# The Effects of Leucine on Mitochondrial Biogenesis and Cell Cycle in A-375 Melanoma Cells.

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In loving memory

of

William and Ella Robison,

& John T. Wilson.

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# Abstract

Mitochondria play a large role in cellular energy metabolism and regulation of cell cycle. A change in or loss of mitochondrial metabolism, termed “mitochondrial dysfunction” has been identified as a hallmark of many different metabolic diseases and disorders, including diabetes, obesity, cardiovascular disease, aging, and cancer. Cancer is a prevalent and complex disease, characterized by the abnormal growth and spread of cells. One theory of cancer, called the “Warburg Effect” classifies cancer as having partial mitochondrial dysfunction accompanied by multiple genetic mutations. Under this theory, cancerous cells reduce their reliance on mitochondria and achieve cellular energy requirements through aerobic glycolysis. Previous studies have demonstrated that mitochondrial biogenesis, an increase in mitochondrial mass or number, can circumvent metabolic disorders and diseases, termed metabolic reprogramming. Previous studies have shown that leucine has a unique signaling role in adipocytes and skeletal muscle cells. Specifically, leucine signaling stimulates mitochondrial biogenesis, which increases cellular respiration and enhances energy partitioning in these cells. Cellular energy metabolism is such that adipocytes increase fat oxidation, and this energy stimulates protein synthesis within muscle cells. In consideration of the multiple impacts of leucine on metabolic diseases, such as obesity, leucine treatment was extended to a cancerous cell line that exhibits the Warburg effect. The results from this research confirm that leucine was able to stimulate mitochondrial biogenesis in these cells. Further, stimulation of mitochondrial biogenesis in this cancerous cell line has no impact on decreasing proliferation and regulating cell cycle.

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# Part One:

# Introduction

Cancer is a prevalent and complex disease, characterized by the abnormal growth and spread of cells (1-3). In 2010, malignant cancer was the 2nd most common cause of death of men and women living in America (1,2). According to the Centers for Disease Control, roughly 77% of cancer incidence occurs in older adults over the age of 55 (1). Thus, age has been shown to play a role in the development of cancer (1,2). Some factors that lead to cancer formation include cellular changes in gene expression and metabolism that result from multiple genetic mutations (1-4). Differences in cancerous and normal tissue have been observed (5 -10). Strikingly, both aged cells and cancerous cells share similarities in metabolism, where they reduce their reliance on mitochondrial function (5,8-10). In normal cells, mitochondria play a large role in regulation of cell cycle, oxidative phosphorylation, fatty acid oxidation, amino acid metabolism, reactive oxygen species (ROS), and calcium homeostasis (3,11-13). Thus, this change in metabolism, termed "mitochondrial dysfunction," has been identified as a hallmark of many different metabolic diseases and disorders, including diabetes, obesity, cardiovascular disease, aging, and cancer (5). Metabolic reprogramming has been shown to circumvent metabolic disorders and promote longevity by decreasing risk of age-related disease. Revival of mitochondria, termed "mitochondrial biogenesis," is the focus of metabolic reprogramming (4).

Previous studies have shown that the essential branched-chain amino acid, leucine, plays a unique signaling role in adipose and muscle tissue. Multiple studies, *in vivo* and *in vitro*, have shown that leucine stimulates mitochondrial biogenesis and promotes energy partitioning (14-18). Considering the role of leucine in modulating mitochondrial abundance and function, the purpose of this research was to determine the effects of leucine on mitochondrial biogenesis, cell proliferation, and cell cycle regulation within cancerous cells.

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# Part Two:

# Literature Review

## Overview

Cancer is a prevalent and complex disease, characterized by the abnormal growth and spread of cells accompanied by multiple genetic mutations (1-2). In addition to these changes, one theory of cancer called the "Warburg Effect," characterizes cancerous cells as having mitochondrial dysfunction, or a loss of mitochondrial abundance and function. Cancerous cells that exhibit the Warburg effect reduce reliance on mitochondria, and gain energy requirements outside of mitochondrial processes (1-4). Mitochondrial dysfunction is recognized as a hallmark of metabolic diseases and disorders, such as obesity, diabetes, and aging (5-7). Strategies for preventing and overcoming metabolic diseases are centered on restoration of abundant and functional mitochondria, termed metabolic reprogramming (5). Dietary and lifestyle strategies can impact metabolic reprogramming to restore mitochondria (5,7-8).

## The Role of Mitochondria in Health

In normal, eukaryotic cells, mitochondria are important for modulating cellular energy metabolism (4,7,8). To maintain its roles in respiration and metabolism, mitochondria house biochemical pathways essential for glucose and lipid metabolism. Mitochondria regulate respiration through the tricarboxylic acid (TCA) cycle and electron transport chain (ETC), whereby cellular-derived reducing equivalents are converted to energy. Accordingly, upkeep of abundant, functional mitochondria is fundamental to life (4, 7-9).

Mitochondrial functions are regulated, in part, by changes in mitochondrial mass, such that decreased mitochondrial number contributes to a loss of function (5,8). "Mitochondrial biogenesis" occurs due to some external conditions including low temperature, caloric restriction of food/glucose, and physical activity (8). These conditions have been shown to increase gene

expression of transcriptional co-activators. Of these, peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) is the best understood, though other co-activators exist including PGC-1β, which modulates similar effects. PGC-1α is a transcriptional co-activator that increases mitochondrial biogenesis and oxidative phosphorylation through the replication and transcription of mitochondrial DNA (mtDNA) (5,8). To accomplish this, PGC-1α binds to nuclear respiratory factors 1 or 2 (NRF-1/2) to mediate these effects (8). Mitochondrial biogenesis, from overexpressed PGC-1α, is an event that has been shown to precede increased respiration within cells *in vitro* and *in vivo* (7). Knock-down of both PGC-1α and β in differentiated brown adipose tissue and mice lead to impaired ability to stimulate mitochondrial processes and result in shorter lifespan/ premature death (7). Additionally, this effect is seen in NRF-1 knock-down (5,8). In addition to NRF-1, other DNA binding factors interacting with PGC-1α include GA-binding protein (GABP), and peroxisome proliferator activated receptors (PPAR)α, δ, and γ. In general, PPARα and PPARδ regulate lipid oxidation, whereas PPARγ increases lipid biosynthesis and storage within adipose tissue (5,7-8).

Caloric restriction, physical activity, and/or severe cold signal through cAMP, nitric oxide (NO), $Ca^{2+}$, or adenosine monophosphate-activated protein kinase (AMPK) to affect PGC-1α's transcriptional activity (7). AMPK responds to low cellular energy in the form of AMP in the cell. Stimulation of AMPK leads to phosphorylation and enhanced activity of PGC-1α (5,7-8). Aside from these activators, many things have to occur to create abundant and functional mitochondria. In addition to the transcription and gene exchange that occurs inside of the cell, mitochondrial membrane biosynthesis must occur (7,8,10).

Mitochondrial DNA (mtDNA) is replicated and transcribed in the mitochondria. In mammalian cells, mtDNA encodes RNA and protein required for the formation of the ETC (7).

This genetic code is essential for maintaining respiration within the cell (5,7). In contrast, most of the DNA needed for mitochondrial-dependent oxidative phosphorylation and other mitochondrial processes are transcribed in the nucleus of the cell (7).

Cell cycle is a highly regulated process consisting of four stages G1, S, G2, and M (5,11). Mitochondrial biogenesis and transcription of mitochondria have been shown to occur early in the cell cycle, during G1 phase. During G1, cell cycle slows. Progression to S phase will not occur without a cellular signal that DNA is correctly encoded (1,7,11). Previous studies have shown that functional mitochondria are required for normal cell cycle, especially for the progression from G1 to S phase (5,7,10). Notably, mtDNA and NRF-1 have been shown to increase during the G1/S phase to G2 phase of cell cycle. In comparison, the S and G2/M phases do not require mitochondrial processes (7). Any cell with DNA damage or compromised function of oxidative phosphorylation will go into cell cycle arrest for DNA repair via the tumor suppressor gene, p53 (12). DNA damage that could not be repaired would be sent to organelle degradation through autophagy or apoptosis (5,7,11).

## Role of Glucose in Cellular Regulation

Dietary glucose consumption is a key regulator of mitochondrial pathways (5,8). Specifically, the level of glucose substrate determines the intercellular signaling between two metabolic pathways.

The first is the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/ mammalian target of rapamycin (mTOR) signaling pathway. In response to insulin and insulin-like growth factor 1 (IGF1), PI3K and Ras become stimulated. Downstream, this stimulates Akt, which stimulates mTOR (5,13). Downstream, IGF-1/PI3K/Akt signaling favors cell survival and metabolism through the inhibition of forkhead box 01/3a (Fox01/3a), a mediator of normal

metabolism, cell cycle arrest, and apoptosis. The IGF-1/PI3K/Akt signal upstream regulates mTOR signaling (4). When growth factor and high glucose substrates are present, the signal of high energy activates mTOR to initiate gene transcription via hypoxia inducible factor 1α (HIF1α), peroxisome proliferator-activated receptor γ (PPAR-γ), and sterol regulatory element-binding protein 1 and 2 (SREBP1/2) to favor glycolytic, adipogenic, and lipogenic enzyme gene transcription, respectively (4,5). These genes antagonize the effects on mitochondrial function and abundance (5).

On the other hand, stresses such as low energy, reduced glucose and DNA damage are communicated through the AMPK/PGC-1α signaling pathway (5). This pathway down-regulates glycolytic energy metabolism by blocking mTOR-mediated transcription and translation of glycolytic, adipogenic, and lipogenic genes in favor of transcription of genes needed for mitochondrial biogenesis, fatty acid oxidation, and oxidative phosphorylation (4,5). AMPK and Akt act opposite to modulate metabolism through sensing nutrients within the cell (5). Similar to AMPK, silent information regulator transcript 1 (SIRT1) senses low cellular $NAD^+$ levels. SIRT1 is an energy sensor dependent upon the low energy abundance of $NAD^+$ in the cell (5,6). SIRT1 is a class III histone deacetylase that is a stimulator of mitochondrial biogenesis through PGC-1α deacetylation (5,13-16). As both sense low cellular energy, SIRT1 and AMPK activation can occur simultaneously (5). Thus, they both have been shown to modulate the downstream effects of oxidative phosphorylation and fatty acid oxidation (5,15).

## Caloric Restriction as a Model of Lifespan and Healthspan

Caloric restriction without malnutrition is the gold standard model of longevity and decreased risk of age-related disease in all organisms (5). Models such as yeast, worms, fruit flies, mice, non-human primates, and humans have demonstrated the effectiveness of caloric

restriction on reducing the incidence of age-related disease to promote lifespan and healthspan (5,6,17-20). Healthspan is the span of time that an organism is free of debilitating disease and disorder. (5,6). When comparing calorically restricted organisms to their normal consumption counterparts, most of the differences are in mitochondrial processes (5,6,19-20). The caloric restriction phenotype includes increased respiration, increased functional electron transport chain, insulin sensitivity, increased fat oxidation, and decreased ROS production *in vitro* and *in vivo* (5,6,17-20).

SIRT1 has come to the forefront of age related disease and longevity studies (15,16). Research completed in yeast lead to the discovery of Sir2, homolog to SIRT1. Caloric restriction, induced through low glucose media, stimulated Sir2 expression and promoted longevity. Further, overexpression of Sir2 showed a longer lifespan within yeast, while knockout of Sir2 caused premature death (18-20). SIRT1 and AMPK, modulators of food restriction pathways, activate PGC-1α to promote mitochondrial function (5). $NAD^+$-induced SIRT1 activation has been demonstrated to increase fatty acid oxidation, gene expression of ETC subunits, and PGC-1α (20). These findings correlate caloric restriction with signaling in the SIRT1/AMPK/PGC-1α axis. Thus, healthy mitochondrial metabolism, supported by mitochondrial abundance and function, are a hallmark of longevity and healthspan. On the other hand, age-related disease and premature death are related to increased, unregulated signaling via PI3K/Akt/mTOR (5,13).

But while caloric restriction has been shown to help modulate mitochondrial metabolism linked to longevity and heathspan, caloric restriction is difficult to adopt by humans (5,6). Accordingly, research to find a caloric restriction mimetic as a therapeutic is widely discussed in the literature. Anderson *et al* postulated that a caloric restriction mimetic would be any compound that activated SIRT1/AMPK/PGC-1α signaling (5). Of these, the most commonly

reviewed substance is the plant-based polyphenol, resveratrol (5,6,21,22). Previous studies using resveratrol have shown that it stimulates mitochondrial biogenesis through its SIRT1 target (5,22). Downstream, this leads to fatty acid oxidation and increased respiration (5,21). A human visceral adipocyte tissue culture study showed that resveratrol stimulated mRNA expression of SIRT1, FOX01, and adiponectin, while down-regulating PPARγ (21). This study demonstrates the energy partitioning effects of resveratrol to mediate blocked adipogenesis via increased Fox0 and decreased PPARγ, while increasing the adipokine, adiponectin, and SIRT1 expression for mitochondrial function (21).

## Cancer as a Metabolic Disorder: The Warburg effect

Abundant and functional mitochondria play a role in cellular energy metabolism, and may be lost in metabolic disorders (1). The theory that cancer is a metabolic disease, or one characterized by impaired cellular energy metabolism, is becoming more accepted (1-3). A theory that classifies most cancer cells as having partial mitochondrial dysfunction is called the "Warburg Effect". Specifically, the energy metabolism of most cancer cells are achieved through aerobic glycolysis/substrate level phosphorylation yielding 2 ATP and lactate, while normal cellular energy metabolism is achieved through mitochondrial oxidative phosphorylation yielding 30/32 ATP (1). Through substrate level phosphorylation, cancer cells achieve energy requirements in an inefficient way, but gain unregulated amounts of glucose and reducing equivalents (NADPH) for proliferation (1-3,23). The products of aerobic glycolysis, lactate and NADPH, are utilized by neighboring cells to convert them to cancerous cells. It is believed that cells that exhibit the Warburg effect switch their metabolism to maintain respiration to avoid signaling for an apoptotic cascade (1).

While the Warburg effect modulates mitochondrial dysfunction, it is disputed whether

cellular mitochondria have a reversible loss of function (1,3). What is certain is that mitochondrial genes are mutated and down-regulated, shutting down oxidative phosphorylation and the TCA cycle (1,13). Previous studies have determined that unregulated signaling via PI3K/Akt/mTOR is a cause for a cell's switch in energy metabolism (13,24,25). This signaling occurs due to the increased ATP and NADH made available through aerobic glycolysis (1). Akt/mTOR-dependent signaling has been shown to initiate glycolytic metabolism that shuts down mitochondrial function. This has been demonstrated to convert normal cells to aerobic glycolytic/Warburg metabolism (24). Another hallmark of cancer cells, multiple genomic mutations, cause the loss of tumor suppressors and overexpression of tumor promoters, helping the cell achieve energy requirements. It has been demonstrated that, in the presence of high glucose, unregulated IGF-1/PI3K/Akt/mTOR signaling will favor transcription of glycolytic, adipogenic, and lipogenic genes (22,24). Tumor promotion studies in mice have shown that tumor progression occurs in cells that have lost the functionality and ability to generate functional mitochondria (1). Accordingly, when the conditions of the Warburg effect are applicable, the focus of cancer research is to promote respiration and induction of oxidative metabolism through the restoration of mitochondrial abundance and function (1,2,13,14).

In addition to glucose, glutamine is a substrate preferred by cancer cells. Glutamine degradation, termed "glutaminolysis", can feed into a cancer cell and give net rise to glucose via gluconeogenesis (1,5), generate NADPH through malic enzyme (25), or participate in nucleotide biosynthesis (5). Glutamine can be used to synthesize glutamate, to feed directly into the TCA cycle via α-ketoglutarate, and is considered a preferred substrate by cancerous cells (1,4,25,26). Previous studies have shown that when glucose is deprived, glutamine degradation is up-regulated; alternatively, when glutamine is restricted, glucose utilization is increased (25).

Human advanced cancer patients exhibit glutamine depleted stores (26). Glutamine utilization has been shown to be regulated according to the cell cycle. Enzymes that break down glutamine are highly expressed in the S phase, and decreases with progression into the G2/M phase (25). Additionally, increased and unregulated IGF-1/PI3K/Akt/mTOR mediates HIF-1α transcription of glucogenic enzymes including hexokinase and GLUT1 (26).

Not all cancer cells exhibit the Warburg Effect, nor is it seen exclusively in cancer cells (7,14). The Warburg Effect is also exhibited in pluripotent stem cells, or cells that undergo constant replication prior to differentiation into mature cells (3,7). Maturation of stem cells occurs in response to growth factors. When examining the differences between stem cells and cell lines after differentiation, it was found that stem cells lack fully developed mitochondria, exhibited reduced mitochondrial function, and contain less DNA (14). Additionally, stem cells exhibit lower oxygen consumption, perhaps due to an increased glycolytic metabolism (4,14). Specifically, it was determined that stems cells have an up-regulation in hexokinase enzyme transcription but low pyruvate dehydrogenase complex(PDC)-specific enzymes. These enzymes are effective in breaking down glucose, but not transporting them to the TCA cycle or ETC (14).

## p53 and Tumor Suppression

The tumor suppressor gene p53 functions in normal cellular energy metabolism through its roles in apoptosis, genetic monitoring, and mitochondrial regulation (1,27-29). Because p53 has important roles in cell cycle regulation, tight regulatory control of p53 occurs in the cell (30). Regulation of p53 expression is maintained through MDM2, a mediator of protein ubiquitination. Additionally, p53 activity seems to be regulated through increases of acetylation on lysine residues (30,31). Differences in acetylation have been determined for p53's roles in cell cycle regulation (30). For example, a weak p53 signal may lead to senescence, while an abundance of

p53 leads to cell cycle arrest (29).

It has been noted that p53 is deleted, mutated, or directly deactivated in the majority of human cancers (1). This event is accompanied by a decrease in mitochondrial respiration and a switch toward aerobic glycolysis (5). In mouse models, p53 deficiency was accompanied by decreased mitochondrial metabolism, reduced respiration, and increased ROS production (5). Persistent mitochondrial dysfunction leads to p53 suppression in cancerous cells (27). Further, p53's target, TIGAR (TP53-induced glycolysis and apoptosis regulator) down-regulates glycolysis by lowering fructose 2,6 bisphosphate (32); in this context, a loss of p53 function would augment glycolysis (1).

While p53 has a role in regulating cellular respiration, p53's signaling pathway leads to one of three of the following events: autophagy, cell cycle arrest or apoptosis. These three signaling pathways are regulated by glucose, growth factors, mTOR signaling, and DNA damage (5,13,27).

In response to low energy, glucose restriction, and DNA damage, autophagy is induced rapidly in normal cells (14). Under these conditions, AMPK is up-regulated, while mTOR is down-regulated. This promotes p53-dependent regulation of autophagy to engulf dysfunctional cellular organelles through lysosomal processes (14,32). This process, necessary for normal cellular turnover, is down-regulated by mTOR (7,13).

In response to reversible DNA damage translated through pro-arrest genes and cofactors, an accumulation of p53 will initiate cell cycle arrest (11). Under these conditions, p53 translocates to the nucleus, where it initiates the transcription of p21 (11,12). Downstream, p21 expresses a cyclin dependent kinase (CDK) that attaches to accumulating cyclins needed for cell cycle progression. The association of CDK 2 decreases bound cyclin A or E to arrest cell cycle.

CDK 4/6 attaches to cyclin D. The decrease in cyclin D or E expression cause G0/G1 phase arrest, while cyclin A arrest occurs in the S phase (1,30). p21 knockout mice are unable to arrest the cell in the G1 phase, while p53 deficient mice made very little p21 (30).

p53 also regulates cell cycle through apoptosis via two different pathways: transcription-dependent apoptosis and transcription-independent apoptosis (1,29,30). Transcription-dependent apoptosis involves p53 translocation to the nucleus, where it initiates apoptotic cofactors and transcribes apoptotic genes BAX, PUMA, and NOXA. At the same time, p53 blocks anti-apoptotic genes, such as survivin (11). On the other hand, p53 regulates transcription-independent apoptosis through mitochondria. This form of apoptosis involves p53 translocation to the mitochondria where it binds to Bcl-2, activates Bax and Bak, and initiates a caspase-mediated apoptosis (11). This process is unique in that it activates the release of cytochrome c from the mitochondria to the cytoplasm for cell death initiation (30).

## SIRT1 and p53

The first known non-histone target for SIRT-1 is p53, though other targets exist including Forkhead box 0 and PGC-1α (15,16). Regulation of p53 activity occurs through acetylation (30). SIRT1 deacteylation of p53 reduces p53 activity, and blocks p53 translocation to the nucleus for transcription-independent regulation of cell cycle and cell cycle arrest. When SIRT1 is activated, p53-mediated regulation of cell cycle occurs through autophagy or mitochondrial-dependent apoptosis to regulate cell cycle (5,30). Due to the negative regulation of p53, SIRT1 has been hypothesized to be a tumor promoter within cancer signaling (30). SIRT1 repression of p53 causes reduced expression of the microRNA (miRNA) 34a, a potential tumor suppressor that helps with p53 induction of apoptosis. Interestingly, miRNA34a can down-regulate SIRT1 expression (30). Additionally, SIRT1 may act as a tumor promoter in cells that exhibit the

Warburg effect. SIRT1 knock-down in 3 glioma cell lines was associated with cell cycle arrest in the G1 phase and apoptosis via caspase 3 and 7 pathways, while overexpression promoted growth and proliferation (34). These findings are similar to longevity studies that show that SIRT1 mediates longer lifespan and knock-down of SIRT1 results in premature death (5,6). One study found that SIRT 1 was significantly up-regulated in skin biopsies that were normal, premalignant, and tumorous (35). Accordingly, these authors determined that niacin (NAD) dietary restriction inhibited SIRT1, and slowed cell differentiation in a skin reconstruction model *in vitro*. These studies demonstrate that SIRT1 expression may be an unwanted event in cell signaling of cells that exhibit the Warburg effect. SIRT1 as a tumor suppressor has been reported as well. In a study by Lynch *et al*, an isoform of SIRT1 regulated p53 through acetylation, but was repressed by p53 under certain conditions in a mouse model (33). This study shows that SIRT-1 and p53 can regulate each other.

## Leucine's Modulation on Energetics

The branched-chain amino acid, leucine, has a unique signaling role in the cellular energy metabolism of normal adipocyte and skeletal muscle cells, *in vivo* and *in vitro*. In these cells, leucine signaling roles include stimulation of mitochondrial biogenesis through SIRT1-mediated processes, fatty acid oxidation, and p53 expression (36-41). Previous studies in the Zemel lab have demonstrated leucine's ability to stimulate mitochondrial biogenesis in adipocyte and skeletal muscle, and this signaling has an energy partitioning effect within the body that favors fatty acid oxidation in adipocytes, while increasing protein muscle synthesis via mTOR activation (38,41). Furthermore, leucine promotes and calcitriol suppresses these effects. Research from our lab has determined that leucine stimulated increases in mitochondrial mass 30% in adipocytes and 50% in skeletal muscle cells (37-40). Similarly, leucine signaling was

found to inhibit adipocyte storage by suppressing fatty acid synthase (FAS) and PPARγ, while stimulating adiponectin release. Instead, skeletal muscle cells utilize energy generated by fatty acid oxidation for protein synthesis (39).

Recent data demonstrate that leucine's stimulation of mitochondrial biogenesis is through leucine's metabolite β-hydroxy-β-methylbutyrate (HMB), independent of mTOR (42-44). Leucine is metabolized to α-ketoisocaproic acid (KIC) via a transamination reaction. KIC has two metabolites, isovaleryl-CoA and HMB. Of these, isovaleryl-CoA is formed most of the time, about 85%, while HMB creation occurs ~15%. Roughly 5-10% of leucine is converted to HMB after calculating metabolic loss (42,43). Additionally, leucine, HMB, and KIC significantly stimulate SIRT1 activity, while the branched-chain amino acid control valine did not (44). Leucine has been shown to stimulate SIRT-1 activity, an activator of PGC-1α to initiate mitochondrial biogenesis within these cells. These results are accompanied by increased gene expression of SIRT1 and the increased mitochondrial component genes, including uncoupling protein 2 (UCP-2), PGC-1α, NRF-1, mitochondrial NADH dehydrogenase (mtND1), and nuclear encoded subunit (NDUFA) (39,41,44,45). These genes verify that an increase in mitochondrial number, rather than mass occur from leucine signaling (40,41).

Leucine is well known to stimulate protein muscle synthesis via mTOR-dependent and independent mechanisms (42,46,47). Leucine is sensed through mTOR, and participates in the ribosomal initiation phase of translation, which encodes mRNA into amino acids for protein (24). Leucine is a substrate for mTOR-dependent stimulation of protein muscle synthesis by causing the association of the eukaryotic initiation factor-4E (eIF4E) and S6 ribosomal protein (24,46). Under mTOR-independent stimulation, protein muscle synthesis is mediated by leucine's activation of eIF4G (the eukaryotic initiation factor-4G) (46). Previous studies from the

Zemel lab have shown that leucine may initiate fatty acid oxidation due to its costly roles in protein muscle synthesis (41). Leucine supplementation has been shown to stimulate protein synthesis via insulin-independent mechanisms. In rats under long-term food deprivation, diets of leucine or leucine + carbohydrate caused increases in insulin. Whereas a leucine + carbohydrate diet increased insulin for 60 minutes, leucine's impact was much smaller and returned to basal amounts in 30 minutes. A small increase in insulin may help protein muscle synthesis occur, as when pancreatic insulin release was kept at fasting basal levels using a somatostatin clamp, skeletal muscle was reduced in this model (46).

## Substrates investigated in Cancer Therapy

In addition to our studies that demonstrate leucine's roles in initiating mitochondrial metabolism through the stimulation of mitochondrial biogenesis, fatty acid oxidation, and p53 signaling, previous studies have shown that leucine supplementation can be helpful in cancer therapy (44,45). Leucine and its metabolite HMB have been implicated directly for its roles in protecting cancer-induced cachexia in *in vivo* models. One showed that leucine supplementation in animal feed was able to dose-responsively maintain muscle mass in cancer cachectic mice (47), a role that may be enhanced with basal insulin conditions needed for the Warburg effect (46). These strategies have been adopted in athletic muscle building (24, 46). Further, a growing body of data promotes the role of a "Warburg Diet", better known as a ketogenic diet (1). Increased gluconeogenesis is observed in cancer-induced cachexia (46). The ketogenic diet is a fat and protein-based diet that has been hypothesized to increase longevity and better outcomes within cancer patients (1,23, 46). Under the conditions of the Warburg Diet, the body can maintain normal functioning through the use of ketone bodies. Leucine's metabolites KIC and HMB offer a ketone body to promote this metabolism (23,46). Of the cancers researched, brain

cancer seems to be the most impacted by glucose restriction (1). Thus, leucine may have a protective role and unique signaling role in tumorigenesis.

On the other hand, leucine has been showed to be a preferred substrate to cancerous cells. In a study using labeled methionine, leucine, and tyrosine, the tumorous uptake of leucine was the highest (46). Authors postulate that branched-chain amino acid degradation may donate nitrogen needed for biosynthesis of glucogenic amino acids for utilization in aerobic glycolysis (46).

Despite the numerous unique signaling roles in normal tissue, leucine's role in protein muscle synthesis via mTOR-dependent mechanisms has received the most attention in the cancer field. Leucine deprivation and toxicity have been used to study its roles in tumorigenesis. In an *in vitro* study in four melanoma cell lines, leucine deprivation was shown to increase caspase-3-mediated apoptosis (48). Further, a leucine-deprivation diet *in vivo* significantly increased apoptosis in melanoma xenografts placed in immunocompromised mice (48). On the other hand, leucine deprivation in 8 different breast cancer cell lines was unable to stop mTOR/Akt signaling (49). One study showed the impact of a toxic dose of leucine on cancer growth. Leucine at doses 10 mM and 60 mM were able to induce cell cycle arrest in the G0/G1 phase in glioma cells, but not at 1 mM. Further, this data shows the ability of leucine (dose 10 mM) signaling to promote cell cycle arrest over alanine, valine, and isoleucine at similar concentrations, but not cause an apoptotic event in cells that exhibit the Warburg effect (50). This dose likely exceeds a level that is normally achievable through diet. In a supplementation study in healthy young males, leucine given at a dose 25 times the estimated average requirement (EAR) (a dose equivalent to 87.5 grams of leucine in a 70kg male) raised blood leucine levels to 2 mM, well below the 10 and 60 mM doses used in the previously described study (51), making them physiologically

unachievable. Moreover, leucine doses in excess of fifteen times the EAR lead to elevated blood ammonia levels that were considered dangerous (51). These data support that suppression of mTOR through leucine deprivation may be beneficial for the treatment of some cancer cell lines, but that normal or increased intakes of leucine may protect from muscle wasting.

Previous studies have shown that a loss of p53 expression occurs with mTOR activation (5,13). A-375 melanoma cells, a cell line that exhibits the Warburg effect, was found to exhibit wild-type p53 (52). A study demonstrated that A-375 melanoma cells administered UV radiation damage had increased p53 and p21. These cells inhibited replication by 75% and sent roughly 15% of cells to apoptosis via nuclear-mediated mechanisms (53). Interestingly, nuclear p53 would be decreased during SIRT1 activity, a signaling role exemplified by leucine (13,30,41). Thus, leucine's signaling roles in these cells may rely on mitochondrial-dependent/ transcription-independent apoptosis.

Accordingly, leucine may have a role in modulating normal energy metabolism through its effects on mitochondrial biogenesis and fat oxidation. These effects, combined with protection from muscle wasting and p53 mediation of cell cycle, may "reverse" the Warburg effect. Leucine's modulation on energetics may lead to tumor suppressive effects in cancer cells exhibiting the Warburg effect by altering cellular respiration through mitochondria. This study aims to determine the effects of leucine and HMB treatment on mitochondrial biogenesis in a cancer cell line that exhibits the Warburg Effect *in vitro*. To accomplish this, the cancer cell line A375 (melanoma) will be treated with leucine, HMB, valine, and alanine and mitochondrial biogenesis will be measured via NAO fluorescence. Mitochondrial biogenesis will be measured throughout one replication of cells. In this study, valine serves as a branched-chain amino acid control and alanine serves as a non-branched-chain amino acid control. Because these cells use

glucose as their primary substrate, this experiment will be repeated in media containing reduced glucose. Additionally, this study will determine the effects of leucine and HMB on cell proliferation and cell cycle arrest in A375 melanoma cells. To accomplish this, the A375 cell line will be treated as previously described. Cell proliferation will be measured via cell number using flow cytometry after 1 and 2 doubling times. Cell cycle arrest will be measured for cell number via flow cytometry and for cell cycle and gene expression via RT-PCR. Cancer cells will be measured for cell cycle regulation via p53, p21, and Cyclin D.

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# Part Three:

# The Effects of Leucine on

# Mitochondrial Biogenesis and Cell Cycle

# in A375 Melanoma Cells

## Abstract

Most cancer cells undergo the Warburg effect, a shift from oxidative to glycolytic metabolism accompanied by suppression of p53. We have demonstrated leucine stimulation of mitochondrial biogenesis, fatty acid oxidation, and p53 expression in both muscle and fat cells. Accordingly, we now sought to determine if leucine stimulates mitochondrial biogenesis and exit from cell cycle in A-375 melanoma cells. Because these cells use glucose as their primary substrate, cells were grown under both standard (11.1 mM) and reduced (5.5 mM) glucose conditions. Increasing glucose reduced mitochondrial mass 50-60%, and accelerated cell proliferation by ~20%. Leucine (0.25, 0.5, 0.75, and 1.0 mM) dose-responsively increased mitochondrial mass 20-40% ($p<0.05$) under low glucose; its metabolite beta-hydroxy-beta-methyl butyrate (50 μM) exerted similar effects, while amino acid controls valine and alanine exerted little effect. Treatments were without significant effect under high glucose conditions. Despite the increase in mitochondrial mass at low glucose, treatments exerted no effect on cell proliferation, p53, p21, or cyclin D. Thus, although both glucose reduction and increased leucine stimulate mitochondrial biogenesis, these effects are insufficient to stimulate p53, induce cell cycle arrest, or reduce proliferation in A-375 melanoma cells.

## Introduction

Cancer is widely accepted as a metabolic disorder, characterized by the loss of functional mitochondria needed for normal cellular energy metabolism (1). The “Warburg effect” is a theory that classifies most cancer cells as having mitochondrial dysfunction that switches cellular energy metabolism from catabolic oxidative metabolism to one of anabolic glycolytic metabolism (1,2,3). While glycolytic metabolism provides an inefficient energy source, this serves to preserve carbons and reducing equivalents needed for further growth and proliferation

(1). In the majority of human cancers, the Warburg effect is accompanied by suppression of the tumor suppressor gene, p53. Under normal cellular energy metabolism, p53 is expressed and controls glucose utilization and monitors normal cell cycle (2,3,4). Thus, p53 deficiency is associated with a metabolic switch away from respiration toward glycolysis (1,3,4,5). When the conditions of the Warburg effect are applicable, the focus of cancer research is to restore mitochondrial function and abundance (1).

A growing body of data from our lab has demonstrated the unique signaling roles of leucine in the cellular energy metabolism of normal adipocyte and skeletal muscle cells, *in vivo* and *in vitro* (6,7,8). In these cells, leucine signaling roles include increased muscle protein synthesis via the mammalian target of rapamycin (mTOR) pathway (9), stimulation of mitochondrial biogenesis and fatty acid oxidation (7,10) as well as for p53 (6). Recent data demonstrate that leucine's stimulation of mitochondrial biogenesis is dependent upon stimulation of SIRT-1 (Silent information regulator transcript 1), a class III histone deacetylase that is $NAD^+$-dependent (7,8,10). Further, the leucine/SIRT-1 signaling pathway acts through leucine's metabolite β-hydroxy-β-methylbutyrate (HMB)(11,12,13). Leucine has been shown to stimulate SIRT-1, an activator of the mitochondrial regulator, PGC-1α (peroxisome proliferator activated receptor gamma co-activator 1 alpha), which is associated with the initiation of mitochondrial biogenesis and fat oxidation in fat and skeletal muscle cells (5,7,8).

The present study examines the effects of leucine and HMB on A-375, a human skin malignant melanoma cell line that exhibits the Warburg effect. Because these cells utilize glucose as their primary substrate (1,2,3,4), cells were grown in both standard glucose conditions (11.1 mM) and reduced glucose conditions (5.5 mM) which model post-prandial and fasting conditions, respectively. This study is predicated on the notion that leucine via mitochondrial

biogenesis will reverse the Warburg effect by shifting cellular energy metabolism towards fat oxidation, accompanied by stimulation of p53 and its downstream effectors. Accordingly, the objective of this project is to (a) determine whether leucine and HMB stimulate mitochondrial biogenesis in this cell line, and (b) to determine the tumor suppressive effects of leucine and HMB, in this cell line.

## Materials and Methods

### *Cell Culture*

A375 cells, a human skin malignant melanoma cell line (American Type Culture Collection) were grown to 100% confluency in either standard glucose conditions or reduced glucose conditions containing 10% fetal bovine serum and 1% antibiotics at 37°C in 5% $CO_2$. Dulbecco's Modified Eagles Medium (Glutamax) was used for standard glucose conditions. These conditions are hyperglycemic in nature containing 11.1 mM glucose, and reflect a blood glucose level similar to that of 200 mg/dL. Cells grown under standard glucose conditions had a doubling time of 6 hours, as determined using flow cytometry. These experiments were repeated in reduced glucose conditions (5.5 mM), using Dulbecco's Modified Eagles Medium. This media differs from standard glucose conditioned medium only in reduced glucose content. Cells grown in reduced glucose conditions had a doubling time of every 9 hours, as determined by flow cytometry.

### *Treatment of cells*

Leucine, HMB, valine, and alanine were freshly diluted in standard glucose medium and reduced glucose medium before treatment. Cells were counted using a hemocytometer and were plated at very low density (36 cells/mL) to prevent overgrowth after adherence. Cells were

washed with fresh medium and re-fed with medium containing one of the following treatments: 0.25, 0.5, 0.75, 1.0 mM leucine, 0.05 mM HMB, 0.5 mM valine, 0.5 mM alanine. HMB served as a positive control in this study. Valine and alanine served as negative controls in this study. Valine served as a branched-chain amino acid control, and alanine served as a non-branched-chain amino acid control. Cells were grown in media as an additional control for treatment comparison. After treatment, cells were incubated at 37°C in 5% $CO_2$ until harvest.

### *Doubling time and Proliferation*

Doubling time and cell proliferation were assessed using flow cytometry. The mitochondrial probe mitotracker green (Invitrogen, Carlsbad, California, USA) was used to analyse doubling time and cell number by fluorescence (excitation 490 nm and emission 516 nm). To prevent mitochondrial toxicity or other structural staining, probes were used according to manufacturer's instructions. Quantitative data was obtained using flow cytometry (BD Accuri C6. Franklin Lakes, NJ).

### *Mitochondrial mass*

The mitochondrial probe nonyl-acrydine orange, NAO (Invitrogen, Carlsbad, California, USA), was used to determine mitochondrial mass via fluorescence (excitation 485 nm and emission 520 nm). First, a dose response curve of leucine was conducted to determine the effects of leucine on mitochondrial biogenesis. Leucine at 0.5 mM is achievable physiologically after a high protein meal, thus treatments (valine and alanine) were administered at comparable concentrations (0.5 mM). It is also noted that roughly 5-10% of leucine is converted to HMB. Accordingly, HMB was delivered at a concentration of 0.05 mM, or 10% of leucine. Quantitative

data was obtained with a fluorescence microplate reader (Packard Instrument, Downers Grove, Illinois, USA). The mean fluorescent intensity was expressed as units per μg of protein.

### *Total RNA Extraction.*

A total cellular RNA extraction kit (Ambion, Inc., Austin, Texas) was used to isolate RNA from cells according to the manufacturer's instructions. The concentration, purity and quality of the RNA was assessed quantitatively via the 260/280 ratio (1.7-2.0) and 260/230 ratio (close to 2.0) acquired from a ND-1000 Spectrophotometer (NanoDrop Technologies Inc., DE).

### *Gene expression.*

Expression of p53, p21, and cyclin D was measured via real-time polymerase chain reaction using an ABI 7300 Real-Time PCR system (Applied Biosystems, Branchburg, NJ) with a TaqMan® core reagent kit according to manufacturer's instructions. Primers and probes were from Applied BiosystemsTaqMan® Assays-on-Demand and used according to manufacturer's instructions. RNA was diluted in the range of 1.5625-25 nanograms and used to establish a standard curve. Expression of each gene was normalized using 18S quantitation, and data for each gene is presented as a ratio to 18S.

### *Doubling time and time-course of this study*

The doubling time of A-375 melanoma cells vary, but are usually 6-12 hours (14). Cell doubling time was determined via flow cytometry to best predict cell cycle to determine the time-course used in this study. Cells grown under standard glucose conditions had a doubling time of 6 hours, while cells grown in reduced glucose conditions had a doubling time of every 9 hours. Accordingly, to measure cell proliferation, cells were tracked at consistent doubling times. Cells grown in standard glucose conditions had doubling time measured at 8 and 16 hours to

capture changes after one doubling time in these cells. Similarly, proliferation of A-375 cells grown in reduced glucose conditions was tracked at 9 and 18 hours.

Mitochondrial biogenesis has been shown to begin early within the G1 phase of cell cycle. Accordingly, cell mitochondrial biogenesis was measured in the middle of the cell cycle, at one doubling time, and after one doubling time of these cells. In standard glucose conditions, mitochondrial biogenesis was measured at 4, 8, and 24 hours. In reduced glucose conditions, mitochondrial biogenesis was measured at 6, 9, and 12 hours. To capture a change in gene expression, cells were harvested in the middle of cell cycle and at the next doubling time. Accordingly, cells grown under standard glucose conditions were harvested at 4 and 8 hours, while cells grown under reduced glucose conditions were harvested at 9 and 18 hours.

### *Statistical analysis.*

All treatment group data is shown as a mean ± SEM, with $n \geq 6$ in experimental groups. Data was evaluated using a one-way ANOVA and Tukey's post-hoc comparison of experimental treatment groups using SPSS software (IBM SPSS Statistics 19. SPSS Inc. Chicago, IL). Treatment was considered significant at $p \leq 0.05$.

## Results

### *Mitochondrial Mass*

Mitochondrial mass was determined in cells treated with various concentrations of leucine (0.25 mM, 0.5 mM, 0.75 mM, and 1.0 mM). Treatments were compared to cells grown in media. Leucine treated cells grown in standard glucose conditions (11 mM glucose) showed no significant change in mitochondrial mass when compared to cells grown in media (Figure 1A).

In contrast, leucine treated cells dose responsively stimulated mitochondrial biogenesis

by 20-40% under reduced glucose conditions when compared to the control (Figure 1B). At 6 hours, all concentrations of leucine significantly stimulated mitochondrial biogenesis ($p<0.01$), and at 9 hours, cells treated with 0.5 mM of leucine, 0.75 mM leucine, and 1.0 mM leucine significantly stimulated mitochondrial biogenesis ($p<0.01$). At 12 hours, cells treated with 0.5 mM and 0.75 mM leucine significantly stimulated mitochondrial biogenesis. At this timepoint, the relative number of mitochondria per cell is reduced due to cells doubling.

Figure 1C shows treatments of leucine, valine, alanine, and HMB grown under standard glucose conditions and compared to the control. Treatments are expressed as a percent of the control. In standard glucose conditions, treatments had no significant induction or reduction in mitochondrial mass at 4 and 8 hours (Figure 1C).

In reduced glucose conditions, all treatments significantly stimulated mitochondrial mass at 6 hours (Figure 1D). At 9 hours, HMB and leucine significantly stimulated mitochondrial mass when compared to the control. At 12 hours, only leucine significantly stimulated mitochondrial mass when compared to the control ($p<0.01$).

These results demonstrate that in reduced glucose conditions, all treatments and doses stimulated mitochondrial mass in these cells at 6 hours. Additionally, leucine treatment at physiological concentrations significantly stimulated mitochondrial mass throughout one replication of A-375 melanoma cells.

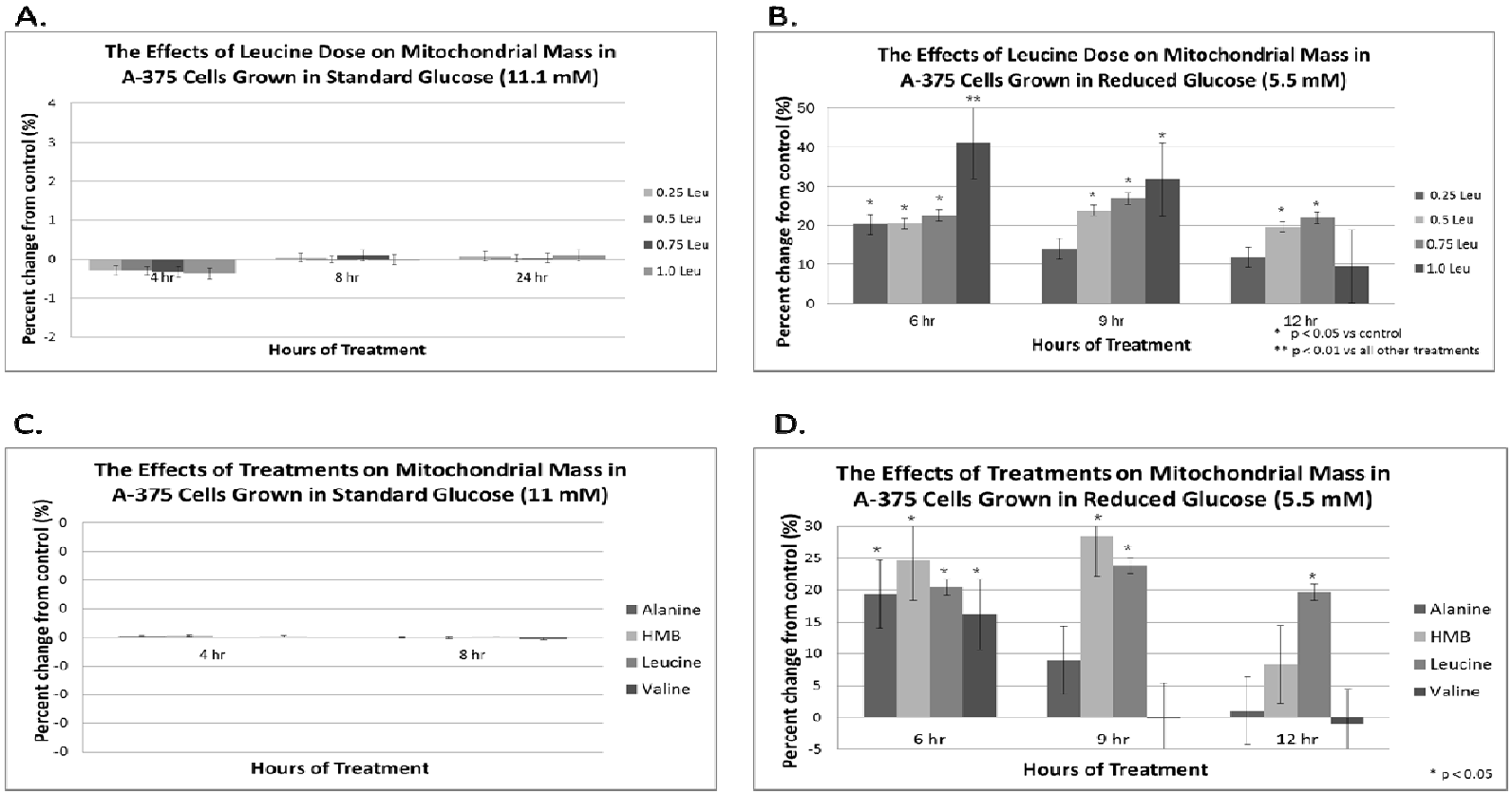



**Figure 1: The effects of treatments on mitochondrial mass in A-375 cells**

A) Dose-response curve of leucine in standard glucose conditions (11 mM). Results are reported as a mean±SE (n=12), and displayed as a percent change from the control (media without leucine added).

B) Dose-response curve of leucine in reduced glucose conditions (5.5 mM). Results are reported as a mean±SE (n=12), and displayed as a percent change from the control (media without leucine added).

C) Effects of treatments alanine, HMB, leucine, and valine in standard glucose conditions (11 mM). Results are reported as a mean±SE (n=12), and displayed as a percent change from the control (media without leucine added).

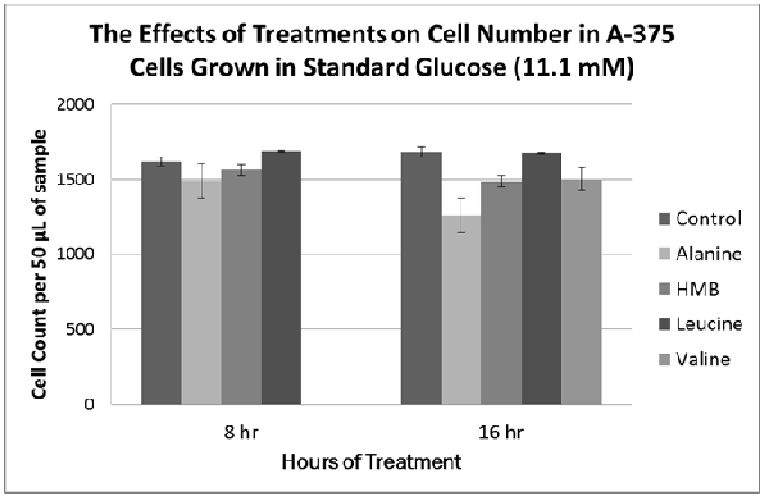
D) Effects of treatments alanine, HMB, leucine, and valine in reduced glucose conditions (5.5 mM). Results are reported as a mean±SE (n=12), and displayed as a percent change from the control (media without leucine added).

*Cell number*

To determine the effects of leucine-stimulated mitochondrial biogenesis on cell proliferation, treated cells were grown in standard and reduced glucose conditions. Cell number was determined at two consecutive doubling times for these cells. Any significant reduction in cell number from the control, and from the first doubling time to the next could indicate a significant treatment effect.

In standard glucose cells, there was no significant treatment effect at 8 and 16 hours (Figure 2A). In reduced glucose A375 cells, there was no significant difference between groups at 9 hours and at 18 hours (Figure 2B). These data show that despite leucine and HMB's ability to stimulate mitochondrial biogenesis in reduced glucose conditions, leucine and HMB-treated cells had no significant reduction in cell number.

A.




B.

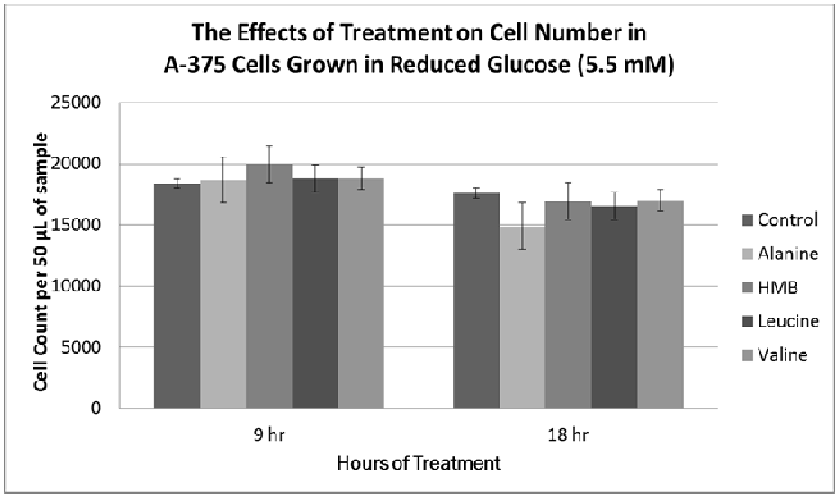



**Figure 2: The effects of alanine, HMB, leucine, valine, and media control on cell number in A-375 cells**
A) Grown in standard glucose conditions (11.1 mM glucose). Results are shown for 8 and 16 hours of treatment, and reported as a total number of cells measured per 50µL sample.
B) Grown in lower glucose conditions (5.5 mM glucose). Results are shown for 9 and 18 hours of treatment, and reported as a total number of cells measured per 50µL sample.

### *Cell cycle Markers*

Treatments had no effect on cell number. Next, we wanted to see the effects of treatments on cell cycle arrest in A-375 melanoma cells. Treated cells were grown in standard and reduced glucose conditions, and mRNA expression for p53, p21, and cyclin D were determined. In standard glucose conditions, cells were harvested in the middle of the cell cycle, and at one doubling time to capture changes in gene expression throughout one replication of the cell cycle. Gene expression for cells grown in reduced glucose conditions was determined at 9 and 18 hours to capture the effects of mitochondrial biogenesis on gene expression.

In A375 cells grown in standard glucose conditions, there was no significant difference between treatment groups for p53 expression at 4 hours and 8 hours (Figure 3A and B). Figure 3A shows mRNA counts. These values are set as a percent of the control in Figure 3B. In reduced glucose A375 cells, there was no significant impact of leucine or HMB on p53 expression at 9 hours and 18 hours (Figure 3C and D). Interestingly, at 9 hours, valine-treated cells had significantly increased p53 expression when compared to the control ($p=0.024$). At 18 hours, valine and alanine had significantly decreased expression of p53 when compared to the control ($p=0.008$). Figure 3C shows mRNA counts, and Figure 3D shows these results as a percent of the control.

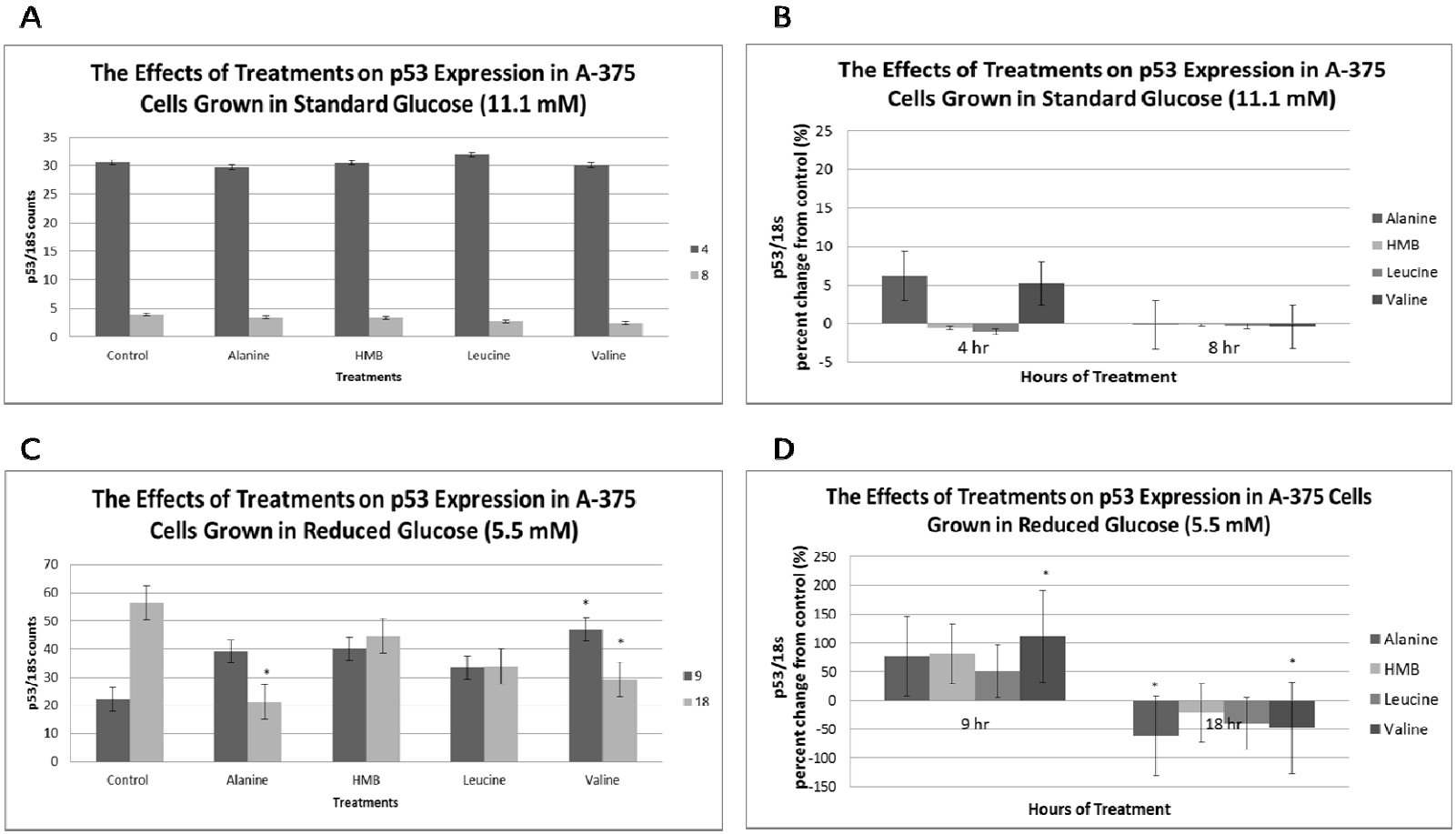



**Figure 3: The effects of treatments alanine, HMB, leucine, valine, and (media) control on p53 expression in A-375 cells**
A) Grown in standard glucose conditions (11.1 mM), showing mRNA counts only.
B) Grown in standard glucose conditions (11 mM) expressed as a percent of the control. Treatment results are shown for 4 and 8 hours of treatment. Results are reported as a p53/18s ratio, and percent change from the control.
C) Grown in reduced glucose conditions (5.5mM), showing mRNA counts only.
D) Grown in reduced glucose conditions (5.5mM), expressed as a percent of the control. Treatment results are shown for 9 and 18 hours of treatment. Results are reported as a p53/18s ratio, and percent change from the control.

Mitochondrial biogenesis occurs early in the cell cycle, in the G1 phase. Accordingly, we measured cyclin D1 gene expression, a marker of G1 cell cycle arrest, at a timepoint in the middle of the cell cycle and after one doubling time of these cells. Significantly reduced cyclin D expression at both time points is a measure of reduced cell cycle progression to the S phase.

In A375 cells grown in standard glucose conditions, there was no significant impact of leucine and HMB treatment on a reduction in cyclin D expression at 4 and 8 hours. The percent fold of the treatments above or below the control follows the trend of p53 expression of cells grown under standard glucose conditions. Figure 4A shows mRNA counts, while Figure 4B shows the treatments as a percent of the control.

In reduced glucose conditions, leucine and HMB treatment had no significant impact on cyclin D expression at 9 and 18 hours (Figure 4C and D). Figure 4C shows mRNA counts, while Figure 4D shows the treatments as a percent of the control. At 18 hours, all treatments reduced gene expression of cyclin D, especially alanine ($p=0.015$), leucine, and valine which reduced cyclin D expression by 61%, 37%, and 46%, respectively from control cells.

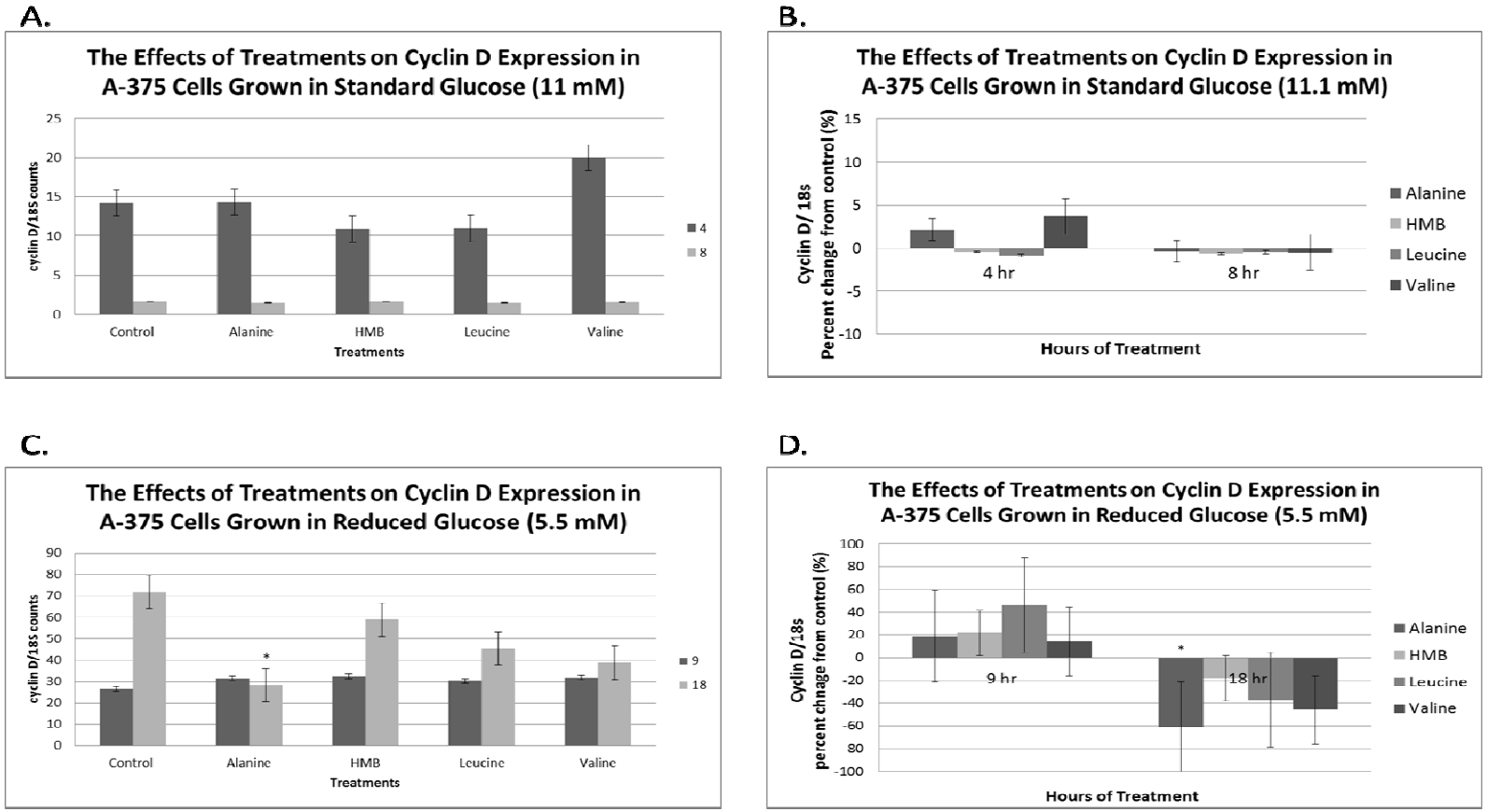



**Figure 4: The effect of treatments alanine, HMB, leucine, valine, and (media) control on cyclin D expression in A-375 Cells**
A) Grown in standard glucose conditions (11.1 mM), showing mRNA counts only.
B) Grown in standard glucose conditions (11 mM) expressed as a percent of the control. Treatment results are shown for 4 and 8 hours of treatment. Results are reported as a cyclin D/18s ratio, and percent change from the control.
C) Grown in reduced glucose conditions (5.5mM), showing mRNA counts only.
D) Grown in reduced glucose conditions (5.5mM), expressed as a percent of the control. Treatment results are shown for 9 and 18 hours of treatment. Results are reported as a cyclin D/18s ratio, and percent change from the control.

Gene expression of p21 and SIRT1 were determined in A375 cells grown in reduced glucose conditions.

In A375 cells grown in reduced glucose conditions, there was no significant impact of leucine and HMB on p21 expression at 9 hours. At 9 hours, all treatments produced an increased expression of p21 in cells when compared to media control. At 18 hours, all treatments produced a decreased expression of p21 in cells when compared to the media control, while alanine, leucine, and valine were significantly decreased by 74%, 52%, and 53%, respectively ($p=0.005$). The trends observed in these cells follow that of p53 expression in cells grown in reduced glucose conditions. Figure 5A shows mRNA counts, while Figure 5B shows treatments as a percent of the control.

Figure 5C shows SIRT1 mRNA counts for A375 cells grown in reduced glucose conditions at 9 hours. When compared to the control, leucine treated cells exhibited a modest increase in SIRT-1 expression at 9 hours, though treatments had no significant impact on SIRT-1 gene expression (Figure 5D).

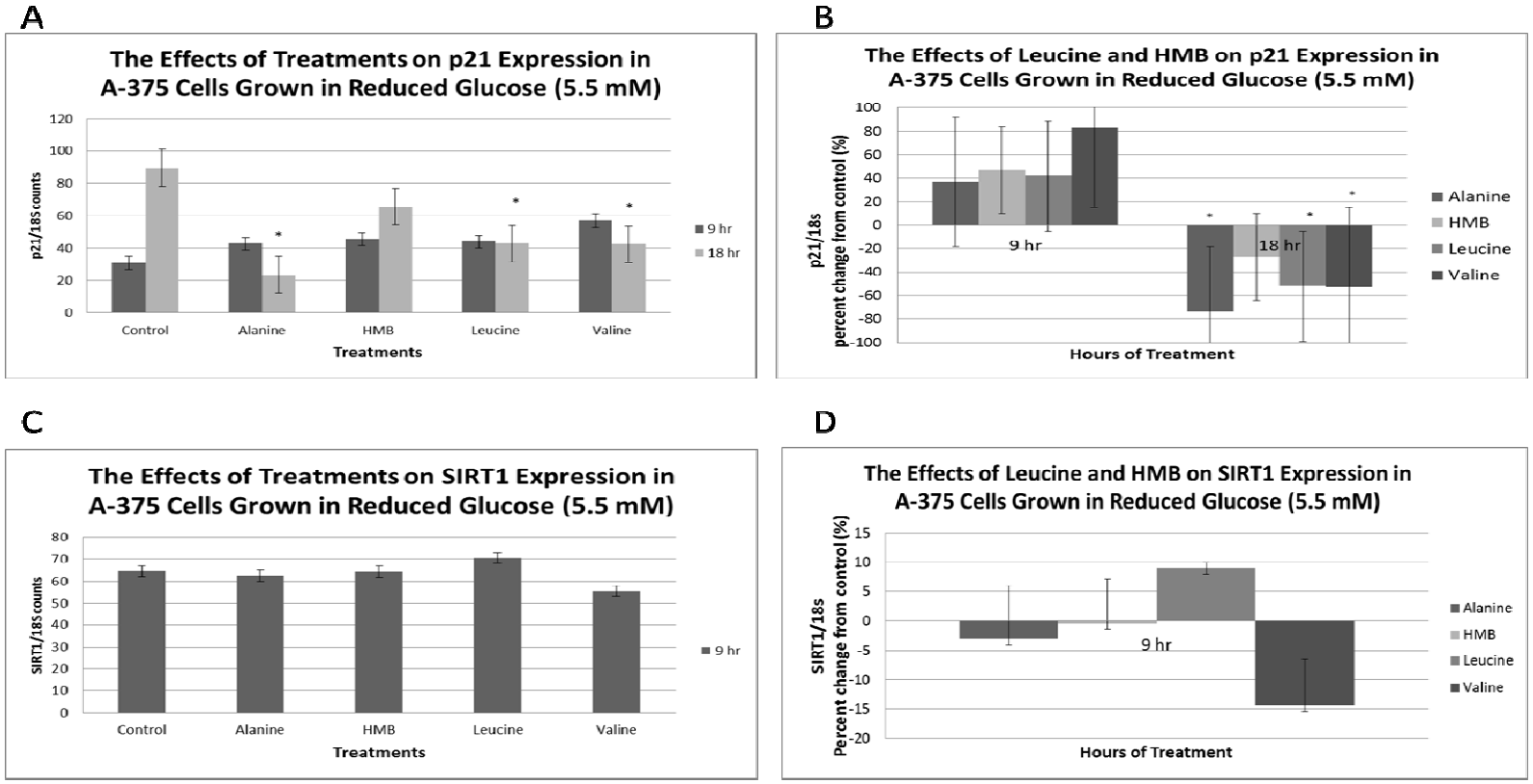



**Figure 5: The Effects of treatments alanine, HMB, leucine, and valine on SIRT1 and p21 expression in reduced glucose conditions (5.5 mM)**

A) p21 expression, showing mRNA counts only.

B) p21 expression shown as a percent of the control. Treatment results are reported as a mean±SE (n=6) and reported as a percent change from the ratio of p21 expression over 18s expression of the control.

C) SIRT1 expression, showing mRNA counts only.

D) SIRT1 expression shown as a percent of the control. Treatment results are reported as a mean±SE (n=6) and reported as a percent change from the ratio of SIRT1 expression over 18s expression of the control.

These data indicate that treatments were able to stimulate mitochondrial biogenesis under reduced glucose conditions, but that leucine and HMB-stimulated mitochondrial biogenesis persisted through one replication of A375 cells. Stimulation of mitochondrial biogenesis was insufficient to stimulate p53 or subsequent exit from cell cycle in these cells.

## Discussion

This study was predicated on the notion that the observed effects of leucine to stimulate mitochondrial biogenesis and correct metabolism in normal tissue would be extended to cancerous cells exhibiting the Warburg Effect. The results show that when glucose is reduced, leucine treated cells were able to stimulate mitochondrial biogenesis throughout one replication of A375 cells. However, early in the cell cycle at 6 hours, alanine and valine, amino acids that served as negative controls, significantly stimulated mitochondrial biogenesis when compared to the control. Additionally, leucine stimulation of mitochondrial biogenesis in these cells was not accompanied by a significant decline in cell number, p53-mediated regulation of cell cycle, or a metabolic shift.

Despite leucine's potential roles in combating metabolic diseases through the restoration of mitochondrial function and abundance (6-11), leucine was without an effect on this cancer cell line. The amino acid controls used in this study, alanine and valine are gluconeogenic (15,16). In addition to its potential roles in metabolic reprogramming, leucine was hypothesized to impact cancer growth due to its previously described signaling roles in normal tissue and classification as an exclusively ketogenic amino acid. Leucine's metabolic products acetoactetate and acetyl CoA cannot serve as a substrate for aerobic glycolysis (16). Our findings show that leucine's impact on cell proliferation and mRNA expression of p53, p21, and cyclin D closely resembles

that of the negative controls. It has been previously suggested that leucine may be a preferred substrate to cancerous cells. In a study using labeled amino acids methionine, leucine, and tyrosine, the tumorous uptake of leucine was the highest (15). Authors postulate that while leucine may not feed directly into glucose metabolism, branched-chain amino acid degradation may donate an amino group needed for biosynthesis of glucogenic amino acids (15).

To put these findings into context, we cannot discount leucine's complex signaling roles to act as a substrate to these A375 melanoma cells. First, leucine is well known as a stimulator of mTOR-mediated protein synthesis within muscle cells. We have previously demonstrated that leucine-stimulated mitochondrial biogenesis was accompanied by p53 expression in normal adipocytes. This event was accompanied by a decrease in fatty acid synthase. But, this signaling role was not extended to muscle tissue. Instead, leucine stimulated mTOR (7), an event known to antagonize the expression of p53 (10). Previous studies have shown that unregulated PI3K/Akt/mTOR signaling are required for sustained glycolysis, a role thought to precede a metabolic shift toward aerobic glycolysis and further mitochondrial dysfunction in tumorous cells (1,10,17,18). One study shows that Akt/mTOR induction converts WM35 melanoma cells to glycolytic/Warburg metabolism (18), a role that leucine deprivation does not attenuate (19). mTOR participates in a tumorigenic role through the induction and transcription-mediated activation of HIF-1α (17). HIF-1α mediates glycolytic gene transcription in the absence of hypoxia to increase glycolytic enzymes, GLUT1, and GLUT3 in cancer cells (1-3,20). Interestingly, malignant melanoma cells contain GLUT1, and exhibit the Warburg effect (18). HIF-1α signaling has been linked to aggressive, metastatic cancers, and serves to keep a constant energy supply (1,2,3,10,16). Additionally, mTOR signaling suppresses p53 and p21 signaling (18). One study showed that A375 melanoma cells had increased proliferation in response to

HIF-induced suppression of p53 (19).

Next, leucine's signaling role for SIRT1-mediated mitochondrial biogenesis may negatively affect p53-mediated regulation of cell cycle. We have previously demonstrated that leucine's ability to stimulate mitochondrial biogenesis is SIRT1 mediated; leucine significantly stimulates SIRT1 activity and expression in normal tissue, while the branched-chain amino acid control valine does not exert a similar effect (13). Our data show that leucine's ability to stimulate mitochondria throughout one replication of A375 cells was accompanied by a modest, but not significant SIRT1 mRNA expression. The time that we measured SIRT1 gene expression in this study was simultaneous with leucine-stimulated mitochondrial biogenesis, an event that SIRT1 activity has been shown to precede (13). SIRT1 is well known to be an energy sensor, with its activation dependent upon low energy in the form of $NAD^+$ availability (13, 21). At 9 hours in reduced glucose conditions, a time point in which only leucine and HMB significantly stimulated mitochondrial biogenesis in this cell line, only leucine treatment is associated with an increase in SIRT1 mRNA expression over the control. Consistent with this finding, when compared to the control, only leucine treatment is associated with significantly stimulated mitochondrial biogenesis at the subsequent measured time point, 12 hours.

Under Warburg conditions, aerobic glycolysis utilizes the pentose phosphate pathway to gain NADPH (1-3). This high cytosolic NADPH would be unfavorable for the activation of SIRT1. Our data demonstrate that in the absence of hyperglycemic conditions, all treatments increase mitochondrial biogenesis, but that leucine dose-responsively stimulates mitochondrial biogenesis and these effects last throughout the A375 cell cycle. A previous study demonstrated that A375 melanoma cells are one of the few melanoma cell lines to exhibit wild-type p53 (22). When A375 melanoma cells were administered with ultraviolet damage, the most common type

of damage for this cell line, p53 and p21 expression increased. These cells inhibited replication by 75% and sent roughly 15% of cells to apoptosis via nuclear-mediated mechanisms (23). Interestingly, the translocation of p53 to the nucleus has been shown to be suppressed during SIRT1 activity (4,5). These findings demonstrate that leucine's stimulation of SIRT1 to mediate mitochondrial biogenesis may be an unwanted signaling role in this cell line.

The use of standard and reduced glucose medium in this study demonstrates that reduced glucose conditions can increase the effectiveness of the treatments when compared to the control. Additionally, these data support an impact of dietary glycemic control may on cancers that exhibit the Warburg effect, as recently suggested (24). A375 cells utilize glucose as their primary substrate, a finding consistent with Akt/mTOR signaling within the cell. Under standard glucose conditions, there was no treatment effect of these amino acids. The level of glucose in media has been shown to play a role in altering outcomes *in vitro* in cancer cell lines. Colon, breast, prostate, and bladder cancer cell lines grown under hyperglycemic conditions (11 mM) showed altered gene expression, including Cyclin A and E, PI3K, protein kinase C α and β, and E-cadherin favoring cell proliferation, migration, tumor cell adhesion, and survival, while cells in reduced glucose (5.5mM) did not show a dramatic up-regulation of these markers. Additionally, proliferation was increased when insulin was added to media (25).

All media used in our study contained all amino acids without the deprivation of any nutrient. The amino acids in the media represented those found in an amino pool. Thus, we cannot discount the utilization of amino acids that could serve as glycolytic precursors to further mediate glycolytic metabolism and proliferation in A375 cells, regardless of amino acid treatment.

On the other hand, a small body of data discusses the "Warburg Diet", a ketogenic diet comprised of high fat and protein that is hypothesized to promote better outcomes in cancer patients (1). Leucine could be used as a metabolic substrate that could counterbalance Warburg metabolism by stimulating mitochondrial biogenesis, fat oxidation, and promoting protein muscle synthesis in the surrounding healthy tissues. Under this theory, leucine could have beneficial long term effects that would not be apparent in a short term study.

Our study has some shortcomings. First, leucine's effects on metabolic shift were not evaluated, such as its role in fat oxidation, reduced lipid storage, mitochondrial function, and insulin sensitivity. While previous data from our lab demonstrate leucine's role in stimulating mitochondrial biogenesis in normal tissue is accompanied by mitochondrial function gene expression (6-9), these were not assessed in our study. Regardless, these data demonstrate that leucine's stimulation of mitochondrial biogenesis was not accompanied by a significant decline in cell number, increase in p53 and p21 mRNA expression, and decline in cyclin D mRNA expression. Due to the absence of impact on cell number or restoration of p53 expression, the insignificant differences between mRNA of p53, p21, and cyclin D indicate that no metabolic shift occurred in response to any treatment in this study.

Previous studies in A375 melanoma cells have shown that these cells have a relatively short doubling time of 6-12 hours (14). Our study relied on flow cytometry to determine doubling time and cell proliferation within this cell line. While these results demonstrate no significant treatment effects on cell proliferation after one doubling time of these cells, we cannot discount the events that may have occurred during the cell cycle and were missed using gene expression during our time course. Our use of flow cytometry and mitochondrial mass dictated times to harvest these cells and determined the time course of this study. While this time

course was an accurate depiction of amino acid signaling roles throughout 24 hours of treatment in these cells, this time course could have been inaccurate due to multiple factors. It has been noted that increased gluconeogenesis has been documented in cancer patients, and is associated with muscle wasting (16). Gluconeogenic amino acids that supply these cells with their primary substrate could significantly accelerate cell growth that would not be accurately captured using the same time point for all treatments. The concentrations of other amino acids in the media invite further investigations on how manipulation could impact this cell line, though the media used in this study represents postprandial conditions most commonly viewed in the American diet.

In conclusion, we have shown that leucine stimulate of mitochondrial biogenesis under lower glucose conditions was not accompanied by stimulation of p53 or subsequent exit from cell cycle. These findings suggest that while beneficial for protection from age-related disease and in cancer–induced cachexia, leucine signaling was unable to reverse the Warburg effect in these cells.

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# Part Four:

# Summary and Conclusions

The hypothesis that metabolic reprogramming can mitigate metabolic diseases and disorders is well accepted. Cancer that exhibits the Warburg effect is hypothesized to be a metabolic disorder. We have previously demonstrated the roles of leucine in anti-obesity and other metabolic diseases within normal tissue through its unique signaling roles in mitochondrial biogenesis, fat oxidation, and oxidative phosphorylation. The present study does not support that leucine-stimulated mitochondrial biogenesis will significantly impact cell proliferation, stimulate p53 expression, regulate cell cycle, or reverse the Warburg effect within A375 melanoma cells.

The finding that leucine's effects on mitochondrial biogenesis are comparable to that of the gluconeogenic, negative controls alanine and valine used in this study were unexpected. These results exemplify leucine's roles as a substrate for cancerous cells. However, these data exemplify the impact of glucose in cancerous cell signaling. These results show that a reduction and control of glucose has a significant impact on cell growth and proliferation.

# Vita

Tia Marie Filhiol was born on May 22, 1987 in Biloxi, Mississippi. After graduating from high school, she attended Auburn University (Auburn, Alabama) where she gained her Bachelor's Degree in Nutrition and Food Science: Dietetics in May 2010. She attended graduate school at the University of Tennessee in Knoxville, Tennessee in August 2010. There, she pursued her combined Master's Degree in Cellular and Molecular Nutrition and Dietetic Internship. In November 2012, she gave her oral Master's thesis defense, and graduated in December 2012.