DISRUPTION OF BRANCHED-CHAIN AMINO ACID CATABOLISM IMPAIRS RAT MYOBLAST SURVIVAL AND DIFFERENTIATION

ZAMEER N. DHANANI

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

GRADUATE PROGRAM IN KINESIOLOGY & HEALTH SCIENCE
YORK UNIVERSITY
TORONTO, ONTARIO, CANADA

APRIL 2016

ABSTRACT

This study investigates the role of branched-chain amino acid catabolism in rat myoblast differentiation. The branched-chain amino acids (BCAAs), particularly leucine, have been consistently shown to possess anabolic and anti-catabolic effects in regards to skeletal muscle hypertrophy and skeletal muscle differentiation. Metabolites of branched-chain amino acid catabolism have also been shown to induce similar effects, suggesting that production of these metabolites may mediate the effect of BCAA presence. However, the role of BCAA catabolism in skeletal muscle differentiation is not known. In skeletal muscle, the first step of BCAA catabolism is mediated by the branched-chain amino transferase-2 enzyme (BCAT2) to produce corresponding branched-chain keto acids (BCKAs). BCKAs can then be further catabolized by the mitochondrial branched-chain α-keto dehydrogenase complex (BCKD) to produce various acyl-CoA derivatives. Our research confirms that the leucine derived BCKA, α-ketoisocaproate (KIC), can positively regulate rat myoblast differentiation and ameliorate conditions of leucine deprivation. Furthermore, we demonstrate that disrupting the enzymes BCAT2 and BCKD that produce KIC and other BCAA metabolites results in impaired myoblast differentiation [MHC and troponin protein reduced by ~70-100%] and proliferation [cell viability reduced by ~15-25%]. Lastly, we show that myoblasts that have impaired BCAA catabolism have elevated levels of apoptosis [cleaved caspase-3 protein increased by ~100%]. Conclusively, our findings demonstrate that BCAA catabolism is an essential process that facilitates myoblast survival and differentiation. This research elucidates mechanisms which regulate skeletal muscle generation and recovery, and thus provides insight into novel targets that may promote muscle development in patients with myopathic diseases.

Acknowledgements

First and foremost, I would like to thank Dr. Adegoke for providing me with this amazing opportunity. Without knowing much about me, he gave me a chance to work in his laboratory and enter the field of academia. His kindness, patience, and knowledge, were some of the many positive factors that facilitated my development into an adequate scientist. I consider myself privileged to have worked under his supervision, and will carry the skillsets he taught me throughout my future career.

To my fellow lab mates past and present, thank you for making those long days in the lab fun and light hearted. My experience would have not been the same without you guys and I consider you all friends for life.

Lastly, thank you to my two wonderful parents. You taught me the importance of education and motivated me to continually strive for higher knowledge. Your love, support, and guidance are what keep me going and I am eternally indebted to you.

TABLE OF CONTENTS

1. **ABSTRACT ..... II**
2. **ACKNOWLEDGEMENTS ..... III**
3. **TABLE OF CONTENTS ..... IV**
4. **LIST OF FIGURES ..... V**
5. **LIST OF ABBREVIATIONS ..... VI**
6. **INTRODUCTION 1**
7. **LITERATURE REVIEW 3**
8. **RESEARCH OBJECTIVES 45**
9. **HYPOTHESES 46**
10. **EXPERIMENTAL DESIGN & MATERIALS 47**
11. **RESULTS 53**
12. **FIGURES 58**
13. **DISCUSSION 73**
14. **CONCLUSION 80**
15. **FUTURE WORK 81**
16. **REFERENCES 83**
17. **DETAILED LABORATORY METHODS AND PROTOCOLS 102**

LIST OF FIGURES

LITERATURE REVIEW

Figure 1: Branched-chain amino acid structure 21

Figure 2: BCAA catabolism supports glutamine/alanine synthesis 30

Figure 3: BCAA catabolism via BCAT and BCKD 31

RESULTS

Figure 4: The leucine metabolite KIC can regulate L6 myoblast differentiation 58

Figure 5: Expression of BCAA catabolic enzymes BCAT2 and BCKD increase during L6 differentiation 61

Figure 6: Intracellular BCAA concentrations do not change during L6 myoblast differentiation 62

Figure 7: BCAT2 disruption prevents L6 myoblast differentiation 63

Figure 8: BCKD disruption prevents L6 myoblast differentiation 65

Figure 9: BCAT2 and BCKDE1α siRNA transfection reduces cell viability 67

Figure 10: Increasing the cell confluency does not rescue differentiation of BCAT2 disrupted myoblasts 68

Figure 11: Supplementing BCKAs to BCAT2 disrupted myoblasts does not rescue myoblast differentiation 69

Figure 12: BCAT2 disruption induces programmed cell death in myoblasts 72

LIST OF ABBREVIATIONS

4E-BP1	eIF4e-binding protein-1
AKT	*also known as* protein kinase B (PKB)
AMPK	AMP-activated protein kinase
AT	alanine transaminase
BCAA	branched-chain amino acids
BCAT2/BCATm	branched-chain amino acid transferase-2 (mitochondrial isoform)
BCAT1/BCATc	branched-chain amino acid transferase-1 (cytosolic form)
BCKA	branched-chain α-keto acid
BCKD/BCKDC/BCKAD	branched-chain α-keto-dehydrogenase complex
BCKDE1α	branched-chain α-keto-dehydrogenase E1 α-polypeptide
BCKDK	branched-chain α-keto-dehydrogenase kinase
bHLH	basic helix-loop-helix
BRG-1	brahma-related gene-1
CDK	cyclin-dependent kinase
DEPTOR	DEP domain-containing mTOR-interacting protein
eIF4E	eurkaryotic translation initiation factor-4E
eIF4F	eurkaryotic translation initiation factor-4F
eIF4G	eurkaryotic translation initiation factor-4G
ERK	extracellular signal-regulated kinase
FGF1	fibroblast growth factor-1
FGF2	fibroblast growth factor-2
FKBP12	12-kDa FK506-binding protein
FoxO	forkhead box-O
GATOR	GAP activity towards Rags
GD	glutamate dehydrogenase
GS	glutamine synthase
GSK-3β	glycogen synthase kinase-3β
HMB	β-hydroxy-β-methylbutyrate
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IGF-1	insulin-like growth factor-1
IGF-2	insulin-like growth factor-2
IRS	insulin receptor substrate
JNK	*jun* N-terminal kinase
KIC	α-ketoisocaproate
KIV	α-ketoisovalerate
KMV	α-keto-β-methylvalerate
Lbx1	ladybird-like homeobox-1
MAPK	mitogen activated protein kinase
MEF2a	myocyte enhancement factor-2a

MHC-1	myosin heavy chain-1
MLST8	mammalian lethal with SEC13 protein 8
MPS	muscle protein synthesis
MRFs	myogenic regulatory factors
MSUD	maple syrup urine disease
mtDNA	mitochondrial DNA
mTORC1	mammalian/mechanistic target of rapamycin complex-1
mTORC2	mammalian/mechanistic target of rapamycin complex-2
MuRF-1	muscle ring finger protein-1
Myf5	myogenic factor-5
Myf4	myogenic factor-4 (myogenin)
NFATC3	nuclear factor of activated T-Cells, cytoplasmic, calcineurin-dependent 3
NRF-1	nuclear respiratory factor-1
PA	phosphatidic acid
Pax3	paired box-3
Pax7	paired box-7
PDCD4	programmed cell death protein-4
PDK	phosphoinositide-dependent kinase
PGC-1α	PPAR γ coactivator of 1α
PHB2	prohibitin-2
PI3K	phosphoinositide 3-kinase
PLD1	phospholipase D1
PPM1K/PP2CM	protein phosphatase 1K
PRAS40	proline-rich AKT substrate of 40 kDa
pRb	retinoblastoma protein
PROTOR	protein observed with Rictor
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
SHH	sonic hedgehog
siRNA	small interfering RNA
shRNA	small hairpin RNA
TGF-β	transforming growth factor-β
TOR	target of rapamycin
UPS	ubiquitin proteasome system
Vps34	vacuolar protein sorting-34

INTRODUCTION

Skeletal muscle differentiation is a process by which precursor muscle cells called myoblasts mature and fuse with one another to form myotubes, which can also further develop to form skeletal muscle fibers. Skeletal muscle differentiation is an important process that enables the formation of new muscle tissue during embryo development or after muscle injury/damage [1]. This process has been found to be regulated by amino acid presence, especially the branched-chain amino acids (BCAAs) [2], which are a group of three amino acids that include leucine, isoleucine, and valine.

Branched-chain amino acids are regarded as important anabolic stimulators of skeletal muscle hypertrophy and growth. In developed muscle, the BCAAs, and in particular leucine, can induce signaling that promotes protein synthesis via the mammalian/mechanistic target of rapamycin complex-1 (mTORC1) [3], [4], and inhibit mechanisms that mediate protein degradation via the ubiquitin-proteasome system [5] . In regards to muscle differentiation, the BCAA leucine has also been shown to be an essential regulator of the myogenic regulatory factors (MRFs) [6], and essential to adequate muscle satellite cell differentiation [7]. However, the mechanisms by which BCAAs exert their anabolic effects in regards to skeletal muscle differentiation have not been completely elucidated.

There is evidence that suggests that BCAAs may exert their regulatory effects via metabolites produced from their catabolism. Previous literature has shown that the leucine metabolites α-ketoisocaproate (KIC) and β-hydroxy-β-methylbutyrate (HMB) can positively regulate skeletal muscle hypertrophy in animals and humans [8]–[13], while HMB has also been shown to positively regulate the differentiation of skeletal muscle myoblasts [14]. It has also been demonstrated that some of the anabolic effects observed with leucine supplementation are due to KIC production, rather than leucine itself [13].

The production of KIC, HMB, and other BCAA metabolites is mediated by the branched-chain aminotransferase enzyme (BCAT) and branched-chain α-keto dehydrogenase complex (BCKD). BCAT reversibly transaminates BCAAs to produce corresponding branched-chain α-keto acids (BCKAs). The BCKD complex mediates the rate-limiting irreversible step that decarboxylates BCKAs to produce corresponding acyl-CoA derivatives. Models of disrupted BCAT and BCKD activity in various animal and cell models result in compromises in skeletal muscle metabolism [15], [16], structure [17], function [18], [19], and endurance capacity [15]. Thus, there is evidence to believe that BCAA catabolism may play a critical role in the regulation of muscle differentiation by BCAAs, although this has not been investigated in previous studies.

Accordingly, this study seeks to identify the importance of BCAA catabolism to the differentiation of skeletal muscle myoblasts. First, we seek to determine if BCAA metabolites other than HMB can also regulate skeletal muscle myoblast differentiation. More importantly, we attempt to determine what the effect of disrupting the BCAA catabolic enzymes, BCAT and BCKD, has on myoblast differentiation. This significance of this research is that it provides insight in to the mechanisms by which nutrition can affect muscle development. Although it is known branched-chain amino acids are important components of an adequate diet that facilitate the growth and maintenance of skeletal muscle, the mechanisms by which this is achieved are not completely known. Additionally, identifying the reasons by which BCAA catabolism facilitates adequate muscle development may lead to the identification of therapies that could treat patients with abnormal muscle metabolism and development resulting from impaired BCAA catabolism.

LITERATURE REVIEW

LITERATURE REVIEW

1. SKELETAL MUSCLE 5
1.1. Skeletal muscle structure and function 5
1.2. Myogenesis 6
1.2.1. Skeletal muscle differentiation 7
1.2.2. Myogenesis via satellite cells 9
2. SIGNALING PATHWAYS THAT REGULATE SKELETAL MUSCLE DIFFERENTIATION 10
2.1. TGFb/SMAD 10
2.2. Notch 11
2.3. MAPK 12
2.4. Akt 13
2.5. mTOR 15
3. AMINO ACID INDUCED REGULATION OF SKELETAL MUSCLE ANABOLISM 18
3.1. Amino acid signaling in skeletal muscle hypertrophy 18
3.2. Amino acid signaling in skeletal muscle differentiation 19
4. BRANCHED-CHAIN AMINO ACIDS 21
4.1. BCAAs and skeletal muscle hypertrophy 22
4.1.1. Mechanisms mediating BCAA effects in existing muscle 22
4.2. BCAAs and skeletal muscle differentiation 25
4.2.1. Mechanisms mediating BCAA effects in skeletal muscle differentiation 27
5. BRANCHED-CHAIN AMINO ACID METABOLISM 28
5.1. BCAA catabolic pathways 29
5.2. BCAT structure and distribution 32
5.2.1. Regulation of BCAT 33
5.3. BCKD structure and distribution 33
5.3.1. Regulation of BCKD 34
5.4. Auxiliary regulation of BCAA metabolism 35
6. BCAA METABOLITES AND SKELETAL MUSCLE ANABOLISM 37
6.1. BCAAs metabolites and skeletal muscle hypertrophy 38
6.2. BCAA metabolites and skeletal muscle differentiation 40
7. DISRUPTION OF BRANCHED-CHAIN AMINO ACID CATABOLISM 40
7.1. Disruption of BCAT 41
7.2. Disruption of BCKD 42
8. **SUMMARY** 44

1.0 Skeletal Muscle

1.1 Skeletal muscle structure and function

Skeletal muscle is the most abundant muscle tissue in the body, making up about 35% of the body's total mass [20]. This organ is not only responsible for providing physical movement and support, but it is also involved in a plethora of metabolic reactions and maintenance of energy homeostasis. Skeletal muscle is a form of muscle tissue that is multi-nucleated, voluntarily activated, and striated. Its striated appearance is due to the presence of repeating functional units called sarcomeres. These sarcomeres contain an alternating pattern of dark and light bands that run along the length of muscle fibers, and it is the presence of the dark band which forms the distinct striations [21].

Within the dark bands are complexes of overlapping 'thick' and 'thin' filaments. The interaction between thick and thin filaments are what give skeletal muscle its contractile properties. The thick filament is made from a protein known as myosin, and resembles 'two hockey-sticks' or 'two golf-clubs' twisted together. The two bulbous regions of the thick filament are referred to as the myosin heads. During a muscle contraction, the myosin heads bind to and pull themselves along the thin filament, which serves to shorten and contract sarcomeres, ultimately contracting the muscle fiber. This is referred to as the cross-bridge cycle. The thin filament is primarily made of actin, which is what the myosin heads form cross-bridges with. However, the thin filament also contains two proteins called troponin and tropomyosin. Under non-contractile conditions, tropomyosin blocks the binding site for myosin on actin and allows relaxation, or lengthening, of the muscle fiber. Conversely, when a muscle contraction is desired, calcium released in the muscle binds to troponin, which causes a conformational change in the

thin filament and moves tropomyosin out of the way of myosin. Thus, myosin can bind to actin and the cross-bridge cycle can occur. (Reviewed in [21]).

It is also important to note that the term ‘muscle fiber’ is synonymous with ‘muscle cell’. A muscle cell is a bundle of individual smaller fibers that run the entire length of the muscle. These smaller fibers are called ‘myofibrils’, and the sarcomere functional units are contained within these smaller fibers. A muscle cell or fiber is surrounded by a plasma membrane, known as the sarcolemma, while the space between myofibrils is known as the sarcoplasm [21]. The reason muscle fibers are multi-nucleated is due to the way are formed, specifically, because they are formed via the fusion of several individual precursor muscle cells. This occurs as part of a process known as myogenesis [21].

1.2 Myogenesis

Myogenesis refers to the formation of new muscle tissue, usually during embryonic development. In a nutshell, myogenesis involves myogenic progenitor cells committing to become myoblasts, which then proliferate and fuse to form muscle. These progenitor cells originate from the dorsal portion of somites, which are divisions of the embryo consisting of mesodermal tissue [22]. Progenitor cells delaminate from somites and migrate to the area where muscle will be developed. For example, limb muscle will originate from progenitor cells delaminated from limb-level somites. A number of genes control the migration of progenitor cells. Progenitor cells in Pax3 (-/-) mutant embryos (a gene that belongs to the paired-box (PAX) family of transcription factors) fail to migrate to the limb [23], and result in embryos that are missing appendicular, tongue, and diaphragm muscles, and greatly weakened body wall muscles [24]. The c-Met tyrosine receptor kinase is also expressed in migrating progenitor cells. Similar to Pax3 mutants, mice lacking c-Met do not form muscles of the limbs and diaphragm [25], and

muscle precursors fail to delaminate [26]. Lbx1 (ladybird-like homeobox-1) disruption has also been found to result in failure of progenitor migration to the limb [27]. Thus, these genes all serve critical functions in progenitor cell migration and the formation of muscle tissue.

Once in the limb, the progenitor cells proliferate and become determined to form myoblasts [28]. This specialization of these precursor muscle cells is marked by an induction of the myogenic regulatory factors (MRFs), which are a family of basic helix-loop-helix (bHLH) transcription factors whose expression in a cell reflects their commitment to a myogenic fate [29]. The expression of the MRFs only occurs after the progenitor cells have migrated. The first MRFs to be expressed in embryonic myoblasts are MyoD and Myf5 [30], [31]. Before becoming programmed to differentiate, myoblasts proliferate, and both MyoD and Myf5 have been shown to be important to this process [32]–[34]. Sonic Hedgehog (SHH) is another protein that has been found to be implicated in myoblast proliferation, and responsible for activating MRF expression after migration [29], [35]. More recently, Brg1 (Brahma-related gene 1), a chromatin remodeling enzyme, was found to be required for mouse primary myoblast survival and proliferation. Brg1 regulates cell proliferation and survival by inducing the transcription of Pax7, which is required for maintaining the viability of progenitor cells [36]. Lastly, the fibroblast growth factors FGF1 and FGF2 can also regulate myoblast proliferation via interacting with cell surface receptors. These growth factors possess mitogenic activity, stimulating myoblast proliferation and preventing differentiation [37], [38] .

1.2.1 Skeletal muscle differentiation

Following the determination and proliferation of myoblasts, depending on environmental cues and internal signaling they can then differentiate and fuse into multi-nucleated myotubes. Myotubes further fuse with one another and mature to form muscle fibers. To enter

differentiation, myoblasts must first withdraw from the cell cycle during the G1 phase [39]. After committing to differentiation, myoblasts are unable to reenter the cell cycle. Cyclin dependent kinases (CDKs) are known to propagate the cell cycle, and p21, a CDK inhibitor has been found to be upregulated during muscle differentiation [40]. The retinoblastoma protein (pRb) is also a target of CDKs, and its inactivation is required for cell cycle progression. Inactivation occurs via phosphorylation by CDKs, and occurs during the G1 phase [41]. In its hypo-phosphorylated form, pRb disrupts G1/S transition by sequestering E2F transcription factors which are required for S phase entry [42]. Thus, it is no surprise that pRb is found to be hypo-phosphorylated in myotubes, which is believed to be in part responsible for permanent withdrawal from the cell cycle [43], [44].

Based on these findings, regulation of the cell cycle is pivotal in determining whether or not myoblasts will differentiate. The myogenic regulatory factor MyoD has been shown to also regulate the cell cycle and thus is also important in the onset of differentiation. MyoD can directly upregulate p21 transcription in murine myoblasts as well as in non-myogenic cell lines [45]. Furthermore, MyoD can directly bind to pRb in its hypo-phosphorylated, active form [43]. This prevents pRb from becoming phosphorylated and thus inactivated, which would promote cell-cycle re-entry. On the other hand, MyoD function can actually be suppressed by the presence of growth factors, ultimately down-regulating myogenic activity [46]. MyoD expression is thus likely involved in cross-talk between cell-cycle signaling pathways, and is a key modulator in determining whether or not myoblasts enter differentiation.

Another myogenic regulatory factor involved in myoblast differentiation is myogenin (Myf4). Myogenin has been found to be upregulated in mono-nucleated myoblasts in the post-mitotic state (p21 positive) [47] , [48]. Upregulation of myogenin then initiates the expression of

muscle-specific structural proteins [48] and cell-to-cell fusion [48]–[50]. Mice that were generated with a homozygous mutation in the myogenin gene survived fetal development, but were postnatally immobile and died soon after birth [51]. These mice also show a severe reduction in skeletal muscle. The essentiality of myogenin in fetal muscle development has also been reported elsewhere [52], and is most important during later stages of myogenesis [53]. Interestingly, in cultured mouse myoblasts it was found that down-regulating myogenin actually caused a reversal of terminal differentiation in differentiated myotubes [54]. siRNA mediated knockdown of myogenin caused cellular cleavage of myotubes back into mono-nucleated cells, and induced cell-cycle re-entry. Furthermore, expressing MyoD simultaneously with myogenin knockdown was not able to prevent this phenomenon, indicating that myogenin itself is crucial in maintaining terminal differentiation [54]. Conclusively, myogenin is a transcription factor that is required for the terminal differentiation and fusion of myoblasts into mature and functional muscle tissue.

1.2.2 Myogenesis via satellite cells

Existing muscle tissue also contains a capacity to regenerate and form new muscle fibers, usually in response to muscle damage. In late fetal development, progenitor cells populate muscle in satellite positions around myofibers and are marked by the expression of Pax7. These ‘satellite cells’ possess an ability to exit quiescence and become myogenic under conditions of stress or trauma. Upon their activation, satellite cells proliferate and differentiate into myoblasts, which can also further proliferate and differentiate to form new muscle fibers. The process has also been found to be regulated by several factors including the MRFs (MyoD, Myf5, myogenin, MRF4), Pax3, and Pax7. (Reviewed in [1])

2.0 Signaling pathways that regulate skeletal muscle differentiation

The myogenic regulatory factors that induce skeletal muscle differentiation are regulated by many upstream signaling pathways. Based on cues such as energy availability, nutritional status, and ligand binding, these signals can be either inhibitor or stimulatory on the transcriptional and post-transcriptional regulation of the MRFs. The onset and progression of skeletal muscle differentiation has been shown to be extensively regulated by peptide growth factors such as insulin-like growth factor (IGF), fibroblast growth factor (FGF), and transforming growth factor β (TGF-β). IGF and FGF signaling has been shown to have a stimulatory effect on myoblast differentiation [55]–[57], while conversely TGF-β has a repressive effect [58], [59]. Myostatin, a member of the TGF-β family of growth factors, has been well characterized as a negative regulator of skeletal muscle anabolism [60], [61], and has been observed to also interfere with myoblast proliferation and differentiation [62], [63]. The presence of myostatin in culture medium causes a down regulation of MyoD, Myf5, myogenin, and p21, while subsequent removal of myostatin allows differentiation to progress and a recovery of these markers [63].

2.1 TGF-β/SMAD signaling in skeletal muscle differentiation

Myostatin is believed to signal through the canonical TGF-β signaling pathway, which begins with myostatin binding to the activin type II receptors [64]. This is followed by the translocation of the type II receptor to the corresponding type I receptor forming a receptor complex [64]. This activated complex then phosphorylates the SMAD2 and SMAD3 proteins [65]. The SMAD family of proteins are intracellular mediators of TGF-β signaling which can modulate gene transcription. After being phosphorylated, SMAD2 and SMAD3 form a hetero-multimeric complex with SMAD4, which is then able to translocate to the nucleus. Upon entry into the nucleus, this complex binds directly to or in complex with components of DNA that

either stimulate or inhibit the transcription of specific target genes. In regards to skeletal muscle differentiation, there is evidence to believe that select SMAD proteins mediate the inhibitory effect of TGF-β signaling on myoblast differentiation. siRNA knockdown of SMAD2/3 in mouse myoblasts caused a 100% increase in myogenin expression, as well as an increase in myosin expression during differentiation [66]. Another study also found that myostatin-induced phosphorylation of SMAD3 causes an inhibitory binding with MyoD protein [63]. Additionally, the expression of a dominant-negative construct of SMAD3 was able to rescue luciferase activity of the MyoD promoter-reporter, which was otherwise inhibited by myostatin [63]. Thus, the SMAD proteins can negatively regulate skeletal muscle differentiation in response to TGF-β signaling.

2.2 Notch signaling

Another pathway which plays an important role in the regulation of embryonic and post-natal skeletal muscle differentiation is the Notch signaling pathway. Notch signaling is believed to be inhibitory on skeletal muscle differentiation. It has been shown that Notch can localize to the nucleus in mouse myoblasts, and bind to the bHLH domain of MyoD, hindering its effects on regulating transcription of myogenic genes [67], [68]. This mechanism can serve to prevent premature myoblast commitment to differentiation. The Notch inhibitor, Numb, is also expressed in myogenic cell lines [69]. It has been found that Numb expression leads to the commitment of progenitor cells to the myoblast cell fate, as well as expression of myogenic regulatory factors such as Myf5 and myogenin [68], [70]. On the contrary, Numb negative cells depict an opposing expression profile with higher levels of the pre-myoblast marker Pax3 [70]. Thus, down-regulation of Notch via Numb is required for myoblast differentiation.

Notch signaling can also act as a mediator in sensing environmental status. Reduced oxygen availability, or hypoxia, has been shown to influence the proliferation and differentiation of progenitor cells in various cell lines. Nervous tissue and adipocyte progenitor cells cultured under hypoxic conditions both show increased proliferation and a preference to maintain an undifferentiated state [71]–[73]. In regards to skeletal muscle differentiation, one study found that culturing mouse myoblasts in 1% O_2 for four days resulted in a four-fold decrease in the number of differentiated cells [74]. This study also concluded the effects of hypoxia on myoblast differentiation occur through Notch signaling. As they observed, adding an inhibitor of Notch cleavage in combination with exposure to hypoxia resulted in a significant attenuation of hypoxia-induced inhibition of differentiation. Notch signaling has also been shown to be implicated in hypoxia-induced effects on proliferation and differentiation in other cell lines [75]–[77].

2.3 MAPK signaling

Extracellular signaling via insulin and insulin-like growth factors have also been found to regulate differentiation through MAPK signaling. The mitogen-activated protein kinases (MAPKs) are a family of proteins that include ERK (extracellular signal-regulated kinase), JNK (*jun* N-terminal kinase), and p38. These protein kinases are activated in response to growth factor presence, with evidence suggesting that the activation of p38 and ERK are more important in differentiating myoblasts. Cultured rat myoblasts that have dysfunctional p38 regulation express an inability to differentiate [78], [79]. Furthermore, abolishing p38 activation also disrupts the phosphorylation and activation of myocyte enhancement factor-2A (MEF2a) [80]. MEF2a is a transcription factor that is able to translocate to the nucleus and positively regulate the expression of myogenic genes [81], as well as form protein-protein interactions with bHLH

transcription factors MyoD and myogenin that increases their functional activity [82]. A binding site for MEF2 within the myogenin promoter region has also been found in cultured mouse myoblasts and embryos, and has been shown to be essential for myogenin transcription [83], [84]. Similarly, transcription of the MyoD gene in *Xenopus* (XMyoDa) is regulated by a MEF2 site that overlaps with the TATA box [85].

MyoD also cannot bind to DNA and regulate transcription without first hetero-dimerizing with E proteins that bind to E-boxes on promoters. E proteins allow binding of transcription factors to DNA elements. Activation of p38 is believed to increase the affinity of MyoD with E47 protein which results in an increase in muscle-specific gene transcription [86].

In addition to p38, ERK1/2 is another MAPK that has regulatory functions in not only in the differentiation of myoblasts, but in their proliferation as well. Growth factors such as IGF-1 and FGF can signal through ERK to maintain myoblast proliferation by preventing cell cycle exit and promoting entry into the S phase [87]–[89]. However, once the cells reach confluency IGF-1 stimulates differentiation, although this is believed to occur through the PI3K/Akt pathway [90]. On the other hand, FGF signaling through ERK continues to promote proliferation and prevent differentiation. It is believed this is achieved by preventing nuclear localization and function of MEF2a [91], as well as inhibiting the transcription of MyoD [92], [93]. Thus, it is no surprise that during skeletal muscle differentiation FGF receptors are lost and ERK activity is diminished [94], [95].

2.4 Akt

Insulin and insulin-like growth factors also can induce Akt signaling within skeletal muscle myoblasts. Akt plays a significant role in growth factor and insulin signaling, and has been found to regulate the phosphorylation of downstream targets that regulate skeletal muscle

differentiation. The Akt pathway is activated by the binding of either insulin to the insulin receptor, or IGF to the IGF receptor. These receptors, being tyrosine kinases, are then able to phosphorylate the insulin-receptor substrates (IRS). Activated IRS then recruits and phosphorylates the lipid kinase phosphatidylinositol 3-kinase (PI3K). Phosphorylated PI3K then produces the membrane-bound $PI(3,4)P_2$ and $PI(3,4,5)P_3$ from PI(4)P and $PI(4,5)P_2$, respectively. The significance of this phosphorylation is that it causes a co-localization of phosphoinositide-dependent kinase-1/2 (PDK1/2) with Akt at the plasma membrane. PDK1 and mTORC2 then phosphorylate Akt at two separate sites, Thr^{308} and Ser^{473}, respectively, which leads to its activation. (reviewed in [90], [96]).

The phosphorylation and activation of Akt allows it to phosphorylate further downstream targets. One such target is glycogen synthase kinase-3β (GSK-3β), which becomes inactivated upon phosphorylation. GSK-3β is believed to have an inhibitory role in skeletal muscle differentiation, in part by antagonizing the activation of MEF2 via p38-MAPK [97]. Suppression of GSK-3β allows the nuclear localization and accumulation of β-catenin and NFATC3, which are genes that have been shown to also upregulate the transcription of myogenic genes [98]–[100]. In myoblasts, the activities of MEF2 and MyoD are also suppressed in part by being bound to the transcriptional repressor prohibitin-2 protein (PHB2). An isoform of Akt, Akt2, is able to mitigate this suppression by binding to and downregulating PHB2 [101]. Akt-mediated inhibitory phosphorylation of the FoxO family of transcription factors also facilitates the onset of differentiation [102]. It has been shown that activation of the forkhead box O (FoxO) transcription factors can attenuate expression of MyoD, and that this preclusion is mediated by an increase in Notch signaling [103]. Lastly, Akt2 can bind to p21, and prevent it from becoming

inhibited via phosphorylation [104]. Recall that p21, a cyclin-dependent kinase inhibitor, promotes cell cycle exit and differentiation.

2.5 mTOR

The mammalian/mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that integrates both intracellular and extracellular signals which regulate skeletal muscle differentiation. In the 1990s, it was discovered that the molecule rapamycin caused toxic and anti-proliferative effects in budding yeast [105]. The proteins TOR1 and TOR2 (target of rapamycin 1 & 2) were found to be mediators of this effect [106], [107]. Further research in mammalian cell lines lead to the discovery and isolation of mTOR, which was homologous to the yeast TORs [108].

mTOR belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family, and forms two distinct complexes, mTORC1 and mTORC2. These complexes differ in their subunits, upstream/downstream signaling, and sensitivity to rapamycin. In addition to mTOR, mTORC1 is comprised of the following proteins: regulatory associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (MLST8), proline-rich AKT substrate of 40 kDa (PRAS40), DEP domain-containing mTOR-interacting protein (DEPTOR), and the Tti1/Tel2 complex of scaffold proteins. On the other hand, mTORC2 is comprised of mTOR, MLST8, DEPTOR, Tti1/Tel2 along with the rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSin1), and protein observed with Rictor 1/2 (Protor1/2) (reviewed in [109]). The RAPTOR protein is what mediates mTORC1 vulnerability to rapamycin, as rapamycin can form a complex with 12-kDa FK506-binding protein (FKBP12) that directly binds to RAPTOR and inhibits mTORC1 activity [108]. Conversely, mTORC2 does not have the RAPTOR protein, but instead a rapamycin-insensitive

RICTOR protein. Thus, mTORC2 is much less sensitive to rapamycin treatment, although prolonged exposure to rapamycin in certain cell types can induce inhibition of mTORC2 [110].

An abundance of research has focused on the role of mTORC1, as it has been found to be an indispensable mediator of numerous processes in the cell. mTORC1 is important in regulating autophagy, lipid synthesis, cell cycle progression, and protein synthesis, in response to nutrient/energy availability and growth factor presence (reviewed in [89], [94]). In regards to skeletal muscle growth and hypertrophy, mTORC1 is believed to integrate the effect of extracellular signals such as growth factors and amino acids, to anabolic mechanisms within the cell. In existing muscle, one of the well characterized effects of mTORC1 activity in response to extracellular anabolic stimuli is increased protein synthesis. This is achieved via the kinase activity of mTORC1, which phosphorylates its downstream targets p70 S6 kinase-1 (S6K1) and eIF4E-binding protein-1 (4E-BP1). The phosphorylation of 4E-BP1 prevents it from inhibitory binding to eIF4E, which enables eIF4E to promote cap-dependent translation. The phosphorylation of S6K1 stimulates mRNA synthesis, cap-dependent translation and elongation, and the synthesis of ribosomal proteins, via the phosphorylation of further downstream targets such as ribosomal protein S6 (rpS6), among others (reviewed in [111]). The role of mTORC2 in skeletal muscle hypertrophy and growth is less clear, although it has been found that mTORC2 is a regulator of actin cytoskeleton reorganization along with cell survival and proliferation [112].

In regards to skeletal muscle differentiation, it has been concretely shown that rapamycin treatment can affect differentiation. Experiments have shown that rapamycin treatment can inhibit differentiation in rat and mouse myoblast lines [113], [114]. Rapamycin has also been shown to abrogate skeletal muscle regeneration in mice [113]. Furthermore, when rapamycin was administered to myoblasts with mutant rapamycin-resistant (RR) mTOR, differentiation was

restored [114]. This supports the idea that rapamycin-sensitive mTOR has important functions in skeletal muscle differentiation. It is not entirely clear how mTOR positively regulates differentiation; however, so far it has been found that both kinase-dependent and kinase-independent mechanisms work to regulate this process. The kinase-independent activity of mTOR is believed to promote IGF-II transcription during differentiation [115]. IGF-II is then secreted by muscle cells and acts as an extracellular signal that activates the IRS/PI3K/AKT pathways, which promotes differentiation [116]. The kinase-dependent function of mTOR has been observed to be critically important in later stages of differentiation involving myoblast fusion and maturation [117]. Further research has shown that this importance is in part due to the fact that mTOR can positively regulate the expression of follistatin [118], a myocyte-secreted factor that antagonizes the effects of myostatin and other TGFβ related cytokines that suppress differentiation [119], [120].

The fact that myoblast differentiation is rapamycin sensitive, suggests that the mTOR-containing complex mTORC1 is likely a key positive regulator of muscle differentiation. However, studies that examine mTORC1 function in muscle differentiation do not paint a clear picture that supports this notion. Knocking out RAPTOR, a component of mTORC1, results in enhanced differentiation of C2C12 mouse myoblasts, while overexpression of RAPTOR results in the inhibition of their differentiation [121]. It was shown that this phenomenon is a result of RAPTOR affecting mTORC1 phosphorylation of IRS-1 on its Serine-307 residue. IRS-1 activity is a key step in insulin and growth factor induced IRS/PI3K/AKT signaling, and phosphorylation on this residue results in IRS-1 destabilization and disruption of this pathway [122]. It was observed that knocking out RAPTOR and disrupting mTORC1 function prevented this inhibitory phosphorylation of IRS-1, facilitating myoblast differentiation through adequate AKT signaling.

Conversely, overexpressing RAPTOR, thus increasing mTORC1 function, promotes destabilizing phosphorylation of IRS-1 and disrupts AKT-mediated myoblast differentiation. In another study, the well characterized target of mTORC1, S6K1, which has been concretely shown to stimulate skeletal muscle hypertrophy, was also found to be dispensable for the adequate differentiation of mouse myoblasts [123]. Thus, it is apparent that mechanisms involving mTORC1 are not consistent across models of skeletal muscle growth and hypertrophy compared to those involving muscle differentiation, as mTORC1 has been found to have a positive role in the former but a dispensable/negative role in the latter. Lastly, the fact that skeletal muscle differentiation was rapamycin sensitive, but not mTORC1 dependent, suggests that other rapamycin-sensitive mTOR complexes (such as mTORC2) regulate muscle differentiation. Accordingly, mTORC2 has been found to positively regulate the terminal differentiation of mouse myoblasts [124].

3.0 Amino acid induced regulation of skeletal muscle anabolism

The mechanisms by which signaling pathways in muscle development are regulated are in large part due to a response to upstream stimuli. These stimuli are mostly nutrient/environmental cues, such as amino acid availability. It has been well established that amino acids serve as the building blocks of peptide-chains which form muscle proteins. However, in addition to this canonical role, it has also been observed that amino acids can act as signaling molecules that drive anabolic signaling mechanisms in skeletal muscle.

3.1 Amino acid signaling in skeletal muscle hypertrophy

In existing muscle, amino acids have most notably been shown to stimulate the activity of mTORC1, which promotes the transcription and translation of myogenic proteins, while also

suppressing proteolytic mechanisms such as autophagy (reviewed in [125]). The upstream signals which connect mTORC1 responsiveness to amino acid presence have also been well studied in existing muscle. These include the small GTPase Rag heterodimers, and the class III PI3K protein Vps34. Upon being activated by the guanine nucleotide exchange factor activity of the Ragulator protein complex, the Rag heterodimers bind to RAPTOR and recruit mTORC1 to the lysosomal surface in response to amino acid presence [126]–[128]. The translocation of mTORC1 to the lysosomal membrane is required for its activation [129]. Vps34 mediates amino acid induced activation of mTORC1 by facilitating the translocation of phospholipase D1 (PLD1) also to the lysosomal region, where its product phosphatidic acid (PA) can activate mTORC1 by displacing the inhibitory subunit DEPTOR [130], [131]. The activation of mTORC1 can then drive skeletal muscle growth and hypertrophy in response to amino acids.

3.2 Amino acid signaling in skeletal muscle differentiation

Although pathways connecting amino acid presence to anabolic mechanisms have been elucidated in existing muscle, such mechanisms have not been as well studied during myogenesis. There is little that is known about how amino acid presence signals to promote anabolic mechanisms during myoblast differentiation. As previously mentioned, the Rag and Vps34 pathways have been shown to act as the amino acid-sensing molecules in existing muscle, which promote growth via mTORC1. However, because mTORC1 is believed to have differential roles when comparing skeletal muscle hypertrophy to skeletal muscle differentiation, the aforementioned model of amino acid sensing might not apply to differentiation. Yoon & Chen (2013) [2] were the first to elucidate the role of mTOR and mTORC1 and its upstream signaling in response to amino acids, in the context of differentiating mouse myoblasts. Interestingly, although they found the levels of the Rags to increase during differentiation, they

concluded the Rags to be negative regulators of myoblast differentiation, as introducing shRNA for RagA and RagB resulted in significantly better myoblast fusion and myogenic protein expression. They found that this was because the Rags were necessary for amino acid induced activation of mTORC1. In congruence with previous literature, they found that activation of mTORC1 during differentiation and in response to amino acids caused destabilizing phosphorylation of IRS-1, ultimately disrupting AKT signaling. This result demonstrated that amino-acid-induced activation of the Rags can promote negative signaling in muscle differentiation. Yoon & Chen (2013) [2] then turned to the Vps34 signaling pathway in an attempt to identify its role in regulating differentiation in response to amino acids. In two separate experiments where Vps34 was disrupted either by using a PI3K inhibitor or by shRNA, it was found that disrupting Vps34 significantly attenuated myoblast differentiation, suggesting Vps34 activity has a positive role on differentiation. In response to amino acids, they found the activity of PLD (target of Vps34) to increase, which ultimately promoted IGF-II transcription. Introducing Torin1 (an inhibitor of mTORC1/mTORC2) did not affect IGF-11 expression; whereas, using rapamycin abrogated IGF-11 expression, suggesting that a rapamycin-sensitive mTOR complex other than mTORC1/2 mediates the anabolic signaling effects of amino acids. The results of this study demonstrated that 1) amino acid presence induces both positive and negative signaling in regards to muscle differentiation, although the positive signaling likely outweighs negative signaling as amino acid presence overall stimulates differentiation 2) the Rag proteins have opposite roles in amino acid sensing when looking at models of skeletal muscle hypertrophy and muscle differentiation 3) Vps34 has a similar positive role in amino acid sensing when comparing muscle hypertrophy and differentiation. This study also provided further evidence to the argument that mTORC1 can act as a negative regulator of myoblast

differentiation, whereas mTOR or another mTOR containing complex is a key positive regulator. However, it was odd that disrupting mTORC2 with Torin1 did not affect IGF-11, as mTORC2 has been shown to positively regulate differentiation [124]. Nonetheless, the mechanisms by which amino acids induce and regulate skeletal muscle differentiation are still far from being clearly understood, and there likely exists other unknown mechanisms that coordinate this phenomenon.

4.0 Branched-chain amino acids

As discussed in previous sections, amino acids can induce signaling pathways that promote skeletal muscle hypertrophy and muscle differentiation. However, the role and potency of individual amino acids in this model is another question that remains to be completely understood. Evidence to date suggests that the presence of certain amino acids can promote muscle anabolism to a greater extent than others. The most notable amino acids which exhibit such effects are the branched-chain amino acids: leucine, isoleucine, and valine. The branched-chain amino acids (BCAAs) are essential amino acids characterized by having a branched aliphatic side chain from the central carbon atom **(Figure 1)**. In humans, the BCAAs constitute ~35% of the essential amino acids in muscle proteins and 14-18% of the total amino acids in muscle proteins [132].

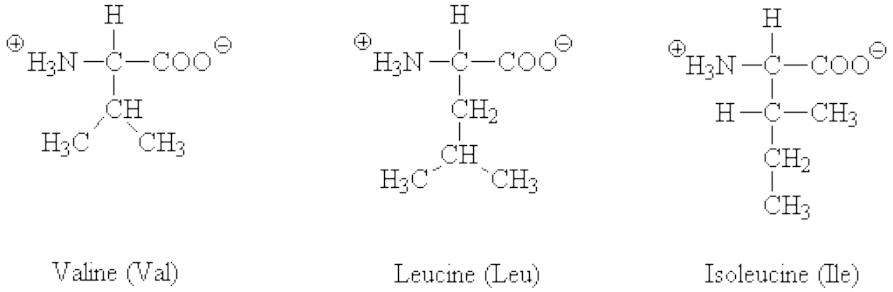


Figure 1: Chemical structure of the three branched-chain amino acids (BCAAs): valine, leucine, and leucine. *Image source: University of Illinois* [218].

4.1 BCAAs and skeletal muscle hypertrophy

Numerous studies have focused on the effects of the branched-chain amino acids on existing skeletal muscle. BCAA supplementation has been shown to promote skeletal muscle protein synthesis [133]–[136], reduce proteolysis [133], [137]–[139], enhance muscle recovery [137], [140], [141], and increase muscle endurance [142], [143]. Because of their ability to promote muscle growth and maintenance and prevent muscle loss, even under conditions of nutrient deprivation, BCAAs are also considered to be anti-sarcopenic, anti-cachectic, and anti-anorectic agents [144]–[146]. Of the three BCAAs, leucine is believed to be a more potent amino acid in mediating the stimulation of protein synthesis in skeletal muscle. In vitro studies show that incubating diaphragm or gastrocnemius muscle with leucine alone is nearly as effective in stimulating protein synthesis as all three BCAAs [133], [147]. Another study in vivo showed that oral administration of isoleucine and valine did not significantly stimulate protein synthesis in fasted rats, whereas leucine did [148]. However, another study demonstrated that intravenous leucine administration does not stimulate protein synthesis in gastrocnemius or heart muscle of fasted rats [149]. Thus, although the method of administration can affect leucine stimulation of protein synthesis in vivo, leucine has been shown to promote protein synthesis without the presence of the other two BCAAs. Nonetheless, the presence of isoleucine and valine are still important in mediating some of the other beneficial effects observed with BCAA administration [150].

4.1.1 Mechanisms mediating BCAA effects in existing muscle

The signaling mechanisms that sense BCAA presence and coordinate responses in muscle have been well studied. In existing skeletal muscle, it is believed the BCAAs can regulate skeletal muscle anabolism in part through mTORC1. In regards to protein synthesis, leucine

supplementation has been shown to increase the phosphorylation of p70-S6K1, 4E-BP1, and S6 in muscle [151]–[153]. As previously described, these proteins are targets of the mTORC1 kinase and result in increased levels of protein synthesis. Thus, leucine can affect mTORC1 signaling to increase levels of protein synthesis [4], [154], [155]. The presence of insulin is also believed to regulate the stimulatory action of leucine on mTORC1. Administration of the insulin inhibitor somatostatin to rats supplemented with leucine resulted in an attenuation of leucine-induced increases in 4E-BP1 and S6K1 phosphorylation, depicting an insulin dependent mechanism via mTORC1. However, leucine-induced assembly of eIF4E and eIF4G was not affected by somatostatin, thus indicating an insulin-independent mechanism for this process.

The mechanism by which leucine causes mTORC1 activation is also becoming increasingly more clear. It has been found that leucine can directly bind to Sestrin-2, a GATOR2 interacting protein that inhibits mTORC1 signaling. The binding of leucine to Sestrin-2 prevents inhibitory binding of Sestrin-2 to GATOR2. This allows the GATOR2 complex to inhibit the action of the GTPase activating protein GATOR1 [156], which would normally inhibit the RagA/B proteins from becoming GTP loaded. Via the action of the v-ATPase and Ragulator guanine exchange factor, RagA/B become GTP loaded under amino acid presence and recruit mTORC1 to the lysosomal surface where it can be activated [3], [129].

BCAA supplementation has also been shown to attenuate atrophy in skeletal muscle. When rats were subjected to a hind-limb suspension protocol to model disuse atrophy, supplementing the rats with an oral BCAA mixture (600mg/(kg day)) significantly reduced the loss in muscle weight and cross-sectional area of the muscle fibers [157]. Furthermore, the rats supplemented with BCAAs had attenuated levels of atrogin-1 and MuRF1 proteins, which are classified as E3 ubiquitin ligases that are important in regulating protein degradation via the

ubiquitin-proteasome system. Another study showed that supplementing individuals with BCAAs reduced levels of atrogin-1 in resting vastus lateralis (VL) muscle, and MuRF1 in both rested and exercised VL muscle [158]. Thus, these results indicate that BCAA supplementation can mitigate losses in muscle protein by down-regulating components of the ubiquitin-proteasome system. The mechanism by which BCAAs regulate the expression of atrogin-1 and MuRF1 is not completely clear. It has been shown that the attenuation of atrogin-1 via leucine occurs in an mTOR dependent manner in C2C12 mouse myoblasts [5]. On the other hand, the mechanism that causes the attenuation of MuRF1 in response to BCAA is not known. Nonetheless, the fact that BCAAs can downregulate atrogin-1 and MuRF1 partially explain why BCAAs can prevent muscle damage and promote faster muscle recovery [159], [160].

The beneficial effect of BCAA supplementation on muscle endurance has also been established. Supplementing individuals with a 0.4% BCAA drink in combination with 4% carbohydrates was shown to increase maximal VO_2 capacity and lactate threshold levels following endurance exercise [161]. Other studies have also shown that BCAA supplementation can increase time to fatigue, lower perceived exertion, lower the respiratory exchange ratio (RER), and increase plasma glucose during exercise [142], [162]–[164]. These effects resulting from BCAA supplementation may be in part explained by the fact that BCAAs can increase mitochondrial biogenesis in skeletal muscle. D'Antona et al. 2010 [165] showed that administering a BCAA mixture to adult cardiac myocytes in-vitro resulted in a dose-dependent increase in mitochondrial biogenesis, as indicated by increases in mtDNA and transcription of mitochondrial biogenic genes PGC-1α, NRF-1, and the β subunit of the mitochondrial H+-ATP synthase. In-vivo experiments by the same group yielded similar results as they observed increases in mitochondrial biogenesis and expression of mitochondrial regulatory genes in heart,

diaphragm, soleus, and tibialis muscles following a 3-month supplementation protocol. Additionally, they found that the increases in mitochondrial content were nearly twice as great in mice that were treated with BCAA supplementation and simultaneous endurance exercise. Increased oxidative damage due to reactive oxygen species (ROS) accumulation is a key factor in muscle damage and aging. Compared to untreated mice, they also found that BCAA supplemented mice showed reduced mitochondrial ROS production, as well as an increased capacity to eliminate superoxides in the mitochondria. Conclusively, increased endurance capacity seen with BCAA supplementation likely results from an increase in mitochondrial content in muscle tissues. The increase in ROS elimination may also contribute to enhanced mitochondrial function and the attenuation of muscle damage.

4.2 BCAAs and skeletal muscle differentiation

Although the beneficial and anabolic effects of BCAAs have been well established in existing muscles tissue, the effects of BCAAs on muscle myogenesis are less known. More specifically, the question of whether BCAAs play a significant role in regulating the skeletal muscle differentiation program is not clear. Averous et al. 2012 [6] attempted to elucidate the effect that leucine withdrawal has on mouse myoblasts that were subjected to a differentiation protocol. They observed that removing leucine from the differentiation media completely prevented the myoblasts from differentiating and caused cell cycle arrest. Additionally, when they examined the regulation of the myogenic regulatory factors (MRFs), they observed that the regulation of Myf5 and MyoD was modified as a result of leucine deprivation. Under control conditions, they found Myf5 protein to increase in expression up to 4h of differentiation, then decrease in expression at 8h and all subsequent time points to day 5. In the absence of leucine, they found that Myf5 expression was similarly increased at 4h, however at 8h and onward they

found its levels to be significantly increased compared to control. Regarding MyoD, in control conditions its protein level remained constant to day 3 of differentiation then decreased upon reaching terminal differentiation. In leucine-deprived cells they observed MyoD protein to be significantly lower at 8h and on to day 5 of differentiation. mRNA analysis showed that Myf5 followed the same pattern as its protein expression in treatment and control conditions; however, MyoD mRNA expression was not modified by either control or leucine-deprived conditions. Their subsequent experiments in primary mouse satellite cells yielded similar observations in both MyoD and Myf5 protein expression under conditions of leucine presence and leucine deprivation. These results indicate that leucine presence is critical in regulating the normal expression pattern of the myogenic regulatory factors during muscle differentiation.

Dai et al. 2015 [7] examined the role of leucine in differentiating primary rat satellite cells. Their work demonstrated that leucine promoted satellite cell differentiation in a dose-dependent manner. Cells cultured in medium containing 0.5 mM leucine resulted in myotubes that were thick, long, and showed rhythmic contraction. However, cells differentiated in medium that only contained 0.1 mM leucine resulted in myotubes that were shorter, thinner, and did not display rhythmic contractions. Thus, these findings also support the notion that the branched-chain amino acid leucine can positively regulate skeletal muscle differentiation.

Unfortunately these two studies are the only ones that have looked at the effects of BCAA alone on skeletal muscle differentiation. The effects of other BCAAs, valine and isoleucine, have not been examined in this manner to date. Clearly, further research is required to examine the full contribution of branched-chain amino acids to skeletal muscle differentiation.

4.2.1 Mechanisms mediating BCAA effects in skeletal muscle differentiation

The pathways mediating the effect of leucine on the skeletal muscle differentiation program were also examined in the aforementioned studies. Averous et al. 2012 [6] demonstrated that although leucine presence was able to regulate mTORC1 activity in mouse myoblasts, neither mTORC1 nor any other rapamycin-sensitive mTOR complex was required for the increase in Myf5 and decrease in MyoD expression they observed as a result of leucine withdrawal. The finding that mTORC1 was not involved in nutrient-induced regulation of differentiation falls in line with previous research that mTORC1 is insignificant in the regulation of myoblast differentiation. However, their finding that another rapamycin-sensitive mTOR complex was also not involved in the regulation of MyoD and Myf5 contradicts previous literature. Previous work by Sun et al. 2010 [118] also in mouse myoblasts showed that MyoD transcriptional levels and protein expression was reduced in the presence of rapamycin, indicating a role for a rapamycin-sensitive mTOR complex in the regulation of MyoD expression. Thus, the role of mTOR in regulating skeletal muscle differentiation is ambiguous, although the literature currently presented suggests mTOR may not be involved in mediating the effect of leucine presence on mouse myoblast differentiation.

The study by Dai et al. 2015 [7] also examined the role of rapamycin-sensitive mTOR in mediating the effect of leucine on rat muscle satellite cell differentiation. Interestingly, their results showed that rapamycin treatment inhibited MyoD expression in response to leucine treatment indicating a role of rapamycin-sensitive mTOR in regulating MyoD. Conversely, rapamycin-sensitive mTOR was not involved in regulating the increase in myogenin protein expression in response to leucine treatment. Thus, they concluded that rapamycin-sensitive

mTOR was critical in mediating at least some part of muscle differentiation in response to leucine.

Clearly, there appears to be some controversy about the role of rapamycin-sensitive mTOR in mediating the effect of leucine on the skeletal muscle differentiation program. Averous et al. 2012 [6] themselves mentioned they could not find an explanation for their contradictory results compared to the similar study by Dai et al. 2015 [7]. There is also a paucity of research that examines the mechanism by which mTOR activation causes transcriptional regulation of Myf5, MyoD, and myogenin during myoblast differentiation, and if any other pathways mediate this interaction. Further investigation is warranted to draw a clearer picture of the mechanisms that regulate muscle differentiation in response to leucine.

5.0 Branched-chain amino acid metabolism

It has been clearly shown that branched-chain amino acids can positively regulate aspects of existing skeletal muscle metabolism and skeletal muscle differentiation. What is not clear is how these effects are achieved, including the intermediates that facilitate this process. In various tissues, including skeletal muscle, all three BCAAs undergo similar multi-step catabolic pathways that yield various metabolites. There is evidence to believe that these metabolites of BCAAs are also responsible for mediating at least some aspects of the anabolic effects observed in response to BCAAs. In this section, evidence will be presented that implicates products of BCAA catabolism in regulating skeletal muscle anabolism, as well as information regarding the characteristics, function, and regulation of the catabolic pathways and their intermediates.

5.1 BCAA catabolic pathways

The first step of leucine, isoleucine, and valine catabolism involves the reversible transamination of these amino acids to their α-keto acid derivatives via the *branched-chain amino transferase* (BCAT) enzyme. Transamination of leucine produces α-ketoisocaproate (KIC), of isoleucine produces α-keto-methylvalerate (KMV), and of valine produces α-ketoisovalerate (KIV). At the same time that the BCAT enzyme removes the amino group from a BCAA, it donates the amino group removed to α-ketoglutarate to produce glutamate. Glutamate can then be converted to glutamine via *glutamine synthetase* (GS). Thus, not only is BCAT important in catabolizing the BCAAs, but it is also important in supporting glutamine synthesis [166]. The reverse reaction of glutamate to α-ketoglutarate by *alanine transaminase* (AT) simultaneously catalyzes the conversion of pyruvate to alanine. The liver can uptake alanine from the blood, and use the alanine to produce pyruvate to be used in gluconeogenesis. Glutamate can also be converted back to α-ketoglutarate in the liver, which removes the amino group and produces urea to be excreted. Thus, BCAA catabolism allows nitrogen cycling that allows various substrates to be produced and used in other reactions in skeletal muscle and the liver. This paradigm is illustrated in **Figure 2**.

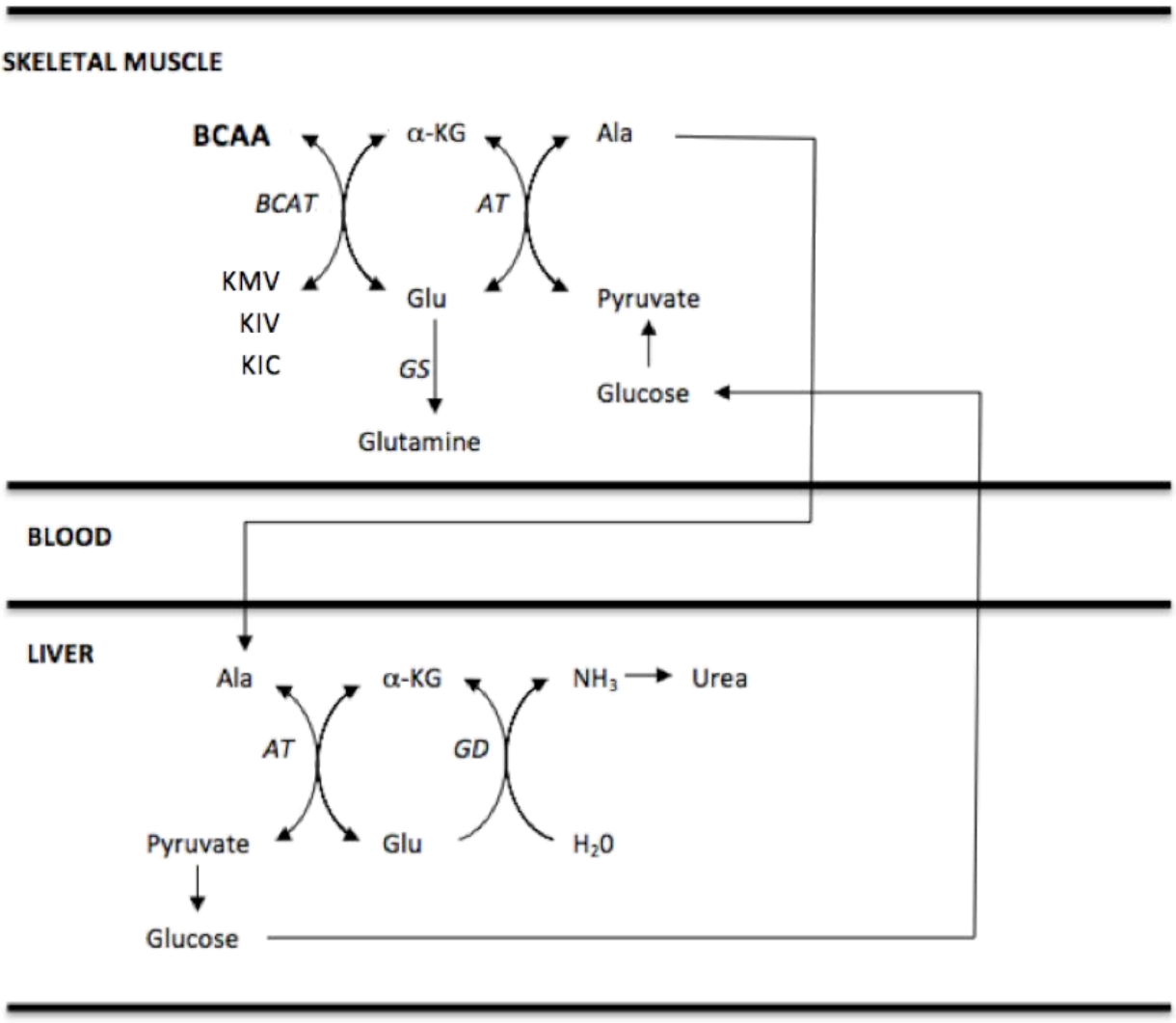


Figure 2: The three BCAAs share a similar initial step in their catabolism. The branched-chain amino transferase (BCAT) enzyme reversibly transaminates BCAAs to yield corresponding α-keto acids. This reaction simultaneously donates an amino group to α-ketoglutarate to yield glutamate (Glu). Glutamate can then be used to produce glutamine in skeletal muscle via glutamate synthetase (GS). Glutamate can also be converted back to α-ketoglutarate using pyruvate, to produce alanine. Alanine can then travel in the blood to the liver where alanine transaminase (AT) can deaminate alanine back to pyruvate to be used in gluconeogenesis. In the liver, glutamate dehydrogenase (GD) disposes of the amino group transferred from alanine to glutamate by producing urea.

The branched-chain α-keto acids (KMV, KIV, and KIC) produced by BCAT in the first transamination reaction can themselves be further catabolized in a separate pathway. The three branched-chain α-keto acids (BCKAs) are irreversibly decarboxylated by the *branched-chain α-keto acid dehydrogenase complex* (BCKDC or BCKD or BCKAD) to produce acyl-CoA derivatives with one less carbon. The subsequent pathways resemble those for fatty acid oxidation, and produce metabolites that can be used in the tricarboxylic acid cycle. KMV (from isoleucine) catabolism ultimately produces propionyl-CoA and acetyl-CoA, which are respectively glucogenic and ketogenic products. KIC (from leucine) catabolism yields acetyl-CoA and acetoacetate, which are ketogenic. KIV (from valine) finally produces succinyl-CoA and is therefore glucogenic. In the liver, KIC can also be catabolized by the KIC-dioxygenase enzyme through a separate pathway to produce the metabolite β-hydroxy-β-methylbutyrate (HMB) [167]. (**Figure 3)**.

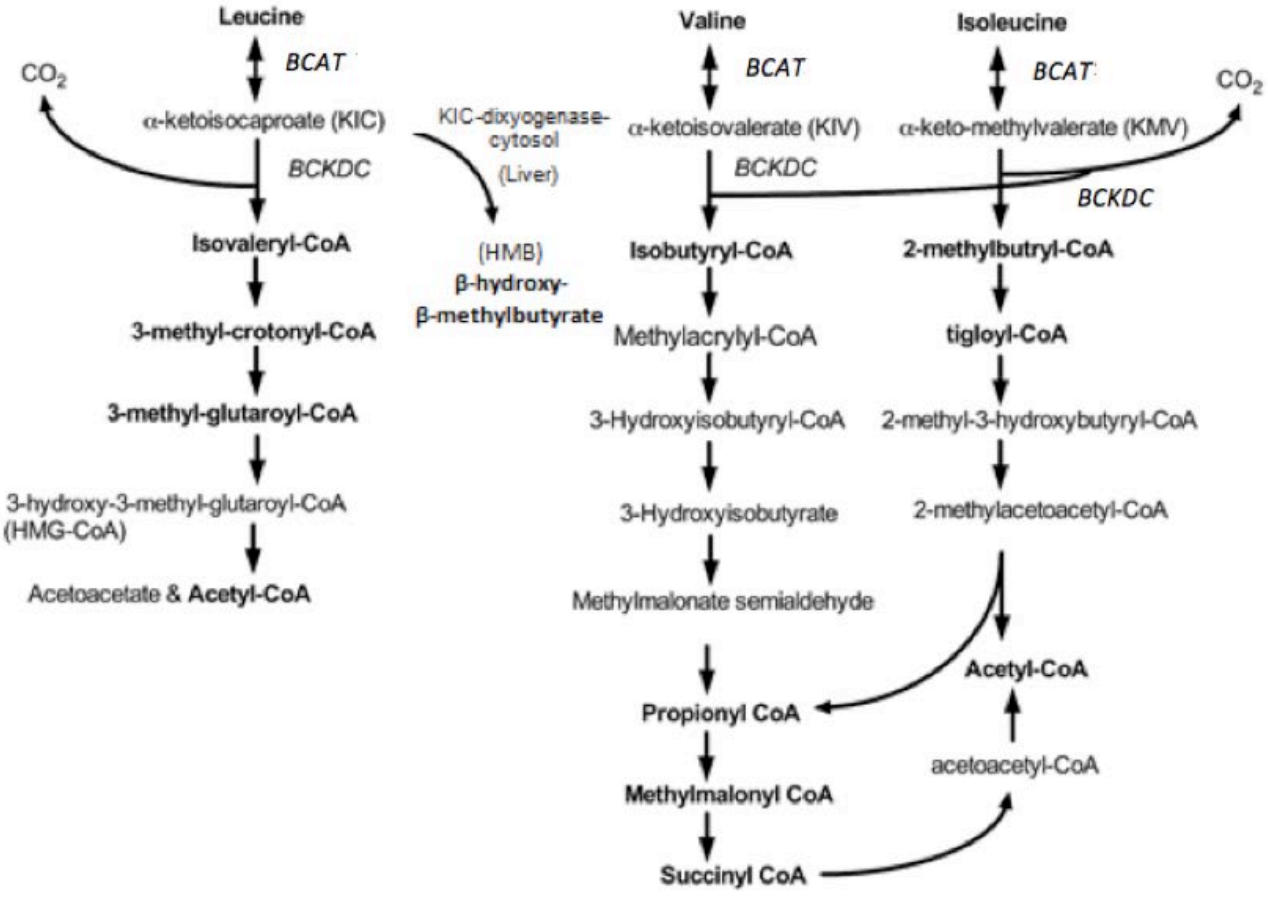


Figure 3. The first step in branched-chain amino acid catabolism is transamination by the BCAT enzyme to produce a corresponding α-keto acid. The BCKD complex then irreversibly decarboxylates keto acids to produce corresponding CoA derivatives. In the liver, KIC can also be metabolized by the KIC-dixoygenase cytosolic enzyme to produce β-hydroxy-β-methylbutyrate (HMB). *Modified from She P, Olson KC, Kadota Y, Inukai A, Shimomura Y, Hoppel CL, Adams SH, Kawamata Y, Matsumoto H, Sakai R, Lang CH, Lynch CJ. Leucine and protein metabolism in obese Zucker rats. PLoS One. 2013;8(3)* [219]

5.2 BCAT structure and distribution

The branched-chain aminotransferase enzyme occupies both the cytosol and mitochondria. The mitochondrial isozyme, BCAT2 or BCATm, exists in most rat tissues but is not found in the liver or brain. The cytosolic isozyme, BCAT1 or BCATc, exists only in the brain, liver, and has been detected at lower levels in rat testes, ovary, and placenta [168]. The Michaelis constants (K_m) for the mitochondrial and cytosolic enzymes differ little in rat brain, kidney, skeletal muscle, and mammary glands [169]. In various tissues, both enzymes also show the highest affinity towards leucine and isoleucine with a K_m ranging from 0.4 to 0.8mM, followed by 1.2 to 4mM for valine [169], [170]. In rats, total BCAT activity per gram of tissue is believed to be highest in the heart and kidney, intermediary in skeletal muscle, and lowest in the liver [171]. In humans, BCAT activity is highest in the kidney, intermediary in the brain and stomach, and lowest in the heart, muscle, liver, and intestines [172]. However, since skeletal muscle represents a much larger portion of tissue relative to the body, skeletal muscle is responsible for the bulk of BCAA transamination. The BCAT enzymes contain a redox-sensitive CXXC center that plays an important role in enabling its catalytic activity. The C's represent cysteine residues while the X's represent other amino acids. The significance of these residues is that they allow the BCAT enzymes to catalyze both forward and reverse transamination between BCAAs and BCKAs [173].

5.2.1 Regulation of BCAT

There are currently no unique mechanisms that have been identified to regulate BCAT2 or BCAT1 activity. Thus, its enzymatic activity primarily depends on the concentration of the enzyme and its substrates [174]. BCAT enzymes operate close to equilibrium in most tissues, with the concentrations of product and substrate being at or below the K_m value [175]. Because the K_m of BCAT for α-ketoglutarate is lower than the K_m for the BCAAs, there is a higher potential for α-ketoglutarate to become the limiting substrate for transamination [174]. In this situation, re-amination of BCKAs via the reverse reaction to reproduce α-ketoglutarate would be expected [174]. Both BCAT2 and BCAT1 also use Vitamin B-6 cofactors to catalyze transamination [176]. Since the rate of reaction for BCAT depends on reactant and product concentrations, BCKAs must be eliminated from the tissue for the forward transamination reaction to proceed. This can occur via transport of the BCKAs out of the cell, or by catabolism of the BCKAs via the second catabolic step. The second catabolic step in BCAA metabolism is mediated by the BCKD enzyme and involves irreversible decarboxylation of BCKAs as previously described.

5.3 BCKD structure and distribution

Once BCAAs are transaminated by the BCAT enzyme, the BCKD enzyme complex can further catabolize the α-keto acid produced. The structure and function of BCKD is said to resemble that of the pyruvate dehydrogenase complex, and is comprised of 3 enzyme subunits [174]. The *branched-chain α-keto acid decarboxylase* is identified as the E1 ($\alpha_2\beta_2$) subunit, the *dihydrolipoyl* transacylase as the E2 subunit, and the *dihydrolipoyl dehydrogenase* as the E3 subunit. It is the E1 subunit that catalyzes the decarboxylation of BCKAs, and uses thiamine diphosphate (ThDP) as a cofactor. BCKD also requires coenzyme A (CoA) and NAD^+ as

cofactors, and is located within the inner mitochondrial wall. Similar to BCAT, BCKD can catabolize the products of all three BCAAs. However, unlike BCAT2 which allows reversible transamination, the decarboxylation by BCKD is an irreversible and rate-limiting step. In rat liver, BCKD has been shown to have a higher affinity towards KIC and KMV (BCKAs of leucine and isoleucine) with a K_m of 15 and 14 μm, respectively. BCKD has a lower affinity for KIV (BCKA of valine), with a K_m of 28 μm [177]. BCKD is present in all cells throughout the body and has been found to have varying activity levels in different tissues and different animals. In rats, BCKD oxidative activity has been found to be highest in the liver, followed by the kidney, and lowest in skeletal muscle, brain, and intestines. In humans, BCKD activity is highest in skeletal muscle, followed by the brain and liver, and lowest in the kidney, and stomach [172]. Unlike BCAT which has no identified regulators, the BCKD complex has been found to be regulated by multiple mechanisms.

5.3.1 Regulation of BCKD

The BCKD complex can be regulated by inhibitory or activating phosphorylation/dephosphorylation by distinct protein kinases. The *branched-chain α-keto acid dehydrogenase kinase* (BCKDK) enzyme phosphorylates the E1 subunit of BCKD and causes inhibition of BCKD activity. There are at least three phosphorylation sites on the E1α subunit that can be phosphorylated by BCKD kinase. Phosphorylation on Ser^{293}, Ser^{332}, and Ser^{337} blocks the binding site for BCKAs and inhibits the function of the BCKD complex [178], [179]. BCKD kinase expression has been found to be regulated by exercise, diet, and hormonal state. BCKD kinase activity decreases in response to exercise, high-protein diets, and insulin withdrawal (reviewed in [180]. The net effect of reduced BCDK kinase activity is increased BCKA oxidation under these circumstances. BCKD kinase can also be directly inhibited by KIC

accumulation [181]. Conversely, BCKD kinase activity can be increased by starvation, low-protein diets, and insulin presence, and results in decreased BCKA oxidation [180], [182]. While BCKD can be inactivated by BCKD kinase, it can be activated by the mitochondrial isoform of *protein phosphatase 1K* (PPM1K, also known as PP2CM). Under conditions of excess BCAA, the phosphorylation of Ser^{293} on the E1α subunit of BCKD becomes dephosphorylated by PPM1K [179]. PPM1K is regulated at the transcriptional level in response to nutrient status. In mice, nutrient deprivation and BCAA depletion causes down-regulation of PPM1K mRNA [183]. Lastly, BCKD activity is also regulated by its end products. NADH and the branched-chain acyl-CoA derivatives produced can competitively inhibit the BCKD complex and prevent further BCKA oxidation [184].

5.4 Auxiliary regulation of BCAA catabolism

Although factors such as nutrient presence, exercise, and insulin can regulate the BCAA catabolic enzymes, pathways that mediate this phenomenon have not been completely elucidated. Recently, Zhen et al. 2016 [185] attempted to identify targets that mediate BCKD activity in response to the BCAA leucine. They found that oral administration of a 2% leucine solution in 7-week-old mice resulted in a significant increase in heart BCKD activity. However, when they provided the leucine solution to mice that were simultaneously treated with rapamycin, they observed that there was no increase in BCKD activity. Thus, this study suggests that a rapamycin-sensitive mTOR complex regulates BCKD responsiveness to BCAAs.

It is known that exercise promotes BCAA oxidation via upregulation of BCAA catabolic enzymes [186]. It is also known that exercise can promote mitochondrial function and biogenesis via the *PPAR γ coactivator of 1α* (PGC-1α) [187]. Hatazawa et al. 2014 [188] sought to determine if PGC-1α can regulate BCAA metabolism by affecting the expression of its key

catabolic enzymes. To do this, they created a model of transgenic mice overexpressing PGC-1α and examined the regulation of BCAT2, BCKD, and BCKDK, in skeletal muscle. Compared to WT mice, they found a significant increase in the mRNA expression of BCAT2, BCKD, but no change in BCKDK. When examining the protein expression, they correspondingly observed an increase in BCKD, but no change in BCKDK. Oddly, the study did not present any data on BCAT2 protein expression. When examining BCAA levels, they found decreased levels of valine and leucine along with increased levels of glutamic acid/glutamate (a metabolite of BCAT2). These findings support the notion that transgenic mice overexpressing PGC-1α have increased BCAA catabolism. Their experiments in C2C12 mouse myoblasts that were overexpressing PGC-1α cells yielded similar results. Thus, this study provides evidence that PGC-1α can control BCAA catabolism by positively regulating the expression of BCAA catabolic enzymes.

Adiponectin is another molecule which has been shown to regulate BCAA catabolism. Adiponectin is an adipocytokine that helps regulate proper glucose and lipid metabolism [189], [190]. In cases of insulin attenuation, such as diabetes, adiponectin levels are decreased [191]. BCAA metabolism can also be regulated by insulin levels [180], [182]. Based on these findings, Lian et al. 2015 [192] tried to determine if there was any connection between adiponectin and BCAA metabolism. They demonstrated that mice that were adiponectin deficient and diabetic exhibited significantly decreased BCKD activity along with decreased PPM1K (PP2CM) protein levels. This was complemented with an increase in BCKD kinase expression, and a concurrent increase in plasma BCAAs and BCKAs. Interestingly, subsequent treatment with adiponectin in these mice restored the expression of PPM1K, BCKD, along with BCKD activity, and BCAA and BCKA levels. Lastly, the adiponectin-mediated amelioration in PPM1K expression and

BCKD activity were eliminated when AMPK (a kinase that plays a role in cell energy homeostasis) was inhibited. Conclusively, the work by Lian et al. 2015 [192] showed that adiponectin-AMPK signaling is required for adequate function of BCAA catabolism. Furthermore, the dysregulation of BCAA metabolism in cases of diabetes may be in part due to the fact that there is reduced adiponectin.

Despite being separate enzymes with distinct functions, Islam et al. have shown that BCATm and BCKD can directly interact with one another to form a structural relationship referred to as a metabolon. Islam et al. 2007 [193] found that human BCATm can directly associate with the E1 decarboxylase component of either rat or human BCKD. NADH, a metabolite of BCKD, dissociates the complex. Furthermore, they found the rate of E1-catalyzed decarboxylation was enhanced 12-fold when BCATm was also present. In a separate study by the same group, they demonstrated that the binding of BCATm to the E1 decarboxylase allows direct channeling of BCKAs from BCATm to BCKD [194]. This evidence suggests that there is direct cooperativity between BCAT and BCKD to facilitate BCAA/BCKA oxidation, and the presence of one can enhance the function of the other.

6.0 BCAA metabolites and skeletal muscle anabolism

As covered in section 4, branched-chain amino acids can positively regulate many aspects of skeletal muscle anabolism. However, it is not entirely clear whether it is BCAAs themselves or products made from BCAAs that regulate muscle anabolism. Interestingly, there is evidence that suggests the metabolites of BCAAs, particularly those of leucine, play important roles in mediating the anabolic effects of BCAAs in skeletal muscle. In this section, studies

examining the roles of the metabolites of BCAAs on skeletal muscle hypertrophy and skeletal muscle differentiation will be reviewed.

6.1 BCAA metabolites and skeletal muscle hypertrophy

One of the most well characterized BCAA metabolites that has anabolic effects on existing skeletal muscle tissue is the leucine metabolite β-hydroxy-β-methylbutyrate (HMB). HMB is a metabolite produced from KIC decarboxylation via the liver KIC-dixoygenase enzyme **(Figure 3)**. In 1996, Nissen et al. [8] were the first to show that supplementing HMB to individuals undertaking resistance exercise enhanced gains in lean body mass and strength, while lowering rates of post-workout muscle proteolysis. Since then, numerous studies have examined the effects of HMB in different cohorts. HMB supplementation has been shown to promote gains in lean body mass, strength, aerobic performance, in trained, untrained, elderly, and sedentary populations (reviewed in [9]). The anabolic effects of HMB in existing muscle have been shown to be mediated in part by the mTORC1 pathway. Eley et al. 2007 [10] demonstrated that HMB supplementation was able to induce protein synthesis in mouse myoblasts. This increase in protein synthesis correlated with an increase in phosphorylation of mTOR along with p70-S6K1 and 4E-BP1 (targets that regulate mRNA translation; see section 2.5). Furthermore, the increase in protein synthesis was abolished in the presence of rapamycin (an mTORC1 inhibitor). Aversa et al. 2011 [11] also showed that HMB supplementation promotes protein synthesis in rats in an mTOR-dependent manner. Similar to leucine, HMB can also suppress dexamethasone and cancer-induced muscle proteolysis via down-regulation of the ubiquitin-proteasome system and its atrogenes MuRF-1 and atrogin-1 [195]–[197]. Clearly, many of the positive effects seen with leucine treatment in muscle (described in section 4) can be emulated by its metabolite HMB.

The leucine metabolite KIC (α-ketoisocaproate) has also been shown to stimulate anabolic mechanisms in muscle. Escobar et al. 2010 [12] sought to compare the effect of leucine vs. KIC administration in neonatal pigs. Infusion of leucine or KIC (400μm/kg) both resulted in a ~60% increase in longissimus dorsi muscle protein synthesis compared to mock infused piglets. Both leucine and KIC also caused a 3-fold increase in 4E-BP1 phosphorylation and eIF4E/eIF4G complex formation in muscle tissues, compared to control. This study suggests KIC can mimic leucine-induced stimulation of protein synthesis and regulation of mTOR signaling. Another study by Tischler et al. 1981 [13] examined the role of leucine and its catabolism in regulating protein synthesis and protein degradation in rat diaphragm and cardiac muscle. They observed that administration of 0.2 to 0.5 mM of leucine was able to both increase protein synthesis and prevent breakdown (measured by ^{14}C labeled amino acid incorporation and release). To determine if leucine catabolism was required for these effects, they inhibited the BCAT2 enzyme with the drug L-cycloserine. Recall that BCAT2 transaminates leucine (along with valine and isoleucine) to produce corresponding BCKAs. L-cycloserine treatment completely blocked the attenuation of protein degradation observed with leucine treatment in both diaphragm and atrial muscle. This result suggests that KIC and/or other metabolites of BCAA catabolism mediate BCAA-induced attenuation of skeletal muscle atrophy. Interestingly, L-cycloserine treatment did not affect leucine-induced stimulation of protein synthesis, suggesting leucine itself is responsible for the stimulation of protein synthesis in rat diaphragm and atrial muscle. Nonetheless, the overall findings of these studies implicate a role for BCAA metabolites, particularly those of leucine, in mediating the anabolic effects seen with BCAA supplementation in skeletal muscle.

6.2 BCAA metabolites and skeletal muscle differentiation

Metabolites of BCAAs have been shown to have similar anabolic properties as their branched-chain amino acid precursors in existing skeletal muscle. In regards to skeletal muscle differentiation, there is evidence to suggest that BCAA metabolites also have anabolic properties. Kornasio et al. 2009 [14] demonstrated that supplementing HMB to serum-starved human and chick myoblasts stimulated their proliferation and differentiation in a dose-dependent manner. In serum-starved chicken and human cells, HMB administration for 17 hours caused increased cell number and DNA synthesis (markers of cell proliferation), along with increased MyoD mRNA and protein accumulation. 24 hours of HMB treatment to serum-starved cells caused an increase in the levels of muscle differentiation factors myogenin and MEF2 in chicken myoblasts. Myosin Heavy Chain (MHC) levels were also increased in both chicken and human myoblasts in a dose-dependent manner. Cell fusion was also observed in the C2 mouse myogenic line in response to HMB treatment. At higher levels of HMB, a significant increase in the number of myotubes containing five or more nuclei was observed. Lastly, HMB treatment was found to increase levels of IGF-1, along with MAPK/ERK signaling and AKT signaling. Thus, this study provides evidence the leucine metabolite HMB can alone promote skeletal muscle myoblast proliferation and differentiation, and upregulate pathways that induce these effects. Unfortunately, this was the only study that was found that examined the specific effect of a BCAA metabolite on skeletal muscle differentiation.

7.0 Disruption of branched-chain amino acid catabolism

Because metabolites of BCAAs have been shown to possess important anabolic properties in skeletal muscle hypertrophy and skeletal muscle differentiation, the enzymes that produce these metabolites likely have great importance in facilitating muscle anabolism. This

section will review studies that have examined the effects of disrupting the BCAA catabolic enzymes (BCAT and BCKD) in muscle.

7.1 Disruption of BCAT

She et al. 2007 [198] conducted a study in which they sought to determine the effect of a whole body BCAT2 knockout in mice. Knockout mice grew at the same rate as wild-type (WT) mice up until ~6 weeks, after which the knockout mice exhibited a 10-15% reduction in body weight compared to WT. The loss in body mass was found to be from reduced epididymal fat and kidney weight. There was no reduction in gastrocnemius muscle, liver, or heart mass. Knockout mice also consumed less of the normal chow diet than WT mice, but consumed more of the BCAA-free chow than WT mice. Although knockout mice consumed less BCAAs, their plasma leucine, isoleucine, and valine levels were increased 14, 21, and 31 fold, respectively. Interestingly, knockout mice were also protected from diet-induced obesity, as feeding mice a high fat diet for 15 weeks resulted in knockout mice that were still significantly leaner than WT mice. Plasma levels of the BCAA transamination product KIC was not changed, whereas KMV and KIV levels were reduced. The leanness of BCAT knockout mice was found to be a result of increased energy expenditure. In regards to the effect of BCAT knockout on skeletal muscle, it was found that both protein synthesis and protein degradation was significantly higher in gastrocnemius muscle, indicating higher rates of protein turnover, although muscle weight was not changed. The increased rate of protein turnover was found to correlate with increased mTOR signaling as indicated by higher phosphorylation of the mTOR downstream targets 4E-BP1 and rpS6. Surprisingly, the results from this study do not convey any negative consequences for skeletal muscle as a result of BCAT knockout. Although there was an increased protein turnover rate, there was no difference in gastrocnemius muscle mass or general

locomotor activity. In fact, BCAT2 knockout provides mice with an advantage of being protected from diet-induced obesity via increased energy expenditure. It should be noted that this study only looked at the change in mass of one muscle (gastrocnemius), and did not measure other functional measurements of muscle such as strength and endurance.

In a separate study, however, She et al. 2010 [15] examined the effect of BCAT2 knockout (KO) on exercise metabolism and endurance in mice. These mice were subjected to inclined treadmill running to exhaustion. They observed that BCAT2 knockout mice had significantly lower running times and running distances. Additionally, it was observed that this impairment in BCAT2 KO mice coincided with a decrease in glutamine levels, and increase in lactate and ammonia production. Thus, BCAT2 disruption likely can impair metabolic homeostasis in skeletal muscle that can negatively affect skeletal muscle performance. They also observed no change to structural components of muscle fibers in BCAT2 KO mice, further suggesting it is impaired muscle metabolism that contributes to exercise impairment.

7.2 Disruption of BCKD

There are multiple studies that have investigated impaired BCKD function, since this enzyme serves as the rate limiting irreversible step in BCAA oxidation and because it is regulated by various mechanisms. Mutations in genes encoding subunits of the BCKD complex or PPM1K can lead to a condition known as maple syrup urine disease (MSUD). The name of the disease is in reference to the sweet smelling urine that results from increased concentrations of BCAAs/BCKAs. Human and animal models of MSUD both show elevated levels of BCAA and BCKAs [199], [200]. Although the disease can be managed by eating a low protein diet, this disease can produce deleterious effects if not treated promptly. Neural cell models of MSUD exhibit increased oxidative stress and mitochondrial dysfunction in response to BCKAs [201],

[202], which can serve as a contributor to brain damage seen in MSUD patients [203]. Neural cells and fibroblasts isolated from patients with MSUD also undergo apoptosis in response to BCKAs [204], [205]. In regards to possible effects on muscle, zebra fish with MSUD show dysfunctional locomotor activity that results in aberrant swimming behavior [18]. Clinical reports also show that adult human patients can develop muscle dystonia [19], and newborns with the disease can display hypotonia and develop muscle spasms [17]. Quadriceps femoris muscle biopsies from these newborns show that some of their muscle fibers have irregular shapes along with nuclei that are abnormally large and also irregularly shaped. There were muscle fibers that also showed indications of myofibrillar destruction indicated by damage to sarcomeres, and in some instances damaged fibers were converted to empty sarcolemmic sacs. Thus, MSUD, which involves dysregulation of the BCKD enzyme, can produce negative consequences for skeletal muscle, among other tissues.

On the other side of the spectrum, hyperactivity of the BCKD complex can also produce negative effects. Joshi et al. 2006 [206] developed a transgenic mouse line that was deficient for the BCKD kinase (BCKDK). Recall that the BCKD kinase mediates inhibitory phosphorylation of the BCKD complex enzyme, thus these mice had hyperactive BCKD activity. These mice had lower plasma and tissue levels of BCAAs, and by 12 weeks of age were 15% smaller than wild-type mice. Muscle, brain, and adipose tissue weights were attenuated in knockout mice, whereas liver and kidney weights were elevated. These mice had abnormal hindlimb flexion throughout life, and developed epileptic seizures after 6-7 months of age, indicating defects in neurological and/or neuromuscular systems.

Conclusively, the negative consequences of BCKD disruption and BCKD hyper activation indicate that adequate BCKD regulation is an important process in many tissues

including skeletal muscle. The role of BCKD disruption in skeletal muscle differentiation is not known, although, the fact that newborn infants with MSUD have defective muscle tissue indicates that defective embryonic myogenesis due to disrupted BCKD function is likely to blame.

SUMMARY

Skeletal muscle differentiation is a process by which precursor muscle cells called myoblasts mature and fuse with one another to form myotubes, which themselves can further develop to form skeletal muscle fibers. Skeletal muscle differentiation is an important process that enables the formation of new muscle tissue during embryo development or after muscle injury/damage.

Skeletal muscle differentiation is regulated by many pathways, some of which are responsive to nutrient presence. Specifically, amino acid presence can regulate and stimulate skeletal muscle differentiation. Out of all amino acids, the branched-chain amino acids, particularly leucine, have been shown to potently stimulate anabolic pathways and suppress atrophic pathways in skeletal muscle hypertrophy and differentiation.

One of the major questions is how BCAAs, particularly leucine, exert their anabolic effects in skeletal muscle differentiation. There is evidence that suggests that it may be in part due to the metabolites that are produced from BCAA catabolism, as these metabolites themselves have been shown to exert similar anabolic and anti-catabolic effects. Furthermore, disruption of BCAA catabolism in models of existing muscle tissue exhibit impaired skeletal muscle function, structure, and metabolism. Thus, BCAA catabolism likely represents a critical process in the maintenance and growth of existing muscle tissue. However, the importance of BCAA

catabolism during skeletal muscle differentiation, where new muscle tissue is being formed, is not known.

RESEARCH OBJECTIVES

The aim of our study was to examine the role of branched-chain amino acid catabolism in skeletal muscle differentiation. Previous research has shown that branched-chain amino acids, particularly leucine, can positively regulate skeletal muscle hypertrophy and skeletal muscle differentiation. Studies that examine metabolites of leucine catabolism also show that these metabolites can induce anabolic effects similar to leucine in both skeletal muscle hypertrophy and skeletal muscle differentiation. Furthermore, models of disrupted BCAA catabolism, such as in maple syrup urine disease, exhibit compromised skeletal muscle structure and function. Thus, we wondered if BCAA catabolism is critical in mediating the anabolic effect of BCAA presence in skeletal muscle differentiation. In this study, we aim to complete the following objectives:

1) Although the leucine metabolite HMB has been shown to regulate skeletal muscle differentiation, the leucine metabolite KIC has not been studied in this context. Thus, we aim to determine if KIC can also regulate skeletal muscle differentiation.

2) Expression levels of enzymes that mediate BCAA catabolism during skeletal muscle differentiation are not known. Hence, we seek to elucidate the expression of both muscle specific isoform of BCAT (BCAT2), and BCKD during skeletal muscle differentiation.

3) The role of BCAA catabolism in skeletal muscle differentiation is not known. We attempt to examine how skeletal muscle differentiation is affected by disrupting the BCAA catabolic enzymes, BCAT2 and BCKD.

To attain these objectives, we utilized an in-vitro model of differentiating rat skeletal muscle myoblasts for a period of five days. Under normal conditions, these myoblasts can

differentiate and fuse with one another to form elongated premature muscle fibers called myotubes. We conducted various experiments throughout this model of differentiation and ultimately attempted to determine if BCAA catabolism regulates this process.

HYPOTHESES

1) Based on literature that suggests KIC can regulate skeletal muscle hypertrophy to a similar extent as leucine, I hypothesize that KIC will be able to also regulate skeletal muscle differentiation.
2) Based on previous literature that suggests that BCAA metabolites can positively regulate skeletal muscle differentiation, I hypothesize that expression of BCAT2 and BCKD will increase during myoblast differentiation so as to produce higher levels of their metabolites.
3) It has been shown that whole-body animal knockouts of BCAT2 demonstrate disrupted muscle energy metabolism and exercise capacity. Thus, I hypothesize that disrupting BCAT2 during skeletal muscle differentiation will disrupt myoblast differentiation. In regards to BCKD, it has been shown that disruption of BCKD negatively affects skeletal muscle structure and function, thus I hypothesize that disrupting BCKD in myoblasts will also negatively affect skeletal muscle differentiation.

EXPERIMENTAL DESIGN & MATERIALS

Cell culture & differentiation protocol: To produce a model of skeletal muscle cell differentiation, we cultured L6 rat myoblasts and differentiated them for a period of 5 days. L6 rat myoblasts were obtained from the American Type Culture Collection. Approximately 5.0 x 10^5 cells were seeded in a 10-cm plate and incubated at 37ºC and 5% CO_2. The growth medium (GM) was α–modified MEM (AMEM) obtained from Wisent Bioproducts (#310-010-CL), supplemented with 10% fetal bovine serum (Gibco #10082147) and 1% antibiotic-antimycotic (Ab) (Wisent #450-115-EL). Cells were passed every 48 hours or at approximately 70% confluency. To initiate differentiation, cells were first grown to ~90% confluency. Cells were then shifted to a differentiation medium (DM) consisting of AMEM, 1% antibiotic-antimycotic, and 2% horse serum (Gibco # 26050088). The differentiation medium was replaced every 24 hours.

Leucine deprivation + KIC rescue experiment: To observe how differentiation was affected by depriving myoblasts of leucine, the following was conducted: 1.5 x 10^5 cells were seeded in 6-well plates using growth media (AMEM + 10% FBS+ 1%Ab). Once cells reached ~90% confluency, the media was shifted to differentiation media consisting of RPMI 1640 (US Biologicals R8999-03), + 2% HS + 1% Ab. RPMI media was used because it lacks L-leucine (along with L-glutamine, L-alanine, and sodium bicarbonate). L-glutamine and sodium bicarbonate were re-added to all RPMI media at concentrations to mimic AMEM. L-leucine and L-alanine were added to respective treatments to replicate AMEM concentrations. RPMI differentiation media was changed every 24 hours. To examine the effect of adding the leucine metabolite KIC to leucine-deprived cells, the following was conducted: cells were differentiated in media that was leucine-free but supplemented with 200 μM of α-ketoisocaproate (KIC)

(Sigma #K0629). The supplemented differentiation media was changed every 24 hours. Following all treatments, cells were washed with cold PBS and harvested with 100µl lysis buffer and stored at -20ºC. The lysis buffer was formulation was: 1mM EDTA, 2% sodium dodecyl sulfate (SDS), 25mM Tris, protease cocktail inhibitor (10 µL/mL) (Sigma #P8340), phosphatase inhibitor cocktail (10 µL/mL) (Sigma #P5726), and DTT (1 µL/ml) .

Western blotting: To obtain protein expression levels of various proteins during the differentiation protocol, samples harvested from each treatment day were subjected to western blots. Sample protein concentrations were first determined using the Pierce BCA Protein Assay Kit (Thermo Scientific #OJI94919). Samples were then mixed with 4x laemmli loading buffer at a ratio of 3 parts sample to 1 part buffer. Samples were run on either 10% or 15% SDS-page gels and transferred to PVDF membranes. MHC-1, BCKDE1α, and troponin were blotted using the 10% gel membranes, whereas, BCAT2, ph-S6, caspase-3, and myogenin were blotted using the 15% gel membranes. Membranes were incubated for 1 hour in 5% non-fat milk at room temperature. Membranes were then washed twice briefly and then once for 5 minutes with TBS-T, then incubated with primary antibody diluted in 2.5% BSA overnight at 4ºC. Following this, membranes were again washed twice briefly and then another three times for 5 minutes each with TBS-T, then incubated in either rabbit or mouse secondary antibody diluted in 5% non-fat milk for 3 hours at room temperature. Membranes were then washed again twice briefly followed by three 5-minute rinses, then incubated with HRP chemiluminescent substrate (Millipore #WBKLS0500). Immediately after, luminescence was detected using the Kodak Image Station 4000mm Pro & Carestream molecular imaging software. *Primary antibodies:* Myosin Heavy Chain-1 1:500 dilution (Developmental Studies Hybridoma Bank #MF20), Troponin 1:500 dilution (Developmental Studies Hybridoma Bank #CT3), Myogenin 1:500

dilution (Developmental Studies Hybridoma Bank #F5D), BCKDE1α 1:2500 dilution (Sigma #SAB2702057), BCAT2 1:3000 (Sigma #B7312), ph-S6 1:3000 dilution (CST #4858), Gamma Tubulin 1:10,000 (Sigma #T6557), Caspase-3 1:2000 dilution (CST #9662). *Secondary Antibodies:* Anti-rabbit IgG-HRP-conjugated (CST #7074), Anti-mouse IgG-HRP-conjugated (CST #7076); 1:10,000 dilutions.

mRNA analysis: To observe mRNA levels of BCAT2 and BCKDE1α during myoblast differentiation, the following was conducted: 1.5×10^5 cells were seeded in 6-well plates using growth media (AMEM + 10% FBS+ 1%Ab). Once cells reached ~90% confluency, the media was shifted to differentiation media consisting of (AMEM + 2%HS + 1%Ab). RNA was isolated from sample wells using an RNA isolation kit (Thermo-Fisher #12183018A). The RNA isolation procedure was carried out according to the manufacturer's instructions except for the fact that the reaction mixture was incubated at 42°C for 60 mins instead of 30 mins. After RNA was isolated it was stored at -80°C. cDNA was synthesized from RNA using a cDNA synthesis kit (Bio-rad #1725038), and the procedure was also followed according to the provided protocol. Quantitative PCR was conducted in 20µl reactions using a kit according to manufacturer's instructions (Bio-Rad #1725271), and analyzed using a real-time detection system (Bio-Rad CFX96™). The following primers were used: BCAT2 [Forward: 5'-TCCAGAACCTCACAGTGC-3' Reverse: 5'-CCTGCTTGTCAAAGTCTG-3'], BCKDE1α [Forward: 5'-GGGCTTGGCTAGATTCA-3' Reverse: 5'-GGGGATCTTCACTGGGGT-3'], HPRT [Forward: 5'-GCTTTCCTTGGTCAAGCAC-3' Reverse: 5'-TCCAACAAAGTCTGGCCTGA-3'].

Measuring intracellular BCAA concentrations: To measure intracellular levels of BCAAs during differentiation the following protocol was followed: Briefly, leucine dehydrogenase can catalyze the oxidative deamination of BCAAs to their corresponding α-keto acid. The reaction

requires NAD^+ and produces NADH in an equal molar ratio to the amount of reactant (BCAA). The production of NADH can be measured via a fluorometer and a standard curve can be generated using different concentrations of BCAAs. This standard curve can then be used to estimate sample concentrations of BCAAs. First, 275 µl of assay buffer containing 0.1 M potassium phosphate, 2% EDTA, and 0.1% β-mercaptoethanol is added to wells of a 96-well plate. Then 10 µl of 120 mM of NAD^+ diluted in 0.1 M sodium carbonate buffer is added to the wells. 14 µl of sample or standard is then added to each well and a blank fluorescence reading is taken. Then, 10 µl of an enzyme solution [7.7857 units of leucine dehydrogenase diluted in (25 mM sodium phosphate buffer + 1 mg/ml BSA)] is added to each well. Another blank reading is taken, followed by a reading after incubation at 37ºC for 15 minutes.

siRNA transfection experiments: To examine the effect of disrupting BCAA catabolic enzymes on myoblast differentiation, we utilized siRNA reverse transfection to disrupt BCAT2 and BCKDE1α. The protocol is as follows: 2.5×10^5 cells were seeded in 6 well plates along with either a control or treatment transfection media. The control medium consisted of scrambled siRNA (Sigma #SIC001) + lipofectamine RNAiMAX (Life Technologies # 13778150) + Opti-MEM (Life Technologies #31985070) + 1 ml growth medium without antibiotic at ratios according to manufacturer protocol. The treatment media consisted of either BCAT2 siRNA (Sigma # NM_022400) or BCKDEIα siRNA (Sigma # NM_012782) + lipofectamine RNAiMAX + Opti-MEM at a similar ratio. Twenty-four hours after transfection, 1 ml of growth medium with antibiotic was added to all wells. Cells were incubated in this media for another 24 hours and grown to ~90% confluency. Cells were then washed twice with warm PBS and shifted to differentiation media. Following treatment, samples were harvested with 100µl lysis buffer, and stored at -20ºC for later analysis.

Cell viability analysis: We measured cell viability to quantify the relative amount of viable cells remaining after BCAT2 and BCKDE1α siRNA transfections. Cell viability was measured in siRNA transfected myoblasts using the CCK-8 cell viability assay kit (Sigma #96992). Briefly, 8.0 x 10^3 cells were seeded along with transfection media in 96-well tissue culture plates. Twenty-four hours after transfection, the transfection solution was removed and replaced with 100µl of growth medium, and 10µl of CCK-8 solution. The absorbance was read at 450nm using a spectrophotometer. 24 hours later, the media of the remaining wells was changed to 100µl of DM, and cell viability was measured at each day of differentiation. Cell viability is expressed relative to control untreated cells that did not receive any siRNA treatment.

Increasing number of cells after siRNA transfection experiment: Because BCAT2 siRNA transfection reduced the number of viable cells, we wondered if adding more cells could rescue BCAT2-disrupted myoblast differentiation. To answer this we conducted the following: 24 hours following reverse transfection of control or BCAT2 siRNA in 6-well plates, we trypsinized 3 wells of the BCAT2 siRNA-treated cells and combined them into one new well. Similarly, for the control siRNA treated cells, we trypsinized 1 well and simply moved the cells into 1 new well. We only used one well for control cells because cell death was minimal in control cells compared to BCAT2 disrupted cells. Once the cells from both treatments were placed in to new wells, they were allowed to grow in GM for another 24 hours. Following this, cells were shifted to DM and their ability to differentiate was observed. The DM was changed every 24 hours.

BCKA rescue experiment in BCAT2 siRNA cells: To examine the effect of adding branched-chain α-keto acids (KIC, KMV, KIV) to BCAT2 disrupted cells, the following was conducted: 2.5 x 10^5 cells were seeded in 6-well plates along with either a control or BCAT2 siRNA transfection mix (described earlier). Twenty-four hours after transfection, 1 ml of regular GM

was added to all wells. In BCAT2 siRNA wells 200μM of KIC, 200 μM of KIV, and 200 μM of KMV was also added. After another 24 hours of incubation, the cells were shifted to DM, and again KIC, KIV, and KMV was re-added to BCAT2 siRNA wells. The DM was changed every subsequent 24 hours, and KIC, KMV, KIV was also re-added to BCAT2 siRNA cells when fresh DM was added.

Statistical analysis: Two-tailed paired *t*-test was used to assess the difference between two groups. One-way ANOVA with Tukey Kramer post-hoc test was conducted on experiments with more than two groups. Values are means ± SEM. $p < 0.05$ was considered significantly different.

RESULTS

The leucine metabolite KIC can regulate L6 myoblast differentiation.

We first sought to demonstrate the essentiality of the branched-chain amino acid leucine to L6 rat myoblast differentiation. Myoblasts differentiated in a differentiation media (DM) that contained all amino acids (CTR) demonstrated a robust ability to differentiate as visible myotubes formed after 5 days (D5) of differentiation (**Fig. 4A**). Myoblasts differentiated in media that lacked the non-essential, non-BCAA amino acid alanine also were able to differentiate well (**Fig. 4A**). However, when we tried to differentiate myoblasts in media that lacked the BCAA leucine, no differentiation was observed as indicated by the absence of myotube formation (**Fig. 4A**). The cells also appeared to enter a non-proliferative and quiescent state. Interestingly, when we added the leucine metabolite α-ketoisocaproate (KIC) to leucine-deprived myoblasts, the myoblasts were able to differentiate and form visible myotubes (**Fig. 4A**).

We also examined the protein expression of various markers of differentiation in these cells. Levels of the myofibrillar proteins myosin heavy chain (MHC) and troponin were both abundantly present in CTR and alanine-deprived conditions by D5 of differentiation (**Fig. 4B**). On the other hand, myoblasts differentiated in leucine-free DM showed no expression of MHC or troponin. However, myoblasts differentiated in the leucine-free DM supplemented with KIC demonstrated robust MHC and troponin expression at D5 (**Fig. 4B**). Protein level of the myogenic regulatory factor myogenin followed a similar expression profile as expression of the myofibrillar proteins. The mTORC1 target S6 (or rpS6) that is phosphorylated in response to nutrient stimulation was also found to be hypo-phosphorylated at D5 in leucine-deprived myoblasts, but phosphorylated in all other conditions (**Fig. 4C**).

Conclusively, these results demonstrate the BCAA leucine is essential for L6 myoblast differentiation, and furthermore, in the absence of leucine, KIC can rescue myoblast differentiation. KIC supplementation can also rescue myofibrillar protein expression, expression of the myogenic regulatory factor myogenin, and nutrient-induced mTORC1 signaling.

Expression of BCAA catabolic enzymes BCAT2 and BCKD increase during L6 differentiation

Because we observed that metabolites of leucine catabolism could regulate L6 myoblast differentiation, we wondered how the enzymes that produce BCAA metabolites are regulated during myoblast differentiation. Expression of BCAT2 (the BCAT isoform found in skeletal muscle) showed a non-significant trend to increase to day 3 (D3) of differentiation (**Fig. 5A**). Expression of the BCKD subunit BCKDE1α showed a significant trend to increase on all days of differentiation, where D4 and D5 were significantly higher compared to D1 of differentiation ($p<0.05$) (**Fig. 5A**). We also investigated mRNA levels of BCAT2 and BCKDE1α during differentiation. We found a non-significant increase in BCAT2 mRNA at D1 and D4 of differentiation (**Fig. 5B**). BCKDE1α mRNA analysis showed a trend to increase between D0 and D2 of differentiation, followed by a significant decrease to D5 ($p<0.05$) (**Fig. 5B**). In summary, our evidence suggests there is an upregulation of BCAT2 and BCKDE1α transcription and protein expression during portions of rat myoblast differentiation.

Intracellular BCAA concentrations do not change during L6 myoblast differentiation

Since we observed that levels of the enzymes responsible for BCAA catabolism increase during differentiation, we wondered if there were concurrent changes in intracellular concentrations of BCAA. We observed no significant difference in levels of BCAAs within myoblasts over the five-day differentiation period (**Fig. 6**).

BCAT2 disruption prevents L6 myoblast differentiation

The increase in BCAT2 and BCKD expression during myoblast differentiation led us to wonder if these enzymes have critical functions that facilitate differentiation. To establish the significance of the BCAT2 enzyme during rat myoblast differentiation, we knocked down BCAT2 protein in myoblasts using reverse siRNA transfection. Forty-eight hours after growing transfected myoblasts, the myoblasts were then placed in differentiation media (D0) and allowed to differentiate for five days. Cell transfected

with control siRNA were able to differentiate well, whereas BCAT2 knockdown cells showed no visible cell-to-cell fusion or myotube formation (**Fig. 7A**). Furthermore, BCAT2 siRNA treated wells showed a visible loss in cell number at D1 and D2 compared to D0 (**Fig. 7A**).

Protein levels of MHC were significantly reduced by over 80% at D3 in BCAT2-siRNA myoblasts ($p<0.05$), and there was no expression of troponin at any days in these cells (**Fig. 7B and C**). Expression of myogenin was also completed abolished by BCAT2 knockdown (**Fig. 7B and C**). Lastly, phosphorylation of S6 was significantly reduced at D2 ($p<0.05$) and showed a trend to be reduced at D3 and D4 as well (**Fig. 7B and C**). Clearly, these results demonstrate that BCAT2 serves an essential role in the differentiation of rat myoblasts.

BCKD disruption prevents L6 myoblast differentiation

We next observed the effect of disrupting the BCKD subunit BCKDE1α in rat myoblasts. Similarly to the previous experiment, we reverse transfected myoblasts with BCKDE1α siRNA, then placed these cells in differentiation media 48 hours later. The results of this experiment are similar to those seen in BCAT2 knockdown. Control transfected cells robustly fused to form healthy myotubes, whereas BCKD knockdown cells showed no visible fusion or myotube formation (**Fig. 8A**). Also similar to BCAT2 knockdown cells, BCKDE1α disrupted cells showed a visible reduction in cell number at D1 and D2 compared to D0 (**Fig. 8A**).

Levels of troponin were also completely abolished in BCKDE1α knocked-down cells, while levels of MHC were significantly reduced by over 90% by D4 ($p<0.05$) (**Fig. 8B and C**). Myogenin levels were also almost completely abolished by BCKDE1α knockdown, and ph-S6 levels were significantly reduced by ~20% at D2 of differentiation ($p<0.05$) (**Fig. 8B and C**). Thus, similar to BCAT2, adequate BCKD function is essential to myoblast differentiation.

BCAT2 and BCKDE1α siRNA transfection reduces cell viability

To quantify the portion of living and/or viable cells in BCAT2 and BCKDE1α knockdowns, we performed a cell viability assay. Myoblasts transfected with two different BCAT2 targeting siRNA oligonucleotides both resulted in a reductions in cell viability at D1 and D2, where myoblasts treated with BCAT2 siRNA-1 at D2 showed significant reductions ($p<0.05$) (**Fig. 9A**). Similarly, myoblasts transfected with two different BCKDE1α targeting siRNA oligonucleotides also both showed a significant reduction in cell viability at D1 and D2 ($p<0.05$) (**Fig. 9B**). These results demonstrate that knockdown of BCAT2 or BCKDE1α result in a reduction in the number of healthy viable cells, which confirms what we visually observed in Figure 7A and 8A. Conclusively, disruption of these enzymes impairs myoblast proliferation and survival. From this figure onward, we attempt to determine the mechanism by which BCAT2 disrupted cells do not differentiate. We focused on BCAT2 as it is the first step of BCAA catabolism, and has additional regulatory functions in conjunction with BCAA catabolism such as glutamine/α-ketoglutarate production.

Increasing the cell confluency does not rescue differentiation of BCAT2 disrupted myoblasts

We next sought to determine if we could rescue the differentiation of BCAT2 disrupted myoblasts by adding a greater number of viable cells after transfection. Since BCAT2 knockdown resulted in a loss of viable cells at D1 and D2 (Fig. 7A & 9A), we wondered if the lack of myoblast fusion and differentiation was due to a lack of cell confluency. To address this question, 24 hours post-transfection we combined three wells worth of BCAT2 deficient cells into one new well (**Fig. 10A**). To control transfected cells, we did not combine wells but we similarly trypsinized them and also transferred them to new wells (**Fig. 10A**). After another 24 hours, we placed these cells in differentiation media (D0).

As expected, increasing the number of cells increased the cell confluency at D1 of differentiation, as there were minimal empty spaces between cells in the BCAT2 siRNA condition (**Fig. 10B**). However, despite there being more cells, BCAT2 disrupted cells still showed an absence of differentiation and

exhibited a marked reduction in cell number at D3 of differentiation (**Fig. 10B**). In conclusion, these findings suggest that the reason BCAT2 deficient myoblasts do not fuse and differentiate is not due to there being a lack of adherent cells.

Supplementing BCKAs to BCAT2 disrupted myoblasts does not rescue myoblast differentiation

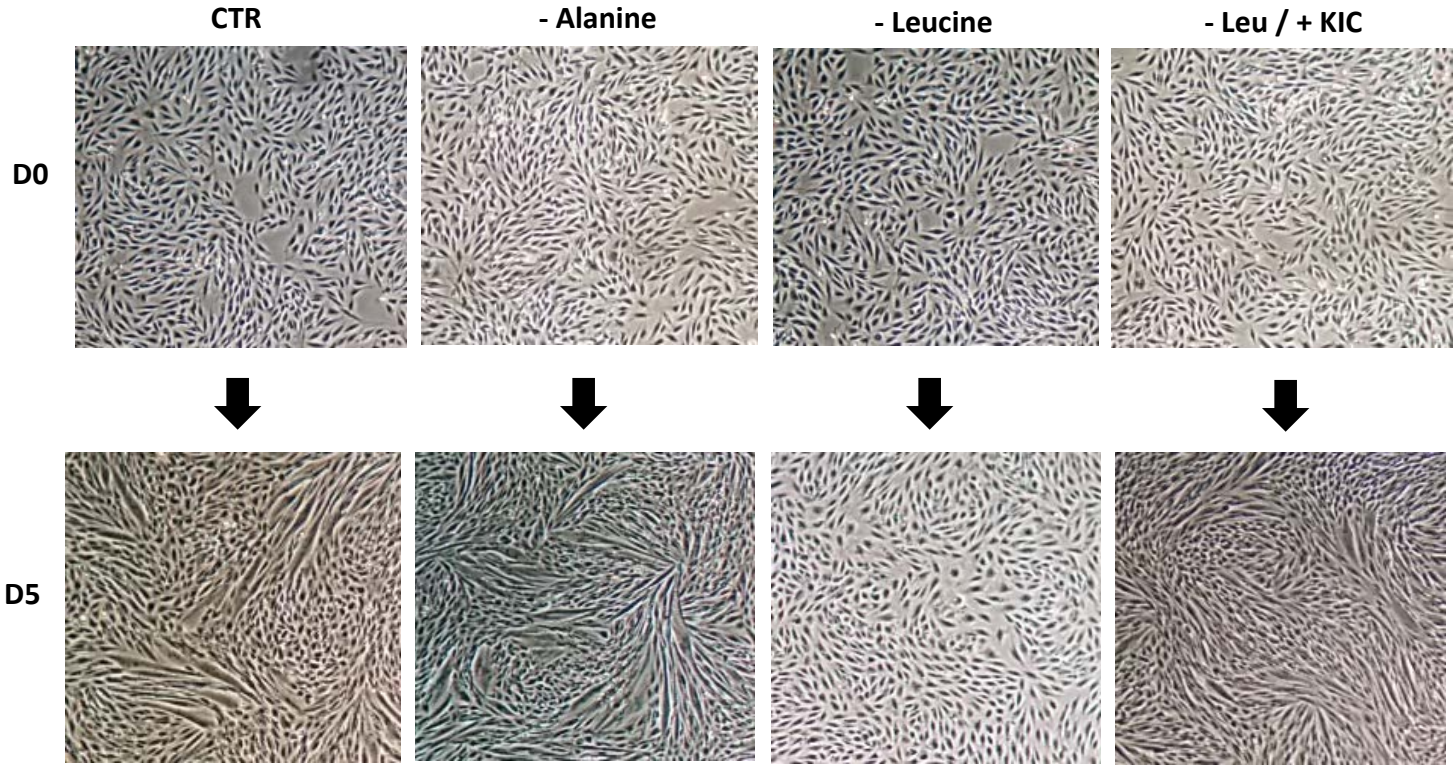
To determine an alternate reason as to why BCAT2 disrupted myoblasts do not differentiate, we wondered if it was due to BCAT2-mediated production of branched-chain α-keto acids (BCKAs) being disrupted (Fig. 3). Thus, we tried to determine if supplementing BCKAs to BCAT2 deficient myoblasts could rescue their differentiation. Supplementing the BCKAs KIC, KMV, and KIV (BCKAs of leucine, isoleucine, and valine, respectively) to BCAT2 siRNA treated cells resulted in no visible amelioration of myoblast fusion or cell death (**Fig. 11A**). Supplementation of BCKAs to BCAT2 disrupted cells also did not rescue the attenuation of myogenic proteins (MHC-1, troponin, myogenin) or mTORC1 signaling (ph-S6) (**Fig. 11B**). Hence, the reason BCAT2 disruption negatively affects myoblast differentiation is likely due to another BCAT2-mediated function other than BCKA production.

BCAT2 disruption induces programmed cell death in myoblasts

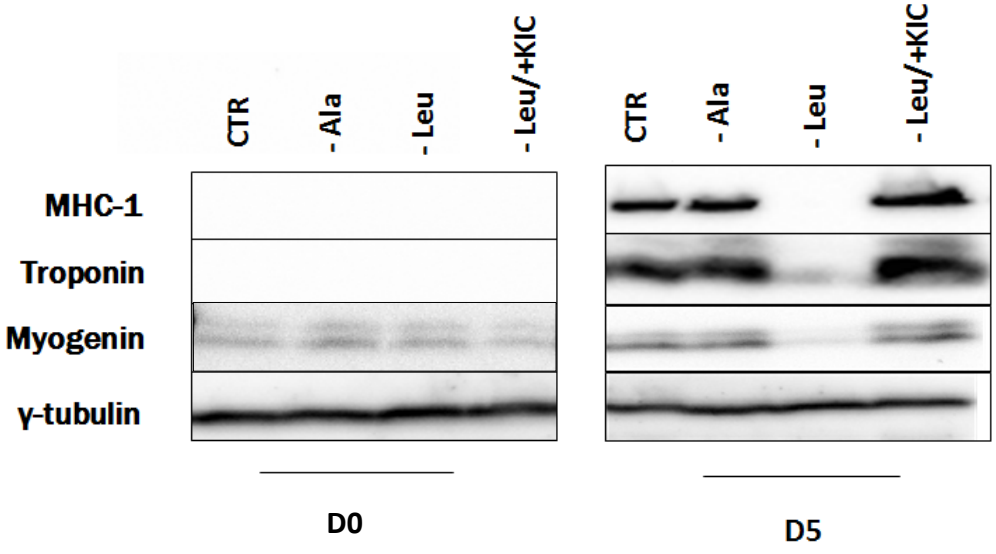
To determine a mechanism by which BCAT2 disruption affects cell survival and differentiation, we wondered if these cells became apoptotic in response to BCAT2 knockdown. Thus, we probed for caspase-3 protein, which when cleaved, induces programmed cell death via apoptosis. We observed that BCAT2 knockdown significantly increased the amount of cleaved caspase-3 at D1 of differentiation ($p<0.05$) (**Fig. 12**). Additionally, supplementing BCKAs to BCAT2 disrupted myoblasts did not rescue levels of cleaved caspase-3 (**Fig. 12**). Thus, these findings demonstrate that BCAT2 disrupted myoblasts become apoptotic when they attempt to differentiate.

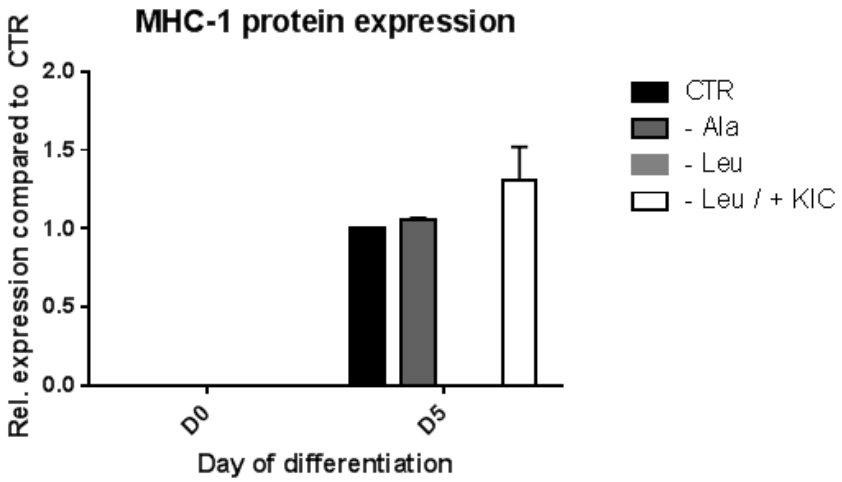
FIGURE 4. ***The leucine metabolite KIC can regulate L6 myoblast differentiation***

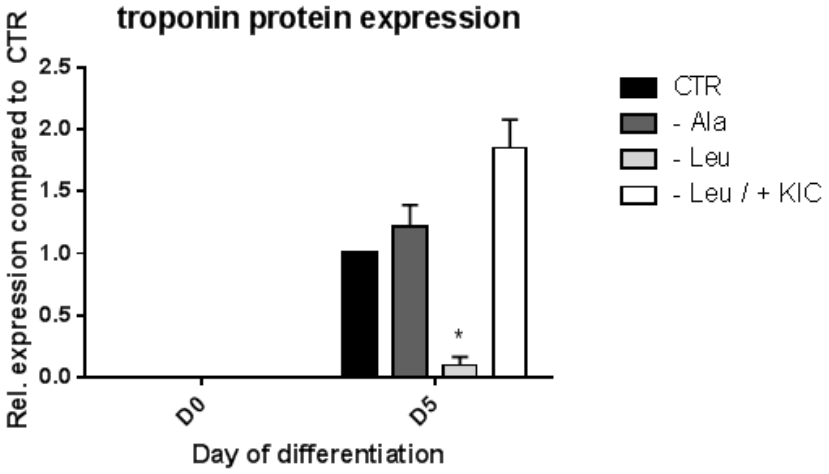
A)

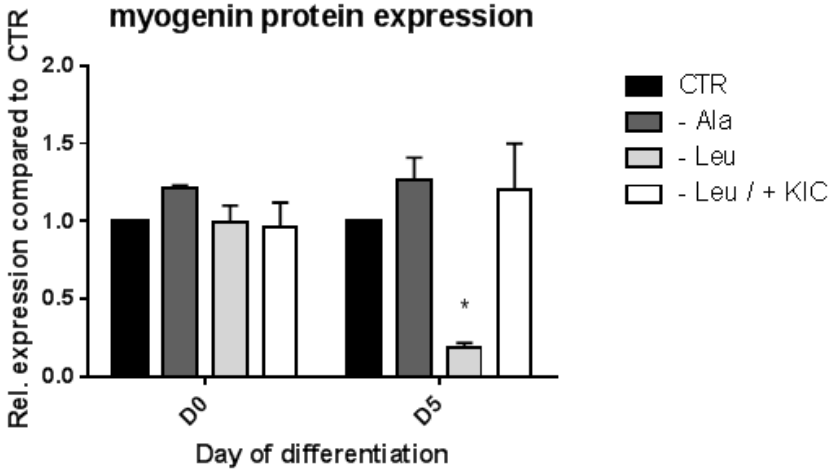


B)




MHC-1 protein expression
Rel. expression compared to CTR
2.0
1.5
1.0
0.5
0.0
D0
D5
Day of differentiation
CTR
- Ala
- Leu
- Leu / + KIC


troponin protein expression
Rel. expression compared to CTR
2.5
2.0
1.5
1.0
0.5
0.0
*
D0
D5
Day of differentiation
CTR
- Ala
- Leu
- Leu / + KIC


myogenin protein expression
Rel. expression compared to CTR
2.0
1.5
1.0
0.5
0.0
*
D0
D5
Day of differentiation
CTR
- Ala
- Leu
- Leu / + KIC

C)

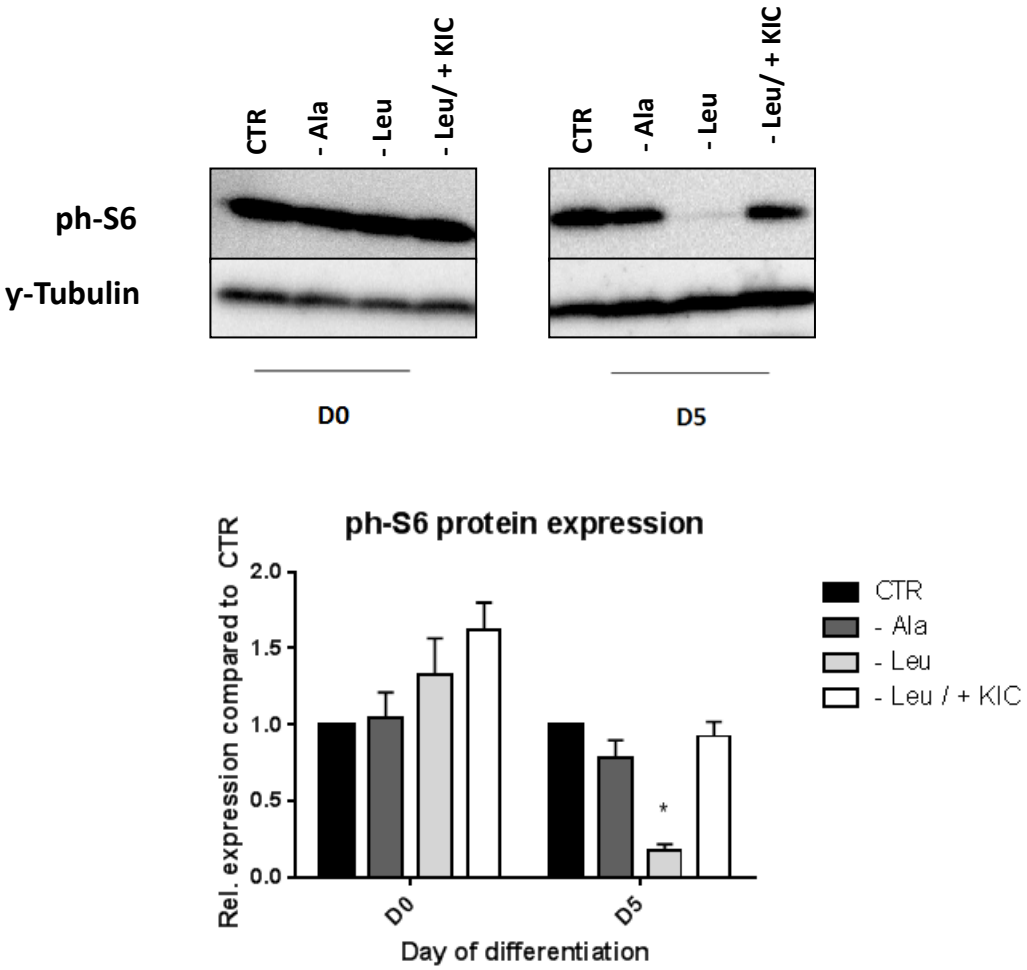
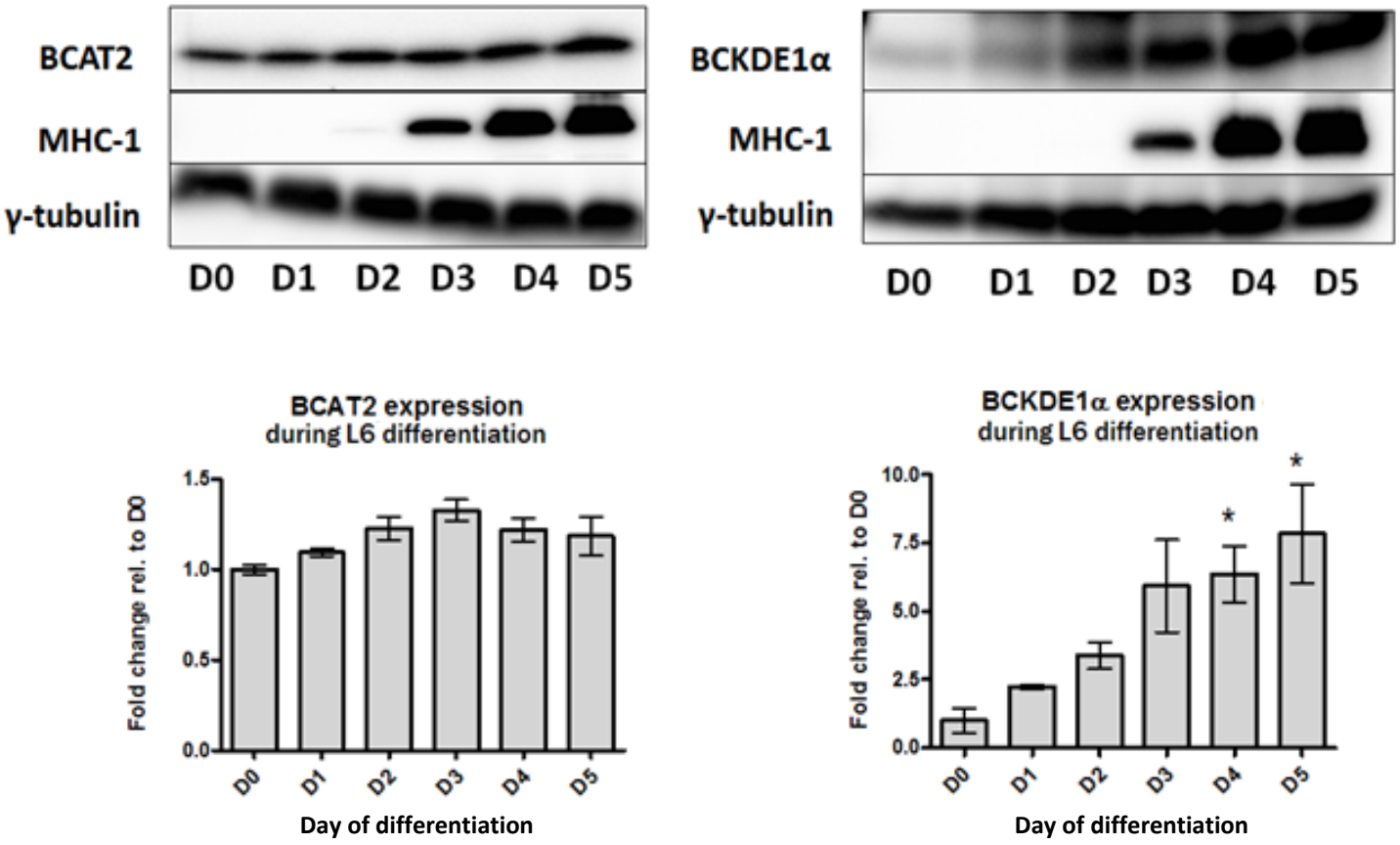


Figure 4. A) L6 rat myoblasts were differentiated in either differentiation media (DM) with all amino acids (CTR), DM without L- alanine, DM without L-leucine, or DM without L-leucine supplemented with 200µM KIC for a period of 5 days. Day 0 (D0) represents the moment immediately before being shifting the cells to differentiation media, and day 5 (D5) represents the moment after allowing the cells to differentiate for five days. Leucine deprived cells showed an inability to differentiate, as indicated by the absence of myotube formation. Supplementing KIC to leucine deprived cells was able to rescue myotube formation. **B)** Cells were harvested on day 0 (D0) and day 5 (D5) and probed for myosin heavy chain-1 (MHC-1), troponin, & myogenin, which are markers of differentiation. The absence of leucine impaired the expression of these proteins after 5 days of differentiation. However, supplementing the leucine-deprived differentiation medium with KIC was able to rescue levels of MHC-1, myogenin, and troponin. ** denotes significantly different compared to all other conditions in the D5 group ($p<0.05$).* **C)** mTORC1 signaling was attenuated by leucine deprivation, but rescued by KIC supplementation. ** denotes significantly different compared to all other conditions in the D5 group ($p<0.05$). n = 3, with 2 replicates per treatment. Data are mean ± SEM.*

FIGURE 5. ***Expression of BCAA catabolic enzymes BCAT2 and BCKD increase during L6 differentiation***

A)



B)

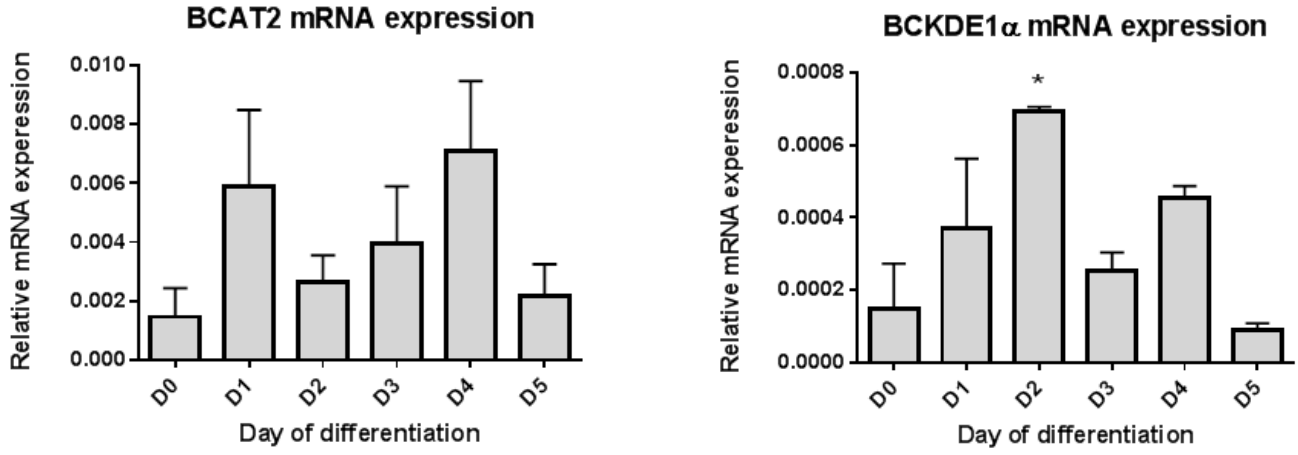


Figure 5. A) BCAT2 (branched-chain aminotransferase-2) and BCKDE1α (branched-chain alpha-keto dehydrogenase E1α) protein expression during a 5-day differentiation of L6 myoblasts. BCAT2 levels showed a non-significant trend to increase to day 3 of differentiation (D3). BCKDE1α showed a consistent increase on each day of differentiation, where day 4 (D4) and day 5 (D5) were significantly greater than day 1 (D1) ($p<0.05$). **B)** BCAT2 mRNA and BCKDE1α mRNA expressed relative to HPRT control mRNA over a 5 day differentiation of L6. BCAT2 showed non-significant increases at D1 and D4, whereas BCKDE1α showed significant elevation on D2 compared to D5 ($p<0.05$). *n = 3, with 2-3 replicates per treatment. Data are mean ± SEM.*

FIGURE 6. ***Intracellular BCAA concentrations do not change during L6 myoblast differentiation***

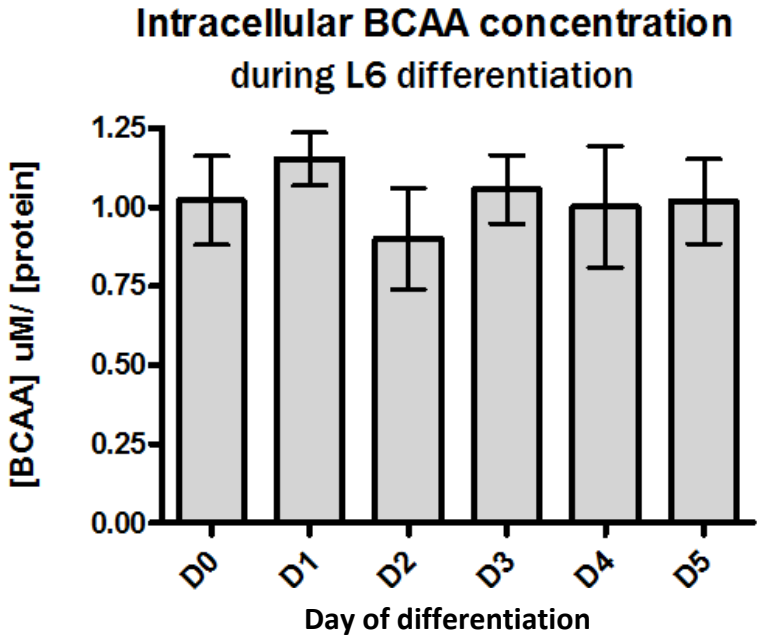
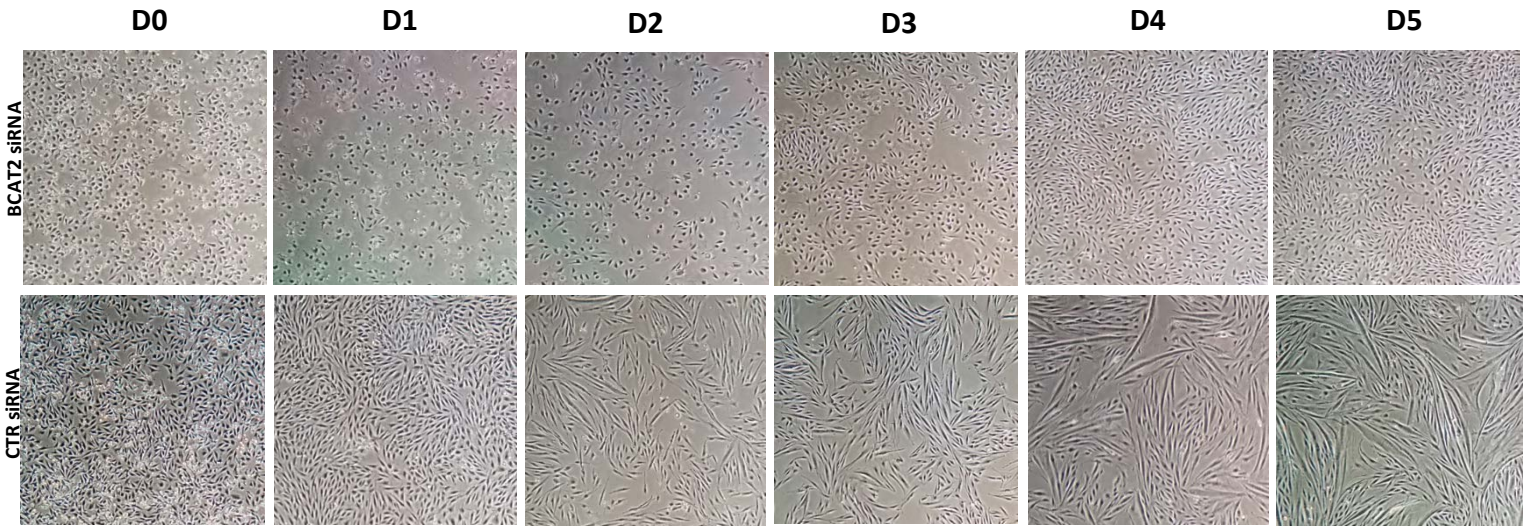


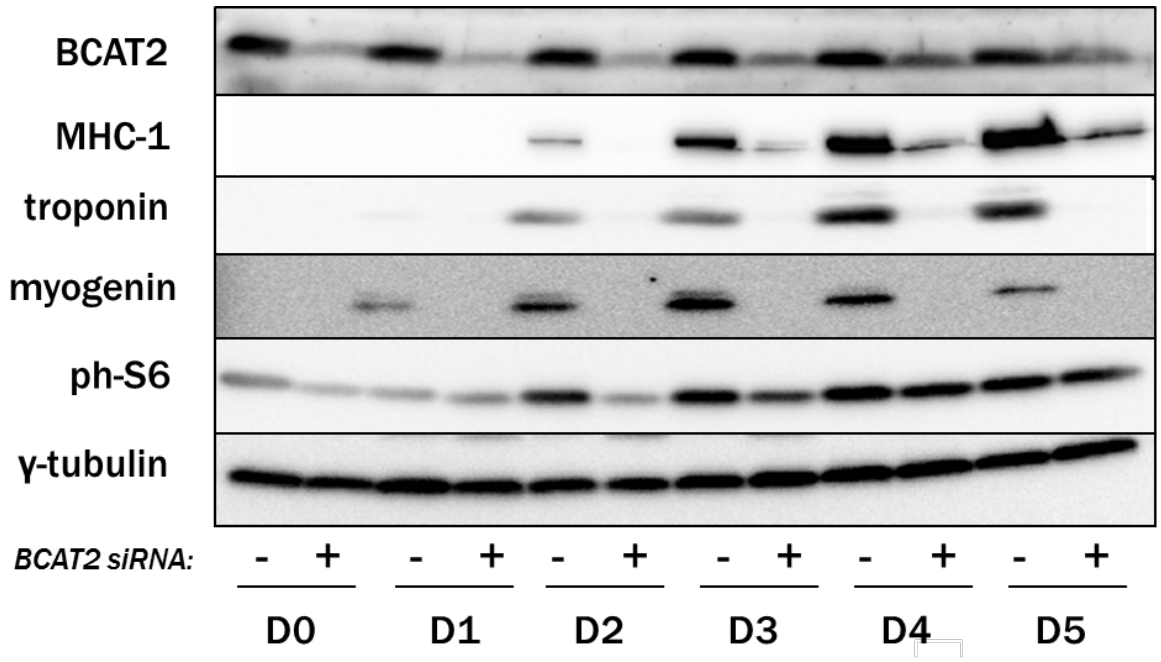
Figure 6. Intracellular BCAA concentration at each day of differentiation was measured using a fluorometric assay. Values were corrected using sample protein concentration. No significant change in intracellular BCAA concentrations was observed during differentiation. *n = 3, with 3 replicates per treatment. Data are mean ± SEM.*

FIGURE 7. ***BCAT2 disruption prevents L6 myoblast differentiation***

A)



B)



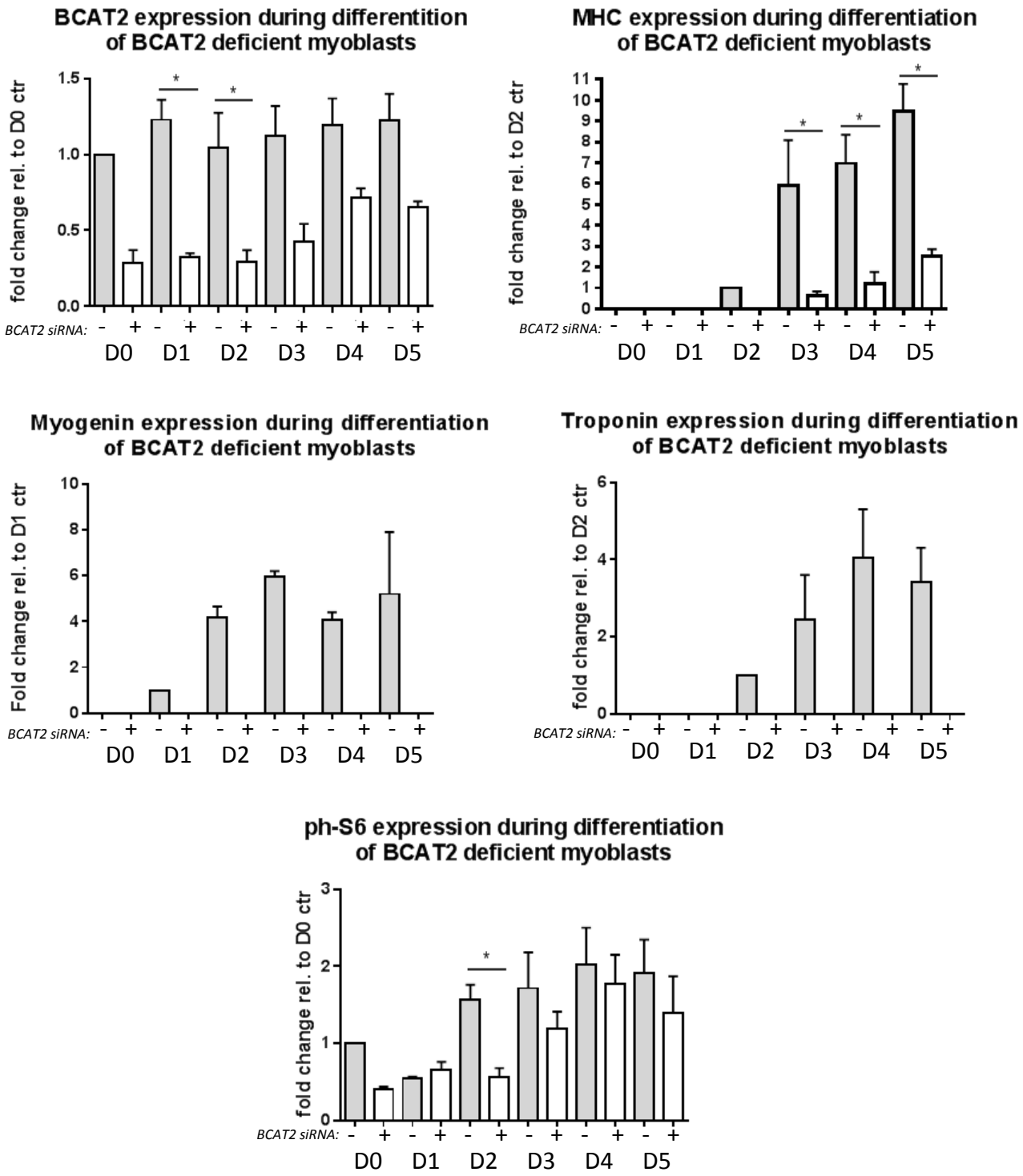
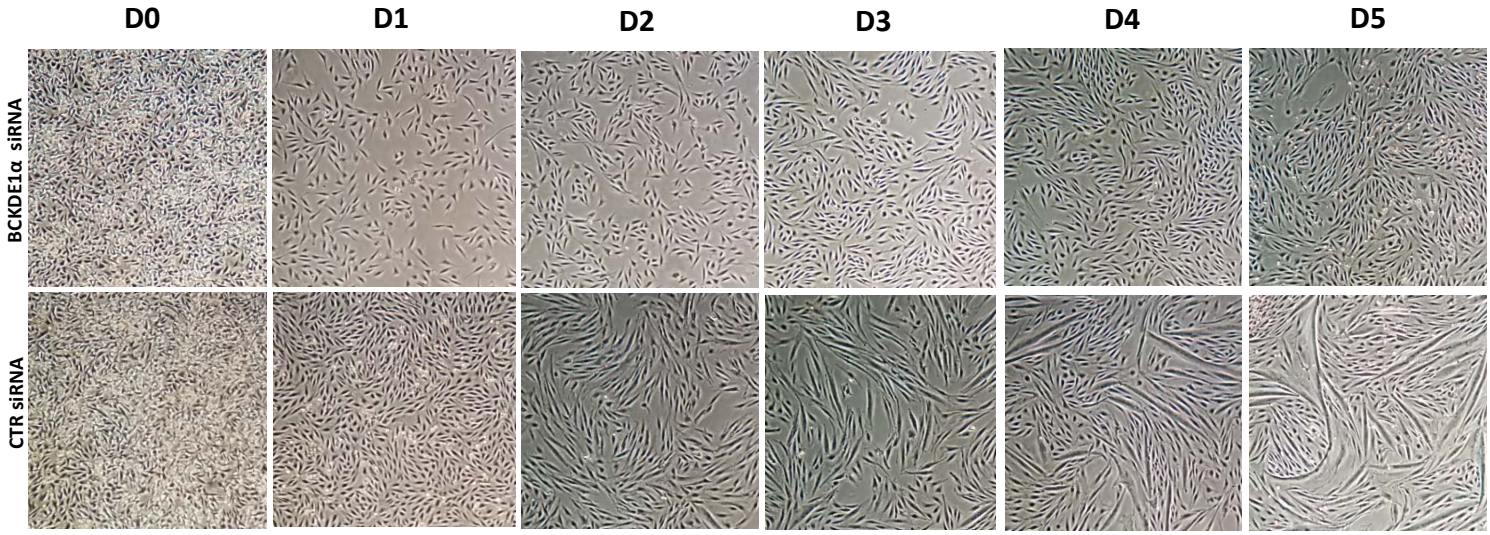


Figure 7. A) L6 rat myoblasts were transfected with BCAT2 siRNA, or control (CTR) siRNA. Myoblasts transfected with BCAT2 siRNA showed no ability to differentiate over 5 days, as indicated by the absence of myotube formation. A visible loss in cell number was also observed at D1 and D2. **B)** Cells were harvested and probed for myogenic proteins MHC-1, troponin, and myogenin. The expression of myogenic proteins was severely attenuated by BCAT2 knockdown. mTORC1 signaling was also impaired by BCAT2 knockdown. ** with bar denotes significantly different ($p<0.05$). n = 3, with 2 replicates per treatment. Data are mean ± SEM.*

FIGURE 8. ***BCKD disruption prevents L6 myoblast differentiation***

A)



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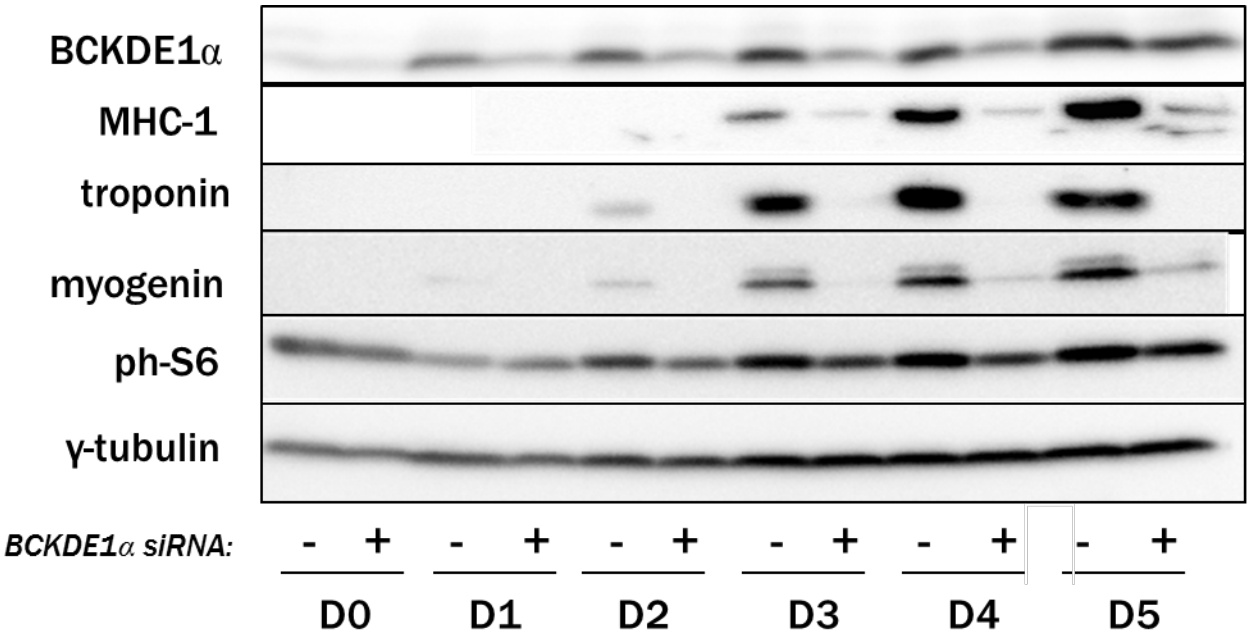


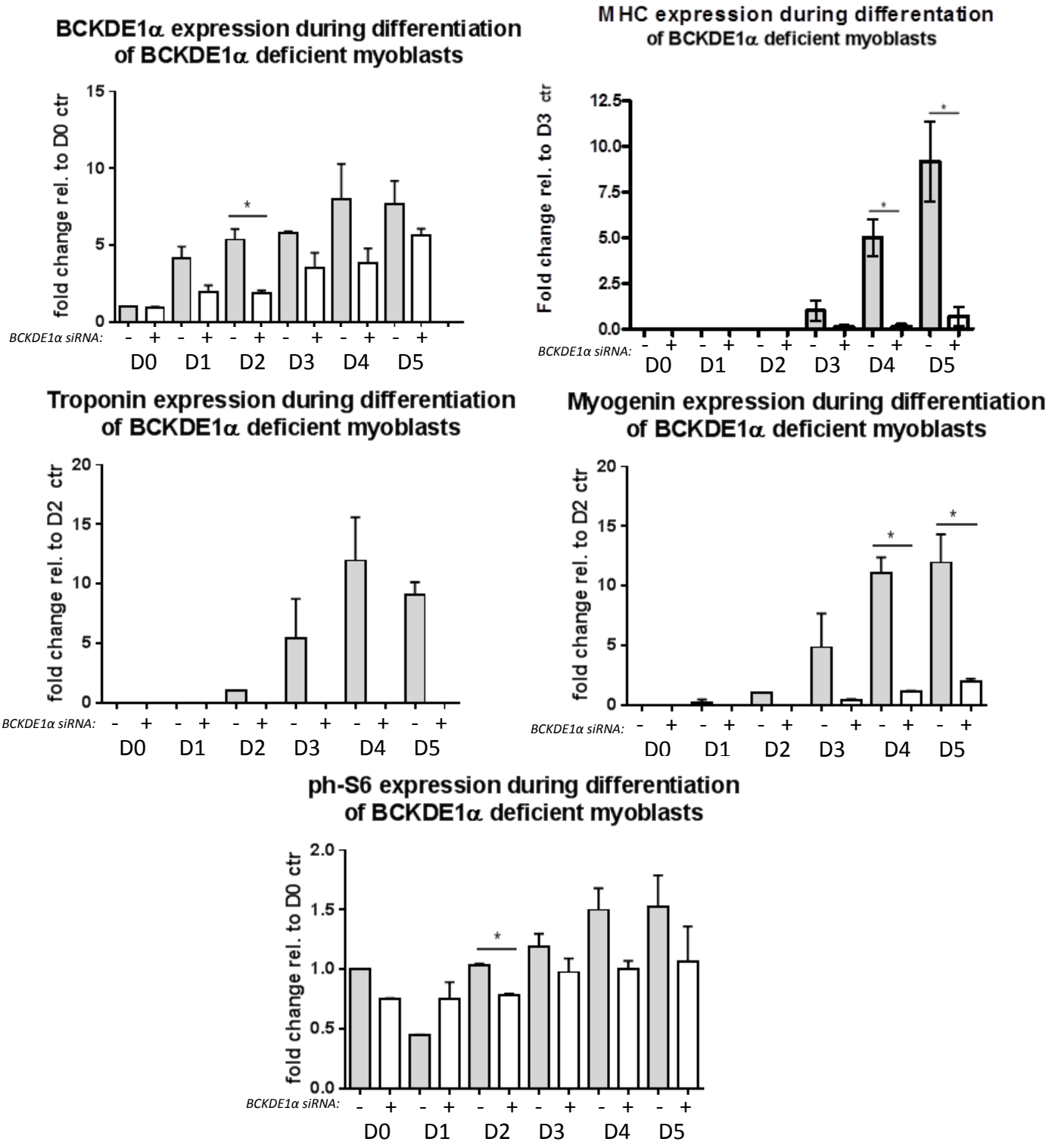
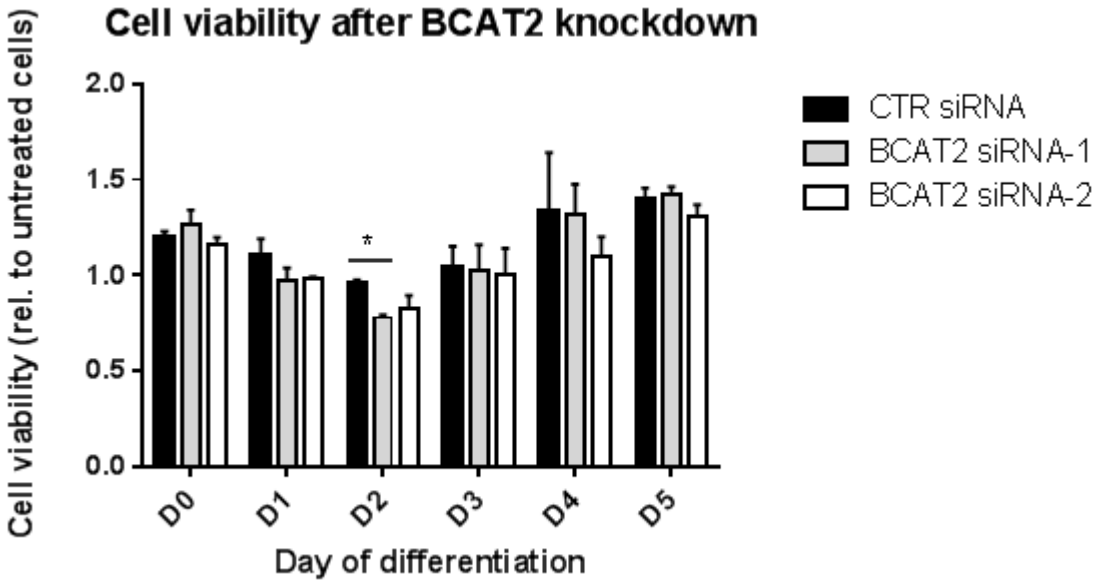


Figure 8. A) L6 rat myoblasts were transfected with BCKDE1α siRNA, or control (CTR) siRNA. Myoblasts transfected with BCKDE1α siRNA showed no ability to differentiate over 5 days, as indicated by absent myotube formation. A visible loss in cell number was also observed at D1 and D2. **B)** Cells were harvested and probed for myogenic proteins MHC-1, troponin, and myogenin. The expression of myogenic proteins was severely attenuated by BCKDE1α knockdown. mTORC1 signaling was also impaired by BCKDE1α knockdown. ** with bar denotes significantly different ($p<0.05$). n = 3, with 2 replicates per treatment. Data are mean ± SEM.*

FIGURE 9. ***BCAT2 and BCKDE1α siRNA transfection reduces cell viability***

A)



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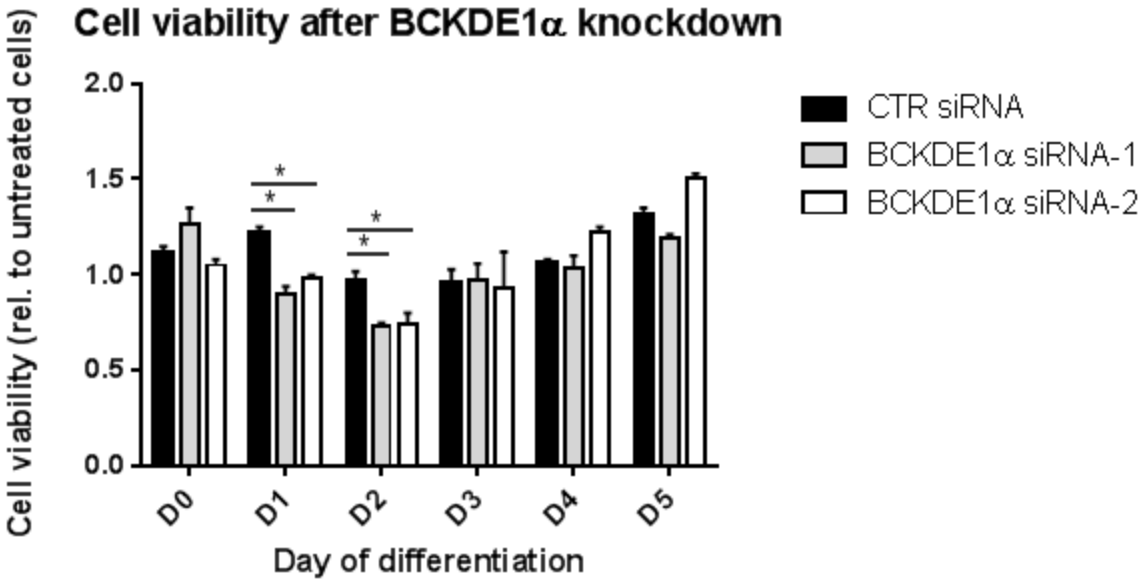
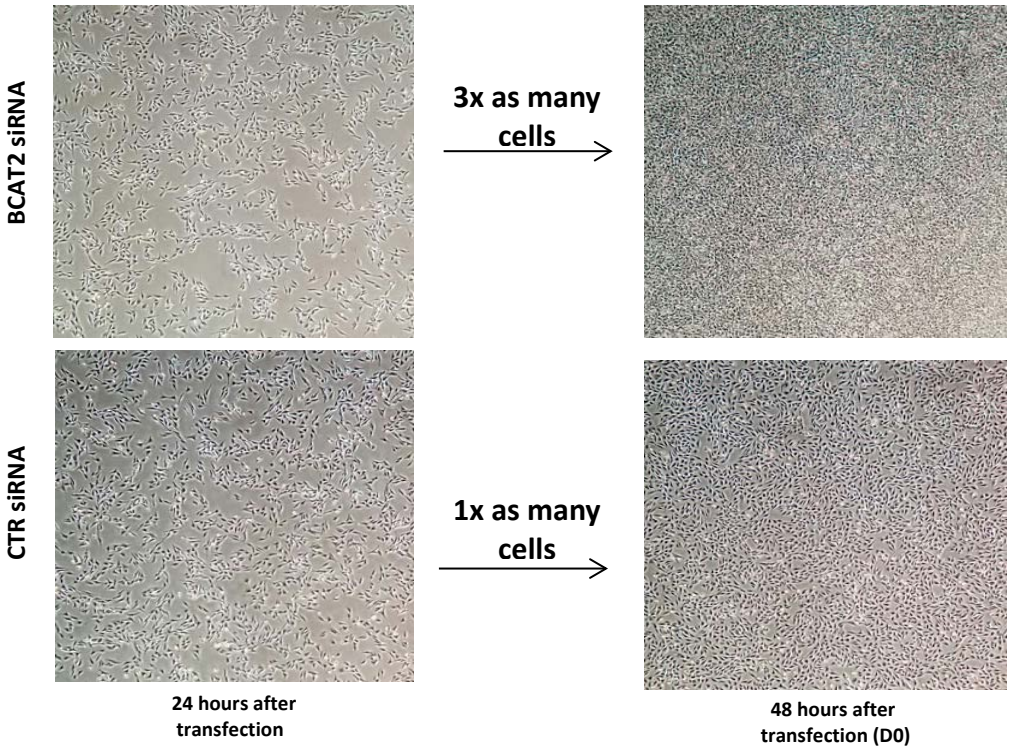


Figure 9. A) Cell viability was measured in wells transfected with two different BCAT2 siRNA oligonucleotides. Both siRNA oligos induced reduced cell viability in treatment wells at day 1 (D1) and day 2 (D2) of differentiation compared to control transfected wells. **B)** Cell viability was measured in wells transfected with two different BCKDE1α siRNA oligonucleotides. Both siRNA oligos induced reduced cell viability in treatment wells also at D1 and D2 compared to control transfected myoblasts. * *with bar denotes significantly different ($p<0.05$). n = 3, with 2 replicates per treatment. Data are mean ± SEM.*

FIGURE 10. ***Increasing the cell confluency does not rescue differentiation of BCAT2 disrupted myoblasts***

A)



B)

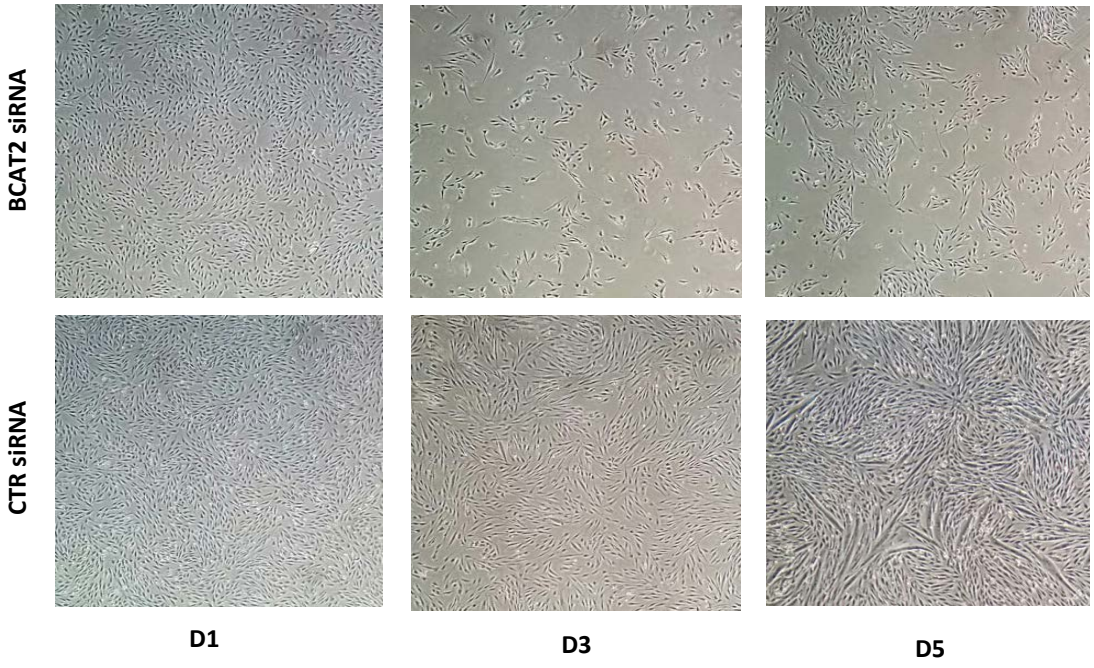
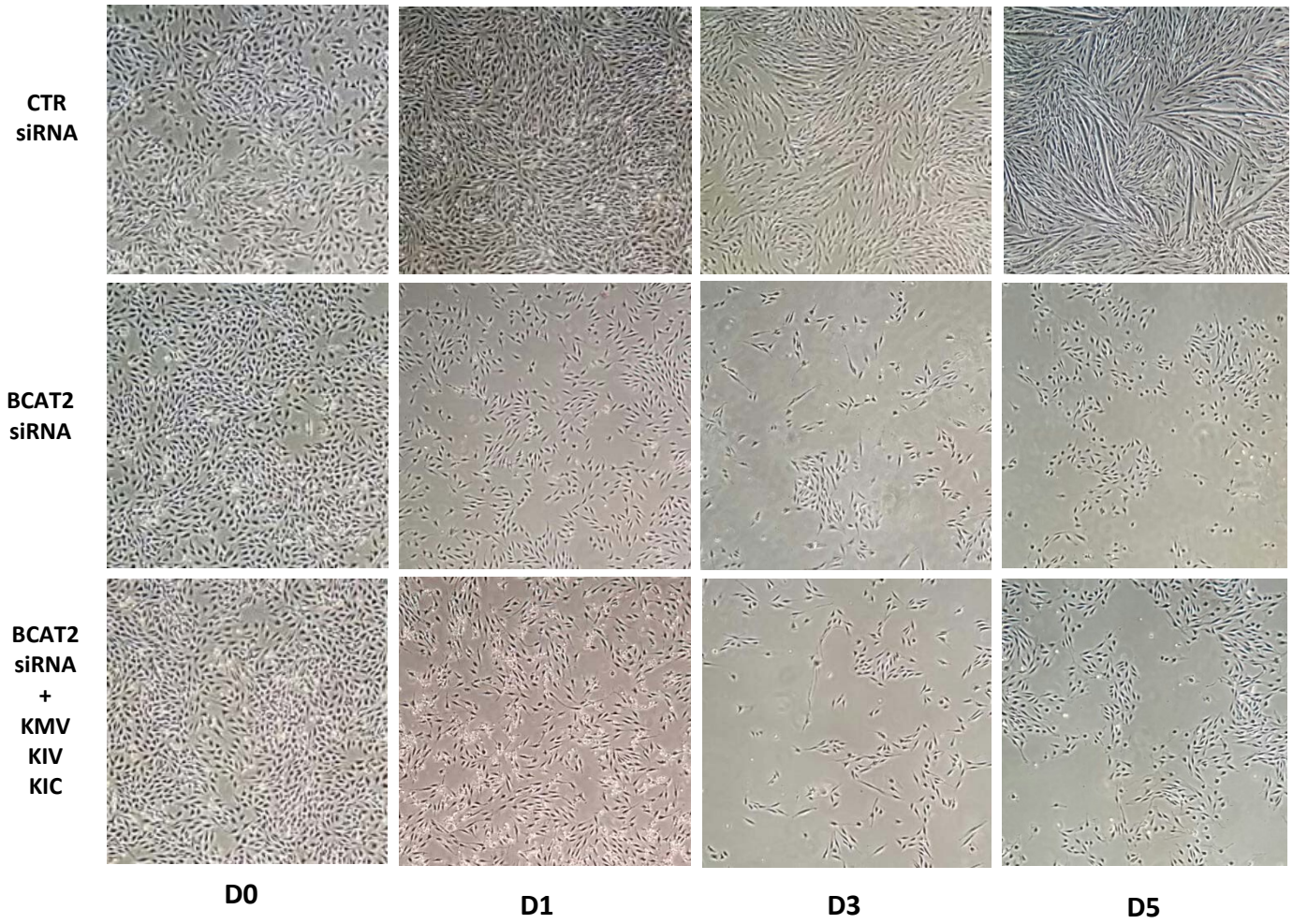


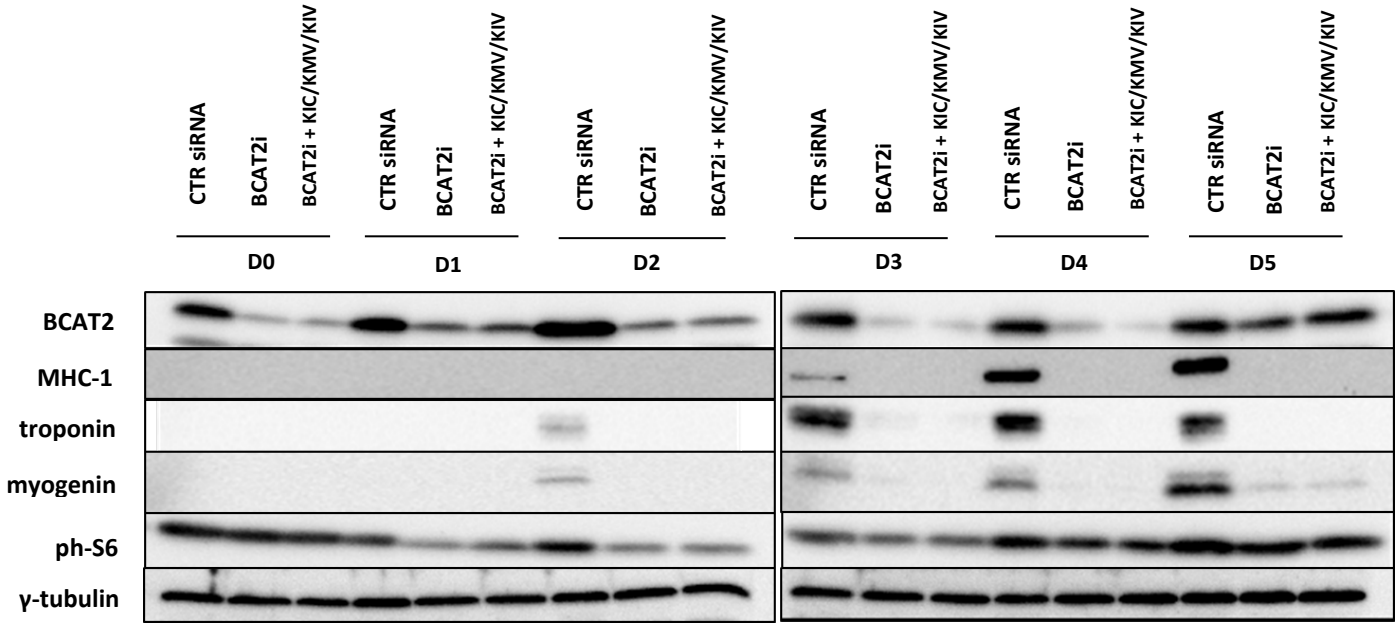
Figure 10. Increasing the cell confluency does not rescue differentiation of BCAT2 disrupted myoblasts **A)** 24 hours after being transfected, cells from 3 BCAT2 transfected wells were combined into one new well. Cells from one control well were also moved into one new well. Twenty-four hours after wells were combined, the cells were shifted to differentiation media**. B)** Increasing the cell number did not rescue myoblast differentiation by day 5 (D5), as absent myotube formation and a loss in cell number was still observed by D3 of differentiation. *Number of experiments = 2.*

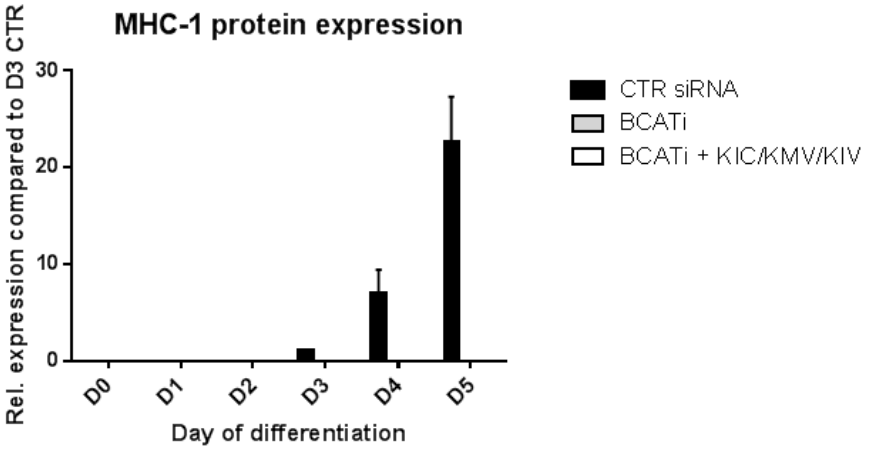
FIGURE 11. ***Supplementing BCKAs to BCAT2 disrupted myoblasts does not rescue myoblast differentiation***

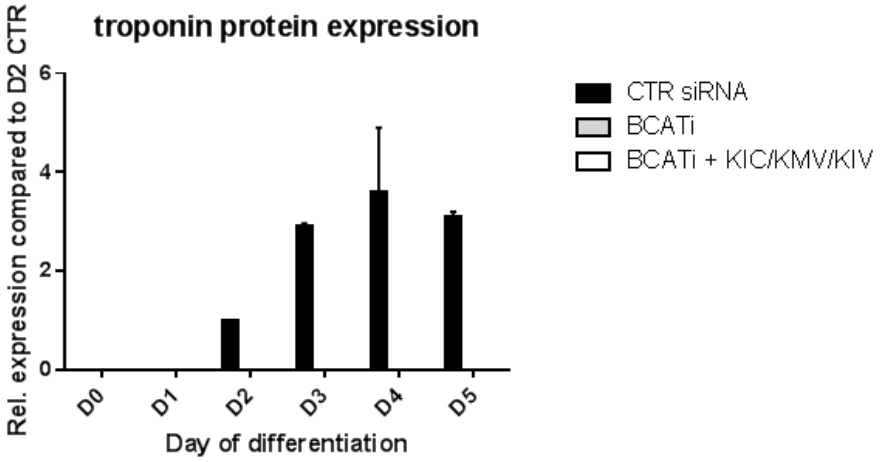
A)



B)


CTR siRNA
BCAT2i
BCAT2i + KIC/KMV/KIV
D0
D1
D2
D3
D4
D5
BCAT2
MHC-1
troponin
myogenin
ph-S6
γ-tubulin


MHC-1 protein expression
Rel. expression compared to D3 CTR
30
20
10
0
D0
D1
D2
D3
D4
D5
Day of differentiation
CTR siRNA
BCATi
BCATi + KIC/KMV/KIV


troponin protein expression
Rel. expression compared to D2 CTR
6
4
2
0
D0
D1
D2
D3
D4
D5
Day of differentiation
CTR siRNA
BCATi
BCATi + KIC/KMV/KIV

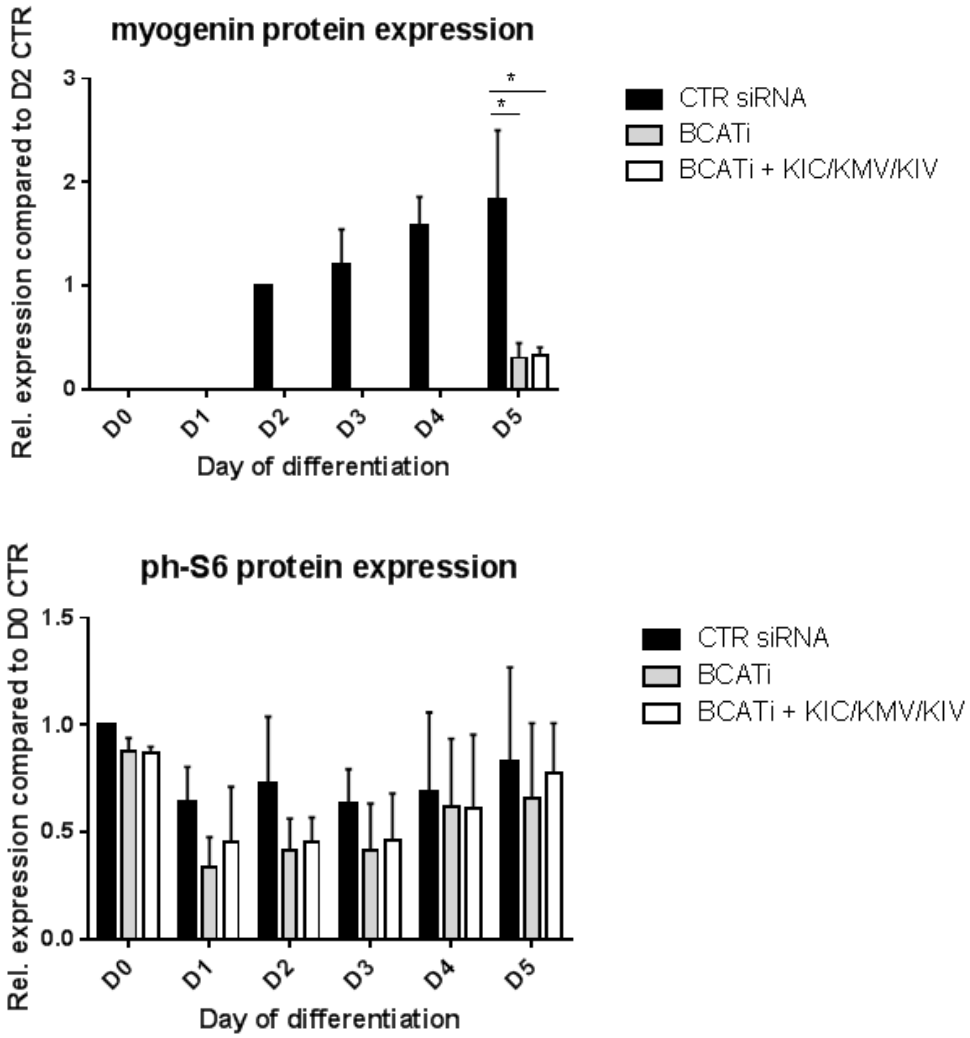


Figure 11. To determine if supplementing BCAT2 siRNA transfected myoblasts with BCKAs could rescue their differentiation, transfected myoblasts were supplemented with 200μM α-ketoisocaproate (KIC), 200μM α-ketomethylvalerate (KMV), and 200μM α-ketoisovalerate (KIV). **A)** Adding BCKAs did not rescue myoblast differentiation, as the absence of myotube formation and a loss in cell number was still observed. **B)** Expression of myogenic proteins MHC-1, troponin, and myogenin, and mTORC1 signaling, were not rescued by BCKA supplementation. BCATi = BCAT2 siRNA. * *with bar denotes significantly different ($p<0.05$). n = 3, with 2 replicates per treatment. Data are mean ± SEM.*

FIGURE 12. ***BCAT2 disruption induces programmed cell death in myoblasts***

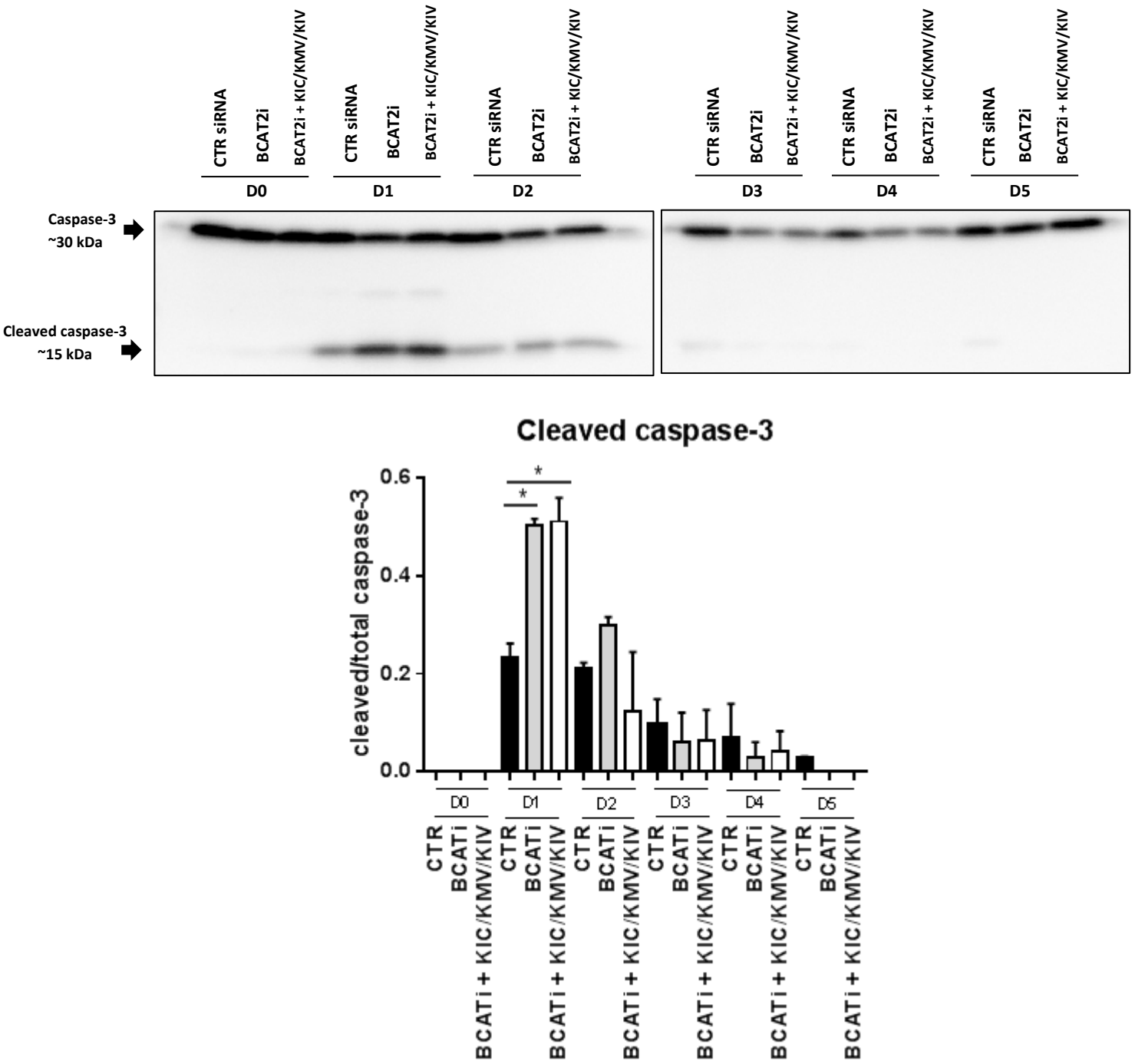


Figure 12. BCAT2 transfected myoblasts show increased apoptotic signaling as indicated by increases in caspase-3 cleavage at day 1 (D1) of differentiation. Addition of 200μM α-ketoisocaproate (KIC), 200μM α-ketomethylvalerate (KMV), and 200μM α-ketoisovalerate (KIV) to BCAT2 transfected cells did not rescue increased apoptotic signaling in myoblasts. BCATi = BCAT2 siRNA. * *with bar denotes significantly different ($p<0.05$). n = 2, with 2 replicates per treatment. Data are mean ± SEM.*

DISCUSSION

In this study, we show that the leucine metabolite KIC can positively regulate rat myoblast differentiation, even in the absence of leucine. This was evident as myotube formation, along with levels of myogenic proteins, were rescued in leucine-deprived-KIC-supplemented cells. Additionally, we observed that mTORC1 signaling was absent in leucine-deprived cells, but was ameliorated in leucine-deprived-KIC-supplemented cells. Our work has also shown that BCAT2 and BCKDE1α protein levels increase during rat myoblast differentiation. Although the pattern of mRNA expression does not follow the pattern of protein expression, levels of BCAT2 and BCKDE1α mRNA also increase at certain points of differentiation. Most interestingly, in our study we found that the disruption of BCAT2 or BCKDE1α in rat myoblasts abolishes their ability to differentiate and BCAT2 disruption induces programmed cell death. This was evident based on the finding that myoblasts from both BCAT2 and BCKDE1α knockdown did not show any visible myotube differentiation and had lower amounts of adherent cells and reduced cell viability at D1 and D2. BCAT2 deficient myoblasts also showed increased caspase-3 cleavage also at D1 and D2. Knockdown of either BCAT2 or BCKDE1α also abolished expression of myogenic proteins and mTORC1 signaling. Lastly, the essentiality of BCAT2 to rat myoblast differentiation is not due to its role in synthesizing BCKAs, as supplementing BCKAs to BCAT2 deficient myoblasts did not rescue their differentiation or apoptotic signaling.

Previous literature has shown that the leucine metabolites KIC and HMB can positively regulate skeletal muscle hypertrophy in animals and humans [8]–[13], while HMB has also been shown to positively regulate the differentiation of mammal skeletal muscle myoblasts as well [14]. However, KIC has not been shown to regulate the differentiation of skeletal muscle myoblasts to date. Our study demonstrates that indeed KIC can positively regulate skeletal

muscle myoblast differentiation, even in the absence of leucine (Fig. 4). Although, the mechanism by which KIC can rescue differentiation likely lies within the ability of the cell to convert KIC back to leucine via the BCAT2 enzyme. Leucine is an essential amino acid, thus, protein synthesis and cell growth cannot continue in leucine-deprived cells, as was also demonstrated by Averous et al. 2012 [6]. The addition of KIC to the cells likely allows them to reversibly transaminate KIC back to leucine, allowing differentiation to occur.

In our experiments, the addition of KIC also restored mTORC1 signaling as indicated by levels of phosphorylated S6 protein (Fig. 4C). Although mTORC1 signaling may be considered inhibitory to myoblast differentiation due to disruption of the IRS/PI3K/AKT pathway [121], mTORC1 signaling is still activated by leucine stimulation in myoblasts [207]. The level of S6 phosphorylation was also similar to that seen in cells cultured in control (leucine-containing) medium. This suggests that indeed KIC supplementation can rescue amino acid nutrient status in the cell in cases of leucine-deprivation.

The expression levels BCAT2 and BCKD have also never been elucidated in a time-dependent manner during myoblast differentiation. Our experiments show that levels of both these enzymes appear to increase during differentiation, with BCKD especially showing a robust significant increase in expression over the five-day differentiation period (Fig. 5A). This result also suggests that BCAA oxidation may be increased during differentiation, although this cannot be said without some uncertainty, as we did not measure enzymatic activity. mRNA expression of these proteins also showed increased levels at earlier stages of differentiation (Fig. 5B), indicating there is an upregulation in the transcription of BCAA catabolic enzymes in response to differentiation. It would be interesting to know if any of the transcriptional myogenic regulatory factors, such as MyoD, can mediate this upregulation.

Because we saw an increase in catabolic enzyme expression, we also wondered if there were corresponding changes in intracellular BCAA levels. Our experiments did not find any significant change in intracellular BCAA levels over the five-day differentiation period (Fig. 6). It should be noted that our variability between replicate experiments was quite high, yielding a large SEM in each group. Thus, further investigation is required to confirm this result. However, if this result is accurate, then it suggests that levels of BCAA remain constant in the cell, even though they may be increasingly catabolized. This suggests transport of BCAAs into the cell may also increase during differentiation to replenish BCAAs catabolized by BCAT2/BCKD. This theory is supported by findings by Moran et al. 2002 [208], who have shown that transcription of amino acid transporters increases during mouse myoblast differentiation.

To establish the importance of BCAA catabolism to rat myoblast differentiation, we disrupted both BCAT2 and BCKDE1α, and observed a severe impairment in myoblast differentiation (Fig. 7 & 8). Not only did these cells not differentiate, but BCAT2 knockdown cells also had increased apoptotic signaling, and both BCAT2 and BKCDE1α knockdown cells resulted in visible cell death/loss around D1 and D2. The amelioration of cell death after D1 and D2 was likely due to the effect of siRNA treatment subsiding, as after D3 protein levels of both BCAT2 and BCKDE1α in knockdowns start to return (Fig. 7B & 8B). Adding more cells to knockdown treatments also did not rescue myoblast fusion, as we still observed reduced cell number (Fig. 10). Thus, the disruption of either of these enzymes in rat myoblasts induces severe detrimental effects that impair myoblast survival, proliferation, and differentiation.

BCAT2 and BCKDE1α knockdown also impaired mTORC1 signaling, as indicated by lower levels of phosphorylated S6 protein (Fig. 7B & 8B). The role of mTORC1 signaling in myoblast differentiation is controversial, as studies suggest that increased mTORC1 signaling is

a positive regulator of skeletal muscle hypertrophy [109], but a negative regulator of myoblast differentiation [121]. We find that mTORC1 signaling is reduced in BCAT2 and BCKDE1α knockdown cells, suggesting that mTORC1 signaling is likely not responsible for disrupting the differentiation of these myoblasts. These results contradict those reported by She et al. 2007 [198], as they found BCAT2 knockdown mice had elevated levels of mTORC1 signaling in muscle tissue. Additionally, they attributed the increase in mTORC1 signaling to elevated plasma BCAAs caused by BCAT2 disruption. We cannot explain the difference in mTORC1 signaling between their model of existing muscle tissue and our model muscle differentiation. However, these results confirm the role of mTORC1 is ambiguous and likely inconsistent when comparing the regulation of muscle differentiation to the regulation of developed muscle tissue.

Why are these enzymes that mediate BCAA catabolism so important to myoblast differentiation? As discussed in the literature review section of this thesis, BCAT2 serves as first enzyme that catabolizes BCAAs to produce corresponding α-keto acids (BCKAs), which can be further metabolized by the BCKD complex. BCAT2 also simultaneously reversibly aminates α-ketoglutarate to produce glutamate, which can then be used for glutamine synthesis. To test whether BCAT2 deficient myoblasts did not differentiate due to disrupted BCKA production, we supplemented these BCAT2 deficient myoblasts with BCKAs (Fig. 11). However, BCKA supplementation did not rescue myoblast differentiation, myogenic protein expression, or apoptotic signaling (Fig. 11 & 12). This suggests that the importance of BCAT2 to rat myoblast differentiation is not due to its role in producing the BCKAs KIC, KMV, KIV, although we cannot rule this out completely as BCKA production may be required in conjunction with some other BCAT2-mediated process. The finding that BCKAs could not rescue differentiation was surprising, as there is convincing evidence that BCKAs, particularly KIC, can regulate many

aspects of skeletal muscle anabolism and muscle differentiation as described earlier. If it is not BCKAs themselves which promote anabolic mechanisms during differentiation, then it is possible that BCKAs facilitate other BCAT2-mediated reactions which are responsible for inducing anabolism. Conclusively, BCKA production may not be the major anabolic component of BCAA catabolism.

Since BCKA production was ruled out, another possibility is the role of BCAT2 in the glutamine/alanine cycle. BCAT2 can use BCAAs to make glutamate, or conversely, it can use BCKAs to produce α-ketoglutarate (Fig. 2). In separate reactions, glutamate can also be a substrate to produce alanine via the alanine transaminase enzyme, or be converted to glutamine via glutamine synthetase (Fig. 2). She et al. 2010 [15] demonstrated that BCAT2 knockout mice have 43-79% declines in muscle concentration of alanine, glutamate, and glutamine. Glutamine is an important precursor for nucleotide synthesis [209], and can positively regulate protein turnover [210]. Alanine is an important gluconeogenic substrate [211]. However, the lack of myoblast differentiation is likely not due to alanine deficiency as in our study we showed that alanine deprivation did not affect differentiation (Fig. 4). Thus, it is possible BCAT2 disrupted cells do not differentiate due to reduced intracellular glutamine formation. Glutamine has also been shown to be responsible for mTORC1 activation [3], and reduced levels of glutamine may explain why we observed lower mTORC1 signaling in BCAT2/BCKDE1α knockdowns. However, it should be noted that the differentiation media myoblasts are cultured in already contains 292 mg/L of L-glutamine, although this may not be enough to rescue glutamine deficiency and/or not enough of it can enter the cell.

There is also a possibility that glutamate and α-ketoglutarate, which can both be interchangeably produced by BCAT2 and alanine transaminase, become limiting in BCAT2

knockdown cells (Fig. 2). α-ketoglutarate production from glutamate has been shown to be a major anaplerotic reaction during exercise which causes expansion of the TCA cycle intermediate pool [212]. Thus, BCAT2-mediated α-ketoglutarate production may contribute to adequate regulation of the TCA cycle and energy homeostasis. However, this may still not explain why BCAT2 deficient myoblast did not differentiate. This is because the differentiation media also contains 75 mg/L L-glutamic acid (glutamate), and even though BCAT2 is not available to convert glutamate to α-ketoglutarate, alanine transaminase was not disrupted and thus should still be able to produce α-ketoglutarate from glutamate (Fig. 2). However, there remains a possibility that alanine transaminase alone cannot keep up with the demand of glutamate/α-ketoglutarate conversion required. Additionally, the amount of glutamate in the differentiation media may not be sufficient enough to rescue glutamate deficiency in knockdown cells.

As we observed, BCKDE1α deficient myoblasts also do not differentiate. The disruption of BCKDE1α activity should theoretically cause an elevation in the BCAA and BCKA intracellular concentrations [202]. High levels of BCAAs/BCKAs have been shown to be responsible for causing neuro-degeneration in patients with maple-syrup urine disease (MSUD) [213]. High levels of KIC have also been shown to induce apoptosis of glial and neuronal cells in culture [214]. Jouvet et al. 2000 [214] also showed that high doses of KIC administration caused impaired cellular respiration as marked by reduced oxygen consumption and mitochondrial death [214]. Another study also showed that the administration of leucine to neuronal cells that are overexpressing the BCKD kinase (thus reducing BCKD activity) results in cell death [16]. Hence, it is possible that the accumulation of BCKAs including KIC in BCKDE1α disrupted myoblasts induces similar cytotoxic effects that cause apoptosis-mediated

death, along with reduced proliferation, and differentiation. An alternate reason that may explain why BCKDE1α deficient myoblast do not differentiate is the possibility that the glucogenic/ketogenic substrates that are formed from BCKA oxidation are impaired. Substrates including isovaleryl CoA, succinyl CoA, and acetyl CoA can be produced from BCKA oxidation via BCKD, and can enter the TCA cycle (Fig. 3). Thus, the disruption of BCKD in myoblasts could theoretically cause decreased production of these substrates in myoblasts, although the contribution of BCKD-derived CoA substrates to anaplerosis and energy production is not known. Additionally, amino acid catabolism is believed to contribute only 10-15% to whole body energy production [215], thus it is not likely that energy production is impaired by BCKD disruption. However, further investigation is warranted to determine what the contribution of these substrates produced from BCKAs /BCAAs are to muscle energy metabolism.

Lastly, the increase in apoptotic signaling at D1 and D2 in BCAT2 knockdown cells likely explains the reduction in cell number, cell viability, and the absence of differentiation. Indeed, increased apoptotic signaling has been associated with cell death [214], along with the suppression of cell proliferation [216] and differentiation [217]. As discussed, there are a number of factors that may explain why disruption of these enzymes inhibits differentiation, and causes programmed cell death of myoblasts. However, further research is required to determine which factors are the likely culprits.

CONCLUSION

In conclusion, our study shows that:

1. The branched chain amino acid leucine is essential to myoblast differentiation. The leucine metabolite KIC can also promote myoblast differentiation, even under conditions of leucine deprivation. This result demonstrates that BCAAs and their corresponding metabolites have regulatory roles in differentiation.
2. Expression of BCAA catabolic enzymes BCAT2 and BCKD increase during differentiation, suggesting that BCAA catabolism has an important role during myoblast differentiation.
3. Disruption of BCAA catabolic enzymes BCAT2 and BCKD results in programmed cell death of myoblasts and impairs their ability to differentiate.
4. The impairment of BCAT2 deficient myoblasts is not due to reduced branched-chain α-keto acid production.

Our study demonstrates that BCAA catabolism plays an important role in the regulation of skeletal muscle development. The significance of this data is that it implicates the BCAA catabolic pathway as a critical regulator of skeletal muscle formation and regeneration. BCAT2 and BCKD are targets that are essential for skeletal muscle development. Thus, developing therapies that ensure the adequate function of these enzymes may promote skeletal muscle generation in patients with myopathic diseases. We also show that for individuals who have mutations in the BCAT2 protein, the intake of branched-chain α-keto acids likely would not ameliorate compromises in skeletal muscle development.

FUTURE WORK

1. **Measure intracellular BCAA/BCKA concentrations in BCAT2 and BCKDE1α deficient cells**

   The knockdown of BCAT2 should theoretically cause an accumulation of intracellular BCAAs leucine, isoleucine, and valine. The knockdown of BCKD should similarly cause an accumulation of BCKAs KIC, KMV, and KIV. Confirming the elevation of these substrates would support the notion that it may be high levels of BCAAs/BCKAs that induce cell toxicity and impair differentiation.

2. **Measure enzyme activity of BCAT2 and BCKD during differentiation**

   We observed an increase in BCAT2 and BCKDE1α expression during myoblast differentiation. This result suggests there likely is an increase in BCAA catabolism during differentiation. To support this notion, finding an increase in enzyme activity would support the idea that BCAA catabolism is increased during differentiation.

3. **Supplement BCAT2 deficient myoblasts with exogenous L-glutamine, L-glutamate, and α-ketoglutarate**

   The impairment of the BCAT2 enzyme should theoretically impair the production of intracellular glutamine, glutamate, and α-ketoglutarate. To determine if this impairment is what causes the disruption of BCAT2-deficient myoblast differentiation, we can supplement the differentiation media with these substrates to determine if it can rescue differentiation.

4. Determine the contribution of BCKA catabolism to anaplerosis

To determine if BCKD deficient myoblasts do not differentiate due to disrupted energy metabolism, we would need to measure the proportion of CoA derivatives made from BCAAs that end up in the TCA cycle. One could do this by introducing [^{14}C] labeled BCAAs into the cell culture media. Following this, you can then measure label flow through the TCA cycle and determine the amount of [^{14}C] labeled TCA substrates that are produced, such as [^{14}C]-succinate or [^{14}C]-malate.

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DETAILED LABORATORY METHODS AND PROTOCOLS

I. Cell passing and differentiation protocol

Reagents:

Growth medium (GM)

AMEM (Wisent #310-010-CL)
10% FBS (Gibco #12484-028)
1% antibiotic (Wisent #450-201-EL)

Differentiation medium (DM)

AMEM (Wisent #310-010-CL)
2% Horse serum (Gibco #26050088)
1% antibiotic

Phosphate buffered saline (PBS) (Wisent #311-010-CL)

1. **Thawing cells**
    a. Retrieve a vial from the -80°C freezer
    b. Hand thaw the vial
    c. Dilute the cells in a 10 mls of growth medium in a 10-cm plate
    d. Mix the cells well by pipetting up and down and swirling the plate
    e. Put the plate in the 37°C incubator
    f. Split the cells the next day
2. **Passing cells**
    a. When cells have reached ~70% confluency it is time to split (about 48 hours later)
    b. Rinse the plate with 5 mls warm PBS
    c. Add 1 ml trypsin, mix the trypsin around the plate
    d. Put the plate in the 37°C incubator for 2 mins
    e. Remove the plate, tap the bottom of the plate lightly to detach remaining cells, check the plate under the microscope to ensure cells have detached
    f. Quench cells with 5 mls growth medium
    g. Add 0.65-1.0 ml of cells to new plate containing 10 mls growth medium
    h. Mix well by pipetting and swirling, then keep the plate in the 37°C incubator
3. **Differentiating cells**
    a. Grow the cells to 90-100% confluency
    b. Remove the growth medium, and place the cells in 10 mls of differentiation media (DM) for a 10 cm plate, or 2 mls for a 6 well plate
    c. Change the DM every day if possible, and every other day at least

II. Cell harvesting/lysis protocol

Lysis buffer formulation (in ddH_20):

a. 1mM EDTA
b. 2% sodium dodecyl sulfate (SDS)
c. 25 mM Tris-HCL pH 7.5
d. 10 µl/ml protease inhibitor (Sigma #P8340)
e. 10 µl/ml phosphatase inhibitor (Sigma #P5726)
f. 1 mM DTT

To harvest cells:

1. Suck out the old differentiation media
2. Rinse the well with PBS (2 mls PBS for 6-well plate, 5 mls PBS for 10-cm plate)
3. Add lysis buffer (75 µl for 6-well plate, 150 µl for 10-cm plate)
4. Use a rubber policeman to spread the lysis buffer around, detach the cells, then collect the lysate in a corner of a well
5. Use a syringe to repeatedly aspirate and expel the lysate, this step is crucial
6. Place the lysate in 1-ml tube and store at -20°C

III. Western blot protocol

STEP 1: Determine the protein concentration using the Pierce™ BCA protein assay kit (Thermo #23225)

STEP 2: Add 4x laemmli sample buffer dye to each sample

1. **Make the 4x laemmli sample buffer dye**
    a. 4ml glycerol
    b. 0.8g SDS
    c. 400 µl of 1M Tris pH 8.0
    d. 400 µl of 0.5M EDTA
    e. Add ddH_20 up to 8 ml
    f. 0.1 mg Bromophenol blue
    g. When ready to use, add β-mercaptoethanol in a 4 part dye: 1 part BME ratio
2. **Add the sample buffer to your samples**
    a. Add the dye in a 3 part sample: 1 part dye ratio
    b. Vortex
    c. Boil the samples at 95°C for 5 mins
    d. Vortex and centrifuge

STEP 3: Make the gels

Buffers:

1. **4X resolving buffer**
    a. 181.5 Tris base
    b. 600 mL water
    c. pH to 8.8 with HCL
    d. volume up to 1 L
    e. store at 4°C
2. **Resolving acrylamide**

a. 0.76g BIS
b. 300 ml acryl 40 (Amresco #0132)
c. 100 mL water
d. Cover bottle with foil and store at 4°C

3. **4X stacking buffer**
    a. 24g Tris base
    b. 240 ml water
    c. pH to 6.8 with HCL
    d. volume up to 400 ml
    e. store at 4°C
4. **Stacking acrylamide**
    a. 1.6g BIS
    b. 150 ml acryl-40
    c. 50 ml water
    d. Cover bottle with foil and store at 4°C
5. **Gel electrophoresis running buffer (10X)**
    a. 120g Tris base
    b. 576 g Glycine
    c. 40g SDS
    d. Volume up to 4L
    e. When ready to use, dilute to 1X in single distilled water in a 1 part water:9 parts 10X buffer ratio
6. **Transfer buffer (10X)**
    a. 30.3 g Tris base
    b. 144.1 g Glycine
    c. Volume to 1 liter
    d. When ready to use, dilute to 1X by mixing:
        i. 100 ml 10X stock
        ii. 700 ml single distilled water
        iii. 200 ml methanol
7. **Ponceau S stain**
    a. 0.1% (w/v) Ponceau S
    b. 0.5% (w/v) glacial acetic acid
8. **Blocking buffer**
    a. 5% (w/v) non-fat milk in TBST
9. **TBST**
    a. 60.57g Tris Base
    b. 87.66g NaCl
    c. 10 mL Tween-20 (Amaresco #M147)
    d. Volume up to 10 L

Procedure to make gels:

1. Clean glass plates with ethanol, ddH_20, and Kimwipes™
2. Assemble the plates into the cast assembly
3. Pour water into the plate assembly to ensure there is no water leakage
4. Empty out the water
5. Mark 1cm below the glass cover plate
6. Make the resolving buffer (refer to chart below)
7. Pour the resolving buffer to the mark on the cover plate

8. Pour a layer of ddH_20 on top of the resolving buffer, and let solidify for 40 mins
9. Once solidified, pour out the excess water from the gel
10. Make the stacking buffer (refer to chart below)
11. Pour the stacking buffer onto the gel, to the rim
12. Insert a well comb
13. Let solidify for about 30 minutes
14. After solidifying, remove the glass plate from the cast assembly, and insert the plates into the electrode assembly

Resolving gel (for one gel)

	10% gel	**15% gel**
4X resolving buffer	1.99 ml	1.99 ml
Resolving acrylamide	2.65 ml	3.98 ml
ddH_20	3.32 ml	1.99 ml
10% APS	40 µl	40 µl
TEMED	8 µl	8 µl

Stacking gel (for one gel)

	10% gel	**15% gel**
4X stacking buffer	0.66 ml	0.66 ml
Stacking acrylamide	0.432 ml	0.432 ml
ddH_20	1.882 ml	1.882 ml
10% APS	19.8 µl	19.8 µl
TEMED	2.6 µl	2.6 µl

Loading the gels:

1. Put the electrode assemblies into the corresponding plastic container
2. Fill the wells up with 1X gel running buffer to ensure the gel remains moist
3. Fill up the plastic container about halfway with running buffer
4. Load the first well of the gel with 5 µl protein ladder (Biorad #161-0374)
5. Load the remaining wells with your samples
6. Fill the electrode assembly with gel running buffer to the top
7. Place a stir bar in the running unit
8. Run the gel initially at 60V until the samples have run through the stacking gel
9. Run the gel at 100V until the dye front has run out of the gel

STEP 4: transfer the proteins to membrane

Transferring the gels to PVDF membranes:

1. Ensure you have enough 1X transfer solution, filter paper, and PVDF (Bio rad #162-0177) membranes cut
2. Place a transfer casket in a tray full of 1X transfer buffer, ensure the red/white side is down
3. Place one sponge soaked in buffer on the red/white side
4. Place one filter paper soaked in buffer on top of sponge, squeegee out any air bubbles
5. Place PVDF membrane on filter paper, squeegee out any air bubbles
6. Remove gel from glass casket, cut off the stacking gel
7. Place the gel on top of your membrane in the transfer casket, make sure gel is thoroughly drenched in transfer buffer, squeegee out any air bubbles
8. Place a filter paper soaked in transfer buffer on top of the membrane, squeegee out any air bubbles
9. Place a sponge soaked in transfer buffer on top of the filter paper, squeegee out any air bubbles
10. Close the transfer casket securely, and place the casket into the transfer unit
    a. Make sure the white/red side is facing the positive terminal, the black side should face the negative terminal
11. Place a stir bar, and run at 23V overnight 4°C. Alternatively, run for 2.5 hours at ~300 mA, making sure unit stays cool.

STEP 5: Obtaining an image

Immunoblotting procedure:

1. Once the gels have transferred, remove the membranes and keep them in TBST
2. Remove TBST, add Ponceau-S solution for 8 minutes to see protein
3. Mark the protein ladder with a pen
4. Cut the membrane to obtain portions you wish to blot
5. Rinse the membranes with TBST
6. Incubate the membranes in blocking buffer
7. Add primary antibody (diluted in 2% (w/v) BSA in TBST)
8. Incubate overnight at 4°C
9. The next day, rinse 2x quickly with TBST
10. Rinse 3X for 5 mins each with TBST
11. Add secondary antibody (diluted 1:10,000 in blocking buffer)
12. Incubate for 3 hours at room temp
13. Rinse 2X quickly with TBST
14. Rinse 3X for 5 mins each with TBST
15. Add 1-ml of immunblot solution to membrane (500µl solution A + 500µl solution B) (Millipore #WBKLS0500)
16. Incubate for approximately 1-10 minutes depending on how strong the signal usually comes out
17. Image for luminescence using Kodak Imagestation™ 4000mm Pro
18. Afterwards, rinse membranes with TBST and store at 4°C

IV. Leucine deprivation + KIC rescue experiment

STEP 1: Make the differentiation media

1. Dissolve 10.04g RPMI 1640 powder (US Biologicals R8999-03) in 800 mls ddH_20 water. This RPMI is missing glutamine, sodium bicarbonate, alanine, and leucine.
2. Add L-glutamine (233.6 mg)
3. Add sodium bicarbonate (1.76 g)
4. Adjust pH to 7.2 with HCL
5. Aliquot the solution into the separate bottles. Then supplement with amino acids. The final concentration of amino acids is meant to mimic those of AMEM.
    a. BOTTLE 1 = CTR DM
        i. Add 200 mls RPMI solution
        ii. Add 10.5 mg L-leucine
        iii. Add 5 mg L-alanine
    b. BOTTLE 2 = DM w/o Alanine
        i. Add 200 mls RPMI solution
        ii. Add 10.5 mg L-leucine
    c. BOTTLE 3 = DM w/o Leucine
        i. Add 350 mls RPMI solution
        ii. Add 8.75 mg L-alanine
6. Add the horse serum and antibiotic to each solution
    a. CTR DM
        i. 4.08 mls HS
        ii. 1 ml AB
    b. DM w/o alanine
        i. 4.08 mls HS
        ii. 1 ml AB
    c. DM w/o leucine
        i. 7.14 mls HS
        ii. 1.75 mls AB

STEP 2: Prepare α-ketoisocaproate (KIC) (Sigma #K0629)

1. Dissolve KIC in ddH_20 to obtain a concentration of 25 mg/ml
2. Filter the solution through a syringe filter to sterilize

STEP 3: Make the plates

1. Seed 150,000 cells in 6 well plates
2. Grow to 90-100% confluency
3. When plates are ready to be differentiated, suck out the GM, then add the respective media we previously made
4. You will have two groups that use the leucine-free DM, however, you will supplement one of those groups with KIC

CTR DM	DM w/o alanine	DM w/o leucine	DM w/o leucine + KIC

5. Add KIC to the last group to make the final concentration of KIC 200 μM in the 2 mls of leucine-free DM
6. Change the DM of each group every day, and supplement KIC to the last group every day
7. Harvest the wells with regular lysis buffer

V. mRNA isolation and qPCR protocol

1. mRNA isolation

a. Grow cells in 10-cm plates to 90-100% confluency
b. Upon reaching confluency, shift the cells to DM
c. When it is time to harvest the cells, suck out the DM, and add 1-ml of Trizol reagent (Thermo Fisher #15596026) to each plate
d. Use a 23-guage syringe to spread the Trizol around the plate and to repeatedly aspirate the lysate
e. Place the lysate in 1-ml tubes, then follow the rest of the instructions provided in the kit (Thermo-Fisher #12183018A).
f. Store RNA at -80°C

2. Check RNA purity

a. Dilute 5 μl of RNA in 200 μl of 10mM Tris buffer pH 7.4 (in sterile ddH_20)
b. Place solution in a cuvette and read in a DNA/RNA spectrophotometer. Record the RNA concentration and 260/280 numbers.
c. The 260/280 should be > 1.8

3. cDNA synthesis

a. To synthesize cDNA, simply follow the instructions provided with the kit (Bio-Rad #1725038)
b. You should load to same amount of RNA from different samples into each reaction. Ideally if you have enough RNA, you can load 7μg, but using smaller concentrations will also work well.
c. Incubate the reaction mixture on the hot plate at 42°C for 60 mins, not 30 mins like mentioned in the instructions
d. Turn up the hot plate to 85°C and let samples incubate for 5 mins
e. Centrifuge sample tubes after synthesis, and store at -20°C

4. qPCR

a. Follow the instructions according to the kit (Bio-Rad #1725271)
b. Use opaque white strip tubes, and clear strip caps
c. Make 20 μl reactions for each sample with the following components:

	For each 20μl reaction
Forward primer	0.75 μl
Reverse primer	0.75 μl
cDNA sample	2 μl
SSOAdvanced SYBRgreen supermix	10 μl
ddH_20 sterile autoclaved	6.5 μl

d. Make sure strip cap is on tightly
e. Vortex strip tubes to mix sample
f. Centrifuge the strip tubes
g. Run the samples in the qPCR real time machine at the following settings:

Enzyme activation	95°C / 2:00 min
Denaturation	95°C / 0:10 sec
Annealing	60°C / 0:20 sec
	40x cycles
	95°C / 0:10 sec
In .05°C increments	65°C / 0:31 sec
	95°C / 0:05 sec

VI. Determining intracellular BCAA concentrations

STEP 1: To determine intracellular BCAA concentrations, we must first harvest the cell culture sample with a unique lysis buffer. This lysis buffer does not contain any SDS, which would otherwise interfere with the enzyme leucine dehydrogenase used in the assay.

Lysis buffer reagents

1. Triton X-100
2. 1.0M Tris-HCL pH 7.5
3. 1.0M NaCl
4. 500mM EDTA
5. 250mM EGTA
6. 200mM Sodium Pyrophosphate
7. 200mM β-glycerophosphate
8. Leupeptin hemisulfate
9. 200mM Sodium orthovanadate
10. PMSF

Final concentrations of reagents in lysis buffer

Prepare the lysis buffer according to these final concentrations. Aliquot and store at -20°C. Immediately before use, add 1µg of PMSF per ml of lysis buffer.

1. **10µl/ml** Triton X-100
2. **20mM** Tris-HCL pH 7.5
3. **150mM** NaCl
4. **1mM** EDTA
5. **1mM** EGTA
6. **2.5mM** Sodium Pyrophosphate
7. **1mM** β-glycerophosphate
8. **1µg/ml** Leupeptin Hemisulfate
9. **1mM** Sodium Orthovanadate
10. Add **1µg/ml** PMSF immediately before use

*Adjust to final volume with ddH_20.

*For Leupeptin and PMSF, first dilute the powders in ddH_20 to make a stock solution. Then from the stock solution calculate how much you need to obtain Xµg.

STEP 2: We will now harvest the sample with the prepared lysis buffer.

1. Rinse the well TWICE with cold PBS
2. Add 75µl of lysis buffer to the well (for 6-well plates) or 150µl of lysis buffer (for 10cm plates)
3. Scrape the well and collect the sample in a 1-ml syringe
4. Release the sample into a 1.5ml tube and repeatedly aspirate and expel the sample with the syringe to breakdown the cell lysate
5. Store at -20°C

STEP 3: Now we centrifuge all the samples to remove any cell debris.

1. Centrifuge samples at 14 x g for 5 minutes
2. Collect the supernatant and place in a new sample tube
3. Store at -20°C for later use or at 4°C if you will use the sample in the same day

STEP 4: BCAA assay theory.

This assay allows us to determine the concentration of all BCAAs in our sample. This assay relies on an enzyme known as Leucine dehydrogenase (LDH). LDH is able to catabolize leucine, valine, or isoleucine according to the following reaction:

After mixing our sample with LDH and NAD^+, we can measure the amount of NADH produced by measuring absorbance at 340nM. After measuring the amount of absorbance, we can determine the amount of BCAA in the sample using a standard curve.

Since the amount of BCAAs in our sample is relatively small, instead of measuring absorbance we used fluorescence which is a more sensitive procedure. NADH absorbs light of wavelength 340±30 nM and emits fluorescence at 460±50 nM.

STEP 4a: Prepare the following buffers in ddH20. Buffers must be made fresh on the day of the assay. Store all buffers at 4°C.

Reagents

1. KH_2PO_4
2. Na_2HPO_4
3. Bovine Serum Albumin (BSA)
4. Na_2CO_3
5. K_2HPO_4
6. EDTA
7. β-mercaptoethanol

Buffers

1. **0.01M KH_2PO_4 pH 6.0** (for dilution of stock BCAA standards)
    a. Dissolve 0.136g in ~95mls ddH_20
    b. Adjust to pH 6.0 with KOH
    c. Adjust volume to 100mls with ddH_20
2. **25mM Na_2HPO_4 pH 7.2 w/ 1mg/ml BSA** (for dilution of leucine dehydrogenase)
    a. Dissolve .445 grams in ~95mls ddH_20
    b. Adjust to pH 7.2 with HCL
    c. Adjust volume to 100mls with ddH_20
    d. Take 20mls of this solution and put into a separate tube. Add 1mg/ml BSA to the 20mls (20mg BSA).
3. **0.1M Na_2CO_3 pH 10.7** (for dilution of β-NAD^+)
    a. Dissolve .529g in ~45mls ddH_20
    b. Adjust to pH 10.7 with HCL
    c. Adjust volume to 50mls with ddH_20
4. **0.1M K_2HPO_4 pH 8.4 + 2mM EDTA + 0.02% β-mercaptoethanol** (known as the "assay buffer")
    a. Dissolve 3.45g K_2HPO_4 in ~190mls ddH_20
    b. Add .148g EDTA powder
    c. Add 40µl β-mercaptoethanol
    d. Adjust to pH 8.4 with HCL
    e. Adjust volume to 200mls with ddH_20

STEP 4b: Make the stock solution of standards. This only needs to be done when you run out of stock 1mM standards in the freezer to use in your assay.

1. Prepare a 1mM solution of leucine powder diluted in buffer 1**:** *0.01M KH_2PO_4 pH 6.0*
2. Aliquot into tubes containing 1ml each of this solution
3. Store at -20°C for use in future assays
4. Each time you perform the assay you will require 1 aliquot of the stock standard

STEP 4c: Prepare the 96-well plate.

1. Obtain a black opaque 96-well plate
2. Clean the plate well with ethanol and dH_20
3. Ensure all wells are completely dry

STEP 4d: Preparation of standard solutions. The standards must be made fresh every time you perform the assay.

1. Obtain 1 aliquot of the 1mM leucine standard
2. Thaw the tube and dilute the 1ml of leucine standard with 1ml of assay buffer (buffer 4) thus giving you a 500µM solution of leucine
3. Now we need to prepare the following additional standards for the assay: 0µM, 100µM, 200µM, 300µM, 400µM at a volume of 500µl each

	Assay Buffer	**500µM leucine**
0µM standard	500 µl	0 µl
100µM standard	400 µl	100 µl
200µM standard	300 µl	200 µl
300µM standard	200 µl	300 µl
400µM standard	100 µl	400 µl

4. Keep the standards at 4°C until you are ready to pipette them into the wells. You should use the standards as soon as possible, ideally within 30 minutes.

STEP 4e: Prepare 120 mM β-NAD^+**.**

Reagents

β-NAD^+ (Sigma Aldrich)

1. To obtain a 120 mM solution, dissolve 79.611mg of β-NAD^+ powder in 920.4µl of buffer 3: *0.1M Na_2CO_3 pH 10.7*
2. Vortex well and immediately place this solution on ice
3. This solution degrades quite quickly and should be used within 30 minutes.

STEP 4f: Prepare leucine dehydrogenase (LDH).

Reagents
Leucine dehydrogenase (Calbiochem)

1. We need to add 10µl of diluted LDH to each well. Calculate how many wells you have [(# of standards + # of samples) x 2 replicates]. For 20 wells you need 200µl of diluted LDH. You should also make extra as you likely will not be exact in pipetting. Therefore for 20 wells make ~230ul.
2. Once you know how much diluted LDH you need, you can prepare the solution by diluting the stock LDH in buffer 2: *25mM Na_2HPO_4 pH 7.2 w/ 1mg/ml BSA*. The final concentration of diluted LDH must be 7.857 Units/ml.
3. Immediately place the diluted solution on ice. This solution should also be used within 30 minutes.

STEP 4g: Add reagents to the wells.

1. Place the 96-well plate on ice
2. To each well add:
    a. 265µl assay buffer (buffer 4)
    b. 10µl of β-NAD^+ solution (step 4e)
    c. 14.2µl of sample or 14.2µl of standard
3. Pipette the wells up and down at each step to thoroughly mix
4. Ensure that no air bubbles enter the well by not pushing the pipette all the way down (it is okay if there is a tiny bit left in the tip). Air bubbles will give false readings.

STEP 4f: Take a blank reading.

Materials
Biotek Synergy HT plate reader

1. Set the fluorometer to read at 340 excitation/460 emission with a sensitivity of 80
2. Take a reading of the plate
3. Make sure once the reading is done to immediately place the plate back on ice

STEP 4g: Add cold diluted LDH.

1. Add 10µl of diluted LDH (step 4f) to each well
2. Pipette the wells up and down to thoroughly mix
3. Ensure that no air bubbles enter the well.

STEP 4h: Take another reading.

1. Set the fluorometer to read at 340 excitation/460 emission with a sensitivity of 80
2. Take a reading of the plate
3. Make sure once the reading is done to immediately place the plate back on ice

STEP 4i: Incubate the plate.

1. Incubate the plate at 37°C for 15 minutes

STEP 4j: Take a final reading.

1. Set the fluorometer to read at 340 excitation/460 emission with a sensitivity of 80
2. Take a reading of the plate

STEP 4k: Interpret the numbers.

Now that you have your data, you need to subtract the second reading with cold LDH from the final 37°C reading for each sample/standard. The data from the first reading can be discarded. The first reading is only necessary because I believe it helps get the machine to give better numbers on subsequent readings.

You can now generate a standard curve with the subtracted fluorescence values you obtained from your standards. Following this, generate an equation for the line of best fit.

Using the equation for your line of best fit, go to the subtracted fluorescence values you obtained for your samples and plug them in to your equation to obtain the concentration of BCAAs.

Equation of the line: $y = mx + b$, where y=fluorescence value and x=BCAA concentration

Lastly, you need to correct the BCAA concentration by the concentration of protein in the sample as determined by performing a Bradford assay.

Important notes

This assay should be carried out without much pause. As soon as you add the cold enzyme to the wells the reaction will have already started (the ice is meant to slow it down). Ideally you would want to add the enzyme to every well simultaneously. However, if you do not have an instrument to do this you can add it one well at a time in a quick manner.

With that being said, I would not recommend trying to run many samples on one plate as it will take too long to add the enzyme to all wells it and may distort your readings. I recommend not running more than 12 samples per assay.

Another thing to keep in mind is you absolutely do not want any air bubbles in the well. To avoid this, do not completely press down on the pipette when you expel any liquid into the well. It is okay to have a little bit left in the tip.

VII. siRNA transfection protocol

1. Prepare cells

a. Grow cells in 10-cm plates, you need 1 plate for every 6-well plate you need to make
b. Trypsinize, centrifuge, and re-suspend cells in 0.8ml GM/10-cm plate used
c. Count cells using a hemocytometer (use 10 μl of cells)
d. Keep the cell solution on ice while you prepare your transfection reagents

2. Make transfection mixtures according to the product instructions, use the following volumes of siRNA, lipofectamine, and optimem.

	Lipofectamine + optimem	**Target siRNA + optimem**	**SCR siRNA + optimem**
BCAT2 transfection	5 μl lipo/well 120 μl optimem/well	2 μl siRNA/well 123 μl optimem/well	2 μl siRNA/well 123 μl optimem/well
BCKDE1α transfection	7 μl lipo/well 118 μl optimem/well	3 μl siRNA/well 122 μl optimem/well	3 μl siRNA/well 122 μl optimem/well

3. After making the transfection mixtures, complete the following:

a. Add 1-ml of GM that contains no antibiotic to each well of the new 6-well plate
b. Add 250 μl of the siRNA mix (Lipo mix + siRNA mix) to each well
c. Add 250,000 cells/well
d. Mix thoroughly by pipetting up and down with 1-ml tip
e. Place in 37°C incubator
f. 24 hours later, add 1-ml regular GM that contains antibiotic
g. After another 24 hours, suck out the GM and add 2-mls of DM
h. Harvest with 75 μl lysis buffer, and change your DM every day
i. Store the samples at -20°C

VIII. Cell viability assay

Reagent: Cell Counting Kit-8 (CCK-8) (Sigma #96992)

1. **Follow a similar protocol as described in the previous section "siRNA transfection protocol"**
    a. Grow cells in 10-cm plates, you need 2 plates for every 96-well plate you need to make
    b. Trypsinize, centrifuge, and re-suspend cells in 0.8ml GM/10-cm plate used
    c. Count cells using a hemocytometer (use 10 μl of cells)
    d. Keep the cell solution on ice while you prepare you transfection reagents

2. **Make transfection mixtures according to the product instructions; use the following volumes of siRNA, lipofectamine, and optimem. Because the wells in a 96-well plate are 30x smaller than a 6-well plate, all the reagent volumes are simply divided by 30.**

	Lipofectamine + optimem	**Target siRNA + optimem**	**SCR siRNA + optimem**
BCAT2 transfection	0.16 μl lipo/well 4 μl optimem/well	0.07 μl siRNA/well 4.1 μl optimem/well	0.07 μl siRNA/well 4.1 μl optimem/well
BCKDE1α transfection	0.23 μl lipo/well 3.93 μl optimem/well	0.1 μl siRNA/well 4.06 μl optimem/well	0.1 μl siRNA/well 4.06 μl optimem/well

3. **After making the transfection mixtures, complete the following:**

a. Add 50 µl of GM that contains no antibiotic to each well of the new 96-well plate
    i. Ensure you make extra wells for non-transfected cells that will just differentiate in regular DM (this serves as the control to be used in the formula below)
b. Add 8.3 µl of the siRNA mix (Lipo mix + siRNA mix) to each well
c. Add 8000 cells/well
d. Mix thoroughly by pipetting up and down
e. Place in 37°C incubator
f. 24 hours later, add 50 µl regular GM that contains antibiotic
g. After another 24 hours, suck out the GM and add 100 µl of DM

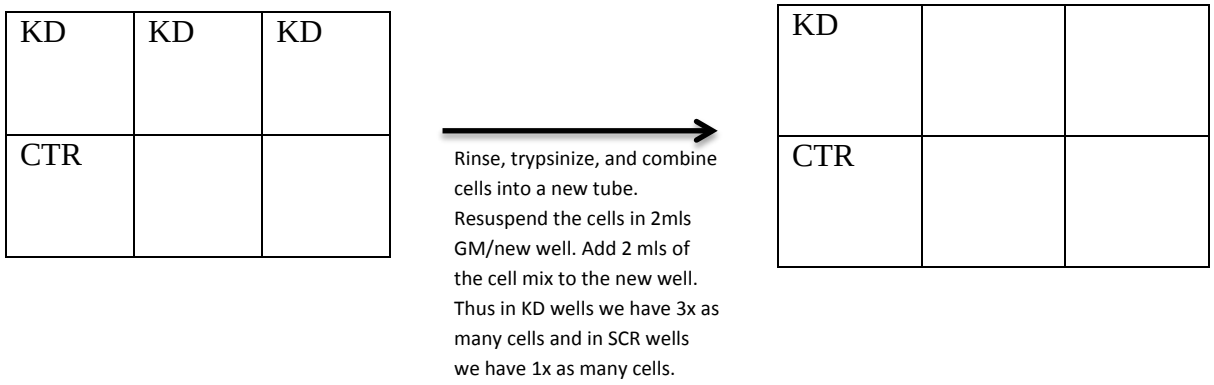
4. Add CCK-8 reagent

a. After adding 100 µl of DM, add 10 µl of CCK-8 reagent
b. Incubate at 37°C for 2 hours
c. Read the plate at 450 nm using a spectrophotometer
d. To get the relative cell viability use this formula
    i. Relative cell viability = *(absorbance of transfected cells)/ (absorbance of cells in regular DM)*
e. Repeat this process every subsequent day on a different set of wells to get the relative cell viability for each day

Note although this reagent is not very toxic to cells, it is best to make duplicate wells for each day of differentiation, and not reuse wells to measure cell viability.*

VIIII. Increasing number of cells after siRNA transfection protocol

1. Conduct siRNA transfection in 6-well plates as described in "siRNA transfection protocol"
2. 24 hours after letting the cells grow in 1-ml of GM w/o antibiotic, suck out the GM, rinse with 2 mls PBS, then add 0.5 mls trypsin to the wells
3. Combine all the target siRNA cells into a new 50 ml tube, and combine all the control siRNA cells into a new 50 ml tube
4. Centrifuge the cells of both tubes at 2000 rpm for 5 minutes
5. Suck out the supernatant, and re-suspend the cells in 2mls GM per new well. For example, if you are re-seeding the cells in 15 new wells, suspend the cells in 30 mls GM. Mix the cells thoroughly by pipetting up and down.
6. Add 2 mls of the cell mixture to a new well
7. **Repeat the protocol if you are making additional replicates*
8. Place the plate in the 37°C incubator
9. After 24 hours, shift the cells to DM
10. Change the DM every 24 hours



X. BCKA rescue experiment in BCAT2 siRNA cells

Reagents:

1. **α-ketoisocaproate (KIC) (Sigma #K0629)**
    a. dilute in autoclaved ddH_20 to make stock concentration of 25 mg/ml
    b. pass solution through sterile syringe filter
2. **α-ketomethylvalerate (KMV) (Sigma #K7125)**
    a. dilute in autoclaved ddH_20 to make stock concentration of 25 mg/ml
    b. pass solution through sterile syringe filter
3. **α-ketoisovalerate (KIV) (Sigma #198994)**
    a. dilute in autoclaved ddH_20 to make stock concentration of 25 mg/ml
    b. pass solution through sterile syringe filter

Procedure:

1. Perform siRNA transfection in 6-well plates as described in "siRNA transfection protocol"
2. Make enough plates to have three treatment groups: SCR siRNA, BCAT2 siRNA, BCAT2 siRNA + BCKAs
3. 24 hours after transfection add 1-ml GM
    a. After adding GM, add sterile KIC, KIV, and KMV to make the final concentration 200 μM for each BCKA
4. After another 24 hours, shift the cells to DM
    a. After adding DM, add sterile KIC, KIV, and KMV to make the final concentration 200 μM for each BCKA
5. Change the DM every 24 hours, and add the BCKAs every day as well
6. Harvest the cells at each day of differentiation with regular lysis buffer