Effect of Branched-Chain Alpha-Keto Acid Dehydrogenase Depletion on Myotube Protein Metabolism

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Abstract

Branched-chain amino acids (BCAAs) are essential amino acids that are crucial for skeletal muscle anabolism. Thus, alterations in their levels are associated with several muscle atrophic diseases such as cancer, chronic inflammatory and neurological disorders. Compared to the effects of these amino acids on skeletal muscle, much less is known on how impairment in BCAA catabolism affects this tissue. BCAA catabolism starts with the reversible transamination by the mitochondrial enzyme branched-chain aminotransferase 2 (BCAT2). This is followed by the irreversible decarboxylation, catalyzed by branched-chain α-ketoacid dehydrogenase (BCKD) complex. Our lab has previously shown that BCAT2 and BCKD catabolic activities are essential for the differentiation of skeletal myoblasts into myotubes *in vitro*. Here, I investigated the effect of depletion of BCAT2 or BCKD in differentiated myotubes. On day 4 of differentiation, L6 myotubes were transfected with the following siRNA oligonucleotides: scrambled (control), BCAT2, or BCKD. Forty-eight hours after transfection, compared to BCAT2 siRNA group, we observed maintained myotube structure in BCKD-depleted cells. BCKD depletion augmented myofibrillar protein levels: myosin heavy chain (MHC, 2-fold) and tropomyosin (4-fold), $p<0.05$, $n=3$. To further analyze the increase in myofibrillar protein content, we examined signaling through mTORC1 (mechanistic target of rapamycin complex 1), a vital complex necessary for skeletal muscle anabolism. BCKD depletion increased the phosphorylation of mTORC1 upstream activator Akt (52%, $p<0.05$, $n=3$), and of mTORC1 downstream substrates by 25%-86%, consistent with the increase in myofibrillar proteins. It was shown that depletion of BCKD increased myofibrillar protein content and anabolic signaling. If these data are confirmed *in vivo,* development of dietary and other interventions that target BCKD abundance or functions may promote muscle protein anabolism in individuals with muscle wasting conditions.

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Table of contents

Abstract ii

Acknowledgements iii

Table of contents iv

List of Tables vi

List of Figures vii

Abbreviations viii

Chapter One: Introduction 1

Chapter Two: Literature Review 2

2.1 Skeletal Muscle 2

2.2 Factors Regulating Skeletal Muscle Mass 2

2.2.1 Factors That Affect Net Muscle Protein Balance 3

2.2.1.1 Exercise 3

2.2.1.2 Nutrition 4

2.2.1.3 Chronic Inflammation 5

2.3 mTORC1 Signaling 6

2.3.1 mTOR Structure 6

2.3.2 Regulation of mTORC1 7

2.3.2.1 Stimulation of mTORC1 by Growth Factors 8

2.3.2.2 Stimulation of mTORC1 by Amino acids 9

2.3.3 mTORC1 and Protein Degradation 11

2.3.3.1 mTORC1 and Ubiquitin-Proteasome System 11

2.3.3.2 mTORC1 and Autophagy-Lysosome System 12

2.4 BCAA Metabolism 13

2.4.1 The Role of BCAAs in Skeletal Muscle Metabolism 13

2.4.2 BCAA Sensing and Uptake in the skeletal muscle 14

2.4.3 The Role of BCAA Catabolites on Skeletal Muscle Metabolism 15

2.4.4 BCAA Catabolic Pathway 17

2.4.4.1 BCAT structure and regulation 19

2.4.4.2 BCKD structure and regulation 20

2.4.5 Regulation of BCAT2 and BCKD in the Skeletal Muscle 23

2.4.5.1 Effect of Disruption of BCAA Catabolism on Whole-body Metabolism 24

2.4.5.2 Effect of Disruption of BCAA catabolism on skeletal muscle metabolism 26

2.4.5.3 The Effect of Disruption of BCAA catabolism in Skeletal Muscle on Skeletal Muscle Metabolism ... 29
Chapter Three: Rationale ... 31
3.1 Research Objectives ... 31
3.2 Hypotheses ... 32
Chapter Four: Manuscript ... 33
Abstract ... 34
Introduction ... 35
Materials and methods ... 37
Reagents ... 37
Cell Culture ... 37
Transfection ... 38
Effect of BCKD Depletion on Myotubes treated with TNF-α ... 39
Cell Harvesting ... 39
Protein Assay and Western Blot Analysis ... 41
Immunofluorescence Microscopy ... 42
Statistical Analysis ... 43
Results ... 44
Discussion ... 57
Limitations of Study ... 61
Chapter Five: Future Directions ... 62
References ... 64
Appendices ... 82
Appendix A: Supplementary Data ... 82
Appendix B: Detailed Laboratory Procedure ... 85

List of Tables

Table 1: Summary of RT-qPCR primers…………………………………………………………...40

Table 2: Primary and Secondary Antibodies for Western Blot Analysis……………………..41

List of Figures

Literature Review

Figure 1: Schematic of mTORC1 signaling pathway……………………………………………10

Figure 2: Schematic of branched-chain amino acid catabolic pathway…………………………18

Manuscript

Figure 1: BCAT2 and BCKD levels in myotubes treated with siRNA…………………………48

Figure 2: myotube structure of BCAT2 and BCKD-depleted cells……………………………..49

Figure 3: Immunofluorescence staining of BCKD-depleted myotubes…………………………50

Figure 4: Myofibrillar protein abundance in BCKD-depleted myotubes……………………….52

Figure 5: mTORC1 signaling in BCKD-depleted myotubes……………………………………54

Figure 6: Effect of BCKD Depletion on Myofibrillar Protein Abundance in Atrophic Condition……………………………………………………………………………………..55

Appendices

Figure 7: mTORC1 signaling following acute starvation in BCKD-depleted myotubes……………………………………………………………………………………..82

Figure 8: TNF-α concentration test……………………………………………………………..83

Figure 9: Effect of BCKD Depletion on mTORC1 signaling in Atrophic Condition…………………………………………………………………………………….84

Abbreviations

4E-BP1	**eIF4e-binding protein-1**
Akt	**also known as protein kinase B (PKB)**
AMPK	**AMP-activated protein kinase**
AP-1	**activating-protein 1**
APN	**adiponectin**
BCAA	**branched-chain amino acids**
BCAT2	**branched-chain amino acid transferase-2 (mitochondrial isoform)**
BCAT1	**branched-chain amino acid transferase-1 (cytosolic form)**
BCKA	**branched-chain α-keto acid**
BCKD	**branched-chain α-keto-dehydrogenase complex**
BCKDE1α	**branched-chain α-keto-dehydrogenase E1 α-polypeptide**
BDK/BCKDK	**branched-chain α-keto-dehydrogenase kinase**
CRP	**c-reactive protein**
DEPTOR	**DEP domain-containing mTOR-interacting protein**
eIF4A	**eurkaryotic translation initiation factor-4A**
eIF4E	**eurkaryotic translation initiation factor-4E**
eIF4F	**eurkaryotic translation initiation factor-4F**
eIF4G	**eurkaryotic translation initiation factor-4G**
ERK	**extracellular signal-regulated kinase**
FADD	**Fas associated protein with death domain**
FoxO	**forkhead box O**
GATOR	**GAP activity towards Rags**
GAP	**GTPase activating protein**
GLN	**glutamine**
HMB	**β-hydroxy-β-methylbutyrate**
HPRT	**Hypoxanthine-guanine phosphoribosyltransferase**
IGF-1	**insulin-like growth factor-1**

IGF-2	**insulin-like growth factor-2**
IGFR	**insulin-like growth factor receptor**
IL-1	**interlukin-1**
IL-6	**interlukin-6**
IR	**insulin receptor**
IRS	**insulin receptor substrate**
JNK	**c-jun N-terminal kinase**
KIC	**α-ketoisocaproate**
KIV	**α-ketoisovalerate**
KLF 15	**kruppel-like factor 15**
KMV	**α-keto-β-methylvalerate**
LAMP	**lysosome-associated membrane protein**
LAT 1	**L-type amino acid transporter 1**
LAT 2	**L-type amino acid transporter 2**
MAPK	**mitogen activated protein kinase**
Mafbx/atrogin-1	**muscle atrophy F-box**
MHC	**myosin heavy chain**
MLST8	**mammalian lethal with SEC13 protein 8**
MPS	**muscle protein synthesis**
MPB	**muscle protein breakdown**
MSUD	**maple syrup urine disease**
MSTN	**myostatin**
mTORC1	**mammalian/mechanistic target of rapamycin complex-1**
mTORC2	**mammalian/mechanistic target of rapamycin complex-2**
MuRF-1	**muscle ring finger protein-1**
mSin 1	**mammalian SAPK interacting protein 1**
NF-κB	**nuclear factor kappa-B**
PDK	**phosphoinositide-dependent kinase**

PGC-1α	**PPAR γ coactivator of 1α**
PI3K	**phosphoinositide 3-kinase**
PPARα	**peroxisome proliferator-activator receptor-α**
PPM1K	**protein phosphatase 1K**
PRAS40	**proline-rich AKT substrate of 40 kDa**
Rag	**RAS-related GTP-binding protein**
RAPTOR	**regulatory associated protein of mTOR**
Rheb	**ras homolog enriched in brain**
RICTOR	**rapamycin-insensitive companion of mTOR**
ROS	**reactive oxygen species**
S6/rpS6	**ribosomal protein S6**
S6K1	**p70 ribosomal protein S6 kinase 1**
siRNA	**small interfering RNA**
TGF-β	**transforming growth factor-β**
TNF-α	**tumor necrosis factor α**
TNFR	**tumor necrosis factor receptor**
TSC	**tuberous sclerosis complex**
TOR	**target of rapamycin**
Ub	**ubiquitin**
UPS	**ubiquitin proteasome system**

Chapter One: Introduction

Muscle wasting is seen in conditions such as sarcopenia (progressive skeletal muscle disorder that involves the loss of muscle mass and function commonly seen as age-related process in older populations)[9,10] cancer cachexia[11], heart failure[12], and chronic inflammatory disease[13]. Loss in skeletal muscle mass is attributed to either the decrease in muscle protein synthesis (MPS) or the increase in muscle protein breakdown (MPB)[18].

MPS and MPB are regulated by exercise and nutrition among other factors. When it comes to nutrition, branched-chain amino acids (BCAAs) are important regulators of anabolic signaling[20]. BCAAs, leucine, isoleucine and valine activate mammalian/mechanistic target of rapamycin complex 1 (mTORC1) to promote anabolic signaling[43]. Previous research has shown that BCAA catabolites, specifically leucine catabolites α-ketoisocaproate (KIC) and β-hydroxy-β-methylbutyrate (HMB) positively regulate muscle protein balance[111, 130].

Disruption in the activity of BCAT2[114,222] and BCKD[224], the first two enzymes in BCAA catabolism, impairs muscle endurance capacity and anabolic signaling in animal models. BCAA catabolic pathway is tightly regulated by the oxidative decarboxylation activity of BCKD (rate limiting step). BCKD activity is regulated by two opposing enzymes, a kinase and a phosphatase[158]. There is evidence to show that the presence of BCAT2 and BCKD complex enzymes is required for skeletal muscle cell differentiation[15]. Therefore, the role of BCAA catabolism, particularly BCAA metabolites and the catabolic enzymes is of great importance to the maintenance of skeletal muscle mass and muscle wasting diseases. This present study examines effect of disrupting BCAA catabolism on differentiated skeletal muscle cells.

Chapter Two: Literature Review

2.1 Skeletal Muscle

Skeletal muscle comprise about 30%-40% of human body mass and majority of whole-body protein[1]. Skeletal muscle is important in the regulation of force and movement production, energy metabolism, glucose homeostasis. Skeletal muscle is a key site for glucose uptake as well as a reservoir for amino acids stored as proteins[2]. Change in muscle mass strength and function has been associated with various metabolic diseases such as cancer cachexia[3]. Thus, the dynamic nature of the skeletal muscle tissue in its strength and mass has garnered research interest.

Skeletal muscle is a multinucleated structure composed of myofibers connected by connective tissues[4]. The muscle fibers are arranged in a small unit called sarcomeres[4]. The sarcomeres have a repeating dark and light band arrangement that gives the muscle a 'striated' appearance[4]. The dark bands contain the myosin 'thick' filaments and actin 'thin' filaments which are responsible for the contractility of the muscle[4]. There are several proteins associated with the muscle fibers[4]. The myosin filament comprises of myosin molecules called myosin heavy chain (MHC), while the actin filaments are associated with regulatory proteins, tropomyosin and troponin[4]. These proteins known as the myofibrillar proteins, are directly involved in the contractility of the muscle. There is a differential expression of these myofibrillar proteins which is transcriptionally regulated[5]. Thus, the absence or dysfunction of these proteins could decrease muscle strengthening and lead to muscle weakness.

2.2 Factors Regulating Skeletal Muscle Mass

The mammalian body regulates its skeletal muscle mass through the maintaining of muscle protein balance. Muscle protein balance is determined by the interplay of two opposing processes, protein breakdown (MPB) and protein synthesis (MPS). If there is a positive protein balance (MPS

exceeds MPB), muscle hypertrophy occurs. On the other hand, if there is a negative protein balance (MPB exceeds MPS), muscle atrophy (loss of muscle mass) occurs[6–8].

Muscle atrophy is seen in conditions such as sarcopenia (progressive skeletal muscle disorder that involves the loss of muscle mass and function commonly seen as age-related process in older populations)[9,10] cancer cachexia[11], heart failure[12], and chronic inflammatory disease[13]. Chronic muscle wasting and weakness is associated with increased protein breakdown (cancer) and contractile dysfunction (chronic inflammatory diseases)[14]. Muscle atrophy can occur as a result of disuse and muscle unloading which decreases physical working capacity of the muscle, described as acute atrophy[6,15]. For example, immobilization and denervation of rats results in the loss of medial gastrocnemius mass which is typically present in muscle atrophy[16,17]. The loss of muscle mass as a result of immobilization has been linked to a decrease in MPS and an increase in proteolysis[18,19].

2.2.1 Factors That Affect Net Muscle Protein Balance

Previous studies have shown that the preservation of muscle protein balance is influenced by physical exercise, nutritional intake and inflammation among others[20,21].

2.2.1.1 Exercise

Exercise training/physical activities have been shown to maintain and improve muscle mass and insulin sensitivity. Exercise varies in the type and the effect it has on the skeletal muscle, but altogether improves physical health. Regular engagement in physical activity has been shown to circumvent age-related muscle wasting (sarcopenia) and chronic diseases[22]. There is a decreased anabolic response (MPS response) seen in the skeletal muscle of aging individuals that leads to smaller muscle strength gain compared to younger individuals. Resistance exercise (RE), a potent anabolic stimulus, has been shown to improve “anabolic sensitivity” on the muscle mass of older

men and women[21–23]. RE leads to greater activation of MPS particularly the myofibrillar proteins in protein fed state. RE also works to reduce muscle protein degradation in fasted state[24]. Endurance training also known as aerobic exercise improves metabolism, cardiovascular health and mitochondrial content[25,26]. Regular endurance training incurs some muscle adaptation such as an increase in oxygen uptake (VO2 max), fiber capillarization, fatty acid oxidation and a decrease in blood glucose utilization[25,26]. Studies have shown the synergistic effect of resistance exercise training and nutrition[21,27].

2.2.1.2 Nutrition

Macronutrients such as carbohydrates, proteins[24,27] and fats[28] have been shown to regulate muscle protein synthesis[29]. Omega-3 polyunsaturated fatty acids combined with strength training have been reported to promote muscle anabolism in aging[28], cancer cachectic[30] and healthy individuals[30]. Micronutrients from food sources including vitamin A[29], D[31], E[29], and minerals[32], have been shown to be directly associated with muscle anabolic signaling. A study in pigs found that high intakes of the mineral selenium increases muscle protein and alters lipid metabolism[32]. A study in aged rats showed that vitamin D deficiency increases adiposity and reduces MPS[31]. Protein-rich food sources have been shown to be good sources of vitamins, minerals, and bioactive compounds (growth factors and peptides) important for muscle anabolism[33,34]. According to the National Health and Nutrition Examination Survey (NHANES), lean beef is a great source of vitamin B12, iron and zinc[33].

Protein Ingestion and Skeletal Muscle Protein Synthesis

Proteins are digested into amino acids which serve as building blocks for protein synthesis in the body. Amino acids are classified as essential and nonessential. Dietary supplementation of nonessential amino acid like glutamine has been shown to stimulate MPS following strength

training[35–37]. The degree at which amino acids stimulate MPS is dependent on dose, the type of amino acid given, and pattern of ingestion[38]. For example, high-quality proteins from milk, whey and soy ingestion promote exercise-induced muscle protein anabolism[24,39].

Provision of dietary protein and amino acids combined with prior resistance exercise has been shown to enhance skeletal muscle protein synthesis and maintain net muscle protein balance[27]. Amino acids such as leucine and arginine attenuate the loss of muscle mass by targeting specific intracellular signalling pathways[20]. These amino acids target mammalian/mechanistic target of rapamycin complex 1 (mTORC1), a serine/threonine protein kinase that promotes MPS and attenuates proteolysis[40]. Branched-chain amino acids (BCAAs), leucine, isoleucine and valine, are essential amino acids with anabolic effects that are important regulators of mTORC1[41–43].

Carbohydrate-Protein Interaction and Protein synthesis

Increased intake of carbohydrates elevates blood insulin levels which enhances net protein balance in leg muscles[44]. In the absence of protein intake, carbohydrate intake does not improve muscle protein metabolism[27,45]. Following resistant exercise, carbohydrate-protein co-ingestion is required to maximize MPS[45]. Carbohydrate restriction (high protein diet) has been accepted to an extent as the dietary approach to reduce the pathogenesis of metabolic syndrome that include cardiovascular disease and type 2 diabetes[46–48]. Obesity and type2 diabetes are linked to decreased muscle protein synthetic response to diet and exercise[49].

2.2.1.3 Chronic Inflammation

Natural progressive loss of skeletal muscle mass seen when individuals age (sarcopenia) has been associated with chronic inflammation[50]. Aging and other inflammatory myopathies have been linked to the upregulation of pro-inflammatory cytokines[50,51]. Some cytokines that are

catabolic in nature (responsible for muscle strength loss) include interlukin-1 (IL-1), interlukin-6 (IL-6), C-reactive protein (CRP) and tumour necrosis factor-α (TNF-α)[52–54]. A population-based study suggests that elevated levels of IL-6 increases the risk of muscle strength loss[52].

Previous studies show that mouse-derived C2C12 (immortalized myoblasts) myotubes treated with TNF-α have time and concentration-dependent decrease in total protein content and MHC levels[55–57]. TNF-α moderates cellular response by interacting with its receptor TNF-α receptors (TNFRs) and Fas associated protein with death domain (FADD) to promote apoptosis. In a second pathway, TNF-α activates c-Jun-N terminal kinases (JNKs) and activator protein 1 (AP-1). JNKs belong to mitogen activated protein kinases family (MAPKs) along with extracellular signal-regulated kinases (ERK 1/2) and p38 MAPKs[58]. JNK activity has been linked to skeletal muscle insulin resistance and suppression of muscle cell differentiation[59,60].

In skeletal muscle cells, TNF-α activates nuclear factor kappa-B (NF-κB) in a third pathway to promote protein degradation[55,56]. Previous studies conducted in rat-derived L6 cells (mononucleated rat myoblasts) suggest that TNF-α-induced catabolism in myotubes require NF-κB activity[55,56,61,62]. In cultured muscle cells, TNF-α has been shown to increase the levels of myostatin (MSTN) through NF-κB signaling[63]. MSTN is a protein of transforming growth factor β-superfamily (TGF-β), secreted by the muscle with an autocrine function in enhancing the loss of skeletal muscle mass[64]. Drug inhibition of MSTN has been shown to repress muscle atrophy in mice with chronic kidney disease[63].

2.3 mTORC1 Signaling

2.3.1 mTOR Structure

mTOR (mammalian/mechanistic target of rapamycin) is the principal protein complex involved in the regulation of fundamental cellular processes such as eukaryotic cell growth, protein

metabolism and autophagy[65]. Deregulation of mTOR has been implicated in cancer, diabetes and in age-related sarcopenia[40,65]. mTOR was discovered two decades ago from a bacterium that was found in soil samples in South Pacific islands. Isolated from the bacteria is a compound called rapamycin that revealed to have the capacity of inhibiting signaling pathways involved in cell growth and proliferation[65].

mTOR is a conserved serine/threonine protein kinase that is made up of at least two complexes in mammalian cells, mTORC1 and mTORC2[40]. The two protein complexes are distinct in subunits components, cellular function, forms of regulation and substrates they regulate[66,67]. mTORC1, one of the complexes that is sensitive to the immunosuppressant drug rapamycin, is involved in promoting anabolism and inhibiting catabolism. mTORC1 contains five subunits, three core subunits including mTOR, regulatory associated protein of mTOR (Raptor) and mammalian lethal with Sec-13 protein 8 (mlST8)[68]. Raptor is a positive regulator of mTORC1 critical for the recruiting of downstream substrates of mTORC1[69]. In addition to the three core components are the two negative regulators of mTORC1: DEP domain containing mTOR interacting protein (DEPTOR) and proline-rich Akt substrate of 40 kDA (PRAS40)[65,70]. mTORC2, the rapamycin insensitive counterpart of mTORC1, consists of mTOR, mlST8, DEPTOR. In addition to these subunits, mTORC2 also contains rapamycin insensitive companion of mTOR (Rictor) in place of Raptor and mammalian SAPK interacting protein 1(mSin 1)[71].

2.3.2 Regulation of mTORC1

The activation of mTORC1 is regulated upstream by factors such as stress, energy levels, oxygen, growth factors (insulin, insulin-like growth factor-1 (IGF-1)) and nutrients (amino acid, glucose, and fatty acids)[65,72]. Activated mTORC1 promotes protein synthesis and mRNA translation initiation via the phosphorylation of its downstream effector substrates[65,70]. Raptor, a

component of mTORC1, binds and promotes the phosphorylation of S6K1 (p70 ribosomal S6 kinase 1) on Thr389. S6K1 then phosphorylates ribosomal protein S6 (rpS6) Ser235/236 to stimulate protein synthesis and cell proliferation[65,73,74]. mTORC1 phosphorylates 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) on Thr37/46, another downstream substrate of mTORC1 that represses of mRNA translation initiation. The phosphorylation of 4E-BP1, prevents its binding to eukaryotic translation initiation factor 4E (eIF4E), a cap-binding protein. This allows eIF4E to form a complex with eIF4F (eukaryotic translation initiation factor 4F) for the initiation of cap-dependent mRNA translation[40]. In addition, upon 4E-BP1 phosphorylation, eIF4A (eukaryotic translation initiation factor-4A) dissociates from 4E-BP1 and interacts with eIF4E and eIF4G (eukaryotic translation initiation factor 4G). This promotes the helicase activity of eIF4A in mRNA translation initiation[73,75–77].

2.3.2.1 Stimulation of mTORC1 by Growth Factors

Growth factors activate mTORC1 through insulin and Ras signaling pathway[78]. Insulin and IGF-1 bind to insulin (IR) and IGF-1 receptors (IGFR)[79]. The binding of insulin/IGF-1 to IR or IGFR stimulates the recruitment of insulin receptor substrate 1 (IRS1). IRS1 activates the phosphorylation of phosphatidylinositol (3,4)-bisphosphate (PIP2) by phosphatidylinositol-3 kinase (PI3K) to form phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 triggers the localization of Akt also known as Protein Kinase B (PKB) to the cell membrane where it is phosphorylated at Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and at Ser473 by mTORC2[76,78,80]. Akt phosphorylates and inhibits tuberous sclerosis complex 1 and 2 (TSC1/2). TSC1 (known as hamartin) and TSC2 (known as tuberin) function as GTPase activating protein (GAP) that directly inhibits small Ras-related GTPase Rheb (Ras homolog enriched in brain) by keeping it in its inactive GDP form[81]. The phosphorylation of TSC1/2 by Akt drives the formation

of active GTP-bound form of Rheb that activates mTORC1. Growth factors also affect mTORC1 signaling through Ras signaling. Activated Ras ultimately leads to the activation of MAPK, ERK1/2 (extracellular signal-regulated kinase) and RSK1 (ribosomal S6 kinase). ERK1/2 phosphorylates and inhibits TSC1/2 thus promoting the activation of mTORC1[8,76,81].

2.3.2.2 Stimulation of mTORC1 by Amino acids

Amino acids, particularly leucine and arginine attenuate the loss of muscle protein and promotes muscle protein synthesis via mTORC1 as previously mentioned[40]. The activation of mTORC1 via amino acid sensing requires the association of mTORC1 with the lysosome[82]. mTORC1 detects amino acids and binds to Rag proteins, a family of four small GTPases. Rag GTPases (Rag A to D) form heterodimers, RagA/ B (GTP loaded) forming a complex with RagC/D (GDP loaded).

Intracellular amino acids stimulate the GTP loading and binding of RagA/B to Raptor component of mTORC1 causing the re-localization of mTORC1 to lysosomal membrane where it interacts with Rheb and becomes activated[83,84]. The Rag GTPases are tethered to the lysosome through a complex called Ragulator in response to amino acid sufficiency[83,84]. The Ragulator complex interacts with the Rag proteins and acts as a guanine nucleotide exchange factor (GEF) to convert RagA/B to GTP loaded active form[85]. mTORC1 is also stimulated by amino acids via V-ATPase (vacuolar associated ATP hydrolase) located on the lysosomal membrane. V-ATPase senses amino acids in lysosomal lumen and interacts with the Ragulator-Rag complex to lead the to activation of mTORC1[82].

The regulation of muscle anabolic signaling via the regulation of mTORC1 by amino acids is especially affected by branched-chained amino acids. A study by Buse in 1981, found BCAAs to be rate limiting for muscle protein synthesis in rats[43].

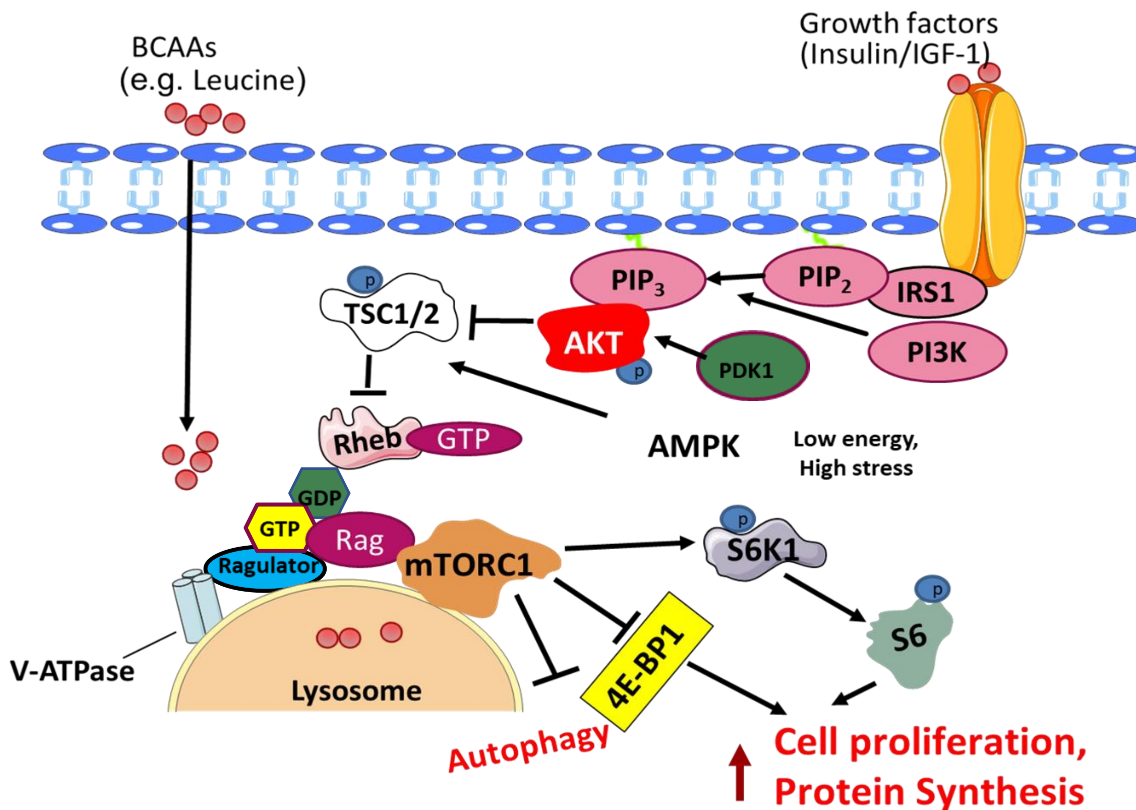

Fig. 1. Overview of mTORC1 complex signaling pathway and protein synthesis. Amino acids, specifically branched-chain amino acids (BCAAs) and growth factors, promote mTORC1 activation. IRS1 when activated by insulin/IGF-1, recruits PI3K. PI3K converts PIP2 to PIP3. PIP3 promotes the localization of AKT to the membrane. AKT is then phosphorylated by PDK1. AKT phosphorylates and inhibits TSC1/2. TSC1/2 phosphorylation activates Rheb and mTORC1. Activated mTORC1 phosphorylates its downstream substrates, p70S6K1 and 4E-BP1 to promote mRNA translation and protein synthesis.

2.3.3 mTORC1 and Protein Degradation

Nutrients and growth factors activate mTORC1 to promote MPS and suppress protein breakdown[86]. Thus, during periods of hypoxia, high stress, and low energy levels in the cell, mTORC1 inhibition occurs, which leads to increase in protein degradation (Fig 1)[86]. The two main systems involved in skeletal muscle protein degradation regulated by mTORC1 is the ubiquitin-proteasome and autophagy-lysosomal systems[87].

2.3.3.1 mTORC1 and Ubiquitin-Proteasome System

The ubiquitin-proteasome system (UPS) selectively degrades various regulatory and misfolded cellular proteins[86–88]. The first and the rate limiting step involves the covalent attachment of ubiquitin (Ub) protein molecules to the protein substrates to be catabolised in a process called conjugation[87,88]. The conjugation process begins with the activation of 76-residue Ub in its C-terminal glycine (Gly) by ubiquitin-activating enzyme (E1)[87,88]. This is followed by the transfer of activated ubiquitin from E1 to ubiquitin-protein ligase (E3) by ubiquitin-conjugating enzyme (E2)[88]. Ubiquitin E3 ligases catalyzes the selective attachment of Ub to substrates, the last step in the conjugation cascade[88,89]. In rat atrophic model, two genes, known as atrogenes have been identified to encode E3 Ub ligases: muscle ring-finger protein -1 (MuRF-1) and muscle atrophy F-box (MAFfbx/Atrogin-1)[16]. Overexpression of MAFbx has been shown to induce muscle atrophy in myotubes[89]. Muscle wasting in cancer cachexia has been linked to the degradation of myofibrillar proteins through UPS[90].

Furthermore, high stress conditions in the cell enhance immune function and the induction of pro-inflammatory cytokines such as TNF-α[91]. In inflammatory state, mTORC1 activity and leucine-stimulated muscle protein synthesis is reduced. The mRNA expression of MuRF-1,

MAFbx and UPS proteolysis is upregulated[92]. Findings have demonstrated that TNF-α inactivates skeletal muscle differentiation markers and increases the levels of MuRF-1 and MAFbx[93].

The second step of UPS includes the degradation of Ub marked proteins by 26S proteasome complex[94]. The 26S proteasome is a "dumbshell-shaped" structure that consists of a catalytic unit, 20S proteasome particle (core of the proteolytic cascade) that is flanked by 19S regulatory subunits[88,95]. The ATPase present in the 19S particle provides energy for the assembly of 26S proteasome, the unfolding and the eventual breakdown of polyubiquitinated substrates[94,96]. The enhanced myofibrillar protein degradation seen in muscle wasting conditions such as fasting and sepsis is suppressed in ATP-depleted rodents[96]. This confirms that the ATPase activity of the proteasome is important for UPS. Clinical suppressor of ATP-ubiquitin-dependent proteolytic pathway, β2-adrenergic agonist (clenbuterol), disrupts excess skeletal muscle protein breakdown seen in cancer cachexia[97,98].

2.3.3.2 mTORC1 and Autophagy-Lysosome System

Autophagy involves the engulfing of damaged intracellular components such as organelles and protein aggregates in a double membrane vesicle called autophagosome[99]. The autophagosome fuses with the lysosome where the cellular components get degraded[99]. Intracellular structures can be recognized by lysosomal membrane receptor, lysosome-associated membrane protein (LAMP 1/2) and directly taken up by the lysosome[100]. Amino acids produced from proteins degraded in the lysosome are removed into the cytoplasm to be reused[99]. Starvation-induced atrophy promotes the activation of 5' Adenosine monophosphate-activated protein kinase (AMPK). Under energy stress, AMPK phosphorylates Unc-51 like autophagy activating kinase (ULK1) on Ser317/777 to promote autophagy in mammalian cells (Fig.1)[101]. AMPK directly phosphorylates TSC2 on Ser1387 and Raptor to suppress mTORC1 activation evidenced by the decreased phosphorylation

of S6K1 and 4E-BP1[102,103]. Impairment in autophagic degradation (excessive or defective) is seen in skeletal muscle sarcopenia. Excessive autophagy promotes cellular stress and loss of skeletal muscle due to enhance protein degradation (glucocorticoid-induced atrophy)[90,104]. Deficiency in basal autophagy leads to the accumulation of protein aggregates. Autophagy degradation impairment has been shown to be circumvented by caloric restriction[90,104]. Thus, it is established that autophagy reduces muscle atrophy by the clearing of dysfunctional organelles and proteins[105].

Attenuated 1GF/P13K/Akt signaling promotes proteolysis via proteasomal and lysosomal pathways by the reduction of mTORC1 activity and the activation of FOXOs (transcription factor of Forkhead box class O)[93,105,106]. Inhibition in Akt signaling ameliorates the activation FOXO1-Ser^{276} and FOXO3, essential for muscle atrophy[107–109]. The downregulation of FOXO1 has been shown to decrease the levels E3 ligases in myotubes[110]. Inhibition of mTORC1 by torin1 and rapamycin is shown to enhance proteolysis via UPS and autophagy[86]. Findings show that mTORC1 regulates the two systems involved in protein degradation.

2.4 BCAA Metabolism

2.4.1 The Role of BCAAs in Skeletal Muscle Metabolism

BCAAs, especially leucine have be shown to have anabolic effect on the skeletal muscle and is able to enhance mRNA translation initiation and reduce proteolysis[41]. Leucine stimulates protein synthesis via mTORC1 pathway through the phosphorylation of 4E-BP1 and P70S6K1[111]. Infusion of leucine has been shown to reduce the plasma concentration of other amino acids, and also reduce proteolysis in humans[112]. A previous study conducted in diabetic rats showed that leucine promotes protein synthesis independent of mTORC1 activity through the activation of eIF4G[113]. Leucine promotes the phosphorylation of eIF4G (Ser-1108) and the binding to eIF4E without the phosphorylation of S6K1 (Thr389) and 4E-BP1 (Thr37/46). This eIF4G-eIF4E

complex bind to mRNA to stimulate translation[113]. The signaling pathway responsible for the mTORC1-independent phosphorylation of eIF4G in the presence of leucine is yet to be studied.

BCAA Supplementation

Branched-chain amino acid supplementation has been shown to affect exercise capacity and metabolism. BCAA supplementation mitigate the enhanced BCAA catabolism and muscle soreness caused by eccentric endurance excercise[114,115]. Furthermore, BCAA administration promotes the recovery of muscle damage in resistance exercise trained males[116]. BCAA supplementation is linked to the increase in lipid oxidation during exercise in glycogen-depleted individuals[117]. In the skeletal muscle of middle-age mice, BCAA decreased reactive oxygen species (ROS) production and increased mitochondrial biogenesis[118]. This is seen by the increase in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) levels, a protein involved in the induction of mitochondrial biogenesis and an increase in oxidative metabolism[118,119]. In addition to the reduction in ROS production, BCAA supplementation, specifically leucine suppresses TNF-α-induced skeletal muscle inflammation[41].

2.4.2 BCAA Sensing and Uptake in the skeletal muscle

BCAAs are taken up into the cell to activate mTORC1 and maintain positive muscle protein balance through specific amino acid transporters. The amino acid transporters present in the skeletal muscle include the L-type amino acid transporter 1 (LAT1) also called solute carrier family 7 member 5 (SLC7A5)[120]. LATs including LAT1 and LAT2 are expressed in skeletal muscle to different degree. Leucine and other BCAAs show their effects on mTORC1 primarily through LAT1[121,122]. LAT1 muscle-specific knockout (mKO) mice show increased expression of LAT2 and decreased amino acid transport[122,123]. This suggests that LAT1 is the primary BCAA transporter. Human LAT1 is an amino acid exchanger transporter that can transport leucine into

the cell and export glutamine out of the cell simultaneously[120,122]. Resistance exercise has been shown to increase muscle amino acid transporter in young and older adults[124]. Immunofluorescent microscopy shows that LAT1 is localized in sarcolemmal membrane with greater intensity in the human type II muscle fibers[122].

Previous research show that leucine-dependent activation of mTORC1 requires amino acid sensors known as sestrins[125]. Sestrins (Sesn1-3) are conserved proteins that build up in cells undergoing stress, genotoxic and oxidative[126,127]. Recent studies show that sestrins, specifically Sesn1 binds to GATOR2 to negatively regulate mTORC1 under leucine deprivation in skeletal muscle[125–127]. In the presence of leucine, Sesn1/2 is activated and dissociated from GATOR2[125,128]. The GATOR2 complex inhibits GATOR1 complex to promote mTORC1 activation via the increase in Rag GTPase activity and translocation to mTORC1 lysosomal surface[125,128]. Sesn1 deficiency in mice increase disuse-induced and aging-induced muscle atrophy[129]. Sestrins preserve skeletal muscle mass by improving mTORC1 sensitivity to nutrient status especially leucine in vivo[125].

2.4.3 The Role of BCAA Catabolites on Skeletal Muscle Metabolism

Although BCAAs promote anabolic signaling, there is evidence to suggest that their catabolic products are involved in protein synthesis and energy production in skeletal muscle[130]. For example, leucine metabolites α-ketoisocaproate (KIC) and β-hydroxy β-methylbutyrate (HMB) have been implicated in leucine-mediated anabolic effect of skeletal muscle. Though this effect is more pronounced in leucine, KIC and HMB improve the activation of mTORC1, p70S6K1 and 4E-BP1[130,131]. A previous study conducted on rat skeletal muscle show that KIC's anabolic effect is not due to its stimulation of muscle protein synthesis, but due to the attenuation of proteolysis[11]. Though a recent study in neonatal pigs show that KIC can stimulate muscle protein

synthesis to the same extent as leucine[132]. Another study proposed that the stimulation of mTORC1 by KIC could be due to the conversion of KIC back to leucine[133]. However, a study in rat heart muscle conducted by Chua et al., 1979 showed that KIC can increase protein synthesis without a change in the intracellular levels of leucine[134].

HMB stimulates muscle protein synthesis and reduces muscle protein breakdown through the PI3K/AKT/mTORC1 pathway[135]. Oral consumption of HMB in humans show an acute increase in muscle anabolism independent of insulin signaling. The mechanism involved is still yet to be understood[130]. Furthermore, HMB has been reported to decrease muscle protein breakdown through muscle protein degradation pathways, ubiquitin-proteasomal and autophagy-lysosomal pathways. HMB attenuates proteolysis through the activation of PI3K/AKT pathway which leads to the phosphorylation of FOXO1 and FOXO3a transcription factors involved in transcriptional regulation of cell cycle arrest and apoptosis[136]. In C2C12 mouse cells, HMB treatment has been shown to increase FOXO phosphorylation and inhibition. This leads to the decrease in MuRF-1 expression and UPS[137]. In the presence of HMB, dexamethasone-induced muscle atrophy in L6 rat myotubes is attenuated and muscle proliferation and protein synthesis is increased via PI3K/AKT and p38/MAPK signaling[138,139]. HMB increases phosphorylation of AKT on Ser473 and Thr308 and is inhibited by wortmannin (PI3K inhibitor), indicating that HMB acts through PI3K/AKT signaling[139]. HMB has an effect in promotion of muscle protein synthesis and in preventing muscle wasting.

Supplementation of HMB in relation with exercise training works to increase oxidative metabolism and muscle strength, decreasing exercise-induced protein degradation in older adults and untrained individuals[140]. Under normal and catabolic conditions, chronic HMB supplementation promotes anabolic effect on protein metabolism via the increase in insulin/IGF-

1 axis activity and IGF-1 mRNA expression[139,141]. High intensity exercise increases the transamination of leucine to KIC at a faster rate compared to the decarboxylation of KIC. This leads to the intracellular accumulation of KIC and anabolic signaling in the skeletal muscle[142]. Leucine metabolites are important supplements with reference to exercise.

2.4.4 BCAA Catabolic Pathway

BCAAs catabolism occurs in the mitochondria through a multi-step process to generate some intermediates that can enter the TCA cycle. The first step of BCAA catabolism involves the reversible transamination by branched-chain aminotransferase 2 (BCAT2) (Fig. 2). This initial step occurs mainly in skeletal muscle[143,144]. This initial reaction yields metabolites known as branched-chain α-keto acids (BCKAs): α-ketoisocaproic (KIC) for leucine, α-keto-β-methylvaleric acid (KMV) for isoleucine and α-ketoisovaleric acid (KIV) for valine[41,144]. The second and the rate limiting step is the irreversible oxidative decarboxylation of BCKAs to branched-chain acyl-CoA ester compounds catalysed by branched-chain α-keto acid dehydrogenase complex (BCKDH, BCKD). After the BCKD reaction, further KIC catabolism leads to acetyl-CoA and acetoacetate. KIC metabolism also produces HMB synthesized by KIC dioxygenase in the cytosol[144]. Further catabolism of KIV leads to succinyl-CoA, KMV to acetyl-CoA and succinyl-CoA[144] via a series of enzymatic reactions. These BCKAs are also substrates for ketogenesis, gluconeogenesis and glycogenesis[144].

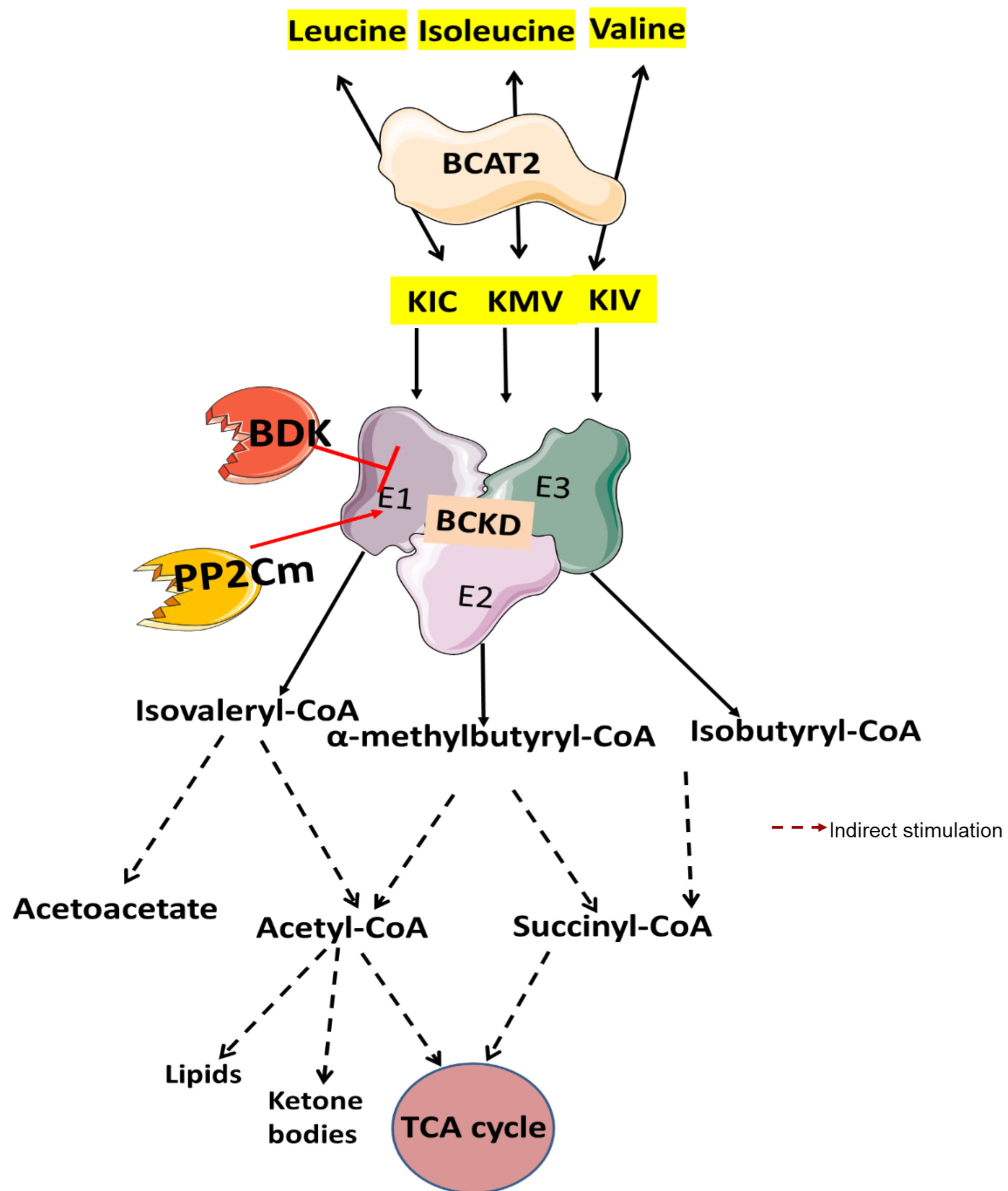

Fig. 2. Schematic of branched- chain amino acid catabolic pathway. The figure depicts the two main BCAA catabolic enzymes in the mitochondrial, BCAT2 and BCKD. BCAT2 catalyses the reversible transamination of the BCAAs to their keto acids. This is followed by the irreversible decarboxylation of the keto acids to CoA derivatives. The derivatives are further metabolized to yield acetoacetate, acetyl-CoA and succinyl-Co-A that can be used for lipid synthesis, ketone production and ATP production. BCAT2= Branched-chain aminotransferase 2; BCKD= Branched-chain ketoacid dehydrogenase; KIC= α-ketoisocaproate; KMV= α-keto-β-methylvalerate; KIV= α-ketoisovalerate.

2.4.4.1 BCAT structure and regulation

The branched-chain aminotransferases (BCATs) are present in the cytosol and in the mitochondria compartments of mammalian cells[145]. Cytosolic isoenzyme, BCAT1 or BCATc is predominantly found in the brain and in low levels in few other organs such as the ovary and placenta of rats[145,146]. Mitochondrial isoenzyme, BCAT2 or BCATm, is generally distributed in all tissues of rodents and humans except for the liver tissue[147,148]. The skeletal muscle mitochondrial has been shown to have highest BCAA transamination capacity by BCAT2, though there is a differential expression in different muscle fiber types[149]. BCAT2-expressing cells also express BCKD (the second enzyme in BCAA catabolic pathway compared to BCAT1-expressing cells)[148]. The distribution of BCAT2 and BCAT1 has been shown not to have an overlap; in human somatic cell, BCAT2 isoenzyme gene is typically found on chromosome 19 and BCAT1 enzyme gene on chromosome 12[148,150]. BCAT1 and BCAT2 transaminate the same hydrophobic substrates, BCAAs, leucine, isoleucine and valine with estimated Michaelis constant (Km) of 1mM for leucine and isoleucine and 5mM for valine[146]. BCAT isoenzymes transfer amino group from BCAAs to α-ketoglutarate to produce branched-chain α-keto acids (BCKAs) and glutamate[151]. The low Km range, 0.6 to 3mM of α-ketoglutarate relative to Km of that of the BCAAs makes α-ketoglutarate a limiting substrate for the transamination reaction[146,151].

Mammalian BCATs have a CXXC motif made up of cysteine residues, Cys315 and Cys318 that regulates substrate specificity for catalysis[152,153]. The cysteine residues are redox-controlled suggesting that BCAT isoenzyme activity is regulated by the redox status of the cell[153]. X-ray crystallography show that disruption in the CXXC motif leads to altered α-keto acid substrate orientation[152]. Nitric oxide modification on redox-active dithiol of the Cys315 and Cys318 residues of BCAT is shown to inactivate the activity of both isoforms of BCAT in a time-dependent

manner[154]. BCAT2 is shown to have a branched-chain α-keto acid transporter feature that transports BCKA's from the muscle into the blood plasma and then the liver in the presence of high levels of BCAAs[155]. In response to BCAA deprivation, BCAT2 is acetylated on the lysine 44 (K44) residue in pancreatic cancer cells[156]. Acetylation of BCAT2 leads to its degradation through UPS and the increase in BCAA availability[156].

2.4.4.2 BCKD structure and regulation

BCKD activity occurs mostly in the liver, with activity low in skeletal muscles, and intermediate in the kidney and heart[157]. BCKD consists of three component parts: heterotetrameric branched-chain α-keto acid decarboxylase (E1α and β), dihyrolipoyl transacyclase (E2) and a homodimeric dihydrolipoyl dehydrogenase (E3)[158,159]. The E1 subunit catalyses the oxidative decarboxylation of the α-keto acids and the reductive acylation of the lipoyl moiety that is covalently bound to the E2 subunit. The E2 subunit contains three folded domains: the core domain, the subunit binding domain and the lipoic acid-bearing domain (lip-LBD)[160]. The binding domain of E2 promotes the attachment of E1 and E3 subunits to the E2 subunits[160]. The E2 subunit transfers the acyl group from the lipoyl moiety to coenzyme A. The E3 subunit is a flavoprotein that re-oxidizes the reduced lipoyl residue of E2[158,161].

BCKD activity is tightly regulated by the phosphorylation and dephosphorylation on residues of the E1 component (Fig. 2). BCKD kinase (BDK, BCKDK) phosphorylates BCKD on Ser293/302 residues of the E1α subunit while mitochondrial targeted 2C-type protein phosphatase (PP2Cm) dephosphorylates BCKD on the same E1α residues[159]. The phosphorylation of BCKD inhibits its activity while the dephosphorylation increases BCKD activity[131,159]. PP2Cm also interacts with E2 subunit of BCKD complex in a substrate-dependent manner (BCKAs) to maintain BCAA homeostasis[159]. Phosphorylated E1β subunit on Ser292 causes a disorder in the

loop conformation of E1β active site[162]. The conformational change prevents the binding of the E2 lip-LBD to E1β and stops the reductive acylation of lip-LBD [162]. The interaction of the C-terminal hinge region of the lip-LBD of E2 transacyclase subunit of BCKD with BDK is crucial for BDK activity[163].

BDK structure and regulation

BDK is a mitochondrial protein kinase that contains a nucleotide-binding domain and a four-helix bundle domain. Its sequence and structure is like that of protein histidine kinases (PHKs)[164,165]. Unlike PHKs, phosphotransfer carried out by BDK on BCKD E1α subunit is not mediated by histidine[164]. BDK is a homotetramer with four 5′-adenylyl-imidodiphosphate-binding sites in a conformation able to bind to ATP[166]. Change in the ATP-binding domain, attenuates BDK catalysis activity, suggesting that it is part of the ATPase Kinase superfamily (protein kinases with intrinsic ATPase activity) rather that the protein histidine kinase family[166]. Crystal structure of BDK show that an allosteric inhibitor such as (S)-α-chloro-phenylpropionic acid [(S)-CPP] binds to a site in the N-terminal domain to cause a movement of helix-bundle and conformational change in BDK to promote full activation of BCKD[167]. BDK mRNA expression has been shown to be highest in the skeletal muscle, intermediate in the brain and kidney and lowest in the liver and small intestine[168]. There is a high ratio of BDK to E2 subunit of BCKD in skeletal muscle compared to other tissues[168].

The activity state of hepatic BCKD complex in rats is affected by its substrates KIC, KIV and KMV. The accumulation of KIC promotes BCAA catabolism by the inhibition of BDK and activation of muscle BCKD complex[169]. Thus, KIC is a potent inhibitor of BDK. KIV and KMV also inhibit BDK but with reduced efficacy compared to KIC[169,170]. BCAA starvation and low protein diet increases BDK mRNA levels to inactivate the BCKD complex. The increased BDK

and decreased BCKD activity seen in protein-starved animals is associated with increased thyroid hormone levels in the blood[171]. Other hormones such as insulin, glucocorticoid and sex hormones have been shown to regulate the expression and activity of BDK in rat liver[131,171]. High glucose levels suppress the activity of the E1α component of BCKD in the islet of the pancrease[172]. Insulin regulates BCAA metabolism via the stimulation of BDK expression in Clone 9 rat cells[173]. These data suggest that BDK expression and BCKD activity is affected by glucose levels.

PP2Cm structure and regulation

PP2Cm is a mitochondrial BCKD phosphatase encoded by protein phosphatase Mg^{2+}/Mn^{2+} dependent 1K (PPM1K) gene and is highly conserved in vertebrates[174,175]. PP2Cm is highly expressed in the liver, brain, heart, kidney and diaphragm but low in skeletal muscle[159]. Loss of PP2Cm has been shown to abrogate the dephosphorylation and increase the hyperphosphorylation of the E1α subunit of BCKD complex in mice. Gene ablation of PPM1K has been associated with numerous diseases including maple syrup urine disease (MSUD) a disorder caused by deficiency in BCKD activity[175,176]. PP2Cm is regulated transcriptionally in response to nutrient status of the cell[159]. Food deprived mice show a decrease in PP2Cm mRNA levels. In cultured cells, removal of BCAA significantly decreased PP2Cm expression while BCAA replenishment increased brought PP2Cm mRNA levels back up[159].

Other regulators of BCAA catabolism

Upregulation of fatty acid oxidation has been shown to enhance BCAA catabolism[170]. Clofibric acid, a drug that promotes fatty acid oxidation, decreases BDK gene expression and activity[177–179]. Circulating free long-chain fatty acids released due to starvation and exercise, bind to peroxisome proliferator-activator receptor-α (PPARα) to activate fatty acid oxidation[170]. PPARα

activation inhibits hepatic BDK expression and promotes BCKD activity[170,171]. This suggests that fatty acid oxidation enhances BCAA catabolism[170,178]. Endurance exercise promotes BCAA catabolism and activation of BCKD complex in human[180] and rat[181] skeletal muscles via the increase in fatty acid oxidation. Exercise has been shown to increase fatty acid oxidation. Prolonged exercise significantly decreased BDK activity and the number bound to BCKD complex in rat liver[170,182,183]. Manipulation in the number of BDK-BCKD interaction is a potential short-term regulatory mechanism for the activity of BCKD complex.

Adiponectin (APN) is characterized as a protein hormone secreted form the adipocyte that is involved in regulating glucose metabolism and fatty acid breakdown[184]. APN-specific knockout mice fed with high fat diet show decreased PP2Cm expression and BCKD activity while BDK expression significantly increased[185]. Plasma BCAA and BCKA levels were elevated in APN-specific knockout mice[185]. APN treatment reverses PP2Cm, BCKD and BDK activity in diabetic mice[185]. APN is a potential regulatory hormone to increase BCAA catabolism in diabetic mammals.

MicroRNAs (miRNA) are non-coding short RNA molecules that regulate gene expression[186]. Interestingly, BCKD expression levels is controlled by human microRNA (miR29b). The miR29b interacts with the mRNA of the E2 subunit of BCKD complex to prevent its translation[187]. MicroRNAs exerts a regulatory role to the levels of BCKD mRNA expression in the cell.

2.4.5 Regulation of BCAT2 and BCKD in the Skeletal Muscle

Specific overexpression of PGC-1 in the skeletal muscle increases the expression of BCAT2 and BCKD complexes. PGC-1α promotes BCAA catabolism and decreases BCAA levels in murine skeletal muscle and C2C12 cultured cells[188].

Metabolic acidosis in pathological conditions increases BCAA oxidation by the stimulation of BCKD activity in the skeletal muscle. In acidosis, plasma and muscle BCAA levels are lower and BCAT transaminase activity is higher. The increased flux through the transaminase produce α-keto acids that activate BCKD complex[189].

Endurance exercise has been shown to increase basal BCAT2 levels compared to resistant exercise. This suggests that endurance exercise increases skeletal muscle BCAA catabolism compared to resistant exercise which is consistent with the study by Wagenmakers et al., 1989[190].

2.4.5.1 Effect of Disruption of BCAA Catabolism on Whole-body Metabolism

Disruption of BCAA metabolism has been seen in diseased states such as type 1 and 2 diabetes, obesity, chronic renal failure and cardiovascular diseases[144].

Type 2 Diabetes mellitus/Obesity

Though BCAAs are a nutrient signal for muscle protein synthesis and cell growth, elevated levels of BCAAs have been shown in insulin resistance disorders such as obesity and type 2 diabetes mellitus (T2DM)[78]. A study illustrated that enhanced BCAA levels seen in obese Zucker rats is due to reduced BCKD complex activity and BCAA catabolism in liver and adipose tissue[191,192]. Insulin resistance can be classified as hyper-activation of mTORC1 activity in the face of excessive nutrient signals such as BCAAs. This leads to reduced insulin signaling (P13K/AKT pathway), an indicator of diabetes and obesity[193]. BCAA-restricted diets have been shown to attenuate insulin resistance in Zucker fatty rats[193].

However, another study showed that defect in BCAA catabolic pathway as indicated by reduction in BCKD activity (elevated BCAA level) promotes insulin sensitivity and glucose tolerance in lean mice[194]. This result was observed in the liver of the lean mice but not in the skeletal muscle[194].

Diabetic mice show defect in BCAA catabolism by way of reduced mitochondrial PP2Cm expression and BCKD activity[195]. Overexpression of PP2Cm in the heart of diabetic mice decreased myocardial ischemia -reperfusion (MI/R injury) and oxidative stress found in diabetic patients[195].

Autism and neurological disorders

BCKD kinase (BDK) mutation has been associated with autism and intellectual disability. Autism spectrum disorder is a structural brain abnormality classified by the impairment in social interaction. Autistic patients present with homozygous BDK mutation that decreases the phosphorylation of the E1α subunit of BCKD and BCAA levels in the plasma. Whole-body BDK knockout in mice enhance abnormal brain amino acid levels (large neural amino acids levels) and neurobehavior. BCAA supplementation reverses the increased levels of other neural amino acid, the neurological defect and normalizes plasma BCAAs in humans[196].

Heart failure/Cardiovascular diseases

Cardiac hypertrophy is a potent indicator of heart failure. Suppression of BCAA catabolism in cardiomyocytes is regulated by Kruppel-like factor 15 (KLF 15)[197]. KLF 15 is a transcription factor that inhibits cardiomyocyte hypertrophic gene expression and protein synthesis. KLF 15-null mice developed cardiac hypertrophy due to loss in the expression of BCAA catabolic genes, such as BCAT2, E1α, E1β and E2 subunits of BCKD complex and PP2Cm[198]. Accumulation of branched-chain keto acids is seen in the myocardium of human failing hearts and hearts of KLF 15-deficient mice[198]. Stimulation of BCAA catabolism by BT2, an allosteric inhibitor of BDK, has been shown to maintain cardiac function and increase the life span of cardiomyocytes[167].

Cancer

Overexpression of the two main BCAA catabolic enzymes, BCAT and BCKD, is seen in various cancers. Elevated levels of cytosolic BCAT (BCAT1) enhances breast cancer cell growth through mTOR-mediated mitochondrial biogenesis[199]. BCAT1 activates mTOR signaling to increase mitochondrial biogenesis and ATP production. This facilitates the growth and colony formation of breast cancer cells[199]. Increased mitochondrial BCAT (BCAT2) and BCAA catabolism is seen in pancreatic ductal adenocarcinoma (PDAC)[200]. Pancreatic tissue specific BCAT2 knockout decreases the progression of pancreatic intraepithelial neoplasia. This suggest that BCAT2 is essential in sustaining BCAA catabolism and mitochondrial respiration in PDAC[200].

Hypertension

Cross-sectional and population-based cohort studies show elevated levels of BCAA is associated with increase incidence of hypertension[201,238,239]. Higher intakes of BCAAs, especially valine is associated with higher risk of newly developed hypertension. Thus, changes in BCAA metabolism affect whole body metabolism[240].

2.4.5.2 Effect of Disruption of BCAA catabolism on skeletal muscle metabolism

Liver Cirrhosis

Decreased BCAA levels and inhibition of protein synthesis is seen in subjects with liver cirrhosis[202]. The decreased plasma BCAA is a result of enhanced BCAA transamination from α-ketoglutarate (α-KG) for ammonia detoxification to form excess GLN in skeletal muscle[202]. Increased glutamine production leads to high levels of ammonia in the blood a deleterious effect in subjects with liver diseases[202]. Leucine, as well as KIC, stimulates glucose uptake in an insulin-independent manner in isolated soleus muscle of rats with liver cirrhosis[203]. Leucine promotes the upregulation of GLU4 and GLUT1 (glucose transporters) as well as mTORC1 signaling[203].

Branched-chain amino acids supplementation is a known requirement for cirrhosis patients, specifically to boost glucose metabolism[203,204].

Chronic renal failure

Chronic renal failure (CRF) is linked to abnormal levels of BCAAs (leucine, isoleucine and valine) and BCKAs (KIC, KIV and KMV) in the blood plasma[42]. CRF patients exhibit decreased BCAAs, specifically valine concentration in the muscle as well as decreased BCKAs (KIC and KIV) in the blood[42,205,206]. Metabolic acidosis, the inability for the kidney to excrete acids, is implicated in the increased protein breakdown and accelerated BCKD activity in muscle which might account for the reduced extracellular BCAA concentration[207]. In response to acidosis, there is an increase in BCAA oxidation and protein degradation in muscle to provide nitrogen for GLN production in the kidney to excrete the acids[208]. Ingestion of amino acids in CRF patients is associated with increased uptake of non-essential amino acids and inadequate uptake of BCAAs by the muscle[209]. Acidosis is a major factor in muscle catabolism.

Maple syrup urine disease

Mutation of PP2Cm gene, PPMIK in the brain has been seen in MSUD (maple syrup urine disease), a hereditary recessive disorder characterized by neurological and developmental dysfunction and a distinct sweet odour in the urine of infants[158,174]. Studies show that the defect in BCAA metabolism is a result of decreased BCKD activity[158,176]. Individuals with type 1A MSUD show a missense mutation in E1α subunit of BCKD complex. The mutation of the C-terminal aromatic residues leads to the loss of kinetics of the E1 subunit of BCKD assemble and catalytic activity[210]. A loss of function of the E1β component of BCKD complex is seen in MSUD. An 11-bp deletion mutation with a change in the reading frame of E1β gene decreases the enzymatic function of BCKD. The lack of E1β subunit is observed to make the E1α unstable[211]. Mutation in

the E2 subunit of BCKD complex is also present in MSUD[212]. Muscle biopsies of MSUD patients presented with skeletal muscle fiber abnormalities[213]. The muscle fibers show evidence of destruction of the myofibrils[213]. In response to BCKA accumulation, MSUD neural cell models show increase in oxidative stress and compromised energy metabolism that contribute to encephalopathy seen in MSUD individuals.

Sepsis and trauma injury

Muscle wasting associated with sepsis and trauma is a result of elevated BCAA oxidation in the muscle and increased release of glutamine (GLN) to the blood[214]. Consumption of GLN by visceral tissues exceeds the release of GLN from the muscle that leads to GLN deficiency[214]. Enhanced release of GLN from muscle decreases GLN intramuscular concentration and attenuates muscle protein synthesis[214]. Fast-twitch muscle fibers are shown to be susceptible to septic stimuli compared to slow-twitch muscle fibers[214]. Inflammatory markers impair BCAA absorption from the gut and into the blood and muscle leading to decreased BCAA stimulated muscle protein synthesis[144].

Type 2 Diabetes/Obesity

Disturbance in BCAA catabolism characterized by the inactivation of BCKD complex in the skeletal muscle has been associated with skeletal muscle insulin resistance[215]. Early-onset type 2 diabetes in mice has been linked to reduced muscle BCAA degradation due to reduced BCKD activity and increased BCAT2 expression[215]. Impaired BCAA catabolism in mitochondria of skeletal muscle causes the release of BCAAs and BCKAs into circulation as seen in T2DM[215]. Recent studies have proposed that BCAA metabolites interfere with fatty acid oxidation in muscle which leads to insulin resistance[216]. Skeletal muscle insulin resistance is linked to excess fatty acid uptake and accumulation[217]. Increase in valine catabolic intermediate, 3-hydroxyisobutyrate (3-

HIB) activates endothelial fatty acid transport, stimulates muscle fatty acid uptake and lipid build-up in the muscle[218]. This finding links increased BCAA catabolic flux and fatty acid uptake to insulin resistance in diabetic individuals[218].

Type 1 Diabetes

Type 1 diabetes, a condition related to the inadequate secretion of insulin from the pancreases, is associated with exaggerated BCKD activity, BCAA catabolism and proteolysis during starvation in the muscle[144,219]. High plasma BCAA, decreased BCAA uptake in the muscle and nitrogen repletion is seen following protein feeding in individuals with type 1 diabetes[220].

Muscle Fatigue

Whole-body BCAT2 knockout (KO) has been shown to decrease exercise tolerance and increase muscle fatigue in mice[114]. There is an accumulation of muscle inosine monophosphate (IMP), a marker of muscle fatigue in BCAT2 KO mice[114,221]. BCAT2 KO mice show significant increase in skeletal muscle protein synthesis and breakdown suggesting higher rates of protein turnover and energy expenditure. These mice show loss in body weight without any change in muscle weight and structure[222]. The increase in protein turnover rate is correlated with the increase in mTORC1 signaling indicated by higher 4E-BP1 and S6 phosphorylation[222].

2.4.5.3 The Effect of Disruption of BCAA catabolism in Skeletal Muscle on Skeletal Muscle Metabolism

The first two enzymes in BCAA catabolic pathway, BCAT2 and BCKD have been shown to be important in skeletal muscle differentiation. Myoblasts depleted of BCAT2 and BCKD enzymes show significant decrease in myoblast differentiation into myotubes and mTORC1 activity[223].

Muscle-specific deletion of BDK (BDK-mKO) in mice with low BCAA concentration has been shown to decrease skeletal muscle protein anabolism[224]. BDK knockout promotes BCKD activation and increases BCAA catabolism. BDK-mKO mice show a decrease in mTORC1 signaling and myofibrillar protein content suggesting that increased BCAA catabolism negatively regulates protein synthesis in skeletal muscle[224]. Controlled-overexpression of BDK via doxycycline treatment attenuates BCKD activity and decreases BCAA catabolism implicated in uncontrollable muscle protein degradation seen in cancer, diabetes and renal failure[225].

Summary

Skeletal muscle structure and contractility is regulated by myofibrillar proteins, MHC, troponin and tropomyosin. Skeletal muscle mass is maintained by muscle protein balance. The interaction of muscle protein synthesis and breakdown regulates muscle protein balance. Amino acids, among other factors, regulate cell proliferation and muscle protein synthesis. Branched-chain amino acids, specifically leucine, stimulate anabolic pathways to increase skeletal muscle hypertrophy.

In addition to BCAAs, their metabolites also show anabolic and anti-catabolic effects on skeletal muscle. Disruption in BCAA metabolism, specifically the impairment in the activity of the first two enzymes in BCAA catabolic pathway (BCAT2 and BCKD), is associated with decreased skeletal muscle function and metabolism.

Chapter Three: Rationale

Skeletal muscle formation and maintenance of protein balance is regulated by various signaling pathways. BCAAs and their metabolites have been shown to have anabolic effect in the regulation of skeletal muscle metabolism[41]. Recent studies from our lab reveal that the BCAA catabolic pathway is essential for skeletal muscle cell differentiation[223,226]. Skeletal muscle cell differentiation starts with the forming of myoblasts from muscle progenitor cells in a process called myogenesis[241]. These myoblasts proliferate, fuse and are differentiated into multinucleated myotubes[241]. Disruption of either BCAT2 or BCKD (two main enzymes involved in BCAA catabolism) in L6 muscle cells prevents the differentiation from myoblasts to myotubes[223]. Therefore, an intact BCAA catabolic pathway is vital for muscle cell differentiation. However, much less has been done to elucidate the role of BCAA catabolic process on differentiated skeletal muscle cells. A study done in mice showed that increasing BCKD activity by way of BDK knockout, decreases protein synthesis[224]. Considering the critical role of BCAA catabolism on myoblasts survival and differentiation, it is imperative to also understand the role BCAA catabolism on myotube survival, protein synthesis and protein breakdown.

3.1 Research Objectives

The goal in this study is to examine the effect of disrupting BCAT2 and BCKD levels on differentiated skeletal muscle cells.

i) To examine the effect of BCAT2 and BCKD depletion on myotube structure and myofibrillar protein content under normal and atrophic conditions.

ii) To examine mTORC1 signaling and insulin signaling (as a measure of glucose metabolism) in BCKD-depleted myotubes.

iii) To examine myotube protein synthesis and protein breakdown in BCKD-depleted cells

3.2 Hypotheses

i) Based on literature that suggests that increased BCAA catabolism results in muscle proteolysis, depleting BCAT2 and BCKD enzymes will enhance myotube structure, protein abundance and attenuate protein breakdown under normal conditions.

ii) Based on previous literature that suggests that increased BCAA catabolism is linked to decreased anabolic signaling, mTORC1 signaling and insulin signaling will be upregulated in BCKD-depleted myotube

Chapter Four: Manuscript

Effect of Branched-Chain Alpha-Keto Acid Dehydrogenase Depletion on Myotube Protein Metabolism

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Keywords: protein synthesis, skeletal muscle metabolism, mTORC1 signaling, myotube, myofibrillar proteins, catabolic condition, branched-chain amino acids, branched-chain α-keto acid dehydrogenase

Abstract

Branched-chain amino acids and their catabolites promote anabolic signaling in skeletal muscle. Previous research has shown that siRNA-mediated knockdown of the first two enzymes in BCAA catabolic process, BCAT2 and BCKD impair L6 muscle cell differentiation. This current study examines the effect of siRNA-mediated depletion of BCAT2 and BCKD on L6 myotubes. It was observed that BCKD-depleted myotubes showed improved myotube structure compared to BCAT2-depleted myotubes. In addition, BCKD-depleted myotubes showed increased myofibrillar protein abundance compared to BCAT2-depleted myotubes and control. BCAT2-depleted myotubes showed a decreasing trend in myofibrillar protein levels compared to control. Due to the low levels of BCKD enzyme in the skeletal muscle and the presence of distinct myotube seen in BCKD-depleted myotubes, we focused more on the effect of BCKD depletion on anabolic signaling. BCKD-depletion increased mTORC1 signaling as shown by the upregulation of the phosphorylation of mTORC1 upstream activator and downstream substrates. These results suggest BCKD enzyme depletion is required to maintain myotube structure and anabolic signaling. Therefore, interventions that target BCKD levels and function can ameliorate muscle protein synthesis in muscle wasting diseases.

Introduction

The skeletal muscle is a multinucleated structure composed of muscle fibers that is crucial for energy and protein metabolism. The contractility of the muscle is regulated my myofibrillar proteins, myosin heavy chain (MHC), troponin and tropomyosin[4]. The loss and dysfunction of these proteins lead to decreased muscle mass. Skeletal muscle mass is regulated by the occurrence of protein turnover[8]. Muscle wasting seen in age-related sarcopenia and cancer cachexia is associated with decreased muscle protein synthesis and increased muscle protein breakdown[18]. Among other factors, protein turnover is controlled by amino acids, especially branched-chain amino acids (BCAAs) which are leucine, isoleucine and valine[20,43].

Branched-chain amino acids are important regulators of skeletal muscle anabolic signalling. BCAAs, specifically leucine, activate the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) to promote muscle protein synthesis and attenuate proteolysis[43]. Though BCAAs promote muscle anabolism, their catabolic products have been shown to also elicit an anabolic effect on skeletal muscle[111,137]. Previous research has shown that leucine metabolites α-ketoisocaproate (KIC) and β-hydroxy-β-methylbutyrate (HMB) positively regulate muscle protein balance.[111,130].

BCAA catabolism starts with the reversible transamination of the BCAAs into branched-chain α-keto acids (BCKAs) by the mitochondrial branched-chain aminotransferase (BCAT2). The second and the rate determining step is the irreversible decarboxylation of BCKAs into acyl-CoA derivatives by the branched-chain α-keto acid dehydrogenase complex (BCKD). BCAT2 activity is regulated by the redox status of the cell[153]. BCKD activity is regulated by the phosphorylation and dephosphorylation on its E1 subunit. Phosphorylation by BDK inhibits BCKD activity whereas PP2Cm dephosphorylates BCKD to activate it[158]. BCAT2 and BCKD protein expressions

are regulated by endurance exercise[180,190], disease condition[189] and upstream regulators such as PGC-1α in the skeletal muscle[188].

Disruption in the activity of BCAT2[114,222] and BCKD[224] has been shown to impair muscle endurance capacity and anabolic signaling in animal models. There is evidence to show that BCAA catabolism is required for skeletal muscle cell differentiation[223]. Impairment in skeletal muscle metabolism has been linked to disruption in BCAT2 and BCKD activity. Thus, the role of BCAA catabolism, particularly BCAA metabolites and the catabolic enzymes is of great importance to the maintenance of skeletal muscle mass and muscle wasting diseases. Accordingly, our study seeks to identify the role of disrupting BCAA catabolism on differentiated skeletal muscle cells. We identify the mechanism of its effect on myofibrillar protein abundance and mTORC1 signalling.

Materials and methods

Reagents

Growth medium (GM) consisted of α-Modification of Eagle's Medium (AMEM) (Wisent #310-010-CL) supplemented with 10% fetal bovine serum (FBS) (Gibco #098450) and 1% Antibiotic-Antimycotic agents (Wisent #15240-062). Differentiation media (DM) consisted of AMEM supplemented with 1% Antibiotic-Antimycotic agents and 2% horse serum (HS) (Gibco #26050088). Phosphate buffered saline (PBS) (#311-010-CL) and trypsin (#325-043-CL) were purchased from Wisent. Protease inhibitor (#P8340) and phosphatase inhibitor (#P5726) were purchased from Sigma Aldrich. Dithiothreitol (DTT) was purchased from Research Organics (#2190DA101X). Tumor Necrosis Factor α was acquired from Shendoah Biotechnology (#300-18). BCAT2, BCKD and scramble (SCR) siRNA oligonucleotides were purchased from Sigma-Aldrich. Lipofectamine RNAiMAX (#13778-150) and Opti- MEM 1X Reduced Serum Medium (#31985-070) were obtained from Life Technologies. Triton was purchased from MP Biomedicals, LLC (#M2528). Pierce BCA Protein Assay Kit (#23225) was purchased from Thermo Scientific. PureLink RNA Mini Kit (#12183018A) was purchased from Life Technologies. iScript Advanced cDNA Kit (#1725037) and SsoAdvanced Universal SYBR® Green Supermix (#1725271) were purchased from Bio-Rad. Experiments were conducted using L6 rat skeletal myoblast purchased from American Type Culture Collection.

Cell Culture

L6 myoblasts were thawed and cultured in T75 flasks purchased from Gibco (#E18033C5) using GM and was placed in an incubator of 37ºC and 5% carbon dioxide (CO_2). Every second day upon reaching 70-80% confluency, cells were passed into a new flask. To seed, cells were expanded from T75 flasks into multiple 10cm plates purchased from Falcon. Once cells reach 70-

80% confluency, myoblasts were trypsinized, counted and seeded 200, 000 cells/well in 6-well plates or 100,000 cells/well in 12-well plates. Cells proliferated for 48 hours or until 90-100% confluency was reached (Day 0), then they were switched to DM. Cells were replenished with fresh DM every 48 hours until Day 4 of differentiation into myotubes. All experiments were conducted on Day 4 myotubes.

Transfection - *BCKD and BCAT2 knockdown*

To examine the effect of disrupting BCAA catabolic enzymes on differentiated myotubes, we used siRNA forward transfection as per manufactures designation (Life Technologies) to disrupt BCAT2 and E1 subunit of BCKD genes. The BCKD siRNA oligo was specifically designed against the E1α subunit of BCKD. This is because BCKD activity is regulated by the phosphorylation and dephosphorylation of its E1α subunit[159]. L6 myoblasts were cultured and grown as previously described. On Day 4 of differentiation, L6 myotubes were transfected with 10µM of scrambled siRNA (negative control, non-specific effects) or 10µM siRNA designed against BCAT2 (no. 1: 5'-GAGUGCAUCCGCCAGCUCA; or no. 2: 5'-CUAUGUGCGGCCGGUGCUU) and BCKD (5'-CAGAUCGUGAUCUGUUACU). The transfection medium contained the siRNAs, lipofectamine RNAiMAX, and Opti-MEM at ratios according to manufacturers protocol. Lastly, for 6-well plates, 250 µl of siRNA-lipid complex was added to wells containing 1ml of antibiotic-free α-MEM containing 2% HS. For 12-well plates, 125 µl of siRNA-lipid complex was added to wells containing 500 µl of antibiotic-free α-MEM containing 2% HS. Twenty-four hours post transfection (Day 5), 1 ml/well of DM (1% Antibiotic-Antimycotic, 2% horse serum (HS) and AMEM) was added into each well of 6-well plates or 500ul/well of 12-well plates[227]. Forty-eight hours after transfection (Day 6), myotubes were harvested for western blot analysis and mRNA isolation or immunofluorescence microscopy.

Effect of BCKD Depletion on Myotubes treated with TNF-α

To induce atrophic condition, myotubes were subjected to cytokine treatment after BCKD siRNA treatment. On Day 4 of differentiation myotubes were treated with scrambled siRNA or siRNA designed against BCKD. Twenty-four hours after transfection, wells containing 1ml of antibiotic-free DM got 1ml/well of DM (total volume of 2mls, control). In the treatment wells, antibiotic-free DM was removed and 2ml/well of Tumor Necrosis Factor α (TNF-α) containing medium (AMEM, 1% Antibiotic-Antimycotic and final concentration of 10ng/ml TNF-α, no horse serum) was added. On day 6 of differentiation (24 hours after TNF-α treatment), myotubes were harvested and stored at -20°C.

Cell Harvesting

Western Blot Analysis

Myotube structure was monitored every day by light microscopy and myotubes were harvested after treatment on Day 6 of differentiation (48hrs after transfection). Following washing with 1ml/well of PBS, 100ul/well of lysis buffer [1mM EDTA, 2% sodium dodecyl sulphate (SDS), 25 mM Tris-HCL pH 7.5, 10μL/mL protease inhibitor, 10μL/mL phosphatase inhibitor and 1mM DTT] was added in each well of 6 well plates. Cells were scraped using cell scraper and with the use of 1ml syringe, the lysate was transferred to a labelled 2ml Eppendorf tubes. Breakdown of the lysate was ensured by repeated uptake and release of lysate in the syringe. Samples were stored in -20°C for future analysis.

mRNA Analysis

To observe mRNA expression of BCKD, E3 ligases muscle ring-finger protein-1 (MuRF-1) and muscle atrophy F-box (MAFfbx) after BCKD siRNA transfection, RT-qPCR analysis was

conducted. Amplification of target genes was normalized to housekeeping gene, Hypoxanthine-guanine phosphoribosyltransferase (HPRT). The cell preparation and transfection are the same as above. Forty-eight hours after transfection, cells were harvested. RNA was isolated using PureLink RNA Mini Kit as directed in the manufacturer's protocol. After RNA extraction, RNA integrity (A260/280 – 1.7-2.0) and concentrations was determined using SmartSpec™ Plus (Bio Rad Laboratories (Canada) Ltd Life Science Group). Purified RNA samples were stored at -80°C until used. cDNA was synthesized using iScript Advanced cDNA Kit for Real-Time (RT) Quantitative Polymerase Chain Reaction (qPCR) following manufacturer's protocol (Bio-Rad). cDNA used was diluted 1.5-fold with autoclaved deionized water. RT-qPCR was performed using SsoAdvanced Universal SYBR® Green Supermix. Summary of primers that were used is found in Table 1.

Table 1: Summary of RT-qPCR primers

Gene Abbreviation	Forward Sequence	Reverse sequence
HPRT	5'-ACAGGCCAGACTTTGTTGGAT-3'	5'-CTTGCCGCTGTCTTTTAGGC-3'
BCKD	5'- CAACGATGTGTTTGCGGTGT-3'	5'-TGCCCGATCCTGTAGGTCAT-3'
MuRF-1	5'-CACCTTCCTCTTGAGTGCCA-3'	5'-CTCAAGGCCTCTGCTATGTGT-3'
MAFbx	5'- GTGAGCGACCTCAGCAGTTA-3'	5'-CATGGCGCTCCTTAGTACTCC-3'

All primers used were purchased from Life Technologies. QPCR products and data were obtained using Real-Time PCR Detection System (Bio Rad CFX96™ and CFX Manager 3.1 (Bio-Rad Laboratories Ltd Life Science Group).

Protein Assay and Western Blot Analysis

To obtain protein expression levels of different proteins in samples after treatments, western blot analysis was conducted. Protein concentration of lysate was determined using Pierce BCA Protein Assay Kit. The absorbance of samples at wavelength 550 nanometers were acquired using KC4 plate reader software (Bio-Tek Instruments Inc.). Samples were then mixed with 4x Laemmli loading buffer at a ratio of 3:1 (sample: loading buffer). To perform SDS-polyacrylamide gel electrophoresis, 25ug of each sample was loaded in each well of either a 10% or 15% polyacrylamide gel, depending on sizes of proteins to be analysed. Proteins that are similar or close in size were analyzed on different gels. For example, p-Akt (60 kDa) and p-S6K1(75 kDa) antigens were ran on different gels. Following gel electrophoresis, proteins now separated on a gel were transferred onto polyvinylidene difluoride (PVDF) membranes overnight at $4^{o}C$. Following transfer, the quality of transfer was assessed by exposure of membrane to ponceauS stain for 15mins. To prevent unspecific antigen binding, membranes were incubated in milk solution (5% milk in Tris Buffered Saline with Tween (TBST)) for 1 hour at room temperature. Each membrane underwent a 3×5minutes wash in TBST. Membranes were incubated in primary antibodies overnight at $4^{o}C$. List of primary and secondary antibodies that were used can be found in Table 2.

Table 2: Primary and Secondary Antibodies for Western Blot Analysis

Primary Antibody	**Purchased From**	**HRP-linked Secondary Antibodies**
BCKD	Cell Signaling #90198	Anti-rabbit (CST #7074)
p-S6K1^{Thr389}	Cell Signaling #9205	Anti-rabbit (CST #7074)
p-S6$^{Ser235/236}$	Cell Signaling #4858	Anti-rabbit (CST #7074)
AKT^{Ser473}	Cell Signaling #9271	Anti-rabbit (CST #7074)

p-SAPK/JNK$^{Thr183/Tyr185}$	Cell Signaling #9255	Anti-rabbit (CST #7074)
MHC	Developmental Hybridoma #MF20	Anti-mouse (CST #7076)
Tropomyosin	Developmental Hybridoma #CH1	Anti-mouse (CST #7076)
Troponin	Developmental Hybridoma #JLT12	Anti-mouse (CST #7076)
Gamma-Tubulin	Sigma Aldrich #T6557	Anti-mouse (CST #7076)

Following incubation, membranes were washed 3×5 minutes in TBST and incubated in horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody diluted in 5 % non-fat milk (1:10000) for 3 hours at room temperature. Following incubation, membranes washed 3×5 minutes in TBST were exposed to HRP chemical luminescent substrate (BioRad #1705060S) for signal visualization. Luminescence signal was obtained using BioRad ChemiDoc XRS+. Images were quantified using Image Lab Software version 7.

Immunofluorescence Microscopy

To examine the endogenous expression level of myofibrillar proteins after siRNA transfection, immunofluorescence microscopy was carried out. L6 myoblasts were grown in 12-well plates on coverslips (Fisher Scientific #092815-9). On Day 4 of differentiation myotubes were treated with scrambled siRNA or siRNA designed against BCKD. On day 6 of differentiation, coverslips were fixed with 1mL/well of a paraformaldehyde (PFA) solution (4% PFA in PBS), permeabilized with 1mL/well of a Triton solution (0.03% Triton X-100 in PBS) and incubated in 400µL/well of a blocking solution (10% horse serum in PBS) for 1 hour. Following incubation, coverslips were incubated overnight in MHC primary antibody solution (2.5µg/ml of MHC in 1% BSA in PBS). The next day, coverslips were then exposed to 500µL/well of a diluted Texas Red anti-mouse IgG (1:100 with 1% BSA in PBS) before DAPI staining (for nuclei exposure) and

coverslip mounting on a microscope slide. Slides were imaged using EVOS FL Auto microscope (Life Technologies). EVOS FL Auto program was used for signal capturing and ImageJ was used to quantify staining.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 7 software. One-way analysis of variance (ANOVA) was performed to assess differences between more than two groups. Two-way ANOVA was used to measure significance of differences between more than two groups, where two factors are considered. Two-tailed, paired t-test analysis was carried out to ensure significance between two dependent groups. Data presented as mean ± SEM.

Results

Protein Expression Levels in Branched-chain aminotransferase (BCAT2) and Branched-chain α-Keto Acid Dehydrogenase (BCKD)-depleted Myotubes

To verify the efficacy of gene knockdown, myotubes were harvested (two days after transfection) differentiation and western blot analysis was conducted. BCAT2 protein levels was significantly decreased in BCAT2-depleted myotubes compared to BCKD-depleted myotubes but not SCR siRNA treated myotubes (Fig. 1A, $p<0.05$). In BCKD-depleted myotubes, there was an increase in BCAT2 expression compared to control (Fig. 1A, C, $p<0.05$). There was a significant decrease in BCKD protein expression in BCKD-depleted myotubes compared to control and BCAT2-depleted myotubes (Fig. 1B, C, $p<0.0001$).

BCKD Depletion Maintains Myotube Structure and Survival

Based on previous findings from our research team which showed that BCAT2 and BCKD are necessary for the differentiation of myoblasts to myotubes[223], we decided to examine the effect of BCAT2 and BCKD depletion on myotube survival. L6 myoblasts were grown and differentiated into myotubes. Two days after transfection, myotubes treated with either scrambled siRNA (Control, Ctrl) or siRNA designed against BCAT2 or the E1 subunit of BCKD were visualized using light microscopy (Fig. 2, n=3). There is little or no myotubes left in BCAT2-depleted cells. The evidence presented here suggests that there is similar myotube structure in BCKD-depleted myotubes and control myotubes.

Immunofluorescence Microscopy Staining of BCKD-depleted Myotubes

As a result of the maintained myotube structure observed in BCKD-depleted myotubes (Fig. 2), we next examined immunofluorescence microscopy (IF) expression of MHC-I (myosin

heavy chain class I). When compared to SCR siRNA-treated myotubes, there was a significant reduction in BCKD levels in BCKD-depleted myotubes (Fig. 3A, $p<0.01$). There was no significant difference in MHC-I staining intensity in BCKD-depleted myotubes compared to SCR siRNA-treated myotubes (Fig. 3B, C). More experiments are required to better understand how BCKD depletion affects immunofluorescence MHC staining.

BCKD Depletion Increases Myofibrillar Protein Content

Due to positive effect on myotube structure seen in BCKD-depleted myotubes (Fig. 2), it was important to examine the abundance of myofibrillar proteins (proteins important for myotube structure): MHC-I, troponin and tropomyosin. Results show that 48 hours following transfection, there was a significant increase in MHC-I protein in BCKD-depleted myotubes compared to control (SCR siRNA) (Fig. 4A, D, $p<0.05$). There was a significant increase in tropomyosin levels in BCKD-depleted myotube compared to control (Fig 4B, D, $p<0.05$). In addition, there was a slight increase in troponin levels in BCKD-depleted myotubes compared to control treated myotubes though this increase was not significant (Fig 4C, D, $p=0.85$). Overall, there was no significant change in myofibrillar protein content, although tropomyosin was 2-fold higher in BCAT2-depleted myotubes. Together these data suggest that the maintained myotube structure in BCKD-depleted myotubes could be due to increased myofibrillar protein content.

mTORC1 Upregulated in BCKD-depleted Myotubes

Due to increase in myofibrillar protein levels, we examined proteins involved in mTORC1 signaling (protein complex that regulates muscle protein synthesis). There is evidence to show that despite the low levels of BCKD enzyme in the skeletal muscle, BCKD regulates muscle anabolic signaling[223,224]. This made us focus more on the effect of BCKD depletion on myotube anabolic

signaling. Phosphorylation of Akt S473, an upstream activator of mTORC1, was increased in BCKD-depleted myotubes relative to SCR siRNA-treated myotubes (control) (Fig 5A, $p<0.05$). There was no significant increase in the levels of downstream effectors of mTORC1 signaling, phosphorylated S6K1 (Thr389) and S6 (S235/236) in BCKD-depleted myotubes compared to control (Fig 5B, C). Compared with control, myotubes treated with BCKD siRNA showed significant increase in phosphorylated 4E-BP1 Thr37/46, another downstream effector of mTORC1 (Fig 5D, $p<0.05$). These findings suggest that the increase in myofibrillar protein abundance could be as a result of increased mTORC1 signaling through the phosphorylation of 4E-BP1. mTORC1 stimulation after acute starvation was not different in control myotubes and BCKD-depleted myotubes (Supplementary Fig. 1).

Effect of BCKD Depletion on Myofibrillar Protein Abundance in Atrophic Condition

Data in Fig 4 showed enhanced myofibrillar protein levels in BCKD-depleted myotubes in normal differentiation medium. We wanted to examine whether BCKD depletion will still augment myofibrillar protein levels in atrophic/catabolic condition. Twenty-four hours following transfection, myotubes were exposed to 10ng TNF-α/ml of AMEM (an inflammatory cytokine) to induce myotube atrophy for twenty-four hours. TNF-α concentration of 10ng/ml induced muscle atrophy best (Supplementary Fig. 2). In control treatment (non-TNF-α), there was no difference in BCKD levels in the two groups. With TNF-α administration, there was a significant decrease in BCKD levels in BCKD-depleted myotubes compared to SCR siRNA-treated myotubes (Fig 6A, F, $p<0.05$). To assess the efficacy of TNF-α treatment, we analysed the protein level of p-JNK on Thr183/Tyr185, an inflammatory marker that is upregulated by other inflammatory cytokines[228]. Compared to control (non-TNF-α), TNF-α did not change p-JNK levels in SCR siRNA-treated myotubes (Fig. 6B, F, $p=0.329$). In comparison with control group (non-TNF-α), there was no

change in p-JNK levels with TNF-α treatment in BCKD-depleted myotubes (Fig. 6B, F). In comparison to SCR siRNA-treated myotubes with TNF-α treatment, BCKD-depleted myotubes treated with TNF-α showed 51% decrease in p-JNK, but this was not found to be a significant change (Fig. 6B, F, n=4, p=0.331). In control (non-TNF-α) treatment, BCKD-depleted myotubes showed no change in MHC levels compared to SCR siRNA-treated myotubes (Fig. 6C, F, n=4, p=0.2317). In BCKD-depleted myotubes, myofibrillar proteins tropomyosin and troponin levels were reduced with TNF-α treatment by 35% and 13% respectively, but this change was not found to be significant (Fig. 6D–F, n=4, no significance). The significant increase in tropomyosin levels in BCKD-depleted myotubes (Fig. 4B) was abolished with TNF-α treatment (Fig. 6D). These data indicate that there is no change in myofibrillar protein abundance with BCKD depletion under atrophic condition. Compared to SCR siRNA-treated myotubes, mTORC1 signaling in BCKD-depleted myotubes was not different with TNF-α treatment (Supplementary Fig. 3).

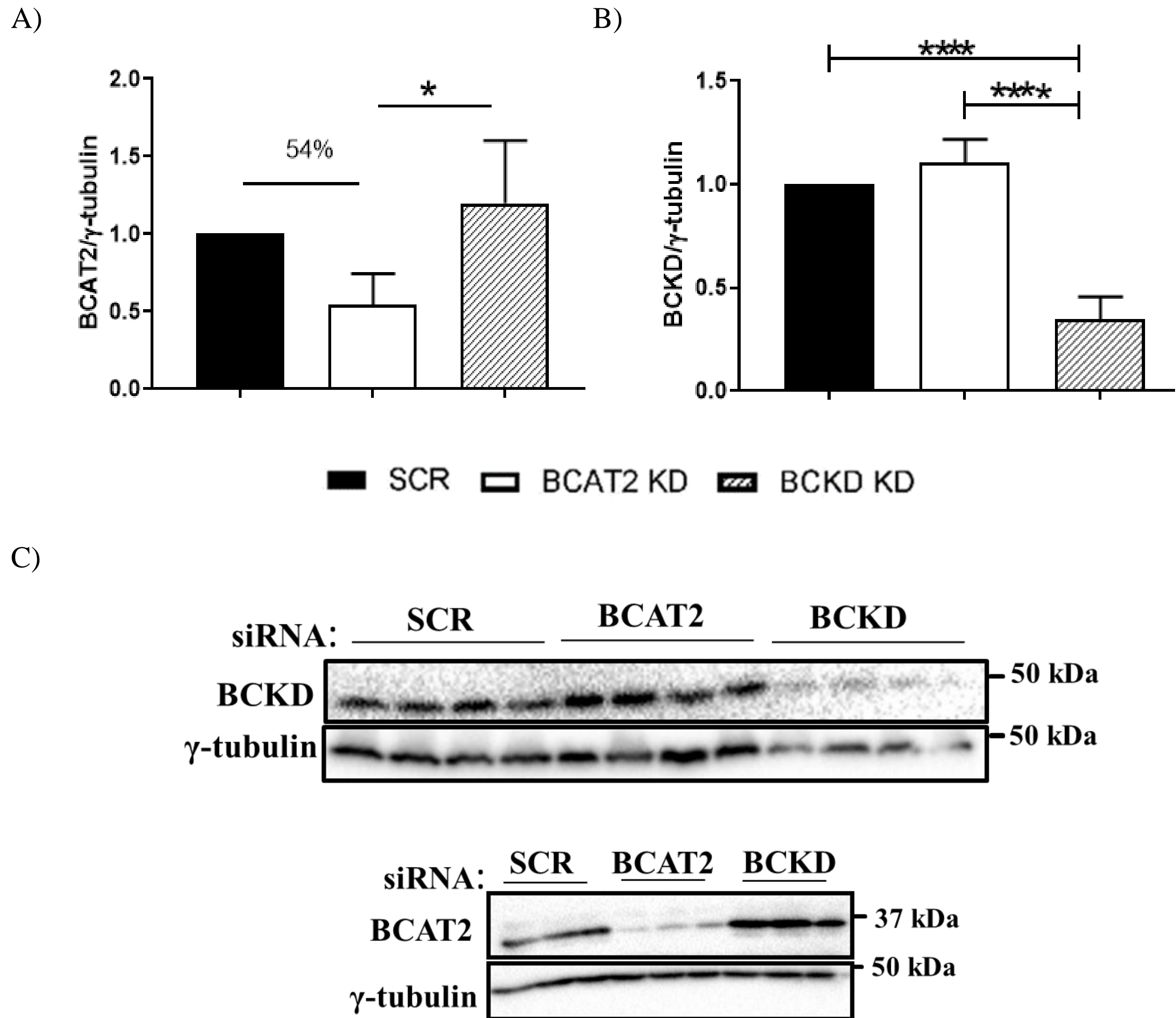


Fig. 1. BCKD and BCAT2 expression in L6 myotubes transfected with BCKD siRNA or BCAT2 siRNA. L6 myoblasts were differentiated until day 4. On Day 4 of differentiation, L6 myotubes in 6-well plates were transfected with siRNA designed against BCKD or BCTA2. Transfected cells were harvested 48 hours later. A) BCKD protein expression B) BCAT2 protein expression C) Representative blots. (*p<0.05, ****p<0.0001, n=4 independent experiments, 3-4 replicates per experiment). Bar graphs show mean ± SEM.

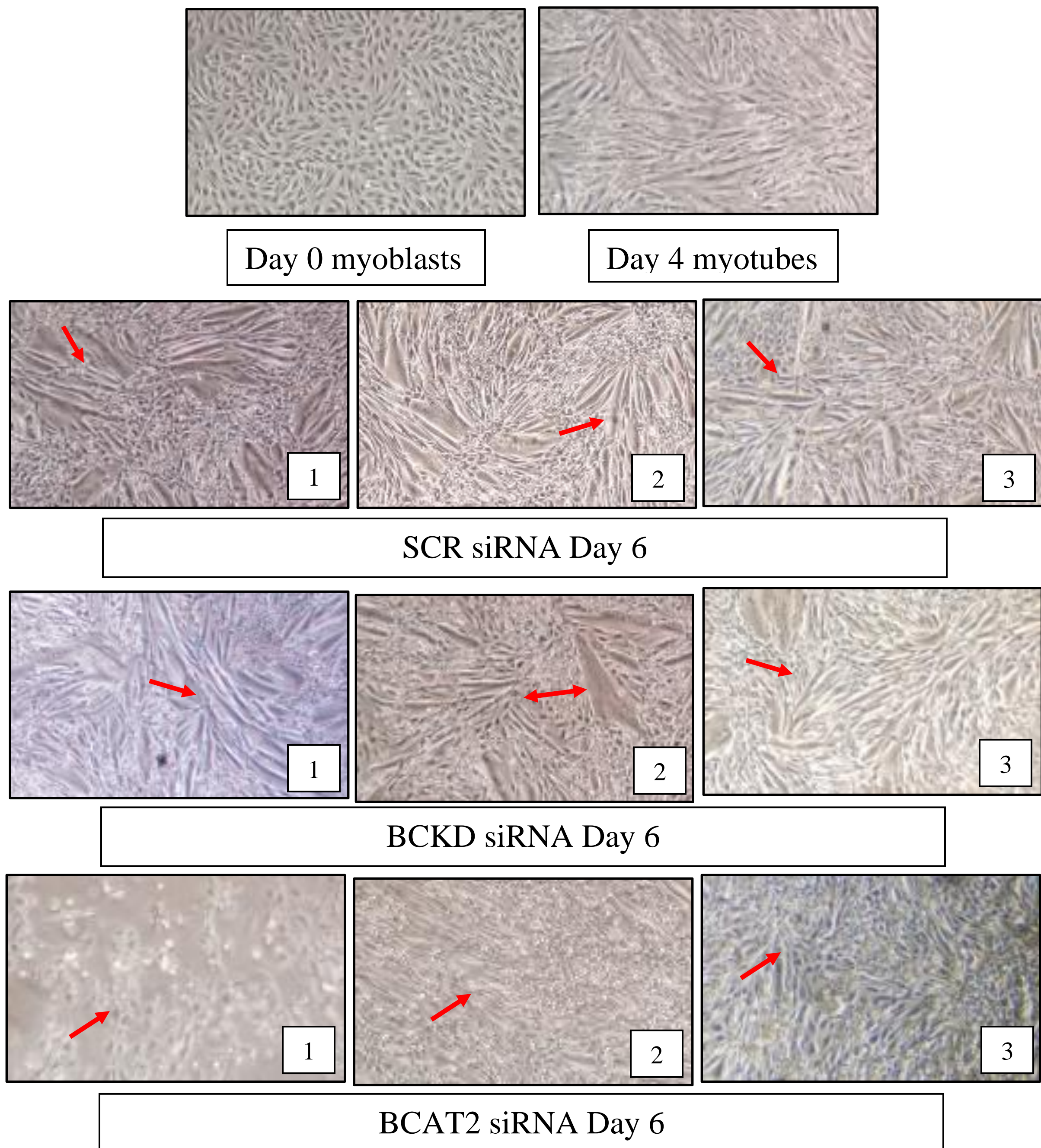
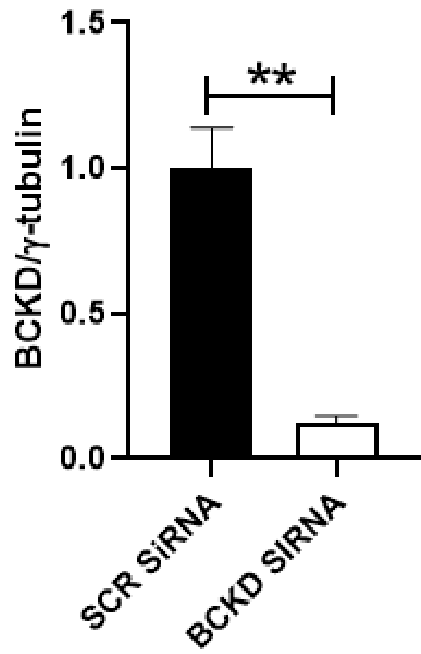
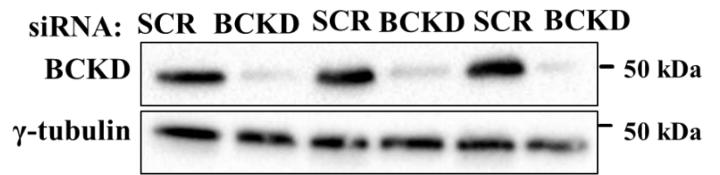


Fig 2. Light microscopy (4X/0.13) of L6 myoblasts and myotubes before transfection (day 4 myotubes) and forty-eight hours after transfection with either scrambled (SCR), BCAT2 or BCKD siRNA. Confluent myoblasts seeded in 6-well plates were differentiated until Day 4. The myotubes were treated with SCR, BCAT2 or BCKD siRNA on Day 4 and harvested forty-eight hours following transfection (Day 6). n=3 independent experiments, images for each treatment from the same independent experiment.

A)





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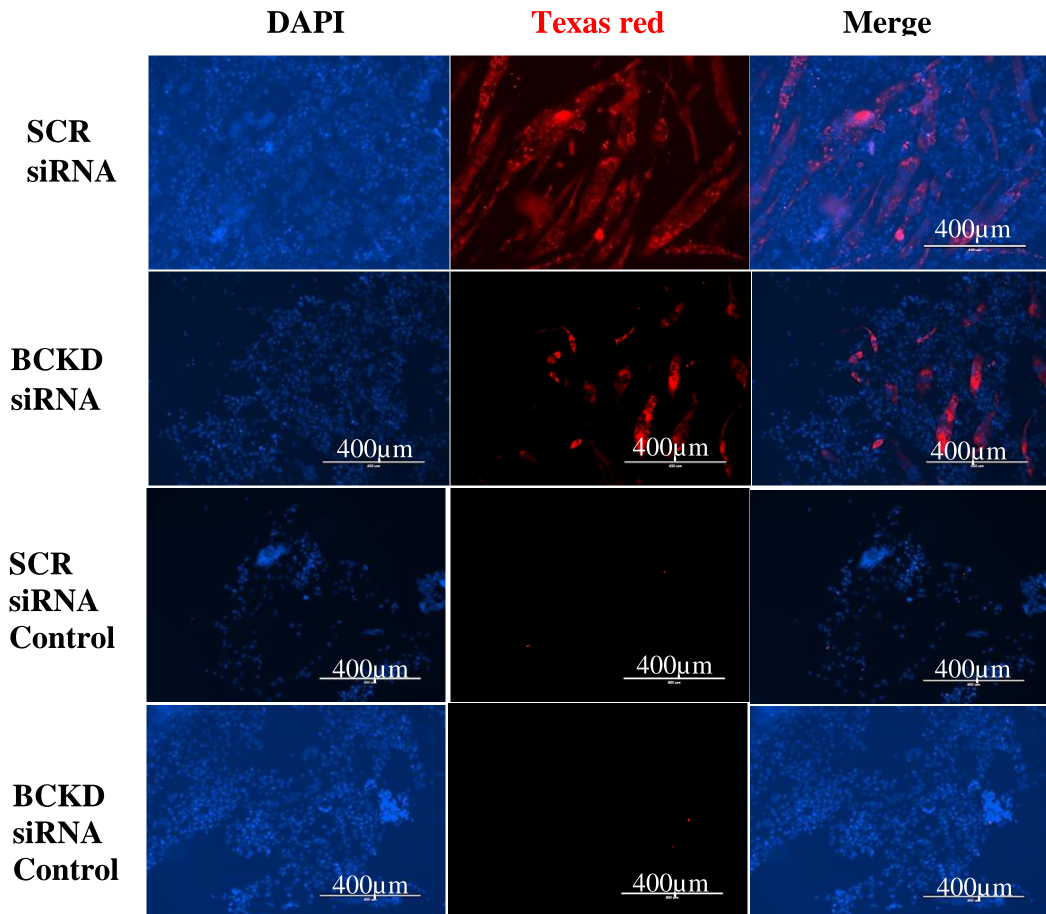

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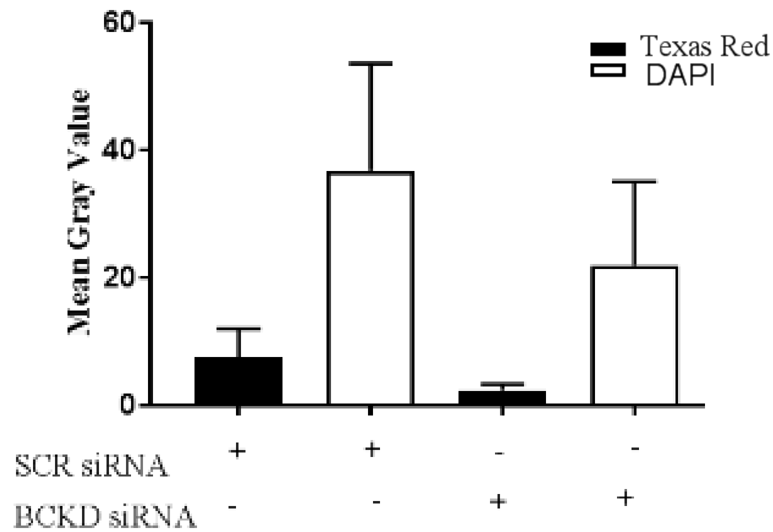
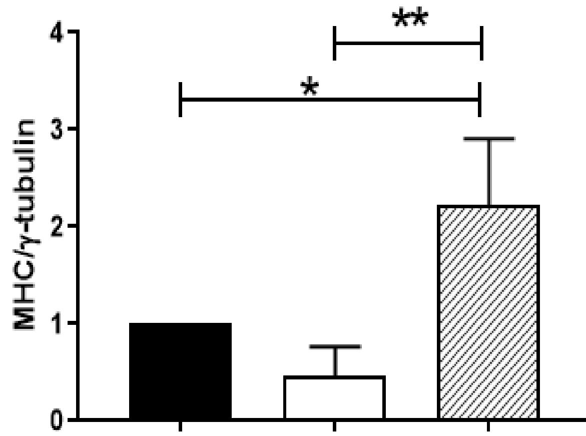


Fig 3. L6 myoblasts were seeded in 12-well plates and differentiated until Day 4. On Day 4, L6 myotubes were transfected with either SCR, or BCKD siRNA for 48hrs. A) BCKD expression. B) Forty-eight hours following transfection, were fixed, permeabilized, blocked and incubated overnight in MHC primary antibody. Next, myotubes were exposed to DAPI for nuclei staining. Texas Red positive immunofluorescence (IF) of MHC is shown in myotubes treated with SCR or BCKD siRNA. Control represents myotubes not exposed to Texas Red Anti-Mouse secondary antibody. C) Quantification of staining intensity. (n=1 independent experiment, 4 replicates per experiment) Bar. 400µm. ** denotes significance, $p<0.01$.

A)



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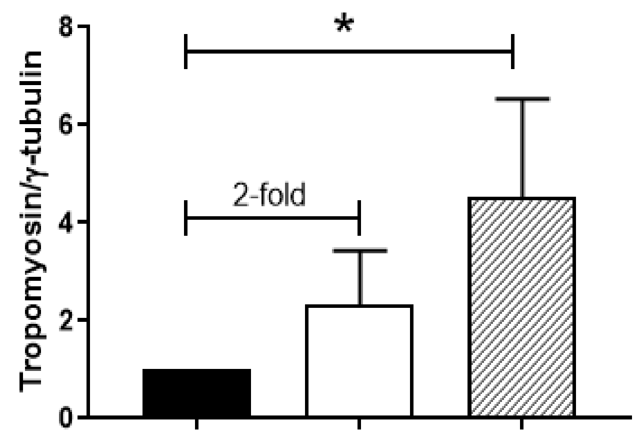
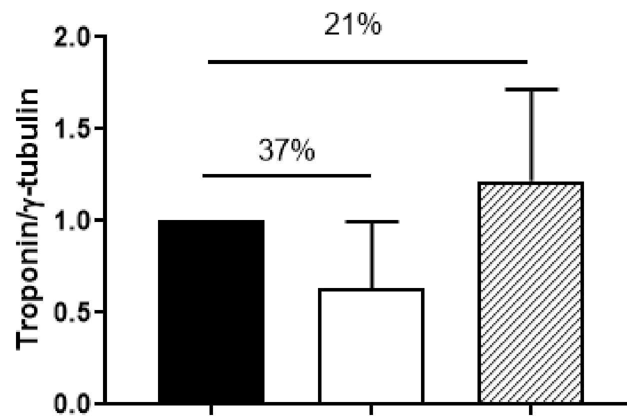


SCR BCAT2 KD BCKD KD

C)



D)

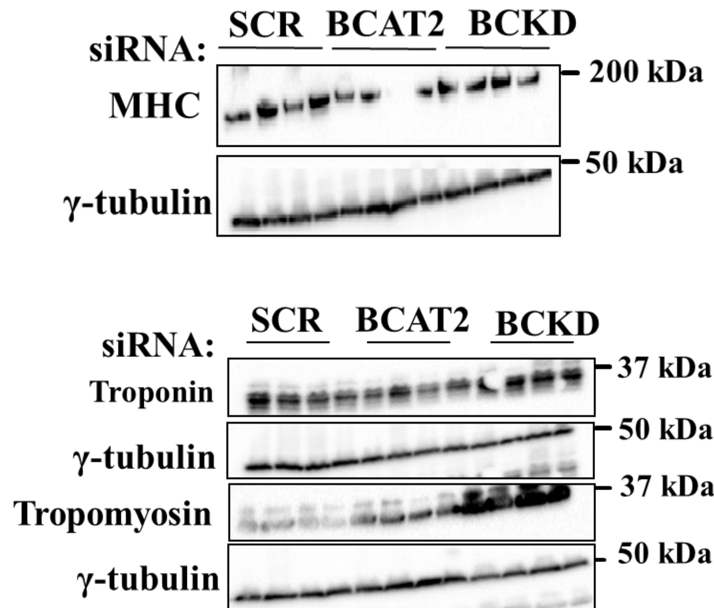
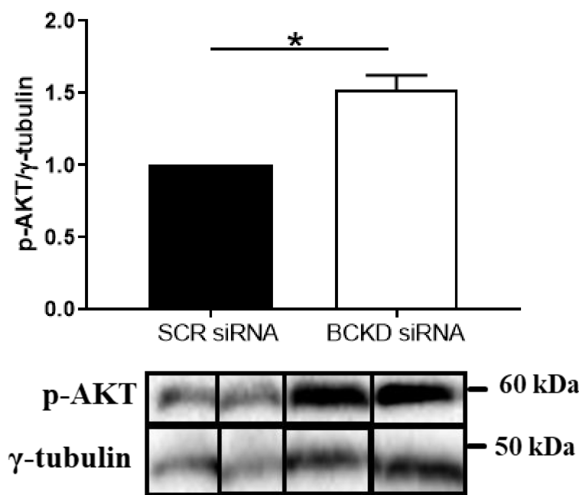


Fig 4. BCKD depleted myotubes have increased myofibrillar protein levels. L6 myotubes were transfected with either SCR, BCAT2 or BCKD siRNA. After cells were harvested forty-eight hours following transfection, western blot analysis was performed. A) MHC protein expression. B) Tropomyosin protein expression. C) Troponin protein levels. D) MHC, Troponin and tropomyosin representative blots. * $p<0.05$ ** $p<0.01$, n=3 three independent experiments. Bar graphs show mean ± SEM.

A)



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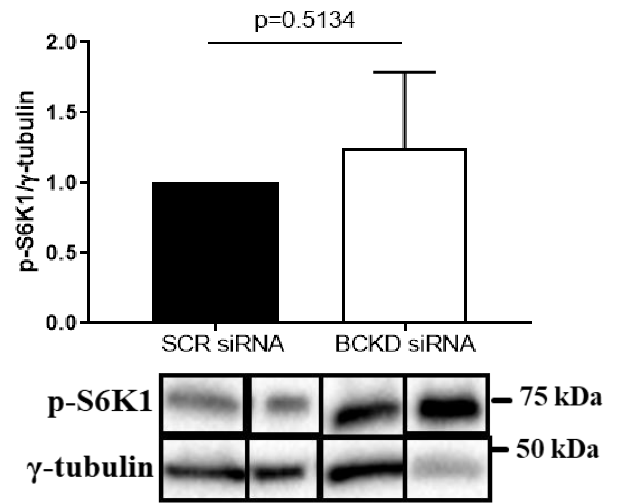

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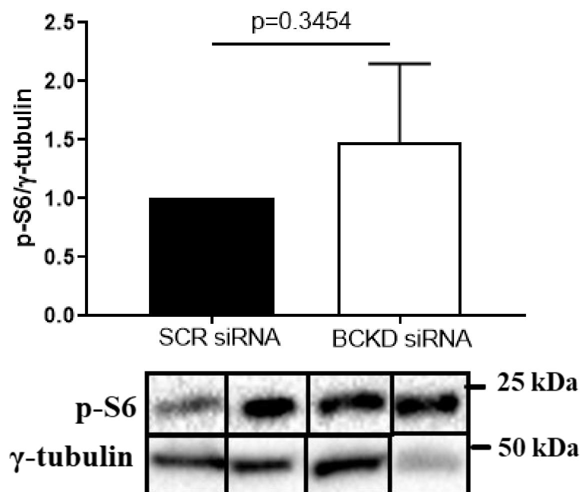


D)

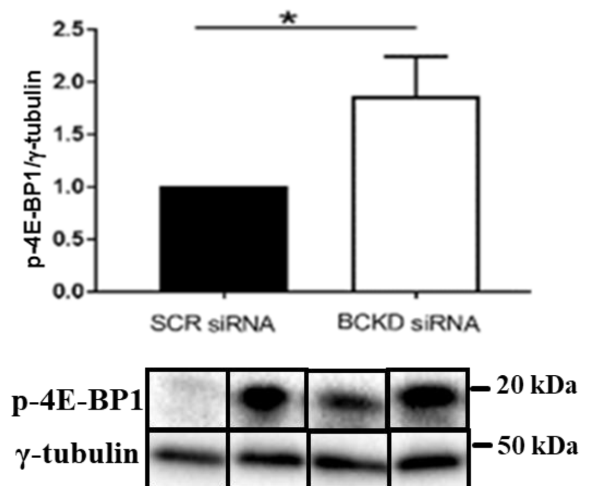
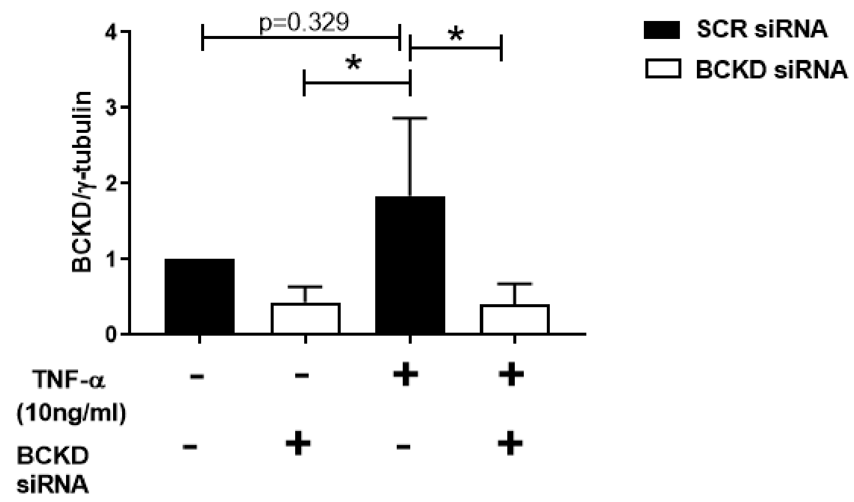
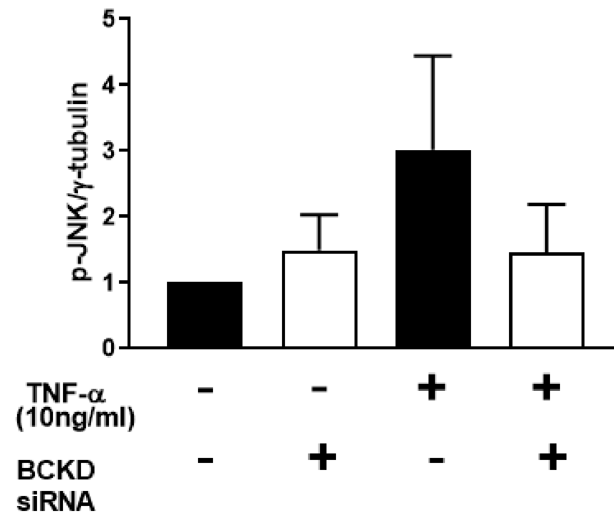


Fig 5. Phosphorylation of mTORC1 upstream activator AKT (Ser473) and downstream substrates, S6K1(Thr 389), S6 (Ser 235/236) and 4E-BP1 (Thr37/46) was enhanced in BCKD-depleted myotubes. L6 myotubes were transfected with either SCR, or BCKD siRNA. Forty-eight hours later, cells were harvested. A) p-AKT expression. B) p-S6K1 expression. C) p-S6 expression. D) p-4E-BP1expression. *$p<0.05$, n=3 independent experiments. Bar graphs show mean ± SEM

A)



B)



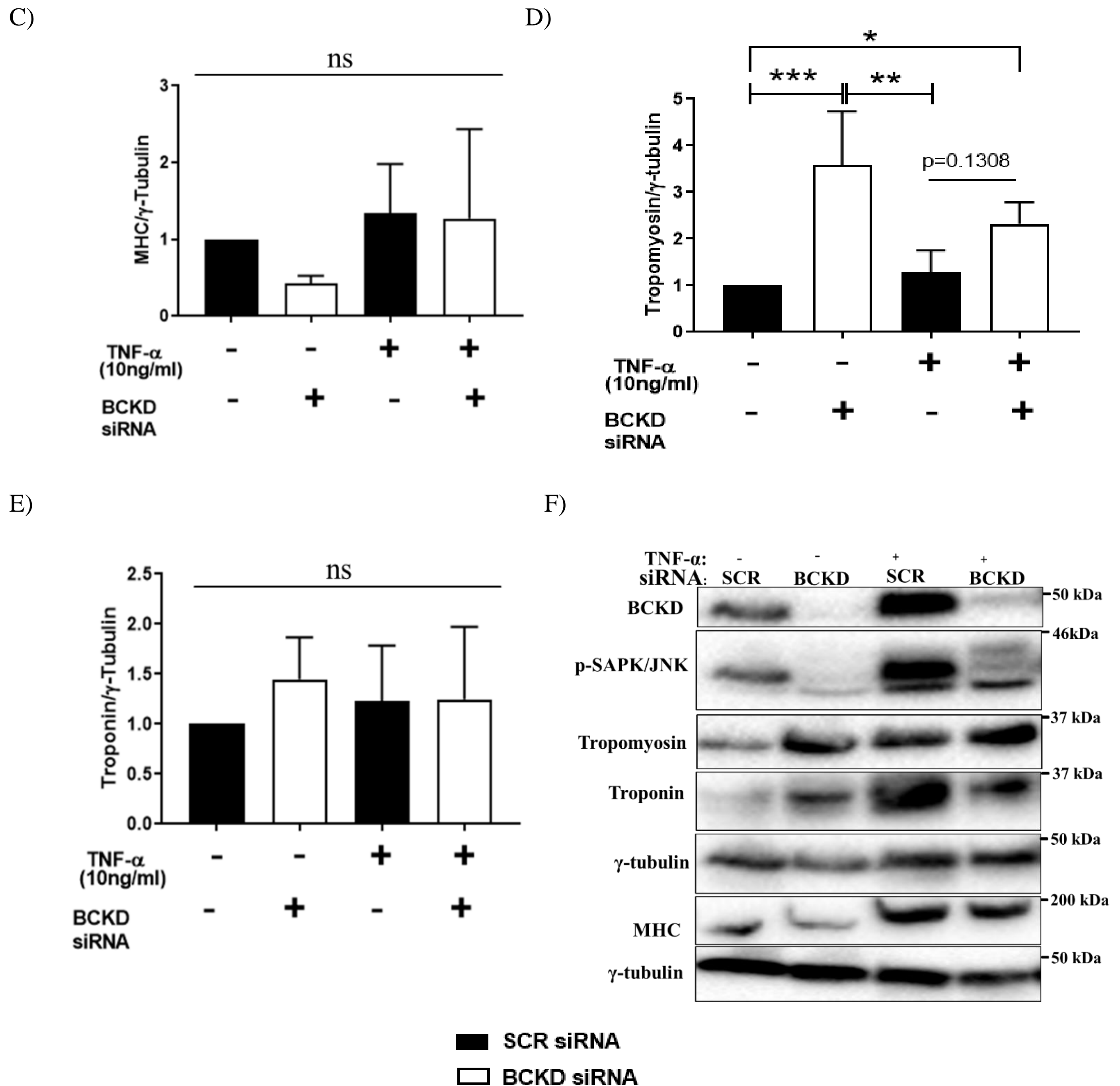


Fig 6: **Myofibrillar protein abundance in BCKD-depleted myotubes under catabolic conditions.** L6 muscle cells were differentiated until Day 4. On Day 4, L6 myotubes were transfected with either SCR, or BCKD siRNA for 48 hrs. Twenty-four hours after transfection, myotubes were treated with TNF-α. A) BCKD protein levels. B) p-JNK protein levels. C) MHC, D) Tropomyosin, E) Troponin protein levels. F) Representative blots. *$p<0.05$, **$p<0.01$, ***$p<0.0001$, n=4 independent experiments. Bar graph shows mean ± SEM.

Discussion

The findings from this study suggest that Branched-chain α-ketoacid dehydrogenase (BCKD) depletion and not Branched-chain aminotransferase (BCAT2) depletion leads to maintained myotube structure, increased myofibrillar protein abundance and anabolic signaling. As opposed to what I hypothesized, BCAT2 depletion destroys existing myotubes and decreases myofibrillar protein abundance.

The maintained myotube structure associated with BCKD depletion could be as a result of enhanced mTORC1 signaling. mTORC1 activation promotes muscle hypertrophy, protein synthesis and anabolic signaling and attenuates protein degradation in skeletal muscle[85]. In concordance with a study by Park et. al, myotube growth requires the kinase activity of mTORC1 which increases the phosphorylation of S6K1 and 4E-BP1[229].

BCKD reaction metabolizes BCKAs to eventually form acyl-CoA that enters the tricarboxylic acid (TCA) cycle[230]. Disruption in BCKD activation and branched-chain amino acid (BCAA) catabolism leads to the intracellular buildup of BCKAs which are nutrients crucial for mTORC1 activation[174]. BCAAs, specifically leucine, is shown to upregulate P13K/Akt/mTORC1 pathway[41]. Beyond BCKD reaction, leucine metabolite KIC is further metabolized into HMB, a reaction catalysed by KIC dioxygenase in the cytosol[144]. This reaction could be enhanced when BCKD enzyme is depleted, leading to more production of KIC and HMB, ultimately increasing mTORC1 signaling. Hence, the improved mTORC1 activation seen in BCKD depleted myotubes could be due to the intracellular build up of BCKAs and HMB.

Fig 1A shows that with BCKD depletion, there is increased level of BCAT2 compared to control. BCAT2 undertakes the first step in BCAA catabolic pathway of reversible transamination,

converting BCAAs to BCKAs and *vice versa*[143,231]. BCKD-depletion promotes the build up of BCKAs that can stimulate BCAT2 upregulation. This observation suggests that there could be a build-up of BCAAs (from BCKAs being converted back to BCAAs) in BCKD-depleted myotubes that leads to the upregulation of mTORC1. Bolus ingestion of BCAAs is shown to increase mTORC1 sensitivity in mice with low BCAA concentration[224]. In L6 cells that are leucine deprived, addition of KIC is able to positively regulate myoblast differentiation likely due to the reversible transamination of KIC back to leucine by the BCAT2 enzyme[223].

BCAT2 and BCKD have been shown to be critical for the differentiation of myoblasts to myotubes[223]. From our findings, it is evident that in addition to myoblast differentiation, BCAT2 is also important to maintain myotube structure, as indicated by the lack of any visible myotube in BCAT2-depleted cells. (Fig 3). Though we did not measure cell viability, we observed visible lower adherent cells in BCAT2-depleted cells. Whole-body deletion of BCAT2 promotes muscle fatigue and decrease myofibrillar protein synthesis[114,232]. BCAT2 transfers the amino group from BCAAs to α-ketoglutarate to produce BCKAs and release glutamine in the cell. Disruption of BCAT2 enzymatic activity is associated with glutamine deficiency which is linked to muscle wasting conditions[214,233]. Thus, the disruption of BCAT2 enzyme is detrimental to myotube survival.

With regards to myotube formation, it has been previously shown that BCKD activity is significantly reduced following the first 48 hours of differentiation (Day 2) and plateaus until Day 6 of differentiation[226]. Thus, while BCKD is needed for the differentiation initiation, its reduced activity by Day 4 to Day 6 of differentiation is necessary for proper myotube formation. This finding could explain the maintained myotube structure and increased myofibrillar protein content observed in BCKD-depleted myotubes, because the knockdown of BCKD further enhances the

decreased levels of BCKD seen during late stages of differentiation. This observation is congruent with the decreased myofibrillar protein level seen with increased BCKD activity *in vivo*[224].

Another reason for the maintained myotube structure and increased myofibrillar protein levels we see in BCKD-depleted myotubes could be as a result of increased glutamine synthesis which as been linked to increased muscle protein[36]. With BCKD knockdown, the first step in the BCAA catabolic pathway which is the reversible transamination of BCAAs and the indirect production of glutamine during BCAA transamination still occurs.

Immunofluorescence microscopy (IF) showed no significant difference in MHC staining intensity in BCKD-depleted myotubes compared to SCR siRNA treated myotubes (Fig 3B, C). Western blot analysis clearly shows the positive effect of BCKD depletion on MHC-I levels (Fig 4A). This discrepancy in IF result could be due to low cell adherence observed from transfecting with siRNA on coverslips. More IF experiments need to be conducted to better understand the discrepancy.

Upon examining the effect of BCKD depletion on myofibrillar protein abundance under atrophic condition, we observe that in SCR siRNA treated myotubes, TNF-α treatment showed a slight increase myofibrillar protein content, though not significant. Inflammatory cytokines such as TNF-α activate MAPKs p38 and c-Jun NH_2-terminal Kinase (JNK) to promote cell differentiation, apoptosis and insulin signaling[234]. Tumor necrosis factor-α inactivates P13k/AKT pathway to induce atrophy in L6 myotubes[93]. In control cells (SCR siRNA-treated myotubes), TNF-α treatment increased p-JNK levels by 2-fold, MHC by 34%, tropomyosin by 27% and 23% troponin increase (although not significant).The slight increase p-JNK level seen is as expected while the slight increase in myofibrillar protein is not consistent with conventional understanding that cytokines such as TNF-α and IL-6 reduce muscle protein synthesis[235].

We found that compared to SCR siRNA treated myotubes, BCKD-depleted myotubes treated with TNF-α showed no significant difference in p-JNK levels because of variability. A study done in cardiac myocyte showed that downregulating BCKD activity by knocking down PPM1K (gene for PP2Cm, a protein that activates BCKD) upregulates p38 MAPK/p-JNK levels[236]. This suggests that in cardiac muscle, BCKD depletion may positively affect p-JNK levels. The non-significant decrease in p-JNK levels could be as a result of increased mTORC1 activation in BCKD-depleted myotubes. mTORC1 activation has been shown to reduce inflammatory markers[81].

Because there is enhanced mTORC1 activation in BCKD-depleted myotubes, it is conceivable that there is an increase in either myofibrillar protein synthesis or decrease in proteolysis. By examining the levels of ubiquitin E3 ligases, Murf-1 and Atrogin-1, proteins involved in ubiquitin-proteasomal degradation pathway, we will determine if protein breakdown is attenuated.

Conclusion

Taken together, this study demonstrates that BCKD depletion enhances myotubes protein metabolism. In L6 myotubes, BCKD knockdown is associated with maintained myotube structure, myofibrillar protein abundance, mTORC1 signaling and a marker of insulin signaling. We show that disruption of BCAA catabolism is critical for the regulation of myotube protein metabolism. Thus, the development of nutritional and hormonal interventions that ensure controlled branched-chain α-keto acid dehydrogenase complex levels in the muscle may promote muscle protein anabolism in individuals suffering from muscle wasting conditions.

Limitations of Study

Our study did not measure protein breakdown which is another signaling pathway that is regulated by mTORC1[86]. Examining protein breakdown involves looking at the mRNA expression of atrogenes (ubiquitin-proteasomal pathway) and the incorporation of radiolabeled methionine. Other than AKT, this study did not look at other upstream regulators of mTORC1 to ascertain that BCAAs activate mTORC1. AKT signaling pathway is mainly activated by growth factors, thus examination of another protein activated by amino acids will be ideal. Sestrins, especially Sesn 1 is an amino acid sensor, specifically BCAAs known to mediate leucine-dependent mTORC1 activation[125]. It remains to be understood if the reason for increases myofibrillar protein levels and mTORC1 signaling is as a result of build-up of BCAA in BCKD-depleted myotubes. We did not measure intracellular BCAA concentration. The focus of future studies will be to unravel and fully understand the mechanisms, whether protein synthesis or breakdown, that promote anabolic signaling in BCKD-depleted myotubes.

Chapter Five: Future Directions

I. **Measure intracellular BCAA/BCKA concentration in BCKD-depleted myotubes.** Theoretically the BCKD knockdown should cause an accumulation of intracellular BCAA and BCKA enough to promote mTORC1signaling. Examining the intracellular levels of BCAAs and BCKAs with BCAA assay kit-colorimetric kit will confirm whether it the high levels of BCAAs and BCKA that promote mTORC1 signaling. BCAAs and NAD+ is required for deamination reaction to produce keto acids and NADH. The production of NADH can be used to make a standard curve for estimation of sample BCAA concentrations[223].

II. **Examine other upstream regulators of mTORC1 activation such as sestrin 1/2**

We observed increased mTORC1 signaling in BCKD-depleted myotubes which was attributed to the build up of BCAAs. To better support the notion that mTORC1 is activated by BCAAs, upstream BCAA sensor and regulator of mTORC1, sestrins 1/2[125] will be analysed using western blot analysis.

III. **Investigate protein synthesis and degradation in BCKD-depleted myotubes using gene expression analysis and pulse chase assay.**

We observed that BCKD depletion increased myofibrillar protein abundance and mTORC1 signaling but the mechanism involved is not known. mTORC1 regulates muscle protein synthesis and protein breakdown[93]. Examining protein synthesis and breakdown will elucidate the mechanism involved.

Gene expression of atrogenes MuRF-1 and MAFbx in BCKD-depleted myotubes as a measure of protein breakdown. Atrogenes are genes involved in muscle atrophy[93]. Pulse Chase assay is a 2-step technique used to measure protein stability and degradation over

time. ^{35}S methionine/cysteine will be incorporated during myotube protein synthesis. The rate of decay (degradation) of labeled protein will be measured when myotubes are exposed to excess cold unlabelled methionine[237].

IV. **Confirm the effect of BCKD depletion on myotube protein metabolism in C2C12 cell line.**

Result showed that BCKD depletion in L6 myotubes increases myofibrillar content and mTORC1 signaling. Also, BCAT2 disruption affects L6 myotube survival. To determine if these results are specific to cell lines, future experiment will confirm the effect of BCAT2 and BCKD depletion on C2C12 mouse myotubes.

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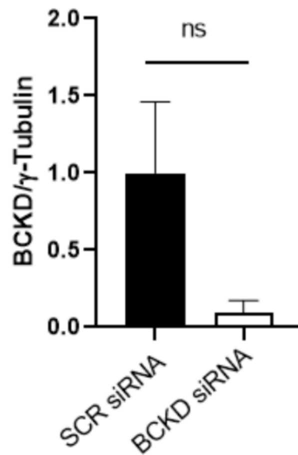
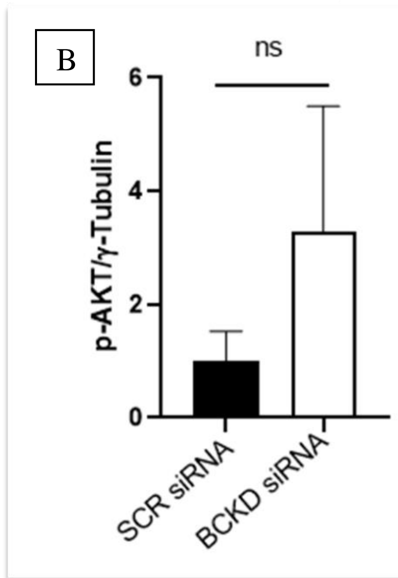
Appendices

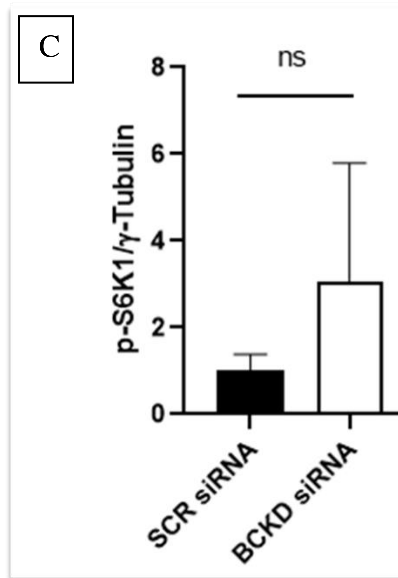
Appendix A: Supplementary Data

1.

A)







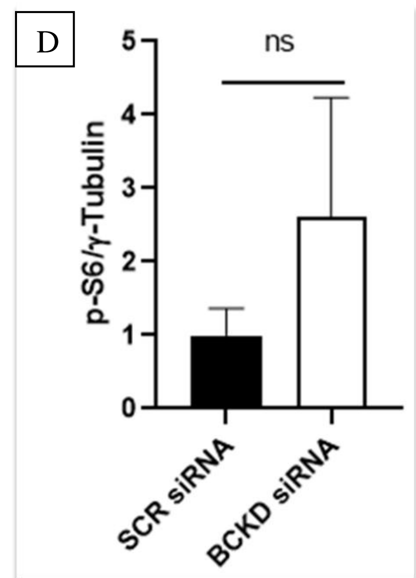


Fig 7. mTORC1 stimulation after acute starvation in BCKD-depleted myotubes. L6 myotubes were transfected with either SCR, or BCKD siRNA. 48h (Day 6) after transfection, myotubes were starved (complete starvation) for 6hrs and stimulated for 30mins (normal differentiation media-serum + AA). A) p-AKT expression. B) p-S6k1 expression. D) p-S6 expression. n=1. Bar graphs show mean ± SEM.

2.

A) B)

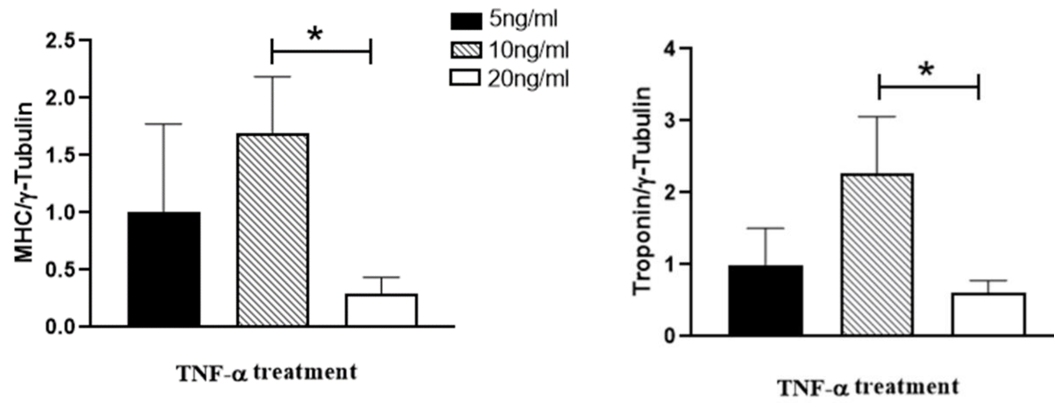
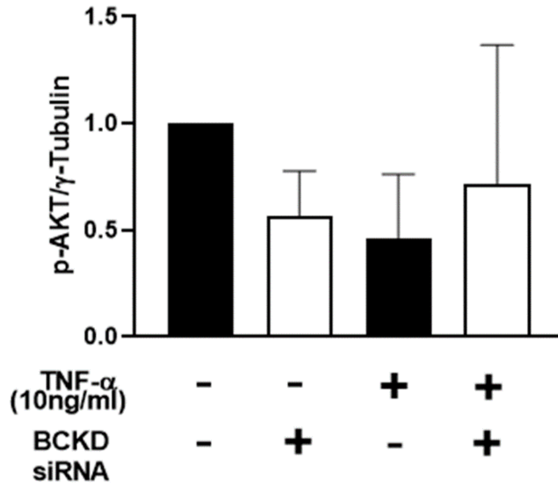


Fig 8. TNF-α concentration test examining myofibrillar protein levels. L6 myotubes were treated with TNF-α on day 4 of differentiation. Cells were harvested forty-eight hours following treatment, western blot analysis was performed. A) MHC protein expression. B) Troponin protein levels. (* $p<0.05$, n=1). Bar graphs show mean ± SEM.

3.

A)



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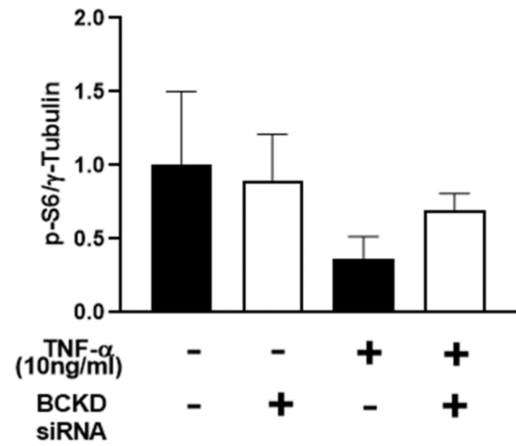

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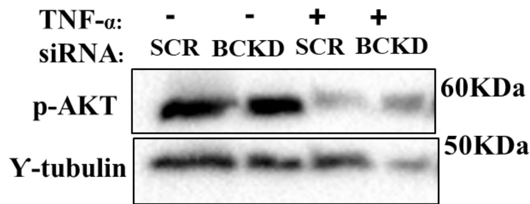

D)

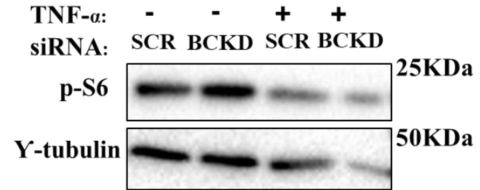

Fig 9: **mTORC1 signaling in BCKD depleted myotubes under catabolic conditions.** L6 muscle cells were differentiated until Day 4. On Day 4, L6 myotubes were transfected with either SCR, or BCKD siRNA for 48hrs. 24hr after transfection, myotubes were treated with TNF-α. A) p-AKT levels. B) p-S6 levels. C) D) Representative blots. n=2, n=1 respectively. Bar graph shows mean ± SEM

Appendix B: Detailed Laboratory Procedure

***RNAi Gene Silencing* Materials**

• Opti-MEM (Life technologies: cat # 31985-070)
• Lipofectamine RNAiMAX reagent (Life technologies: cat #13778-150)
• siRNA scramble and BCAT2 (Sigma Aldrich)
• Growth Medium (GM) without antibiotics (AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 10% FBS (Cat # 12484-028))
• Growth Medium (GM) with antibiotics (AMEM (Wisent Inc. Cat # 310-010-CL) supplemented with 10% FBS (Cat # 12484-028) and 1% Ab-Am (Wisent Inc. Cat # 450-115-EL))
• 15 ml polypropylene conical tubes (BD Falcon Ref #372096)
• 6 well plates (Cat#08-772-1B)
• 12 well plates (Cat#665180)

Procedures:

1. In the cell culture hood, prepare three 15 ml tubes and label "A", "B", "C", and D one 50 ml tube labelled as "Optimem"

A- Lipofectamine+ Opti-MEM
B- BCAT2 siRNA+Opti-MEM
C- Scramble siRNA+Opti-MEM
D- BCKD siRNA+Opti-MEM

2. Pour 18ml Optimum solution into the designated 50 ml-Optimum tube (without touching the mouth of the bottle).
3. Add 120 µl of Opti-MEM/well + 5 µl of Lipofectamine/well to tube "A".
4. Add 2 µl of BCAT2 siRNA/well and 123 µl of Opti-MEM/well to tube "B".
5. Add 2 µl of scramble siRNA/well and 123 µl of Opti-MEM/well to tube "C".
6. Add 3 µl of scramble siRNA/well and 122 µl of Opti-MEM/well to tube "C"
6. Add tube "A" to tube "B" and tube "C" in 1:1 ratio. (For example, 125µl of "A" to tube "B" and 125µl of tube "A" into tube "C" and 125 of tube "A" into "D").
7. Wait at least 5 minutes.
8. While waiting, add 1 mL/well and 0.5ml/well of GM without antibiotics into the 6 well and 12 well plates respectively, and add 125000 or 250000 cells to each 12 well or 6 well respectively.
9. Add 250 µl or 125µl/well of diluted tube "B", "C", and "D" to 6 well plate wells or 12 well plate wells respectively.
10. Following 24 hours, add 1 mL/well of GM (with antibiotics and pro-inflammatory factors) into the 6 well and 0.5mL/well of GM (with antibiotics and pro-inflammatory factors) into the 12 wells.