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PANCREATIC CANCER AND CACHEXIA: EFFECTS OF LEUCINE AND HMB SUPPLEMENTATION

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PANCREATIC CANCER AND CACHEXIA: EFFECTS OF LEUCINE AND HMB SUPPLEMENTATION

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PANCREATIC CANCER AND CACHEXIA: EFFECTS OF LEUCINE AND HMB SUPPLEMENTATION

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Pancreatic cancer has the highest mortality rate of all major cancers with a 5-year survival rate of 8%. A major contributor to this statistic is the lean muscle loss that occurs with cachexia, a condition found in 80% of pancreatic cancer patients. The effects of two potential cachexia treatments—the branched-chain amino acid, leucine, and its metabolite, β-hydroxy-β-methylbutyrate (HMB)—have been analyzed in multiple studies. However, most of these studies have determined their effects on muscle tissue only, while their effects on pancreatic tumor growth remain unknown. The objective of this current study was to evaluate the impact of dietary leucine and HMB supplementation on pancreatic ductal adenocarcinoma (PDAC) in pro-tumor and anti-tumor environments and identify potential mechanisms of inhibition or enhancement.

Using C57BL/6 male mice, we demonstrated that dietary leucine supplementation enhanced pancreatic tumor growth in both the pro-tumor environment of overweight mice and the anti-tumor environment of lean mice.

Leucine supplementation increased the amount of circulating glucose available for the tumor in the overweight mice, while it increased tumor mechanistic target of rapamycin (mTOR) activation in the lean mice.

Next, we determined that dietary HMB supplementation preserved muscle mass through increased muscle mTOR activation. HMB supplementation also inhibited pancreatic tumor growth and enhanced the efficacy of the chemotherapy gemcitabine in the pro-tumor environment of obese C57BL/6 mice. HMB supplementation downregulated numerous olfactory receptor genes that were upregulated in the tumors of obese mice, and HMB also increased cytotoxic CD8+ T cell infiltration in these tumors.

Additional experiments were conducted to determine the potential mechanisms for the effects of leucine and HMB on pancreatic cancer. We showed leucine supplementation increased, while HMB supplementation decreased, both human and murine pancreatic cancer cell proliferation and mTOR signaling. Leucine and HMB also had differential effects on the regulation of certain olfactory receptor genes, and octanal, an olfactory receptor agonist, could mimic the repressive effects of HMB supplementation on pancreatic cancer cell proliferation.

Collectively, these findings suggest that HMB has the most potential for cancer cachexia treatment due to its ability to preserve muscle mass, reduce tumor growth, and enhance the effects of chemotherapy. The *in vivo* results suggest leucine and HMB can affect tumor indirectly by manipulating physiology, and the *in vitro* findings suggest they can also affect cancer cells directly.

Table of Contents

Page Number

List of Figures

Page number

Chapter I

Introduction

Obesity, Cachexia and Cancer

The prevalence of obesity, defined as a body mass index (BMI) of ≥ 30 kg/m^2, has remained high in the United States (US) for decades with 1 in 3 adults and almost 1 in 5 children obese (1). Obesity is a well-established risk and progression factor for many cancers (including pancreatic cancer) and contributes to 15-30% of cancer deaths in the US (2).

While obesity can enhance tumor growth, another condition called cancer cachexia, can weaken the patient. Cachexia, which is characterized by significant muscle loss, is estimated to be the cause of death in 20% to 40% of cancer patients and is present at the highest incidence in gastric, pancreatic, and lung cancer patients (3). The combination of obesity and cachexia results in the poorest prognosis due to simultaneous enhancement of tumor progression and metabolic and immune dysregulation that weaken the body and increase mortality risk (4).

Due to the obesity epidemic in Western society, a growing proportion of pancreatic cancer patients at the start of therapy have a body mass index (BMI) in the overweight or obese range. Many of these individuals have sarcopenic obesity, a condition characterized by the presence of high fat mass but low muscle mass (sarcopenia). Recent studies have reported that obesity in the presence of sarcopenia is predictive of morbidity and mortality in pancreatic cancer patients, at least in part because sarcopenic obesity increases the odds of developing cancer cachexia (5).

Given the consistently high prevalence of obesity and no approved medications to treat cachexia and muscle loss in cancer patients, mortality rates will continue to be high in these patients (3). Therefore, it is crucial for research to be performed that aims to identify potential treatments for cachectic muscle loss that are safe to administer to cancer patients.

Diet and Changing the Physiological Environment

Obesity is associated with cancer growth because of its ability to alter the body's physiological environment. Obesity is associated with increased growth signaling and increased systemic inflammation in humans, and the diet-induced obesity (DIO) regimen induces a similar pro-tumor environment in animal models (2,6). This translates to DIO-induced enhancement of tumor growth in these animal models (7-9). The DIO regimen is a diet with 60% total calories from fat and is usually compared to a control diet with only 10% total calories from fat (7).

In contrast, calorie restriction (CR) acts as a potent inhibitor of cancer growth in animal models by creating an anti-tumor environment and inhibiting the same growth and inflammatory pathways enhanced by DIO (10). The CR regimen restricts total calorie intake by 30% relative to *ad libitum*-fed controls, while maintaining isonutrient conditions to avoid malnutrition (11).

The pro- and anti-tumor effects of the DIO and CR diets are consistent across species, tumor type, and method of tumor induction, suggesting that systemic circulating factors may be mediators of the growth and inflammatory pathways important to cancer growth (10-11). In addition, these pathways are also relevant when

studying potential cachexia treatments, because most cachexia research has focused on how aberrations in growth signaling and inflammation affect muscle growth and breakdown (12). Due to the importance of these pathways for both tumor growth and muscle preservation, the next section will discuss them in detail.

Mechanisms Associated with Diet Effects on Tumor and Muscle

The obesigenic DIO diet creates a pro-tumor environment associated with high levels of multiple circulating factors that enhance either growth signaling or inflammatory pathways within tissues. Insulin and insulin-like growth factor-1 (IGF-1), and adiponectin affect growth signaling, while the hormone leptin and pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), IL-6, IL-17A affect inflammatory signaling.

Defining the factors that modulate growth signaling, insulin is a peptide hormone synthesized by pancreatic β-cells in response to elevated blood glucose (13). IGF-1 is a peptide growth factor produced by the liver and is responsible for facilitating growth and development of many tissues (14). Adiponectin is a hormone secreted by adipocytes involved in regulating glucose and fatty acid breakdown, and it is often seen as an antagonist to leptin (15). While these hormones have different functions, their effects on cancer can be largely attributed to their actions on one pathway involving the mechanistic target of rapamycin (mTOR).

Circulating insulin and IGF-1, which are positively associated with adiposity, attach to their respective receptors on the cell surface, and activation of these receptor tyrosine kinases stimulates PI3K to produce lipid messengers. They activate Akt, which

then leads to the phosphorylation and activation of mTOR and downstream regulator of protein translation, ribosomal S6 kinase 1 (S6K1) (15-16). This mTOR-induced increase in global protein translation is associated with increased tumor growth (18), and inhibition of the mTOR pathway inhibits cancer growth in various *in vivo* and *in vitro* models (19-22). On the other hand, adiponectin, which is negatively-correlated with adiposity, reduces mTOR signaling through activation of AMP-activated kinase (AMPK) (13). AMPK inhibits mTOR by phosphorylation of the tuberous sclerosis complex 2 (TSC2). This stabilizes the TSC1-TSC2 complex and allows it to inhibit Ras homolog enriched in brain (Rheb), which is then unable to activate mTOR (23). Adiponectin can also inhibit inflammatory pathways by inhibiting nuclear factor κ light-chain enhancer of B cells (NF-κB), which is discussed below (24).

Defining the factors that modulate inflammatory pathways, leptin is a peptide hormone produced by adipocytes and primarily functions as an energy sensor (25). TNF-α is produced largely by macrophages and is involved in the regulation of immune cells (26). IL-1β is produced by macrophages and undergoes further processing to become active in mediating the inflammatory response (27). IL-6 is secreted by T cells and macrophages to stimulate the immune response (28). IL-17A is produced by T-helper cells and helps in the recruitment of monocytes and neutrophils to sites of inflammation (29). Although the original functions for leptin and pro-inflammatory cytokines are different, their effects on cancer are largely due to their effects on transcription factors.

Leptin binds to its receptor, which is similar to a class I cytokine receptor (13), and activates Janus kinases (JAKs). These kinases phosphorylate each other and

intracellular end of their receptors in order to create docking sites for signal transducers and activators of transcription (STATs). JAK-induced phosphorylation of STATs activate them, and they translocate to the nucleus and bind to DNA (30). The STAT transcription factors then enhance transcription of various genes involved in cell growth, proliferation, and survival. Due to the wide array of genes it regulates, STAT dysregulation is often associated with cancer (31). STAT activation is also tied to inflammation through a few inflammatory cytokines (IL-6, IFN-γ, IL-10) (30). However, the most well-studied and prominent of inflammatory pathways is the canonical NF-κB pathway.

NF-κB is normally attached to inhibitor of κB (IκB) proteins in the cytoplasm of cells. When cells are exposed to pro-inflammatory cytokines, the IκB kinase (IKK) complex activates and phosphorylates the IκB proteins. This targets the IκB proteins for ubiquitin-dependent breakdown and frees NF-κB, which translocates to the nucleus and enhances the transcription of multiple genes (32). Many of these genes code for proteins involved in immunity, proliferation, and the inhibition of apoptosis. Through transcription of these genes, NF-κB activation promotes tumor growth, while NF-κB inhibition reduces tumor growth in various cancer models (32-38) *in vivo* and *in vitro* cancer models.

Although there are numerous pathways that affect tumor growth and often cross-talk between these pathways, mTOR and NF-κB are specifically highlighted, because they have significant roles in skeletal muscle as well. There is extensive literature supporting the role of the mTOR pathway in regulating skeletal muscle hypertrophy by increasing protein translation and synthesis (39-40). mTOR signaling promotes muscle

growth regardless of animal model or type of muscle atrophy studied, including disuse (41), denervation (42), disease-induced (43). When mTOR activation is inhibited, muscle wasting occurs (44).

NF-κB signaling, on the other hand, promotes muscle atrophy. In muscle, NF-κB specifically enhances the transcription of muscle RING finger 1 (MuRF-1), which is an atrogene. MuRF-1 is a ubiquitin ligase, involved in the rate-limiting step of the ubiquitination process of the ubiquitin-proteasome system. This makes it a crucial enzyme for proteasome-dependent degradation of proteins, so it is upregulated during muscle breakdown (45). Mice lacking MuRF-1 are resistant to muscle atrophy induced by denervation (46) or dexamethasone (47), and if NF-κB is inhibited, muscle atrophy induced by denervation (48) or hindlimb unloading (49) is inhibited.

In summary, mTOR signaling promotes tumor and muscle growth, while inflammatory NF-κB signaling promotes tumor growth and muscle breakdown. Due to the significance and tissue specificity of mTOR and NF-κB action, potential treatments for cancer cachexia should be evaluated for their effects on these pathways.

Nutritional Supplementation to Preserve Muscle Mass

There is considerable support in the literature for using nutritional supplementation to improve cachectic symptoms in cancer patients, and while a number of nutritional regimens have been suggested, much of the research focuses on supplements that improve muscle growth or inhibit muscle breakdown (50-51). This focus on muscle has led researchers to study amino acids, the building blocks of protein, and ascertain whether they have effects on growth and inhibition signaling as

well. The goal is to discover a supplement that can be not only an activator of growth or inhibitor of breakdown, but also utilized itself for protein synthesis.

The branched-chain amino acids, leucine, isoleucine, and valine, are found at high percentages in skeletal muscle, and can increase human muscle protein synthesis almost to the same extent as complete meals (52). Leucine alone stimulates human muscle protein synthesis, although for a shorter duration (52). It is also the leucine content of dietary proteins that is the determining factor for muscle protein synthesis in rats (53). This is because, leucine activates mTOR within skeletal muscle (54). Thus, leucine supplementation is a popular potential treatment for cachexia.

Due to rising interest in leucine, research began to be conducted on its metabolites. One in particular, β-hydroxy-β-methylbutyrate (HMB), increases muscle mTOR activation and muscle growth (55-56). HMB also inhibits muscle NF-κB activation and inhibits muscle atrophy (56-57). Thus, HMB supplementation, which is actually better tolerated than leucine (58), is another popular potential treatment for cachexia. However, due to the focus of cachexia research on muscle, not much is known about how these supplements affect tumor growth. If these supplements are going to be administered to cancer patients with cachexia, examining their effects on tumor growth is important.

Analyzing Pancreatic Cancer Growth In Vivo and In Vitro

To determine the effects of leucine and HMB on tumor growth, the type of cancer, animal model, and method of tumor induction needed to be selected. Pancreatic cancer, C57BL/6 male mice, and Panc02 cell subcutaneous injection were chosen

based on the following reasons: 1) Cachexia occurs in 80% of pancreatic cancer patients (59). 2) Diet, both obesigenic and calorie restrictive, affect pancreatic tumor growth (60-61). 3) Pancreatic cancer is dependent on both the mTOR and NF-κB pathways for growth (60-61). 4) C57BL/6 mice are well-known to respond physically to different diets and have been successfully used to study pancreatic cancer (60-61). 5) Male C57BL/6 mice do not have an estrous cycle, which can affect growth hormone levels and indirectly affect pancreatic tumor growth (62). 6) Panc02 cells are syngeneic to the C57BL/6 mice (60). 7) Subcutaneous tumors are easily palpated, monitored, excised, and measured ex vivo (60-61). 8) There are no studies of leucine or HMB supplementation in a pancreatic tumor model.

Pancreatic cancer cells were used to further confirm effects found in the animal studies. Two murine (Panc02 and NB508) and two human (Panc-1 and MiaPaca-2) pancreatic cancer cell lines were used. The Panc02 line was originally derived from a chemically-induced PDAC in the pancreas of C57BL/6 mice (63). The NB508 line was originally derived from the Kras/INK4A transgenic mouse, which has a mutant K-ras and a heterozygous deletion for INK4A. These genes are the most commonly mutated in spontaneous human PDAC (64). The Panc-1 and MiaPaca-2 cell lines were originally derived from different human PDACs (65-66). The data presented in Chapters II, III, and IV demonstrate the effects of leucine and HMB supplementation on *in vivo* and *in vitro* pancreatic cancer growth and identify potential mechanisms underlying these effects.

Potential Interplay Between Diet and Cytotoxic T Cells

While understanding how diet and supplementation affect tumor growth is important, understanding how they affect the physiological environment can be vital when interpreting results. High levels of inflammatory markers are often associated with obesity, and this chronic inflammation can affect immunologic cells, including dendritic, natural killer, and T cells (67). Cytotoxic CD8+ T cells are associated with reduced pancreatic tumor growth and a more positive prognosis if they infiltrate the tumor (68). Unfortunately, the pro-tumor obese environment is associated with decreased cytotoxic CD8+ T cell levels (67,69). This may be due, in part, to dysregulated NF-kB signaling, which impacts T cell differentiation. NF-kB activity is important for CD8+ T cell synthesis specifically, because the inhibition of NF-kB activity results in lower numbers of CD8+ T cells both in the thymus and in circulation (70). TNF-α inhibition results in the same reduction of CD8+ T cell-mediated activity (71).

Although the aforementioned effects seem contradictory to the obesity-induced low T cell numbers, negative feedback loops can be activated during chronic inflammation. IL-2, a promoter of T cell synthesis, upregulates the transcription of its own inhibitor, FOXP3 (72). NF-kB itself also enhances the transcription of its own repressor (IκBα) (73). HMB supplementation has been shown to inhibit inflammatory and NF-kB signaling in various tissues, so it may also play a role in cytotoxic T cell regulation as well. Whether HMB supplementation can affect the immune system is unknown and will be explored in Chapter III.

Potential Interplay Between Diet and Olfactory Receptors

Identifying how diet and supplementation may change pancreatic cancer cells is also important to understand the entire story. The pro-tumor, obese environment can also affect gene regulation in tissues, specifically cancer-related genes and olfactory receptor genes. Mice on a high-fat diet had upregulation of olfactory receptors and cancer-related genes in subcutaneous fat tissue and gastrocnemius muscle compared to mice on a non-fat diet (74). The upregulation of cancer-related genes makes sense, because it is well-known that obesity enhances tumor growth (13). However, the reason for obesity-induced upregulation of olfactory receptor genes is unknown.

The olfactory receptor gene family is the largest in the mammalian genome, and olfactory receptor expression can be detected in non-olfactory tissues (75). Thus, it is reasonable to assume they may play non-olfactory roles. In fact, cancer cells may be utilizing them for their own purpose, as olfactory receptor expression is highly dysregulated in various cancer cells, including prostate and pancreatic (76-77). This data is correlative, however, and no mechanistic conclusion can be attained through the literature. Chapter IV will explore the effects of HMB and leucine on olfactory receptor expression and also whether olfactory receptor expression can affect pancreatic cancer cells.

Specific Aims

The overall goal of this project was to determine the impact of leucine and HMB supplementation, two potential cancer cachexia treatments, on pancreatic tumor growth in a mouse model and to elucidate potential mechanisms underlying these effects. Different diets were included to provide either anti-tumor or pro-tumor environments, because these nutritional supplements may be provided to patients with different diet-induced physiological environments. The individual specific aims were as follows:

1) Characterize the effects of dietary leucine supplementation on pancreatic tumor growth in lean (CR) and overweight (control) mice (Chapter II). We evaluated diet- and leucine-induced changes on physiological parameters (body weight, lean mass, fat percentage, serum hormones, glucose tolerance), and on pancreatic tumor growth in C57BL/6 mice. Immunohistochemistry was performed to determine the effects of CR and leucine on tumor cell proliferation (Ki-67) and mTOR activation (p-S6). MTT assays and western blot analyses were performed to determine the effects of CR and leucine on Panc02 cell proliferation and mTOR activation.

2) Characterize the effects of dietary HMB supplementation on pancreatic tumor growth and muscle breakdown in overweight (control) and obese (DIO) mice treated with gemcitabine (Chapter III). We evaluated diet- and HMB-induced changes on physiological parameters (body weight, serum hormones, serum cytokines, muscle size) and on pancreatic tumor growth in C57BL/6 mice. Immunohistochemistry was performed to determine the effects of DIO, gemcitabine, and HMB on tumor cell proliferation (Ki-67) and mTOR activation (p-S6). A microarray was performed to determine the effects of DIO and HMB on tumor gene expression, and immunohistochemistry and qPCR were done to validate and further explore the results.

3) Determine the mechanisms by which leucine and HMB supplementation affect murine and human pancreatic cancer cells (Chapter IV). We evaluated leucine- and HMB-induced changes in murine (Panc02 and NB508) and human (Panc-1 and MiaPaca-2) cells. MTT assays and western blot analyses were performed to determine the effects of supplementation on pancreatic cancer cell proliferation and mTOR signaling, respectively. Seahorse experiments were performed to determine effects on cellular metabolism, and a microarray was done to determine effects on gene expression. As a follow up to the microarray results, MTT assays with olfactory receptor agonists and qPCR for specific olfactory receptors were performed to validate and further explore the results

Chapter II

Leucine supplementation differentially enhances pancreatic cancer growth in lean and overweight mice (78)[1].

Introduction

Effective prevention and treatment strategies are urgently needed for pancreatic cancer, the 4th leading cause of cancer-related death in both men and women in the United States (79). Less than 15% of pancreatic cancer patients have localized disease amenable to curative resection, and the overall 5-year survival rate in affected patients is less than 5% (80). Obesity is an established pancreatic cancer risk and progression factor in humans and animal models (81-83). In contrast, calorie restriction (CR) prevents or reverses obesity and related metabolic perturbations and pancreatic tumor development and/or progression in experimental models (84-85,87-89); the impact of CR on human pancreatic cancer has not been well studied. CR results in a negative energy balance state and exerts its antitumor effects, at least in part, through decreased mammalian target of rapamycin (mTOR) signaling in many epithelial tissues (84, 85-88). mTOR acts as a nutrient sensor that regulates protein synthesis, cell survival, and proliferation in response to growth factor levels, nutrient availability, and intracellular energy status. We have previously established that rapamycin (a selective mTOR

[1] Liu KA, Lashinger LM, Rasmussen AJ, Hursting SD. Leucine supplementation differentially enhances pancreatic cancer growth in lean and overweight mice. Cancer Metab. 2014;2:6. Lashinger contributed to ideas, animal study, and editing. Rasmussen contributed to animal study. Hursting contributed to ideas, finances, writing, and editing.

inhibitor), and metformin (an indirect inhibitor of mTOR signaling through its effects on gluconeogenesis and associated activation of AMPK-regulated signals), partially mimic the tumor inhibitory effects of CR on transplanted pancreatic tumor growth (89).

Branched-chain amino acids (BCAAs), which account for over 20% of total dietary protein intake, are known activators of the mTOR pathway in muscle and epithelial tissues (90-92). Of the three BCAAs, leucine exerts the most potent effect on mTOR activation and enhancement of protein synthesis in various tissues, including skeletal muscle (93-94). Athletes commonly use leucine supplementation to activate mTOR-regulated protein synthesis and accelerate muscle repair and regeneration after injuries or intense bouts of exercise (95). Leucine supplementation is also increasingly being recommended to reduce the muscle wasting that occurs with cancer cachexia (95). Cachexia is characterized by involuntary weight loss and muscle wasting, is associated with increased morbidity and mortality, and frequently occurs in pancreatic cancer patients (97). Increased muscle protein synthesis in response to leucine-induced mTOR activation has been shown to inhibit muscle wasting in mouse models of cancer cachexia and in cancer patients (98-101). However, the rates of protein synthesis increase to a much greater extent in tumors than in muscle (100), suggesting that while leucine supplementation may protect against cancer-associated cachexia, it may also enhance the progression of the cancer.

Unfortunately, studies of the effects of leucine supplementation on cancer are limited. Long-term leucine supplementation (2% of diet, w/w) promoted bladder

cancer development in rats treated with a known bladder carcinogen (102-103), but no studies have connected leucine supplementation with tumor growth. In the present study, we tested the effect of leucine supplementation on transplanted Panc02 mouse pancreatic cancer growth and mTOR signaling in the context of lean mice (fed a CR diet regimen) or overweight mice (fed a high calorie control diet regimen). Our findings suggest that leucine enhances pancreatic tumor progression in lean and overweight mice, and the underlying mechanisms may differ by weight status.

Materials and Methods

Mice and dietary interventions

All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Eighty-eight male C57BL/6 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA, USA) at 6 to 8 weeks of age, and upon arrival were singly-housed in a semibarrier facility at the University of Texas at Austin Animal Resource Center and fed a chow diet during a one-week acclimation period. Mice were then randomized to receive one of four diets for 27 weeks: (i) AIN-76A control diet consumed *ad libitum* (control, n = 22); (ii) 30% CR diet (CR, n = 22); (iii) control diet with leucine supplementation (5% of diet, w/w) consumed *ad libitum* (control + LEU, n = 22); or (iv) 30% CR diet with leucine supplementation (CR + LEU, n = 22). The AIN-76A control diet, when consumed *ad libitum*, results in an overweight phenotype characterized by steady weight gain, while the CR results in a lean phenotype characterized by weight maintenance (104-105). Both CR diets were administered as a daily aliquot providing 70% of the total energy but 100% of the vitamins, minerals, amino acids and fatty acids consumed by the controls. Leucine was purchased from AIDP, Inc. (City of Industry, CA, USA) and was incorporated into the AIN-76A diet premix to provide 50 g/kg feed, or 5% dietary leucine supplementation. This dose of leucine is commonly used in animal studies of muscle regeneration (106-107). All diets were purchased from Research Diets (New Brunswick, NJ, USA).

Energy intakes and body weights for each mouse were recorded weekly for 21 weeks, and then glucose tolerance tests (GTTs) were performed on a randomly selected subset of animals (n = 10/group). After injecting a 20% (w/v) glucose solution, blood glucose levels were measured with a Contour glucometer (Bayer HealthCare LLC, Mishawaka, IN, USA) at baseline, 15, 30, 60, and 120 minutes. Also after the rats were on the diet for 21 weeks, quantitative magnetic resonance (qMR) analysis (EchoMRI, Houston, TX) was done on a randomly selected subset of animals (n = 10/group) to obtain percent body fat and lean mass. At 22 weeks on the diet, all the mice were fasted for 12 hours and blood samples were collected from the retro-orbital venous plexus. After coagulating at room temperature for 30 minutes, blood samples were centrifuged at 9,300 × g for 5 minutes. Serum was separated, then snap-frozen and stored at −80°C until assayed for hormones. At 23 weeks on the diet, randomly selected mice (control, n = 7; control + LEU, n = 6; CR, n = 6; and CR + LEU, n = 7) were fasted for 12 hours and anesthetized by CO_2 inhalation. Blood was collected by cardiac puncture, and pancreata were collected and stored for analyses other than those outlined in this manuscript. One mouse from the control + LEU group died, and one mouse from the CR group died before week 23. All remaining mice (n = 15/group) were subcutaneously injected into the right flank with 500,000 Panc02 cells (kindly provided by Dr. J. Schlom, National Cancer Institute, Bethesda, MD, USA) suspended in serum-free McCoy's 5A medium. Once palpable, tumors were measured weekly with calipers, and tumor volume was approximated using the formula for an ellipsoid ($4/3\pi r_1^2 r_2$).

At 27 weeks on the diet, mice were fasted for 12 hours and then anesthetized by CO_2 inhalation. They then underwent cardiac puncture for blood collection and were subsequently killed by cervical dislocation. Pancreatic tumors were harvested and either snap-frozen in liquid nitrogen and stored at −80°C, or fixed with 10% neutral-buffered formalin overnight, switched to 70% ethanol, paraffin embedded, subsequently used for immunohistochemical analyses

Serum hormones

After study termination, serum insulin and leptin levels were analyzed using Lincoplex™ bead-based multiplexed assays (Millipore, Billerica, MA, USA; MADPK-71 K-07). Serum adiponectin and IGF-1 were quantified by singleplex assay kits (Millipore; MADPK-71 K-ADPN and RMIGF187K, respectively). All assays were analyzed using a BioRad Bioplex™ analyzer (BioRad, Hercules, CA, USA) according to manufacturer's directions.

Immunohistochemical analyses

Formalin-fixed tissues were embedded in paraffin, cut into 4-µm thick sections, and processed for immunohistochemistry at the Histology Core Laboratory at The U.T. M.D. Anderson Cancer Center, Science Park Research Division (Smithville, TX, USA). Antibodies used for immunohistochemistry were optimized by core personnel using positive and negative controls for each analysis. Slides were deparaffinized in xylene and sequentially rehydrated in ethanol to water. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes. Antigen retrieval required microwaving slides

with 10 mM citrate buffer. Nonspecific binding was blocked by treating sections with Biocare blocking reagent (Biocare Medical, Concord, CA, USA) for 30 minutes at room temperature, followed by incubation with primary antibody diluted in blocking buffer overnight at 4°C. The following primary antibodies and dilutions were used: phospho-S6 ribosomal protein$_{S235/236}$ (Cell Signaling, Danvers, MA, USA; 1:100); phospho-mTOR$_{Ser2448}$ (Cell Signaling; 1:50); phospho-ACC$_{Ser79}$(Cell Signaling; 1:50); Ki-67 (Dako, Carpinteria, CA, USA; 1:200); cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500); and cleaved caspase-3 (R&D Systems, Minneapolis, MN, USA; 1:500). Slides were washed twice in PBS, incubated for 30 minutes with secondary antibody, washed two times with PBS, stained with diaminobenzidine (BioGenex, Fremont, CA, USA) and counterstained with hematoxylin. Images were captured by the Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA) and staining was quantified using the Aperio ImageScope (Aperio Technologies). For Ki-67, phospho-mTOR, and cyclin D1 quantification, automated algorithms were used to determine negative or positive nuclear staining. The percentage of positive cells was obtained with 20× objective in pancreatic tumor sections. Positive staining was defined as 1+, 2+, and 3+ for cyclin D1 and phospho-ACC. Positive staining for phospho-mTOR was defined as only 2+ and 3+ due to its high baseline phosphorylation. Cleaved caspase-3 (CC3) was quantified as the average area of positively stained cells, with positive staining defined as at least 70% of a 100 μm × 100 μm section. For all proteins, the positive staining was averaged per group (n = 5/group).

In vitro studies

Panc02 cells were cultured in a 37°C incubator under 5% CO_2 with McCoy's 5A media with glutamine (HyClone, Logan, UT, USA) and 3 g/L glucose but without BCAA, and then supplemented with penicillin/streptomycin, nonessential amino acids, sodium pyruvate, HEPES, 10% heat-inactivated FBS (HyClone), and physiological levels of leucine, isoleucine, and valine (MP Biomedicals, Santa Ana, CA, USA) (108). For western blotting, approximately 100,000 Panc02 cells were seeded in 6-well plates and allowed to settle overnight in McCoy's 5A media with 10% FBS. Cells were then treated with McCoy's 5A plus 10% FBS with or without leucine supplementation and McCoy's 5A plus 1% FBS with or without leucine supplementation (MP Biomedicals, Santa Ana, CA, USA). For western blot analysis, cells were treated for 20 min with 0.3 mM leucine after 3 hours of media pretreatment.

Western blotting

Panc02 cells were lysed on ice for 1 hour in RIPA buffer (Sigma, St. Louis, MO, USA) with protease inhibitor tablet (Roche Applied Sciences, Indianapolis, IN, USA) and phosphatase inhibitor cocktails II and III (Sigma). Protein lysates (40 µg) were resolved by SDS-PAGE using 6%, transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) overnight at 25 volts and blocked for 1 hour at room temperature with LI-COR Blocking Buffer (LI-COR Biotechnologies, Lincoln, NE, USA). Membranes were incubated overnight at 4°C with primary antibody (from Cell Signaling unless otherwise stated) diluted in 5% BSA (Santa Cruz) and specific for: phospho-ACC_{Ser79} (1:1000), β-actin (1:10000; Sigma), phospho-

AMPKα$_{T172}$ (1:1000), cleaved caspase-3 (1:1000), phospho-mTOR$_{Ser2448}$ (1:1000), phospho-p70S6K$_{T389}$ (1:1000), and phospho-S6 ribosomal protein$_{Ser235/236}$ (1:1000). β-actin was used as a loading control for all antibodies. After three washes (5 minutes each) in 0.1% Tween-20/PBS (PBS-T), membranes were incubated for 1 hour at room temperature in species-specific secondary antibody (LI-COR) diluted (1:5000) in LI-COR Blocking Buffer. Following two washes in PBS-T and one wash in PBS, membranes were scanned using the Odyssey infrared fluorescent imaging system (LI-COR). Densitometry was performed using LI-COR software (LI-COR). Relative levels of proteins were calculated from three biological replicates.

Cell proliferation assay

Cell viability was measured by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Cell Proliferation Assay Kit (Trevigen, Gaithersburg, MD; 4890-025-K). In 96-well plates, Panc02 (1500 cells/well) in media were allowed to adhere overnight. Each well was filled with fresh treatment media supplemented with different amounts of FBS (10% or 1%) and leucine (0 or 0.3 mM). The cells were then incubated for 24 h at 37°C, exposed to fresh treatment media after the removal of old media, and incubated for an additional 24 h. MTT was added at a 1:10 ratio for 2 h, then the liquid was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to lyse the cells and dissolve the solid residue. The optical density of each well at 570 nm and 690 nm, a reference wavelength, was determined using the Synergy 2 Multi-Detection Microplate

Reader and Gen5 Data Analysis Software (Fisher Scientific, Pittsburgh, PA). Relative cell viability was then calculated using the absorbance of cells grown in media with 10% FBS and no leucine supplementation for normalization. Data shown represent the average of three biological replicates.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (GraphPad Software, La Jolla, CA). Temporal differences between groups with respect to body weight and energy intake were assessed using repeated measures analysis; final measurements were compared using one-way ANOVA and Newman-Keul's post hoc test of significance. Blood glucose levels at each time point were compared using one-way ANOVA followed by Newman-Keul's post hoc test of significance, and overall blood glucose differences were compared by performing a one-way ANOVA on calculated areas under the curve followed by Newman-Keul's post hoc test of significance. Pretumor serum hormone levels, percent body fat, lean mass, and fasting glucose at week 21 were compared by one-way ANOVA followed by Newman-Keul's post hoc test of significance. Final measurements of *ex vivo* tumor volume and immunohistochemical staining of all antibodies were also compared among the groups by one-way ANOVA followed by Newman-Keul's post hoc test of significance. To compare the effects of leucine supplementation in media with either 10% FBS or 1% FBS, western blot densitometry and relative cell viabilities at their respective time points were compared by two-tailed t-tests. Results were considered significant if $p < 0.05$.

Results

Effects of calorie restriction and leucine supplementation on body composition, glucose, homeostasis, and serum hormones

Male C57BL/6 mice were fed a control diet with or without leucine supplementation, or a 30% CR diet with or without leucine supplementation, for 27 weeks (including 21 weeks of diet before GTTs and qMRs were performed). Relative to the control mice, the CR mice had significantly reduced caloric intake ($n = 22$/group; $p < 0.001$), body weight ($n = 22$/group; $p < 0.001$), body fat ($n = 10$/group; $p < 0.001$), and lean mass ($n = 10$/group; $p < 0.001$), irrespective of leucine supplementation (Figure 2.1A-D).

At 21 weeks of study, the CR group without leucine supplementation, relative to controls without leucine supplementation, displayed enhanced glucose clearance as assessed by GTT ($n = 10$/group; $p < 0.05$), with blood glucose concentrations peaking in 15 minutes in CR mice and 30 minutes in control mice following glucose bolus (Figure 2.1E). Leucine supplementation significantly decreased glucose clearance in the context of the high-calorie control diet ($n = 10$/group; $p < 0.001$), but did not significantly alter glucose uptake in the context of the CR diet ($n = 10$/group; $p > 0.05$) (Figure 2.1E). Even at 6 weeks, leucine supplementation showed the same trend of inhibiting glucose clearance in mice on the control diet (see Figure 2.6). The CR diet group without leucine supplementation showed significantly lower fasting serum glucose levels relative to the controls (control group, $n = 14$; CR group, $n = 15$; $p < 0.001$) (Figure 2.1F), and leucine supplementation further reduced glucose levels in the CR mice

(n = 15/group; $p < 0.001$), but did not affect glucose levels in control mice (control group, n = 14; control group with leucine supplementation, n = 15; $p > 0.05$).

The CR mice without leucine supplementation, relative to the control mice without leucine supplementation, had significantly lower serum levels of IGF-1 (control group, n = 9; CR group, n = 10; $p < 0.001$) (Figure 2.1H) and leptin (control group, n = 9; CR group, n = 10; $p < 0.001$) (Figure 2.1I) and higher levels of adiponectin (control group, n = 9; CR group, n = 10; $p < 0.001$) (Figure 2.1J) but did not have significantly altered levels of insulin (control group, n = 9; CR group, n = 10; in $p > 0.05$) (Figure 2.1G). Leucine supplementation lowered IGF-1 in the control group (n = 9/group; $p < 0.001$) and reduced adiponectin (n = 10/group; $p < 0.05$) in the CR mice, but caused no other alterations to the levels of the other energy-responsive serum hormones measured in either diet group (Figure 2.1G-H).

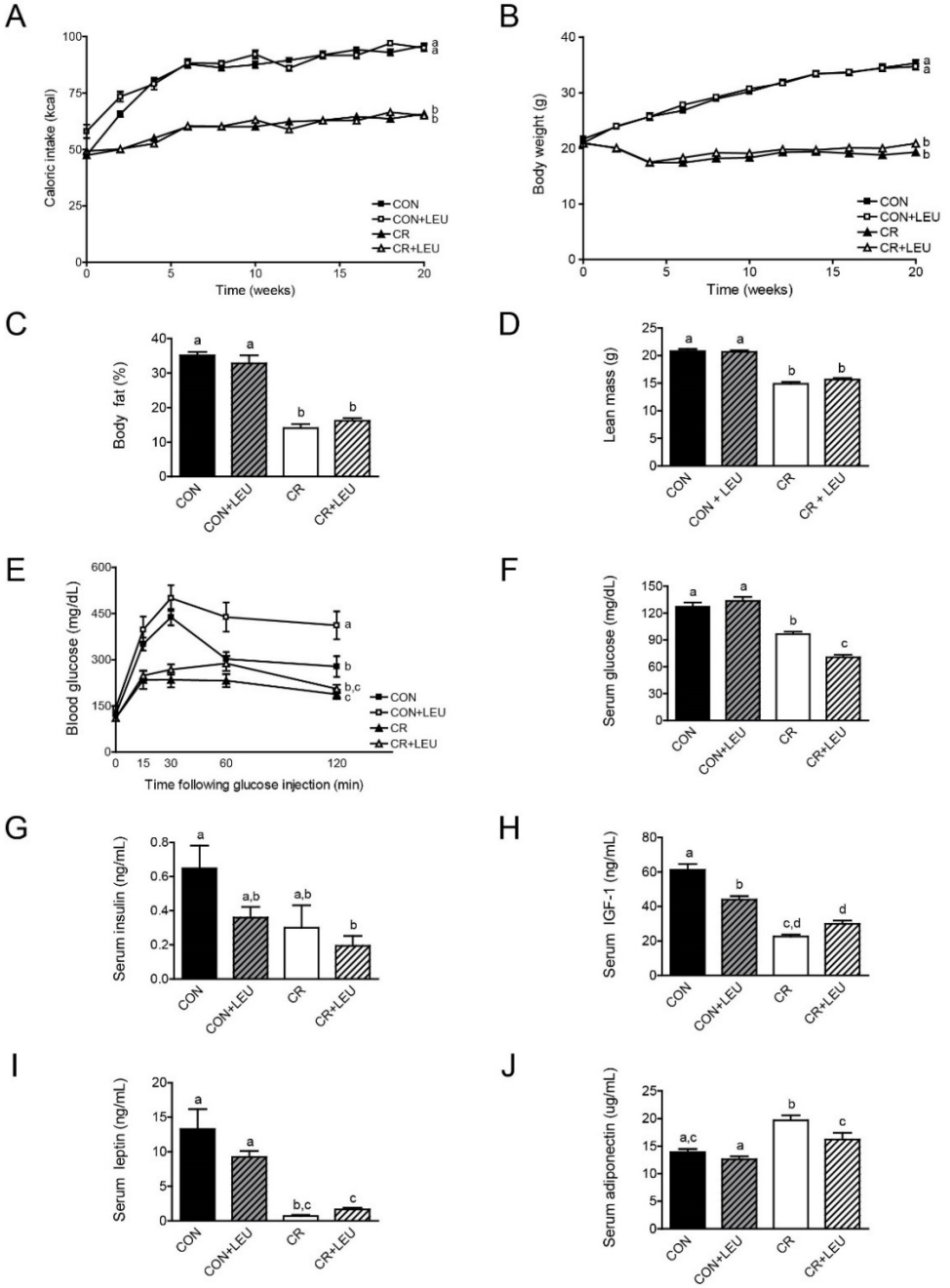

Figure 2.1. Effects of calorie restriction (CR) and/or leucine (LEU) supplementation on body composition, glucose tolerance, and hormones. (A) Caloric intake and **(B)** body weight of C57BL/6 male mice on control and CR diets with and without leucine supplementation reported until glucose tolerance test (GTT) and quantitative magnetic resonance imaging (qMRI) were performed. **(C)** qMRI quantification of body fat and **(D)** lean mass between mice fed control or CR diets with and without leucine supplementation for 21 weeks. **(E)** GTT performed after 21 weeks on diet. **(F)** Fasting glucose levels after 21 weeks on diet (prior to tumor injection. **(G-J)** Serum hormone analyses after 21 weeks on diet (prior to tumor injection) of **(G)** insulin, **(H)** IGF-1, **(I)** leptin, and **(J)** adiponectin. All data are presented as the mean with error bars indicating the SD **(A,B)** or SEM **(C-J)**. Differences are considered significant if $p < 0.05$. Within the same graph, bars with different letters are significantly different. Abbreviations: CON, control diet; CR,calorie restriction diet; LEU, leucine-supplemented diet.

Effects of calorie restriction and/or leucine supplementation on Panc02 tumor growth and apoptosis

To interrogate whether leucine supplementation modulates murine pancreatic cancer cell growth in control mice, and/or impacts the anticancer response to CR, we injected mice from each diet group with Panc02 cells at week 23 and monitored tumor growth during the next 4 weeks. The final mean *ex vivo* tumor volume from CR mice, both with and without leucine supplementation, was significantly smaller than control mice ($n = 14$/group; $p < 0.001$). However, leucine supplementation resulted in significantly larger tumors in both the control and CR diet groups, relative to each diet's respective nonsupplemented group (control group and control with leucine supplementation group, $n = 14$; $p < 0.01$) (CR group, $n = 14$; CR group with leucine supplementation, $n = 13$; $p < 0.001$) (Figure 2.2A).

The influence of energy balance and leucine supplementation on cell proliferation was assessed in tumor tissues by immunohistochemical staining against Ki-67 (Figure 2.2B). While CR significantly reduced cell proliferation, relative to control diet, in nonsupplemented mice ($n = 5$/group; $p < 0.001$), leucine supplementation significantly increased cell proliferation relative to the respective nonsupplemented mice within both diet groups ($n = 5$/group; $p < 0.001$). The amount of Ki-67 staining in the leucine-supplemented CR group was augmented to the level of the nonsupplemented control group ($n = 5$/group; $p < 0.001$).

Leucine supplementation in the CR group enhanced tumor proliferation more than it did tumor burden (Figure 2.2A,B), suggesting that final tumor size was

influenced by both proliferation and apoptosis. Based on immunohistochemical analysis of tumors, we found no appreciable levels of CC3 in tumors from mice not supplemented with leucine; however, leucine supplementation in both the control and CR diet groups resulted in marked CC3-positive areas ($n = 5$/group; $p < 0.05$) (Figure 2.2B). Leucine supplementation in the CR group resulted in much higher levels of apoptosis with 9.8 percent of the tumor composed of apoptotic areas in the CR group compared to 1.6 percent in the control group (Figure 2.2B). This increase in apoptosis could explain why leucine supplementation in the CR group, despite an equivalent level of proliferation as the control group, resulted in restrained tumor growth. Although apoptosis occurred in tumors of mice that consumed leucine-supplemented diets, 0.3 mM leucine supplementation *in vitro* did not significantly affect CC3 levels (Figure 2.7) due to 1% FBS only partially modeling CR through growth factor reduction.

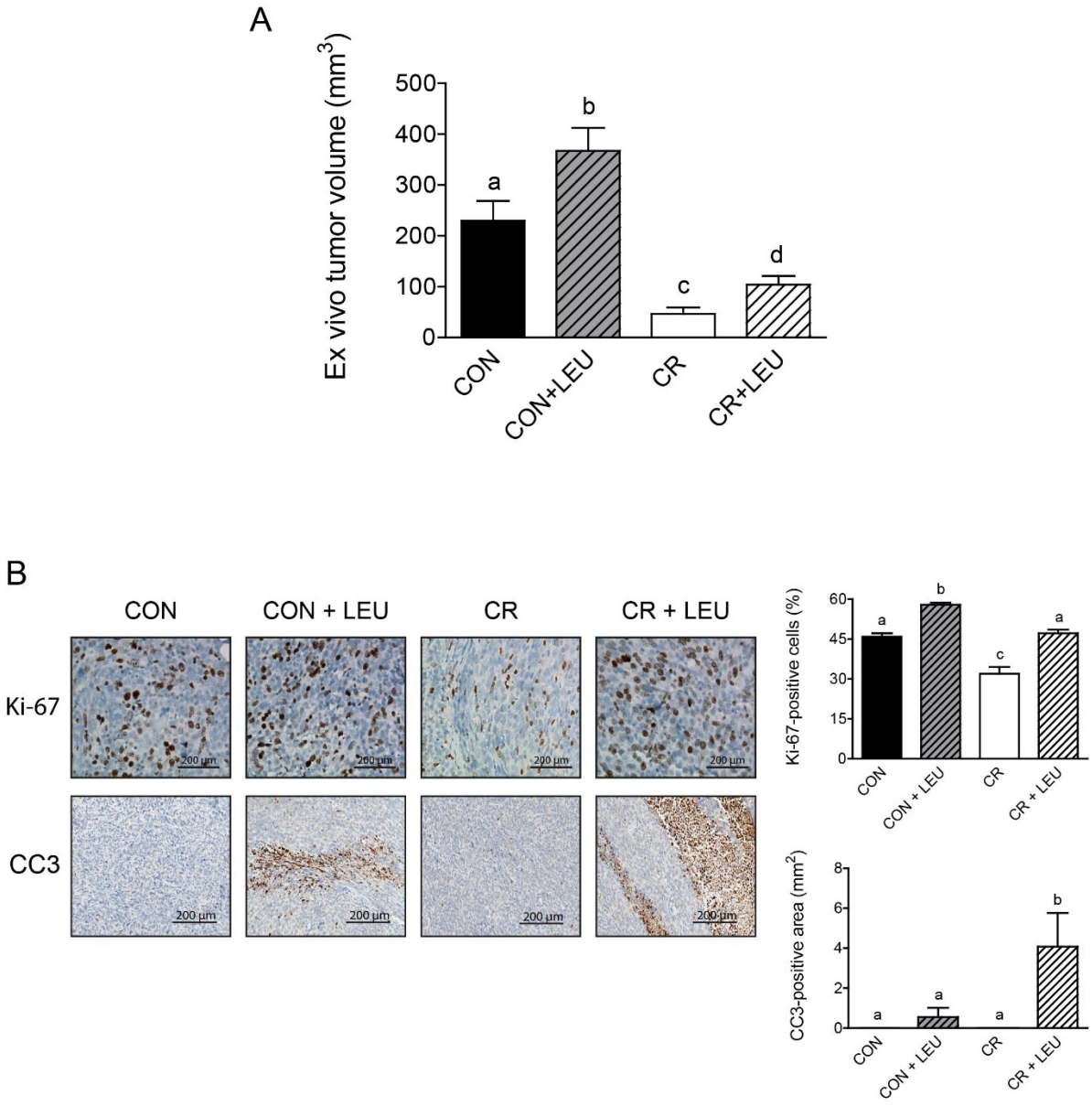

Figure 2.2. Effects of leucine supplementation on Panc02 tumor growth and apoptosis. **(A)** Differences in tumor volume between mice on control and calorie restriction (CR) diets with and without leucine (LEU) supplementation 4 weeks after tumor cell injections. **(B)** Comparison of immunohistochemical analyses performed on tumor sections for Ki-67 and cleaved-caspase 3 (CC3). Scale bars represent 200 µm. Tumor volume is presented as mean ± SD, and Ki-67 and CC3 data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$. Within the same graph, bars with different letters are significantly different. Abbreviations: CON, control diet; CR,calorie restriction diet; LEU, leucine-supplemented diet.

CR and leucine supplementation have differential effects on energy responsive signaling intermediates

The effects of energy balance and leucine supplementation on mTOR signaling were assessed by immunohistochemical analyses of the levels of phospho (p)-mTOR, p-ACC (a marker of AMPK activity, an upstream inhibitor of mTOR), and p-S6 and cyclin D1 (both downstream mTOR targets). Based on this analysis, we found that tumors from CR mice without leucine supplementation, relative to tumors from control mice without leucine supplementation, displayed increased levels of p-ACC ($n = 5$/group; $p < 0.05$) and reduced levels of p-mTOR ($n = 5$/group; $p < 0.05$) and its downstream effector, p-S6 ribosomal protein ($n = 5$/group; $p < 0.001$). Additionally, tumors from CR mice, relative to tumors from control mice, showed significantly reduced levels of cyclin D1 ($n = 5$/group; $p < 0.05$) (Figure 2.3A).

Leucine supplementation in the control diet did not significantly alter amounts of these energy responsive intermediates. However, leucine supplementation in the CR diet significantly reduced p-ACC ($n = 5$/group; $p < 0.001$) and increased p-mTOR ($n = 5$/group; $p < 0.05$), p-S6 ($n = 5$/group; $p < 0.05$), and cyclin D1 ($n = 5$/group; $p < 0.05$) to levels comparable to the non-supplemented control group (Figure 2.3A-B).

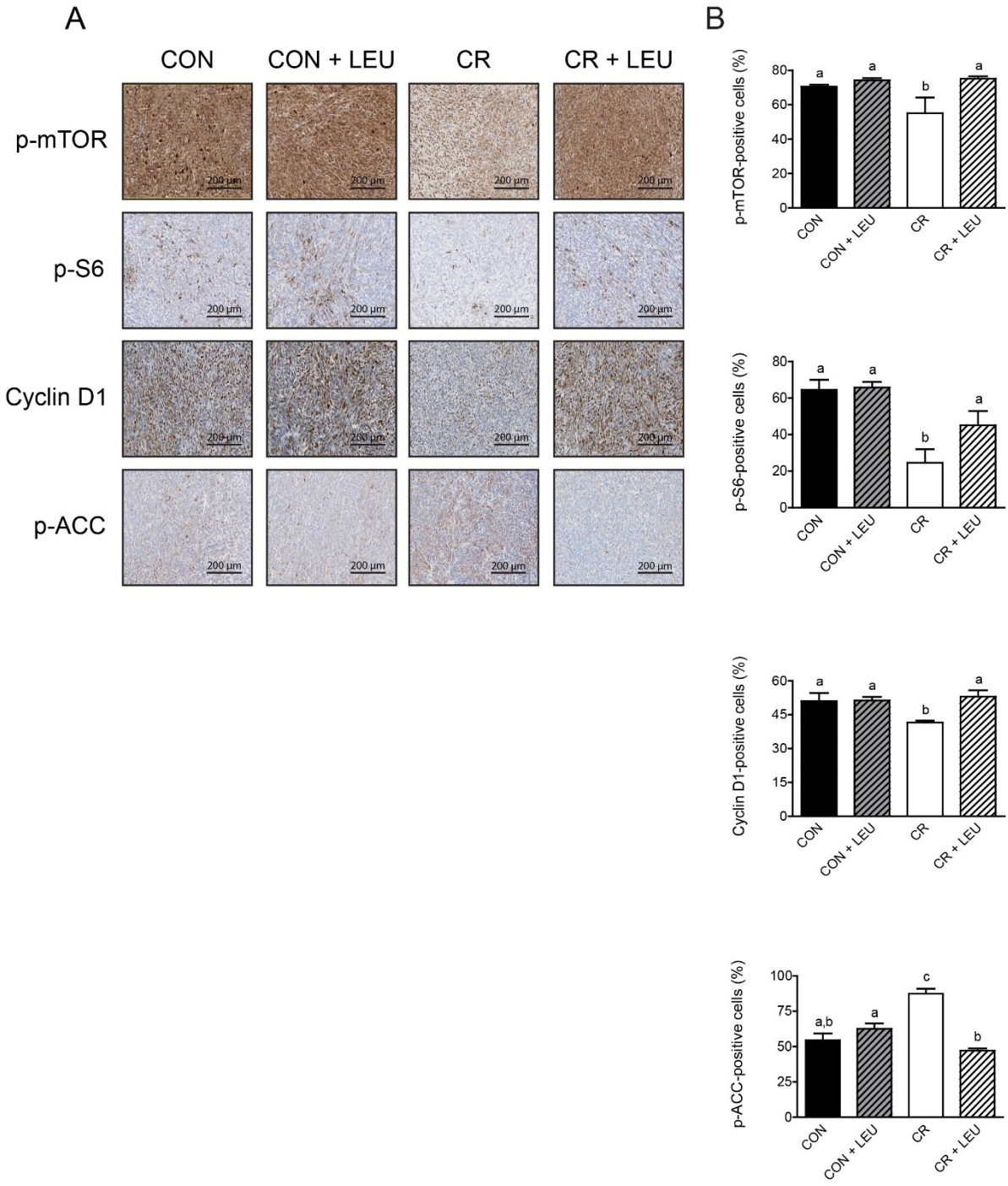

Figure 2.3. Effects of calorie restriction (CR) and/or leucine (LEU) supplementation on energy responsive signals in Panc02 tumors. (A) Comparison of immunohistochemical analyses on tumor sections for phospho-mTOR, phospho-S6, cyclin D1, phospho-ACC. Scale bars represent 200 µm. All data are presented as the mean ± SEM. Differences are considered significant if $p < 0.05$. Within the same graph, bars with different letters are significantly different. Abbreviations: CON, control diet; CR,calorie restriction diet; LEU, leucine-supplemented diet.

Effect of leucine supplementation on Panc02 cell lines

To confirm the proliferative effect of leucine supplementation seen *in vivo*, *in vitro* analyses were performed using the Panc02 cell line. To model the growth factor restrictive environment in CR mice relative to the overweight control mice as seen in Figure 2.1H, we grew the cells in media with either 1% FBS or 10% FBS. Supplementing media with 1% FBS has been used to mimic serum growth factor reduction found in calorie-restricted mice (109). In the growth factor-rich environment of media with 10% FBS, cell viability was significantly increased by ~30% with 0.3 mM leucine supplementation ($p < 0.05$) (Figure 2.4A). Leucine supplementation also increased cell viability by ~30% in the growth factor-restricted environment of media with 1% FBS ($p < 0.01$) (Figure 2.4B). These 30% increases are similar to the increases in Ki-67 seen when comparing mice on leucine-supplemented diets to their respective nonsupplemented controls (Figure 2.2B). This 0.3 mM concentration of leucine were chosen based on experiments showing that: i) serum leucine increased by 0.3 mM in mice consuming a leucine-supplemented diet (110); and ii) cell viability of Panc02 cells significantly increased with 0.3 mM leucine supplementation *in vitro* (Figure 2.8).

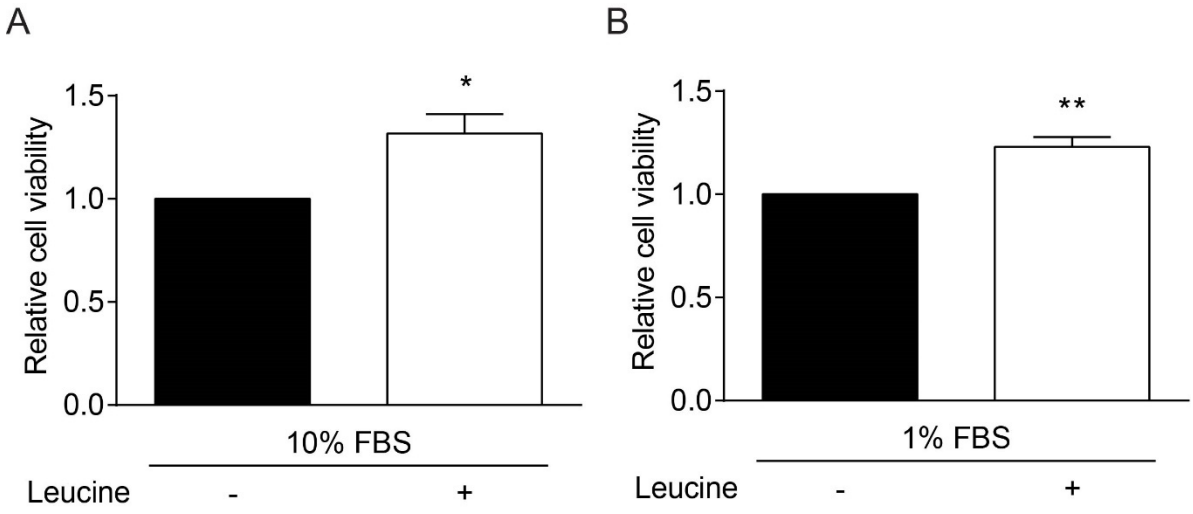

Figure 2.4. Effects of leucine supplementation on viability of Panc02 tumor cells. (A-B) Comparison of relative viability of cells grown in media with either **(A)** 10% fetal bovine serum (FBS) or **(B)** 1% FBS as assessed by MTT assays after 48 hours of 0.3 mM leucine supplementation (* = $p < 0.05$, ** = $p < 0.01$). All data are presented as the mean ± SEM. Differences are considered significant if $p < 0.05$.

Effect of leucine supplementation on mTOR pathway intermediates

In order to understand the differential response to leucine supplementation between the diet groups with respect to mTOR signaling, *in vitro* analyses were performed using Panc02 cell lines. Western blot analyses for the energy responsive intermediates p-AMPK, p-ACC, p-mTOR, p-p70S6K, and p-S6 revealed that the effects of leucine supplementation on cell signaling intermediates were impacted by growth factor availability. In the growth factor-rich environment of media with 10% FBS, supplementation with 0.3 mM leucine had no effect on phosphorylated AMPK, ACC, mTOR, p70S6K, or S6 ribosomal protein (Figure 2.5A,B). In the 1% FBS setting, leucine supplementation had no effect on phosphorylated AMPK or ACC, but did significantly increase phosphorylated mTOR ($p < 0.05$) and its downstream effector S6 ribosomal protein ($p < 0.05$). Another downstream effector of mTOR, p-p70S6K, was also increased with leucine supplementation in the 1% FBS setting, although the difference was not statistically significant (Figure 2.5A,C).

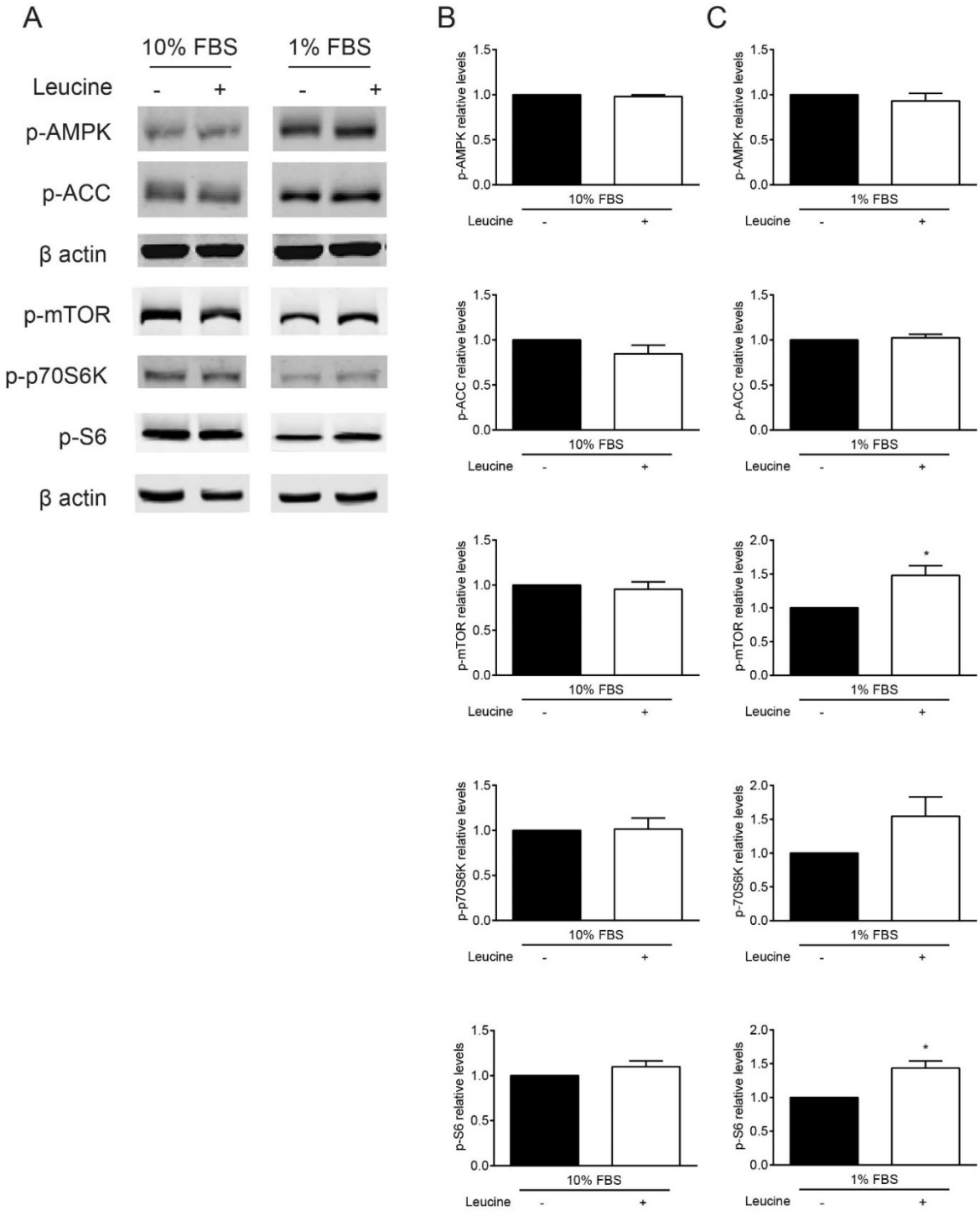

Figure 2.5. Effects of leucine supplementation on energy responsive signals of Panc02 tumor cells. (A-C) Western blot analysis of phosphorylated AMPK, ACC, mTOR, p70S6K, and S6 after 20 minutes of 0.3 mM leucine administration after pretreatment with respective media for 3 hours. Data shown are representative blots from three biological replicates, and images for each protein are from the same blot. **(B-C)** Relative phosphorylation of p-AMPK, p-ACC, p-mTOR, p-p70S6K, and p-S6 in cells grown in media with either **(A)** 10% FBS or **(B)** 1% FBS with or without leucine supplementation (* = $p < 0.05$). All data are presented as the mean ± SEM. Differences are considered significant if $p < 0.05$.

Discussion

Findings in this report demonstrate for the first time that dietary leucine supplementation increases growth of pancreatic tumors. More specifically, we show that leucine supplementation not only enhances the pro-tumorigenic nature of a high calorie, high carbohydrate control diet, but also partially overcomes the well-established anticancer effects of CR. The mechanisms underlying these leucine-induced protumor effects may be diet-dependent, suggested by increased glucose availability in overweight mice and increased activation of the mTOR protein synthetic pathway in CR mice.

Mice administered the leucine-supplemented control diet developed the largest tumors and had the highest level of tumor cell proliferation of all four groups. The increased tumor burden observed in the leucine-supplemented control group (relative to controls without leucine supplementation) occurred without significant changes in tumoral apoptosis or mTOR activation, as evidenced by unchanged levels of both p-AMPK, an upstream inhibitor of mTOR, and p-S6, a downstream effector of mTOR. In overweight control mice, high basal levels of circulating IGF-1 and tumoral mTOR activity are consistently found (Lashinger 2011). This high level of activity likely blunted any further increase in mTOR activation in response to leucine supplementation in the control diet, suggesting a biological threshold was attained. This concept of a biological threshold for mTOR phosphorylation was substantiated using Panc02 cancer cells *in vitro*, because mTOR activation was only enhanced in response to leucine under growth factor

restrictive conditions (1% FBS) and not growth factor-abundant conditions (10% FBS). The enhanced tumor growth in the leucine-supplemented control group cannot be explained by changes in mTOR signaling in the tumor, but was associated with greater glucose availability (reduced fasting insulin levels and diminished glucose clearance). High levels of glucose have been shown to increase proliferation in multiple pancreatic cancer cell lines by stimulating glucose consumption and metabolism (111-112). Although the noted effects on insulin levels in the control group contradict the putative characteristics of leucine as an insulin secretagogue and enhancer of blood glucose disposal in patients with type 2 diabetes (113), recent evidence suggests that leucine's effects on glucose sensitivity differ depending on physiologic context, i.e., diabetic versus non-diabetic state (114). In a physiologic scenario, leucine stimulates mTOR activity in the β-cells of the pancreas and promotes proliferation and thus insulin secretion (114). On the other hand, chronic β-cell hyperfunction, a consequence of excessive leucine exposure, results in accelerated β-cell apoptosis and eventual secretory deficiency through a negative feedback loop involving the mTOR-dependent inhibition of IRS-1 (115). Indeed, a diet consisting of high levels of leucine combined with saturated fatty acids results in insulin resistance in rodents (116), and chronic infusion of amino acids at high concentrations induces insulin resistance in humans (117). Leucine supplementation did not induce insulin resistance in mice on the CR regimen. CR has been shown to decrease basal p70S6K activation, which may have protected against mTOR-dependent β-cell hyperfunction (118). Taken together, our data suggest that control tumors obtained

a leucine-induced growth advantage because of increased glucose availability as a consequence of either impaired insulin secretion or function.

Mice administered the CR diet without leucine supplementation had the smallest tumors and lowest level of tumor cell proliferation, while mice fed the leucine-supplemented CR regimen (relative to CR mice without leucine supplementation) had increased tumor growth to levels intermediate between the unsupplemented mice on the CR and control diets. Leucine supplementation in the CR diet, relative to CR alone, also increased tumor cell proliferation (to the levels observed in control mice), and increased apoptosis. It is not uncommon to observe increases in both cell proliferation and cell death in the same tumor, as seen in the tumors of mice on the CR diet. In fact, a number of dominant oncogenes that increase proliferation through induction of aberrant growth signals, also induce apoptosis (119). Thus, leucine-induced dysregulation of growth signals, such as mTOR activation, in a setting of low-energy substrates and growth factors in response to a CR regimen, may explain the observed increases in apoptosis, tumor cell proliferation (to control levels), and partial rescue of tumor burden in the leucine-supplemented CR mice. This rescue of tumor burden was only partial, because leucine significantly increased proliferation. As previously stated, high levels of mTOR activity support proliferation and survival of pancreatic cancer cells, and CR consistently results in decreased activation of mTOR in pancreatic tumors (89). In contrast to tumors from the leucine-supplemented control group, we found that tumors from the leucine-supplemented CR group demonstrated marked

increases in mTOR activation, as evidenced by lower levels of p-AMPK and higher levels of p-S6 and cyclin D1, without changes in fasting insulin levels and glucose clearance. The maintenance of physiologic insulin secretion in the CR mice was perhaps due to the protection of β-cells by chronic CR, a strategy that has been shown to increase β-cell proliferation in rats (118). Taken together, our data suggest that CR tumors obtained a leucine-induced growth advantage because of increased mTOR activation.

In conclusion, this report establishes that dietary leucine supplementation, irrespective of energy balance status, promotes pancreatic tumor growth. These findings suggest caution regarding the clinical use of leucine supplementation for purposes of lean muscle enhancement in cachectic cancer patients.

Supplementary Figures

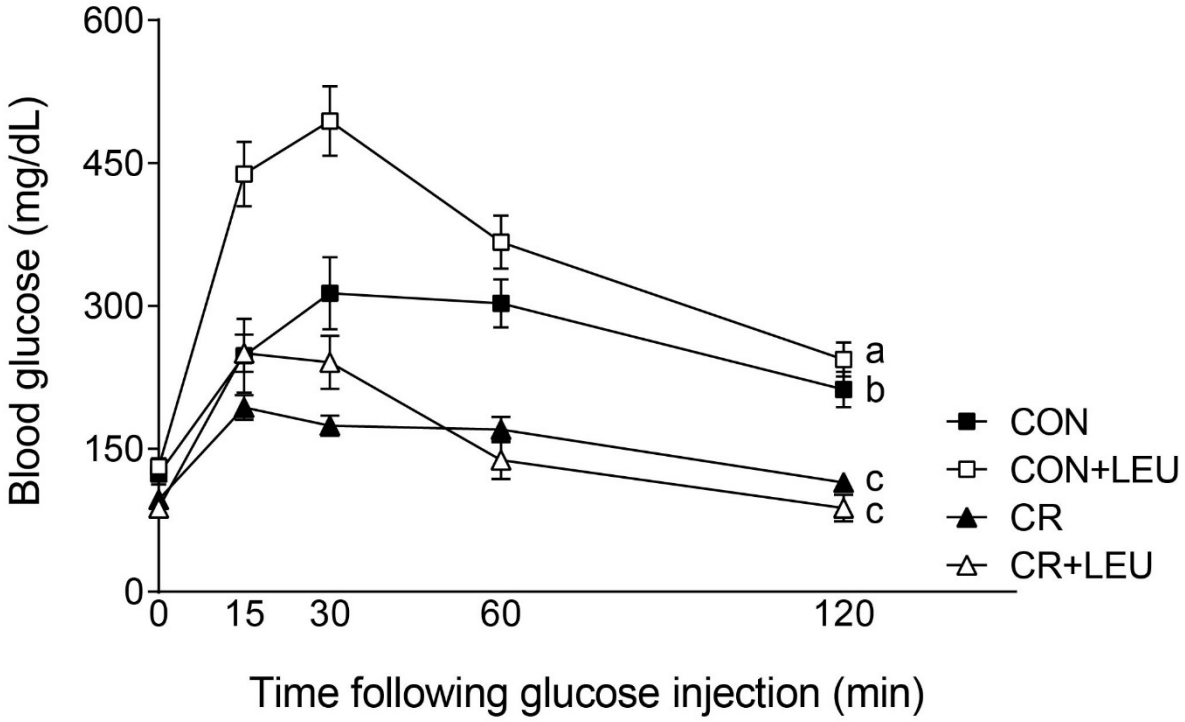

Figure 2.6. Effects of leucine supplementation on glucose tolerance at 6 weeks. Glucose tolerance test (GTT) performed after 6 weeks on diet (n = 10/group; $p < 0.001$ between control with leucine supplementation and the calorie restriction (CR) groups; $p < 0.05$ between the control groups). All data are presented as the mean ± SEM. Differences are considered significant if $p < 0.05$.

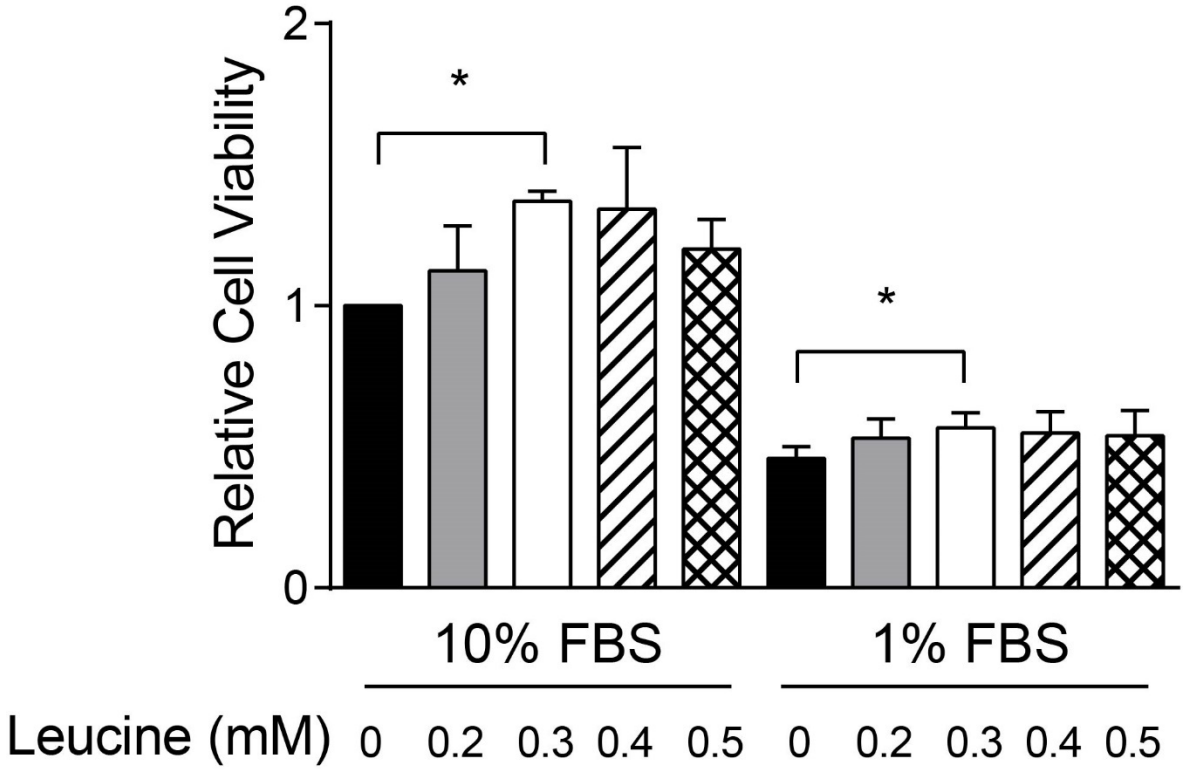

Figure 2.7. Effects of single BCAA supplementation on apoptosis of Panc02 tumor cells. Western blot analysis of cleaved caspase-3 protein levels after 24 hours of either 0.3 mM leucine, isoleucine, or valine administration. Data shown are representative blots from three biological replicates. Relative protein levels of cleaved caspase-3 were quantified by densitometry using LI-COR Odyssey software. All data are presented as the mean ± SEM. Differences are considered significant if $p < 0.05$.

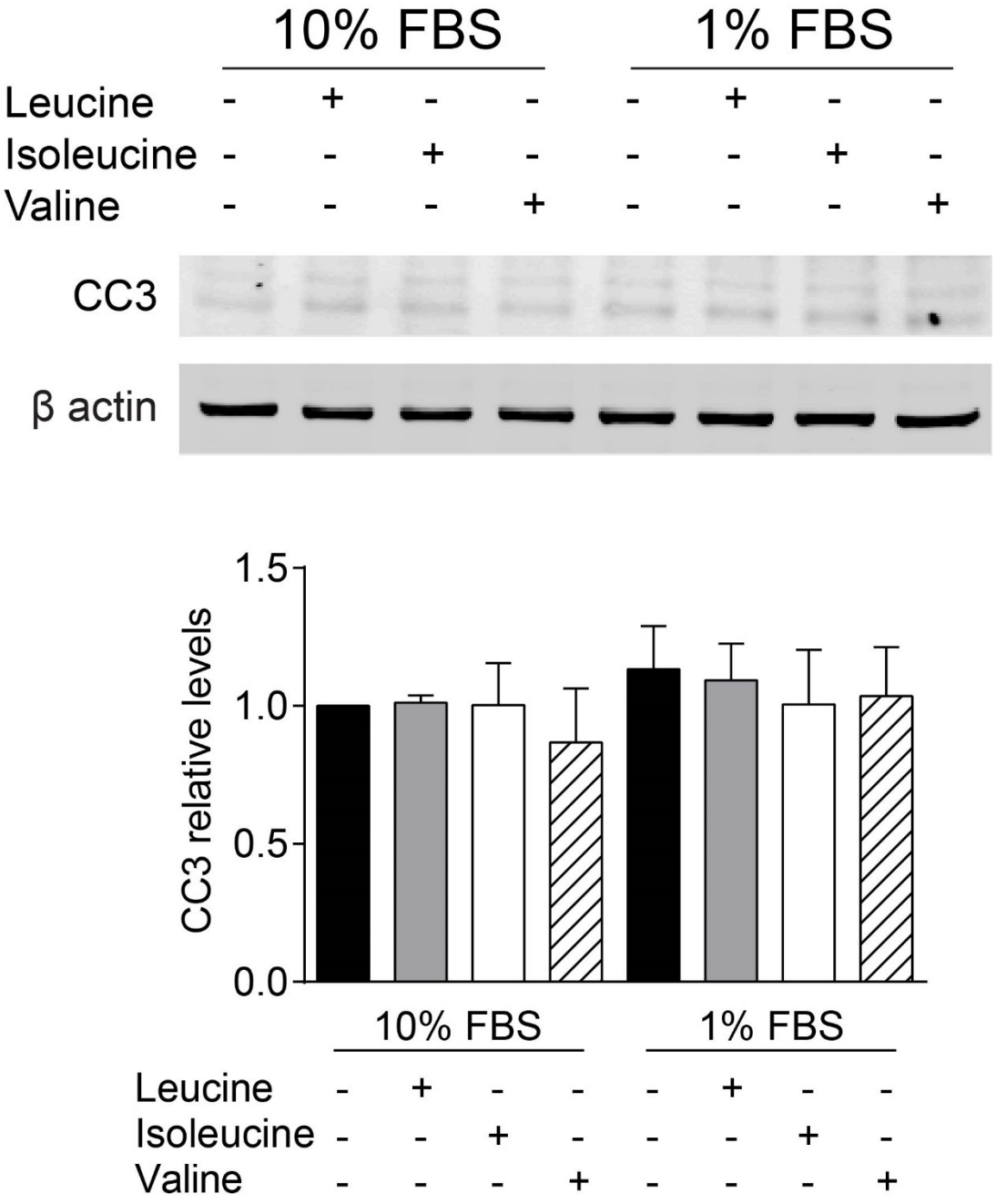

Figure 2.8. Effects of different doses of leucine supplementation on Panc02 tumor cell viability. Comparison of relative cell viability as assessed by MTT assays after 48 hours of leucine supplementation (* = $p < 0.05$). All data are presented as the mean ± SEM. Differences are considered significant if $p < 0.05$.

Chapter III

β-Hydroxy-β-methylbutyrate supplementation inhibits pancreatic tumor growth and preserves muscle mass

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related deaths in the United States. A major contributor to this statistic is cancer cachexia, which is present in about 80% of pancreatic cancer patients (49). Cachexia is characterized by involuntary weight loss and breakdown of adipose and muscle tissue. This condition lowers quality of life, reduces response to gemcitabine, the primary chemotherapy for pancreatic cancer, and increases mortality rate (120-121). The negative effects of cachexia are specifically tied to muscle loss, because muscle breakdown is an independent predictor of reduced cancer survival, even after controlling for body mass index (BMI), age, and tumor stage (4). Low muscle mass is also an independent prognostic factor of poor overall and recurrence-free survival of pancreatic cancer patients after resection (122).

Due to the obesity epidemic in Western society, a growing proportion of pancreatic cancer patients at the start of therapy have a body mass index (BMI) in the obese range ($\geq$30 kg/m2). Many of these individuals have sarcopenic obesity, a condition characterized by the presence of high fat mass but low muscle mass (sarcopenia). Recent studies have reported that obesity in the presence of sarcopenia is predictive of morbidity and mortality in pancreatic cancer patients (4),

at least in part because sarcopenic obesity increases the odds of developing cancer cachexia (5). Obesity is connected to these outcomes through hormones involved in growth signaling, such as insulin and insulin-like growth factor-1 (IGF-1) and through pro-inflammatory cytokines, such as TNF-α. Obese mice, compared with lean mice, have higher circulating levels of these factors, which activate specific intracellular signaling pathways (123-125).

Insulin and IGF-1 bind to their respective receptors on the cell surface, which leads to the activation of several downstream signaling pathway components including PI3K, Akt, and the mechanistic target of rapamycin (mTOR). mTOR then phosphorylates and activates its downstream proteins 4EBP1 and ribosomal protein S6, both of which increase protein translation and eventually lead to cell growth and proliferation. Therefore, if mTOR is activated within tumor cells, it increases tumor growth, and if mTOR is activated within muscle fibers, it increases muscle fiber size. Rapamycin, a specific mTOR inhibitor, reduces pancreatic tumor growth and inhibits muscle growth in mice (89, 126).

Pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), are produced mainly by macrophages (127) and bind to their cell surface receptors leading to the degradation of the inhibitor of NF-κB (IK-kB) and the subsequent translocation of NF-κB to the nucleus, where it regulates the transcription of many different genes, including genes involved in cell survival and the cell cycle (128). Thus, NF-κB activation within PDAC cells is typically pro-tumorigenic, and inhibition of NF-κB reduces PDAC growth in mice (129). In muscle tissue, NF-κB specifically increases the transcription of the atrogene MuRF-1, a protein involved

in the ubiquitin-proteasome system of muscle breakdown, which leads to muscle atrophy (130). Inhibition of NF-κB prevents cancer-induced muscle atrophy (131).

The PI3K/Akt/mTOR and NF-kB signaling pathways, which are highly activated in an obese environment, may explain why patients with sarcopenic obesity often have enhanced tumor growth coupled with muscle loss. A major challenge in treating PDAC patients with cachexia is finding interventions that take into account effects on both tumor and muscle. However, as muscle loss is the driving force behind the negative consequence of cachexia, most treatments have focused on either increasing muscle protein or inhibiting muscle protein breakdown (132). This focus on protein has led many researchers to study the building blocks of protein, the amino acids and their metabolites. β-hydroxy-β-methylbutyrate (HMB), the metabolite of the branched-chain amino acid leucine, has begun to emerge as a strong potential candidate for increasing muscle mass (132). HMB supplementation increases muscle growth through mTOR activation (133) and also attenuates muscle protein degradation through the prevention of NF-κB activation (56). However, mTOR and NF-κB are involved in tumor growth as well, and the effects of HMB supplementation on PDAC growth are unknown.

To establish that HMB exerts positive effects on muscle and PDAC, in the context of gemcitabine (an FDA-approved chemotherapy drug for PDAC; 134), we tested the effects of HMB supplementation on the growth of muscle and transplanted Panc02 mouse pancreatic tumors with and without gemcitabine chemotherapy. The study was performed on both normo-weight and diet-induced obese (DIO) mice to assess the impact of obesity, shown previously to enhance

PDAC growth (60), on the anti-cachetic and anti-cancer effects of HMB ± gemcitabine.

Materials and Methods

Mice and dietary interventions

All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. All diets were purchased from Research Diets (New Brunswick, NJ, USA). One hundred twenty male C57BL/6 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA, USA) at 6 to 8 weeks of age, and upon arrival were doubly-housed in a semi-barrier facility at the Dell Pediatric Research Institute and fed the control diet during a one-week acclimation period.

Mice were then singly-housed and randomized (n = 60 per group) to either continue on the control (CON) diet with 10% kcal from fat (D12450J from Research Diets; New Brunswick, NJ, USA) or receive the diet-induced obesity (DIO) diet with 60% kcal from fat (D12492 from Research Diets) for 10 weeks. The diets, consumed *ad libitum*, matched in both type and amount of protein, vitamins, and minerals, and only differed in the percentage of fat and carbohydrate.

Energy intake and body weight for each mouse were recorded weekly. At the beginning of week 10, randomly selected mice (n = 30 per diet group) were fasted for 6 h, and then tail clips for fasting glucose and retro-orbital bleeds for blood collection were performed. Blood glucose levels were measured with a Contour glucometer (Bayer HealthCare LLC, Mishawaka, IN, USA). After coagulating at room temperature for 30 minutes, blood samples were centrifuged at 12,000 x g for 5 minutes. Serum was separated, then snap-frozen and stored at -80°C until assayed for hormones and pro-inflammatory cytokines. After a 48h rest

period, all mice were subcutaneously injected into the right flank with 250,000 Panc02 cells (generously provided by Dr. J. Schlom, National Cancer Institute, Bethesda, MD, USA) suspended in serum-free McCoy's 5A medium (Sigma, St. Louis, MO, USA).

Also at week 10, dietary HMB supplementation began (CON, n = 30; CON + HMB, n = 30; DIO, n = 30; DIO + HMB, n = 30). Ca-HMB (HMB) was purchased from Metabolic Technologies, Inc. (Ames, IA, USA) and was incorporated into the diets at a concentration of 1% (w/w) dietary HMB supplementation to achieve a daily amount of 0.5 g HMB/kg body weight. This amount was chosen based on the calculations done by Kim, et al, who converted the human supplemental dose (6 g/day) to an appropriate rodent dose after taking into account rodent metabolic rate and average daily food consumption (135).

Once palpable, tumors were measured biweekly with calipers and 3 gemcitabine (50 mg/kg each) injections were administered intraperitoneally every 3 days. At week 15, all mice were fasted for 6-8 h and then anesthetized by CO_2 inhalation. They underwent tail clips for fasting glucose, cardiac punctures for blood collection, and then were killed by cervical dislocation. Pancreatic tumors and gastrocnemius muscle were harvested and either snap-frozen in liquid nitrogen and stored at -80°C, or fixed with 10% neutral-buffered formalin for 48 hours, switched to 70% ethanol, and paraffin embedded. The frozen tissue was subsequently used for PCR and western blot analysis, and the paraffin-embedded tissue was used for immunohistochemical analysis.

Serum Hormones and Cytokines

Serum collected at week 10 (before dietary HMB supplementation and tumor cell injection) was analyzed for hormones and cytokines. Insulin levels were measured using the Bio-Plex Pro Mouse Diabetes Insulin Single Plex Assay (Bio-Rad, Hercules, CA, USA) IGF-1 levels were measured using the Millipore MILLIPLEX Rat/Mouse IGF-1 Single Plex Assay (Millipore, Billerica, MA, USA), and the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-10, IL-17A, IFN-γ, and TNF-α were measured using the Bio-Plex Pro Mouse Cytokine Th17 Panel A 6-Plex Assay (Bio-Rad). Analysis was performed on the Bio-Rad Bio-Plex 200 Analysis System (Bio-Rad).

Tumor and Muscle H&E and Immunohistochemistry

Paraffin-embedded tumors and skeletal muscle were cut into 4-μm thick sections, and processed for either hematoxylin and eosin (H&E) or immunohistochemical (IHC) staining at the Histology Core Laboratory at the UT-MD Anderson Cancer Center, Science Park Research Division (Smithville, TX, USA). The muscle tissue was cut into cross-sections, so the thickness of each individual fiber could be quantified. All antibodies used for immunohistochemistry were optimized by core personnel using positive and negative controls for each analysis.

Slides were deparaffinized in xylene and sequentially rehydrated in ethanol to water. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 minutes, and antigen retrieval was achieved by microwaving slides

with 10 mM citrate buffer. Nonspecific binding was blocked by treating sections with Biocare blocking reagent (Biocare Medical, Concord, CA, USA) for 30 minutes at room temperature, followed by incubation with primary antibody diluted in blocking buffer overnight at 4°C. The following primary antibodies and dilutions were used: phospho-S6 ribosomal protein$_{S235/236}$ (Cell Signaling, Danvers, MA, USA; 1:100); and Ki-67 (Dako, Carpinteria, CA, USA; 1:200). Slides were washed twice in PBS, incubated for 30 minutes with secondary antibody, washed two times with PBS, stained with diaminobenzidine (BioGenex, Fremont, CA, USA) and counterstained with hematoxylin.

Images were captured by the Aperio ScanScope XT (Aperio Technologies, Vista, CA, USA) and staining was quantified using the Aperio ImageScope (Aperio Technologies). Automated algorithms were used to determine positive nuclear or cytoplasmic staining of Ki-67 and phospho-S6, respectively. The percentage of positive cells (or positive intensity) was obtained with 20x objective in 4 different areas of the PDAC sections. Necrotic sections were not included. For all proteins, the positive staining was averaged per treatment group (n = 6 randomly selected slides per group).

Gene Expression Microarray Analysis

Total RNA samples were extracted by RNeasy Kit (Qiagen, Valencia, CA, USA) from homogenized tumors and hybridized to the Affymetrix Mouse Gene 2.1 ST 24-Array plate. The quality of the RNA sample was checked using Agilent bioanalyzer. The mRNA expression raw data were quantile normalized and the

signal values were transformed to the $\log_2$ value. The comparative analysis between different group samples was carried out using the t-test (p-values) and the Benjamini-Hochberg False Discovery Rate (FDR) correction (adjusted p-values) using R. Differentially expressed genes between experimental groups were determined if the p-values were < 0.05 and if the fold changes were > 1.5. The expression level of selected genes were combined and zero-transformed to the control samples, then subjected for hierarchical clustering and viewed by Java Treeview.

Quantitative RT-PCR

Real-time quantitative PCR was performed to validate the microarray results. Total RNA was reverse-transcribed to cDNA using High-Capacity cDNA reverse transcription kit (Fisher Scientific, Waltham, MA, USA) with random hexamers. The relative level of gene expression was measured and normalized to β-actin by qPCR using PowerUp SYBR Green PCR Mix (Applied Biosystems, Austin, TX, USA).

Quantitative PCR was also done to determine how HMB supplementation affected muscle degradation signaling. Total RNA was isolated from gastrocnemius muscle (n = 5 per group) using TRIzol reagent (Invitrogen, Grand Island, NY, USA), and 2 µg of RNA per sample was reverse transcribed using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). RNA extraction and reverse transcription were performed according to the respective manufacturer's protocol. Gene expression levels were quantified by mixing

optimized amounts of Power SYBR Green PCR Master Mix (Applied Biosystems), forward and reverse primers, and cDNA in 96-well plates and performing RT-PCR reactions in an Eppendorf instrument (Santa Clara, CA). Relative differences in gene expression were normalized to the housekeeping gene, GAPDH, and analyzed using the $2^{-\Delta\Delta CT}$ method, as described by Schmittgen and Livak (136). All primers used are listed in Figure 3.7.

Muscle Fiber Size

Cross-sectional images of H&E-stained gastrocnemius muscle were analyzed using Fiji (ImageJ from NIH). Muscle breakdown due to cancer cachexia is largely restricted to fast-twitch (type II) glycolytic fibers (137), so only fast-twitch muscle fibers were analyzed. This was done by marking regions of the small and dark slow-twitch fibers, the large and light fast-twitch fibers, and the slide background to train the Advanced Weka Segmentation classifier included in Fiji and help it distinguish between these fibers before analysis (138).

Muscle fiber size was determined using the minimal Feret's diameter, because this parameter has been proven to be the most robust against experimental errors, such as the orientation of the sectioning angle (139). There were 200-400 muscle fibers analyzed per slide (n = 6 per group) resulting in a total of greater than 8000 muscle fiber measurements. The minimal Feret's diameters of each fiber were averaged for each treatment group.

Western Blot Analyses

Protein lysates were obtained by cutting frozen gastrocnemius muscle into 10-30 mg pieces on dry ice and then placing them into bead beater tubes with 0.5 mL of freshly prepared RIPA buffer (Sigma) with a protease inhibitor tablet (Roche Applied Sciences, Indianapolis, IN, USA) and phosphatase inhibitor cocktails II and III (Sigma). Then, the tubes were half-filled with 2.5mm diameter zirconia/silica beads (Biospec Products, Bartlesville, OK, USA). Homogenization was performed using the Mini-BeadBeater-1 (Biospec Products), where the tubes were shaken at 4800 rpm for 10 seconds and left on ice for 30 seconds. The cycle of shaking and cooling was repeated 5 times until homogenization was complete. After quick centrifugation, the protein homogenate was placed into a new Eppendorf tube and left to incubate in ice for 30 minutes with occasional vortexing. To shear DNA, the samples were then sonicated 5 times for 10 seconds with a 30 second rest on ice between each sonication. Then, the samples were centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was transferred to a new tube.

Protein lysates (100 µg) were resolved by SDS-PAGE using precast 6% polyacrylamide gels followed by transfer to PVDF membranes (Bio-Rad) overnight at 25 volts and blocked for 1 hour at room temperature with LI-COR Blocking Buffer (LI-COR Biotechnologies, Lincoln, NE, USA). Membranes were incubated overnight at 4°C with primary antibody (from Cell Signaling unless otherwise stated) diluted in 5% BSA (Santa Cruz) with 0.2% Tween-20. The primary antibodies were specific for: GAPDH (1:10,000), MuRF-1 (0.1 µg/mL; Abcam), phospho-S6 ribosomal protein$_{Ser235/236}$ (1:500), and S6 ribosomal protein (1:1000).

GAPDH was used as a loading control for all antibodies. After three washes (10 minutes each) in 0.1% Tween-20/PBS (PBS-T), membranes were incubated for 1 hour at room temperature in species-specific secondary antibody (LI-COR) diluted (1:5000) in LI-COR Blocking Buffer with 0.2% Tween-20 and 0.01% SDS.

Following two washes in PBS-T and one wash in PBS, membranes were scanned using the Odyssey infrared fluorescent imaging system (LI-COR). Densitometry was performed using LI-COR software (LI-COR). Raw values were compared between groups only if they were on the same membrane, and then those values, normalized to GAPDH levels, were used to calculate the overall relative levels of proteins (n = 4 per group). Values of phosphorylated proteins normalized to GAPDH were also normalized to levels of their respective non-phosphorylated forms.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism (GraphPad Software, La Jolla, CA). All comparisons between the CON group and the DIO group were analyzed using unpaired, two-tailed t-tests. Final differences between groups with respect to body weight were compared using one-way ANOVA and Tukey's post hoc test of significance. Fasting glucose, serum hormone levels, and serum cytokine levels were compared by one-way ANOVA followed by Tukey's post hoc test of significance. Significance regarding complete tumor regression was obtained by comparing the number of mice with tumor to mice without tumor and analyzing using Fisher's exact test. Final measurements of *ex vivo* tumor weight

and volume, immunohistochemical staining of phospho-S6, and muscle fiber size (Feret's diameter) were also compared among the groups by one-way ANOVA followed by Tukey's post hoc test of significance. Western blot densitometry was compared by one-way ANOVA followed by Fisher's LSD test of significance. Results were considered significant if $p < 0.05$.

Results

Effects of the high-fat, diet-induced obesity regimen (DIO) on body weight, fasting glucose, and hormones

Relative to the control (CON) mice, the obese (DIO) mice had significantly higher body weight (n = 57-58 per diet group; $p < 0.05$) (Figure 3.1A), higher fasting glucose (n = 27-30 per diet group; $p < 0.05$) (Figure 3.1B), higher fasting insulin (n = 25-30 per diet group; $p < 0.0001$) (Figure 3.1C), and higher fasting IGF-1 (n = 24-25 per diet group; $p < 0.0001$) (Figure 3.1D) at week 11 of the study.

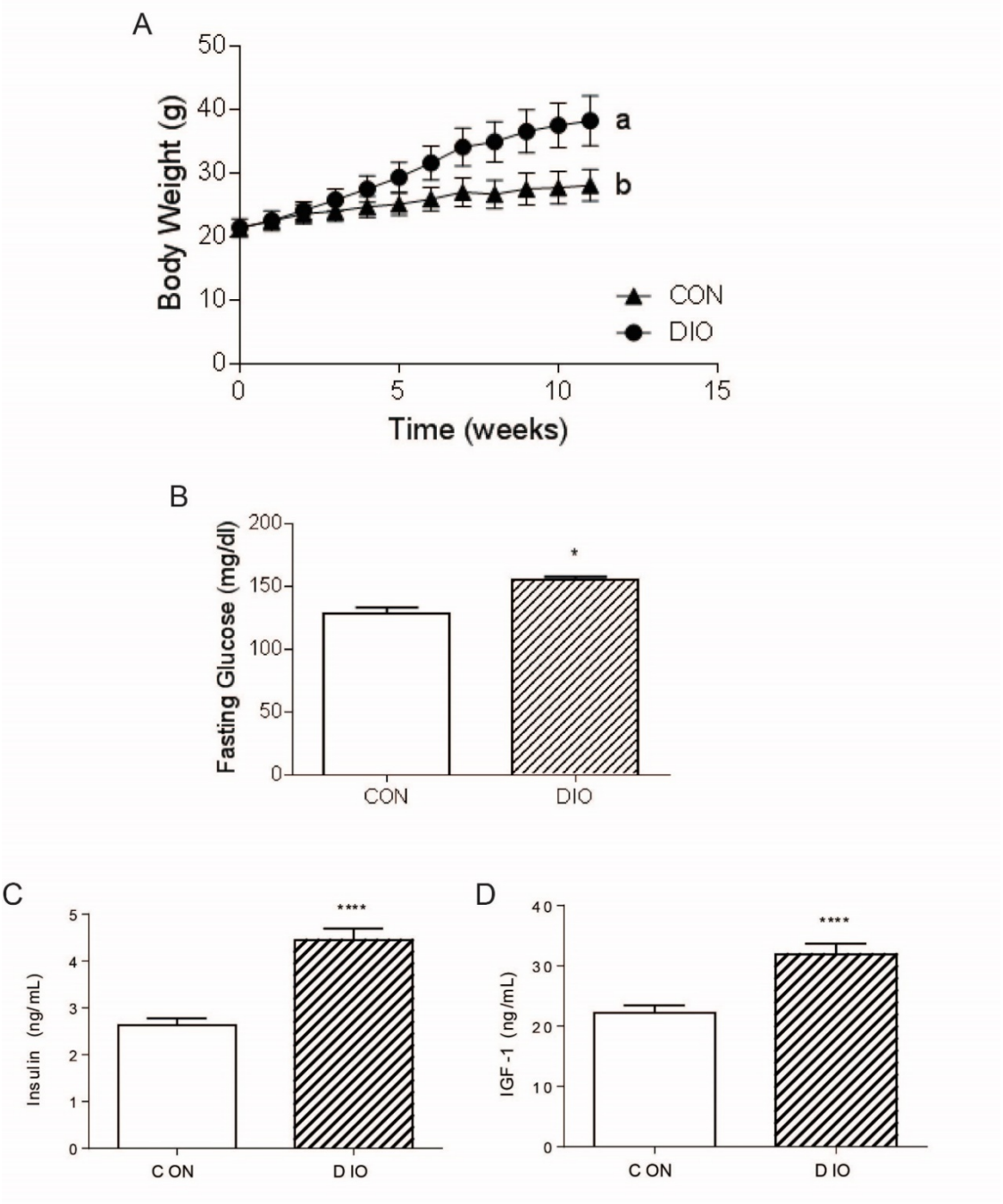

Figure 3.1. Effects of the high-fat, diet-induced obesity regimen (DIO) on body weight, fasting glucose, and hormones. (A) Body weight (n = 57-58 per diet group) of C57BL/6 male mice on control and DIO diets reported until week 10 on diet prior to tumor cell injection. **(B-D)** Serum analyses of fasting **(B)** glucose (n = 27-30 per diet group), **(C)** insulin (n = 25-30 per diet group) and **(D)** IGF-1 (n = 24-25 per diet group). All data are presented as the mean ± SD **(A)** or mean ± SEM **(B-D)**. Differences are considered significant if $p < 0.05$. If groups were compared to all other groups, different letters were used to indicate significantly different values. If groups were compared only to a control group, * were used to indicate significance, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Effects of HMB supplementation on skeletal muscle size and signaling

At the end of the study DIO mice had smaller muscles compared with the CON mice, and HMB supplementation increased muscle size regardless of diet ($n > 1,000$ fibers per group; $p < 0.05$ between bars with different letters) (Figure 3.2A). HMB supplementation decreased MuRF-1 expression in the CON mice, but increased MuRF-1 expression in the DIO mice ($n = 5$ per group; $p < 0.05$ between bars with different letters) (Figure 3.2B). Neither diet nor HMB supplementation had an effect on protein levels of MuRF-1 ($n = 4$ per group; $p > 0.05$) (Figure 3.2C-E). A marker of muscle growth and mTOR signaling, phospho-S6, increased in the DIO mice compared with the CON mice, although this difference did not achieve statistical significance. HMB supplementation increased phospho-S6 levels regardless of diet ($n = 4$ per group; $p < 0.05$ with *) (Figure 3.2F-H).

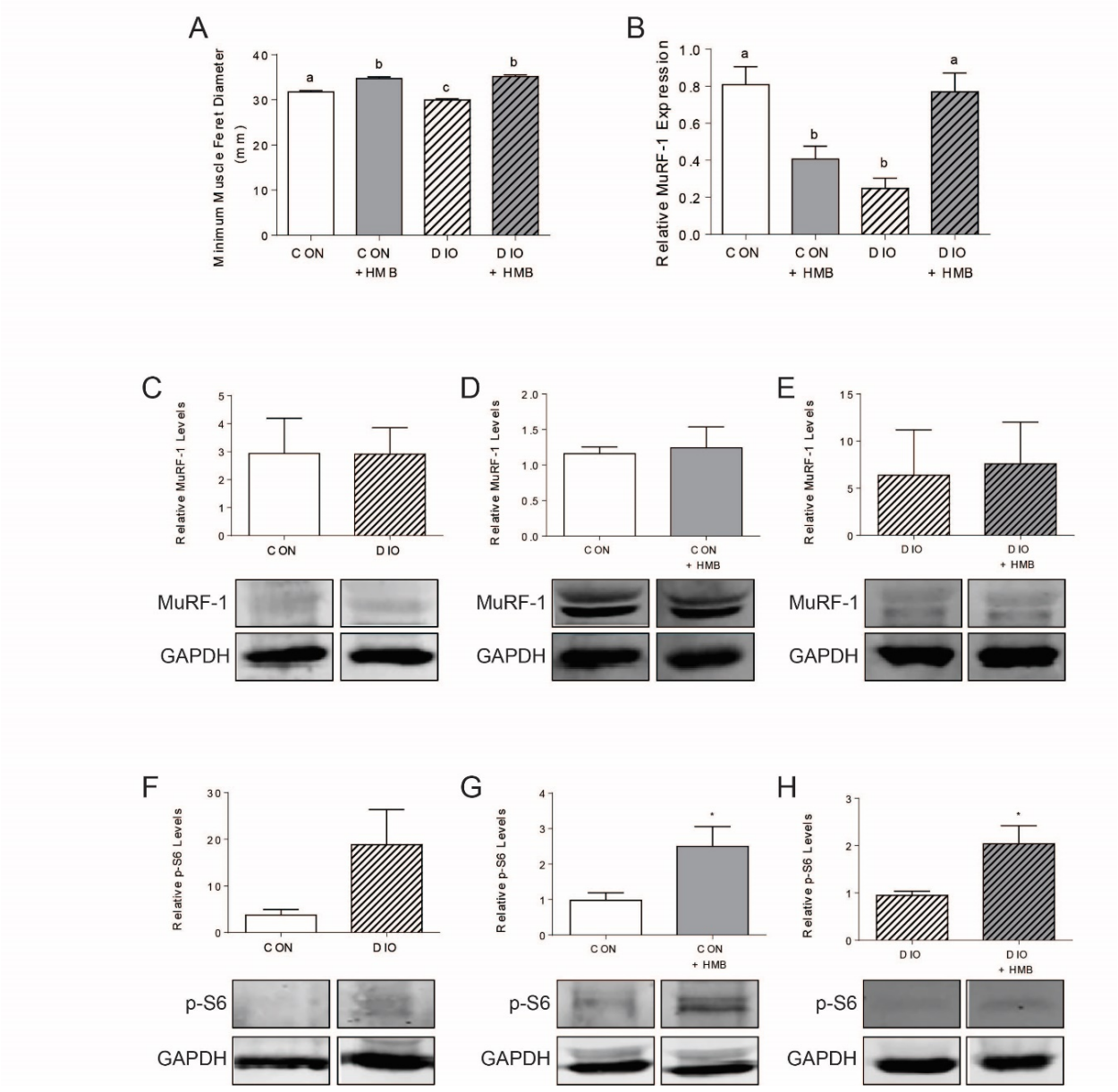

Figure 3.2. Effects of HMB supplementation on skeletal muscle size and signaling. (A-B) Results of HMB supplementation within both the control and DIO diets at study termination on muscle **(A)** minimal Feret's diameter, the most robust measurement that correlates with muscle fiber size (n > 1,000 per group) and **(B)** gene expression of the atrogene, MuRF-1 (n = 5 per group). **(C-E)** Protein levels of the atrogene MuRF-1 when comparing **(C)** control and DIO diets only, **(D)** HMB supplementation within the control diet, and **(E)** HMB supplementation within the DIO diet (n = 4 per group). **(F-H)** Levels of phospho-S6, a protein downstream of active mTOR, when comparing **(F)** control and DIO diets only, **(G)** HMB supplementation within the control diet, and **(H)** HMB supplementation within the DIO diet (n = 4 per group). All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$. If groups were compared to all other groups, different letters were used to indicate significantly different values. If groups were compared only to a control group, * were used to indicate significance, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Effects of HMB supplementation on glucose levels, Panc02 tumor growth, and tumor signaling

DIO mice had increased glucose levels compared with the CON mice, and HMB supplementation decreased glucose regardless of diet, although not statistically significantly in the DIO mice ($n = 13\text{-}15$ per group; $p < 0.05$ between bars with different letters) (Figure 3.3A). The DIO mice also had significantly larger tumors than the CON mice, and HMB supplementation significantly decreased tumor size in the DIO mice ($n = 13\text{-}15$ per group; $p < 0.05$ between bars with different letters) (Figure 3.3B). The results for tumor cell proliferation were similar to tumor volume. The DIO diet increased Ki-67 significantly compared with the CON diet. Moreover, HMB supplementation significantly decreased Ki-67 in the DIO mice ($n = 6$ per group; $p < 0.05$ between bars with different letters) (Figure 3.3C). The DIO diet increased phospho-S6 significantly compared with the CON diet, while HMB supplementation had no significant effect on phospho-S6 in the CON or DIO mice ($n = 6$ per group; $p < 0.05$ between bars with different letters) (Figure 3.3D).

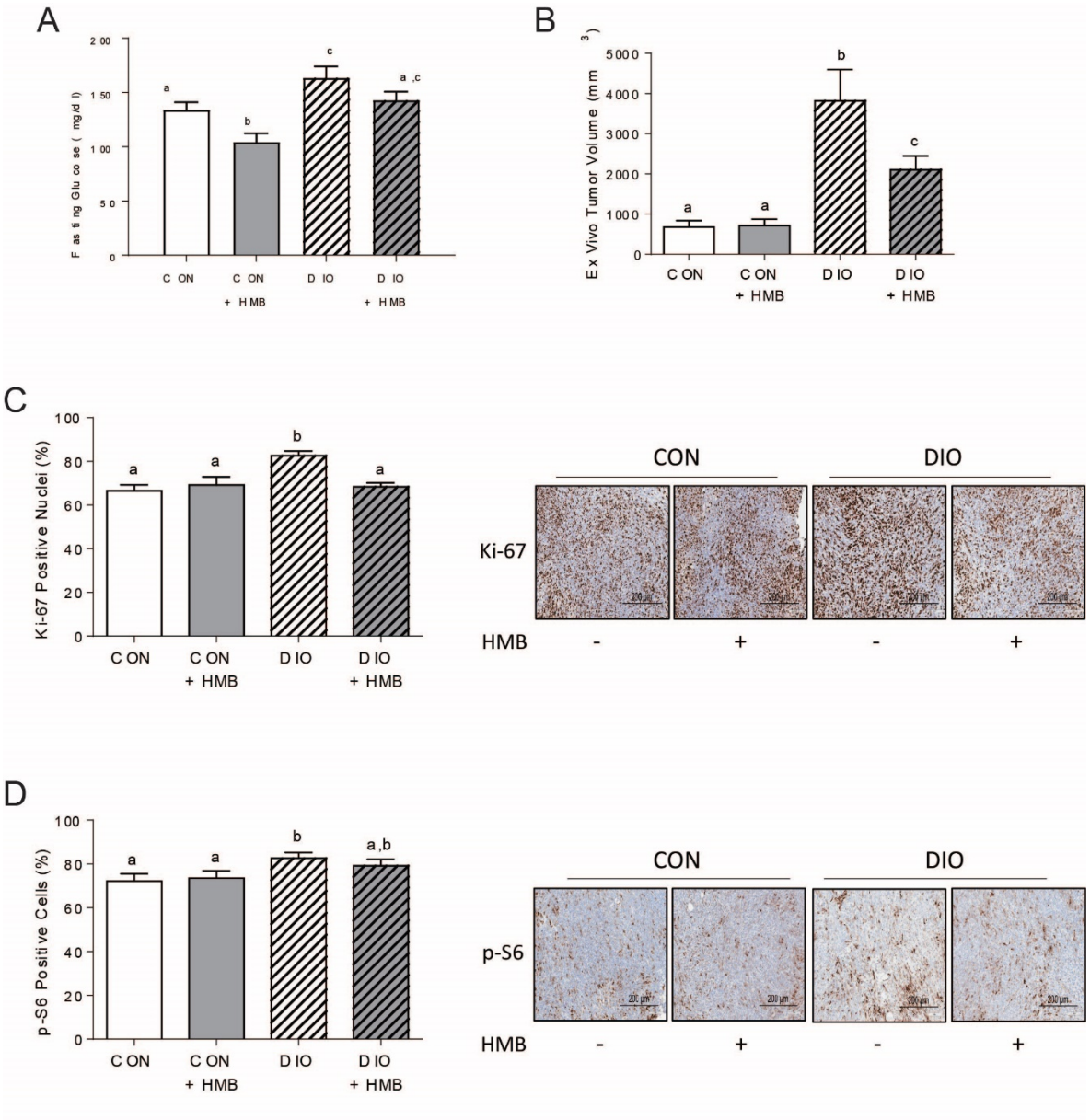

Figure 3.3. Effects of HMB supplementation on glucose levels, Panc02 tumor growth, and tumor signaling. (A-D) Results of HMB supplementation within both the control and DIO diets at study termination on **(A)** fasting glucose (n = 13-15 per group), **(B)** tumor volume (13-15 per group), **(C)** a marker of proliferation, Ki-67 (n = 6 per group), and **(D)** a protein downstream of active mTOR, phospho-S6 (n = 6 per group). Scale bars represent 200 µm. All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$, as indicated by different letters within the same graph.

Effects of HMB supplementation on Panc02 tumor response to gemcitabine

To determine whether dietary HMB supplementation affects the efficacy of gemcitabine treatment, we added HMB into the diets of half the mice in each original diet group (CON or DIO) and injected Panc02 tumor cells into all mice at week 11. After two weeks of tumor growth, we administered gemcitabine intraperitoneally every 3 days until study termination. The comparisons were done within each respective diet group (CON or DIO) in order to distinguish between any diet-differential effects of HMB on gemcitabine treatment.

After the first two weeks of tumor growth, palpations were performed twice weekly and tumors were present in all mice. However, at the end of the growth period, four weeks post tumor-injection, mice had tumors that had regressed so much in size, they were not found when surgery was performed. This complete tumor regression occurred in 5 out of 14 of the CON mice treated with gemcitabine alone and in 5 out of 15 of the CON mice treated with gemcitabine and HMB ($p < 0.05$) (Figure 3.4A). Complete tumor regression did not occur in the DIO mice with gemcitabine alone, but it did occur in 3 out of 14 of the DIO mice treated with gemcitabine and HMB, although results did not achieve statistical significance ($p > 0.05$) (Figure 3.4B).

At study termination, we compared the effects of either gemcitabine alone or gemcitabine with HMB supplementation on tumor volume and Ki-67, a marker of proliferation. The tumors excised from the CON mice were small and variable, and there were no statistically significant differences between the groups, although gemcitabine alone and in combination with HMB did decrease the average tumor

volume (n = 13-15 per group; $p > 0.05$) (Figure 3.4C). The difference between tumor size was much easier to distinguish in the DIO mice. Gemcitabine treatment decreased tumor volume, but not significantly; however, the addition of HMB supplementation to gemcitabine decreased average tumor volume significantly 3.5-fold (n = 13-15 per group; $p < 0.05$ between bars with different letters) (Figure 3.4D).

In the CON mice, gemcitabine treatment alone increased Ki-67 (n = 6 per group; $p < 0.05$ between bars with different letters) (Figure 3.4E). In the DIO mice, both gemcitabine alone and in combination with HMB decreased Ki-67 levels (n = 6 per group; $p < 0.05$ between bars with different letters) (Figure 3.4F).

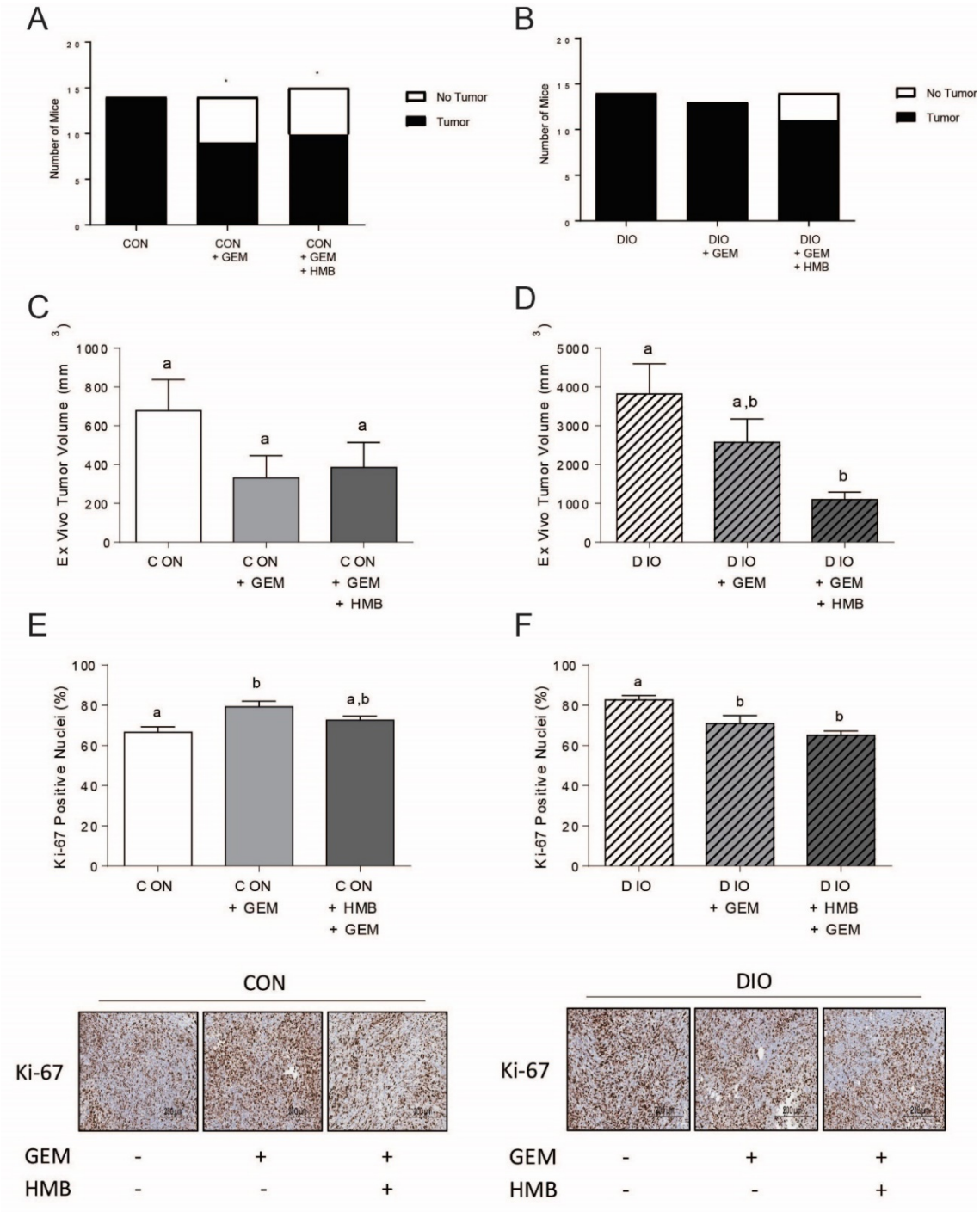

Figure 3.4. Effects of HMB supplementation on Panc02 tumor response to gemcitabine. (A-F) Results of treating mice in the same diet group with gemcitabine alone (GEM) or in conjunction with HMB supplementation at study termination on **(A-B)** complete tumor regression (n = 13-15 per group), **(C-D)** tumor volume (n = 13-15 per group), and **(E-F)** a immunohistochemical marker of proliferation, Ki-67 (n = 6 per group). Scale bars represent 200 µm. All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$, as indicated by different letters within the same graph.

Transcriptional response to diet and HMB supplementation

After demonstrating HMB inhibited tumor growth and had no detrimental effects on chemotherapy, we investigated possible mechanisms. The literature suggested two possibilities: 1) HMB could increase mTOR and growth signaling, and 2) HMB could inhibit NF-κB and inflammatory signaling. We showed HMB supplementation did not have a significant effect on tumor mTOR activation (Figure 3.3d), and although HMB could be inhibiting inflammatory signaling within the tumor, we showed no effect of diet on circulating pro-inflammatory cytokines (Figure 3.8). This led us to perform a microarray, a global analysis, on tumors from mice consuming the CON or DIO diet with and without HMB supplementation.

Although some genes were similarly regulated by the DIO diet and HMB supplementation (Figure 3.5A), a significant number of genes were upregulated by the DIO diet and downregulated by HMB supplementation (Figure 3.5B-C). Most of the genes suppressed by HMB were in the olfactory receptor family, and a few genes were involved in tumor proliferation and metastasis (Cldn9 and Pax2) and lipogenesis (Acsm1 and Lpcat4). Overall, the DIO diet increased expression of these genes in the tumor, while HMB supplementation decreased expression of these genes.

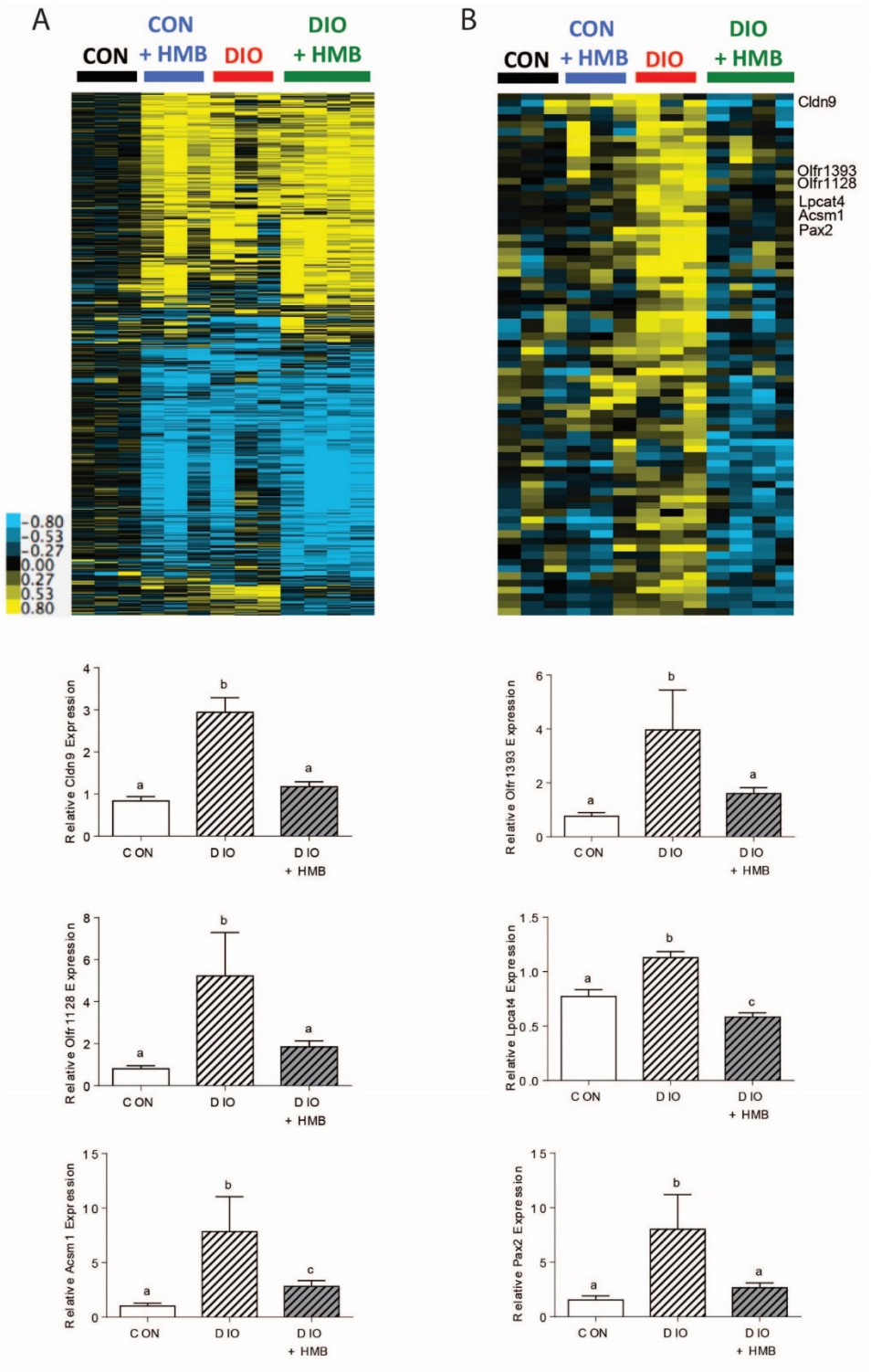

Figure 3.5. Transcriptional response to diet and HMB supplementation. (A-B) Heatmap of **(A)** all and **(B)** specifically chosen transcriptional response of tumor tissue from mice consuming the control or DIO diet with or without HMB supplementation. **(B)** qPCR validation of specific genes upregulated by the DIO diet and downregulated by HMB supplementation.

HMB supplementation-specific transcriptional response

This cluster of genes, which refers to a family of related genes, was upregulated by HMB supplementation regardless of diet (Figure 3.6A). The majority of these genes were either T cell receptor genes or involved in T cell-specific activity (CD4). Immunohistochemistry performed on tumor tissue for CD3, a universal T cell marker, revealed significant T cell infiltration of the tumor in response to HMB supplementation (Figure 3.6B). Further, quantitative PCR performed on tumor tissue for CD8a and CD8b showed the DIO diet significantly decreased cytotoxic CD8+ T cell expression, while HMB in the DIO diet significantly increased cytotoxic CD8+ T cell expression within the tumor (Figure 3.6C).

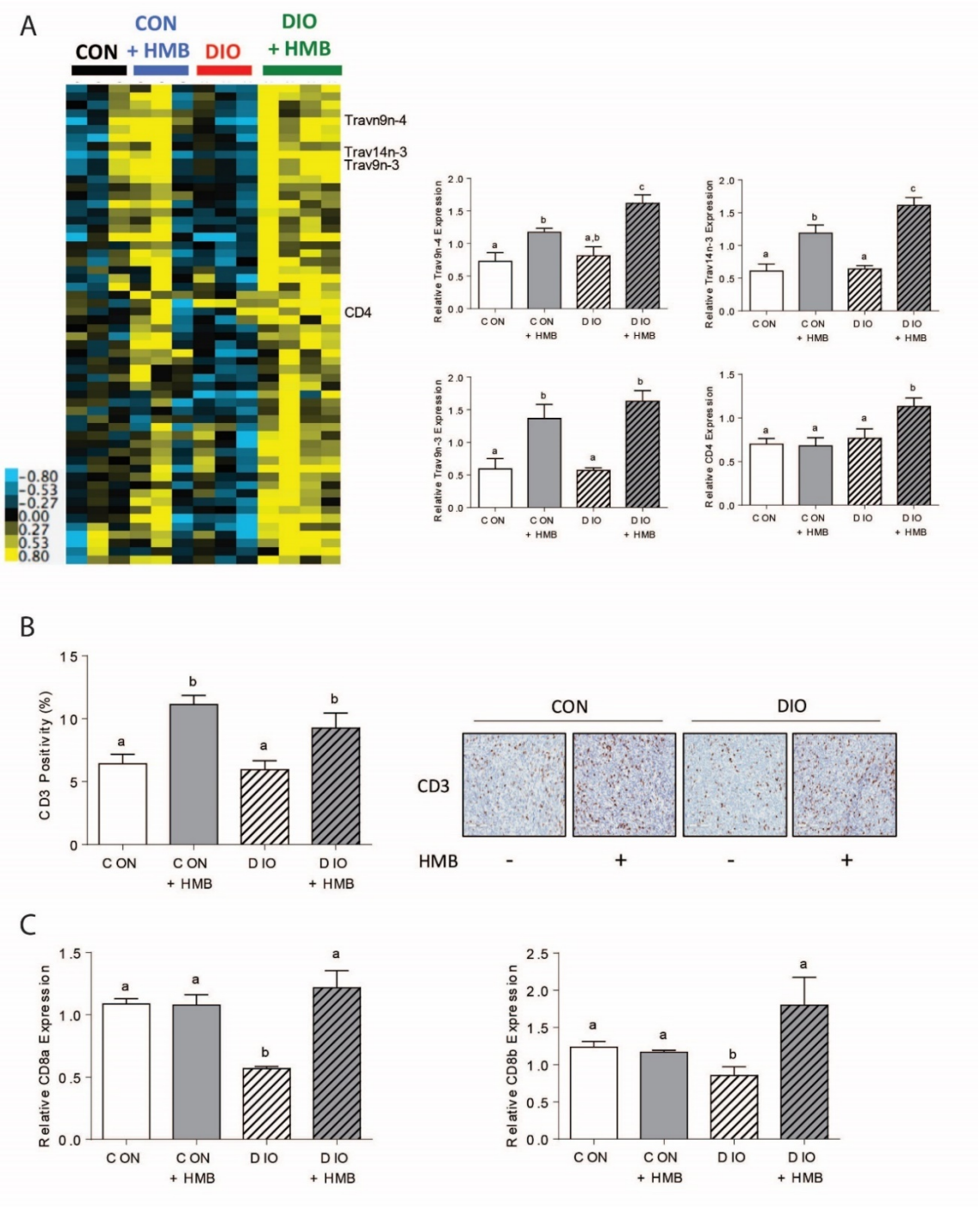

Figure 3.6. HMB supplementation specific transcriptional response. **(A)** Heatmap of transcriptional response of tumor tissue from mice consuming the control or DIO diet with or without HMB supplementation. qPCR validation of specific genes downregulated by the DIO diet and upregulated by HMB supplementation. **(B)** Immunohistochemistry of a marker of T cell infiltration, CD3 (n = 6 per group). Scale bars represent 100 µm. **(C)** qPCR of cytotoxic CD8+ T cell gene expression within the tumor tissue. All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$, as indicated by different letters within the same graph.

Discussion

Our findings demonstrate for the first time that dietary HMB supplementation decreases the growth of pancreatic tumors and preserves skeletal muscle mass in DIO mice. More specifically, we show that HMB supplementation not only inhibits the pro-tumorigenic environment of a high-fat, diet-induced obesity regimen (DIO), but also increases mTOR activation and growth only in muscle but not in tumor tissue. The mechanisms underlying these effects of HMB on tumor growth may be diet-dependent, suggested by a greater effect on glucose levels in the CON tumors and a greater effect on mTOR activation in the DIO tumors.

HMB supplementation in the CON mice exerted its own effects on muscle and tumor. In this diet group, HMB supplementation significantly increased muscle fiber size, decreased MuRF-1 gene expression, had no effect on MuRF-1 protein levels, and increased muscle mTOR activation. All these results support the enhanced muscle growth, except for the MuRF-1 protein levels. It is not uncommon when mRNA levels are unable to predict protein levels, especially when the protein is involved in a cyclical event, such as muscle regeneration (140). Decreased atrogene expression, but no significant difference in protein, may indicate HMB was beginning to inhibit atrophy and that the effects had not yet become apparent at the protein level. With respect to tumor, HMB had no effect on tumor size, proliferation, or tumor mTOR activation, although it did decrease glucose levels. Since this diet did not result in high circulating growth factors, the tumors did not grow quickly, even in the untreated group. Thus, even if HMB were

exerting an anti-tumorigenic effect by decreasing available glucose (77), it may not have been apparent in the slow-growing CON tumors.

HMB supplementation in the DIO mice exerted its own effects on muscle and tumor as well. In this diet group, HMB supplementation significantly increased muscle fiber size, increased MuRF-1 gene expression, had no effect on MuRF-1 protein levels, and increased muscle mTOR activation. All these results support enhanced muscle growth, except for the MuRF-1 gene expression levels. In an obese setting, especially with consistently high glucose and insulin (Figure 3.1B-C), insulin resistance can occur, and this can cause muscle degradation (141). HMB has been shown to attenuate insulin resistance (142), and thus, attenuate the associated muscle breakdown. HMB may have slowly increased muscle synthesis over time, eventually leading to a large amount of protein synthesis near the end of the 4-week supplementation period. Then, when protein levels became high in the muscle, MuRF-1 expression was transiently increased to recycle some of the excess protein. MuRF-1 expression has been shown to be transient (143). Taken together, this may explain the HMB-induced increase in MuRF-1 gene expression with no change in protein levels. With respect to tumor, HMB significantly decreased tumor size and tumor cell proliferation, while slightly decreasing glucose and tumor mTOR activation. All these results support each other and show dietary HMB supplementation alone can affect pancreatic cancer.

HMB supplementation did not harm gemcitabine efficacy within the CON diet. Complete tumor regression occurred in a significant number of mice on the CON diet when they were treated with gemcitabine, and their average tumor size

was lower than the untreated mice. The addition of HMB did not change these outcomes, and HMB even managed to slightly decrease tumor cell proliferation seen in the gemcitabine-treated CON mice. The gemcitabine-induced increase in Ki-67 was surprising. In this case, the CON tumors that did not regress may have become gemcitabine-resistant. Pancreatic cancer cells able to survive high-dose gemcitabine treatment end up expressing increased levels of stem cell genes, show characteristics similar to epithelial-mesenchymal transition, and retain proliferative capability (144). Due to their resistance, the remaining CON tumors may have become more aggressive and proliferated at a faster rate.

HMB supplementation enhanced gemcitabine efficacy within the DIO diet as evidenced by tumor regression and decreased tumor size. HMB may have been particularly synergistic with gemcitabine in this setting, because of the specific environment established by the obesigenic diet. Obesity is associated with resistance to gemcitabine in pancreatic cancer (Figure 3.4C-D) and breast cancer (145). This obesity-induced chemoresistance is associated with various factors, one of which is increased production of IL-1β within the tumor microenvironment, leading to the activation of the angiotensin II type-1 receptor (AT1) signaling pathway and elevated desmoplasia. Desmoplasia is an overproduction of extracellular matrix tissue, which blocks the penetration of gemcitabine into tumors (146). Although we found no evidence of the DIO diet increasing circulating inflammatory markers (Figure 3.8), this does not preclude the involvement of inflammation within or near the tumor. IL-1β can be produced by pancreatic stellate cells, tumor-associated macrophages, and pancreatic tumor cells (147). HMB has

been shown to decrease inflammatory signaling within cancer cells (56), so HMB may have been able to overcome some resistance to gemcitabine and increase its efficacy by reducing local inflammatory signaling.

As revealed by the gene expression microarray analysis, the DIO diet compared with the CON diet upregulated genes involved in olfaction and tumor proliferation and metastasis, while HMB supplementation within the DIO diet downregulated these genes. Although olfactory receptors (ORs) have been traditionally relegated to olfaction, studies have shown the existence of ectopic ORs, or ORs outside of the olfactory sensory system, suggesting possible nontraditional roles. In fact, our results support previous work showing a high-fat diet, similar to our DIO diet, upregulated olfactory and cancer-related genes in fat and muscle tissue of mice (73). Recent research has also shown connections between ORs and some cancers. Increased expression of ORs has also been seen in various cancers, including gastrointestinal neuroendocrine, prostate, and lung cancer (148). Upregulation of OR51E1 promotes prostate tumor growth and correlates with cancer progression (149-150), and a mutation in OR2W3 is associated with pancreatic ductal adenocarcinoma development (76). Further, knockdown of the OR2A1 and OR2A4 receptors in HeLa cervical cancer cells and in HCT116 colon cancer cells inhibits cell division (151). Our data showed the DIO diet increased OR gene expression and pancreatic tumor growth, while HMB supplementation decreased OR gene expression and tumor growth. Taken together, this suggests HMB may inhibit pancreatic tumor growth by reducing tumor-specific ORs.

The microarray further revealed HMB supplementation upregulated T cell receptor genes, genes involved in T cell activity, and overall T cell infiltration in both diet groups. The DIO diet compared with the CON diet specifically decreased cytotoxic CD8+ T cell tumor infiltration, while HMB increased cytotoxic CD8+ T cell tumor infiltration in the DIO group. For over ten years, researchers have shown the positive correlation between cytotoxic CD8+ T lymphocyte infiltration in tumors and enhanced survival of patients with colorectal cancer, breast cancer, melanoma, ovarian cancer, and non-Hodgkin's lymphoma (152). As such, the cytotoxic CD8+ T cells have been known as a key component of an effective anti-tumor immune response (153). Our data showed the DIO diet decreased cytotoxic CD8+ T cell tumor infiltration and increased pancreatic tumor growth, while HMB increased cytotoxic CD8+ T cell tumor infiltration and decreased tumor growth. Taken together, this suggests HMB may inhibit pancreatic tumor growth by stimulating the immune system and generating T cells that localize and kill tumor cells.

Although our transplant model of pancreatic cancer provides insight into how HMB supplementation affects tumor growth, it cannot provide any information with respect to tumor development. In order to study how HMB affects the development of PDAC, future studies could be performed in our spontaneous model of pancreatic cancer, the LSL-Kras(G12D)/Pdx-1-Cre/Ink4a/Arf(lox/+) mice. These mice have an activating *Kras* mutation and an *Ink4a/Arf* deficiency, the combination of which promotes the formation of pancreatic intraepithelial neoplasia (PanIN) lesions and eventually PDAC (123, 154-155). Moreover, our transplant model is also limited, because it is not a model of cancer cachexia. There are no

models of pancreatic cancer cachexia available, however, there are well-established models of colon and breast cancer cachexia. These models involve the subcutaneous injection of specific cell lines (C 26 for colon, and Walker 256 for breast) that cause cachexia. To study how HMB affects cancer cachexia and cachectic symptoms (weight loss, muscle breakdown, hypoglycemia, hyperlacticidemia, hypertriacylglycerolemia, and loss of glycogen stores) one of these models could be selected.

In conclusion, this report establishes that dietary HMB supplementation preserves muscle mass, irrespective of diet, has no detrimental effects on gemcitabine treatment, and inhibits pancreatic tumor growth, reduces tumor-specific olfactory receptor expression, and increases cytotoxic CD8+ T cell tumor infiltration in an obese setting. These findings suggest HMB is a strong candidate for the purpose of lean muscle enhancement in cachectic cancer patients, although caution should still be exercised due to the differences between murine and human metabolism. Additional research is needed to ascertain the impact amino acids and their metabolites have on cancer growth and muscle repair; the identification of natural supplementation approaches to spare muscle in cachectic cancer patients; and the link between diet, supplementation, mTOR signaling, olfactory receptors, and immunity.

Supplementary Figures

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Cldn9	GTATCGTACTCACCGCAGGG	AGCCCCCAGTTCTCTCTTGA
Olfr1393	TTCTGTGAGATGCCTGTTTTCCT	AGGCACTGCAACAATCACCAC
Olfr1128	ACACATCCTGTACATTCCAACTG	ATGTATCTGAGCAGGAGAGGGC
Lpcat4	GCCTCTCCCTCTGTCAGTTC	GGTATCTGGGAGGTGCTTCG
Acsm1	CTGAAGTGGAGTGTGGGGAC	ATGCGATACCCGGAAGCATT
Pax2	GCGAGGAAGATGTGTCTGAGG	TCGGGATAGGAAGGACGCTC
Trav9n-4	GATGCCCAAGCTCAGTCAGT	AAGGTGTCGCAGAGTAGGAA
Trav14n-3	TGGGAAGGAGAGACCGCAAT	TCGGACACTGAACGTATGGC
Trav9n-3	GGAGTGAATGGCTTTGAGGC	GAAGTACACAGCCCAGTCGC
CD4	ATCCAGAGGGGTGAACCAGA	TGCCTGGCGCTGTTGG
CD8a	TACCACAGGAGCCGAAAGCG	CCTGGCGGTGCCATTTTACA
CD8b	AGACTCAAGACGGCCCTTTC	GGAAGGACATCAACCACAGTC
MuRF-1	ACGAGAAGAAGAGCGAGCTG	CTTGGCACTTGAGAGAGGAAGG
GAPDH	CGTGTTCCTACCCCCAATGT	ATGTCATCATACTTGGCAGGTTTCT

Figure 3.7. List of murine oligonucleotide primers used for qPCR and microarray validation.

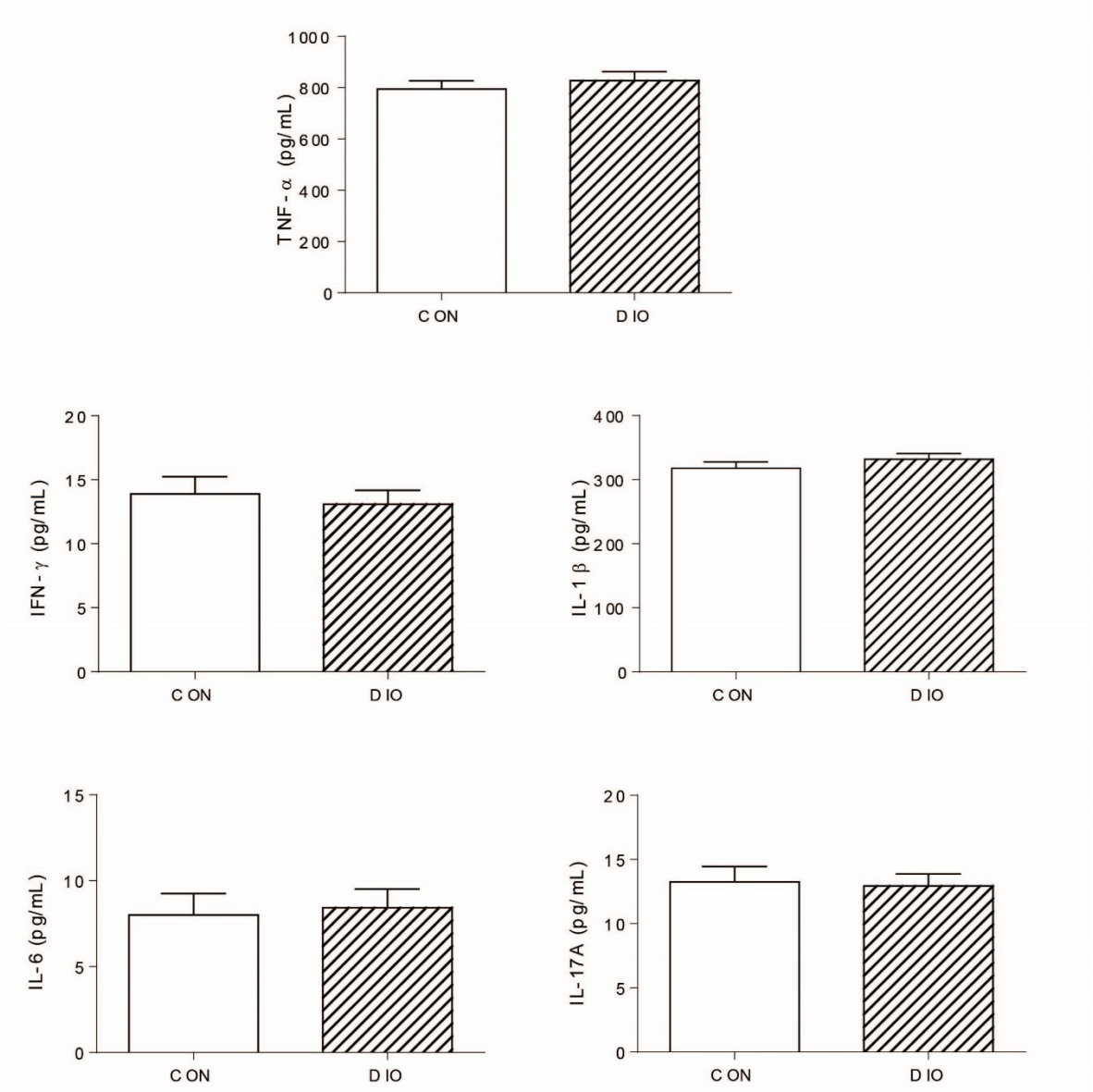

Figure 3.8. Levels of pro-inflammatory cytokines in serum of mice on the CON or DIO diet after 10 weeks (n = 28-32 per diet group, $p > 0.5$).

Chapter IV

Leucine and HMB supplementation affect pancreatic cancer cell proliferation and olfactory receptor expression

Introduction

Pancreatic cancer is projected to be the 2nd leading cause of cancer-related deaths by the year 2020 (156). This is due, in part, to cachexia, which occurs in 80% of pancreatic cancer patients (49). Cachexia is characterized by involuntary weight loss and significant breakdown of lean muscle and adipose tissue, and it reduces the patients' quality of life, their response to chemotherapy, and their survival rate (120). The muscle breakdown especially contributes to these consequences, as it is an independent predictor of reduced cancer survival, after controlling for weight, age, and tumor stage (4). Muscle breakdown is also an independent prognostic factor of poor overall and recurrence-free survival of pancreatic cancer patients even after resection of the tumor (122).

Due to the significant correlations between muscle breakdown and poor outcomes, the research for cachexia treatment focuses on compounds that affect muscle protein synthesis/breakdown (49-50). This focus on protein led researchers to study amino acids, the building blocks of protein. Branched-chain amino acids increase muscle protein synthesis almost to the same extent as an entire meal, and the branched-chain amino acid leucine alone can stimulate muscle protein synthesis (51). Increased focus on leucine led to the discovery of one of its metabolites, β-hydroxy-β-methylbutyrate (HMB). Leucine and HMB are particularly

relevant as potential cachexia treatments, because they lead to the phosphorylation and activation of the mechanistic target of rapamycin (mTOR) in skeletal muscle (53). mTOR is a serine/threonine protein kinase integral to protein synthesis through its phosphorylation of several downstream proteins involved in protein translation, including p70S6 kinase (p70S6K), ribosomal protein S6 (S6), and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (157). When it is activated in muscle, protein synthesis and hypertrophy occur (158). However, mTOR activation is correlated with increased pancreatic tumor growth (77), while mTOR inhibition decreases pancreatic tumor growth and has been shown to cause tumor regression in a specific subtype (60,159).

If leucine and HMB are to be administered to pancreatic cancer patients with cachexia to enhance muscle growth through mTOR signaling, studies need to be conducted to ascertain their effects on both muscle and tumor tissue. As most studies focus on the effects of leucine and HMB on muscle, we chose to determine the effects of these supplements on pancreatic ductal adenocarcinoma (PDAC) tumor growth using a murine transplant model (77, Chapter III). We found leucine supplementation increased PDAC tumor growth in lean and overweight mice, while only enhancing mTOR signaling within the tumors of lean mice (77). Then, we found HMB supplementation decreased PDAC tumor growth, while having no significant effect on mTOR signaling within the tumor. Instead, HMB downregulated multiple olfactory receptor (OR) genes within the tumor (Chapter III).

Although ORs are traditionally believed to play roles only in the olfactory system, a few studies have shown dysregulation of ORs in various cancer types (148). Upregulation of OR51E1 enhances prostate tumor growth (149-150). Knockdown of the OR2A1 and OR2A4 receptors in cervical and colon cancer cells inhibits cell proliferation (151). Further, a mutation in OR2W3 is correlated with PDAC (76). These findings support the idea that ORs could regulate tumor growth.

In the present study, our goals were to investigate if the direct effects of leucine and HMB on pancreatic cancer growth were: 1) similar to the *in vivo* results, 2) associated with mTOR signaling, 3) associated with OR gene expression, and 4) comparable to the effects of common OR agonists. We used two murine (Panc02 and NB508) and two human (Panc-1 and MiaPaca-2) PDAC cell lines to evaluate the effects of leucine and HMB on cell growth and growth signaling. These cells were also treated in media with different concentrations of fetal bovine serum (FBS) to simulate an *in vitro* growth factor-rich and growth factor-restricted environment, similar to the internal environments of the obese/overweight and lean mice, respectively. We then evaluated the effects of leucine and HMB on gene expression to determine their regulation, if any, of specific ORs. Finally, we determined the effects of OR agonists on pancreatic cancer cell proliferation. Our findings suggest that leucine and HMB are able to directly affect pancreatic cancer cell growth and growth signaling and that their effects are connected to OR regulation.

Materials and Methods

Cell culture treatments

The murine PDAC cells, Panc02 and NB508, and the human PDAC cells, Panc-1 and MiaPaca-2, were cultured in a 37°C incubator under 5% CO_2 in RPMI media with glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 3 g/L glucose. The media was supplemented with penicillin/streptomycin, nonessential amino acids, sodium pyruvate, HEPES, and 10% heat-inactivated FBS (Sigma-Aldrich).

For MTT assays, Panc02 and NB508 cells (1,500 cells/well) and Panc-1 and MiaPaca-2 cells (3,000 cells/well) were seeded in 96-well plates. After 24 hours, the old media was removed and fresh RPMI media was added, supplemented with different amounts of FBS (10% or 1%), leucine or HMB (0, 1, 2, or 4 mM), and OR agonists (5 µM, 10 µM, 50 µM, or 100 µM). The cells were then incubated for 48 h at 37°C. Leucine was from MP Biomedicals (Santa Ana, CA, USA), HMB was from Metabolic Technologies, Inc. (Ames, IA, USA), and all OR agonists were from Sigma-Aldrich. Optimal doses of leucine and HMB were used in later experiments.

For western blotting, Panc02 and NB508 cells (200,000 cells/well) and Panc-1 and MiaPaca-2 cells (400,000 cells/well) were seeded in 6-well plates. After 24 hours, the old media was removed and fresh RPMI media was added, supplemented with different amounts of FBS (10% or 1%). After 4 hours of pretreatment in new media, cells were treated for 30 min with 4 mM of leucine or HMB.

For the microarray, Panc02 cells (100,000 cells/well) were seeded in 6-well plates. After 24 hours, the old media was removed and fresh RPMI media with 1% FBS was added, supplemented with different amounts of leucine or HMB (4 mM). The cells were then incubated for 24 h at 37°C.

Cell proliferation assay

MTT reagent, thiazolyl blue tetrazolium bromide (Sigma), was added to treated cells at a 1:10 ratio for 2 hours, then 180 µL of liquid was aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to lyse the cells and dissolve the purple formazan crystals. The optical density of each well at 540 nm and 690 nm, a reference wavelength, was determined using the Synergy 2 Multi-Detection Microplate Reader and Gen5 Data Analysis Software (Fisher Scientific, Pittsburgh, PA, USA). Relative cell viability was then calculated using the absorbance of cells grown in media without treatment for normalization. Data shown represent the average of three biological replicates.

Western blot analysis

Treated cells were lysed on ice for 1 hour in RIPA buffer (Sigma) with protease inhibitor tablet (Roche Applied Sciences, Indianapolis, IN, USA) and phosphatase inhibitor cocktails II and III (Sigma). Protein lysates (20 µg) were resolved by SDS-PAGE using 6%-12% gels, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) for 15 hours at 25 volts and blocked for 1 hour at room temperature with LI-COR Blocking Buffer (LI-COR Biotechnologies,

Lincoln, NE, USA). Membranes were incubated overnight at 4°C with primary antibodies diluted in 5% BSA (Santa Cruz). The primary antibodies were from Cell Signaling, Danvers, MA, USA, unless otherwise stated, and were specific for: β-actin (1:1000), mTOR (1:1000), phospho-mTOR$_{Ser2448}$ (1:1000), p70S6K (1:1000), phospho-p70S6K$_{T389}$ (1:1000), S6 ribosomal protein (1:1000), and phospho-S6 ribosomal protein$_{Ser235/236}$ (1:1000). After three 5-minute washes in 0.1% Tris-Buffered Saline Tween-20 (TBST), membranes were incubated for 45 minutes in species-specific secondary antibody (LI-COR) diluted (1:5000) in LI-COR Blocking Buffer. Following two washes in TBST and one wash in TBS, membranes were scanned using the Odyssey infrared fluorescent imaging system (LI-COR). Densitometry was performed using LI-COR software, and relative levels of proteins were calculated from three biological replicates. Raw values were compared between groups only if they were on the same membrane. β-actin was used as a loading control for all antibodies, and then all phosphorylated proteins were normalized to their respective non-phosphorylated protein.

Microarray analysis

Total RNA samples were extracted by RNeasy Kit (Qiagen, Valencia, CA, USA) from homogenized tumors and hybridized to Affymetrix Mouse Gene 2.1 ST 24-Array plate. The quality of the RNA sample was checked using Agilent bioanalyzer. The mRNA expression raw data were quantile normalized and the signal values were transformed to the $\log_2$ value. The comparative analysis between different group samples was carried out using the t-test (p-values) and the

Benjamini-Hochberg False Discovery Rate (FDR) correction (adjusted p-values) using R. Differentially expressed genes between experimental groups were determined if the p-values were < 0.05 and if the fold changes were > 1.5. The expression level of selected genes were combined and zero-transformed to the control samples.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism (GraphPad Software, La Jolla, CA). When multiple treatment doses were used in the same experiments, pancreatic cancer cell viability was compared by one-way ANOVA followed by Fisher's LSD tests of significance to compare all treated groups to the non-treated group. Western blot densitometry was analyzed using unpaired, two-tailed t-tests, where the leucine- and HMB-supplemented groups were compared to the non-supplemented group. Results were considered significant if $p < 0.05$.

Results

Effects of leucine supplementation on PDAC cell proliferation

To determine the effects of direct leucine supplementation on PDAC cells, Panc02, NB508, Panc-1, and MiaPaca-2 cells were supplemented with different doses of leucine (0, 1, 2, 4 mM) within serum-rich (10% FBS) and serum-restricted (1% FBS) environments. In the serum-rich environment, leucine increased Panc02 cell proliferation, but it actually inhibited MiaPaca-2 proliferation at a lower dose ($n = 3$; $p < 0.05$) (Figure 4.1A). Then, in the serum-restricted environment, leucine increased Panc02, NB508, and MiaPaca-2 proliferation ($n = 3$; $p < 0.05$) (Figure 4.1B). Overall, leucine supplementation enhanced PDAC cell proliferation at 4 mM, especially in the serum-restricted environment. The 4 mM dose of leucine was subsequently used in later experiments.

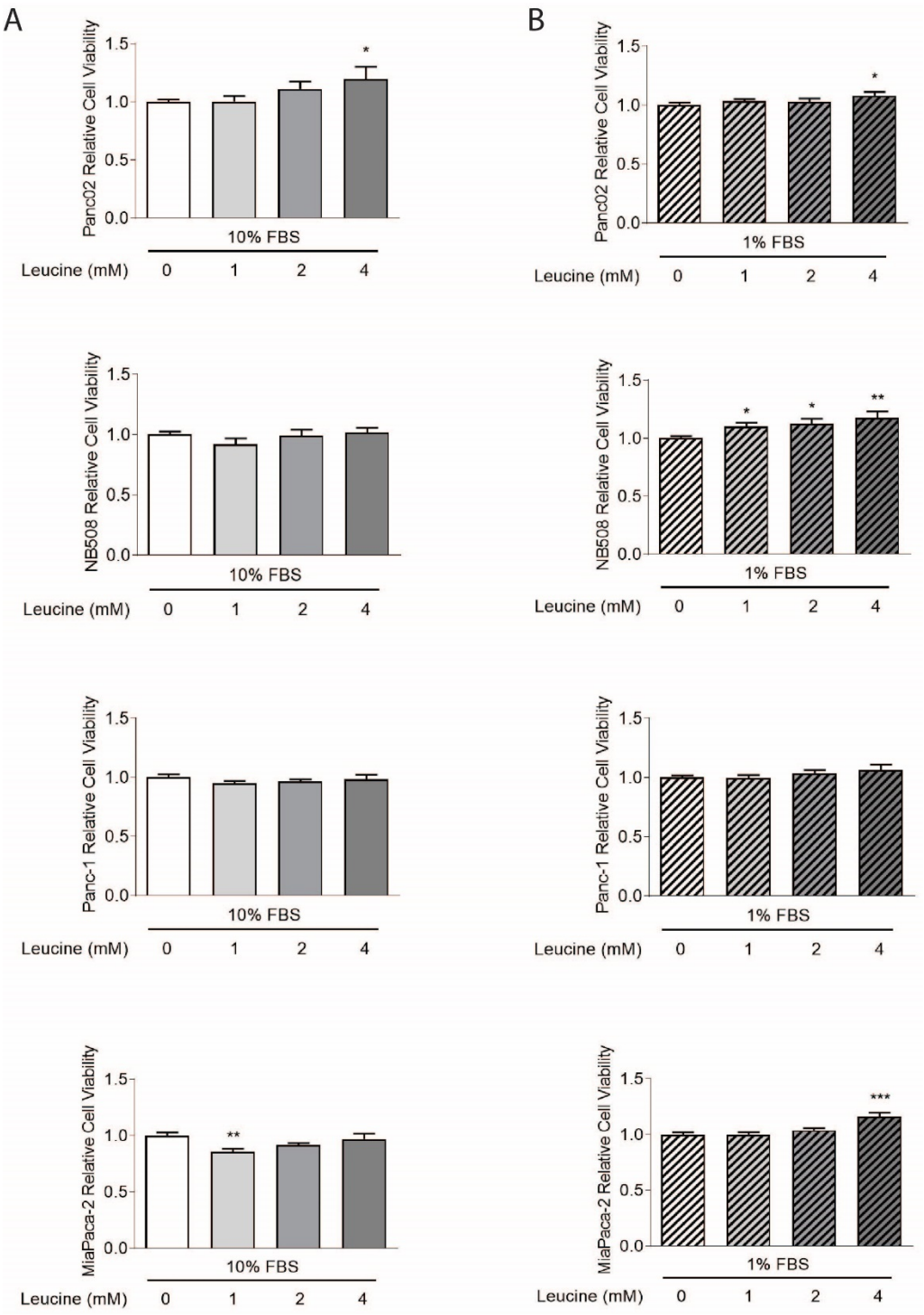

Figure 4.1. Effects of leucine supplementation on PDAC cell proliferation. (A-B) Results of MTT assays performed on murine and human PDAC cells after supplementation with leucine for 48 hours in either a **(A)** serum-rich or **(B)** serum-restricted environment (n = 3 biological replicates). All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$. Groups were compared only to the non-supplemented control group, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Effects of HMB supplementation on PDAC cell proliferation

To determine the effects of direct HMB supplementation on PDAC cells, Panc02, NB508, Panc-1, and MiaPaca-2 cells were supplemented with different doses of HMB (0, 1, 2, 4 mM) within serum-rich (10% FBS) and serum-restricted (1% FBS) environments. In the serum-rich environment, HMB decreased Panc02, Panc-1, and MiaPaca-2 proliferation ($n = 3$; $p < 0.05$) (Figure 4.2A). Then, in the serum-restricted environment, HMB significantly decreased Panc02, NB508, Panc-1, and MiaPaca-2 proliferation ($n = 3$; $p < 0.05$) (Figure 4.2B). Overall, HMB supplementation inhibited PDAC cell proliferation, especially at 4 mM in the serum-restricted environment. The 4 mM dose of HMB was subsequently used in later experiments.

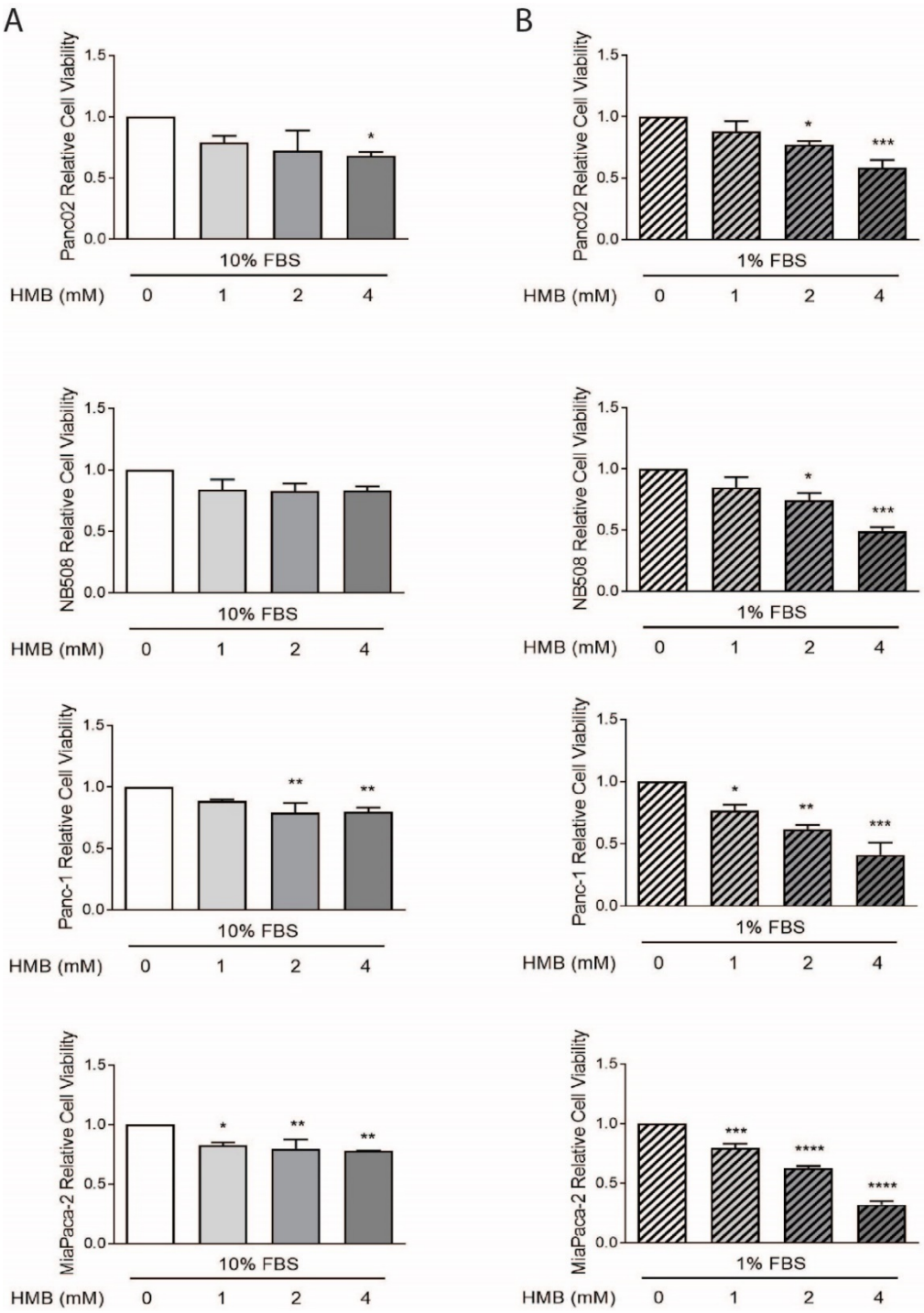

Figure 4.2. Effects of HMB supplementation on PDAC cell proliferation. (A-B) Results of MTT assays performed on murine and human PDAC cells after supplementation with leucine for 48 hours in either a **(A)** serum-rich or **(B)** serum-restricted environment (n = 3 biological replicates). All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$. Groups were compared only to the non-supplemented control group, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Effects of leucine and HMB supplementation on murine PDAC mTOR signaling

To determine the effects of direct leucine and HMB supplementation on mTOR signaling within murine PDAC cells, Panc02 and NB508 cells were supplemented with leucine and HMB (4 mM) within serum-rich (10% FBS) and serum-restricted (1% FBS) environments. In the serum-rich environment, leucine did not affect mTOR signaling ($n = 3$; $p > 0.05$) (Figure 4.3A), and HMB only significantly decreased NB508 p-mTOR levels ($n = 3$; $p < 0.05$) (Figure 4.3A). In the serum-restricted environment, leucine did not have a statistically significant effect on mTOR signaling ($n = 3$; $p > 0.05$) (Figure 4.3B), although it seemed to increase mTOR signaling. On the other hand, HMB decreased p-mTOR and p-S6 in the NB508 cells ($n = 3$; $p < 0.05$). Overall, the effects of leucine and HMB supplementation were more pronounced in the serum-restricted environment. Leucine supplementation trended in the direction of increased mTOR signaling, although it did not reach statistical significance, while HMB supplementation decreased mTOR signaling in the NB508 cells.

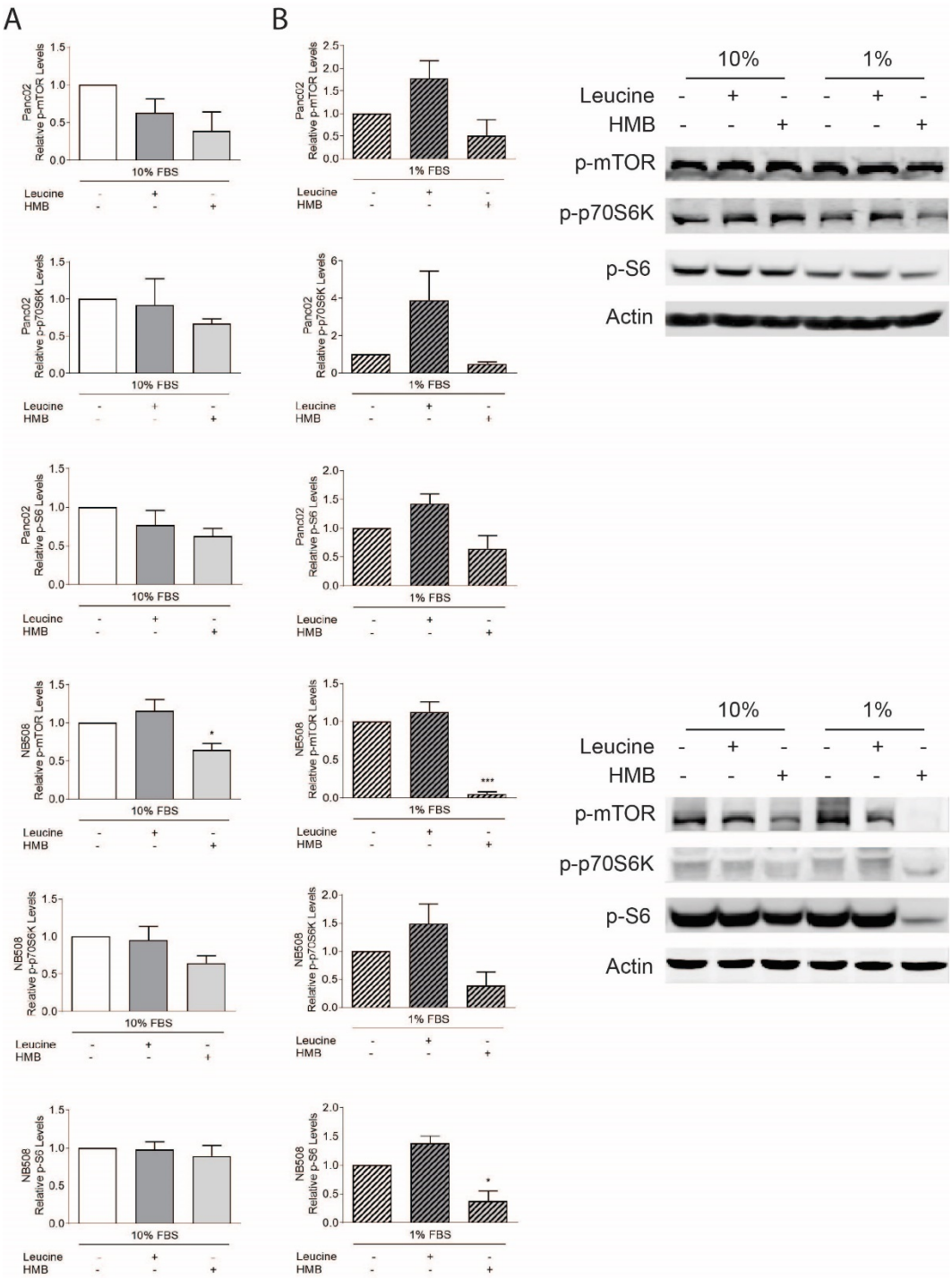

Figure 4.3. Effects of leucine and HMB supplementation on murine PDAC mTOR signaling. (A-B) Results of western blot analyses performed on murine pancreatic cancer cells after supplementation with 4 mM leucine or HMB for 30 minutes after pretreatment in either a **(A)** serum-rich or **(B)** serum-restricted environment for 4 hours (n = 3 biological replicates). All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$. Groups were compared only to the non-supplemented control group, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Effects of leucine and HMB supplementation on human PDAC mTOR signaling

To determine the effects of direct leucine and HMB supplementation on mTOR signaling within human PDAC cells, Panc-1 and MiaPaca-2 cells were supplemented with leucine and HMB (4 mM) within serum-rich (10% FBS) and serum-restricted (1% FBS) environments. In the serum-rich environment, leucine did not affect mTOR signaling ($n = 3$; $p > 0.05$) (Figure 4.4A), and HMB only decreased Panc-1 p-mTOR signaling ($n = 3$; $p < 0.05$) (Figure 4.4A). Then, in the serum-restricted environment, leucine significantly increased Panc-1 p-p70S6K and p-S6 and MiaPaca-2 p-mTOR and p-S6 levels, while HMB significantly decreased Panc-1 p-S6 and MiaPaca-2 p-mTOR levels ($n = 3$; $p < 0.05$) (Figure 4.4B). Overall, the effects of leucine and HMB supplementation were once again stronger in the serum-restricted environment. Leucine increased mTOR signaling, while HMB decreased mTOR signaling in human PDAC cells.

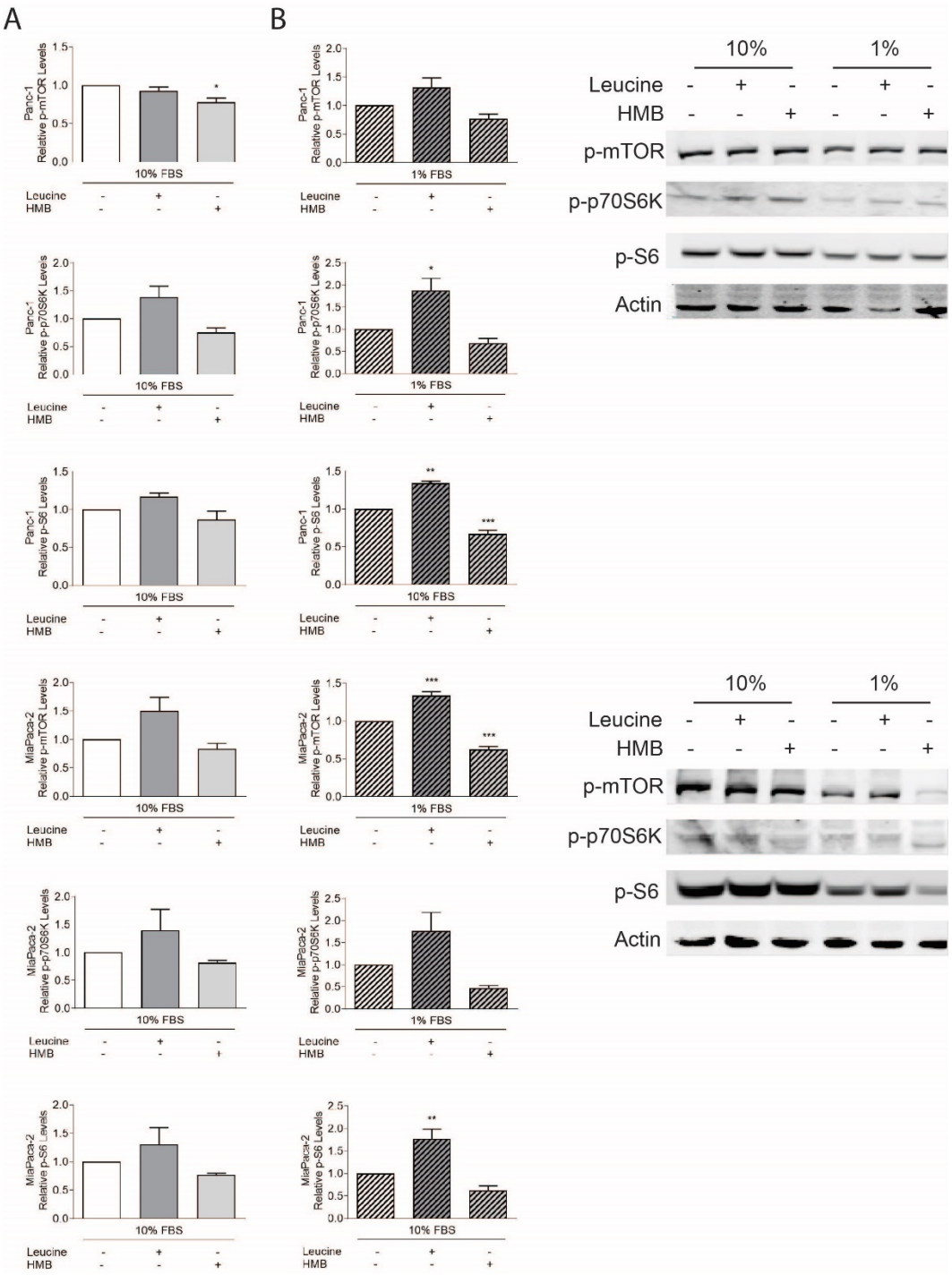

Figure 4.4. Effects of leucine and HMB supplementation on human PDAC mTOR signaling. (A-B) Results of western blot analyses performed on human pancreatic cancer cells after supplementation with 4 mM leucine or HMB for 30 minutes after pretreatment in either a **(A)** serum-rich or **(B)** serum-restricted environment for 4 hours (n = 3 biological replicates). All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$. Groups were compared only to the non-supplemented control group, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Transcriptional response of Panc02 cells to leucine and HMB supplementation

Although the proliferation and mTOR signaling results were consistent as a whole—leucine increased proliferation and mTOR signaling, while HMB decreased proliferation and mTOR signaling—the Panc02 cells were not as consistent with these overall results. Panc02 cell proliferation was enhanced with leucine and inhibited with HMB; however, mTOR signaling was not as strongly affected in the Panc02 cells as it was in the other cells. This suggested leucine and HMB were possibly working through other mechanisms as well as growth signaling. To investigate their effects on global gene expression, a microarray was performed on Panc02 cells supplemented with leucine or HMB in a serum-restricted (1% FBS) environment.

As seen in Figure 5A, leucine and HMB did regulate many genes differently in Panc02 cells. Specifically, leucine downregulated Mir721, Fosb, Olfr487, Olfr582, Atp1a4, and Ap3s1-ps2, while HMB upregulated these genes. Then, HMB downregulated Mir709, Olfr1138, Olfr402, Adamts9, Mir337, Trav7-6, Fxyd6, Lair1, Olfr1034, and Mir669m-1, while leucine upregulated these genes (Figure 4.5B). One-third of these differentially regulated genes were the OR genes, Olfr487, Olfr582, Olfr1138, Olfr402, and Olfr1034 (Figure 4.5C). HMB has been shown to decrease Panc02 tumor growth and OR expression previously (Chapter III), so it is plausible OR regulation may be one more mechanism by which HMB and leucine affect PDAC cells.

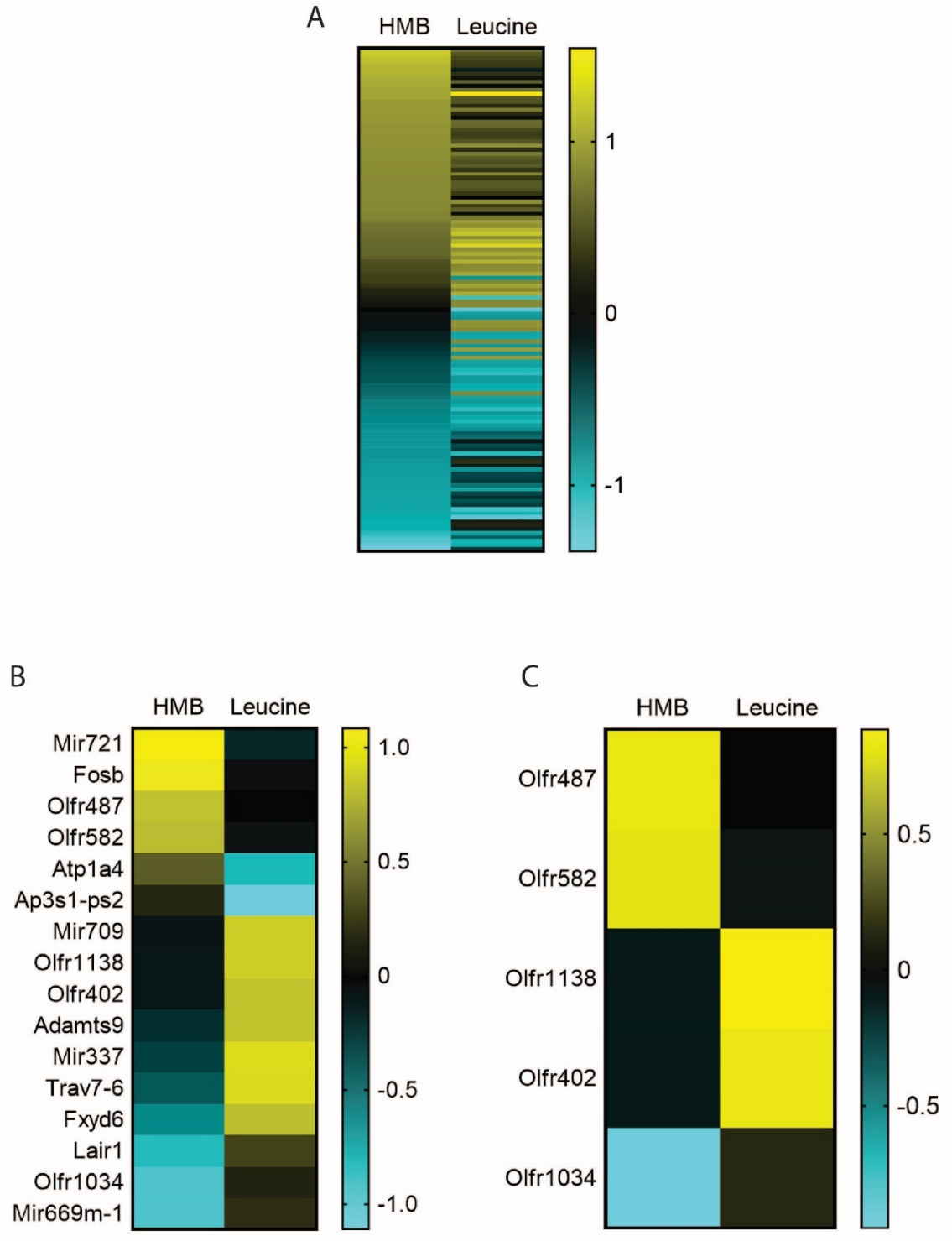

Figure 4.5. Transcriptional response of Panc02 cells to leucine and HMB supplementation. (A-C) Results of a microarray performed on Panc02 cells after 24 hour supplementation with leucine or HMB in a serum-restricted environment. Heat maps shown display gene expression for (A) all identifiable genes, (B) genes differentially regulated by HMB and leucine, and (C) specific olfactory receptor genes differentially regulated by HMB and leucine.

Effects of amyl acetate on PDAC cell proliferation

To interrogate whether it was plausible that a few ORs could affect PDAC cell proliferation, we first determined if an OR agonist had an effect. OR agonists are commonly used in studies to activate many ORs simultaneously at doses ranging from 10 µM to 1 mM, although the most common concentration is 100 µM (160-163). Panc02, NB508, Panc-1, and MiaPaca-2 cells were treated with various doses of the OR agonist, amyl acetate (0, 5, 10, 50, 100 µM), in both serum-rich (10% FBS) and serum-restricted (1%) environments.

In the serum-rich environment, amyl acetate increased NB508 proliferation at 100 µM ($n = 3$; $p < 0.05$) (Figure 4.6A), while it decreased Panc02 and MiaPaca-2 proliferation in the serum-restricted environment ($n = 3$; $p < 0.05$) (Figure 4.6B). This shows amyl acetate can affect both murine and human PDAC cell lines, although the effects are environment-dependent.

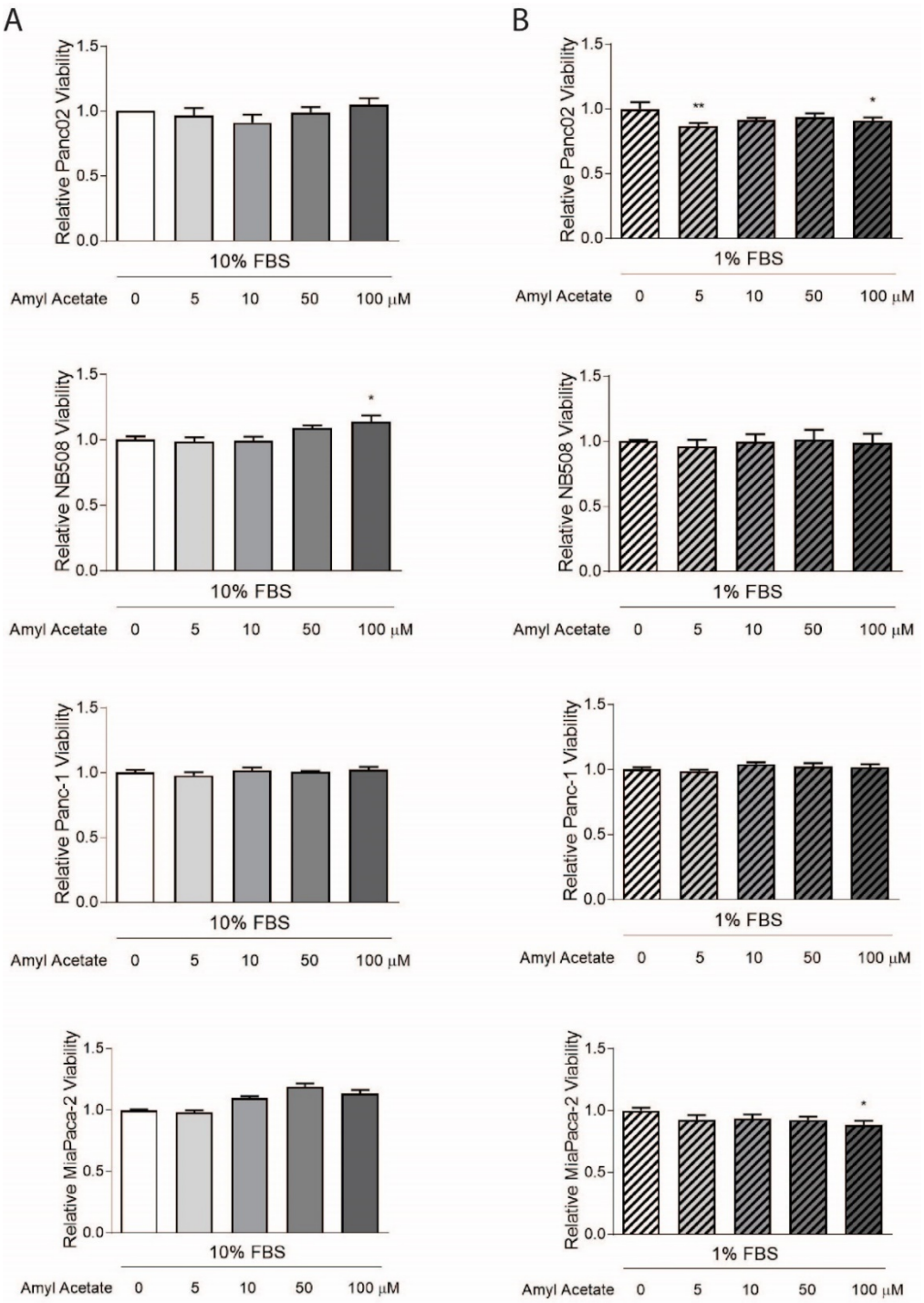

Figure 4.6. Effects of amyl acetate on PDAC cell proliferation. (A-B) Results of MTT assays performed on murine and human PDAC cells after supplementation with an OR agonist, amyl acetate, for 48 hours in either a **(A)** serum-rich or **(B)** serum-restricted environment (n = 3 biological replicates). All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$. Groups were compared only to the non-supplemented control group, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Effects of octanal on PDAC cell proliferation

To further explore the effects of OR agonists on PDAC cell proliferation, Panc02, NB508, Panc-1, and MiaPaca-2 cells in serum-rich (10% FBS) and serum-restricted (1%) environments were treated with various doses of octanal (0, 5, 10, 50, 100 μM), another OR agonist commonly used in studies (160-163). Octanal at 100 μM decreased Panc02, NB508, Panc-1, and MiaPaca-2 proliferation in the serum-rich ($n = 3$; $p < 0.05$) (Figure 4.7A) and serum-restricted environment ($n = 3$; $p < 0.05$) (Figure 4.7B). Octanal was able to inhibit growth of both murine and human PDAC cells in both types of environments, although the growth-inhibitive effects were more pronounced in the serum-restricted environment. These effects of octanal are similar to the growth-inhibitive effects of HMB.

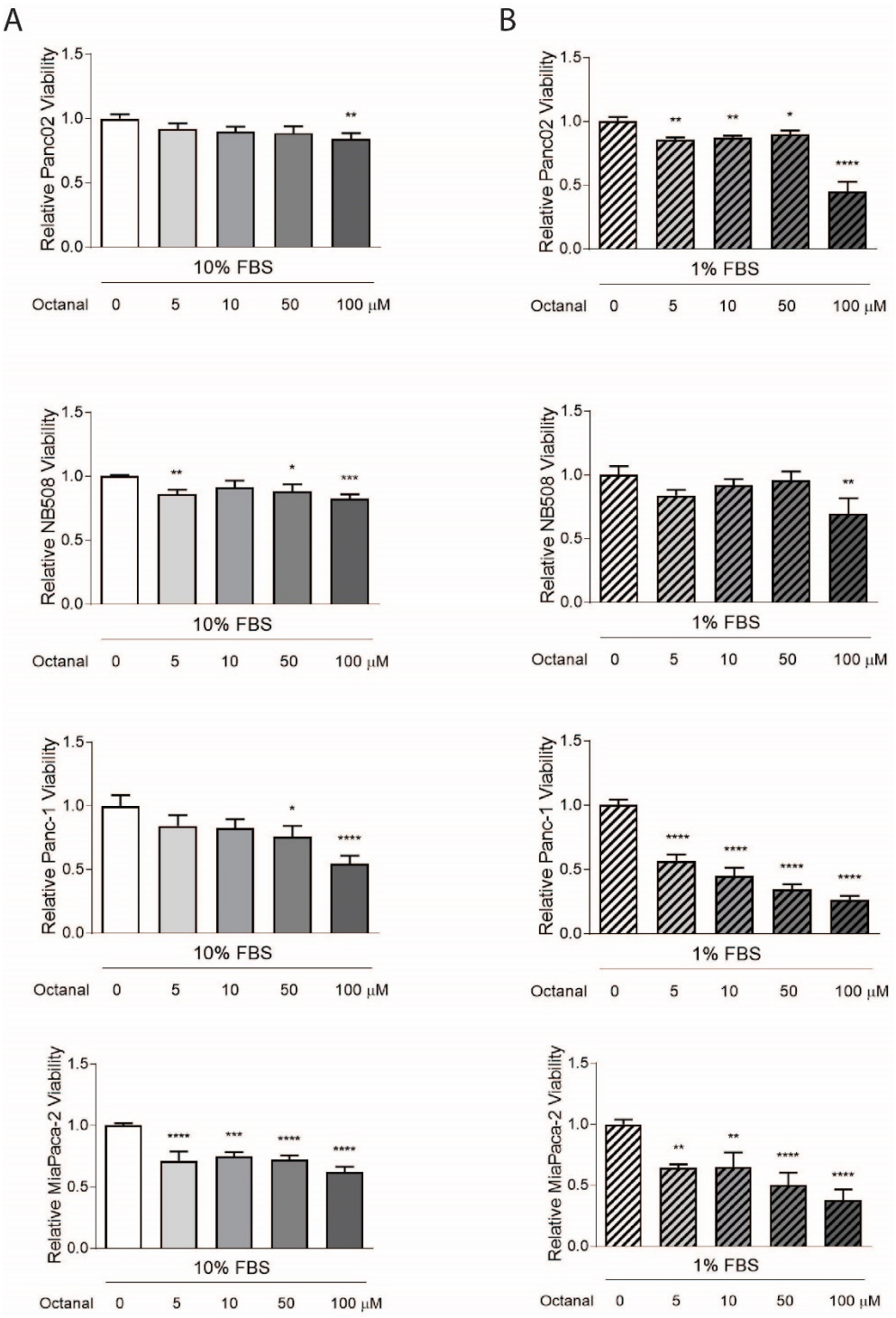

Figure 4.7. Effects of octanal on PDAC cell proliferation. (A-B) Results of MTT assays performed on murine and human PDAC cells after supplementation with an OR agonist, octanal, for 48 hours in either a **(A)** serum-rich or **(B)** serum-restricted environment (n = 3 biological replicates). All data are presented as mean ± SEM. Differences are considered significant if $p < 0.05$. Groups were compared only to the non-supplemented control group, where * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Discussion

The current study was conducted to determine if the direct effects of leucine and HMB supplementation on PDAC growth were: 1) similar to the *in vivo* results, 2) associated with mTOR signaling, 3) associated with OR gene expression, and 4) comparable to the effects of common OR agonists. We first began by supplementing leucine and HMB to two murine (Panc02 and NB508) and human (Panc-1 and MiaPaca-2) cell lines in serum-rich (10% FBS) and serum-restricted (1% FBS) environments, which simulated *in vitro* obese/overweight and lean environments.

In the serum-rich environment, leucine increased Panc02 and NB508 proliferation, but it did not have a significant effect on mTOR signaling. This is consistent with our previous results, where dietary leucine supplementation enhanced Panc02 tumor growth in overweight mice without affecting mTOR activation (77). Leucine did not increase mTOR activation, because mTOR was already activated at a high level due to the serum-rich environment. Leucine, however, did not have similar results in the human cells, because it inhibited MiaPaca-2 proliferation. The MiaPaca-2 cells are human rather than murine, so they may have characteristics that make susceptible to leucine in a different way than the Panc02 and NB508 cells. It is known that the uptake of leucine occurs through a sodium-insensitive, L-type amino acid transporter (LAT1), and that this transporter exists in various cancer cell lines, including C6 glioma and and the breast cancer cell lines, MDA-MB-231 and MCF-7 (164-166). The MiaPaca-2 cells may have a larger amount of the LAT1 transporter, thus making them more sensitive to leucine supplementation. In an environment rich with growth factors, the addition of a large amount of leucine may have caused a negative

feedback loop and the inhibition of growth. It is common for signaling pathways to have negative feedback loops as checkpoints. In fact, the mTOR pathway, a central growth pathway, can be inhibited by one of its downstream proteins, S6K (167).

Then, in the serum-restricted environment, leucine increased Panc02, NB508, and MiaPaca-2 proliferation along with Panc-1 and MiaPaca-2 mTOR signaling. This is again consistent with the results of our previous study, which showed leucine enhanced Panc02 tumor growth in lean mice while increasing mTOR activation (77). Although leucine did not significantly increase mTOR signaling within the Panc02 cells, it did activate this growth pathway in the human cells. These results suggest that leucine may be utilized for growth differently in murine PDAC cells and human cells. In the serum-restricted environment, human PDAC cells may use leucine mainly to stimulate mTOR, and the murine PDAC cells may oxidize leucine for energy instead. Breast and liver tumors have been shown to contain active forms of the enzymes involved in branched-chain amino acid catabolism, BCAAT and BCKDH (168-169), so it is plausible PDAC cells catabolize leucine as well.

On the other hand, HMB had similar effects in both the serum-rich and serum-restricted environments. In the serum-rich environment, HMB decreased Panc02, Panc-1, and MiaPaca-2 proliferation, while decreasing NB508 and Panc-1 mTOR signaling. In the serum-restricted environment, HMB decreased Panc02, NB508, Panc-1, and MiaPaca-2 proliferation, while decreasing NB508, Panc-1, and MiaPaca-2 mTOR signaling. These results are consistent with our previous findings that dietary HMB supplementation decreased Panc02 tumor growth in obese mice (Chapter III). However, the finding that HMB supplementation decreases mTOR signaling in

pancreatic cancer cells is novel. HMB has been shown to increase mTOR activation, especially in skeletal muscle and the myotube cell lines, C2C12 and L6 (170-171), and due to the similarity of its actions to leucine, there is support for HMB as an mTOR-enhancer. However, Sabatini et al. demonstrated that the mTOR pathway has a sensor for leucine specifically, which explains why leucine consistently increases mTOR in various tissues (172). No studies have shown HMB may have a similar mTOR pathway-specific sensor.

While both leucine and HMB affected mTOR signaling in the serum-restricted environment, their effects on mTOR were not always consistent with their effects on PDAC cell proliferation, especially in the Panc02 cells. Thus, our next step was to explore other potential mechanisms through the analysis of global gene expression. A microarray was performed on Panc02 cells in serum-restricted media supplemented with leucine or HMB, and they were found to differentially regulate various genes. One-third of these genes coded for ORs, which suggests OR expression is correlated with PDAC proliferation and may even play a role in PDAC growth. The association between OR expression and PDAC growth is consistent with our finding in the PDAC tumors of obese mice, where obesity enhanced both OR expression and PDAC growth, and HMB decreased both OR expression and PDAC growth (Chapter III). However, in the Panc02 cells, HMB did not universally downregulate ORs. Instead, leucine and HMB had opposing effects on Olfr487, Olfr582, Olfr1138, Olfr402, and Olfr1034. This suggests specific ORs may have different roles to play in PDAC proliferation. Multiple studies have demonstrated aberrant OR expression in cancerous tissues (148). OR51E1 is increased in various cancers, including gastrointestinal neuroendocrine

cancer (173-174), prostate cancer (149-150), and somatostatin receptor SSTR-negative lung carcinoid tumors (175). OR51E2 is upregulated in prostate cancer and is thought to promote tumor growth (176-177), although Neuhaus et al. demonstrated that activation of OR51E2 inhibited prostate cancer cell proliferation (178). Moreover, different mutations in OR2W3 are associated with PDAC and autosomal dominant retinitis pigmentosa (76,179).

Before investigating if specific ORs affected PDAC proliferation, we first tested whether simultaneous changes in multiple ORs could affect cell growth. To do this, all PDAC cell lines were treated with two commonly used OR agonists, amyl acetate and octanal. Amyl acetate increased NB508 proliferation in the serum-rich environment, but it decreased Panc02 and MiaPaca-2 proliferation in the serum-restricted environment. Octanal was more consistent in that it decreased Panc02, NB508, Panc-1, and MiaPaca-2 proliferation in both the serum-rich and serum-restricted environments. These results show that changing the expression of multiple ORs can affect PDAC cell proliferation, and different OR agonists have distinct effects on PDAC cells. This is due to their ability to regulate different ORs, which suggests PDAC cell proliferation is affected by specific ORs. Amyl acetate treatment was similar to leucine supplementation with respect to its environment-dependent effects on PDAC proliferation, while octanal mirrored HMB supplementation with respect to its growth-inhibitory effects on PDAC cells. Although the nutritional supplements and OR agonists may not regulate the same ORs, their similar effects on proliferation suggest ORs may be another link connecting leucine, HMB, and PDAC growth.

In conclusion, this report establishes that leucine supplementation increases PDAC cell proliferation and mTOR signaling in the serum-restricted environment. On the other hand, HMB supplementation decreases PDAC cell proliferation and mTOR signaling in both the serum-rich and serum-restricted environments. Leucine and HMB then differentially affect OR expression within Panc02 cells, and two OR agonists have different effects on PDAC growth. These findings confirm HMB, not leucine, is a strong candidate for cancer cachexia treatment, because it inhibited both murine and human PDAC cell proliferation. Moreover, a potential mechanism for leucine and HMB may involve specific ORs and OR gene regulation.

Chapter V

Final conclusions and future directions

Due to the obesity epidemic in the United States and many other parts of the world, an increasing number of pancreatic cancer patients at the start of treatment are overweight or obese. Many of these patients have sarcopenic obesity, which is characterized by high fat mass and low muscle mass. This combination increases the chances of developing cancer cachexia, which is present in 80% of pancreatic cancer patients, and is predictive of morbidity and mortality (58,5). These consequences are due to both obesity, which creates a pro-tumor environment through various circulating factors, and muscle loss, which reduces response to chemotherapy and increases mortality rate (2-8). An ideal treatment for these patients would treat both the cancer and cachexia, as they are strongly connected, but unfortunately therapeutic regimens that effectively target both the cancer and the cachexia are not currently available. In practice, the treatment would most likely have to be two-pronged; for example chemotherapy for the cancer and potentially nutritional supplementation for the cachexia (49-50). Two potential cachexia treatments are the branched-chain amino acid (BCAA) leucine and its metabolite, β-hydroxy-β-methylburtyrate (HMB) due to their growth-enhancing and breakdown-inhibiting effects in muscle (51-56). However, we previously found in a preclinical model that leucine promotes pancreatic cancer growth (77), while to our knowledge no studies to date have explored the effects of HMB on pancreatic tumor growth.

The overall aim of the project presented herein was to determine the impact of leucine and HMB supplementation on PDAC tumor growth within different diet-induced

physiological environments (lean versus obese) or with and without chemotherapy (gemcitabine) and to elucidate potential mechanisms underlying these effects. From these studies, we were able to demonstrate the following: 1) leucine had no effect on lean mass in lean and overweight mice (Chapter II); 2) leucine enhanced Panc02 tumor growth in lean and overweight mice and increased PDAC cell proliferation in a serum-restricted environment (Chapter II, IV); 3) leucine increased mTOR signaling within the Panc02 tumors of lean mice and increased mTOR signaling within PDAC cells in a serum-restricted environment (Chapter II, IV); 4) leucine affected olfactory receptor (OR) expression in PDAC cells (Chapter IV); 5) HMB preserved muscle mass in normo-weight and obese mice (Chapter III); 6) HMB inhibited Panc02 tumor growth in obese mice and decreased PDAC cell proliferation in both a serum-rich and serum-restricted environment (Chapter III, IV); 7) HMB increased the efficacy of the chemotherapeutic agent gemcitabine, in obese mice (Chapter III); 8) HMB had no effect on mTOR signaling within the Panc02 tumors of normo-weight and obese mice (Chapter II); 9) HMB decreased mTOR signaling in PDAC cells in both serum-rich and serum-restricted environments (Chapter IV); 10) HMB stimulated T cell infiltration into the Panc02 tumors of normo-weight mice and specifically cytotoxic CD8+ T cell infiltration into the Panc02 tumors of obese mice (Chapter III); 11) HMB downregulated many OR genes in the Panc02 tumors of obese mice and differentially regulated OR genes in Panc02 cells in a serum-restricted environment (Chapter III, IV). Taken together, these findings suggest that leucine enhances pancreatic tumor growth, and therefore may not be a good candidate for cancer cachexia in PDAC patients. HMB, however, inhibits pancreatic tumor growth while preserving muscle mass, and therefore may be a good

candidate for the cancer cachexia in PDAC patients. Specifically, leucine increases mTOR signaling, especially in lean mice (that typically have low levels of circulating growth factors that can activate mTOR signaling), while HMB either has no effect on Panc02 tumor mTOR activation or (depending on the cell line and dose) decreases PDAC cell mTOR signaling. Moreover, HMB increases cytotoxic CD8+ T cell infiltration and decreases OR expression in Panc02 tumors. Both leucine and HMB regulated OR expression in PDAC cells, although we did observe differential effects of these two agents on specific receptors. Thus, there may be multiple mechanisms by which leucine and HMB differentially affect PDAC.

While the data presented fulfills the project aims, further *in vivo* and *in vitro* studies could be performed to provide a more comprehensive evaluation of the effects of leucine and HMB on muscle and tumor. Additional *in vitro* studies could be conducted to elucidate potential mechanisms of leucine and HMB action on PDAC cells. Specifically, a continuation of Chapter IV would involve performing qPCR for the specific ORs discovered using the microarray on PDAC cells supplemented with leucine, HMB, amyl acetate, and octanal. This would both confirm the microarray results and determine if these ORs are regulated by leucine, HMB, and the OR agonists. Then, silencing of these ORs through specific siRNA could be performed on the PDAC cells. Following the silencing with MTT assays would determine if these ORs are mechanistically involved in PDAC cell proliferation. Thus, leucine and HMB would be linked to PDAC proliferation through their ability to regulate specific OR gene expression.

Another continuation of Chapter IV would involve determining the effects of leucine and HMB on metabolic parameters, specifically glycolysis and mitochondrial respiration. These pathways are of interest, because pancreatic cancer cells are known to exhibit high levels of glycolysis over mitochondrial respiration. This is called the Warburg effect, and studies suggest it may be due in part to mitochondrial respiration injury (180). Leucine and HMB have both been shown to enhance the efficiency of mitochondrial biogenesis in muscle. Leucine induces peroxisome proliferator-activated receptor beta/delta (PPARβ/δ) in myotubes and mediates PPARβ/δ-dependent mitochondrial biogenesis (181), while HMB increases expression of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α), which induces genes involved in mitochondrial metabolism and biogenesis (182). Although the effects of leucine and HMB on pancreatic cancer cell metabolic flux has, to my knowledge, not previously been assessed, I hypothesize that HMB in particular may be able to shift PDAC cells to favor mitochondrial respiration, interrupting the Warburg effect and depriving these cells of the building blocks required for cell proliferation they normally obtain through glycolysis (183). To determine any shifts in cellular energetics, PDAC cells would be treated with leucine or HMB and subjected to the Seahorse XF Extracellular Flux Analyzer, which measures oxygen consumption rates (OCR) and extracellular acid release (ECAR) simultaneously. The OCR correlates with mitochondrial respiration, while ECAR correlates with glycolysis. This experiment would provide insight on how leucine and/or HMB may be changing the internal metabolic landscape of PDAC cells and thus, how those cells grow and respond to changes in their microenvironment.

Although investigating possible avenues for the effects of leucine and HMB on PDAC cells gives valuable mechanistic data, a clearer understanding of how these supplements may work in cancer patients would be gained through *in vivo* studies. Our studies used a previously-established C57BL/6 murine model of subcutaneous PDAC that did not have cachexia for several reasons. These mice were responsive to high-energy and low-energy diets that would establish pro-tumor and anti-tumor environments; they could easily grow tumors from the syngeneic Panc02 and thus, maintain an intact immune system; and they have been used to demonstrate PDAC-dependency on mTOR signaling (60). These factors were relevant when studying the effects of leucine and HMB on tumor growth. However, now that leucine and HMB have been shown to play roles in both muscle and pancreatic tumor growth, a pancreatic cancer cachexia model should be established. Most murine models of cancer cachexia are transplant models, where the injected cancer cells subcutaneously form tumors that cause weight loss from 20%-40% proportional to tumor size after 3 weeks, wasting of adipose and muscle tissues, hypoglycemia, hyperlacticidemia, hypertriacylglycerolemia, and depletion of glycogen stores (184-187). There are colon (MAC16 and C 26) and breast cancer (Walker 256) cells that cause cachexia, but no pancreatic cancer cells. To create a PDAC cell line that causes cachexia, established cells lines from transgenic models of spontaneous PDAC (UN-KC-6141, UN-KPC-960, and UN-KPC-961) could be injected subcutaneously into obese C57BL/6 mice, or another mouse line that is not a transgenic model of PDAC. This ensures all cachectic symptoms would be attributed to the injected tumor, and the obese state should increase the changes of cachexia occurring. Tumor cells from the mice with the most

significant weight loss would then be cultured and injected into new obese mice. If 20%-40% weight loss occured after 3 weeks, the tumor would be excised, and if the weight loss is reversed, this would be a PDAC cell line that causes cachexia. Further analysis on adipose tissue, muscle tissue, and serum markers would have to be done to confirm. Tumor cells from these obese mice could be cultured and established as a PDAC cachectic cell line.

The next suggested studies would be done after characterization and proven repeatability of the murine cachectic model of PDAC. Due to the positive results in Chapter III, dietary HMB supplementation and gemcitabine treatment should be repeated in this model to confirm effects of HMB on tumor, muscle, and chemotherapy. Novel additions to this experiment could be further analysis of gastrocnemius muscle by measuring weight and fiber type through IHC for markers of Type I vs. Type II. Cachexia is known to disproportionately affect Type II (fast-twitch) muscle (188). In addition, the effects of HMB on local inflammatory pathways within tumor and muscle could be assessed through western blot analysis for the p65 subunit of NF-κB in the nucleus or through the visualization of NF-κB through immunofluorescence. Downstream analysis of various NF-κB-regulated genes could then be done to confirm. These experiments would help support HMB as a strong candidate for cachexia treatment, because they would confirm that HMB can affect these pathways in a cachectic model of PDAC as well as in other models of cancer cachexia.

After the characterization of HMB in a PDAC cachexia model, additional mechanistic studies could be done to explore the effects of HMB on the immune system. In Chapter III, HMB supplementation significantly increased the amount of

cytotoxic CD8+ T cell infiltration into Panc02 tumors. However, there is little evidence to suggest why this occurred. From our study, it is evident that HMB supplementation increased tumor T cell infiltration at a much higher rate in the obese mice compared to the normo-weight mice, so it must be due to the pro-tumor obese environment. One aspect of this environment that can be connected to the immune system is chronic inflammation. Although we saw no increase in pro-inflammatory cytokine levels in the serum of obese mice compared to normo-weight mice, this was prior to tumor injection. Pancreatic cancer cells have been shown to secrete cytokines of their own and contribute to systemic inflammation (189). The tumors of obese mice were larger and could have secreted more cytokines, causing a shift towards a more inflammatory environment. Increased TNF-α and NF-κB activation in this environment could have promoted CD8+ T cell synthesis (69-70), and then HMB could have promoted T cell infiltration by stimulating specific pathways within the tumor. T cells normally function by scanning cells for major histocompatibility complex (MHC) molecules presented on the cell surface and destroying cells with foreign MHC molecules. Defects in the MHC system, such as loss or downregulation of antigen processing and antigen-presenting molecules, are found in various tumors and help them avoid recognition by cytotoxic CD8+ T cells (190). Reduction or loss of human leukocyte antigen (HLC class I) and transporter for antigen presentation (TAP) occurs in the majority of pancreatic cancer cell lines (191). HMB may be preventing the loss of HLC and TAP and subsequently increasing tumor cytotoxic CD8+ T cell infiltration. To determine if this is true, HMB should be supplemented in the diet of mice without tumors and mice with the PDAC cachectic tumors. Then, serum should be obtained for cytokine analysis as well as

tumor for HLC and TAP analysis (qPCR, western blot, IHC). This data would provide evidence for the mechanism behind HMB-induced cytotoxic CD8+ T cell infiltration in PDAC tumors.

Similar to how adjunct treatment can strengthen a chemotherapy regimen, HMB could be a stronger candidate for cachexia treatment when combined with another treatment. One option is another popular nutritional supplement, omega-3 fatty acids, which are often found at high quantities in fish oil supplements. Long term omega-3 fatty acids supplementation has been shown to enhance the anabolic (growth-promoting) stimuli from amino acids, protein, insulin, and physical activity (192). In an animal model of cancer cachexia, lifelong supplementation with fish oil rich in omega-3 fatty acids decreased tumor growth by approximately 60%, increased survival, and prevented weight loss. This fish oil supplementation also inhibited hypoglycemia, hyperlacticidemia, hypertriacylglycerolemia, and preserved glycogen stores (187). Previous reports also show the anti-cancer effects of omega-3 fatty acids are due to various mechanisms, including suppression of NF-κB, activation of AMPK and SIRT1, and modulation of cyclooxygenase (COX) activity (193). These studies suggest that the combination of long-term omega-3 fatty acid supplementation in the form of fish oil and short-term HMB supplementation would be beneficial both for muscle growth and tumor growth. This could be done in the murine model of PDAC cachexia to determine the effects of omega-3 fatty acids and HMB on weight loss, body composition (adipose and muscle mass), hypoglycemia, hyperlacticidemia, hypertriacylglycerolemia, and glycogen stores. Downstream effects on Akt/mTOR, NF-κB, SIRT1, and COX signaling could then be analyzed to confirm mechanism. This data would provide evidence for

the positive effects of combining HMB and fish oil (omega-3 fatty acids) on PDAC cachexia.

The data presented in the current project suggests that leucine enhances pancreatic tumor growth, mTOR signaling, and OR expression in PDAC, while HMB inhibits pancreatic tumor growth, mTOR signaling, and OR expression, and increases cytotoxic CD8+ T cell infiltration into the tumor. Findings from the proposed studies would identify specific ORs involved in PDAC cell proliferation and regulated by leucine and HMB and characterize the effects of leucine and HMB on glycolysis and mitochondrial respiration within PDAC cells. Results from the proposed studies would also create and characterize a PDAC cachectic model that is similar to other cancer cachexia models, provide insight into a novel immunity-based mechanism for the tumor-inhibiting effects of HMB, and determine the efficiency of combination therapy with HMB and omega-3 fatty acids. Findings from these experiments would provide further insight into the varied mechanisms of cancer cachexia, leucine, and especially HMB, which has been administered to elderly and clinical populations safely and effectively for up to 12 months (194). These results could then be used in translational cancer treatment studies.

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