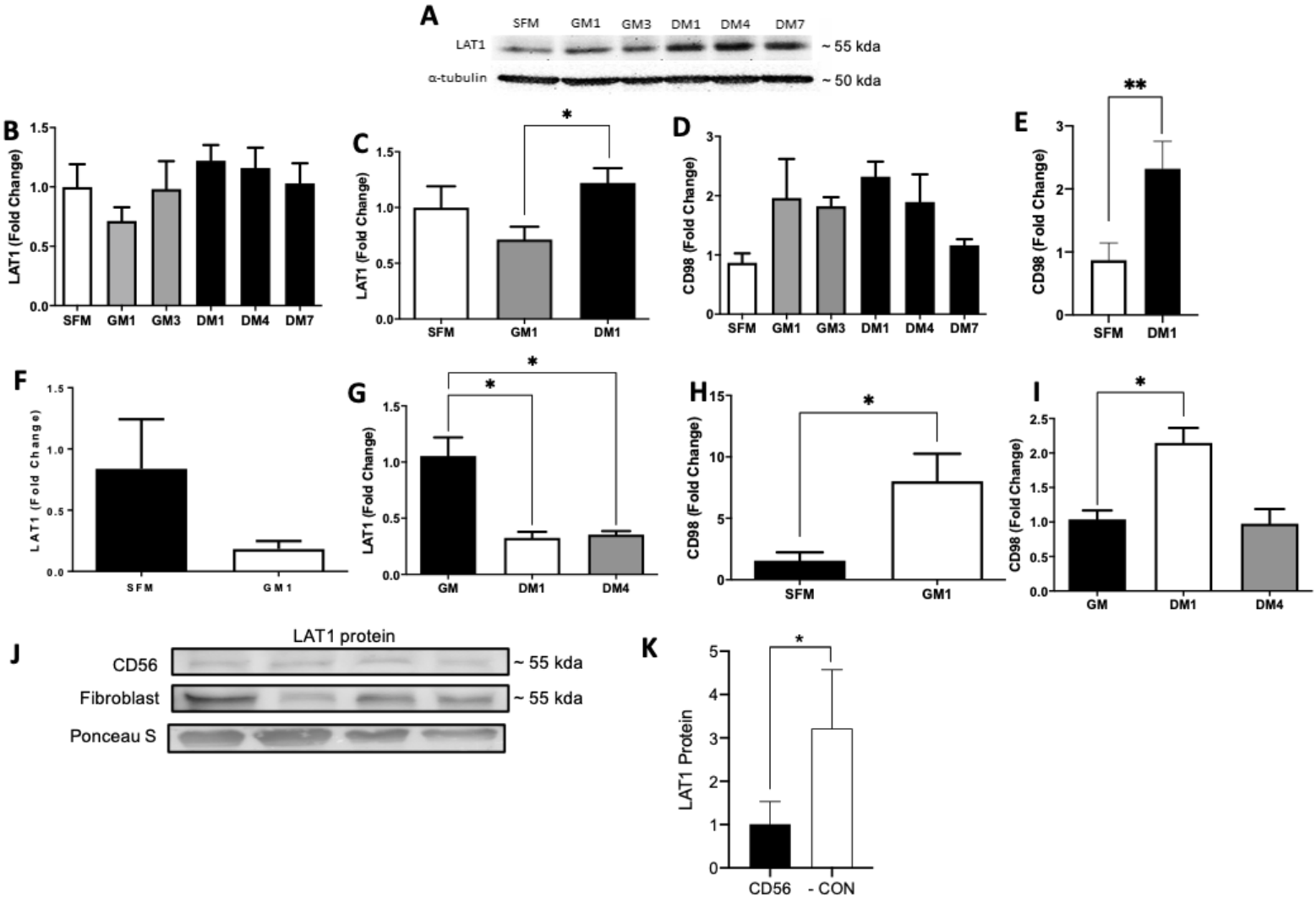
Figure 1. Changes in LAT1 and CD98 transporter gene expression throughout myogenesis. C2C12 myoblasts were made quiescent by culture in serum-free media (SFM) for 24 hours, followed by stimulation with growth media (GM) for 1-3 days, and then induced to differentiate by treatment with differentiation media (DM) for 1-5 days. (A) Western blot analysis of LAT1 and loading control α-tubulin during different stages of myogenesis. (B) Quantifications of LAT1 protein content, displaying LAT1 content was not significantly different at any point during myogenesis. (C) At key transitional stages of myogenesis (i.e. quiescence (SFM) to proliferation (GM1) to differentiation (DM1)), LAT1 content was found to be significantly higher at DM1 compared to GM1. Data are mean ± SEM for LAT1 with n=5 for SFM, and n=8/group for GM1 and DM1. (D) Western blot analysis of CD98 and loading control α-tubulin during different stages of myogenesis, where CD98 was not significantly different at any stage during myogenesis. (E) Comparison of CD98 content was completed at SFM and DM1, as these time points correspond to distinct differences in LAT1 content, showing a significant difference existed here as well. Data are mean ± SEM for CD98 with n=3, **$p<0.05$ vs. GM1. (F) LAT1 gene expression during quiescence (SFM) and early proliferation (GM1). (G) LAT1 gene expression during late proliferation (GM) and early differentiation (DM1 and DM4), data are mean ± SEM for LAT1 with n=5, *$p<0.05$ GM vs. DM1, and GM vs DM4. (H) CD98 gene expression during quiescence (SFM) and early proliferation (GM1) data are mean ± SEM for CD98 with n=3, *$p<0.05$ SFM vs. GM1. (I) CD98 gene expression during late proliferation (GM) and early differentiation (DM1 and DM4), data are mean ± SEM for CD98 with n=6, *$p<0.05$ GM vs DM1. Isolation and culture of human fibroblasts and myoblasts were then conducted to demonstrate the existence of LAT1 on human samples. (J) Western blot analysis of sorted cells, (K) quantification of protein density with paired t-test. *p-value of 0.02 n=4

Figure 2. LAT1 inhibition reduced C2C12 myoblast proliferation and differentiation. (A) C2C12 myoblasts were cultured in growth media with or without 25 mM of BCH, the LAT1 inhibitor, for 24, 48, and 72 hours. Myoblast content was significantly lower in BCH media at 24, 48, and 72 hours compared to CON. Data are mean ± SEM of n=8/group. *p<0.05 vs. control. (B) C2C12 myoblasts were also cultured in differentiation media (DM) with or without 25 mM of BCH for 1, 3, and 5 days. Quantification of protein content showed a significant decrease in myoblast differentiation in BCH versus control. Data are mean ± SEM of n=6/group, *p<0.05 vs. control. To investigate the morphological changes in C2C12 cells, (C) phase-contrast representative images of CON, and BCH-treated C2C12 cells were taken, scale bar corresponds to 200 μm. (D) Cells were subjected to immunofluorescence staining with MHC antibody (MF20 – red color indicative of myotubes) and visualized with anti-mouse IgG (H+L) Alexaflour 594 goat. The nuclei were counterstained with DAPI (blue), scale bar was 200 μm with 10x lens used. Morphological changes were characterized. (E) Differentiation index; the percentage of cells expressing myosin heavy chain relative to total cell number, (F) fusion index; the ratio of the nuclei number in myocytes with two or more nuclei versus the total number of nuclei, (G) maturation index; the ratio of myotubes with > 5 nuclei versus the total number of nuclei, (H) nuclei per myotube (I) density; the number of myonuclei per area analyzed, and (J) the myotube per area. Results are means ± SEM. *p < 0.05 vs treated group, n=4.

Figure 3. LAT1 content is not increased in proliferating myoblasts following *in vitro* leucine supplementation. (A) representative western blot analysis of a LAT1 time course, (B) C2C12 myoblasts were cultured in growth media with 5 mM leucine for a time course of 0, 0.5, 1, and 2 hours. LAT1 content was not significantly different across the time course. Data are expressed as mean ± SEM with n=9.

Figure 4. Dexamethasone (DEX) induced morphological changes in C2C12 cells. Cells were cultured in growth media, differentiated, then treated with a vehicle of 10^{-6} M DEX in 2% horse serum for 24 hrs. (A) Cells were subjected to immunofluorescence staining with MHC antibody (MF20 – red color indicative of myotubes) and visualized with anti-mouse IgG (H+L) Alexaflour 594 goat. The nuclei were counterstained with DAPI (blue). Scale bar, 200 μm with 10x lens. (B) Differentiation index; the percentage of cells expressing myosin heavy chain relative to total cell number. (C) Fusion index; the ratio of the nuclei number in myocytes with two or more nuclei versus the total number of nuclei (D) Maturation index; the ratio of myotubes with > 5 nuclei versus the total number of nuclei, (E) Nuclei per myotube, (F) Density; the number of myonuclei per area analyzed, and (G) myotube per area. Results are means ± SEM. *$p < 0.05$ vs treated group, n=3. (H) Phase-contrast representative images of CON and DEX-treated C2C12 cells, scale bar corresponds to 200 μm with 10x lens. (I) Difference of intensity for western blot bands for CON and DEX-treated C2C12 cells**,** from left to right – standard sample (blank), 3 control, 3 DEX conditioned wells. (J) Myotube diameter of DEX treated groups were significantly lower compared to CON, *$p < 0.05$. (K) Quantification of western blot bands displayed no significant difference, however with a $p= 0.0563$, DEX treated group had a decreasing trend. Results are means ± SEM n=6.

Figure 1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SFM
GM1
GM3
DM1
DM4
DM7
LAT1
~ 55 kda
α-tubulin
~ 50 kda
B
LAT1 (Fold Change)
C
D
CD98 (Fold Change)
E
F
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K
LAT1 Protein
CD56
- CON

Figure 2

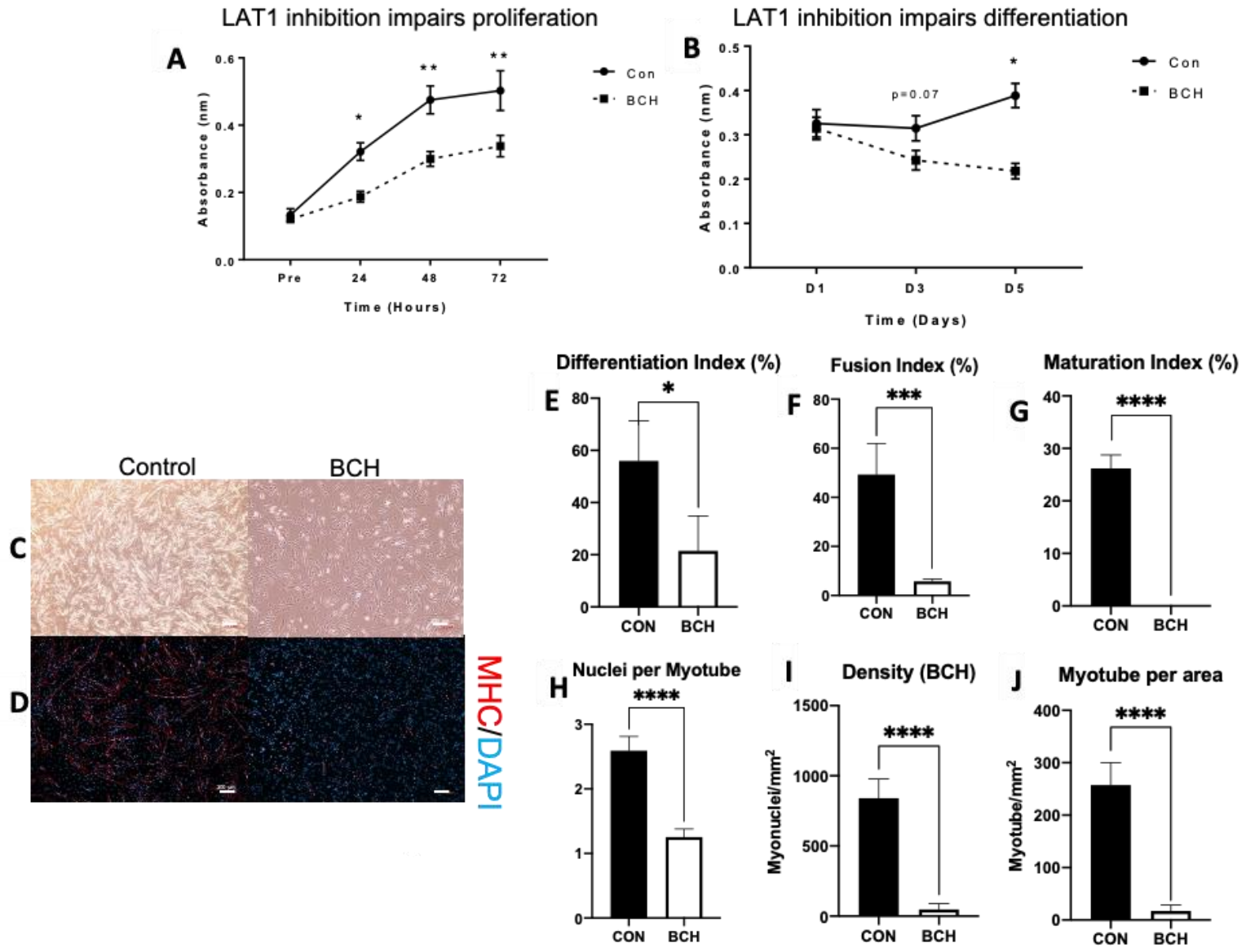

LAT1 inhibition impairs proliferation
A
Absorbance (nm)
Time (Hours)
Pre
24
48
72
Con
BCH
LAT1 inhibition impairs differentiation
B
p=0.07
Time (Days)
D1
D3
D5
Control
BCH
C
D
MHC/DAPI
Differentiation Index (%)
E
Fusion Index (%)
F
Maturation Index (%)
G
CON
BCH
Nuclei per Myotube
H
Density (BCH)
I
Myonuclei/mm²
Myotube per area
J
Myotube/mm²

Figure 3

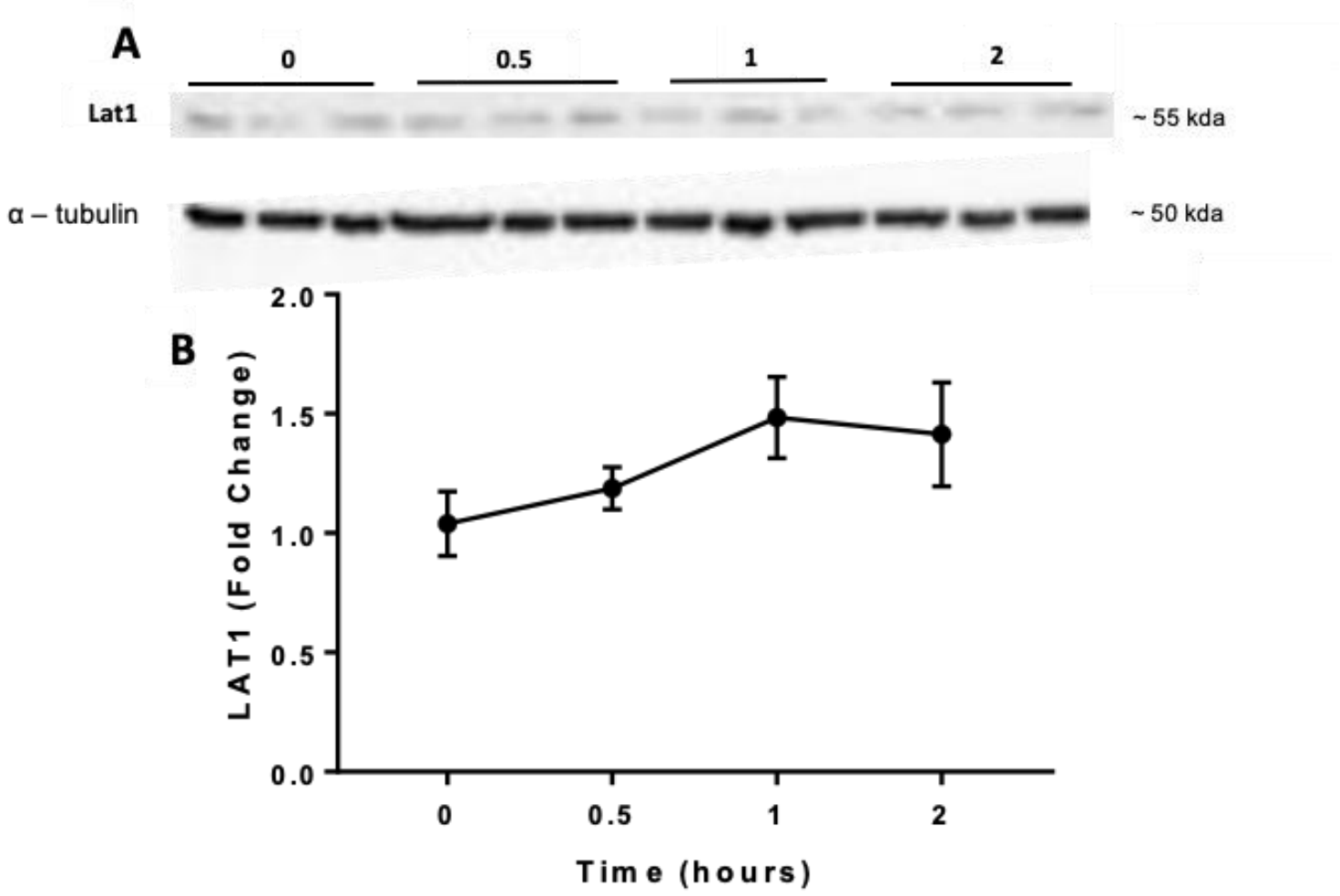
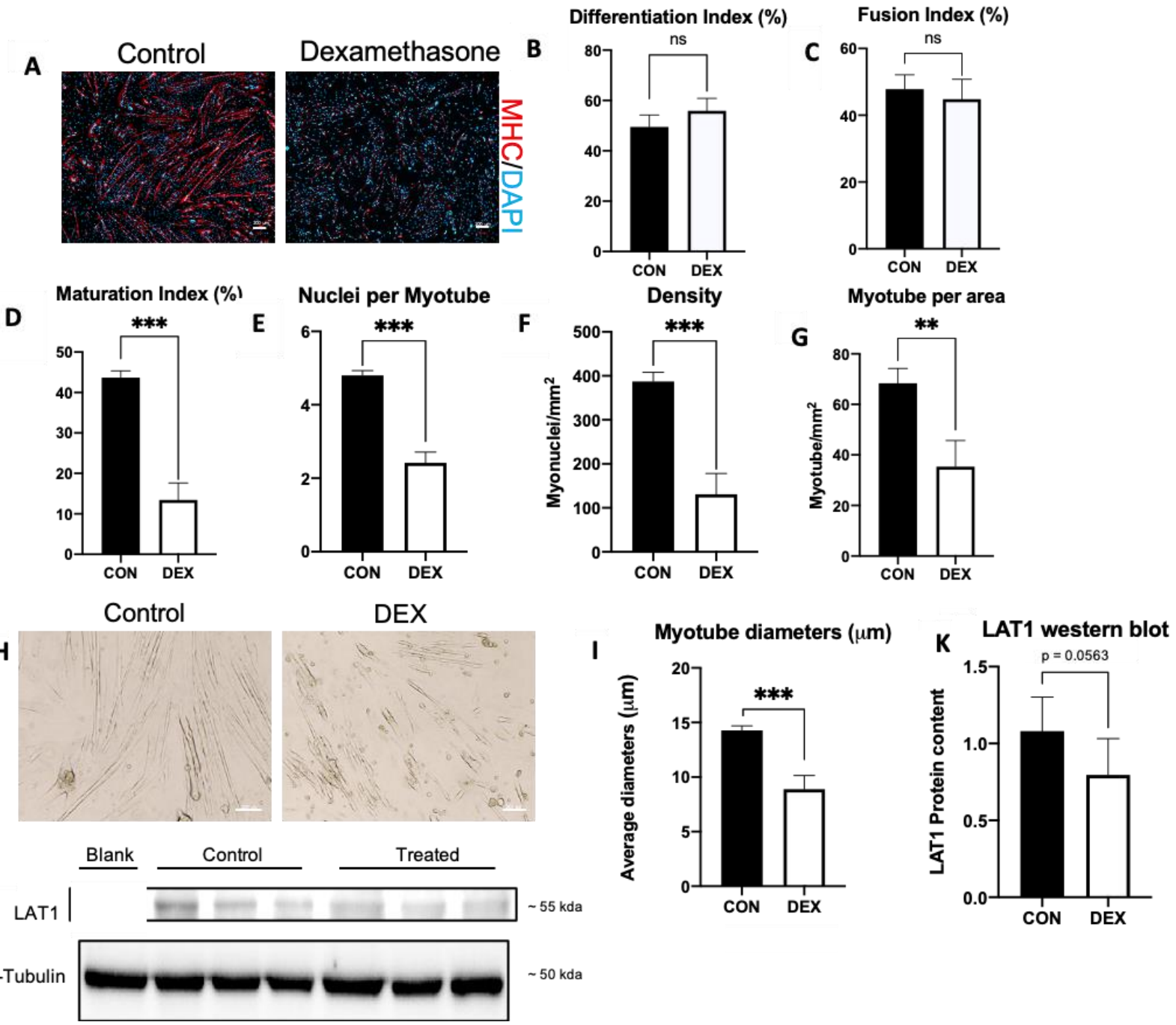

A
0
0.5
1
2
Lat1
~ 55 kda
α – tubulin
~ 50 kda
B
LAT1 (Fold Change)
2.0
1.5
1.0
0.5
0.0
0
0.5
1
2
Time (hours)

Figure 4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Control
Dexamethasone
MHC/DAPI
B
Differentiation Index (%)
ns
CON
DEX
C
Fusion Index (%)
ns
CON
DEX
D
Maturation Index (%)
***
CON
DEX
E
Nuclei per Myotube
***
CON
DEX
F
Density
Myonuclei/mm²
***
CON
DEX
G
Myotube per area
Myotube/mm²
**
CON
DEX
H
Control
DEX
I
Myotube diameters (μm)
Average diameters (μm)
***
CON
DEX
J
Blank
Control
Treated
LAT1
~ 55 kda
α-Tubulin
~ 50 kda
K
LAT1 western blot
p = 0.0563
LAT1 Protein content
CON
DEX

CHAPTER 3

General Discussion

MuSC dysfunction or depletion leading to reduced muscle regeneration (7) underlies several chronic muscle-wasting condition including fasting, disuse, side effects of pharmaceutical therapy, disease, and aging (3). Amino acid supplementation, specifically with leucine, has been investigated as a means of preventing muscle atrophy (15). LAT1 an amino-acid transporter, is the exclusive transporter for leucine, which requires the co-transporter glycoprotein CD98 for proper function (13). Leucine directly initiates protein synthesis (2, 5, 17, 22) by activating mTORC1 (8); therefore making its sole transporter LAT1 important for regulating skeletal muscle remodeling (8). Thus, by examining LAT1, leucine's only avenue into cells, we aimed to determine if LAT1 was expressed on myoblasts and if this expression changed throughout the different phases of myogenesis *in vitro*, if LAT1 played a role in regulating myoblast differentiation, proliferation, and if LAT1 expression on myoblasts is regulated by anabolic and catabolic conditions *in vitro*. Our results demonstrate that LAT1 was expressed on myoblasts *in vitro* and plays a pivotal role for myogenesis. We observed through pharmacological inhibition of LAT1, that it played a significant role in myoblast proliferation and differentiation. Finally, we noted that treating myoblasts with leucine *in vitro* did not alter LAT1 content, whereas the atrophic conditions induced by glucocorticoids (in this case, DEX) resulted in lower LAT1 expression. Together, these results suggest that LAT1 is an important regulator of myoblast function.

Characterization of LAT1 presence in human myoblast

After confirming the presence of LAT1 in our *in vitro* studies using C2C12 myoblasts, which are an immortalized cell line derived from mouse MuSCs (19), we wanted to demonstrate that LAT1 was also expressed by human myoblasts. We first attempted an immunofluorescence approach using human muscle biopsies and co-staining for LAT1 and Pax7 (Supplemental Figure 6). We found our images to be similar to published results of LAT1 staining in human muscle

biopsies (8); however, we considered our images not satisfactory as they produced a large amount of non-specific staining. To overcome this obstacle and still achieve our objective of determining the presence of LAT1 on human myoblast, we used stocks of sorted human myoblasts previously isolated by our lab for western blotting analysis of LAT1. We confirmed that human myoblasts express LAT1. As a control, we used human fibroblasts, which are non-myogenic cells that aid in forming structural framework of tissues (20). We know that fibroblasts are the main connective tissue cells in the body and help form structural framework of tissues, as well as playing a significant role in tissue repair. Interestingly, LAT1 content was significantly higher in fibroblasts compared to myoblasts. LAT1 plays a major role in transport for tyrosine within fibroblasts (9, 14, 21). It is possible that a high abundance of LAT1 on fibroblasts could be required for fibrogenic homeostasis. These findings about LAT1 content on fibroblasts bring rise to questions about the role of leucine and other amino acids in regulating fibroblasts, leaving speculation to be further investigated.

Effects of Atrophic conditions on LAT1

As previously stated, glucocorticoids (GCs) have been associated with negative effects on myoblasts *(4)*. We demonstrated above that GCs induce atrophy and reduce LAT1 expression. Previous studies have also taken this a step further and displayed that amino acids could inhibit muscle atrophy (18). Cancer-induced cachexia has also been associated with whole-body muscle wasting (loss of muscle, fat mass, and body weight) which cannot be undone by feeding (10). Thus, to replicate these conditions *in vitro*, C2C12 myotubes were treated with Lewis lung carcinoma condition media (LCM), which are highly cachectic (23), inducing atrophy and cachexia (12, 16) (Supplemental Fig. 5). The utilization of Lewis lung carcinoma (LLC) cells within mice has been observed to induce muscle wasting (6). Additionally, the cachectic media generated from LLC cells, can also be utilized on myotubes to induce wasting directly *in vitro*, ultimately disturbing the protein synthesis and protein degradation regulation systems (6). The model we utilized is a cultured experiment, whereas skeletal muscle is incubated in cancer cell conditioned medium

(CM), to observe mechanisms of wasting in muscle cells (10, 16). We observed that LCM induced atrophy on C2C12 cells (Supplemental Fig. 5). There was a significant decrease in differentiation, fusion and maturation indices of LCM treated groups compared to CON ($p<0.05$. Supplemental Fig. 5B-5D). As well as a significant decline of LCM treated groups for nuclei per myotube, myonuclei density, myotube per area and myotube diameters compared to CON ($p<0.05$. Supplemental Fig. 5E-5G, 5I). This finding is in line with other studies that observed catabolic responses in cultured myotubes of LLC tumor-bearing mice (23). Puppa and associates observed LCM decreased myosin heavy chain expression, and higher doses of LCM (25%) was sufficient to reduce myotube diameter (16). We utilized a ratio of 50%.

Limitations

One of the biggest limitations of this study was the suboptimal immunofluorescent images that were obtained from our human muscle samples in our search to determine the presence of LAT1 in human myoblasts. Differences in antibodies or the utilization of old samples could have been the cause of these suboptimal images. This lack of clear imaging was an important limitation of our study because by visualizing LAT1 in human skeletal muscle we would have been able to take our analysis further than gene expression and protein levels, but add location as a proxy for functional information to our results (8). We would have been able to possibly locate LAT1 near the sarcolemmal membrane, similar to Hodson and colleagues (8).

Also, due to Covid-19, we were unable to completely analyze and compare the protein difference between atrophic condition experiment of LLC. The LCM condition experiment would validate the LLC model highlighted above and investigate further how LAT1 would be affected by LCM vs CON. We hypothesized that LCM treated group would undergo atrophy, and be greatly affected morphologically by the LLC model.

Future Research

Our current research has demonstrated that the inhibition of LAT1, through the use of BCH, negatively impacts myogenesis. Future research could take this a step further and completely ablate LAT1 in MuSCs. The Clustered Regularly Interspaced Short Palindromic Repeats/Cas (CRISPR-associated) (CRISPR-Cas9) system is a mediated gene-editing tool, which cleaves DNA, making ablation or deletion possible (11). One would hypothesize that with the ablation of LAT1 there would be a significant decrease in leucine transport, proliferation, differentiation, myogenesis as a whole, and mTORC1 activation (1). Bothwell and colleagues (1) used CRISPR-Cas to reduce LAT1 gene expression up to 90% in epithelial (HUH7) and mesenchymal (SK-Hep1) hepatocellular carcinoma (HCC) cell lines and observed that this suppression failed to substantially alter proliferation and stimulation of mTORC1 growth. The data suggest that *in vitro* human epithelial and mesenchymal HCC cells adapt to LAT1 knockout (1). It is possible that utilizing myoblasts instead of cancer cell lines like Bothwell and colleagues (1) could yield different results, displaying a possible difference in LAT1 dependent on location and cell type.

Conclusion

The novel contribution of our work is that myoblasts express LAT1 throughout *in vitro* myogenesis with peak expression corresponding to a time when myofibrillar proteins are actively being synthesized. Further, we show for the first time that pharmacological LAT1 inhibition impaired myoblast proliferation and differentiation, and that LAT1 was not altered in myoblasts by leucine supplementation but reduced in catabolic conditions *in vitro*. Together, these results indicate a novel role for LAT1 in muscle hypertrophy through its function as a key amino acid sensor on myoblasts that participates in regulating myogenesis. These results are significant because they provide the proof-of-principle data to support future work moving towards the development of novel therapies for combating muscle atrophy and other muscle-wasting conditions by directly targeting LAT1 in myoblasts to improve muscle regeneration.

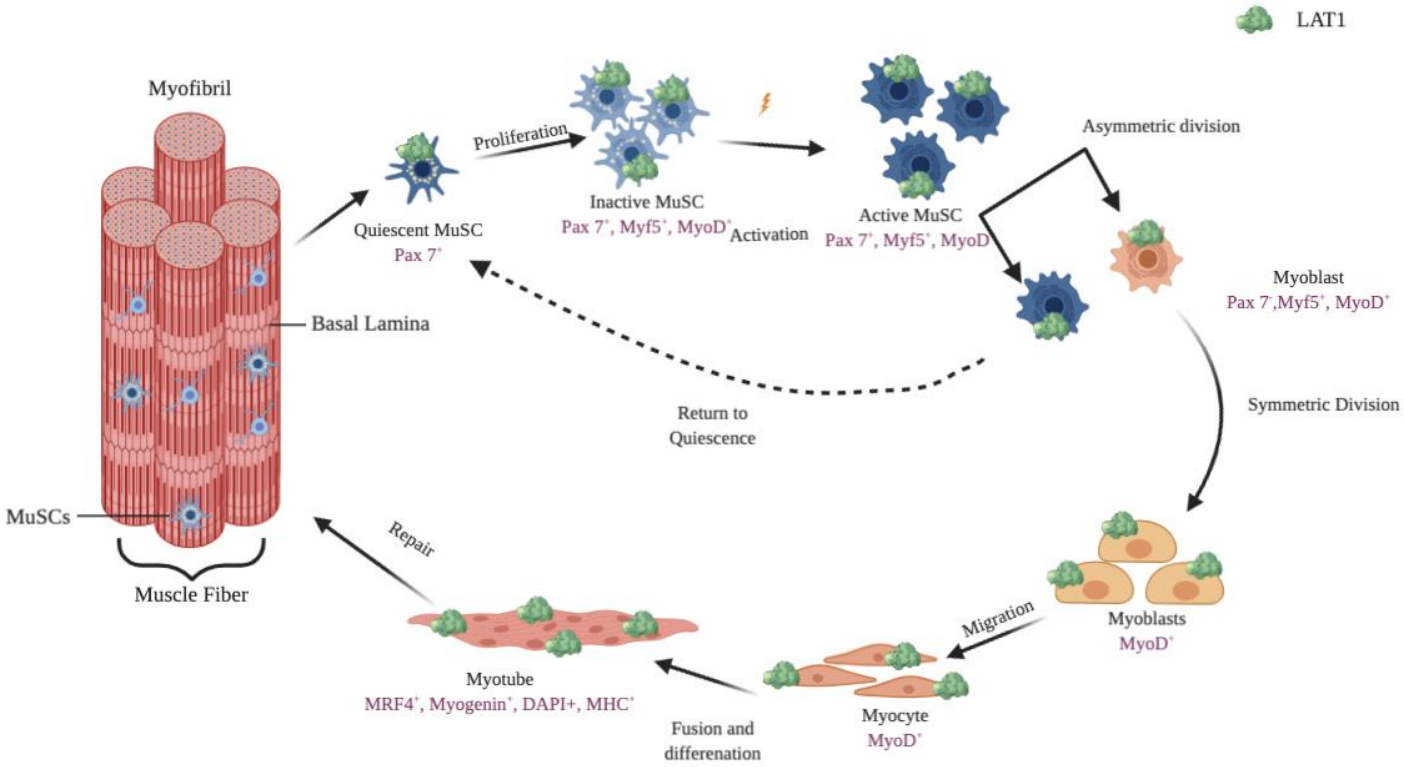

Figure 1. LAT1 is expressed throughout myogenesis. the L-type amino acid transporter 1 (LAT1), the sole transporter of Leucine intracellularly, has been observed to be expressed throughout all stages of myogenesis *in vitro*, with the peak expression being displayed at the beginning of myogenic differentiation. LAT1 is a key amino acid sensor and could provide future information in regards to therapy directed at improving muscle regeneration.

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Appendix
Appendix A – Supplemental Figures

Supplemental Figure 1. Comparative cell cycle analysis of C2C12 cells in (A) growth media (GM), (B) serum free media (SFM), and (C) GM prior to 24hrs in SFM. Comparison of populations in each cell cycle phase in GM and SFM (D), and between cells in GM prior to 24hrs in SFM and SFM only (E). Significant difference between GM vs. SFM in G_0/G_1 phase, GM vs. SFM in S phase, SFM + GM vs. SFM in G_0/G_1 phase, SFM + GM vs. SFM in S phase, and SFM + GM vs. SFM in G_2/M (****p < .0001). Significant difference between GM vs. SFM in G_2/M phase (**p <.01). Values represented as mean ± S.E.M.

Supplemental Figure 2. Isolation and culture of human mesenchymal progenitors and satellite cells. (A) FACS analyses via flow cytometry showing the expression of PDGFR**α –** PECy5 (x-axis) and CD56-FITC (y-axis). Gene expression through transcription factors of Pax3 (B), (C) Pax7, (D) MyoD1, and (E) PDGFRa. n = 2-7/ per group.

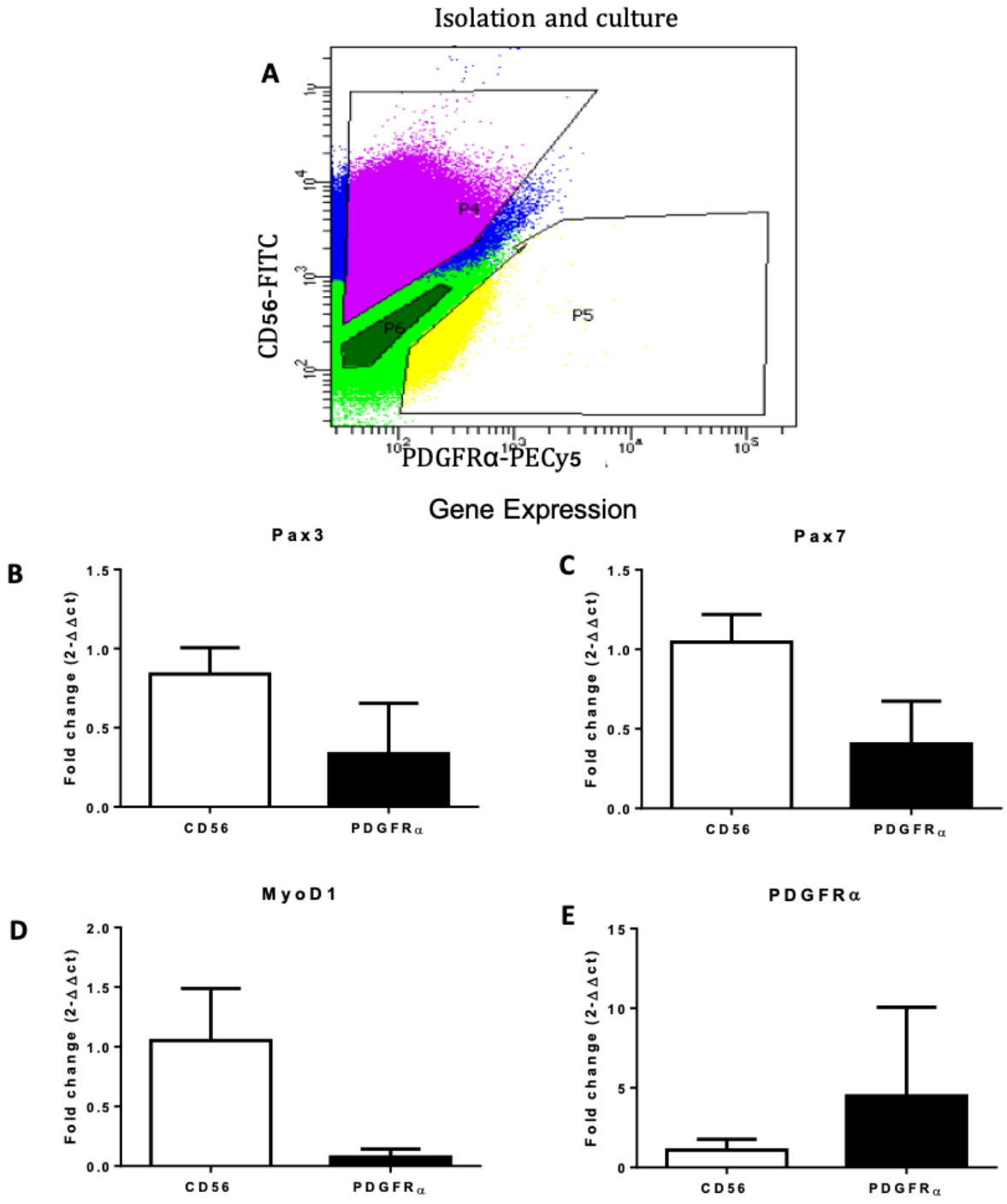
Supplemental Figure 3. Leucine dose-response of C2C12 cells. Doses of 0 mM, 0.63 mM, 1.24 mM, 2.5 mM, and 5 mM of leucine (x-axis) and fold change (y-axis) were analyzed from western blot of dose response. n = 6/ per group.

Supplemental Figure 4. Myoblast BCH dose response. The protein content of proliferating C2C12 myoblasts represented by absorbance at 590 nm treated with varying concentration of LAT1 inhibitor, BCH, after 3 (GM3) days in growth media. Significant difference of 47% between the 0mM and 25mM condition in protein content ($p<0.05$). N=8/group for B), data presented as mean ± SEM.

Supplemental Figure 5. Lewis Lung Carcinoma conditioned media (LCM) induced morphological changes in C2C12 cells. Cells were cultured in growth media, differentiated, then treated with a vehicle, LCM for 48 hrs. (A) Cells were subjected to immunofluorescence staining with MHC antibody (MF20 – red color indicative of myotubes) and visualized with anti-mouse IgG (H+L) Alexaflour 594 goat. The nuclei were counterstained with DAPI (blue). Scale bar of 200 μm with 10x lens used. Morphological changes were characterized. (B) Differentiation index; the percentage of cells expressing myosin heavy chain relative to total cell number. (C) Fusion index; the ratio of the nuclei number in myocytes with two or more nuclei versus the total number of nuclei, (D) Maturation index; the ratio of myotubes with > 5 nuclei versus the total number of nuclei, (E) Nuclei per myotube, (F) Density; the number of myonuclei per area analyzed, and (G) myotube per area. Results are means ± SE. *p < 0.05 vs treated group, n=3. (H) Phase-contrast representative images of CON and LLC treated C2C12 cells. Scale bar corresponds to 200 μM with 10x lens used. Myotube diameter (I) of C2C12 cells after 48 h of LLC treatment. Results are means ± SE (n = 3). Statistical analysis determined *p < 0.05, p = 0.0328

Supplemental Figure 6. Immunofluorescence stains of human muscle biopsies and co-staining for LAT1 and Pax7. Scale bar of 200 μm.

Supplemental Figure 1.



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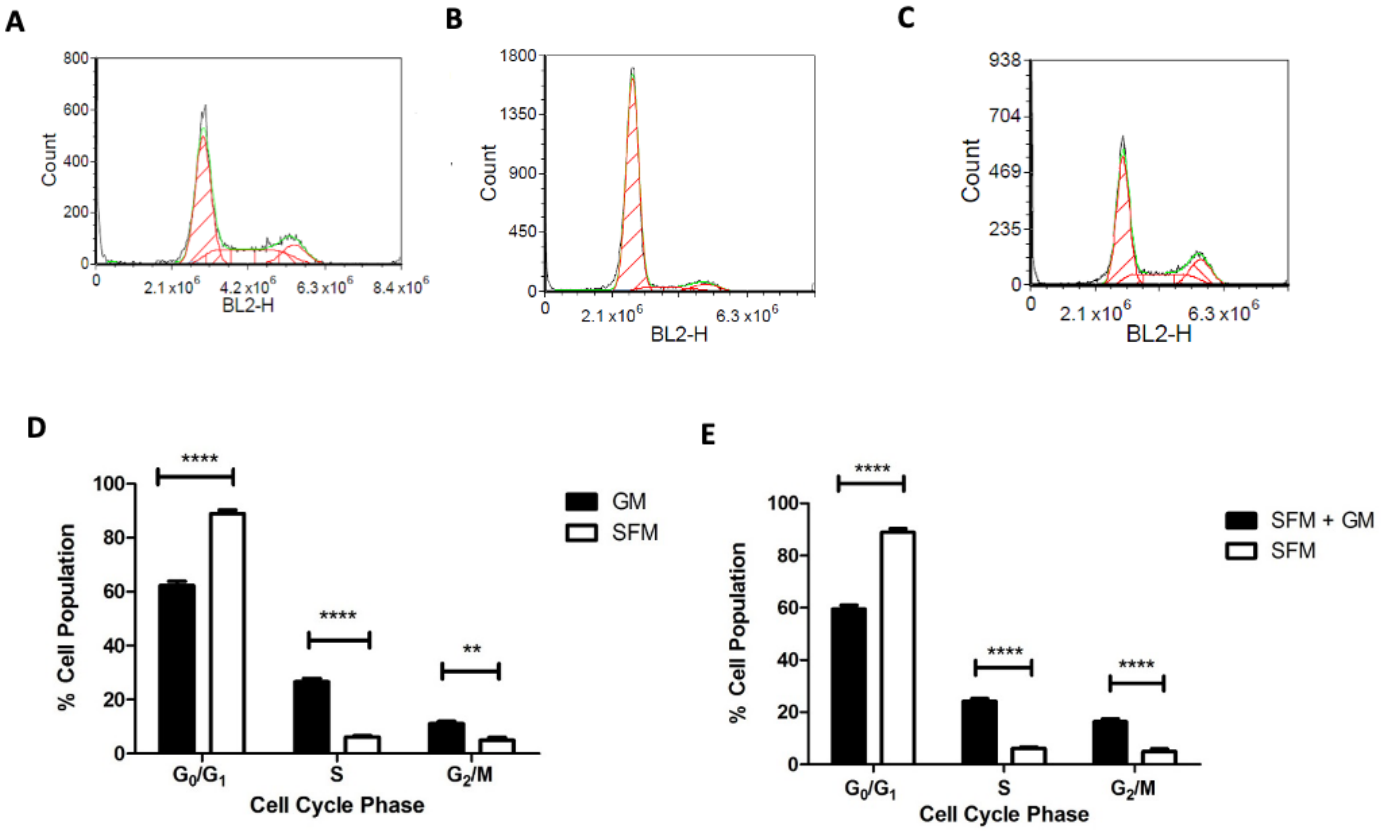

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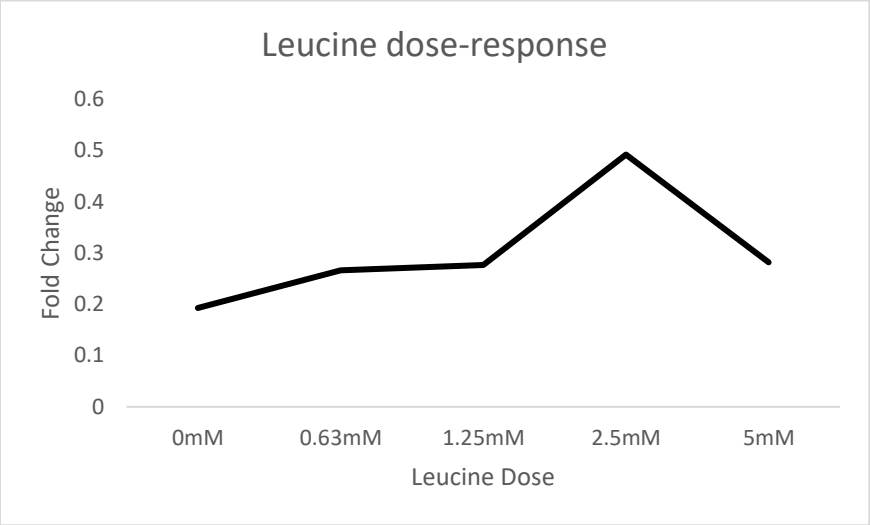

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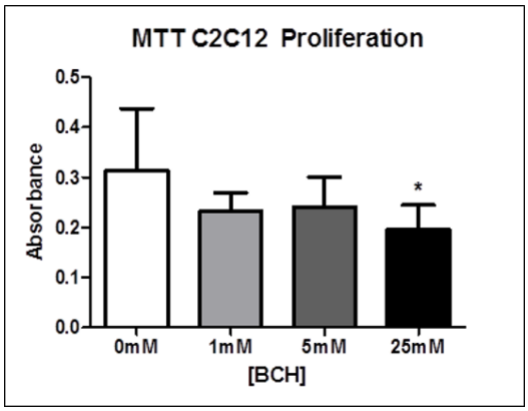
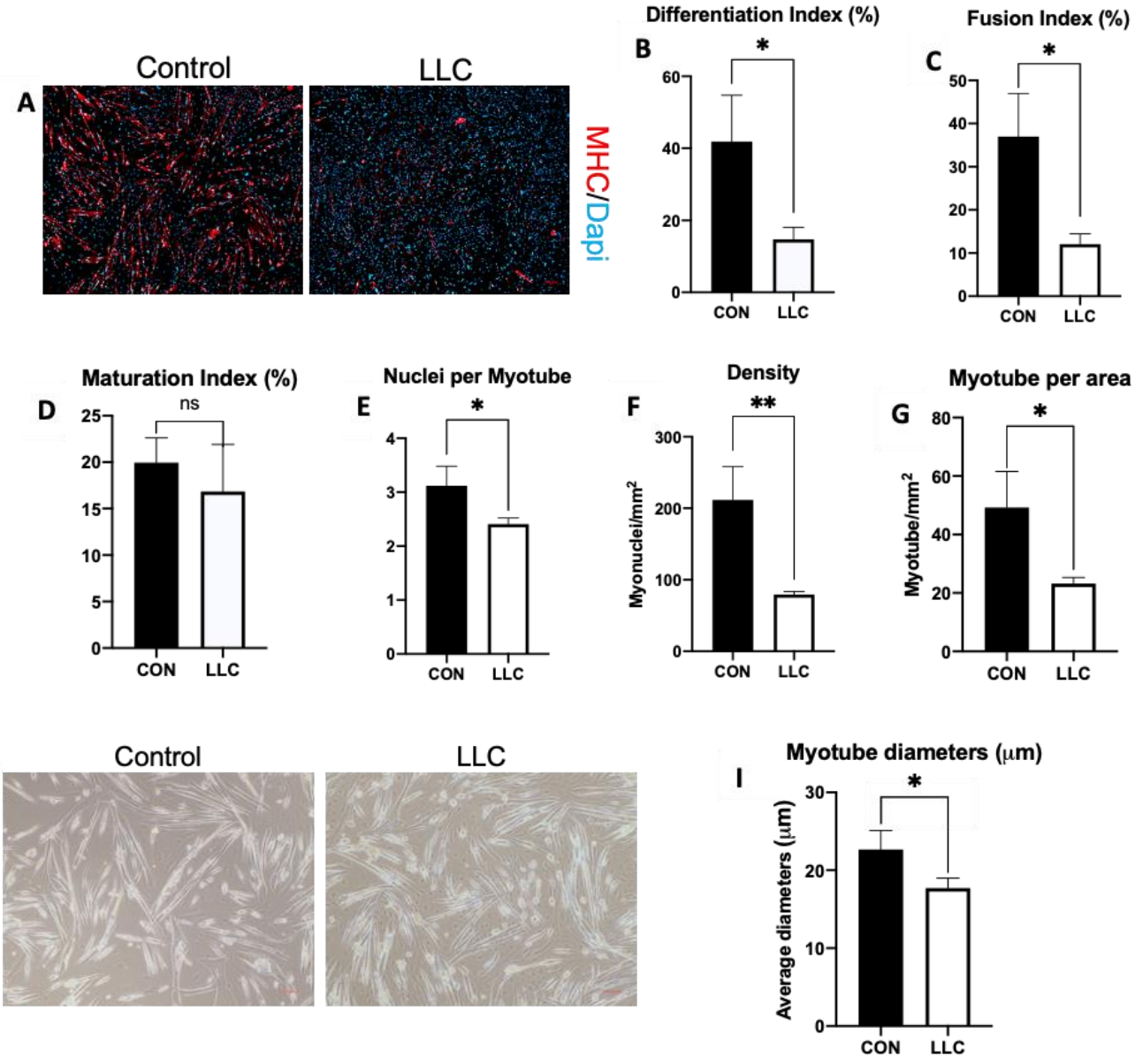


Supplemental Figure 5.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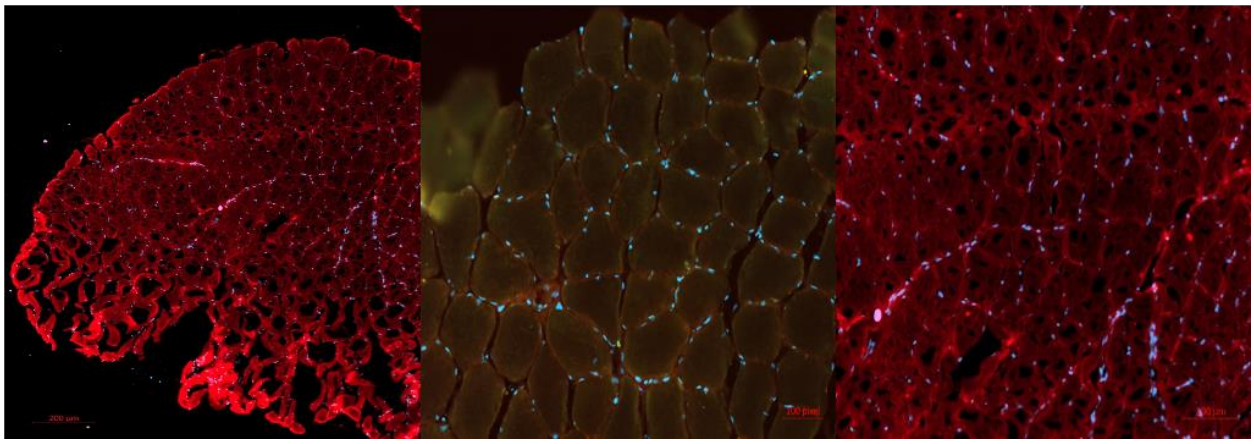
Control
LLC
MHC/Dapi
B
Differentiation Index (%)
*
60
40
20
0
CON
LLC
C
Fusion Index (%)
*
50
40
30
20
10
0
CON
LLC
D
Maturation Index (%)
ns
25
20
15
10
5
0
CON
LLC
E
Nuclei per Myotube
*
4
3
2
1
0
CON
LLC
F
Density
**
Myonuclei/mm2
300
200
100
0
CON
LLC
G
Myotube per area
*
Myotube/mm2
80
60
40
20
0
CON
LLC
H
Control
LLC
I
Myotube diameters (μm)
*
Average diameters (μm)
30
20
10
0
CON
LLC

Supplemental Figure 6.



Appendix B – Supplemental Methods

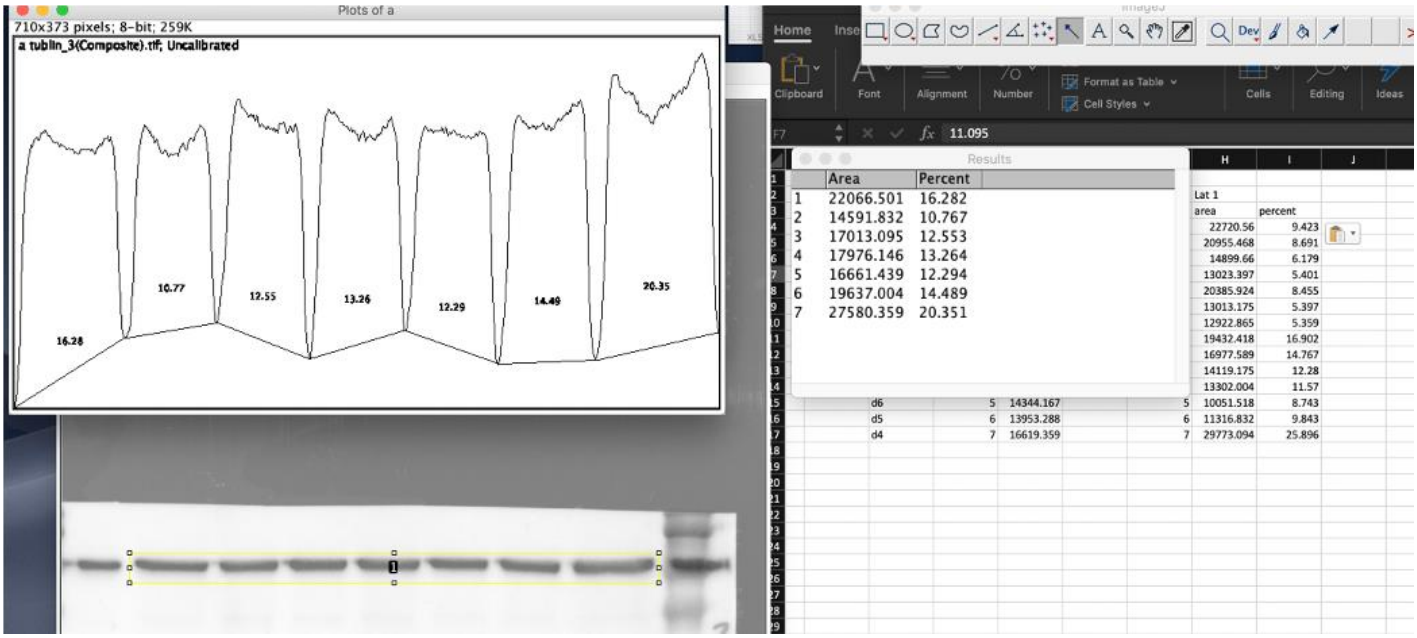
Cell Cycle Analysis

Cell cycle analysis was assessed after 24 hours in SFM, and after 24 hours SFM plus 24 hours GM. Cells were washed in phosphate buffered saline (PBS) then centrifugation at 200 x g for 5 min at 4˚C. Cells were fixed by resuspension in 1 mL of ice-cold PBS then added drop wise into 9 mL of ice-cold ethanol while gently vortexed. Cells were stored at -20˚C for 24hrs and then centrifuged at 200 x g for 10 min at 4˚C. After washing, cells were resuspended in 500 µl PI/Triton X-100 solution and then left to incubate at room temperature for 30min in the dark. Data was acquired by Attune acoustic focusing cytometer (Applied Biosystems, Grand Island, NY). After doublet discrimination, cell cycle phase was determined by FCS-Express analysis software.

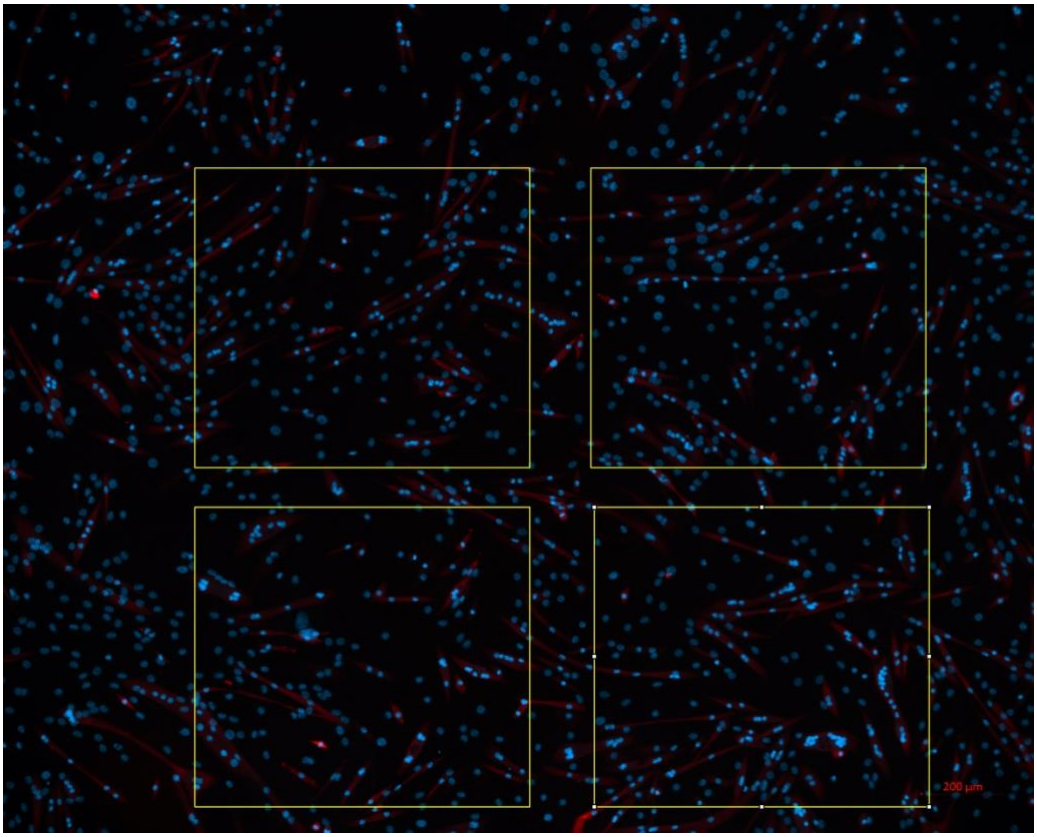
Supplemental Figure 6. Western blot analysis. Displaying how western blot band densities were calculated, and background adjustment was incorporated.

Supplemental Figure 7. Myosin heavy chain immunofluorescence myotube staining. Displaying ROI where myotube comparisons were calculated.

Supplemental Figure 7.



Supplemental Figure 8.



Appendix C – Protocols used in Thesis

Cell Culture (C2C12)

Make Growth Media (50 mL)

i. Heat DMEM, Pen/Strep and FBS in water bath (37$_o$C)
ii. Pipette 44.5 mL of DMEM into 50 mL Falcon tube
iii. Add 10% FBS (5 mL), and 1% Pen/Strep (0.5 mL)

Changing Media

i. Remove old growth media
ii. Rinse cells with warm saline
iii. Remove and discard used saline
iv. Fill plates with 8-10 mLs of GM

Cell Splitting

i. Heat growth media in water bath (37$_o$C), saline and 0.25% trypsin
ii. Remove old growth media from cells
i. Rinse cells with warm saline
ii. Remove and discard used saline
iii. Add roughly 600-1000 µL of 0.25% trypsin to cells
iv. Incubate cells for 5 mins
v. Gently tap plate of cells to make sure cells have detached from dish
vi. Add ~3 mL fresh media to plate
vii. Trituate cells roughly 8-10 times
viii. Remove cells from dish, placing them into 15 mL centrifuge tubes
ix. Spin centrifuge tubes for 5 mins, at 1500 rpm at 4$_o$C
x. Remove supernatant and resuspend cells with GM
xi. Split cells into new plates
xii. Top each plate up with 8-10 mLs of media (dependent on dish the cells are being regrown in)

Cell Counting

i. Follow procedure for cell splitting up to just after trypsinization
ii. Add 10 mLs GM to cells
iii. Triturate x 10
iv. Transfer to 50 mL Falcon
v. Centrifuge at 2800 g and 4oC for 3 min (if no pellet, spin again)
vi. Remove supernatant
vii. Resuspend pellet in 3 mLs of GM
viii. Trituate
ix. Add 10 µL to each side of hemocytometer
x. View under inverted scope
xi. Count all 4 grids (make sure cells are evenly distributed in all 4 grids)
xii. Use only 3 grids
xiii. Calculations:
    - Ave # of cells/ grid = grid 1+grid 2+grid 3/3
    - # cells/mL = Ave x 10_4
    - total # cells = cells/mL x 3 (3 b/c resuspended in 3 mLs)
    - $C_1V_1=C_2V_2$ to get final volume to add (subtract 3 mLs from V2)

Make Freeze Media (50 mL – 20% FBS, 10% DMSO in DMEM)

i. Heat DMEM, and thaw FBS in water bath (37$_o$C)
ii. Pipette 20% FBS, 10% DMSO in DMEM into 50 mL Falcon tube
iii. Add 10% FBS (5 mL), and 1% Pen/Strep (0.5 mL)

Freezing Cells
i. Make freeze media
ii. Rinse with warm saline and trypsinze (same as procedure for splitting cells)
iii. Add roughly 5 mL GM to dish
iv. Transfer cells to 15 mL Falcon
v. Centrifuge at 500 rpm for 5 min at 4$_o$C
vi. Remove supernatant (keep pellet)
vii. Resuspend cells in 3 mL freeze media
viii. Trituate
ix. Add roughly 1.5 mL of cells to each cyro vial
x. Store in –80 for 24 hours
xi. Transfer to liquid nitrogen for storage

Thawing Cells
i. Fill plate with GM
ii. Thaw cells in hand (keep cells slushy, do not let them fully thaw)
iii. Pipette cells into plate
iv. Rinse the vial in which cells came from with growth media to ensure all cells have been removed
v. Transfer all cells from plate to Falcon tube
vi. Spin at 1500 rpm for 5 min
vii. Remove supernatant
viii. Resuspend pellet in growth media and add to new plate

DEX induced atrophy
Make Differentiation Media (50 mL)
i. Heat DMEM, Pen/Strep and Horse Serum (HS) in water bath (37$_o$C)
ii. Pipette 48.5 mL of DMEM into 50 mL Falcon tube
iii. Add 2% HS (1 mL), and 1% Pen/Strep (0.5 mL)

Differentiation (Myotubes)**:**
1. C2C12 cells were cultured in GM
2. Grow C2C12 cells until they are ~80-90 % confluent
3. Change GM to DM (10% FBS was replaced by 2% HS)
4. Remove old media, wash with PBS, and add new DM every 2 – 3 days
5. Following 5 days of differentiation, treat C2C12 with DEX (Sigma-Aldrich, MO, USA) at 10^{-6} M for 24 hours
6. Image cells with Evos
7. Scrap cells and proceed with Bradford and western blot protocol

Proliferation (Myoblasts)**:**
1. Grow C2C12 cells until they are ~50-75 % confluent in 6 well plates (x2)
2. Add DEX to media to growth media for 24 hours
3. Control plate is just growth media
4. Proceed to image
5. Scrap cells and proceed with Bradford and western blot protocol

LLC induced atrophy

Note: For the condition medium, we always recommend using fresh ones rather than frozen, but you could try both.

To prepare the CM:

1. Grow the LLC cells until they are ~80-90 % confluent
2. Change to fresh growth media and collect that media after 48h
3. Spin down collected cachectic media to get rid of dead cells and prepare CM in 1:1 ratio to the media of the cells to be treated.
4. Treat myotubes or myoblasts for 48h then analyze
5. Depending on your assay, you could change the ratio of CM and the more cancer secretiome, the more atrophy

LLC Induced Atrophy Differentiation (myotubes)**:**

6. Grow C2C12 cells in GM until they are ~80-90 % confluent in 6 well plates (x2)
7. Change media to differentiation media for roughly 5 days
8. Image cells with Evos
9. Once cells are differentiated, treat with CM for 48 hours
10. Proceed to image again
11. Scrap cells and proceed with Bradford and western blot protocol

LLC Induced Atrophy Proliferation (Myoblasts)**:**

12. Grow C2C12 cells until they are ~50-75 % confluent in 6 well plates (x2)
13. Change media to growth media 1:1 with cachectic media for 1-2 days (depends on confluence)
14. Control plate is just growth media
15. Proceed to image again
16. Scrap cells and proceed with Bradford and western blot protocol

MTT Assay

1. Plate 1,000-100,000 cells per well in a 96-well plate and incubate with the appropriate stimulus for the desired time (usually 6-48 hours).
2. Remove medium and wash cells with PBS.
3. Add MTT made up in medium to a final concentration of 0.5 mg/mL.
4. Incubate for 30 minutes to 4 hours at 37°C, until intracellular purple formazan crystals are visible under microscope
5. Remove MTT and add solubilizing solution and triturate.
6. Incubate at room temperature or 37°C for 30 minutes to 2 hours, until cells have lysed and purple crystals have dissolved.
7. Measure absorbance at 570 nanometers.

Myosin Heavy chain staining for cultured Cells

8. Wash coverslips/plate twice with PBS for 5 Min
9. Fix cells with 4% formaldehyde/PBS for 10 min @ RT
10. Wash twice with PBS for 5 Min
11. Permeabilize cells with 0.5% Triton X-100 (in PBS) for 10 min
12. Wash twice with PBS for 5 Min
13. Block the cell with 10% goat serum (in PBS) for 30 min

14. Incubate with primary Ab against myosin heavy chain (diluted in blocking solution 1:1 Ratio) for 1 h (MF20 at 2.5 µg g/ml)
15. Wash twice for 5 min with PBS
16. Incubated with secondary Ab (Alexa flour 594 goat, anti-mouse) for 1 h diluted in blocking solution.1:300 Ratio
17. Wash twice with PBS for 5 min
18. Counterstain with DAPI (300nm) for 5 min
19. Wash twice with PBS for 5 min
20. Mount with mounting media
21. Visualize with cell discover

Extraction of Protein from cells:

1) Protein is extracted at 4_oC in 150 µl of the appropriate buffer.

**If the cells are in freeze media (-80C), taw them (few seconds in the water path 37C) and transfer it to a new 15ml with Growth Media in order to wash them. Centrifuge it 1000g 4C 5min. Remove the supernatant and add 150ul of RIPA BUFFER, vortex and continue with step 3.*

2) Put 150 µl of buffer in each tube, and vortex until pellet is in solution
3) Place on inverter in cold room (4degrees room) for 30 minutes
4) Microcentrifuge at 14,000rpm for 2-5 minutes
5) Collect supernatant in a 1.5 µl microcentrifuge tube on ice (this is your extracted sample)
6) Make aliquots if necessary and save 5 µl of each sample for the Bradford assay.
7) Snap Freeze in liquid nitrogen and store in -80

RIPA BUFFER: add one tablet of protease and phosphatase inhibitor in 10ml of Ripa Buffer.

Western Blot: LAT1

Sample preparation

1. Remove a small volume of lysate to perform a protein quantification assay (Bradford assay). Determine the protein concentration for each cell lysate.
2. Determine how much protein to load and add an equal volume 4X Laemmli buffer and ddH_20 (use a excel spreadsheet for the calculation).
3. Denature your samples, boil each cell lysate in sample buffer at 95°C for 5 min. Lysates can be aliquoted and stored at -20°C for future use. (Proteins are more stable and protein degradation is avoided. If the lysate is stored at -80 for future use, it has to be boiled again at 95 ° C 5 min). After denature vortex the sample and spin-down.

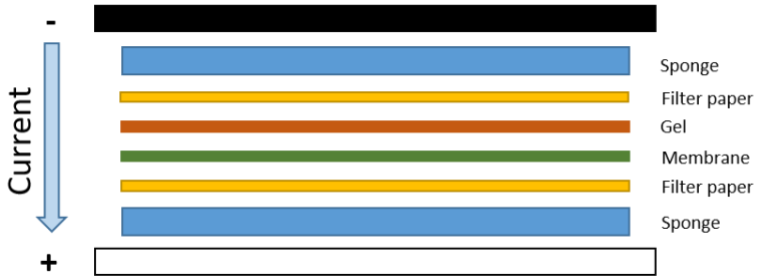
Loading and running the gel

1. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker.
2. Run the gel for 1–2 h at 100 V at room temperature.

Transferring the protein from the gel to the membrane

1. The membrane can be either nitrocellulose or PVDF. Activate **PVDF with methanol** for 5 to 10 sec and rinse with ddH20 first and then with transfer buffer before preparing the stack.

Prepare the stack as follows:



2. Transfer for 1h 120 V. The time and voltage of transfer may require some optimization.
3. After transfer, check if the transfer of the proteins from the gel to the membrane works using Ponceau S staining before the blocking step. (You can use Ponceau S for normalization, take a picture).
4. Wash the membrane 3 times 5 min with T-BST in order to remove the Ponceau S.

*PVDF membrane, 10% running gel, 4% stacking gel

Day 1:

1. Block: 5% BSA in 1x TBST - 1 hour @RT
2. 1' Ab: LAT1 (Protein Tech Cat#13752-1-AP Rabbit Polyclonal) 1:2000 in 2.5% BSA – O/N in cold room on shaker

Day 2:

1. Wash 1x TBST – 5 x 3 min
2. 2' Ab: anti-Rabbit HRP 1:10,000 in 2.5% BSA – 1 hour @RT
3. Wash 1x TBST – 5 x 3 min
4. ECL detection: 1:1 (equal parts of both solutions) – 5mins
5. Image
    a. Colorimetric
    b. Chemiluminescence
6. Wash 1x TBST – 3 x 3 min
7. Strip: 5mL restore stripping buffer per well – 20min @ RT
8. Wash 1x TBST – 3 x 3 min
9. Block: 5% BSA in 1x TBST – 1 hour @ RT
3. 1' Ab: alpha-tubulin (loading control ab4074) 1:5,000 in 2.5% BSA - O/N in cold room on shaker

Day 3:

1. Wash 1x TBST – 5 x 3 min
2. 2' Ab: anti-Rabbit HRP 1:10,000 in 2.5% BSA – 1 hour @RT
3. Wash 1x TBST – 5 x 3 min
4. ECL detection: 1:1 (equal parts of both solutions) – 5mins
5. Image
    a. Colorimetric
    b. Chemiluminescence
6. Wash in 1 x TBST – 2 x 2min
7. Save in fridge in 1x TBS (**NOT** TBST) or ddH_2O

LAT1/Pax7/Laminin Stain

NOTE:

I. Laminin and DAPI used from this stain to determine CSA, myonuclei, and myonuclear domain
II. Place all slides in humidifying chamber to prevent slides from drying out
III. Standard Block (1% BSA, 0.1% Triton X-100, 5% GS)
IV. Conjugation list: LAT1(rabbit) - Alexa Flour 594 anti-rabbit, PAX7 - Alexa Flour 488 anti-mouse (DHSB, mouse), Laminin (rat) - Alexa Flour 647 anti-rat(Thermo Fisher, anti-rat, MAI-06100, DAPI

1.Allow sections to thaw and dry
2. Wash 1 x PBS, 2x3 min
3. Fix 4% PFA, 15 min @RT
4. Wash 1xPBS, 10 min
5. Wash in 1 x PBST (0.2% Tween), 10 min @ RT
6. DAKO – 30 min @ RT
7. 1°Ab LAT1(rabbit) 1:50 (Proteintech-SCL7A5 Rabbit Polyclonal Antibody, Catalog #: 13752-1-AP) in 5% Goat Serum (1 x PBS and 0.2% Triton) – overnight @ 4^{0}C
8. Wash in PBST 3 x 10 min
9. Wash in PBST 1 x 5 min

In the dark

10. 1st secondary antibody -Alexa Flour 594 anti-rabbit, 1:200 in 5% GS – 1 hour @ RT
11. Wash in PBST 3 x 10 min
12. Place slides in 2% PFA – 10 min, wash in 1 x PBS - 1 x 10 min
13. Wash in 1 x PBST – 1 x 10 min
14. Re-block with 5% GS and 0.2% Triton – 1hour @ RT
15. 2nd primary antibody Ab PAX7 (mouse) [neat] - overnight @ 4^{0}C
16. Wash in PBST 2 x 10 min
17. Wash in PBST 1 x 5 min
18. 2nd Secondary Ab Alexa Flour 488 anti-mouse 1:400 in 1% BSA – 2 hour @ RT
19. Wash in PBST 3 x 10 min
20. 3rd primary Ab Laminin (rat) 1:200 (ThermoFisher Scientific, Laminin monoclonal antibody A5, Catalog #: MA1-06100) in 5% Goat Serum– 1 hour @ RT
21. Wash in 1 X PBST – 3 x 5 min
22. 3rd secondary Ab Alexa Flour 647 anti-rat 1:200 in 1% BSA- 1 hour @ RT
23. Wash in 1 x PBST 1 x 10 min
24. DAPI 1 x 5 min. Wash in 1 x PBST 1x 10 min
25. Coverslip. 1 drop of paramount over each section (eliminate air bubbles under coverslip). Seal with nail polish

ImageJ Guide

***NB: When ROI's are listed in ROI manager, highlight all ROI's. Then, press more and save. This will save all ROI's and you will be able to open individual ROI's again for the image and edit them if necessary!

Merging Images

1. Load photos into Image J. You can drag and drop the photos into ImageJ.
2. Press image (this should be at the very top of your screen)
3. Select Image → _Colour → _Merge channels
4. Once you press merge channels, a window will pop up.

5. Select the colours you want your images to appear as. I.e. DAPI as blue and laminin as red.
6. Press OK. The photo will merge and you can proceed.

How to count fibers (150 per section)

1. Set scale
    a. a Click line bar
    b. Draw line over the scale
    c. Press analyze
    d. Click set scale
    e. Change the known distance to what the scale bar reads
    f. Change the units to microns

2. Set measurements
    a. Click Analyze
    b. Click set measurements.
    c. Select area and Feret's diameter

3. Use the kidney bean looking free hand selection.
4. Draw a circle around the fibers
5. Hit "t" and it will add it to your ROI
6. Click show all and label
7. Press measure in window that opens up when you press "t" (this will show you the area and the Ferets)
8. Record the area and the Ferets in an excel document for each section

*Tip: measure all of them and then export. Be sure to select the right fiber when measuring.

*If fibers look elongated or very big avoid these.

How to measure myonuclei

1. Laminin represents the basal lamina. This is what you circled when you were measuring cross sectional area.
2. The blue dots represent nuclei. Not all nuclei will count as myonuclei.
3. Myonuclei are any nuclei that are at least 50% beneath the basal lamina.
4. You will need to manually count the number of nuclei for each fiber you measured. You can use the counter on ImageJ. It is the box that looks like stars.
5. Also make note of centrally located nuclei (CLN). They are very rare, and you may not see any.

Tip: when you zoom in on the photo, if you want to scroll around, hold down the space bar and click and drag the photo.